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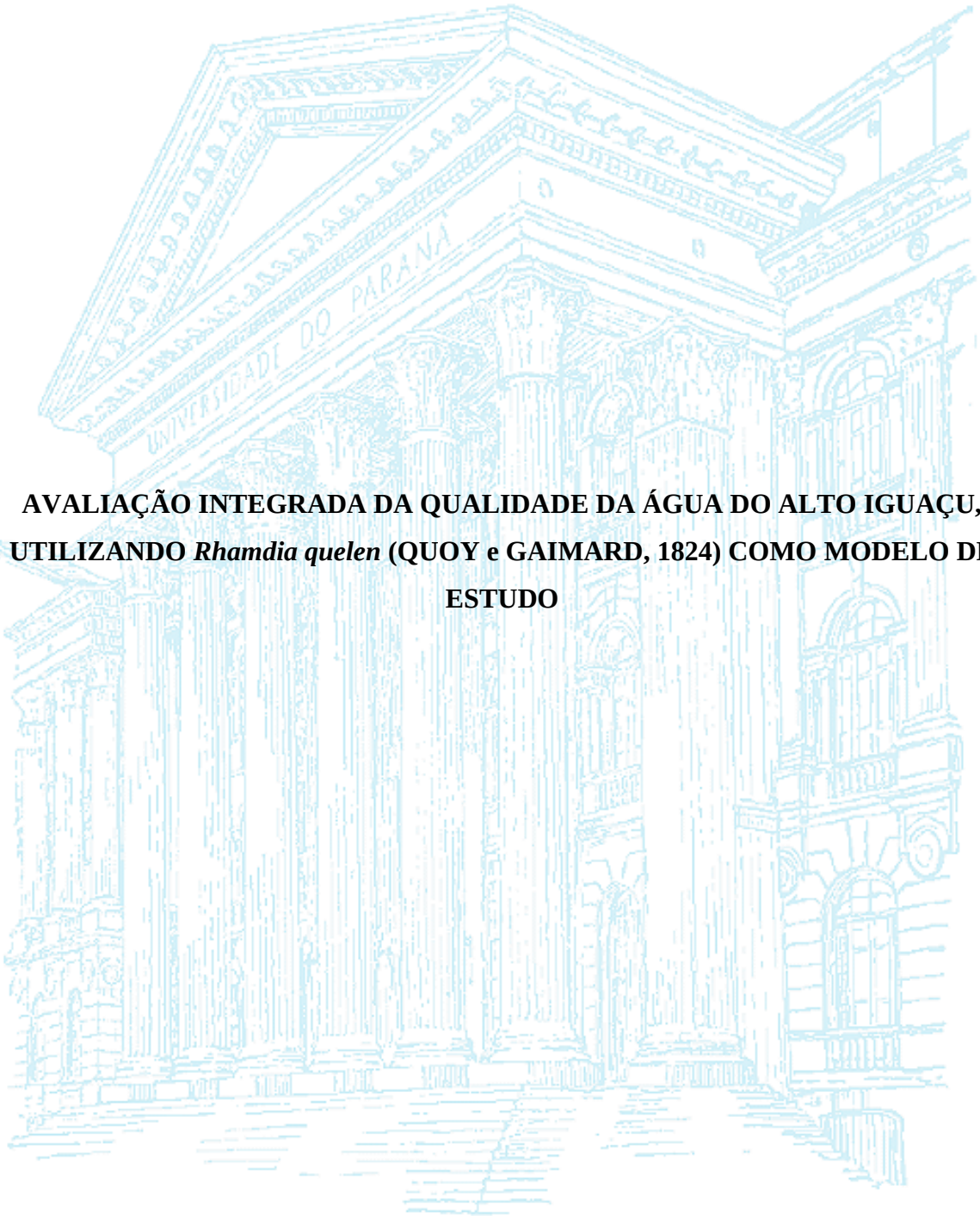

AVALIAÇÃO INTEGRADA DA QUALIDADE DA ÁGUA DO ALTO IGUAÇU, UTILIZANDO *Rhamdia quelen* (QUOY e GAIMARD, 1824) COMO MODELO DE ESTUDO

CURITIBA
2015

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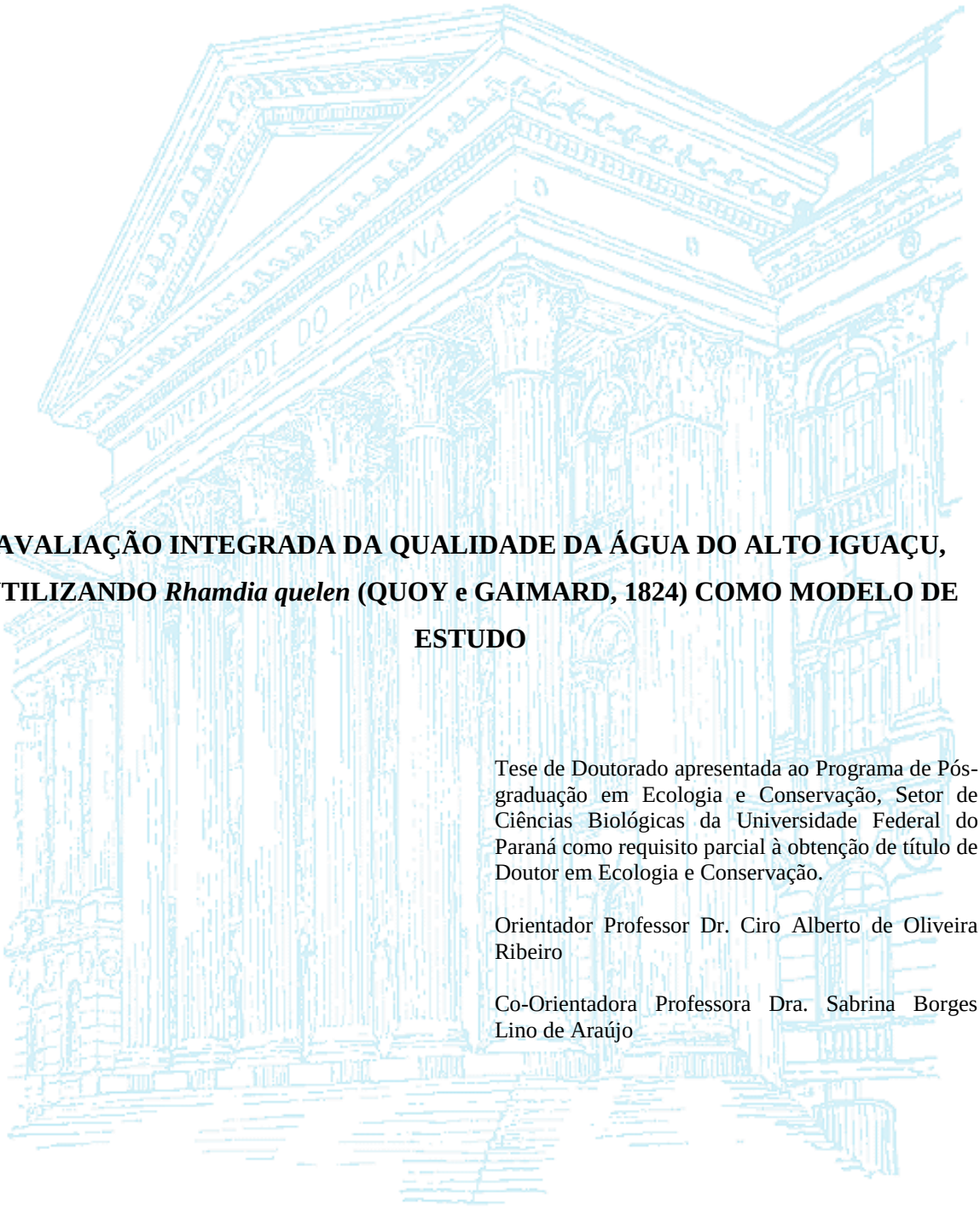

AVALIAÇÃO INTEGRADA DA QUALIDADE DA ÁGUA DO ALTO IGUAÇU, UTILIZANDO *Rhamdia quelen* (QUOY e GAIMARD, 1824) COMO MODELO DE ESTUDO

Tese de Doutorado apresentada ao Programa de Pós-graduação em Ecologia e Conservação, Setor de Ciências Biológicas da Universidade Federal do Paraná como requisito parcial à obtenção de título de Doutor em Ecologia e Conservação.

Orientador Professor Dr. Ciro Alberto de Oliveira Ribeiro

Co-Orientadora Professora Dra. Sabrina Borges Lino de Araújo

CURITIBA
2015

Ministério da Educação
UNIVERSIDADE FEDERAL DO PARANÁ
Setor de Ciências Biológicas
PROGRAMA DE PÓS-GRADUAÇÃO EM
ECOLOGIA E CONSERVAÇÃO

PARECER

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

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

Curitiba, 17 de setembro de 2015.

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DEDICATÓRIA

"In the end we will conserve only what we love; we will love only what we understand; and we will understand only what we have been taught."

Baba Dioum.

Dedico a todos os dispostos.

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Encerro aqui, um importante ciclo da minha vida passados 1460 dias, 16 estações, 4 projetos, incontáveis idas a Paulo Lopes, dois empregos, muitas amizades, belas histórias, experimentos fracassados e inestimáveis experiências.

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EPÍGRAFE

"O ser humano é uma parte do todo a que chamamos Universo, uma parte limitada pelo tempo e pelo espaço. Experimenta-se a si mesmo, os seus pensamentos e os seus sentimentos como fatos separados do resto, o que é uma espécie de ilusão de ótica da sua consciência. Tal ilusão é para nós uma forma de prisão porque nos reduz aos nossos desejos pessoais e nos constrange a reservar o nosso afeto para algumas pessoas, as mais próximas. O nosso esforço deveria consistir em libertarmo-nos desta amarra, alargando o nosso círculo de compaixão, de modo a abranger todas as criaturas vivas e a natureza em toda a sua beleza. "

Albert Einstein

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RESUMO

A falta de planejamento na urbanização e industrialização é responsável pelos efeitos negativos na água e hábitats, que quando adicionados à precariedade do saneamento degradam a qualidade da água e aumentam os riscos de exposição. O monitoramento dos sistemas hídricos, segundo os critérios oficiais, em sua maioria, tem sido baseado em parâmetros físico-químicos. No entanto, a Resolução Conama nº 357/05 instituiu o uso de testes ecotoxicológicos para avaliar o efeito deletério de agentes físicos ou químicos nos organismos aquáticos, que embora não obrigatório, constitui um parâmetro importante de avaliação de qualidade de água e limites para descarga de efluentes. O rio Iguaçu é o maior e mais importante rio do Paraná, no entanto, vem sofrendo alterações na sua composição natural devido ao lançamento de efluentes em seu curso. O presente estudo avaliou, através da exposição de embriões e larvas de *Rhamdia quelen*, primeiramente a eficiência de tratamento de uma estação piloto de Recuperação de Água (URA) instalada na ETE Atuba Sul, que possui uma vazão de 1.120 L/s no rio Atuba sendo este o principal tributário do rio Iguaçu. Posteriormente, foi analisada, a qualidade da água do Alto Iguaçu em 5 pontos, sendo: Cabeceira (confluência dos rios Iraí e Atuba), São José dos Pinhais, Fazenda Rio Grande, Araucária e Guajuvira. Os efeitos letais e sub-letais nos embriões e larvas expostos ao esgoto não tratado e tratado com tecnologia RALF foram altos quando comparados como o tratamento de reuso, indicando a ineficiência do tratamento convencional para este teste. O tratamento de reuso mostrou-se eficiente também na remoção de metais tóxicos. A água do alto rio Iguaçu apresentou concentrações significativamente altas de hidrocarbonetos policíclicos aromáticos (HPAs) em todos os pontos analisados, principalmente, acenaftileno, naftaleno, fuoreno, criseno e benzo[a]antraceno) e metais tóxicos, incluindo o chumbo. Os embriões e larvas expostas à água Alto Iguaçu, apresentaram uma alta taxa de mortalidade e deformidades em todos os pontos avaliados, indicando que, de acordo com o teste utilizado, não existe um gradiente de poluição no rio devido às inúmeras fontes poluidoras. Os dados experimentais foram modelados para avaliar a propagação dos efeitos dos poluentes nos estágios iniciais de *R. quelen* para o nível populacional. De acordo com o modelo teórico proposto, o impacto dos poluentes promove uma diminuição na densidade da população e em cenários mais drásticos, pode aumentar as chances de extinções locais.

Palavras-chave: Ecotoxicologia, embriotoxicologia, *Rhamdia quelen*, contaminação ambiental

ABSTRACT

The lack of planning in the urbanization and industrialization is responsible for negative effects on water and habitats, which when added to the precarious sanitation degrade water quality and increase the risk of exposure. The monitoring of water systems, according to the official criteria, for the most part, has been based on physical and chemical parameters. However, the Conama Resolution nº 357/05 instituted the use of ecotoxicological tests to evaluate the deleterious effect of physical or chemical agents in aquatic organisms, which although not required, is an important parameter for evaluating water quality and limits for discharge effluents. The Iguaçu River is the largest and most important river Paraná, however, has undergone changes in its natural composition due to discharge of effluents into its course. This study evaluated, by exposing embryos and larvae *Rhamdia quelen*, first the effectiveness of treatment of a water reclamation pilot station (URA) installed in ETE Atuba Sul, which has a flow rate of 1,120 L/s in the Atuba River which is the main tributary of the Iguaçu. It was later analyzed, the Upper Iguaçu water quality in 5 points, as follows: Headwater (confluence of the Irai and Atuba rivers), São José dos Pinhais, Fazenda Rio Grande, Araucaria and Guajuvira. The lethal and sub-lethal effects from embryos and larvae exposed to untreated sewage and treated UASB technology were higher when compared to reuse treatment, indicating that conventional sewage treatment is it not efficient for this test. The treatment reuse is better than UASB to removal toxics metals. Water from the Upper Iguaçu River was significantly high concentrations of polycyclic aromatic hydrocarbons (PAHs) in every point, especially, acenaphthylene, naphthalene, fuoreno, chrysene and benzo[a]anthracene) and toxic metals, including lead. Embryos and larvae exposed to high Iguaçu water, showed a high rate of mortality and deformities in all evaluated points, indicating that, according to the test used, there is no pollution gradient in the river due to the numerous pollution sources. The experimental data were modeled to evaluate the spread of the effects of pollutants in the initial stages of *R. quelen* for the population level. According to the theoretical model proposed, the impact of pollutants promotes a decrease in the density of the population and more drastic scenarios, can increase the chances of local extinctions.

Keyboards: Ecotoxicology, embryo toxicology, *Rhamdia quelen*, environmental contamination.

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LISTA DE ABREVIAÇÕES E SIGLAS

Ag	–	Prata
$CaCl_2$	–	Cloreto de cálcio
Cd	–	Cádmio
CETESB	–	Companhia de Tecnologia de Saneamento Ambiental
COMEC	–	Coordenação da Região Metropolitana de Curitiba
Co	–	Cobalto
Cr	–	Cromo
Cu	–	Cobre
DBO	–	Demanda Bioquímica de Oxigênio
ETE	–	Estação de Tratamento de Esgoto
RMC	–	Região Metropolitana de Curitiba
Fe	–	Ferro
HPA	–	Hidrocarbonetos Policíclicos Aromáticos
HPF	–	Horas Pós Fertilização
IAP	–	Instituto Ambiental do Paraná
IBGE	–	Instituto Brasileiro de Geografia e Estatística
IPARDES	–	Instituto Paranaense de Desenvolvimento Econômico e Social
KCl	–	Cloreto de potássio
$MgSO_4$	–	Sulfato de magnésio
Mn	–	Manganês
$NaHCO_3$	–	Bicarbonato de sódio
Pb	–	Chumbo
RALF	−	Reator Anaeróbio de Manto de Lodo e Fluxo Ascendente
SANEPAR	−	Companhia de Saneamento do Paraná
SUDERHSA	−	Superintendência de Desenvolvimento de Recursos Hídricos e Saneamento
UASB	−	*Upflow Anaerobic Sludge Blanket*
UFPR	−	Universidade Federal do Paraná

1. **INTRODUÇÃO**

1.1. RECURSOS HÍDRICOS

A água é considerada um recurso natural abundante no planeta. Porém, somente 0,003 por cento do volume total de água pode ser, sob o ponto de vista técnico e econômico, explorável (BRAGA *et al*. 2002). Dos percentuais disponíveis, destaca-se a pequena parcela referente aos cursos d'água, que constituem como principal manancial de abastecimento à população humana, mas paradoxalmente, água é o corpo receptor mais utilizado para o lançamento e descarte de efluentes.

A humanidade consome hoje, mais água do que todos os outros bens e matérias-primas combinados. Quanto maior o consumo de água, maior a produção de efluentes que deterioram sua qualidade e comprometem seu potencial de uso à jusante. Com a crescente demanda, o fator qualidade passou a ser tão importante quanto à quantidade, e atualmente, em muitas situações tem se tornado o fator limitante (ANDREOLI, 2003).

Além da degradação, o aumento exponencial do consumo está interferindo no ciclo hidrológico, na medida em que as atividades humanas alteram os fatores que controlam a dinâmica da água (ANDREOLI, 2003). A impermeabilização do solo, por exemplo, impede a infiltração da água de chuva e consequentemente, o reabastecimento dos lençóis freáticos. A retirada da vegetação ripária altera a velocidade do escoamento superficial e a purificação da água. Ainda sim, as atividades antrópicas têm provocado alterações no clima, que proporcionam mudanças no regime de chuvas e temperatura, interferindo com o processo de evaporação e com isso refletindo na produção e recuperação da água disponível.

