UNIVERSIDADE FEDERAL DO PARANÁ

PALOMA KACHEL GUSSO CHOUERI

USO DO BAGRE AMARELO (*Cathorops spixii*) COMO MODELO BIOLÓGICO DE EXPOSIÇÃO E EFEITO DE CONTAMINANTES NO COMPLEXO ESTUARINO-LAGUNAR CANANÉIA-IGUAPE-PERUÍBE

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Tese apresentada como requisito parcial à obtenção do grau de doutor, pelo Curso de Pós-Graduação em Ecologia e Conservação, Setor de Ciências Biológicas da Universidade Federal do Paraná.

Orientador: Prof. Dr. Ciro Alberto de Oliveira Ribeiro
Co orientador: Prof. Dr. Denis Moledo de Sousa Abessa

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PARECER

Os abaixo-assinados, membros da banca examinadora da defesa da tese, a que se submeteu **Paloma Kachel Gusso Choueri** para fins de adquirir o título de Doutora em Ecologia e Conservação, são de parecer favorável à **APROVAÇÃO** do trabalho de conclusão da candidata.

Secretaria do Programa de Pós-Graduação em Ecologia e Conservação.

Curitiba, 27 de março de 2015.

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“A dúvida é se a civilização pode mesmo travar esta guerra contra a vida sem se destruir e sem perder o direito de se chamar de civilizada.”

Rachel Carson

RESUMO

Diversas áreas protegidas marinhas e estuarinas (AMP) apresentam níveis moderados de contaminação porém, nestas áreas, as respostas biológicas nem sempre são evidentes. Desta forma, as avaliações de risco ambiental não devem se restringir às analises químicas do sedimento, mas também se faz necessário avaliar parâmetros biológicos que sejam capazes de indicar precocemente os potenciais efeitos de xenobióticos, como os biomarcadores. O primeiro objetivo deste trabalho foi utilizar um conjunto de biomarcadores bioquímicos (GPx, GST, GSH, danos em DNA, LPO, AChE) e somáticos (fator de condição) em diferentes órgãos-alvo (fígado, rim e brânquias) da espécie de peixe *Cathorops spixii*, e relacionar com os níveis de metais e As bioacumulados nos tecidos e com os hidrocarbonetos policíclicos aromáticos (PAHs) presentes na bile. Assim, foi avaliada a qualidade do ambiente aquático da AMP de Cananéia-Iguape-Peruíbe (APA-CIP) exposta a atividades de mineração pretérita e assentamentos urbanos. O segundo objetivo foi aplicar diferentes ferramentas moleculares e estruturais para investigar possíveis efeitos genotóxicos em *C. spixii* coletados na APA-CIP. Já o terceiro objetivo foi avaliar as correspondências, complementaridades ou conflitos entre as várias linhas de evidência na qualidade do sedimento da APA-CIP. Nestas análises são incluídos biomarcadores, biacumulação e linhas-de-evidência “clássicas”, através de uma abordagem de “peso de evidências”. O quarto objetivo foi estimar o potencial risco de exposição para a saúde humana através do consumo de *C. spixii* provenientes da APA-CIP. Os resultados confirmaram a utilidade do uso de biomarcadores na avaliação da biota exposta às fontes de poluição localizadas ao longo da APA-CIP. O fígado foi o órgão considerado mais sensível em termos de resposta subcelular, enquanto que as brânquias foram mais sensíveis para avaliar os biomarcadores de efeito. Os resultados indicaram que a APA-CIP é influenciada por duas fontes de contaminação: descargas provenientes do rio Ribeira de Iguape (RIR) e de áreas urbanas da cidade de Cananéia. Os resultados observados apartir do uso de diferentes ferramentas na avaliação da genotoxicidade mostraram que micronúcleos e alterações nucleares estão mais associadas com os níveis de metais bioacumulados nos tecidos do que as análises de danos em DNA. A aplicação dos índices de biomarcadores dentro de uma abordagem de peso-de-evidências mostrou que os índices que incorporam as lesões histopatológicas em *C. spixii* foram melhor associados com a toxicidade do sedimento, enquanto que os índices de biomarcadores, que incluem apenas as respostas subcelulares e celulares, foram melhor associados com níveis de metais bioacumulados. Os níveis de metais e As no músculo e fígado não se associaram com os níveis de contaminantes nos sedimentos, sugerindo diferentes níveis de biodisponibilidade para *C. spixii* ou a um mecanismo celular de eliminação destes compostos. As concentrações de Cd, Pb e As encontradas no músculo de *C. spixii* foram superiores aos níveis máximos permitidos para o consumo humano. Desta forma, o estudo mostra que existe risco de exposição para populações humanas e que mais atenção deve ser dirigida à proteção das AMPs, a fim de minimizar os riscos para a biota residente e para população humana.

Palavras chave: biomonitoramento, “peso-de-evidência”; qualidade do sedimento; qualidade de água, biomarcadores; avaliação de risco ambiental; risco a saúde humana

ABSTRACT

Many marine and estuarine protected areas present moderate levels of contamination therefore biological responses are not so evident. Thus, environmental risk assessments should not be restricted to chemical sediment analysis, but it is also necessary to evaluate biological parameters that are able to early indicate the potential effects of xenobiotics, such as biomarkers. The first aim of current work was to investigate the role of a set of biomarkers (GPx, GST, GSH, DNA damage, LPO, AChE and condition index) in different target organs (liver, kidney and gills) of a demersal fish (*Cathorops spixii*) and its relationship with contaminants (metal body burdens on liver and muscle tissues and PAHs in bile), in order to assess the environmental quality of the Marine Protected Area of Cananéia-Iguape-Peruíbe (APA-CIP), affected by former mining activities and urban settlements. The second goal was to use different structural and molecular tools to detect possible genotoxic effects in *C. spixii* from the APA-CIP. The third aim was to assess correspondences, complementarity, or conflicts among multiple sediment quality LOEs, including biomarkers analyzes, through a WOE sediment-quality assessment. The fourth aim was to estimate the potential risk of the consumption of *C. spixii* from APA-CIP for human health. The results confirmed the usefulness of the biomarker approach for the identification of both seasonal and spatial variations in pollution sources around APA-CIP. The liver was found to be more responsive in terms of its antioxidant responses, whereas gills were found to be more responsive to biomarkers of effect. APA-CIP seems to be influenced by two sources of contamination: Ribeira de Iguape River (RIR) mouth and urban areas of Cananéia city. The results of different genotoxicity responses showed that micronucleus and nuclear alterations were more frequently associated with the metal body burdens than the analyses of DNA damage, which suggests that the first analyses are less vulnerable to the effects of confounding factors in mildly contaminated areas. The used of biomarker indices within a WOE approach showed that the biomarker index that incorporates histopathological lesions in resident fish was better associated with sediment toxicity and contamination, whereas the biomarker indices that included only sub-cellular and cellular responses were better associated with metals and As body burdens. Metals and As burdens in muscle or liver tissue, in turn, did not associate with sediment levels of these contaminants, suggesting that *C. spixii* have a mechanism of internal metal regulation. Cd, Pb, and As were found at concentrations above action levels for human consumption. The study of the risk of *C. spixii* consumption showed that, depending on the level of exposure of the local population, the levels of metals and As in *C. spixii* pose risk to human health. Taken altogether, the results of this study showed that more attention should be directed to the protection of MPA in order to minimize risks to the resident biota and sensitive human population.

Key words: weight-of-evidence; factor analysis; sediment quality; histopathology; antioxidant responses; genotoxicity; environmental risk assessment, human health risk

LISTA DE SIGLAS

AChE - Acetylcholinesterase

AMP - Área Marinha Protegida

APA-CIP - Área de Proteção Ambiental de Cananéia-Iguape-Peruíbe

BRI - Biomarkers Response Index

CRisk - Cancer risk

CR_{mm} - Maximum allowable fish consumption rate ($meals.month^{-1}$)

FA/PCA - Factor analysis with principal component analysis as the extraction method

GPx - Glutathione peroxidase

GSH - Non-protein reduced thiols

GST- Glutathione S-transferase

IBR - Integrated Biomarkers Response

LOEs - Linhas de evidência (lines of evidence)

LPO - Lipid peroxidation

METs - Metallothionein-like protein

MN - Micronucleus

MPAs - Marine Protected Areas

NA - Nuclear alterations

PAHs - Hidrocarbonetos Policíclicos Aromáticos (Polycyclic Aromatic Hydrocarbons)

RIR - Rio Ribeira do Iguape (Ribeira do Iguape River)

SEM-AVS - Acid Volatile Sulfides

SQT - Tríade de Qualidade em Sedimentos (Sediment Quality Triad)

SPMDs - Semipermeable Membrane Devices

THQ - Target Hazard Quotient

WBR - Weighted Biomarkers Response

$WBR_{Effects}$ - Weighted Biomarkers Response of Effects

$WBR_{AOxResp}$ - Weighted Biomarkers Response of Antioxidant Responses

WOE - “Peso de Evidências” (weight-of-evidence)

SUMÁRIO

1. INTRODUÇÃO GERAL

1.1 ÁREAS MARINHAS PROTEGIDAS E CONTAMINAÇÃO AMBIENTAL

Historicamente, as populações humanas tem habitado zonas costeiras e são componentes naturais dos ecossistemas costeiros. No entanto, especialmente nas últimas décadas, a intensificação das atividades humanas no litoral causou alterações na estrutura e função dos ecossistemas costeiros. Estima-se que aproximadamente 50% das áreas costeiras no planeta estão ameaçadas por atividades relacionadas ao desenvolvimento econômico (UNEP, 2002). Entre os principais fatores relacionados à mudança no estado dos sistemas marinhos e costeiros pode-se listar: as mudanças climáticas, o uso de fertilizantes e defensivos agrícolas, gestão e as mudanças do uso da terra, doenças, espécies invasoras, e a poluição urbana e industrial (UNEP, 2006).

Neste contexto, as áreas marinhas protegidas (AMP) foram estabelecidas para preservar o ambiente marinho e assegurar o bem-estar e do patrimônio para as gerações futuras (SCHERL *et al.,* 2006). A lógica por trás do conceito de AMP é que, reduzindo a perda de habitat e a mortalidade devido á exploração, os processos ecossistêmicos são mantidos e os indivíduos podem sobreviver por mais tempo e produzir mais descendentes, e, desta forma, as populações podem crescer até atingirem um estado de equilíbrio teoricamente próximo do clímax. No entanto, as AMPs costumam ser planejadas, criadas e gerenciadas seguindo os mesmos conceitos e teorias historicamente utilizadas para áreas protegidas terrestres, embora hajam diferenças substanciais nos processos ecossistêmicos, percepções históricas e marcos regulatórios entre os ambientes marinhos e terrestres (HOUDE, 2001). Por exemplo, populações marinhas, comunidades e ecossistemas estão ligados a uma escala mais ampla que a maioria dos organismos terrestres, uma vez que organismos marinhos podem se deslocar por longas distâncias para se estabelecer, reproduzir, alimentar etc. (Convenção das Nações Unidas sobre o Direito do Mar (CNUDM, 1982).

Além disso, a água não respeita as fronteiras e contaminantes podem ser introduzidos a partir das zonas adjacentes à área das AMPs (PALMER, ALLAN e BUTMAN *et al.*, 1996, BOERSMA *et al.*, 1999; POZO *et al.*, 2009). É amplamente aceito que as AMPs são vulneráveis à poluição, além da pesca, alteração de habitat, e ás alterações climáticas (JAMESON *et al.*, 2002; CICIN-SAIN e BELFIORE, 2005) e, portanto, o monitoramento ambiental destas áreas deve considerar os impactos causados por esse tipo de estresse. No entanto, a maioria das avaliações biológicas realizadas na AMP consideram apenas os efeitos diretos ou indiretos relacionados a pesca (GARCÍA-CHARTON *et al*., 2000; FRASCHETTI *et al*., 2002). Quando realizados estudos ligados à contaminação ambiental, estes geralmente se concentram em medir os níveis de contaminantes em matrizes ambientais, tais como água, sedimentos e biota (por exemplo, MICHEL *et al*., 2001; CHOU *et al*., 2004; POZO *et al*., 2009; PERRA *et al*., 2011). São poucas as inciciativas de estudos de avaliação dos efeitos dos poluentes sobre a biota, que deveria, de fato, ser protegida nas AMPs (por exemplo, TERLIZZI, 2004; PINSINO, TORRE E SAMMARINO, 2008; RODRIGUES, ABESSA E MACHADO, 2013; ARAUJO *et al.,* 2013; CRUZ *et al*., 2014).

1.2 AVALIAÇÃO DA QUALIDADE DOS SEDIMENTOS

Dos contaminantes que alcançam os ecossistemas aquáticos, grande parte se deposita nos sedimentos, complexada à matéria orgânica, sais inorgânicos, ou à fração fina da matriz sedimentar. Isso faz com que os sedimentos atuem como uma espécie de depósito de contaminantes, podendo apresentar concentrações químicas muito superiores àquelas encontradas na coluna d’água (PETROVIC e BARCELÓ, 2004; INGERSOLL *et al*., 2003).

Por outro lado, as substâncias químicas depositadas nos sedimentos podem eventualmente solubilizar-se novamente, especialmente a partir de alterações físico-químicas que levem a uma diminuição do caráter redutor do ambiente sedimentar (HOFFMAN *et al*., 2003), como por exemplo, a ressuspensão dos sedimentos (natural ou resultado de atividade humana) e bombeamento de água com maior concentração de oxigênio em consequência da atividade biológica bentônica, entre outros eventos. Os contaminantes acumulados nos sedimentos - muitas vezes durante longos períodos – podem, desta forma, entrar novamente em solução através de processos físico-químicos como a ressolubilização e difusão, e assim comprometer a qualidade de água do ponto de vista ambiental e de saúde humana (CHAPMAN e WANG, 2001). A contaminação dos sedimentos pode não apenas afetar os organismos bentônicos diretamente como também ser incorporada à biota bentônica e transferida através da cadeia trófica dos sistemas aquáticos, expondo organismos de níveis tróficos superiores, incluindo seres humanos (BORGMAN *et al*., 2001; GRAPENTINE *et al*., 2002; CHAPMAN e ANDERSON, 2005).

Nas últimas décadas, a contaminação dos sedimentos tem, cada vez mais frequentemente, causado danos ambientais e prejuízos econômicos. Entre as atividades humanas afetadas por este problema, as mais importantes são a pesca (uma vez que as substâncias químicas nos sedimentos podem se transferir e acumular em espécies economicamente importantes), recreação (por impedimento das atividades de banho, pesca desportiva), turismo (perda de atrações naturais, beleza cênica, produção de maus odores) e atividades portuárias e navegação. Além disso, a própria saúde pública pode ser afetada ou ameaçada, considerando o processo de transferência dos contaminantes retidos no sedimento para a biota aquática, sobretudo peixes, crustáceos e moluscos de importância econômica (CHOUERI *et al*., 2010).

Atualmente existe uma crescente preocupação global com o conhecimento do estado ecológico real dos ambientes estuarinos; portanto, foram realizados esforços consideráveis para fornecer ferramentas eficazes para os planos de gestão ambiental (BORJA *et al*., 2010; LYONS *et al*., 2010).

Ainda que tradicionalmente as legislações nacionais recomendem ou obriguem unicamente a utilização de caracterizações físico-químicas, atualmente o meio científico afirma de forma consonante que a integração de duas ou mais categorias de análises produz resultados mais confiáveis sobre a qualidade dos sedimentos (CHOUERI *et al*., 2010; CESAR *et al*., 2007, 2006; ABESSA *et al*., 2005; HUNT *et al*., 2001; DELVALLS, FORJA e GÓMEZ PARRA, 1998; CARR *et al*., 1996; CHAPMAN, 1992). Este tipo de abordagem vem ganhando espaço também na esfera regulatória, uma vez que convenções internacionais para a proteção do meio marinho (LONDON CONVENTION, 1972; OSPAR, 1992), além de agências ambientais de Estados Unidos, Canadá, União Européia e Brasil (ex.: USEPA, 1994;

ENVIRONMENT CANADA, 2002; EUROPEAN COMMISSION, 2000; CETESB, 2007) vêm recomendando e aplicando abordagens integradas que incluem análises químicas e biológicas/ecológicas na avaliação da qualidade ambiental de sedimentos (INGERSOLL, 1995).

Em estudos ambientais, a abordagem de "peso de evidências" (WOE- weight-of-evidence) é o processo de integração de informações de múltiplas linhas de evidência (LOE) para chegar a uma conclusão sobre um ambiente ou estressor (BURTON Jr *et al*., 2002). As LOE mais comumente utilizadas em estudos de qualidade ambiental ou avaliações de risco são aquelas que compõem a Tríade de Qualidade em Sedimentos (SQT) (LONG e CHAPMAN, 1985; CHAPMAN, 1990): (i) a química do sedimento, (ii) a estrutura da comunidade bentônica infaunal, e (iii) ensaios de toxicidade em laboratório (BURTON Jr et al, 2002;. CHAPMAN e HOLLERT, 2006).

A SQT oferece respostas às três perguntas referenciais em estudos de avaliação ambiental de ecossistemas aquáticos (RIBA *et al*., 2004):

- Quais contaminantes estão presentes na área de estudo e em que concentrações?
- Estão estes contaminantes disponíveis para a biota?
- Quais os efeitos biológicos provocados por estes contaminantes?

Artigos pretéritos (por exemplo, CHAPMAN, 2000; CHAPMAN e HOLLERT, 2006) incentivaram a evolução da SQT, mantendo a sua essência multidimensional, mas incorporando novos LOE em sua estrutura básica. Por exemplo, níveis de contaminantes nos tecidos (BORGMANN *et al*., 2001) e potencial de biomagnificação (GRAPENTINE *et al*, 2002;. CHAPMAN e ANDERSON, 2005) foram propostos para integrar as avaliações da qualidade dos sedimentos. Recentemente, Torres *et al*. (2014) avaliaram diversas LOE aplicadas na avaliação da qualidade de sedimento, entre as quais, a proporção entre metais extraídos simultaneamente e sulfetos voláteis ácidos (SEM-AVS) em sedimentos; níveis de hidrocarbonetos poliaromáticos (HPA) em dispositivos membrana semipermeável (SPMDs); bioacumulação em ostras nativas e transplantadas; além dos componentes SQT clássicos (níveis de contaminantes nos sedimentos, toxicidade de sedimentos e índices para descrição da estrutura da comunidade bentônica).

Apesar do consenso geral (IJC 1987; CHAPMAN *et al*., 1992; BURTON and MACPHERSON, 1995) de que estas linhas de evidência são geralmente de grande utilidade para a avaliação da qualidade do sedimento, elas contemplam apenas efeitos em níveis elevados de organização (populações e comunidades). Desta forma, o uso de ferramentas mais sensíveis, como por exemplo biomarcadores, pode permitir uma tomada de decisão mais preventiva, pois considera os efeitos dos contaminantes a longo prazo (MARTIN-DIAZ *et al*., 2008).

1.3 Biomarcadores

Os biomarcadores, que de acordo com Depledge (1993) são variações bioquímicas, celulares, fisiológicas ou comportamentais que podem ser avaliadas em tecidos ou fluidos corporais ou em nível de organismo, avaliando precocemente evidências de exposição e/ou efeitos de um ou mais contaminantes. Esta ferramenta tem se mostrado confiável e foi bastante difundida nos últimos dez anos em estudos ambientais (BONNINEAU *et al*., 2012). Tais respostas podem antecipar danos potenciais em níveis mais elevados de organização biológica, como a morte de organismos, alterações em populações, comunidades ou ecossistemas (CHEUNG *et al.*, 1997).

Considerando a hipótese fundamental da teoria dos biomarcadores que preconiza que efeitos em níveis hierárquicos superiores (BAYNE *et al*., 1985) são sempre precedidos por modificações prévias em processos biológicos, é possível então que a identificação destas alterações mais sutis possa alertar sobre efeitos ambientais mais amplos. Desta forma, a análise de biomarcadores é considerada mais adequada para avaliações ambientais uma vez que, ao contrário de outras técnicas que apenas detectam o dano quando já existente, estas respostas sub-letais antecedem a ocorrência de danos ambientais em maior escala, possibilitando desta forma uma gestão preventiva – e não somente remediadora – da contaminação dos ecossistemas aquáticos.

Os biomarcadores podem ser divididos em três classes (WHO, 1993; NRC, 1987):

i) ***biomarcadores de exposição***: são alterações biológicas que podem ser mensuráveis e que evidenciam a exposição dos indivíduos aos contaminantes.

ii) ***biomarcadores de efeito***: inclui a mensuração de alterações bioquímicas, fisiológicas, ou de qualquer outro tipo, ocorridas em tecidos ou fluidos corporais de um organismo, e que estejam reconhecidamente associadas a um estresse real ou potencial à saúde do organismo;

iii) ***biomarcadores de susceptibilidade***: indicam a capacidade, inerente ou adquirida, de um organismo em responder à exposição a uma substância xenobiótica e/ou metal-traço específico;

A título de ilustração considera-se, por exemplo, como *biomarcadores de susceptibilidade*, alterações genéticas e modificações em receptores químicos que alterem a susceptibilidade de um organismo à exposição a substâncias xenobióticas e metais-traço. Os *biomarcadores de efeito*, por sua vez, são aqueles que estão associados a prejuízos às funções essenciais para funcionamento normal de células, tecidos e organismos, como por exemplo, danos em DNA e inibição de enzimas como a acetilcolinesterase (AChE) – diretamente associada à função neurotransmissora (THOMPSON, 1999). Por último, os *biomarcadores de exposição* em si não informam se o organismo experimentou algum efeito deletério, mas indicam sua exposição a algum tipo de estresse, e.g., a produção de metalotioneína, substância envolvida na homeostase de metais no interior das células (LIU, LIU e KLASSEN, 2001), indica a exposição do organismo a metais-traço. Da mesma forma, de acordo com a Organização Mundial de Saúde (WHO, 1993) e o Conselho Nacional de Pesquisas Estadunidense (NRC, 1987), a acumulação de certos contaminantes ambientais persistentes em tecidos de animais pode ser considerada como um biomarcador de exposição a estas substâncias.

No campo da ecotoxicologia aquática, diversos métodos para análise de biomarcadores têm sido desenvolvidos e extensivamente aplicados em diferentes organismos, principalmente em peixes e moluscos bivalves, mas também poliquetas (HANNAM *et al.*, 2008; GERACITANO, MONSERRAT e BIANCHINI, 2004), copépodos (RAISUDDIN *et al.*, 2007) e larvas de decápodos (SNYDER e MULDER, 2001, entre outros).

O amplo estudo de biomarcadores em peixes é explicado pela resposta a compostos tóxicos de maneira similar aos grandes vertebrados, podendo assim, serem utilizados para analisar potenciais carcinogênicos e teratogênicos em humanos. Além disso, os peixes estão entre os maiores veículos de transferência de contaminantes para os seres humanos (AL-SABTI e METCALFE, 1995).

Entretanto, apesar do enorme número de artigos científicos e livros sendo publicados a respeito de biomarcadores, este enfoque ainda é apenas ocasionalmente utilizado como uma ferramenta ecotoxicológica na prática do manejo ambiental (HANDY, GALLOWAY e DEPLEDGE*,* 2003, MARTIN-DÍAZ *et al.,* 2008).

Atualmente existem iniciativas de integração de biomarcadores em programas de monitoramento da poluição aquática nos EUA (e.g. *Status National and Trends Program* da NOAA), norte da Europa (*North Sea Task Force Monitoring Master Plan*) e Mediterrâneo (programa de biomonitoramento do Mar Mediterrâneo lançado pelo PNUMA). Além disso, a convenção Oslo-Paris (OSPAR), de âmbito europeu e destinada à proteção do ambiente marinho contra a poluição, recentemente incluiu a recomendação de uso de biomarcadores em seu ‘Programa de Monitoramento Conjunto’. No Brasil, a utilização de biomarcadores pelas agências ambientais ainda é incipiente, sendo raros os casos de sua aplicação em projetos regulares de avaliação e monitoramento da qualidade de sistemas aquáticos. Devido às vantagens inerentes à utilização do monitoramento de efeitos da contaminação em níveis biológicos mais baixos (sub-indivíduo), há uma necessidade de que agências ambientais brasileiras se equiparem àquelas de países da Europa e América do Norte, que já vêm utilizando alguns biomarcadores (ex.: integridade da membrana lisossômica) no monitoramento regular da qualidade de sistemas aquáticos (ALVAREZ-GUERRA *et al*., 2007).

A integração dos biomarcadores como LOE no âmbito das avaliações de “pesos de evidências” para avaliação da qualidade/risco ambiental tem sido utilizada em estudos de áreas marinhas altamente contaminadas em todo o mundo (por exemplo, mostrando várias violações das diretrizes de qualidade do sedimento) (GALLOWAY *et al*., 2004. MARTÍN-DIAZ *et al*., 2004, 2008; PEREIRA *et al*., 2012, 2014; PIVA *et al*., 2011; SOUZA, DUARTE e PIMENTEL, 2013). Na avaliação de risco em caso de risco particular, como por exemplo em derramamentos agudos e crônicos de petróleo (JIMÉNEZ-TENORIO *et al*., 2008; MORALES-CASELLES *et al*., 2008, 2009), injeção e armazenamento de CO_2 (REGUERA *et al*., 2009), infiltrações naturais de hidrocarbonetos (BENEDETTI *et al*., 2014), e avaliação de efeitos ambientais do naufrágio do navio Costa Concórdia (REGOLI *et al*., 2014).

Apesar destes estudos atestarem a utilidade dos biomarcadores no âmbito de uma avaliação de peso de evidências em ambientes marinhos, tais estudos só foram realizados em ambientes altamente impactados. Para Áreas Marinhas Protegidas (AMP), que são, em certos casos, submetidos a entradas não muito altas, porém contínua de contaminantes (PALMER, ALLAN e BUTMAN, 1996; POZO *et al*., 2009), nenhuma informação pode ser encontrada sobre a adequabilidade das ferramentas de biomarcadores integradas à avaliação baseada em pesos de evidências.

É importante considerar que, mesmo em locais onde a contaminação é moderada, uma exposição a longo prazo pode afetar a saúde dos organismos aquáticos (NIPPER *et al*., 1998). Os impactos ecológicos de efeitos subletais ainda são pouco conhecidos, uma vez que eles geralmente são mais sutis e tardam mais para se manifestar, sendo, portanto, mais difíceis de serem quantificados (BARBEE, GANIO e SWEARER, 2014). Além disso, especialmente em ambientes estuarinos, avaliações biológicas em geral são sensíveis a vários fatores interferentes, o que pode culminar em diagnósticos falso-positivos. Estuários estão sob um “permanente estresse” natural devido à variação periódica de parâmetros como salinidade, entrada de matéria orgânica, entre outros. Isto faz com que seja particularmente difícil a diferenciação entre estresse induzido naturalmente e estresse antropogênico (ELLIOTT e QUINTINO, 2007; GONÇALVES *et al*., 2013).

Particularmente, as análises de biomarcadores são sensíveis não apenas às variáveis ambientais relacionadas com a sazonalidade e eventos naturais (RODRIGUES *et al*., 2013;. VINAGRE *et al*., 2012), mas também a fatores interferentes de origem biológica, tais como infecções bacterianas e parasitárias nos organismos, variação fisiológica sazonal, restrição alimentar, idade, estado reprodutivo e variação entre indivíduos (VAN DER OOST *et al.*, 2003;. AU, 2004; SOLÉ *et al*., 2010; TOMASELLO *et al*., 2012). Em áreas moderadamente contaminadas, estes fatores interferentes podem mascarar os resultados e gerar falsos diagnósticos da qualidade/risco ambiental, fato que dificilmente ocorre em áreas altamente contaminadas, nas quais os efeitos biológicos induzidos pela poluição tendem a ser mais evidentes. Dada as vantagens que as análises de biomarcadores trazem para uma avaliação de risco/qualidade ambiental, tornam-se importantes os estudos que empreguem esta ferramenta nestes cenários, para que sejam esclarecidas suas potencialidades e minimizadas suas limitações.

Idealmente, um *framework* de Peso-de-Evidências deve ser o mais quantitativo possível; ser lógico e transparente; ser compreensível por pessoal não especializado; incorporar julgamentos sobre a qualidade, extensão e congruência das informações em cada LOE; e, construir em um vasto leque de conhecimentos interdisciplinares para abranger as ligações entre exposição e efeitos primários (BURTON Jr *et al*., 2002; CHAPMAN, MAC DONALD e LAWRENCE, 2002). O uso integrado das informações ambientais (que engloba a avaliação da exposição e efeitos, e caracterização de risco – CHAPMAN, 2007) deve permitir uma determinação clara do risco de impactos ecológicos provocados por fatores químicos ou outros, em última análise, informando e subsidiando o processo de tomada de decisão na gestão ambiental (CHAPMAN, MAC DONALD e LAWRENCE, 2002). O framework de Peso-de-Evidências também deve ser capaz de determinar se informações suficientes foram obtidas e se a diferenciação entre perigo (a possibilidade de impacto) (*hazard*) e risco (probabilidade de impacto) (*risk*) foi adequadamente realizada (CHAPMAN, MAC DONALD e LAWRENCE, 2002).

1.4 RISCO À SAÚDE HUMANA

As abordagens de “peso de evidências” também preconizam que as avaliações dos efeitos biológicos devem ser complementadas com estudos de exposição aos contaminantes. Uma forma de se avaliar a exposição é analisar os níveis de contaminantes nos tecidos de organismos potencialmente expostos. Os estudos de bioacumulação são, de fato, considerados importantes em análises de Risco Ambiental (DEN BESTEN *et al*., 2003). Este tipo de avaliação baseia-se no estudo da evolução da concentração de contaminantes em organismos e pode ser utilizado como um indicador adicional da biodisponibilidade de contaminantes. Outra importante vantagem desta Linha-de-Evidência é a possibilidade de relacionar a degradação ambiental e poluição resultado da contaminação com o risco à saúde humana.

Substâncias contaminantes podem afetar seres humanos através da contaminação de organismos aquáticos utilizados como alimento (RAJAGURU *et al*., 2002). A ingestão de água e alimentos contaminados é a forma mais comum de exposição de populações humanas a xenobióticos, sendo os peixes e crustáceos reconhecidamente os maiores veículos nessa transferência de contaminantes aos humanos. Desta forma, estudos que quantifiquem os níveis de contaminantes em tecidos de organismos normalmente consumidos por humanos são úteis, não apenas do ponto de vista de estudar riscos ecológicos associados à contaminação, mas também por indicarem o potencial risco de exposição para populações humanas a substâncias presentes no ambiente (ANKLEY *et al.*, 1986).

1.5 ESPÉCIE BIOINDICADORA

As espécies marinhas que vivem em contato com os sedimentos, e se alimentam no *habitat* bentônico, são especialmente importantes nos estudos de avaliação da qualidade dos ambientes aquáticos, tendo em vista que estes organismos ficam em contato direto com contaminantes depositados neste compartimento ambiental. Desta forma, não apenas estão mais expostos à contaminação presente nesta matriz, como também podem servir de elo para transferência destes contaminantes para outros níveis tróficos (organismos predadores, detritívoros, decompositores).

O bagre amarelo (*Cathorops spixii*) (Agassiz, 1829) (Figura 1) é uma espécie demersal e habita no estuário todo seu ciclo de vida (AZEVEDO *et al*., 1999). Apresenta hábito detritívoro e utiliza invertebrados e pequenos peixes como itens principais de sua dieta (FISHBASE, 2006). Esta espécie tem sido considerada um importante recurso para os pescadores tradicionais da costa sulamericana tropical e subtropical do Atlântico (REIS, 1986; MELO e TEIXEIRA, 1992; ALVAREZ-LEÓN e REY-CARRASCO, 2003), e é amplamente consumido pela população no local de estudo.

FIGURA 1- *Cathorops spixii* COLETADO NA ÁREA DE PROTEÇÃO AMBIENTAL DE CANANÉIA-IGUAPE-PERUÍBE. FONTE: O autor (2014)

1.6 ÁREA DE PROTEÇÃO AMBIENTAL DE CANANÉIA-IGUAPE-PERUÍBE

A Área de Proteção Ambiental de Cananéia-Iguape-Peruíbe (conhecida como a APA-CIP) (latitudes 24^{o}40S e 25^{o}05S) é um ecossistema estuarino-lagunar reconhecido pela Unesco como parte da Reserva da Biosfera da Mata Atlântica, devido à sua relevância para conservação ambiental. Desde 2000, a região tem sido parte da lista global de Sítios do Patrimônio Mundial da UNESCO. Além disso, a APA-CIP é considerada uma área prioritária para futura inclusão na lista das zonas úmidas de importância internacional do Brasil no âmbito da Convenção de Ramsar (BRASIL, 2012).

