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CENTRO DE BIOCIÊNCIAS
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ROMULO NEPOMUCENO ALVES

# Biomarcadores ecotoxicológicos em *Danio rerio* para o monitoramento da poluição em rios de Pernambuco

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Tese apresentada ao Programa de Pós-graduação em Biologia Animal, da Universidade Federal de Pernambuco, como parte dos requisitos para à obtenção do grau de Doutor em Biologia Animal.

**Área de Concentração:** Biologia Animal

Orientador: **Dr. Paulo Sérgio Martins de Carvalho**
Co-orientadora: **Dra. Eliete Zanardi Lamardo**

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# RESUMO

Ecossistemas aquáticos são o principal destino de rejeitos das atividades urbanas, industriais e agrícolas, diminuindo a qualidade da água e a saúde da biota. Estágios iniciais de desenvolvimento de peixes são uma ferramenta importante para a detecção da toxicidade de sustâncias isoladas ou de misturas, e alterações nessa fase podem estar diretamente relacionadas à presença de contaminantes no ambiente. O presente estudo teve como objetivo investigar a ecotoxicidade das águas superficiais de onze bacias hidrográficas de Pernambuco para a fase embriolarval do zebrafish (*Danio rerio*) através da análise de efeitos letais e subletais, e avaliar correlação da toxicidade com os índices de qualidade da água (IQA) e de estado trófico (IET). Inicialmente, embriões foram expostos a amostras de dez bacias hidrográficas divididas em região norte do estado (Grupo 1), região central e metropolitana (Grupo 2), e região sul (Grupo 3), totalizando 55 pontos de coleta. Posteriormente, foi realizada exposição a amostras de 10 pontos do Rio Capibaribe, numa escala espacial menor, mas significativa. A toxicidade foi expressa por taxas de letalidade, atrasos no desenvolvimento embriolarval com base no índice "General Morphology Score" (GMS), e por frequências de anomalias do desenvolvimento. Na escala espacial maior mortalidade significativa foi observada em 7 das 10 bacias analisadas, variando entre 10% e 40%. A análise dos efeitos subletais indicou toxicidade significativa em 50% das amostras. A maior toxicidade foi detectada nas bacias do Grupo 2 (62%) com GMS variando entre 14,1 e 17,6. Para a bacia do Rio Capibaribe foi observado menor impacto em áreas rurais (GMS médio 16,9), impacto intermediário em locais com influência urbana e agrícola (GMS médio 16,4), e os maiores impactos em locais mais urbanizados (GMS médio 14,9). Hidrocarbonetos policíclicos aromáticos (HPA) foram detectados tanto em áreas rurais quanto urbanizadas do Rio Capibaribe. Em todo o estudo as alterações de desenvolvimento mais frequentes incluíram o não insuflamento da bexiga natatória, atraso na eclosão, não protrusão da boca, estase sanguínea e não desenvolvimento das nadadeiras peitorais, com destaque para o não insuflamento da bexiga natatória que apresentou frequência de 100% em algumas estações de coleta. Além disso, as maiores frequências de estase sanguínea foram detectadas em amostras com maiores concentrações de $NH_3$, corroboradas por uma correlação positiva, sugerindo a existência de uma relação causal. Também foram detectadas correlações significativas entre o GMS, o IQA e o IET, indicando que a toxicidade subletal ao longo das bacias analisadas é maior em locais com maiores níveis de contaminação por esgoto doméstico e maior risco de eutrofização. Este estudo demonstra o potencial dos estágios iniciais da vida

do zebrafish como um modelo ecotoxicológico para avaliar a toxicidade de águas superficiais de rios tropicais, podendo assim contribuir para um melhor entendimento entre o potencial tóxico das fontes poluidoras e os efeitos adversos sofridos pela biota residente nesses ambientes. Além disso, evidencia possíveis impactos que as espécies residentes dos ambientes estudados podem estar sofrendo, se fazendo necessário um manejo adequado desses impactos e um maior controle das atividades desenvolvidas no entorno da área de estudo.
PALAVRAS-CHAVE: ictiotoxicidade; *Danio rerio*; rios tropicais; hidrocarbonetos aromáticos policíclicos; águas superficiais.

# ABSTRACT

Aquatic ecosystems are the main destination for waste from urban, industrial and agricultural activities, reducing water quality and the health of the biota. The early stages of fish development are an important tool for detecting the toxicity of isolated substances or mixtures, and changes in this stage of development can be directly related to the presence of contaminants in the environment. The present study aimed to investigate the ecotoxicity of surface waters from eleven river basins throughout Pernambuco for the embryolarval stage of zebrafish (*Danio rerio*) through the analysis of lethal and sublethal effects, possible sources of contaminants, and to evaluate correlation of toxicity with the water quality (WQI) and trophic status (TSI) indices. On a wider spatial scale, significant mortality was observed in 7 of the 10 basins analyzed, ranging between 10% and 40%. Analysis of sublethal effects based on the General Morphology Score (GMS) indicated significant toxicity in 50% of the samples. The highest toxicity was detected in the basins of group 2, indicating that 61% of the samples presented sublethal toxicity, with GMS varying between 14.1 and 17.6. Subsequently, zebrafish were exposed to samples from 10 sampling sites along the Capibaribe River, on a relatively small but significant spatial scale. For the Capibaribe River basin, a spatial pattern of lesser impact was observed in rural areas, in terms of contamination by domestic sewage, eutrophication and sublethal ecotoxicity (average GMS 16.9), an intermediate impact in places with urban and agricultural influence (GMS average 16.4), and the greatest impacts in more urbanized locations (average GMS 14.9). Polycyclic aromatic hydrocarbons (PAH) were detected both in rural and urbanized areas of the Capibaribe River. Throughout the study, the most frequent developmental alterations included delayed hatching, non-protrusion of the mouth, blood stasis, lack of pectoral fin formation and failure in swim bladder inflation, reaching a frequency of 100% at some sites. In addition, both on a smaller and broader spatial scale, the highest frequencies of blood stasis were detected in samples with higher concentrations of $NH_3$, corroborated by a positive correlation, suggesting the existence of a causal relationship. Significant correlations between the GMS, WQI and TSI were also detected in the two spatial scales, indicating that sublethal toxicity along the analyzed watersheds is greater in places with higher levels of contamination by domestic sewage and greater risk of eutrophication. This study demonstrates the potential of the early stages of zebrafish life as an ecotoxicological model to assess the toxicity of surface waters of tropical rivers, thus contributing to a better understanding between the toxic potential of polluting sources and the adverse effects suffered by the biota residing in these environments. In addition, it shows possible impacts that the resident species of the

studied environments may be suffering, making it necessary to have an adequate management of these impacts and a greater control of the activities developed in the surroundings of the study area.
KEYWORDS: ichthyotoxicity; *Danio rerio*; tropical rivers; polycyclic aromatic hydrocarbons; surface water.

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## Fundamentação teórica



## Manuscrito I

**Manuscrito II**

# LISTA DE ABREVIATURAS E SÍGLAS

ANA – Agência Nacional de Águas

ANOVA – Análise de variância

APA – Áreade Proteção Ambiental

APAC – AgênciaPernambucana de Águas e Clima

CI – Controle interno

CPRH – AgênciaEstadual de Meio Ambiente e RecursosHídricos

DBO – Demanda Bioquímica de Oxigênio

FET – Fishembryo acute toxicity test

HPA – Hidrocarbonetos policíclicos aromáticos

HPF – Horas Pós Fertilização

IET – Índice de Estado Trófico

IDM – Índice de desenvolvimento morfológico

IBGE – Instituto Brasileiro de Geografia e Estatística

IQA – Índice de Qualidade da Água

GMS – General Morphology Score

OD – Oxigênio Dissolvido

OECD – Organizationfor Economic Co-operation and Development

POP – Poluentesorgânicos persistentes

RMR – Região Metropolitana do Recife

UF – Unidades da Federação

SUMÁRIO

# 1 FUNDAMENTAÇÃO TEÓRICA

## 1.1 ECOSSISTEMAS AQUÁTICOS

Os ecossistemas aquáticos costeiros são ambientes que tendem a ser mais férteis, sendo considerados como áreas provedoras de recursos e atrativas para a ocupação humana. Esses ambientes foram altamente explorados para a ocupação e desenvolvimento urbanos ao longo do tempo, o que causou impactos negativos na saúde desses ecossistemas. As águas continentais, costeiras e marinhas são contribuintes importantes dos serviços essenciais, como ciclagem de nutrientes e produção primária (PERNAMBUCO, 2019b). Ambientes aquáticos são sistemas vulneráveis à contaminação e tendem a receber elevadas cargas de compostos pela drenagem a partir do ambiente terrestre (DI GIULIO, R. T.; HINTON, D. E., 2008). Além disso, possuem uma cadeia alimentar bem delimitada o que proporciona um aumento na acumulação de contaminantes persistentes nos níveis mais altos, como os predadores de topo de cadeia (DI GIULIO, R. T.; HINTON, D. E., 2008).

Dentre esses ambientes, as águas continentais sofreram uma maior pressão das ocupações urbanas pois se tratam de fonte de água doce, essencial para a sobrevivência humana. Essas águas são utilizadas para o consumo doméstico, agrícola, industrial, e até geração de energia, e contribuem indiretamente na prestação de serviços (alimentos, bioquímicos, recursos genéticos) e culturais (como recreação e ecoturismo, benefícios estéticos e educacionais) (PERNAMBUCO, 2019b). Os impactos da ocupação humana no entorno dos rios começam com a erradicação da cobertura vegetal e exposição do solo, causando erosões e facilitando a lixiviação de resíduos urbanos para esses ambientes. Esses resíduos contêm diversas classes de contaminantes, provenientes da indústria, agricultura, urbanização, transporte, turismo e vida cotidiana e a proteção dessas águas deve desempenhar um papel fundamental nas políticas de salvaguarda ambiental, visto que são os ecossistemas mais ameaçados, ainda mais do que as marinhas ou terrestres (CRISTIANO; LACCHETTI; MANCINI; CORTI *et al.*, 2019). Atualmente, acredita-se que cerca de 100.000 moléculas são introduzidas em meios aquáticos intencionalmente e mais frequentemente não intencionalmente (LAETZ; HECHT; INCARDONA; COLLIER *et al.*, 2015). Conceitualmente, os efluentes líquidos são os contaminantes aquáticos, poluentes do ar são os contaminantes atmosféricos e os resíduos sólidos são os contaminantes terrestres, mas após diversos processos naturais, o meio aquático aparece como destino final para a maioria desses contaminantes antrópicos. Desta forma, os sedimentos aquáticos, depositados ou ainda em suspensão, configuram o principal sumidouro

destes compostos (AMIARD-TRIQUET, 2015). De acordo com a European Union (EU) Water Framework Directive (WFD), a qualidade química da água é determinada pelo monitoramento das águas superficiais quanto à presença de 45 grupos de substâncias prioritárias, no entanto a compreensão dos riscos desses contaminantes requer uma mudança de paradigma, que permite novos métodos de monitoramento holístico que não dependem apenas da análise química de substâncias prioritárias, mas, em contraste, consideram os efeitos biológicos em primeiro lugar (DE BAAT; KRAAK; VAN DER OOST; DE VOOGT *et al.*, 2019).

Dentre as substâncias que podem causar efeitos negativos nos ambientes aquáticos estão as dioxinas e PCBs, metais pesados, a amônia, que pode ser mais tóxica para peixes em sua forma não ionizada ($NH_3$) (RANDALL; TSUI, 2002) e os hidrocarbonetos policíclicos aromáticos (HPA), que apresentam um potencial de toxicidade elevado, podendo causar alterações enzimáticas, genéticas, alterações na função cardíaca, no desenvolvimento embrio-larval, no comportamento e na reprodução de peixes (INCARDONA; CARLS; DAY; SLOAN *et al.*, 2009; INCARDONA; COLLIER; SCHOLZ, 2004; KENNEDY, 2014; PAYNE; MATHIEU; COLLIER, 2003).

## 1.2 BACIAS HIDROGRÁFICAS DE PERNAMBUCO

No estado de Pernambuco a cobertura hídrica é composta por 16 bacias hidrográficas que são formadas por rios principais e alguns grupos de rios menores (afluentes). Em 2019 a população estimada do estado era de 9.557.071 habitantes, com uma taxa de urbanização de 83% (IBGE, 2020), mas com a prevalência de um maior número de habitantes no meio rural em algumas bacias hidrográficas. Não diferente dos demais estados do país, em Pernambuco a ocupação da terra também se deu no entorno dos rios, o que causou diversos impactos nesses ecossistemas. Os principais impactos antrópicos causados nesses ambientes foram a destruição da vegetação ciliar, implantação de cultivos agrícolas, alteração do curso dos rios para fins de irrigação, drenagem de áreas alagadas (brejos) para o estabelecimento de plantios, introdução de espécies exóticas, poluição da água pelo despejo de esgotos, resíduos sólidos e rejeitos industriais (PERNAMBUCO, 2019a). Dentre as fontes desses impactos, a agricultura irrigada e a urbanização aparecem como as ações que mais afetam negativamente a saúde dos ambientes aquáticos da região (PERNAMBUCO, 2019a). Apesar do relevante impacto da agricultura irrigada nos rios do estado, Pernambuco não se destaca no percentual de áreas irrigadas no seu território frente às demais Unidades da Federação (UF), ocupando apenas a 14ª. posição entre as 27 UFs, com 0,7% (1.460 $km^2$) de todo seu território com áreas irrigadas. Entretanto, cerca de 30% dessa área é destinada à plantação de cana-de-açúcar (ANA, 2017), o que tornou essa

monocultura objeto de estudos quanto aos impactos gerados nos ecossistemas da região (ARRUDA-SANTOS; SCHETTINI; YOGUI; MACIEL *et al.*, 2018; GUNKEL; KOSMOL; SOBRAL; ROHN *et al.*, 2007; SILVA; SILVA; TEIXEIRA; BEDOR *et al.*, 2020). Em relação ao saneamento básico e tratamento de esgoto, Pernambuco se destaca entre os estados com menor percentual de municípios com tratamento de esgoto, tendo apenas 34,2% dos municípios com esse serviço (SNIS, 2019).

As bacias hidrográficas e rios de Pernambuco são divididos de acordo com o seu escoamento. As bacias e rios que escoam para o Rio São Francisco são denominadas de bacias e rios interiores e os que escoam para o Oceano Atlântico são denominadas de bacias e rios litorâneas (PERNAMBUCO, 2019b). Devido aos impactos causados nas bacias hidrográficas do estado, a Agência Estadual de Meio Ambiente e Recursos Hídricos (CPRH) iniciou o monitoramento da qualidade da água em rios litorâneos em outubro de 1984 no Rio Pirapama, em 1986 no rio Ipojuca e no Rio Beberibe e, ao longo dos anos, foi sendo ampliado, chegando hoje a 84 pontos de monitoramento. Essa rede de monitoramento abrange áreas dos rios Goiana, Botafogo, Igarassu, Timbó, Paratibe, Beberibe, Capibaribe, Jaboatão, Ipojuca, Pirapama, Maracaípe, São Francisco, Sirinhaém e Una, e conta com a avaliação de diversos parâmetros físico-químicos (Tabela 1). Para fins de monitoramento, a CPRH utiliza como base a Resolução CONAMA 357/2005 para verificar se o corpo d'água está sofrendo algum tipo de contaminação e faz uma integração desses dados utilizando dois indicadores de qualidade da água: o Índice de Qualidade da Água (IQA) (NOORI; BERNDTSSON; HOSSEINZADEH; ADAMOWSKI *et al.*, 2019) e o Índice de Estado Trófico (IET) (PIRES; TUCCI; CARVALHO; LAMPARELLI, 2015)

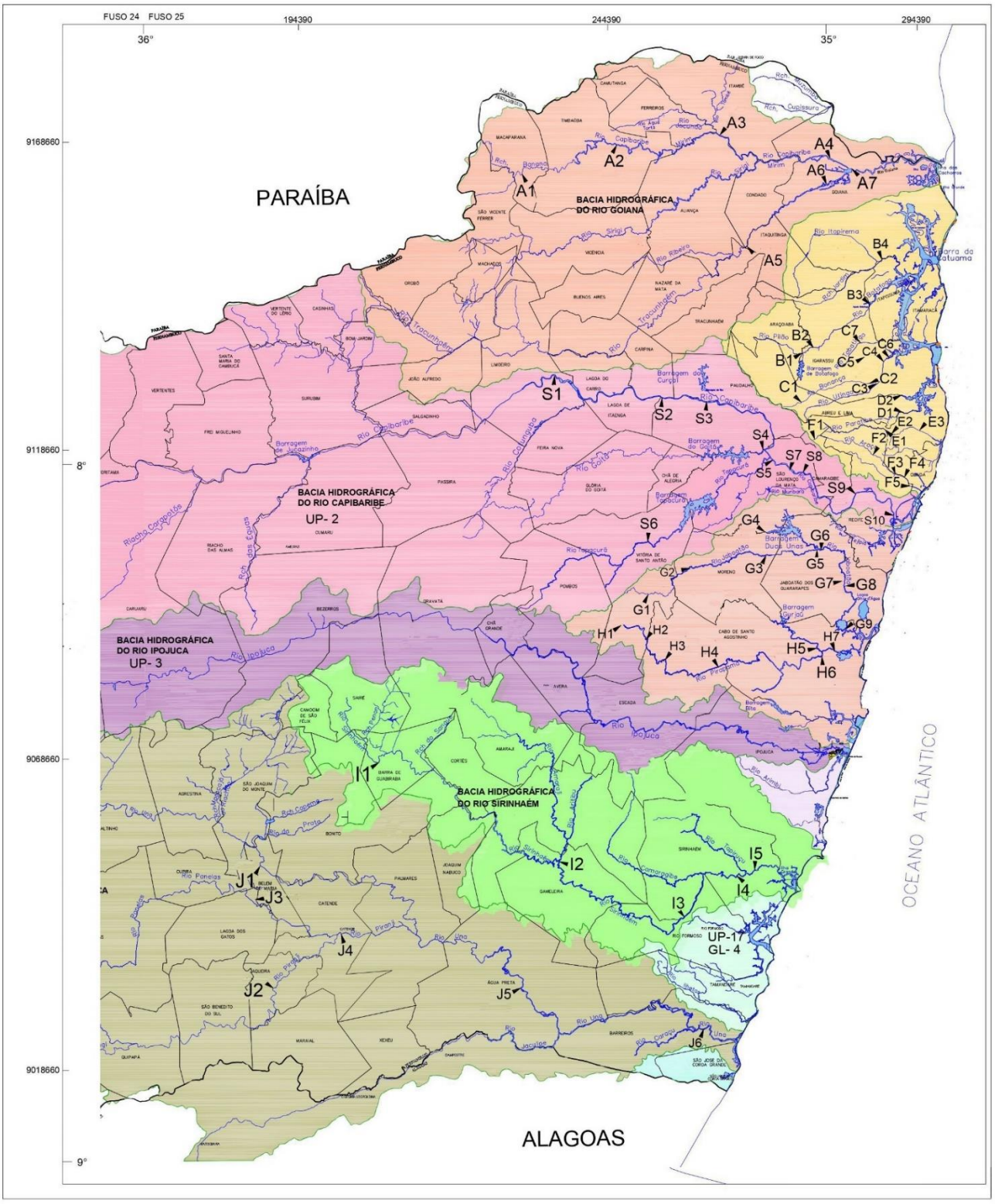
Tabela 1 – Conjunto de parâmetros monitorados pela CPRH nos 84 pontos de amostragem em rios.

| Parâmetros Gerais | Metais | Ecotoxicidade |
|---|---|---|
| pH | Cádmio ($mg.L^{-1}$) | *Vibrio Fischeri* |
| Condutividade ($\mu S\ cm^{-1}$) | Chumbo ($mg.L^{-1}$) | *Daphnia magna* |
| OD ($mg\ O_2\ L^{-1}$) | Cobre ($mg.L^{-1}$) | |
| DBO ($mg.L^{-1}$) | Cromo ($mg.L^{-1}$) | |
| Turbidez (UNT) | Ferro ($mg.L^{-1}$) | |

| | |
|---|---|
| Fósforo (mg P $L^{-1}$) | Manganês ($mg.L^{-1}$) |
| Coliformes Termotolerantes (MPN 100 $mL^{-1}$) | Níquel ($mg.L^{-1}$) |
| Salinidade (ups) | Zinco ($mg.L^{-1}$) |
| Clorofila ($\mu.L^{-1}$) | Mercúrio ($mg.L^{-1}$) |
| Amônia ($mg.L^{-1}$) | |
| Nitrato ($mg.L^{-1}$) | |
| Nitrito ($mg.L^{-1}$) | |
| Sólidos Totais ($mg.L^{-1}$) | |
| Sólidos Suspensos ($mg.L^{-1}$) | |
| Sólidos Dissolvidos ($mg.L^{-1}$) | |

Dos 84 pontos de amostragens em rios feitos pela CPRH, 65 foram analisados neste estudo, excluindo apenas as estações do Rio Ipojuca por ser objeto de estudo de outro projeto de pesquisa, do Canal de Santa Cruz e do rio Maracaípe, devido à alta salinidade das amostras e do rio São Francisco, devido à indisponibilidade de amostras no período de desenvolvimento deste trabalho. Dessa forma, foram analisadas amostras de águas das bacias dos rios Goiana, Botafogo, Igarassu, Timbó, Paratibe, Beberibe, Capibaribe, Jaboatão, Pirapama, Sirinhaém e Una (Figura 1). Vale salientar que atualmente o monitoramento dessas bacias é realizado juntamente com a Agência Pernambucana de Águas e Clima (APAC) e inclui além das 84 amostras de rios, o monitoramento de 54 reservatórios localizados nas bacias interiores, totalizando 138 estações de amostragem. A APAC, a partir do Programa de Estímulo à Divulgação de Dados de Qualidadede Água – QUALIÁGUA, monitora a qualidade de água dos 54 reservatórios e de mais 41 pontos de amostragem em rios, sendo todas as análises laboratoriais realizadas na CPRH. O QUALIÁGUA é uma iniciativa da Agência Nacional de Águas (ANA), e tem entre os seus objetivos a padronização dos critérios e métodos de monitoramento de qualidade de água em todo o país.

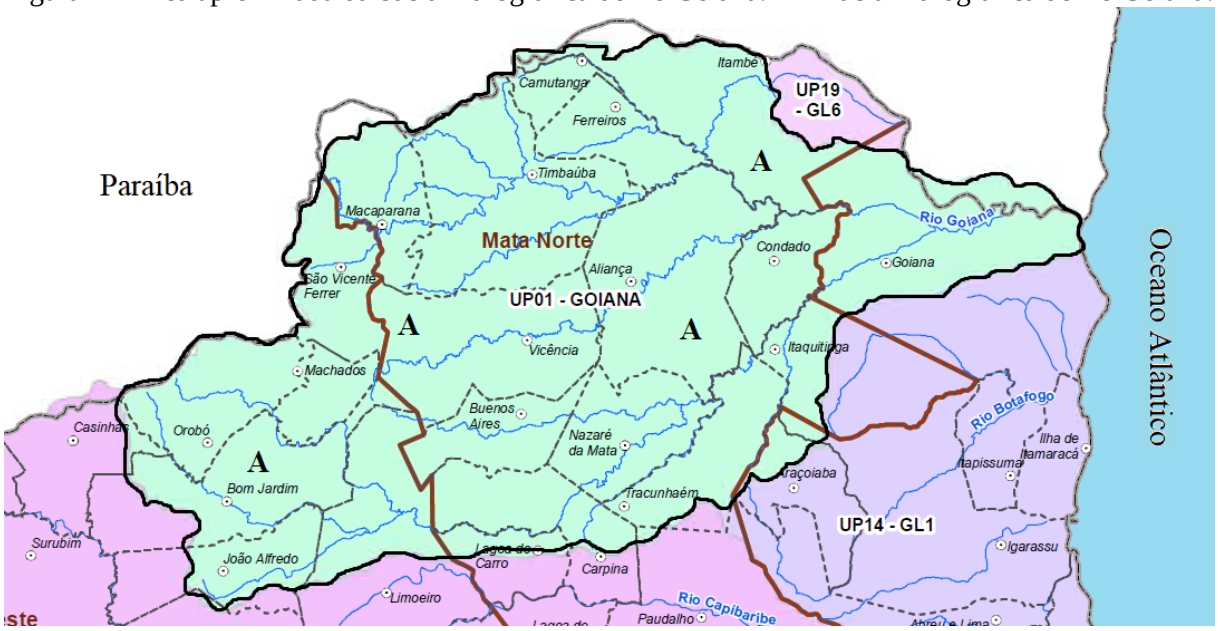
Figura 1 – Área de amostragem com os 65 pontos de coleta nas 11 bacias hidrográficas no estado de Pernambuco abordadas nesse estudo.




Fonte: (CPRH, 2020) (adaptada).
A – Rio Goiana, B – Rio Botafogo, C – Rio Igarassu, D – Rio Timbó, E – Rio Paratibe, F – Rio Beberibe, S – Rio Capibaribe, G – Rio Jaboatão, H – Rio Pirapama, I – Rio Sirinhaém e J – Rio Una.

A bacia hidrográfica do rio Goiana (A) (figura 2) possui uma área de drenagem de 2.878,30 km², está localizada entre os estados de Pernambuco e Paraíba, e possui 26 espaços territoriais de municípios total ou parcialmente inseridos em sua área, com uma população total de 465.549 habitantes, 61% das quais estão em áreas urbanas. A bacia tem como principal curso de água o rio Goiana, com aproximadamente 19 km de extensão, que se forma a partir da confluência do rio Capibaribe Mirim (aproximadamente 93 km de extensão) e o rio Tracunhaém (127 km de extensão) (ANDREZA TACYANA FELIX, 2020). A bacia do rio Goiana sofre pressão de atividades urbanas, industriais e agrícolas, com destaque para o cultivo da cana-de-açúcar para a produção de açúcar e álcool.

Figura 2 – Área aproximada da bacia hidrográfica do rio Goiana. A - Bacia hidrográfica do rio Goiana.




Fonte: (PERNAMBUCO, 2019b) - adaptada.

A bacia hidrográfica do rio Botafogo (B) (figura 3) possui área total de aproximadamente 477 km$^2$ e está localizada ao norte do estado de Pernambuco, tendo como limite superior a bacia do rio Goiana e a bacia hidrográfica do rio Igarassu ao sul. A bacia do rio Botafogo abrange áreas de 5 municípios do estado (Araçoiaba, Tracunhaém, Igarassu, Itaquitinga, Goiana), sofrendo uma pressão populacional de aproximadamente 90 mil habitantes com a maior parte de sua área em áreas rurais, e os principais usos das suas águas são a irrigação de canaviais, abastecimento urbano e industrial (CPRH, 2018; LEÃO; PASSAVANTE; DA SILVA-CUNHA; SANTIAGO, 2008).

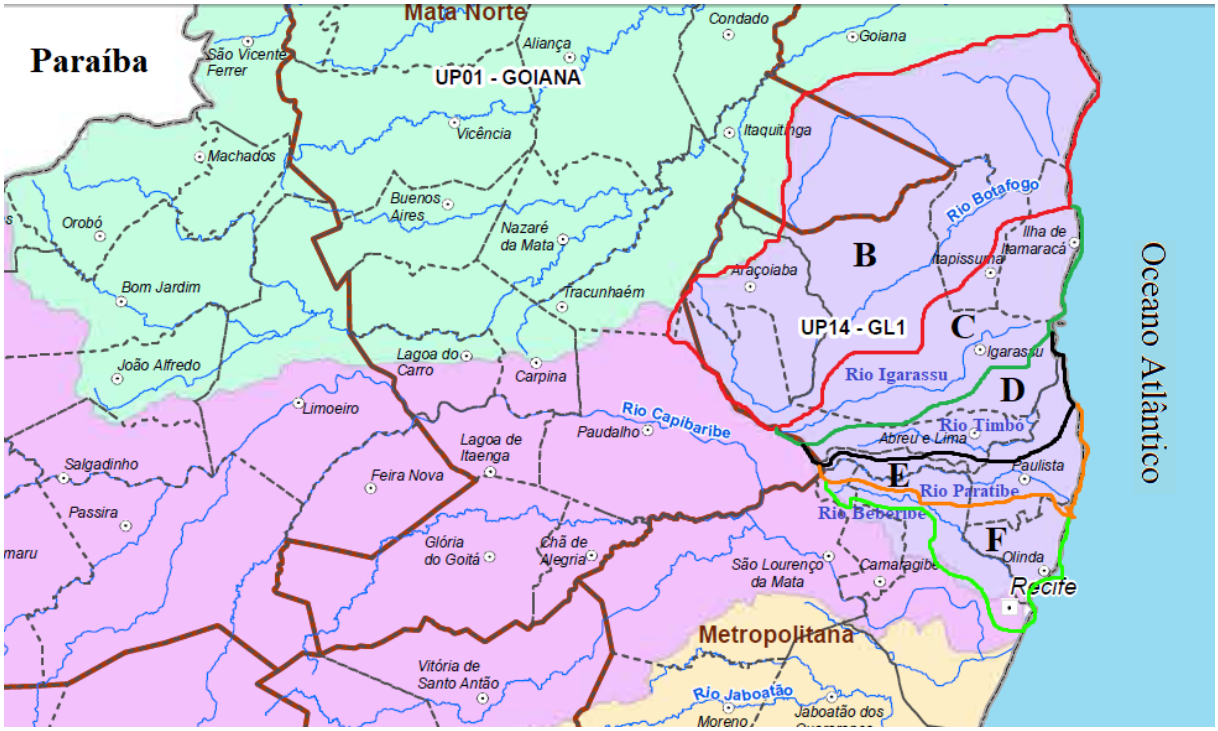
A bacia do rio Igarassu (C) (figura 3) tem uma área total de aproximadamente 143 km$^2$, abrangendo o município de Igarassu (81%) e Abreu e Lima (19%), limitada ao norte com a bacia do rio Botafogo e ao sul, com as bacias dos rios Timbó e Paratibe. As atividades

predominantes nessa bacia são agrícolas e urbanas, atendendo a uma população de aproximadamente 125 mil pessoas. Em seu curso médio existe um remanescente de Mata Atlântica, com sessenta hectares de extensão, onde são desenvolvidas pesquisas de animais selvagens ameaçados de extinção (Refúgio Ecológico Charles Darwin) (CPRH, 2018).

A bacia hidrográfica do Rio Timbó (D) (figura 3) com 104 $km^2$ de área, está localizada logo abaixo da bacia do rio Igarassu e acima da bacia do Rio Paratibe, abrange os municípios de Abreu e Lima, Igarassu e Paulista, e está sob pressão de uma população de aproximadamente 120 mil habitantes. Apesar de sofrer uma forte pressão urbana e industrial, que faz com que as suas águas sejam utilizadas principalmente para a recepção de efluentes domésticos e industriais, o Rio Timbó está inserido em Área de Proteção Ambiental (APA) desde 1986 (CPRH, 2018; FEITOSA; FLORES; MELO; SANTANA *et al.*, 2016). A bacia do rio Paratibe (E) (figura 3) possui uma área total de cerca de 118 $km^2$, limitada ao norte com as bacias dos rios Timbó e Igarassu, e ao sul com a bacia do Rio Beberibe (CPRH, 2018). Assim como o Rio Timbó, a bacia do Rio Paratibe é predominantemente urbana, abrangendo os municípios de Paulista e Olinda, sob pressão de uma população de aproximadamente 400 mil habitantes. Apesar de suas águas serem utilizadas para o abastecimento público, o Rio Timbó é o principal receptor de efluentes domésticos e industriais da região (SANTANA; COELHO JUNIOR, 2017).

A bacia do Rio Beberibe (F) (figura 3) tem 79 $km^2$ de área total, está inserida na região metropolitana do Recife (RMR), abrangendo os municípios de Camaragibe, Olinda e Recife, sob pressão de uma população de cerca de 1 milhão de habitantes. Devido ao déficit de saneamento básico em Olinda e Recife, bem como à ocupação urbana das encostas das margens, e por ser um dos principais receptores de efluentes domésticos e industriais em sua região, o rio Beberibe é considerado um dos mais poluídos do estado (CPRH, 2018; VAINSENCHER, 2009).

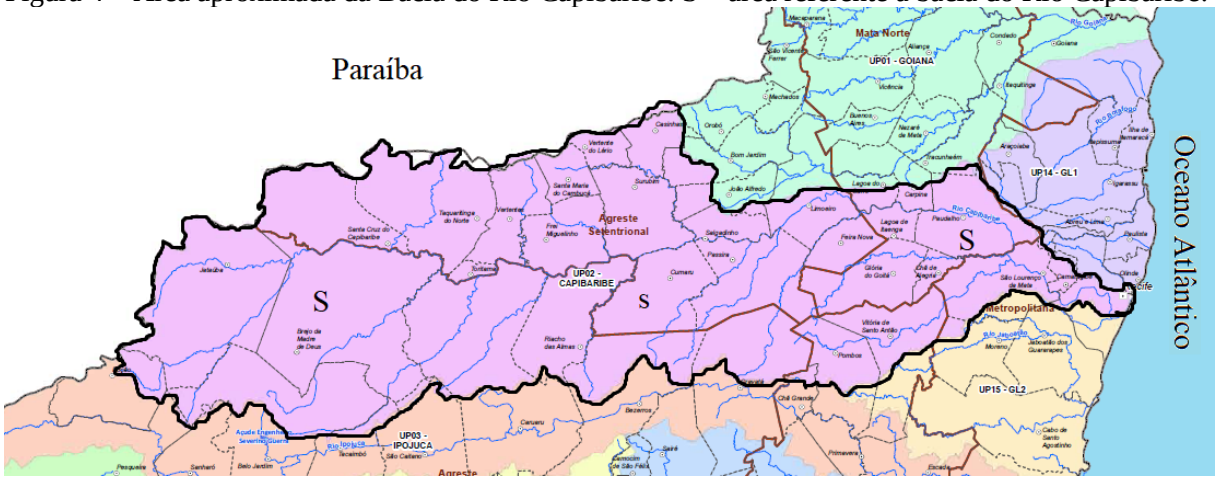
Figura 3 – Áreas aproximadas das bacias hidrográficas dos rios Botafogo, Igarassu, Timbó, Paratibe e Beberibe. B – Rio Botafogo, C – Rio Igarassu, D – Rio Timbó, E – Rio Paratibe e F – Rio Beberibe.