Neste contexto, a observação dos limites impostos pela capacidade de suporte[1] do ambiente natural (físico, químico e biológico) é essencial para a que a intervenção antrópica não comprometa irreversivelmente a integridade e o funcionamento apropriado dos processos naturais (ANDRADE E ROMEIRO, 2009). O que observa-se hoje para algumas regiões é um desequilíbrio ambiental que indica que foi ultrapassado o limite regulador do ambiente devido a uma sobrecarga em sua capacidade suporte. Segundo Filet (1995), o ambiente está perdendo ou já perdeu a capacidade ou habilidade de acomodar, assimilar e incorporar os impactos das atividades antrópicas.

A capacidade suporte dos ecossistemas aquáticos poderia ser otimizada e a degradação ambiental minimizada, através do tratamento de esgotos, hoje, responsáveis pelo maior aporte material orgânica nos cursos d'água (Von Sperling, 1996). Porém, as pesquisas apontam que no Brasil apenas 55,2% da população urbana é atendida pelos serviços de coleta, sendo que os índices de tratamento de esgotos são ainda mais baixos (IBGE, 2011a), como exemplo, a região Sul que coleta 61,1% do esgoto e trata apenas 21,7%.

A Lei Federal Nº 9.433, de 8 de janeiro de 1997, que institui a Política Nacional de Recursos Hídricos e o Sistema Nacional de Gerenciamento de Recursos Hídricos, conhecida por "Lei das Águas", contraditoriamente, permite outorga para o lançamento de efluentes em corpos hídricos, mesmo sem tratamento, de acordo com o uso estabelecido para o corpo receptor (Classe), conforme o Art. 12, III:

> Art. 12. Estão sujeitos a outorga pelo Poder Público os direitos dos seguintes usos de recursos hídricos:
>
> (...)
>
> III - lançamento em corpo de água de esgotos e demais resíduos líquidos ou gasosos, tratados ou não, com o fim de sua diluição, transporte ou disposição final.

[1] Não importa se o recurso é renovável ou não-renovável, o ecossistema sempre tem uma capacidade máxima de suporte relacionada ao tempo que aquele recurso leva para se regenerar naturalmente (exemplo: fixação de nitrogênio pelos microrganismos no solo). Podemos dizer que a capacidade de suporte de utilização de um recurso natural foi ultrapassada a partir do momento em que ele começa a ser consumido mais rapidamente do que sua capacidade de reposição.

De acordo com a legislação vigente, os pré-requisitos necessários para abertura de processos administrativos de outorga de lançamento de efluentes, junto às agências reguladoras, são baseados na vazão e demanda bioquímica de oxigênio (DBO)[2] do corpo hídrico receptor e na concentração da DBO do efluente, baseando no conceito ecológico de autodepuração. Corpos hídricos poluídos por lançamento de matéria orgânica biodegradável sofrem um processo natural de recuperação denominado autodepuração, a qual é regida por um conjunto de processos físicos, através da diluição e sedimentação; químicos por oxidação; e biológicos por meio da decomposição da matéria orgânica (BRAGA et al., 2002).

No entanto, não existe uma depuração absoluta devido ao incremento da concentração de certos produtos e subprodutos da decomposição além de outros ditos persistentes. Outro fator importante é que a capacidade de depuração dos corpos d´água é finita. Adicionado ao déficit de saneamento nos grandes centros urbanos, a baixa qualidade do tratamento dado ao esgoto, que muitas vezes não consegue atender a legislação lançando efluentes em desacordo com as normas estabelecidas e ainda, a falta de fiscalização, principalmente quando se refere às águas residuais provenientes da indústria, o problema torna-se mais complexo. Fato que pode ser observado, pela quantidade de rios considerados mortos no Brasil, como o Tietê e Pinheiros em suas passagens pela cidade de São Paulo e os rios Iguaçu e Belém na área de Curitiba e região metropolitana, apenas para citar duas regiões brasileiras.

É importante salientar que os compostos orgânicos biorresistentes e os compostos inorgânicos, incluindo os metais pesados, não são afetados pelo mecanismo da

[2] DBO é uma variável de qualidade que permite quantificar indiretamente a matéria orgânica presente no corpo d'água ou o seu potencial poluidor. A matéria orgânica presente nos esgotos é a causadora principal do problema da poluição das águas, pois é responsável pelo consumo, por meio dos microrganismos decompositores, do oxigênio dissolvido. A DBO pode ser de origem natural (advinda de vegetais, animais e microrganismos) ou de origem antropogênica, como de despejos domésticos e industriais (VON SPERLING, 1996).

autodepuração. (BRAGA et al., 2002). Estes contaminantes podem permanecer ativos no ambiente natural por longos períodos, sendo conhecidos por poluentes orgânicos persistentes (POPs), devido à alta resistência à degradação química e biológica. A combinação entre a baixa solubilidade em água e a alta capacidade de adsorção à matéria orgânica, leva ao acúmulo desses compostos ao longo da cadeia alimentar, afetando ecossistemas e representando um grande risco para saúde pública (FLORES, 2004).

O Brasil detém aproximadamente 18% do potencial hídrico superficial utilizável no planeta (SETTI, 2001) e ainda sim, enfrenta junto com mais 40% da população mundial, a crise da água no século XXI (UNESCO, 2015), relacionada, sobretudo com a má gestão do recurso natural mais utilizado no planeta. Além da escassez de água para abastecimento público, a falta desse recurso tem graves implicações ambientais, econômicas, sociais e políticas. De acordo com Sistema Nacional de Informações sobre Saneamento (SNIS, 2008), 37% da tratada é desperdiçada. As florestas, responsáveis pela estabilidade dos ciclos hidrológicos perdem espaço para o uso urbano de forma completamente desordenada. Assim, além do crescimento populacional causar uma grande demanda de uso por esse recurso, este é responsável também pelo incremento da poluição, devido à grande quantidade de efluentes, sedimentos e lixo que são carreados para dentro dos rios. A perda das características naturais causadas pelas transformações nos cursos hídricos que atravessam grandes cidades (SHEPP e CUMMINS, 1997), apresentam consequências como a perda da capacidade de autodepuração e profunda alteração da sua biota aquática (TUNDISI, 2003). Estes fatores refletem na perda da diversidade biológica local e enfraquecem a capacidade suporte dos ecossistemas aquáticos naturais no provisionamento de serviços ecossistêmicos relacionados não somente aos recursos hídricos, mas à segurança ambiental das populações humanas pelo comprometimento da produção de alimentos.

No Brasil, a gestão dos recursos hídricos ainda não exige que a avaliação e o monitoramento da qualidade da água sejam realizados através de testes ecotoxicológicos, apesar destes se apresentarem como métodos indicadores mais diretos e eficazes para aferir a saúde ambiental e o risco de exposição humana. Ainda, de acordo com Lied e Rodrigues (2001), o controle de substâncias tóxicas não tem se constituído em objetivo importante na construção e operação de estações de tratamento de esgotos. Tem-se dado atenção quase que exclusiva a remoção de DBO, sólidos e outros poluentes convencionais e ao atendimento dos parâmetros presentes na Resolução Conama 357/2005.

1.2. SANEAMENTO AMBIENTAL

Redes coletoras de esgotos são fundamentais para a saúde e para o desenvolvimento econômico. No entanto, apenas 55,2% dos municípios brasileiros dispõem de coleta por meio de rede sanitária (IBGE, 2011a).

No Brasil, grande parte da água consumida, cerca de 80%, é transformada em esgoto e em média, apenas 36% do esgoto gerado nas cidades recebem algum tipo de tratamento. Cada brasileiro consome cerca de 150 litros de água/dia, produzindo 120 litros de esgoto/dia. Associando ao fato de que o tratamento da água e esgoto não prevê com eficiência a detecção e muito menos a eliminação de compostos, tais como hormônios naturais e sintéticos, bem como substâncias químicas artificiais que possivelmente interferem no sistema endócrino dos seres vivos, o risco em potencial de exposição das populações humanas tem aumentado consideravelmente.

A cidade de Curitiba apresenta uma população total estimada em 1.764.540 habitantes (IBGE, 2011a). Atualmente cerca de 60% desta população é atendida pelos serviços de coleta e 48% com coleta e tratamento de esgoto. Os serviços de coleta e

tratamento de esgoto da cidade são operados pela Companhia de Saneamento do Paraná (SANEPAR), somando 27 estações de tratamento de esgoto (ETEs), localizadas nos municípios de Curitiba (ETEs), Araucária (ETEs), São José dos Pinhais (ETEs) e Colombo (ETE).

O sistema de tratamento de esgoto predominante é o anaeróbio, utilizado em 98% das ETEs, realizado através de reatores RALF (reator anaeróbio de lodo fluidizado), atendendo 62,28% da população com tratamento de esgoto. O sistema anaeróbio está presente, principalmente, nas ETEs de pequeno e médio porte (com capacidade de projeto para até 50.000 habitantes). A ETE Belém é o único sistema aeróbio atualmente em operação na RMC (PEGORINI *et al*. 2003).

A maior estação de tratamento de esgoto da Sanepar é a ETE Atuba Sul (**Figura 1**), situada no bairro Cajuru. A estação atende cerca de 580 mil habitantes de 14 bairros de Curitiba e parte dos municípios de Pinhais e São José dos Pinhais. A ETE Atuba Sul possui uma vazão nominal de 1.120 L/s, obtendo como subproduto uma parte líquida chamada de efluente e uma parte sólida chamada lodo.

Figura 1. Estação Atuba Sul localizada no bairro Cajuru em Curitiba/PR.
FONTE: Google Earth, 2015.

1.3. REUSO DA ÁGUA NO BRASIL

A Resolução nº 54 do Conselho Nacional de Recursos Hídricos (CNRH, 2005), no seu artigo 2° descreve as seguintes definições:

> I - Água residuária: esgoto, água descartada, efluentes líquidos de edificações, indústrias, agroindústrias e agropecuária, tratados ou não;
> II - Reúso de água: utilização de água residuária;
> III - Água de reúso: água residuária, que se encontra dentro dos padrões exigidos para sua utilização nas modalidades pretendidas;
> IV - Reúso direto de água: uso planejado de água de reúso, conduzida ao local de utilização, sem lançamento ou diluição prévia em corpos hídricos superficiais ou subterrâneos;

O reuso da água no Brasil não prevê o uso direito não potável de água de reuso, sendo considerado como uma prática de racionalização e conservação de recursos hídricos, ainda em caráter de implementação, de forma a regular a oferta e a demanda de recursos hídricos.

A crise da água já afeta severamente algumas regiões do planeta, seja pela escassez ou indisponibilidade do recurso devido a poluição. Diante desse conflito, uma alternativa para reduzir a demanda sobre mananciais de abastecimento público de água e reduzir a contaminação dos rios, pode ser a reutilização de efluentes tratados para usos não potáveis ou mesmo através do uso da tecnologia de reciclagem da água para implementar melhorias no tratamento convencional dado aos efluentes, que são os grandes causadores da atual degradação dos recursos hídricos.

Em 2010, a Companhia de Saneamento do Paraná (SANEPAR) implantou uma estação piloto de recuperação de água (URA - Unidade de Reuso de Água) na ETE Atuba Sul (**Figura 2 e Figura 3**). O efluente, após passar pelo tratamento anaeróbico (RALF) é direcionado para a URA, atravessando uma outra etapa de limpeza, constituído por 5 tratamento adicionais: (1) flocodecantador; (2) filtro de carvão e areia; (3) ozonização, (4)

filtro de carvão ativado sem cloro e (5) filtro de carvão ativado com cloro, conforme os fluxogramas apresentados nas **Figura 4** Figura **5**.

Figura 2. Reator Anaeróbio de Manto de Lodo e Fluxo Ascendente (Ralf) ETE Atuba Sul.
FONTE: Franco, 2010.

Figura 3. Vista geral da Unidade de Recuperação de Água (URA) instalada na ETE Atuba Sul.
FONTE: Franco, 2010.

Neste sentido, o reuso de água propõe o uso racional ou eficiente da água, o qual compreende também o controle de perdas e desperdícios e a minimização da produção de efluentes e do consumo de água. Ao liberar as fontes de água de boa qualidade para abastecimento público e outros usos prioritários, o uso de esgotos não contribui apenas para a conservação dos recursos, mas acrescenta uma dimensão econômica ao planejamento dos recursos hídricos. O reuso reduz a demanda sobre os mananciais de água devido à substituição da água potável por uma água de qualidade inferior. Essa prática, atualmente muito discutida, posta em evidência e já utilizada em alguns países é baseada no conceito de substituição de mananciais (CETESB, 2013).

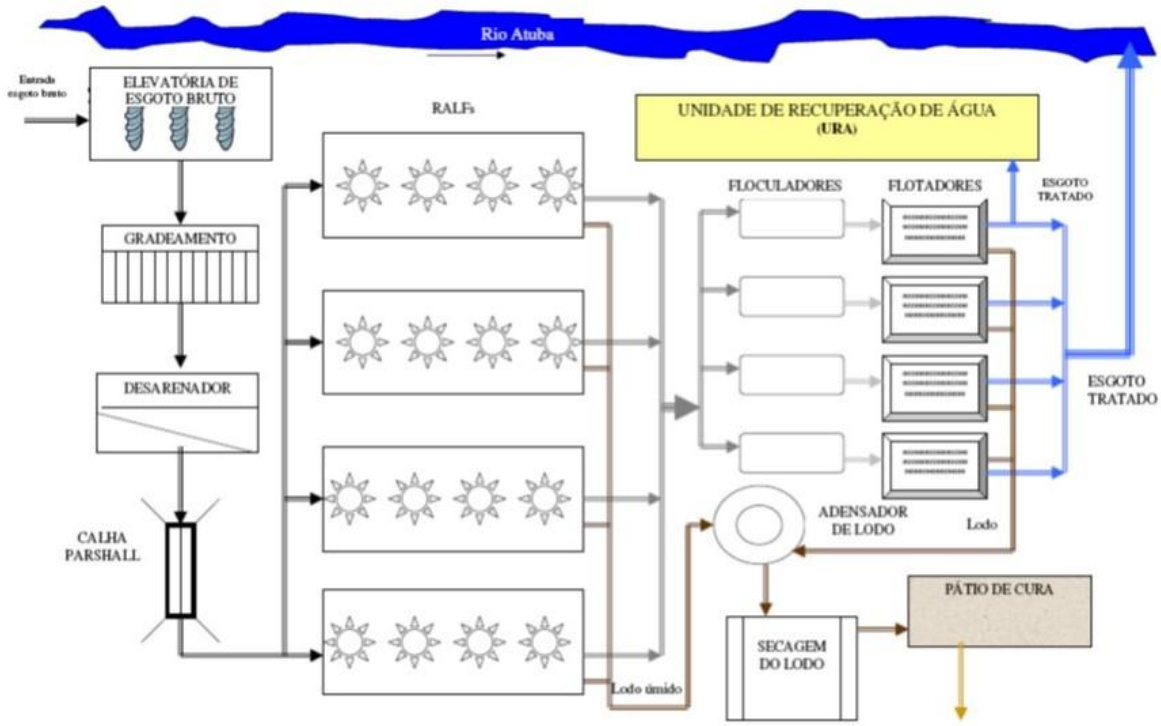


Figura 4. Fluxograma da ETE Atuba Sul.
FONTE: Franco, 2010.

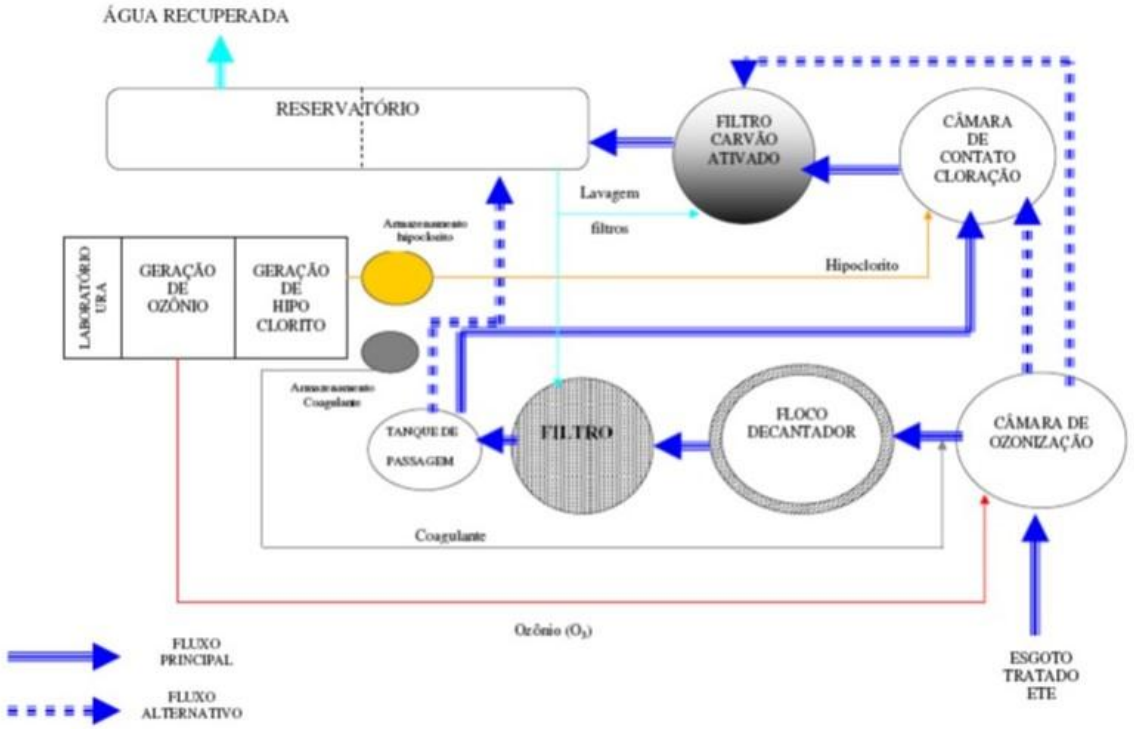


Figura 5. Fluxograma da URA instalada na ETE Atuba Sul.
FONTE: Franco, 2010.

