UNIVERSIDADE FEDERAL DO PARANÁ

LILIAN DALAGO SALGADO

AVALIAÇÃO INTEGRADA DOS NÍVEIS DE CONTAMINANTES DOS SEDIMENTOS E DAS RESPOSTAS BIOLÓGICAS DE DUAS ESPÉCIES DE PEIXES RESIDENTES DO COMPLEXO ESTUARINO-LAGUNAR DE IGUAPE-CANANÉIA.

CURITIBA

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Orientadora: Profª. Drª. Helena Cristina da Silva de Assis

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Curitiba, 22 de Fevereiro de 2019.

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“Imagine o quanto mais avançada a sociedade seria hoje se as mulheres, que compreendem a metade do poder cerebral do mundo, fossem socialmente e intelectualmente emancipadas desde o começo da civilização.”

Neil deGrasse Tyson

RESUMO

O Complexo Estuarino-Lagunar de Iguape-Cananéia (CELIC) está entre as áreas de maior relevância ambiental do Atlântico Sul. Entretanto, passou por diversas alterações que modificaram os parâmetros das águas estuarinas e afetaram sua qualidade ambiental. A área hoje encontra-se sob crescente pressão antrópica e enfrenta inúmeros problemas de gestão ambiental. Este estudo visou contribuir para a avaliação da qualidade ambiental local, por meio de um estudo integrado, unindo-se dados relativos à análise química dos sedimentos (metais, hidrocarbonetos policíclicos aromáticos - HPA e produtos farmacêuticos e de higiene pessoal - PFHP) e da avaliação das respostas biológicas de duas espécies de peixes estuarino-residentes. As duas coletas para cada espécie estudada, juntamente com os sedimentos dos mesmos pontos, se deram entre 2016 e 2017 contemplando os períodos frio-seco e quente-chuvoso. O peixe *Atherinella brasiliensis* foi amostrado nas proximidades da Cidade de Cananéia e de Subaúma, enquanto o peixe *Gobioides broussonnetii* foi amostrado ao norte da Ilha de Cananéia, em Subaúma e próximo à Cidade de Iguape. Os peixes tiveram sua saúde avaliada por meio do uso de biomarcadores bioquímicos (AChE; SOD; CAT; GPx; GST; EROD; GSH; MT; LPO), histopatológicos e de genotoxicidade (quebras do DNA e alterações morfológicas nucleares - AMN) em diferentes tecidos (sangue, cérebro, brânquias, músculo, fígado e rim). A análise dos sedimentos revelou a presença das três classes de contaminantes estudadas, mostrando contaminações de baixas a moderadas em todo o sistema. As concentrações de contaminantes tiveram variações temporais e espaciais com maiores valores nos pontos de maior presença antrópica (cidades de Cananéia e Iguape), especialmente no período frio-seco. Contribuições de mineração pretérita, agricultura, queima de biomassa e combustíveis fósseis, presença de embarcações e despejo de lixo e esgoto foram evidenciadas, com influências das áreas urbanas e do maior tempo de permanência das águas no estuário no período seco. Nos peixes, variações espaciais e sazonais nos biomarcadores seguiram o mesmo padrão, com respostas mais pronunciadas nos animais coletados próximo à Cananéia e Iguape no período seco. Para o *A. brasiliensis* foram observados AMN no sangue e necrose no fígado. Para *G. broussonnetii,* aneurismas e elevação do epitélio lamelar foram observados em brânquias, necrose e centros de melanomacrófagos – CM nos rins e esteatose e CM no fígado. Os resultados das análises multivariadas e do índice de integração de biomarcadores revelaram piores condições ambientais em Cananéia e Iguape. Ainda, revelaram que a contaminação dos sedimentos foi parcialmente responsável pelas respostas biológicas em ambas espécies. Os resultados suportaram a associação entre as alterações encontradas nos peixes com a presença de metais, HPA e PFHP como estressores. Assim, reforça-se a existência de efeitos subletais à ictiofauna local, que podem, ao longo do tempo, influenciar na saúde e na sobrevivência dos organismos e levar a alterações em níveis de organização biológica mais elevados que podem contribuir para a deterioração do estado ecológico da região. A abordagem integrada usada por este estudo contribui para um panorama atual da qualidade ambiental do CELIC que pode futuramente contribuir para a gestão da área.

Palavras-chave: Metais. Hidrocarbonetos policíclicos aromáticos. Produtos farmacêuticos e de higiene pessoal. Biomarcadores. Índice integrado de biomarcadores.

ABSTRACT

The Estuarine-Lagoon Complex of Iguape-Cananéia (ELCIC) is among the areas of greater environmental relevance in the South Atlantic. However, several changes altered the parameters of the estuarine waters and affected its environmental quality. The area today is under increasing human pressure and faces numerous environmental management problems. This study aimed to contribute to the assessment of local environmental quality through an integrated approach, combining data on the chemical analysis of sediments (metals, polycyclic aromatic hydrocarbons - PAHs and pharmaceuticals and personal care products - PPCPs) and the evaluation of the biological responses of two estuarine-resident fish species. The two sample campaigns for each studied species, together with the sediments of the same points, occurred between 2016 and 2017 contemplating the cold-dry and hot-rainy periods. The *Atherinella brasiliensis* fish was sampled near the cities of Cananéia and Subaúma, while the *Gobioides broussonnetii* fish was sampled in the north of the Cananéia Island, in Subaúma and near Iguape City. The fish had their health conditions evaluated through the analysis of biochemical (AChE; SOD; CAT; GPx; GST; EROD; GSH; MT; LPO), histopathological and genotoxicity biomarkers (DNA strand breaks and nuclear morphological abnormalities - NMA) in different tissues (blood, brain, gills, muscle, liver and kidney). The sediment analysis revealed the presence of the three classes of the studied contaminants, reveling low to moderate contaminations throughout the system. The concentrations of contaminants had temporal and spatial variations with higher values at the points of greater anthropic presence (Cananéia and Iguape cities), especially in the cold-dry period. Contributions from past mining, agriculture, burning of biomass and fossil fuels, the presence of boats and garbage and sewage disposal were evidenced, with influences of the urban areas and the longer permanence of the waters in the dry period. In fish, spatial and seasonal variations in the biomarkers followed the same pattern, with more pronounced responses in the animals collected near Cananéia and Iguape in the dry period. For *A. brasiliensis*, NMA in blood and necrosis in liver were observed. For *G. broussonnetii*, aneurysms and lifting of the lamellar epithelium in gills, necrosis and melano-macrophagous centers – MC in kidney and steatosis and MC in liver were observed. The results of the multivariate analysis and the integrated biomarkers response index revealed worse environmental conditions in Cananéia and Iguape. In addition, they revealed that the contamination of the sediments was partially responsible for the biological responses in both fish species. The results supported the association among the alterations found in the fish with the presence of metals, PAHs and PPCPs as stressors. Thus, reinforcing the existence of sublethal effects on the local ichthyofauna which can, over time, influence in the health and the survival of the organisms and lead to changes in higher biological levels, that may contribute to the deterioration of the regional ecological status. The integrated approach used in the present study contributes to a current overview on the environmental quality of the ELCIC, which can further contribute for the management of the area.

Key words: Metals. Polycyclic aromatic hydrocarbons. Pharmaceutical and personal care products. Biomarkers. Integrated biomarker response index.

SUMÁRIO

1 INTRODUÇÃO GERAL

A degradação dos ecossistemas marinhos e costeiros está intimamente relacionada com o aumento populacional e os impactos negativos das atividades antrópicas, sejam elas de origem doméstica, industrial, agrícola ou comercial (VIKAS; DWARAKISH, 2015). Atualmente, estima-se que cerca de 40% da população mundial viva a menos de 100 Km do mar, o que causa pressões constantes sobre estas regiões (THE OCEAN CONFERENCE, 2017). Assim, objetivando uma melhor gestão destas áreas, com o equilíbrio do uso humano e o mantenimento de ecossistemas saudáveis, áreas marinhas protegidas (AMP) têm sido criadas e implementadas ao redor do mundo (SMITH; SMITH, 1994; MARINE CONSERVATION INSTITUTE, 2019).

As AMP são consideradas como uma das ferramentas de manejo mais simples e robustas para a conservação dos ambientes marinhos e costeiros, as quais se baseiam no principio de que ao se remover alguns usos de um determinado ambiente, certos ecossistemas podem recuperar sua função real, as populações podem ser reforçadas e os habitats protegidos (MARINE CONSERVATION INSTITUTE, 2019). Deste modo, as AMP incluem diferentes tipos de áreas de preservação que variam em seus níveis de proteção e tipos permitidos de uso.

No entanto, apenas 4,8% dos oceanos encontram-se protegidos, sendo que muitas destas áreas protegidas são pobremente administradas ou não assistidas (LOPES; VILLASANTE, 2018; TOSIC et al., 2018; MARINE CONSERVATION INSTITUTE, 2019). Ainda, a eficácia das AMP está inevitavelmente ligada às ações em suas fronteiras, o que faz com que por muitas vezes o cumprimento de seus objetivos de conservação não seja efetivamente concretizado (SMITH; SMITH, 1994; MACEDO; MEDEIROS, 2018).

O Brasil conta com uma extensa área litorânea, de aproximadamente 8.500 km, aonde encontram-se 16 das 28 metrópoles do país. No último ano, o país estendeu seu território de áreas marinhas protegidas de 1,5% para 25%, do seu total de 3,6 milhões de km^2 de território marinho, com a criação de 4 novas AMP nos arquipélagos São Pedro e São Paulo (Pernambuco) e Trindade e Martim Vaz (Espírito Santo) (MINISTÉRIO DO MEIO AMBIENTE, 2018). Contudo, a maior parte do território nacional sob proteção em AMP referem-se a áreas de mar aberto e nas zonas costeiras, onde estão os ecossistemas sob maior pressão, a porcentagem ainda é muito pequena (WWF, 2018).

Desta maneira, apesar dos avanços do último ano, é necessário ainda continuar investindo em novas AMP buscando-se garantir uma maior representatividade ecológica no desenho da rede de áreas protegidas no Brasil (PACHECO et al., 2018). Adicionalmente, a maioria das unidades de conservação nacionais são de uso sustentável e a administração destas áreas enfrenta inúmeros conflitos sociais e ambientais que fazem com que muitas delas sigam sendo precariamente geridas (MORAIS; ABESSA, 2014; LOPES; VILLASANTE, 2018; MACEDO; MEDEIROS, 2018; PACHECO et al., 2018).

No país, ainda se observam diferentes cenários socioeconômicos e ambientais que mostram uma realidade conflitante. Por um lado, o Brasil está entre as dez maiores economias do mundo, o que reflete em um grau de consumo elevado e problemas ambientais de economias mais desenvolvidas, como a presença de inúmeras novas substâncias no ambiente. Por outro lado, ainda existem problemas em relação ao saneamento básico em diversas regiões do país, o que faz com que o Brasil também apresente problemas ambientais típicos de países com menor desenvolvimento, como a existência de epidemias de diferentes doenças (MONTAGNER et al., 2017; MACEDO; MEDEIROS, 2018). Deste modo, a fabricação e o uso de diferentes produtos em larga escala, somados a ineficiência nos processos de coleta e de tratamento dos efluentes e de resíduos, permitiram que diferentes classes de contaminantes impactassem nossa costa (AZEVEDO et al., 2012; TOSIC et al., 2018; TRAMONTE et al., 2018).

Os contaminantes podem chegar às águas costeiras por fontes naturais ou antrópicas, seja por via atmosférica, pela precipitação pluvial, pelo escorrimento para os cursos de água, lixiviação do solo ou pelo descarte direto, feito tanto de maneira acidental quanto intencional (VIKAS; DWARAKISH, 2015; TOSIC et al., 2018). Os contaminantes que despertam maior preocupação são aqueles que apresentam persistência no ambiente, biodisponibilidade, capacidade de passar pelos processos de bioacumulação e/ou biomagnificação e que apresentam efeitos tóxicos aos seres vivos (LU et al., 2018).

Os metais e os hidrocarbonetos policíclicos aromáticos (HPA) apresentam essas propriedades, fazendo com que as suas concentrações sejam reguladas por diferentes agências ao redor do mundo (LU et al., 2018; SUN et al., 2018). No Brasil, o Conselho Nacional do Meio Ambiente - CONAMA orienta os níveis permitidos desses contaminantes em águas superficiais (Resolução 357/05; CONAMA, 2005) e nos sedimentos de dragagem (Resolução 454/12; CONAMA, 2012). No entanto, produtos farmacêuticos e de higiene pessoal (PFHP) têm sido cada vez mais detectados em amostras ambientais, e embora sua presença possa representar algum risco para os ecossistemas e a saúde humana, eles não são incluídos nos programas de monitoramento de rotina já que não são legislados (MONTAGNER et al., 2017; KRAMER et al., 2018).

Os diferentes ecossistemas costeiros apresentam características distintas de diversidade, produtividade e estabilidade das comunidades biológicas que os formam, em função da grande variedade de fatores ambientais (SMITH; SMITH, 1994). Os estuários são ambientes de transição entre a rede de drenagem continental e o ambiente marinho, que constituem um típico exemplo de alta produtividade e biodiversidade (DAME, 2008). Estas regiões oferecem inúmeros serviços ambientais, entre eles o suprimento de água potável e de recursos pesqueiros, sendo áreas de intensa ocupação humana (DAME, 2008). Assim, a presença de contaminantes nestas regiões é uma perturbação que pode desencadear uma série de reações químicas e biológicas, que ao longo do tempo podem levar a perda da qualidade, da biodiversidade e dos serviços ambientais prestados por estas áreas (GUSSO-CHOUERI et al., 2018; LU et al., 2018; SUN et al., 2018).

Contudo, a avaliação dos impactos causados por contaminações em ambientes aquáticos vai além da simples presença dos contaminantes nestas áreas, e tomam grande complexidade em função das diferentes características dos ecossistemas e da presença de múltiplos estressores nestas regiões (BREITBURG; RIEDEL, 2005; KROON et al., 2017). A biodisponibilidade e a toxicidade dos contaminantes em misturas complexas podem ser alteradas como resultado de suas interações com as condições ambientais (pH, salinidade, potencial redox, agentes complexantes orgânicos e inorgânicos), que fazem com que estes transitem entre os compartimentos ambientais, e de sinergias entre os próprios contaminantes (BREITBURG; RIEDEL, 2005; GU et al., 2015).

Nas últimas décadas o conhecimento e as pesquisas acerca da contaminação ambiental tiveram grande evolução. Em um primeiro momento, o gerenciamento e os estudos estiveram focados na medição dos níveis de contaminantes em diferentes compartimentos aquáticos, principalmente na água e nos sedimentos. O conhecimento destas concentrações é de suma importância e pode possibilitar a identificação das principais fontes de contaminação e indicar o risco de exposição da biota, quando as concentrações medidas são comparadas com as diretrizes de qualidade e as legislações ambientais vigentes (CHOUERI et al., 2009; BIRCH, 2018).

Dentre os compartimentos aquáticos, a água geralmente atua como corpo receptor para os contaminantes. No entanto, devido a flutuações nas condições ambientais e ao fato do tempo de permanência para muitos contaminantes ser pequeno na coluna d’água, havendo sua remoção ou transferência para o compartimento sedimentar ou a biota, as concentrações da coluna d´água apresentam alto grau de variação o que implica na limitação da confiabilidade dos resultados (TOSIC et al., 2018). Deste modo, os sedimentos mostram-se como bons indicadores da qualidade ambiental devido suas elevadas capacidades de sorção e acumulação, desempenhando um importante papel no destino final de contaminantes. Geralmente, as concentrações nos sedimentos são maiores do que na água, o que faz com que os sedimentos também representem um potencial latente de degradação da coluna d´água, mesmo após de interrompidas as emissões (GU et al., 2015; OMAR et al., 2018).

No entanto, os dados químicos, isoladamente não são capazes de fornecer informações sobre os efeitos biológicos. Assim, a medição da mistura de efeitos dos contaminantes faz-se necessária em um segundo momento, surgindo como auxilio no preenchimento da lacuna entre a presença de contaminantes em um ambiente e seus possíveis impactos aos seres vivos (MARANHO et al., 2015). Estas medições podem se dar de diferentes maneiras, como por exemplo: por meio do emprego de estudos ecológicos (MARTÍN; BAYLE, 2018), de testes de toxicidade (CRUZ et al., 2019), do uso das ciências ômicas (SNAPE et al., 2004) e do uso de biomarcadores (MARANHO et al., 2012).

Os biomarcadores podem ser definidos como alterações quantificáveis em componentes moleculares ou celulares, processos, estruturas e funções relacionadas à exposição a substâncias químicas, refletindo desta maneira a saúde dos organismos e indicando a presença, o efeito e o grau de contaminação de um ambiente (WALKER, 1995; SILVA DE ASSIS, 1998; DALZOCHIO et al., 2016). Essas respostas têm sido amplamente utilizadas para fornecer a conexão entre níveis externos de exposição a contaminantes, níveis internos de contaminação dos tecidos e efeitos adversos precoces em organismos (BURKINA et al., 2015; KROON et al., 2017). Apesar de não serem ferramentas obrigatórias nas legislações ambientais, são considerados como importantes indicadores do estado de saúde ambiental em pesquisas avançadas e estratégias eficazes de gestão (KROON et al., 2017).

Numerosos estudos têm aplicado o uso de diferentes biomarcadores para monitorar diversas fontes de contaminação antrópica em áreas costeiras usando peixes como espécies indicadores (AZEVEDO et al., 2012, RIBEIRO et al., 2013; GUSSO-CHOUERI et al., 2016; SANTOS et al., 2018). Os peixes são reconhecidos como importantes bioindicadores dos sistemas aquáticos, devido ao seu contato direto com os contaminantes da água, sua capacidade de bioacumulação, por terem ampla distribuição geográfica e ocuparem diferentes níveis tróficos (KROON et al., 2017). Assim, a relevância ecológica destas respostas faz com que assumam um eficiente papel no biomonitoramento aquático (MARANHO et al., 2012; BURKINA et al., 2015).

O objetivo do presente estudo foi utilizar uma abordagem integrada com dados relativos a análise química dos sedimentos e das respostas biológicas de exemplares da ictiofauna de uma AMP do sul do estado de São Paulo, o Complexo Estuarino-Lagunar de Iguape-Cananéia (CELIC). A investigação teve como finalidade contribuir para um melhor entendimento de qualidade ambiental do local, bem como, avaliar a relação entre a presença de contaminantes e os efeitos nocivos encontrados na biota, indicando os possíveis estressores.

A proposta da pesquisa envolveu a determinação de metais, HPA e PFHP nos sedimentos e a avaliação da saúde de duas espécies de peixes estuarino-residentes, sendo uma espécie pelágica (*Atherinella brasiliensis*) e uma espécie demersal (*Gobioides broussonnetii*), com a finalidade de investigar duas diferentes formas de exposição da ictiofauna aos contaminantes dos sedimentos.

A escolha das espécies em estudo se deu por ambas possuírem importância ecológica e social na região. Estes peixes desempenham um importante papel na cadeia trófica local, servindo como alimento para outras espécies de peixes e mamíferos (BORDIGNON, 2006; DIAS et al., 2009; LOPES et al., 2012). Também são alvo da pesca recreacional e artesanal na área (BERVIAN; FONTOURA, 2007; CONTENTE et al., 2011), servindo como isca para a pesca de espécies de peixes de maior valor comercial, mas que também acabam sendo, por muitas vezes, consumidas pelos ribeirinhos, especialmente pelas famílias de baixa renda (SANTOS; SAMPAIO, 2013). Ainda, estas espécies possuem relevância para estudos de avaliação de impactos ambientais, podendo assim, serem consideradas como modelos biológicos adequados para avaliações ecotoxicológicas. A saúde dos peixes foi investigada por meio do uso de diferentes biomarcadores (bioquímicos, histopatológicos e de genotoxicidade) em diferentes tecidos (sangue, cérebro, brânquias, músculo, fígado e rins).

A seleção dos contaminantes avaliados foi feita em decorrência da viabilidade das análises químicas e de sua importância para a saúde ambiental e coletiva. Cinco pontos amostrais distribuídos ao longo do sistema estuarino foram investigados nos dois principais períodos climáticos da região (estações quente-chuvosa e fria-seca) com a finalidade de se indicar o período com maior estresse ambiental.

Este documento é composto por uma introdução ao tema, que se segue da exposição da problemática da área em estudo, seguida por dois capítulos referentes as duas pesquisas realizadas, as quais são apresentadas sob a forma de artigos científicos. O **Capítulo I** envolve a integração das análises dos contaminantes nos sedimentos e o uso da espécie de peixe *Atherinella brasiliensis* como sentinela para a avaliação da saúde ambiental do CELIC. O **Capítulo II** envolve a avaliação dos efeitos tóxicos da contaminação ambiental do CELIC sobre a espécie de peixe *Gobioides broussonnetii*. Por fim, as considerações finais apresentam uma avaliação geral dos resultados obtidos pelo estudo e de sua amplitude, levando em consideração suas contribuições para a população e autoridades locais no desenvolvimento de políticas públicas que busquem preservar ou melhorar a qualidade ambiental, de vida e da saúde pública na região. As referências bibliográficas são apresentadas ao final de cada capítulo, enquanto às usadas na introdução geral e nas considerações finais são apresentadas no final do documento.

1.1 A PROBLEMÁTICA DA ÁREA EM ESTUDO

O Complexo Estuarino-Lagunar de Iguape-Cananéia (CELIC) situa-se no extremo sul do litoral de São Paulo. O local faz parte de uma das AMP brasileiras, a Área de Proteção Ambiental de Cananéia-Iguape-Peruíbe (APA-CIP), a qual foi criada em 1984 (BRASIL, 1984, 1985). A região compreende a um sistema de canais, entre quatro grandes ilhas (Cardoso, Cananéia, Comprida e Iguape). Esta área está inserida na maior mancha contínua de Mata Atlântica do Brasil, que abriga grande biodiversidade, e inclui espécies raras, endêmicas e ameaçadas de extinção (CUNHA-

LIGNON et al., 2009). Os cenários naturais do local contam com praias, ilhas, lagoas, serras, dunas, restingas e as mais extensas e mais preservadas áreas de mangues do litoral paulista (SCHAEFFER-NOVELLI et al., 1990).

Atualmente, os principais municípios da região são: os núcleos históricos de Cananéia e de Iguape, e Ilha Comprida. A cidade de Cananéia se localiza na porção sul do estuário e possui atividade econômica baseada na pesca, no turismo, na agricultura e na pecuária. A cidade de Iguape localiza-se ao norte e apresenta ainda, além das atividades já citadas, as práticas comerciais e industriais, especialmente do setor pesqueiro, como fontes da economia. Já o município da Ilha Comprida apresenta urbanização muito reduzida, com pequenos vilarejos ao longo de sua extensão com maior concentração urbana em sua porção norte (CUNHA-LIGNON et al., 2009; MORAIS; ABESSA, 2014).

A região foi declarada como Reserva da Biosfera da Mata Atlântica, no ano de 1991 pela Organização das Nações Unidas para a Educação, a Ciência e a Cultura – UNESCO. É ainda atualmente reconhecida pela União Internacional para Conservação da Natureza - IUCN como o terceiro ambiente de importância quanto à produtividade marinha no Atlântico Sul. Dada a sua importância, a região apresenta ainda diversas outras áreas de conservação ambiental, federais e estaduais, que circundam o CELIC (MORAIS; ABESSA, 2014). No entanto, apesar de deter patrimônio natural, paisagístico, histórico e cultural a região é considerada como uma das mais pobres do estado, necessitando da demanda de grandes investimentos no campo social, em infraestrutura e regularização fundiária (CUNHA-LIGNON et al., 2009; MORAIS; ABESSA, 2014).

Esta região abrigou ainda um dos exemplos mais claros de degradação ambiental causada por atividades de origem antrópica em um ambiente costeiro, com a abertura de um canal artificial na área, sofrendo assim fortes alterações ambientais pretéritas à criação da AMP. Estas alterações permitiram que o sistema apresente características ambientais bastante distintas entre seus setores. Próximo à Ilha de Cananéia existe forte influência marinha. Neste local a contribuição de água doce é feita por diferentes pequenos rios que somam uma bacia de drenagem de cerca de 1.339 km^2. Isto faz com que se observem características típicas de ambiente estuarino nesta região. Já nas proximidades de Iguape as características são tipicamente fluviais devido ao aporte de água doce feito pelo rio Ribeira de Iguape e seus afluentes, que contam com uma bacia de drenagem total de 23.350 km^2 (BONETTI-FILHO; MIRANDA, 1997; MAHIQUES et al., 2009).

Este rio, passou a adentrar o sistema estuarino pela abertura de um canal artificial, chamado de Valo Grande. A construção foi realizada em 1852 e hoje permite o desvio de 70% das águas do rio para dentro do estuário (MAHIQUES et al., 2013). Isso fez com que o rio se tornasse o maior contribuinte de água doce para o CELIC, causando várias alterações nas condições estuarinas, entre elas, na salinidade, na sedimentação e no aporte de contaminantes (TRAMONTE et al., 2018).

O Rio Ribeira de Iguape possui 470 km de extensão, sendo 220 km em território paranaense e 250 km em território paulista. Sua bacia conta com vários depósitos minerais, que foram particularmente explorados durante o século passado, especialmente entre os anos de 1945 a 1995 (MAHIQUES et al., 2013). Diferentes minas nos dois estados extraíram Pb, Zn, Au, Ag, As e Cu, e operaram durante anos descartando os rejeitos e a escória do forno de fundição indiscriminadamente no rio, sendo que a maior delas ficava localizada na cidade de Adrianópolis - PR (TRAMONTE et al., 2018). Estima-se que durante o período de mineração o rio recebeu cerca de 5,5 toneladas por ano de resíduos ricos em metais (GUIMARÃES; SÍGOLO, 2008). Isso ocasionou extensa contaminação da água, dos sedimentos, dos solos, dos alimentos e das populações humanas residentes nas proximidades das minas de extração, especialmente por Pb (CUNHA, 2003; LAMMOGLIA et al., 2010; ABESSA et al., 2014).

Devido à diminuição da lucratividade e aos problemas ambientais causados, em 1996 as atividades de mineração foram encerradas (CUNHA, 2003). Após o fechamento das minas, os resíduos foram depositados nas margens do rio na forma de pilhas de rejeitos, ficando por muitos anos expostas ao clima e consequentemente à lixiviação (ABESSA et al., 2014). Deste modo, os metais sofreram mobilidade através dos sólidos suspensos e, ao longo dos anos, atingiram o CELIC (MAHIQUES et al., 2013; SALGADO; AZEVEDO, 2018).