Fonte: (PERNAMBUCO, 2019b) - adaptada.

A bacia hidrográfica do Rio Capibaribe (S) (figura 4) está localizada entre as bacias dos rios Beberibe e Jaboatão, sendo a maior bacia hidrográfica do estado com uma área de 7.454,88 km² (7,58% da área do estado), 280 km de extensão, abrangendo 42 municípios (RIBEIRO NETO; SCOTT; LIMA; MONTENEGRO *et al.*, 2014), dentre eles a Região Metropolitana do Recife (RMR), sexta maior conurbação do país (IBGE, 2020). Essa região está sujeita a uma série de impactos antrópicos, sob pressão de uma população de cerca de mais de 4 milhões de habitantes, tendo como as principais fontes de poluição os resíduos dos canaviais, os efluentes das atividades industriais e os resíduos urbanos.

Figura 4 – Área aproximada da Bacia do Rio Capibaribe. S – área referente à bacia do Rio Capibaribe.

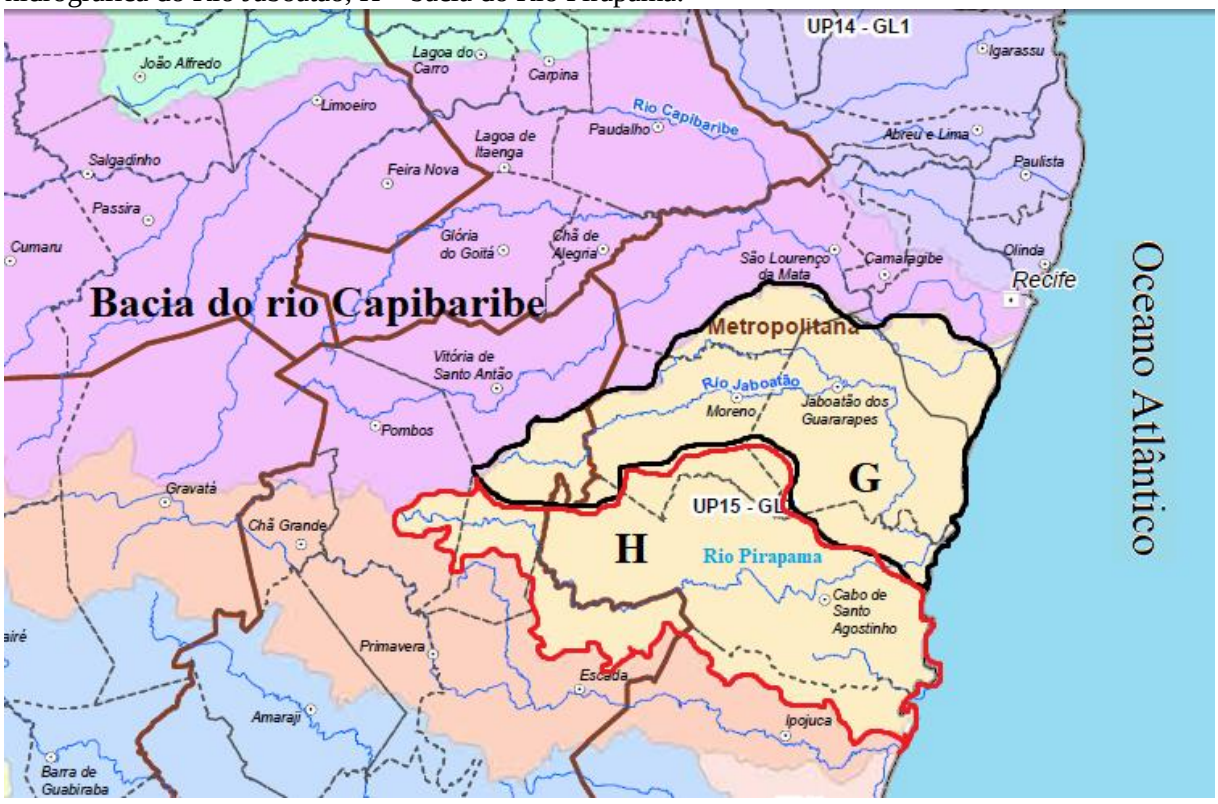



Fonte: (PERNAMBUCO, 2019b) - adaptada.

A bacia hidrográfica do rio Jaboatão (G) (figura 5) está localizada entre a bacia do Rio Capibaribe ao norte e do Rio Pirapama ao sul. Possui uma área de 422 km$^2$, abrangendo seis importantes municípios do estado (Vitória de Santo Antão, Cabo de Santo Agostinho, Moreno, São Lourenço da Mata, Jaboatão dos Guararapes e Recife), com uma população de 446.426 habitantes dividida entre áreas rurais e agrícolas, e áreas urbanas. As principais pressões antrópicas sofridas pelo Rio Jaboatão advêm das atividades domésticas, industriais e sucroalcooleiras (CPRH, 2018; SOUZA; TUNDISI, 2003).

A bacia hidrográfica do Rio Pirapama (H) (figura 5) possui uma área de aproximadamente 600 km², está localizada na porção sul da RMR e na Zona da Mata Pernambucana, entre as bacias dos rios Jaboatão (ao norte) e Ipojuca (ao sul). Abrange a área de sete municípios, incluindo Cabo de Santo Agostinho, Jaboatão dos Guararapes e Ipojuca (VIANA; MONTENEGRO; SILVA; DA SILVA *et al.*, 2019). As pressões exercidas nesta bacia estão relacionadas principalmente às atividades industriais, ao cultivo da cana-de-açúcar e às densidades urbanas desenvolvidas em seu entorno com uma população de aproximadamente 1.160.000 habitantes (CPRH, 2018; IBGE, 2020).

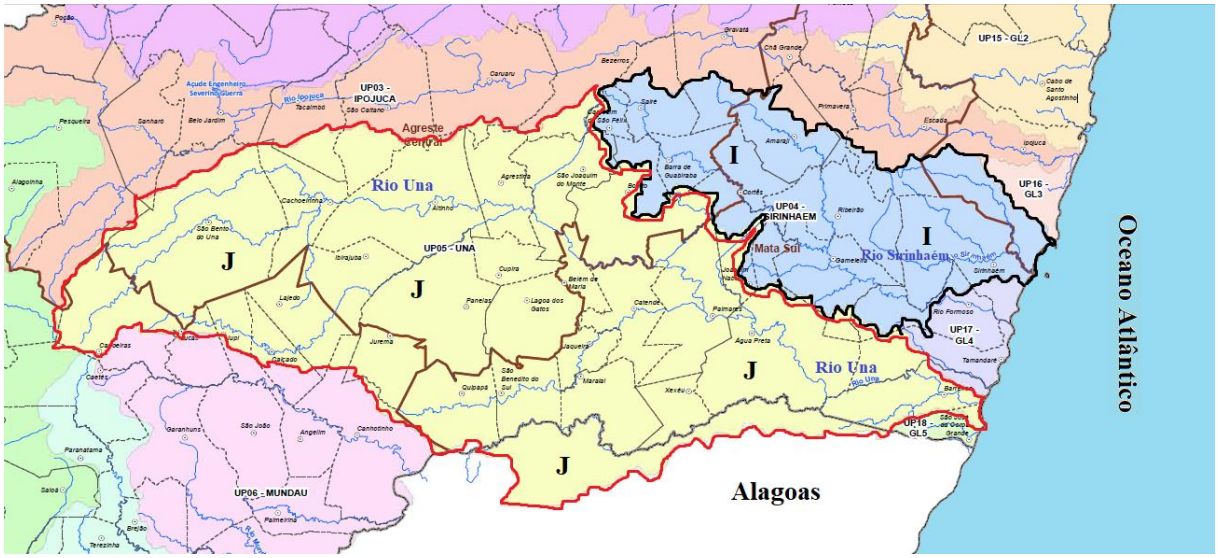
Figura 5 – Área aproximada das bacias hidrográficas dos rios Jaboatão e Pirapama. G – Bacia hidrográfica do Rio Jaboatão, H – bacia do Rio Pirapama.




Fonte: (PERNAMBUCO, 2019b) – adaptada.

A bacia do Rio Sirinhaém (I) (figura 6) está localizada entre as bacias dos rios Ipojuca (ao norte) e Una (ao sul), com área de 2.069,60 km², abrange 19 municípios, com aproximadamente 40% de sua área inserida em áreas rurais. Essa bacia recebe pressão principalmente de atividades urbanas e rurais, com uma população de aproximadamente 182.000 habitantes, dos quais 81.000 estão em áreas rurais (CPRH, 2018). A bacia do Rio Una (J) (figura 6) está localizada logo abaixo da bacia do rio Sirinhaém, na porção sul do estado de Pernambuco. Possui uma área de aproximadamente 6.292,89 km2, abrangendo cerca de 43 municípios sob pressão de uma população de 553.259 habitantes, 43% dos quais são rurais (CPRH, 2018). A bacia do Rio Una apresenta uma diversidade de usos da água, com algumas áreas destinadas à recreação, pesca, irrigação e abastecimento industrial e público, com destaque para a atividade sucro-alcooleira (TAVARES; CORRÊA; SOUZA; SCARIOTTO *et al.*, 2017).

Figura 6 – Áreas aproximadas das bacias hidrográficas dos rios Sirinhaém e Una. I – bacia do Rio Sirinhaém, J – bacia do Rio Una (PERNAMBUCO, 2019b) – adaptada.




Fonte: (PERNAMBUCO, 2019b) – adaptada.

## 1.3 ECOTOXICOLOGIA

O desenvolvimento tecnológico no último século promoveu expressivos avanços em diversos setores da economia mundial, tais como indústria, agricultura, mineração e petroquímica. Esse crescimento econômico trouxe consigo um aumento significativo na produção de diferentes compostos químicos, como os fertilizantes, inseticidas e fármacos. Por falta de conhecimento ou fiscalização adequada, muitas vezes estes produtos são descartados nos ecossistemas aquáticos sem um tratamento apropriado. A eficiência destes compostos associada a altas demandas da sociedade por mais produtos, promovem uma produção e

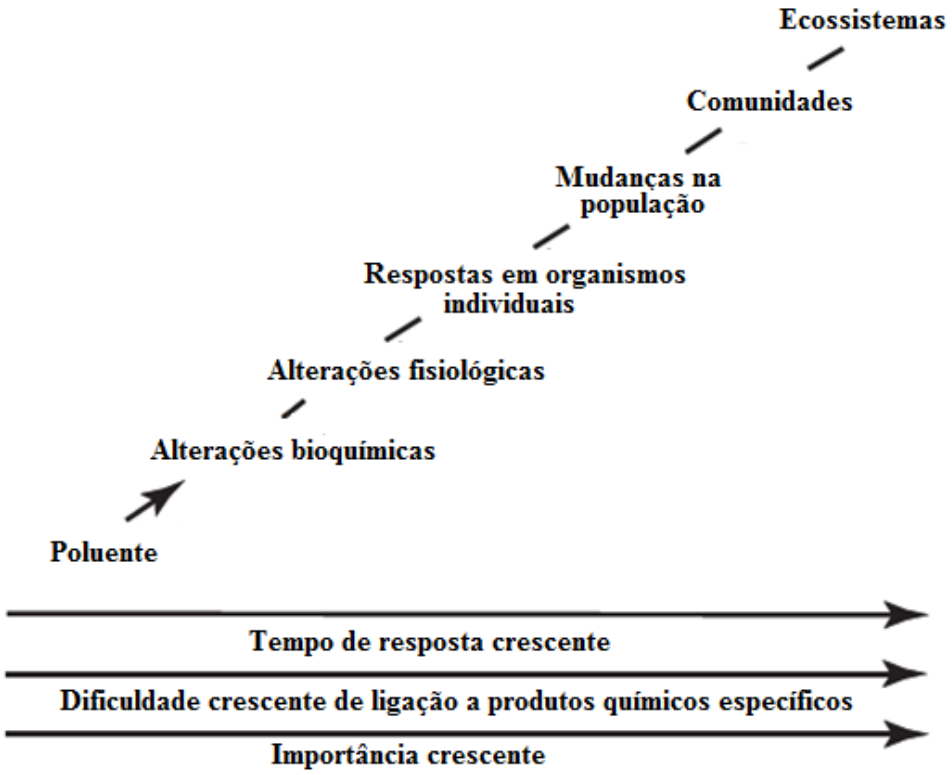
consequente descarte cada vez maior, gerando assim uma alta concentração de compostos xenobióticos nos ecossistemas com efeitos tóxicos desconhecidos. Além disso, diversos outros processos de carreamento, tais como o escoamento urbano e agrícola, descarga de esgoto doméstico e percolação, também levam substâncias potencialmente tóxicas e seus derivados para os corpos d'águas, gerando misturas complexas que muitas vezes apresentam potencial tóxico imprevisível.

Devido à essa quantidade de produtos químicos sintéticos em uso atualmente, a exposição a misturas de produtos químicos tóxicos resultantes de atividades humanas é comum em ambientes aquáticos, e seria logisticamente impossível avaliar empiricamente a toxicidade de cada combinação possível para as espécies aquáticas (LAETZ; HECHT; INCARDONA; COLLIER *et al.*, 2015). Essas misturas impactam as águas superficiais causando assim a "síndrome do fluxo urbano" em corpos d'água próximos às áreas urbanas, que é caracterizada por uma má qualidade da água, declínios na abundância e diversidade de espécies aquáticas, e à proliferação de taxa não nativos e tolerantes à poluição (WALSH; ROY; FEMINELLA; COTTINGHAM *et al.*, 2005). Atualmente, um dos principais desafios de conservação em bacias hidrográficas urbanas é o escoamento de águas pluviais urbanas, que devido à constante impermeabilização do solo com diversos tipos de coberturas artificiais, tais como ruas, estradas, calçadas e edificações, carreiam misturas complexas de produtos químicos tóxicos que causam problemas significativos relacionados à quantidade e qualidade das águas (LAETZ; HECHT; INCARDONA; COLLIER et al., 2015).

Dentre os componentes dessas misturas, e que se tornaram foco dos testes ecotoxicológicos ao longo do tempo, estão os hidrocarbonetos policíclicos aromáticos (HPA), devido ao alto potencial tóxico que possuem. Estes compostos tendem a se adsorver a partículas suspensas na coluna d'agua e são posteriormente integrados aos sedimentos, o que os tornam uma matriz complexa com aumento gradativo da toxicidade (INCARDONA; CARLS; TERAOKA; SLOAN *et al.*, 2005; WÖLZ; BRACK; MOEHLENKAMP; CLAUS *et al.*, 2010). Nesta matriz ainda é possível encontrar uma série de outras espécies de contaminantes como os metais pesados, dioxinas e pesticidas que são igualmente perigosos para os ecossistemas aquáticos (DA SILVA; CANTALICE; DO NASCIMENTO; SINGH *et al.*, 2017; SEVERO; MARINS; CEREZER; COSTA *et al.*, 2020; WEI; LEUNG; WONG; GIESY *et al.*, 2011). Esta mistura certamente aumenta o potencial danoso para os organismos que vivem diretamente relacionados com os sedimentos.

Nesse contexto, a partir da década de 1960, os problemas causados ao meio ambiente começaram a entrar na pauta de discussões da sociedade, e muito se deve à publicação do livro "Silent Spring", de Rachel Carson em 1962, considerado um dos livros mais importantes do século XX. Rachel tratou de forma didática essa problemática, conseguindo sensibilizar grande parte da população da época ao relatar estudos de inúmeros cientistas que demonstravam como o uso em larga escala de pesticidas organoclorados poderia causar efeitos tóxicos em espécies não alvo, tendo uma grande repercussão mundial (ROMÉO E GIAMBÉRINI, 2013). Com isso, em 1969 foi sugerido pela primeira vez o termo Ecotoxicologia, conceituado como um ramo da toxicologia que investiga os efeitos tóxicos de poluentes químicos nos ecossistemas, abrangendo desde sua entrada no ambiente até suas implicações nos organismos, populações e comunidades expostos (TRUHAUT, 1977). Esta abordagem vem sendo utilizada desde então em estudos de monitoramento ambiental. Atualmente, a Ecotoxicologia pode ser definida como a ciência que estuda os efeitos deletérios não intencionais causados por compostos químicos nos ecossistemas e organismos que os constituem (WALKER, 2005), tendo como paradigma a relação dose-resposta entre o contaminante ao qual o organismo foi exposto e o efeito biológico causado por essa exposição. Estes efeitos podem ser quantificados desde o nível subcelular, passando pelo indivíduo, até as comunidades e ecossistemas (RAND, 1995) (Figura 7).

Figura 7. Características fundamentais das respostas biológicas dos organismos aos contaminantes em diferentes níveis de organização biológica.




Fonte: (TRUHAUT, 1977) – adaptada.

Desse modo, testes ecotoxicológicos foram desenvolvidos para avaliar o potencial tóxico de substâncias isoladas, de misturas de substâncias e de amostras ambientais. No final dos anos 1960/70 começou-se a utilizar células de vertebrados para identificar e compreender os efeitos potenciais impostos aos humanos por produtos químicos em geral. Rapidamente esta abordagem foi aplicada para a avaliação da qualidade da água, sendo as células de mamíferos substituídas por de linhagens de células de peixes, como alternativa ao teste de letalidade de peixes, usado no monitoramento de efluentes industriais no final da década de 1980 (SCHIRMER, 2006). Posteriormente, testes com invertebrados foram sendo introduzidos, sendo o teste padronizado com *Daphnia* sp. (OECD, 2004) um dos mais utilizados atualmente. Estes testes com invertebrados são bem aceitos na comunidade científica devido ao baixo custo de realização e por esses animais possuírem um ciclo de vida curto e tamanho reduzido (ARAÚJO-CASTRO; SOUZA-SANTOS; TORREIRO; GARCIA, 2009). A CPRH vem realizando testes ecotoxicológicos, desde 2004, em amostras de águas dos rios de Pernambuco utilizando *Daphnia magna*. Hoje, cerca de 35% das estações de coletas da sua rede de monitoramento, principalmente em estações de captação da Companhia Pernambucana e Saneamento (COMPESA), são monitoradas ecotoxicologicamente com o teste padronizado com *D. magna*, que consiste em expor indivíduos jovens com menos de 24 horas de idade por 48 horas a amostras de água, seguida da quantificação das taxas de imobilização dos indivíduos (OECD, 2004).

Entretanto, apesar dos testes ecotoxicológicos com invertebrados terem uma boa aceitação na comunidade científica, testes utilizando peixes vêm sendo utilizados cada vez mais na análise de risco e monitoramento ambiental. Os peixes ocupam hoje uma posição de destaque no campo da ecotoxicologia, provavelmente mais do que qualquer outra classe de organismos (Di Giulio, 2008), fazendo com que seu uso no monitoramento ambiental seja cada vez mais necessário, e se tornando uma ferramenta indispensável para avaliar a saúde desses ecossistemas. Desse modo, o monitoramento das águas das bacias hidrográficas de Pernambuco realizado pela CPRH e APAC, baseado principalmente em dados físico-químicos, e com alguns poucos testes ecotoxicológicos, pode estar fornecendo informações incompletas sobre a saúde desses ecossistemas e o impacto de contaminantes na saúde da biota residente.

## 1.4 TESTES ECOTOXICOLÓGICOS COM PEIXES

No final da década de 1980 e início de 1990 o monitoramento de ambientes aquáticos era baseado exclusivamente na quantificação química da água (ROMÉO; GIAMBÉRINI, 2012). Entretanto, esse tipo de monitoramento não avalia os possíveis efeitos deletérios na biota

residente e que está exposta aos contaminantes ali presentes. Desta forma, foram criadas ferramentas que avaliam como a contaminação química prejudica as comunidades dos ecossistemas atingidos (AMIARD-TRIQUET; AMIARD, 2013). Frente a essa problemática, animais aquáticos começaram a ser utilizados em testes ecotoxicológicos, principalmente moluscos (BAYNE; MOORE; WIDDOWS; LIVINGSTONE *et al.*, 1979; HAGGER; DEPLEDGE; GALLOWAY, 2005), invertebrados (CARVALHO; ZANARDI; BURATINI; LAMPARELLI *et al.*, 1998; GALLOWAY; DEPLEDGE, 2001; GALLOWAY; MILLWARD; BROWNE; DEPLEDGE, 2002; OECD, 2004; RÉGIS; SOUZA-SANTOS; YOGUI; MORAES *et al.*, 2018; UNTERSTEINER; KAHAPKA; KAISER, 2003) e peixes (ALVES; MARIZ; PAULO; CARVALHO, 2017; CARLS; HOLLAND; LARSEN; COLLIER *et al.*, 2008; CARVALHO; TILLITT, 2004; INCARDONA; CARLS; TERAOKA; SLOAN *et al.*, 2005).

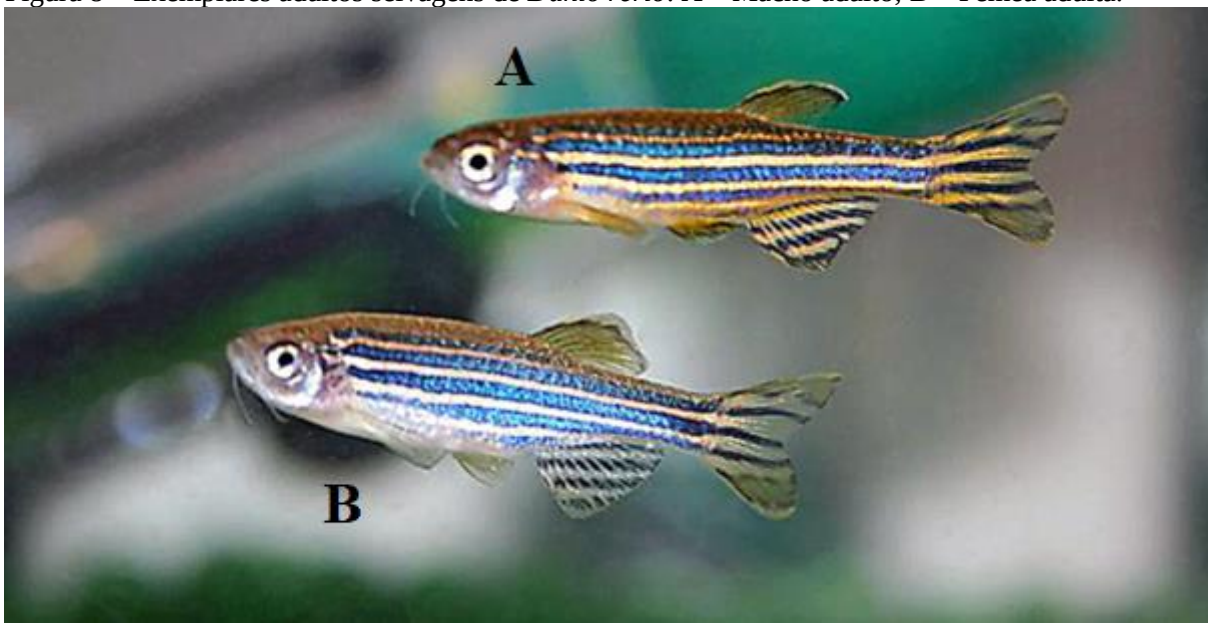
Atualmente é conhecida uma biodiversidade de aproximadamente 32.000 espécies de peixes que habitam oceanos, estuários, rios, lagos e riachos, sendo uma diversidade maior que a soma de todas as outras espécies de vertebrados conhecidas (HELFMAN, 2013; NELSON; GRANDE; WILSON, 2016). Os peixes têm relevância social, econômica e ecológica, exercendo as mais variadas funções dentro dos ecossistemas aquáticos, apresentam respostas claras sobre a saúde do ambiente e têm sido utilizados com destaque ao longo da história da ecotoxicologia (DI GIULIO, R.; HINTON, D., 2008). Além disso, os peixes, em muitos casos, funcionam como organismos-sentinela, que são organismos residentes nos ambientes potencialmente contaminados capazes de revelar precocemente a presença e a toxicidade de poluentes químicos (BERTHET, 2013). Com isso, parâmetros ecotoxicológicos avaliados em peixes se tornaram ferramentas importantes para a análise do impacto e do risco ambiental dos contaminantes químicos, sendo hoje o monitoramento biológico da poluição baseado na avaliação de parâmetros ecotoxicológicos, com efeitos letais e subletais em peixes, uma ferramenta indispensável para o monitoramento aquático (ROMÉO; GIAMBÉRINI, 2012).

## 1.5 ZEBRAFISH COMO MODELO BIOLÓGICO

O peixe *Danio rerio*, conhecido popularmente como paulistinha ou “zebrafish”, é um peixe teleósteo de água doce, pertencente à família Cyprinidae, originário do sul asiático, sendo de fácil criação e manutenção em cativeiro. Indivíduos adultos vivem de dois a quatro anos, possuem comprimento em torno de quatro centímetros, em sua forma selvagem possui cauda arredondada e nadadeiras pouco alongadas, reprodução goocorística com fertilização externa dos ovos. Exibem dimorfismo sexual aparente onde os machos são mais delgados apresentando coloração alaranjada na maturidade sexual, e as fêmeas apresentam uma região ventral maior

com coloração prateada podendo produzir 200 ovos férteis e uma única desova(SIEBEL, 2015) (figura 8). O zebrafish é hoje um dos principais modelos biológicos de vertebrados utilizado para o desenvolvimento de metodologias de biomonitoramento e avaliação de parâmetros ecotoxicológicos em laboratório a nível letal e subletal, incluindo biomarcadores morfológicos e comportamentais (EMBRY; BELANGER; BRAUNBECK; GALAY-BURGOS *et al.*, 2010). Estes parâmetros são avaliados nas suas fases iniciais de desenvolvimento embrio-larval e aplicados no monitoramento de águas superficiais (VANLANDEGHEM; MEYER; COX; SHARMA *et al.*, 2012).

Figura 8 – Exemplares adultos selvagens de *Danio rerio*. A – Macho adulto; B – Fêmea adulta.




Fonte: brainn.org.br

Dentre as características que tornaram o zebrafish um dos principais modelos biológicos estão o baixo custo de manutenção e reprodução em laboratório, o rápido desenvolvimento, ovos e embriões translúcidos e um vasto repertório de ferramentas desenvolvidas que descrevem atributos importantes da biologia desta espécie (SIEBEL, 2015). Além disso, por ter seu genoma mapeado, o *D. rerio* também se tornou um modelo amplamente utilizado em variados experimentos toxicológicos no âmbito da biomedicina e farmacologia, pois apresenta 71% de genes ortólogos com a espécie humana (HOWE; CLARK; TORROJA; TORRANCE *et al.*, 2013), facilitando pesquisas no âmbito molecular (DAVIS, 2004; GRUNWALD, 2013).

Dentre os testes padronizados com o zebrafish, o “fish embryo acute toxicity test (FET)”, previsto na norma OECD 236 de 2013, é hoje um dos mais aceitos e utilizados na avaliação da toxicidade de contaminantes isolados ou em misturas, inclusive de amostras ambientais. Esse teste de toxicidade surgiu como uma alternativa aos testes utilizando peixes jovens e adultos, a

exemplo da norma OECD 203, e consiste em determinar a toxicidade aguda ou letal de produtos químicos em estágios embrionários do zebrafish (LAMMER; CARR; WENDLER; RAWLINGS *et al.*, 2009). Entretanto, um teste de toxicidade baseado apenas em mortalidade pode mascarar impactos menos drásticos na biota exposta, o que gera uma necessidade de testes mais sensíveis e que avaliem efeitos tanto letais quanto subletais dos indivíduos expostos aos contaminantes.

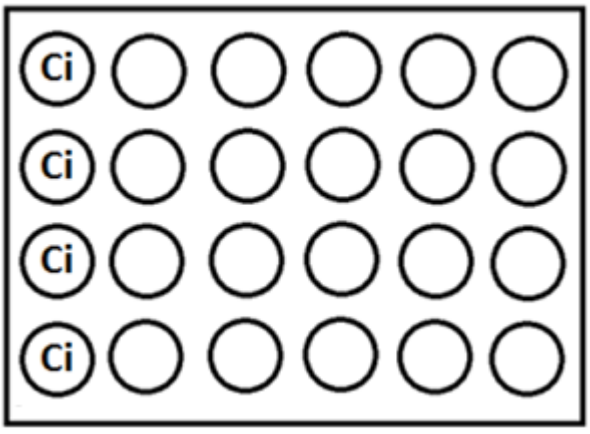
### 1.5.1 General Morphology Score (GMS)

O General Morphology Score (GMS), ou índice de desenvolvimento morfológico (IDM), consiste numa pontuação dada para alguns "endpoints", ou marcos morfológicos, observados durante o desenvolvimento embriolarval do zebrafish entre 0-96 horas pós-fertilização (hpf). Cada indivíduo é avaliado cuidadosamente ao longo das 96h de exposição a fim de verificar alterações no desenvolvimento normal dos marcos morfológicos. Dessa forma, espera-se que embriões expostos a algum contaminante apresente alteração e/ou retardo no seu desenvolvimento, e a sua pontuação ao final das 96h de exposição seja menor do que a pontuação de um embrião saudável que se desenvolve perfeitamente (BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.*, 2015).

### 1.5.2 Desenho experimental

Como modelo de exposição foi adotada a norma (OECD, 2013), que consiste na exposição dos embriões a contaminantes em placas de poliestireno de 24 poços, por 96 horas, onde vinte indivíduos são expostos ao contaminante a ser testado e quatro são mantidos em água limpa (controle interno) (figura 9).

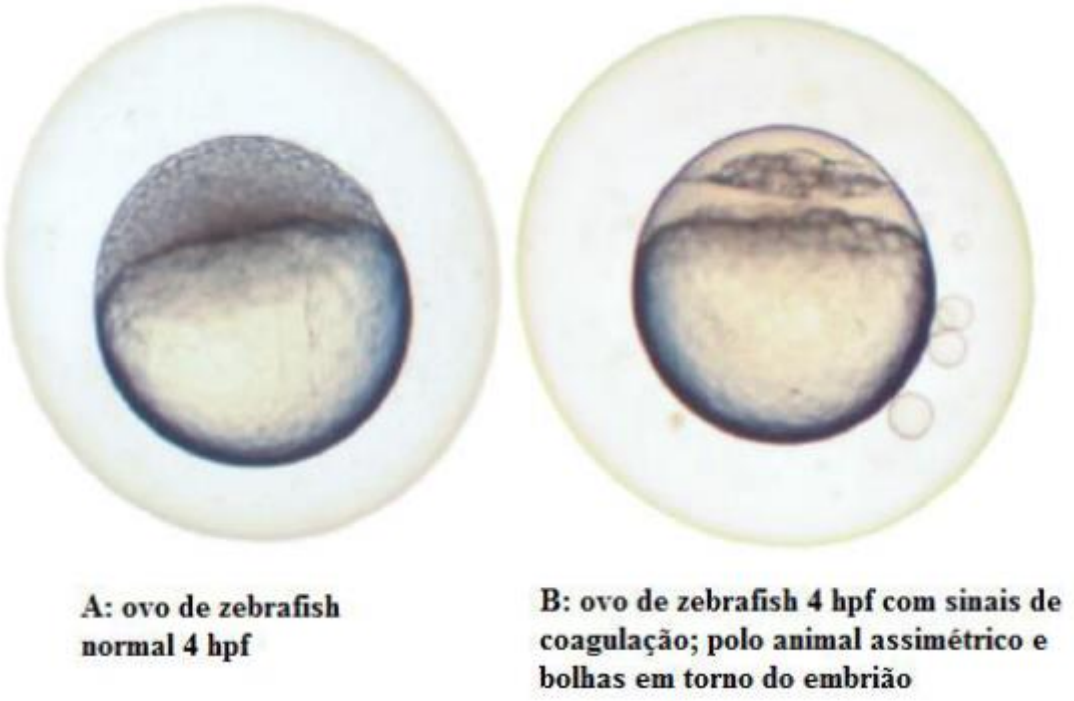
Figura 9. Esquema da placa de exposição utilizada no GMS, com 20 poços destinados aos embriões expostos ao contaminante e 4 utilizadas para o controle interno (CI).




Fonte: (OECD, 2013)

Na manhã de início dos testes, preferencialmente, entre 3 e 4 hpf, os ovos fertilizados sem sinais de coagulação (córion redondo e transparente) devem ser previamente selecionados. Os ovos que apresentarem os primeiros sinais de coagulação, como formação de bolhas no ovo (especialmente em torno do polo animal), assimetria do polo animal ou do vitelo, devem ser descartados (BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.*, 2015) (figura 10).