1.4. ECOTOXICOLOGIA

Bioindicadores são espécies vegetais e animais que por qualquer particularidade ecológica são indicadores precoces de modificações abióticas ou bióticas do ambiente devido a algum tipo de atividade antrópica (RAMADE, 1998). Portanto, os bioindicadores representam um modelo adequado para o estudo dos efeitos causados por um determinado contaminante ou pelo conjunto deles.

Embora a área biogeográfica neotropical seja a região mais rica do planeta para a manutenção das espécies de peixes de água doce que totaliza cerca de 8.000 espécies (SCHAEFER, 1998), existem poucos estudos publicados sobre os efeitos da urbanização sobre a fauna aquática neotropical (WERNER e HITZFELD, 2012).

Existem vários efeitos de contaminantes possíveis de serem mensuráveis que podem avaliar o comprometimento da aptidão ecológica de indivíduos, das populações ou mesmo de um ecossistema. O impacto de um contaminante ou no caso de esgotos, caracterizados por apresentarem-se como misturas tóxicas complexas, podem ser dependes da sensibilidade relativa das espécies, intensidade e o tempo de exposição. No entanto, os efeitos crônicos, sob a forma de danos sub-letais para os organismos podem ser observados quando expostos a concentrações traços encontrados no ambiente. Estes em geral incluem o comprometimento reprodutivo (SUMPTER, 2005), danos no sistema imunológico (ARKOOSH *et al.* 2001), genéticos (SHUGART, 1995), na biologia do desenvolvimento e em mudanças comportamentais (WEIS e WEIS 1995, SANDAHL *et al*. 2007). Os efeitos indiretos afetam a aptidão ecológica e, finalmente, a sobrevivência, uma vez que o indivíduo deve ser capaz de competir com sucesso por comida, evitar os predadores e lidar com

patógenos e outros estressores ambientais. Tais efeitos não são facilmente detectados e podem atuar por longos períodos de tempo antes de serem observados.

De acordo com Forbes *et al*. (2001), a grande crítica aos ensaios ecotoxicológicos padrões é que os resultados são medidos em indivíduos, e há incertezas quanto à forma como esses efeitos serão interpretados a nível de populações. Uma outra preocupação é que os efeitos sub-letais, às vezes estatisticamente não detectados em diferentes fases do ciclo de vida do indivíduo (por exemplo, sobrevivência, taxa de desenvolvimento, reprodução) podem ser magnificados e serem a causa de grandes impactos sobre a dinâmica populacional (HALBACH *et al*. 1983).

A embriotoxicidade pode ser descrita como a avaliação da ação de compostos tóxicos que ocasionam efeitos em embriões. O conhecimento do potencial tóxico de agentes químicos sobre a biota aquática pode auxiliar no estabelecimento de limites permissíveis de várias substâncias químicas para a proteção da vida nestes ecossistemas. De acordo com Bertoletti e Zagatto (2006), a determinação desta toxicidade auxilia na avaliação do impacto momentâneo que esses poluentes podem causar à biota. O embrião em desenvolvimento é geralmente considerado o estágio mais sensível do ciclo de vida de um peixe (HALLARE et al., 2006). Devido a sua imobilidade, os embriões são mais afetados que os adultos pelos agentes tóxicos ambientais e, devido a sua imaturidade fisiológica, possuem os mecanismos de defesa pouco eficientes na proteção do organismo. Além disso, os embriões têm gasto energético muito grande para sua formação e a sua exposição à poluentes demanda um gasto extra de energia para a biotransformação dos mesmos. Muitos poluentes podem ser tóxicos aos embriões, ocasionando alterações em processos fisiológicos, má formação e até mesmo danos genéticos (WESTERFIELD, 2000).

O conhecimento das fases iniciais de desenvolvimento larval pode ser útil no estudo dos efeitos de contaminantes presentes na água, mesmo em pequenas quantidades que eventualmente podem alcançar os ambientes aquáticos (PEREIRA *et al*. 2005).

O jundiá (*Rhamdia quelen)* (QUOY E GAIMARD, 1824) é uma espécie de peixe nativa da América do Sul (Teleostei, Heptaridae) (**Figura 6**). A espécie é onívora, apresenta um hábito migratório através de distâncias curtas (ZANIBONI FILHO e SCHULZ, 2003), habita águas calmas perto de vegetação, e é de hábito noturno (BALDISSEROTTO e RADÜNZ NETO, 2004). No momento em que eles atingem a fase adulta, as fêmeas e os machos podem chegar a 66,5 centímetros e 52 centímetros de comprimento, respectivamente (BENADUCE *et al*. 2006). A sua vida reprodutiva varia entre 18 anos (machos) e 21 anos (fèmeas) (WEIS e CASTELLO, 1983). De acordo com Borges *et al*. (2005), *R. quelen* atinge a maturidade sexual no primeiro ano de vida e não apresenta cuidado parental.

Adicionalmente, a escolha da espécie deveu-se, principalmente, a ocorrência do gênero registrada na área de estudo (BASTOS e ABILHOA, 2004). Entretanto, outras características da espécie como: (1) abundância e facilidade de obtenção por meio da piscicultura; (2) conhecimento da sua biologia e (3) facilidade em adaptar-se aos ensaios laboratoriais formam considerada importantes para a realização do estudo.

Figura 6. *Rhamdia quelen* (Quoy e Gaimard, 1824) - Teleostei, Heptaridae.
FONTE: Fishbase, 2010.

Neste estudo foram utilizados os estágios de desenvolvimento de *Rhamdia quelen*, até 96 horas pós fertilização (hpf) descritos por Rodrigues-Galdino *et. al*. (2010), conforme **Tabela 1**.

Tabela 1. Estágios do desenvolvimento *Rhamdia quelen*.

Estágio	**Tempo**[3]	**Características observadas**
Zigoto	0 hpf*	Óvulo fecundado que apresenta processo de divisão celular.
Embrião	24 hpf	Estágios de desenvolvimento dentro do córion, desde a fecundação até a eclosão.
Larva	24 a 96 hpf	Estágios de desenvolvimento após a eclosão até o esgotamento das reservas vitelínicas.
Pós-larva	7 dpf**	Indivíduos que esgotaram as reservas vitelínicas até a completa metamorfose. Apresenta aspecto de adulto, porém com tamanho reduzido e imaturo sexualmente. Alimenta-se e nada verticalmente na coluna d'água.
Juvenil	21 dpf	Apresenta bexiga natatória cheia, grande crescimento (biomassa), sexualmente imaturo.
Adulto	21 dpf a 21 anos	Fase reprodutiva.

* Hpf = horas pós-fertilização. ** Dpf = dias pós-fertilização.

[3] A duração de cada período varia de acordo com a temperatura de incubação, sendo que existe uma relação negativa entre o tempo de desenvolvimento e a temperatura. Para este trabalho será adotado a temperatura de 24ºC.

2. OBJETIVOS

2.1. OBJETIVO GERAL

Avaliar a qualidade da água com fins ao diagnóstico do impacto da atividade urbana, industrial e agrícola nestes ecossistemas, bem como o risco de exposição à biota.

2.2. OBJETIVOS ESPECÍFICOS

- Validar e testar ensaios com embriões e larvas da espécie *Rhamdia quelen* como metodologia a ser utilizada para avaliação de qualidade da água;
- Aplicar o método padronizado dos estágios iniciais de *Rhamdia quelen* como indicadores da qualidade do efluente tratado com a tecnologia de reuso e a tecnologia RALF, avaliando a eficiência da estação piloto de Unidade de Recuperação de Água (URA) implantada em Curitiba;
- Avaliar a qualidade da água do alto rio Iguaçu em Curitiba e RMC e o risco associado de exposição experimentado pela biota nativa e a população humana circundante;
- Propor um modelo matemático teórico para investigar o impacto da poluição da água a nível da população, utilizando os dados experimentais dos embriões e larvas de *Rhamdia quelen* expostas as condições de poluição resultantes dos tratamentos de esgoto (RALF e Reuso) e da bacia o alto rio Iguaçu.
- Realizar análises físico-químicas no efluente e na água do alto rio Iguaçu e correlacioná-las com os efeitos letais e sub-letais observados nos experimentos de exposição dos embriões larvas de *Rhamdia quelen*.

3. METODOLOGIA

3.1. ÁREA DE ESTUDO

O presente estudo foi realizado no rio Iguaçu, dentro da sub-bacia do Alto Iguaçu, aqui, delimitado das nascentes do rio Iguaçu (rios Atuba e Iraí) até o município de Guajuvira, região rural de Araucária, perfazendo a extensão de 40 km do rio Iguaçu, na sua porção mais urbana.

O rio Iguaçu é o maior e um dos mais importantes rios do Paraná, formado pela confluência entre os rios Iraí e Atuba na parte leste da cidade de Curitiba, próximo aos municípios de Pinhais e São José dos Pinhais (**Figura 7**). O curso do rio segue o sentido leste/oeste onde em algumas regiões servindo de divisa natural entre o Paraná e Santa Catarina. Sua foz ocorre no rio Paraná, na cidade de Foz do Iguaçu, fazendo fronteira com a Argentina e formando as Cataratas do Iguaçu.

Figura 7. Vista aérea da confluência entre os rios Iraí e Atuba formando o Iguaçu no bairro Cajuru e a elevada urbanização presente na região.
FONTE: Google Earth, 2015

As cabeceiras do rio Iguaçu estão localizadas na bacia do Altíssimo Iguaçu, em uma área de 565 km^2, constituindo os mananciais de abastecimento da RMC, formados pelos rios Iraí, Iraizinho, do Meio, Piraquara, Palmital, Itaqui e Pequeno (ANDREOLLI *et al.* 1999).

A bacia do Alto Iguaçu é formada pelas cabeceiras do rio Iguaçu (bacia do Altíssimo Iguaçu) até as corredeiras situadas no município de Porto Amazonas, compreendendo uma área de 3.662 km^2, totalizando 26 afluentes principais, dentre os quais Atuba, Belém, Barigui, Iraí, Iraizinho, Itaqui, Miringuava, Miringuava Mirim, Padilha, Palmital, Passaúna, Pequeno, Piraquara e Ressaca (**Figura 8**).

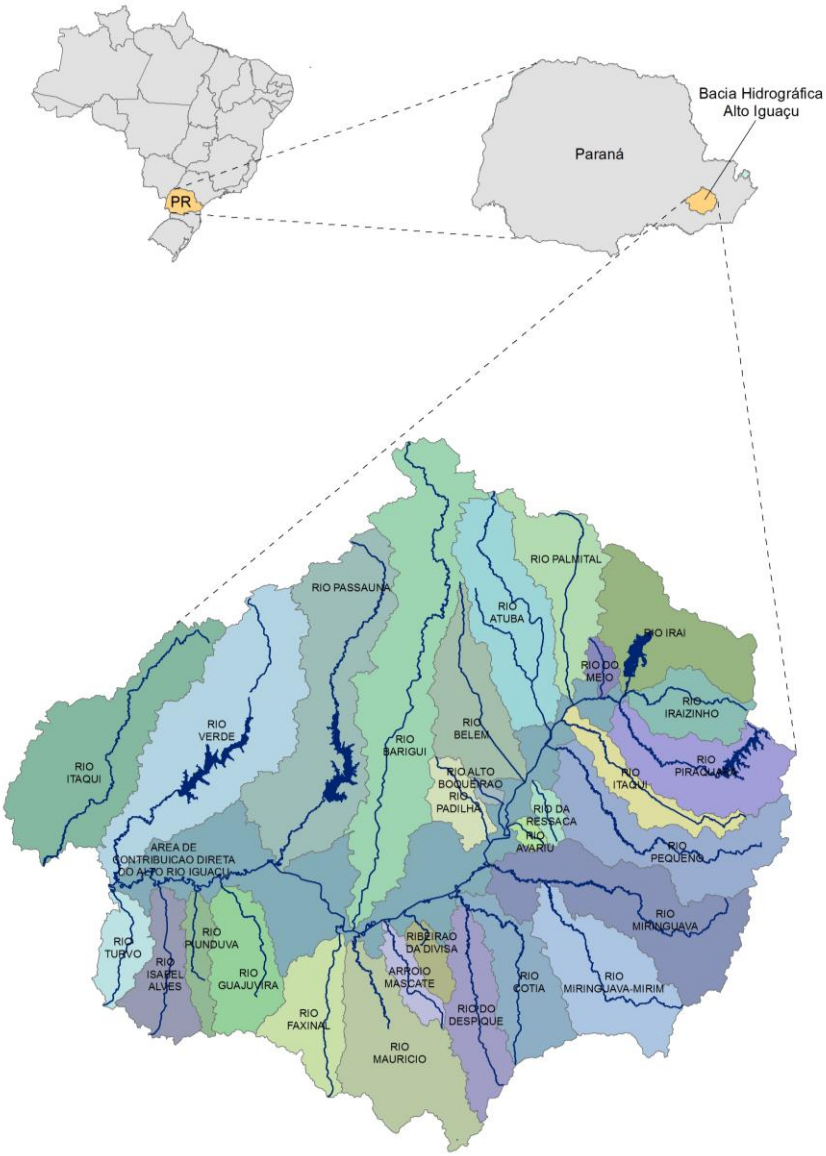


Figura 8. Localização das bacias do Altíssimo e Alto Iguaçu.
FONTE: Adaptado SUDERHSA, 2000.

A população humana presente nas bacias (Altíssimo e Alto Iguaçu) é de aproximadamente 3 milhões de habitantes distribuídos em 14 dos 25 municípios da Região Metropolitana de Curitiba (RMC). Segundo COMEC (2002), a RMC apresentou a maior taxa de crescimento populacional dentre as regiões metropolitanas do Brasil, e a bacia em estudo concentra 30% da população urbana do estado, com baixos índices de atendimento e tratamento de esgoto.

Associado a este crescimento populacional, essas bacias vêm sofrendo grande pressão da ocupação urbana sobre os seus mananciais (formados pelas cabeceiras do rio Iguaçu). Atualmente, são mais de 543 mil pessoas residindo em áreas de mananciais, o que corresponde a cerca de 20% da população total da RMC, sendo que, grande parte dessa população não conta com infraestrutura urbana, como construções regulares, serviços de saneamento, incluído coleta e tratamento de esgoto e lixo (COMEC, 2002).

Os rios formadores da bacia do Alto Iguaçu estão incluídos na classe 2 conforme disposto pela Portaria da SUREHMA n° 20/1992. No entanto, estudos apontam que a maior parte dos cursos de água na bacia do Alto Iguaçu, possui condições atuais ruins, e encontram-se na sua grande maioria pior que aqueles avaliados como Classe 4 (PORTO *et al.* 2007). Entre as principais causas apontadas neste diagnóstico, destacam-se a deterioração da água por descarte de efluentes domésticos e industriais. Com base no Cadastro de Usuários dos Recursos Hídricos da Região Metropolitana de Curitiba, efetuado pela SUDERHSA, hoje Instituto das Águas Paraná, foi observado 306 estabelecimentos industriais poluidores dos recursos hídricos localizados na RMC. De acordo com o mesmo cadastro, praticamente 20% desses estabelecimentos, ou seja, 57 indústrias são responsáveis pelo lançamento de efluentes *in natura*.

O Instituto Brasileiro de Geografia e Estatística (IBGE, 2008b), através de um levantamento denominado “Indicadores de Desenvolvimento Sustentável", apontou o rio Iguaçu como o segundo rio urbano mais poluído do país (**Figura 9**), devido ao baixo índices de qualidade de água (IQA)[4] apresentado, principalmente pelas atividades urbanas, elevado

[4] O Índice de Qualidade das Águas (IQA) foi criado em 1970, nos Estados Unidos, pela National Sanitation Foundation (Agência Nacional de Água, 2015). O IQA é um indicador de qualidade da água obtido a partir de uma fórmula matemática que usa como variáveis (parâmetros) temperatura, pH, oxigênio dissolvido, demanda bioquímica de oxigênio, coliformes termotolerantes, nitrogênio total, fósforo total, turbidez e resíduos totais dissolvidos, medidos na água. Quanto maior o valor do IQA, melhor a qualidade da água.

número de habitantes habitando as cabeceiras do rio e a falta de saneamento existente no rio e em seus afluentes.

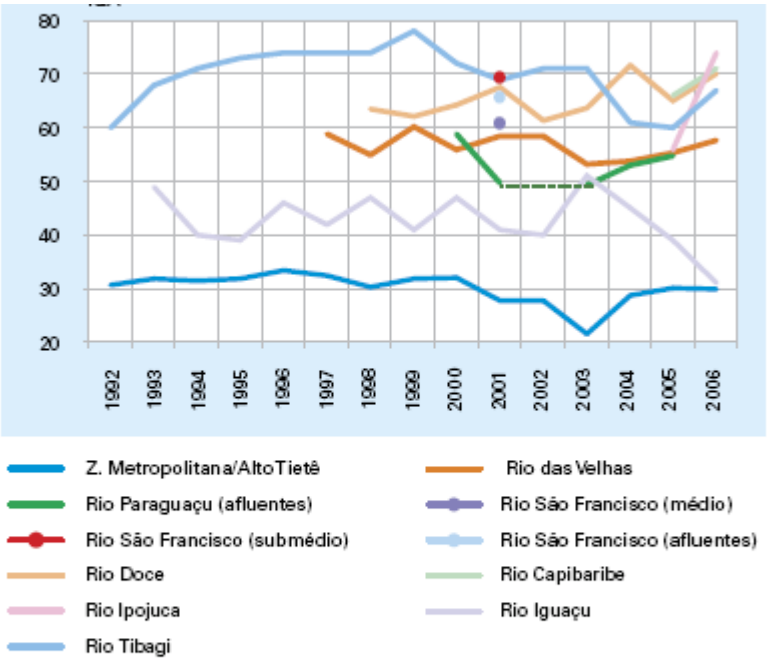


Figura 9. Média anual dos índices de qualidade de água dos principais rios do Brasil.
FONTE: Adaptado IBGE, 2008b.