O rio ainda drena importantes áreas agrícolas dos estados de São Paulo e do Paraná e recebe efluentes de diferentes origens das cidades circundantes (ABESSA et al., 2014; MORAIS; ABESSA, 2014). Além disso, licenciamentos permitiram a implantação de diferentes indústrias cimenteiras para exploração de calcário na cidade de Adrianópolis - PR, liberando inclusive a utilização do espaço das antigas minas de extração de metais, o que permite uma nova remobilização destes solos (BREMBATTI, 2014).

Atualmente, o Canal Valo Grande exerce influência sobre todo CELIC e prove aporte substancial de lama e água doce que permitem a entrada de diferentes contaminantes (GUIMARÃES; SÍGOLO, 2008; TRAMONTE et al., 2018; CRUZ et al., 2019). Somados a este cenário, têm-se ainda a contribuição das cidades litorâneas e das atividades antrópicas de dentro do sistema estuarino no aporte de contaminantes. Entre estas pode-se citar o despejo de dejetos e de efluentes domésticos, as atividades agrícolas e de maricultura e a presença de embarcações (MORAIS; ABESSA, 2014; GUSSO-CHOUERI et al., 2015). Deste modo, estudos anteriores da qualidade ambiental já revelaram níveis acimas dos permitidos pelas legislações ambientais para alguns metais e níveis baixos à moderados de hidrocarbonetos policíclicos aromáticos para os sedimentos do CELIC (ANTONELLI et al., 2017; SALGADO; AZEVEDO, 2018; TRAMONTE et al., 2018; CRUZ et al., 2019).

Apesar da degradação das condições estuarinas ter determinando um gradiente espacial de perda de abundância, riqueza e diversidade funcional da ictiofauna em direção à Iguape, há abundância regular de recursos ao longo de todo o ano no CELIC, o que permite que os recursos pesqueiros sejam sua base econômica (MENDONÇA; KATSURAGAWA, 2001; CONTENTE, 2013). A pesca artesanal constitui uma importante atividade econômica e praticamente todo o produto de alto valor comercial é direcionado para o comércio (MENDONÇA; KATSURAGAWA, 2001). Assim, as populações locais, por muitas vezes, acabam por consumir as espécies menos visadas comercialmente (GUSSO-CHOUERI et al., 2018).

Em resposta à necessidade de uma profunda investigação da qualidade ambiental do local, estudos sobre bioacumulação e efeitos sobre a biota aquática tiveram início nas últimas décadas na região. Na ictiofauna foram observadas preocupante bioacumulação de metais (AZEVEDO et al., 2013; GUSSO-CHOUERI et al., 2015, 2018) e HPA (AZEVEDO et al., 2012), sendo evidenciado também que estes contaminantes podem estar induzindo estresse sobre a biota aquática (CRUZ ET AL., 2019), incluindo na saúde dos peixes (FERNANDEZ et al., 2011; AZEVEDO et al., 2013; GUSSO-CHOUERI et al., 2016).

Uma vez que esses organismos estão sujeitos cronicamente a estas e outras exposições, existe a possibilidade de que em longo prazo, as contaminações levem a efeitos mais drásticos, com significância a nível populacional, de comunidades e até mesmo ecossistêmico. Deste modo, estudos sobre as contaminações ambientais e suas implicações para as diferentes espécies do CELIC continuam tendo grande importância e garantem que um monitoramento contínuo da qualidade ambiental da região seja feito, sendo de interesse da população em geral e dos órgãos ambientais e de saúde.

2 CAPITULO I

Integrated assessment of sediment contaminant levels and biological responses in sentinel fish species *Atherinella brasiliensis* from a sub-tropical estuary in South Atlantic

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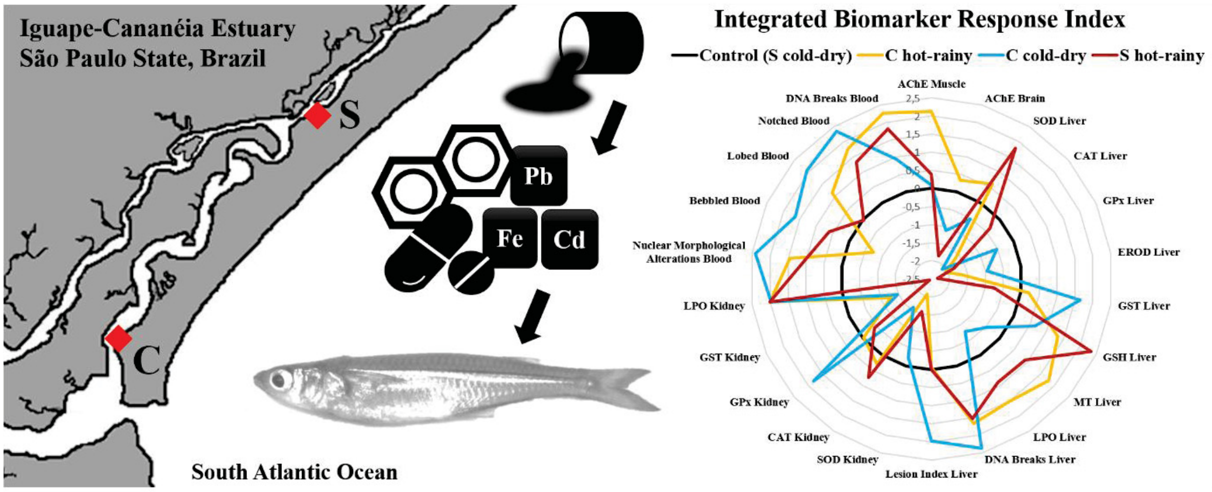
2.1 ABSTRACT

This study combined data of the concentrations of metals, polycyclic aromatic hydrocarbons (PAHs) and pharmaceuticals and personal hygiene products (PPCPs) in the sediments and the biological responses of the *Atherinella brasiliensis* fish in two different sites and climate seasons in the Estuarine-Lagoon Complex of Iguape-Cananéia, Southeast Brazil. The presence of metals, PAHs, and PPCPs were observed in the sediments demonstrating the contamination throughout the system with contributions of sewage and residues disposal, oil and combustion of biomass and fossil fuels. Higher contaminations were identified in the point of greater human presence (C - Cananéia City), especially during the cold-dry season. The influence of anthropic activities and variations in the estuarine conditions, such as lower hydrodynamics during the lower rainfall period, were observed. In fish, spatial and seasonal changes in the parameters of oxidative stress and biotransformation, genotoxicity and histopathological alterations followed the same trend, with more pronounced responses in C in the cold-dry season. The biological responses of the fish have revealed adverse effects in the local species population and have indicated the presence of metals, PAHs and PPCPs as stressors. The multivariate analysis and the integrated biomarker response index (IBR) corroborated with these results, also indicating that site C had the worst environmental quality. The present study provides new information about the contamination of the sediments of Estuarine-Lagoon Complex of Iguape-Cananéia and the chronic exposure to contaminants in *A. brasiliensis*. Therefore, contributing to a better understanding of the local environmental quality with data that can support protective management of the area.

Key words: Metals; Polycyclic aromatic hydrocarbons; Pharmaceutical and personal care products; Biomarkers; Integrated biomarker response index; Field study.

2.2 GRAPHICAL ABSTRACT



2.3 HIGHLIGHTS

- Sediments of the ELCIC are contaminated by metals, PAHs and PPCPs.
- Higher human presence and lower rainfall increased the sediment contaminant levels.
- Genotoxicity, histopathological and biochemical alterations were observed in fish.
- Sediment contaminants were partially responsible for the biological responses in fish.
- The integrated biomarker response index is effective in field studies.

2.4 INTRODUCTION

Worldwide, estuaries and other coastal environments have been receiving different contaminants and their associated breakdown products, many of which are still of unknown toxicity and effects (Lu et al., 2018). In Brazil, the most relevant sources of contaminants to these areas are: urban occupation, poor waste management, inappropriate disposal and inefficient treatment of sewage, environmental accidents and activities of agricultural, industrial, port, petrochemical and mining origins (Mahiques et al., 2013; Dias et al., 2013; Barbieri et al., 2014; Morais and Abessa, 2014).

Metals and polycyclic aromatic hydrocarbons (PAHs) have persistence in the environmental, bioavailability, capacity to pass through bioaccumulation and/or biomagnification processes and present toxic effects to living beings (Gu et al., 2018a; Lu et al., 2018; Sun et al., 2018). Nonetheless, pharmaceutical and personal care products (PPCPs) have been increasingly detected in environmental samples and may also pose some risk to the ecosystem and human health (Burkina et al., 2015; Omar et al., 2018).

The knowledge of the contaminant levels in the environmental compartments, such as water and sediments, can help to identify the main sources of contamination and indicate potential adverse biological effects when compared to quality guidelines and international or national laws (Choueri et al., 2009; Gu et al., 2015). However, these chemical measurements may not suitably reflect the bioavailability and the toxicity of the contaminants to the organisms (Birch, 2018). As so, the use of biomarkers helps to fill the gap between the presence of contaminants and its possible biological alterations (Burkina et al., 2015; Maranho et al., 2015; Kroon et al., 2017). Therefore, many studies have been applying the use of different biomarkers and its integration by the *Integrated Biomarker Response* index (IBR) for monitoring different sources of anthropogenic contamination in aquatic environmental using fish as bioindicators (e.g. Azevedo et al., 2012; Mela et al., 2013; Gusso-Choueri et al., 2015; Gu et al., 2018b).

The *Atherinella brasiliensis* fish is a resident species of estuarine systems of southeastern Brazil (Contente et al., 2011; Souza-Bastos; Freire, 2011). This species plays an important part in trophic chains as food for other fishes, birds, bats and dolphins (Bordignon, 2006; Dias et al., 2009; Lopes et al., 2012) and it is a target food fish for recreational and artisanal fisherman (Bervian; Fontoura, 2007; Contente et al., 2011). Thus, this fish has been widely used as sentinel for studies of assessments of environmental impact in coastal areas (Dias et al., 2009; Fernandez et al., 2011; Souza-Bastos; Freire, 2011; Ribeiro et al., 2013).

The Estuarine-Lagoon Complex of Iguape-Cananéia (ELCIC), Southeastern Brazil, is characterized by a low population density, a lack of large-scale economic activities and the presence of several terrestrial and aquatic protected areas (Morais; Abessa, 2014). The climate of the region is divided in two well-defined seasons with rainy summers, with maximum temperature in February and higher precipitation rates from January to March, and dry winters, with minimum temperature in July and little precipitation rates from July to August (Cunha-Lignon et al., 2009). Due to its relevance for environmental conservation it is recognized by UNESCO as part of the Biosphere Reserve of the Atlantic Rainforest. However, this region went through several changes in the last centuries which affected its environmental quality, being today under increasing anthropic pressure.

Previous contributions reported expressive levels of metals and PAHs in the sediments (Antonelli et al., 2017; Salgado; Azevedo, 2018) and different responses to oxidative and physiological stress in the ichthyofauna (Azevedo et al., 2012; Gusso-Choueri et al., 2015, 2016). Nonetheless, the studies with contaminants in the region are still incipient and little is known about the effects of the environmental contamination in the aquatic biota.

A multiple analysis of contaminants in the sediments had never been investigated in the ELCIC, as the PPCPs levels. That combined with a multi-biomarker approach and the use of the integrated biomarker response index in a sentinel fish species can be useful to elucidate the stressors and their effects, also allowing their comparison to others studies in estuarine areas. Therefore, the purpose of this study was to assess the levels of contaminants in the sediments (metals, PAHs and PPCPs), responses of different biomarkers and the application of the IBR in *A. brasiliensis* from this sub-tropical estuary.

2.5 MATERIALS AND METHODS

2.5.1 Sampling

Two sites in the Estuarine-Lagoon Complex of Iguape-Cananéia, São Paulo, Brazil (24º50` to 25º10`S / 47º25` to 48º00`W; Figure 1) were sampled, representing sites with different anthropic pressures. Cananéia City - C (25°02`27.73” S /

47°54`47.44” W) represents one of the biggest urban centers in the region, while Subaúma - S (24°53`45.48” S / 47°48`20.43” W) has a smaller human occupation. Sampling campaigns corresponded to the hot-rainy season (March) and cold-dry season (September) periods in 2016.

Sediment samples for chemical analysis were collected at a depth of 20 cm, with a Petersen dredge that was thrown three times yielding composite samples of approximately 200 g. In the same day, 20 to 30 fish of the species *Atherinella brasiliensis* were collected per site with the use of fishing nets (SISBio License No. 50365-3) to assess their health conditions. They were anesthetized with benzocaine 0.1% and the blood was taken by heart puncture for the analysis of biomarkers of genotoxicity. The fish were then euthanized by medullary section and submitted to biometry. The gills were taken to analysis of histopathological biomarkers and autometallography. The brains, muscles and kidneys to analysis of biochemical biomarkers. Livers to analysis of genotoxicity, biochemical and histopathological biomarkers and autometallography. The tissues were kept on liquid nitrogen for transportation to the laboratory and stored at −80°C until the analysis. The liver and gills taken for analysis of histopathology were fixated in Alfac solution.

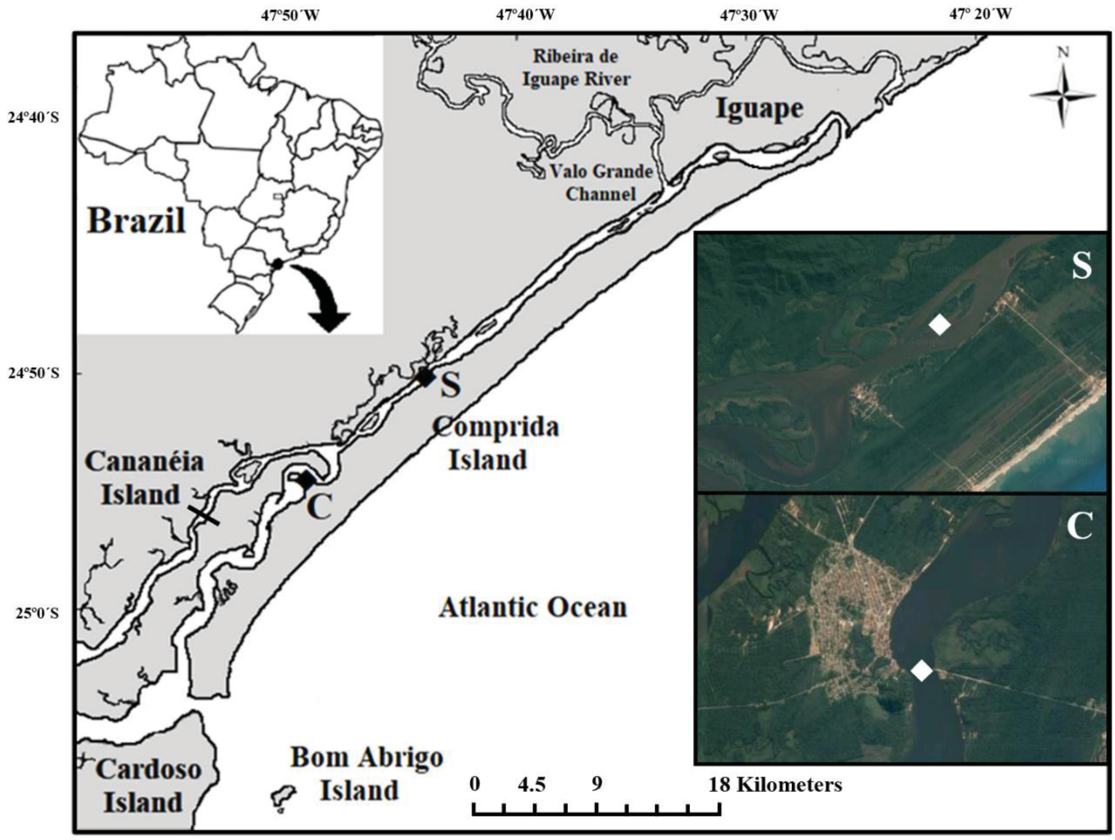


Figure 1. Map of the Estuarine-Lagoon Complex of Iguape-Cananéia and its location in Brazil, highlighting the sampled points and its aerial view (C - Cananéia and S - Subaúma).

2.5.2 Chemical analysis

2.5.2.1 Determination of metals

For determinations of metals, the sediments samples were stored in whirl-pack plastic bags and frozen at -20°C until analysis. Each sample of sediment was dried in drying oven at 40 °C for 48 h or until completed dried, homogenized and divided in triplicates of 1g. The analysis followed the method proposed by the Environmental Protection Agency (EPA, 1996). The procedures were performed by acid digestion with HNO_3, H_2O_2 and HCl. All the reagents utilized were of an analytic degree and the materials used were previously decontaminated by being washed with Extran® detergent and deionized water and bath in 10% nitric acid for 24h.

The determinations of the Fe, Zn, Mn, Cu, Cr, Ni, Cd and Pb were done by flame atomic absorption spectrometry (FAAS) model GBC, Avanta, respecting the characteristics of the method for the different metals (Supplementary material A). The standard solutions were prepared with successive dilutions using stock solutions of each metal (1000 mg/L^{-1}), using 13% v/v HNO_3. The quality of the results was accompanied with the use of an analytic blank and the determination performed in triplicate. The exactitude of the method was verified by addition and recovery tests performed in fish muscular tissues, with satisfactory recovery rates that ranged from 82% to 108% in all investigations.

2.5.2.2 Determination of pharmaceuticals and personal care products (PPCPs)

For the determinations of PPCPs, the sediments samples were stored in whirl-pack plastic bags and frozen at -20°C until analysis. These samples were dried in drying oven at 40 °C for 48 h or until completed dried, homogenized and divided in triplicates of 1g. The sediment analysis was done by the methodology presented by Kramer et al. (2018). All solvents used were HPLC grade and all materials used in the analyzes were decontaminated by being washed with Extran® detergent and deionized water, and bathed in 5% hydrochloric acid. The non-volumetric glassware was decontaminated in a muffle at 550 ° C for 5 hours.

The PPCPs determinations were performed in Gas Chromatographic (Agilent Technologies 7890) tandem Mass Spectrometry (GC-MS/MS) fitted with a triple quadrupole mass spectrometer (Agilent Technologies 7000), with an autosampler (Agilent Technologies 80), and with a HP-5Msi capillary column (30m x 0.25mm x 0.25 µm). The oven temperature program was initially at a temperature of 100°C, held for 2 min, then increased to 180°C at a ramp rate of 15°C min^{-1}, after that raised to 270°C at a rate of 6°C min^{-1}, and 5°C min^{-1} until 310°C, being held for 3 min. Total time of analysis was 33.3 min. The injector and the transfer line temperature were 280°C, and the capillary column pressure was $8.91x10^6$ Pa, with a helium flow rate of 1.2 mL min^{-1}; 1µL of the samples was injected on splitless mode. The ionization method was Electron Impact (EI) with 70 eV of energy and 300°C of source temperature.

The following pharmaceuticals and personal care products (PPCPs) were analyzed: Lipid regulators - fenofibrate (FNF) and gemfibrozil (GFZ); β-blockers - propranolol (PRL) and metoprolol (MTL); Analgesics and anti-inflammatories - salicylic acid (SA), acetylsalicylic acid (ASA), ibuprofen (IBU), diclofenac (DCF), naproxen (NPX) and fenoprofen (FNP); Stimulants - caffeine (CAF); Estrogens - ethinylestradiol (EE2), estradiol (E2) and estrone (E1); Antiseptics - triclosan (TRC), methylparaben (MEP), ethylparaben (ETP), propylparaben (PRP), butylparaben (BTP), and benzylparaben

(BZP). All chemical standards were purchased from Sigma Aldrich and the characteristics of the method to the PPCPs analyzed are shown in the supplementary material B.

2.5.2.3 Determination of Polycyclic Aromatic Hydrocarbons (PAHs)

For determination of PAHs, the sediments samples were stored in aluminum containers, previously decontaminated by heating in muffle at 400˚ C for 1 h, and frozen at -20°C until analysis. These samples were dried in drying oven at 40 °C for 48 h or until completed dried, homogenized and divided in triplicates of 10g. The method proposed by Mater et al. (2004) was applied. All solvents used were HPLC grade. All materials used in the analyzes were decontaminated by being washed with Extran® detergent and deionized water then bathed in 5% hydrochloric acid. The non-volumetric glassware was decontaminated in a muffle at 550 ° C for 5 hours as a way of eliminating organic residues.

The determinations of PAHs were held by Gas Chromatographic tandem Mass Spectrometry (GC-MS/MS) with a gas chromatograph (Agilent Technologies 7890) fitted with a triple quadrupole mass spectrometer (Agilent Technologies 7000), with an autosampler (Agilent Technologies 80). 1 µL of the sample was injected on splitless mode using a HP-5Msi capillary column (30m x 0.25mm x 0.25 µm). The carrier gas used was Helium at a flow rate of 1.2mL/min. The furnace temperature was programmed in two ramps, from 40˚C to 250˚C at a rate of 120˚C min^{-1}, remaining 0.5 minute and another from 250˚C to 310˚C. The temperature of the injector and the transferline was set to 280˚C and the source ion temperature to 300˚C. For the mass spectroscopy analysis, the multiple reaction monitoring was used, with the fragmentation occurring through electron impact at 70 eV. The total analysis time per sample was 15.5 min.

Sixteen priority PAHs were analyzed: Naphthalene (Nap); Fluorene (Fl); Acenaphthene (Ace); Acenaphthylene (Acy); Anthracene (Ant); Phenanthrene (Phe); Fluoranthene (Flu); Pyrene (Pyr); Chrysene (Chr); Benzo(a)anthracene (BaA); Benzo(b)fluoranthene (BbF); Benzo(k)fluoranthene (BkF); Dibenzo(a,h)anthracene (DBahA); Benzo(a)pyrene (BaP); Indeno(1,2,3-cd)pyrene (InP); Benzo(g,h,i)perylene (BghiP). All chemical standards were purchased from Sigma Aldrich and the characteristics of the method to the PAHs analyzed are given in the supplementary material C.

2.5.3 Biological responses

2.5.3.1 Condition factor (K)

The condition factor (K) was used as indicator of environmental stress, as presented by Bolger and Connolly (1989). The K demonstrates the length-weight relationship that was expressed by K = fish total weight / (fish total length)^3 * 100.

2.5.3.2 Biochemical biomarkers

The brain and muscle were weighed and homogenized in potassium phosphate buffer (0.1 M pH 7.5) and then were centrifuged at 12,000 × g for 20 minutes at 4°C. The supernatants were used for the measurement of the acetylcholinesterase activity (AChE) by the method of Ellman et al. (1961), modified for microplates by Silva de Assis (1998) at 415 nm.

The liver and kidney were weighed and homogenized in potassium phosphate buffer (0.1 M pH 7.0) 1:10 (w/v), and then were centrifuged at 15,000 × g for 30 minutes at 4°C. The supernatants were used for the measurement of the activities of the superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase activity (GPx), ethoxyresorufin-O-deethylase activity (EROD), glutathione S-transferase (GST) and the concentrations of glutathione (GSH), metallothioneins and lipoperoxidation (LPO). SOD activity was measured using the method described by Gao et al. (1998) at 440 nm. CAT activity was determined by the method presented by Aebi (1984) at 240 nm. GPx activity followed the method by Paglia and Valentine (1967) at 340 nm. EROD activity was determined according to Burke and Mayer (1974) at 482 nm. GST activity was measured according to the method of Keen et al. (1976) at 340 nm. GSH concentrations were determined by the method of Sedlak and Lindsay (1968) at 415 nm, while the metallothioneins (MT) were measured by the method of Viarengo et al. (1997) at 412 nm. LPO damages were measured by the method presented by Jiang et al. (1992) at 570 nm.

All biochemical analyses were normalized to protein concentration by the method presented by Bradford (1976). The calibration curve was obtained with bovine serum albumin as the standard. The biochemical analyses were carried out on a BioTek ELx800 Absorbance Microplate Reader (BioTek Instruments, Inc.).

2.5.3.3 Genotoxicity biomarkers

The comet assay based in the Single-Cell Gel Electrophoresis (SCGE) were performed in erythrocytes and liver cells for the visualization of the DNA strand breaks (DNA ST). The method described by Singh et al. (1988) was applied, with modifications for the erythrocytes (Ferraro et al., 2004; Cestari et al., 2004) and for the tissues cells by Ramsdorf et al. (2009).

The micronucleus test (MN) and nuclear morphological abnormalities (NMA) were investigated in erythrocytes where the blood microscopy slides were examined and scored based on the presence of both typical MN and NMA. The MN test was based on Heddle (1973) and Schmid (1975) methodology. The NMA analysis (blebbed, notched, lobed and vacuolated) was performed according to Carrasco et al. (1990).

2.5.3.4 Histopathological biomarker

The samples of liver and gills were fixated in Alfac solution (70% ethanol, 4% formaldehyde and 5% acetic acid) for 16 h and then stored in 70% alcohol until the inclusion procedures. The tissues were then dehydrated in a graded series of ethanol baths and embedded in Paraplast Plus resin (Sigma®). Sections of 3-5μm were stained in hematoxylin/eosin and observed in Zeiss Axiophoto photomicroscope. Histopathological indexes were estimated for the liver using a semi-quantitative protocol (Bernet et al., 1999), modified by Mela et al. (2013). The liver and gill indexes were calculated on the basis of two factors: the extension of the pathological change (score value, a) and its pathological importance (importance factor, w). The score value (1-6) was assigned according to the percentage of tissue exhibiting a certain alteration: 0 = less than 5%; 1 = 5-20%; 2 = 21-40%; 3 = 41-50%; 4 = 51-60%; 5 = 61-80%; and 6 = 81-100%. The importance factor (1-3) reflects the reversibility of the alteration after removing the stressor (1 = easily reversible; 2 = reversible in most cases; 3 = generally irreversible). The organ index (Iorg) was calculated as the sum of the five reaction patterns of an organ. It was determined as:

$$I_{org} = \sum_{rp} \sum_{alt} (a_{org\ rp\ alt} \times w_{org\ rp\ alt})$$

Where: org is the organ (constant), rp: reaction pattern, alt: alteration, a: score value, and w: importance factor.

2.5.3.5 Autometallography

Samples of liver and gills were submitted to autometallography analysis for the investigation of accumulation and distribution of nonspecific metals in these tissues. The samples were collected and fixated with 3 % glutaraldehyde (in 0.1 M sodium cacodylate buffer, pH 7.4) for 24 h at 4 ˚C and rinsed with buffer (0.1 M sodium cacodylate buffer, 2 % NaCl, pH 7.4), dehydrated in a graded series of ethanol baths and embedded in Paraplast Plus (Sigma®). Then, autometallography was performed in the tissue sections (3-5 µm) according to the protocol proposed by Danscher et al. (1987) with modifications by Rossi et al. (2014). The procedures were developed in a dark room where the cuts received a silver solution (Emulsion L4, Cambridge Nuclear Emulsion TAAB) for 30 minutes. The sections were then stained with hematoxylin/eosin and observed under the Leica® DME light microscope.