Figura 10. Ovo de zebrafish normal 4 hpf (A) e ovo com sinais de coagulação (bolhas e assimetria) 4hpf (B).



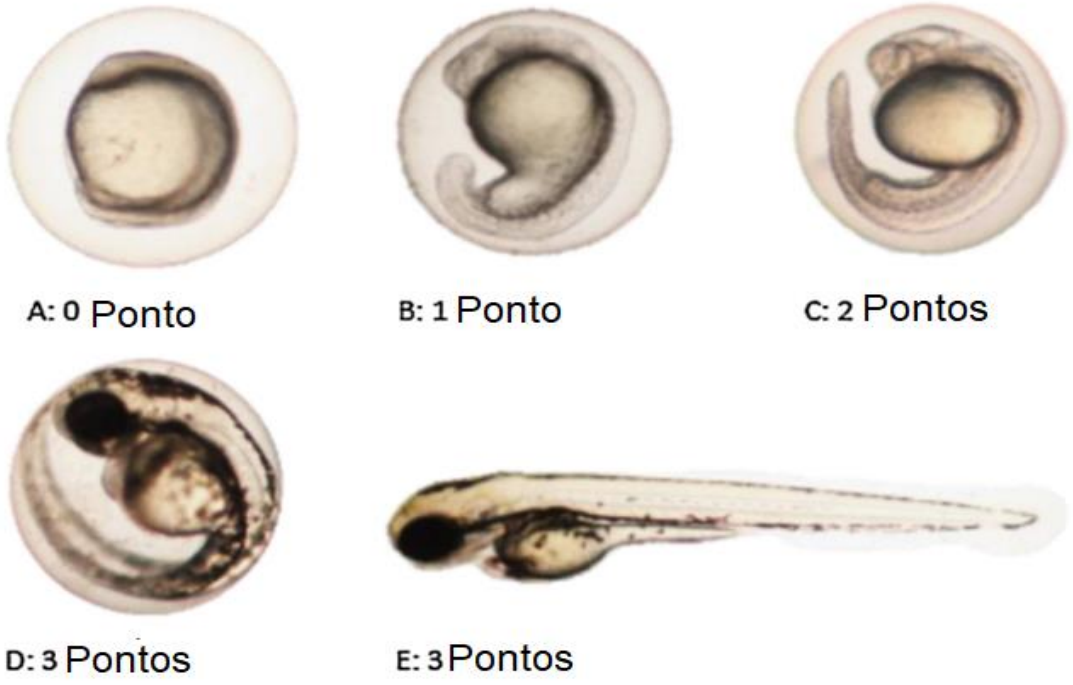

Fonte: (Beekhuijzen, 2015).

Após a montagem das placas com os embriões fertilizados e saudáveis, o teste é iniciado, sendo feitas avaliações do desenvolvimento embriolarval a 24, 48, 72 e 96 horas de exposição em um estereomicroscópio. Todos os desvios em relação ao esperado, como por exemplo não desenvolvimento da boca ou das nadadeiras peitorais, resultarão num menor número de pontos, gerando um índice GMS mais baixo (BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.*, 2015). Uma larva saudável e com seu desenvolvimento completo receberá uma pontuação máxima de 18; portanto, qualquer valor abaixo disso será indicativo de falha ou retardo no desenvolvimento embriolarval. Nas primeiras 24h de exposição são observados e pontuados o destacamento da cauda, a formação dos somitos, o desenvolvimento dos olhos, movimentos e circulação.

### 1.5.3 Destacamento da cauda

Ao completar 24 hpf os embriões que apresentarem cauda destacada até a extensão do saco vitelínico recebem um ponto (Figura 11B). Um ponto adicional é dado quando a cauda se destaca além da extensão do saco vitelínico (Figura 11C). O descolamento total da cauda a partir de 48 hpf também gera adição de mais um ponto (Figura 11D) (BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.*, 2015).

Figura 11. Descolamento da cauda do embrião de zebrafish entre 12 e 72 hpf evidenciando A- indivíduo em torno de 10 hpf; B- indivíduo com cerca de 16 hpf; C- indivíduo com cerca de 24 hpf; D- indivíduo com cerca de 48 hpf e D- indivíduo com cerca de 72 hpf.

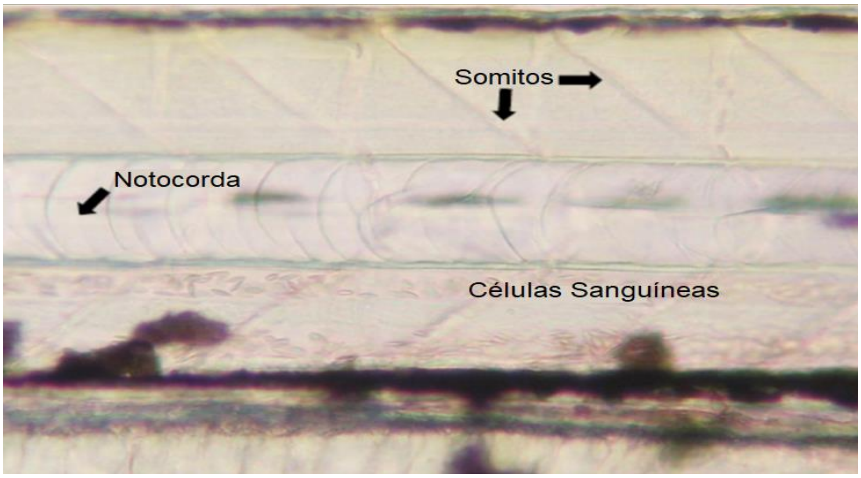



Fonte: (Beekhuijzen, 2015).

### 1.5.4 Formação dos somitos

A formação de somitos no embrião é pontuada com 0 ou 1, sendo 1 quando o somito é visível e 0 quando não estão visíveis no segundo dia após a fertilização (24 hpf a 30 hpf) (BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.*, 2015) (Figura 12). Os somitos se diferenciarão em Miótomos (originando a musculatura dorsal) e Esclerótomo (originando as vertebras em volta da notocorda) (BEEKHUIJZEN et al., 2015).

Figura 12. Região dorsal do embrião do zebrafish. As setas evidenciam a presença dos somitos.

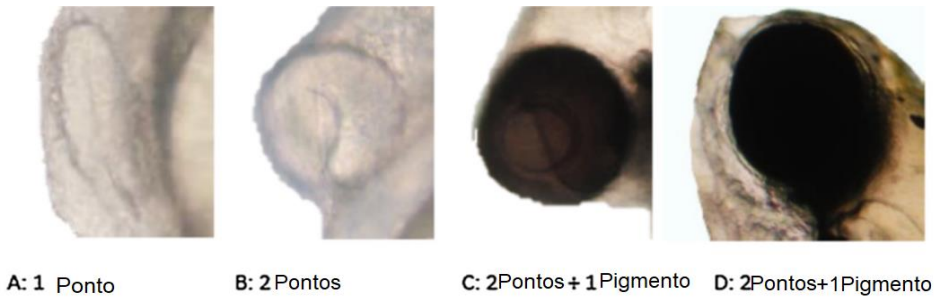



Fonte: (Beekhuijzen, 2015).

### 1.5.5 Desenvolvimento dos olhos

Esta característica é observada durante 48 h do teste. Durante as primeiras 24 horas, o embrião recebe pontuação 0 quando não há olhos visíveis. Quando os olhos são visíveis, mas planos, recebe pontuação 1 (Figura 13A). Após o aparecimento de uma esfera, o desenvolvimento do olho recebe 2 pontos (Figura 13B). A partir das 48 horas após a exposição, se os olhos apresentarem pigmentação (figura 13C, D), será acrescido 1 ponto para esse "*endpoint*" (BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.*, 2015) (figura 13).

Figura 13. Desenvolvimento dos olhos de embrião do zebrafish 96hpf. A e B - desenvolvimento normal nas primeiras 24 horas; C - desenvolvimento normal 48hpf; D - desenvolvimento normal 96 hpf.




Fonte: (Beekhuijzen, 2015).

### 1.5.6 Movimento

O movimento receberá 1 ponto 24 hpf quando o embrião/larva mover a cauda ou o corpo inteiro. Após o desenvolvimento das nadadeiras peitorais, o movimento de uma ou de ambas será considerado como movimento, garantindo assim a pontuação normal para esse marco. Caso não haja movimento espontâneo pode-se gerar um estímulo com pipeta pasteur de plástico, mas sem exercer contato com o embrião, apenas com a inserção de ar no volume da solução do poço.

Caso não haja movimento após essa ação o indivíduo receberá a pontuação 0 (zero) (BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.*, 2015).

### 1.5.7 Circulação

A circulação sanguínea receberá 1 ponto quando for observada em algumas células 24 hpf, ou quando começam os batimentos cardíacos e a circulação fica mais evidente em 48 hpf (BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.*, 2015).

No período seguinte (48 horas pós exposição) os marcos do desenvolvimento que são observados são o batimento cardíaco, a pigmentação da cabeça e do corpo, e a pigmentação da cauda.

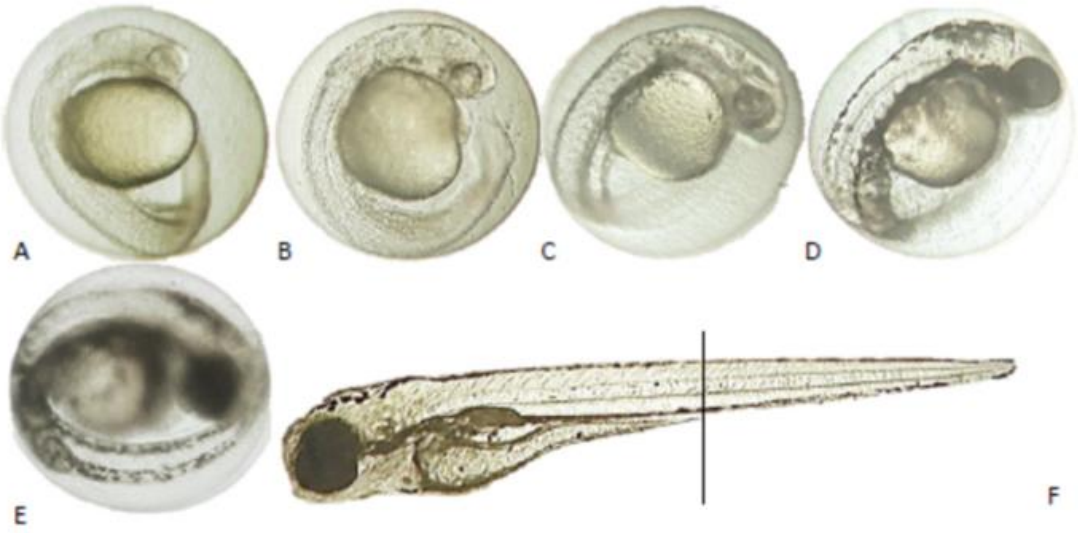
### 1.5.8 Batimento cardíaco

O batimento cardíaco é pontuado com 0 ou 1 de acordo com a ausência ou presença, respectivamente. Para melhor visualização do batimento cardíaco sugere-se manter a observação por pelo menos 1 minuto, até que um ou mais batimentos cardíacos distintos sejam anotados. Caso não seja detectado, o batimento cardíaco é considerado ausente, indicando assim a morte do embrião ou larva (BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.*, 2015).

### 1.5.9 Pigmentação da cabeça e do corpo, e da cauda

A pigmentação da cabeça e do corpo, bem como da cauda, é pontuada com 1 ponto quando presente e 0 pontos quando ausente. A pigmentação da cabeça e do corpo é avaliada como presente quando o pigmento é visível entre a cabeça e a extremidade do saco vitelínico, e a pigmentação da cauda é avaliada como presente quando o pigmento é visível a partir da extremidade da extensão do saco vitelínico para a cauda (figura 14) (BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.*, 2015).

Figura 14. Pigmentação da cabeça e do corpo, e da cauda. A: pigmentação ausente. B, C, D: pigmentação visível para a cabeça e o corpo. E: pigmentação da cauda. F: diferenciação entre corpo e cauda.



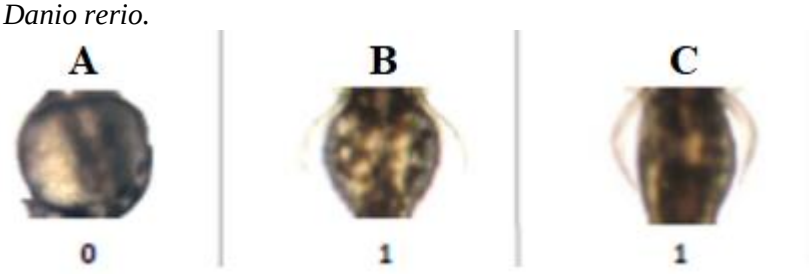

Fonte: (Beekhuijzen, 2015).

Após 72 horas de exposição outros três "*endpoints"* são observados no GMS, o desenvolvimento das nadadeiras peitorais, a protrusão da boca e a eclosão. Dessa forma, após a inserção desses três marcos do desenvolvimento, um embrião com seu desenvolvimento normal possui uma pontuação total de 15 pontos.

### 1.5.10 Nadadeira peitoral

As nadadeiras peitorais originam-se aproximadamente entre o coração e o fígado, estendendo-se posteriormente ao longo de cada lado da larva. A pontuação referente às nadadeiras será dada mesmo que a larva apresente apenas uma nadadeira. Dessa forma, uma larva com uma ou duas nadadeiras receberá 1 ponto, e uma larva sem ambas as nadadeiras receberá a pontuação 0 (zero) (BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.*, 2015) (figura 15).

Figura 15. Desenvolvimento normal da nadadeira peitoral em 48 hpf (A), 72 hpf (B) e 96 hpf (C) em embrião/larva de *Danio rerio.*

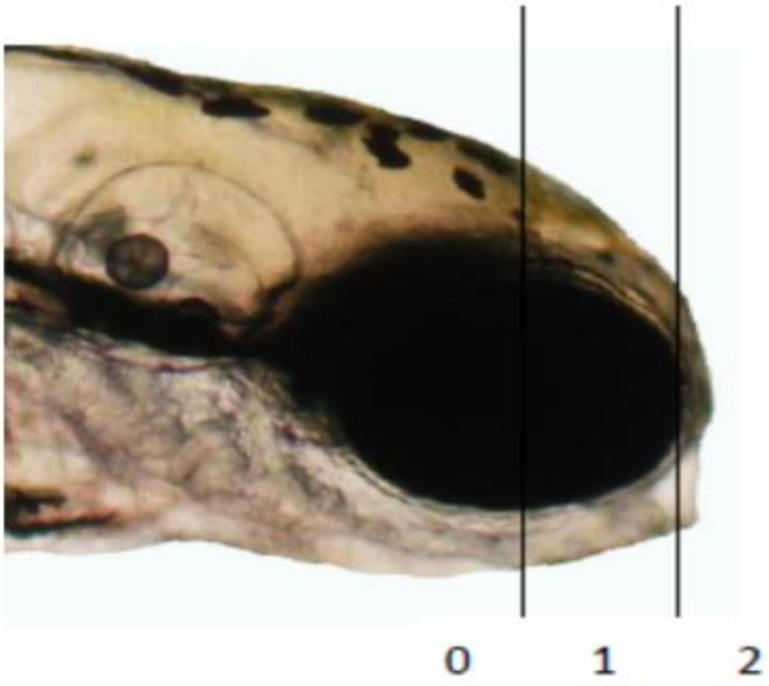



Fonte: (Beekhuijzen, 2015).

### 1.5.11 Boca protrusível

Esse *endpoind* recebe pontuação em dois tempos amostras, sendo em 72 hpf e em 96 hpf. Será acrescido 1 ponto em 72 hpf e quando for observado que a mandíbula inferior do embrião/larva atinge toda a extensão do olho, e mais 1 ponto em 96 hpf quando a mandíbula inferior atinge a frente do olho ou mais. Uma boca protrusa está ausente (pontuação é 0) quando não é observada mandíbula inferior ou quando a mandíbula inferior atinge apenas cerca de metade do olho (ao nível da pupila) (BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.*, 2015) (Figura 16).

Figura 16. Desenvolvimento normal da boca de larva de *D. rerio.*

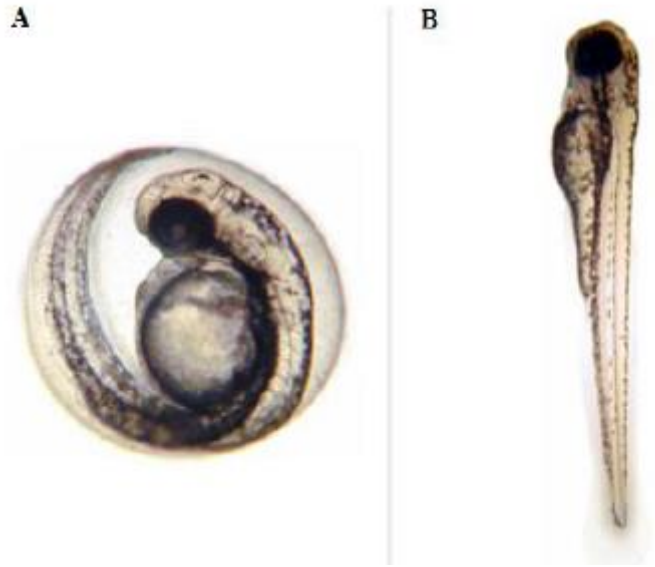



Fonte: (Beekhuijzen, 2015).

### 1.5.12 Eclosão

A eclosão é considerada aplicável quando o corpo do embrião não está mais cercado pelo córion e recebe pontuação 1, sendo considerado larva a partir deste momento (figura 17). Quando há eclosão antes ou depois das 72 hpf não haverá pontuação, mas essa observação deverá ser anotada, pois o tempo de exposição da larva (sem o córion) ao contaminante poderá influenciar nos efeitos deletérios observados (BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.*, 2015).

Figura 17. Embrião não eclodido envolto pelo córion (A); Larva eclodida sem a proteção do córion (B).




Fonte: (Beekhuijzen, 2015).

Por fim, em 96 hpf, acrescenta-se os dois últimos *"endpoints"* para análise do GMS (extensão do saco vitelínico quase vazio e insuflamento da bexiga natatória), que pontuarão juntamente com o segundo estágio do desenvolvimento da boca, totalizando 13 marcos do desenvolvimento morfológicos a serem analisados. Desse modo, em 96 hpf serão acrescidos até 3 pontos para cada indivíduo, totalizando uma pontuação máxima de 18 para uma larva com desenvolvimento normal.

### 1.5.13 Extensão do saco vitelínico

A avaliação deste *"endpoint"* é feita observando se a espessura do saco vitelino não excede o coração, permitindo a visualização de um espaço onde a bexiga natatória estará situada (BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.*, 2015) (figura 18). Caso a espessura do saco vitelínico não exceda o coração, a larva receberá 1 ponto, caso o saco vitelínico não tenha sido completamente absorvido e sua espessura esteja excedendo o coração, a larva receberá pontuação zero.

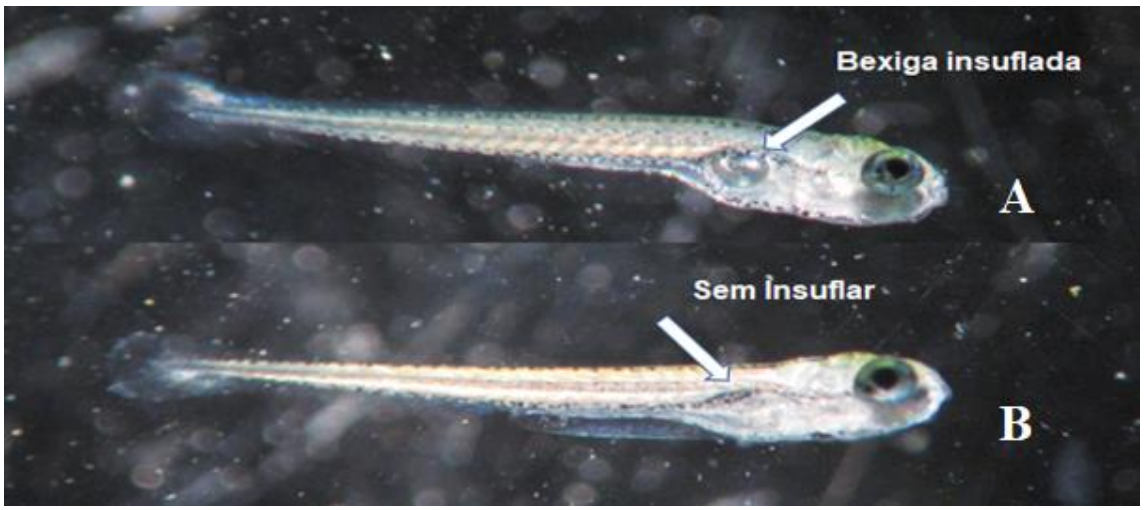
Figura 18. Extensão do saco vitelínico e espaço para insuflamento da bexiga entre as 72 e 96 hpf. A- 96hpf com o saco vitelínico na altura no coração e B- 72 hpf com o saco vitelínico excedendo a linha do coração


Fonte: (Beekhuijzen, 2015).

### 1.5.14 Insuflamento da bexiga natatória

A bexiga natatória (figura 19) tende a ficar insuflada entre 72 hpf e 96 hpf (HAGENAARS; STINCKENS; VERGAUWEN; BERVOETS *et al.*, 2014), sendo imprescindível que haja a absorção completa do saco vitelínico de forma que a abertura do espaço onde se posiciona a bexiga fique disponível. Na proposta do GMS (BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.*, 2015) não há previsão da avaliação deste marco morfológico. Entretanto, devido à sua importância na viabilidade das larvas, este *endpoint* será acrescentado ao GMS de forma que ao final das 96hpf uma larva saudável alcance a pontuação máximo de 18.

Figura 19. Larva de *Danio rerio* saudável em 96 hpf. A – larva com bexiga natatória insuflada; B –larva com bexiga natatória não insuflada.




Fonte: (Mariz, 2020).

### 1.5.15 Análise estatística do GMS

Após a análise de todos os marcos do desenvolvimento e computado as devidas pontuações para cada embrião/larva, é necessário realizar uma análise de variância (ANOVA) para verificar se o retardo observado no desenvolvimento de alguns embriões e larvas é estatisticamente significativo. Entretanto, mesmo que não haja diferença estatística entre alguma amostra testada e o controle, qualquer falha ou retardo no desenvolvimento pode ser prejudicial e decisivo para a sobrevivência do embrião/larva. Dessa forma, sugere-se que também seja feito um levantamento do percentual de ocorrência de cada anomalia para cada amostra ou concentração testada.

## 1.6 OBJETIVOS

Esta tese foi dividida em dois grandes objetivos e apresentados na forma de manuscritos.

## 1.7 MANUSCRITO I

Zebrafish as a biological model for assessing water quality along ten tropical hydrographic river basins (Revista pretendida: Environmental Monitoring and Assessment).

### 1.7.1 Objetivo Geral

Investigar se o estágio inicial de desenvolvimento *D. rerio* é um modelo biológico viável para avaliar a toxicidade das águas superficiais de rios tropicais.

### 1.7.2 Objetivo Específico

- Avaliar a toxidade para embriões e larvas de *D. rerio* das 55 amostras coletadas em 10 bacias hidrográficas de Pernambuco

- Verificar a possível correlação entre **os** padrões de toxicidade observados, os índices de qualidade de água (IQA e IET) e contaminantes químicos.

## 1.8 MANUSCRITO II

Contamination and Toxicity of Surface Waters Along Rural and Urban Regions of the Capibaribe River in Tropical Northeastern Brazil (Environmental Toxicology and Chemistry - volume 40, number 11—pp. 3063–3077, 2021).

### 1.8.1 Objetivo geral

Avaliar a toxicidade das águas superficiais do Rio Capibaribe para embriões e larvas do *D. rerio* e verificar a contaminação por HPA dessas amostras.

### 1.8.2 Objetivos Específicos

- Realizar exposição controlada de embriões e de larvas de *D. rerio* a amostras de águas superficiais de 10 estações de coleta do Rio Capibaribe, com foco em *"endpoints"* letais e subletais durante o período inicial de desenvolvimento.

- Investigar a presença e concentração dos 16 HPA prioritários segundo a USEPA.

# 2 MANUSCRITO I

Zebrafish as a biological model for assessing water quality along ten tropical hydrographic river basins.

## 2.1 ABSTRACT

Tropical rivers are the main destinations for tailings from urban, industrial and agricultural activities. The present study aimed to investigate if early stages of zebrafish (*Danio rerio*) development is a viable biological model to assess the toxicity of surface waters of tropical rivers, and whether that toxicity could be correlated to standard water quality indexes. Zebrafish embryos were exposed to surface water samples from 55 sites from 10 hydrographic basins of rivers in northern (Group 1), middle (Group 2) and southern (Group 3) Pernambuco State, northeastern Brazil. Lethality rates, sublethal toxicity quantified as delays in embryo-larval development based on the general morphology score (GMS), and frequencies of developmental abnormalities were analyzed. Significant mortality was observed in 7 of the 10 analyzed basins. The mortality rate varied between 10% and 40%. The GMS indicated significant delay in embryo-larval development in 50% of the collected surface water samples. 41% of surface water samples from Group 1 indicated significant sublethal toxicity, with an average GMS of 17.0, varying between 15.4 and 17.7, and were classified at an intermediate level of ecotoxicity. Highest toxicity was detected in Group 2 basins within Recife metropolitan area, where 61% of the surface water samples caused sublethal toxicity, with lowest average GMS of 16.4, varying between 14.1 and 17.6. 47% of surface water samples from group 3 basins caused sublethal toxicity in the study, with an average GMS of 17.1, ranging from 16.2 to 17.6. Most frequent developmental abnormalities included non-inflation of the swim bladder, delayed hatching, non-protrusion of the mouth and blood stasis, with emphasis on the non-inflation of the swim bladder, which showed a frequency of 100% in some sampling stations. In addition, highest frequencies of blood stasis were detected in samples with highest $NH_3$ concentrations, corroborated by a positive correlation suggesting the existence of a causal relationship. A significant correlation was detected between water quality indexes and GMS, as well as sublethal abnormalities during zebrafish development, with a greater toxic effect being observed in samples collected in areas of greater urban density and greater contamination by domestic sewage. This study demonstrates that the early stages of the zebrafish is a viable ecotoxicological model to assess the toxicity of surface waters and can contribute to a better

understanding between the chemical composition and the adverse effects suffered by fish early life stages in tropical rivers.

## 2.2 INTRODUCTION

Synthetic chemicals used in pesticides, pharmaceuticals, residential and industrial settings have been increasing in both variety and volume at a more rapid rate than other stressors, including $CO_2$ emissions and nutrient pollution (BROOKS; SABO-ATTWOOD; CHOI; KIM *et al.*, 2020). Freshwater aquatic ecosystems are the main destinations for chemicals from urban activities, agriculture and industries, which are the main contributors to eutrophication and changes in the chemical composition of these habitats (TRIPATHI; HUSAIN; AHMAD; HASAN *et al.*, 2021)

It is estimated that around 100,000 molecules are introduced into aquatic environments from different sources, where mixtures of several classes of contaminants are present concurrently, and can interact additively, synergistically or antagonistically (AMIARD-TRIQUET, 2015). Chemical analyses of these environments provide only information about the nature of a limited number of target substances and their concentrations in the environment, disregarding their bioavailability and potential harmful effects on biota. The responses of whole organism bioassays to surface water samples are caused by the combined action of mixtures of all the bioavailable compounds and their metabolites present, thus overcoming the limitations imposed by the chemical analysis of a limited number of target compounds (BRACK; AIT-AISSA; BURGESS; BUSCH *et al.*, 2016; DE BAAT; KRAAK; VAN DER OOST; DE VOOGT *et al.*, 2019). In view of this, ecotoxicological tests using biological models to identify the toxicity of unknown substances and new substances, or environmental samples has become an important tool in the risk assessment for aquatic ecosystems and human health. Fishes represent approximately half of the vertebrate biodiversity and have social, economic and ecological relevance. Water quality has been associated with fish health for more than a century, and the development of methods using fishes as biological models for environmental monitoring has been gaining prominence throughout the history of Ecotoxicology (DI GIULIO; HINTON, 2008; MINIER; RACHID; LEPAGE; AMIARD TRIQUET *et al.*, 2015).

Acute toxicity tests with vertebrates are an integral part of identifying environmental hazards and assessing the risks of chemicals to monitor the quality of effluents and surface waters (STRÄHLE; SCHOLZ; GEISLER; GREINER *et al.*, 2012). However, the acute lethal effect test on juvenile and adult fish is not compatible with most animal welfare legislation (LAMMER; CARR; WENDLER; RAWLINGS *et al.*, 2009), which led to other biological

model options being adopted as an alternative to lethal fish testing. An important approach to replace these tests was the use of fish cell cultures, but an underestimation of in vivo toxicity by cell based ecotoxicity assays by up to three orders of magnitude became a major disadvantage (SCHIRMER, 2006). Another ecotoxicological test that gained importance was the immobility test with *Daphnia sp.* (OECD, 2004). The test consists of exposing young individuals of *Daphnia* sp less than 24 hours old for 48 hours to water samples, followed by the quantification of immobilization rates of the individuals (OECD, 2004). However, the need for a standardized ecotoxicological test with fish that respected the 3R's (RUSSELL; BURCH, 1960) principle was still necessary. With that, in May 2013, the acute toxicity test with zebrafish *Danio rerio* fish embryos (FET) appeared (OECD, 2013). However, due to the acute lethality caused by pollutants having become a rare event, there has been a growing concern about the sublethal effects of chemicals emitted to the environment at low concentrations. Zebrafish early stages, i.e. embryos and early larvae, have become a very successful vertebrate model to assess the lethal and sublethal toxic effects on aquatic organisms and to subsequently perform a valid ecosystem monitoring (CRISTIANO; LACCHETTI; MANCINI; CORTI *et al.*, 2019).

The General Morphology Score (GMS) based on the quantification of delays in the embryonic development of zebrafish *Danio rerio* proved to be an important tool in the evaluation of sublethal toxicity of contaminants to embryos and larvae during the first 96 hours of life (BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.*, 2015). The GMS consists of observing morphological developmental milestones every 24 hours of exposure, which begins with the formation of somite and goes on to the development of other main structures, such as the development of pectoral fins and mouth, and inflation of the swimming bladder. For each developmental milestone reached, the individual receives increasing scores totaling 17 points for a normal development (BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.*, 2015). On the other hand, development milestones that have not been met generate reductions in the GMS, which correspond to a certain degree of development delay (BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.*, 2015). Concomitantly with the use of GMS to assess toxicity in aquatic ecosystems impacted by human activities, the use of water quality indexes, such as the Water Quality Index (WQI) from the National Sanitation Foundation (NOORI; BERNDTSSON; HOSSEINZADEH; ADAMOWSKI *et al.*, 2019) and the eutrophication oriented Trophic State Index (TSI), can be used in monitoring programs to assess possible sources of contamination in these environments (PIRES; TUCCI; CARVALHO; LAMPARELLI, 2015).

In northeastern Brazil, within the state of Pernambuco, the hydrographic basins of the coastal rivers include a total area of approximately 13,180.00 $km^2$ and serves a population of approximately 4,542,200 inhabitants (CPRH, 2018). These basins are under pressure from various anthropogenic activities, such as agricultural activities for sugarcane plantations, textile and chemical industries, weaving and urban activities. Runoff from rural and urbanized areas produces increasing amounts of waste to the surface waters of these tropical rivers, which serve as a system for the disposal of this waste. Among these residues, nitrogenous materials stand out for causing an increase in the production of ammonia in these environments, which can be more toxic to fish in its unionized $NH_3$ form (DYER; PENG; MCAVOY; FENDINGER *et al.*, 2003; RANDALL; TSUI, 2002). Thus, this study aimed to investigate if early stages of zebrafish (*Danio rerio*) development is a viable biological model to assess the toxicity of surface waters of tropical rivers, and whether that toxicity could be correlated to standard water quality indexes and chemical contaminants.