Desta forma, o Capítulo 1 avaliou a eficiência da Unidade de Recuperação de Água, instalada na maior Estação de Tratamento de Esgotos de Curitiba (ETE Atuba Sul), localizada as margens do rio Atuba, principal formador do rio Iguaçu (**Figura 10**), onde foi analisado cada fase do tratamento de reuso, de acordo com a **Tabela 2**.

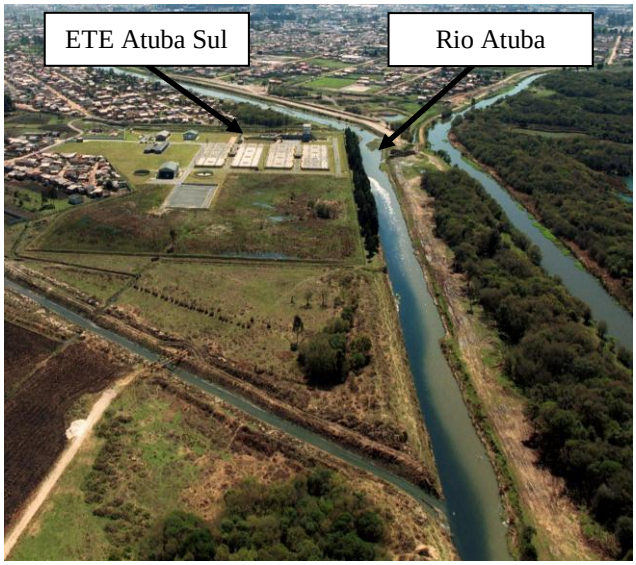


Figura 10. Rio Atuba e Estação de Tratamento de Esgotos (ETE) Atuba Sul, área de estudo do capítulo 1. FONTE: Google Earth, 2015

Tabela 2. Identificação dos tratamentos analisados de acordo com o fluxograma da Unidade de Recuperação de Água (URA) instalada na ETE Atuba Sul.

Tratamentos	Grupos Experimentais
1	Afluente da ETE Atuba Sul - Esgoto sem tratamento
2	Afluente da URA - Esgoto tratado pelo RALF
3	URA Fase 1 - Pós Flocodecantador
4	URA Fase 2 - Pós Filtro de Carvão e Areia
5	URA Fase 3 - Pós Ozonificação
6	URA Fase 4 - Pós Filtro de Carvão Ativado
7	URA Fase 5 - Pós Filtro de Carvão Ativado com Cloro
8	Controle composto por água reconstituída

No Capítulo 3 foram avaliados 5 pontos do rio Iguaçu, numerados em forma sequencial e crescente no sentido da foz, de acordo com a **Tabela 3**, definidos pela entrada de importantes tributários, como o rio Barigui e estações de amostragem coincidentes com as áreas monitoradas pelo Instituto Ambiental do Paraná (IAP, 2005). As coordenadas de localização geográfica foram obtidas com o equipamento de *Global Positioning System* (GPS).

O efluente proveniente da URA da ETE Atuba Sul e água do rio Iguaçu foram mantidos refrigerados imediatamente após as coletas e filtrados no laboratório para remoção do material particulado em suspensão (**Figura 11**. Filtragem das soluções testadas..

Tabela 3. Identificação e coordenadas geográficas das áreas amostradas.

Pontos	Áreas Amostradas	Coordenadas Geográficas	
1	Cabeceira	-49,189958	-25,483819
2	São José dos Pinhais	-49,219111	-25,528458
3	Fazenda Rio Grande	-49,415955	-25,596101
4	Araucária	-49,315918	-25,622896
5	Guajuvira	-49,514061	-25,600794

Figura 11. Filtragem das soluções testadas.

Figura 12. Ponto 1 – Cabeceira, localizado na ponte na BR-277, após a captação de água da Sanepar.

Figura 13. Ponto 2 - São José dos Pinhais, localizado próximo ao Parque Naútico.

Figura 14. Ponto 3 - Fazenda Rio Grande, localizado à montante do Aterro da Caximba.

Figura 15. Ponto 4 – Araucária, localizado na ponte na BR-476 (Rodovia do Xisto).

Figura 16. Ponto 5 – Guajuvira, localizado na área rural de Araucária.

Figura 17. Coleta dos parâmetros físico-químicos da água com a sonda multiparâmetros no Rio Iguaçu.

Figura 18. Fixação da água coletada *in loco*.

3.2. DESENHO EXPERIMENTAL

As larvas utilizadas para condução dos ensaios laboratoriais deste estudo foram obtidas na Estação de Piscicultura Panamá (**Figura 19**), localizada no município de Paulo Lopes, 45 km ao sul de Florianópolis, em Santa Catarina, cujas as atividades são estocagem de reprodutores, produção de alevinos e pesquisa.

Para realizar os testes de toxicidade, os gametas utilizados foram originados de um único casal de progenitores, reduzindo assim, variabilidade genética no experimento. Desta forma, um exemplar macho e uma fêmea de *Rhamdia quelen* com características externas de

maturação sexual foram coletados dos tanques da piscicultura e mantidos em tanques de 500 L com areação constante e com temperatura média de 24º C controlada por aquecedores com termostato (**Figura 20**). A desova das fêmeas foi induzida com extrato de hipófises de carpa de 2.5 mg cada uma (**Figura 21**), sendo que as doses foram calculadas de acordo com o peso de cada animal. Após a segunda inoculação hormonal foi calculado o momento da desova. Sendo este valor a somatório das temperaturas obtidas nas medições a cada hora após a segunda aplicação do hormônio até a desova.

No momento da desova, os exemplares são retirados da água e tem o seu ventre pressionado suavemente para liberação dos gametas em recipientes de plástico. Posteriormente, os gametas (femininos e masculinos) foram coletados e fertilizados (**Figura 22** a **Figura 25**). Os gametas são misturados a seco para aproveitar que a micrópila permaneça aberta mais tempo aumentando assim a possibilidade de fecundação. Posteriormente, realiza-se a hidratação com água do tanque (**Figura 25**) onde se encontram os progenitores para manter as mesmas condições abióticas.

Após a fertilização, os embriões foram transportados até o Departamento de Biologia Celular da Universidade Federal do Paraná, em sacos plásticos acrescidos de oxigênio (**Figura 26**).

Figura 19. Instalações da Estação de Piscicultura Panamá.

Figura 20. Tanques para manutenção dos progenitores e indução da desova

Figura 21. Inoculação hormonal nas fêmeas de *Rhamdia quelen*.

Figura 22. Coleta dos gametas femininos de *Rhamdia quelen.*

Figura 23. Coleta de gametas masculinos de *Rhamdia quelen.*

Figura 24. Fertilização mecânica.

Figura 25. Hidratação dos ovos.

Figura 26. Oxigenação para tansporte até o laboratório da UFPR.

No laboratório da UFPR, os embriões foram observados em estereoscópico (×0.67–6.7, Olympus) e após 8 horas da fertilização (hpf), durante a fase de gástrula, segundo Rodrigues-Galdindo et al (2010), foi iniciado os ensaios de toxicidade.

Somente os embriões viáveis foram transferidos individualmente para placas de cultivo celular com 96 poços (**Figura 27** e **Figura 28**), contendo 200 µl das soluções testes (Capítulo 1 foram utilizados os efluentes da ETE Atuba Sul; Capítulo 3 foram utilizadas a água de 5 pontos amostrados do rio Iguaçu) diluídas em água reconstituída (0,0065 g/l $CaCl_2$, 0,1335 g/l $MgSO_4$, 0,0004 g/l KCl, 0,0105 g/l $NaHCO_3$) em 33 e 50%.

Figura 27. Placa de cultivo de 96 poços contendo 200 µl das soluções testadas.

Figura 28. Transferência individual dos embriões viáveis 8 hpf para a placa de 96 poços.

Figura 29. Incubadora com as placas de cultivos mantidas a 24º C.

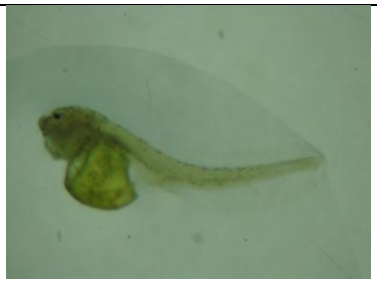
Figura 30. Coleta das larvas em tubos eppendorfs com solução fixadora 96 hpf.

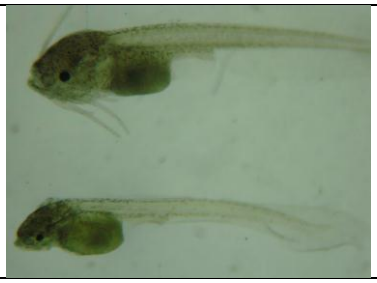
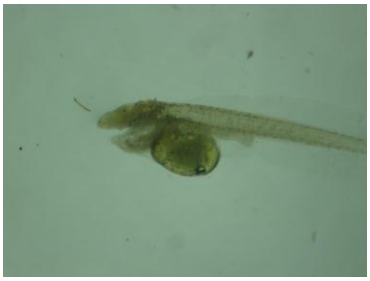
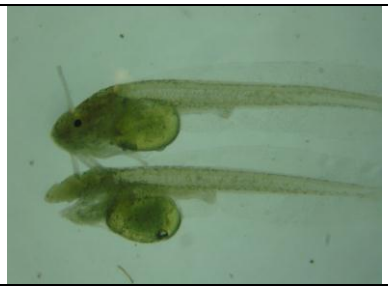
O tempo de exposição dos embriões e larvas foi 24, 48, 72 e 96 horas, representados por uma placa de cultivo cada. Adicionalmente, uma placa foi acrescentada ao experimento somente para análise da taxa de eclosão (24 hpf) e da taxa de sobrevivência (96 hpf). Logo, cada tratamento avaliado continha 6 placas (24, 48, 72, 96 hpf, sobrevivência e análise química). As placas foram mantidas na incubadora a 24º C durante todo o experimento (**Figura 29**).

A cada 24 horas, 100µl da solução teste de cada poço contendo um embrião eram trocadas para manutenção da concentração dos componentes da água semelhante ao inicial e, principalmente, proporcionando um incremento de oxigênio e a remoção da amônia, que é tóxica para as larvas. As análises de sobrevivência de cada tratamento também foram contabilizadas a cada 24 horas.

Após 88 horas de exposição (o experimento iniciou-se 8 hpf e finalizou as 96 hpf) às soluções presentes nas placas de cultivo, as larvas foram coletadas e fixadas em Paraformaldeído 4% diluído em tampão PBS 0,1M (PFA 4%) e mantidas refrigeradas a 4º C para as análises das deformidades (**Tabela 4**) e captura de imagens dos indivíduos em microscopia de luz (microscópios tipo lupa e composto).

Tabela 4. Categorias das deformidades analisadas nas larvas 96 hpf.

Categorias	**Deformidades**	
Espinha	Enrolamento da cauda Cifose ou lordose	

Nadadeiras	Nadadeiras atrofiadas ou danificadas	
Craniais/Faciais	Edema ocular Ausência de olhos	
Torácicas	Edema cardíaco Distensão e/ou atrofia corporal	

FONTE: Adaptado de POWERS *et al.* (2011).

3.3. MODELAGEM ECOLÓGICA APLICADA

No Capítulo 2 foi construído um modelo baseado no indivíduo - I*ndividual Based Model* (IBM) para avaliação da propagação dos efeitos dos poluentes na dinâmica populacional, que posteriormente, também foi aplicado no Capítulo 3. Desta forma, foi criado um programa e a linguagem computacional utilizada foi o Fortran - *IBM Mathematical Formula Translation System*. Embriões, larvas, juvenis e adultos em diferentes idades foram explicitamente modelados e submetidos a eventos estocásticos.

CAPÍTULO 1

The first stages of development of the neotropical catfish Rhamdia quelen (Teleostei, Heptaridae): fast and promising bioindicators of recycled water quality

Autores

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The first stages of development of the neotropical catfish *Rhamdia quelen* (Teleostei, Heptaridae): fast and promising bioindicators of recycled water quality

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Abstract

Urban pollution is a cause of both water degradation and the extinction of freshwater fish populations. In Brazil, the most common reason for urban water pollution is the lack of sewage collection and treatment. Water recycling technology is a global trend and a promising alternative to sewage treatment and water recovery, both of which are currently highly recommended to reduce pollution and improve the industry practices and water supplies for irrigation. A series of chemical and physical analyses are required to monitor sewage treatment, but few have been established for use as biological parameters, biomarkers, or treatment quality parameters. In the current study, the use of *Rhamdia quelen* embryos and larvae is proposed as a way to evaluate the effectiveness of water recycling in eliminating sewage toxicity. Eight hours post fertilization (hpf), eggs were exposed to each of the five steps of water recycling treatment. The efficiency of the water recycling treatment was demonstrated by higher hatching rates (24hpf) and survival rates (96hpf) in

specimens exposed to treated water when compared to results in wastewater treated with UASB technology. The morphological deformities were present in the larvae exposed to samples untreated from non-treated sewage, treated sewage with UASB, and the first steps of recycling water treatment although the injuries were no observed in the final treatment of recycled water and control showed no injuries. Thus, the use of fish embryos to evaluate the risk of exposure to treated or non-treated sewage is very promising because of the applicability of these kinds of results to government environmental policies, as well as to the discussion about the current water quality standards. As many efforts as possible are necessary to preserve and protect water resources, and these efforts include the establishment of policies in order to encourage more rigorous quality control of water from sewage treatment stations.

Keywords: Recycled water, Biomarkers, Embryo toxicology, Environmental contamination, *Rhamdia quelen*.

1. Introduction

Water is a both strategic and economical resource for developing guides for the use or occupation of land, and is even one of the driving elements of urban expansion. Historically, the largest urban centers are developed around large rivers (Trevisan, 2004).

Water quality worsens as urban expansion increases. At the same time, the quality of the water availability for human consumption decreases. According to Asano (2007), water recycling is an appropriate way to treat sewage so that the water can be reused in various ways, such as irrigation, industrial needs, and recreational activities. Thus, the application of water recycling technologies can reduce pressure on the human water supply by minimizing the production of wasted water and the disposal of wastewater into natural aquatic

ecosystems, such as rivers. In many places around the world, including Israel (Furtran, 2013), Europe (Köck-Schulmeyer et al., 2011) and the United States (EPA, 2014), water recycling technologies are largely discussed; however, in other countries, such as Brazil, these uses are still scarce according to the Brazilian Institute of Geography and Statistics, or IBGE (2011). Additionally, primary and secondary sewage treatment is not the most efficient method for the elimination or even neutralization of chemicals from urban, industrial, and agriculture activities (Fatta-Kassinos et al., 2011). These compounds expose both humans and regional biota to toxic properties and can be carcinogenic, teratogenic, and mutagenic (Tchobanoglous et al., 2003) if they are not completely removed by the sewage treatment process.

Aquatic ecotoxicology studies the effects of chemicals through experiments with biomarkers in animals models (Kendall et al., 2001). These studies aid in the prediction of risk of exposure for regional biota and human populations. Despite the recognized value biomarkers, they are not the focus of environmental monitoring programs, nor are they emphasized in quality control programs for Brazilian sewage treatments. The current study proposes a set of parameters that involve the first stages of development in fish to evaluate the efficiency of urban wastewater treatment, including water recycling technologies. Tundisi and Tundisi (2010) report that, although the Brazilian National Water Resources Council defines the criteria for water recycling practices, a legal framework to regulate water recycling in Brazil is still needed. Thus, it is important to develop scientific criteria or even tools to monitor the efficiency of applied water recycling technologies before establishing the destination of the water after treatment.

Fish are recognized as models for experimental studies, because they allow for the evaluation of chemical exposure and the risk of exposure among biota. However, a consensus was recently reached in which the use of adult animals was replaced by acute

exposure during the first stages of life post-fecundation, represented by embryos and larvae (Embry at al., 2010). Tests with fish embryos have been found to be a powerful tool for ecotoxicological assessment, since they allow for testing on a small scale (Rocha et al., 2011) and since they offer findings that can be extrapolated to adults (Chorus, 1987; McKim, 1977; Woltering, 1984).According to Hallare et al. (2005), fish embryos offer additional advantages, such as sensitivity, reproducibility, and adaptability to laboratory tests.

In the current study, the early stages of development of the freshwater catfish *Rhamdia quelen* (Quoy and Gaimard, 1824) were used to evaluate the efficiency of a wastewater recycling treatment. This omnivorous species is native to South America and exhibits migratory habits (Zaniboni-Filho and Schulz, 2003). It is also widely utilized by fish farms in southern Brazil. Many studies have recently used *R. quelen* as model to investigate exposure to chemicals present in freshwater aquatic ecosystems (Kreutz et al., 2008; Pimpão et al., 2013). Thus, the main steps of the present study were first to test the methodology of using specimens in the early stages of development to evaluate water quality, and second, to investigate the efficiency of a specific water recycling technology. In addition, the findings were compared to tests involving UASB technology in order to better discuss the impact on local biota and human populations in the wetlands and the Iguaçu River basin near the Brazilian city of Curitiba.