O principal contribuinte de água doce para o estuário do CIP é o rio Ribeira de Iguape. Aproximadamente 70% do curso do rio flui em direção às águas do complexo lagunar através de um canal artificial, conhecido localmente como Canal do Valo Grande. A bacia do rio Ribeira de Iguape é uma província metalogênica com depósitos de Pb e Zn naturais (MORAES *et al*., 2003). A atividade de mineração foi intensamente realizada nesta área ao longo do século XX até a década de 1990, quando as minas foram fechadas. Desde então, altos níveis de metais (Pb, Zn, Cu, Cr) e arsênio (As) foram registrados nas águas do rio Ribeira de Iguape, bem como em sedimentos de corrente e em suspensão (EYSINK *et al.,* 1998;. CORSI e LANDIN, 2003; MORAES *et al*., 2003; GUIMARÃES E SÍGOLO, 2008; ABESSA *et al.,* 2014).

No complexo Estuarino-Lagunar da APA-CIP, os níveis de metais nos sedimentos que costumavam ser baixos, aumentaram substancialmente após a construção do Canal do Valo Grande (MAHIQUES *et al*., 2009). Apesar deste incremento de metais nos sedimentos estuarinos, estes contaminantes foram encontrados em níveis apenas moderados (AZEVEDO *et al.*, 2011; CRUZ *et al*., 2014), segundo os valores-guia internacionais de qualidade de sedimento (LONG e CHAPMAN, 1995; *ENVIRONMENT CANADA E MINISTÈRE DU DÉVELOPPEMENT DURÁVEL, DE L'ENVIRONNEMENT ET DES PARCS DU QUÉBEC*, 2005).

A contaminação por metais através de atividades de mineração tem sido uma grande preocupação ambiental em escala global e uma das mais graves ameaças para o ambiente aquático em muitos países (ZHUANG *et al*., 2014; KROLL *et al*., 2005). Esta preocupação é, em parte, porque as atividades de mineração comumente têm sido realizadas de forma não controlada (RYBICKA, 1996). Além disso, os resíduos e rejeitos podem afetar gravemente o entorno das minas (FERNÁNDEZ-CALIANI *et al*., 2008), uma vez que os poluentes são propensos a serem transportados através da água (por exemplo, a drenagem ácida de minas) e do ar (deposição atmosférica, partículas levadas pelos ventos), podendo-se acumular em diferentes compartimentos ambientais. Metais das atividades de mineração podem, portanto, direta ou indiretamente, afetar a biota e seres humanos (e.g. MOLINA-VILLALBA *et al*., 2014; TAYLOR *et al*., 2014; CAMIZULI *et al*., 2014: RIBA *et al*., 2005).

Além da contaminação proveniente do alto Rio Ribeira de Iguape, a APA-CIP também abrange três cidades (Iguape, 30.259 hab; Ilha Comprida, 9.025 hab.; Cananéia, 12.601 hab.) (IBGE, 2014), as quais não possuem infraestrutura de saneamento adequado. Os resíduos são descartados diretamente nos rios ou no sistema lagunar, pois o tratamento é insuficiente para a demanda local (MORAIS e ABESSA, 2014). Um estudo recente mostrou que, na APA-CIP, as áreas urbanas constituem fontes potenciais de poluição para os sedimentos (CRUZ *et al*., 2014).

2. HIPÓTESES

A avaliação do desempenho das AMP é fundamental, não só para a própria proteção da biodiversidade, mas também por conta de que o fracasso da AMP pode minar o apoio público e político para a conservação (MORA e SALE 2011). Desta forma, no presente estudo foram testadas duas hipóteses, a primeira metodológica e a segunda relacionada a qualidade ambiental da APA-CIP, conforme apresentado a seguir:

i) As análises com biomarcadores, integradas no contexto de uma avaliação clássica de “peso de evidências” para avaliação da qualidade do sedimento (testes de toxicidade de sedimentos e propriedades físico-químicas de sedimentos), são adequadas para avaliar ou monitorar a qualidade ambiental dos ecossistemas aquáticos costeiros moderadamente contaminados, como geralmente encontrado em Áreas Marinhas Protegidas.

ii) A Área de Proteção Ambiental de Cananéia-Iguape-Peruíbe (APA-CIP) está sujeita a aportes de metais e hidrocarbonetos policíclicos aromáticos (HPA) provenientes do Rio Ribeira de Iguape, podendo haver acumulação nos diferentes compartimentos ecológicos (ex.: sedimentos e biota) e produção de efeitos adversos sobre a biota e seres humanos.

3. OBJETIVOS

Neste contexto, esta tese foi dividida em 4 capítulos em forma de artigo científico.

O **primeiro artigo** investiga o papel de um conjunto de biomarcadores (respostas antioxidantes, danos em DNA, peroxidação lipídica) avaliados em diferentes órgãos (fígado, rim e brânquias) e integrados aos níveis de contaminantes em tecidos (metais e arsênio) e metabólitos de HPA em bile, em uma espécie de bagre residente na APA-CIP (*Cathorops spixii*) a fim de avaliar a qualidade ambiental de uma Área Marinha Protegida submetida a níveis moderados de contaminação.

No **segundo artigo** se buscou avaliar se os níveis de contaminantes nos tecidos poderiam estar relacionados a efeitos genotóxicos nos indivíduos residentes na APA-CIP através de diferentes ferramentas de análise de respostas genotóxicas, tanto em nível molecular como em nível celular.

No **terceiro artigo,** o objetivo foi avaliar as correspondências, complementaridades ou conflitos entre as várias linhas de evidência de qualidade de sedimentos, incluindo análise de biomarcadores e linhas-de-evidência “clássicas”, através de uma abordagem de “peso de evidências” para a avaliação da qualidade do sedimento em uma área protegida moderadamente contaminada (APA-CIP). O estudo incluiu a avaliação da exposição (níveis de contaminantes nos sedimentos e em peixes residentes, além de biomarcadores específicos e não-específicos de exposição), avaliação de efeitos (biomarcadores de efeitos e estado geral de saúde de peixes residentes, testes de toxicidade de sedimentos) e caracterização da qualidade ambiental (integração das LOEs individuais por meio de análise multivariada).

O **quarto artigo** estima o risco potencial de metais (Cu, Mn, Zn, Cr, Ni, Cd, Pb) e arsênio (As) para a saúde humana através da ingestão de peixes coletados na Área Marinha Protegida de Cananéia-Iguape-Peruíbe. Desta forma, os níveis de metais e As dosados no tecido muscular (parte comestível) de *C. spixii* foram primeiramente comparados com os Níveis de Ação nacionais e internacionais sobre o consumo humano. A avaliação do risco humano foi mais detalhada através da estimativa do “Quociente de Perigo” (Target Hazard Quotient - THQ) para metais e As, e o risco de câncer a partir da exposição ao As (USEPA, 2000).

CAPÍTULO I

Submetido para "Environmental Science and Pollution Research"

Assessing Pollution in Marine Protected Areas: The role of a multi-biomarker and multi-organ approach

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ABSTRACT

Marine Protected Areas (MPAs) are vulnerable to many anthropic pressures, including pollution. However, environmental quality monitoring in these areas traditionally relies on only water chemistry and microbiological parameters. The goal of the current study was to investigate the role of a set of biomarkers (GPx, GST, GSH, DNA damage, LPO, AChE and condition index) in different target organs of fish (liver, kidney and gills) and during different seasons, in order to assess the environmental quality of an MPA. Chemical analyses were also performed on liver and muscle tissues to evaluate metal body burdens, and PAHs were identified in bile. A demersal fish (*Cathorops spixii*) that is widely consumed by the local population was used as bioindicator species, and the results were integrated using multivariate analysis. The use of the biomarker approach allowed for the identification of both seasonal and spatial variations in pollution sources around the Environmental Protected Area of Cananéia-Iguape-Peruíbe (APA-CIP). Higher metal body burdens associated with biological responses were found in the sites under the influence of urban areas during the dry season, and they were found in the sites under the influence of the Ribeira de Iguape River (RIR) during the rainy season. The liver was found to be more responsive in terms of its antioxidant responses, whereas gills were found to be more responsive to biomarkers of effect. These results show that this set of biomarker analyses in different organs of fish is a useful tool for assessing chemical pollution in an MPA.

Keywords: Environmental monitoring; Contamination; Metal body burdens; PAHs metabolites in bile; Oxidative stress; Neurotoxicity; Genotoxicity; WOE approach

1. INTRODUCTION

Marine protected areas (MPAs) have been established to preserve the marine environment (Scherl et al. 2006). The rationale behind the MPA concept is that, by reducing habitat loss and mortality due to anthropogenic pressures, individuals can survive longer and produce more offspring, and populations can grow. However, MPAs are usually planned, created and managed following same concepts and theories historically used for terrestrial protected areas, despite the substantial differences in ecosystem processes, historical perceptions and regulatory frameworks between marine and terrestrial environments (Houde 2001). For example, marine populations, communities, and ecosystems are all connected to a broader landscape and seascape: holoplanktonic organisms, the early life stages of several species, diadromous fish, many forage fish species, and highly migratory species can all travel for long distances to settle, spawn, feed, or nurse (UNCLOS 1982; Carr et al. 2003).

Furthermore, water rarely respects man-made boundaries, and contaminants may be introduced into an MPA from adjacent areas (Palmer et al. 1996; Boersma et al. 1999; Pozo et al. 2009). It is widely accepted that MPAs are vulnerable to pollution, as well as to fishing, habitat alteration, and climate change (Cicin-Sain and Belfiore 2005; Keller et al. 2009).

Most biological assessments carried out in MPAs consider only direct or indirect effects of fishing on the biota (refer to García-Charton et al. 2000 and Fraschetti et al. 2002 for reviews). Still, pollution studies in MPAs usually focus on measuring contaminant levels in environmental matrices such as water, sediments, and organisms (e.g. Michel et al. 2001; Conti and Cecchetti 2003; Chou et al. 2004; Pozo et al. 2009; Perra et al. 2011; King et al. 2013; García-Alvarez et al. 2014) rather than the effects of pollutants on the biota that is supposed to be protected (e.g. Terlizzi et al. 2004; Pinsino et al. 2008; Rodrigues et al. 2013; Araujo et al. 2013; Cruz et al., 2014)

The knowledge of the chemical concentrations in a given environment provides important information on the risks of pollution. However, chemical data alone is not capable of providing information on biological effects, since the bioavailability and toxicity of chemicals in complex mixtures may be altered as a result of both contaminant synergies (Beyer et al. 2014) and interactions between contaminants and environmental conditions (Chapman and Wang 2001). Thus, the use of ecotoxicological tools (i.e., biological-based assessment tools) is imperative in order to directly assess the health of aquatic organisms within an MPA, since these tools shift the focus of the assessment from the agents (contaminants) to the targets (biological/ecological responses).

Effects of pollution are particularly difficult to assess in MPAs, since such areas are usually subjected to low to moderate levels of contamination and biological or ecological responses are therefore not as evident (Choueri et al. 2009). Under these unfavorable conditions, effects may not necessarily be lethal, but the conditions can deteriorate the health status of the biota and can affect populations in the long term. Sensitive biological responses are potentially suitable tools for assessing or monitoring the environmental quality of mildly contaminated MPAs.

Biochemical and cellular responses measured in organisms' tissues (i.e., biomarkers) can determine the health status of an individual and thus aid in the detection of the first signs of injury caused by pollutants. Biomarkers combined with metal body burdens of resident organisms have been widely used in aquatic pollution monitoring (Van der Oost et al. 2003; Au 2004), though most biomarker-based environmental quality studies were performed exclusively in highly anthropized sites (Malins et al. 2006; Ramos-Gómez et al. 2011; Ben Ameur et al. 2012; Maranho et al. 2013; Pereira et al., 2014). We hypothesize that biomarkers are adequate for assessing or monitoring the environmental quality of MPAs.

The evaluation of MPA performance is a critical issue, not only for the protection biodiversity itself, but also because the failure of MPAs could erode public and political support for conservation (Mora and Sale 2011). The goal of the current study was to investigate the role of a set of biomarkers (antioxidant responses, DNA damage, lipid peroxidation, metal body burdens in different organs and total PAHs in bile) in fish in order to assess environmental quality of an MPA subjected to moderate levels of contamination. To achieve that, a demersal fish (*Cathorops spixii*) that is widely consumed by the local population was used as a bioindicator species.

2. MATERIALS AND METHODS

2.1 Study Area

The Cananéia-Iguape-Peruíbe Environmental Protected Area (known as the APA-CIP) (24^{o}40'S and 25^{o}05'S) is an estuarine-lagoon ecosystem recognized by UNESCO as part of the Biosphere Reserve of the Atlantic Rainforest due to its relevance for environmental conservation. Since 2000, the region has been part of the global list of UNESCO's World Heritage Sites; in addition, the APA-CIP is considered a area of priority for future inclusion on the list of Brazilian wetlands of international importance within the scope of the Ramsar Convention (Brazil 2012).

Two well-defined climate seasons dominate in this region: a drier winter and a rainier summer, with minimum precipitation rates occurring from July to August (monthly average of 95.3mm) and maximum rates from January to March (monthly average of 266.9mm). Monthly mean temperatures range from a maximum of 28 ºC (February) to a minimum of 20 ºC (July). Tides are semidiurnal, and mean tidal amplitude is 0.82m (Cunha-Lignon et al. 2009).

The main freshwater contributor to the CIP estuary is the Ribeira de Iguape River (RIR). Approximately 70% of the course of the river flows toward the lagoon waters through an artificial channel known locally as Valo Grande. The river basin is a metallogenic province with natural Pb and Zn deposits (Moraes et al. 2004). Mining activities were performed in this area for many decades during the 20th century, but the mines were closed down in the 1990s. Since then, high levels of metals (Pb, Zn, Cu, Cr) and arsenic (As) have been recorded in the river waters, as well as in both bottom and suspended sediments (Eysink et al. 1998; Corsi and Landin 2003; Moraes et al. 2004; Guimarães and Sígolo 2008). In the estuarine sediments, metals were found at only moderate levels (Mahiques et al. 2009) as defined by the international Sediment Quality Guidelines (Long et al. 1995; Environment Canada and *Ministère du Développement durable, de l'Environnement et des Parcs du Québec*, 2007).

Apart from its important natural features, the APA-CIP also encompasses three cities (Iguape, 30,259 inhab.; Ilha Comprida, 9,025 inhab.; Cananéia, 12,601 inhab.) (IBGE 2014) that lack proper sanitation infrastructure. Waste is discharged in rivers, in groundwater, or directly into the lagoon system, since treatment is insufficient for local demand (Morais and Abessa 2014). Cruz et al. (2014) showed that the superficial runoff from the cities located in the vicinities of the APA-CIP may constitute a source of contaminants to the estuarine sediments.

2.2 Fish Collection and Sample Preparation

The madamango sea catfish (*Cathorops spixii*) is a demersal fish that lives in a wide salinity range and preys mainly upon zoobenthos (crustaceans and polychaetes in particular) and small fishes (Fishbase, 2014). Adult individuals migrate from coastal zones to lower reaches of estuaries to spawn and the early juvenile development occur in bays and estuaries (Araújo 1988). In addition to its ecological relevance, this species is of socioeconomic interest, as it is widely consumed by the local population (Fávaro et al. 2005).

C. spixii was collected using a bottom otter trawl (2 minutes trawling) and fifteen specimens with homogeneous length were kept for the analyses at each sampling site (Figure 1). Five animals were set aside for metal body burden analyses and ten specimens were set aside for biomarker analyses. To account for seasonal variation, individuals were collected during three seasons with different amounts of rainfall: (i) the partially dry season (P) (May 2012); (ii) the dry season (D) (August 2012); and (iii) the rainy season (R) (March 2013). The average rainfall during these seasons was 192mm, 111mm and 390mm, respectively (CEPAGRI, 2014). In the first sampling campaign (during the partially dry season), four sampling stations distributed along the APA-CIP area (P2 to P5) were set up. In the subsequent campaigns, two additional sampling stations were included (P1 and P6), in order to enable a better understanding of the influence of important contaminants sources in APA-CIP. The scope of metals body burdens analyses in *C. spixii* was enlarged as well. Metals analyses was limited to axial muscle in the first sampling campaign, but liver was included in the subsequent campaigns (dry and rainy seasons). Before dissection, the collected specimens were kept in local water, under aeration until transportation to the laboratory.

Before euthanized by spinal cord section, individuals were anesthetized with benzocaine in water, then weighted and measured. Fish gills, kidney, liver, bile and axial muscle tissues were dissected, frozen and stored at -80 °C until biochemical analyses. Axial muscle and liver tissues used in metal body burden analyses were stored in plastic vessels at -20ºC.

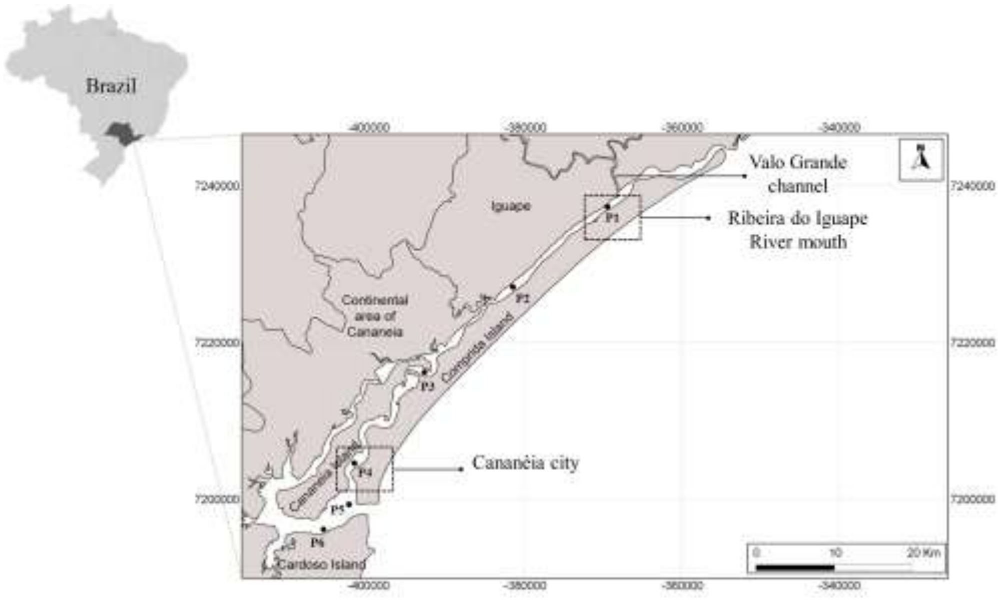


Fig. 1 Sampling stations located within the APA-CIP, Brazil

2.3 Condition Factor

Fulton's condition factor was calculated according to the formula: KF = (W / L^3 x 100), where KF = Fulton condition factor, W = body weight in grams, and L = total body length in cm.

2.4 Determining Biomarkers

Gills, kidney, liver, and muscle tissues were kept on ice and homogenized at 10% W/V in Tris-HCl buffer (TRIS 50mM; EDTA 1mM; DTT 1mM; Surose 50mM; KCl 150mM; PMSF 1mM, pH 7.6). Homogenates were centrifuged at 10.000×g for 20 min at 4 °C, and in in the case of the liver, gills, and kidney, aliquots of the supernatants were kept for the analyses of Glutathione S-transferase (GST) and Glutathione peroxidase (GPx) activities, as well as for the quantification of non-protein reduced thiols (GSH), lipid peroxidation (LPO), and DNA strand breaks. In the case of the muscle tissue, supernatants were used for acetylcholinesterase (AChE) activity analyses. Liver, kidney, and gill tissue samples were also set aside for metallothionein-like protein (MTs) activity analysis, homogenized with 20mM Tris–HCl buffer supplemented with 0.5M sucrose, 0.01% β-mercaptoethanol, and centrifuged at 15.000×g for 30min at 4 °C.

GST (Keen et al. 1976) and GPx activities (Sies et al. 1979) were determined spectrophotometrically at 340nm. GSH levels were measured spectrophotometrically at 415nm (Sedlak and Lindsay 1968). AChE activity analysis was performed at 415nm using the colorimetric method by Ellman et al. (1961). The concentration of metallothionein-like protein (MTs) was established based on cysteine residue titration of a partially purified MT extract, and the concentration was quantified using Ellman's reagent containing DTNB at 412nm (Viarengo et al. 1997).

Levels of lipid peroxidation (LPO) were determined by quantifying the concentration of 2-thiobarbituric acid reactive substrates (TBARS) through fluorescence (λex 532nm and λem 556nm) (Wills et al. 1987). DNA strand breaks were measured using an alkaline precipitation assay (Olive 1988; Gagné and Blaise 1995). The assay is based on the potassium-dodecylsulphate precipitation of protein-bound genomic DNA, which leaves protein-free DNA strand breaks in the supernatant. These DNA strands are quantified using fluorescence (λex 360nm and λem 450nm) after staining with Hoescht dye. Standard solutions of salmon sperm DNA were used for calibration. Protein concentrations were determined spectrophotometrically at 595nm (Bradford 1976), with BSA as the standard. All biomarker analyses were performed in a microplate reader (Biotek-Synergy™ HT).

2.5 Chemical Analyses

2.5.1 Metal Body Burden

Concentrations of As in muscle and liver tissues were determined using an atomic absorption spectrophotometer (Varian®, AA 240Z) equipped with a graphite furnace (AAS-GF) (Model, GTA 120). Metals were quantified using flame atomic absorption spectroscopy (FAAS) (Varian®, AA 240FS). All analyses were performed according to standard method 200.9 (USEPA 1994). Detection limits for As were 5.88 µg kg^{-1} and detection limits for metals (Cu, Mn, Zn, Cr, Co, Ni, Cd, Pb) were 0.034 mg kg^{-1}, 0.0697 mg kg^{-1}, 0.0525 mg kg^{-1}, 0.112 mg kg^{-1}, 0.146 mg kg^{-1}, 0.0623 mg kg^{-1}, 0.042 mg kg^{-1}, and 0.0602 mg kg^{-1}, respectively. Standard curves were prepared using reference material (Qhemis High Purity®). Recovery rates ranged from 80% to 120% in all investigations. Metal concentrations were expressed in mg kg^{-1} dry weight.

2.5.2 Polycyclic Aromatic Hydrocarbons in Bile

The metabolites of polycyclic aromatic hydrocarbons (PAHs) in the bile of *C. spixii* were quantified via fixed-wavelength fluorescence in the spectrofluorometer (Sunrise-Tecan) at wavelengths of 288/330 nm, 334/376 nm, 364/406 nm, and 380/422 nm (λex/λem), which correspond respectively to naphthalene-type (2 rings), pyrene-type (4 rings), benzo(a)pyrene-type (5 rings), and benzo(ghi)perylene-type (6 rings) (Aas et al. 2000; Oliveira Ribeiro et al. 2005). PAH concentrations were determined through a comparison with a standard curve for each group of rings. The results were expressed as units of PAH mg $prot^{-1}$.

2.6 Statistical Analyses

First, biomarker data and total PAH contents in bile were tested for normality (Kolmogorov-Smirnov's test) and homoscedasticity (Bartlett's method). The statistical differences of mean values of each data series (n=10) that met Analysis of Variance (ANOVA) assumptions were tested through ANOVA followed by a post hoc Tukey's test. Non-parametric statistical tests (Kruskal-Wallis test, with Dunn's multiple comparisons as post-test) were used to compare data series that violated ANOVA assumptions. The significance level was set at p=0.05

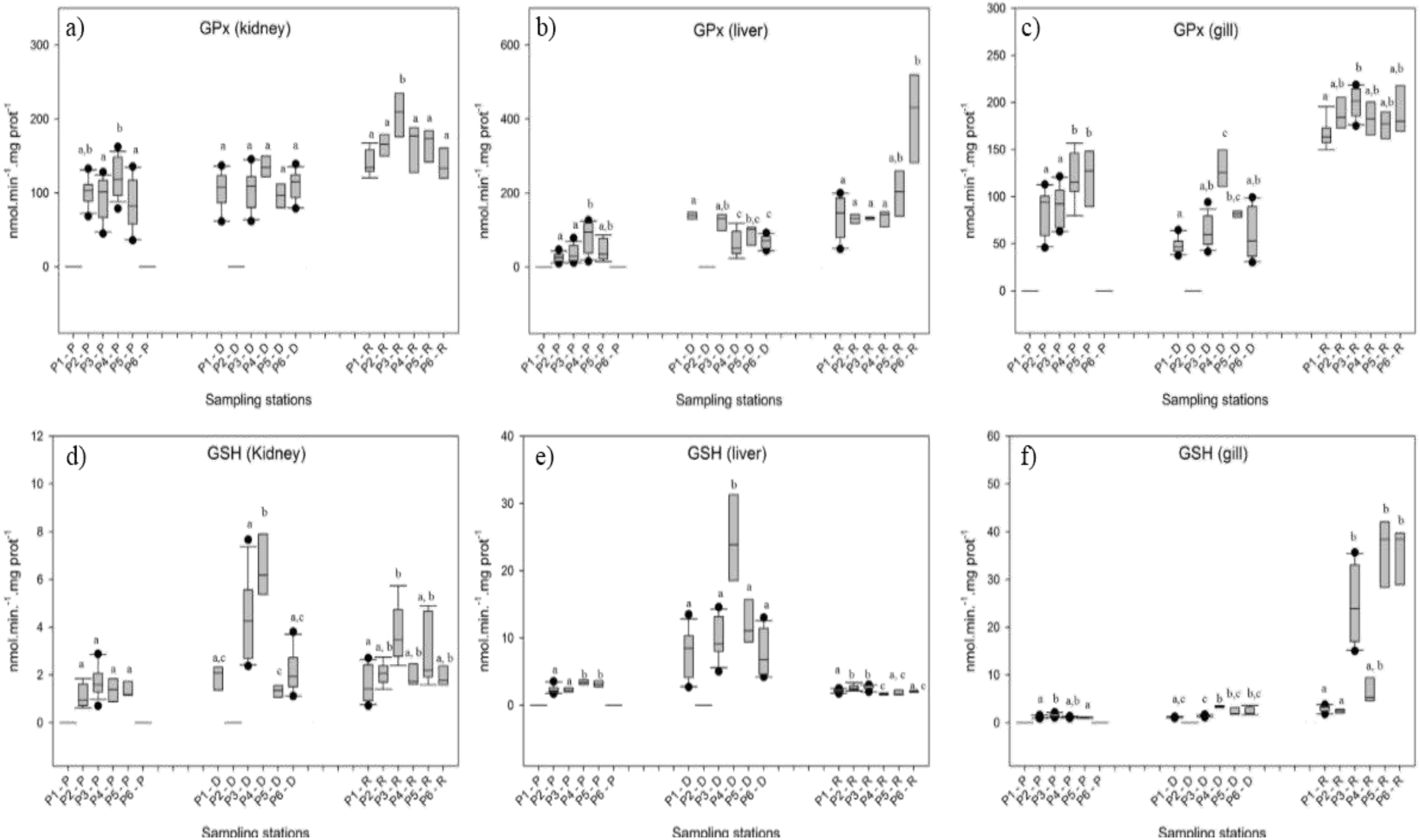
Factor analysis (with principal component analysis as the extraction method) (FA/PCA) was used to highlight associations among the variables investigated in this study during each of the three seasons (partially dry, dry, and rainy). Associations between the different biomarkers (GST, GSH, GPX, MTs, LPO, DNA damage, and AChE), metal loads (Cu, Mn, Zn, Cr, Co, Ni, Cd, Pb) and As loads in liver and muscle tissues, and total PAH metabolites in bile were assessed. The variables that failed to present significant variation among sampling stations were removed from the original datasets. The variables were autoscaled (standardized) so as to be treated with equal importance. The selected variables to be interpreted were those associated with the factors with a loading ≥0.50, a value which is more conservative than the loading cut-off recommended by Tabachnic and Fidell (1996). The relevance of the observed associations to each of the 6 sampling stations (cases) was estimated by calculating the factor score from each case for the centroid of all cases for the original data. All the statistical and multivariate analyses were performed using the STATISTICA 12 software (StatSoft Inc. USA).

3. RESULTS

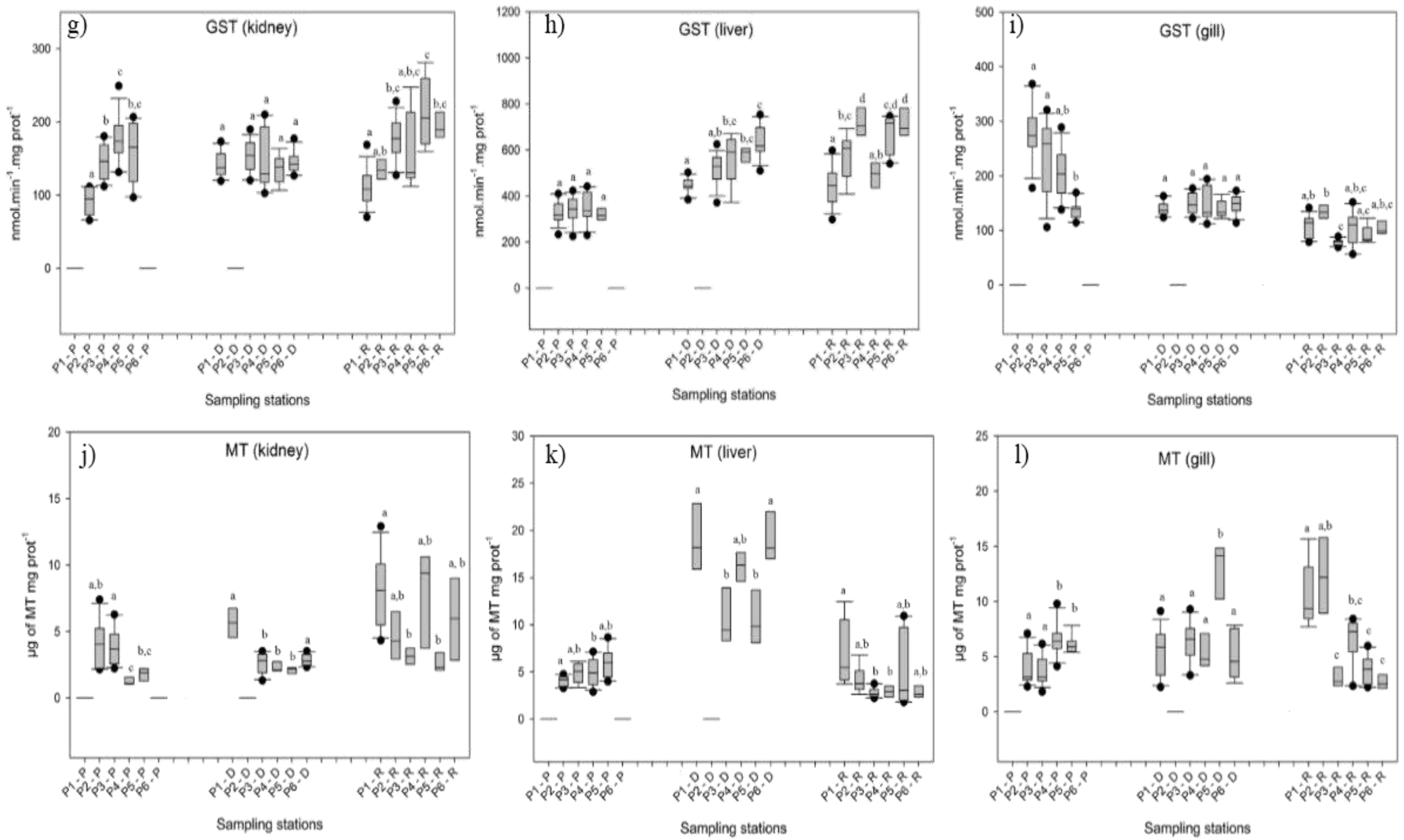
The responses of the biomarkers of exposure found in the liver and gills of *C. spixii* specimens collected along the APA-CIP revealed both seasonal and spatial variations (Figures 2a to 2l). During the periods of lower rainfall (dry, partially dry), biomarkers of exposure (MT, GST, GPX and GSH) presented significantly higher values among the fish collected at the estuarine sections influenced by the city of Cananéia (P4, P5 and P6); during the rainy season, these responses are more pronounced in fish sampled from the region under the influence of the RIR (P1, P2, P3).

Biomarkers of effect (LPO, DNA damage, and AChE) found in *C. spixii* liver tissue also followed these seasonal and spatial variations (Figures 3a to 3g). In addition, liver genotoxicity responses were higher in specimens sampled during the dry and partially dry seasons in the stations under the influence of Cananéia (P4, P5, P6); during the rainy season, LPO levels in the liver were higher ($p<0.05$, ANOVA). AChE activity was inhibited, particularly in the stations under the influence of the RIR ($p<0.05$, ANOVA). Responses of biomarkers found in kidney tissue generally followed a pattern of response based on the sample’s proximity to the two main sources of contamination (the RIR and Cananéia).