## 2.3 MATERIALS AND METHODS

### 2.3.1 Study area and sampling sites

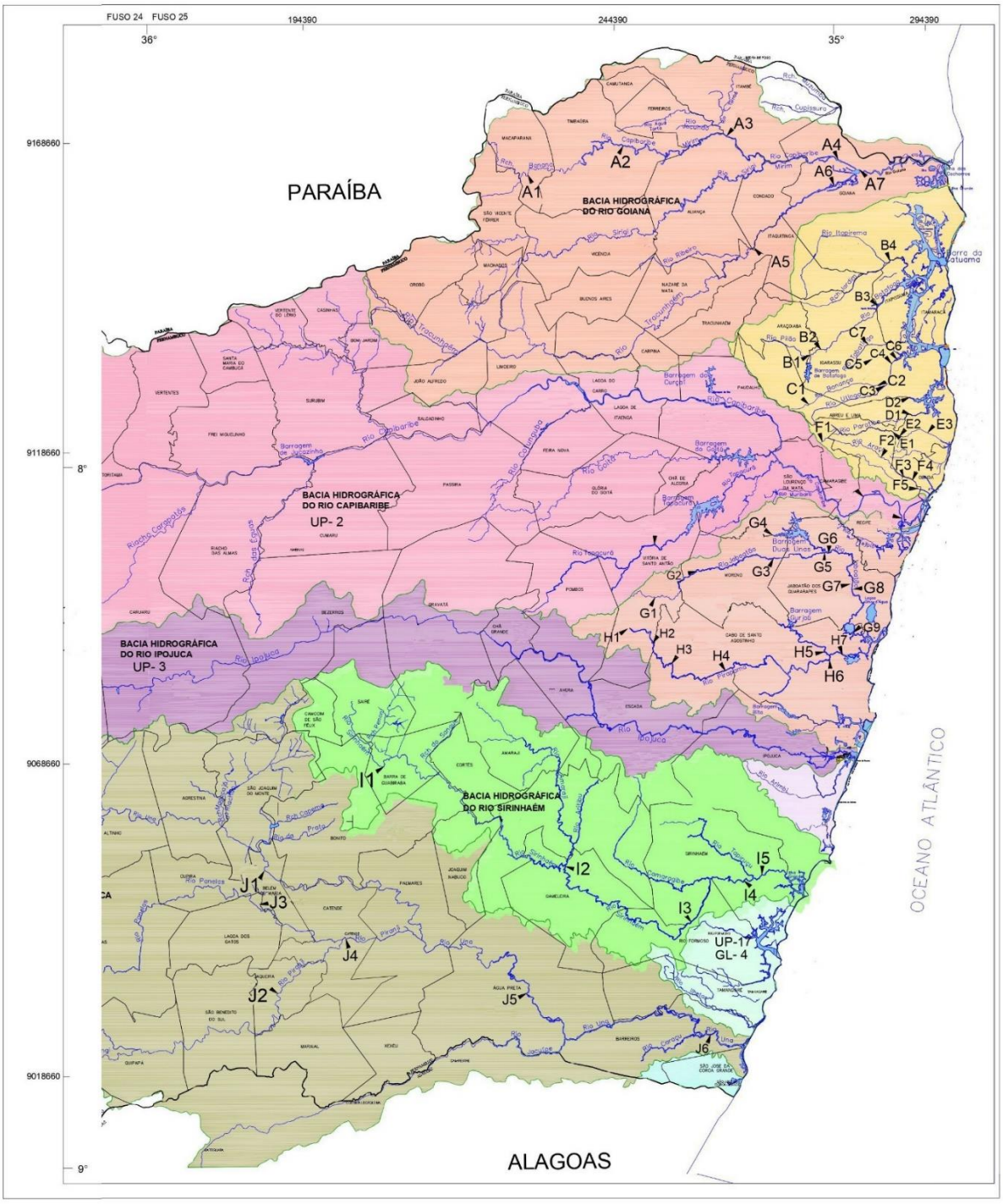
The study area (Fig. 1) was composed of ten hydrographic basins in the state of Pernambuco, northeastern Brazil. The basins were divided in three groups. Group 1 is formed by Goiana River hydrographic basin (GRB), the northernmost basin, Botafogo River basin (BRB) and Igarassu River basin (IRB) in the north-south direction, which are basins influenced by agricultural, industrial and urban pressures. Group 2 is formed by Timbó River (TRB), Paratibe River (PRB), Beberibe River (BeRB) and Jaboatão River (JRB), which are basins located closer to the city of Recife metropolitan region (RMR) and which are influenced by greater pressure from industrial and urban activities. Group 3 is formed by Pirapama River basin (PiRB), Sirinhaém River basin (SRB) and Una River basin (URB), which are basins located south of RMR and influenced mostly by agricultural and industrial activities.

A different number of sites were analyzed along each river basin, according to Table 1. Sites at each river basin were coded with letters from A-C (Group 1), D-G (Group 2) and H-J (Group 3) in the north-south direction (Fig. 1 and Table 1).

Surface water sampling was carried out between March and September 2018, and two samples were collected for each site. Altogether, sampling was carried out at 55 sites distributed along the 10 river basins (Table 1). At each site, water samples were collected with aluminum buckets and transferred immediately to 1 L amber bottles, transported in ice to the laboratory

and kept in refrigerators at 4° C, for a maximum of four days, until the beginning of the toxicity tests.

Figure 1 – Location of sampling stations along the basins covered in this study.




Source: (CPRH, 2020).

Goiana River Basin (A), Botafogo River Basin (B), Igarassu River Basin (C), Timbó River Basin (D), Paratibe River Basin (E), Beberibe River Basin (F), Jaboatão River Basin (G), Pirapama River Basin (H), Sirinhaém River Basin (I) and Una River Basin (J).

Table 1. Number of sites analyzed and sampling dates at Pernambuco State hydrographic basins.

| Hydrographic basin | Number of sites | Sampling (2018) |
|---|---|---|
| Goiana River (A) – G1 | 7 | March (T1)<br>June (T2) |
| Botafogo River (B) – G1 | 4 | March (T1)<br>June (T2) |
| Igarassu River (C) – G1 | 7 | April (T1)<br>July (T2) |
| Timbó River (D) – G2 | 2 | May (T1)<br>August (T2) |
| Paratibe River (E) – G2 | 3 | May (T1)<br>August (T2) |
| Beberibe River (F) – G2 | 5 | May (T1)<br>August (T2) |
| Jaboatão River (G) – G2 | 9 | July (T1)<br>October (T2) |
| Pirapama River (H) – G3 | 7 | April (T1)<br>July (T2) |
| Sirinhaem River (I) – G3 | 5 | June (T1)<br>September (T2) |
| Una River (J) – G3 | 6 | June (T1)<br>September (T2) |

Letters in parentheses represent the location of the basin in the north-south direction.
T1 – first sampling; T2 – second sampling.

### 2.3.2 Characteristics of analyzed river basins

Within Group 1, GRB (A) has a drainage area of 2,878.30 km², is located between the states of Pernambuco and Paraíba, and has 26 territorial spaces of municipalities totally or partially inserted in its area, with a total population of 465,549 inhabitants, 61% of which are urban areas. GRB (A) is under pressure from urban, industrial and agricultural activities, with emphasis on the cultivation of sugar cane and ethanol production. BRB (B) has a total area of approximately 477 $km^2$ and covers areas of 5 municipalities in the state, suffering a population pressure of approximately 90 thousand inhabitants with most of its area in rural areas. Its main uses of water are irrigation in sugarcane plantation, urban and industrial supply (CPRH, 2018). IRB (C) has a total area of approximately 143 $km^2$. The predominant forms of occupation in this basin are agricultural and urban, supporting a population of approximately 125 thousand people. There is a predominance of urban areas, having in its middle course a remnant of the Atlantic Forest, with sixty hectares of extension, destined to the research of wild animals endangered (Refúgio Ecológico Charles Darwin) (CPRH, 2018).

Within Group 2, TRB (D) has 104 $km^2$ in area and is located just below IRB. TRB (D) is under pressure from a population of approximately 120 thousand inhabitants. The main use

of its waters is for the reception of domestic and industrial effluents. On the other hand, Timbó River is within an Environmentally Protected Area since 1986 (CPRH, 2018; FEITOSA; FLORES; MELO; SANTANA *et al.*, 2016). PRB (E) has a total area of about 118 km$^2$ (CPRH, 2018). Like TRB, PRB is a predominantly urban basin, under pressure from a population of approximately 400 thousand inhabitants. The use of its waters is for public supply, and reception of domestic and industrial effluents. BRB (F) has 79 km$^2$ of total area, is inserted in the metropolitan region of Recife (RMR), under pressure from a population of about 590,000 inhabitants, it is also an almost exclusively urban basin (FREITAS; PAIVA; FILHO; CABRAL *et al.*, 2015). Due to the deficit in basic sanitation in Olinda and Recife cities, as well as the urban occupation of the slopes of the margins, and being one of the main recipients of domestic and industrial effluents in its region, the Beberibe River is considered one of the most polluted in the state (CPRH, 2018; VAINSENCHER, 2009). JRB (G) is one of the most extensive in the region with 422 km$^2$ of area, covering six important municipalities in the state (Vitória de Santo Antão, Cabo de Santo Agostinho, Moreno, São Lourenço da Mata, Jaboatão dos Guararapes and Recife), under a population of 446,426 inhabitants divided between rural agricultural and urban areas. In this way, the main anthropic pressures suffered by JRB come from domestic, industrial and sugar-alcohol activities (CPRH, 2018; SOUZA; TUNDISI, 2003).

Within Group 3, PRB (H) has an area of approximately 600 km². The pressures exerted on this basin are mainly related to industrial and urban activities, and the cultivation of sugar cane. The urban densities in its surroundings is of approximately 1,160,000 inhabitants (CPRH, 2018; IBGE, 2020). SRB (I) covers 19 municipalities, in an area of 2,069.60 km², with approximately 40% of its area inserted in rural areas. SRB receives pressure mainly from urban and rural activities, with a population of approximately 182,000 inhabitants, of which 81,000 refer to rural areas (CPRH, 2018). URB (J) is located just below the SRB in the southernmost portion of the state of Pernambuco. URB has an area of approximately 6,292.89 km$^2$, covering about 43 municipalities, under pressure from a population of 553,259, 43% of which are rural (CPRH, 2018). URB presents a diversity of water uses, with some areas destined to recreation, fishing, irrigation and industrial and public supply, with emphasis on the sugar-ethanol industry (TAVARES; CORRÊA; SOUZA; SCARIOTTO *et al.*, 2017).

### 2.3.3 *Danio rerio* cultivation and embryo exposure

Adult breeders of *Danio rerio* were fed three times a day with *Artemia* sp nauplii and commercial fish feed with 40% protein once a day (first feed) until one day before spawning preparation. A group of 3 males and 6 females was separated the afternoon before spawning in

a 15 L aquarium, and after spawning and morning fertilization, the selection of fertilized and viable eggs was made by direct observation in a 50x magnification stereomicroscope. Coagulated or opacity eggs were discarded, only spawnings with a fertilization rate greater than 90% were used in essays. Animal management and embryo exposure were performed according to a protocol approved by the Animal Experimentation Ethics Committee of the Federal University of Pernambuco (UFPE). The water used to create and maintain the breeders was monitored daily with a YSI Professional Plus multiparametric probe. The pH varied from 7.8 to 8.0, the dissolved oxygen varied from 5.3 to 7.0 mg $L^{-1}$ and the temperature was maintained at 28 ± 0.5 °C (mean ± standard deviation).

### 2.3.4 *Danio rerio* exposure and toxicity tests

Ecotoxicological tests with fertilized eggs exposed to surface water samples were performed according to the Fish Embryo acute Toxicity test (FET) (OECD, 2013). The tests consisted of exposing zebrafish embryos with up to 3h post-fertilization (hpf) to samples collected at the 55 sites covered in this study, and for control, clean water was used in each of the sampling periods (T1 and T2). Exposure of embryos was performed in 24-well polystyrene plates, one embryo per well with a 2.5 mL total water volume. In each plate, 20 embryos were exposed to water samples, while 4 embryos were kept in clean water as internal plate controls. The control plates and internal controls were filled with culture water with pH 7.5 ± 0.5, dissolved oxygen 6 ± 1 mg $L^{-1}$ and temperature 27 ± 0.5 ºC. During all tests, 70% of the exposure water volume in each well was renewed daily. The samples were analyzed for pH, temperature, and dissolved oxygen with a multi-parameter YSI Professional Plus meter before starting each test. All surface water samples were aerated, if necessary, to ensure that the dissolved oxygen was above 5 mg $L^{-1}$. When egg coagulation was observed, absence of somites after 24 hpf, absence of heartbeat and/or lack of movement, the embryo or larva was considered dead (OECD, 2013). Mortality rates were calculated by the ratio between the total number of deaths after 96 hours of exposure and the total of 20 individuals exposed for each sample.

### 2.3.5 General Morphology Score (GMS)

Sublethal effects or delays in embryonic and larval development were evaluated using the General Morphology Score (GMS) (BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.*, 2015). The GMS is based on the computation of partial scores attributed for each embryo or larvae as they develop basic morphological structures considered developmental hallmarks. Every 24 h, up to 12 types of developmental hallmarks were evaluated and alterations recorded as sublethal indicators of effects on embryos or larvae,

during the 96h exposure period. Each morphological or developmental hallmark reached received a specific score in such a way that a healthy larva at the end of 96 h receives 18 points, after the inclusion of the hallmark swim bladder inflation as a final point in the GMS index proposed by BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.* (2015). The morphological hallmarks of development observed were: embryo development; detachment of the tail during early somite formation; eye development and pigmentation; presence of movement of the embryo or larva; presence of blood circulation; presence of heartbeat; pigmentation of the head and body; pigmentation of the tail; presence of pectoral fins, hatching, presence of a protruding mouth, consumption of yolk sac reserves and presence of an inflated swim bladder. Any delay in the development of any of these structures at expected time intervals leads to a decrease in the GMS index and indicates delay in development. A classification of the GMS index calculated in zebrafish exposed to surface water samples was applied to facilitate the interpretation of the different degrees of developmental delay observed at each site, basin or group of sites, following the criteria described in Table 2.

Table 2. Ranges of the GMS index calculated for zebrafish exposed to surface water samples and associated degrees of developmental delay.

| GMS ranges | Developmental delay category |
|---|---|
| 18.0 - 17.5 | No effect |
| 17.4 - 17.0 | Light |
| 16.9 - 16.0 | Moderate |
| 15.9 - 15.0 | Severe |
| 14.9> | Very Severe |

2.6 Frequency of abnormalities after the 96h exposure

Morphological abnormalities were recorded every 24 hours for each embryo during the test, and their frequencies were calculated after the end of the exposure by dividing the total number of larvae that presented the abnormality by the total number of live larvae present in the sample. These abnormalities were: slow blood circulation (blood stasis) at 24 hpf, hatching delay indicated by a live larva inside the egg after 72 hpf, lack of a pectoral fin at 96 hpf, lack of a protrusible mouth at 96 hpf, incomplete yolk sac absorption, and absence of swim bladder inflation at 96 hpf.

2.3.6 Ammonia analysis

Total ammonia was analyzed using the indophenol method (KOROLEFF, 1976), adapted to 96-well microplates. Indophenol absorbance was measured at 673 nm with a spectrophotometer Spectramax M3, Molecular Devices. A calibration curve was prepared with

9 concentrations of total ammonia (0, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 mg $NH_4.L^{-1}$) from ammonium chloride ($NH_4Cl$) stock solution (NH4Cl, 99.5% purity, Sigma-Aldrich). Ammonia exists mainly in the non-ionized form ($NH_3$) and ammonium ion ($NH_4^+$), where speciation between these two forms is controlled by pH and temperature. As the pH increases, the $NH_3$ (more toxic form) proportion also increases, and the conversion between the 2 forms was calculated according to EMERSON; RUSSO; LUND e THURSTON (1975).

### 2.3.7 Water quality index (WQI) and Trophic State Index (TSI)

The Water Quality Index (WQI) from National Sanitation Foundation (BROWN; MCLELLAND, 1970) was calculated based on CETESB (2017). The WQI ranges from 0 to 100, with the following categories being proposed: 0 to 19 - very bad (VB), 20 to 36 - bad (BD), 37 to 51 - acceptable (AC), 52 to 79 - good (GD) and 80 to 100 - excellent (EX). Its calculation consists of nine parameters which included percentage of dissolved oxygen (DO) saturation, pH, temperature, total solids (TS), five-day biochemical oxygen demand at 20ºC (BOD5,20), turbidity (Turb), total phosphate (TP), total nitrogen, and fecal coliforms (FC).

The Trophic State Index (TSI) classifies aquatic ecosystems in different degrees of eutrophication potential, according to the following scale: ≤ 47 - ultra-oligotrophic (UO), 47 to 52 - oligotrophic (OL), 52 to 59 - mesotrophic (MT), 59 to 63 - eutrophic (EU), 63 to 67 - supereutrophic (SE) and > 67 - hypereutrophic (HE). TSI was calculated according to CARLSON (1977), based on total phosphorus concentration (P).

### 2.3.8 Statistical analysis

Mortality rates and frequencies of abnormalities were compared with the control using the two-sample t test for proportions using the Statistic Calculator software (StatPac, Northfield, USA). The average GMS indices obtained from the embryos exposed to samples from different river basins were analyzed separately by Kruskall-Wallis followed by the Dunn test to detect significant differences compared to the control. Normality was verified by (Kolmogorov Smirnov test) and homoscedasticity by Levene's median test. Pearson's correlation coefficients between water quality indices, toxicity endpoints mortality rate, GMS index, frequency of abnormalities and ammonia concentrations were evaluated. These procedures used Sigmaplot software version 12 (Jandel Scientific, Erkrath, Germany).

A Principal Component Analysis (PCA) using the PAST program (Paleontological Statistics, ver. 2.17c) was used to examine spatial patterns of water quality indexes (WQI and TSI), GMS index and the frequency of the main anomalies observed in the sampled locations. The eigenvalues and the percentage variation were determined for each component to judge

their contribution to the PCA, and the factorial scores were used to judge which variables were most prominent in each component.

## 2.4 RESULTS AND DISCUSSION

Our study indicates that the sublethal toxicity endpoints focused on early stages of zebrafish development and the integrated GMS index were efficient in classifying sites within a large spatial area within analyzed river basins at different degrees of environmental degradation. Furthermore, correlations between the GMS index and WQI and TSI water quality indices indicate that sublethal toxicity within analyzed river basins is higher at sites with higher levels of sewage contamination and eutrophication risk. Although it was possible to observe lethal toxicity in some sites, the sublethal effects integrated by the GMS index proved to be more efficient to detect toxic surface water samples from areas impacted by domestic, industrial and agricultural pressures.

Group 1 river basins are inserted in rural and urban areas, suffering pressure from sugar cane plantation and ethanol production, textile and chemical activities (CPRH, 2018). According to the WQI, sites along these basins were generally classified between “acceptable” and “good”, an intermediate range that reflects lower domestic sewage contamination. According to the TSI, sites along these basins were classified between “oligotrophic” to “mesotrophic” at sites C1, C2 and C3 with smaller eutrophication risk, and between “mesotrophic” and “supereutrophic” at other sites (Table 3). Zebrafish early life mortality rates were significantly elevated after exposure to water from 19.4% of all samples analyzed from G1. Within this group river basins, zebrafish early life mortality rates were significantly elevated after exposure to water from 28.5% of samples along GRB (A) and 37,5% of samples along BRB (B), reaching 40% mortality at site A3-T1 and 25% mortality at site B4-T1, highest mortality rates at each basin. Mortality rates were not elevated at sites along IRB (C) (Table 4).

Table 3. Water quality indices and un-ionized ammonia quantified in surface waters from Goiana (A), Botafogo (B) and Igarassu (C) (Group 1 river basins). Timbó (D), Paratibe (E), Beberibe (F) and Jaboatão (G) (Group 2 river basins). Pirapama (H), Sirinhaém (I) and Una (J) (Group 3 river basins).

| Sites Group 1 | WQI | TSI | $NH_3$ (mg $L^{-1}$) | Sites Group 2 | WQI | TSI | $NH_3$ (mg $L^{-1}$) | Sites Group 3 | WQI | TSI | $NH_3$ (mg $L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A1T1 | 48 (AC) | 67 (SE)) | 0,05 | D1-T1 | 30 (BD) | 71 (HI) | 0,01 | H1-T1 | 62 (GD) | 57 (ME) | 0 |
| A1T2 | 54 (GD) | 67 (SE) | 0,01 | D1-T2 | 28 (BD) | 74 (HI) | 0,01 | H1-T2 | - | - | 0,01 |
| A2T1 | 38 (AC) | 65 (SE) | 0,02 | D2-T1 | 35 (AC) | 68 (HI) | 0 | H2-T1 | 40 (AC) | 72 (HI) | 0 |
| A2T2 | 38 (AC) | 68 (HI ) | 0,02 | D2-T2 | 27 (BD) | 70 (HI) | 0 | H2-T2 | 64 (GD) | 62 (EU) | 0 |
| A3T1 | 35 (BD) | 71 (HI) | 0,03 | E1-T1 | 45 (AC) | 73 (HI) | 0 | H3-T1 | 61 (GD) | 59 (EU) | 0 |
| A3T2 | 46 (AC) | 67 (HI) | 0 | E1-T2 | 63 (GD) | 58 (ME) | 0 | H3-T2 | 57 (GD) | 61 (EU) | 0 |
| A4T1 | 45 (AC) | 68 (HI) | 0,02 | E2-T1 | 63 (GD) | 56 (ME) | 0 | H4-T1 | 63 (GD) | 60 (EU) | 0 |
| A4T2 | 58 (GD) | 66 (SE) | 0 | E2-T2 | 42 (AC) | 71 (HI) | 0 | H4-T2 | 72 (GD) | 61 (EU) | 0 |
| A5T1 | 49 (AC) | 68 (HI) | 0,02 | E3-T1 | 23 (BD) | 64 (SE) | 0,02 | H5-T1 | 41 (AC) | 67 (HI) | 0 |
| A5T2 | 52 (GD) | 68 (HI) | 0 | E3-T2 | 23 (BD) | 74 (HI) | 0 | H5-T2 | 50 (AC) | 64 (SE) | 0 |
| A6T1 | 46 (AC) | 67 (SE) | 0,01 | F1-T1 | 59 (GD) | 53 (ME) | 0 | H6-T1 | 54 (GD) | 62 (EU) | 0 |
| A6T2 | 64 (GB) | 64 (SE) | 0 | F1-T2 | 58 (GD) | 57 (ME) | 0 | H6-T2 | 62 (GD) | 62 (EU) | 0 |
| A7T1 | 40 (AC) | 69 (HI) | 0,01 | F2-T1 | 73 (GD) | 53 (ME) | 0 | H7-T1 | 53 (GD) | 51 (OL) | 0 |
| A7T2 | 56 (GD) | 65 (SE) | 0 | F2-T2 | 66 (GD) | 59 (ME) | 0 | H7-T2 | 48 (AC) | 64 (SE) | 0 |
| B1T1 | 53 (GD) | 62 (EU) | 0 | F3-T1 | 18 (VB) | 75 (HI) | 0,02 | I1-T1 | 67 (GD) | 53 (ME) | 0 |
| B1T2 | 61 (GD) | 56 (ME) | 0 | F3-T2 | 20 (BD) | 77 (HI) | 0,12 | I1-T2 | 67 (GD) | 67 (HI) | 0 |
| B2T1 | 57 (GD) | 58 (ME) | 0,01 | F4-T1 | 26 (BD) | 72 (HI) | 0,07 | I2-T1 | 61 (GD) | 59 (EU) | 0 |
| B2T2 | 57 (GD) | 60 (EU) | 0 | F4-T2 | 22 (BD) | 73 (HI) | 0,2 | I2-T2 | 62 (GD) | 71 (HI) | 0 |
| B3T1 | 50 (AC) | 56 (ME) | 0,01 | F5-T1 | 18 (VB) | 74 (HI) | 0,01 | I3-T1 | 63 (GD) | 58 (ME) | 0 |
| B3T2 | 60 (GD) | 57 (ME) | 0 | F5-T2 | 19 (VB) | 78 (HI) | 0,16 | I3-T2 | 71 (GD) | 66 (SE) | 0 |
| B4T1 | 42 (AC) | 53 (ME) | 0 | G1-T1 | 60 (GD) | 56 (ME) | 0 | I4-T1 | 68 (GD) | 58 (ME) | 0 |
| B4T2 | 42 (AC) | 60 (EU) | 0 | G1-T2 | 57 (GD) | 55 (ME) | 0 | I4-T2 | 70 (GD) | 61 (EU) | 0 |
| C1T1 | 66 (GD) | 56 (ME) | 0 | G2-T1 | 65 (GD) | 60 (EU) | 0 | I5-T1 | 55 (GD) | 64 (SE) | 0 |
| C1T2 | 66 (GD) | 61 (EU) | 0,01 | G2-T2 | 73 (GD) | 53 (ME) | 0 | I5-T2 | 71 (GD) | 62 (EU) | 0 |
| C2T1 | 74 (GD) | 51 (OL) | 0 | G3-T1 | 43 (AC) | 67 (SE) | 0 | J1-T1 | 60 (GD) | 62 (EU) | 0 |
| C2T2 | 67 (GD) | 58 (ME) | 0 | G3-T2 | 60 (GD) | 66 (SE) | 0,01 | J1-T2 | 71 (GD) | 61 (EU) | 0 |
| C3T1 | 70 (GD) | 51 (OL) | 0 | G4-T1 | 57 (GD) | 64 (SE) | 0 | J2-T1 | 70 (GD) | 57 (ME) | 0 |
| C3T2 | 44 (AC) | 61 (EU) | 0 | G4-T2 | 66 (GD) | 62 (EU) | 0 | J2-T2 | 73 (GD) | 55 (ME) | 0 |
| C4T1 | 49 (AC) | 62 (EU) | 0 | G5-T1 | 59 (GD) | 67 (HI) | 0 | J3-T1 | 77 (GD) | 51 (OL) | 0 |
| C4T2 | 59 (GD) | 59 (EU) | 0 | G5-T2 | 48 (AC) | 67 (HI) | 0,01 | J3-T2 | 77 (GD) | 56 (ME) | 0 |
| C5T1 | 52 (GD) | 53 (ME) | 0 | G6-T1 | 39 (AC) | 68 (HI) | 0 | J4-T1 | 53 (GD) | 60 (EU) | 0 |
| C5T2 | 39 (AC) | 56 (ME) | 0 | G6-T2 | 39 (AC) | 72 (HI) | 0,06 | J4-T2 | 71 (GD) | 56 (ME) | 0 |
| C6T1 | 21 (BD) | 64 (SE) | 0 | G7-T1 | 55 (GD) | 59 (ME) | 0 | J5-T1 | 68 (GD) | 60 (EU) | 0 |
| C6T2 | 22 (BD) | 67 (SE) | 0 | G7-T2 | 68 (GD) | 56 (ME) | 0 | J5-T2 | 73 (GD) | 56 (ME) | 0 |
| C7T1 | 69 (GD) | 53 (ME) | 0 | G8-T1 | 46 (AC) | 65 (SE) | 0 | J6-T1 | 66 (GD) | 51 (OL) | 0 |
| C7T2 | 55 (GD) | 60 (EU) | 0 | G8-T2 | 56 (GD) | 65 (SE) | 0,01 | J6-T2 | 71 (GD) | 56 (ME) | 0 |
| - | - | - | - | G9-T1 | 43 (AC) | 68 (HI) | 0 | - | - | - | - |
| - | - | - | - | G9-T2 | 48 (AC) | 66 (SE) | 0 | - | - | - | - |

WQI = water quality index; TSI = trophic state index; WQI (water quality index): 0 to 19 - very bad (VB), 20 to 36 - bad (BD), 37 to 51 - acceptable (AC), 52 to 79 - good (GD) and 80 to 100 - excellent (EX); TSI (trophic state index): ≤ 47 - ultra- oligotrophic (UO), 47 to 52 - oligotrophic (OL), 52 to 59 - mesotrophic (MT), 59 to 63 - eutrophic (EU), 63 to 67 - supereutrophic (SE) and > 67 - hypereutrophic (HE).
T1 and T2 refer to first and second sampling date for each site, specified in Table 1.

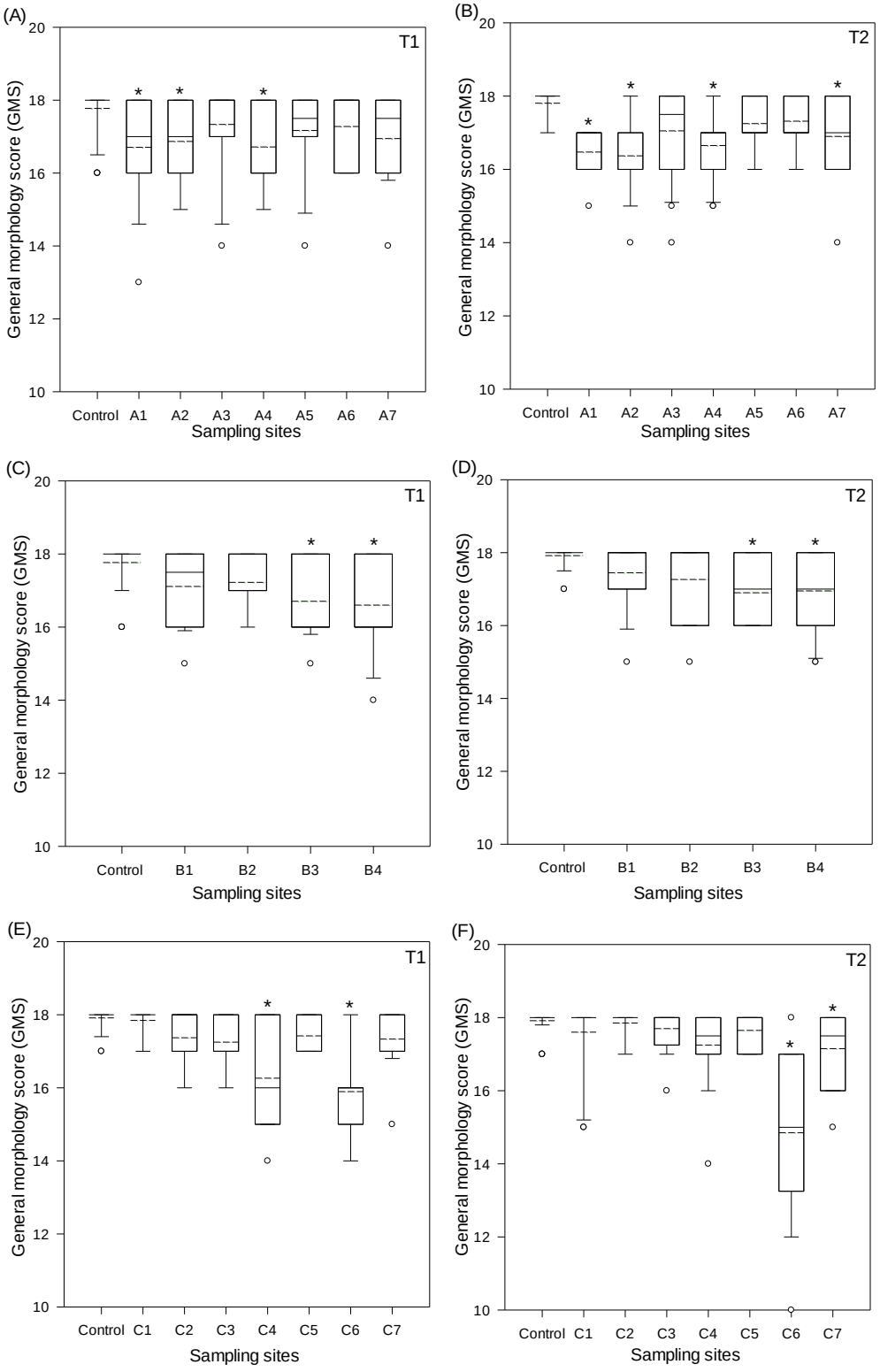
Table 4. Mortality rates (%) of zebrafish embryos and larvae exposed to surface water samples along different sites from Goiana (A), Botafogo (B) and Igarassu (C) River Basins.

| Sites along Group 1 | Mortality rates | t-test comparison with control |
|---|---|---|
| Control-T1 | 4.2% | - |
| Control-T2 | 2.1% | - |
| A1-T1 | 15.0% | NS, p > 0.05 |
| A1-T1 | 5.0% | NS, p > 0.05 |
| A2-T1* | 25.0% | t(66)=2.610, p=0.0112 |
| A2-T2 | 5.0% | NS, p > 0.05 |
| A3-T1* | 40.0% | t(66)=3.832, p=0.0033 |
| A3-T2 | 0.0% | NS, p > 0.05 |
| A4-T1* | 30.0% | t(66)=3.832, p=0.0033 |
| A4-T2 | 0.0% | NS, p > 0.05 |
| A5-T1 | 10.0% | NS, p > 0.05 |
| A5-T2* | 20.0% | t(66)=2.601, p=0.015 |
| A6-T1 | 10.0% | NS, p > 0.05 |
| A6-T2 | 0.0% | NS, p > 0.05 |
| A7-T1 | 10.0% | NS, p > 0.05 |
| A7-T2 | 0.0% | NS, p > 0.05 |
| Control-T1 | 2.1% | - |
| Control-T2 | 0.0% | - |
| B1-T1 | 10.0% | NS, p > 0.05 |
| B1-T2* | 10.0% | t(66)=2.224, p=0.0296 |
| B2-T1 | 10.0% | NS, p > 0.05 |
| B2-T2 | 5.0% | NS, p > 0.05 |
| B3-T1* | 15.0% | t(66)=2.086, p=0.0409 |
| B3-T2 | 0.0% | NS, p > 0.05 |
| B4-T1* | 25.0% | t(66)=3.056, p=0.032 |
| B4-T2 | 0.0% | NS, p > 0.05 |
| Control-T1 | 2.1% | - |
| Control-T2 | 2.1% | - |
| C1-T1 | 5.0% | NS, p > 0.05 |
| C1-T2 | 0.0% | NS, p > 0.05 |
| C2-T1 | 5.0% | NS, p > 0.05 |
| C2-T2 | 0.0% | NS, p > 0.05 |
| C3-T1 | 0.0% | NS, p > 0.05 |
| C3-T2 | 0.0% | NS, p > 0.05 |
| C4-T1 | 5.0% | NS, p > 0.05 |
| C4-T2 | 0.0% | NS, p > 0.05 |
| C5-T1 | 5.0% | NS, p > 0.05 |
| C5-T2 | 0.0% | NS, p > 0.05 |
| C6-T1 | 5.0% | NS, p > 0.05 |
| C6-T2 | 0.0% | NS, p > 0.05 |
| C7-T1 | 10.0% | NS, p > 0.05 |
| C7-T2 | 0.0% | NS, p > 0.05 |

*:statistically significant difference based on Student two sample t-test for comparison between site and control mortality rates. T1 and T2 refer to first and second sampling date for each site, specified in Table 1.