2. Materials and Methods

2.1. Influence of the Area of Study

The Atuba River is 23 km in length. It crosses part of the Brazilian city of Curitiba (located in the state of Paraná) and is highly polluted. According to Superintendent of Water

Resources Development and Environmental Sanitation, or SUDERHSA (2007a), this sub-basin receives the largest amount of untreated effluent: approximately 4,259 kg BOD/day, the majority of which comes from the Atuba Sul Wastewater Treatment Plant. However, both the Atuba and Iraí Rivers are tributaries of the Iguaçu River, which itself is the largest river in the state of Paraná (**Figure 1**). According to IBGE (2008 and 2010), the Iguaçu River has been described as the second most polluted urban river in Brazil. However, data from the SUDERHSA (2007) indicates that the Upper Iguaçu River basin is the most important source of water for greater Curitiba. Furthermore, a rich wetlands region is located around the Atuba Sul Wastewater Treatment Plant. According to the Brazilian Ministry of the Environment (2007), it is of high biological importance, and it requires immediate action to conserve its biodiversity. The water and sewage used in the current study were sampled from a pilot water recycling plant that release its discharge directly into the Atuba River (**Figure 1**).

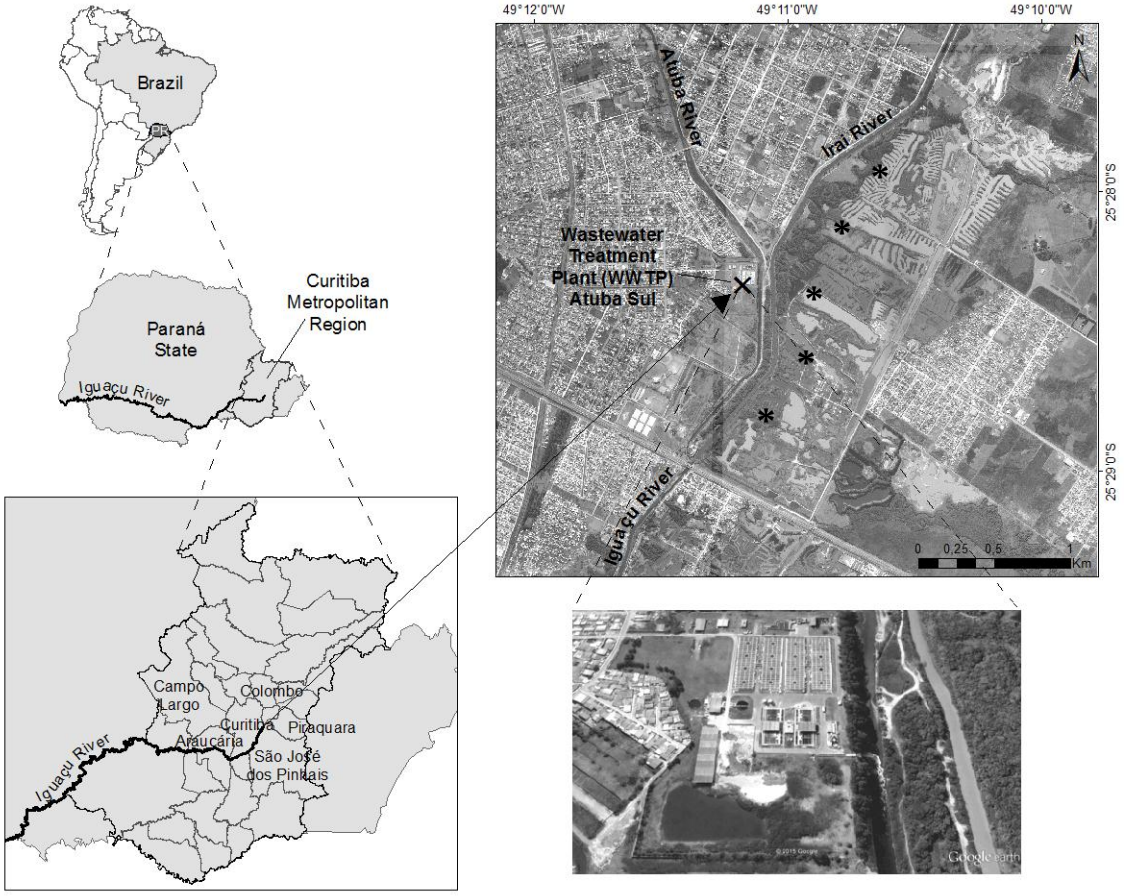

Figure 1. Map of the region studied, including the Atuba Sul Wastewater Treatment Plant (Arrow), located on the Atuba River in Curitiba, Paraná, Brazil. *indicates wetland region.

2.2. Sewage Sampling and Treatment

The Atuba Sul Wastewater Treatment Plant uses Upflow Anaerobic Sludge Blanket (UASB) technology for sewage treatment, according to the Sanitation Company of Paraná State (SANEPAR, 2014). The water recycling technology tested in the current study consists of 5 steps after biological treatment (UASB): flake settling, sand and charcoal filtering, ozonation, activated carbon filtering, and chlorination (**Figure 2**). According to Brazilian laws (Resolution nº 54, National Water Resources Council, 2005), this technology does not meet the criteria for the development of non-potable recycled water.

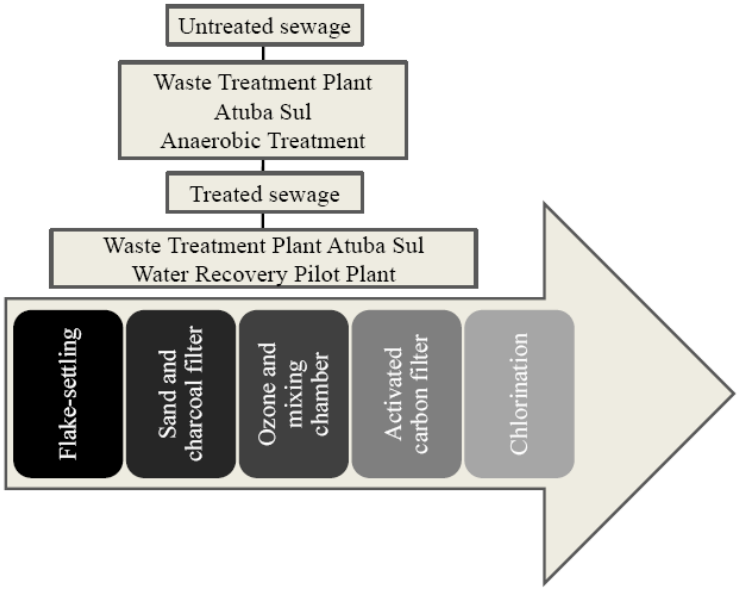

Figure 2. Water recycling steps consisting of additional treatment at the water recovery pilot plant located within the Atuba Sul Wastewater Treatment Plant, Curitiba, Paraná, Brazil.

The samples from untreated sewage, UASB treated sewage, and sewage treated with the aforementioned recycling steps (**Figure 2**) were immediately filtered through filter paper 85g/m^2 in size and with a porosity of 6.00 microns to remove suspended solids. Next, the samples were diluted to 33% and 50% in reconstituted water (0.0065 g/l $CaCl_2$, 0.1335 g/l $MgSO_4$, 0.0004 g/l KCl, 0.0105 g/l $NaHCO_3$) and kept under refrigeration at 4ºC. The dissolved oxygen and pH parameters were measured using the Oxygen Meter DO-5510 at a temperature of 24ºC.

2.1.1. Metals Testing using Chemical Analysis

The samples used for metals testing were collected in 1-L sterile polyethylene bottles, fixed in nitric acid (HNO_3), 1:1 and kept under refrigeration at 4°C. The treatment used for to determine the presence and concentration of metals in the samples was measured using atomic absorption (flame emission) spectrophotometry following to the protocol from Sao Paulo State Environmental Technology Company (CETESB, 1978).

2.2. Experimental Design

The fertilized *Rhamdia quelen* eggs were obtained from a fish farm (www.pisciculturapanama.com.br) and transported to the Cellular Toxicology Laboratory at the Federal University of Paraná. The fertilized eggs were selected under an Olympus stereo microscope (× 0.67 to 6.7). Only eggs in the gastrula stage that were viable 8 hours post fertilization (hpf) were considered, following Rodrigues-Galdino et al. (2010).

For each treatment, ninety-six eggs were individually transferred into 96-well culture plates according to MacPhail et al. (2009). Each well contained 200 µl of the solution diluted to one of the concentrations (33% or 50%). The tests were conducted in triplicate, with 288 embryos per concentration per exposure time (24, 48, 72 and 96 hpf), with a total of 2,880 embryos per assay.

The reconstituted water mentioned above was used as a medium to dilute the samples to 33% and 50%, as well as in the control group, at room temperature according to ASTM (1993). After the first day, 50% of the solution medium was replaced with new samples of the medium every 24 hours to promote better levels of water oxygenation and ammonia removal. Hatching rates were considered at 24 hpf. Larva survival was determined at 96 hpf, and deformities were counted at 24, 48, 72 and 96 hpf. The experimental procedures were performed under a constant temperature of 24ºC. For hatching and survival rate analysis, the larvae were counted individually in the 96-wells plate using a Leica® DM/IL inverted light microscope. For the morphological evaluation, the larvae were collected and immersed in Karnovsky's fixative (glutaraldehyde 2.5%, paraformaldehyde 2.5% in a sodium cacodylate buffer 0.1M, $CaCl_2$ 0.001 M, pH 7.2). After the fixation period (a minimum of 24 hours), the samples were washed with 0.1M sodium cacodylate buffer (2x) and then stored in 1 ml plastic Eppendorf® vials at 4 °C until analysis. In order to record toxic effects, such as morphological alterations, damages, and deformities, the larvae were carefully transferred

to microwell plates and photographed using a Sony® Cyber Shot camera and an Olympus® light microscope. The deformities were classified into five categories according to Powers et al. (2011): (1) spine; (2) fins; (3) cranial/facial; (4) thorax and (5) abdominal (**Table 1**).

The Committee for the Ethics for Animal Experiments from the Biological Sciences Department of the Federal University of Paraná (CEUA/BIO), certified that the procedures using animals in this study were in agreement with the Brazilian Guidelines for the Care and Use of Animals for Scientific and Teaching purposes established by the Brazilian National Council for Control of Animal Experimentation (CONCEA), as well as with the international guidelines for animal experimentation.

2.4. Statistical Analyses

The rates of survival and deformities were analyzed using the Friedman test in the R software version 3.0.2 (R Development Core Team, 2013) and assuming a value of $p < 0.05$ for statistical significance.

Table 1. Category deformities observed in *R. quelen* after exposure to different sewage treatment.

Category	Description
Spine	Stunted skeletal growth Kink in tail Column lordosis or kyphosis
Fins	Stunted fins
Cranial/facial	Ocular edema Absence of eyes
Thorax	Distension Cardiac edema
Abdominal	Distension Emaciation

Powers (2011) with adaptations.

3. Results

Table 2 summarizes the abiotic parameters (temperature, oxygen rates, and pH) measured in the sampling. The lowest oxygen concentrations was found in the untreated and treated sewage samples, but this concentration increased during the recycling treatment and reached its highest value (four times higher) during the activated carbon filter step. pH levels did not vary enough to compromise bioavailability processes during the experiment.

Table 2. Oxygen concentration (mg/L) and pH of samples of untreated sewage, UASB treated sewage, and recycled water.

	Treatment Steps	**pH**	**Oxygenic (mg/L)**
	Untreated sewage	6.0	1.0
UASB Treatment	Treated sewage	6.0	1.6
Recycling Water Treatment	Flake-settling	7.0	4,2
	Sand and charcoal filter	7.0	4.6
	Ozonation	7.0	6.2
	Activated carbon filter	7.0	6.1
	Chlorination	7.0	4.2

The metals tested in the samples (Cu, Mn, Zn, Fe, Co, Cr, Ag, Cd and Pb) have been reported as biomarkers of human activities. After the recycling treatment, the data showed a reduction in all of the metals detected after the UASB sewage treatment (**Table 3**).

Table 3. Analysis of metals in untreated sewage, treated sewage with UASB, and recycled water. The results were compared to the Brazilian legislation (Resolution 357 from the National Environment Council). Values written as mg/L.

Treatment Steps	**Cu**	**Mn**	**Zn**	**Fe**	**Co**	**Cr**	**Ag**	**Cd**	**Pb**
Untreated sewage	0,01	0,19	0,15	12,01	0,04	ND	ND	ND	ND
Treated sewage	ND*	0,12	0,24	3,45	0,02	ND	ND	ND	ND
Recycling Water Treatment	0	0,21	0,04	0,76	0,02	0	0	0	0
Resolution 357/05	0,009	0,10	0,18	0,30	0,05	0,05	0,01	0,001	0,01
Ordinance 518/04	2	0,10	5	0,30	--	0,05	--	0,005	0,01

*ND = not detected

The value can only be in the form of trace, below the detection limit with values in ppb or ppt.

However, iron and manganese were found to differ from the values established by Brazilian law, even after the recycling treatment (**Figure 3**).

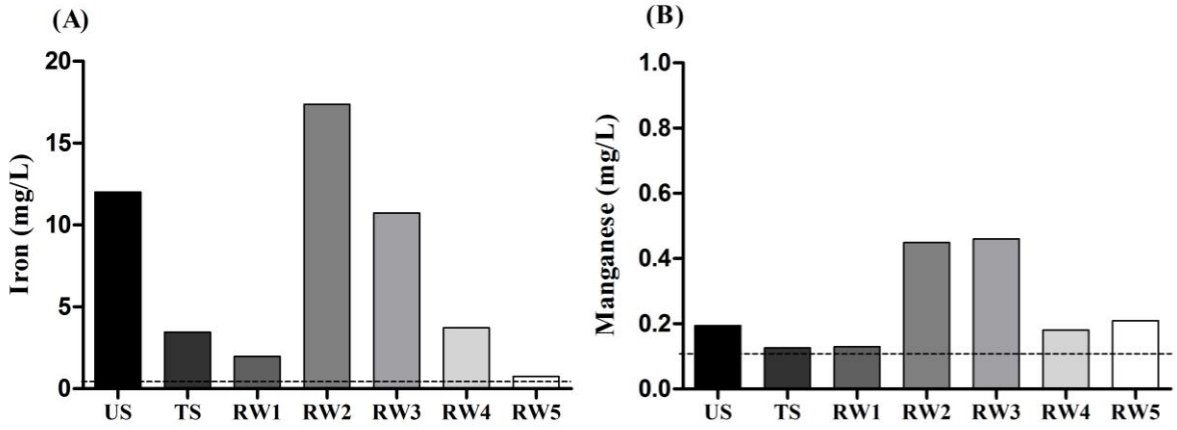

Figure 3. Testing for iron (A) and manganese (B) in sewage treatment sample (UN = untreated sewage; TS = treated sewage) and in the recycled water (RW). RW1= flake-settling, RW2 = sand and charcoal filtering, RW3 = ozonation, RW = activated carbon filtering, RW5 = Chlorination. The dotted line represents the standard set by Brazilian law (National Environment Council Resolution No. 357/05.

The survival rate of embryos and larvae exposed to the treatments differed significantly among all time variables and both dilutions. This result shows that time is crucial in determining the mortality of specimens exposed to pollutants from sewage treated and untreated sewage (**Figure 4**).

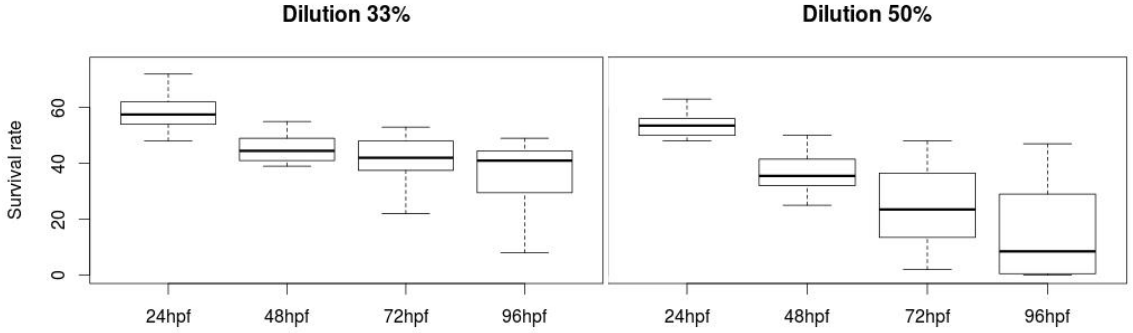

Figure 4. Survival rate of *R. quelen* larvae exposed to treated sewage, untreated sewage, and recycled water at diluted to 33% and 50% ($p<0.0001$).

The results of the embryo hatching rates (24 hpf) were found to be similar at both dilution levels in the untreated sewage, treated sewage, and recycled water. In the recycled water, the hatching rates after dilution to 33% concentration was higher than observed in the 50% dilution group. However, the hatching rates increased with the addition of recycled water, and after the final step of the recycling treatment, the hatching rates at both dilution values were closer to those of the control group (conditions without contaminants). Statistical differences ($p<0.05$) were found between the dilution values (**Figure 5**).