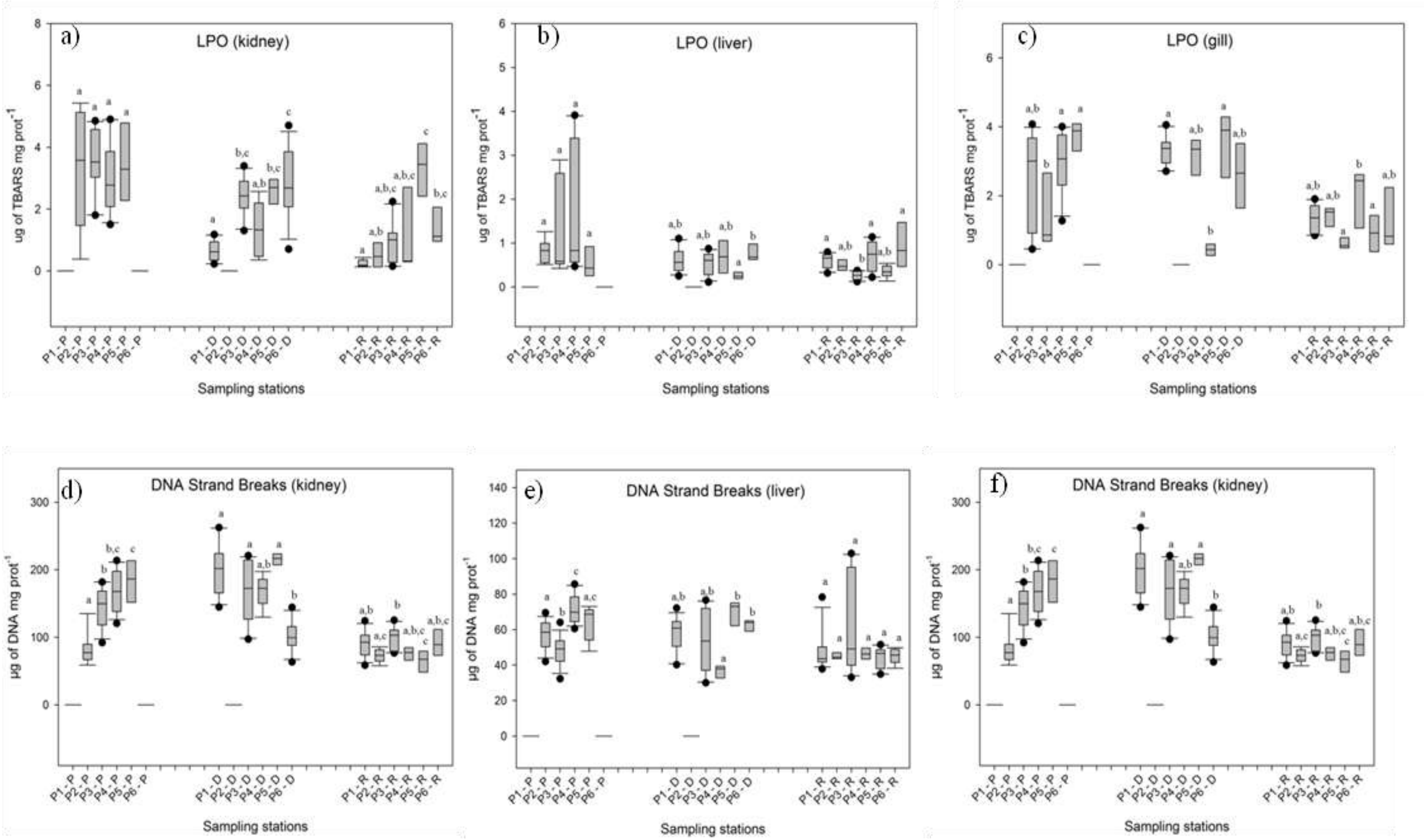
Fulton’s condition factor results (Figure 3h) showed that specimens from P1 (during both the dry season and the rainy season) and P2 (during the rainy season) were more well nourished ($p<0.05$, ANOVA). No spatial differences were observed in the partially dry season ($p>0.05$, ANOVA).



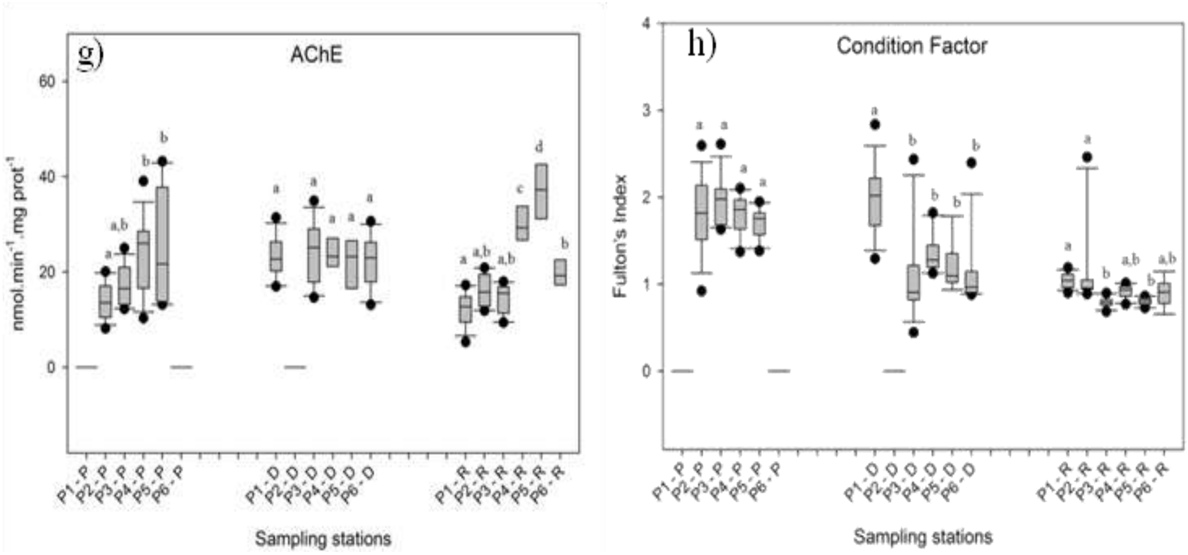
Figs. 2a to 2f Responses of biomarkers of exposure in kidney, liver, and gill tissues from *Cathorops spixii* from the APA-CIP collected during the partially dry season (P), the dry season (D), and the rainy season (R). The site with a line instead of a boxplot means that no organisms were sampled. Data are presented as boxes with boundaries that indicate 25th and 75th percentiles; a line within the box marks the median value; whiskers below and above the box indicate 10th and 90th percentiles; the outliers are presented with black dots. The use of different letters above the data indicates significant differences during the same season (p = 0.05)



Figs. 2g to 2l Responses of biomarkers of exposure in kidney, liver, and gill tissues from *Cathorops spixii* from the APA-CIP collected during the partially dry season (P), the dry season (D), and the rainy season (R). The site with a line instead of a boxplot means that no organisms were sampled. Data are presented as boxes with boundaries that indicate 25th and 75th percentiles; a line within the box marks the median value; whiskers below and above the box indicate 10th and 90th percentiles; the outliers are presented with black dots. The use of different letters above the data indicates significant differences during the same season (p = 0.05)



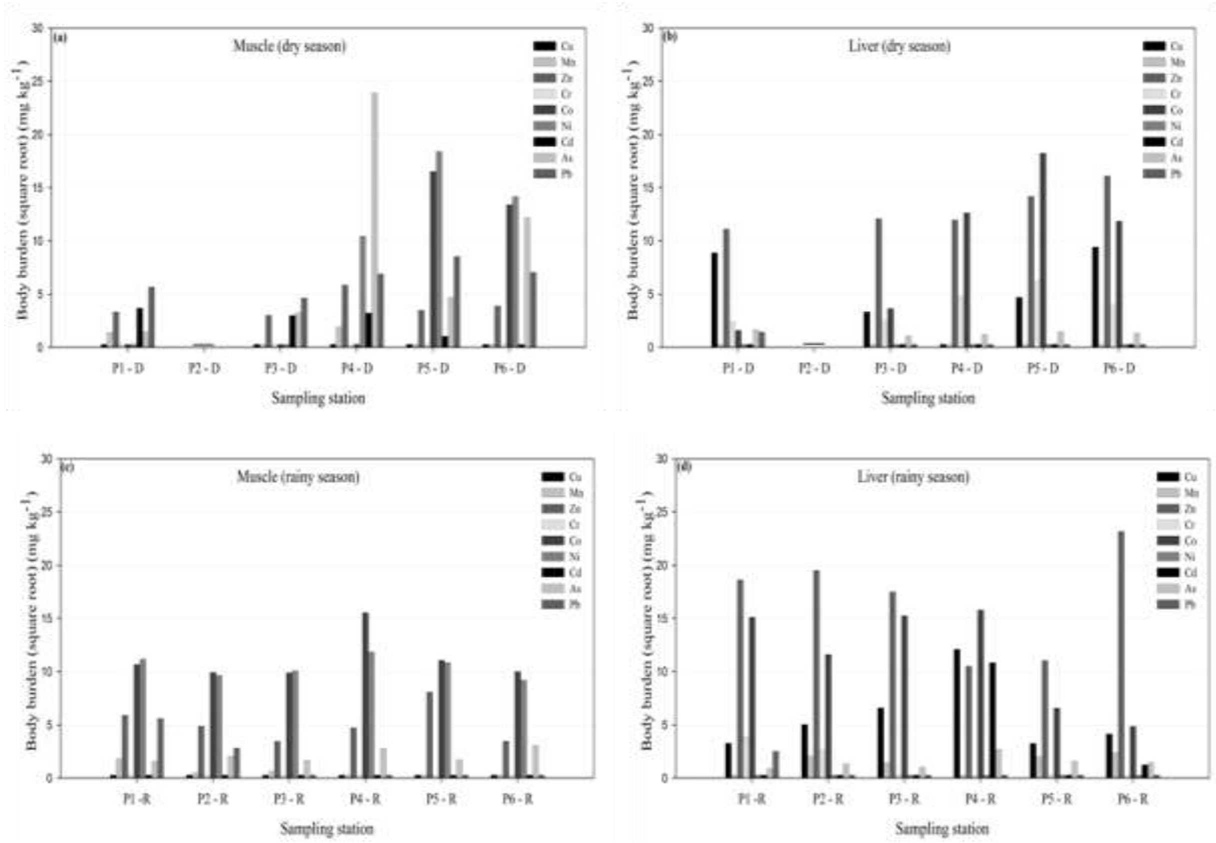
Figs. 3a to 3f Responses of biomarkers of effects in kidney, liver, and gill tissues from *Cathorops spixii* from the APA-CIP collected during the partially dry season (P), the dry season (D), and the rainy season (R). The site with a line instead of a boxplot means that no organisms were sampled. Data are presented as boxes with boundaries that indicate 25th and 75th percentiles; a line within the box marks the median value; whiskers below and above the box indicate 10th and 90th percentiles; the outliers are presented with black dots. The use of different letters above the data indicates significant differences during the same season (p = 0.05)



Figs. 3g and 3h Responses of biomarkers of effects in muscle tissues and condition factor from *Cathorops spixii* from the APA-CIP collected during the partially dry season (P), the dry season (D), and the rainy season (R). The site with a line instead of a boxplot means that no organisms were sampled. Data are presented as boxes with boundaries that indicate 25th and 75th percentiles; a line within the box marks the median value; whiskers below and above the box indicate 10th and 90th percentiles; the outliers are presented with black dots. The use of different letters above the data indicates significant differences during the same season (p = 0.05)

Biological responses were consistent with As and metal concentrations in liver and muscle tissues of *C. spixii*. (Figures 4a to 4d). In general, As, Mn, Zn, Cd, and Pb concentrations in muscle were higher in stations closer to the city (P4, P5 and P6) during the dry season and decreased during the rainy season. The opposite was found in analyses of Co and Ni levels in specimens from the station closest to the city (P4): levels were higher during the rainy season. A similar pattern was found in the analyses of metals in liver tissue: in general, metal concentrations in the dry season tended to be elevated in stations closer to the outskirts of the city (P5 and P6), although the station closest to the city (P4) showed higher levels of As, Cu, Co, and Cd during the rainy season compared to the dry season. In analyses of muscle and liver tissues from specimens collected closer to the RIR, higher metal concentrations (Mn, Zn, Co, Cr, Ni) were found during the rainy season than during the dry season

(Figures 4a to 4d). Exceptions were only Cd in muscle, and Cu and As in the liver, of which higher concentrations were found during the dry season.



Figs. 4a to 4d Metal body burdens (muscle and liver) from *Cathorops spixii* sampled in the APA-CIP during both the dry season, and the rainy season. Data were transformed (square root) for better visualization.

Total PAH metabolites in bile also showed a seasonal trend, with higher concentrations reported during the rainy season ($p<0.05$, ANOVA) (Figure 5) for stations under the influence of both the river and the city. During the dry season, there was an increase in total PAH metabolites in bile in specimens from P3 and P4 compared to the other sampling stations, whereas during the rainy season, specimens from P1 and P4 presented higher levels.

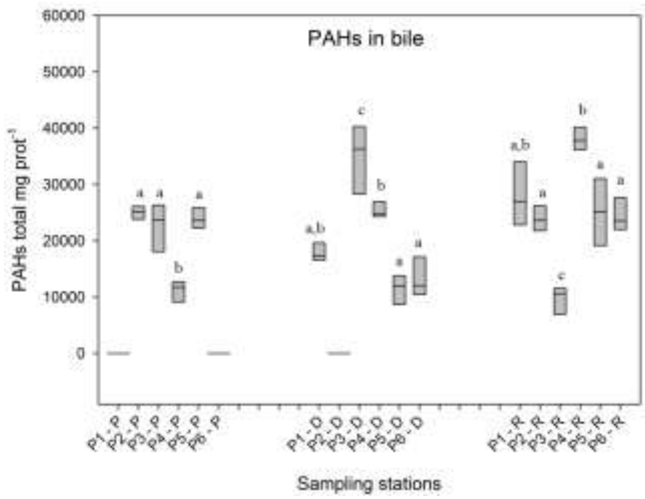


Fig. 5 Total PAH metabolites in the bile of *Cathorops spixii* sampled in the APA-CIP during the partially dry season (P), the dry season (D), and the rainy season (R). The site with a line instead of a boxplot means that no organisms were sampled. Data are presented as boxes with boundaries that indicate 25th and 75th percentiles; a line within the box marks the median value. The use of different letters above the data indicates significant differences during the same season (p = 0.05)

Three different datasets were used to perform FA/PCA: samples from the (i) partially dry, (ii) dry, and (iii) rainy seasons. Total explained variance of each FA/PCA was 100% for the partially dry season dataset, 87.9% for the dry season dataset, and 93.4% for the rainy season dataset.

Three factors were extracted from the original dataset of the partially dry season. The first factor (F1, which explained 62.2% of the variance) (Table 1) reflected contaminants (Cd and As) found in muscle and associated with MTs in the gills and liver tissues, several antioxidant responses in different organs, and DNA damage in the renal cells. Factor score analysis showed that these associations were more relevant (i.e., presented higher scores) in specimens from stations under the influence of the urban area (P4 and P5) (Table 2). Also in F1, another group of associated variables (with negative values) included Pb in muscles, PAH metabolites in bile, MT levels in kidneys, genotoxicity in gills, and neurotoxicity (AChE inhibition).

This group of variables was found to have the highest factor score for P2, which is the site that is under the greatest influence of the RIR. The second factor (F2, 25.1% of explained variance) related, in one group, metals in muscle (Pb and Zn) and antioxidant response in liver and kidney tissues (GPx) (with the highest factor score estimated for P4), and, in another group, levels of Mn were found to be related to MTs in the liver (highest score for P5). The third factor (F3, 12.7% of explained variance) associated As, Mn, and Zn levels in muscle tissues, MTs, oxidative damage and antioxidant response in the gills, in addition to an indication of antioxidant activity and genotoxicity in the liver. This factor shows the highest score for P5, followed by P2 (Table 2). Another group of variables associated with F3 included antioxidants and genotoxic responses in the gills, without, however, any relationship with the contaminants quantified in organisms. These associations are relevant for P3.

Table 1 Loadings of each of the sampling stations in Partially dry; Dry and Rainy seasons evaluated in the APA-CIP after varimax rotation for the three factors obtained in the PCA[a]. The variance of the principal factors is given in percentage of the total variance in the original data matrix. NM is non mesured.

	Partially dry season			Dry season			Rainy season			
Factors	**F1**	**F2**	**F3**	**F1**	**F2**	**F3**	**F1**	**F2**	**F3**	**F4**
Variance	62.2%	25.1%	12.7%	37.8%	35.0%	15.1%	36.9%	27.1%	15.7%	13.8%
KIDNEY										
MTs	-0.83	-	-	-0.53	-	0.74	-	-0.66	0.52	-
GPx	-	0.97	-	-	-	-	-	-	-0.83	-
GSH	-	-	-	0.87	-	-	-0.50	-	-0.72	-
GST	0.99	-	-	-	-	-	-0.81	0.50	-	-
LPO	-	-	-	-	-	0.83	-	-	-	0.72
DNA dam	0.97	-	-	-	0.61	-0.73	-	-	-	-0.73
LIVER										
MTs	0.84	-0.53	-	-	-0.78	-	0.99	-	-	-
GPx	-	0.84	-	-0.69	-	-0.57	-	-	0.76	-
GSH	0.78	-	0.57	0.99	-	-	-	-	-	-0.81
GST	-	-	-	-	-	0.88	-0.78	0.59	-	-
LPO	-	-	-	-	0.92	-	-	-	0.92	-
DNA dam	-	-	0.89	0.84	0.52	-	-	-	-	-
GILLS										
MTs	0.75	-	0.60	-	0.93	-	0.70	-	-	-
GPX	0.67	-	0.75	0.96	-	-		-	-	-0.85
GSH	-	-	-0.98	0.91	-	-	-0.69	0.57	-	-
GST	-0.78	-	-0.56	-	-	-	0.58	-	-	-
LPO	-	-	0.99	-0.95	-	-		-0.91	-	-
DNA dam	-0.83	-	-0.56	-0.90	-	-	-0.86	-	-	-
GENERAL CONDITION INDEX										
C. factor	-	-	-	-	-	0.87	-0.82	-	-0.51	-
MUSCLE										
AChE	-0.86	-	-	-	-	-	0.53	-	-	-0.78
Mn	-	-0.71	0.63	0.76	-	-	0.93	-	-	-
Zn	-	0.82	0.58	0.92	-	-	-	-	-	0.93
Co	-	-	-	-	0.75	0.54	-	-0.95	-	-
Ni	-	-	-	-	0.82	0.53	-	-0.76	-	-
Cd	0.92	-	-	-	-	-0.84	-	-	-	-
As	0.78	-	0.54	0.92	-	-	-	-	0.84	-
Pb	-0.56	0.77	-	-	0.86	-	0.98	-	-	-
LIVER										
PAH bile	-0.50	-0.87	-	-	-0.63	-	-	-0.67	0.54	-
Cu	NM	NM	NM	0.72	-	-	-	-0.96	-	-
Mn	NM	NM	NM	-	-	-	-0.52	0.68	-	-
Zn	NM	NM	NM	-	-	0.87	-	0.62	0.53	-0.56
Cr	NM	NM	NM	-	0.90	-	0.99	-	-	-
Co	NM	NM	NM	-	0.89	-	-	-0.64	-	-
Cd	NM	NM	NM	-	-	-	-	-0.97	-	-
As	NM	NM	NM	0.50	-	-0.57	-	-0,89	-	-
Pb	NM	NM	NM	-	-	-0.83	0.92	-	-	-

[a] Only variables with loadings > 0.5 were considered components of the factors

Three factors were extracted from the original dry season dataset (Table 1). In one group, F1 (37.8% of explained variance) represented metal levels in muscles associated with antioxidant responses (GSH in the kidney, liver and gills, and GPx in the gills) (with highest score for P4). F1 also associated another group of variables: DNA damage and LPO in the gills, DNA damage in liver tissue, and levels of As and Cu in the liver (highest score for P1, followed by P6). F2 (35.0%) (Table 1) associated metal levels in the liver (Cr, Co) and muscle (Pb, Ni and Co) with increased MTs in gills and genotoxicity in liver and kidney tissues. These associations are highly relevant only in P5 (Table 2). Another group of variables showed a relationship between levels of PAH metabolites in bile and damage in liver cells (genotoxicity and lipid peroxidation) (Table 1). The estimated scores of this factor are especially high for stations P3 and P6 (Table 2). F3 (15.0%) revealed strong associations between levels of some metals in the tissues (Zn in the liver, Ni and Co in muscles), oxidative damage in kidney cells, and a decreased condition factor. These responses were particularly relevant for specimens from P6 (Table 2). In other associations with F3, other contaminants in tissues (Pb and As in liver tissues and Cd in muscle tissues) were linked to MTs and genotoxic damage in the kidney, and these associations were relevant for specimens from P1.

Four factors were extracted by the FA/PCA applied to the rainy season dataset (Table 1). F1 (36.9% of explained variance) reflected the association of metal burdens in the tissues (Pb in muscle and liver tissues, Mn in muscle tissues, and Cr in liver tissues), MTs in the liver and gills, and an indication of antioxidant response in gills (GST) and of neurotoxicity (AChE inhibition). This group of variables was found to have higher scores for the sampling stations under higher influence of the RIR (P1 and P2) (Table 2). Another group of variables was also related to F1: Mn in the liver (associated with decreased condition factor), the presence of DNA damage in the gills, and antioxidant responses in different organs (GSH in the gills and kidneys, GST in the liver and kidneys). These associations are relevant to P3, P4 (moderately), P5 and P6 (Table 2). F2 (27.1%) reflects the associations between Cd, Co, Cu and As levels in the liver, Ni and Co in muscle tissues, MTs in kidney tissues, lipid peroxidation in the gills, and PAH metabolites in bile (Table 1). Only P4 (the sampling station under highest influence of the urban area) was found to have a high score for this group of variables (Table 2). Another group of variables associated with

F2 included Mn and Zn and antioxidant responses (GST in liver and kidney tissues, and GSH in the gills); high scores were estimated mainly for stations under urban influence (P5 and P6), but also in the case of P3, which is under the influence of RIR. Factor 3 (15.7%) revealed the association of Zn in the liver and As in muscle tissues, MTs in the kidneys, an indication of antioxidant response (GPx) and oxidative damage (LPO) in the liver, and PAH metabolites in bile. Higher F3 scores were estimated for P4 and P6. F4 (13.8%) loadings showed an association of Zn in muscle and LPO in kidney, and, in another group, Zn in the liver was found to be associated with antioxidant responses in the liver (GSH), with antioxidant responses in the gills (GPx), and with genotoxicity in kidney and neurotoxicity (AChE inhibition). Estimation of F4 scores showed that a high level of Zn in muscle associated with oxidative damage in the kidney is relevant for specimens from P5, while Zn in the liver associated with antioxidant responses and damage are relevant for specimens from P2 and P3.

Table 2 Factor scores estimated for each of the sampling stations in Partially dry (P); Dry (D) and Rainy (R) seasons evaluated in the APA-CIP to the centroid of all cases for the original data.

Station	F1	F2	F3	F4
Partially Dry season				
P2P	-1.444	-0.001	0.406	-
P3P	0.106	-0.280	-1.469	-
P4P	0.602	1.341	0.297	-
P5P	0.735	-1.059	0.765	-
Dry season				
P1D	-0.709	-0.343	-1.484	-
P3D	-0.175	-0.714	0.115	-
P4D	1.739	-0.170	-0.099	-
P5D	-0.229	1.752	0.143	-
P6D	-0.625	-0.524	1.324	-
Rainy season				
P1R	1.876	0.195	0.040	0.169
P2R	0.370	0.152	0.039	-0.550
P3R	-0.603	0.296	-1.376	-1.183
P4R	-0.332	-1.977	0.316	0.115
P5R	-0.579	0.506	-0.633	1.775
P6R	-0.732	0.829	1.614	-0.326

4. DISCUSSION

During the dry season, biomarker responses and metal burdens in muscle and liver tissues were generally higher in the sections of the APA-CIP around the city of Cananéia. In addition, *C. spixii* from areas closer to the RIR also were found to possess increased molecular damages in lipids and DNA. Previous studies in coastal areas subjected to seasonal rainfall variation reported that, although the amount of contaminants may decrease with lesser continental inputs during the dry season, the concentration of contaminants in the aquatic system may increase if freshwater inflow is diminished (Sainz et al. 2004; Fianko et al. 2007; Costas et al. 2011).

During the rainy season, biomarker responses and metal burdens in liver and muscle tissues from fishes are more evident in fish from sampling stations under the highest influence of the RIR. Increased rainfall during the rainy season leads to a higher contribution of the RIR to the APA-CIP, which may remobilize metals from the sediment and/or carry metals from land-based sources into the estuary. Previous studies have shown the major role played by rainstorms in the release of contaminants from surface soils into the RIR and its tributaries within the RIR basin (Corsi and Landim 2003; Cunha et al. 2007; Costa et al. 2009; Abessa et al. 2014).

Metals bioavailability in aquatic organisms is closely related with salinity, being the main controlling factor for the partitioning of contaminants between sediments and overlying or interstitial waters in estuaries (Chapman and Wang, 2001). The influence of salinity on the geochemical behaviour, and also the bioavailability of metals, is dependent of two counteractive processes: (i) desorption due to increase complexation with sea water anions (Cl^- and SO_4^{2-}) and/or increasing competition for particle sorption sites with sea water cations (Na^+, K^+, Ca^{2+}, Mg^{2+}); (ii) coagulation, precipitation and flocculation (Chapman and Wang, 2001). Furthermore, according to the biotic ligand model, water hardness affects the toxicity of metals, once that Ca^{2+}, Mg^{2+} have a ‘protecting’ status relative to the toxicity of divalent cationic metals (Paquin et al. 2002). In the current study, *C. spixii* were collected in salinities ranging from 0 to 20 in the sites under higher influence of the RIR (P1, P2, and P3) and from 26 to 32 in the sites closer to the city (P4, P5, and P6), similar ranges of salinity also were reported in previously works at this same estuary (Bérgamo, 2000; Jesus, 2010). The salinity in the current study may be a confounding factor, either increasing or ameliorating the bioavailability and toxicity of metals depending on the sampling site or the sampling season.

Although the RIR basin is a natural metal-rich geological area, evidence suggests that the increased levels of metals in fish tissues observed in this study were due to human activities in the river basin. High levels of metals (Pb, Zn, Cu, Cr, As) recorded in river waters, bottom sediments, and suspended sediments were largely linked to mining activities in the river headwaters (Eysink et al. 1998; Corsi and Landin 2003; Moraes et al. 2004, Guimarães and Sígolo 2008). In the estuary, metals in sediments have been generally found only at moderate levels (Mahiques et al. 2009; Azevedo et al. 2011; Cruz et al. 2014) however, these values have increased substantially since the opening of the Valo Grande channel, and even more so after industrial mining activities began (Mahiques et al. 2009). These reports show that metals in the APA-CIP are largely from anthropogenic sources.

Our results suggest the influence of rainfall seasonality on the bioaccumulation of metals and bioavailability of PAHs, though some physiological disturbances may also indicate that some changes are due to seasonal differences. Some DNA damages in fishes exposed to low temperatures may be explained by decreased protein synthesis, which results in less efficient DNA repair mechanisms (Benincá et al. 2013; Pellacani et al. 2006; Buschini et al. 2003). Although the austral winter presented lower water temperatures (21 ºC) if compared to the rainy season (28 ºC), this difference could not explain the effects on *C. spixii* described in the current study. In fact, it is more reasonable to assign DNA damages to pollutant exposure, as demonstrated above, than to suggest that lower temperatures alone could give rise to these effects on DNA.

Data integration using FA/PCA revealed that metal concentrations in the body of *C. spixii* are associated with antioxidant responses, oxidative damage, neurotoxicity and genotoxicity. Zn, As, and Mn followed by Pb, Co and Cd were the contaminants that were most frequently associated with biomarker responses in *C. spixxi*. Other metals, including Ni, Cu, and Cr, showed some association with biomarkers, though less frequently. Concerning results that corroborate our study, a previous study showed that Pb and Cd levels in the muscle tissues of two catfish species (*C. spixii* and *Genidens genidens*) from the APA-CIP were comparable to a highly polluted estuary located within the Santos Estuarine System (Azevedo et al. 2012).

The induction of antioxidant enzymes present in different organs of *C. spixii* were associated with Zn, As, Mn and Pb that had bioaccumulated in liver and muscle tissues, a finding which suggests both acute and chronic exposure. The relationship between metal exposure and antioxidant response has long been established (Livingstone, 2003). Metals can stimulate the production of reactive oxygen species (ROS) through different pathways, such as NADPH oxidases, metal-catalysed oxidation systems via Fenton reactions (Luschak 2001, Sharma and Dietz 2009), or even the depletion of major antioxidants in the cell, particularly thiol-containing molecules (Atli and Canli 2010; Stohs et al., 2001). Consequently, increased ROS production triggers the cell defense mechanisms by increasing the activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), GST, and GPx, and also by increasing the expression of free-radical scavenger molecules (e.g. GSH). These responses can be used as biomarkers of a cellular pro-oxidant state.

The induction of antioxidant responses has been validated for cases of waterborne exposure in both field and laboratory studies with fish, as reported by Nunes et al. (2014) in the gills of *Anguilla anguilla* exposed to Pb, Cu, and Cd, by Eroglu et al. (2014) in the liver of *Oreochromis niloticus* exposed to Cd, Cu, Cr, Pb and Zn, and by Eyckmans et al. (2011), who described the induction of antioxidant responses in different freshwater fishes exposed to Cu. According to Fonseca et al. (2014), marine or estuarine fish species respond to waterborne or sediment exposure to metal, and metal was found to induce antioxidant responses in *Dicentrarchus labrax*, *Solea senegalensis* and *Pomatoschistus microps*. Similar findings were reported Souza et al. (2013) in a study on *Centropomus paralellus* from two estuaries along the Brazilian coast.

In the current study, the induction of antioxidant mechanism in all of the tissues considered was most evident in the liver, followed by the gills and less frequently in the kidney. Previous studies on fishes also found that antioxidant responses in the liver are more responsive to pollution than in other organs, as described in a study on *Zacco platypus* after waterborne exposure to Cu (Kim et al. 2014), as well as in a study on *O. niloticus*, whose liver presented a higher sensitivity after metal exposure than its kidney (Atli and Canli 2010). The liver is a key organ in evaluating the effects of pollutants in fish (Bennet, et al. 1999). Hepatocytes are known to exhibit a more efficient antioxidant mechanism because the multiple functions increase the metabolism in the tissue and, consequently, function as a constant source of oxidant species (Gul et al. 2004; Avci et al. 2005).

In the gills of *C. spixii*, antioxidant activity was more responsive to metal bioaccumulation than it was in the kidney. Fish gills are areas of extensive epithelium that exhibit high permeability to ions per unit of surface area, and they are continuously ventilated to allow for the exchange of gases, ions, salts, and water with the environment (Hill et al. 2004). Therefore, this organ is in direct contact with contaminants present in the aquatic milieu, a factor which justifies studies using this tissue. The current data show that antioxidant responses are associated with an increase of LPO in the gills, a finding which demonstrates the failure of these antioxidant responses to neutralize oxidative species. However, the prevalent pattern is one of increased gill damage (DNA damage and/or LPO) associated with decreased antioxidant activity in this organ, a result which clearly reflects the risk of exposure experienced by fishes living in the estuary around both the RIR and the diffuse and point sources located within the city of Cananéia. The susceptibility of the gills to pollutants, as described in the current study, means that there is a real risk of exposure for the local biota and an environmental health problem. Other studies have corroborated the same findings in the gills of other fish, including as *Solea solea* from the Tunisia coast (Jebali et al. 2013), *O. niloticus* from heavily contaminated sites in Egyptian lakes (Abdel-Moneim et al. 2012), and *Anguilla anguilla* after exposure to metal-rich effluent from a bleached kraft pulp mill (Santos et al. 2004). The increased LPO in the gills suggests that the gills have a less developed antioxidant mechanism compared to the liver.

As the current study on *C. spixii* describes, the liver presents more efficient antioxidant responses, and thus partially protects itself from oxidative damage, while the gills are more susceptible to oxidative damages. Meanwhile, the kidney possibly do not represent a good target organ because fewer associations of antioxidant responses, macromolecular damages and metal body burdens were observed in FA/PCA.