Delays in zebrafish embryolarval development were observed based on significantly decreased GMS scores from fish exposed to 41.6% of analyzed water samples from G1 sites when compared to controls (Fig. 2). Mean GMS for controls ranged from 17.8 to 17.9 across the whole study (Fig. 2, 3 and 4). The GMS decreased significantly in at least one sampling time from eight sites A1, A2, A4, A7, B3, B4, C4 and C6, of the 18 sites analyzed (44%), with mean GMS ranging between 15.4 and 16.8 (Fig. 2). Among these sites, A1, A2 and C6 stood out with the lowest mean GMS values of the group, equal to 16.6, 16.6 and 15.4, respectively, and with the highest frequencies of anomalies observed in exposed individuals. At these sites, non-inflation of the swim bladder ranged between 53% and 100%, and failure in yolk sac absorption ranged between 26% and 55% (Table 5). A1 is located downstream of the slaughterhouse in the city of Macaparana and site A2 is located downstream of the tannery in the city of Timbaúba. In addition to the influence of these activities, both sites are influenced by urban and sugarcane cultivation, which is a common activity throughout the Goiana River basin. In these regions, it is common to burn sugarcane leaves to facilitate harvesting, which generates negative environmental impacts, as this practice releases hydrocarbons into the atmosphere. These hydrocarbons are later deposited in the soil and, since there is practically no riparian vegetation near the cultivated areas, they are more easily leached into aquatic environments (ARRUDA-SANTOS; SCHETTINI; YOGUI; MACIEL *et al.*, 2018). Sites A4 and A7 had mean GMS of 16.7 and 16.9 respectively, and non-inflation of the swim bladder and failure in absorption of the yolk sac ranged between 20% and 70% (Table 5). These two sites are downstream from the city of Goiana, which receives urban and sugarcane-growing tailings in its course. They are located close to the sites studied by ARRUDA-SANTOS; SCHETTINI; YOGUI; MACIEL *et al.* (2018) where total polycyclic aromatic hydrocarbons (HPA) at concentrations of 0.51, 0.29 and 0.21 μg $L^{-1}$ (expressed as equivalents of Carmópolis oil) were detected. Although the detected HPA concentrations indicate a low local contamination (ARRUDA-SANTOS; SCHETTINI; YOGUI; MACIEL *et al.*, 2018), these contaminants cannot be ruled out as one of the potential sources of the toxicity detected in the samples analyzed in this study.

Figure 2. General morphology score (GMS) values for zebrafish embryos and larvae exposed to surface water samples along different sites from Goiana (A), Botafogo (B) and Igarassu (C) River Basins. (n = 20 zebrafish per site).




Box plot dashed horizontal lines indicate mean values, box plot continuous horizontal lines (lower, middle, and upper) indicate quartiles of 25, 50 (median), and 75%, respectively. * Statistically significant differences between sites at each sampling time compared to the respective control based on Kruskall Wallis test. A: (KW $H_{10}$ = 22.5, p=0.002; Dunn p <0.05), B (KW $H_{10}$ = 53.3, p <0.001; Dunn p <0.05), C (KW $H_{10}$ = 25.1, p <0.001; Dunn p <0.05), D (KW $H_{10}$ = 19.4, p <0.001; Dunn p <0.05), E (KW $H_{10}$ = 52.5, p <0.001; Dunn p <0.05), F (KW $H_{10}$ = 71.7, p <0.001; Dunn p <0.05). T1 and T2 refer to first and second sampling date for each site, specified in Table 1.

Table 5. Frequency (%) of recorded abnormalities during zebrafish development after exposure to surface waters from Goiana (A), Botafogo (B) and Igarassu (C) River basins

| Sites along river basins | Blood stasis 24 hpf | Hatching delay 72 hpf | Lack of pectoral fin 96 hpf | Lack of protrusible mouth 96 hpf | Incomplete Yolk sac absorption 96 hpf | Swim bladder not inflated 96 hpf |
|---|---|---|---|---|---|---|
| Control 1 | 0% | 3% | 0% | 0% | 3% | 5% |
| Control 2 | 0% | 7% | 0% | 0% | 0% | 4% |
| A1T1 | 0% | 6% | 0% | 12%* | 47%* | 59%* |
| A1T2 | 0% | 11% | 0% | 5% | 26%* | 100%* |
| A2T1 | 0% | 0% | 0% | 7% | 33%* | 53%* |
| A2T2 | 0% | 10% | 0% | 15%* | 55%* | 75%* |
| A3T1 | 0% | 0% | 0% | 8% | 17% | 33%* |
| A3T2 | 0% | 20% | 0% | 5% | 10%* | 50%* |
| A4T1 | 0% | 7% | 0% | 7% | 50%* | 50%* |
| A4T2 | 0% | 20% | 0% | 10%* | 20%* | 70%* |
| A5T1 | 0% | 0% | 6% | 11%* | 22%* | 67%* |
| A5T2 | 0% | 6% | 0% | 0% | 13% | 56%* |
| A6T1 | 0% | 0% | 0% | 11%* | 28%* | 33%* |
| A6T2 | 0% | 16% | 0% | 5% | 11%* | 47%* |
| A7T1 | 0% | 0% | 0% | 6% | 39%* | 39%* |
| A7T2 | 0% | 10% | 10%* | 5% | 25%* | 55%* |
| Control T1 | 0% | 2% | 4% | 0% | 0% | 4% |
| Control T2 | 0% | 4% | 0% | 0% | 0% | 0% |
| B1T1 | 0% | 0% | 0% | 11%* | 39%* | 50%* |
| B1T2 | 0% | 0% | 0% | 6% | 11% | 39%* |
| B2T1 | 0% | 17%* | 6% | 0% | 17%* | 39%* |
| B2T2 | 0% | 0% | 0% | 5% | 26%* | 42%* |
| B3T1 | 0% | 12% | 12% | 12%* | 41%* | 59%* |
| B3T2 | 0% | 20% | 10% | 10% | 15% | 35%* |
| B4T1 | 0% | 7% | 13% | 13%* | 47%* | 60%* |
| B4T2 | 0% | 25%* | 5% | 5% | 10% | 40%* |
| Control 1 | 0% | 4% | 0% | 0% | 0% | 4% |
| Control 2 | 0% | 2% | 0% | 0% | 0% | 4% |
| C1T1 | 0% | 5% | 0% | 0% | 0% | 11% |
| C1T2 | 0% | 0% | 0% | 0% | 10%* | 20%* |
| C2T1 | 0% | 16% | 5% | 5% | 0% | 32%* |
| C2T2 | 0% | 10% | 0% | 0% | 0% | 5% |
| C3T1 | 0% | 20% | 5% | 0% | 0% | 45%* |
| C3T2 | 0% | 0% | 0% | 0% | 5% | 20%* |
| C4T1 | 0% | 26%* | 11% | 16% | 37%* | 63%* |
| C4T2 | 0% | 10% | 0% | 0% | 0% | 30%* |
| C5T1 | 0% | 5% | 11% | 0% | 0% | 37%* |
| C5T2 | 0% | 10% | 0% | 0% | 0% | 20%* |
| C6T1 | 0% | 21% | 0% | 21%* | 74%* | 74%* |
| C6T2 | 5% | 35%* | 10% | 35%* | 40%* | 90%* |
| C7T1 | 0% | 6% | 0% | 5% | 6% | 50%* |
| C7T2 | 0% | 30%* | 0% | 0% | 10%* | 45%* |

*:statistically significant difference based on Student two sample t-test for comparison between site and control mortality rates. T1 and T2 refer to first and second sampling date for each site, specified in Table 1.

Also, within Group 1, sites C4 and C6 from Igarassu River basin caused moderate (GMS 16.8) and severe (GMS 15.4) developmental delay for exposed individuals, respectively, with emphasis on C6, which had the lowest GMS in group 1, reaching 14.8 in C6-T2 (Table 2). C6 also had the highest frequency of abnormalities in the group, reaching 100% of individuals without an inflated swim bladder and 35% of individuals with a non-protrusible mouth (Table 5). Both sites are located within the city of Igarassu and receive domestic sewage effluents and waste from the textile, metallurgical, food, chemical, sugar-alcohol and beverage industries

(LEÃO; PASSAVANTE; DA SILVA-CUNHA; SANTIAGO, 2008). In addition, C6 is located downstream of a pulp and paper industry, and an aluminum industry, which may be releasing waste from their activities directly into the river, which together with domestic waste, may be contributing to the highest degree of toxicity to zebrafish embryos detected in the samples. In addition, SOUZA; NASCIMENTO e MELO (2013) found in their work high concentrations of metals (Fe, Mn, Cd, Pb and Cu) and radioactive elements (Th and U) in mussels collected in at an arm Igarassu River, downstream of our C6 site. Previous studies have shown that zebrafish embryos exposed to concentrations of copper (JOHNSON; CAREW; SLOMAN, 2007), iron (HASSAN; KWONG, 2020) and uranium (BOURRACHOT; SIMON; GILBIN, 2008) exhibited, among other deleterious effects, a delay in hatching and changes in yolk sac area length. Similar effects were also observed in our study, where the C6 site showed frequencies of 21% and 35% of hatching delay, and 40% and 74% of failure in yolk sac absorption. Although we do not have data on these elements in water samples from the region, the occurrence of high concentrations of these contaminants in mussels is an indication that these contaminants may be present in the surface waters of the Goiana River and can contribute to the detected toxicity.

Group 2 river basins (G2) are located in predominantly urban areas and receive a high load of domestic and industrial effluents (CPRH, 2018), and received the lowest scores for WQI and TSI. According to the WQI, sites along these basins were classified between “acceptable” to “very bad”. Sites F3, F4 and F5 were classified by the WQI in categories “bad” to “very bad”, and the highest concentrations of $NH_3$ were found, which reflects the high domestic sewage load received by sites within these basins. According to the TSI, sites along these basins were classified between “supereutrophic” and “hyperotrophic” (Table 3). Within Group 2 river basins, zebrafish early life mortality rates were significantly elevated after exposure to water from 33% of samples along PBR(E) and JBR (G), and 60% of samples along BeBR, reaching 25% mortality at sites G6-T1 and G6-T2. Mortality rates were not elevated at sites along TRB (D) (Table 6). The delays in zebrafish embryolarval development were observed from fish exposed to water from samples from 60.5% of all samples analyzed from G2 river basins. Among 19 sites analized within G2, 13 sites (68%) indicated a mean GMS of 16.4, which represent moderate degree of developmental delay. Within G2 river basins, GMS decreased significantly at all analyzed samples (T1 and T2) from sites D1, D2, E2, E3, F3, F4, F5, G5, G6 and G9, with mean GMS equal to 15.9, 16.3, 16.8, 16.1, 14.1, 15.4, 14.5, 16.4, 16.5 and 16.6, respectively (Fig. 3). The lowest mean GMS among all analyzed sites was observed at sites F3 and F5 (GMS 14.1 and 14.5, respectively), indicating a very severe developmental

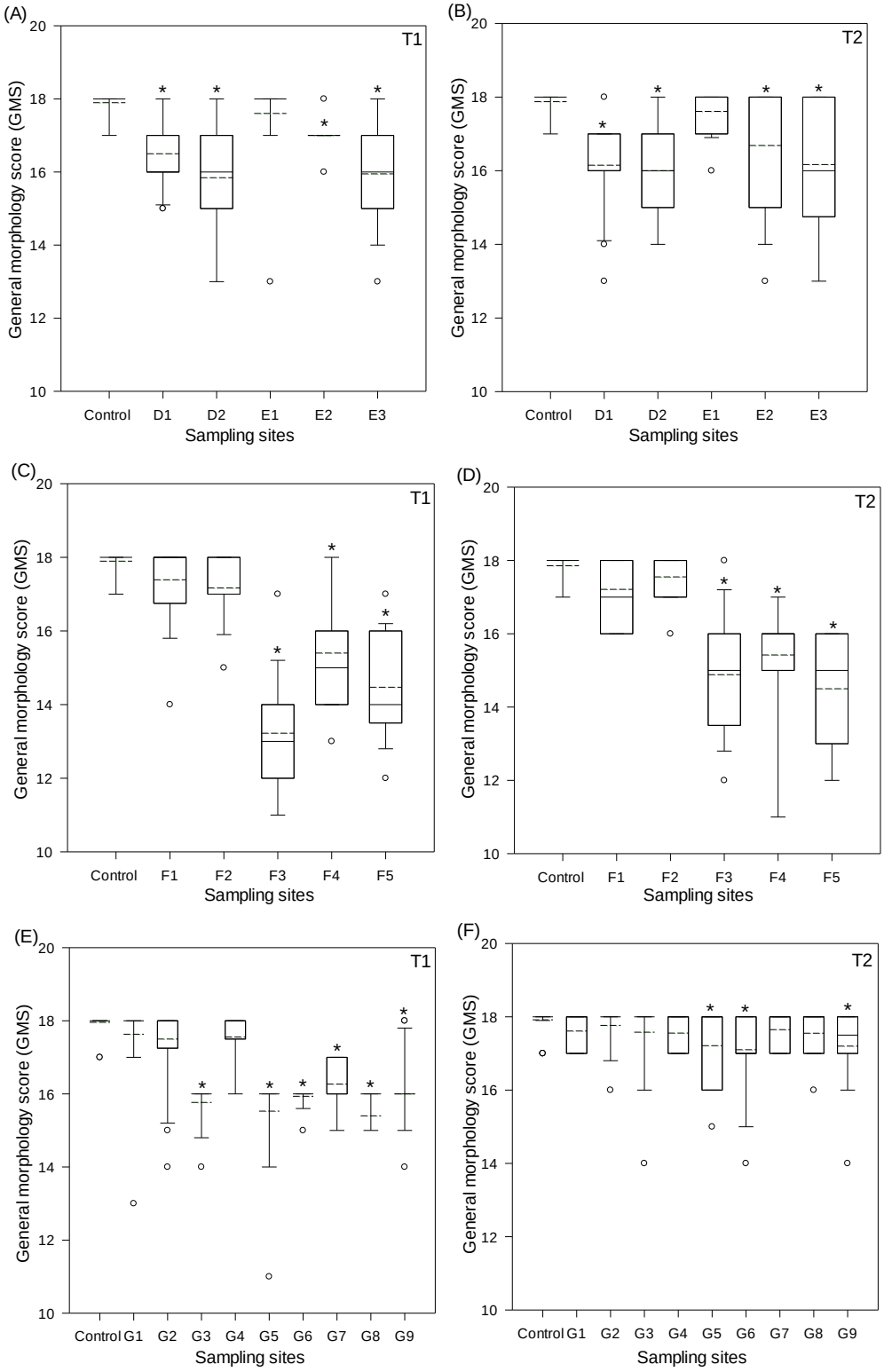
delay, followed by sites D1 and F4 (GMS 15.9 and 15.4, respectively), indicating a severe developmental delay.

Table 6. Mortality rates (%) of zebrafish embryos and larvae exposed to surface water samples along different sites from Timbó (D), Paratibe (E), Beberibe (F) and Jaboatão (G) River Basins.

| Sites along Group 2 | Mortality rates | t-test comparison with control |
|---|---|---|
| Control-T1 | 0.0% | - |
| Control-T2 | 0.0% | - |
| D1-T1 | 0.0% | NS, p > 0.05 |
| D1-T2 | 0.0% | NS, p > 0.05 |
| D2-T1 | 5.0% | NS, p > 0.05 |
| D2-T2 | 5.0% | NS, p > 0.05 |
| Control-T1 | 0.0% | - |
| Control-T2 | 0.0% | - |
| E1-T1 | 0.0% | NS, p > 0.05 |
| E1-T2* | 10.0% | t(66)=2.224, p=0.0296 |
| E2-T1 | 0.0% | NS, p > 0.05 |
| E2-T2 | 5.0% | NS, p > 0.05 |
| E3-T1 | 5.0% | NS, p > 0.05 |
| E3-T2* | 10.0% | t(66)=2.224, p=0.0296 |
| Control-T1 | 0.0% | - |
| Control-T2 | 0.0% | - |
| F1-T1* | 10.0% | t(66)=2.224, p=0.0296 |
| F1-T2 | 5.0% | NS, p > 0.05 |
| F2-T1* | 10.0% | t(66)=2.224, p=0.0296 |
| F2-T2 | 0.0% | NS, p > 0.05 |
| F3-T1* | 10.0% | t(66)=2.224, p=0.0296 |
| F3-T2* | 15.0% | t(66)=2.745, p=0.0078 |
| F4-T1 | 0.0% | NS, p > 0.05 |
| F4-T2 | 5.0% | NS, p > 0.05 |
| F5-T1* | 15.0% | t(66)=2.745, p=0.0078 |
| F5-T2* | 10.0% | t(66)=2.224, p=0.0296 |
| Control-T1 | 2.1% | - |
| Control-T2 | 0.0% | - |
| G1-T1 | 0.0% | NS, p > 0.05 |
| G1-T2* | 10.0% | t(66)=2.224, p=0.0296 |
| G2-T1 | 0.0% | NS, p > 0.05 |
| G2-T2* | 10.0% | t(66)=2.745, p=0.0078 |
| G3-T1* | 15.0% | t(66)=2.086, p=0.0409 |
| G3-T2 | 0.0% | NS, p > 0.05 |
| G4-T1 | 10.0% | NS, p > 0.05 |
| G4-T2* | 10.0% | t(66)=2.224, p=0.0296 |
| G5-T1 | 0.0% | NS, p > 0.05 |
| G5-T2 | 0.0% | NS, p > 0.05 |
| G6-T1* | 25.0% | t(66)=3.056, p=0.032 |
| G6-T2 | 0.0% | NS, p > 0.05 |
| G7-T1* | 25.0% | t(66)=3.056, p=0.032 |
| G7-T2 | 0.0% | NS, p > 0.05 |
| G8-T1 | 0.0% | NS, p > 0.05 |
| G8-T2 | 0.0% | NS, p > 0.05 |
| G9-T1 | 0.0% | NS, p > 0.05 |
| G9-T2 | 0.0% | NS, p > 0.05 |

*:statistically significant difference based on Student two sample t-test for comparison between site and control mortality rates. T1 and T2 refer to first and second sampling date for each site, specified in Table 1.

Figure 3. General morphology score (GMS) values for zebrafish embryos and larvae exposed to surface water samples along different sites from Timbó (D), Paratibe (E), Beberibe (F) and Jaboatão (G) River Basins. (n = 20 zebrafish per site).




Box plot dashed horizontal lines indicate mean values, box plot continuous horizontal lines (lower, middle, and upper) indicate quartiles of 25, 50 (median), and 75%, respectively. * Statistically significant differences between sites at each sampling time compared to the respective control based on Kruskall Wallis test. A: (KW H10 = 81.55, p <0.001; Dunn p <0.05), B (KW H10 = 60.36, p <0.001; Dunn p <0.05), C (KW H10 = 99.52, p <0.001; Dunn p <0.05), D (KW H10 = 99.16, p <0.001; Dunn p

<0.05), E (KW H10 = 149.7, p <0.001; Dunn p <0.05), F (KW H10 = 28.8, p <0.001; Dunn p <0.05). T1 and T2 refer to first and second sampling date for each site, specified in Table 1.

Site D1 is located in the Timbó River Basin and is surrounded by an industrial complex involving textile, metallurgical, non-metallic minerals, food products, plastics and perfumery activities (CPRH, 2018). In addition, D1 is under pressure from a strong residential occupation that uses its water for the disposal of domestic sewage, mainly due to the deficient urban sewage system in the region (SANTANA; COELHO JUNIOR, 2017). The residues from the activities developed in the industrial complex and the domestic sewage discharged upstream of D1 are possibly the main sources of degradation of this environment and contributors to the harmful effects presented by the exposed individuals.

Three of the five sites analyzed in Beberibe River basin (G2) indicated severe developmental delay for exposed zebrafish embryos and larvae, with a mean GMS of 15.7 for the entire basin, which was the lowest value among all the studied basins. In addition, BRB received the lowest scores for water quality indices, with most samples ranging from very poor to poor according to WQI, and hypereutrophic for TSI. Sites F3, F4 and F5 had mean GMS of 14.1, 15.4 and 14.5, respectively, these three sites showed significant increases in all frequencies of pathologies when compared to the control, highlighting the endpoint non-inflation of the swim bladder which varied between 95% and 100%, non-protrusion of the mouth reaching 83%, delayed hatching ranging between 20% and 65%, and blood stasis ranging between 20% and 35% (Table 7). Sites F1 and F2 are in the upper and middle reaches of the Beberibe River, which are inserted in remnants of the Atlantic Forest, which allowed these sites to have good water quality. Sites F3, F4 and F5 are located in an area of dense urbanization and receive a significant load of domestic sewage, which results in a considerable increase in the level of pollution in this river (VERAS; CABRAL; PAIVA; BARCELLOS *et al.*, 2016; VERAS; PAIVA; DUARTE; NAPOLEÃO *et al.*, 2019), aggravated by the direct disposal of waste from small industries in its surroundings. Previous studies have detected the presence of intestinal parasites (mainly protozoa and helminths) (FREITAS; PAIVA; FILHO; CABRAL *et al.*, 2015) and pharmaceutical products (diclofenac and paracetamol) (VERAS; PAIVA; DUARTE; NAPOLEÃO *et al.*, 2019) in water samples from the Beberibe River, indicating it is the direct destination of untreated domestic waste in the river. Although Beberibe River is considered one of the most polluted rivers in the region, it supplies water to about 100,000 people (FREITAS; PAIVA; FILHO; CABRAL *et al.*, 2015).

Drugs widely used by the population are part of the group of emerging contaminants that have caused increasing environmental concern for not being completely removed from

water treatment before disposal and for being able to generate endocrine, renal and reproductive disruption in teleost fish (XIA; ZHENG; ZHOU, 2017). Among these drugs, diclofenac is one of the most common and was detected in water samples from the Beberibe River at concentrations ranging from 22 µg $L^{-1}$ to 193 µg $L^{-1}$ (VERAS; PAIVA; DUARTE; NAPOLEÃO *et al.*, 2019) at a site upstream of F3. Previous studies have found that diclofenac is capable of causing oxidative stress in zebrafish embryos at concentrations between 0.5 µg $L^{-1}$ and 500 µg $L^{-1}$ (BIO; NUNES, 2020) and decreased the hatch rate after exposure to 24.1 µg $L^{-1}$ diclofenac for 56 h (ZHANG; YUAN; WERDICH; ZHAO, 2020). Thus, the presence of drugs detected in the samples from the Beberibe River may be one of the sources of the toxicity detected in the present study, as a delay in hatching and a reduction in the hatching rate were also observed in all five sites sampled from the Beberibe River.

Fish exposed to sites G5, G6 and G8 in the Jaboatão River basin showed severe developmental delay at T1, with GMS equal to 15.5, 15.9 and 15.4, respectively. These three points are in areas with high urban density, G5 and G6 are in the city of Jaboatão dos Guararapes and G9 in Cabo de Santo Agostinho City. These sites receive mainly untreated domestic effluents, discharges from sugarcane mills and residues from small industries in their surroundings (SOUZA; TUNDISI, 2003).

Table 7. Frequency (%) of recorded abnormalities in zebrafish development after exposure to surface waters from Timbó (D), Paratibe (E), Beberibe (F) and Jaboatão (G) River Basins.

| Sites along river basins | Blood stasis 24 hpf | Hatching delay 72 hpf | Lack of pectoral fin 96 hpf | Lack of protrusible mouth 96 hpf | Incomplete Yolk sac absorption 96 hpf | Swim bladder not inflated 96 hpf |
|---|---|---|---|---|---|---|
| Control 1 | 0% | 2% | 0% | 2% | 0% | 0% |
| Control 2 | 0% | 4% | 0% | 0% | 0% | 4% |
| D1-T1 | 21%* | 32%* | 5% | 11% | 32%* | 79%* |
| D1-T2 | 15%* | 10% | 5% | 10% | 65%* | 85%* |
| D2-T1 | 0% | 45%* | 0% | 0% | 20%* | 80%* |
| D2-T2 | 10%* | 10% | 0% | 5% | 55%* | 100%* |
| Control-T1 | 0% | 4% | 0% | 0% | 0% | 0% |
| Control-T2 | 0% | 2% | 0% | 0% | 0% | 4% |
| E1-T1 | 0% | 15%* | 0% | 0% | 0% | 0% |
| E1-T2 | 0% | 11% | 0% | 0% | 6% | 39%* |
| E2-T1 | 0% | 5% | 0% | 0% | 0% | 89%* |
| E2-T2 | 0% | 0% | 5% | 30%* | 20%* | 50%* |
| E3-T1 | 26%* | 84%* | 11%* | 11%* | 26%* | 21%* |
| E3-T2 | 17%* | 11% | 11%* | 29%* | 65%* | 65%* |
| Control-T1 | 0% | 4% | 0% | 0% | 2% | 4% |
| Control-T2 | 0% | 0% | 2% | 0% | 4% | 6% |
| F1-T1 | 0% | 6% | 6% | 11%* | 11% | 33%* |
| F1-T2 | 0% | 0% | 0% | 0% | 21%* | 53%* |
| F2-T1 | 0% | 6% | 0% | 6% | 11% | 56%* |
| F2-T2 | 0% | 10%* | 0% | 0% | 5% | 40%* |
| F3-T1 | 22%* | 61%* | 61%* | 83%* | 78%* | 100%* |
| F3-T2 | 35%* | 0% | 18%* | 35%* | 76%* | 100%* |
| F4-T1 | 20%* | 20% | 25%* | 35%* | 55%* | 80%* |
| F4-T2 | 21%* | 4% | 11% | 21% | 63%* | 95%* |
| F5-T1 | 0% | 65%* | 65%* | 71%* | 47%* | 76%* |
| F5-T2 | 26%* | 22%* | 44%* | 39%* | 78%* | 100%* |
| Control-T1 | 0% | 4% | 0% | 0% | 0% | 4% |
| Control-T2 | 0% | 2% | 0% | 0% | 0% | 4% |
| G1-T1 | 0% | 0% | 0% | 0% | 16%* | 26%* |
| G1-T2 | 0% | 22%* | 0% | 0% | 0% | 39%* |
| G2-T1 | 0% | 0% | 0% | 5% | 15%* | 30%* |
| G2-T2 | 0% | 18%* | 0% | 0% | 6% | 18% |
| G3-T1 | 0% | 0% | 12%* | 24%* | 94%* | 94%* |
| G3-T2 | 0% | 16%* | 0% | 5% | 11%* | 16% |
| G4-T1 | 11%* | 0% | 0% | 6% | 28%* | 28%* |
| G4-T2 | 11%* | 6% | 0% | 0% | 0% | 39%* |
| G5-T1 | 0% | 5% | 11%* | 21%* | 100%* | 100%* |
| G5-T2 | 0% | 11% | 0% | 0% | 26%* | 53%* |
| G6-T1 | 0% | 0% | 0% | 7% | 100%* | 100%* |
| G6-T2 | 0% | 10% | 0% | 16%* | 16%* | 58%* |
| G7-T1 | 0% | 0% | 7% | 20%* | 53%* | 100%* |
| G7-T2 | 0% | 5% | 5% | 5% | 5% | 35%* |
| G8-T1 | 5% | 10% | 10%* | 15%* | 95%* | 95%* |
| G8-T2 | 5% | 5% | 0% | 0% | 5% | 40%* |
| G9-T1 | 5% | 0% | 0% | 15%* | 90%* | 90%* |
| G9-T2 | 0% | 10% | 0% | 5% | 20%* | 20%* |

*:statistically significant difference based on Student two sample t-test for comparison between site and control mortality rates. T1 and T2 refer to first and second sampling date for each site, specified in Table 1.

Group 3 river basins are located in predominantly rural areas and suffer pressure mainly from sugar cane and ethanol production (CPRH, 2018). Sites along these basins received the best scores for the WQI and were mostly classified as “good”. According to the TSI, sites along these basins were classified mostly between “oligotrophic” and “eutrophic” (Table 3). Within Group 3 river basins, zebrafish early life mortality rates were significantly elevated after

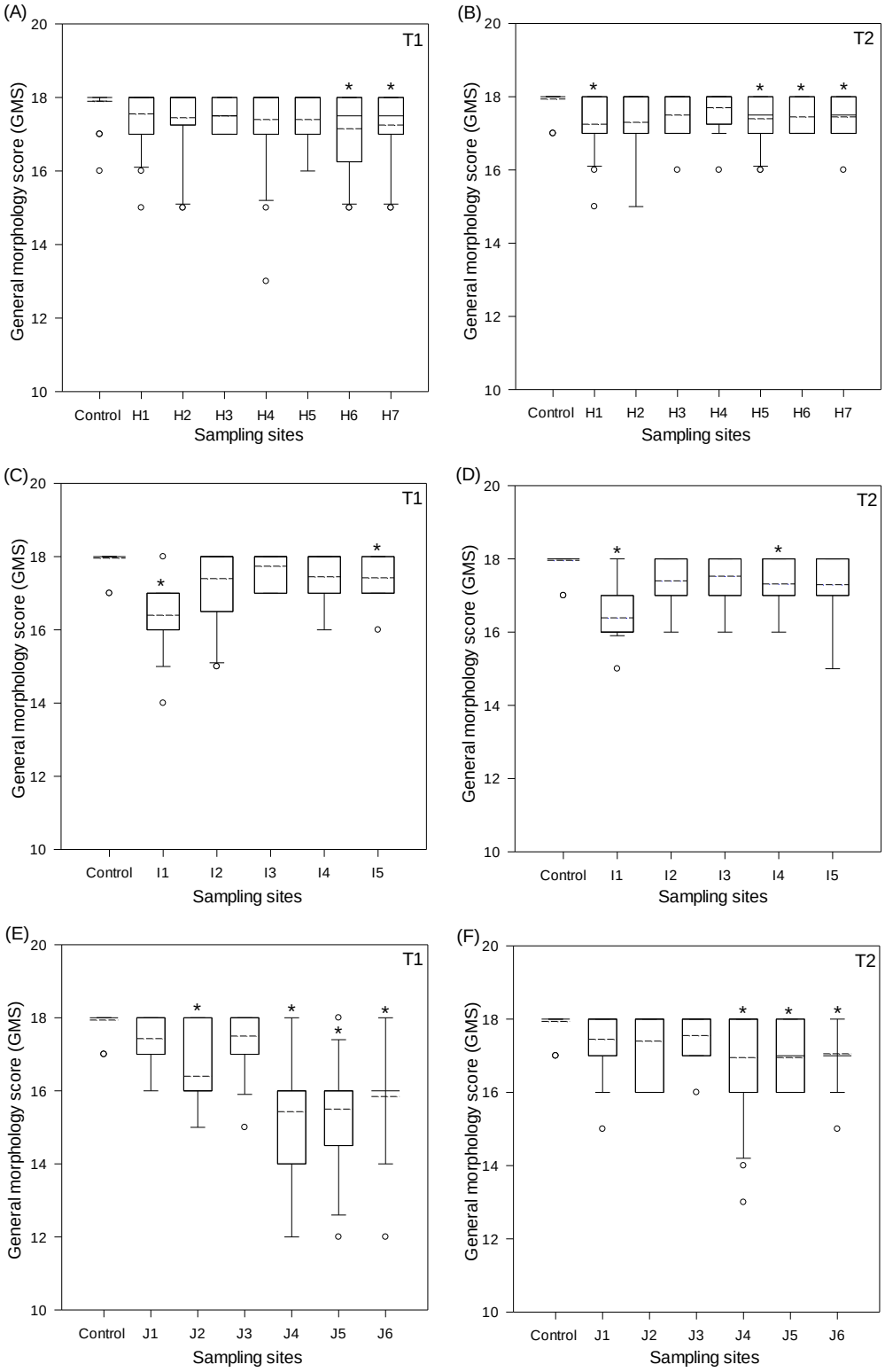
exposure to water from 20% of samples along SRB (I) and 25% of sites along URB (J), reaching 30% mortality at site J4-T1. Mortality rates were not elevated at sites along PRB(H) (Table 8). Regarding sublethal effects, significant toxicity was observed in 17 of the 36 samples analyzed (47%) in the basins of this group and only five sites (I1, J2, J4, J5 and J6) (13.8%) presented moderate developmental delay for exposed individuals, with mean GMS of 16.4, 16.9, 16.2, 16.2 and 16.4 respectively (Fig. 4).