2.5.4 Data analysis

The Shapiro-Wilk normality test and the Bartlett homogeneity test preceded data analyses. ANOVA two-way analysis followed by Fisher's test (LSD), and the Permutational Variance analysis (PERMANOVA) followed by the bootstrap analysis of multiple comparison of the averages were used to analyze the differences among the biological responses of the fish from the different sampled sites and the metal concentrations in the sediments. The biomarkers were also analyzed using the Principal Coordinates Analysis (PCoA; Gower, 1966) and the Permutational Multivariate Analysis of Variance procedure (PERMANOVA; Anderson, 2001), which were used to summarize and show general patterns. The correlation between the PCoA axis and the biomarkers were analyzed by Spearman. Finally, the Redundancy Analysis (RDA) was used to investigate the influence of the concentrations of metals, PAHs and PPCPs detected in the sediments in the biological responses of the fish. For this analysis, log transformation was used to minimize the problem of non-normality of the data. Then the scale of the variables was standardized by rescaling all the variables to an average of zero and standard deviation of one, to reduce the variation. The contaminants of the sediments that had values below the limit of detection (LD) were not included. Statistical analyses were performed in R software R 3.2.2 (R Core Team 2015). The decision rule was $p<0.05$ for all analyzes.

There is no specific federal legislation dealing with the maximum admitted limits for the concentrations of metals and PAHs in estuarine sediments nor for the levels of PPCPs in environmental samples in Brazil. Therefore, the concentrations of metals and PAHs found in the sediments were compared to the limits determined by the Resolution nº 344, from 25th March 2004, of the Brazilian National Council for the Environment (CONAMA, 2004). This establishes the general guidelines and the minimum procedures for the assessment of the matter to be dragged from Brazilian jurisdiction waters, and gives also other steps. The comparison values were of class I, where a limit is established below which is foreseen as a low probability of adverse effects to aquatic life.

The biomarkers results were calculated by the integrated biomarker response index (IBR), described by Beliaeff and Burgeot (2002) and modified by (Sanchez et al., 2013). This version of IBR is based on the principle of reference deviation between a disturbed and an undisturbed state. Since there is no baseline biomarker data for *A. brasiliensis*, the responses obtained in fish from the sampling point showing the least effects were considered as the reference condition.

2.6 RESULTS AND DISCUSSION

2.6.1 Chemical analysis of the sediments

Spatial and seasonal variations in the concentrations of the analyzed contaminants were observed, indicating the influence of the anthropic activities in the contamination of the ELCIC.

The concentrations of metals in the sediments were obtained in the following order of magnitude: Fe>Mn>Cr>Zn>Ni>Cu>Cd>Pb (Table 1). The concentrations of Fe were higher in Subaúma (S) during the hot-rainy season and in Cananéia (C) in the cold-dry season. The higher Mn values were measured in S during the hot-rainy season. The Cr, Zn and Ni had higher concentrations in C in the cold-dry-season. The concentrations of Cr (84.84 µg g^{-1}) and Ni (22.99 µg g^{-1}) in C surpassed the limits of 81 µg g^{-1} and 20,9 µg g^{-1} during the cold-dry period, respectively, established by the Brazilian Environmental Agency. The Cu, Cd and Pb concentrations were higher in the same site on both periods. In addition, the Pb was not detected in S during the cold dry-season. Regarding the seasons, the Fe, Zn, Cu, Cr and Ni concentrations were higher in the sediments during the cold-dry season, as Mn were higher during the hot-rainy period.

The analysis of PPCPs in the sediments detected fenoprofen, fenofibrate, caffeine, propranolol and metoprolol in both sites and seasons (Table 1). Fenoprofen was detected in site C in both seasons, varying from 14.81 $\mu g\ g^{-1}$ to 21.83 $\mu g\ g^{-1}$, and in site S during the hot-rainy period in concentrations <0.039 $\mu g\ g^{-1}$. Fenofibrate was detected in site C during the hot-rainy season in concentrations <0.003 $\mu g\ g^{-1}$. Caffeine was detected in concentrations <0.018 $\mu g\ g^{-1}$ in site C during both seasons and in S in the hot-rainy period. Propranolol was detected in all sites and seasons in concentrations varying from 5.41 $\mu g\ g^{-1}$ in S in the cold-dry period to 66.47 $\mu g\ g^{-1}$ in C in the hot-rainy season. Metoprolol was detected in site C during the cold-dry period in concentrations of 31.34 $\mu g\ g^{-1}$. The others lipid regulators and analgesics and anti-inflammatories analyzed were not detected, as none of the estrogens and antiseptics used in personal care products.

The presence of different PAHs was observed in the sediments in distinct concentrations among the analyzed sites and seasons (Table 1), as well as different probable origins of the PAHs were identified (Supplementary material D). A higher number of compounds were detected during the cold-dry season. Site C presented higher values for the ∑PAHs in both seasons, with a value of 406.26 $\mu g\ g^{-1}$ for the cold-dry period and of 10.83 $\mu g\ g^{-1}$ for the hot-rainy period, with higher values for ∑ PAHs 5-6 in both periods and compounds of probable mixed origins, from petroleum and combustion of petroleum and biomass. Site S presented a higher value for the ∑PAHs during the cold-dry (4.13 $\mu g\ g^{-1}$) than the hot-rainy season (0.67 $\mu g\ g^{-1}$), with higher values for the ∑ PAHs 2-4 in both periods and probable origins from petroleum. None of the sites surpassed the values established by the Environmental Agency in the sum of the legislated compounds of 3000 $\mu g\ g^{-1}$.

Table 1. Concentrations of metals, pharmaceuticals and personal care products (PPCPs) and of individuals polycyclic aromatic hydrocarbons (PAHs), sum of the compounds with 2 to 4 aromatic rings (∑PAHs 2-4), sum of the compounds with 5 or 6 aromatic rings (∑PAHs 5-6), sum of the total PAHs analyzed (∑PAHs) and sun of the PAHs present in the Brazilian legislation (∑PAHs Legislation) in the sediments (in $\mu g\ g^{-1}$).

Contaminants		**Sites and seasons**			
		hot-rainy		**cold-dry**	
		C	**S**	**C**	**S**
		Metals			
	Fe	2836.48 ± 18.71^{a}	3299.68 ± 37.05^{b}	$3668.11 \pm 24.15^{c*}$	$3256.32 \pm 75.18^{b*}$
	Mn	$41.39 \pm 1.65^{a*}$	$114.34 \pm 7.25^{b*}$	65.25 ± 0.99^{c}	60.85 ± 8.46^{c}
	Zn	5.88 ± 0.99^{a}	14.19 ± 0.24^{b}	$74.39 \pm 2.07^{c*}$	$18.09 \pm 2.41^{b*}$
	Cu	0.48 ± 0.15^{a}	2.22 ± 0.53^{b}	$26.36 \pm 0.77^{c*}$	$5.59 \pm 0.77^{d*}$
	Cr	10.29 ± 4.32^{a}	14.12 ± 3.03^{a}	$\mathbf{84.84} \pm 29.95^{b*}$	$17.70 \pm 2.69^{a*}$
	Ni	5.68 ± 0.69^{a}	8.13 ± 0.66^{b}	$\mathbf{22.99} \pm 2.51^{c*}$	$4.15 \pm 0.01^{a*}$
	Cd	0.39 ± 0.42^{a}	0.29 ± 0.14^{b}	0.65 ± 0.09^{a}	0.05 ± 0.07^{b}
	Pb	0.21 ± 0.36^{a}	0.04 ± 0.01^{b}	0.04 ± 0.01^{b}	<LD
		Pharmaceuticals and personal care products (PPCPs)			
Lipid regulators	**FNF**	<LQ	<LD	<LD	<LD
	GFZ	<LD	<LD	<LD	<LD
B-blockers	**PRL**	66.47	15.05	7.37	5.41
	MTL	<LD	<LD	31.34	<LD
Analgesics and anti-inflammatories	**SA**	<LD	<LD	<LD	<LD
	ASA	<LD	<LD	<LD	<LD
	IBU	<LD	<LD	<LD	<LD
	DCF	<LD	<LD	<LD	<LD
	NPX	<LD	<LD	<LD	<LD
	FNP	14.82	<LQ	21.83	<LD
Stimulants	**CAF**	<LQ	<LQ	<LQ	<LD
Estrogens	**EE2**	<LD	<LD	<LD	<LD
	E2	<LD	<LD	<LD	<LD
	E1	<LD	<LD	<LD	<LD

Antiseptics	**TRC**	<LD	<LD	<LD	<LD
	MEP	<LD	<LD	<LD	<LD
	ETP	<LD	<LD	<LD	<LD
	PRP	<LD	<LD	<LD	<LD
	BTP	<LD	<LD	<LD	<LD
	BZP	<LD	<LD	<LD	<LD
Polycyclic aromatic hydrocarbons (PAHs)					
PAHs 2-4	**Nap**	<LD	<LD	0.32	<LD
	Fl	<LD	<LD	<LD	<LQ
	Ace	2.31	<LD	<LD	2.31
	Acy	1.64	<LD	1.79	1.64
	Ant	0.46	<LD	26.97	<LQ
	Phe	0.35	0.49	23.44	<LQ
	Flu	<LD	<LD	16.47	<LQ
	Pyr	<LD	<LD	1.12	<LQ
	Chr	<LD	0.16	<LD	0.16
	BaA	<LD	0.02	0.29	0.02
PAHs 5-6	**BbF**	3.08	<LD	43.05	<LQ
	BkF	2.98	<LD	45.16	<LQ
	DBahA	<LD	<LD	81.62	<LQ
	BaP	<LD	<LD	63.65	<LQ
	InP	<LD	<LD	54.30	<LQ
	BghiP	<LD	<LD	48.07	<LQ
Sum	**∑PAHs 2-4**	4.76	0.67	70.41	4.13
	∑PAHs 5-6	6.06	0	335.85	0
	∑PAHs	10.82	0.67	406.26	4.13
	∑PAHs Legislation	4.76	0.67	215.53	4.13

Data for metals expressed in mean ± standard deviation.
LD - Limit of Detection; LQ - Limit of Quantification.
a b c d Indicates statistical differences between the sites ($p<0.05$).
* Indicates statistical seasonal differences between the periods ($p<0.05$).
Values for metals above the established by the Brazilian Environmental Agency - CONAMA, 2004 are in bold.
The PAHs present in the Legislation of the Brazilian Environmental Agency - CONAMA, 2004 are underlined.

The presence of higher pharmaceutical products, metals and PAHs concentrations observed in the sediments of site C during both seasons reveled the contributions made by the urban center of the Cananéia City. Nonetheless, the presence of these contaminants was also observed in the sediments of the S site, indicating the anthropic influence of the small villages near Subaúma.

Only few studies have assessed the occurrence and distribution of PPCPs in coastal sediments around the world (e.g. Li et al., 2012; Moreno-González et al., 2015; Omar et al., 2018). Most PPCPs are mainly hydrophilic and tend to be present in the dissolved fraction, however, some of them can undergo specific interactions with solid fractions and as a result can be transferred to sediments (Moreno-González et al., 2015). Domestic sewage is referred as the main source of pharmaceuticals and personal care products in aquatic systems, and higher concentrations are often observed close to large urban centers (Moreno-González et al., 2015; Ide et al., 2017; Paíga and Delerue-Matos, 2017; Kramer et al., 2018; Omar et al., 2018). Therefore, the occurrence of pharmaceutical products in the sediments of the study area attests the local contamination by domestic effluents (Li et al., 2012; Paíga and Delerue-Matos, 2017; Kramer et al., 2018).

Among the detected compounds, PRL had the highest frequency of detection and concentrations, followed by the CAF and FNP that were found in tree of the analyzed sites, and by MTL and FNF that were detected in only one of the analyzed points. This result is probably related to the widespread consumption of these products, once these compounds have been frequently detected in aquatic systems, and to their physical and chemical proprieties and kinetics in the environment (Lin et al., 2010; Aznar et al., 2013; Moreno-González et al., 2015; Maranho et al., 2015; Ide et al., 2017; Paíga and Delerue-Matos, 2017; Kramer et al., 2018).

Propranolol and metoprolol are selective β-blockers mainly used in heart diseases (Maszkowska et al., 2014; Rubirola et al., 2014). In the aquatic environment both show great persistent (Lin et al., 2010; Rubirola et al., 2014; Smith et al., 2018). Between the two compounds, propranolol is the most hydrophobic one (Maszkowska et al., 2014), and its detection in all seasons may probably be due to the fact that this drug has a chronic use. Still, caffeine is one of the most consumed substances in the world being found in foods, drinks, tobacco and medicines (Paíga; Delerue-Matos, 2017). In the environment caffeine is highly biodegradable (Kramer et al., 2018), as so, its continuous input causes these concentrations to not decrease in the environmental matrices (Ide et al., 2017).

Fenofibrate is a lipid regulator used to control the levels of cholesterol and triglycerides in the blood (Aznar et al., 2013), while fenoprofen is a non-steroidal anti-inflammatory drug commonly used in painkillers pills (Maranho et al., 2015). On the study of PPCPs of wastewater treatment in the Paranaguá estuary, a coastal region near our study area in Southeast Brazil, Kramer et al. (2018) found that fenofibrate was among the products of higher input mass in the wastewater, reveling its patterns of consumption by the local population. In this same study, fenoprofen was among the pharmaceuticals with the highest persistence in the effluents and sludge sediments after the treatment, demonstrating its low removal by the conventional treatment (Kramer et al., 2018).

The cities of the ELCIC region have an inadequate treatment and an insufficient collection of sewage, which cause effluents to be dumped *in natura* in the rivers or directly into the sea (Barbieri et al., 2014; Morais; Abessa, 2014). In addition, the disordered human occupation and the deficiency in the removal of these compounds together with the predominance of dumps as final disposal of solid waste contribute to the presence and persistence of PPCPs in this environment and can also contribute to the input of metals and PAHs to the estuary (Barbieri et al., 2014; Morais; Abessa, 2014; Gusso-Choueri et al., 2015).

The sediments of site C during the cold-dry season presented higher concentrations for most metals (Fe, Zn, Ni, Cu, Cd and Pb), including metals of high toxicity as Pb and Cd. These sediments also had Cr and Ni concentrations above the limit determined in the legislation of the Brazilian Environment Agency for briny-salty waters, inferring that these concentrations could cause adverse effects on the local biota (CONAMA, 2004). These results reinforce the contribution of the human's activities in the input of metals to this area, corroborating with previous studies (Morais and Abessa, 2014; Gusso-Choueri et al., 2015, 2018; Salgado and Azevedo, 2018).

Besides the incorrect garbage and sewage disposal, the existence of agriculture and aquaculture activities and the presence of vessels constitute potential sources of metals throughout the estuary (Barbieri et al., 2014; Morais and Abessa, 2014; Salgado and Azevedo, 2018). Additionally, metals display distinct distribution pattern in the sediments once they are associated with different fractions of sediments that make them more or less available to the aquatic organisms (Gu et al., 2015). A high bioavailability of metals in the sediments of the ELCIC were previously evidenced by Salgado and Azevedo (2018) by the method of the simultaneously extracted metals and acid-volatile sulfide (SEM/AVS), that considered these sediments as potentially toxic with probability to cause adverse effects to the aquatic biota.

The occurrence of PAHs in the environment may be due to natural and anthropic sources, being the last one more dominant (Sun et al., 2018). Thus, the main origins of its emissions may be pyrogenic, by the incomplete burning of organic matter, or petrogenic, from petroleum (Antonelli et al., 2017; Ke et al., 2017). Pyrolysis can give rise to a variety of PAHs depending on the combustion temperature. At low temperatures, such as wood burning or coal combustion, low molecular weight PAHs are more abundant and at higher temperature, such as vehicular emissions, compounds of high molecular weight are dominant, such as chrysene, fluoranthene, pyrene and benzo[a]anthracene (Yunker et al., 2002; Antonelli et al., 2017). In the petrogenic origin low molecular weight compounds are predominant with two to three aromatic rings and its alkylated homologues (Yunker et al., 2002; Ke et al., 2017).

The presence of high molecular mass compound between 5 to 6 aromatic rings (Benzo[b]fluoranthene to Benzo[ghi]perylene) were found in higher concentrations than the lower molecular weight compounds with 4 aromatic rings (Chrysene to Benzo[a]anthracene), indicating mixture of PAHs formed during the pyrolysis, primally at high temperatures. Therefore, these results indicate the contributions of the incineration of biomass and of the burning of fossil fuels, from vehicles and motorboats, as the main pyrolytic sources of PAHs to both areas. The presence of low molecular weight and alkylated PAHs compounds (Naphthalene to Phenanthrene) are probably from oil spill, derivative from the presence of boats in all the system and from the marina and the ferry from the Cananéia City to the Comprida Island.

The higher PAHs concentrations detected in the sediments of site C are probably due to various anthropogenic activities in this region. Additionally, the dominance of 5-6 rings PAHs was observed in this site, indicating that pyrolytic source played a significant role in the origin of PAHs in this area. Minors PAHs concentrations were observed in S, reflecting the little human presence on this area, and a dominance of 2-4 rings PAHs was observed in this site, indicating its petrogenic origins. In addition, the concentrations of PAHs in the sediments of the ELCIC were in accordance with the limits established by the environmental agency, not causing big concern (CONAMA, 2004).

Nonetheless, the climate seasonality and the rainfall regime interfered in the levels of contaminants in the sediments and consequently in their bioavailability. The presence of high concentrations of PAHs, metals and PPCPs in the sediments during the cold-dry season suggests the influence of the changes in the climate and the estuarine conditions in this period. The lower rainfall leads to a lower hydrodynamic, and consequently, to a lesser renewal of the waters of the system (Britcha, 2000). This, may affect the concentrations of the contaminants in the sediments, leading them to high concentrations, due to the less dispersion and the longer time of permanency of the waters in the estuary. Other studies focused on the investigation of contaminants in the ELCIC also evidenced differences in the concentrations among different annual periods (Antonelli et al., 2017; Salgado; Azevedo, 2018).

2.6.2 Biological responses

The *A. brasiliensis* fish from ELCIC exhibited biological responses in different intensity degrees, in accordance with the levels of contaminants obtained for the sediments. Thereby, demonstrating that all analyzed sites were affected by anthropic activities.

In brain and muscle, the AChE activities were similar between sites and seasons (Table 2). In gills, the histopathological analysis did not evidence any alteration nor the accumulation of metals were seen through autometallography analysis.

In liver, the accumulation of metals was also not observed, however, therefore alterations of the antioxidant system were observed (Table 2), genotoxicity (Table 3) and histological alterations (Figure 2). The fish liver of C point presented lower CAT and GPx activities and higher MT concentrations in both periods. Similar values were obtained from hepatic tissue for SOD, EROD and GST activities, GSH concentrations and LPO. However, regarding the time of the year, the interaction between both points showed higher values for hepatic SOD activities, GSH and MT concentrations and LPO values in the hot-rainy season. Nevertheless, the fish in both points presented lower EROD and GPx activities in this period.

Additionally, higher hepatic DNA strand breaks and a higher value for the lesion index in the liver (Table 3; Figure 2) were observed in C during the cold-dry season, as well as both parameters were higher during this same period. In this tissue the presence of vacuolization of the hepatocytes represented 50% of the total alterations, the necrosis represented 44%, and the dilation of the sinusoids represented 6%.

Table 2. Biochemical biomarkers responses in brain, muscle, liver and kidney of *A. brasiliensis*.

Biomarkers	Sites and seasons			
	hot-rainy		cold-dry	
	C	S	C	S
AChE (brain)	83.81 ± 5.07 [a]	75.09 ± 3.20 [a]	77.90 ± 3.34 [a]	82.40 ± 4.61 [a]
AChE (muscle)	86.02 ± 4.96 [a]	65.37 ± 4.09 [a]	62.51 ± 4.49 [a]	61.58 ± 4.24 [a]
SOD (liver)	216.21 ± 19.10 [a*]	288.14 ± 18.36 [a*]	162.40 ± 14.62 [a]	185,39 ± 13.43 [a]
CAT (liver)	88.72 ± 12.65 [a]	159.46 ± 13.39 [b]	72.23 ± 6.48 [a]	175,81 ± 23.24 [b]
GPx (liver)	14.10 ± 3.09 [a]	16.12 ± 3.37 [b]	29.76 ± 7.11 [c*]	38.02 ± 3.98 [d*]
EROD (liver)	0.09 ± 0.01 [a]	0.07 ± 0.01 [a]	0.13 ± 0.02 [a*]	0.19 ± 0.03 [a*]
GST (liver)	4.49 ± 0.39 [a]	3.89 ± 0.37 [a]	5.54 ± 0.54 [a]	4.35 ± 0.33 [a]
GSH (liver)	52.43 ± 2.33 [a*]	57.24 ± 4.38 [a*]	49.32 ± 5.11 [a]	46.61 ± 2.73 [a]
MT (liver)	84.74 ± 13.18 [a*]	42.96 ± 6.29 [a*]	14.18 ± 2.16 [a]	20.61 ± 3.49 [a]
LPO (liver)	15.72 ± 3.14 [a*]	12.40 ± 1.02 [a*]	6.59 ± 1.36 [a]	8.82 ± 1.25 [a]
SOD (kidney)	774.59 ± 181.90 [a]	1027.92 ± 323.52 [a]	2141.19 ± 605.09 [a*]	2446.35 ± 600.34 [a*]
CAT (kidney)	14.09 ± 2.20 [a*]	16.90 ± 2.87 [a*]	6.34 ± 1.22 [a]	12.38 ± 0.79 [a]
GPx (kidney)	26.77 ± 11.87 [a]	25.63 ± 7.33 [a]	32.26 ± 9.83 [a]	26.83 ± 6.32 [a]
GST (kidney)	18.49 ± 2.16 [a]	13.41 ± 1.49 [a]	17.47 ± 5.02 [a]	26.23 ± 2.36 [a]
LPO (kidney)	27.50 ± 7.70 [a]	27.79 ± 12.86 [a]	26.57 ± 10.51 [a]	2.59 ± 0.55 [b]

Data expressed in mean ± standard error of the mean.
[a b c d] Indicates statistical differences between the sites ($p<0.05$).
* Indicates statistical seasonal differences between the periods ($p<0.05$).
AChE (nmol min^{-1} mg^{-1} protein), SOD (SOD mg $protein^{-1}$), CAT (µmol min^{-1} mg $protein^{-1}$), GPx (nmol min^{-1} mg $protein^{-1}$), EROD (nmol resorufin min^{-1} mg $protein^{-1}$), GST (µmol min mg $protein^{-1}$), GSH (µg GSH mg $protein^{-1}$), MT (µg MT mg $protein^{-1}$) and LPO (nmol min^{-1} mg $protein^{-1}$).

Table 3. Genotoxicity in liver and blood of *A. brasiliensis*. DNA strand breaks (DNA ST) in liver and blood, total nuclear morphological abnormalities (TNMA) and blebbed, notched and lobed nuclear abnormalities in blood.

Biomarkers	Sites and seasons			
	hot-rainy		cold-dry	
	C	S	C	S
DNA ST (liver)	124.0 (92.75; 177.5)[a]	(92.25; 155.8)[a]	190.0 (126.0; 225.0)[b*]	56.50 (37.25; 85.75)[c*]
DNA ST (blood)	261.0 (226.8; 302.5)[a*]	229.0 (193.3; 274.0)[a*]	191.5 (98.25; 315.0)[b]	116.0 (86.0; 211.0)[c]
TNMA (blood)	70.0 (43.0; 142.0)[a]	25.50 (45.50; 126.80)[a]	159.50 (93.50; 240.50)[b*]	40.50 (19.0; 55.25)[a*]
Blebbed (blood)	12.0 (3.50; 19.50)[a]	10.50 (3.75; 45.0)[b]	30.0 (17.0; 55.0)[b*]	15.50 (8.50; 24.50)[a*]
Lobed (blood)	3.50 (1.0; 9.0)[a]	1.0 (0.0; 5.0)[b]	8.50 (4.0; 19.75)[a]	2.0 (0.0; 6.0)[b]
Notched (blood)	61.0 (40.25; 105.80)[a]	38.50 (13.0; 84.25)[a]	122.0 (75.0; 169.0)[b*]	18.0 (9.0; 26.0)[c*]

Data expressed in median (quartile 1; quartile 3).
[a b c] Indicates statistical differences between the sites ($p < 0.05$).
* Indicates statistical seasonal differences between the periods ($p < 0.05$).

In the kidney, SOD, CAT, GPx and GST activities were similar in both sites and seasons as LPO were lower in S during the cold-dry period (Table 2). The interaction of the values of the two different sites revealed that in the cold-dry season renal SOD activities were higher and CAT activities were lower.

Following the same tendency of the results of the others biomarkers, higher values of total nuclear morphological abnormalities (TNMA) were observed in C in the cold-dry season. This same response was obtained for blebbed, notched and lobed abnormalities, and no vacuolated abnormality nor micronuclei was observed. Additionality, lower DNA strand breaks were observed in S during the cold-dry period. The influence of the season was observed for the total NMA that were more present in the blood of the fish during the cold-dry season, while the DNA damages where more present among the animals in the hot-rainy season (Table 3).

In addition, the condition index (K) values varied from 0.62 to 0.61 in site C and from 0.69 to 0.64 in site S, during the hot-rainy and the cold-dry periods, respectably. Therefore, the K value was higher in S site during the hot-rainy season, being this period also significantly different between the cold-dry seasons.