The results regarding neurotoxicity showed that Mn and Pb in muscle tissues and Zn, Pb, and Cr in liver tissues are related with a decrease in AChE activity. Other researchers have highlighted the use of AChE activity in fish as a useful biomarker for studying the effect of toxic metals, such as neurotoxic chemicals, in the field (Payne et al. 1996; Fasulo et al. 2010). The current study found that Pb is most clearly associated with neurotoxicity. Pb is recognized as an environmentally toxic chemical that causes many biological consequences such as mutation, chromosome aberration, cancer, neurotoxicity, and birth defects (Hong et al. 2007; Nunes et al. 2014). Similar to the current study, previous studies have reported AChE inhibition in fishes exposed to Pb. The decrease in AChE activity has been attributed to the fact that metals can bind to the functional groups of the enzyme, an action which leads to enzyme activity inhibition and a consequent overstimulation of the acetylcholine (ACh) receptors (de Lima et al. 2013 Richetti et al. 2011). The physiological consequences include several adverse effects, such as movement impairment, respiratory failure (WHO/IPCS/INCHEM, 1986), or even behavioral changes such as a loss of prey capture ability (Mager et al. 2010). Other results of ACh receptor overstimulation include an increase in peristaltic movement, muscle weakness, tremor, hypertension, and cardiovascular collapse (Boelsterli 2007).

The lowest levels of AChE activity were usually observed in fish collected in the areas around the RIR that contribute to metal exposure, including the area where mining activities were intense in the river headwaters for decades. The high quantity of metals reflects the fact that contaminants have been introduced into the APA-CIP from land-based sources. Additionally, this hydrographical basin is an important agricultural area in Brazil, which means that other contaminants, like pesticides, could potentially affect AChE activity not measured in the present study. Pesticides in drinking water and fish from RIR have already been reported in previous studies (Tardivo and Rezende 2005; Marques et al. 2007). Two classes of pesticides in particular can affect AChE activity: organophosphorous compounds and carbamates, which are used as an insecticide and an herbicide, respectively (Payne et al. 1996). DDTs were also used in the region on a large scale until the early 1980s, and metabolites are still present (Yogui et al. 2003).

The increased levels of metallothionein-like proteins in different organs were associated with higher metal body burdens in *C. spixii*. The metal scavenging role of MTs is well established, and the induction of their expression is a recognized biomarker of exposure to metals (Van deer Oost et al. 2003; Viarengo et al. 2000; Stegeman et al. 1992). In the current study, the association between MT expression and metal bioaccumulation was more evident in the gills of specimens from sites closer to the city of Cananéia during dryer seasons and in those closer to the RIR during the rainy season. The data clearly show that the levels of MT expression are correlated with the levels of metal exposure, a result which corroborates the use of this biomarker in areas affected by metals. The higher metal body burdens in *C. spixii* associated with high levels of liver MTs in sites closer to the river during the rainy season may be explained by the release of metals that had accumulated in the sediment during the mining activities in the past. The explanation of the same result in specimens from sites closer to the city (P4 and P5) during the dry season could be the concentration of metals resulting from urban activities, since the influence of RIR waters is minimal or negligible at these sites, during this season.

On the other hand, during the driest season, levels of metals in the organisms seem to decrease MTs levels in the liver. The literature also reports decreased MT levels in fishes as a consequence of exposure to high metal concentrations (Carvalho et al. 2012; Romeo et al. 1997). This finding may be explained by an increased demand of cysteine residues for GSH synthesis during metal detoxification (Roméo et al. 1997).

In the gills of *C. spixii*, the strong evidence of high levels of MTs with metal bioaccumulation and lipid peroxidation suggests that this organ is an important target for metals in the APA-CIP. These findings show that metals are relevant contaminants in the estuary and that the activities along the river basin and in the cities located on the coast are important sources of pollutants. Therefore, from these results, and in light of a previous study showing high levels of metals (Pb, Zn, Cu and Cr) in suspended sediments at the upper RIR as well as at its mouth (Guimarães and Sígolo 2008), it is possible to argue that metal-associated suspended particles may be an important uptake route of these contaminants for fishes in the protected area.

MTs behaviour in both the liver and the gills, which is associated with metal body burdens, is in accordance with the other biomarkers in that it reflects two main sources of pollution in the APA-CIP: the city of Cananéia and the Ribeira do Iguape River. Additionally, the seasons also sometimes play an important role in the bioaccumulation of metals, depending on the site studied. The current findings corroborate the study by Cruz et al. (2014), who concluded that the contamination from both the RIR and urban areas (the city of Cananéia) are relevant sources of pollution in the APA-CIP.

Although metals may be the main class of contaminants affecting fish health in the APA-CIP, the presence of PAHs in the bile of *C. spixii* indicates that fishes living in the estuary were exposed to PAHs. PAH metabolites in fish bile are useful for indicating chronic exposure to pyrogenic or petrogenic PAHs (Oliveira Ribeiro et al. 2005; Osório et al. 2014). The increase of LPO in the liver and gills and the genotoxicity in the gills were associated with high levels of PAHs in specimens from the estuary. However, these associations were more discreet than the associations between metals and damages in macromolecules. The increased levels of PAH metabolites in bile were found in individuals from the studied sites closer to the city of Cananéia (sites 4, 5 and 6). This finding strongly suggests that PAHs from urban sources, when combined with metals, lead to LPO increases and DNA damage in the liver of *C. spixii* specimens living in the APA-CIP. Sources of PAH in this area include nautical structures (marinas, decks, nautical garages, and small docks), ferry boat stations, a nautical gas station, municipal sewage discharges, and urban drainage (Cruz et al. 2014). PAHs are of concern in MPAs since they are potentially carcinogenic (Shailaja and D'Silva 2003) and may induce hepatic lesions, physiological disorders, and biochemical disorders in fish (Oliveira Ribeiro et al. 2005).

The biological effects seen in fish from the estuary after natural exposure are due to a diversity of pollutants confirm the potential risk of exposure as described previously by Rodrigues et al. (2013) and Araújo et al. (2013). In the APA-CIP, the present study shows that, in addition to the pollution from RIR inputs, the city located within the Environmental Protected Area is another important pollution source that affects this important ecosystem.

The current study shows that rainfall seasonality plays an important role in the estuarine pollution of the APA-CIP in terms of the risk of exposure for the biota. The evidence includes the association of metal burdens in muscle and liver tissues, as well as the association of PAH levels in the bile of *C. spixii* with the biomarker responses in target tissues. The spatial distribution of biomarker responses and pollutants that bioaccumulated in fish tissues changed throughout the year, a result which indicates that levels and/or bioavailability of these contaminants change according to the season, although physiological variations can also play a role in some of the measured biological responses. Although the climate in the region is classified as tropical rainforest with high humidity throughout the year, the present study shows that the differences in rainfall between seasons are enough to influence the environmental quality of the APA-CIP.

Water chemistry and microbiological parameters are the water quality indicators recommended by the International Union for Conservation of Nature and Natural Resources (IUCN) for pollution assessment in MPAs (Pomeroy et al. 2005). Biological indicators are also recommended, but they are not specific to assess the health of fish exposed to contaminants. The results of the current study suggest that biomarker analyses in fish are useful tools for assessing effectiveness and for environmental quality monitoring of MPAs subjected to contamination pressure. This finding is important because they suggest that, even if contaminants in MPAs are at only moderate levels, they are still harmful to fishes in many instances. In these cases, shifting the focus of the assessment from agents (e.g. measuring contaminant levels) to targets (biota), but not disregarding any of those information, can provide useful information for protective management.

It is important to evaluate sublethal responses combined with metal body burdens in tissues and PAHs in bile in order to assess how pollution affects the environment. This study adds to the knowledge on the effectiveness of MPA management. Such tools provide information for making sound decisions regarding environmental health status in vulnerable or valuable marine areas, and they support the development of MPAs and policies for them.

5. CONCLUSIONS

The current study shows that a set of biomarker analyses using different fish organs is a useful tool for assessing chemical pollution in a estuarine (or coastal) MPA. This is the first study on MPAs in which both biomarker responses and metal body burdens, or PAHs, in *C. spixii* reflected seasonal variation. During the dry season, antioxidant responses, LPO, genotoxicity, and MTs, combined with metal body burdens, clearly showed that fish were affected in areas under the influence of the city of Cananéia (P4, P5 and P6); during the rainy season, the evidence is clearly present in sampling stations under the highest influence of the RIR (P1, P2, P3). In addition, the current study shows that PAHs from the city may have been causing LPO, as well as DNA damages. The studies of AChE activity reinforce these data and include a new finding: lead may be the most relevant neurotoxicity-causing metal in *C. spixii*. Liver tissue was more responsive in terms of antioxidant responses, whereas the gills were found to be more responsive to biomarkers of effects. The combined use of contaminant body burdens and sensitive biological tools allowed determining that different contamination sources can affect MPAs. The current study showed that, when planning MPAs, the influence of contamination sources and their effects must be considered, as well as strategies to control them in order to avoid negative impacts to the native biota and ecosystem processes.

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CAPÍTULO II

Capítulo formatado de acordo com as normas da revista “Marine Pollution Bulletin”

Assessing Genotoxic effects in fish from a Marine Protected Area influenced by former mining activities and other stressors

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ABSTRACT

The goal of the current study was to evaluate different genotoxicity tools to assess an MPA affected by former mining activities and urban settlements. *Cathorops spixii* was analyzed for genotoxic effects (i) in molecular level (DNA damage on peripheral blood, kidney, liver, and gills tissues), (ii) chromosome level as micronucleus (MN) and nuclear alterations (NA) tests in erythrocytes. Sampling was done during the dry and rainy seasons and the genotoxicity data were integrated with levels of metals and As in liver and muscle tissues, or even with the levels of PAHs metabolites in bile, trough Factor Analysis. Among all analyses employed, the results showed that MN and NA were more frequently associated with the bioaccumulated metals than the analyses of DNA damage. These finding suggests that the first analyses are less vulnerable to the effects of confounding factors in mildly contaminated areas. The use of different genotoxicity responses allowed the identification of sources of pollution in the MPA, and was important to detect environmental risks related to genotoxicity and cytogenotoxicity of the complex mixtures of contaminants in a mildly contaminated MPA.

Keywords: Estuary, Weight of Evidence, Environmental Quality Assessment, Biomarkers, Bioaccumulation, Multivariate Approach.

1. INTRODUCTION

Metal contamination of aquatic ecosystems due to mining activities has been a great environmental concern on a global scale and one of the most serious threats to the aquatic environments worldwide (Zhuang et al., 2014). This concern is partly because mining activities have commonly been performed in an uncontrolled way (Rybicka, 1996). Additionally, mine wastes and tailings can severely affect the surroundings of the mines (Fernández-Caliani et al., 2009), since pollutants tend to be transported mainly via water (e.g. acid mine drainage) and air (atmospheric deposition, wind-blown particulate matter), and can accumulate in various environmental compartments. Metals from mining activities can therefore impact the biota and human beings (e.g. Molina-Villalba et al., 2014; Taylor et al., 2014; Camizuli et al., 2014: Riba et al., 2005).

The Ribeira de Iguape River (RIR), located in the Southeastern Brazil (Figure 1), is an important region of mining activities and represents an example of uncontrolled mining activity. Lead mines operated during the 20th century and their residues were discharged into the river or on the river banks. High levels of metals (Pb, Zn, Cu, Cr) and arsenic were recorded in river waters, bottom sediments, and suspended sediments in the river headwaters (Eysink et al. 1998; Moraes et al. 2003, Guimarães and Sígolo 2008). The RIR flows towards the Estuarine System of Cananeia-Iguape-Peruíbe (APA-CIP), a protected area, which was recognized as a UNESCO World Natural Heritage site. Metals in the APA-CIP estuarine sediments, which were found at low levels in the past, have increased substantially after the construction of an artificial connection between the river and the estuary (Mahiques et al. 2009). Recent studies have been reported metals at moderate levels in the sediments from the estuary, which has been attributed to the former mining activities as well as the contribution of contamination from urban settlements in the APA-CIP (Azevedo et al. 2011; Cruz et al. 2014).

A multitude of ecotoxicological approaches and tools, from molecular to ecological responses, have been proposed in the last decades for environmental assessment or monitoring (Van Straalen, 2003). Their application, however, has been tested and validated only in highly contaminated sites (e.g. Torres et al., 2014; Choueri et al., 2010; Galloway et al., 2004; Adams and Greeley, 2000). Mildly contaminated sites can also affect the health of aquatic biota, since the organisms are subjected to a long-term exposure (Nipper et al. 1998). This is the case of many marine protected areas influenced by urban or industrial settlements, which may be subjected to not too high but continuous inputs of contaminants from outer MPA boundaries (Araujo et al., 2013; Perra et al., 2011; Pozo et al., 2009; Chou et al., 2004).

However, in mildly contaminated areas, cause-and-effect relationships between contamination and toxicity may not be straightforward as they are in highly contaminated sites, especially in a complex physical and chemical milieu as estuaries (Choueri et al., 2009). Biological responses must be sensitive to low levels of contaminants, but still representative of an actual or potential risk to the individual organism or the population. Genotoxic responses may trigger a damaging chain of biological changes (e.g. reproduction disturbances, growth inhibition, carcinogenesis) that can be forwarded to ensuing generations and they may lead to a loss of genetic diversity (Mitchelmore and Chipman, 1999; Jha, 2004; Barsiene et al., 2013).

Different types of DNA damage may occur when organisms are exposed to environmental contamination, like single- and double-strand breaks, inter-strand and intra-strand cross-links, DNA adducts and DNA protein cross-links (Wood et al. 2001). Some metals and PAHs (especially high molecular weight PAHs) are known to cause genotoxicity. DNA damage caused by such contaminants can be characterized in three phases: (i) it first initiates with the formation of adducts with toxic molecules, (ii) followed by secondary modifications of DNA, including single- and double-strand break, changes in DNA repair, base oxidation, and cross-links (Fonseca et al., 2014). In an advanced stage, (iii) cells present altered functions, cell proliferation, mutagenesis and eventually carcinogenesis (Monserrat et al. 2007).

The quantification of DNA strand breaks (by electrophoresis or fluorescence measurement) is considered a sensitive indicator of genotoxicity at chromosome level (Olive 1998; Gagné and Blaise, 1995; Silva et al., 2012; Maranho et al., 2012; Parolini et al., 2013). At cellular level, micronuclei and nuclear abnormalities formation in fish erythrocytes have also been successfully used as indicators of genotoxicity caused by environmental contamination (e.g. Souza and Fontanetti, 2006; Hoshina et al., 2008). Several studies reported increased frequencies of micronuclei and nucleus abnormalities in fish cells after exposure to different metals under both field and laboratory conditions (Al-Sabti and Metcalfe, 1995; Çavaş et al., 2005; Çavas et al., 2008; Isani et al., 2009; Yadav and Trivedi, 2009).

The Marine Strategy Framework Directive (2008/56/CE) has recently proposed the use of genotoxic endpoints as a part of the characterization of biological status of marine water bodies. In addition, different genotoxicity assays have been used in environmental monitoring programs (e.g. ICES Working Group on Biological Effects of Contaminants; Mediterranean Pollution Programme) (Davies and Vethaak, 2012). Although such approach has been widely used for environmental quality assessment/monitoring, no attempts have been made to assess the suitability of such tools for monitoring mildly contaminated sites or MPAs. This is important since genotoxic responses can be affected by, for example, natural environmental conditions, diet and hormonal status (Mitchelmore and Chipman, 1998), and such confounding factors may be exacerbated in mildly contaminated environments.

The current study aimed to evaluate different genotoxicity tools in fish to assess an MPA affected by land-based former mining activities as well as urban settlements. *Cathorops spixii,* a demersal fish, was analysed for genotoxic effects through (i) the quantification of DNA damage in different tissues by means of two different methodologies (the comet assay with blood tissue, and the alkaline precipitation assay with kidney, liver, and gills tissues), (ii) cytogenotoxicity assessment, performed through the fish micronucleus (MN) and nuclear alterations (NA) test in erythrocytes. The relationship with environmental contamination is provided by integrating genotoxicity data with metals body burdens (liver and muscle) and levels of PAHs metabolites in bile by using a multivariate approach.

2. MATERIALS AND METHODS

2.1 Study Area

The Cananéia-Iguape-Peruíbe Environmental Protected Area (APA-CIP) (24^{o}40’S and 25^{o}05’S) (Figure 1) is an estuarine-lagoon ecosystem recognised by Unesco as part of Biosphere Reserve of the Atlantic Rainforest due to its relevance to environmental conservation. It is a priority area to be included in the list of Brazilian wetlands of international importance within the scope of the Ramsar Convention (Brazil, 2012) and the region is part of the global list of Unesco’s World Heritage Sites. A dry winter (July to August, monthly average rain of 95.3mm) and a rainy summer (January to March, monthly average rain of 266.9mm) are well defined in the region. Monthly mean temperature ranges from a maximum of 28 ºC (February) and a minimum of 20 ºC (July).

The main freshwater contributor to the estuarine lagoon is the Ribeira de Iguape river (RIR) since the opening of a water channel (named “Valo Grande”), which deviates about 70% of the river course towards the lagoon waters. For many years, mine tailing and metallurgical slags of blast furnace were directly dumped into the RIR. In the early 1990's, after the entry into force of Brazilian environmental laws, mining industry halted to release the residues into the river, but started to dispose them on the river banks, exposed to the weathering and subjected to lixiviation (Guimarães and Sigolo, 2008; Abessa et al., 2014).

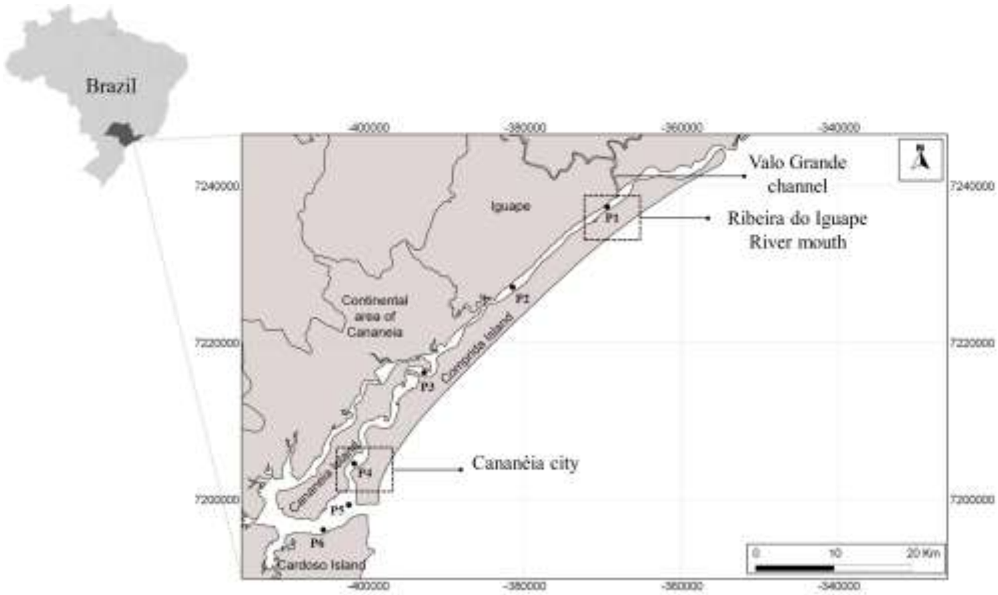


Fig. 1 Sampling stations located within the APA-CIP, Brazil

High levels of metals (Pb, Zn, Cu, Cr) and As were recorded in the river waters, as well as bottom and suspended sediments (Eysink et al., 1998; Corsi et al., 2003; Moraes et al., 2003, Guimarães and Sígolo, 2008), although in the estuarine lagoon metals were found at only moderate levels in sediments (Mahiques et al., 2009) when compared to the international Sediment Quality Guidelines (Long et al., 1995; Environment Canada and *Ministère du Développement durable, de l'Environnement et des Parcs du Québec*, 2007).

Apart from the contribution of former mining activities, other source of contaminants to the APA-CIP is the three cities placed within the limits of the protected area (Iguape, Ilha Comprida, and Cananéia), with a total estimated population of approximately 51,900 inhabitants (IBGE, 2014) without adequate sanitation infrastructure (Morais and Abessa 2014).

2.2 Fish collection and sample preparation

Cathorops spixii (madamango sea catfish) is a demersal fish that preys mainly upon the zoobenthos (especially crustaceans and polychaetes) and small fishes (Fishbase, 2014). This species tolerates a wide range of salinity and it is widely consumed by the local population in the estuarine-lagoon complex (Fávaro et al., 2005).

The sampling sites were set with the aim of encompassing the main potential contaminant sources along the APA-CIP (Figure 1). Thus, site P1 is the closed to the RIR mouth and site P4 is the closed to Cananéia city. Fifteen specimens were collected at each sampling site (Figure 1) with a bottom otter trawl. To account for seasonal variation, two sampling campaigns were conducted: one during (i) the dry season (D) (August 2012), and the other during (ii) the rainy season (R) (March 2013). The average rainfall in these seasons was 111mm and 390mm, respectively (CEPAGRI, 2014).

The collected specimens were kept in local water, under aeration until transportation to the laboratory located at Cananéia city. Five animals were set aside for metal body burden analyses and ten individuals for genotoxic analyses and bile fluid extraction. Before euthanized by spinal cord section, individuals were anesthetised with benzocaine (0.01%) in water, then weighted and measured, and the peripheral blood to genotoxic biomarkers (comet, micronucleus, and nuclear alterations assays) was withdrew from the caudal vein using heparinized syringes. Fish gills, kidney, and liver were dissected, frozen and stored at -80 °C for the DNA strand break assessment. Axial muscle and liver used in metal body burden analyses were stored in plastic vessels at -20ºC until the analyses. Bile fluid was stored in glass vessels kept at -80 °C for PAHs metabolites analyses.

Fulton's condition factor was calculated according to the formula: KF = (W / L3 x 100), where KF = Fulton condition factor, W = body weight in grams, and L = total body length in cm.

2.3. Genotoxicity assessment

2.3.1 Comet assay in blood

Comet assay followed the procedures described by Singh et al. (1988) and Ferraro et al. (2004). Blood was diluted in fetal bovine serum and stored on ice (protected from light) for 24h. Microscope slides were prepared with a blood cell suspension (10µl) in low-melting-point agarose (120µl) at 37 °C followed by incubation in lysis solution at 4 °C for 7 days. After lysis, the slides were placed in a solution of NaOH (10M) and EDTA (200mM), with pH>13, for 20 min for DNA denaturation. Electrophoresis was carried out at 25V and 300 mA for 25 min at 4 °C, and slides were neutralized for 15 min with 0.4M Tris, pH 7.5, fixed in 95% ethanol for 5min, and stained with ethidium bromide (0.02 µg ml^{-1}). DNA damage was scored using a Leica® epifluorescence microscope at a magnification of 400x. For each fish, 100 nucleoids were visually analyzed according to the method described by Ramsdorf et al. (2009).

2.3.2 Fish micronuclei (MN) and nuclear abnormalities (NA) test in peripheral blood

The analysis of erythrocytes NA is a variant of the standard MN test and it includes the quantification of a number of alterations in cell nuclei that may generate micronuclei. Thus, such analyses may complement the traditional MN scoring (Ayllon and Garcia-Vasquez 2000; Çavas and Ergene-Gozukara 2005; Costa and Costa 2007; Costa et al., 2008). Nuclear abnormalities may be a consequence of effects caused by clastogenic pollutants, leading to problems in chromosomal attachments or gene amplification (Omar et al 2012).

Blood aliquots were immediately smeared on glass microscopy slides and before air-drying the layer was fixed with absolute ethanol (30min) and stained with acridine orange. For each animal 2,000 erythrocytes were examined under Leica® epifluorescence microscope at 1,000x magnification and scored for the presence of both typical MN and NA. MN test were performed in accordance with Heddle (1958) and Schimd (1975) and NA (blebbed, lobed, vacuolated and notched) were characterized in accordance with Carrasco et al. (1990).

2.3.3 Alkaline precipitation assay in kidney, liver and gills

Kidney, liver, and gills tissues were kept on ice throughout the analyses. Liver and kidney were analyzed because they are target organs in metabolism and accumulation of contaminants (Bernet et al.,1999), whereas gills exhibit high permeability to ions, and they are continuously ventilated to allow for the exchange of gases, ions, salts, and water with the environment (Hil, 2005).

Tissues were homogenized at 10% W/V in Tris-HCL buffer (TRIS 50mM; EDTA 1mM; DTT 1mM; Sucrose 50mM; KCl 150mM; PMSF 1mM, pH 7,6). The homogenates were centrifuged at 10.000×g for 20 min at 4 °C, and aliquots of the supernatant were kept for the analysis.

DNA strand breaks were measured through the alkaline precipitation assay (Olive 1988; Gagné and Blaise 1995). The assay is based on the potassium-dodecylsulphate precipitation of protein-bound genomic DNA, which leaves protein-free DNA strand breaks in the supernatant. These stranded DNA is quantified by fluorescence (λex 360nm and λem 450nm) after staining with Hoescht dye. Standard

solutions of salmon sperm DNA were used for calibration. Protein concentrations were determined spectrophotometrically at 595nm (Bradford 1976), with BSA as the standard. All biomarkers analyses were performed in a microplate reader (Biotek-Synergy™ HT). The results were expressed as µg DNA mg prot^{-1}.

2.4 Metals body burden

Concentrations of arsenic (As) in muscle and liver were determined by atomic absorption spectrophotometer (Varian®, AA 240Z) equipped with a graphite furnace (AAS-GF) (Model, GTA 120). Metals were quantified by flame atomic absorption spectroscopy (F-AAS) (Varian®, AA 240FS). All analyses were performed according to standard METHOD 200.9 (USEPA, 1994). Detection limits for As was 5.88 µg kg^{-1} and for metals (Cu, Mn, Zn, Cr, Co, Ni, Cd, Pb) were 0.034 mg kg^{-1}, 0.0697 mg kg^{-1}, 0.0525 mg kg^{-1}, 0.112 mg kg^{-1}, 0.146 mg kg^{-1}, 0.0623 mg kg^{-1}, 0.042 mg kg^{-1}, 0.0602 mg kg^{-1}, respectively. The method limits of quantification (LOQ) was 0.178 mg/kg^{-1} for As, .0.004 mg/kg^{-1} for Cu, 0.537 mg/kg-^{1} for Mn, 0.720 mg/kg^{-1} for Zn, 0.345 mg/kg-^{1} for Cr, 0.390 mg/kg^{-1} for Co, 0.204 mg/kg^{-1} for Ni, 0.059 mg/kg^{-1} for Cd and 0.545 mg/kg^{-1} for Pb.

Standard curves were prepared using reference material (Qhemis High Purity®). Quality control for analytical procedures was performed using standard addition and recovery rates ranged from 80% to 120% for all elements investigated. Metal and As concentrations were expressed in mg kg^{-1} dry weight.

2.5 Polycyclic aromatic hydrocarbons metabolites in the bile

PAHs may had different anthropogenic sources: 2 to 4-ring PAHs are considered from petrogenic sources of pollution, while the 5 to 6-ring PAHs are related to pyrolytic sources (Olajire et al., 2005). The metabolites of polycyclic aromatic hydrocarbons (PAHs) in the bile of *C. spixii* were quantified through fixed-wavelength fluorescence in the spectrofluorometer (Sunrise-Tecan) at wavelengths 288/330 nm, 334/376 nm, 364/406 nm and 380/422 nm (λex/λem) which correspond respectively to naphthalene-type (2 rings), pyrene-type (4 rings), benzo(a)pyrene-type (5 rings), and benzo(ghi)perylene-type (6 rings) (Aas et al., 2000; Oliveira Ribeiro et al., 2005). The PAH concentrations were determined through comparison with a standard curve for each group of rings. The results were expressed as units of PAH mg $prot^{-1}$.

2.6 Statistical analyses

First, data sets of specimens length, condition factor, comet scores, MN and NA frequencies in blood; DNA strand breaks in kidney, liver, and gill tissues; and total PAHs contents in bile were tested for normality (Kolmogorov-Smirnov's test) and homoscedasticity (Bartlett's method). Data sets that met Analysis of Variance (ANOVA) assumptions were tested for difference between the means through ANOVA followed by post hoc Tukey test. Non-parametric statistical tests (Kruskal-Wallis test, with Dunn's multiple comparisons as post-test) were used to compare data series that violated ANOVA assumptions.

Pearson correlation analysis was performed with the data of levels of individual PAHs metabolites in bile of each season data matrix, in order to assess significant correlations among the individual PAHs along the sampling sites. The significance level was set at $p=0.05$ for all analyses.

2.6.1 Multivariate Approach

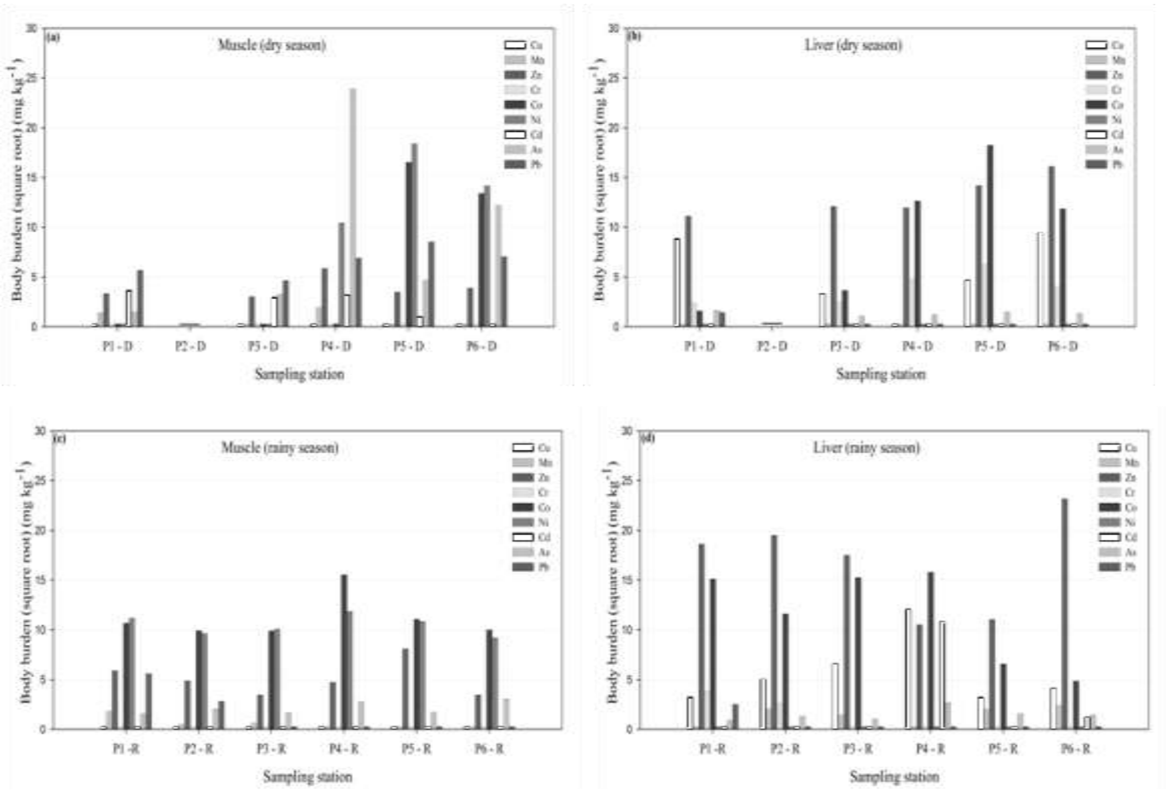
Factor Analysis with Principal Component Analysis (FA/PCA) as the extraction procedure was used to highlight the associations between the genotoxicity responses (DNA strand breaks in kidney, liver, and gill tissues; comet scores, frequencies of micronuclei, blebbed, lobed, vacuolated and notched nuclei, and total nuclear abnormalities in blood), contaminants body burdens (Cu, Mn, Zn, Cr, Co, Ni, Cd, Pb, and As) and total metabolites of PAHs in bile at each season (dry and rainy). The variables were autoscaled (standardized) so as to be treated with equal importance. Only factors with eigenvalues greater than 1.0 were interpreted (Kaiser criterion). The selected variables to be interpreted were those associated with the factors with a loading ≥ 0.60, a value which is more conservative than the loading cut-off recommended by Tabachnic and Fidell (1996). The relevance of the observed associations to each of the 6 sampling stations (cases) was estimated by calculating the factor score from each case to the centroid of all cases for the original data. All the analyses were performed using the STATISTICA 12 software (StatSoft Inc., USA).