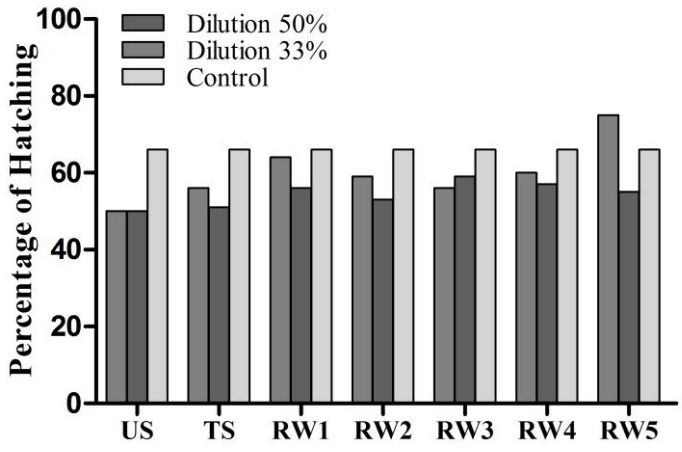

Figure 5. Hatching rates of *Rhamdia quelen* exposed to untreated sewage (UN), treated sewage (TS), and recycled water (RW1= flake settling, RW2 = sand and charcoal filtering, RW3 = ozonation, RW4 = activated carbon filtering and RW5 = Chlorination). Letters indicate significant differences between the treatments ($p < 0.05$).

The results of our study show that larvae mortality to be dependent on dilution levels in all of the treatments considered. In addition, larva survival rates 96 hpf were significantly

higher in the 77% dilution groups in both untreated sewage and recycled water when compared to the 50% dilution groups (**Figure 6**). An increased larva survival rate was found at both dilution levels when the recycling process was applied to the sewage (RW4). At a dilution value of 50%, survival rates were below both the values in the control treatment and the natural rates of survival for the species *R. quelen*, while the values from the 77% dilution group were similar to those of the control group and of the recycled water group. As expected, after the chlorination treatment high mortality rates were observed among the larvae.

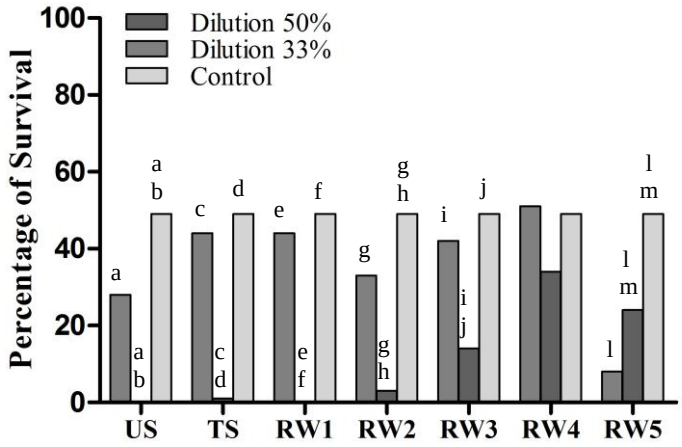

Figure 6. Survival rates of *Rhamdia quelen* exposed to the untreated sewage (UN), treated sewage (TS), and recycled water (RW1= flake settling, RW2 = sand and charcoal filtering, RW3 = ozonation, RW4 = activated carbon filtering, and RW5 = Chlorination). Letters indicate significant differences between the treatments ($p < 0.05$).

In all of the groups tested, survival rate was found to be dependent upon both duration and dilution values. When the samples were less diluted, the recycled water group experienced the worst result 96 hpf. The larva survival rates (96 hpf) were very low in the UASB treated sewage group, particularly at 50% dilution, in which mortality rates reached

almost 100% of the population. When survival rates among the UASB treatment samples and the recycled water samples were compared, the survival rate was 1.5 times higher among the recycled water sample specimens than among the specimens exposed to the UASB treatment at both dilution levels. These findings show that, when diluted to 33%, the recycled water produced in the best conditions for larva survival – the conditions closest to those of the control (which were conditions with a lack of pollutants).

Table 4. Hatching rate (24 hpf) and survival rate (96 hpf) of larvae exposed to untreated sewage, treated sewage with UASB, and recycled water. The results are shown as percentages. Letters indicate significant differences between the treatments ($p < 0.05$).

	Dilution 33%				**Dilution 50%**			
Treatment Steps	**24 hpf**	**48 hpf**	**72 hpf**	**96 hpf**	**24 hpf**	**48 hpf**	**72 hpf**	**96hpf**
Untreated sewage (US)	50a	42	40	28a	50f	39	2f	0f
Treated sewage (TS)	56	44	44	44	51g	38	18g	1g
Flake-settling (RW1)	63b	50	50	43b	56h	36h	10h	0h
Sand and charcoal filter (RW2)	59c	41c	39c	33c	53i	35i	24i	3i
Ozonation (RW3)	56	47	44	41	59j	31j	19j	14j
Activated carbon filter (RW4)	60	57	55	55	57l	48	47	34l
Chlorination (RW5)	75d	46d	23d	8d	55	26	25	24m
Control (CT)	65acde	52	50e	50e	65fgn	52	50n	50fghijmn

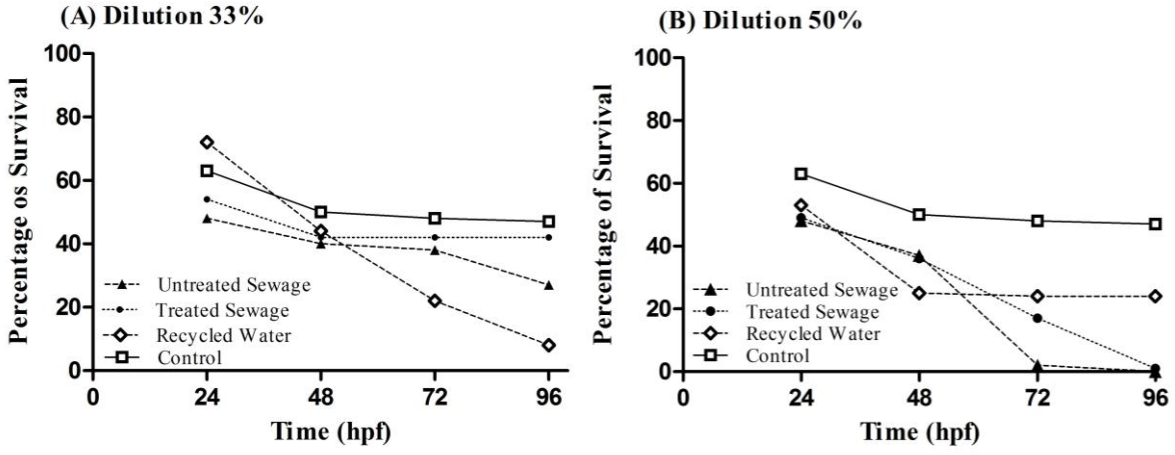

Figure 7. Survival rate of *R. quelen* larvae (96 hpf) exposed to untreated sewage, treated sewage, recycled water, and the control sample diluted (A) 33% and (B) 50%.

After 96 hours of exposure to untreated sewage, treated sewage, and recycled water, the quantitative analysis of the larva deformities revealed a lower frequency of deformities at both dilution levels. At 77% dilution, the frequency of lesions was higher than in specimens from the 55% dilution group. Meanwhile, the treated sewage (TS) and recycled water groups were found to have the highest number of lesions with an average of 43% of individuals affected (**Figure 7**). Different from the other groups, the specimens in last step of the recycled water treatment (RW4) and in the control groups exhibited lower frequencies of abnormalities. No significant statistical differences ($p<0.05$) were found among the groups at the different dilution values.

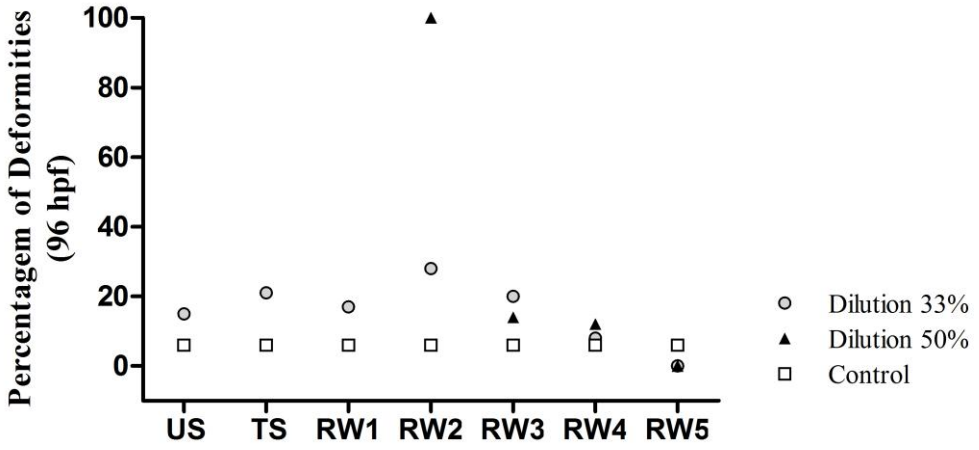

Figure 8. Deformities observed in *R. quelen* larvae after 96 hours of exposure to untreated sewage (UN), treated sewage with UASB (TS), and recycled water (RW1 = flake settling, RW2 = sand and charcoal filtering, RW3 = ozonation, RW4 = activated carbon filtering, and RW5 = Chlorination). The treatments US, TS and RW1 (dilution 50%) were no survivors.

Among the categories assessed, deformities in the skeletal system (notochord), such as lordosis (dorsoventral curvature in the vertebral column), kyphosis (ventredorsal curvature), and scoliosis (lateral curvature) were the most frequent, and were present in individuals from all treatment groups, including the control group. Injuries such as stunted

skeletal growth were found at a low frequency in the specimens, and no kinks in tails were found. Cranial/facial abnormalities, such as a lack of eyes and underdeveloped jaws, were observed in larvae. Meanwhile, in the thorax, the most common injury was cardiac edema (**Figure 9**).

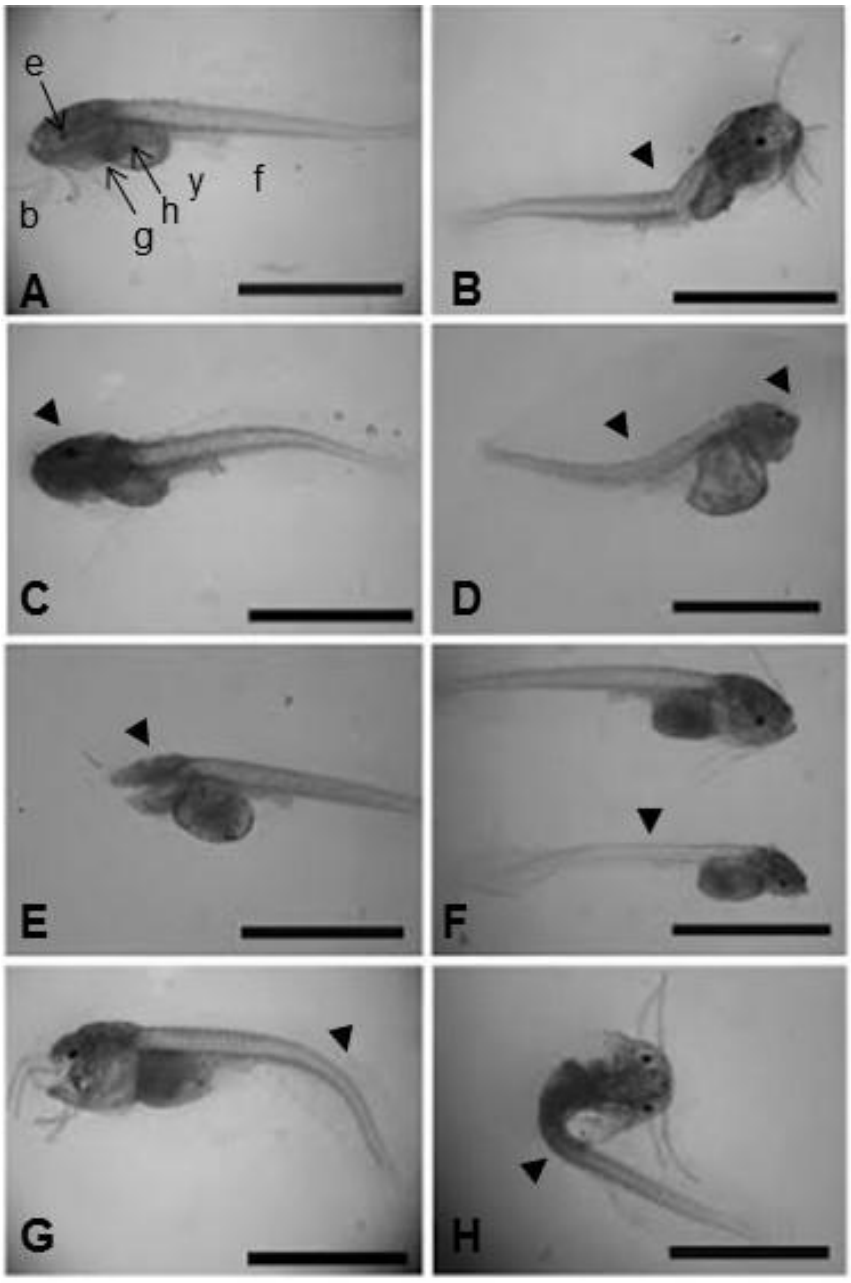

Figure 9. Deformities observed in *R. quelen* larvae after 96 of exposure to untreated sewage (US), treated sewage with UASB (TS), and recycled water (RW) at 33 and 50% dilution. (A) Normal larvae without alterations. (B, C, D) Skeletal or muscle structure malformations (►). (B) Severe damage in the vertebral

column while (C) and (D) also lacked barbells (►) and (D) was found to have cardiac edema (Δ). (E) Cranial structure deformities with malformation of the head and a lack of eyes (►) and barbells. (G) Skeletal or muscle structure malformation (►). (H) Larvae with muscular or skeletal atrophy, including head alterations (►). e = eye, f = embryonic fin, g = gill arches, h = heart (pericardial cavity), y = yolk, b = barbells. Scale: 1.0 mm.

4. Discussion

The experimental results based on embryos and larvae of *Rhamdia quelen* demonstrated limited effectiveness of UASB technology for decreasing the potential toxicity of sewage. The results of this study suggest that the reason for this limited effectiveness is likely due to the failure of this treatment to remove contaminants. The data also shows that all effects in all treatments considered depended on dilution levels. The specimens exposed to the recycled water sample, however, experienced significantly higher hatching and survival rates, particularly at 77% dilution – at this level, hatching rates reached 75%. These results reflect both the efficiency of recycled water and the need for new studies.

Larva survival rates were found to be most dependent only dilution levels when each group was compared to the control group. The values were very low in the treated sewage when it was diluted to 33% of its original concentration, but much lower when compared to the higher dilution level tested (50%). At 77% dilution, mortality was almost 100% among specimens exposed to untreated sewage, to treated sewage with UASB, and up to the RW2 step in the water recycling treatment.

When survival rates among the UASB treatment samples and the recycled water samples were compared, the survival rate higher among the recycled water sample specimens than among the specimens exposed to the UASB treatment at both dilution levels. This value is closed to the *R. quelen* survival rate in the absence of pollutants (about 60%) as described by Ferreira et al. (2001) and also in fish farm conditions (80%) (Behr et al., 1999).

These findings show that the recycled water resulted in the best larva survival rates – rates which were similar to those seen among specimens exposed to the control conditions and among specimens raised in the absence of pollutants.

The exception was the final step of the recycling treatment, which includes chlorine as a sterilizing agent against pathogens. After this step was performed, the mortality rate was higher than after step 4. In other words, the survival rate improved during step for but decreased after step 5 was applied. In this study, chlorine proved to be very harmful to the early developmental stages of *R. quelen* specimens. However, its use in the discharge of effluents into natural ecosystems is required by Brazilian regulatory agencies. According to Tortora et. al. (2012), chlorine may also be dangerous to human health, since it can react with water contaminants and create carcinogenic compounds, such as trihalomethanes organochlorides (THM). It is also associated with residual toxicity in the local aquatic biota (Gonçalves, 2003). However, chlorine use is still the most widely used disinfectant agent in sewage treatments because of its relatively low cost and high effectiveness (Tortora et al, 2012) compared to other mechanisms, such as disinfectants (Ozonation) and UV radiation. The results of our study corroborate the high chorine toxicity in biota and reflect the need for further discussion on its use in sewage treatment.

Water recycling proved to be effective in removing metals overall, but neither iron nor manganese reached the Brazilian standards set for release of treated effluents into nature. Iron is present in the coagulation process of water recycling, and in the flake-settling process of sewage treatment (Asano, 2007). These features can explain the high concentration of the metal in the test samples. According to the World Health Organization (WHO), iron does not constitute a toxic metal; however, a maximum concentration of 2 mg/L in the public water supply is legally permitted. The presence of manganese may be associated with urbanization sources, such as mining and industrial activities (Morillo and Usero, 2008) and

this metal is toxic to organisms at high concentrations (Bertoletti and Zagatto, 2006). Nagamatsu (2013) describes that the Mg decreased the egg hatching rates and larva survival of *R. quelen* exposed in concentration of 10 mg/L, at which point cephalic deformities and atrophies occurred as well. Although water recycling is able to reduce the concentration of this metal, the legal limits for metal concentrations in water and the metal removal process in sewage treatment must both be discussed. This argument is supported by the negative impacts on both the biota and water quality due to metals' high persistence, bioaccumulation, and biomagnification rates, all of which potentially increase the toxicity of this class of contaminants.