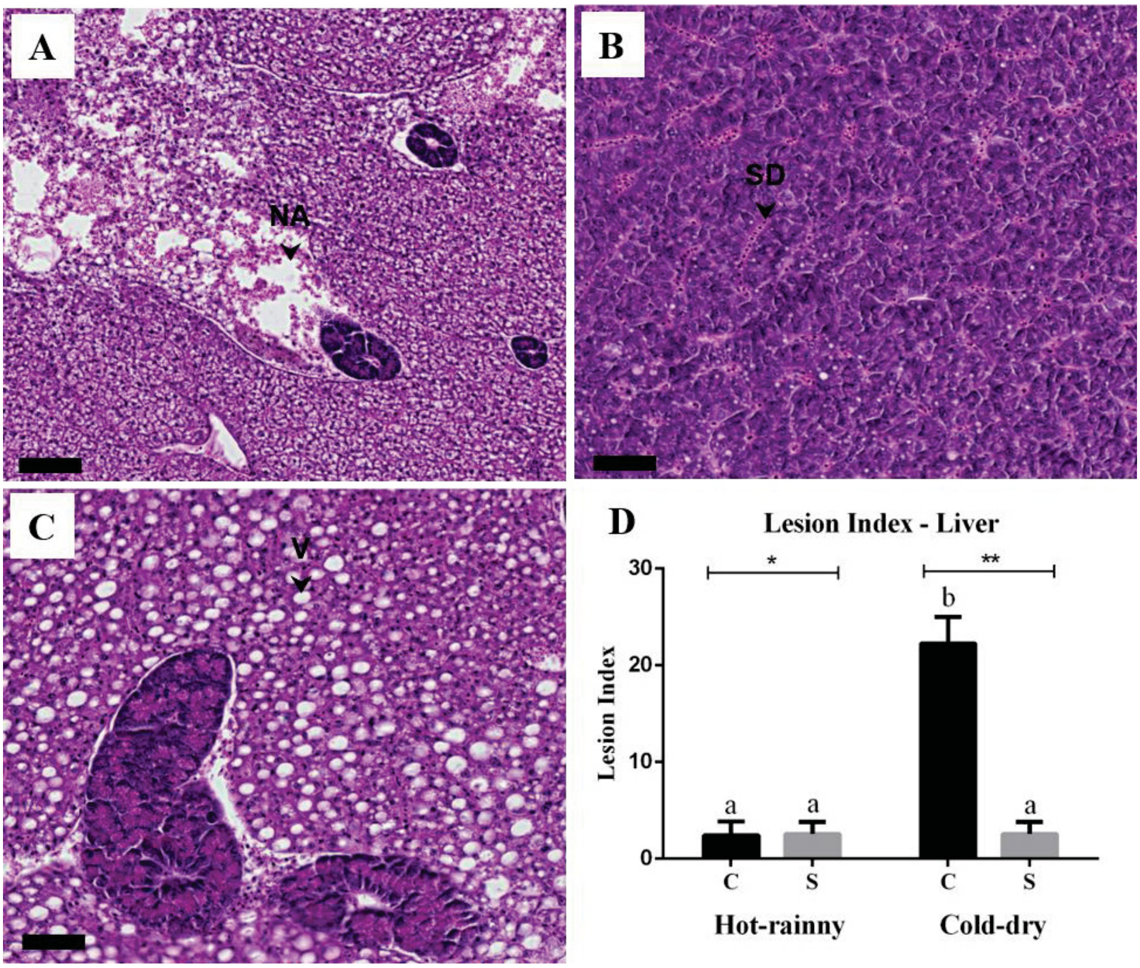


Figure 2. Histopathological alterations observed in liver of *A. brasiliensis* fish from the ELCIC and lesion index of the liver in the fish from Cananéia (C) and Subaúma (S) during hot-rainy and cold-dry seasons. A: Necrosis area (NA); B: Sinusoids dilatation (SD); C: Vacuolization of the hepatocytes (V); D: Lesion index of the liver.
Scale bars = 20 µm.
[a b] Indicates statistical differences between the sites ($p<0.05$).
* - ** Indicates statistical seasonal differences between the periods ($p<0.05$).

Thus, the fish collected near Cananéia City presented a more pronounced response than the fish from Subaúma in both periods and the cold-dry season was the most stressful period to the *A. brasiliensis* in the studied area.

Among the analyzed tissues the blood and liver showed more conspicuous responses, being considered as the best predictors in this investigation. Both are important tissues for the evaluation of the effects of pollutants on fish (Fernandez et al., 2011; Souza-Bastos; Freire, 2011). Genotoxicity of both tissues and alterations in the enzyme activities and the presence of lesions in the hepatic tissue may lead to depletory effects, compromising biological functions and altering the pattern of transportation, metabolism and storage of substances on the organisms (Fernandez et al., 2011; Mela et al., 2013; Ribeiro et al., 2013; Souza-Bastos; Freire, 2011; Prodocimo et al., 2015).

The lower hepatic CAT and GPx activities from site C in both seasons suggest an inhibition of these enzymes to the exposure to environmental contaminants, as metals and PAHs. The CAT and GPX are groups of antioxidant enzymes that form part of a cellular defense system that inhibit and detoxify oxyradical formation. Both has function in the degradation of the hydrogen peroxide, being important to prevent cellular damages (Kroon et al., 2017). The activities of these enzymes in fish differ among species, tissue, contaminant, concentration and duration of exposure (Kroon et al., 2017).

Metals and PAHs can decrease the major antioxidants activities in the cell (Gusso-Choueri et al., 2015) and previous studies showed a decreased in CAT and GPX activities at metal and PAHs contaminated areas (Benedetti et al., 2012; Souza et al., 2013). Recent studies also demonstrated that a range of pharmaceuticals products, including drugs of the classes of the β-blockers, lipid regulators, anti-inflammatory and stimulants, that were detected in the ELCIC sediments by the present study, can alter the antioxidant and biotransformation enzymes as so may constitute a threat for different fish species (Burkina et al., 2015; Barreto et al., 2018; Mathias et al., 2018; Santos-Silva et al., 2018).

The higher MT concentrations were measured in the liver of the animals of C site during the hot-rainy seasons, but the accumulation of metals was not evidenced in gills and livers by the autometallography analysis. MTs are a family of proteins with specific function on the regulation, sequestration and detoxification of metals including Cu, Zn, Cd and Hg (Viarengo et al., 1997). Thus, the presence of Cu, Zn and Cd seen in the sediments of site C during the hot-rainy season are probably the cause of the increased MT concentrations in liver. Significant increase in hepatic MT levels was observed in fish exposed to metals by contaminated sediment in bioassays (Benedetti et al., 2012) and in field studies (Gusso-Choueri et al., 2015). The same response was observed for *C. spixii* of the ELCIC, where increased levels of MT were correlated with higher metal body burdens, being more evident in the gills of the fish in sites closer to the Cananéia City also as a result of the urban activities (Gusso-Choueri et al., 2015).

The higher DNA damages and lesion index in the liver of the specimens of site C during the cold-dry season may be associated with the alterations found for the enzymes of antioxidant defense, demonstrating the failure of these antioxidant responses. A similar pattern of histopathological alterations was described by Azevedo et al. (2013) on the analysis of the livers of *C. spixii* specimens from the Cananéia estuary, where the authors observed necrosis as the most common alteration, with a higher prevalence among the animals during the cold-dry season.

The vacuolization of the hepatocytes was the most present alteration in liver, and is mainly caused by steatosis that represents an abnormal lipid accumulation. The presence of this alteration has been related to PAHs exposure, and had also being evidenced for *A. brasiliensis* specimens of the Brazilian coast (Fernandez et al., 2011; Ribeiro et al., 2013). Fernandez et al. (2011) observed the presence of steatosis in the fish of both polluted and non-polluted environments. Nonetheless, Ribeiro et al. (2013) related these alterations as a result of a disturbance in the cellular metabolism caused by the presence of organic compounds, as PAHs, in a most polluted area. In this study the presence of PAHs in the sediments can be responsible for the abnormal lipid accumulation in the liver of the animals, as they can interfere with vesicles transport and as consequence lead to the accumulation of secretion product (Fernandez et al., 2011).

The presence of necrosis in the hepatic tissues of fish is strongly associated with oxidative stress (Mela et al., 2013) and has also been linked to metals (Mela et al., 2013; Rossi et al., 2014) and PAHs (Ribeiro et al., 2013) exposures. Equally, Fernandez et al. (2011) observed higher prevalence of necrosis in *A. brasiliensis* fish from the area under higher influence from urban and harbor activities. The dilation of the sinusoids was the lest present alteration, nonetheless, these alterations may also be linked to the presence of environmental contaminants, as it can occur due to metal exposure (Rossi et al., 2014).

The presence of DNA strand breaks in blood and liver and of NMA in blood of the fish from both points indicates possible contributions of the different environmental contaminants with genotoxic proprieties such as metals and PAHs. The occurrence of DNA breaks may not be persistent as it is possibly reversible, since these lesions in the DNA are amenable to be repaired (Ferraro et al., 2004; Kroon et al., 2017). This damage cannot be attributed to a specific exposure (Ferraro et al., 2004; Ramsdorf et al., 2009), however, field studies have reported a significant increase in DNA damage following metal (Azevedo et al., 2013; Gusso-Choueri et al., 2016, 2015) and PAHs (Fernandez et al., 2011; Ribeiro et al., 2013) exposure in fish from sub-tropical estuaries.

The highest incidence of liver lesions and genotoxic responses in liver and blood of *A. brasiliensis* specimens near Cananéia City are probably resulted from a continuous and multiple exposure to contaminants which was evidenced in the chemical analysis of the sediments, including the contributions of metals of high toxic potential as Cd and Pb. These results indicate possibility harmful consequences for the organisms, such as the impairment of the liver functions, that can lead to adverse consequences for growth, health and reproduction (Fernandez et al., 2011; Mela et al., 2013). These responses also were influenced by the seasonal variations of the estuarine environmental conditions, as the lesions index, the DNA strand breaks in liver and the NMA in blood were more present in the fish during the cold-dry season and a higher presence of DNA stand breaks were seen in hepatocytes during the hot-rainy period.

Meanwhile, the kidney provided fewer information, being this finding also observed by Gusso-Choueri et al. (2015) on analyzing the biological responses of *C. spixii* in the same region. Nonetheless, fewer macromolecular damages were observed in the liver of the fish from S site, evidenced by a lower LPO. These results are in accordance to the atrophic activities and contamination pattern in the sediments. In addition, the condition index indicated that the fish sampled at S point were in better condition during the summer, which was also evidenced by Ribeiro et al. (2013) in *A. brasiliensis* fish from the least impacted site among those analyzed, indicating that the somatic indexes may provide practical parameters to evaluate fish’s health conditions.

The seasonal variations observed for SOD, GPX and EROD activities, GSH and MT concentrations and DNA damage and histopathological lesions in liver as for SOD and CAT activities and LPO in kidney can be related to the variations of the estuarine environmental conditions, that consequentially alter the concentrations and/or the bioavailability of the contaminants. These seasonal variations of the biological parameters revealed that climate and rainfall seasonality also play an important role in the exposure of the aquatic organisms to the environmental contamination in the ELCIC.

The Permanova results showed that the fish exhibited different biological responses among all analyzed sites and seasons, with site S during the cold-dry season being the most different one. The first PCoA axis explained 34.0% and the second axis 15.8% (Figure 3). AChE in muscle and brain, CAT, GPx and LPO in liver and kidney, GST, GSH, MT, LI and DNA ST in liver, as well as DNA ST, TNMA, BNA, LNA and NNA in blood were positively related with the first axis, while renal and hepatical SOD, hepatical EROD and renal GST were negatively related. SOD, CAT, EROD, GST, GSH in liver and kidney, GPx and LI in liver, as well as TNMA, BNA, LNA and NNA in blood were positively related with the second axis (Supplementary material E).

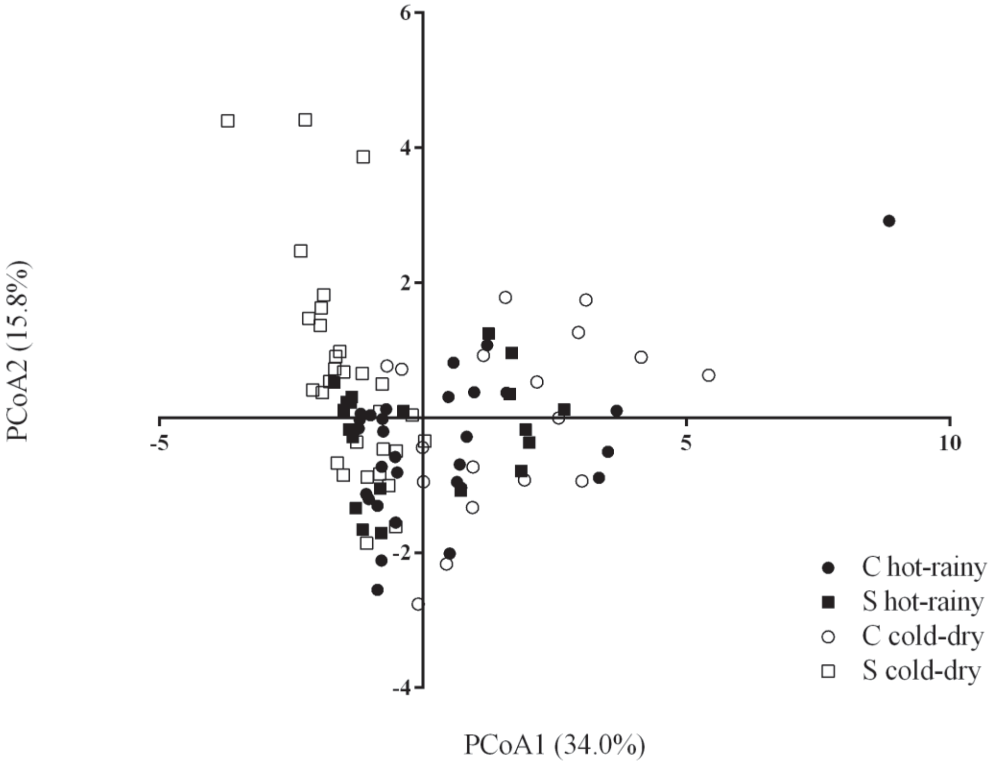


Figure 3. Multivariate analyses of biomarkers in *A. brasiliensis* from Cananéia (C) and Subaúma (S) during hot-rainy and cold-dry seasons.

2.6.3 Integrated Biomarker Response Index (IBR)

The responses obtained in fish of site S during the cold-dry season were used as reference condition since there is no baseline biomarker data for *A. brasiliensis*. The IBR values demonstrated a lower value at site S during the hot-rainy season (26.91), and higher values at C site, during the hot-rainy (28.04) and the cold-dry (29.65) seasons (Figure 4; Supplementary material F), indicating that the level of contamination at C were higher than at S site. These results corroborated with the metals, PAHs and PPCPs concentrations found in the sediments and with the Permanova and PCoA results, which indicated a difference in the biological responses of the fish between the sites, supporting the highest difference of site S during the cold-dry period.

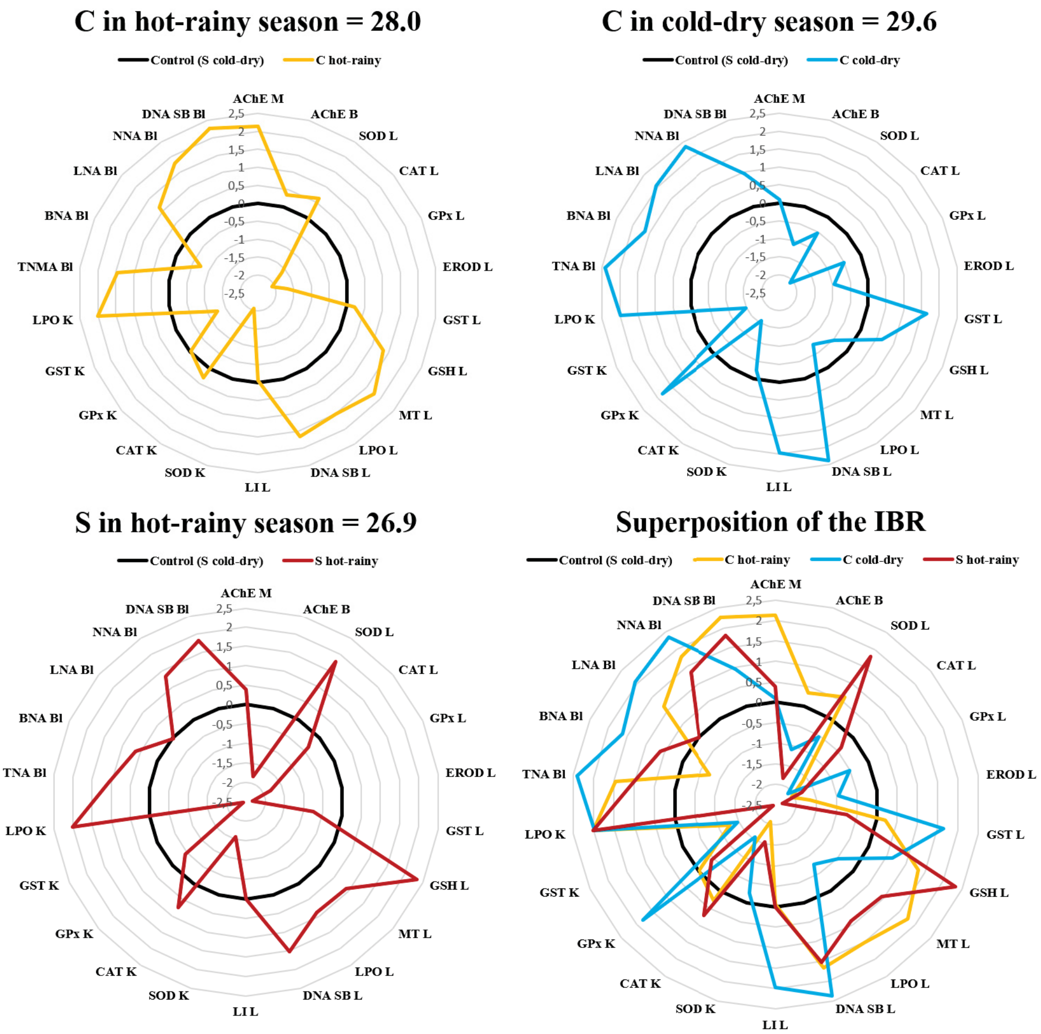


Figure 4. Integrated biomarker response index (IBR) in *Atherinella brasiliensis* based on the following biomarkers: AChE, SOD, CAT, GPx, EROD, GST, GSH, MT, LPO, DNA strand breaks (DNA SB), lesion index (LI), total nuclear morphological abnormalities (TNMA) and blebbed (BNA), lobed (LNA) and notched (NNA) abnormalities in different tissues (B= brain, M= muscle, L= liver, K=kidney, Bl = blood). Biomarkers results are represented in relation to the reference group from site S in the cold-dry season. The area above 0 reflects induction of the biomarker and below 0 indicates reduction of the biomarker.

2.6.4 Integrated analysis

The RDA results show that the concentrations of metals, PAHs and PPCPs detected in the sediments explained 13% of the responses observed in the fish, 12% of those were explained by the combination of all detected contaminants and 1% was explained only by the metals (Supplementary material G). Thus, the combination of all contaminants explained the majority of the biological responses, evidencing the influence of multiple stressors in the health conditions of the *A. brasiliensis* fish in the ELCIC.

Additionally, it was possible to determine that Cd, Cu, Pb and Fe were the major stressors among metals, while fluorene, pyrene and Benzo[k]fluoranthene were the major stressors among PAHs and caffeine, metoprolol and fenoprofen were the major stressors among PPCPs. These results indicated that others stressors not included in the present study may be present in this environment and could be contributing to the depletory effects observed in the fish. Still, contaminants from others environmental matrixes, such as water, could have larger representativity for this fish species, considering its pelagic habit.

Nevertheless, these results contribute with new information about the presence and the behavior of the contaminants in the investigated estuarine region and their possible contribution on adverse effects for the local fish. The observed sublethal effects on the ichthyofauna are able to infer in consequences that can lead to alterations of the fitness of the organisms, in the structure of the communities and the deterioration of the ecological state through the time. The present integrated approach supplies useful information about the local environmental quality that can be further used for protective management of the area on the way to mitigate the anthropic influence.

2.7 CONCLUSIONS

The present study provided fundamental information about the chronic exposure to contaminants in *A. brasiliensis* from the ELCIC. The presence of metals, PAHs, PPCPs and other contaminants from anthropic activities in the sediments can represent a significant hazard to this fish species. Our integrative approach to the chemical analysis of the sediments and the biological responses in a sentinel fish species proved to be useful for the environmental evaluation of coastal zones. These responses combined can reveal the pattern of contaminants distribution, their main sources and the main adverse effects in the health of the fish. In addition, the integrated biomarker response index is effective in field studies as they related well with the levels of environmental contaminants.

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2.10 SUPPLEMENTARY MATERIAL

A: Characteristics of the analysis of metals.

Metal	**IL (mg/L)**	r^2	**LD ($\mu g\ L^{-1}$)**	**LQ ($\mu g\ L^{-1}$)**
Fe	2.0 – 20.0	0.926	10	33
Zn	0.5 - 5	0.979	3.5	11
Mn	1.0 – 10.0	0.885	0.4	1.4
Cu	0.5 - 5	0.997	0.4	1.5
Cd	0.5 - 5	0.992	2	7
Cr	0.5 - 5	0.993	1.8	6
Pb	1.0 – 10.0	0.987	4	13
Ni	0.5 - 5	0.985	2	6

IL - Intervals of linearity; r^2 - linearity of the method; LD - Limit of Detection;
LQ - Limit of Quantification.

B. Characteristics of the analysis of PPCPs.

Compounds	Abb.	Fragment mass (m/z)	LD $\mu g\ g^{-1}$	LQ $\mu g\ g^{-1}$	Rec. (%)
Fenofibrate	FNF	360 – 273.1	0.001	0.003	61
Gemfibrozil	GFZ	201.1 – 129	0.063	0.209	86
Propranolol	PRL	144 – 115	0.003	0.010	32
Metoprolol	MTL	223.3 – 72	0.002	0.008	20
Salicylic Acid	SA	267.1 – 209	0.006	0.019	30
Acetylsalicylic Acid	ASA	195 – 177	0.001	0.003	51
Ibuprofen	IBU	295 – 280	0.014	0.047	87
Diclofenac	DCF	367 – 242.1	0.016	0.055	60
Naproxen	NPX	302.2 – 185	0.006	0.019	79
Fenoprofen	FNP	270.1 – 196	0.012	0.039	50
Caffeine	CAF	194 – 109	0.005	0.018	88
Ethinylestradiol	EE2	425.2 – 193.1	0.005	0.018	43
Estradiol	E2	416.3 – 326.2	0.003	0.010	59
Estrone	E1	342.2 – 257.1	0.003	0.010	74
Triclosan	TRC	362 – 347	0.077	0.256	82
Methylparaben	MEP	224.1 – 209.1	0.004	0.014	61
Ethylparaben	ETP	238.2 – 223.1	0.007	0.023	82
Propylparaben	PRP	252 – 195	0.012	0.039	95
Butylparaben	BTP	266.5 – 210	0.023	0.077	87
Benzylparaben	BZP	300 – 193.1	0.008	0.026	99

PPCPs - Pharmaceutical and Personal Care Products; Abb. - Abbreviation; LD - Limit of Detection; LQ - Limit of Quantification; Rec – Recovery.

C. Characteristics of the analysis of PAHs.

Compounds	**Abb.**	**LD** $\mu g\ g^{-1}$	**LQ** $\mu g\ g^{-1}$
Naphthalene	Nap	0.03	0.102
Fluorene	Fl	0.02	0.066
Acenaphthene	Ace	0.001	0.005
Acenaphthylene	Acy	0.001	0.003
Anthracene	Ant	0.001	0.004
Phenanthrene	Phe	0.012	0.039
Fluoranthene	Flu	0.002	0.008
Pyrene	Pyr	0.001	0.008
Chrysene	Chr	0.001	0.003
Benz[a]anthracene	BaA	0.001	0.003
Benzo[b]fluoranthene	BbF	0.002	0.008
Benzo[k]fluoranthene	BkF	0.001	0.003
Dibenzo[a,h]anthracene	DahA	0.003	0.010
Benzo[a]pyrene	BaP	0.009	0.003
Indene[1,2,3-cd]pyrene	IcdP	0.001	0.003
Benzo[g,h,i]perylene	BghiP	0.007	0.022

PAHs - Polycyclic Aromatic Hydrocarbons; Abb. - Abbreviation; LD - Limit of Detection; LQ - Limit of Quantification.

D. Main reasons applied in the diagnosis of PAH sources.

PAHs	Sites and seasons				Probable origin Yunker et al. (2002)
	hot-rainy		cold-dry		
	C	S	C	S	
∑PAHs2-4/ ∑PAHs5-6	0.0008	-	0.0002	-	>1 pyrogenic <1 petrogenic
Ant/(Phe+Ant)	0.5679	-	0.5350	-	>0.1 pyrogenic <0.1 petrogenic
Flu/(Flu+Pyr)	-	-	0.5946	-	>0.50 pyrogenic <0.50 petrogenic
BaA/(BaA+Cry)	-	0.1111	1	0.1111	>0.35 combustion of petroleum and biomass <0.20 petrogenic
IcdP/(IcdP+BghiP)	-	-	0.5304	-	>0.50 combustion of biomass <0.20 petrogenic 0.20 – 0.50 combustions of liquid fuel

- Reason not calculated due to values below the limit of detection <LD.
Ant: Anthracene; Phe: Phenanthrene; Flu: Fluoranthene; Pyr: Pyrene; BaA: Benzo[a]anthracene; Cry: Chrysene; BghiP: Benzo[g,h,i]perylene; IcdP: Indene[1,2,3-cd]pyrene.

E. Correlations biomarkers and PCoA axis.

Biomarkers	Tissue	Axis 1	p	Axis 2	p
AChE	muscle	0.2754*	0.0098*	-0.0959	0.3765
	brain	0.1866	0.0782	-0.0846	0.4277
SOD	liver	-0.2154	0.0616	0.2128	0.0650
	kidney	-0.1215	0.3723	0.2833	0.0344
CAT	liver	-0.5087*	<0.0001*	0.5498*	<0.0001*
	kidney	0.1153	0.4253	0.1145	0.4284
GPx	liver	0.6609*	<0.0001*	0.5731*	<0.0001*
	kidney	0.1509	0.3464	-0.4087*	0.0080*
EROD	liver	-0.4381*	0.0007*	0.4882*	0.0001*
GST	liver	0.0629	0.6020	0.2225	0.0621
	kidney	-0.3676*	0.0102*	0.2445	0.0940
GSH	liver	0.1256	0.3475	0.4625*	0.0003*
MT	liver	0.0193	0.9058	-0.1734	0.2847
LPO	liver	0.0623	0.6276	-0.0627	0.6249
	kidney	0.5480*	0.0038*	-0.4571*	0.0189*
DNA SB	liver	0.3499*	0.0049*	-0.2987*	0.0174*
	blood	0.1776	0.0818	-0.2748*	0.0065*
LI	liver	0.5879*	0.0013*	0.2952	0.1349
TNMA	blood	0.8620*	<0.0001*	0.0586	0.5875
BNA	blood	0.6793*	<0.0001*	0.2015	0.0568
LNA	blood	0.7090*	<0.0001*	0.1638	0.1390
NNA	blood	0.8247*	<0.0001*	0.0046	0.9656

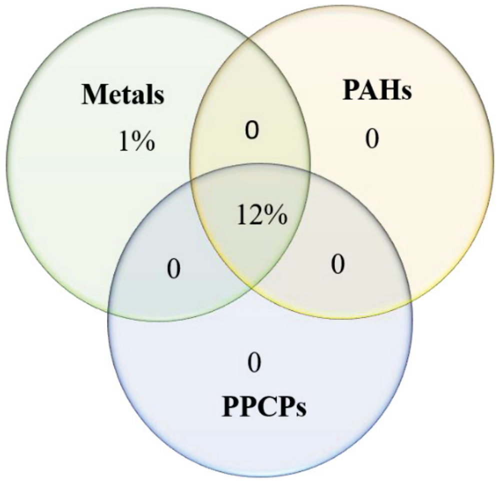
* $p<0.05$
AChE (acetylcholinesterase), SOD (superoxide dismutase), CAT (catalase), GPx (glutathione peroxidase), EROD (ethoxyresorufin-O-deethylase), GST (glutathione transferase), GSH (glutathione), MT (metallothionein), LPO (lipoperoxidation), DNA SB (DNA strand breaks), LI (lesion Index), TNMA (Total nuclear morphological abnormalities), BNA (blebbled nuclear morphological abnormalities), LNA (lobed nuclear morphological abnormalities) and NNA (nuclear morphological abnormalities).