3. RESULTS

The length of the specimens collected in the different sampling stations along the APA-CIP, during the dry season, ranged from 16.4cm (±2.2) in P5 to 20.5cm (±5.9) in P4. During the rainy season these values ranged between 14.9cm (±1.10) in P1 and 25.6cm (±4.6) in P5. Significant differences were found only during the rainy season: specimens from P3 and P5 presented higher values than those from P1 and P4 ($p<0.05$) while animals from P5 presented values higher than those from P2 ($p<0.05$).

During the dry season the condition factor (K) ranged from 1.03 (±0.22) in P6 to 1.95 (±0.39) in P1 while in the rainy season the K factor values ranged from 0.79 (±0.06) in P3 to 1.05 (±0.09) in P1. The condition factor was significantly different in fishes from the APA-CIP during both dry and rainy season. In the dry season, individuals from P1 showed significantly higher values than those sampled in the other stations ($p<0.05$); and according to the results from rainy season, the values in P1 and P2 were significantly higher than animals from P3 and P5 ($p<0.05$).

Metals (Cu, Mn, Zn, Cr, Co, Ni, Cd, Pb) and As bioaccumulated in liver and muscle tissues of *C. spixii* are showed in Figures 2a to 2d. During the dry season, metal burdens in muscle and liver were generally higher in the sections of the APA-CIP around the city of Cananéia, whereas during the rainy season these values were higher in individuals from stations affected by RIR.



Figs. 2a to 2d Bioaccumulation of metals and As in muscle and liver of *Cathorops spixii* sampled in the APA-CIP during both dry an rainy seasons. Data were transformed (square root) for better visualization.

The levels of PAHs (2-ringed, 4-ringed, and 5-ringed) in bile are presented in Figures 3a and 3b, and were significantly correlated to each other (p<0.05) along the sampling sites during the winter. In summer, only 5-ringed and 6-ringed was significantly correlated. During the dry season, P3 showed increased values for all analyzed PAHs but significant differences were detected only for 4- and 5-ringed PAHs (ANOVA, p<0.05). 4-ringed PAHs were significantly higher in P4, and six-ringed was higher in P1 and P6 (ANOVA, p<0.05). During the rainy season, 4-, 5- and 6-ringed PAHs show a general trend of decreasing from P1 to P3, then increasing from P3 to P6. 2-ringed PAHs did not follow this pattern showing the highest value in P4 (ANOVA, p<0.05).

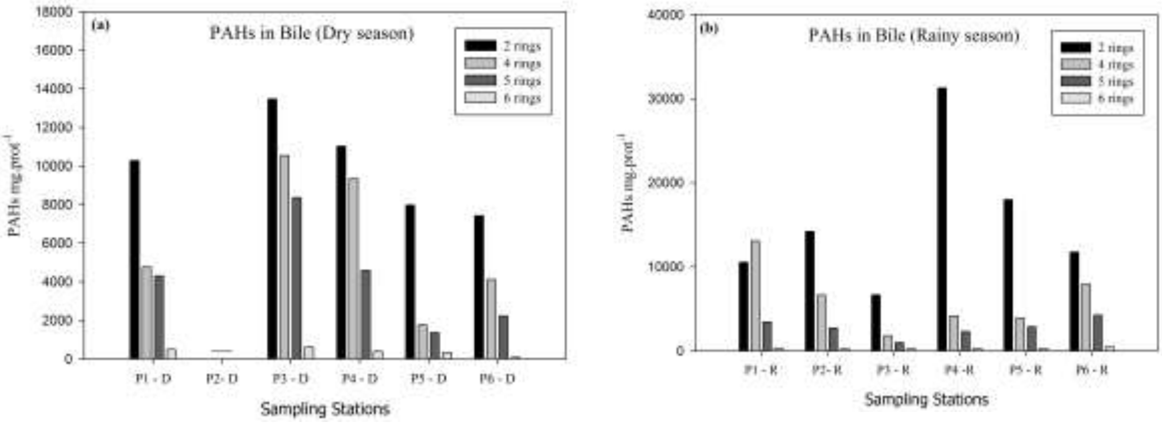
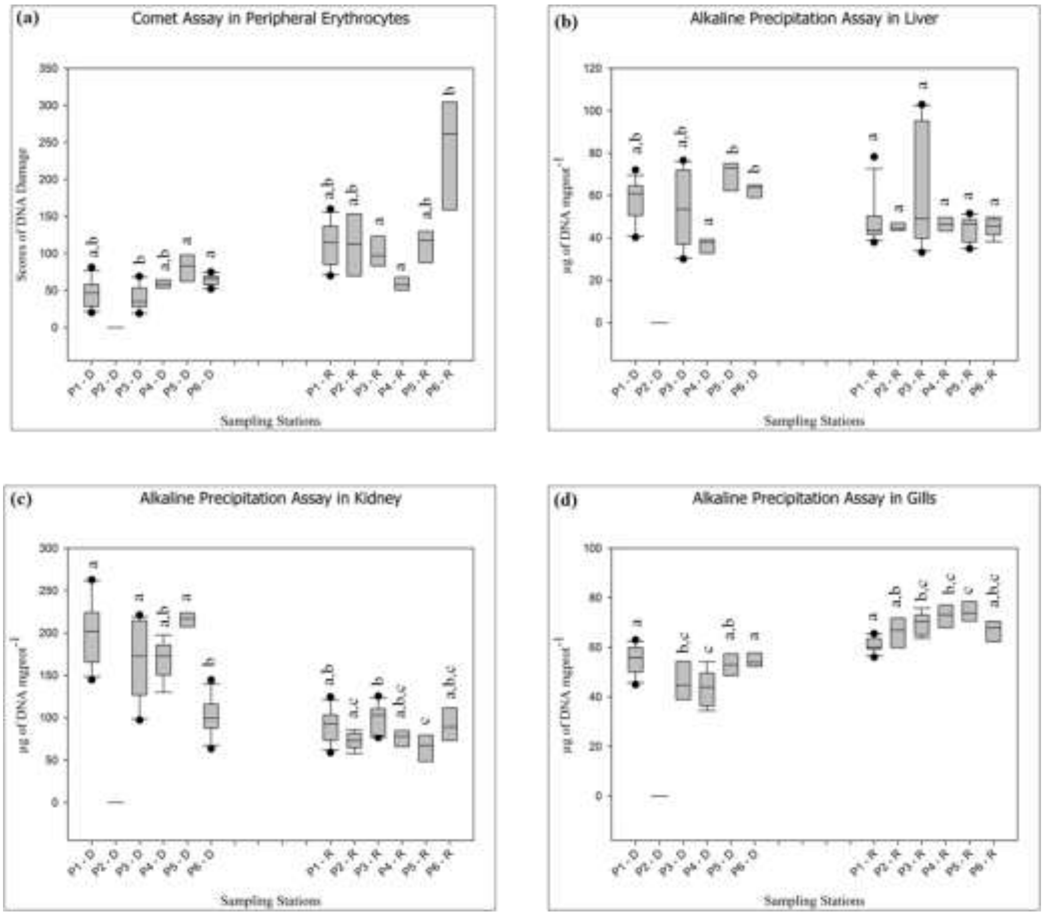


Fig. 3a and 3b PAH in bile of *Cathorops spixii* sampled in the APA-CIP during the dry season (D) and the rainy season (R). Site with a line instead of a bar means that no organisms were sampled

Comet scores (Figure 4a) were significantly higher in P5 and P6 during the dry season while P6 showed the highest score during the rainy season (ANOVA; *p*<0.05). Micronuclei frequency did not showed differences among studied sites or even between seasons, but it was also statistically higher in P1 (during both the dry and the rainy seasons) (*p*<0.05) (Table 1). Specimens from P5 showed the highest frequencies of blebbed, notched, and total nuclear abnormalities during both seasons (Table 1), although the means did not presented significant differences among studied sites (p>0.05).

Likewise comet score values, DNA strand breaks results in liver tissue were significantly higher in P5 and P6 during the dry season (*p*<0.05), but no significant differences were observed during the rainy season among sites (*p*>0.05) (Figure 4b). The DNA damage in kidney tissue was higher in P1, P3 and P5 during the dry season, and in P1 and P3 during the rainy season (Figure 4c) (*p*<0.05). In gills, DNA strand break levels were higher in P1 and P6 during the dry season (Figure 4d) and, during the rainy season, P3, P4 and P5 presented higher values (*p*<0.05) with a clear trend of increase from P1 to P5.



Figs. 4a to 4d DNA damages in target tissues of *Cathorops spixii* (blood, liver, kidney and Gills). site with a line instead of a boxplot means that no organisms were sampled Data are presented as boxes with boundaries that indicate 25th and 75th percentiles and a line within the box showed the median values. The use of different letters show significant differences during the same season (p = 0.05).

Table 1 Frequency of micronucleated cells and nuclear abnormalities in *Cathorops spixii* sampled in APA-CIP.

	Micronuclei		Blebbed		Lobed		Vacuolated		Notched		Total nuclear abnormality	
Dry season												
P1	1.92	±2.10	5.85	±3.58	0.08	±0.28	2.23	±2.95	7.77	±3.59	17.85	±8.06
P3	0.00	±0.00	6.53	±5.36	0.07	±0.26	1.13	±1.73	4.93	±2.22	12.67	±6.52
P4	1.20	±1.87	5.80	±4.83	0.00	±0.00	2.50	±5.87	7.00	±5.42	16.50	±9.62
P5	0.13	±0.35	17.25	±17.04	0.00	±0.00	1.88	±2.70	13.63	±8.63	32.88	±24.77
P6	0.14	±0,36	5.21	±5.37	0.00	±0.00	2.07	±1.94	7.00	±6.37	14.64	±12.29
Rainy season												
P1	0.93	±1.16	14.80	±15.69	0.00	±0.00	3.80	±5.65	28.73	±12.38	48.27	±19.52
P2	0.60	±0.70	21.00	±16.08	0.00	±0.00	2.10	±3.21	18.30	±5.74	42.00	±14.82
P3	0.09	±0.30	21.00	±15.85	0.00	±0.00	1.55	±2.25	19.64	±11.28	42.27	±23.07
P4	0.00	±0.00	22.00	±7.57	0.00	±0.00	0.57	±0.79	14.71	±4.99	37.29	±11.16
P5	0.00	±0.00	32.10	±35.42	0.00	±0.00	1.80	±5.03	22.50	±18.49	56.40	±12.08
P6	0.14	±0.37	27.71	±13.05	0.00	±0.00	0.86	±1.86	14.86	±9.17	43.57	±15.67

Two different datasets were used to perform FA/PCA: (i) the dry and (ii) the rainy seasons. The total explained variance of each FA/PCA was 84% for the dry season data and 86.41% for the rainy season data. The data of the dry season yielded three new variables (factors) (Table 2). Factor 1 (F1) (which explained 44.7 6% of the variance) showed association between DNA damage in blood (comet scores), blebbed and notched nuclei, metal body burdens in muscle (Co, Ni, and Pb) and liver (Co, Cr, and Zn). Score analysis showed that such associations presented high relevance in sites under the influence of the urban area (especially in P5) (Table 3). Still associated with F1, other group of variables included micronucleus induction, some metals (Cd in muscle and Pb in liver) and all analyzed PAHs in bile (Table 2). Such associations showed higher scores in P1, and P3 (Table 3) while F2 (22.11% of explained variance) associated genotoxicity (DNA strand breaks in liver and gills, and frequency of vacuolated nuclei) with As and metals (Cu and Pb) in liver (Table 2), with higher scores in P1>P6>P5 (Table 2). Other group associated with F2 was As and metals in muscle (Mn and Zn) including 2- and 4-ringed PAHs, without relationship to genotoxicity. F3 (18.24% of explained variance) are related with genotoxic results (DNA damage in kidney, notched, blebbed and total nuclear abnormalities) and levels of Cr in liver. This association was relevant mainly in P1 with a minor score in P5. Still in F3, another group of variables included the frequency of vacuolated nuclei associated with metals in liver (Zn and Cr) with the highest score in P6.

Table 2 Loadings of dry season after varimax rotation for the three factors obtained in the PCA[a]. The variance of the principal factors is given in percentage of the total variance in the original data matrix

	Dry season			Rainy season		
Factors	**F1**	**F2**	**F3**	**F1**	**F2**	**F3**
Variance	44.70%	22.12%	18.24%	38.40%	27.70%	16.82%
Mn muscle	-	-0.498	-	-0.951	-	-
Zn muscle	-	-0.745	-	-	-	-
Co muscle	0.911	-	-	-	-0.982	-
Ni muscle	0.970	-	-	-	-0.794	-
Cd muscle	-0.910	-	-	-	-	-
Pb muscle	0.903	-	-	-0.986	-	-
As muscle	-	-0.777	-	-	-	-0.828
Cu liver	-	0.843	-0.486	-	-0.912	-
Mn liver	-	-	-	-	0.639	-0.506
Zn liver	0.819	-	-0.481	-	0.602	-0.597
Cr liver	0.850	-	0.479	-0.970	-	-
Co liver	0.908	-	-	-	-0.510	0.578
Cd liver	-	-	-	-	-0.973	-
As liver	-	0.793	-	-	-0.923	-
Pb liver	-0.628	0.617	-	-0.948	-	-
PAHs (2 rings)	-0.615	-0.746	-	-	-0.943	-
PAHs (4 rings)	-0.579	-0.776	-	-0.854	-	-
PAHs (5 rings)	-0.821	-	-	-	-	-0.847
PAHS (6 rings)	-0.775	-	-	-	-	-0.872
Total nuclear abnormality	-	-	0.897	-	-0.502	-
Micronuclei	-0.638	-		-0.963	-	-
Blebbed nuclei	0.444	-	0.732	0.513	-	-0.825
Vacuolated nuclei	-	0.590	-0.566	-0.574	-	0.500
Notched nuclei	0.544	-	0.830	-0.957	-	-
DNA damage in kidney	-	-	0.979	-	-	-
DNA damage in liver	-	0.789	-	-	-	0.681
DNA damage in gills	-	0.983	-	0.900	-	-
DNA damage in bood	0.922	-	-	-	0.549	-0.805

[a] Loadings considered as a components of the factors in dry season are >0.44 and in rainy season >0.50.

The FA/PCA applied to the rainy season data matrix rearranged the data into three new variables (Table 2). F1 (explained 38.4% of the variance in the original dataset) showed genotoxicity responses (frequencies of micronucleus, notched and vacuolated nuclei) associated with 4-ring PAHs in bile, and bioaccumulated metal (Mn and Pb in muscle and Cu and Pb in liver tissues) (Table 2). The estimated scores for this factor are higher in the sites affected by Ribeira do Iguape river (mainly P1) (Table 3). F2 (27.7% of explained variance) is associated with DNA damage in blood cells and bioaccumulated Zn and Mg in liver tissues. Such association occurred with a high score in P6. Other association with F2 included total nuclear abnormalities, As and accumulated Cd, Cu, Co in liver, and Co and Ni in muscle, including 2-ring PAHs. This was relevant only in P4. F3 (16.8%) associated genotoxicity (DNA damage in erythrocytes, and blebbed nuclei frequency), PAHs in bile (5- and 6-rings), As contents in muscle, and Mn and Zn in liver (Table 2). These relationships were strongly relevant in P6 (Table 3).

Table 3 Factor scores estimated for each of the sampling stations in Dry (D) and Rainy (R) seasons evaluated in the APA-CIP to the centroid of all cases for the original data.

Station	**F1**	**F2**	**F3**
Dry season			
P1D	-1.124	1.104	0.333
P3D	-0.830	-0.481	-0.082
P4D	-0.034	-1.469	-0.033
P5D	1.182	0.292	1.280
P6D	0.806	0.554	-1.498
Rainy season			
P1R	-1.935	0.049	0.176
P2R	-0.158	0.311	-0.053
P3R	0.520	0.675	1.248
P4R	0.366	-1.989	-0.044
P5R	0.825	0.349	0.461
P6R	0.382	0.606	-1.787

4. DISCUSSION and CONCLUSION

The results of the current study showed that contaminants from land-based activities, i.e. a former mining area and urban sewage discharge, are, in general, inducing genotoxic effects in fish from the APA-CIP. The FA/PCA demonstrated that relationships between bioaccumulated metals (mainly Cr, Zn, Co, Pb and Cu) and genotoxic effects were constantly found during both the dry and rainy seasons.

Chromium was the metal most frequently associated with genotoxicity in the current study. Previous studies corroborate the genotoxic effects of Cr in other fish, including *Carassius auratus gibelio* exposed to three doses of chromium (Cr(VI) and Cr(III)) (Al Sabti et al., 1994), *Oreochromis niloticus* exposed to water from a chromium-containing tannery effluent discharge site (Matsumoto et al., 2006), *Oncorhynchus mykiss* after exposure to environmental concentrations (50, 100, and 200 µg/L) of waterborne chromium (VI) (Li et al., 2011) and *Heteropneustes fossilis* exposed to $K_2Cr_2O_7$.

However, in the studied area, a previous investigation showed that Cr is natural in the estuarine sediments and is not related with mining operations (Mahiques et al., 2009). But other metals bioaccumulated mainly in liver tissue were associated with genotoxic responses in the studied area (e.g. Zn, Co, Cu, Pb). Pb in muscle was also frequently related to genotoxicity. Previous studies reported genotoxic effects in *Hoplias malabaricus* (Ramsdorf et al., 2009) and *Prochilodus lineatus* (Monteiro et al., 2011) in response to inorganic lead (PbII) exposure. This metal has also been related with lethality in fish as well as decreased reproductive performance, growth, and behavior alterations under sub lethal exposure (Burden et al. 1998).

Copper exposure has also been related to increased genotoxicity in marine and freshwater fish, such as *Sparus aurata* (Gabbianelli et al., 2003) and *O. niloticus* in vivo (Harabawy and Mosleh, 2013) but the existence of strong mechanisms to limit internal Cu variation in fish suggest that this essential metal (McGeer et al., 2003; Orieux et al., 2011) is presented in very high bioavailable concentrations.

The relation of Zn with genotoxicity in fish is, therefore, not as evident in the literature as described to other toxic metals. However, previous studies reported modification in hematological parameters and induced disturbances in both specific and non-specific immune mechanisms of fishes (Witeska and Kosciuk 2003).

Toxic metallic ions present in general a well-known potential to cause DNA and chromosome damage due to their ability to increase levels of reactive oxygen species (ROS) in cells. This process is explained directly by via Fenton or Haber–Weiss-type reactions, or indirectly, via interaction with ROS scavenger molecules, such as glutathione and cystein (Atli and Canli 2010; Stohs et al., 2001; Gurer and Ercal, 2000). Such processes may change the cell to a pro-oxidant state leading to oxidative DNA lesions as base oxidation and DNA strand breaks (Livingstone et al., 2000; Akcha et al., 2004). Adittionally, some toxic metals, depending on the level of contamination, can be involved in diseases such as cancer (Linder, 2001; Costa et al., 2008; Ramsdorf et al., 2009; Fatima et al., 2014). Metals as Cr and Pb, can also interact directly to DNA molecules by covalent binding (Zhitkovich et al., 1996; Hong et al., 2007), causing damage and, eventually, cell death through apoptosis (Rudolph and Cervinka, 2006).

The current results showed that the individual genotoxicity analyses, when analysed by only univariate statistical methods, did not respond directly to the levels of bioaccumulated metals in the tissues. Micronuclei incidence was found most at relatively low levels while NA were more frequent. The analyses of MN and NA were more frequently associated with the bioaccumulated metals than other lesions as DNA strand breaks. This can be partially explained because DNA strand break detects damage at molecular while MN/NA analyses quantify damage at chromosome level (Wirzinger et al., 2007). At a molecular level, DNA damage assays (assessed through comet assay or alkaline precipitation) detects alkali-labile sites or DNA lesions that are repairable; in the other hand, at a chromosome level (MN and NA), the analyses detect fixed mutations which are persistent for at least one mitotic cycle (Kassie et al. 2000). Therefore, the induction of MN and NA from cell injury and mitotic errors may be a result of not-repaired DNA damage, and it may be accompanied by cell death as apoptosis and necrosis (Omar et al., 2012). Although the MN levels observed in the current study were low, the assessment of genotoxicity at chromosome level tends to be better associated with contamination exposure than the molecular-level assessment. These results suggest that the molecular levels are less vulnerable to natural environmental variations common in estuaries. In addition, at moderate levels of environmental contamination, the effects tend to be more difficult to differentiate from the effects of environmental contaminants.

I addition, the MN results indicate that some chromosome responses may be also related with seasonal differences, e.g. water temperature. As can be seen in Table 1, MN cells frequencies in the different sampling sites were higher during the dry season (which corresponds to the austral winter with lowest water temperatures during the year) compared with rainy season (austral summer). This may suggest that the mechanism of chromosome repair may be more effective during the rainy season, when water temperature is higher. A previous study showed a role of low temperature in DNA damage, probably because protein synthesis decreased due the low RNA translation rates, thus DNA repair mechanisms can be slower an less efficient leading to genetic damage (Beninca et al., 2013; Pellacani et al., 2006; Buschini et al., 2003). Although literature show evidences of mechanisms of repairing genetic material at only molecular level, our results suggest that the repair mechanisms also act at chromosome level.

Metals bioavailability in aquatic organisms is closely related with salinity, being the main controlling factor for the partitioning of contaminants between sediments and overlying or interstitial waters in estuaries (Chapman and Wang, 2001). The influence of salinity on the geochemical behaviour, and also the bioavailability of metals, is dependent of two counteractive processes: (i) desorption due to increase complexation with sea water anions (Cl^- and SO_4^{2-}) and/or increasing competition for particle sorption sites with sea water cations (Na^+, K^+, Ca^{2+}, Mg^{2+}); (ii) coagulation, precipitation and flocculation (Chapman and Wang, 2001). Furthermore, according to the biotic ligand model, water hardness affects the toxicity of metals, once that Ca^{2+}, Mg^{2+} have a ‘protecting’ status relative to the toxicity of divalent cationic metals (Paquin et al. 2002).

Another factor that can influence these differences concerns to bioaccumulated metal is the use of different tissues for the analyses. The genotoxic responses assessed in peripheral blood were more frequently associated with metal body burdens than the responses in kidney, liver, and gills tissues. Variation in damage of genetic material is expected due to differential background levels of DNA single-strand breaks in tissues with different activity of excision repair, metabolism, antioxidant concentrations, or other factors (Lee and Steinert 2003; Ali et al., 2008). The higher responsiveness of blood tissue was also reported by Ramsdorf et al. (2009). The authors discussed that alterations occur during a time interval equivalent to the cell cycle of organ; thereby cells from peripheral circulation reflect events that have occurred in a time equal to the lifespan of the circulating erythrocytes, which tend to be greater than the lifespan of cells in kidney, liver, and gill tissues.

The FA/PCA results showed that, during the dry season, fish from the sites located closer to the RIR mouth (P1 and P3) showed increased levels of PAHs in bile, Pb in liver, and Cd in muscle associated with higher micronuclei frequency. The site closer to RIR (P1) showed, additionally, increased levels of As, Cu, and Pb in liver, vacuolated nuclei, and higher DNA damage in liver and gills. During the rainy season, again P1 showed increased Pb levels in both liver and muscle associated with other metals (Mn in muscle and Cr in liver), 4-ring PAH, micronuclei, vacuolated nuclei, notched nuclei, and DNA damage in gills.

Such results suggests that *C. spixii* from the sites under influence of the RIR were affected by the continuous input of metal from the RIR basin. Other authors reported the contribution of the RIR for the contamination of the APA-CIP environment. Mahiques et al. (2009) performed a historical analysis of the sediments of the APA-CIP and reported that, previously to the opening of the Valo Grande Channel, some metals as Pb, Cu, Zn and Cr were found in low levels in the APA-CIP sediments. The levels of these metals, however, increased significantly after the RIR flow, was deviated into the estuary through the Valo Grande Channel. The metal contamination in the RIR has been attributed to the presence of mining wastes from former mining activities (Au, Ag and specially Pb) at the upper river course. Guimarães and Sígolo (2008) found high levels of Pb, Zn, Cu and Cr in suspended sediments from the upper to the mouth of the RIR in the APA-CIP.

Despite the high metal contamination in the RIR reported by previous studies, the levels of metals in the sediments of the APA-CIP have been found at moderate levels (Mahiques et al., 2009). However, the current data showed that these metals may be available to demersal fishes, such as the *C. spixii*, and they can cause genotoxicity. The bioavailability of metals to *C. spixii* is corroborated by a previous study that reported the levels of Pb, Cd, Hg, Cu and Zn in the muscle tissue of *C. spixii* from the APA-CIP equivalent to levels found in specimens from a highly polluted estuary in Brazil (Santos-SP) (Azevedo et al., 2012). The demersal behaviour and benthic foraging habits of this species may contribute to the exposition of these organisms to the contaminated sediment, which, even at moderate levels, are affecting the health of the fishes.

The site closest to Cananéia city (P4) showed increased As and bioaccumulated metals in muscle (Mn and Zn) and 2- and 4-ring PAHs, but, in the dry season no association with genotoxicity was found. In the rainy season, however, As and metals not only in liver (Cu, Co, and Cd), but also in muscle (Co and Ni), were associated with increased 2-ring PAH and total nuclear abnormalities. Other sites under the influence of the city (P5, P6) showed genotoxicity responses associated with bioaccumulated metals in both muscle and liver during the dry season. During the rainy season, the genotoxicity effects do not appear in fishes from P5, but they are relevant in P6, in association with metals in liver (Mn and Zn), As in muscle, and 5- and 6-ring PAHs. These results suggest that the city of Cananéia and mining activities are important sources of pollution concern to toxic metals and PAHs to the APA-CIP. The current findings corroborate the study of Cruz et al. (2014), which related the presence of contaminants and toxicity of APA-CIP sediments with the urban runoff from the city of Cananéia.

Notwithstanding metals seem to be the main class of contaminants related with genotoxicity in the APA-CIP, despite of PAHs in bile also has been related with genotoxicity at chromosome level (MN and NA) or even DNA damage in gill tissues in areas impacted by RIR. The higher correlation between PAHs metabolites in bile found during the dry season indicates that these compounds have the same source of pollution in this season as urban activities, since there is a slight increase in PAHs in bile of *C. spixii* from areas closer to the city (Figure 3a). Data of PAHs in bile during rainy season is not well correlated as the dry-season data, indicating that there is a differential input of individual PAHs along the APA-CIP. This is clearly demonstrated in Figure 3b, where a substantial increase of 2-ringed PAHs (usually associated with petrogenic sources) can be observed in P4 the closer site to Cananéia city. Sources of PAHs in the area impacted by urban activities include also the nautical structures (marinas, decks, nautical garages, and small docks), ferry boat stations, nautical gas station and urban drainage (Cruz et al. 2014).

In the current study 4-ringed PAHs metabolites in bile (related to pyrolytic sources) were the most related with genotoxicity, especially regarding rainy season. Closer to Cananéia city, also 2-ringed PAH is related to the total nuclear abnormalities, again during the rainy season. It is well-known that PAHs are associated with strong mutagenicity and carcinogenicity (Myers et al., 2003). These events include mechanisms of chromosomal breakage (leading to the formation of MN and other NA), but are still not fully understood (Costa et al., 2008). Other studies described the association between induction of MN and NA in fish exposed to PAHs (Çavas and Ergene-Gozukara, 2005; Ergene et al., 2007; Costa et al., 2008).

The associations described in the current study among bioaccumulated As and metals and levels of PAHs in bile with genetic biomarkers (DNA breakage, micronuclei frequency and other nuclear abnormalities) in *C. spixii*, provided evidences that these contaminants, even in a moderately contaminated area, causes genotoxic effects and potentially affect the health of fishes in the APA-CIP. Genotoxicity responses in *C. spixii* were suitable to identify sources of pollution. Additionally, the use of different organs was very important to detecting environmental risk of exposure to biota related to genotoxicity and complex mixtures of contaminants in the environment.

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CAPÍTULO III

Capítulo formatado de acordo com as normas da revista "Environment Pollution"

Assessing moderately contaminated estuaries: the use of a multilevel-biomarker approach in fish within an WOE-based sediment quality assessment

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ABSTRACT

Previous studies in highly impacted environments have attested the usefulness of biomarker tools as a part of Weight of Evidence assessment (WOE) in marine environments. However, once that isolated responses of biomarkers are often difficult to interpret due to many confounding factors, the integration of biomarker data into multiple-biomarker indices for the evaluation of contaminant-induced stress is a helpful tool to aid in the interpretation of the responses. The aim of this study was to evaluate the suitability of an weighted index of biomarker responses (WBR) within a WOE approach to assess a global relevant Marine Protected Area (the Cananéia-Iguape-Peruíbe Environmental Protected Area, Brazil), which is subjected to moderate levels of contaminants. The research included: (i) exposure assessment, through the estimation of indices integrating non-specific biomarkers of exposure (GST, GPx, GSH), as well as metallothionein levels, PAHs in bile, levels of metals and As in sediments and in resident fish (liver and muscle tissues); (ii) effects assessment in both resident fish (estimation of indices integrating biomarkers of effects - LPO and DNA damage in target organs, macromolecular damages, histopathology, general health condition - and activity of AChE in muscle) and sediments (toxicity tests - copepod fecundity and sea urchin embryolarval development); (iv) environmental quality characterization as integration of individual Lines of Evidences (LOEs) through multivariate analysis (FA/PCA). The application of WBR is a promising approach to MPA environmental quality assessment, simplifying the interpretation of the individual biomarker responses to environmental contaminants. The WBR that incorporates histopathological data (WBReffects in liver) in resident fish was better associated with sediment toxicity and contamination. On the other hand, the biomarker indices that included only sub-cellular and cellular responses were better associated with bioaccumulated metals and As. An internal mechanism of detoxification (either accumulation and/or depuration) can explain the lack of relationship between damages in tissues and sediment contamination. The inclusion of biomarker indices based on sub-cellular, cellular, and histological responses within the WOE approach, was useful to discriminate the different sites within a range of environmental degradation in this moderately contaminated estuary-lagoon.

Key words: weight-of-evidence; factor analysis; sediment quality; biomarkers; environmental risk assessment.

1. INTRODUCTION

In environmental studies, the weight-of-evidence (WOE) approach is the process of integrating information from multiple lines of evidence (LOEs) to reach a conclusion about an environment or stressor (Burton Jr et al., 2002). The most common LOEs used in environmental quality studies or risk assessments are those that compose the Sediment Quality Triad (SQT) (Long and Chapman 1985; Chapman 1990): (i) sediment chemistry, (ii) benthic infaunal community structure, and (iii) laboratory-based toxicity assays (Burton Jr et al., 2002; Chapman and Hollert, 2006).

Subsequent discussion papers (e.g. Chapman, 2000; Chapman and Hollert, 2006) have encouraged the evolution of the SQT, maintaining its multidimensional essence but incorporating novel LOEs into its basic framework. For example, contaminant body burdens (Borgmann et al. 2001) and potential for biomagnification (Grapentine et al., 2002; Chapman and Anderson, 2005) were proposed to integrate WOE-based sediment quality assessments. Recently, Torres et al. (2014) assessed multiple LOEs applied to sediment quality assessments, among them the ratio of simultaneously extracted metals and acid volatile sulfides (SEM-AVS) in sediments; levels or PAHs in semipermeable membrane devices (SPMDs); bioaccumulation of native and transplanted oysters; besides the classical SQT components (levels of contaminants in sediments, sediment toxicity, and indices for benthic community structure description).

In the last ten years, the use of biomarkers became widespread in environmental studies (Bonnineau et al., 2012). Its integration within the scope of WOE-based environmental quality/risk assessments has been utilized in studies of highly contaminated marine areas worldwide (e.g. showing several exceedences of sediment quality guidelines) (Galloway et al., 2004; Martín-Diaz et al., 2004, 2008; Pereira et al., 2012, 2014; Piva et al., 2011; Benedetti et al., 2012; Carreira et al., 2013; Souza et al. 2013) and to establish risk assessment in case of particular hazards, such as acute and chronic oil spills (Jimenez-Tenorio et al., 2008; Morales-Caselles et al., 2008, 2009), CO_2 injection and storage (Reguera et al., 2009), natural hydrocarbons seepage (Benedetti et al., 2014), and the Costa Concordia ship-wreck (Regoli et al., 2014).

Although there are previous studies attesting the usefulness of biomarker tools within the scope of a WOE assessment in marine environments, such studies were only carried out in highly impacted environments. For Marine Protected Areas (MPA), which are, in some instances, subjected to a not too high but continuous input of contaminants from outer their boundaries (Palmer et al. 1996; Boersma and Parrish, 1999; Pozo et al. 2009), no information can be found about the suitability of biomarker tools integrated in WOE-based assessment. Even in sites where contamination is mild, a long term exposure can affect the health of aquatic organisms (Nipper et al. 1998). The ecological impacts of sublethal effects are still poorly understood because they can be more subtle and therefore harder to detect and measure, taking longer times to manifest (Barbee et al., 2014).