Table 8. Mortality rates (%) of zebrafish embryos and larvae exposed to surface water samples along different sites from Pirapama (H), Sirinhaém (I) and Una (J) River Basins.

| Sites along Group 3 | Mortality rates | t-test comparison with control |
|---|---|---|
| Control-T1 | 0.0% | - |
| Control-T2 | 2.1% | - |
| H1-T1 | 0.0% | NS, p > 0.05 |
| H1-T2 | 0.0% | NS, p > 0.05 |
| H2-T1 | 0.0% | NS, p > 0.05 |
| H2-T2 | 0.0% | NS, p > 0.05 |
| H3-T1 | 0.0% | NS, p > 0.05 |
| H3-T2 | 0.0% | NS, p > 0.05 |
| H4-T1 | 0.0% | NS, p > 0.05 |
| H4-T2 | 0.0% | NS, p > 0.05 |
| H5-T1 | 0.0% | NS, p > 0.05 |
| H5-T2 | 0.0% | NS, p > 0.05 |
| H6-T1 | 0.0% | NS, p > 0.05 |
| H6-T2 | 0.0% | NS, p > 0.05 |
| H7-T1 | 0.0% | NS, p > 0.05 |
| H7-T2 | 0.0% | NS, p > 0.05 |
| Control-T1 | 0.0% | - |
| Control-T2 | 0.0% | - |
| I1-T1 | 0.0% | NS, p > 0.05 |
| I1-T2* | 10.0% | t(66)=2.224, p=0.0296 |
| I2-T1 | 0.0% | NS, p > 0.05 |
| I2-T2 | 0.0% | NS, p > 0.05 |
| I3-T1 | 5.0% | NS, p > 0.05 |
| I3-T2 | 5.0% | NS, p > 0.05 |
| I4-T1 | 0.0% | NS, p > 0.05 |
| I4-T2 | 0.0% | NS, p > 0.05 |
| I5-T1 | 0.0% | NS, p > 0.05 |
| I5-T2* | 15.0% | t(66)=2.745, p=0.0078 |
| Control-T1 | 2.1% | - |
| Control-T2 | 2.1% | - |
| J1-T1* | 15.0% | t(66)=2.086, p=0.0409 |
| J1-T2 | 0.0% | NS, p > 0.05 |
| J2-T1 | 10.0% | NS, p > 0.05 |
| J2-T2 | 0.0% | NS, p > 0.05 |
| J3-T1 | 10.0% | NS, p > 0.05 |
| J3-T2 | 0.0% | NS, p > 0.05 |
| J4-T1* | 30.0% | t(66)=3.47, p=0.0009 |
| J4-T2 | 0.0% | NS, p > 0.05 |
| J5-T1* | 15.0% | t(66)=2.086, p=0.0409 |
| J5-T2 | 0.0% | NS, p > 0.05 |
| J6-T1 | 5.0% | NS, p > 0.05 |
| J6-T2 | 5.0% | NS, p > 0.05 |

*:statistically significant difference based on Student two sample t-test for comparison between site and control mortality rates. T1 and T2 refer to first and second sampling date for each site, specified in Table 1.

Figure 4. General morphology score (GMS) values for zebrafish embryos and larvae exposed to surface water samples along different sites from Piarapama (H), Sirinhaém (I) and Una (J) River Basins. (n = 20 zebrafish per site).




Box plot dashed horizontal lines indicate mean values, box plot continuous horizontal lines (lower, middle, and upper) indicate quartiles of 25, 50 (median), and 75%, respectively. *: statistically significant differences between sites at each sampling time compared to the respective control based on Kruskall Wallis test. A: (KW H10 = 20.9, p <0.001; Dunn p <0.05), B (KW H10 = 30.12, p <0.001;

Dunn p <0.05), C (KW H10 = 57.03, p <0.001; Dunn p <0.05), D (KW H10 = 46.43, p <0.001; Dunn p <0.05), E (KW H10 = 79.9, p <0.001; Dunn p <0.05), F (KW H10 = 37.17, p <0.001; Dunn p <0.05). T1 and T2 refer to first and second sampling date for each site, specified in Table 1.

Along its course, Una River receives domestic, industrial and mainly sugarcane effluents, with the most significant use of its water focused on public supply, and irrigation of short and long-cycle crops (MELO; RIBEIRO; JUNIOR; PASTICH, 2018; SILVA; SILVA JUNIOR; LIMA, 2018). Among the pathologies observed, non-inflation of the swim bladder varied between 40% (J4) and 93% (J5), non-absorption of the yolk sac varied between 5% (J6) and 93% (J5), and hatching delay ranged between 5% (J6) and 35% (J5) (Table 9). Sites J4 and J6 are located downstream of the cities of Catende and Barreiros, respectively, and are the two sites along URB that suffer the greatest pressure from urbanization, receiving domestic effluents from the region and tailings from the sugarcane plantation from its surroundings. Highest frequencies of abnormalities were detected in fish exposed to J5 within URB, even though it is in a predominantly rural area. J5 is influenced by sugarcane cultivation areas, which are associated with the intensive use of fertilizers rich in phosphorus, vinasse, among other fertilizers, which also increase BOD (MELO; RIBEIRO; JUNIOR; PASTICH, 2018), in addition to waste generated by the burning of sugarcane leaves before harvest.

Table 9. Frequency (%) of recorded abnormalities in zebrafish larvae after exposure to surface waters from Pirapama (H), Sirinhaém (I) and Una (J) River Basins.

| Sites along river basins | Blood stasis 24 hpf | Hatching delay 72 hpf | Lack of pectoral fin 96 hpf | Lack of protrusible mouth 96 hpf | Incomplete Yolk sac absorption 96 hpf | Swim bladder not inflated 96 hpf |
|---|---|---|---|---|---|---|
| Control-T1 | 0% | 4% | 0% | 0% | 0% | 4% |
| Control-T2 | 0% | 2% | 0% | 0% | 0% | 2% |
| H1-T1 | 0% | 0% | 0% | 10%* | 5% | 35%* |
| H1-T2 | 0% | 0% | 0% | 10%* | 5% | 60%* |
| H2-T1 | 0% | 0% | 5% | 25%* | 10%* | 35%* |
| H2-T2 | 0% | 0% | 0% | 15%* | 10%* | 40%* |
| H3-T1 | 0% | 0% | 0% | 0% | 0% | 50%* |
| H3-T2 | 0% | 0% | 0% | 0% | 5% | 40%* |
| H4-T1 | 0% | 0% | 5% | 10%* | 5% | 30%* |
| H4-T2 | 0% | 0% | 0% | 0% | 5% | 25%* |
| H5-T1 | 0% | 0% | 0% | 10%* | 5% | 45%* |
| H5-T2 | 0% | 0% | 0% | 0% | 5% | 50%* |
| H6-T1 | 0% | 5% | 0% | 10%* | 20%* | 50%* |
| H6-T2 | 0% | 0% | 0% | 0% | 0% | 55%* |
| H7-T1 | 0% | 0% | 0% | 5% | 15%* | 50%* |
| H7-T2 | 0% | 5% | 0% | 0% | 0% | 40%* |
| Control-T1 | 0% | 2% | 0% | 2% | 0% | 0% |
| Control-T2 | 0% | 4% | 0% | 0% | 0% | 4% |
| I1-T1 | 0% | 15%* | 0% | 0% | 15%* | 95%* |
| I1-T2 | 0% | 0% | 0% | 5% | 68%** | 95%* |
| I2-T1 | 0% | 15%* | 0% | 5% | 40%* | 80%* |
| I2-T2 | 0% | 5% | 0% | 5% | 70%* | 80%* |
| I3-T1 | 0% | 26%* | 0% | 0% | 0% | 0% |
| I3-T2 | 0% | 20%* | 0% | 0% | 20%* | 40%* |
| I4-T1 | 0% | 40%* | 0% | 0% | 15%* | 95%* |
| I4-T2 | 0% | 16% | 0% | 0% | 63%* | 100%* |
| I5-T1 | 0% | 37%* | 5% | 5% | 5% | 95%* |
| I5-T2 | 0% | 11% | 0% | 33%* | 78%* | 94%* |
| Control-T1 | 0% | 2% | 0% | 0% | 0% | 0% |
| Control-T2 | 0% | 6% | 0% | 0% | 0% | 2% |
| J1-T1 | 0% | 14%* | 0% | 0% | 0% | 43%* |
| J1-T2 | 0% | 15% | 10%* | 10%* | 5% | 20% |
| J2-T1 | 0% | 7% | 0% | 7% | 67%* | 73%* |
| J2-T2 | 0% | 25%* | 5% | 5% | 5% | 35%* |
| J3-T1 | 0% | 6% | 6% | 6% | 19%* | 38%* |
| J3-T2 | 0% | 10% | 0% | 0% | 0% | 30%* |
| J4-T1 | 0% | 29%* | 14%* | 29% | 79%* | 79%* |
| J4-T2 | 0% | 20% | 10%* | 10%* | 20%* | 40%* |
| J5-T1 | 0% | 29%* | 14%* | 29%* | 93%* | 93%* |
| J5-T2 | 0% | 35%* | 0% | 0% | 5% | 65%* |
| J6-T1 | 0% | 11% | 5% | 11%* | 89%* | 84%* |
| J6-T2 | 0% | 5% | 5% | 5% | 5% | 79%* |

*:statistically significant difference based on Student two sample t-test for comparison between site and control mortality rates. T1 and T2 refer to first and second sampling date for each site, specified in Table 1.

Highest ecotoxicity based on GMS, worst water quality for human consumption based on WQI and greatest risk of eutrophication based on TSI was verified at sites along Group 2 river basins, which are located within or close to the metropolitan region of Recife in highly urbanized areas. Lowest ecotoxicity based on GMS and better water quality based on WQI and TSI was verified at sites along Groups 1 and 3 basins in the northern and southern portions of the state of Pernambuco, respectively. Frequencies of developmental abnormalities in at least one of the analyzed endpoints in zebrafish were significantly higher than controls at all 55

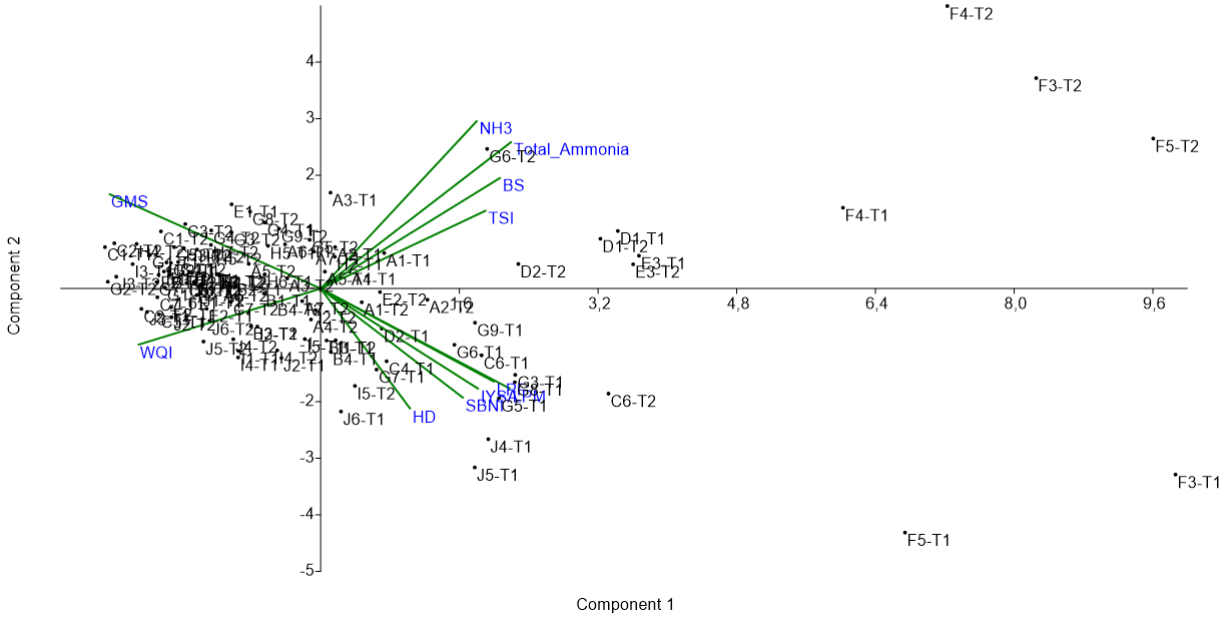
analyzed sites, except for site C2-T2 (Table 5). Among these, statistically significant increase in the frequency of larvae with uninflated swim bladder was detected at the highest frequencies especially in sites that are inserted in areas with greater urban density, especially in group 2 river basins such as TRB (D), BRB (F) and JRB (G) (Table 6). Increased frequencies of blood stasis were verified exclusively in Group 2 basins, reaching 26% at E3 and F5 (Table 7), which are sites that suffer from greater urban pressure and that have high concentrations of $NH_3$. Highest frequencies of absent pectoral fins and delayed hatching were also observed in samples from basins E and F (Table 7). Although a higher frequency of abnormalities was verified in these Group 2 river basins within more urbanized areas, elevated frequencies of late hatching (35%), uninflated swim bladder (95%), incomplete yolk sac absorption (70%) and absent protrusible mouth (35%) was verified in Group 1 and Group 3 river basins within rural areas, such as sites C, I and J (Table 5 and Table 9). This predominance of higher toxic effects in more urbanized areas along with low WQI and high TSI rankings suggests that zebrafish toxicity increases in places where large amounts of domestic sewage are being released. This pattern was corroborated by the PCA analysis which indicated the direct relationship between GMS and WQI, grouping the sampling sites with higher toxicity and worst WQI water quality classifications close to these two axes. On the other hand, the sites with higher eutrophication risk and higher toxicity were grouped opposite to these two axes and close to the TSI axis (Table 10 and Fig. 5). This pattern was also confirmed by a positive Pearson correlation coefficient of 0.69 between GMS and WQI indices, and an inversely proportional correlation of 0.52 between GMS and TSI indices (Table 11). Positive correlations were detected between sublethal developmental abnormalities during zebrafish development and $NH_3$, especially between $NH_3$ and blood stasis ($r > 0.653$, $p < 0.01$, Table 11), which suggests a causal relationship between the presence of $NH_3$ and blood stasis in the analyzed rivers, as observed by ALVES; MARIZ; DE MELO ALVES; CAVALCANTI *et al.* (2021).

Table 10. Factor loading scores for the first and second components of principal component analysis of water quality indices, general morphology score and frequency of abnomalities for all sampling sites along Goiana (A), Botafogo (B), Igarassu (C) (Group 1 river basins), Timbó (D), Paratibe (E), Beberibe (F) and Jaboatão (G) (Group 2 river basins), Pirapama (H), Sirinhaém (I) and Una (J) (Group 3 river basins).

| GMS/WQI/AF | Axis 1 | Axis 2 |
|---|---|---|
| GMS | -0.3752 | 0.2570 |
| WQI | -0.3240 | -0.1526 |
| TSI | 0.2933 | 0.2122 |
| Total Ammonia | 0.3383 | 0.3989 |
| $NH_3$ | 0.2774 | 0.4561 |
| IYSA | 0.2798 | -0.2726 |
| SBNI | 0.2539 | -0.2971 |
| LPM | 0.3361 | -0.2720 |
| HD | 0.1591 | -0.3272 |
| LPF | 0.3078 | -0.2532 |
| BS | 0.3187 | 0.3013 |

GMS (General morphology score); WQI (Water quality index); TSI (Trophic State Index); AF (anomalies frequency); $NH_3$ (unionized ammonia); IYSA (Incomplete Yolk sac absorption); SBNI (Swim bladder not inflated); LPM (Lack of protrusible mouth); HD (Hatching delay); LPF (Lack of pectoral fin); BS (Blood stasis).

Figure 5. Biplot of the principal components 1 and 2. The data inside the biplot show the specific sampling sites, water quality index (WQI), trophic state index (TSI), general morphology score (GMS), total ammonia, $NH_3$ and the developmental abnormality endpoints.




IYSA (Incomplete Yolk sac absorption); SBNI (Swim bladder not inflated); LPM (Lack of protrusible mouth); HD (Hatching delay); LPF (Lack of pectoral fin); BS (Blood stasis).

Table 11. Pearson correlation coefficients and significance (p values) between water quality indexes, zebrafish ecotoxicity endpoints and $NH_3$ evaluated in surface water along Goiana, Botafogo, Igarassu, Timbó, Paratibe, Beberibe and Jaboatão, Pirapama, Sirinhaém and Una River Basins

| | WQI | TSI | NH3 | MOR | IYSA | SBNI | LPM | HD | LPF | BS |
|---|---|---|---|---|---|---|---|---|---|---|
| GMS | **0.69** | **-0.53** | **-0.45** | -0.20 | -0.82 | -0.80 | -0.78 | -0.46 | -0.74 | -0.58 |
| | **<0.0001** | **<0.0001** | **<0.0001** | 0.03 | **<0.0001** | **<0.0001** | **<0.0001** | **<0.0001** | **<0.0001** | **<0.0001** |
| WQI | | **-0.74** | **-0.48** | -0.17 | **-0.55** | **-0.48,** | **-0.62** | **-0.30** | **-0.50** | **-0.60** |
| | | **<0.0001** | **<0.0001** | 0.08 | **<0.0001** | **<0.0001** | **<0.0001** | **<0.0001** | **<0.0001** | **<0.0001** |
| TSI | | | **0.47** | 0.05 | **0.47** | **0.38** | **0.52** | 0.12 | **0.34** | **0.51** |
| | | | **<0.0001** | 0.59 | **<0.0001** | **<0.0001** | **<0.0001** | 0.20 | **<0.0001** | **<0.0001** |
| NH3 | | | | 0.07 | **0.34** | **0.35** | **0.36** | 0.02 | **0.38** | **0.65** |
| | | | | 0.48 | **<0.0001** | **<0.0001** | **<0.0001** | 0.81 | **<0.0001** | **<0.0001** |

Indexes: WQI (water quality index); TSI (trophic state index); GMS (general morphology score).
Anomalies: MOR (mortality); IYSA (incomplete yolk sac absorption); SBNI (swim bladder not inflated); LPM (lack of protrusible mouth); HD (hatching delay); LPF (lack of pectoral fin); BS (blood stasis).
Toxic contaminant: $NH_3$ (un-ionized ammonia).
Highlighted values with Pearson correlation coefficients statistically significant.

Finally, the immobility test with *Daphnia magna* (OECD, 2004) was applied to 38 of the 110 surface water samples (34.5%) analyzed for zebrafish toxicity in this study. Of these 38 samples, only two samples (5.2%) (F3-T2 and F5-T2) indicated significant toxicity to *D. magna*, while 11 samples (29%) indicated lethal toxicity to *D. rerio* embryos and larvae, including F3-T2 and F5-T2, which demonstrates a greater sensitivity of zebrafish early life stages to detect lethal effects in the analyzed surface water samples. Furthermore, if we compare the toxicity detected in the *D. magna* test with the toxicity detected in the GMS-based sublethal tests, 17 samples (45%) out of 38 showed some degree of toxicity to exposed zebrafish embryos and larvae, including F3-T2 and F5-T2, indicating an even greater sensitivity of zebrafish embryos to detect sublethal effects in the analyzed surface water samples. Previous studies have also detected greater sensitivity in early stages of zebrafish when compared to the *D. magna* immobility test in 16% of 82 compounds tested (RAWLINGS; BELANGER; CONNORS; CARR, 2019), in 9.5% of tests involving 223 compounds (Teixidó et al., 2020) and in samples of surface waters of a tropical river (ALVES; MARIZ; DE MELO ALVES; CAVALCANTI *et al.*, 2021).

## 2.5 CONCLUSION

The use of zebrafish developmental toxicity endpoints based on the GMS was efficient in classifying sites within a large spatial area within analyzed river basins at different degrees of environmental degradation. Severe developmental delay was identified at sites within basins

inserted in exclusively urban areas. Moderate to light developmental delay was verified at sites within basins in less urbanized or exclusively rural areas. Furthermore, correlations between the GMS index and WQI and TSI water quality indices indicate that sublethal toxicity within analyzed river basins is higher at sites with higher levels of sewage contamination and eutrophication risk. The GMS index is a useful tool to be integrated in monitoring the quality of surface waters of tropical rivers.

# 3 MANUSCRITO II

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Contamination and Toxicity of Surface Waters Along Rural and Urban Regions of the Capibaribe River in Tropical Northeastern Brazil

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## 3.1 ABSTRACT

The Capibaribe River provides water to a population of 1.7 million people in the Brazilian Northeast, while receiving agricultural runoff, industrial and domestic effluents along its 280 km. The present study aimed to evaluate the ecotoxicity of surface waters along 10 sites in rural and urban areas using zebrafish *Danio rerio* early-life stages and relate it to water quality indices and chemical abiotic variables. Lethality rates, delays in embryo-larval development quantified by the General Morphology Score (GMS) and frequencies of developmental abnormalities were analyzed. A correlation was detected between Zebrafish GMS and Water Quality Index (WQI), sensitive to domestic sewage contamination, and Trophic State Index (TSI), focused on eutrophication. These indices agreed in identifying a spatial pattern of smaller impact in terms of ecotoxicity, domestic sewage contamination and eutrophication risk at three sites in rural areas (mean GMS 16.9), an intermediate impact at four sites with urban and agricultural influence (mean GMS 16.4), and greatest impacts were detected at three more urbanized sites (mean GMS 14.9). Most frequent developmental abnormalities included non-inflation of the swim bladder, delayed hatching, non-protrusion of the mouth, blood stasis and non-development of pectoral fins. Toxic $NH_3$ concentrations varied spatially, with higher concentrations in urban sites, and blood stasis correlated positively with $NH_3$, suggesting a causal relationship. Polycyclic aromatic hydrocarbons were detected both in rural and urbanized sites, contributing to detected toxicity. The present study demonstrates the potential of zebrafish early-life stages as an ecotoxicological model that may contribute to a better understanding of surface water quality and ecotoxicity in tropical river systems.

Keywords: developmental toxicity; effects-based monitoring; freshwater toxicology; ammonia; polycyclic aromatic hydrocarbons

## 3.2 INTRODUCTION

Surface water quality is a serious problem in developing countries due to contamination by domestic and industrial wastewater effluents and agricultural and urban runoff (LAETZ; HECHT; INCARDONA; COLLIER *et al.*, 2015). Adult and early-life stages of fishes have been used as sentinel species together with other biological methods to assess water quality since the 19$^{th}$ century (MINIER; RACHID; LEPAGE; AMIARD TRIQUET *et al.*, 2015), establish legal water quality criteria, and monitor environmental risk (DI GIULIO; HINTON, 2008).

Although *Danio rerio* is native to South Asia, zebrafish embryo-larval bioassays are gradually being used worldwide as efficient and sensitive tools for assessing the ichthyotoxicity of surface waters (CHEN; CHEN; CHEN; LIU *et al.*, 2015; VANLANDEGHEM; MEYER; COX; SHARMA *et al.*, 2012; WILSON; CASTRO; CHAVES; ESPINOSA *et al.*, 2021) and aquatic sediments (LI; CHEN; LIU; WU, 2016). Zebrafish early-life stages provide a sensitive testing model that is amenable to high-throughput screening of chemical contaminants, due to low cost easily maintained high fecundity breeders, and a transparent chorion allowing observation of developing key organ systems in embryos (CAPELA; GARRIC; CASTRO; SANTOS, 2019). Zebrafish embryos and early larvae can replace or reduce experiments with adult fish, an objective being increasingly applied in ecotoxicology to evaluate the toxicity of many chemicals using lethal and sublethal endpoints (BRAUNBECK; KAIS; LAMMER; OTTE *et al.*, 2014; HOLLERT; KEITER, 2015). The General Morphology Score (GMS) is an integrated index that focuses on the quantification of delays in zebrafish embryo-larval development during the first 96 h of life that can be used to assess the sublethal toxicity of environmental contaminants on zebrafish development (BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.*, 2015; HERMSEN; VAN DEN BRANDHOF; VAN DER VEN; PIERSMA, 2011).

The Capibaribe River Basin (CRB) in northeastern Brazil covers 42 municipalities along the 280 km of the main river course and is the main source of freshwater for a population of 1.7 million people (RIBEIRO NETO; SCOTT; LIMA; MONTENEGRO *et al.*, 2014). The Capibaribe River (CR) is under pressure from a diverse socioeconomic perspective along its course, and its intermittent upper and medium reaches is dominated by agriculture, livestock, and small-scale textile production (COLLIER; ALMEIDA NETO; ARETAKIS; SANTOS *et al.*, 2015; COLLIER; DE ALMEIDA NETO; DE ALMEIDA; ROSA FILHO *et al.*, 2019; RIBEIRO NETO; SCOTT; LIMA; MONTENEGRO *et al.*, 2014). It is perennial after the city

of Limoeiro and flows 96 km along its lower reaches through rural areas with sugarcane plantations, and smaller urban areas, until reaching the highly urbanized city of Recife, the capital of the state of Pernambuco, and the Atlantic Ocean (Figure 1). The runoff from rural and urbanized areas produces significant amounts of nitrogenous material including ammonia, toxic to fish in its unionized $NH_3$ form (EDDY, 2005; RANDALL; TSUI, 2002). Polycyclic aromatic hydrocarbons (PAH) are ubiquitous in freshwater, estuarine and coastal aquatic environments (MOJIRI; ZHOU; OHASHI; OZAKI *et al.*, 2019) and may cause significant toxicity to fishes (KENNEDY, 2014), especially during teleost embryo-larval development (CHERR; FAIRBAIRN; WHITEHEAD, 2017; INCARDONA, 2017).

Changes in river surface water quality over space and time have been analyzed worldwide by integrative indices such as the Water Quality Index (WQI) from National Sanitation Foundation (NOORI; BERNDTSSON; HOSSEINZADEH; ADAMOWSKI *et al.*, 2019). Capibaribe River surface waters are monitored by Pernambuco State Environmental Agency (CPRH), which uses the WQI and the eutrophication oriented Trophic State Index (TSI) (PIRES; TUCCI; CARVALHO; LAMPARELLI, 2015). However, these indices based on physico-chemical monitoring methods, are not sufficient to evaluate the quality of aquatic ecosystems, since a limited number of target substances are generally analyzed (VAN DER OOST; MCKENZIE; VERWEIJ; SATUMALAY *et al.*, 2020).

Therefore, the present study focused on the use of early-life stage *D. rerio* developmental endpoints and the integrated GMS index to evaluate the ecotoxicity of surface waters from 10 sites along the perennial lower course of the Capibaribe River, ranging from predominantly rural upstream to more urbanized downstream sites. We tested whether increased ecotoxicity along the CR correlates with degradation of water quality based on chemically based indices WQI and TSI, or with increasing unionized $NH_3$ and PAH concentrations. Additionally, this study will test if zebrafish can be a useful ecotoxicological model to characterize surface water ecotoxicity in tropical river systems.

## 3.3 MATERIALS AND METHODS

### 3.3.1 Study area and sampling sites

Capibaribe River flows from its headwaters in a semiarid region (550 mm $yr^{-1}$ rainfall) to the Atlantic Ocean (1228 mm $yr^{-1}$ rainfall) (Figure 1) (PERNAMBUCO, 2010). Sampling sites were located within the lower perennial course of CR, which are subject to anthropogenic impacts within CRB. The 10 sampling sites were numbered sequentially from S1 furthest

upstream to S10 closest to the ocean (Figure 1). Site S1 is located downstream of Limoeiro city in an area where the river course becomes perennial and flows approximately 89 km until Atlantic Ocean. Sites S2 and S3 are influenced by sugarcane plantations, as site S4 located in Goitá River (GR), 350m before it flows into CR. Sites S5 and S7 are located within the urbanized area of São Lourenço da Mata City, and where the state water supply company pumps water for human consumption. Site S6 is on the Tapacurá River, an important affluent of the Capibaribe River, downstream from Vitória de Santo Antão city and upstream of Tapacurá reservoir. Site S8 is at São Lourenço da Mata City, within a large metropolitan area, and sites S9 and S10 are located within the highly urbanized city of Recife (population 1.5 M people), within the CR estuarine area. This final portion of the CR, from S8 to S10, is affected by intense urbanization and production and trade of chemicals, pharmaceuticals, plastics, beverages, among others (PERNAMBUCO, 2010). The volume of domestic and industrial wastewater entering this final river portion is comparable to the entire river discharge during the dry season (SCHETTINI; MIRANDA; VALLE-LEVINSON; TRUCCOLO *et al.*, 2016).

Surface water samples were collected at each site in May 2018 (T1), August 2018 (T2), and in May 2019 (T3). Water samples were collected with aluminum buckets and transferred immediately to separate 1L amber bottles for biological tests and PAH analysis, closed with airtight leak proof phenolic lined cap closures. Samples were transported on ice to the laboratory and refrigerated at 4° C, for a maximum of seven days, until the beginning of the toxicity tests and chemical analysis.

### 3.3.2 D. rerio cultivation and embryo exposure

A population of adult breeders of *D. rerio,* maintained at the Aquatic Ecotoxicology Laboratory, were fed *Artemia* sp nauplii once per day and commercial fish food with 40% protein 3 times per day. Fertilized eggs were obtained from a group of 3 males and 6 females which were separated the previous afternoon in a 15 L aquarium. After early morning spawning and fertilization, selection of fertilized and viable eggs was made by direct observation using a stereomicroscope Zeiss Stemi 2000 at 50x magnification. Eggs that showed coagulation or opacity were discarded. Fertilization rate was higher than 90% in all experiments. Animal handling and embryo exposures were performed in accordance with protocols approved by the Ethics Committee for Animal Experiments from Federal University of Pernambuco (UFPE). Water used for maintenance of breeders was monitored daily with a YSI Professional Plus multiparametric meter. The pH varied from 7.8 to 8.1, dissolved oxygen (DO) varied from 5 to 7 mg $L^{-1}$, and the temperature was maintained at 28 ± 0.5 ° C (mean ± standard deviation).

### 3.3.3 *D. rerio* exposure and 96 h toxicity tests

Exposures of fertilized eggs to surface water samples were performed according to (OECD, 2013). The tests involved exposing zebrafish embryos at less than 3 h post-fertilization (hpf) to surface water from the sampling sites and to laboratory control clean water at each of the sampling periods (T1, T2 and T3). The embryos were exposed in a volume of 2.5 mL inside wells of 24-well plates. In control plates 24 embryos were exposed in wells filled with laboratory culture water at pH 7.2 ± 1, DO 6 ± 1 mg $L^{-1}$ and temperature 26.5 ± 0.5 ºC. For each surface water sample plate, 20 embryos were exposed to the surface water samples in 20 wells, while 4 embryos were kept in clean water in the remaining 4 wells as additional internal plate controls. For each sampling period, 2 control plates with 24 embryos each were analyzed and compared with the surface water sample plates for the different sites. During tests 70% of exposure water was renewed daily, and water samples were analyzed for pH, temperature, and DO with a YSI Professional Plus multiparametric meter. All surface water samples were aerated, if necessary, to assure DO was above 5 mg $L^{-1}$. During the experiments surface water samples were kept refrigerated in 1 L amber bottles closed with airtight leak proof phenolic lined cap closures. Approximately 2 hours before water renewal in wells, samples were removed from the refrigerator to allow temperature to equalize with experimental plates.

Wells were observed once daily under an inverted microscope (Digilab) at 40 x and 100 x magnification. Death was assigned to embryos and larvae when egg coagulation, absence of somites after 24 hpf, absence of heartbeat and/or lack of movement was observed in any well (OECD, 2013). Mortality rates in embryos exposed to surface water samples were calculated by the ratio between the total accumulated number of deaths after 96 h of exposure and the total of 20 individuals exposed for each sample. Control mortality was calculated by the ratio between the total accumulated number of deaths after 96 h of exposure and the total of 24 individuals exposed for each control plate.

### 3.3.4 General Morphology Score (GMS)

The sublethal developmental toxicity index GMS (BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.*, 2015) is based on the integration of partial scores attributed to each embryo or larva as they develop basic morphological structures during the first 96 hours of development. Both controls and treated embryos receive points for each developmental hallmark achieved, including detachment of the tail during early somite formation; eye development and pigmentation; presence of movement of the embryo or larva; presence of blood circulation; presence of heartbeat; pigmentation of the head and body;

pigmentation of the tail; presence of pectoral fins; hatching; presence of a protruding mouth and consumption of yolk sac reserves. We included the presence of an inflated swim bladder at 96 hpf as an additional hallmark not used in (BEEKHUIJZEN; DE KONING; FLORES-GUILLÉN; DE VRIES-BUITENWEG *et al.*, 2015). In our study a perfectly developed larva at 96 hpf will have a maximum GMS score of 18 points, therefore, a decrease in the index indicates delayed development.

### 3.3.5 Frequency of abnormalities

Developmental abnormalities that indicate specific and relevant alterations during early development were checked and recorded every 24 h for each embryo during testing, and their frequencies were calculated after 96 h of exposure, dividing the total number of live larvae presenting the abnormalities by the total number of live larvae present in the sample. These abnormalities included: slow blood circulation (blood stasis) at 24 hpf, hatching delay indicated by a live larva inside the egg after 72 hpf; lack of pectoral fin at 96 hpf, lack of protrusible mouth at 96 hpf, incomplete yolk sac absorption and absence of swim bladder inflation at 96 hpf.