F. Reference levels of the biomarkers results of *A. brasiliensis* fish from site S during the cold-dry season for the IBR analysis.

Biomarkers	**Tissue**	**Mean ± SD**
AChE	muscle	61.59 ± 23.06
	brain	82.41 ± 22.84
SOD	liver	185.39 ± 69.83
	kidney	2446.36 ± 2879.14
CAT	liver	175.81 ± 111.46
	kidney	12.38 ± 3.87
GPx	liver	38.02 ± 21.05
	kidney	26.8 ± 26.04
EROD	liver	0.19 ± 0.12
GST	liver	4.35 ± 1.46
	kidney	26.23 ± 11.05
GSH	liver	46.61 ± 13.66
MT	liver	20.61 ± 9.89
LPO	liver	8.82 ± 5.45
	kidney	2.59 ± 1.75
DNA SB	liver	64.16 ± 33.40
	blood	142.21 ± 70.88
LI	liver	2.57 ± 3.20
TNMA	blood	42 ± 28.8
BNA	blood	17.70 ± 14.69
LNA	blood	2.5 ± 2.76
NNA	blood	18.30 ± 11.34

AChE (nmol min^{-1} mg^{-1} protein), SOD (SOD mg $protein^{-1}$), CAT (μmol min^{-1} mg $protein^{-1}$), GPx (nmol min^{-1} mg $protein^{-1}$), EROD (nmol resorufin min^{-1}·mg $protein^{-1}$), GST (μmol min mg $protein^{-1}$), GSH (μg GSH mg $protein^{-1}$), MT (μg MT mg $protein^{-1}$), LPO (nmol min^{-1} mg $protein^{-1}$), DNA SB (Score of DNA strand breaks), LI (Lesion Index - Score of histopathological alterations), TNMA (Score of total nuclear morphological abnormalities), BNA (Score of blebbled nuclear morphological abnormalities), LNA (Score of lobed nuclear morphological abnormalities) and NNA (Score of notched nuclear morphological abnormalities).

G. Multivariate analysis of the concentrations of metals, PAHs and PPCPs in the sediments and the biological responses of *A. brasiliensis* in the ELCIC.



3 CAPÍTULO II

Sediment contamination by metals and pharmaceuticals and personal care products of a Marine Protected Area and their toxic effects in the Violet Goby fish (*Gobioides broussonnetii* - Gobiidae).

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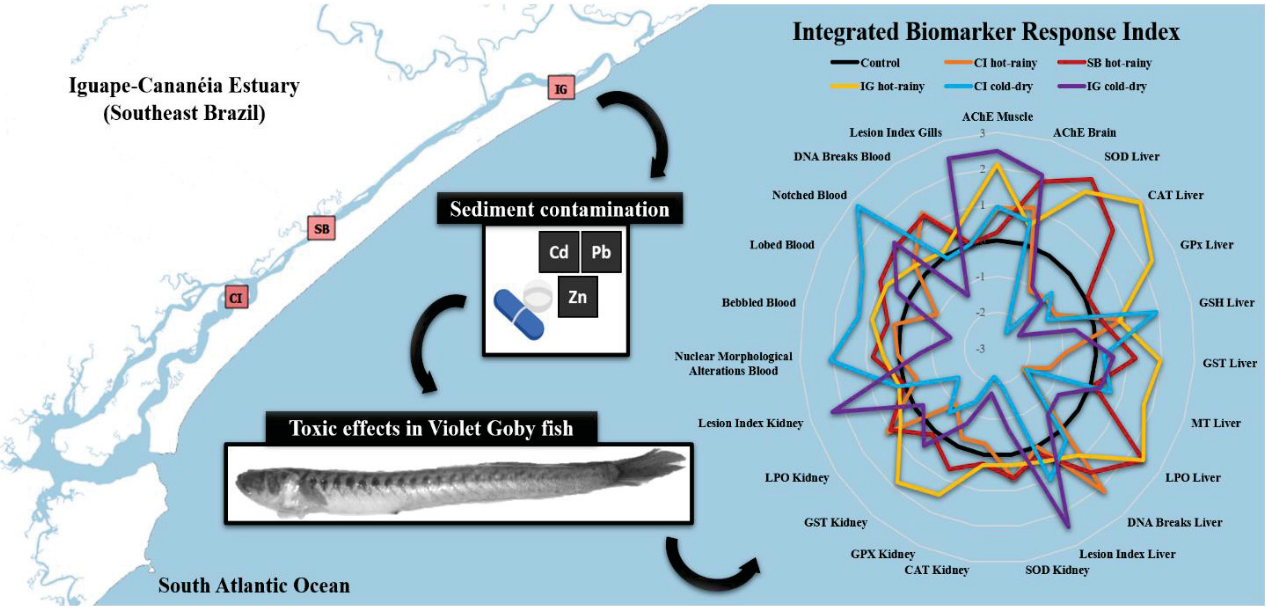
3.1 ABSTRACT

The Estuarine-Lagoon Complex of Iguape-Cananéia (ELCIC) is a Marine Protected Area, located in the Southeastern Brazil. Nowadays, the area is under the negative influences of the poor infrastructure conditions of its coastal cities and of the Ribeira de Iguape River (RIR). This river was heavily polluted by mining and also receives inputs of agricultural and urban areas. In the last decade, the regional decline in the population of the *Gobioides broussonnetii* fish has been reported and assigned to the contaminations from the RIR. The aim of this study was to evaluate the health of the Violet Goby fish and if the species responds to the sediment contaminations by metals and pharmaceuticals and personal care products (PPCPs). Three sites under different anthropic pressures, in two different seasons, were analyzed by the chemical analysis of the sediments and of biochemical, histopathological and genotoxicity biomarkers in fish. Higher sediment contamination was observed near the Iguape City (IG), that houses the mouth of the RIR. The metals levels were considered low to moderated and decreased with greater distance of the RIR outfall. The PPCPs levels were increased by anthropic presence and were higher near IG and Cananéia Island (CI). Contributions of past mining, agriculture, nautical activities and sewage and residues disposal were identified. Higher contaminants values were observed during the cold-dry season, suggesting the influences of the lower hydrodynamics during the lower rainfall season. In fish, the biological responses followed the same spatial and seasonal pattern. More pronounced changes in the antioxidant, biotransformation, histopathological and genotoxic biomarkers were observed in IG and CI. The values of the multivariate analysis and the integrated biomarkers response index also indicated worse environmental conditions of these sites. The results indicated a negative influence of anthropic activities in the sediment contamination, as well to the health of the Violet Goby fish. This study presented the first ecotoxicological data for the species and suggests that the chronic exposure can negatively influence the health of this local fish population. The data contribute to the understanding of the local environmental quality and can be further applied for the management of the MPA.

Key words: Field study; Metals; Pharmaceutical and personal care products; Biomarkers; Integrated Biomarker Response Index.

3.2 GRAPHICAL ABSTRACT



3.3 HIGHLIGHTS

- Contamination by metals and PPCPs were observed in the sediments of the MPA.
- Anthropic presence and lower rainfall increased the sediment contaminant levels.
- Genotoxicity, biochemical and histopathological alterations were observed in fish.
- Higher sediment contaminant levels increased toxic effects in *G. broussonnetii.*

3.4 INTRODUCTION

The Estuarine-Lagoon Complex of Iguape-Cananéia (ELCIC) is part of the Environmental Protection Area of Cananéia-Iguape-Peruíbe (APA-CIP), a Marine Protec Area (MPA) in the Southeast of Brazil (Gusso-Choueri et al., 2016). This region is recognized as a UNESCO World Natural Heritage Site, which constitute an important environment for marine productivity in the South Atlantic (Morais and Abessa, 2014). It has regular abundance of resources throughout the year, which allows fishing resources to be their economic base (Mendonça; Katsuragawa, 2001). However, this region went through several changes in the last centuries which affected its environmental quality (Tramonte et al., 2018). These changes especially occurred before the creation of the MPA, however the region is currently under increasing human pressure (Cruz et al., 2019).

The Ribeira de Iguape River (RIR) became the major freshwater contributor for this estuary after the opening of an artificial channel, named Valo Grande Canal, in the Iguape City in the 19th century (Mahiques et al., 2013). This river was polluted by the residues former mining activities containing Pb, Zn, Cu and As, and the input of the waters of the RIR into the estuarine system dramatically changed the estuarine conditions of the area. The opening of the artificial channel lead to alterations in the water parameters of the estuary (as pH and salinity), increased the supply of nutrients and allowed the entry of different classes of contaminants, including metals and persistent organic pollutants (Abessa et al., 2014; Salgado; Azevedo, 2018; Tramonte et al., 2018). These alterations have caused the contamination of the sediments and biodiversity loss near Iguape City (Mahiques et al., 2013; Morais and Abessa, 2014; Tramonte et al., 2018; Cruz et al., 2019).

Nonetheless, the region faces many conflicts of interest upon the uses of the territory and natural resources, demanding for large investments in the social field, infrastructure and land regularization (Barbieri et al., 2014; Morais and Abessa, 2014). As so, other factors linked to anthropic presence may be contributing to the input of contaminants throughout this estuarine system, which also can constitute a threat to the health of the aquatic organisms.

Previous contributions reported expressive concentrations of metals, moderated levels of polycyclic aromatic hydrocarbons (PAHs) and the presence of pharmaceuticals and personal care products (PPCPs) in the sediments (Antonelli et al., 2017; Salgado and Azevedo, 2018; Salgado et al., 2018; Cruz et al., 2019) and aquatic organisms of the ELCIC (Azevedo et al., 2012; Gusso-Choueri et al., 2018). Additionally, different biological responses to these exposures were described in the ichthyofauna (Fernandez et al., 2011; Azevedo et al., 2012; Gusso-Choueri et al., 2015, 2016; Salgado et al., 2018), since these organisms are permanently exposed to such contaminants. Even so, the studies with contaminants in the region are still incipient and little is known about how environmental contamination affects the different species of these MPA.

The Violet Goby (*Gobioides broussonnetii*) is the largest member of the Gobiidae family which can reach up to 60 cm in total length (Menezes et al., 2003). Its estimated distribution extends from the Atlantic coast of the United States to the Paraná State, in Brazil, including the Gulf of Mexico and the Caribbean coasts of Colombia, Venezuela and French Guyana (Murdy, 1998; Mata-Cortes et al., 2004; Ross and Rhode, 2004; Rodríguez and Villamizar, 2006; Passos et al., 2012). It’s a demersal fish of social and economic importance to traditional fisheries of the ELCIC region, as it is mainly used as bait for the fishery of more commercial fish species. Nonetheless, the species can also serve as source of food, especially for the low-income community, as observed in other areas of Brazil (Santos; Sampaio, 2013). In addition, the ingestion of the Violet Goby also may represent a potential risk for human health in the ELCIC due to the concentrations of metals, as described for the consumption of the local *Cathorops spixii* fish (Gusso-Choueri et al., 2018).

Over the last decade the regional decline in the *G. broussonnetii* fish population has been reported by the local people and fishermen in the studied area, including the observation of events of high mortality. The contamination caused by the RIR in the area (Mahiques et al., 2013; Tramonte et al., 2018; Cruz et al., 2019) has been referred as one of the causes of the decline of this population.

Little data is available on the biology and ecology of this species, with the exception of some systematics (Murdy, 1998; Pezold, 2004), feeding ecology (Mata-Cortes et al., 2004; Rodríguez; Villamizar, 2006), salinity effect (Reis-Filho; Oliveira, 2014) and parasitism (Azevedo et al., 2013; Velasco et al., 2012; Videira et al., 2013). Thus, the purpose of this study was to assess, for the first time, the health conditions and the responses of this fish species using different biomarkers and, to investigate the contamination by metals and PPCPs in the sediment of the ELCIC

3.5 MATERIALS AND METHODS

3.5.1 Sampling

Tree sites, representing sites with different atrophic pressures, in the Estuarine-Lagoon Complex of Iguape-Cananéia, São Paulo State, Brazil (24º50` to 25º10`S / 47º25` to 48º00`W; Figure 1) were sampled. Iguape - IG (24º42`17.82" S / 47º28`56.47" W) is located near the biggest urban center of the region, the Iguape City, which homes the Mouth of the Ribeira de Iguape River trough the Valo Grande Canal. Subaúma - SB (24º53`45.48" S / 47º48`20.43" W) is located in an area of little human occupation and represents the point of the inversion of the waters of the estuary. The site close to the Cananéia Island - CI (25º02`27.73" S / 47º54`47.44" W) is located near a small human occupation, the Pedrinhas Village in the Comprida Island, which also can suffer influences of the other main urban center of the area, the Cananéia City.

The region displays two well-defined climate seasons, with dry winters (mean pluviosity of 95.3 mm month^{-1} and mean temperature of 20 °C) and rainy summers (mean pluviosity of 266.9 mm month^{-1} and mean temperature of 28 °C), where little annual temperature occurs in July and highest annual temperature occurs in February (Cunha-Lignon et al., 2009). Thereby the sampling campaigns correspond to the two different periods, the cold-dry season (September 2016) and the hot-rainy season (February 2017). Nonetheless, in order to investigate these seasonal differences between the sampling campaigns, the environmental variables of water and air temperature, pH and salinity were obtained *in loco*. The precipitation data of the region corresponding to the sampling months were obtained from the Integrated Agrometeorological Information Center (CIAGRO), a governmental organ of the São Paulo State in Brazil (2019).

Sediment samples were collected to a depth of 20 cm, with a Petersen dredge that was thrown three times yielding composite samples of approximately 200 g, to perform chemical investigations. In the same day, about 10 fish of the species *G. broussonnetii* were collected per site with the use of fishing nets (SISBio License No. 50365-3) to assess their health conditions. They were anesthetized with benzocaine and the blood was taken from the caudal vein for the analysis of biomarkers of genotoxicity. After euthanasia by medullary section the fish were submitted to biometry. The gills were collected for the analysis of histopathological biomarkers and autometallography. The brains and muscles were collected for biochemical biomarker analysis. The kidneys were used for the analysis of biochemical and histopathological biomarkers and autometallography. Livers were used for genotoxicity analysis, biochemical and histopathological biomarkers and autometallography. The tissues were kept on liquid nitrogen for transportation to the laboratory and stored at -80°C until the analysis. The gills, kidneys and livers for the histopathology analyses were fixed in Alfac solution.

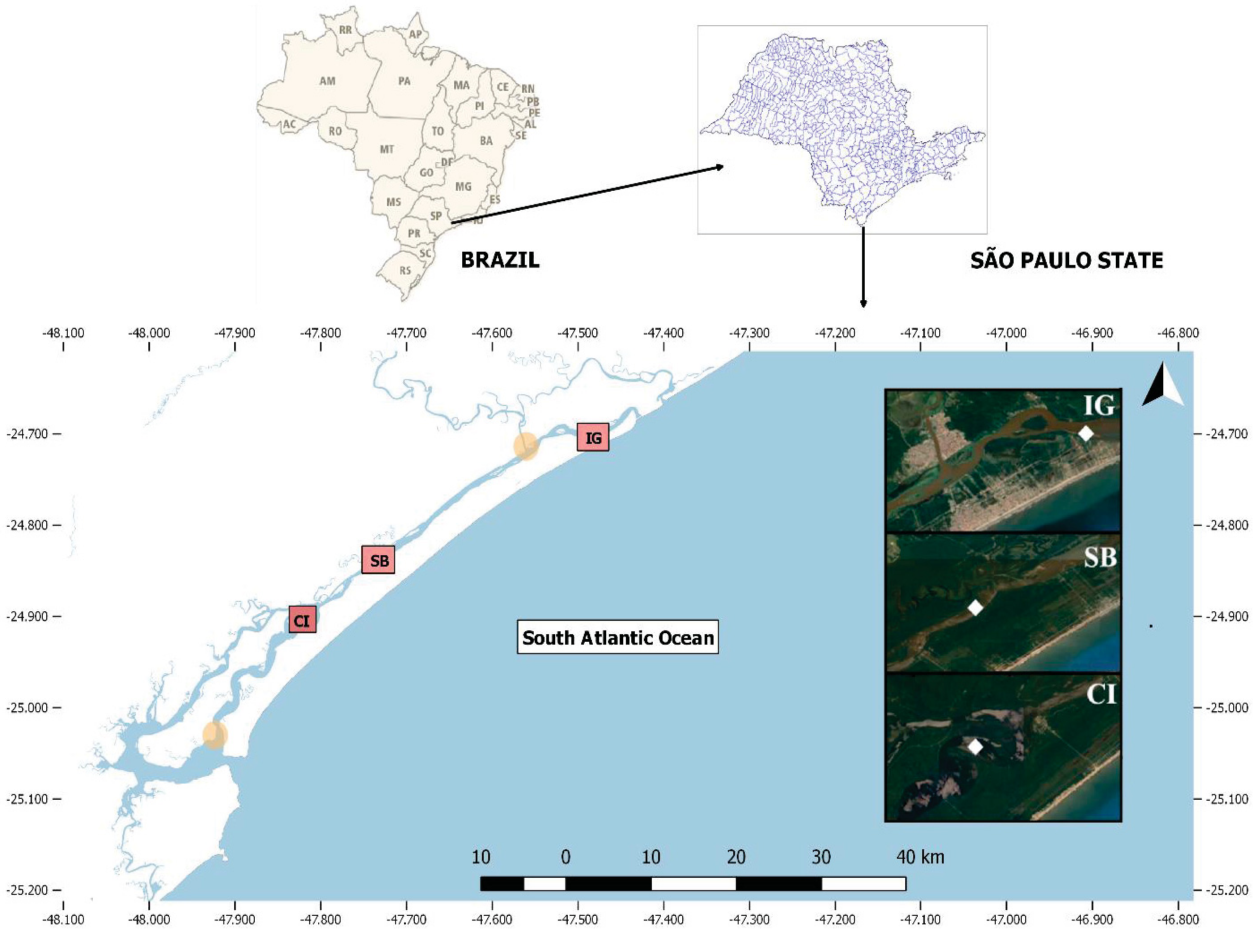


Figure 1. Map of the Estuarine-Lagoon Complex of Iguape-Cananéia and its location in Brazil and the São Paulo State, highlighting the sampled sites and their aerial view (CI - Cananéia Island; SB - Subaúma; IG - Iguape). The main urban centers of the region are circled in orange, Cananéia City at South and Iguape City at North.

3.5.2 Chemical analysis of the sediments

3.5.2.1 Determination of metals

The sediments samples were stored in whirl-pack plastic bags and frozen at -20°C until the analysis of the determinations of metals. Each sample of sediment was dried in drying oven at 40 °C for 48 h or until completed dried, homogenized and divided in triplicates of 1g. The analysis followed the method presented by the Environmental

Protection Agency (EPA, 1996). The procedures were performed by acid digestion with HNO_3, H_2O_2 and HCl. All materials used were previously decontaminated by being washed with Extran® detergent and deionized water and bath in 10% nitric acid for 24h and the reagents utilized were of an analytic degree.

A flame atomic absorption spectrometry (FAAS) model GBC, Avanta, was used to held the determinations of the Fe, Zn, Mn, Cu, Cr, Ni, Cd and Pb, respecting the characteristics of the method for the different metals (Supplementary material A). The standard solutions were prepared with successive dilutions using stock solutions of each metal (1000 mg/L^{-1}), using 13% v/v HNO_3. The determination was performed in triplicate and an analytic blank were used to accompanied the quality of the results. The exactitude of the method was verified by addition and recovery tests performed in fish muscular tissues, with satisfactory recovery rates that ranged from 82% to 108% in all investigations.

3.5.2.2 Determination of pharmaceuticals and personal care products (PPCPs)

The sediments samples were packed in aluminium foil and stored at -20°C until the PPCPs analysis. These samples were dried in drying oven at 40 °C for 48 h or until completed dried, homogenized and divided in triplicates of 1g. The methodology presented by Kramer et al. (2018) was used for sediment analysis. All materials used in the analyzes were washed with Extran® detergent and deionized water and bathed in 5% hydrochloric acid, and all solvents used were HPLC grade. The non-volumetric glassware was decontaminated in a muffle at 550 ° C for 5 hours.

The Gas Chromatographic (Agilent Technologies 7890) tandem Mass Spectrometry (GC-MS/MS) fitted with a triple quadrupole mass spectrometer (Agilent Technologies 7000), with an autosampler (Agilent Technologies 80), and with a HP-5Msi capillary column (30m x 0.25mm x 0.25 µm) was used to perform the PPCPs determinations. The temperature program of the oven was initially at a temperature of 100°C, held for 2 min, then increased to 180°C at a ramp rate of 15°C min^{-1}, after that raised to 270°C at a rate of 6°C min^{-1}, and 5°C min^{-1} until 310°C, being held for 3 min. The analysis total time was of 33.3 min. The injector and the transfer line temperature were 280°C, and the capillary column pressure was 8.91×10^6 Pa, with a helium flow rate of 1.2 mL min^{-1}; 1µL of the samples was injected on splitless mode. The ionization method was Electron Impact (EI) with 70 eV of energy and 300°C of source temperature.

The following pharmaceuticals and personal care products (PPCPs) were analyzed: Lipid regulators - fenofibrate (FNF) and gemfibrozil (GFZ); β-blockers - propranolol (PRL) and metoprolol (MTL); Analgesics and anti-inflammatories - salicylic acid (SA), acetylsalicylic acid (ASA), ibuprofen (IBU), diclofenac (DCF), naproxen (NPX) and fenoprofen (FNP); Stimulants - caffeine (CAF); Estrogens - ethinylestradiol (EE2), estradiol (E2) and estrone (E1); Antiseptics - triclosan (TRC), methylparaben (MEP), ethylparaben (ETP), propylparaben (PRP), butylparaben (BTP), and benzylparaben (BZP). All chemical standards were purchased from Sigma Aldrich and the characteristics of the method to the PPCPs analyzed are shown in the supplementary material B.

3.5.3 Biological responses

3.5.3.1 Condition factor (K)

The condition index (K) demonstrate the length-weight relationship that was expressed by K = fish total weight / (fish total length)^3 * 100. It was used as indicator of the physiological state of the fish, as presented by Bolger and Connolly (1989).

3.5.3.2 Biochemical biomarkers

The brain and muscle were weighed and homogenized in potassium phosphate buffer (0.1 M pH 7.5) and centrifuged at 12,000 × g for 20 minutes at 4°C. The supernatants were used for the measurement of the acetylcholinesterase activity (AChE) by the method presented by Ellman et al. (1961), modified for microplates by Silva de Assis (1998) at 415 nm.

The kidney and liver were weighed and homogenized in potassium phosphate buffer (0.1 M pH 7.0) 1:10 (w/v), and centrifuged at 15,000 × g for 30 minutes at 4°C. The supernatants were used for the measurement of the activities of the superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase activity (GPx), glutathione S-transferase (GST) and the concentrations of glutathione (GSH), metallothioneins and lipoperoxidation (LPO). SOD activity followed the method described by Gao et al. (1998) at 440 nm. CAT activity was determined by the method presented by Aebi (1984) at 240 nm. GPx activity was measured by the method of Paglia and Valentine (1967) at 340 nm. GST activity was measured according to the method presented by Keen et al.

(1976) at 340 nm. GSH concentrations were determined according Sedlak and Lindsay (1968) at 415 nm, while the metallothioneins were measured by the method described by Viarengo et al. (1997) at 412 nm. The level of LPO were measured by the method of Jiang et al. (1992) at 570 nm.

All biochemical biomarkers were normalized to protein concentration following the method presented by Bradford (1976). The calibration curve was obtained with bovine serum albumin as standard. The biochemical analyses were carried out on a BioTek ELx800 Absorbance Microplate Reader (BioTek Instruments, Inc.).

3.5.3.3 Genotoxicity biomarkers

The micronucleus test (MN) and nuclear morphological abnormalities (NMA) were investigated in erythrocytes. The blood microscopy slides were examined and scored based on the presence of both typical MN and NMA. The MN test was based on Heddle (1973) and Schmid (1975) methodology. The NMA analysis (blebbed, notched, lobed and vacuolated) was performed according to Carrasco et al. (1990).

The comet assay based in the Single-Cell Gel Electrophoresis (SCGE) was performed in erythrocytes and liver cells for the visualization of the DNA strand breaks (DNA ST). The method described by Singh et al. (1988) was applied, with modifications for the erythrocytes (Ferraro et al., 2004; Cestari et al., 2004) and liver cells by Ramsdorf et al. (2009).

3.5.3.4 Histopathological biomarker

The samples of gills, kidney and liver were fixated in Alfac solution (70% ethanol, 4% formaldehyde and 5% acetic acid) for 16 h and then stored in 70% alcohol until the inclusion procedures. The tissues were dehydrated in a graded series of ethanol baths and embedded in Paraplast Plus resin (Sigma®). Sections of 3-5μm were stained in hematoxylin/eosin and observed in Zeiss Axiophoto photomicroscope. Histopathological indexes were estimated for the organs using a semi-quantitative protocol presented by Bernet et al. (1999), and modified by Mela et al. (2013). The liver, kidney and gill indexes were calculated on the basis of two factors: the pathological importance (importance factor, w) and the extension of the pathological change (score value, a). The importance factor (1-3) reflects the reversibility of the alteration after removing the stressor (1 =

easily reversible; 2 = reversible in most cases; 3 = generally irreversible). The score value (1-6) was assigned according to the percentage of tissue exhibiting a certain alteration: 0 = less than 5%; 1 = 5-20%; 2 = 21-40%; 3 = 41-50%; 4 = 51-60%; 5 = 61-80%; and 6 = 81-100%. The organ index (Iorg) was calculated as the sum of the five reaction patterns of an organ. It was determined as:

$$I_{org} = \sum_{rp} \sum_{alt} (a_{org\ rp\ alt} \times w_{org\ rp\ alt})$$

Where: org is the organ (constant), rp: reaction pattern, alt: alteration, a: score value, and w: importance factor.

3.5.3.5 Autometallography

For the investigation of accumulation and distribution of nonspecific metals, samples of gills, kidneys and livers were submitted to autometallography analysis. The samples were collected and fixated with 3 % glutaraldehyde (in 0.1 M sodium cacodylate buffer, pH 7.4) for 24 h at 4 ˚C and rinsed with buffer (0.1 M sodium cacodylate buffer, 2 % NaCl, pH 7.4). Then, the samples were dehydrated in a graded series of ethanol baths and embedded in Paraplast Plus (Sigma®). The autometallography was performed in the tissue sections (3-5 µm) according to the protocol described by Danscher et al. (1987) with modifications by Rossi et al. (2014). The procedures were developed in a dark room where the cuts received a silver solution (Emulsion L4, Cambridge Nuclear Emulsion TAAB) for 30 minutes. Then, the sections were stained with hematoxylin/eosin and observed under the Leica® DME light microscope.