In addition, especially in estuarine environments, biological assessments are sensitive to many confounding factors, which can lead to false-positive diagnostics. Estuaries are under a natural “permanent stress” (in Environmental Risk Assessment this is known as “the estuarine quality paradox”) due to periodic variation of parameters such as salinity, organic matter input and others, which makes the differentiation between natural from human-induced stress particularly difficult (Elliott and Quintino, 2007; Gonçalves et al 2013). In addition, biomarker analyses are sensitive not only to environmental variables related to seasonality and natural events (Freire et al., 2011; Rodrigues et al., 2012; Vinagre et al., 2012; Madeira et al., 2013), but also to biological factors such as starvation, bacterial infections, parasitic infestation, physiological seasonal variation, age and reproductive status, types of tissue and individual variation (van Der Oost et al., 2003; Au, 2004; Solé et al., 2010; Tomasello et al., 2012). In mildly contaminated areas, these findings may have greater importance in misleading the diagnostic of environmental quality/risk assessment than in highly contaminated areas where pollution-induced biological effects tend to be clearer.

Ideally, WOE frameworks should be as quantitative as possible; logical and transparent; readily understandable by non-expert personnel; incorporate judgments about the quality, extent and congruence of the information in each LOE; and, draw on a broad range of interdisciplinary expertise to encompass the primary exposure and effect linkages (Burton Jr et al., 2002; Chapman et al., 2002). The integrated use of the environmental information (encompassing the assessment of exposure and effects, and characterization of risk – Chapman, 2007) should allow a clear determination of the risk of ecological impacts from chemical or other stressors, ultimately subsidizing informed and sound decision-making process (Chapman et al., 2002). The WOE framework should also be able to determine if sufficient information has been gathered and to appropriately differentiate between hazard (the possibility of impact) and risk (the probability of impact) (Chapman et al., 2002).

In the current study it was hypothesized that biomarkers analyses, integrated within the scope of a ‘classical’ WOE-based sediment quality assessment (sediment toxicity tests and sediment physical-chemical properties) are adequate for assessing or monitoring the environmental quality of moderately contaminated coastal environments, like MPAs. In this context, the objective of the current study was to assess correspondences, complementarity, or conflicts among multiple sediment quality LOEs, including biomarkers analyzes, through a WOE sediment-quality assessment in a moderately contaminated estuarine-lagoon protected area (Cananéia-Iguape-Peruíbe, Southern Brazil). The research included the exposure assessment, effects assessment, toxicity tests and environmental quality characterization.

The inclusion of the biomarker approach within the WOE scope should provide useful information for subsidizing environmental decision-making in the management of MPAs.

2. MATERIALS AND METHODS

2.1 Study Area

The Cananéia-Iguape-Peruíbe Environmental Protected Area (hereafter referred as the APA-CIP) (between latitudes 24°40’S and 25°05’S) integrates the global list of UNESCO’s World Heritage Sites as part of the Biosphere Reserve of the Atlantic Rainforest, and it is considered a priority area for future inclusion on the list of Brazilian wetlands of international importance within the scope of the Ramsar Convention (Brazil 2012).

The main freshwater contributor to the CIP estuary is the Ribeira de Iguape River (RIR). Approximately 70% of the course of the river flows toward the lagoon waters through an artificial channel locally known as “Valo Grande”. The river basin is a metallogenic province with natural Pb and Zn deposits (Moraes et al. 2003). Metal mining and smelting activities were performed in this area for many decades during the 20th century, but the mines were closed down in the 1990s. Since then, high levels of metals (Pb, Zn, Cu, Cr) and arsenic (As) have been recorded in the river waters, as well as in both bottom and suspended sediments (Eysink et al. 1988; Corsi and Landin 2003; Moraes et al. 2003; Guimarães and Sígolo 2008). The metal contamination of lagoonal-estuarine sediments has increased substantially after the “Valo Grande” channel was opened (Mahiques et al. 2009).

Apart from its important natural features, the APA-CIP also encompasses three cities (Iguape, 30,259 inhab.; Ilha Comprida, 9,025 inhab.; Cananéia, 12,601 inhab.) (IBGE 2014). Waste is discharged in rivers, groundwater, or directly into the lagoon system, since sewage treatment is insufficient for local demand (Morais and Abessa 2014).

2.2 Fish and sediment sampling

Six sampling sites were set along the APA-CIP area with the aim of encompassing the main potential contaminant sources along the APA-CIP (Figure 1). Thus, site P1 is the closest to the RIR mouth and site P4 is the closest to the Cananéia city. To account for seasonal variation, sediment and fish were synoptically sampled during two seasons with different amounts of rainfall: (i) the dry season (D) (August 2012); and (ii) the rainy season (R) (March 2013). The average rainfall during these seasons was 111mm and 390mm, respectively (CEPAGRI, 2014). Sediments were sampled with a 0.036 m^2 stainless steel Van Veen grab sampler and kept chilled during field work. In the laboratory, aliquots for toxicity tests were kept refrigerated at 4ºC, while those for physical-chemical analyses were stored at -20ºC.

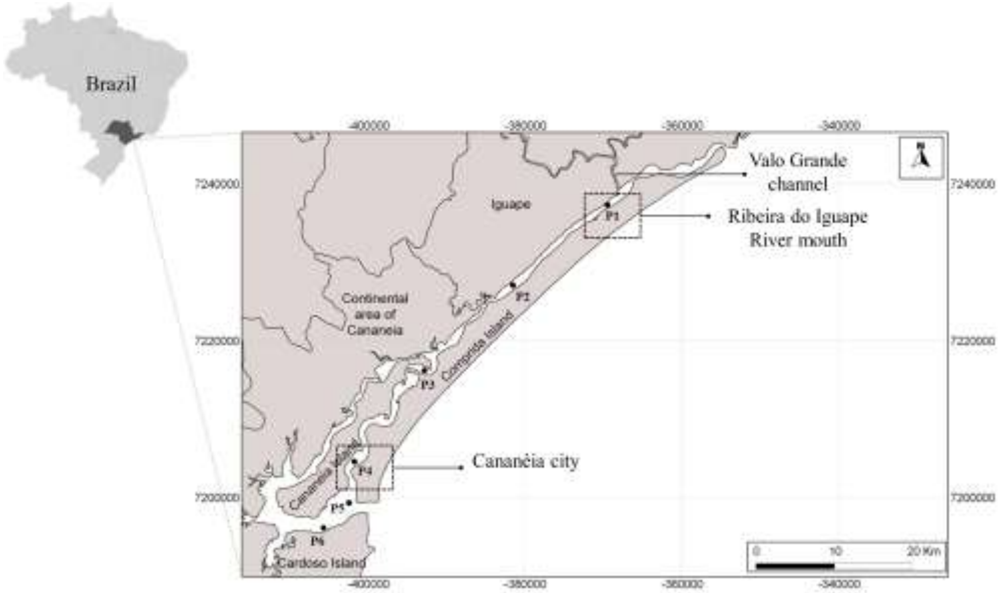

Fig. 1 Sampling stations located within the APA-CIP, Brazil

Fifteen specimens of a demersal catfish (*Cathorops spixii*) were collected in each of the sampling sites using a bottom otter trawl. The individuals were kept alive during field work and, in laboratory, they were anesthetized with benzocaine 0.01% in water, and then weighted, measured, and the peripheral blood was withdraw from the caudal vein using heparinized syringes to the analysis of biomarkers in blood (comet, micronucleus, and nuclear alterations assays). The individuals were then euthanized by spinal cord section and fish kidney, liver, gills, and axial muscle tissues were dissected for biomarker analyses, and bile was extracted for PAH metabolites quantification. Tissues and bile were stored at -80 °C until the analyses. For histopathological analyses, samples of liver tissue were dissected and immediately preserved in ALFAC fixative solution for 16h. Axial muscle and liver tissues used in metal and As body burden analyses were stored in plastic vessels at -20ºC.

2.3 Sediment characterization

Sediment grain size distribution was analyzed based on the protocol proposed by Mudroch and MacKnight (1994) and the results were classified based on the Wentworth scale. The calcium carbonate ($CaCO_3$) contents in each sample were measured using the method described by Hirota and Szyper (1976). Organic matter (OM) contents in the sediment samples were estimated using the ignition method (Luczak et al. 1997).

2.4 Metals and As quantification in sediments and fish tissues

The acid extraction of metals (Cd, Cu, Ni, Pb, Zn, Cr, Fe and Mn) from sediments was performed based on the technique adopted by the USEPA for wet sediment samples (Allen et al. 1993; Machado et al., 2004) and determined by ICP-OES (induced coupled plasma – optical emission spectrophotometry) (Spectro Ultima 2). The precision of the analysis was estimated by comparing the difference between the mean concentration and that obtained by one of the replicates. Values of precision were 100% to Cr, Cd and Zn; 90% to Pb and Mn; 88% to Fe; 92% to Ni; and 99% to Cu. Metal and As concentrations in sediments were expressed in mg kg^{-1}.

Metals and As loads in axial muscle and liver tissues (Cu, Mn, Zn, Cr, Co, Ni, Cd, Pb) were performed according to standard method 200.9 (USEPA 1994). Metals were quantified using flame atomic absorption spectroscopy (FAAS) (Varian®, AA 240FS). The concentration of As was determined using an atomic absorption spectrophotometer (Varian®, AA 240Z) equipped with a graphite furnace (AAS-GF) (Model, GTA 120) equipped with a transverse Zeeman corrector for background correction, automatic sampling (model PSD 120). Standard curves were prepared using reference material (Qhemis High Purity®) and recovery rates ranged from 80% to 120% in all investigations. Metal and As concentrations in tissues were expressed in mg kg^{-1} dry weight.

2.5 Polycyclic Aromatic Hydrocarbons in bile

The metabolites of polycyclic aromatic hydrocarbons (PAHs) in the bile of *C. spixii* were quantified via fixed-wavelength fluorescence at wavelengths 288/330 nm, 334/376 nm, 364/406 nm and 380/422 nm ($\lambda ex/\lambda em$) in the spectrofluorometer (Model, Sunrise-Tecan), following method described in Aas et al. (2000) and Oliveira Ribeiro et al. (2005).

2.6 Biomarkers analyses

2.6.1. Biomarkers at subcellular and cellular level

Kidney, liver, gills and muscle tissues used in biochemical analyses (i.e. activities of glutathione S-transferase (GST), glutathione peroxidase (GPx) and acetylcholinesterase (AChE), levels of non-protein reduced thiols (GSH) and lipid peroxidation (LPO)) were homogenized at 10% W/V in Tris-HCL buffer (TRIS 50mM; EDTA 1mM; DTT 1mM; sucrose 50mM; KCl 150mM; PMSF 1mM, pH 7.6). Homogenates were centrifuged at 10.000×g for 20 min at 4 °C. Liver, kidney, and gill tissue samples were also set aside for metallothionein-like protein (MTs) activity analysis, homogenized with 20mM Tris–HCl buffer supplemented with 0.5M sucrose, 0.01% β-mercaptoethanol, and centrifuged at 15.000×g for 30min at 4 °C.

GST (Keen et al., 1976) and GPx activities (Sies et al., 1979) were determined spectrophotometrically at 340nm. GSH levels were measured spectrophotometrically at 415nm (Sedlak and Lindsay 1968). AChE activity analysis was performed at 415nm using the colorimetric method (Ellman et al., 1961). The concentration of metallothionein-like protein (MTs) was quantified using Ellman's reagent containing DTNB at 412nm (Viarengo et al., 1997). Levels of lipid peroxidation (LPO) were determined by quantifying the concentration of 2-thiobarbituric acid reactive substrates (TBARS) through fluorescence (λ_{ex} 532nm and λ_{em} 556nm) (Wills et al., 1987). Protein concentrations were determined spectrophotometrically at 595nm (Bradford, 1976), with BSA as the standard. All biomarkers analyses were performed in a microplate reader (Biotek-Synergy™ HT).

DNA strand breaks were measured in homogenized tissues of kidney, liver, and gills tissues through the alkaline precipitation assay (Olive 1988; Gagné and Blaise 1995) by fluorescence (λ_{ex} 360nm and λ_{em} 450nm). In the peripheral blood, DNA damage was evaluated by the comet assay through the alkaline single gel electrophoresis (SGCE), following procedures described by Singh et al. (1988) and Ferraro et al. (2004); the damages were scored following methods described in Ramsdorf et al. (2009). Fish micronuclei (MN) analysis was performed in accordance with Heddle (1973) and Schimd (1975) and nuclear abnormalities (NA) were characterized in accordance with Carrasco et al. (1990) by smearing blood aliquots on glass microscopy slides, fixing with absolute ethanol (30min) and staining with acridine orange. For each animal 2,000 erythrocytes were examined under Leica®

epifluorescence microscope at 1,000x magnification and scored for the presence of both typical MN and NA.

2.6.2 Biomarker at tissue level

After fixing (as described in section 2.2), liver samples were dehydrated in a graded series of ethanol and embedded in Paraplast-Plus (Sigma®) (Oliveira Ribeiro et al., 2005). Sections of 5µm were performed, stained with hematoxylin/eosin, and assessed under light microscope. The hepatic histopathological index, which is based on importance and extension of the observed pathologies, was calculated according to Bernet et al. (1999).

2.6.3 Biomarker of general health status

Fulton's condition factor was calculated according to the formula: CF = (W/L^3 x 100), where CF = Fulton condition factor, W = body weight (g), and L = total body length (cm).

2.7 Sediment toxicity tests

Two different sediment matrices were assessed with toxicity tests: (i) whole sediment (WS); and (ii) sediment-water interface (SWI). In the first assay, the endpoint was the fecundity of the benthic copepod *Nitocra* sp. (Lotufo and Abessa, 2002) (photoperiod 12:12h light:dark; temperature 25 °C±2; salinity 17). After the exposure time (7d) under static conditions, the number of adult females and their offspring (nauplii and copepodits) were counted using a stereomicroscope. In the second assay, the embryo-larval development test with *Lytechinus variegatus* (ABNT, 2006) was performed (photoperiod 12:12h light:dark; temperature 25 °C±2; salinity 34), with modification for using reduced volumes of experimental medium (Cesar et al., 2004). After an incubation period of 24h under static conditions, the percentage of normal embryos was estimated.

Reference sediments were collected in a non-contaminated site located at Ilhabela, São Paulo (Araújo et al., 2013; Cruz et al., 2014). Four replicates were done for each test. Physical-chemical parameters (pH, salinity, dissolved oxygen and temperature) of the overlying water in the test chambers were checked at the beginning and at the end of each assay.

2.8 Data treatment

2.8.1 Weighted Biomarkers Response (WBR)

Once that isolated responses of biomarkers are often difficult to interpret due to many confounding factors, including seasonality, the integration of biomarker data into multiple-biomarker indices for the evaluation of contaminant-induced stress is a helpful tool to aid in the interpretation of the responses (Beliaeff and Burgeot, 2002; Haager et al., 2009).

The Weighted Biomarkers Response (WBR) was built upon previous biomarker indices, i.e. the Integrated Biomarkers Response (IBR) (Beliaeff and Burgeot, 2002; Sanchez et al., 2013) and the Biomarkers Response Index (BRI) (Hagger et al., 2008, 2009). For a better description of the *C. spixii* biomarker response at each season and sampling site: (i) the WBR was standardized by the mean value at each season (see details below), in order to avoid the influence of physiological seasonal variations in the response to environmental contaminants; (ii) WBR was estimated separately for each of the studied organ (kidney, liver, gill, and peripheral blood tissues); (iii) WBR differentiates between effects and exposure, as biomarkers of effects (DNA damage and LPO in the kidney and gill tissues; DNA damage, LPO, and histopathology in the liver tissue; and the comet score, MN, and NA in the blood tissue) and exposure (antioxidant responses, i.e. GST, GPX, and GSH, in kidney, liver or gill) were integrated separately ($WBR_{Effects}$ and $WBR_{AOxResp}$, respectively); (iv) different weight to each response was attributed in the WBR calculation; thus, subcellular responses weighted 1 (W=1) (GST, GPX, GSH, LPO, DNA damage), cellular responses weighted 2 (W=2) (MN and NA), and histological responses weighted 3 (W=3) (histopathological index). The MT and AChE data were subjected to the same standardization process, but they were not integrated into the WBR, in order to allow the assessment of these biomarkers individually in the integration with the other variables.

For the WBR calculation, the data was standardized based on the IBR (Sanchez et al., 2013), with modifications. Individual biomarker data (X_i) was firstly divided by the mean value at the season (dry or rainy) ($\overline{X}$) and was subjected to a log transformation to reduce variance (Y_i = log ($X_i/\overline{X}$). Y_i was subsequently standardized by subtracting the new mean value ($\overline{Y}$), and dividing it to the standard deviation (σ) (Z_i = (Y_i-$\overline{Y}$)/σ). This step created an average line centered on 0 for each biomarker data, i.e. the closer the value was from 0, the closer it was from the mean value of that season. Therefore, the negative values were lower, and the positive values were higher than the mean value. These values were the final scores for MT and AChE, which were not integrated to any other biomarker. The final score for $WBR_{Effects}$ of each station was given by $WBR_{Effects} = \sum W.Z_{i\ (Effects)}$ (W is the weighting factor). For the calculation of the final score of $WBR_{AOxResp}$, the values entered the final calculation as their absolute value ($|Z_i|$) since negative values might represent an inhibition of the antioxidant system as a response of environmental contamination. Thus, the final score of antioxidant responses was given by $WBR_{AOxResp} = \sum W.|Z_i|_{(AOxResp)}$.

2.8.2 Multivariate approach

Factor analysis (with principal component analysis as the extraction method) (FA/PCA) was used to highlight associations among the variables measured in this study. Associations between the different biomarkers scores for each organ (MT, AChE, $WBR_{Effects}$ and $WBR_{AOxResp}$), metals (Cu, Mn, Zn, Cr, Co, Ni, Cd, Pb) and As loads in liver and muscle tissues, PAH metabolites in bile, sediment physical-chemical characteristics (percentage of fines, carbonates and OM, and levels of Cd, Cr, Cu, Ni, Pb, Zn, Fe and Mn,), and sediment toxicity (sea-urchin embryolarval development and copepod fecundity) were assessed. The selected variables to be interpreted were those associated with the factors with a loading ≥0.45, a value which is more conservative than the loading cut-off recommended by Tabachnic and Fidell (1996). The relevance of the observed associations to each of the 6 sampling stations (cases) was estimated by calculating the factor score from each case for the centroid of all cases for the original data. All the statistical and multivariate analyses were performed using the Statistica 12 software (StatSoft Inc. USA).

3. RESULTS

The original biomarker data used for the calculation of the WBR index was fully presented in the Paper 1 of this Thesis. Tables 1,2 and 3 shows the biomarkers scores, metals and As burdens in muscle and liver, total PAH metabolites in bile, sediment toxicity, sediment properties, and levels of metals in sediments in each sampling site (P1 to P6) during the dry (D) and rainy (R) seasons. Biomarkers responses, metals and As body burdens, and total PAHs were fully discussed in the Papers 1 and 2 of this Thesis. In general, $WBR_{AOxResp}$ are higher in sites P1 and P4 in the dry season, while in the rainy season P3 shows the highest scores. $WBR_{Effects}$ are, in general, higher in the sites closer to the city both during the dry (P5) and the rainy (P4, P5 and P6) seasons, although some sites closer to the RIR mouth (P1, P2, and P3) showed higher $WBR_{Effects}$ to some organs tissues (gill and liver in the dry season, and liver and kidney in the rainy season).

Table 1. Original dataset matrix showing WBR and biomarkers scores in each sampling site (P1 to P6) during the dry (D) and rainy (R) seasons.

	Sampling Stations										
Variables	**P1 – D**	**P3 - D**	**P4 - D**	**P5 – D**	**P6 – D**	**P1 - R**	**P2 - R**	**P3 - R**	**P4 - R**	**P5 - R**	**P6 – R**
AChE score	-0.037	1.220	0.770	-1.048	-0.905	-1.128	-0.509	-0.704	0.961	1.431	-0.051
Kid-MT score	1.680	-0.745	-0.476	-0.611	0.152	1.082	-0.168	-0.945	0.959	-1.346	0.418
Liv-MT score	0.836	-1.051	0.364	-1.088	0.939	1.627	0.166	-0.826	-0.748	0.619	-0.837
Gill-MT score	-0.419	-0.224	-0.370	1.756	-0.743	1.025	1.249	-0.881	0.272	-0.557	-1.109
Kid-WBR$_{AOxResp}$	1.396	2.507	3.406	3.368	0.930	3.721	1.074	3.595	0.619	2.091	2.312
Liv-WBR$_{AOxResp}$	3.357	1.692	2.352	1.803	2.430	1.963	1.975	2.737	2.616	1.721	3.022
Gill-WBR$_{AOxResp}$	3.753	2.149	3.438	1.315	1.137	2.085	3.414	3.526	0.901	2.803	1.445
Kid-WBR$_{Effects}$	-0.858	0.482	-0.330	1.598	-0.892	-0.830	-1.445	1.216	-0.016	-0.007	1.082
Liv- WBR$_{Effects}$	0.045	3.466	-0.029	-5.324	1.843	0.712	4.419	-2.613	1.535	-1.997	-2.055
Gill- WBR$_{Effects}$	1.266	-0.354	-3.094	1.044	1.138	-1.255	0.063	-1.270	2.219	0.453	-0.210
Bld- WBR$_{Effects}$	-1.035	-2.459	-0.431	4.944	-1.019	1.260	-0.658	-1.119	-3.955	3.144	1.328
Condition fator (CF)	1.952	1.093	1.333	1.210	1.028	1.051	0.980	0.794	0.921	0.803	0.900

Table 2. Original dataset matrix showing metals (mg kg^{-1} DW) and As (µg kg^{-1} DW) burdens in muscle and liver, total PAH metabolites in bile (mg prot^{-1}) in each sampling site (P1 to P6) during the dry (D) and rainy (R) seasons

	Sampling Stations										
Variables	**P1 – D**	**P3 - D**	**P4 - D**	**P5 – D**	**P6 – D**	**P1 - R**	**P2 - R**	**P3 - R**	**P4 - R**	**P5 - R**	**P6 – R**
Cu mus	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100
Mn mus	2.015	0.130	3.815	0.130	0.130	3.400	0.272	0.444	0.130	0.130	0.130
Zn mus	11.145	9.193	34.518	12.244	15.365	34.989	24.015	12.102	22.488	65.575	12.120
Cr mus	0.080	0.080	0.080	0.080	0.080	0.080	0.080	0.080	0.080	0.080	0.080
Co mus	0.110	0.110	0.110	273.000	180.000	114.333	98.808	98.241	242.080	122.700	100.421
Ni mus	0.090	0.090	109.348	339.333	201.292	125.444	93.271	102.130	140.892	117.900	85.013
Cd mus	13.636	9.039	10.558	1.111	0.110	0.110	0.110	0.110	0.110	0.110	0.110
Pb mus	32.389	21.641	47.878	73.217	49.835	31.556	8.056	0.110	0.110	0.110	0.110
As mus	2.171	10.730	5725,47	21.976	149.231	2.549	4.263	2.761	7.861	3.017	9.511
Cu liver	79.062	11.231	0.100	22.243	88.926	10.825	25.491	43.766	146.700	10.783	17.374
Mn liver	0.130	0.130	0.130	0.130	0.130	0.130	4.334	2.151	0.130	4.086	5.801
Zn liver	123.932	146.816	143.591	201.473	259.694	347.425	381.068	306.714	110.888	122.574	537.845
Cr liver	5.720	6.077	22.909	39.280	16.218	14.900	6.897	0.080	0.080	0.080	0.080
Co liver	2.586	13.462	160.000	333.586	140.809	229.000	135.260	233.523	250.000	43.414	23.693
Ni liver	0.090	0.090	0.090	0.090	0.090	0.090	0.090	0.090	0.090	0.090	0.090
Cd liver	0.110	0.110	0.110	0.110	0.110	0.110	0.110	0.110	1.180	0.110	1.585
Pb liver	2.126	0.110	0.110	0.110	0.110	6.392	0.110	0.110	0.110	0.110	0.110
As liver	2.783	1.230	1.515	2.184	1.846	0.848	1.791	1.111	7.348	2.548	2.198
Total PAHs in bile	19827.014	32987.368	25348.049	11436.386	13840.499	27405.964	23852.421	9720.727	38033.006	25117.404	24420.257

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able 3. Original dataset matrix showing sediment toxicity (% of abnormal sea-urchin embrio-larval development or mean offspring size per female), sediment properties (%), and metals in sediments (mg kg^{-1}) in each sampling site (P1 to P6) during the dry (D) and rainy (R) seasons.

	Sampling Stations										
Variables	**P1 – D**	**P3 - D**	**P4 - D**	**P5 – D**	**P6 – D**	**P1 - R**	**P2 - R**	**P3 - R**	**P4 - R**	**P5 - R**	**P6 – R**
Sed-Seaurchin toxicity	10.250	9.500	8.000	8.250	4.250	22.750	92.500	34.250	22.000	12.000	10.250
Sed-Copepod fecundity	33.842	43.733	24.333	45.763	17.230	20.233	1.175	5.525	20.863	20.211	26.395
Sed-Mud	0.855	1.625	19.053	2.682	40.396	4.553	98.165	8.536	59.341	16.446	10.156
Sed-OM	0.472	0.598	4.329	1.218	5.440	0.200	13.800	0.600	0.600	16.800	2.000
Sed-Carbonates	4.503	2.646	12.155	9.178	9.452	0.000	8.200	1.600	8.800	5.000	1.830
Cd sed	0.012	0.008	0.019	0.010	0.018	0.021	0.168	0.010	0.037	0.012	0.011
Cr sed	0.339	1.607	2.120	0.179	2.882	1.141	8.589	0.393	3.842	2.429	1.302
Cu sed	0.395	1.967	0.649	2.298	0.262	0.875	7.988	0.290	1.607	0.023	0.164
Fe sed	1211.387	1366.262	3610.820	271.664	3225.715	2846.755	17537.942	1772.216	6472.689	2679.882	2460.768
Mn sed	53.665	10.942	115.866	1.784	43.323	87.341	221.201	16.135	149.247	34.730	26.022
Ni sed	0.010	0.660	0.010	0.010	0.325	0.010	0.010	0.010	0.010	0.010	0.010
Pb sed	1.466	7.427	4.210	0.857	3.716	8.271	32.839	2.233	8.679	1.516	1.834
Zn sed	0.040	13.706	8.729	0.040	10.830	5.069	32.615	4.145	14.406	14.158	11.622

Sediment physical-chemical and toxicity characterization was fully discussed in Cruz (2014). In summary, sediments were predominantly sandy along the APA-CIP, with the exception of P2 and P4 which showed muddier characteristics. Substantial variations in the sediment grain size between seasons were found in the sites closer to the Cananéia City (southern estuary). Organic matter contents in sediments were, in general, higher during the rainy season, with higher values in P2 and P5. Low contents of carbonates in sediments were found in all sites along the APA-CIP, ranging from 0 to 16%. Levels of metals in sediments were higher in sites closer to the RIR mouth (P1 and P2), while sediments from the vicinities of the city (P4) exhibited intermediate values in comparison with the other sites. Higher sediment toxicity was observed during the rainy season in the sites P1, P2, P3, and P4 to sea-urchin embryo-larval development, and P2 and P3 to *Nitocra* sp.

The FA/PCA extracted four factors (Table 2) from the original dataset. The scores of each factor for each of the sampling site were showed in Table 3. F1 (26.55% of the explained variance) showed the association between $WBR_{Effects}$ in the liver tissue, chronic sediment toxicity (*Nitocra* sp and *L. variegatus*) and sediment physical-chemical characteristics (mud, OM, Cr, Cr, Cu, Fe, Mn, Pb and Zn). This group of variables was found to have higher scores in sampling stations P2 and P4 during the rainy season. Another group of variables was also related to F1 (with opposite signal): $WBR_{Effects}$ and $WBR_{AOxResp}$ in the kidney tissue. This association was relevant in P1, P3 and P5 during dry season and P3 and P6 in the rainy season (Table 3).

F2 (14.64% of the explained variance in data original dataset) shows a relationship between induction of AChE and total HPAs in bile (Table 2). Such associations are especially relevant in P1 and P3 during the dry season and in P5 and P6 in the rainy season (Table 3). In addition, F2 shows an inverse relation between AChE and metals body burden (Co, Ni, Pb in muscle and Co, Cr in liver), which is relevant in P5 and P6 during the dry season, and in P1 in the rainy season (Table 3). $WBR_{Effects}$ in the blood tissue and MT score in gill tissue were also associated with these metals in the organisms (with the same signal) (Table 2).

F3 (explained 13.83% of the variance in the original dataset) associated, in one group of variables, the $WBR_{Effects}$ in the kidney tissue with some metals in fish tissues (Co in muscle, Mn and Cd in liver), with higher scores were found in P5 (dry season) and P3, P5 and P6 (rainy season). In another group of variables, F3 associated $WBR_{AOxResp}$ in the gill tissue, MT scores in the liver tissue, condition factor, and metals body burden in muscle tissue (Cd, Mn, Pb, As) and liver (Pb) (Table 2). These associations were relevant mainly in P1 during the both seasons (dry and rainy) and in P4, during the dry season (Table 3).

F4 (explained 11.83% of the variance in the dataset) showed relationships between $WBR_{Effects}$ in the gill tissue, MT in kidney tissue, and some metals accumulated in the liver tissue (Cu and As) (Table 2). Higher scores for F4 were found in P1 and P6 in the dry season and P4 in the rainy season (Table 3). Another association showed in F4 was $WBR_{Effects}$ in the blood and $WBR_{AOxResp}$ in the gill and kidney tissues related with loads of Mn in liver (Table 2). This group of variables shows relevance in P3 and P5 in the rainy season (Table 3).

Table 2. Loadings of dry and rainy season after Varimax rotation for the four factors obtained in the PCA[a]. The variance of the principal factors is given in percentage of the total variance in the original data matrix.

	F1	F2	F3	F4
% variance	26.55	14.64	13.85	11.83
AChE score	-0.006	**0.594**	-0.067	0.123
Kid-MT score	0.019	0.130	0.316	**0.620**
Liv-MT score	0.211	0.034	**0.588**	-0.099
Gill-MT score	0.357	**-0.736**	0.158	0.050
Kid-$WBR_{AoxResp}$	**-0.486**	-0.248	0.207	**-0.678**
Liv-$WBR_{AoxResp}$	-0.231	0.408	0.023	0.386
Gill-$WBR_{AoxResp}$	0.227	0.360	**0.480**	**-0.471**
Kid-$WBR_{Effects}$	**-0.658**	-0.175	**-0.544**	-0.184
Liv-$WBR_{Effects}$	**0.674**	0.389	0.281	0.276
Gill-$WBR_{Effects}$	0.042	-0.130	-0.411	**0.708**
Bld-$WBR_{Effects}$	-0.275	**-0.541**	-0.123	**-0.553**
Condition factor	-0.220	0.068	**0.743**	0.315
Mn mus	-0.083	0.017	**0.832**	-0.176
Zn mus	0.218	0.043	0.037	-0.372
Co mus	-0.001	**-0.718**	**-0.555**	0.339
Ni mus	-0.134	**-0.930**	-0.245	0.042
Cd mus	-0.252	0.393	**0.734**	0.147
Pb mus	-0.317	**-0.677**	**0.543**	0.079
As mus	-0.029	-0.057	**0.547**	-0.111
Cu liver	0.064	0.035	-0.176	**0.898**
Mn liver	0.328	0.301	**-0.608**	**-0.476**
Zn liver	0.192	-0.004	-0.374	-0.388
Cr liver	-0.213	**-0.833**	0.393	-0.060
Co liver	-0.002	**-0.823**	-0.078	0.100
Cd liver	-0.077	0.264	**-0.594**	0.363
Pb liver	-0.072	-0.067	**0.464**	-0.095
As liver	0.111	0.041	-0.330	**0.805**
Total PAHs in bile	0.275	**0.450**	0.023	0.344
Sed-SeaurchinTox	**0.882**	-0.052	-0.107	-0.189
Sed-CopepodTox	**0.656**	0.120	-0.179	-0.220
Sed-Mud	**0.922**	-0.097	-0.183	0.260
Sed-OM	**0.602**	0.037	-0.173	-0.381
Sed-Carbonates	0.287	-0.434	0.206	0.348
Cd sed	**0.963**	-0.101	-0.024	-0.035
Cr sed	**0.975**	0.002	-0.112	0.084
Cu sed	**0.844**	-0.243	-0.017	-0.035
Fe sed	**0.986**	-0.028	-0.068	0.023
Mn sed	**0.891**	-0.016	0.193	0.216
Ni sed	-0.135	0.257	0.063	0.106
Pb sed	**0.953**	-0.043	0.030	-0.035
Zn sed	**0.892**	0.194	-0.262	-0.061

[a] Only variables with loadings > 0.45 (in bold text) were considered components of the factors

Table 3. Factor scores estimated for each of the sampling stations in Dry (D) and Rainy (R) seasons evaluated in the APA-CIP to the centroid of all cases for the original data.