Among the compounds found in the Atuba River, Osawa (2013) described the occurrence of antihypertensive agents due discharges of ETE Atuba Sul, Ide (2014) showed the presence of caffeine, gemfibrozil and fenofibrate in the WWTP downstream and high concentration of estradiol was found in the output of the WWTP effluent Atuba, showing the inefficiency of the sewage treatment to remove this kind of compounds. Additionally, the anaerobic treatment (UASB) is not efficient in removing ammonia nitrogen contributing to the pollution of Atuba River due to the input of organic matter from wastewater without treatment or even to the deficit sewage treatment. All those factors are strongly contributing to the reduction of dissolved oxygen in the river. According Von Sperling (1996), raw domestic sewage presents the following metals in composition, Cd, Ca, Cr, Co, Cu, Pb, Mg, Hg, Mo, Ni, Se, Na, Zn and Fe. Some metals as Fe and Mg are necessary nutrients for the biological treatment. Additionally SUDERHSA (2000b) indicated that the effluent from the upper Iguaçu basin contains metals, mainly zinc and chromium, as also cadmium, lead and nickel. These findings showed that to urban dejects toxic metals are abundant and are important biomarkers of urban activities. On this sense, as metals are hard to be eliminated by conventional treatment of sewage, this class of contaminants was choose to monitoring

the efficiency of the recycling method tested. In addition, this method is very consolidated in a diversity of matrix, making easy the comparative studies or even the methodologies to metals analysis.

Several studies indicate that effluents from treated sewage can have toxic effects on aquatic organisms (Hodgson, 2004; Klaassen, 2008; Di Giulio and Hinton, 2008). In addition, Tebo (1986) report that 79% of domestic and 62% of industrial sewage in the United States in the 80s had toxic effects on the environment and on human health, even after conventional treatment. In Brazil, problems are already serious because of the weaknesses in the country's sewage collection system, which reaches less than 50% of the population. To make matters worse, the treatment has been found to be ineffective for removing the toxic effluents, even when the treatment plant meets national emission standards (Bertoletti and Zagatto, 2006). Nevertheless, neither the control of toxic substances or the toxicity of effluents are traditionally presented as the most important subjects in the operation of sewage treatment plants. Physical, chemical, and biological parameters should be more adequately considered in order to achieve better results in terms of effluent quality and water resource protection.

According to the Environmental Institute of Paraná State, or IAP (2009) and Yamamoto (2012), the Atuba River receives a high load of sewage effluents, which mainly come from the Atuba Sul Wastewater Treatment Plant. Other studies have demonstrated the ineffectiveness of the anaerobic treatment process (Yamamoto, 2012) and the drug removal process (Osawa et al., 2013) at this plant. The IAP (2009) also reported that, in samples from the Atuba River Treatment Plant , man physicochemical, bacteriological, and ecotoxicological parameters are not in accordance with those established by federal Brazilian environmental laws. The test samples in the current study are from the station that discharges the largest source of sewage effluents into both the Atuba River and to a very

ecologically relevant wetland area nearby. Alarmingly, these wetlands have been used to supply water to human populations during droughts, as described by Iwamura et al. (2011).

Klumpp and Von Westernhagen (1995) reported that the natural rates of embryonic malformations are less than 10%, but that this rate may increase significantly with high levels of chemical stress. In the present study, the deformity rates observed in the *R. quelen* larvae exposed to sewage treated in different ways showed higher than natural deformity rates. Only the specimens in the control group and in the group exposed to the last two stages of the water recycling treatment presented levels of deformity below 10%. According to López-Barrera (2013), many deformities in the early stages of development of *R. quelen* specimens exposed to silver nanoparticles were concentrated in the spine and brain region or in the spinal column; Nagamatsu (2013) also reported a high frequency of deformities and severe lesions in larvae of *R. quelen* exposed to metals.

Fishes are highly sensitive to chemical pollutants during the embryo–larval stages of development (Kendall et al., 2001). These stages are known to be among the most chemically sensitive life stages (McKim, 1977). Many studies indicate that early exposure to contaminants may affect the embryonic and larval development of the fish, and may also eventually lead to mortality. Debruyn et al. (2007) found an increase in the occurrence and severity of embryonic and larval abnormalities in *Coregonus clupeaformis* that were exposed to polycyclic aromatic hydrocarbons (PAHs). These abnormalities included several skeletal deformities (lordosis and scoliosis), ocular deformities, and jaw deformities. Adema-Hannes and Shenker (2008) reported an increase in mortality rates, in yolk sac edema, and in skeletal deformities among *Coryphaena hippurus* specimens after their exposure copper chloride ($CuCl_2$), while Von Westernhagen et al., (1988) described that the fish embryos exposed to pesticides experienced an increase in deformities.

In general, the susceptibility of fish to pollutants is influenced by environmental factors such as pH (Ferreira et al., 2001; Alvaro et al, 2007), organic matter present, and temperature (Galdino-Rodrigues, 2010). In the current study, there were no significant differences in pH levels among the test groups when the temperature was kept at 24ºC. Thus, the deformities as described in the present study were understood as the toxic effects of chemicals still present in treated sewage. Additionally, according to Prochazka (2005), embryonic and larval deformities due to chemical exposure may be also inherited, or, in other words, a consequence of parental exposure.

Thus, the release of urban, industrial, and agricultural effluents directly into continental waters affects the early stages of the development among fish species and may even have lethal effects. However, reduced survival rates among individuals at any stage of life can also be interpreted by some non-lethal and non-measurable effects, including environmental exposure and chronic exposure (Jorgensen et al, 2002; Carvalho and Fernandes, 2006). These kinds of effects develop in adults individuals, and they can be extrapolated to the rest of the species population and its communities (Yu, 2004). Findings such as the inhibition of growth, changes in reproduction, differing immune responses, and changes in behavior have all been described (Van der Oost et al., 2003; Portz et al., 2006). According to Oba et al. (2009), the reduced tolerance of environmental stressors developed in fish during the early stages of their development as a result of chemical exposure may lead to individual pathologies or even death in adulthood. Additionally, the deformities found in the fins and spine (as shown in the present study) reduce the abilities needed for swimming and escape from predators (Pinder and Gozlan, 2004). These alterations certainly make specimens more susceptible to both environmental changes and predation, thus reducing the survival rates of juveniles and adults.

According to Agostinho et al. (2005), pollution is one of the most pervasive causes of the direct loss of biodiversity in Brazilian freshwater ecosystems. *R. quelen* is described as an abundant fish species in the Iguaçu River basin (Ingenito et al., 2004; Silva et al, 2011), but the species population has been systematically decreasing in number and even disappearing from urban areas. According to Vitule and Abilhoa (2009), the reason is likely exposure to urban and industrial effluents.

According to the United Nations (UN), two million tons of waste are dumped into the environment daily. This waste includes industrial waste, chemicals, and urban and agricultural waste (fertilizers and herbicides). In light of this information, water recycling is a viable option for decreasing water pollution created by large urban centers. Recycled water can be more adequately used for power generation, industrial processes and irrigation, but not for human consumption. Currently, the discussion water recycling is often limited by the large financial investments required, and little attention has been given to environmental issues (Hespanhol, 2010). However, long-term costs can be considered very low when treatment effectiveness is considered; recycled water is returned to rivers, without damage to aquatic ecosystems or, just as importantly, to human health.

The early stages of the development of fish and their embryos are not protected by legislation, according to the 86/609/EEC European directive (Embry et al., 2010). Brazil also fails to mention the early stages of vertebrates as being protected by law (Machado et al., 2008). The present results and others have consistently shown that the laws must be revised, since the high mortality rates that occur in this phase can significantly affects the juvenile and adult populations that follow. Another crucial factor is that pollutants continue to damage chronically exposed populations, which become more vulnerable as fewer individuals reach the reproductive stage of adulthood. Additionally, studies on the ecology of larvae in freshwater ecosystems are scarce (Santin et al., 2009; Leis and Trnski, 1989),

despite the fact that an abundance of ichthyoplankton is essential for the conservation of fish stocks.

5. Conclusions

This has shown that recycled sewage water can allow for a more adequate return of water to the environment. Water reclamation has positive long-term impacts on public health, local and national economies, aquatic ecosystem quality. However, the implementation of technology capable of the large-scale production of safe water from requires changes to legislation, as well as investment in research to establish and assess the risks of exposure. Finally, the biological methods used to evaluate the effectiveness of this kind of treatment must be tested further to confirm their reliability.

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CAPÍTULO 2

A theoretical approach to the evaluation of wastewaters risk for the silver catfish Rhamdia quelen (Teleostei, Heptapteridae)

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A theoretical approach to the evaluation of wastewaters risk for the silver catfish *Rhamdia quelen* (Teleostei, Heptapteridae)

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Abstract

Modeling is being used with greater frequency by ecotoxicologists to understand the effects of pollutants at the population level. This experiment first studied embryos and larvae of the silver catfish (*Rhamdia quelen*) exposed the pollution conditions of untreated sewage, of sewage treated using UASB technology, and of sewage treated using recycling water technology, which has featured numerous environmental, economic, and social benefits as demand for water for public supply has increased. We therefore propose a stochastic individual-based model (IBM) to evaluate the effect of the pollutants on population dynamics among adults of the species using three hypothetical scenarios: (I) the pollutants impact only the embryo-larval stage; (II) pollutants impact both the larval and juvenile stages; and (III) pollutants impact both the larval and adult stages. The model results showed that, in the first hypothetical scenario, the decline in the number of individuals in the

embryo-larval stage does propagate through the population. When we consider the perturbation of the pollutant in the embryo-larval and juvenile stages, we can observe that the population is still quite resistant to perturbations. However, in the third scenario, the system becomes very sensitive and may easily lead to local population extinction.

Keywords

Individual-Based Model (IBM), Embryo Toxicology, Wastewater Contamination, *Rhamdia quelen.*

1. Introduction

The importance of water conservation has increased in recent years as water availability and quality have decreased. According United Nations World Water Assessment Programme (WWAP, 2015), most economic models do not value the essential services provided by freshwater ecosystems, often leading to unsustainable use of water resources and ecosystem degradation. Pollution from untreated residential and industrial wastewater and agricultural run-off also weakens the capacity of ecosystem to provide water-related services. Lack of water supply and sanitation takes a huge toll on health and well-being and comes at a large financial cost, including a sizable loss of economic activity. Over 80% of wastewater worldwide (and 90% or more in developing countries) is not collected or treated (WWAP, 2012), and it is discharged untreated directly into rivers, lakes or the oceans, causing major environmental and health risks (Corcoran et al., 2010). In Brazil, the sanitation rate is precarious: only 55% of municipalities are served by sewage collection systems, and only 28% of municipalities are served by sewage treatment plants (IBGE, 2010). In the southern region of the country, 29% of the population is served by sewage collection, and it is estimated that 78% of the collected sewage is treated (SNIS, 2009).

Sewage pollution is the main cause of degradation of aquatic ecosystems due to the entry of toxic persistent organic pollutants (POPs), as well as the presence of metals, polycyclic aromatic hydrocarbons (PAHs), drugs, hormones, and organic pollutants, all of which have presented high toxicity, resistance to biodegradation, biological accumulation, and long-distance transport (Jones and Voogt, 1999; Yao et al., 2014).

Effective management of water resources and reduction of water pollution will require promotion of reuse, treatment of wastewater to an appropriate level for the intended reuse option, and integration of sanitation systems with overall water resource and urban planning and design (Lüthi et al., 2011). The term "water recycling" came about in the 1980s, when water supplies were becoming a more expensive burden on industrial production costs (Mancuso, 2003). In several countries, including Israel (Furtran, 2013), Japan (Mancuso, 2003), the USA (EPA, 2014), and various members of the EU (Köck-Schulmeyer et al., 2011), water recycling technology is widely utilized for various applications. These applications include agriculture, industry, recreational use, household use, aquaculture, and recharging groundwater aquifers (Westerhoff, 1984). In Brazil, however, these applications are still scarce (IBGE, 2010), Recently, the application of water recycling technology has increased alongside demand for water, which has largely resulted from a water crisis in large urban centers across the country (CETESB, 2010). There are many advantages of applying recycled water, such as the reduction of domestic and industrial sewage discharge into water bodies (Hespanhol, 1997), the decreased pollution of water supply sources (Bernardi, 2003), a lower demand for surface water and groundwater, and the increased availability of water for more demanding uses, such as public supply.

Fish are important components of aquatic ecosystems and are particularly vulnerable because of their high exposure to contaminants throughout their lives, and also because of the permeability of their gills and skin (Di Giulio and Hilton, 2008). The intake of pollutants

can also occur through the sediment and through bioaccumulation among top-chain animals. Thus, fish have been widely used as a model in ecotoxicology studies (Braunbeck et al., 1998; Norrgren, 2012) to understand the potential for bioaccumulation, the persistence of chemical pollutants, and the effects of these pollutants on both the environment and public health.

The silver catfish *Rhamdia quelen* (Quoy and Gaimard, 1824) is a Neotropical fish native to South America (Teleostei, Heptaridae) that is commonly raised in fish farming. This availability has provided a series of studies on the species' biology, and particularly on its reproductive process. *R. quelen* is omnivorous, presents a migratory habit across short distances (Zaniboni-Filho and Schulz, 2003), inhabits calm waters near vegetation, and is nocturnal (Baldisserotto and Radünz Neto, 2004). By the time they reach the adult phase, females and males can reach 66.5cm and 52cm in length, respectively (Benaduce et al., 2006). Their life span ranges from 18 years (males) to 21 years (females) (Weis and Castello, 1983). According to Borges et al. (2005), *R. quelen* reaches sexual maturity in the first year of life and does not exhibit parental care. The hatching of larvae occurs 24 hours post fertilization (hpf), and the opening of the mouth occurs 96 hpf, at which point the juveniles start feeding on exogenous resources (Rodrigues Galdino et al., 2010). According to Andreatta (1979), the rate of fertilization can reach close to 95% in artificial systems. The species presents a low annual growth rate and low natural mortality (Gubiani et al, 2012), temperature fluctuation resistance and low levels of dissolved oxygen (Zaions and Baldisserotto, 2000; Carneiro, 2002), and tolerance when exposed to natural contaminants, xenobiotics, and changes to abiotic factors.

Perturbation at the individual level has been studied at length by ecotoxicologists; however, there are few studies that connect this knowledge to impacts at the population level (Stark, 2005). The integrated use of tools such as ecotoxicology assays and modeling

increases the potential detection of the causes and effects of stressors on aquatic ecosystems (Buss, 2008; Beketov and Liess, 2012). Mathematical models are an abstract representation of nature and can aid in the understanding of how a perturbation at the individual level can propagate to the population level, to the community level, or even to the ecosystem level (Murray, 2002). According to Gledhill and Kirk (2011), modeling studies are important tools for broadening the scope of ecotoxicological studies. In recent years, individual-based modeling (IBM) has been used with greater frequency to analyze ecological processes due to its flexibility, its use of detailed parameters, and the possibility of variation among individual parts (Giacomini, 2007).

In this study, we consider the initial stages of development of *R. quelen* to formulate the theoretical model for three reasons: (I) the embryo–larval stages of fishes are highly sensitive to chemical pollutants (Kendall et al., 2008); (II) some studies have reported that results can be extrapolated to toxicological studies in the field (McKim, 1977; Woltering, 1984; Chorus, 1987); and (III) there are time constraints on experiments that include every phase of adulthood.

We propose a mathematical model to investigate the impact of water pollution at the population level. First, we utilized experimental data on *R. quelen* embryos and larvae exposed the pollution conditions resulting from the sewage. Then, we used the literature to parameterize the model. We built a stochastic model to evaluate the effect of the pollutants at the population level. Due the lack of data on the impact of pollutants on juvenile and adult populations, we investigated the damage at the population level based on three main hypothesized scenarios: (I) the pollutants impact only the embryo-larval stage; (II) pollutants impact both larval and juvenile stages; and (III) pollutants impact both larval and adult stages. We have considered different impact intensities and discuss them in terms of loss in population density.

2. Methods

2.1. Experimental Approach

We used experimental data on the initial life cycle stages of *R. quelen* when specimens were exposed to untreated sewage, to sewage treated using UASB technology, and to sewage treated using water recycling technology, which consists of five additional steps: (1) flake settling; (2) sand and charcoal filtering; (3) ozonation, (4) activated carbon filtering, and (5) chlorination to illustrate the results of the theoretical model and to parameterize a simulation version to assess effects of pollution in the population.

In the study by Brito et al. (2015), dilutions and other parameters were tested to evaluate the effectiveness of the treatments that were applied to the effluents. In the present study, we utilized the lowest concentration of the effluent (33%) diluted in reconstituted water (0.0065 g/l CaCl2, 0.1335 g/l MgSO4, 0.0004 g/l, and KCl 0.0105 g/l NaHCO3) to evaluate the effects of pollutants according to method for evaluating acute toxicity of wastewater samples and acute toxicity in fish (ABNT, 2011) and only the final water recycling technology (steps 4 and 5).

The fertilized eggs were obtained from a fish farm, and only viable eggs were selected for study under an Olympus stereo microscope (× 0.67 to 6.7) and exposed to treatments 8 hours post fertilization (hpf) while in the gastrula stage, following Rodrigues-Galdino et al. (2010). For each treatment, eggs were individually transferred into 96-well culture plates according to MacPhail et al. (2009). The wells contained 200µl of the solution diluted by 33%. The tests were conducted in triplicate with 288 embryos per exposure time (24, 48, 72, and 96 hpf). In addition, oxygen levels, pH levels, and metals were measured in all treatments in order to evaluate the effects of their interactions with toxic effects on *R. quelen* embryos (Brito et al., 2015).

The hatchability rates (24 hpf) and survival rates (96 hpf) were higher among individuals incubated in the effluent from wastewater recycling plants than among those kept in the treatment with UASB technology. The lowest survival rates were found among specimens kept in untreated sewage, according with the data demonstrated in Table 1.

A temperature of 24°C was recorded in all of the samples, and the physical and chemical parameters were higher in treated and untreated sewage samples than in samples that had received the water recycling treatment. The analyses of oxygen levels, pH levels, and metals are summarized in Table 2.