### 3.3.6 Ammonia analysis

Total ammonia was analyzed in refrigerated (4 °C) surface water samples within 24 h of field collection, and at the beginning of the toxicity tests, within 7 days after collection, to evaluate whether a decrease in ammonia concentrations would be occurred prior to initiation of toxicity tests. Total ammonia was analyzed using the indophenol method (KOROLEFF, 1976) adapted to 96-well microplates. A calibration curve was prepared with 9 concentrations of total ammonia using ammonium chloride ($NH_4Cl$) stock solution ($NH_4Cl$, 99.5% purity, Sigma-Aldrich): 0, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 mg $NH_4$ $L^{-1}$ and absorbance was measured at 673 nm with a spectrophotometer (Spectramax M3, Molecular Devices). Calculation of unionized ammonia $NH_3$ concentration of water samples were based on temperature, pK and pH according to equations from (Emerson et al. 1975):

(1) $pK = 0.09018 + (2729.92/(273.2 + T))$

(2) fraction of $NH_3 = 1 / 1 + (10^{(pK-pH)})$

(3) concentration of $NH_3$ (mg $L^{-1}$) = fraction of $NH_3$ x total ammonia (mg $L^{-1}$)

where T is temperature in degrees Celsius

*Physical-Chemical parameters and water quality indices*

The Water Quality Index (WQI) from National Sanitation Foundation (BROWN; MCLELLAND, 1970) was calculated based on (CETESB, 2017). WQI consists of nine parameters including percentage of DO saturation, pH, total solids (TS), five-day biochemical oxygen demand (BOD) at 20 $^{o}$C ($BOD_{5,20}$), turbidity (Turb), total phosphorus (P), total nitrogen (Nt), and thermotolerant coliforms (TC). WQI ranges from 0 to 100, and the number is directly proportional to increased water quality. DO, $BOD_{5,20}$, P, Nt, and TC were analyzed in surface water samples based on (APHA-AWWA-AWEF, 2017) and were provided by Pernambuco State Environmental Agency (CPRH, 2018). Trophic State Index (TSI) was calculated according to Lamparelli (2004) based on total phosphorus. TSI is inversely proportional to increased eutrophication.

### 3.3.7 Polycyclic aromatic hydrocarbons

Water samples were analyzed for PAH during T3 only. Samples were liquid-liquid extracted according to (ARRUDA-SANTOS; SCHETTINI; YOGUI; MACIEL *et al.*, 2018). In summary, 20 mL of *n*-hexane (pesticide grade) were added to the 1 L amber glass bottle immediately after water sampling. 100 μL of a deuterated PAH MIX (Cerilliant, containing acenaphthene-d10, phenanthrene-d10 and chrysene-d12, at 1000 ng $mL^{-1}$) were also added as surrogate standards in all samples. The bottle was vigorously agitated for 2 min to extract hexane-soluble PAH. Anhydrous $Na_2SO_4$ (previously heated at 450 °C) was added to the extract for binding traces of water. The extracts were concentrated to 1 mL in a rotary evaporator and 100 μL of deuterated PAH fluorene-d10, benzo[a]anthracene-d12 and benzo[a]pyrene-d12 (Absolute Standards INC at 1000 ng $mL^{-1}$) were added to each extract as internal standards in order to calculate the recovery of surrogates. PAH were analyzed in a gas chromatograph (GC-Agilent Technologies, model 7820) coupled to mass spectrometry (MS-Agilent Technologies, model 5975C) in selected ion monitoring (SIM) mode, while the MS electron ionization source was operated at 70 eV. The GC oven was equipped with an HP-5 ms capillary column (30 m × 0.25 mm × 0.25 μm) and the injections (1 μL) were performed under constant flow. GC injector and MS transfer line temperatures were set at 300 °C. The oven temperature was programmed as follows: at 60 °C, rate 15 °C $min^{-1}$ to 150 °C, rate 5 °C $min^{-1}$ to 220 °C and rate 10 °C $min^{-1}$ to 300 °C, with a final retention of 10 min. The analytical curve was prepared with five different concentrations (1.0 to 800 ng $mL^{-1}$) of each compound (Absolute Standards INC, 2000 μg $mL^{-1}$), considering the ratio between the responses of the analyte of interest and surrogates (100 ng $mL^{-1}$). Quantification was done based on the ratio

between responses from analytes and surrogates in samples. Recoveries of surrogates were calculated based on the ratio of their areas to the internal standard, and ranged from 41 to 105%, which are satisfactory according to (Lauenstein and Cantillo 1998). The limit of quantification was calculated as the ratio of the lowest concentration in the analytical curve to the volume of extracted water, resulting in 1.0 ng $L^{-1}$ for all analytes. The 16 priority PAH listed by the United States Environmental Protection Agency (USEPA) plus 2-methylnaphthalene were quantified.

### 3.3.8 Statistical analysis

Mortality rates and frequencies of abnormalities from fish exposed to each surface water sample site and controls were compared by one-way analysis of variance followed by Dunnet´s multiple comparison tests to evaluate differences between each site and controls when ANOVA significant differences were detected. Mean GMS index obtained from embryos exposed to water from each site and sampling period was analyzed using a non-parametric Permutational Multivariate Analysis of Variance (PERMANOVA) with PRIMER version 6 (Primer-E Ltd, Plymouth, U.K.) based on (Clarke and Gorley 2006). The similarity matrix was constructed using Euclidean distance with 9999 permutations to assess whether there were significant differences in the GMS index among sampling sites. Pairwise comparisons between treatments at each sampling period were performed when significant results were obtained ($p < 0.05$). Mean GMS index obtained from embryos at different sites for each sampling period were also separately analyzed by Kruskall-Wallis followed by the Dunn test to detect significant differences compared with control. Normality was checked by Kolmogorov Smirnov test and homoscedasticity by Levene median test. Pearson correlation coefficients between water quality indices, toxicity endpoints (mortality rate, GMS index, frequency of abnormalities) and surface water ammonia and PAH concentrations were calculated using Sigmaplot software version 12 (Jandel Scientific, Erkrath, Germany). A cluster analysis was performed using the Euclidean distance applied to the sites according to their similarities and proximity in relation to the GMS using PAST software (Paleontological Statistics, ver. 2.17c).

A Principal component analysis was used to examine potential interrelationships between water quality indices (WQI and TSI), GMS index and the frequency of developmental abnormalities observed in exposed zebrafish. Potential interrelationships were then confirmed or rejected with a Spearman correlation test. The eigenvalues and the percentage variation were determined for each component to judge their contribution to the PCA, and the factorial scores were used to judge which variables were most prominent in each component.

## 3.4 RESULTS

### 3.4.1 Mortality

Mortality rates (mean ± standard deviation) of zebrafish exposed to CR surface water samples varied significantly and reached highest levels at S3 (33.3 % ± 46.8 %) and S6 (26.7 % ± 18.4 %), where a significant difference from control mortality (2.1 % ± 3.1 %) was detected (Anova $F_{10,88} = 2.95$, $p = 0.003$, followed by Dunnett´s test, $p < 0.05$, Table 1). S3 is within an urbanized area also influenced by agriculture, and S6 is in the CR affluent Tapacurá River, downstream of Vitória de Santo Antão City. Mean mortality rates of zebrafish exposed to CR surface water samples at other sites were also higher than controls, reaching 21% at S10, in the urban area of Recife, but a high variability prevented detection of significant differences.

### 3.4.2 GMS index

GMS of zebrafish exposed to CR surface waters were statistically different among sampling times (Pseudo-F = 4.12; p = 0.021), and sites (Pseudo-F = 39.46; p = 0.0001) but generally indicated a consistent pattern of development delay effects at all sites over time (Figure 2D). No significant effects for interaction of spatial and temporal factors were detected (Pseudo-F = 0.89; p = 0.59). The statistically significant temporal effect detected a higher GMS at T1 (mean 16.46) compared with T2 (mean 16.13, df = 2, Permanova, p = 0.009), suggesting a less severe developmental delay effect in the first sampling time T1 compared with T2, but no differences were detected between GMS at T1 and T3 (mean 16.13, df = 2, Permanova, p = 0.06), or between T2 and T3 (df = 2, Permanova, p = 0.88). Significant sublethal toxicity indicating a delay in embryo-larval development based on GMS was detected at all but two sites within CRB. No effects on GMS were detected at any time at S2 and S7 (Figures 2A, 2B and 2C). Mean GMS for controls ranged from 17.8 to 17.9, and GMS of zebrafish exposed to CR surface water samples from sites S1, S6, S8, S9 and S10 were significantly lower than controls at all sampling times (Kruskall-Wallis followed by Dunn test, $p < 0.05$). Most severe developmental delays and lowest GMS were detected in fish exposed to S6 (mean from 14.3 to 15.3), S9 (mean from 14.9 to 15.3) and S10 (mean from 13.9 to 15.3). An intermediate degree of developmental delay was detected in fish exposed to S1 and S8 (mean from 15.9 to 16.4), and S3 and S4 (mean from 16.1 to 17.0). Absent or less severe developmental delay was observed at S2, S5 and S7 (mean from 16.4 to 17.1) (Figure 2A, 2B, 2C, 2D). Cluster analysis identified three main groups, the first group being formed by sites S6, S9 and S10, the second group consisting predominantly of S1, S3, S4 and S8, and the third group formed by S2, S5 and S7 (Figure 3). These groupings corroborate the spatial differences detected by PERMANOVA, where no

statistically significant differences were detected between S1, S3, S4 and S8, between S2, S5 and S7, and between S6, S9 and S10.

### 3.4.3 Developmental abnormalities

Major types of abnormalities observed during embryo-larval development were blood stasis at 24 h, delayed hatching at 72 hpf, absence of pectoral fin, non-protrusion of the mouth, incomplete yolk sac absorption and uninflated swim bladder at 96 hpf (Table 2). A statistically significant increase in the frequency of larvae with blood stasis was detected in 53%, 24% and 43% of larvae at sites S6, S9 and S10, which are inside highly urbanized regions. Frequency of hatching delays was higher at all sites, but not statistically different when compared to controls. Frequency of absent pectoral fin was higher at sites S6 (12%) and S10 (15%) when compared to controls. Frequency of absent protrusible mouth was significantly higher at sites S1 (34%), S6 (39%), S9 (31%) and S10 (40%) compared to controls. Frequency of incomplete yolk sac absorption was significantly higher at sites S1 (51%), S6 (63%), S9 (89%) and S10 (98%) compared to controls. Frequency of larvae with uninflated swim bladders was significantly higher than controls at all sites but S2 and S7, and highest frequencies were detected at sites S6 (83%), S9 (96%) and S10 (98%).

### 3.4.4 Water quality and indices

Water quality variables and indices for all sites in the three sampling periods are presented in Table 3. Sites 1 - 4 were classified as acceptable based on WQI water quality categories, which mainly reflects the contamination of water bodies caused by domestic sewage releases. In terms of eutrophication potential, S1 was classified as hyper-eutrophic, S2 and S4 were eutrophic, and S3 was super-eutrophic based on TSI. S5 and S7 are sites where water is pumped for urban supply, and both were classified, based on WQI, as good and as eutrophic, based on TSI. S8 is located downstream of São Lourenço da Mata City, in the beginning of the metropolitan region of Recife. S8 is in a transition region with rural and urban influences and was classified as good according to the WQI, but super-eutrophic based on TSI. S6 was classified as very bad and S9 and S10 as bad, according to WQI and all three sites were classified as hypereutrophic based on TSI. Highest BOD, total ammonia and $NH_3$ values were verified in S6, S9 and S10, and mean BOD, total ammonia and $NH_3$ at these sites were approximately 3x, 11x and 56x higher than the mean of other sites together. Mean TC, P, and conductivity at S6, S9 and S10 were 2.5x, 3x and 4.5x higher than mean of other sites, respectively.

Total ammonia and $NH_3$ concentrations of surface water samples analyzed during toxicity tests, varied from 95% to 100% of concentrations measured 24 h after sampling, indicating that a maximal loss of 5 % of ammonia occurred during the short period of sample storage under refrigeration.

Low concentrations of DO were detected at all sites, varying from < 0.5 to 7 mg $O_2$ $L^{-1}$. DO concentrations above 5.0 mg $O_2$ $L^{-1}$ were detected only at S5 in T3, S10 and S8 in T2. DO concentrations below 2 mg $O_2$ $L^{-1}$ were detected in S1, S6 and S9 at all sampling times. All surface water samples collected had salinity below 0.1 practical salinity units (PSU).

### 3.4.5 Spatial and temporal patterns of surface water quality

PCA of measured water quality variables and the main ecotoxicological effects observed at all sampling sites indicated that approximately 68% of the variability in the observed variables was explained by the first two components, with eigenvalues 6.03 and 1.42, respectively (Figure 4). GMS, lack of protrusible mouth, blood stasis, total ammonia and $NH_3$ (factor loadings ≥ |0.31|) were the major contributing factors in component one. For component two, hatching delay (HD) (factor loading ≥ |0.68|) was the major contributing factor. PCA analyses indicated a separation between urban sites and those in rural areas. Sites S6, S9 and S10 were grouped along axis 1 variables, indicating the highest values of TSI, total ammonia, $NH_3$ and all six abnormalities observed. In addition, lowest values of WQI and GMS were verified at S6, S9 and S10, located at opposite sides of these two vectors. Sites S2, S5 and S7 were grouped along the variables WQI and GMS on axis 2, indicating the highest values for both. Sites S1, S3, S4 and S8 were grouped with intermediate values along axes 1 and 2.

### 3.4.6 Polycyclic aromatic hydrocarbons

Total PAH concentration (ΣPAH) ranged from 181 (S2) to 1,079 ng $L^{-1}$ (S1), in samples collected during T3. There was a predominance of phenanthrene, followed by naphthalene and pyrene (Table 4). The highest phenanthrene concentration (971 ng $L^{-1}$) was observed at S1, where it represented 90% of ∑PAH. At other sites, its concentration was greater than or equal to 70% of ∑PAH, except at S10, where it was 42% of ∑PAH. Among PAH with four or more rings, only fluoranthene and pyrene were detected and present in all samples with the highest concentrations were reported at S10 (9.1 ng $L^{-1}$) and S6 (47.7 ng $L^{-1}$), respectively. S10 was the only site where 13 PAH were detected out of 17 PAH analyzed. Diagnostic ratios were applied to infer the predominant PAH source or prevalent process contributing PAH to the aquatic system. The ratio between the sum of compounds with two or three rings (low molecular weight

ΣPAH-LMW) and the sum of compounds with four to six rings (high molecular weight ΣPAH-HMW) was calculated. LMW-PAH are dominant among PAH of petrogenic origin and HMW-PAH are predominantly formed during combustion processes of typical pyrolytic origin. The ratios LMW/HMW were greater than 1 at all sites, ranging from 1.93 to 62.3, suggesting a prevalence of petrogenic PAH sources. Ratios between anthracene and anthracene plus phenanthrene (Ant/178) were less than 0.1 at all sites, also suggesting petrogenic sources (Yunker et al. 2002). Ratios between fluoranthene and fluoranthene plus pyrene (Fl/Fl + Py) were less than 0.5 at all sites (min 0.1, max 0.37), further evidence of petrogenic sources (Budzinski et al. 1997).

Using all data collected from CRB sites at all time points, a positive or directly proportional Pearson correlation coefficient GMS and WQI ($r = 0.58$, $p = 0.0008$), and a negative or inversely proportional correlation between GMS and TSI was observed ($r = -0.669$, $p = 0.00009$) (Table 5). No significant correlations were observed between zebrafish mortality rates and water quality indices, $NH_3$ or PAH (Table 5). However, both WQI and TSI were significantly correlated ($p \leq 0.002$) with frequency of abnormalities LPM an BS. Furthermore, GMS was negatively correlated with toxic contaminants $NH_3$, 2-methylnaphthalene, fluorene, chrysene, and with ΣPAH-HMW ($r > 0.5$, $p < 0.01$, Table 5). Frequency of LPM was positively correlated with $NH_3$, FLU, CRI and ΣPAH-HMW. Frequency of HD was positively correlated with fluorene and ΣPAH-HMW. Frequency of BS was positively correlated with $NH_3$, 2-methylnaphthalene, fluorene, chrysene and ΣPAH-HMW ($r > 0.5$, $p < 0.01$, Table 5). A positive correlation between toxic contaminants $NH_3$ and fluorene was observed ($r > 0.5$, $p < 0.01$, Table 5).

## 3.5 DISCUSSION

Our study indicates that sites along the perennial lower course of the Capibaribe River are impacted to different degrees by land-use based on results of ecotoxicity testing with zebrafish early-life stages and on water quality indices WQI and TS. PAH and $NH_3$ were considered relevant contributors to detected zebrafish developmental toxicity.

### 3.5.1 Zebrafish as a model species for water quality monitoring

Extrapolation of toxicity data from standard test species to predict impact to a wider range of species in ecosystems is a complex issue (SPURGEON; LAHIVE; ROBINSON; SHORT *et al.*, 2020). The use of a sensitive species to evaluate toxicity is essential, and this requirement has been directed towards the use of representative taxa from multiple trophic levels such as

algae, Daphnia and fish (RAWLINGS; BELANGER; CONNORS; CARR, 2019). The zebrafish *Danio rerio* is exotic to our study region, but it is a sensitive model species in Ecotoxicology. Zebrafish lethal endpoints based on (OECD, 2013) used in this study were more sensitive than Daphnia immobility and algae growth inhibition tests in 16% of 82 compounds tested (RAWLINGS; BELANGER; CONNORS; CARR, 2019) and are considered good surrogates for acute fish toxicity tests (BELANGER; RAWLINGS; CARR, 2013). Fish early-life stage toxicity tests including zebrafish acute and sublethal developmental endpoints used in this study were more sensitive than Daphnia reproduction and algae growth inhibition tests in 9.5% of tests involving 223 compounds (TEIXIDO; LEUTHOLD; DE CROZE; LÉONARD *et al.*, 2019). Zebrafish embryo-larval bioassays focused on lethal and sublethal endpoints are increasingly being used worldwide as efficient and sensitive tools for assessing the ichthyotoxicity of surface waters and sediments in North America (VANLANDEGHEM; MEYER; COX; SHARMA *et al.*, 2012), Central America (WILSON; CASTRO; CHAVES; ESPINOSA *et al.*, 2021), Europe (SCHWEIZER; DIETERICH; CORRAL MORILLAS; DEWALD *et al.*, 2018) and Asia (CHEN; CHEN; CHEN; LIU *et al.*, 2015).

### 3.5.2 Spatial water quality variation and fish developmental effects

Zebrafish exposed to S1 waters presented a significant developmental delay based on GMS at all time points, and no developmental delay was observed in zebrafish exposed to S2 at any time (Figure 2). This decrease in toxicity suggests that Carpina Reservoir and its dam which releases water from the top of the reservoir is trapping toxic chemical contaminants from upstream S1 to downstream S2. There is a clear drop in ΣPAH at S2 (Table 4), as well as an improvement in water quality based on WQI and TSI from S1 to S2 (Table 3). Reduced PAH water concentrations were found at sites below the Three Gorge River dam in China, possibly due to deposition of PAH associated to suspended solids deposited in sediments trapped by the dam (WANG; HARRIS; ESPINOZA; MCCLAIN *et al.*, 2012).

S5 and S7 are located within the urbanized area of São Lourenço da Mata City where the state water supply company collects water for treatment and distribution for human consumption. There is a clear pattern of less severe developmental delay at S2, S5 and S7 expressed as an overall mean GMS of 16.9 at these least impacted sites, confirmed by the grouping of these three sites in cluster analysis (Figure 3) and through their grouping to the left of axis 2 through PCA analysis, with highest values for GMS and WQI (Figure 4).

S1, S2, S3 and S4 are influenced by sugar cane agriculture, but include some smaller urbanized areas with a diverse array of industrial activities such as textile, tannery, and ethanol production (CPRH, 2018). A significant developmental delay was observed at S3 (mean GMS = 16.7) (Figure 2), as well as significant increase in developmental abnormality SBNI (81%), downstream from Paudalho City (population of 56,000), a region dominated by sugarcane plantations. No mortality was detected at S4, located 350 m before Goitá River (GR) flows into CR, but a significant developmental delay was observed (mean GMS = 16.5), as well as significant increase in SBNI (69%).

S8 is downstream of São Lourenço da Mata City and within the second largest conurbation among the cities involved in this study. Mean GMS was 16.4, equal to the overall mean GMS for sites S1, S3 and S4. Sublethal ecotoxicity parameters are similar at S1, S3, S4 and S8, and these sites were grouped in the cluster analysis (Figure 3) and showed intermediate values along axes 1 and 2 in PCA analysis (Figure 4), indicating an intermediate impact in water quality.

S6, S9, and S10 undergo the greatest anthropogenic pressures as they are near densely urbanized areas with the largest populations. S6 is located on Tapacurá River downstream from Vitória de Santo Antão City (population of 138k people), and upstream of Tapacurá reservoir. Contaminants from this city are probably trapped within the reservoir, as they do not influence downstream reaches of CR at S7 after Tapacurá reservoir (Figure1). ΣPAH at S6 was 579 ng $L^{-1}$ and $NH_3$ reached highest concentrations detected in this study. At S6 mean WQI was lowest (19) among all sites, and sublethal ecotoxicity followed the same pattern with a mean GMS of 14.8. S9 and S10 are located within the highly urbanized city of Recife (population of 1.5M people) (IBGE, 2020). S6, S9, and S10 were considered the most impacted in terms of sublethal toxicity expressed by GMS (Figure 2). This pattern is also indicated in Figure 4, where S6, S9 and S10 are located opposite to the GMS vector and close to the vectors for developmental abnormality frequencies.

### 3.5.3 $NH_3$, PAH and zebrafish toxicity

Concerning possible toxic contaminants in the analyzed mixtures, the significant negative correlation observed between $NH_3$ and GMS suggests $NH_3$ is involved in zebrafish sublethal developmental toxicity along CR (Table 5). $NH_3$ is toxic and can induce numerous effects in fish including loss of equilibrium, gill hyperplasia, and mortality, reaching aquatic ecosystems from multiple sources including domestic sewage, agricultural fertilizers, and industrial processes (FAIRCHILD; ALLERT; SAPPINGTON; WADDELL, 2005; USEPA, 2013). The

significant positive correlation observed between WQI and GMS (Table 5) suggests that zebrafish developmental toxicity increases at sites where larger amounts of domestic sewage and associated contaminants such as $NH_3$ are being released. Frequencies of blood stasis and lack of protrusible mouth (Table 2) presented a positive correlation with $NH_3$ (Table 5). Laboratory-based experiments estimated the lowest observed effect concentration (LOEC) of 0.15 mg $NH_3$ $L^{-1}$ in water for the endpoint frequency of exposed zebrafish larvae with blood stasis at 24 hpf (C. F. Mariz Jr. and P.S.M. Carvalho, unpublished results). Highest $NH_3$ was detected at S6, where this LOEC threshold was always exceeded in T1, T2 and T3, and where highest frequencies of BS were observed. High $NH_3$ was also detected at S9 and S10 above this LOEC (Table 3), where high frequencies of BS were also observed (Table 2). Blood circulation is necessary for the differentiation of the mesenchymal smooth muscle and the organization of the external mesothelium in the bladder, which can be fundamental for the proper inflation of the swim bladder (WINATA; KORZH; KONDRYCHYN; KORZH *et al.*, 2010). In addition, zebrafish need to rise to the surface to capture an initial amount of air so that the swim bladder can be inflated (LINDSEY; SMITH; CROLL, 2010), and the high frequencies of fish without protrusible mouths at S1, S6, S9 and S10 might have compromised the process of initial swimbladder inflation and contributed to the high frequencies of fish without inflated swimbladders at the same sites (Table 2). This specific abnormality can compromise the larva's viability if it is not reversed, since the development and inflation of the swim bladder guarantees the normal development of most species (CZESNY; GRAEB; DETTMERS, 2005), conferring neutral buoyancy that is essential for proper swimming and initial capture of exogenous food, as well as to escape from predators.

The chronic benchmark expressed as the effective concentration of $NH_3$ resulting in 20% reduction in either growth or mortality of fish (EC20s) ranges from 0.07 mg $NH_3$ $L^{-1}$ in the most sensitive bluegill *Lepomis macrochirus* to 0.48 mg $NH_3$ $L^{-1}$ in channel catfish *Ictalurus punctatus* (FAIRCHILD; ALLERT; SAPPINGTON; WADDELL, 2005). Highest $NH_3$ concentrations detected at sites S6 and S10 significantly exceed 0.20 mg $NH_3$ $L^{-1}$, roughly in the middle range of the above-mentioned chronic benchmarks for growth and mortality in fishes, whereas at S9 $NH_3$ concentrations range from 18% to 74% of this value. Furthermore, USA EPA's chronic ambient water quality criteria (AWQC) for protecting freshwater organisms from long term sublethal effects of ammonia which is 1.9 mg $L^{-1}$ total ammonia nitrogen at pH 7.0 and temperature 20 °C, equivalent to 0.008 mg $NH_3$ $L^{-1}$ (USEPA, 2013). If we consider the latter most restrictive criteria, our results indicate that resident aquatic

organisms from a significantly larger number of sites within CRB would be at risk due to $NH_3$ toxicity. These more restrictive chronic benchmarks are based on solid scientific information and are significantly lower than the LC50-96h of 2.07 mg $NH_3$ $L^{-1}$ for zebrafish exposed from fertilized eggs (C. F. Mariz Jr. and P.S.M. Carvalho, unpublished results). Mortality rate was 95% in zebrafish exposed to water samples from S3-T3 (Table 1), with 0.018 mg $NH_3$ $L^{-1}$ (Table 3), a concentration that is 0.9 % of the zebrafish $NH_3$ LC50-96h (concentration that kills 50% of exposed zebrafish from fertilized egg until 96 hpf) of 2.07 mg $NH_3$ $L^{-1}$ (C. F. Mariz Jr. and P.S.M. Carvalho, unpublished results). Therefore, $NH_3$ concentrations would not explain the 95% mortality detected at S3-T3. However, at S6-T2, where a 45% mortality was observed (Table 1), the $NH_3$ concentration of 1.56 mg $NH_3$ $L^{-1}$ (Table 3) was equivalent to 75 % of zebrafish $NH_3$ LC50-96h, suggesting that $NH_3$ is a major contributor to the detected lethality. At S6-T3 and S10-T2, where zebrafish mortalities of 30 % and 45 % were detected (Table 1), $NH_3$ concentrations of 0.45 and 0.22 mg $NH_3$ $L^{-1}$ (Table 3) represented 23% and 11% of zebrafish $NH_3$ LC50-96h, respectively, suggesting that $NH_3$ is contributing to the detected lethality. Overall, the highest mortality rates detected tended to be associated with the highest $NH_3$ surface water concentrations, although no significant positive correlation between these 2 variables was found (Table 5).

Developing fish embryos are sensitive to the toxic effects of dissolved PAH that affect heart development and induce morphological abnormalities (CHERR; FAIRBAIRN; WHITEHEAD, 2017). Zebrafish embryos exposed for 96 h to urban stormwater runoff with total PAH concentrations ranging from 2 to 23 μg $L^{-1}$ and dominated by 3-ring (phenanthrenes) and 4-ring (chrysenes and fluoranthenes) developed delayed hatching, reduced swimbladder inflation and reduced growth (MCINTYRE; DAVIS; INCARDONA; STARK *et al.*, 2014), similar to the sublethal effects we observed in zebrafish exposed to CR surface waters. The significant correlations between GMS and MNA, FLU, CRI and HMW-PAH (Table 5) suggest PAHs as relevant contributors to the detected developmental toxicity. Phenanthrene was the major PAH detected in CR surface waters, but its LC50-120h for zebrafish early-life stages is 310 μg $L^{-1}$ (VERGAUWEN; SCHMIDT; STINCKENS; MAHO *et al.*, 2015), more than 2 orders of magnitude higher than the highest concentration of phenanthrene observed at S1 (971 ng $L^{-1}$, Table 4), reducing the probability of this PAH being responsible for the lethal effects detected.

PAH were detected at all sites, but an unexpected spatial pattern was observed. Highest concentrations of ΣPAH were detected at predominantly rural areas in S1 and S4, and slightly lower ΣPAH were found in the more urbanized sites S6, S8, S9 and S10 (Table 4). Phenanthrene

accounted for 70 % or more of the ΣPAH at most sites, including the upstream reaches represented by S1, S2, S3 and S4. ΣPAH at all sites are above typical values for rivers with low PAH contamination (10 to 50 ng $L^{-1}$) and accordingly would be classified as moderately polluted (ΣPAH 250 to 1,000 ng $L^{-1}$) (CAO; LIU; LUAN; LI *et al.*, 2010). The final effluent of oil-water-separators from gasoline stations within Recife metropolitan region were shown to be significant sources of PAH, including phenanthrene, to pluvial systems that drain to the same estuarine complex as the Capibaribe River (ALVES; MARIZ JR; PAULO; CARVALHO, 2017). Highest ΣPAH and phenanthrene concentrations verified at S1 can be related to the 10 gas stations located within 400 m of CR margins in urban Limoeiro city upstream of S1 functioning as point sources. Three gas stations are present close to the river upstream of S3, where ΣPAH is above the mean ΣPAH for all sites of 638 ng $L^{-1}$, and no gas stations are present within 400 m of any other site (Table 4). Although a significant number of gas stations are close to CR at S1, it is important to emphasize that agricultural activities and domestic sewage could also be contributing to PAH input. The prevalence of phenanthrene at most sites contributes to the predominance of low molecular weight PAH, and the ratio between LMW/HMW PAH in samples is more than 20 at most sites, indicating that PAH sources within the studied area are mostly petrogenic, corroborated by ratios of Ant/178 and Fl/Fl + Py isomers (Table 4). The consistent indication of petrogenic origin of PAH at S1 corroborates the hypothesis that gas stations in Limoeiro City could be significant local sources of PAH. At S10 the ratio between LMW/HMW is 1.9, and although it also indicates petrogenic origin, the gradual decrease on this ratio along CR suggests that other sources are contributing with PAH input, possibly a mix of petrogenic and pyrolytic sources, a hypothesis that is corroborated by a previous study that quantified PAH in sediments from CR close to S10 and reported a mixture of petrogenic and pyrolytic PAH sources (MACIEL; DE SOUZA; TANIGUCHI; BÍCEGO *et al.*, 2015).

It is reasonable that other contaminants not measured in this study might be influencing the detected zebrafish developmental toxicity. There is a lack of information about chemical contaminant concentrations in the upper reaches of CR, but in the lower reaches close to sites S9 and S10 sediment contaminated by metals, PAH, chlorinated pesticides DDT and DDE, and polychlorinated biphenyls were associated with toxicity in laboratory tests to nauplii of the meiofaunal copepod *Tisbe biminiensis* (RÉGIS; SOUZA-SANTOS; YOGUI; MORAES *et al.*, 2018), and estrogens have been detected and associated with behavioral effects in male *Poecilia vivipara* (MELO; DE PAULO; MONTAGNER; CARVALHO, 2021).

Although local fish species should be used to confirm trends in toxicity detected with zebrafish in this study, it is reasonable to assume that the detected effects in this study could happen to early-life stages of local fish species. A community level study detected less structured fish assemblages characterized by lower species richness, evenness and diversity observed within the region of our sites S4 and S8, which were characterized as anthropized sites by (COLLIER; DE ALMEIDA NETO; DE ALMEIDA; ROSA FILHO *et al.*, 2019), and where CR surface waters caused developmental effects in zebrafish in this study. Sites analyzed in the present study are part of the Pernambuco State Environmental Agency Water Quality Monitoring Network. Undiluted surface waters from S5, S6 and S7 were not toxic to *Daphnia magna* neonates after a 48h exposure based on the immobilization test (OECD, 2004), the only sites where the test was applied by the agency (CPRH, 2018). If we consider *Daphnia magna* immobilization an effect comparable to mortality, zebrafish mortality results from the present study concur with the absence of severe toxicity at S5 and S7. However, at S5-T3 CR surface waters caused 30% mortality to zebrafish, and at S6 our results detected mortalities of 45% and 30% at S6-T2 and S6-T3, indicating that zebrafish was more sensitive than *Daphnia magna* in terms of lethal effects. Furthermore, zebrafish sublethal endpoints applied in the present study detected a large set of significant effects along CR which are not even evaluated by the *D. magna* 48h immobilization test.

Our results indicate that Capibaribe River surface waters cause increasing zebrafish sublethal developmental toxicity from more rural upstream to more urbanized downstream reaches closer to Recife metropolitan region, helping to prioritize river reaches which require further chemical characterization to identify other relevant contaminants causing toxicity. The use of zebrafish lethal and sublethal developmental endpoints was also useful to characterize and rank the toxicity of river surface waters in more urbanized and industrialized areas such as the Huangpu River in China (ZHANG; LI; CHEN; ZHANG *et al.*, 2015) and the Matasnillo and Curundú rivers in Panama (WILSON; CASTRO; CHAVES; ESPINOSA *et al.*, 2021). A significant number of other studies increasingly corroborate the relevance of this model as an important effect-based method for river water monitoring strategies (CRISTIANO; LACCHETTI; MANCINI; CORTI *et al.*, 2019).

## 3.6 CONCLUSION

Application of zebrafish developmental toxicity endpoints to Capibaribe River surface waters identified a smaller impact in terms of ecotoxicity in rural areas at sites S2, S5 and S7, an intermediate impact at sites with urban and agricultural influence S1, S3, S4 and S8, and

greatest impacts were detected at more urbanized sites S6, S9 and S10. Endpoints focused on zebrafish early development can improve surface water quality monitoring in tropical river systems.