Station	F1	F2	F3	F4
Dry season				
P1 - D	-0.572	0.964	1.415	1.002
P3 - D	-0.429	1.078	0.190	-0.029
P4 - D	-0.052	0.020	1.678	-0.544
P5 - D	-0.958	-2.485	-0.338	-0.012
P6 - D	-0.010	-0.517	0.028	0.764
Rainy season				
P1 - R	-0.038	-0.516	0.973	-0.614
P2 - R	2.775	-0.263	-0.073	-0.472
P3 - R	-0.554	0.259	-0.745	-0.962
P4 - R	0.449	0.058	-0.893	2.309
P5 - R	-0.010	0.509	-0.778	-1.053
P6 - R	-0.600	0.894	-1.456	-0.388

4. DISCUSSION

In general, the levels of metals in sediments were lower than the regional sediment quality guidelines (SQGs) already proposed to sediments of two Brazilian estuarine systems close to the APA-CIP (Choueri et al., 2009a) except by P2 sediments. In this case, the levels of Pb (32.84 mg kg^{-1}) and Cu (7.89 mg kg^{-1}) were above the highest proposed benchmarks, and Zn (32.61 mg kg^{-1}), which is considered a moderate contamination. Compared against international guidelines, only Pb in P2 was found in concentrations exceeding the TEL (Threshold effect level) (CCME, 2002). This situation corroborates previous studies showing that the sediments in the estuarine-lagoon environment are moderately contaminated by metals (Mahiques et al. 2009; Azevedo et al. 2012; Cruz et al. 2014).

The FA/PCA revealed that sediment contamination was associated with fine-grained and organically-rich sediments in depositional areas of the estuarine-lagoon. This well-known relationship has been frequently observed in previous WOE-based studies (Cesar et al., 2007; Choueri et al., 2009b; Ramos-Gomez et al 2011; Araújo et al., 2013; Cruz et al., 2014) and it is due to the higher adsorption capacity of fine particles and organic matter to pollutants. In addition, the finer particles are more likely to be resuspended and transported to regions far from their point of origin (USEPA, 2002). Previous studies performed in the APA-CIP already reported high levels of contaminants in fine sediments and organic matter, and this was attributed to the metal-rich mining residues from the upper Ribeira River (Guimarães and Sígolo, 2008; Amorim el at. 2008; Mahiques et al 2011).

The WOE approach and LOEs integration through multivariate analysis used in the current study revealed that the different levels of biological responses (sub-cellular, cellular, tissue or organism levels) are preferentially associated with contaminants in one of the two different matrices evaluated (sediment and body tissues). Sub-cellular and cellular responses in all studied tissues (kidney, liver, gill, and blood) were more strongly associated with metals and As body burdens. On the other hand, sediment contamination was better associated with most severe biological effects on higher levels of organization (from tissues to organism).

In a sub-cellular level, antioxidants responses in gill were associated with loads of metals in *C. spixii* tissues (Cd, Mn, and As in muscle tissue, and Pb in muscle and liver tissues) (variables associated with F3) (Table 2). Manganese in liver was also associated with antioxidant responses in gill and kidney tissues (variables associated with F4) (Table 2). Metals can stimulate the production of reactive oxygen species (ROS) through different pathways (Livingstone, 2001; Luschak 2001; Sharma and Dietz 2009), including the depletion of major antioxidants in the cell (Stohs et al., 2001; Atli and Canli 2010). High levels of ROS trigger cell defense mechanisms, i.e. the increase of the activity of antioxidant enzymes and levels of free-radical scavenger molecules (e.g. GSH). These responses can be used as biomarkers of a cellular pro-oxidant state.

MT-like proteins activity (represented as the MT scores), neurotoxicity (AChE inhibition represented by low values of the AChE score), and integrated sub-cellular and cellular effects (represented by $WBR_{Effects}$) in the different tissues were also better associated with metal contents in the organism than in the sediments. This suggests the existence of mechanisms of regulating metal uptake, or internal metal regulation (detoxification and/or depuration) in *C. spixii*.

MT in gill, liver and kidney tissues were associated with levels of different metals and As both in muscle and liver (associated with F2, F3 or F4) (Table 2). The increased levels of metallothionein-like proteins (MTs) associated with higher metal body burdens in *C. spixii* may be due to the well-known role of MTs of scavenging metals in the cell and participation in the processes of metal regulation and depuration (Stegeman et al. 1992; Viarengo et al. 2000; Van deer Oost et al. 2003).

AChE inhibition was associated with Ni and Cr in the muscle and liver tissues, respectively, and Pb and Co in both muscle and liver tissues (associated with F2) (Table 2). Decreased AChE activity in organisms exposed to metals has been attributed to the binding of metals to functional groups of the enzyme, leading to a loss of enzyme function and ultimately to an overstimulation of acetylcholine (ACh) receptors (de Lima et al. 2013; Richetti et al. 2011). The inhibition of AChE activity has been related to movement impairment, respiratory failure (WHO/IPCS/INCHEM, 1986), increase in peristaltic movement, muscle weakness, tremor, hypertension, cardiovascular collapse (Boelsterli, 2007), and, in fish, a loss of ability to capture prey was reported (Mager et al., 2010).

Sub-cellular and cellular damage ($WBR_{Effects}$) in kidney, gill, and blood tissues were associated with metals in the muscle and liver tissues. Oxidative damage in DNA and cell lipids of aquatic organisms, which consequently may generate genotoxicity and disturbances in membrane fluidity (Heath, 1995), has been related to metal exposure (Livinstone, 2001; Loro et al., 2012; Machado et al., 2013; Nunes et al., 2014). Oxidative damage can occur when antioxidant and detoxifying systems are deficient to neutralize the active intermediates produced by xenobiotics and their metabolites (Vasseur and Cossu-Leguille, 2003). Apart from causing oxidative stress, some metallic ions, such as Cr and Pb, can also directly interact with DNA by covalent binding (Zhitkovich et al., 1996; Hong et al., 2007), causing genotoxicity and eventually cell apoptosis (Rudolph and Cervinka, 2006).

$WBR_{Effects}$ in liver (which includes lipid peroxidation, DNA damage, and histopathology) was the biomarker response most related with sediment contamination, together with SWI and WS toxicity. It is known that liver plays a major role in the uptake, biotransformation and excretion of pollutants (Bernet et al., 1999); however, when the metabolism of contaminants produces highly reactive and toxic metabolites, they can affect the structural integrity of DNA (Shugart, 2000), disrupts normal cellular processes, leading to loss of cell and histological integrity (Regoli et al., 2004; Costa et al., 2011). The better association between $WBR_{Effects}$ in liver with sediment contamination and toxicity, compared to the $WBR_{Effects}$ and $WBR_{AOxResp}$ in other organ tissues, suggests that the inclusion of the histopathological assessment (which shows effects at higher level of biological organization) increased the association between biomarkers, environmental contaminant levels, and traditional ecotoxicological endpoints at organism level.

It is noteworthy that, in one hand, the higher level of biological responses (the index that includes histopathological assessment of *C. spixii* liver, as well as sea-urchin early development, and copepod fecundity) are better associated with levels of metals and As in sediments. But, the findings including only sub-cellular and cellular responses are better associated with metals and As body burdens. It is suggested that the defense responses triggered by the internal metal and As concentrations and effects (e.g. increasing of MTs levels, induction of antioxidant responses, and other responses not measured in the current study), might be able to halt the toxicity. Therefore, in some sites, the deleterious effects were not found in higher levels of biological organization (e.g. histopathological effects).

The biomarkers responses in *C. spixii* did not show a simple linear pattern. The absence of association between higher internal levels of contaminants and the most severe effects may be a result of the development of pollutant-sequestering detoxifying systems. This mechanisms could minimize the toxicity of metals, keeping them in inclusion bodies (e.g. granules), or bound to heat-stable proteins which are not toxic to the organism (refer to Vijver et al. 2004 and Adams et al. 2011 for reviews). On the other hand, aquatic organisms can also start excreting metals after internal levels reach threshold toxicity (Adams et al., 2011). This could explain why it was observed more severe effects in specimens with lower contaminant body burdens.

The lack of association between sediment contamination and levels of contaminants in *C. spixii* muscle or liver tissues is odd as well. This suggests that, at moderate levels of environmental contamination in estuaries, no linear pattern is found between contaminant levels in sediments and organisms tissues. Many factors can affect the potential association between environmental levels and internal levels of metals and As in *C. spixii*, as observed in the current study. Metals and As burdens in organisms tissues are influenced by the capacity of the organism to cope (store, detoxify, or eliminate) with the metal challenge. In addition, levels of contaminant body burdens may be influenced by different environmental and physiological parameters, such as ecological needs, metabolic rates, feeding patterns, concentration of the element, temperature, salinity, pH, and seasonal changes (Allen-Gil and Martynov 1995; Terra et al., 2007; Copat et al., 2012). Therefore, metal uptake and elimination rates are not necessarily constant (Adams et al., 2011), but caution is need tissues residue of metals and As are used to assess exposure in mildly contaminated environments.

Table 4 summarizes the relevance of the assessed LOEs for each of the sampling sites based on the FA/PCA scores. A seasonal and spatial pattern is apparent for some LOEs, i.e. in P2 and P4, during the rainy season where sediments are muddier, presenting higher levels of contaminants, toxicity, and levels of damages in liver of *C. spixii* comparativelly with other studied sites. Such results highlight the influence of seasonal environmental variability on the environmental quality in the estuary-lagoon. Lower fish condition is also found predominant in the rainy season (P3 to P6), though it is important to consider a possible normal physiological variation as a confounding factor.

Table 4. Summary of the relevance of the assessed LOEs for each of the sampling sites based on the FA/PCA scores.

Lines of evidence		**Dry season**					**Rainy season**					
		P1-D	P3-D	P4-D	P5-D	P6-D	P1-R	P2-R	P3-R	P4-R	P5-R	P6-R
Kidney	Biomarker response											
	Induced MT					x				x		
	Antioxidant response	x			x				x		x	x
	Induced effects	x			x				x	x	x	x
Liver	Biomarker response											
	Induced MT	x		x			x					
	Antioxidant response											
	Induced effects							x		x		
Gill	Biomarker response											
	Induced MT				x							
	Antioxidant response	x		x			x				x	
	Induced effects					x				x		
Blood	Induced effects				x						x	
Muscle	AChE inhibition				x	x	x					
Organism	Low CF								x	x	x	x
Muscle	Higher levels of contaminants											
	Cd, Mn, Pb and As	x		x			x					
	Co, Ni and Pb				x							
	Co only								x	x	x	x
Liver	Higher levels of contaminants											
	Pb only	x					x					
	Cu and As	x				x				x		
	Co and Cr				x							
	Mn and Cd								x	x	x	x
Bile	Higher levels of PAH metabolites	x	x									x
Sediment	Toxicity											
	SWI toxicity							x		x		
	WS toxicity							x		x		
Sediment	Physical-chemical characteristics											
	Higher mud and OM contents							x		x		
	Higher levels of contaminants							x		x		

As a diagnosis of the environmental quality in the studied area, P2 and P4, in the rainy season, can be considered as the most degraded sites in the APA-CIP. In this case the WOE assessment showed that the organisms are exposed to contaminants (higher sediment contamination), and subjected to effects at higher levels of biological organization (histological damages in liver of resident fish, abnormal sea-urchin development exposed to SWI, decreased copepod fecundity exposed to WS). P2 and P4 are under the influence of the most important contaminant sources in the APA-CIP, the RIR and the Cananéia city, respectively. The current finding corroborate the study of Cruz (2014), who assessed sediments from site P2 through a whole sediment Toxicity Identification Evaluation (TIE), identifying the metals and ammonia as the main toxicants in this site. The current results also indicated that the environmental quality is worse in the rainy season, which may be related with a higher river and superficial runoff, carring contaminants to the estuary-lagoon and the increased seasonal population in the city during the rainy season.

The indices integrating sub-cellular and cellular responses or effects suggest these sites as moderately impacted. During both dry and rainy season, the fish from the sites closer to the RIR (P1) and nearby Cananéia city (P5) showed sub-cellular responses and effects in kidney, gill tissues, blood cells, and inhibited AChE. In P1, it is also noteworthy that liver MT is induced in both seasons, associated with higher metals and As burdens in muscle and liver tissues of *C. spixii*. The sites that the WOE assessment indicated as the lower impacted were P3 (both seasons) and P6 (in the rainy season), since the biological effects were either absent or circumscribed to the kidney.

In the current study, the biomarker findings showed harmonization with ‘classical’ LOEs of biological responses (i.e. toxicity responses at organismal level) only when histopathological of liver responses were included in the index. Costa et al. (2012) also pointed out that biomarkers that reflect histological lesions are more consistent to discriminate between exposure to contaminated and uncontaminated sediments. Other responses, however, cannot be disregarded. Sub-cellular and cellular effects in different tissues, associated with metals and As levels in muscle and liver, were useful to provide indications of the environmental quality in the other stations. Consequently, it was possible to identify intermediate classifications between the most and the least impacted sites.

The environmental quality diagnosis of the APA-CIP through the assessment of biological responses at different levels of biological organization in the WOE approach minimized missing toxicological effects, which would not be detected if only classical LOEs were used. At moderately contaminated environments, a simple 'pass-fail' approach based on the traditional LOEs might not reveal subtle effects which can be important for a scientific-based management decision in a MPA.

The use of biological responses at different levels are encouraged in a WOE assessment of environmental quality in moderately contaminated areas. However, due to the difficulties of performing an ample set of analyses in the day-by-day management of MPAs, the focus on biological responses at a lower (biomarkers of exposure and/or effects) or a higher levels of biological organization (histopathology, embryolarval development, fecundity) is possible depending on the objectives of the WOE assessment or monitoring. If the objective is to identify most degraded sites, then the histopathological and/or classical ecotoxicological tools, integrated with chemical levels in tissues and/or sediments can provide enough environmental information. However, if the objective is to better detail biological effects, including early warning signals which can anticipate disturbs in the ecological fitness of target species, then the use of the WBR and biomarkers scores within a WOE approach is highly recommended.

5. CONCLUSIONS

The current study showed that the biological responses at higher level of biological organization were better associated with levels of metals in sediments, whereas the biomarker findings including only sub-cellular and cellular responses were better associated with bioaccumulated metals and As.

Levels of metals and As in muscle or liver tissue did not associate with sediment levels of these contaminants, suggesting that *C. spixii* have a mechanism of internal metal regulation after a threshold toxicity, or these metals are not bioavailable. Therefore, caution is needed when using metals and As residues in tissue to assess exposure in mildly contaminated environments.

The inclusion of biomarker indices based on sub-cellular, cellular, and histological responses in the WOE approach was useful to discriminate the different sites within a range of environmental degradation in moderately contaminated estuary-lagoon.

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Capítulo IV

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Metals and arsenic in catfish from a Marine Protected Area (MPA) under past and present human pressures: consumption risk factors to the local population

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ABSTRACT

The risk of metals and As in seafood for traditional populations living in a MPA is seldom assessed, although the risk of human exposure to contaminants is one of the indicators associated with socioeconomic goals of Marine Protected Areas (MPA). The current study aimed to estimate the potential risk of metals (Cu, Mn, Zn, Cr, Ni, Cd, Pb) and arsenic (As) for human health through the ingestion of fish locally harvested in a Marine Protected Area (MPA), the Cananéia-Iguape-Peruíbe Environmental Protected Area (APA-CIP). *Cathorops spixii*, a catfish widely consumed by local population, was collected along the estuary in three seasons with different rain regimes. Metals and As bioaccumulation in muscle tissue were quantified and compared against national and international action levels regarding human consumption. In addition, the target hazard quotient (THQ) for metals and As, the cancer risk (CRisk) for As, and the number of eligible meals per month were estimated. Cd, Pb, and As were found at concentrations above action levels for human consumption. Depending on the level of exposure of the local population, the consumption of *C. spixii* pose risk to human health. Highest THQs were estimated for fish collected in sites closer to the main contamination sources in the APA-CIP, i.e. the RIR mouth (P1) and the city of Cananéia (P4, P5, and P6). Although As in *C. spixii* showed low risk of causing systemic effects, the cancer risks estimated based on these levels of As are high, especially in the sites under the influence of the city. The exposure of the local population to metal and As contaminated seafood cannot be disregarded in environmental studies and management of the APA-CIP.

Keywords: toxic metals, human exposure; consumption limits; allowable daily consumption; mining activity.

1. INTRODUCTION

Fish is an important source of protein to human population, providing around 37% of the total animal protein consumed by the world’s population (FAO, 2011). However, toxic substances released to human activities in aquatic ecosystems increase the bioavailability to fish and biota in general. Seafood are natual veichle of human population exposure to metals (Begúm et al., 2013; Copat et al., 2012).

Mining and smelting activities are a serious threat to the aquatic environment in many countries (Kroll et al., 2005). In some instances, such activities can affect more the surroundings of the mines than the mining operations themselves (Fernández-Caliani et al., 2009). Pollutants from mines may be transforted by water and air, that can accumulate in a myriade of environmental compartments (Camizuli et al., 2014; Ruelas-Inzunza et al., 2011; Riba et al., 2005) including fish (e.g. Park and Curtis, 1997; Moiseenko and Kudryavtseva, 2001; Riba et al., 2005).

Metal accumulated in edible tissues of fish poses health risks to consumers. Indeed, the diet can be an important route of exposure in the case of populations indirectly exposed to mining activities (Fréry et al., 2001; Castro-Gonzalez and Méndez-Armenta, 2008; Marrugo-Negreti et al., 2008; Zhuang et al., 2014). Therefore, studies focused on the quality of edible fish are relevant for characterizing human health risks in areas under the influence of mining activities (Subotić et al., 2013).

Although metal contamination and their adverse health effects have long been studied, human exposure to these elements is still present (Fréry et al., 2001; Marrugo-Negreti et al., 2008; Tang et al. 2013; Sow et al. 2013; Zhuang et al., 2014). The calculation of risk factors for the population is considered a more reliable approach than solely comparing against consumption limits provided by legal documents such as EC (2006), USEPA (2000), FAO/WHO (2014), Mercosul (2011, 1994) and Brazil (1998, 2013). These national and international rules do not include sensitive subpopulations, or even people with increased susceptibility to toxicological effects (pregnant women and children) (USEPA, 2000).

The Environmental Protected Area of Cananéia-Iguape-Peruíbe (CIP), Southeastern Brazil, is recognized as a World Natural Heritage Site by UNESCO (2000). Additionally is considered priority area for future inclusion on the list of Brazilian wetlands of international importance within the scope of the Ramsar Convention (Brazil 2012). Apart from its ecological relevance, one of the objectives of the CIP is protecting its cultural and historical value for traditional people, such as the traditional fishermen (known as “Caiçaras”), Maroons (known as “Quilombolas”), and native americans. Although the APA-CIP is legally protected, this lagoonal-estuarine environment showed increased metal contamination from former mining activities located in the Ribeira de Iguape River basin (RIR). The coastal modification creating an artificial navigational channel now connect the river with the lagoon increasing the release of metals on the estuary (Mahiques et al., 2009; Guimarães and Sigolo, 2008; Abessa et al., 2014).

The current study aimed to estimate the potential risk of metals (Cu, Mn, Zn, Cr, Ni, Cd, Pb) and arsenic (As) for human health through the ingestion of fish locally harvested in a Marine Protected Area (MPA). To achieve such goal, *Cathorops spixii*, a catfish widely consumed by local population (Fávaro et al., 2005), was sampled along the estuary in three seasons with different rain regimes. Metals and As burdens in edible muscle tissue were firstly compared against national and international action levels regarding human consumption. The human risk assessment was further detailed by estimating the target hazard quotient (THQ) for metals and As, and the cancer risk for As were calculated (USEPA, 2000). Lastly, the number of eligible meals per month was estimated in order to minimize chronic systemic effects.

The quality of human health is one of the indicators associated with socioeconomic goals of Marine Protected Areas (MPA), as suggested by Pomeroy et al. (2005). However, the risk of dietary metals and As for people living in a MPA is seldom assessed. The results of the current work may provide information for human health protection in Marine Protected Areas.

2. MATERIAL AND METHODS

2.1 Study Area

The Cananéia-Iguape-Peruíbe Environmental Protected Area (known as the APA-CIP) (24^{o}40’S and 25^{o}05’S) presents two well-defined climate seasons: a drier winter (mean temperature of 20 °C and mean pluviosity of 95.3mm.month^{-1}) and a rainier summer (mean temperature of 28 °C and mean pluviosity of 266.9mm.monthly^{-1}). Tides are semidiurnal, and mean tidal amplitude is 0.82m (Cunha-Lignon et al. 2009).

The main freshwater contributor to the CIP estuary is the Ribeira de Iguape River (RIR). Approximately 70% of the course of the river flows toward the lagoon waters through an artificial channel locally known as “Valo Grande”. The river basin is a metallogenic province with natural Pb and Zn deposits (Moraes et al. 2003). Mining activities were performed in this area for many decades during the 20th century, but the mines were closed down in the 1990s. Since then, high levels of metals (Pb, Zn, Cu, Cr) and arsenic (As) have been recorded in the river waters, as well as in both bottom and suspended sediments (Eysink et al. 1998; Corsi and Landin 2003; Moraes et al. 2003; Guimarães and Sígolo 2008). The metal contamination of lagoonal-estuarine sediments has increased substantially after the "Valo Grande" channel opening (Mahiques et al. 2009).

Apart from its important natural features, the APA-CIP also encompasses three cities (Iguape, 30,259 inhab.; Ilha Comprida, 9,025 inhab.; Cananéia, 12,601 inhab.) (IBGE 2014). Waste is discharged in rivers, groundwater, or directly into the lagoon system, since sewage treatment is insufficient for local demand (Morais and Abessa 2014).

2.2 Fish Collection and Sample Preparation

The madamango sea catfish (*C. spixii*) has demersal habits and spends its whole life cycle in muddy-bottom estuaries (Azevedo et al, 1999). This species has been considered as an important artisanal fishing resource in tropical and sub-tropical South American Atlantic coasts (Reis, 1986; Melo and Teixeira 1992; Alvarez-Leon and Rey-Carrasco, 2003), and it is widely consumed by the local population.

The sampling sites were set with the aim of encompassing the main potential contaminant sources along the APA-CIP (Figure 1). Thus, site P1 is the closest to the RIR mouth and site P4 is the closest to the Cananéia city. The specimens were collected with a bottom otter trawl during three seasons with different amounts of rainfall: (i) the partially dry season (P) (May 2012); (ii) the dry season (D) (August 2012); and (iii) the rainy season (R) (March 2013). The average rainfall during these seasons which sampling was performed was 192mm, 111mm and 390mm, respectively (CEPAGRI, 2014). In the first sampling campaign (during the partially dry season), four sampling stations distributed along the APA-CIP area (P2 to P5) were set up. In the subsequent campaigns, two additional sampling stations were included (P1 and P6), in order to enable a better understanding of the influence of important contaminants sources in APA-CIP. The total number of fish sampled was n=225.

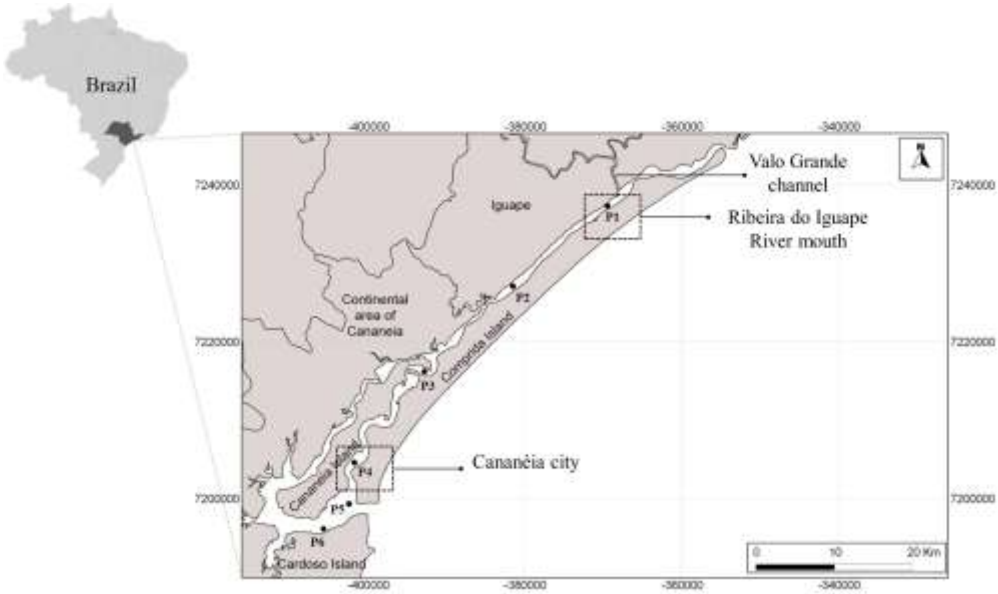

Fig. 1 Sampling stations located within the APA-CIP, Brazil

Before dissection, the collected specimens were kept in local water, under aeration until transportation to the laboratory. Before euthanized by spinal cord section, individuals were anesthetized with benzocaine in water, then weighted and measured. The axial muscle used in metal body burden analyses was stored in plastic vessels at -20 ºC.

2.3 Analyses of Metal Body Burdens

Concentrations of As in muscle and liver tissues were determined using an atomic absorption spectrophotometer (Varian®, AA 240Z) equipped with a graphite furnace (AAS-GF) (Model, GTA 120) and a transverse Zeeman corrector for background correction, automatic sampling (model PSD 120). Metals were quantified using flame atomic absorption spectroscopy (FAAS) (Varian®, AA 240FS). All analyses were performed according to standard method 200.9 (USEPA 1994). Detection limits for As were 5.88 µg kg^{-1} and detection limits for metals (Cu, Mn, Zn, Cr, Co, Ni, Cd, Pb) were 0.034 mg kg^{-1}, 0.0697 mg kg-1, 0.0525 mg kg^{-1}, 0.112 mg kg^{-1}, 0.146 mg kg-^{1}, 0.0623 mg kg^{-1}, 0.042 mg kg^{-1}, and 0.0602 mg kg^{-1}, respectively. The limits of quantification (LOQ) was estimated at 0.178 mg kg^{-1} for As, 0.897 mg kg^{-1} for Cu, 0.537 mg kg^{-1} for Mn, 0.720 mg kg^{-1} for Zn, 0.345 mg kg^{-1} for Cr, 0.390 mg kg^{-1} for Co, 0.204 mg kg^{-1} for Ni, 0.059 mg kg^{-1} for Cd and 0.545 mg kg^{-1} for Pb.

Standard curves were prepared using reference material (Qhemis High Purity®) and recovery rates ranged from 80% to 120% in all analyses. Metal concentrations were expressed in mg kg^{-1} dry weight. All glassware was acid washed and rinsed with Milli-Q water.

2.4 Data Analysis

A cluster analyses were made to assess the association between metals and As in the three seasons (intermediate, dry and rainy) on the basis of the average values for each collection site. The data was firstly standardized, so the variables were treated with equal importance. Cluster analysis was based on the correlation matrix created by computing the Pearson's r statistic, using Bray-Curtis as the similarity measure.

2.4.1 Estimation of target hazard quotient (THQ) and cancer risk (CRisk)

For the estimation of target hazard quotient (THQ), cancer risk (CRisk), and safe consumption rates, the concentration of metals and As results in edible tissues were converted in mg kg^{-1} wet weight assuming a water content of 80% in the muscle tissues (Begum et al., 2013).

Mean of metal body burdens in muscle tissues (edible parts) of *C. spixii* were used to estimate the potential health risk to population at each sampling site. Arsenic consumption limits calculations were performed as inorganic arsenic contents (3% of the total organic arsenic) (FSA, 2004).

Risk factors (THQ and CRisk) were calculed to standardize fish and sea food consumption advisories for minimizing the risk of both cancer and non-cancer endpoints (USEPA, 1989, 2000). In the current study, THQ and CRisk were calculated as recommended by USEPA (2000). It was assumed that no portion of the metal in the muscle was lost or magnified during the cooking process, and consequently the ingestion dose is equal to the absorbed contaminant dose (Moreau et al., 2007). The estimation of the THQ for metals and As, calculated according equation (1), considers the ratio between exposure and the reference dose. Values of exposure higher than the reference dose (i.e. THQ above 1) suggest that systemic effects may occur (USEPA, 1989).

(1) THQ= (EF x ED x MS x C)/(RfD x BW x AT)

where EF is the exposure frequency (365 days year^{-1} for people who eat fish seven times a week, 52 days year^{-1} for people who eat fish once a week, and 12 days year^{-1} for people who eat fish once a month); ED is the exposure duration (70 years for adults and 6 years for children); MS is the food meal size (0.227kg for adults and 0.114kg for children); C is the metal concentration in fish (mg kg^{-1}, wet weight); RfD is the oral reference dose (mg kg^{-1} day^{-1}) (obtained from USEPA's Integrated Risk Information System) (USEPA- IRIS, 2014); BW is the body weight (70kg for adults and 16kg for children); AT is the average time of exposure (days) to the chemical (365 days year^{-1} x ED). The average fish ingestion rates, body weight, and lifetime of the target population were set in accordance with the values provided by USEPA (1989, 2000).

Once the inorganic As (estimated as 3% of the total As according to FSA, 2004) is classified by the USEPA as a human carcinogen (USEPA, 1989; 2002), the lifetime cancer risk (CRisk) (equation 2) was estimated to this compound in the current study. CRisk was estimated by using the cancer slope factor (CSF) of the chemical provided by USEPA's IRIS (2014). CRisk above the acceptable lifetime risk value (10^{-5}) indicates a probability greater than 1 chance over 100,000 of an individual develop cancer (USEPA, 1989; USEPA, 2000).

(2) CRisk = (EF x ED x MS x C x CSF)/(BW x AT)

The RfD is defined by USEPA (1987) as an estimate of daily exposure to the human population, including sensitive subgroups, that is likely to be without an appreciable risk of deleterious effects during a lifetime. CSF, in turn, since the agency assumes that carcinogens do not have safe thresholds, is defined as the "cancer potency", usually the upper 95 percent confidence limit on the linear term in the multistage model used by USEPA (1996) based on data obtained in an epidemiological study or a chronic animal bioassay (USEPA, 2000).

2.4.2 Estimation of safe consumption rates (CR)

The consumption rate (i.e. maximum allowable number of fish meals that could be consumed over a month which would not be expected to cause any chronic systemic effects) (CR_{mm}) was evaluated for Ni, Pb, Cd and As according to USEPA (2000). First, the consumption rate limit (CR_{lim}) (maximum safe consumption rate) was estimated according to equation 3, and expressed in kg fish d^{-1}. It was assumed that no other source of metals exists in the diet of consumers.

(3) CR_{lim} = RfD x BW/C

where RfD is the reference dose for each metal (mg kg^{-1} day^{-1}); BW is the body weight (kg); C is the measured concentration of a chemical in the samples of fish tissue (mg kg^{-1}). The safe fish intake in a weekly basis (CR_{lim}^{*}) was subsequently calculated by simply multiplying the CR_{lim} by seven, and, lastly, the maximum safe number of fish meals in a monthly basis (according to Moreau et al., 2007) was calculated (see equation 4):

(4) CR_{mm}= (CR_{lim}^{*} x T_{ap})/MS

where CR_{mm} is the maximum allowable consumption rate (meals $month^{-1}$), CR_{lim}^{*} is the maximum weekly consumption rate of fish (kg $week^{-1}$), T_{ap} is the average time period in a month (4.3 week/$month^{-1}$), and MS is the meal size. Number of meals higher than 16 $month^{-1}$ was considered with no obvious human health risk (USEPA, 2000).

3. RESULTS AND DISCUSSION

The concentrations of metals (Cu, Mn, Zn, Cr, Co, Ni, Cd, Pb) and arsenic (As) (mg kg^{-1} wet weight) in the muscle of *C. spixii* from APA-CIP are presented in Table 1. The concentration of contaminants in the tissue showed a notable seasonal trend, as can be confirmed by cluster analysis (Figure 2).