3.5.4 Data analysis

The Shapiro-Wilk normality test and the Bartlett homogeneity test preceded all the statistical analyses. To analyze the differences among the metals concentrations in the sediments and the biomarkers responses of the fish from the different sites, ANOVA two-way analysis followed by Fisher's test (LSD) and the Permutational Variance analysis (PERMANOVA) followed by the bootstrap analysis of multiple comparison of the averages were used. The biomarkers also were analyzed using the Principal

Coordinates Analysis (PCoA; Gower, 1966) and the Permutational Multivariate Analysis of Variance procedure (PERMANOVA; Anderson, 2001), which were used to summarize and show general patterns. The Spearman´s Test was used to analyze the correlations between the PCoA axis and the biomarkers.

Finally, to investigate the influence of the concentrations of metals and PPCPs of the sediments in the biomarkers responses of the fish, the Redundancy Analysis (RDA) was used. For this analysis, log transformation was used to minimize the problem of non-normality of the data. Then, to reduce its variation, the scale of the variables was standardized by rescaling all the variables to an average of zero and standard deviation of one. The contaminants of the sediments that had values below the limit of detection (LD) were not included. Statistical analyses were performed in R software R 3.2.2 (R Core Team 2015). The decision rule was $p<0.05$ for all analyzes.

The biomarkers also were integrated through the calculation of the integrated biomarker response index (IBR), described by Beliaeff and Burgeot (2002) and modified by (Sanchez et al., 2013). This version of IBR is based on the principle of reference deviation between a disturbed and undisturbed state. Since there is no baseline biomarker data for G*. broussonnetii*, the responses obtained in fish from the SB sampling site during the cold-dry season, which presented the lowest genotoxic and histopathological effects, were considered as the reference condition.

Once there is no specific federal legislation dealing with the maximum admitted limits for the concentrations of metals in estuarine sediments in Brazil, the results of the concentrations of Zn, Cr, Cu, Ni, Cd and Pb at each site were compared to the limits determined by the Resolution nº 454 of the Brazilian National Council for the Environment (CONAMA, 2012) which establishes the general guidelines and minimum procedures for the evaluation of the matter to be dragged from Brazilian jurisdiction waters. This resolution establishes two quality criteria, where values below the quality criterion I indicate non-expected toxicity of the sediments to aquatic life, values above the quality criterion I indicate the possibility of toxicity of the sediments and values above the quality criterion II indicate probable toxicity of the sediments. In addition, these results were also compared with the site-specific Sediment Quality Guidelines (SQGs) presented by Choueri et al. (2009). These SQGs were developed specifically for two estuarine and port areas of Southeastern Brazil (Santos Estuary System and Paranaguá Estuary System), which are located between the study area, taking into consideration the specific environmental conditions of each locality. Therefore, the values are more restrictive than those applied in Brazilian legislation. These SQGs allow the inference whether the area is classified as highly, moderately or not polluted (Choueri et al., 2009).

3.6 RESULTS AND DISCUSSION

3.6.1 Seasonal differences between the sampling campaigns

The environmental variables of water and air temperature, pH, salinity and mean precipitation for the sampling months are presented in supplementary material C. The sampling campaign carried out during the hot-rainy season showed higher mean precipitation, water and air temperature and lower salinity in relation to cold-dry season sampling.

3.6.2 Chemical analysis of the sediments

The results of the metals and PPCPs concentrations in the sediments presented both spatial and seasonal variations (Table 1). The concentrations of metals were obtained in the following order of magnitude: Fe>Mn>Zn>Cr>Ni>Cu>Pb>Cd. The concentrations of Fe were higher in IG (Iguape) in both seasons and in SB (Subaúma) during the hot-rainy season ($p<0.001$). The Mn values were higher in SB during both seasons ($p<0.001$).

The concentrations of Zn, Ni and Cu were higher in IG in both seasons ($p<0.001$), while Cr was higher in IG in both seasons and in SB during the cold-dry season. In addition, the Cu and Cr concentrations in IG surpassed the level of 34 µg g^{-1} and of 81 µg g^{-1}, respectively, established as quality criteria I by the Brazilian Environment Agency for brackish waters, during the cold-dry (Cu: 42.89 µg g^{-1} and Cr: 94.31 µg g^{-1}) and hot-rainy seasons (Cu: 39.34 µg g^{-1}; CONAMA, 2012). Nonetheless, the Ni concentrations were above the limit of 20.9 µg g^{-1} determined in the legislation in all sites during the hot-rainy season and in SB and IG during the cold-dry season (from 27.32 to 53.39 µg g^{-1};CONAMA, 2012).

The Cd was not detected in CI (Cananéia Island) and SB during the hot-rainy season, and had similar values among the other sites. The Pb was not detected in SB during the cold dry-season and IG in the hot-rainy season, and also had similar values among the others sites ($p>0.05$).

The concentrations of Fe and Mn in the sediments are not included in the Brazilian environmental legislation nor in the SQGs specifically developed for the estuaries in Southeastern Brazil, thus preventing further comparisons (Choueri et al., 2009; CONAMA, 2012). In the comparison of the results to the Brazilian legislation, the concentrations of Zn, Cd and Pb in the sediments of the ELCIC were inferior to the class I values, indicating low probability of toxic effects to the biota (CONAMA, 2012). Nonetheless, regarding the Ni concentrations, the results indicate possible toxicity of the sediments in all of the tree analyzed sites, with special attention to site IG, which also presented possible toxicity regarding to the Cu and Cr concentrations (CONAMA, 2012). However, in none of the analyzed sites and seasons the concentrations of metals in the sediments exceeded the values established by the legislation in the quality criteria II (CONAMA, 2012).

Table 1. Concentrations of metals and pharmaceuticals and personal care products (PPCPs) in the sediments (in μg g^{-1}).

Contaminants		Sites and seasons					
		Cold-dry			Hot-rainy		
		CI	SB	IG	CI	SB	IG
Metals							
	Zn	56.23 ± 3.58^{a}	52.08 ± 7.72^{a}	137.7 ± 13.03^{b}	32.96 ± 2.69^{c}	86.86 ± 6.44^{d}	134.49 ± 9.31^{b}
	Cu	14.83 ± 0.26^{a}	14.62 ± 0.60^{a}	**42.89** ±5.07^{b}	10.19 ± 0.71^{a}	25.40 ± 2.23^{c}	**39.34** ± 4.07^{b}
	Cr	35.23 ± 10.29^{a}	47.44 ±17.61^{a}	**94.31** ±18.35^{b}	58.97 ±12.33^{a}	79.99 ±28.32^{b}	78.31 ±14.54^{b}
	Ni	14.49 ± 0.89^{a}	**27.32** ±13.34^{a}	**49.53** ±11.12^{b}	**28.99** ± 9.47^{a}	**37.07** ±12.37^{a}	**53.39** ± 9.00^{b}
	Cd	0.23 ± 0.28^{a}	0.24 ± 0.26^{a}	< LD	< LD	< LD	0.09 ± 0.01^{a}
	Pb	0.25 ± 0.31^{a}	< LD	0.43 ± 0.25^{a}	0.24 ± 0.42^{a}	0.20 ± 0.35^{a}	< LD
Pharmaceuticals and personal care products (PPCPs)							
Lipid regulators	FNF	<LD	<LD	180.72	<LD	<LD	133.15
	GFZ	<LD	<LD	<LD	<LD	<LD	<LD
B-blockers	PRL	11.80	<LQ	10.11	19.75	9.22	4.79
	MTL	<LD	<LD	<LQ	<LD	<LD	<LD
Analgesics and anti-inflammatories	SA	<LD	<LD	<LD	<LD	<LD	<LD
	ASA	<LD	<LD	<LD	<LD	<LD	<LD
	IBU	<LD	<LD	<LD	<LD	<LD	<LD
	DCF	<LD	<LD	<LD	<LD	<LD	<LD
	NPX	<LD	<LD	<LD	<LD	<LD	<LD

	FNP	<LQ	<LD	26.73	27.37	<LD	<LD
Stimulants	**CAF**	<LQ	<LQ	<LD	<LD	<LQ	<LD
Estrogens	**EE2**	<LD	<LD	<LD	<LD	<LD	<LD
	E2	<LD	<LD	<LD	<LD	<LD	<LD
	E1	<LD	<LD	<LD	<LD	<LD	<LD
Antiseptics	**TRC**	<LD	<LD	<LD	<LD	<LD	<LD
	MEP	<LD	<LD	<LD	<LD	<LD	<LD
	ETP	<LD	<LD	<LD	<LD	<LD	<LD
	PRP	<LD	<LD	<LD	<LD	<LD	<LD
Antiseptics	**BTP**	<LD	<LD	<LD	<LD	<LD	<LD
	BZP	<LD	<LD	<LD	<LD	<LD	<LD

Data for metals expressed in mean ± standard deviation.
FNF – Fenofibrate; GFZ – Gemfibrozil; PRL – Propranolol; MTL – Metoprolol; SA - Salicylic Acid; ASA - Acetylsalicylic Acid; IBU – Ibuprofen; DCF – Diclofenac; NPX – Naproxen; FNP - Fenoprofen; CAF - Caffeine; EE2 – Ethinylestradiol; E2 – Estradiol; E1 - Estrone; TRC – Triclosan; MEP – Methylparaben; ETP - Ethylparaben; PRP – Propylparaben; BTP – Butylparaben; BAP - Benzylparaben.
LD - Limit of Detection; LQ - Limit of Quantification.
[a b c d] Indicates statistical differences among the sites ($p<0.05$).
* Indicates statistical seasonal differences between the seasons ($p<0.05$).
Values for metals above the established by the Brazilian Environmental Agency - CONAMA, 2012 are in bold.

In comparison with the SQGs presented by Choueri et al. (2009), the Zn concentrations in the sediments of sites CI and SB during both seasons were considered from not polluted to moderately polluted when compared to the Santos Estuarine System (SES) and moderated to highly polluted when compared to the Paranaguá Estuarine System (PES). Nonetheless, the site IG was considered highly polluted in both seasons in the comparisons to both estuarine systems. The Cu concentrations in all sampled sites and seasons were considered as not polluted to moderately polluted compared to the SES and highly polluted compared to the PES. For the Cr concentrations, the CI and SB sites during the cold-dry season were considered as not polluted to moderately polluted compared to both estuarine systems, while the others sites and seasons were considered as highly polluted. For the Ni concentrations only the sediments of site IG during the cold dry season were considered as moderately polluted in comparison to the SES, while the others sites and seasons where considered as highly polluted.

According to the SQGs for both estuarine areas, the Cd and Pb concentrations in the sediments of the ELCIC in all sites and seasons indicated a not polluted environment (Choueri et al., 2009). Thereby, these comparisons indicated that all three analyzed sites could be considered as highly polluted by metals in at least one of the seasons: Site IG were highly polluted by Zn, Cu, Cr and Ni while sites SB and CI were highly polluted by Cu, Cr and Ni.

The higher concentrations of metals observed by the present study near Iguape City (Fe, Zn, Cu, Cr and Ni) and Subaúma (Fe, Mn and Cr) indicate that in addition to the pedogenic concentrations, the waters of the Ribeira de Iguape River (RIR) are probably still the main contributor to the entry of metallic elements in this estuarine system. Thus, indicating the continental supply of these metals. These results are consistent with the contamination gradient found on previous studies, which reveled higher contamination of the sediments by different metals in the central and northern portions of the ELCIC, due to the higher influence of the Mouth of the RIR in these regions (Mahiques et al., 2013; Salgado and Azevedo, 2018; Tramonte et al., 2018; Cruz et al., 2019).

The Valo Grande Canal, is an artificial canal located at the Iguape City, which allows the entry of about 70% of the RIR waters into the estuarine system, providing substantial supply of mud and fresh water. It also allows the entry of many metallic contaminants from former mining activities in the RIR basin, agricultural areas and various different urban centers (Guimarães and Sígolo, 2008; Mahiques et al., 2013; Abessa et al., 2014; Cruz et al., 2019).

Regarding to the contamination by the mining activities, the metals contributions came mainly from the incorrect disposal of these residues (Mahiques et al., 2009, 2013). Mining companies operated for years in the basin of the RIR discharging the mining residues indiscriminately into the river until 1995, as so contaminating the water and the sediments along its course, especially with Pb (Guimarães and Sígolo, 2008; Mahiques et al., 2013; Abessa et al., 2014; Tramonte et al., 2018). After the closure of the mines, the residues were deposited marginally in the river in the form of tailings piles, being exposed to the winds and rains and consequently the leaching. As a consequence, unknown quantities of metals began to be continuously introduced into the river and, over the years, reached ELCIC through suspended solids in the RIR waters (Guimarães and Sígolo, 2008; Cruz et al., 2019). Previous publications indicated that the mining residues contained high concentrations of Pb, Cu, Cr, Zn and Ba (Guimarães and

Sígolo, 2008) and that the Pb, Cu and Zn were the main elements of concern in the ELCIC (Mahiques et al., 2009, 2013; Tramonte et al., 2018; Cruz et al., 2019).

The suspended solids coming from the RIR are deposited in the sediments of the ELCIC probably in higher intensity in the Iguape City (near site IG) and in the point of the inversion of the waters of the estuary, in Subaúma (near site SB), where a big sand bench exists. In the study by Salgado and Azevedo (2018) higher concentrations of Fe, Mn, Zn, Cu and Pb were found close to Iguape City. Cruz et al (2019) found higher concentrations of Cd, Cr, Cu, Fe, Pb, Mn and Zn in the same region. Although many publications showed that the Pb was present in moderate to high levels in certain regions of the estuary (Salgado et al., 2018; Tramonte et al., 2018; Cruz et al., 2019), the present study observed low presence and concentrations of the element. This may be due to the different techniques applied in the studies and/or due to the characteristics of the sampled sediments.

The literature demonstrated that the sediment composition of the ELCIC has great relation in the accumulation of metals, as the sediments are predominantly sandy, mainly composed of very fine sands, whit some sites containing high quantity of mud (Salgado and Azevedo, 2018; Cruz et al., 2019) and organic matter (Tramonte et al., 2018; Cruz et al., 2019). This last condition was observed in the sediments near of Subaúma, where high concentrations of some metals were described (Tramonte et al., 2018; Salgado; Azevedo, 2018; Cruz et al., 2014; 2019).

Though the sediment metal concentrations observed in the present study were considered low to moderate according to the Brazilian legislation, there is a possibility of toxicity of these sediments to the local biota. In the analysis of the bioavailability of metals in the region (Salgado and Azevedo, 2018), all sites presented SEM/SVA ratio>1 in at least one of the analyzed periods, with higher toxicity observed close to Iguape City. Cruz et al. (2019) also found SEM / SVA ratio>1 close to Iguape City and in the northern portion of Cananéia Island. Studies involving toxicity tests with the ELCIC sediments demonstrated that metals were one of the main factors responsible for the sediment toxicity and reveled that the most toxic areas are not necessarily those closest to the Valo Grande Canal, but occurs especially in the depositional areas, which contains finer sediments (Cruz et al., 2014; 2019). Thereby, the ELCIC remains weakened by the loading of mineral residues received and the entrance of metals can still persist, due to the contributions of the existing tailings piles on the banks of the RIR.

Regarding to the other anthropic activities which may be contributing to the input of metals into the estuary, the agriculture is also highlighted as a source, once the economy of many communities in the basin of the RIR are based on tea, rice and banana agriculture that demand the use of fertilizers in these plantations (Salgado and Azevedo, 2018). In addition, according to the elements that were above the quality criteria I in the sediment samples in this study (Ni, Cu and Cr) defined by CONAMA (20112), the nautical activity probably plays a relevant role in the input of these metals into the estuary (Morais and Abessa, 2014; Salgado et al., 2018). Moreover, contributions of metals can also occur through incorrect effluents and residuals disposal throughout the system extension (Salgado et al., 2018), in greater intensity in areas of large human presence, as the Iguape City, which characterizes the major urban center of the area and houses of the few industrial activities in the region (Guimarães; Sígolo, 2008; Mahiques et al., 2013).

The analysis of PPCPs in the sediments reveled levels of fenoprofen, fenofibrate, caffeine, propranolol and metoprolol in different sampled sites in both seasons (Table 2). Propranolol had the highest frequency of detection being found in all sites and seasons, in concentrations from 11.80 to 19.74 $\mu g\ g^{-1}$ in CI, from <0.010 to 9.22 $\mu g\ g^{-1}$ is SB and from 4.78 to 10.11 $\mu g\ g^{-1}$ in IG. Fenoprofen and caffeine were found in tree of the six analyzed sites. Fenoprofen was detected in CI during both seasons, from <0.039 $\mu g\ g^{-1}$ to 27.37 $\mu g\ g^{-1}$ and in IG in the cold-dry season in the concentration of 26.73 $\mu g\ g^{-1}$. Caffeine was found in concentrations <0.018 $\mu g\ g^{-1}$ in CI and IG during the cold-dry season and in SB during the hot-rainy season. Fenofibrate had the highest concentrations in the sediments and was found in IG at both seasons, in concentrations from 133.15 to 180.72 $\mu g\ g^{-1}$. Metoprolol was only detected in site CI during the hot-rainy season in concentrations <0.008 $\mu g\ g^{-1}$. The others lipid regulators, analgesics and anti-inflammatories analyzed were not detected, as none of estrogens and antiseptics used in personal care products.

The main source for PPCPs in aquatic systems are domestic effluents (Moreno-González et al., 2015; Paíga and Delerue-Matos, 2017; Kramer et al., 2018). The widespread consumption of this products and their physical and chemical proprieties, as they kinetics in the environment are generally related with the presence of PPCPs in the sediments (Li et al., 2012; Moreno-González et al., 2015). Its detections in environmental matrices allow the understanding of the consumption pattern by the local populations (Vazquez-Roig et al., 2010).

Propranolol and metoprolol are selective β-blockers mainly used for the treatment of heart diseases, and its detections in the sediments of all sites may probably be due to the fact that it has a chronic use (Moreno-González et al., 2015). Caffeine is a substance of exclusive human use, being one of the most consumed in the world through diverse foods and drinks (Paíga; Delerue-Matos, 2017). Fenoprofen is a non-steroidal anti-inflammatory drug commonly used in painkillers pills, being among the pharmaceuticals products with the highest persistence in the effluents and sludge sediments from wastewater treatment (Aznar et al., 2013; Kramer et al., 2018). Fenofibrate is a lipid regulator used to control the levels of cholesterol and triglycerides in the blood (Aznar et al., 2013; Ide et al., 2017). It was among the pharmaceuticals of higher input mass in the wastewater treatment in the Paranaguá estuary, a coastal region near our study area (Kramer et al., 2018).

The local ELCIC population has a limited access to medicines, due to the fact that this region is the least developed in the state of São Paulo in socio-economic terms, in health care, sanitation infrastructure and services, education levels and household incomes. It can contribute to the occurrence of few substances in the sediment (IBGE, 2010; Cruz et al., 2019). According to the population census of the region, the population of the area is mainly comprised of adults and seniors, who may be the main users of the pharmaceuticals detected in this study (IBGE, 2010).

These detections corroborated with the study held by Salgado et al. (2018) which described the presence of the same pharmaceuticals in the sediments of the ELCIC and attributed this presence to sewage discharge. The effluents of the coastal cities of the region are many times discharged *in natura* into the rivers or directly into the sea due to insufficient treatment and collection of sewage (Barbieri et al., 2014; Morais; Abessa, 2014). Additionally, the disordered human occupation of the area, the deficiency removal of these compounds in conventional sewage treatment together with the predominance of dumps as final disposal of solid waste caused by the poor management of the area can be contributing to the presence of PPCPs and metals in this environment (Salgado et al., 2018).

Nevertheless, the levels of contaminants of the sediments can suffer the interference of the climate and rainfall seasonality, which alter the water parameters, such as temperature and salinity, as observed in the present study. This, can consequently interfere in the bioavailability of the contaminants (Cruz et al., 2019). The presence of higher concentrations of metals and PPCPs observed in the sediments during the cold-dry season compared to the hot-rainy season, suggested the influence of these changes. During the cold-dry season the smaller amount of rain leads to a lower hydrodynamic in the ELCIC (Britcha, 2000). Thereby, the concentration of contaminants in the ELCIC can be increased during the cold-dry period due to the lower dispersion of the contaminants, the lower renewal of the estuary waters, the smaller dilution and the possible accumulation of some contaminants near the emissions sources. Previous researches reported seasonal changes in the concentrations of contaminants in different seasons of the year in the region (Antonelli et al., 2017; Salgado et al., 2018; Salgado; Azevedo, 2018; Cruz et al., 2014; 2019), and also identified high levels of metals, PPCPs and PAHs during the cold-dry season (Salgado; Azevedo, 2018).

3.6.3 Biomarkers responses

Biomarkers responses in gills, liver, kidney, blood, brain and muscle of *G. broussonnetii* specimens revealed both seasonal and spatial variations. Alterations in the antioxidant and biotransformation systems, histopathologies and genotoxicity were observed in the fish of all sites and seasons. More pronounced responses were observed in the animals near the locals of higher human presence, in CI and IG sites, where the fish of site IG presented a more compromised health in accordance to the results for the metals and PPCPs sediment contaminations.

In gills, the accumulation of metals was not observed through autometallography analysis but the histopathological analysis evidenced a higher value for the lesion index in IG during the cold-dry season ($p<0.001$; Figure 2), which could be caused by the higher presence of metals in this region. In this tissue the presence of aneurism represented 65% of the total alterations as the lifting of the epithelium represented 35%.

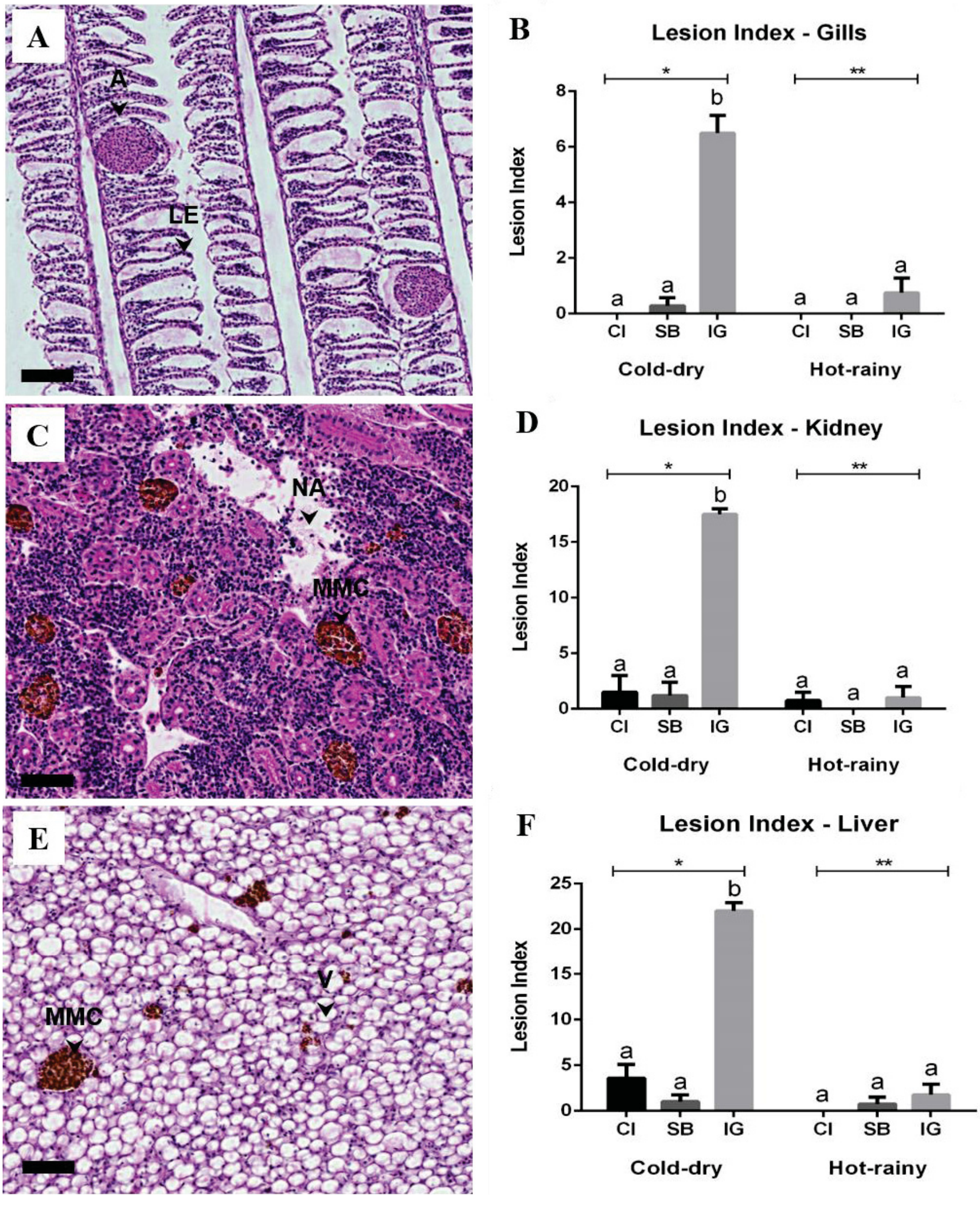


Figure 2. Histopathological alterations and lesion indexes observed in *G. broussonnetii* fish from the ELCIC in Cananéia Island (CI), Subaúma (SB) and Iguape (IG) during the hot-rainy and cold-dry seasons. A: Aneurism (A) and lifting of the lamellar epithelium in gills. B: Lesion index of the gills. C: Necrosis area (NA) and melano-macrophages center (MMC) in kidney. D: Lesion index in kidney. E: Melano-macrophages center (MMC) and vacuolization of the hepatocytes (V). F: lesion index in liver.
Scale bars = 20 µm.
[a b] Indicates statistical differences among the sites ($p<0.05$).
* - ** Indicates statistical seasonal differences between the seasons ($p<0.05$).

The lifting of the epithelium could serve as a mechanism of defense, once the epithelial separation of the lamellae increases the distance across which waterborne pollutants must diffuse to reach the bloodstream, however also impairing oxygen uptake (Figueiredo-Fernandes et al., 2007). Moreover, aneurisms in gills are a later and worrying response that can occur from changes in the normal cell structure, that consequentially lead to the loss of their support function and to the emergence of lamellar aneurysms (Flores-Lopes and Thomaz, 2011). These both alterations can affect the fish respiration (Fernandes and Mazon, 2003). The presence of these alterations have been extensively reported in gills of fish exposed to metals (Fernandes and Mazon, 2003; Figueiredo-Fernandes et al., 2007; Flores-Lopes and Thomaz, 2011) and also in studies that evaluated the impact of environmental contamination by domestic and industrial wastes in fish, with pronounced responses in more polluted areas (Flores-Lopes; Thomaz, 2011; Mela et al., 2013; Ribeiro et al., 2013).