## 3.7 ACKNOWLEDGEMENTS

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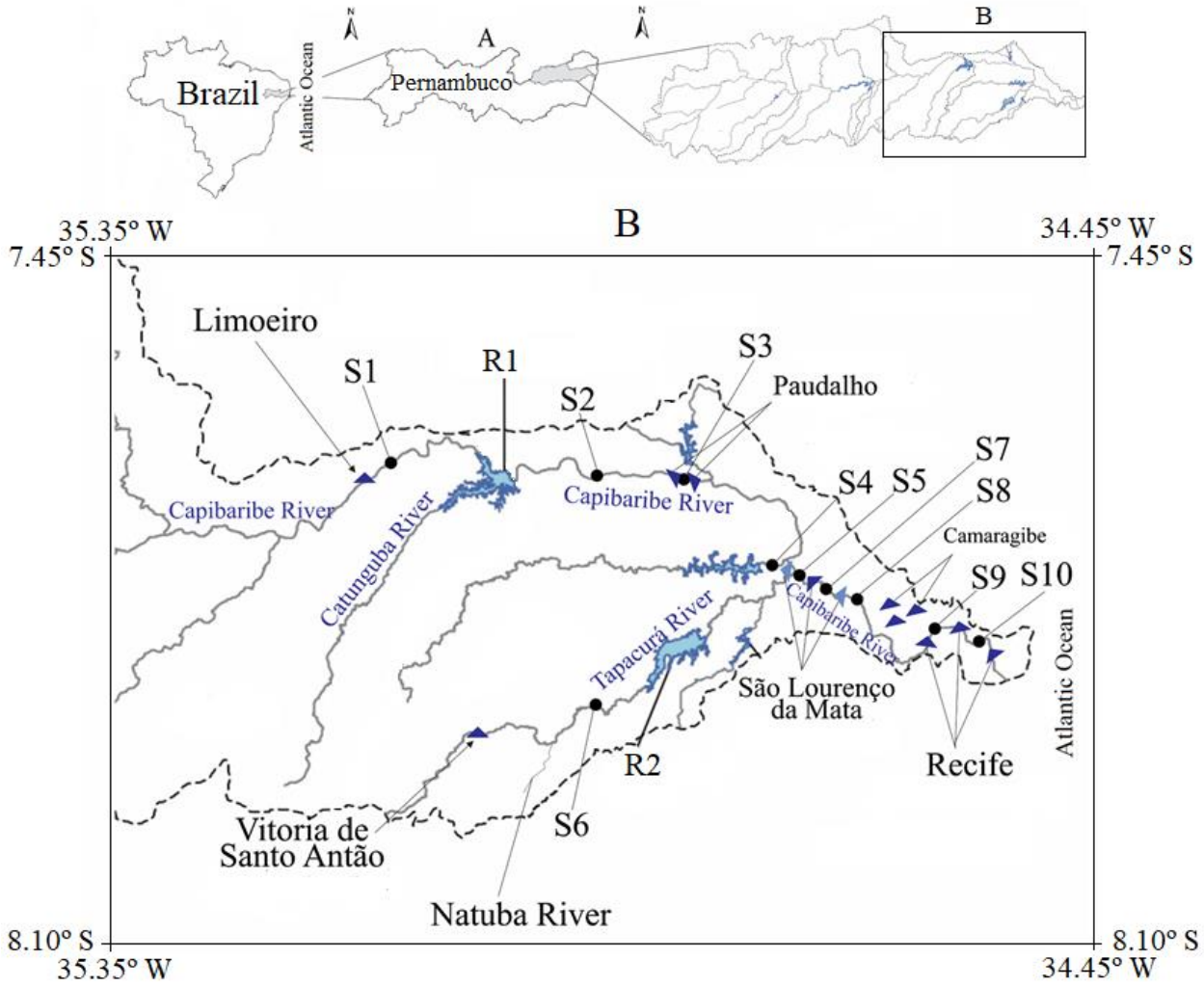
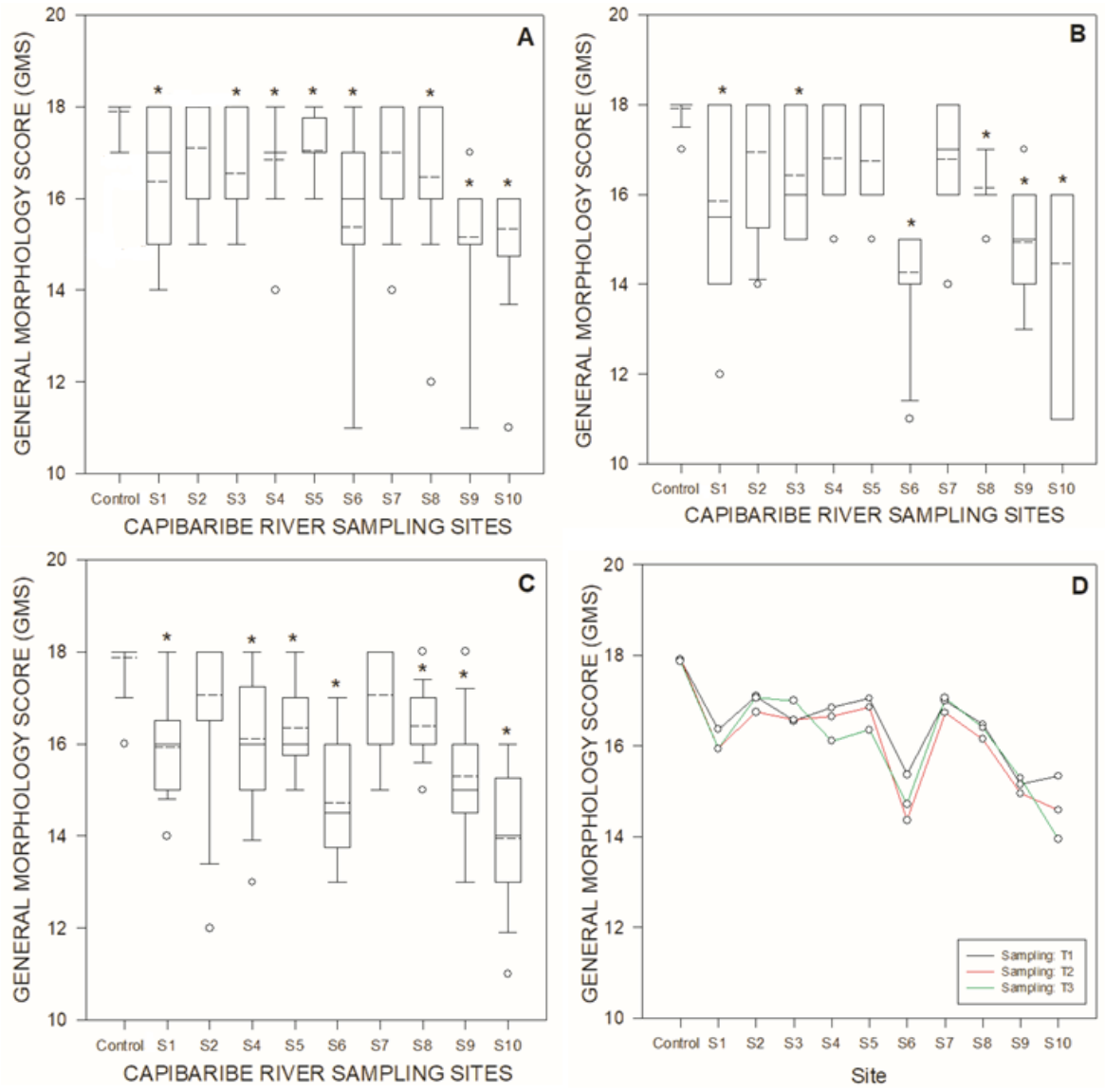
Figure 1 - Location of Capibaribe River Basin (enclosed by dotted lines) within the State of Pernambuco, Brazil (A) and sampling stations (S1-S10) analyzed along the perennial lower portion of Capibaribe River (B).



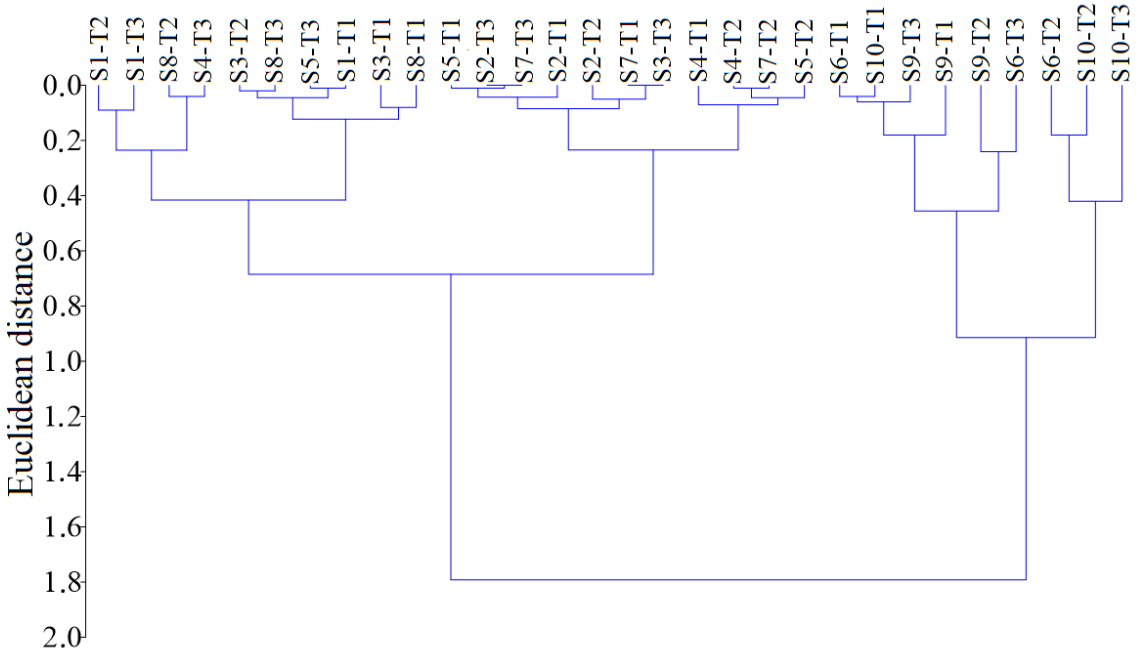

S2, S5 and S7: least impacted sites in rural areas; S1, S3, S4 and S8: sites with an intermediate impact with mixed urban and rural/agricultural influence; S6, S9 and S10: most impacted sites in heavily urbanized areas. R1: Carpina Reservoir, R2: Tapacurá Reservoir.

Figure 2 - General morphology score (GMS) values for zebrafish embryos and larvae exposed to surface water samples from Capibaribe River Basin and control water. (n = 20 zebrafish per site).



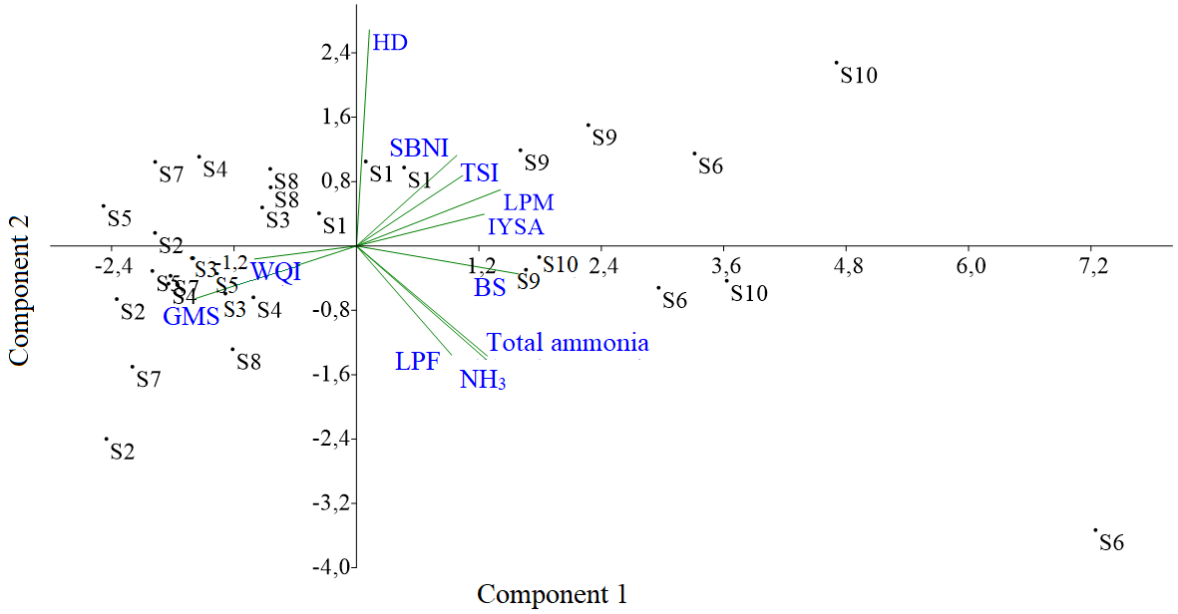

Box plot dashed horizontal line indicate mean values, and box plot continuous horizontal lines (lower, middle and upper) indicate quartiles of 25%, 50% (median) and 75%, respectively. A: May 2018 (T1), B: August 2018 (T2) and C: May 2019 (T3). D: Plot of mean GMS values for each sampling site and control at T1, T2 and T3. *: statistically significant differences between sites at each sampling time compared to the respective control based on Kruskall Wallis test. A: (KW H10 = 96.3, p <0.001; Dunn p <0.05), B (KW H10 = 80.75, p < 0.001; Dunn p < 0.05), C (KW H10 = 106.4, p < 0.001; Dunn p < 0.05).

Figure 3 - Cluster analysis (Euclidean distance) for zebrafish early life stage development Global Morphology Score data for each sampling point along different sites and sampling times in the Capibaribe River.




S: site; T1: May 2018, T2: August 2018, T3: May 2019

Figure 4 - Biplot of principal components 1 and 2. Data inside the biplot refer to the different sampling sites, water quality index (WQI), trophic state index (TSI), general morphology score (GMS), total ammonia, NH3 and the main observed anomalies.




IYSA (Incomplete Yolk sac absorption); SBNI (Swim bladder not inflated); LPM (Lack of protrusible mouth); HD (Hatching delay); LPF (Lack of pectoral fin); BS (Blood stasis).

Table 1: Mortality rates (%) of zebrafish embryos and larvae exposed to lower Capibaribe River Basin surface water samples along different sites in May 2018 (T1), August 2018 (T2), and May 2019 (T3).

| Site | Mortality rates (%) | Mortality rates (%) mean ± standard deviation |
|---|---|---|
| Control-T1 | 2.1 | |
| Control-T2 | 0.0 | 2.1 ± 3.1% |
| Control-T3 | 4.0 | |
| S1-T1 | 5.0 | |
| S1-T2 | 0.0 | 6.6 ± 8.0% |
| S1-T3 | 15.0 | |
| S2-T1 | 0.0 | |
| S2-T2 | 0.0 | 6.6 ± 10.6% |
| S2-T3 | 20.0 | |
| S3-T1 | 0.0 | |
| S3-T2 | 5.0 | 33.3 ± 46.8%* |
| S3-T3 | 95.0 | |
| S4-T1 | 0.0 | |
| S4-T2 | 0.0 | 3.1 ± 6.3% |
| S4-T3 | 10.0 | |
| S5-T1 | 0.0 | |
| S5-T2 | 0.0 | 10.1 ± 15.1% |
| S5-T3 | 30.0 | |
| S6-T1 | 5.0 | |
| S6-T2 | 45.0 | 26.7 ± 18.4%* |
| S6-T3 | 30.0 | |
| S7-T1 | 0.0 | |
| S7-T2 | 5.0 | 6.6 ± 78.8% |
| S7-T3 | 15.0 | |
| S8-T1 | 5.0 | |
| S8-T2 | 0.0 | 9.8 ± 12.5% |
| S8-T3 | 25.0 | |
| S9-T1 | 0.0 | |
| S9-T2 | 0.0 | 5.0 ± 7.5% |
| S9-T3 | 15.0 | |
| S10-T1 | 10.0 | |
| S10-T2 | 45.0 | 21.4 ± 18.9% |
| S10-T3 | 10.0 | |

*: statistically significant differences between sites compared to the control (ANOVA $F_{10,88} = 2.95$, $p = 0.003$, followed by Dunnet test, $p < 0.05$).

Table 2: Frequency (%, mean ± standard deviation) of recorded abnormalities during zebrafish development after exposure to surface waters from the Capibaribe River Basin.

| Sites Capibaribe River | Blood stasis 24 hpf | Hatching delay 72 hpf | Absent pectoral fin 96 hpf | Protrusible mouth absent 96 hpf | Incomplete Yolk sac absorption 96 hpf | Uninflated Swim bladder 96 hpf |
|---|---|---|---|---|---|---|
| Control | 0 ± 0 % | 0 ± 0 % | 0 ± 0 % | 0 ± 0 % | 1 ± 2 % | 9 ± 1 % |
| S1 | 2 ± 3 % | 25 ± 11 % | 1 ± 2 % | **34 ± 7 %*** | **51 ± 7 %*** | **65 ± 18 %*** |
| S2 | 0 ± 0 % | 20 ± 14 % | 4 ± 7 % | 3 ± 3 % | 28 ± 6 % | 30 ± 9 % |
| S3 | 0 ± 0 % | 17 ± 16 % | 0 ± 0 % | 0 ± 0 % | 34 ± 3 % | **81 ± 16 %*** |
| S4 | 3.7 ± 6 % | 27 ± 24 % | 1 ± 3 % | 7 ± 3 % | 27 ± 4 % | **69 ± 13 %*** |
| S5 | 0 ± 0 % | 28 ± 19 % | 2 ± 4 % | 2 ± 3 % | 16 ± 2 % | **65 ± 12 %*** |
| S6 | **53 ± 22 %*** | 23 ± 21 % | **12 ± 5 %*** | **39 ± 4 %*** | **63 ± 10 %*** | **83 ± 3 %*** |
| S7 | 0 ± 0 % | 27 ± 25 % | 2 ± 3 % | 5 ± 5 % | 25 ± 12 % | 54 ± 14 % |
| S8 | 0 ± 0 % | 26 ± 18 % | 4 ± 6 % | 4 ± 6 % | 41 ± 12 % | **87 ± 17 %*** |
| S9 | **24 ± 5 %*** | 28 ± 16 % | 7 ± 8 % | **31 ± 8 %*** | **89 ± 1 %*** | **96 ± 3 %*** |
| S10 | **43 ± 17 %*** | 31 ± 28 % | **15 ± 16 %*** | **40 ± 2 %*** | **98 ± 3 %*** | **98 ± 3 %*** |

*****: statistically significant differences between sites compared to the control (ANOVA followed by Dunnet test, $p < 0.05$). hpf: hours post fertilization

Table 3: Water quality variables and indexes quantified in lower Capibaribe River Basin surface water samples along different sites in May 2018 (T1), August 2018 (T2), and May 2019 (T3).

| Site | Sampling | DO ($mg\ O_2\ L^{-1}$) | BOD ($mg\ O_2\ L^{-1}$) | Total Ammonia ($mg\ L^{-1}$) | $NH_3$ ($mg\ L^{-1}$) | P ($mg\ P\ L^{-1}$) | TC (MPN 100 $mL^{-1}$) | pH | Conductivity ($\mu S\ cm^{-1}$) | WQI | TSI |
|---|---|---|---|---|---|---|---|---|---|---|---|
| S1 | T1 | 1.70 | 3.60 | 1.12 | 0.012 | 1.51 | <180 | 7.20 | 2387 | AC (51) | HE (74) |
| S1 | T2 | 1.70 | 5.50 | 0.31 | 0.005 | 1.55 | <180 | 7.30 | 3270 | AC (42) | HE (74) |
| S1 | T3 | 1.50 | 3.00 | 0.13 | 0.002 | 0.00 | 2100 | 7.60 | 3070 | BD (34) | - |
| S2 | T1 | <0.5 | 6.70 | <0.07 | - | 0.27 | 780 | 7.00 | 1340 | GD (55) | SE (65) |
| S2 | T2 | 2.90 | 5.90 | 0.15 | 0.002 | 0.58 | <180 | 7.30 | 1422 | GD (55) | HE (69) |
| S2 | T3 | 1.70 | 10.20 | 0.56 | 0.009 | 0.03 | 1700 | 7.40 | 1856 | AC (47) | MT (54) |
| S3 | T1 | 2.70 | 6.70 | 0.54 | 0.004 | 0.22 | 4800 | 7.00 | 940 | AC (46) | SE (64) |
| S3 | T2 | 1.90 | 3.40 | 2.75 | 0.019 | 0.45 | 7900 | 6.90 | 828 | AC (39) | HE (68) |
| S3 | T3 | 0.90 | 17.00 | 2.13 | 0.018 | 0.57 | <180 | 7.30 | 891 | BD (35) | HE (69) |
| S4 | T1 | <0.5 | 14.70 | <0.07 | - | 0.13 | 3300 | 6.90 | 246 | AC (38) | EU (61) |
| S4 | T2 | <0.5 | 2.10 | <0.07 | - | 0.37 | 2200 | 6.60 | 373 | BD (34) | SE (67) |
| S4 | T3 | 4.00 | 4.00 | 0.27 | 0.001 | 0.09 | 200 | 7.10 | 221 | BD (63) | MT (59) |
| S5 | T1 | 1.30 | 23.10 | 0.25 | 0.003 | 0.18 | <180 | 7.10 | 442 | GD (39) | SE (63) |
| S5 | T2 | 3.00 | 2.30 | 0.13 | 0.001 | 0.12 | 780 | 6.80 | 350 | AC (57) | EU (61) |
| S5 | T3 | 5.40 | 1.50 | <0.07 | - | 0.00 | 1400 | 7.00 | 319 | GD (67) | - |
| S6 | T1 | <0.5 | 19.80 | 7.66 | 0.332 | 1.46 | 35000 | 6.70 | 876 | BD (22) | HE (74) |
| S6 | T2 | <0.5 | 46.20 | 49.20 | 1.563 | 2.31 | 4600 | 6.80 | 946 | VD (17) | HE (76) |
| S6 | T3 | <0.5 | 37.40 | 9.06 | 0.448 | 2.81 | 2000 | 7.50 | 1145 | VB (18 | HE (77) |
| S7 | T1 | 4.60 | 3.40 | 0.33 | 0.003 | 0.11 | 1300 | 6.70 | 446 | GD (57) | EU (60) |
| S7 | T2 | 3.60 | 4.40 | 0.12 | 0.001 | 0.17 | 2700 | 6.60 | 352 | GD (52) | EU (62) |
| S7 | T3 | <0.5 | 11.10 | 0.67 | 0.006 | 0.23 | 7000 | 6.70 | 437 | AC (37) | SE (64) |
| S8 | T1 | 3.80 | 3.60 | 1.08 | 0.004 | 0.12 | <180 | 6.70 | 435 | GD (53) | EU (61) |
| S8 | T2 | 7.00 | 4.00 | 2.85 | 0.015 | 0.55 | 3400 | 6.80 | 382 | GD (58) | HE (69) |
| S8 | T3 | 2.80 | 5.50 | 6.01 | 0.028 | 0.40 | 14000 | 7.30 | 380 | AC (41) | SE (67) |
| S9 | T1 | 1.10 | 20.60 | 2.21 | 0.037 | 0.34 | 3900 | 6.50 | 385 | BD (31) | SE (66) |
| S9 | T2 | 1.80 | 8.40 | 5.40 | 0.098 | 0.87 | 4000 | 6.80 | 461 | AC (39) | HE (71) |
| S9 | T3 | <0.5 | 13.20 | 5.20 | 0.148 | 0.54 | 14000 | 7.10 | 492 | BD (31) | HE (68) |
| S10 | T1 | 0.80 | 18.10 | 4.70 | 0.095 | 0.69 | 3900 | 6.50 | 1134 | BD (29) | HE (70) |
| S10 | T2 | 5.40 | 10.90 | 6.77 | 0.220 | 1.43 | 4700 | 7.60 | 2238 | AC (46) | HE (74) |
| S10 | T3 | <0.5 | 15.30 | 11.90 | 0.289 | 1.76 | 1700 | 7.20 | 1525 | BD (28) | HE (75) |

DO (dissolved oxygen); BOD (biochemical oxygen demand); P (Phosphorus); TC (thermotolerant coliforms); WQI (water quality index): 0 to 19 - very bad (VB), 20 to 36 – bad (BD), 37 to 51 - acceptable (AC), 52 to 79 - good (GD) and 80 to 100 - excellent (EX). TSI (trophic state index): ≤ 47 - ultra-oligotrophic (UO), 47 to 52 - oligotrophic (OL), 52 to 59 - mesotrophic (MT), 59 to 63 - eutrophic (EU), 63 to 67 - supereutrophic (SE) and > 67 – hypereutrophic (HE).

Table 4: Individual and total PAH concentrations (ng $L^{-1}$) quantified in lower Capibaribe River Basin surface water samples along different sites in May 2019 (T3).

| PAH | S1 | S2 | S3 | S4 | S5 | S6 | S7 | S8 | S9 | S10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Naphthalene | 65.0 | 9.0 | 19.0 | 9.2 | 103.8 | 86.9 | 12.8 | 12.9 | 22.5 | 73.2 |
| 2-Methylnaphthalene | ND | ND | 1,9 | ND | 1.1 | 9.2 | ND | 1.3 | 19.1 | 16.3 |
| Acenaphthylene | ND | ND | 2.0 | ND | ND | ND | ND | ND | ND | ND |
| Acenaphthene | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Fluorene | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Phenanthrene | 971 | 158 | 636 | 881 | 647 | 405 | 252 | 749 | 610 | 165 |
| Anthracene | 15.0 | 5.0 | 10.9 | 14.8 | 8.2 | 10.0 | 5.5 | 5.6 | 7.3 | 4.5 |
| Fluoranthene | 2.5 | 2.1 | 2.5 | 2.9 | 2.1 | 9.0 | 2.8 | 2.9 | 5.3 | 9.1 |
| Pyrene | 23.0 | 4.0 | 6.8 | 18.0 | 14.0 | 47.7 | 8.0 | 16.4 | 19.1 | 15.1 |
| Benzo[a]anthracene | ND | ND | ND | ND | ND | ND | ND | ND | ND | 17,4 |
| Chrysene | ND | ND | 1.5 | ND | ND | 3.1 | <LOQ | 1.8 | 2.0 | 18.5 |
| Benzo[b]fluoranthene | ND | ND | ND | ND | ND | ND | ND | ND | ND | 14.5 |
| Benzo[k]fluoranthene | ND | ND | ND | ND | ND | ND | ND | ND | ND | 19.5 |
| Benzo[a]pyrene | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Indeno[1,2,3-cd]pyrene | ND | ND | ND | ND | ND | ND | ND | ND | ND | 10.6 |
| Dibenzo[a,h]anthracene | 3.0 | 2.0 | ND | 2.0 | ND | 7.8 | 4.0 | 5.1 | ND | 14.8 |
| Benzo[g,h,i]perylene | ND | ND | ND | ND | ND | ND | ND | ND | ND | 14.7 |
| ΣPAH | 1079 | 181 | 680 | 927 | 776 | 579 | 286 | 795 | 685 | 393 |
| Number of gas stations* | 10 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ∑PAH LMW | 1051 | 173 | 669 | 905 | 760 | 511 | 270 | 768 | 659 | 259 |
| ∑PAH HMW | 28 | 8 | 11 | 23 | 16 | 68 | 15 | 26 | 26 | 134 |
| LMW/HMW | 37.5 | 20.9 | 62.3 | 39.6 | 47.1 | 7.6 | 18.5 | 29.4 | 25.0 | 1.9 |
| Ant/178 | 0.015 | 0.032 | 0.017 | 0.017 | 0.013 | 0.024 | 0.021 | 0.007 | 0.012 | 0.027 |
| Fl/Fl+Py | 0.10 | 0.35 | 0.27 | 0.14 | 0.13 | 0.16 | 0.26 | 0.15 | 0.22 | 0.38 |

ND (not detected, considered 0 ng $L^{-1}$), Limit of quantification (LOQ) = 1.0 ng $mL^{-1}$); LMW (low molecular weight); HMW (high molecular weight)
Ant/178 = anthracene/anthracene + phenanthrene; Fl/Fl+Py = fluoranthene/fluoranthene + pyrene.
*: within 400m of Capibaribe River margins upstream of Capibaribe sites, verified by Google Earth maps.

Table 5: Pearson correlation coefficients and significance (p values) between water quality indices, zebrafish ecotoxicity endpoints and toxic contaminants evaluated in surface water along Capibaribe River Basin.

| | TSI | GMS | MOR | IYSA | SBNI | LPM | HD | LPF | BS | NH3 | MNA | FLU | PIR | CRI | SPAH | LPAH | HPAH |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WQI | -0.43 | **0.58** | -0.25 | -0.31 | -0.42 | **-0.55** | -0.08 | -0.18 | **-0.65** | **-0.56** | -0.55 | -0.67 | -0.46 | -0.41 | 0.24 | 0.30 | -0.49 |
| | 0.024 | **0.001** | 0.186 | 0.098 | 0.020 | **0.002** | 0.663 | 0.334 | **0.001** | **0.001** | 0.100 | 0.033 | 0.178 | 0.244 | 0.501 | 0.403 | 0.149 |
| TSI | | **-0.67** | 0.26 | **0.48** | 0.47 | **0.64** | 0.16 | 0.27 | **0.60** | **0.49** | 0.50 | 0.52 | 0.40 | 0.53 | 0.26 | 0.17 | 0.52 |
| | | **0.001** | 0.183 | **0.009** | 0.012 | **0.001** | 0.423 | 0.171 | **0.001** | **0.009** | 0.204 | 0.191 | 0.333 | 0.173 | 0.533 | 0.682 | 0.186 |
| GMS | | | -0.16 | | | | | | | **-0.61** | **-0.80** | **-0.92** | -0.62 | **-0.76** | -0.06 | 0.06 | **-0.89** |
| | | | 0.389 | | | | | | | **0.001** | **0.005** | **0.001** | 0.054 | **0.010** | 0.873 | 0.867 | **0.0005** |
| MOR | | | | 0.00 | 0.35 | 0.05 | -0.30 | 0.27 | 0.26 | 0.35 | -0.20 | -0.21 | -0.17 | -0.17 | 0.05 | 0.09 | -0.28 |
| | | | | 0.990 | 0.057 | 0.779 | 0.113 | 0.145 | 0.162 | 0.060 | 0.584 | 0.565 | 0.630 | 0.638 | 0.883 | 0.807 | 0.431 |
| IYSA | | | | | | | | | | 0.35 | 0.72 | 0.64 | 0.39 | 0.57 | 0.15 | 0.06 | 0.67 |
| | | | | | | | | | | 0.062 | 0.019 | 0.048 | 0.267 | 0.084 | 0.683 | 0.878 | 0.035 |
| SBNI | | | | | | | | | | 0.24 | 0.49 | 0.44 | 0.38 | 0.42 | 0.54 | 0.47 | 0.44 |
| | | | | | | | | | | 0.201 | 0.151 | 0.207 | 0.282 | 0.232 | 0.106 | 0.175 | 0.203 |
| LPM | | | | | | | | | | **0.51** | 0.76 | **0.86** | 0.49 | **0.79** | -0.05 | -0.16 | **0.89** |
| | | | | | | | | | | **0.004** | 0.011 | **0.002** | 0.153 | **0.007** | 0.900 | 0.657 | **0.001** |
| HD | | | | | | | | | | -0.08 | 0.71 | **0.86** | 0.54 | 0.73 | -0.12 | -0.22 | **0.85** |
| | | | | | | | | | | 0.677 | 0.021 | **0.002** | 0.107 | 0.016 | 0.748 | 0.535 | **0.002** |
| LPF | | | | | | | | | | 0.39 | -0.05 | 0.16 | 0.02 | 0.13 | -0.47 | -0.48 | 0.17 |
| | | | | | | | | | | 0.035 | 0.885 | 0.658 | 0.947 | 0.713 | 0.171 | 0.165 | 0.641 |
| BS | | | | | | | | | | **0.82** | **0.79** | **0.96** | 0.50 | **0.86** | -0.19 | -0.31 | **0.95** |
| | | | | | | | | | | **0.001** | **0.006** | **0.001** | 0.143 | **0.001** | 0.592 | 0.381 | **0.000** |
| NH3 | | | | | | | | | | | 0.69 | **0.96** | 0.76 | 0.57 | -0.23 | -0.32 | 0.75 |
| | | | | | | | | | | | 0.027 | **0.001** | 0.011 | 0.083 | 0.527 | 0.370 | 0.012 |

Indices: WQI (water quality index); TSI (trophic state index); GMS (general morphology score). Anomalies: MOR (mortality); IYSA (incomplete yolk sac absorption); SBNI (swim bladder not inflated); LPM (lack of protrusible mouth); HD (hatching delay); LPF (lack of pectoral fin); BS (blood stasis). Toxic contaminants: $NH_3$ (un-ionized ammonia); MNA (2-Methylnaphthalene); FLU (Fluoranthene); PYR (Pyrene); CHR (Chrysene); SPAH (sum of the 16 priority PAH + 2-methylnaphthalene); LPAH (low molecular weight PAH); HPAH (high molecular weight PAH). Values in **bold** highlight Pearson correlation coefficients that are statistically significant ($p \leq 0.01$).

## 4 CONSIDERAÇÕES FINAIS

Os resultados deste trabalham demonstram a capacidade dos estágios iniciais de desenvolvimento de *Danio rerio* de detectarem toxicidade letal e subletal em amostras de águas superficiais de rios tropicais inseridos em áreas exclusivamente urbanas, parcialmente urbanas e rurais. Além disso, os dados obtidos demonstraram que os testes com embriões do zebrafish são mais sensíveis para detectar toxicidade nessas amostras do que os testes de imobilidade com *Daphnia magna.* Essas informações são subsídios para que os testes ecotixocológicos com embriões e larvas de *D. rerio*, baseados em efeitos letais e subletais, seja introduzido nas estratégias de monitoramento de rios pela Agência Pernambucana de Meio Ambiente (CPRH).

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