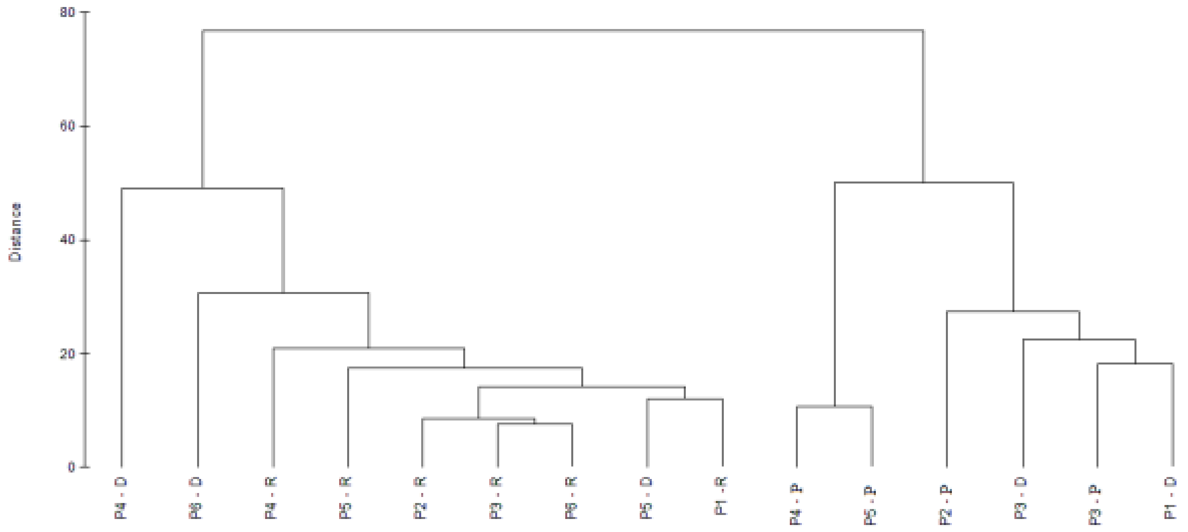

Fig. 2 Cluster analysis (Bray-Curtis distance; Group Average Link) with standardized data of metals and As levels in muscle tissue of C. spixii sampled along the APA-CIP in three seasons with different rain regimes: the partially dry season (P); the dry season (D); and the rainy season (R)

In general, As, Mn, Zn, Cd, and Pb concentrations in muscle were higher in the stations closer to the city (P4, P5 and P6) during the dry season and decreased during the rainy season. In the other hand, specimens collected closer to the RIR (P1, P2 and P3) presented higher metal concentrations (Mn, Zn, Co, Cr, Ni) during the rainy season.

Increased environmental metal concentrations in the dry season, as observed in the sites closer to the city in the current study, has been observed in previous studies in coastal areas subjected to seasonal variation of rainfall, as the concentration of contaminants in the aquatic system may increase if freshwater inflow is diminished during the dry season (Sainz et al. 2004; Fianko et al. 2007; Costas et al. 2011). In the other hand, increasing environmental metal concentrations, as observed in the sites closer to RIR mouth in the current study, may also be expected in sites subjected to runoff of metals from land-based sources, as well as metal remobilization from bottom sediments by rainstorms. Previous studies have shown the major role played by rainstorms in the release of contaminants from surface soils into the RIR and its tributaries within the RIR basin (Corsi and Landim 2003; Cunha et al. 2007; Costa et al. 2009; Abessa et al. 2014).

The levels of metals and As found in the axial muscle of *C. spixii* in the current study are, in general, higher than those found in muscle of estuarine fishes as reported by other studies performed at polluted sites (e.g. Souza et al., 2013; Vasanthi et al., 2013). In the current study (Table 1), levels of Cd (annual means in sampling stations ranging from <0.11 to 1.78 mg kg^{-1} w.w.), Pb (from 3.80 to 6.94 mg kg^{-1} w.w.) and total As (from 0.0004 to 1.45 mg kg^{-1} w.w.) were higher than the action levels set by Mercosul (2011) and the Brazilian Sanitary Vigilance Authority (ANVISA) (Brasil, 2013) (both established a range from 0.05 to 0.3 mg kg^{-1} w.w. for Cd, 0.3 mg kg^{-1} w.w. for Pb, and 1.0 mg kg^{-1} w.w. for total As), FAO/WHO (2014) (1.0, 2.0, and 1.0 mg kg^{-1} w.w. for Cd, Pb, and total As, respectively), and EC (2002, 2006) (Cd = ranging from 0.1 to 0.30 mg kg^{-1} w.w.; Pb = 0.30 mg kg^{-1} w.w.).

The high levels of As and Cd found in *C. spixii* from APA-CIP are concerning because these contaminants have a known carcinogenic potential while Pb is known to cause neurotoxicity and other disorders (USEPA, 2000; Squadrone et al., 2013). It is worth noting that Pb is the main element related to mining acitivities in the RIR watershed. Previous studies reported high levels of Pb in the blood of children and adults living nearby the closed Pb refinery (Paoliello et al., 2002), and increased levels of As (compared to the reference area) in the urine of adult and children population (Figueiredo et al., 2007). Sakuma et al. (2010) also conducted studies of arsenic exposure of children from places in the RIR watershed. The results concluded that the presence of arsenic in their urine is both due to residual anthropogenic arsenic from mining activities and geologic arsenic from arsenopirite outcrops that naturally increases the levels of As in the environment.

Higher levels of metals and As body burdens in species that live and feed in direct contact with sediments (like *C. spixii*) are expected since such organisms are directly exposed to sediment-associated contamination (Storelli 2008; Storelli and Baroni, 2013). Similarly to the current research, a study of bioaccumulation in Ariidae catfish from the APA-CIP also concluded that the levels of Cd and Pb in the muscle tissue of *C. spixii* and *Genidens genidens* indicated the presence of an influence of a contaminant source of metals in the area (Azevedo et al., 2012).

Table 1. Metals and As concentrations (mg kg^{-1} w.w.) in muscle tissues of *Cathorops spixii* sampled during three seasons with different pluviometric regimes the partially dry season (P); the dry season (D); and the rainy season (R) in the APA-CIP. Action levels for human consumption established by different health agencies are also given (mg kg^{-1} w.w.) (the exceedances are presented in bold). LOQ values were used in annual means in case of detection below the LOQ.

Sampling station-Season	Cd	Cr	Cu	Mn	Ni	Pb	Zn	As
P1 - D	**2.727**	<0.081	<0.100	0.403	<0.0926	**6.478**	2.229	0.0004
P1 - R	<0.114	<0.081	<0.100	0.680	25.089	**6.311**	6.998	0.0005
P1 -Anual Mean	**1.421**	<0.081	<0.100	0.542	12.591	**6.394**	4.613	0.0005
P2 - P	<0.114	<0.081	<0.100	0.432	<0.0926	**7.343**	2.197	0.0005
P2 - R	<0.114	<0.081	<0.100	0.054	18.654	**1.611**	4.803	0.0008
P2 -Anual Mean	<0.114	<0.081	<0.100	0.243	9.373	**4.477**	3.500	0.0007
P3 - P	**1.657**	<0.081	<0.100	0.200	<0.0926	**7.064**	0.830	0.0013
P3 - D	**1.808**	<0.081	<0.100	<0.127	<0.0926	**4.328**	1.839	0.0021
P3 - R	<0.114	<0.081	<0.100	0.089	20.426	<0.109	2.420	0.0005
P3 -Anual Mean	**1.193**	<0.081	<0.100	0.139	6.870	**3.834**	1.696	0.0013
P4 - P	**3.086**	<0.081	<0.100	0.257	<0.0926	**7.293**	3.373	0.0233
P4 - D	**2.112**	<0.081	<0.100	0.762	21.870	**9.576**	6.904	**1.4510**
P4 - R	<0.114	<0.081	<0.100	<0.127	28.178	<0.109	4.498	0.0015
P4 -Anual Mean	**1.770**	<0.081	<0.100	0.382	16.713	**5.659**	4.925	0.3900
P5 - P	**4.543**	<0.081	<0.100	1.183	<0.0926	**6.166**	1.550	0.0116
P5 - D	**0.222**	<0.081	<0.100	<0.127	67.867	**14.643**	2.449	0.0044
P5 - R	<0.114	<0.081	<0.100	<0.127	23.580	<0.109	13.115	0.0006
P5 -Anual Mean	**1.626**	<0.081	<0.100	0.479	30.513	**6.973**	5.705	0.0055
P6 - D	<0.114	<0.081	<0.100	<0.127	40.258	**9.967**	3.073	0.0298
P6 - R	<0.114	<0.081	<0.100	<0.127	17.003	<0.109	2.424	0.0019
P6 -Anual Mean	<0.114	<0.081	<0.100	<0.127	28.630	**5.038**	2.748	0.0149
Action Levels								
ANVISA (2013)	0.05 to 0.3	-	30	-	-	0.3	-	1.0
FAO/WHO (2014)	1.0	-	30	-	-	2.0	-	1.0
EC (2006)	0.1 to 1.0	-	-	-	-	0.2 to 2.0	-	-

Levels of As found in muscle of *C. spixii* from the APA-CIP are concerning as well, especially in the sampling sites close to the urban site (Cananéia city). The levels of the semimetal found in the current study were within the range reported in a study with seafood (fish, shellfish, and cephalopods) from local fish markets in a place in SE Spain (levels ranging from 0.04 to 20.02 mg kg^{-1} w.w.) (Delgado-Andrade et al., 2003). In the APA-CIP, similarly to the current research, a previous study found As in muscle tissue of *C. spixii* at a higher level in the vicinities of Cananéia city compared to the level found in specimens from the mouth of the RIR (Kuniyoshi et al., 2011). The As bioaccumulated was also similar to those observed in current study.

The enrichment of metal and As in the RIR basin and downstream may have a contribution from non-anthropogenic sources, since this is a geochemically anomalous environment (Figueiredo et al., 2007). Despite of that, the activities of metal mining and Pb smelting have been accounted for Ag, Ba, Cd, Cu, Pb, and Zn contamination in the river and the lagoon-estuary (Moraes et al., 2003; Figueiredo et al., 2007; Guimarães and Sígolo, 2008; Mahiques et al., 2009). Mahiques et al. (2009) performed a historical analysis of the sediments of the APA-CIP and reported that the levels of Pb, Cu, Zn and Cr increased significantly after the RIR flow was deviated into the estuary through the Valo Grande channel.

The amount of contaminants in aquatic organisms potentially responds to different factors, such as the exposure period, the concentration of the element, temperature, salinity, pH and seasonal changes (Terra et al., 2007; Copat et al., 2012; Greenfield et al., 2013). Such variables can influence in the geochemical process of metallic ions as well as in the physiology of aquatic organisms. These findings may influence the bioavailability, uptake, metabolism, and excretion of metals (Chapman et al., 1998; Chapman and Wang, 2001; Paquin et al., 2000; Vijver et al. 2004). Salinity is especially important to modulate As body burdens in marine fish. It has been observed that the accumulation and retention of As (specifically arsenobetaine) is related to osmotic regulation (Clowes and Francesconi, 2004; Amlund and Berntssen, 2004) since arsenobetaine has a chemical structure similar to a well-known organic osmolyte, the glycine betaine. For marine fish, a positive correlation between salinity and arsenic burdens in three fish species (*Clupea harengus*, *Gadus morhua*, *Platichthys flesus*) from the Baltic and the North Sea was shown (Larsen and Francesconi, 2003). In the current study, *C. spixii* were collected in salinities ranging from 0 to 20 in the sites under higher influence of the RIR (P1, P2, and P3) and from 26 to 32 in the sites closer to the city (P4, P5, and P6), while the highest levels of As were found in fish from the sites with the highest salinity. Although the influence of urban wastewater as a point source of As in APA-CIP is probable due to the presence in many household products (Carbonell-Barrachina, 2000), the natural background and the retention of As as arsenobetaine for osmotic regulation may have a contribution to the levels in fishes from the vicinities of the Cananéia city.

Risk factors (THQ and CRisk) of metals and inorganic As in the muscle tissue of *C. spixii* collected at the sampling sites along the APA-CIP are presented in Table 2. It is assumed that THQ values greater than one, and CRisk above $1x10^{-5}$, are of concern, because there is a high risk of developing chronic systemic effects (assessed by THQ) or cancer (assessed by CRisk) due to the intake of the evaluated contaminants (USEPA, 2000). The estimation of Risk factors indicates the risk to the human health of consumption *C. spixii* due not only to the THQ values of Cd, Ni, and Pb, depending on the exposure level (number of days of consumption per year), but also due to the high value of CRisk of As to children.

In general, every-day consumption (365 days $year^{-1}$) of *C. spixii* presents potential risks of chronic systemic effects for children and adults due to the presence of Cd, Ni, and Pb in fish collected all along the estuary. The estimation also indicates that children may be at risk of chronic systemic effects even in the scenario of eating fish once a week (56 days $year^{-1}$), mainly because of the high levels of Pb and Cd in the muscle tissue of *C. spixii*. For As, the THQ for every-day consumption is above the threshold only at sites closer to the city.

Table 2. Target hazard quotient in the different sites of the APA-CIP for all elements analyzed, and cancer risk estimate for inorganic As (assumed as thee 3% of the total As concentration), at different levels of exposure. THQ>1 and CR>10^{-5} are reported in bold.

Sampling station	Exposure level (days.year^{-1})	Cd		Pb		As		As Cancer Risk	
		THQ adult	THQ child	THQ adult	THQ child	THQ adult	THQ child	CR adult	CR Child
P1	365	**4.6**	**10.1**	**5.2**	**11.4**	<1	<1	1. 21 x 10^{-13}	2.82 x 10^{-7}
	56	<1	**1.4**	<1	**1.6**	<1	<1	1.73 x 10^{-14}	4.02 x 10^{-8}
	12	<1	<1	<1	<1	<1	<1	3.99 x 10^{-15}	9.29 x 10^{-9}
P2	365	<1	<1	**3.6**	**8.0**	<1	<1	2.84 x 10^{-13}	4.32 x 10^{-7}
	56	<1	<1	<1	**1.1**	<1	<1	4.05 x 10^{-14}	6.16 x 10^{-8}
	12	<1	<1	<1	<1	<1	<1	9.34 x 10^{-15}	1.42 x 10^{-8}
P3	365	**3.9**	**8.5**	**3.1**	**6.8**	<1	<1	9.89 x 10^{-13}	8.07 x 10^{-7}
	56	<1	**1.2**	<1	**1.0**	<1	<1	1.41 x 10^{-13}	1.15 x 10^{-7}
	12	<1	<1	<1	<1	<1	<1	3.25 x 10^{-14}	2.65 x 10^{-8}
P4	365	**5.7**	**12.6**	**4.6**	**10.1**	<1	<1	8.28 x 10^{-8}	**2.33 x 10^{-4}**
	56	<1	**1.8**	<1	**1.4**	<1	<1	1.18 x 10^{-8}	**3.33 x 10^{-5}**
	12	<1	<1	<1	<1	<1	<1	2.72 x 10^{-9}	7.67 x 10^{-6}
P5	365	**5.3**	**11.6**	**5.7**	**12.4**	<1	<1	1.67 x 10^{-11}	3.31 x 10^{-6}
	56	<1	**1.7**	<1	**1.8**	<1	<1	2.38 x 10^{-12}	4.72 x 10^{-7}
	12	<1	<1	<1	<1	<1	<1	5.48 x 10^{-13}	1.09 x 10^{-7}
P6	365	<1	<1	**4.1**	**9.0**	<1	<1	1.21 x 10^{-10}	8.93 x 10^{-6}
	56	<1	<1	<1	**1.3**	<1	<1	1.73 x 10^{-11}	1.27 x 10^{-6}
	12	<1	<1	<1	<1	<1	<1	3.99 x 10^{-12}	2.94 x 10^{-7}

Although THQ values for As are not concerning, the cancer risk of this chemical (table 2) is above the acceptable lifetime risk to children in the estimated exposure levels of 365 and 56 days year^{-1} for fish sampled in P4. This means that the human populations who consume this fish in any of these scenarios have a probability of developing cancer that is greater than 1 over 100,000 individuals (USEPA, 1989; USEPA, 2000).

It is important to emphasize the CRisk of As is calculated over an estimation of the amount of inorganic As from the measured levels of total As, since organic As compounds (such as the arsenobetaine, discussed above) are considered to be nontoxic and therefore not a threat to human health (ATSDR, 1998). No studies have been carried out in the APA-CIP to assess the relationship between contaminated seafood and cancer incidence, but previous studies in other places associated chronic inorganic As exposure via contaminated food with of an increasing of the number of diseases, such as cancer in the skin, lung, bladder and kidney (Castro-González and Méndez-Armenta, 2008; Sirot et al., 2009).

An important aspect of the assessment of risks to human health through exposure to potentially harmful substances in fish is the estimation of the allowable daily consumption of such substances (Moreau et al., 2007). This information is given in terms of maximum safe number of meals in a certain period of time, which is more suitable to communicate with local people and decision-makers since it is easy to understand. The maximum safe number of fish meals month^{-1} (monthly consumption rate, CR_{mm}) is presented in Table 3 for each chemical at each sampling site along the APA-CIP. An integrated CR_{mm} value considering the lowest CR_{mm} among all chemicals at each sampling station is also presented. The maximum safe number of fish meals ranged from 5-8 meals month^{-1} to adults, and 2-4 meals month^{-1} to children. The maximum safe number of meals is lowest for *C. spixii* collected in the sites closer to the Cananéia city, intermediate for the site closer to the RIR, and highest to the sites between far from main contamination sources in the APA-CIP. It is noteworhty that the CR_{mm} only takes into account the reference dose of the chemical for systemic effects; if the cancer slope was taken into account, the number of maximum number of fish meals would be considerably smaller because of the levels of As in the fish muscle tissue.

The results of the current study pointed out that the levels of metals and As in edible parts of an widely consumed fish have a potential risk of affecting human health through the consume of contaminated fish. It is important to bear in mind that this study considered only one fish species, which showed metals and As in muscle at levels considerably higher than other species as reported by previous studies. Individuals often eat several species of fish and other seafood in their diets, and therefore further studies are required to measure the contamination levels in the different organisms used for local consumption, as well as to detail the consumption patterns. Other uncertainty about the human health risk of consuming fish and seafood to people from APA-CIP is the lack of data about levels of contaminants other than metals and As (such as hydrocarbons and pesticides) in edible tissues of the organisms. In addition, in a broader sense, a more accurate assessment of human health risk in this area must consider nonfish sources of exposure to contaminants (other food items, drinking and bathing water, soil, air).

Nonetheless, the estimated risk factors presented in the current study indicated that there is potential risk of people who consume *C. spixii* developing chronic systemic effects and/or cancer in the APA-CIP. Small-scale fisheries are one of the main economic and subsistence activity in this area (Mendonça and Katsuragawa, 2001; Barcellini et al., 2013). “Caiçaras” (traditional fishermen), “Quilombolas” (maroons), and Native Americans frequently consume their own fish catch as an important source of protein in their diets, and *C. spixii* is widely consumed in the region. Furthermore, it is important to have in mind that, for traditional people, eating fish is not only a dietary choice, but it integrates their lifestyle and culture (USEPA, 2000). Therefore, metal and As contents found in the edible part of *C. spixii* is potentially a health issue for the traditional population, especially for children who are even more susceptible than adults.

Table 3. Maximum allowable fish consumption rate (meals.month^{-1}) (CR_{mm}) for adults (meal size = 227 g) and children (meal size = 114 g) in the different sites of the APA-CIP (P1 to P6).

Sampling station	Cd		Pb		Ni		As		Integrated CR_{mm}	
	Adult	Child	Adult	Child	Adult	Child	Adult	Child	Adult	Child
P1	7	3	6	3	15	7	>16	>16	6	3
P2	>16	>16	8	4	>16	9	>16	>16	8	4
P3	8	4	10	4	>16	12	>16	>16	8	4
P4	5	2	7	3	11	5	>16	>16	5	2
P5	6	3	5	2	6	3	>16	>16	5	2
P6	>16	>16	7	3	6	2	>16	>16	6	2

4. CONCLUSIONS

The current study reported the presence of Cd, Ni, Mn, Pb, Zn, and As muscle tissue of *C. spixii*. Cd, Pb, and As were found at concentrations above national and international action levels for human consumption. Depending on the level of exposure of the local population (number of days per year that a fish meal is consumed), the consumption of *C. spixii* pose risk to human health. Highest THQs were estimated for fish collected in sites closer to the main contamination sources in the APA-CIP, i.e. the RIR mouth (P1) and the city of Cananéia (P4, P5, and P6). Although As in *C. spixii* muscle shows low risk of causing systemic effects, there is cancer risks estimated based on these levels of As in *C. spixii* from the sites under the influence of the Cananéia city.

The present study on the health risks posed by the consumption of metal and As contaminated seafood by a sensitive population who lives in a MPA is not exhaustive. However, these findings raise concerns about the local population's health. This study showed that the exposure of the local population to metal and As contaminated seafood cannot be disregarded in environmental studies and management of the APA-CIP. Further studies are needed (e.g. feeding habits of the exposed population, levels of contaminants in other food items, levels of contaminants in the adult and child population) to detail at what extent such population are exposed to metal contamination in food and possibly subsidize management actions.

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4.CONCLUSÕES GERAIS

Os resultados deste estudo indicaram que a poluição ambiental na APA-CIP é causada por duas fontes principais de contaminação: i) o rio Ribeira do Iguape (RRI) que, a partir do canal do Valo Grade, introduz na APA-CIP água e sedimentos contaminados decorrentes de atividades pretéritas de mineração; e ii) a cidade de Cananéia, que introduz contaminantes diretamente no complexo estuarino lagunar provenientes de diferentes atividades humanas.

O presente estudo mostrou que a sazonalidade deve ser considerada em estudos integrados de qualidade ambiental envolvendo biomarcadores e bioacumulação. As concentrações de metais e As no músculo e fígado de *C. spixii* foram mais elevadas nas estações mais próximas a cidade de Cananéia (P4, P5 e P6) durante a estação seca, e diminuíram durante a estação chuvosa. Ao contrário, indivíduos coletados nas estações de amostragem mais próximas do RIR (P1, P2 e P3) apresentaram concentrações de metais mais elevadas durante a estação chuvosa. Percebeu-se também que, durante a estação seca, respostas antioxidantes, LPO, genotoxicidade e MTs, combinadas com níveis de metal no fígado e no músculo dos peixes foram mais presentes em áreas sob a influência da cidade de Cananéia (P4, P5 e P6). Durante a estação chuvosa, por outro lado, as respostas foram mais evidentes nas estações de amostragem sob maior influência do RIR (P1, P2, P3).

Os resultados obtidos no presente estudo também mostram que um conjunto de análises de biomarcadores utilizando diferentes órgãos alvo de peixes é uma ferramenta útil para avaliar a qualidade ambiental de uma área marinha protegida (AMP), Complexo Estuarino Lagunar de Cananéia-Iguape-Peruíbe (APA-CIP), sujeita a níveis moderados de contaminação.

Quando consideradas as respostas dos biomarcadores de atividade antioxidante e efeito nos diferentes órgãos avaliados (fígado, rim e brânquias), o tecido do fígado foi considerado o mais sensível em termos de respostas antioxidantes, enquanto que as brânquias foram órgãos mais sensíveis às respostas relacionadas aos biomarcadores de efeito.

Em relação à neurotoxicidade, os resultados mostraram que os níveis mais baixos da atividade da AChE foram observados geralmente em exemplares provenientes das áreas sob influência do RIR, o que pode estar relacionado a exposição aos metais e As provenientes das atividades de mineração ocorridas no alto Ribeira ao longo de décadas. A maior concentração de metais nos organismos coletados em estações próximas ao Rio Ribeira sugere na estação chuvosa que estes contaminantes são em grande parte introduzidos na APA-CIP a partir de fontes terrestres. Considerando ainda as respostas de atividade de AChE, é importante ressaltar que, sendo esta bacia hidrográfica uma importante área agrícola, outros contaminantes como pesticidas, ainda que não quantificados neste estudo, podem potencialmente serem responsáveis pela neurotoxicidade observada.

Diversos metais quantificados no músculo e fígado de *C. spixii* foram relacionados à genotoxicidade (e.g. Cr, Zn, Co, Cu, Pb). Dentre estes, o cromo foi o metal mais fortemente relacionado com as respostas genotóxicas, embora este metal, em estudos anteriores, não tenha sido relacionado às atividades pretéritas de mineração e sim à sua presença natural naquele ambiente. As alterações em nível celular (formação de micronúcleos (MN) e de anomalias nucleares (AN) no sangue), que normalmente persistem maior tempo no organismo, foram as respostas mais freqüentemente relacionadas aos metais nos fígado e no músculo quando comparadas a respostas genotóxicas em nível molecular (danos em DNA no sangue, fígado, rim e brânquias).

Observou-se ainda que o sangue foi o tecido que apresentou a maior relação entre os metais medidos e o aumento do dano em DNA em relação aos outros tecidos avaliados (fígado, rim e brânquias).

Os níveis de PAHs em bile foram também relacionados à genotoxicidade em nível cromossômico (MN e AN) e dano em DNA principalmente na brânquia. Além disso, os níveis de PAHs podem ter sido responsáveis pelo aumento da peroxidação lipídica nos indivíduos devido à ativação do Sistema P450 de detoxificação celular. A principal fonte de PAHs para o estuário aparenta ser a cidade de Cananéia, os quais, segundo estudos pretéritos, podem ser introduzidos na APA-CIP através das estruturas náuticas (marinas, garages, decks, etc.), do ferry boat, de efluentes municipais e drenagem urbana.

Quando realizada a abordagem de “peso de evidências” através da integração das diferentes linhas de evidência clássicas de qualidade de sedimento (toxicidade e físico química do sedimento), bioacumulação e respostas de biomarcadores por meio de análise multivariada, os resultados revelaram que as respostas sub-celulares e celulares em todos os órgãos estudados (rim, fígado, brânquia e sangue) foram mais fortemente associadas aos níveis de metais nos tecidos. Por outro lado, a contaminação dos sedimentos foi associada a efeitos biológicos mais severos, em níveis mais elevados de organização (a partir de danos em tecidos até efeitos em nível de organismo).

Observou-se, no presente estudo, a ausência de um padrão linear entre aumento de níveis de contaminantes no organismo e subseqüente aumento da severidade da lesão observada. Isto indica, por um lado, que nem todos os metais podem estar metabolicamente disponíveis para o organismo, ou exercendo efeitos tóxicos, como resultado do desenvolvimento de um sistema de seqüestro de contaminantes pelo organismo. Este mecanismo minimizaria a toxicidade, mantendo os contaminantes nos tecidos do corpo, mas em uma forma relativamente inerte. Por outro lado, outra hipótese é que os organismos aquáticos também podem começar a excretar metais após os níveis internos atingirem uma toxicidade limiar. Isto poderia explicar porque foram observados efeitos mais graves em amostras com menores concentrações de contaminantes nos tecidos.

A inclusão de índices de biomarcadores baseados nas respostas sub-celulares, celulares, e histológicas na abordagem de “peso de evidências”, aliados a da avaliação ecotoxicológica tradicional em nível de organismo, foi útil para discriminar os diferentes níveis de degradação ambiental na APA-CIP. Como um diagnóstico da qualidade ambiental na área estudada, P2 e P4, durante a estação chuvosa, podem ser considerados como os locais mais degradados da APA-CIP. Isto porque a avaliação de “peso de evidências” mostrou que os organismos destes pontos estão expostos a contaminantes (maior contaminação dos sedimentos) e sujeitos a efeitos em níveis mais elevados de organização biológica (danos histológicos no fígado de *C. spixii*, desenvolvimento anormal de larvas de ouriço do mar e diminuição da fecundidade de copépodos). P2 e P4 estão sob a influência das fontes de contaminantes mais importantes da APA-CIP, o RIR e a cidade de

Cananéia respectivamente, o que também corrobora a influência destas fontes de contaminantes na APA-CIP.

Quando considerada a avaliação de risco à saúde humana frente ao consumo de *C. spixii*, este estudo observou a presença Cd, Pb, e As em concentrações acima dos níveis permitidos para o consumo humano com base em legislações nacionais e internacionais que regulam o setor. Ademais, dependendo do nível de exposição da população local (número de dias por ano em que o peixe é consumido), o consumo de *C. spixii* pode colocar em risco a saúde humana. Altos valores de “THQs” (*Target Hazard Quocient*) foram estimados para os peixes coletados nos locais mais próximos das principais fontes de contaminação da APA-CIP, ou seja, a desembocadura do RIR (P1) e a cidade de Cananéia (P4, P5 e P6). Embora, os níveis de Arsênio aparentam ter baixo risco de efeitos sistêmicos, os riscos de câncer estimados com base nesses níveis de As são elevados, especialmente nos locais sob a influência da cidade.

5. CONSIDERAÇÕES FINAIS

Os efeitos biológicos observados nos peixes da APA-CIP sugerem o risco potencial de exposição da biota frente aos contaminantes. Os resultados do presente estudo mostraram que a APA-CIP recebe contaminantes provenientes tanto de fontes externas aos limites da Área de Proteção Ambiental (APA), principalmente metais e outros contaminantes provenientes do alto Ribeira, como provenientes de fontes internas a APA, adicionados a laguna/estuário através de efluentes e escoamento superficial da cidade de Cananéia. Tais resultados ressaltam a importância de se controlar não somente as fontes contaminantes dentro das APAs, mas também fontes externas aos seus limites jurisdicionais.

A química da água e parâmetros microbiológicos são atualmente os indicadores de qualidade de água recomendados pela União Internacional para a Conservação da Natureza e dos Recursos Naturais (IUCN) para a avaliação da poluição nas áreas Marinhas Protegidas (POMEROY *et al*., 2005). Os indicadores biológicos também são recomendados, mas eles não são específicos para avaliar a saúde dos peixes expostos aos contaminantes. Os resultados do presente estudo sugerem que as analises de biomarcadores em peixes são ferramentas úteis para avaliar a eficácia e para o monitoramento da qualidade ambiental nas áreas marinhas protegidas submetidas à contaminação. Esta observação é importante pois sugere que, mesmo que os contaminantes em áreas marinhas protegidas estejam em níveis apenas moderados, eles ainda podem afetar a saúde da biota residente e conseqüentemente impactar seu ajustamento ecológico. Nestes casos, mudando o foco da avaliação dos agentes (por exemplo, a medição dos níveis de contaminantes) para os alvos (biota), mas não desconsiderando qualquer dessas informações, pode-se fornecer subsídios importantes para o gerenciamento e proteção destas APAs.

Adicionalmente, é importante avaliar as respostas subletais combinadas com os níveis de contaminantes nos tecidos, a fim de avaliar a forma como a poluição afeta o meio ambiente. Outras linhas de evidências, como níveis de contaminantes ambientais e toxicidade, também são importantes para um melhor entendimento dos níveis de degradação do ambiente, especialmente quando se trata de áreas moderadamente contaminadas. A falta de associação, no entanto, entre a contaminação dos sedimentos e níveis de contaminantes em *C. spixii*, e a ausência de linearidade entre aumento de níveis de contaminantes nos organismos e subseqüente aumento da severidade da lesão, sugerem a presença de mecanismos complexos de regulação de metais em *C. spixii*, o que pode levar a interpretações equivocada dos resultados se avaliadas isoladamente as linhas de evidência. Isso reforça a importância de abordagens integradas e que considerem diferentes matrizes ambientais (organismos e sedimentos), especialmente em áreas moderadamente contaminadas.

Atualmente ainda há uma lacuna no entendimento das relações entre contaminantes medidos no ambiente (como, por exemplo, no sedimento), bioacumulação e respostas biológicas, para cenários de exposição moderadamente contaminados. Este conhecimento deve ser incentivado e aprofundado de modo com que se possa avaliar com mais clareza como os níveis de contaminantes ambientais podem afetar a bioacumulaçao na biota local e também as respostas destes indivíduos frente a esta contaminação moderada.

Quando abordada a avaliação de risco a saúde humana frente ao consumo de peixe proveniente de diferentes áreas da APA-CIP, os resultados do presente estudo indicam que se deve ter preocupação em relação a saúde da população local. Estudos mais detalhados ainda são necessários (para, por exemplo, entender detalhadamente os hábitos alimentares da população exposta, os níveis de contaminantes em outros itens alimentares, além dos níveis de contaminantes na população adulta e infantil). No entanto, as análises iniciais apresentadas neste estudo mostram que a população que consome alimentos provenientes da APA-CIP está exposta a níveis altos de metais e arsênio. O presente estudo reforça que a população local não pode ser desconsiderada na gestão ambiental de AMPs.

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