In liver, the accumulation of metals was also not observed by autometallography, however, the others biomarkers response revealed alterations to the antioxidant and biotransformation systems, genotoxicity and histological alterations, which also could be linked to the fish's responses to higher metal contaminated sites. The fish of site IG presented lower hepatic CAT and GPX activities in both seasons ($p<0.05$; Table 2), suggesting an inhibition of these activities in the exposure to environmental contaminants.

The CAT and GPX take part in the cellular defense system which inhibit and detoxify oxyradical formation, with functions in the degradation of the hydrogen peroxide, preventing cellular damages (Kroon et al., 2017). Metals can decrease these antioxidant defenses, which may indicate excessive consumption of these enzymes in tissues and/or a reduction in their synthesis for reasons such as damage to the molecular components involved in the synthesis or damage to the genes involved in this process (Cao et al., 2012; Beg et al., 2015; Gusso-Choueri et al., 2016). The decreased CAT and GPX activities were observed in fish species at metal contaminated sites (Benedetti et al., 2012; Souza et al., 2013). In addition, a previous research in the ELCIC also observed the decreased activity of these enzymes in the liver of the *Atherinella brasiliensis* fish, indicating diminished ability in the antioxidant defense of the fish (Salgado et al., 2018).

Moreover, studies demonstrated that different pharmaceuticals can alter the antioxidant and biotransformation enzymes, including drugs of the classes of the β-blockers (Burkina et al., 2015), lipid regulators (Burkina et al., 2015; Barreto et al., 2018), anti-inflammatory (Mathias et al., 2018) and stimulants (Santos-Silva et al., 2018). These classes of pharmaceuticals drugs were detected in the sediments of the ELCIC by the present study and can also be causing stress to the health of the local biota.

Table 2. Biochemical biomarkers responses in brain, muscle, liver and kidney of *G. broussonnetii*.

Biomarkers	Sites and seasons					
	Cold-dry			Hot-rainy		
	CI	SB	IG	CI	SB	IG
AChE (brain)	60.79 ± 6.33 ^{a}	51.58 ± 2.71 ^{a}	85.64 ± 16.28 ^{b}	67.59 ± 2.18 ^{a}	81.46 ± 8.20 ^{b}	45.0 ± 17.33 ^{a}
AChE (muscle)	96.95 ± 17.60 ^{a}	81.27 ± 9.44 ^{a}	130.06 ± 14.12 ^{b}	95.75 ± 14.32 ^{a}	84.89 ± 11.72 ^{a}	121.14 ± 20.31 ^{b}
SOD (liver)	256.45 ± 13.26 ^{a}	331.52 ± 29.28 ^{a}	299.75 ± 21.31 ^{a}	295.09 ± 28.76 ^{a}	256.53 ± 14.97 ^{a}	267.11 ± 27.87 ^{a}
CAT (liver)	76.09 ± 8.33 ^{a*}	97.01 ± 11.39 ^{a*}	66.52 ± 6.32 ^{b*}	70.59 ± 8.16 ^{a}	53.85 ± 6.03 ^{a}	37.44 ± 6.21 ^{b}
GPx (liver)	34.47 ± 4.35 ^{ab}	43.80 ± 4.83 ^{a}	28.75 ± 3.46 ^{b}	36.43 ± 4.03 ^{ab}	43.05 ± 5.41 ^{a}	28.71 ± 4.75 ^{b}
GST (liver)	29.92 ± 4.77 ^{a}	28.56 ± 2.54 ^{a}	31.88 ± 5.77 ^{a}	23.47 ± 3.31 ^{a}	36.8 ± 4.74 ^{a}	42.94 ± 6.19 ^{a}
GSH (liver)	2.19 ± 0.08 ^{a}	1.23 ± 0.11 ^{b}	1.03 ± 0.20 ^{b}	1.60 ± 0.52 ^{a}	1.35 ± 0.18 ^{b}	0.97 ± 0.30 ^{b}
MT (liver)	21.23 ± 4.15 ^{a}	16.77 ± 3.63 ^{a}	18.79 ± 4.50 ^{a}	10.40 ± 1.86 ^{a}	16.15 ± 5.55 ^{a}	31.23 ± 8.39 ^{a}
LPO (liver)	20.23 ± 4.21 ^{a*}	38.31 ± 6.10 ^{a*}	30.15 ± 7.81 ^{a*}	19.62 ± 3.99 ^{a}	17.37 ± 3.45 ^{a}	17.36 ± 4.13 ^{a}
SOD (kidney)	175.01 ± 18.85 ^{a}	347.27 ± 51.25 ^{a}	268.90 ± 40.34 ^{a}	441.24 ± 59.81 ^{a*}	439.44 ± 34.61 ^{a*}	384.16 ± 44.33 ^{a*}
CAT (kidney)	7.56 ± 2.45 ^{a}	26.68 ± 5.16 ^{a}	9.83 ± 2.19 ^{a}	22.28 ± 7.97 ^{a*}	30.97 ± 3.68 ^{a*}	22.56 ± 3.79 ^{a*}
GPx (kidney)	22.28 ± 6.46 ^{a}	44.74 ± 5.96 ^{a}	31.34 ± 6.88 ^{a}	39.05 ± 15.18 ^{a*}	63.15 ± 10.06 ^{a*}	93.74 ± 23.92 ^{a*}
GST (kidney)	37.86 ± 5.69 ^{a}	43.52 ± 4.97 ^{a}	48.47 ± 2.03 ^{b}	35.29 ± 4.17 ^{a}	42.46 ± 3.53 ^{a}	63.44 ± 10.82 ^{b}
LPO (kidney)	16.05 ± 2.43 ^{a}	25.91 ± 3.71 ^{a}	23.99 ± 5.10 ^{a}	36.29 ± 7.36 ^{a}	34.91 ± 4.86 ^{a}	21.54 ± 3.92 ^{a}

$^{a\ b\ c\ d}$ Indicates statistical differences among the sites ($p<0.05$).
* Indicates statistical seasonal differences between the seasons ($p<0.05$).
AChE (nmol min^{-1} mg^{-1} protein), SOD (SOD mg protein^{-1}), CAT (µmol min^{-1} mg protein^{-1}), GPx (nmol min^{-1} mg protein^{-1}), GST (µmol min mg protein^{-1}), GSH (µg GSH mg protein^{-1}), MT (µg MT mg protein^{-1}) and LPO (nmol min^{-1} mg protein^{-1}).

In addition, a high value for the lesion index in liver ($p<0.001$) was observed in IG during the cold-dry season (Figure 2). In this tissue the presence of the vacuolization of the hepatocytes represented 78% of the total alterations as the melano-macrophages centers represented 22%. The vacuolization of the hepatocytes is mainly caused by steatosis which represents an abnormal lipid accumulation. This accumulation in the liver of the fish can result from a disturbance in the hepatocytes cellular metabolism caused by the presence of xenobiotics, such as metals and polycyclic aromatic hydrocarbons (Fernandez et al., 2011; Mela et al., 2013; Ribeiro et al., 2013). The melano-macrophages centers also can appear as a response of an inflammatory process induced by the exposure to metals (Mela et al., 2013).

Thus, both conditions can affect the liver normal functions and can be further responsible for liver impairment, being of some concern as they were previously reported in fishes from areas nominated affected by human activities (Fernandez et al., 2011; Ribeiro et al., 2013). As well, both histopathological alterations were previously observed in fishes from the ELCIC region on the livers of *Cathorops spixii* (Azevedo et al., 2013) and of *Atherinella brasiliensis* (Salgado et al., 2018), reinforcing the idea that the environmental contamination by metals and others contaminants can lead to these alterations in the local ichthyofauna.

Similar mean values were obtained in the hepatic tissue for SOD and GST activities and MT concentrations ($p>0.05$; Table 2). However, regarding the time of the year, the interaction of the three sites showed lower means values for hepatic CAT activities ($p<0.001$) during the hot-rainy season as higher LPO in the cold-dry seasons ($p<0.05$; Table 2).

Nevertheless, the fish from site CI presented higher hepatic GSH concentrations in both seasons ($p<0.05$; Table 2). The GSH provides the major pathways for conjugation of electrophilic compounds and metabolites, playing a key role in the detoxification of xenobiotics by reacting with compounds, replacing hydrogen, chlorine and nitro groups among others (Kroon et al., 2017). Some laboratory toxicity tests evidenced an increase in GSH concentrations in metals exposure in fish (Cao et al., 2012; Hariharan et al., 2016), however, these alterations can also occur in response to others pollutants (Kroon et al., 2017).

Higher DNA strand breaks in liver ($p<0.001$; Table 3) were observed in CI during the hot-rainy season, suggesting the presence of genotoxic pollutants in this area, such as metals and organic pollutants (Gusso-Choueri et al., 2016). Site CI is also influenced by the anthropic activities, as the presence of metals and pharmaceuticals products were observed in these sediments by the present study. These contaminants probably come from the urban areas of the Pedrinhas Village and the city of Cananéia, which may also contribute with other stressors. Nonetheless, all sampled sites had significantly higher DNA strand breaks during the cold-dry season, demonstrating genotoxicity in this period.

Table 3. Genotoxicity in liver and blood of *G. broussonnetii*. DNA strand breaks (DNA ST) in liver and blood, total nuclear morphological abnormalities (TNMA) and blebbed, notched and lobed nuclear abnormalities in blood.

Biomarkers	Sites and seasons					
	Cold-dry			Hot-rainy		
	CI	SB	IG	CI	SB	IG
DNA ST (liver)	22.50 (20.50; 28.25) [a]	15.0 (8.0; 21.0)[a]	11.0 (2.0; 12.0)[a]	221.0 (132.0; 370.50)[b*]	90.0 (67.50; 143.50)[c*]	48.0 (29.0; 61.50)[c*]
DNA ST (blood)	39.0 (25.50; 94.50) [a]	43.0 (17.75; 100.50)[a]	13.50 (3.0; 27.50)[a]	229.0 (190.0; 294.0) [b*]	199.0 (171.0; 253.0) [b*]	75.0 (81.0; 55.0)[a*]
TNMA (blood)	299.25 ± 127.72[a]	69.03 ± 13.18[b]	44 ± 11.03[b]	70 ± 21.32[b]	120.5 ± 23.60[b]	49.75 ± 11.86[b]
Blebbed (blood)	31.50 (89.25)[a]	13.50 (4.0; 25.25)[b]	2.50 (1.0; 8.75)[b]	12.0 (8.0; 35.0)[b]	17.0 (10.50; 35.75)[b]	6.50 (2.0; 10.50)[b]
Lobed (blood)	38.0 (16.25; 158.8)[a]	11.0 (1.0; 42.75)[b]	21.0 (10.25; 48.25)[b]	6.0 (3.0; 18.25) [b]	29.0 (20.50; 82.0)[b]	6.50 (4.25; 20.75)[b]
Notched (blood)	128.50 (309.0;	11.0 (5.0; 37.0)[b]	9.50 (30.50; 118.80)[b]	27.0 (14.0; 42.0)[b]	44.50 (26.50; 83.50)[b]	28.50 (12.25; 47.0)[b]

Data expressed in median (quartile 1; quartile 3).
[a b c] Indicates statistical differences among the sites ($p < 0.05$).
* Indicates statistical seasonal differences between the seasons ($p < 0.05$).

The occurrence of DNA breaks may not be a persistent damage, as it is possibly reversible (Ferraro et al., 2004; Kroon et al., 2017). Although, it cannot be attributed to a specific exposure (Ferraro et al., 2004; Ramsdorf et al., 2009), several field studies reported a significant increase in DNA damage following metal exposure in fishes (Ferraro et al., 2004; Fernandez et al., 2011), including species of the ELCIC (Azevedo et al., 2013; Gusso-Choueri et al., 2015, 2016; Salgado et al., 2018).

In kidney, GST activity was higher in IG in both seasons ($p<0.05$), while SOD, CAT, GPX activities and LPO levels had similar values among the sites ($p>0.05$; Table 2). Regarding the time of the year, the interaction of the sites showed higher means values for renal SOD and GPX activities during the hot-rainy season ($p<0.001$).

The GST is a catalyst required for the conjugation of electrophilic compounds by GSH in the process of biotransformation of xenobiotics. It provides essential functions in the intracellular transport as well in the defense against oxidative damage and peroxidative DNA products (Kroon et al., 2017). Previous investigations have examined the response of the GST activity in fish to elevated metals levels in water or sediment and encountered positive responses (Piva et al., 2011; Beg et al., 2015). In the study with the *Cynoglossus arel* fish, Beg et al. (2015) found higher GST activity in the animals from polluted locations. Nonetheless, these responses can be attributed to the presence of contaminants besides metals, such as organic pollutants (Schipper et al., 2009), that in the case of the present study, can reach site IG through the Valo Grande Canal.

Additionally, the accumulation of metals was observed through autometallography analysis in the kidney of the animals of site IG during the cold-dry season (Supplementary material D). As no chemical analysis for metals determinations were performed in this tissue, little can be affirmed about the accumulation. However, it can be related to the mechanisms that control and/or mitigate the metals toxic effects in organisms, such as the presence of metallothioneins in this tissue.

Metallothioneins are cytosolic metal-binding proteins, that are primally synthesized in the liver and the kidney, whose biological function are related to the regulation of essential metals, such Zn and Cu, and with the detoxification of toxic metal such as Cd and Hg (Coyle et al., 2002). As a higher presence of Zn was observed in the sediments of site IG during the cold-dry season, a higher bioavailability of the metal could permit a higher bioaccumulation of Zn in the kidney of the fish. This organ plays an important role in the excretion of xenobiotics of the body as it is responsible for the filtration of blood. This could have led to an increase in the MT levels, collaborating to the observation of the black silver deposits in the renal tissue.

Moreover, a higher value for the index lesion was observed in the kidney of the animals of site IG during the cold-dry season ($p<0.001$), where the presence of necrosis represented 69% of the total alterations as the melano-macrophages centers represented 31% (Figure 2). Necrosis is a degenerative lesion of the tissue, characterized by cell lysis followed by tissue disorganization (Azevedo et al., 2013; Ribeiro et al., 2013). It is considered an irreversible lesion which is strongly associated with oxidative stress in fish (Mela et al., 2013) and has also been linked to the exposure to metals (Mela et al., 2013; Rossi et al., 2014). The presence of necrosis and of melano-macrophages centers in the renal tissue could be related to the alterations in the biotransformation parameters and the presence of metals previously discussed for the organ, as result of the higher contamination described in site IG.

In blood, higher DNA strand breaks were observed in CI and SB at the hot-rainy season, while TNMA and all blebbed, notched and lobed alterations were higher in CI during the cold-dry season ($p<0.01$; Table 3), as so, indicating possible contributions of different environmental contaminants with genotoxic proprieties in both seasons (Gusso-Choueri et al., 2016). Nuclear abnormalities are a more preoccupant damage as they are irreversible (Cestari et al., 2004; Ferraro et al., 2004), and these genotoxic events have also been identified in fishes exposed to metals (Ramsdorf et al., 2009; Kroon et al., 2017).

In the muscle AChE activity was higher in IG in both seasons ($p<0.01$), as in brain it was higher in IG during the cold-dry season and in SB in the hot-rainy-season ($p<0.05$; Table 2). The studies conducted by Zang et al (2002; 2012) demonstrated that AChE is induced in various types of apoptotic cells and expressed identically to the synapse type AChE. Many environmental chemicals such as metals, persistent organic pollutants, pesticides and cyanobacterial toxins can induce apoptotic pathways in different tissues of fish (Minovski et al., 2019). These affirmations corroborate with the histological findings from the present study in liver and kidney. However, AChE activity also could be inhibited in the other studied sites in the ELCIC by the presence of anticholinesterasic pollutants such as organophosphate and carbamates compounds not measured in this study (Golombieski et al., 2008; Parlak, 2018).Further, the values for the condition index (K) varied from 0.18 to 0.14 in site CI and from 0.16 to 0.14 in site IG during the hot-rainy and the cold-dry seasons, respectably, and were 0.20 in site SB in both seasons. Therefore, the K indicates that the fish sampled at site SB were in better health conditions ($p<0.05$), suggesting an environment of a less compromised quality. Other study conducted in a estuarine area in the Brazilian Southeast coast evidenced a higher K for the fish from the least impacted site, indicating that this response could provide a practical parameter to evaluate the health conditions of the fish (Ribeiro et al., 2013).

Moreover, enzymatic biomarkers also respond to other stressors, such as physical-chemical parameters (temperature, salinity, dissolved oxygen), reproductive cycles and fish nutritional status (Kroon et al., 2017). As so, the seasonal variations observed for all biological parameters indicated that climate and rainfall seasonality, as changes in the water parameters (temperature, pH and salinity) are important factors in these responses in the Violet Goby fish as they also influence in the exposure of the aquatic organisms to the environmental contaminations in the ELCIC (Gusso-Choueri et al., 2015; Salgado et al., 2018).

The multivariate analysis results agreed with the data perilously discussed. The PERMANOVA results showed that the fish exhibited different biological responses among all analyzed sites and seasons ($p<0.001$), with greater differentiation of IG and CI sites. The first PCoA axis explained 23.6% and the second axis 20.4% (Figure 3). AChE in muscle, SOD, GST, MT and LI in liver, CAT, GPx, GST and LI in kidney, as well as LI in gills were positively related with the first axis. Meanwhile, AChE in brain, hepatical CAT, GSH and DNA ST, renal SOD and LPO, as well as DNA ST, TNMA, BNA, LNA and NNA in blood were negatively related. AChE in brain and muscle, CAT, GST, MT and LI in liver, LI in kidney and gills, as well as TNMA, LNA and NNA in blood were positively related with the second axis (Supplementary material E).

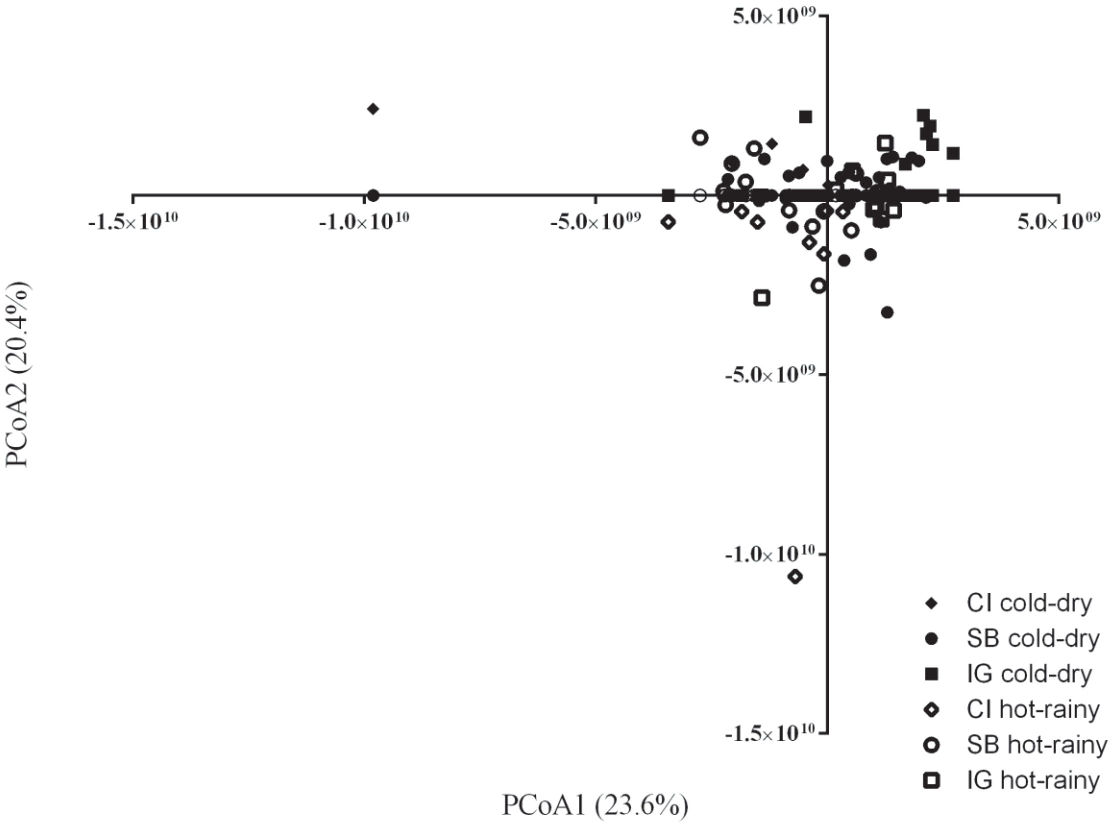


Figure 3. Multivariate analyses of biomarkers in *G. broussonnetii* from Cananéia Island (CI), Subaúma (SB) and Iguape (IG) during hot-rainy and cold-dry seasons.

3.6.4 Integrated biomarker response index (IBR)

Once there is no baseline biomarker data for *G. broussonnetii,* the responses obtained in the fish of site SB during the cold-dry season were used as reference condition. The IBR values supported the previous results and demonstrated lower values at CI (19.2) and SB (19.5) during the hot-rainy season, with higher value in IG (26.1) for this same season. In addition, higher values were observed in the sites during the cold-dry season, where IG (28.0) and CI (27.9) present the worst environmental conditions among all sites (Fig. 5; Supplementary material F).

The star plots also indicate that the histopathological analysis (lesion indexes of gills, liver and kidney) and the genotoxic damages (DNA strand breaks and NMA in liver and blood) were the most representative biomarkers to differentiate the animals of the SB site during the cold-dry season from the others analyzed fish, as they showed greater variations in comparison to the referenced conditions. These biological results clearly indicated that the levels of contaminations were higher at site IG, followed by site CI, corroborating with the human presence in these areas and the metals and PPCPs concentrations found in the sediments.

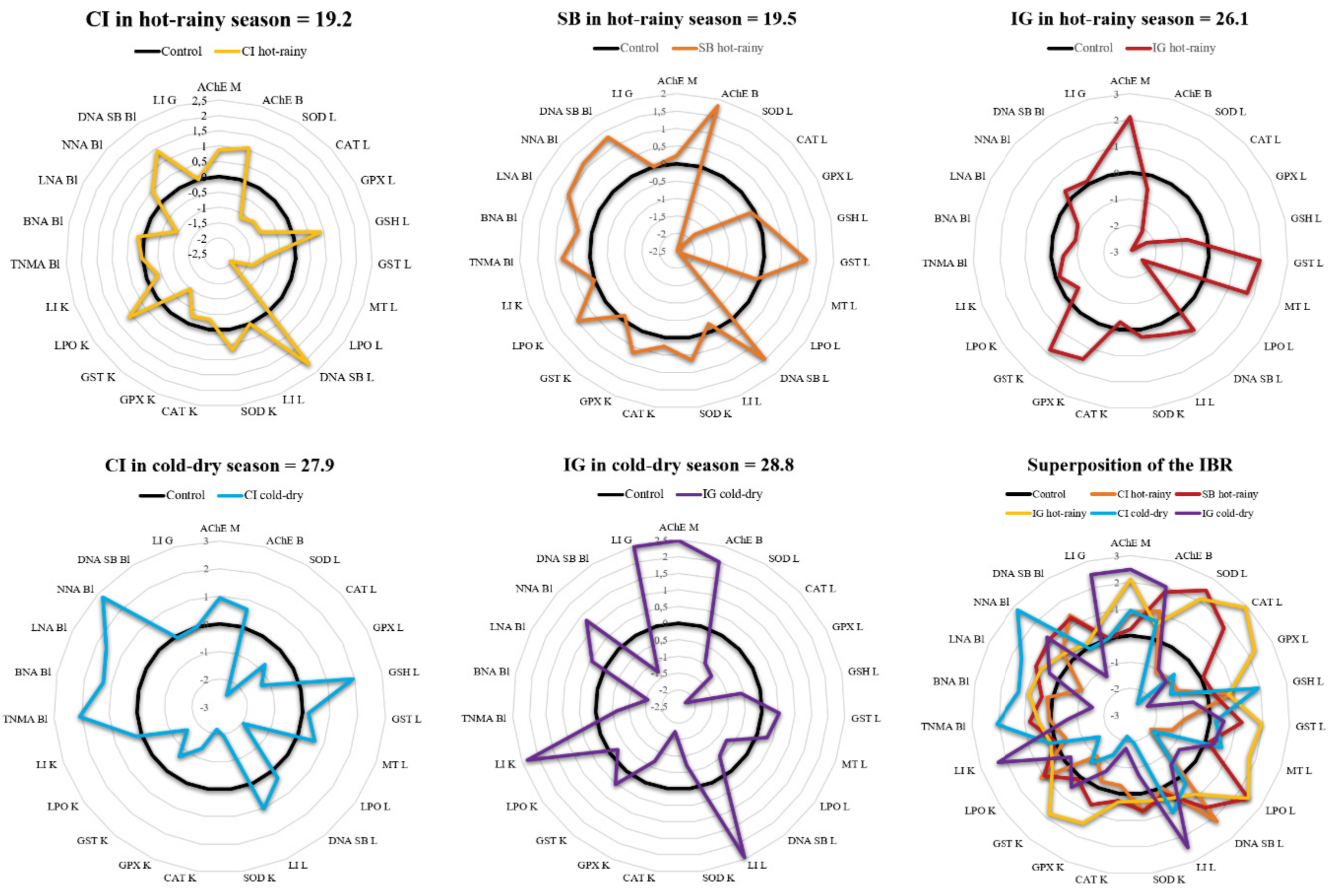


Figure 5. Integrated biomarker response index (IBR) in *G. broussonnetii* based on the following biomarkers: AChE, SOD, CAT, GPx, GST, GSH, MT, LPO, DNA strand breaks (DNA SB), lesion index (LI), total nuclear morphological abnormalities (TNMA) and blebbed (BNA), lobed (LNA) and notched (NNA) abnormalities in different tissues (B= brain, G = gills, M= muscle, L= liver, K=kidney, Bl = blood,). Biomarkers results are represented in relation to the baseline group SB in cold-dry season.

3.6.5 Integrated analysis

The RDA results showed that combined the concentrations of metals and PPCPs detected in the sediments explained 16% of the responses observed in the fish ($p<0.001$), while separated neither of the contaminants classes were considerable ($p<0.001$; Supplementary material G). In addition, it was possible to indicate that among the metals the Zn, Cr, Cu, Ni and Cd were the major stressors ($p<0.01$), while all the PPCPs (CAF, MTL, PRL, FNF and FNP) were representative ($p<0.01$).

These results suggest the influence of multiple stressors in the health conditions of the *G. broussonnetii* in the ELCIC, as the combination of all analyzed contaminants explained the majority of the biological responses. In addition, they indicated that others stressors that were not addressed in the present research may be present in this environment and could also be contributing to the sublethal effects observed in the Violet Goby fish Moreover, contaminants from others environmental matrixes, such as water, could have larger representability for the fish.

The sublethal effects observed in the fish can, over time, influence in the survival and the fitness of the organisms, leading to alterations in higher biological levels, such as the population structure. It was observed that even at moderated levels the contaminations by metals and PPCPs, as other substances from anthropic activities, can be harmful to the health of this species in the study area. Nonetheless, the interference of other factors as sudden changes in the environmental conditions, the involvement of pathogens and overfishing may also be considered as causes for the decline of the species population level.

The combination of chemical analysis of the sediments and biomarkers in fish used in this study supplies important information about the local environmental quality. The main compromised areas regarding the presence of metals and PPCPs were pointed and the possible toxic effects in the Violet Goby fish were elucidate. These data can be further used for environmental and social management of the area, on the way to diminish the anthropic influence in the aquatic organisms and to assist on the preservation of the *G. broussonnetii* fish, for the maintenance of the ecological state of the region and the local fishing.

3.7 CONCLUSIONS

The present study provided the first ecotoxicological information's for *G. broussonnetii.* The results demonstrated that the presence of metals and pharmaceuticals drugs in the sediment, as others possible contaminants, may have toxic effects on the health of this fish species in the Brazilian Marine Protec Area of the Estuarine-Lagoon Complex of Iguape-Cananéia.

Higher metals and PPCPs concentrations were observed in the sediments of the sites of higher human presence (Iguape City and Cananéia Island) as well more pronounced alterations the fish biomarker responses. The worse condition was found close to the Iguape City, that houses the Mouth of the Ribeira de Iguape River (RIR), its main freshwater contributor. These results suggested that although the contaminations from the RIR and from the Iguape City, had more negative effects on the health of the

Violet Goby fish, the anthropic activities throughout the extension of the system are collaborating to the input of contaminants. Thereby, as other factors, these contaminations may be contributing for the decline observed in this local fish population over the last years.

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3.10 SUPPLEMENTARY MATERIAL

A: Characteristics of the analysis of metals.

Metal	**IL (mg/L)**	**r^2**	**LD ($\mu g\ L^{-1}$)**	**LQ ($\mu g\ L^{-1}$)**
Fe	2.0 – 20.0	0.926	5	15
Zn	0.5 - 5	0.979	3	10
Mn	1.0 – 10.0	0.885	6	18
Cu	0.5 - 5	0.997	4	13
Cd	0.5 - 5	0.992	4	13
Cr	0.5 - 5	0.993	5	16
Pb	1.0 – 10.0	0.987	5	16
Ni	0.5 - 5	0.985	2	6

IL - Intervals of linearity; r^2 - linearity of the method; LD - Limit of Detection; LQ - Limit of Quantification.

B. Characteristics of the analysis of PPCPs.

Compounds	Abb.	Fragment mass (m/z)	LD $\mu g\ g^{-1}$	LQ $\mu g\ g^{-1}$	Rec. (%)
Fenofibrate	FNF	360 – 273.1	0.001	0.003	61
Gemfibrozil	GFZ	201.1 – 129	0.063	0.209	86
Propranolol	PRL	144 – 115	0.003	0.010	32
Metoprolol	MTL	223.3 – 72	0.002	0.008	20
Salicylic Acid	SA	267.1 – 209	0.006	0.019	30
Acetylsalicylic Acid	ASA	195 – 177	0.001	0.003	51
Ibuprofen	IBU	295 – 280	0.014	0.047	87
Diclofenac	DCF	367 – 242.1	0.016	0.055	60
Naproxen	NPX	302.2 – 185	0.006	0.019	79
Fenoprofen	FNP	270.1 – 196	0.012	0.039	50
Caffeine	CAF	194 – 109	0.005	0.018	88
Ethinylestradiol	EE2	425.2 – 193.1	0.005	0.018	43
Estradiol	E2	416.3 – 326.2	0.003	0.010	59
Estrone	E1	342.2 – 257.1	0.003	0.010	74
Triclosan	TRC	362 – 347	0.077	0.256	82
Methylparaben	MEP	224.1 – 209.1	0.004	0.014	61
Ethylparaben	ETP	238.2 – 223.1	0.007	0.023	82
Propylparaben	PRP	252 – 195	0.012	0.039	95
Butylparaben	BTP	266.5 – 210	0.023	0.077	87
Benzylparaben	BZP	300 – 193.1	0.008	0.026	99

PPCPs - Pharmaceutical and Personal Care Products; Abb. - Abbreviation; LD - Limit of Detection; LQ - Limit of Quantification; Rec – Recovery.

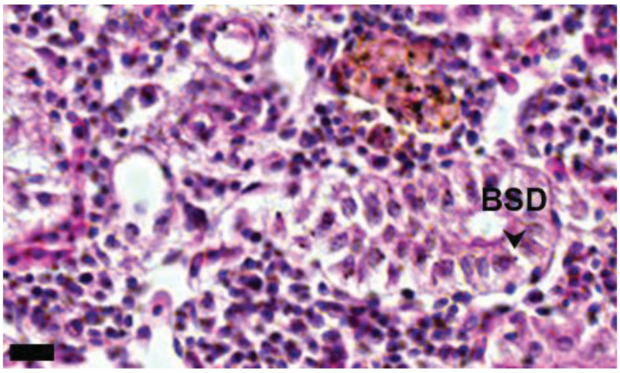
C: Environmental variables obtained *in loco* during the sampling campaigns and mean precipitation data of the ELCIC region.

Site	Mean precipitation (mm)	Air temperature (ºC)	Water temperature (ºC)	pH	Salinity (g/kg)
Sampling campaign 1 - September 2016 - Cold-dry season					
Cananéia Island (CI)	112.1	18.2	19	7.92	17.71
Subaúma (SB)	n.a	15	18.8	7.08	11
Iguape (IG)	120.3	12.3	19.3	7.24	21
Sampling campaign 2 – February 2017 – Hot-rainy season					
Cananéia Island (CI)	263.3	25.4	23.6	6.94	13
Subaúma (SB)	n.a	24	22.5	9.26	1.1
Iguape (IG)	214.9	25	23.7	7.27	0.8

Mean precipitation data obtained from the Integrated Agrometeorological Information Center (CIAGRO, 2019); n.a – data not available.

D: Autometallography evidencing metals deposits in cross-section of kidney from *G. broussonnetii* from site IG during the cold-dry season showing black silver deposits (BSD) counterstained with Hematoxylin-eosin.

Scale bars = 20 μm



E. Correlations biomarkers and PCoA axis.

Biomarkers	Tissue	Axis 1	p	Axis 2	p
AChE	muscle	0.0707	0.05783	0.0405	0.7503
	brain	-0.0837	0.4969	0.1007	0.4141
SOD	liver	0.1179	0.3457	-0.2220	0.0733
	kidney	-0.0107	0.9428	-0.5243*	0.0002*
CAT	liver	-0.0675	0.5930	0.1898	0.1299
	kidney	0.0641	0.6484	-0.3190*	0.0199*
GPx	liver	-0.1477	0.2728	-0.2200	0.1001
	kidney	0.1952	0.1936	-0.2232	0.1360
GST	liver	0.0730	0.5598	0.2967*	0.0155*
	kidney	0.2066	0.1338	0.0123	0.9296
GSH	liver	-0.2505	0.0536	-0.2408	0.0638
MT	liver	0.3671*	0.0111*	0.1928	0.1942
LPO	liver	0.2616*	0.0353*	-0.1025	0.4166
	kidney	-0.1715	0.2544	-0.4023*	0.0056*
DNA SB	liver	-0.4761*	0.0006*	-0.4246*	0.0026*
	blood	-0.6087*	<0.0001	-0.6363*	<0.0001*
LI	liver	0.5277*	0.0002*	0.5613*	<0.0001*
	kidney	0.4235*	0.0112*	0.3610*	0.0331*
	gills	0.6678*	<0.0001*	0.5523*	0.0001
TNMA	blood	-0.7378*	<0.0001*	0.1398	0.2832
BNA	blood	-0.7783*	<0.0001*	-0.0082	0.9517
LNA	blood	-0.6479*	<0.0001*	0.2788*	0.0295*
NNA	blood	-0.5986*	<0.0001*	0.1322	0.3015

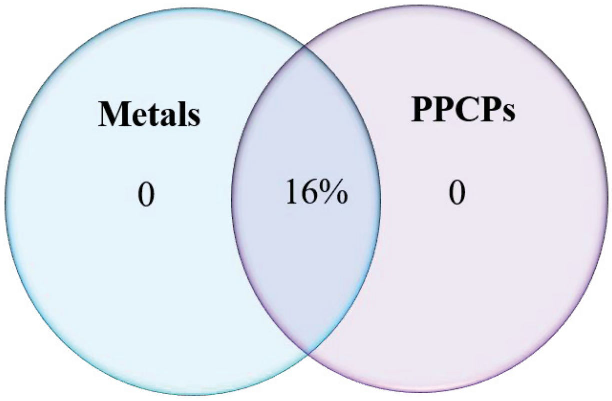
* p<0.05
AChE (acetylcholinesterase), SOD (superoxide dismutase), CAT (catalase), GPx (glutathione peroxidase), GST (glutathione transferase), GSH (glutathione), MT (metallothionein), LPO (lipoperoxidation), DNA SB (DNA strand breaks), LI (lesion Index), TNMA (Total nuclear morphological abnormalities), BNA (blebbled nuclear morphological abnormalities), LNA (lobed nuclear morphological abnormalities) and NNA (nuclear morphological abnormalities).

F: Reference levels of the biomarkers results of *G. broussonnetii* fish from site S during the cold-dry season for the IBR analysis.

Biomarkers	Tissue	Mean ± SD
AChE	muscle	81.28 ± 48.15
	brain	51.58 ± 14.10
SOD	liver	331.52 ± 149.31
	kidney	347.270 ± 198.51
CAT	liver	97.00 ± 56.98
	kidney	26.68 ± 20.02
GPX	liver	43.80 ± 22.15
	kidney	44.74 ± 23.86
GST	liver	16 ± 10.55
	kidney	1 ± 2.13
GSH	liver	1.23 ± 0.55
MT	liver	16.77 ± 12.59
LPO	liver	38.30 ± 31.11
	kidney	43.52 ± 20.49
DNA SB	liver	16 ± 10.54
	blood	59.96 ± 50.32
LI	liver	1 ± 2.13
	kidney	1.2 ± 2.67
	gills	0.28 ± 0.75
TNMA	blood	69.03 ± 67.24
BNA	blood	15.76 ± 12.78
LNA	blood	21.65 ± 23.63
NNA	blood	22.48 ± 23.73

AChE (acetylcholinesterase activity: nmol min^{-1} mg^{-1} protein), SOD (superoxide dismutase activity: SOD mg $protein^{-1}$), CAT (catalase activity: µmol min^{-1} mg $protein^{-1}$), GPx (glutathione peroxidase activity: nmol min^{-1} mg $protein^{-1}$), EROD (ethoxyresorufin-O-deethylase activity: nmol resorufin min^{-1}·mg $protein^{-1}$), GST (glutathione S-transferase activity: µmol min mg $protein^{-1}$), GSH (glutathione levels: µg GSH mg $protein^{-1}$), MT (metallothionein levels: µg MT mg $protein^{-1}$), LPO (lipoperoxidation: nmol min^{-1} mg $protein^{-1}$), DNA SB (Score of DNA strand breaks), LI (Lesion Index: Score of histopathological alterations), TNMA (Score of total nuclear morphological abnormalities), BNA (Score of blebbled nuclear morphological abnormalities), LNA (Score of lobed nuclear morphological abnormalities) and NNA (Score of notched nuclear morphological abnormalities).

G: Multivariate analysis (RDA) of the concentrations of metals and PPCPs in the sediments and the biological responses of *G. broussonnetii* in the ELCIC.



4 CONSIDERAÇÕES FINAIS

Em estudos que visam a avaliação da qualidade de ambientes aquáticos, as investigações químicas das matrizes ambientais, sejam da água ou dos sedimentos, são indispensáveis. Estas permitem averiguar a presença, os níveis, a biodisponibilidade e também auxiliam no entendimento da cinética e dos possíveis efeitos adversos dos contaminantes no ecossistema de estudo. Contudo, a avaliação das respostas biológicas dos organismos do local, são igualmente relevantes, pois também podem indicar a presença e o grau da contaminação do ambiente, revelando sobretudo os efeitos, nos mais diferentes níveis biológicos. Assim, ambas as frentes de investigação podem ser consideradas vantajosas possibilitando a aplicação de ações preventivas para a conservação de um dado ambiente, antes que efeitos drásticos e irreversíveis ocorram (CHOUERI et al., 2009; MARANHO et al., 2012).

Deste modo, o presente estudo empregou uma abordagem integrada para a investigação da qualidade ambiental do Complexo Estuarino-Lagunar de Iguape-Cananéia (CELIC), utilizando-se tanto das análises químicas dos sedimentos estuarinos quanto da análise de biomarcadores nos peixes *Atherinella brasiliensis* e *Gobioides broussonnetii,* analisando em sua totalidade na região, cinco diferentes pontos (Cidade de Cananéia – C; Cidade de Subaúma – S; Ilha de Cananéia – CI; Subaúma – SB; Iguape - IG) em duas diferentes estações do ano (período frio-seco e período quente-chuvoso).

A respeito das avaliações químicas dos sedimentos, a presença de 45 diferentes contaminantes foi investigada e revelou a existência de contaminações baixas a moderadas no sistema pelas três classes de contaminantes abordadas (metais, produtos farmacêuticos e de higiene pessoal - PFHP e hidrocarbonetos policíclicos aromáticos - HPA). As concentrações destes contaminantes tiveram variações temporais e espaciais com maiores valores nos pontos de maior presença antrópica (cidades de Iguape e Cananéia), especialmente durante o período frio-seco. As maiores concentrações vistas nas proximidades das áreas urbanas, provavelmente se dão devido à influência das diferentes atividades antrópicas realizadas nestes locais. Já as maiores concentrações vistas no período de menor precipitação se devem especialmente à menor hidrodinâmica e maior tempo de permanência das águas no estuário durante o período seco.

Dentre os oito metais analisados nos sedimentos do CELIC (Fe, Zn, Mn, Cr, Cu, Ni, Cd e Pb), apenas o Fe e o Mn não são legislados pela Resolução n. 454 do Conselho Nacional do Meio Ambiente – CONAMA (CONAMA, 2012). Neste estudo, as concentrações de nenhum dos metais legislados ultrapassaram os valores de Classe II, os quais são indicados como limiar de toxicidade provável para os sedimentos, em nenhum dos pontos e períodos investigados. Apenas o Ni, o Cr e o Cu excederam, em alguns pontos, as concentrações de Classe I que são sugeridas como limiar de possível toxicidade para os sedimentos.

No estudo realizado com o *A. brasiliensis*, as concentrações de Ni (22,99 µg g^{-1}) e Cr (84.84 µg g^{-1}) nos sedimentos foram ligeiramente maiores do que os valores de 20,9 µg g^{-1} ug e 81 µg g^{-1}, respectivamente orientados pelo CONAMA. Esta situação foi observada em somente um dos pontos analisados, na Cidade de Cananéia (C) durante o período seco. Ainda, este mesmo ponto apresentou, no geral, as maiores concentrações de metais nos sedimentos.

Já, no estudo com o *G. broussonnetii*, as concentrações de Ni nos sedimentos, com exceção do ponto CI na estação fria-seca, ultrapassaram a orientação do CONAMA em todos os outros pontos (CI, SB e IG) e períodos analisados, variando de 27,32 µg g^{-1} a 53,39 µg g^{-1}. Ainda, as concentrações de Cr excederam a orientação do órgão ambiental em Iguape (IG) durante o período seco (94,31 µg g^{-1}), enquanto as concentrações de Cu excederam no mesmo ponto o valor orientado de 34 µg g^{-1}, em ambas as estações, com variação de 39,34 µg g^{-1} a 42,89 µg g^{-1}. Adicionalmente, nesta investigação o ponto IG foi o que apresentou, em geral, as maiores concentrações de metais nos sedimentos, seguido do ponto de Subaúma (SB). Assim, estes resultados sugerem possível toxicidade destes sedimentos (CONAMA, 2012).

Dentre os dezesseis HPA estudados, treze são dados como prioritários e aparecem na legislação ambiental brasileira (CONAMA, 2012). De acordo com as concentrações individuais dos HPA e do somatório total dos HPA legislados, os valores encontrados nos sedimentos não excederam os níveis orientados pelo órgão ambiental no estudo com o *A. brasiliensis* (CONAMA, 2012). No entanto, a presença destes compostos foi observada em todos os pontos estudados, com maiores valores vistos nas proximidades da Cidade de Cananéia (C) durante o período seco. Desta maneira, por meio dos valores das concentrações de metais e HPA nos sedimentos foi possível observar que a presença humana contribui para as contaminações vistas no estuário.

Contribuições de diferentes atividades antrópicas puderam ser identificadas. No aporte de metais, contribuições das atividades antrópicas atuais e das antigas atividades de mineração foram evidenciadas, especialmente nas proximidades da entrada do Rio Ribeira de Iguape (RIR), que se dá pelo Canal Valo Grande em Iguape (IG) e no ponto de Subaúma (SB), aonde há a inversão das águas do estuário e grande acúmulo de sedimentos que chegam pelo RIR. Para o aporte de HPA, a queima de biomassa e de combustíveis fósseis, e o derrame de óleo e de combustível, oriundos da presença dos postos de abastecimentos e das embarcações, foram identificadas como as principais prováveis fontes destas contaminações ao longo do sistema.

Os resultados obtidos por esta pesquisa corroboram com estudos realizados anteriormente na região, os quais demonstram a influência negativa do canal Valo Grande em Iguape e das cidades litorâneas no aporte de metais (TRAMONTE et al., 2018; SALGADO; AZEVEDO, 2018; CRUZ et al., 2014; 2019) e HPA (ANTONELLI et al., 2017) para este ambiente aquático por meio das atividades citadas anteriormente. Os dados também corroboram com os estudos prévios que apontam toxicidade dos sedimentos do CELIC (SALGADO; AZEVEDO, 2018; CRUZ et al., 2014; 2019).

Já para os PFHP, a presente investigação trouxe os primeiros dados para a região em estudo. Dentre as vinte e uma substâncias investigadas, somente a presença de cinco fármacos foram detectadas nos sedimentos amostrados, sendo que as mesmas substâncias foram encontradas nos dois diferentes estudos. Entre os fármacos detectados se encontram a cafeína, os β-bloqueadores metoprolol e propranolol, o anti-inflamatório e analgésico fenoprofen, e o regulador lipídico fenofibrato. Estes resultados possivelmente refletem a cinética destes contaminantes em ambientes aquáticos e o padrão de consumo pela população local.

Tendo em vista que a região do litoral sul de São Paulo é considerada como uma das menos populosas e mais pobres do estado, carecendo de investimentos na área de saúde e saneamento básico (IBGE, 2010; MORAES; ABESSA, 2014), o baixo nível populacional e o acesso limitado da população local a medicamentos pode ter se refletindo nas baixas detecções e concentrações das substâncias analisadas. No entanto, o aporte destes fármacos ao estuário é provavelmente feito pelo lançamento de esgotos domésticos, podendo também sofrer influência da deposição incorreta de lixo, devido à insuficiência de sistemas de coleta e tratamento de efluentes e de resíduos das cidades litorâneas do CELIC. Estes últimos fatores podem ainda contribuir para o aporte de metais e de HPA para dentro do sistema estuarino.

Nos peixes, a avaliação das condições de saúde dos animais se deu através da análise de biomarcadores bioquímicos, histopatológicos e de genotoxicidade, as quais foram realizadas em diferentes órgãos e totalizaram 25 investigações por espécie. Alterações espaciais e sazonais em enzimas do sistema antioxidante e de biotransformação, genotoxicidade e alterações histopatológicas foram observadas mais pronunciadamente nos animais amostrados em Iguape (IG) e em Cananéia (C), especialmente durante o período frio-seco. Ou seja, nos pontos e no período aonde foram encontradas as maiores contaminações nos sedimentos. Assim, se sugere que as atividades antrópicas, em todos os pontos analisados, podem causar impacto negativo na ictiofauna, contudo, em diferentes níveis de intensidade.

Diferentes respostas biológicas e diferentes níveis de comprometimento da saúde do peixe demersal *G. broussonnetii* e do peixe pelágico *A. brasiliensis* foram observadas, possivelmente em função dos diferentes hábitos de vida de cada espécie, que podem se refletir em diferentes modos de exposição aos contaminantes dos sedimentos. No entanto, pode-se observar que ambas as espécies apresentaram alterações biológicas que podem estar ligadas à exposição crônica aos contaminantes estudados no CELIC. As respostas dos biomarcadores foram variadas, de diferentes intensidades, e observadas em todos os diferentes tecidos avaliados (cérebro, músculo, brânquias, fígado, rins e gônadas).

Os efeitos mais drásticos para o *A. brasiliensis* foram vistos no sangue e no fígado, que incluíram danos irreversíveis ao DNA em eritrócitos (alterações morfológicas nucleares) e manifestações graves como necrose hepática, vistas em maio número nos animais de Cananéia (C) do que de Subaúma (S). Esta espécie tem sido muito usada em estudos da avaliação do impacto ambiental em áreas costeiras do Brasil nos últimos anos (DIAS et al., 2009; FERNANDEZ et al., 2011; SOUZA-BASTOS; FREIRE, 2011; RIBEIRO et al., 2013; SANTOS et al., 2018), o que permitiu a comparação dos resultados encontrados com outras regiões. Os resultados vistos para a espécie indicam que os animais do CELIC apresentaram alterações que também foram vistas em áreas nomeadamente contaminadas por esgotos domésticos e com concentrações elevadas de metais e HPA, como por exemplo na região portuária de Paranaguá, PR (RIBEIRO et al., 2013; SANTOS et al., 2018).

Já para o *G. broussonnetii* as manifestações mais significativas se deram nas brânquias, com a observação de aneurismas e elevação do epitélio lamelar, nos rins com a observação de pontos de necroses e a formação de centros de melanomacrófagos e no fígado com a observação de centros de melanomacrófagos e grandes áreas de esteatose. Para essa espécie estas alterações histopatológicas estiveram em maior número nos animais amostrados nas proximidades de Iguape (IG). No entanto, os animais amostrados ao norte da Ilha de Cananéia (CI), próximo a um pequeno vilarejo chamado de Vila de Pedrinhas, apresentaram maior número de danos ao DNA e alterações morfológicas nucleares no sangue, revelando a contribuição deste vilarejo no aporte de substâncias genotóxicas. Em Subaúma, também foram observadas alterações histopatológicas e genotoxicidade nos peixes, contudo em menor intensidade durante o período seco. Apesar de não ser observada ocupação humana relevante neste local, a área também sofre com o acúmulo de metais vindos do RIR (SALGADO; AZEVEDO, 2018; CRUZ et al., 2019) que podem vir a causar efeitos adversos nos peixes.

Esta investigação foi pioneira na avaliação de biomarcadores na espécie *G. broussonnetii* e foi impulsionada pelo objetivo de investigar a saúde dos peixes frente a diminuição populacional da espécie na região visto pela população residente e por pescadores locais nos últimos anos. A pesquisa sugeriu que há a influência de efeitos tóxicos da presença de metais e PFHP sobre a saúde desta espécie no CELIC, inferindo que não só as contaminações que chegam através do Rio Ribeira de Iguape, mas também que as atividades antrópicas ao longo da extensão do sistema, podem estar contribuindo para o declínio observado nesta população. Contudo, outros fatores não avaliados por esta pesquisa também podem ter contribuição neste processo de declínio, como a interferência de mudanças repentinas nas condições ambientais, o envolvimento de patógenos, a sobrepesca e a presença de outros estressores não avaliados.

Os resultados da análise multivariada das respostas biológicas (PCoA) e do Índice Integrado de Respostas de Biomarcadores (IBR) corroboraram com os resultados vistos anteriormente para as contaminações dos sedimentos e para as respostas biológicas em ambos os estudos. Assim, estes resultados confirmaram maios estresse a saúde dos peixes e piores condições ambientais nas proximidades dos centros urbanos de Cananéia para o *A. brasiliensis* e de Iguape para *G. broussonnetii*.

A segunda forma da integração dos resultados foi o uso da análise multivariada de redundância (RDA), utilizando-se tanto dos dados biológicos quanto dos dados químicos dos sedimentos. Esta análise mostrou que a contaminação dos sedimentos foi parcialmente responsável pelas respostas biológicas nos peixes de ambas as espécies, com maior representatividade destas contaminações para o *G. broussonnetii*. Deste modo, tais resultados mostraram que possivelmente os efeitos adversos dos peixes tenham ainda a contribuição de diferentes estressores ou variações ambientais não abordados nesta investigação. Pode-se especular ainda que a exposição às contaminações ambientais por via hídrica tenha também boa representatividade nas alterações vistas nos peixes do CELIC.

De maneira geral, a abordagem integrativa utilizada por este estudo se mostrou bastante útil na avaliação ambiental de áreas costeiras. A consistente relação dos resultados validou a associação entre as alterações encontradas nos peixes com a presença de metais, HPA e PFHP como agentes estressores. Assim, estas respostas combinadas puderam revelar o padrão de distribuição de contaminantes, suas principais fontes de emissão, os principais efeitos adversos nos organismos aquáticos e a representatividades destas contaminações sobre as espécies investigadas. Assim, este estudo contribui para um panorama atual da qualidade ambiental do CELIC, com dados que podem futuramente apoiar a gestão da área no desenvolvimento de políticas públicas que preservem e/ou melhorem a qualidade ambiental, de vida e de saúde pública da região.

A área em estudo abriga a APA CIP (Área de Preservação Ambiental de Cananéia-Iguape-Peruíbe), um sistema estuarino legalmente reconhecido como região de alta prioridade para a conservação (BRASIL, 1984, 1985), que como os dados revelaram já apresenta sinais de degradação ambiental. Deste modo, fica evidente a necessidade de uma maior atenção das autoridades do poder público (municipal e estadual), dos governos e da sociedade como um todo, para a adoção de condutas mais eficientes no controle da poluição. Investimentos em infraestrutura de coleta e tratamento de esgoto e de resíduos, fiscalização do uso da AMP, medidas para conscientização e educação ambiental para moradores e turistas da região, são exemplos de ações que podem ser implementadas objetivando mitigar a influência antrópica. Por fim, levando-se em consideração o valor da área como patrimônio da humanidade, traz-se a atenção para a importância de um monitoramento contínuo do local e dá-se incentivo à futuros estudos científicos que permitam um maior entendimento da situação ambiental local na busca da preservação deste ambiente e dos serviços prestados pela região.

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