2.2. Theoretical Approach

We have proposed a model to study the impact of pollutants on fish populations. The model was built by dividing the fish life cycle into three phases. Phase (A) is the embryo-larval stage, which corresponded to the first 96 hours post fertilization and which is characterized by the presence of the yolk vesicle. Phase (B) is the juvenile phase, which is measured from the complete absorption of the yolk (96 hpf) to one year of age. In this phase, the individuals begin exogenous feeding and horizontal swimming movements (Luz et al., 2001), but are not yet reproductive. Phase (C) is the adult phase, which is established as of one year of age. During phase (C), reproduction occurs but depends on age (see details below).

During the first year of life (the larval and juvenile stages), individuals may die from natural causes or from the effects of pollutants. Individuals survive into the adult phase, during which the natural and pollutant pressures still exist and at which point they can reproduce. The viable offspring go through phases A and B while their surviving parents become one year older and may participate in another reproduction event. It is important to

note that the model considers asynchronous events, which means that different stages and adult ages coexist through time (Figure 1**)**. The model does not consider any other negative pressure (such as predation or environmental variability). As a consequence, the model simplification probably overestimates the survival rate in real conditions.

Due to the level of details, we choose an individual-based model (IBM) to describe these dynamics. This approach also allows for random events based on individual characteristics. Below, we outline the events imposed upon the population according to the stage of each individual:

A) Embryo-larval (0 hpf to 96 hpf):

Each embryo has a probability S^A of surviving to the juvenile stage. The maximum value of S^A is equivalent to a situation in which pollutants have no effect, and which can be understood as the natural embryo survival rate. For silver catfish, Ferreira et al. (2001) reports that $S^A = 0.6$ in cases of fish-farming or in the absence of pollutants. When the larvae are exposed to other deleterious incidents (such as pollutants), the probability of survival decreases and may even reach zero (Brito et al., 2015). We investigate the population dynamics for $0 \leq S^A \leq 0.6$, and we highlight the cases in which the probability of survival matches the experimental results (presented in the next section). The surviving individuals go on to Phase B: the juvenile phase.

B) Juvenile (96 hph to 1 year)

In this phase, the individuals begin exogenous feeding and horizontal swimming movements (Luz et al., 2001), but are not yet reproductive. During the juvenile stage, individuals begin to compete. Each individual has a probability S^B of surviving in this phase that is proportional to the density of individuals competing for the same resources (Gledhill and Kirk 2011):

$$S^B = \frac{b}{1 + e^{a(n_t - K)}} \quad (1)$$

where b is the maximum survival rate in the absence of interspecific competition, K is the carrying capacity for one-year-old fish, and n_t is number of larvae at time t. Fish farming creates a situation in which the individuals have an abundance of food and competition is therefore minimized. In the model, we assume that b=0.8 (Piaia et al., 2000). In the field, the juvenile survival (S^B) decreases as the number of individuals increases because of limited resources (equation 1). The impact of pollutants in this phase was modeled by reducing b, which means that individual survival probability decreases regardless of the number of competitors. We investigated all dynamics considering $0 \leq b \leq 0.8$.

The carrying capacity was restricted to one-year-old fish, because we assumed that juveniles do not compete with adults and that morphological constraints (e.g. mouth size) restrict the size of prey (Gerking, 1994; Brazil-Souza et al., 2009). In this scenario, larger items will be consumed by larger adults with higher food plasticity. Adults are also able to migrate across small distances, which enables foraging in other microhabitats. In the model, K is an arbitrary value because all results were normalized by this value (see next subsection).

C) Adult (1 to 21 years)

From 1 to 21 years of age, individuals may die from natural causes but may reproduce before death (Gomes et al., 2000). We assume a constant annual mortality rate of d = 0.35 (Gubiani et al, 2012), which means that each individual has a probability of survival of S^C = 1 – d. Surviving individuals can then reproduce. As in the previous phases, the impact of the pollutants in this phase is modeled by reducing the value of S^C value. We considered $0 \leq S^C \leq 0.65$.

The fecundity of individuals depends on their weight and, consequently, on age. Individuals were separated into age groups with an annual interval according to the number of eggs F_i that a female of age i will produce, as predicted by Olaya-Nieto et al. (2010):

$$F_i = 5683 W_i^{2.37}, \quad (2)$$

where W_i is a female's weight at age i. The relation between weight and age can be described following the Beverton-Holt model (Benaduce et al., 2006):

$$W_{i+1} = \frac{r W_i}{1 + \alpha W_i}, \quad (3)$$

where $r = 1.5$ is the rate of weight increase per year and $\alpha = 0.17$ is a growth inhibition constant (Benaduce et al., 2006). The number of embryos generated by the adult population is:

$$n_0^A = \frac{1}{2} f \sum_{i=1}^{I_{max}} F_i n_i^C, \quad (4)$$

where I_{max} is the maximum reproductive age, n_i^C is the number of individuals in stage C (adults) at age i, and f is fecundity, which was assumed to be 0.75. The term ½ appears because a 1:1 natural sex ratio is assumed (Olaya-Nieto et al., 2010); therefore, only the half of the population (the female half) produces eggs.

We do not impose any competition among adults; we could have done it by using a method similar to that applied to phase B (equation 1), but considering a carrying capacity for the adults. However, we lack data on juvenile and adult carrying capacities, and this capacity is likely to vary among rivers. Our simplification can be considered reasonable for situations in which competition among juveniles is much greater than among adults. Although it may sound speculative, we can still support the model if we keep in mind that

this model overestimates the survival rate of a real situation. Table 3 summarizes the parameters involved in the model found in the literature.

2.2.1 Scenarios Investigated

We utilized the model to predict how populations would be impacted by pollutants when : (I) the pollutants are restricted to the embryo-larval stage (varying $0 \leq S^A \leq 0.6$ and maintaining the maximum values of $b = 0.8$ and $S^C = 0.65$); (II) the pollutants affect larvae and juveniles (computing all combinations when $0 \leq S^A \leq 0.6$ and $0 \leq b \leq 0.8$); and (III) the pollutants affect larvae and adults (computing all combination when $0 \leq S^A \leq 0.6$ and $0 \leq S^C \leq 0.65$). The variation in the parameters was determined using an interval of 0.01 units.

2.2.2 Simulations and Data Analyses

For each parameter combination, we considered a initial population composed of 20 individuals (we tested different initial conditions and observed that the asymptotic behavior was not sensitive the variations). We then computed the relative population density, which we define as the adult population size divided by the average population size in a non-pertubative scenario (without pollutants; $S^A = 0.6$, $S^B = 0.8$ and $S^C = 0.65$):

$$p_{rel} = \frac{n^C}{\langle n^C_{np} \rangle} \tag{5}$$

where $n^C = \sum_{i=1}^{Imax} n_i^C$ is the sum of adult population and where

$$\langle n^C_{np} \rangle = \frac{1}{m_s (t_f - t_c)} \sum_{i=1}^{m_s} \sum_{t=t_c}^{t_f} n_i^C(t) \tag{6}$$

.

is the average population size in a non-pertubative scenario counted over $m_s=10$ simulations and considering the last 100 of a total of 200 years ($t_f=200$ $t_c=100$). We determined the last

100 years to be enough to eliminate the transient element (dependency on initial condition), and we also determined 10 simulations to be sufficient to achieve the average value. It is important to note that, when pollutants are not considered, the average size of the relative population is equal to 1 and may decrease (population density decay) when pollutants are considered.

The results presented herein relied on a initial population composed of n^C = 20 individuals and a carrying capacity of K=150. We tested different initial population sizes (n^C= 10, 20, 50, 100) and carrying capacities (K = 50, 75, 100, 150, 200) and found that asymptotic behavior was not sensitive to these aspects.

3. Results

The experimental study showed that perturbations in water composition can have an intense impact on the embryo-larval stage (phase A). In Figure 2 is shown the evolution over 200 years after considering the impacts of different pollutants. In a non-perturbative scenario, population density increases to reach a certain density, at which point it oscillates around a fixed value (the average size of the relative population, as shown in equation 5). When pollutants are considered, population density may decrease.

In the first modeled scenario, we consider different intensities of pollutants and restrict their impact to phase A. In all of the experimental treatments, a decline in the number of individuals in the embryo-larval stage was found, but it did not prevent adult population growth (Figure 3, when b or S^C is at maximum values). The water recycling treatment with chlorine (S^A = 0.08% of survivors), though quite aggressive against individuals in the embryo-larval stage, still resulted in only a slight decrease in population density (10%) relative to the non-perturbative system (S^A = 0.6).

When we consider the perturbation of the pollutant in phases A and B, the population is still quite resistant to perturbations (note the large blue area in Figure 3): for a large combination of S^A and b, the population density is similar to the population density in the absence of the pollutants. Only for low survival rates in S^A and b was population decline or extinction observed. Even if the effect of the pollutants on juveniles was as intense as the effect on specimens in the embryo-larval stage when observed experimentally (i.e., the proportional reduction in survival rates is the same), the population still reached maximum density. However, when the recycling treatment with chlorine was applied, the population went extinct (Figure 3a).

When we analyze the scenario in which the effect of the pollutants is visible in phases A and C, the system becomes very sensitive to disturbances. While in the previous situation only a small combination of values caused extinction, almost all of the combinations of values impacted the population size in this scenario (note the large red area in Figure 3b). If the effect of the pollutants on specimens in the embryo-larval stage is equivalent in adults, all treatments would affect the size of the adult population and would thus keep its population below 50% in almost all treatments, with the exception of the recycling treatment (Figure 3b).

The illustration of population evolution over time for one of the simulations (Figure 2) shows that, when the adults are proportionally as impacted as the larvae, the non-perturbative scenario (S^A = 0.60 and S^C = 0.65) and the water recycling treatment without chlorine (S^A = 0.55 and S^C = 0.60) slightly affected the population dynamics. The population quickly increased and reached its asymptotic regime (close to 10 years). The sewage treated conventionally with primary and secondary treatments (UASB treatment, S^A = 0.44 and S^C = 0.48) kept the adult population at a very low density throughout the 200-year period, presenting a value above 50% only occasionally. The untreated sewage (S^A = 0.28 and S^C =

0.30) kept the adult population at very low densities (less than 5%), which would probably prevent the continuation of the species over generations. The effluent from the recycling station that contained chlorine (Recycled water with chlorine, S^A = 0.08 and S^C = 0.09) completely limited the growth of the adult population, which went extinct in a short period of time. As a result, no individuals were represented at this stage; that is to say, the population went extinct in a short period of time because there were no individuals of reproductive age.

4. Discussion

Rhamdia quelen embryos and larvae exposed both to untreated sewage and to sewage treated with UASB technology experienced high mortality rates, a result which reflects the toxic potential of wastewater, even after it is treated (Hespanhol, 1997). The finding also suggests that the biological treatment is unable to reduce or eliminate the toxicity from urban sewage, as described by Ryu and Yoon (2011), Köck-Schulmeyer et al. (2011), and Zhao et al. (2011). Meanwhile, after the application of the additional water recycling treatment, the larval survival rate increased and approached the survival rates seen in the control treatment. This result indicates reduced effects of pollutants and is consistent with reports by Tortora et al. (2012) and FAO (2014). The water recycling technology employed was found to present better results than conventional treatment in terms of survival rates and also in terms of physical and chemical parameters; the water recycling treatment increased oxygen concentrations, pH levels, and metal removal. However, manganese and iron were found at levels above the legal limits established by the Brazilian legislation (Brito et al., 2015).

The early life cycle stages of fish are highly sensitive to chemical exposure and have therefore been widely used in effluent toxicity protocols and since 2005. Fish embryo

toxicity testing has even been made mandatory for routine sewage surveillance in Germany (Embry et al., 2010). Currently, little is known about the effects of pollutants on the embryonic development of native species like *R. quelen*, though exposure during critical windows of development can have severe and permanent consequences that can manifest later in life (Hamlin and Guillette, 2011).

The experimental data demonstrates that different intensities of pollutants cause high mortality rates during the embryo-larval stage, even at low concentrations. However, the theoretical model predicts that the adult population is not affected when the pressure of the pollutants is limited to the embryo-larval stage (phase A). Indeed, each female can produce thousands of eggs each year, and if only one egg reaches 1 year of age, breeding can begin and the population balance is maintained. However, if the animals are living in water polluted by chemicals, the success would be hindered, and would thus have negative effects on the health, development, reproduction, and survival of the species (Soso, 2006).

When the effects of pollutants were assumed to be limited to the first year of life (impact on phases A and B), a slight impact on the population density was observed. In the juvenile stage, the competition for resources limits juvenile population growth (Brandão-Gonçalves and Sebastien, 2013). Thus, the mortality rate as a result of exposure to toxicity ends up diminishing competition by reducing individuals at 1 year of age. Again, if at least one individual reaches adulthood and reproduces, the population density is not affected. However, when we considered the impact of pollutants on larvae and adults (phases A and C), population dynamics oscillated. A small decrease in survival probability among the adult population can lead to a reduction in population size, which may not exceed 50% (UASB-treated sewage), 25% (non-treated sewage) or 8% (recycling water treatment with chlorine). The only conditions in which population density does not decrease is in non-pertubative conditions (without pollutants) and when water recycling treatment technology (without

chlorine) is applied. This result indicates that the water recycling treatment is very effective. Thus, the *R. quelen* population would be more susceptible to local extinction as a result of demographic fluctuations, internal mechanisms (demographic stochasticity), or external mechanisms (environmental stochasticity) (Pimm et al., 1988; Pimm, 1991; Lande, 1993).

Low population density also has important effects on genetic events, such as genetic drift and inbreeding in small potentiated populations (Futuyma, 1992), as well as on demographic stochasticity (including fluctuations in the sex ratio), and the "Alle effect" on the shortage of meetings among receptive partners during mating periods (Courchamp et al, 1999). As consequence, the persistence capacity of a small population may be lower than that of an average-sized population, due the low reproductive success leading to a collapse of the population can be the most prominent effect.

According to Van Der Oost et al. (2003), exposure to contaminants interferes with growth, reproduction, immune response, and behavior, and also reduces the species' ability to tolerate stressful environmental agents. Exposure can therefore potentially lead to serious pathological conditions or death (Oba et al., 2009). In the present study, when the model restricted the impact to the embryo-larval stage, the consequences in natural conditions were certainly underestimated: first, because we accounted only for lethal effects for modeling purposes, and not for sub-lethal effects such as deformities in embryos and larvae, which would reduce the species' ability to respond to environmental stressors; second, because we exposed the embryos and larvae to a low dilution concentration (33%). In the natural environment with the highest concentration of the pollutant, the lethal effects on *R. quelen* embryos and larvae would be worse, and the probability of local extinction would be higher. We chose to attempt to propagate the impact of the pollutant to the other phases in order to the model more realistic, despite not knowing which mortality rate to consider in the other phases.

However, when we compare the effect of pollutants on both larvae and adults, we may be overestimating mortality rates due to the capacity of physiological adjustments (adaptive mechanisms) that could minimize the negative effects on the population. We also chose not to consider environmental variability, competition, predation, or intraspecific and interspecific variation (such as the individual ability metabolize xenobiotics), all of which impacts species mortality rates. On the other hand, the current results are consistent with recent studies that reflect changes in diversity and composition of fish assemblages. According to Felipe and Súarez (2010), these changes are a result of changes to the structure of habitats, which have been caused by the dumping of organic and industrial waste around Brazil. According to Von Westernhagen et al. (1988), adults that experience no lethal effects of chemical contamination can produced reduced-quality oocytes and spermatogonia; thus, contamination can interfere with various stages of development as a result of parental exposure. Organochlorine, organometals, and heavy metals can affect vitelogenesis and gonad development in females (Black et al., 1988; Cameron et al, 1992; Von Westernhagen et al., 1988) and can decrease sperm quality (Singh et al., 2008). According to Zhang et al. (2008), exposure to pollutants also affects the frequency of spawning plus the number and quality of eggs, thus affecting reproductive success and the maintenance of species. In this study, we considered all individuals to be capable of reproduction.

According to Brazilian legislation (CONAMA, 2005) post-treatment chlorination is mandatory in sewage treatments. However, the use of chlorine to combat pathogenic agents is highly toxic to fish in their early life cycle stages and, according to the model, can result in a drastic reduction in population density. In Brazil, water recycling does not result in potable water. However, there is a growing interest in recycling sewage water from urban, industrial, and agricultural activities. This practice is common in many countries, whether they are developed or developing (FINEP, 2014). This interest is justified in large part by

the increasing scarcity of water resources that are adequate in quantity and quality for different applications. The use of water recycling technology to produce potable water requires a large financial investment, but this value seems low when treatment efficacy is considered: this treatment would increase the return of water of the highest quality to rivers, without damage to aquatic ecosystems or human health.

Pollution is one of the causes of extinction in continental aquatic ecosystems (Tundisi, 2011). In Brazil, despite legislation mandating treatment, less than 30% of the urban sewage is treated (PNUD, 2014). This lack of treatment significantly decreases water quality and the conservation of natural aquatic resources (Martinelli et al., 2002). Additionally, the loss of species and changes to community structures have each been associated with pollution and with the eutrophication of aquatic ecosystems (Marques and Barbosa, 2001).

Species extinction is due to both natural and environmental selective pressures. The present model demonstrated that the abrupt input of chemicals into aquatic environments could introduce new harsh conditions for biota such as fishes and can potentially accelerate the conditions that cause local extinction. According to Dawson et al. (2011), habitat loss is the main cause of extinction. When combined with other factors, such as pollution and climate change, it can have synergistic effects, as was also reported by Hogan (2010) in a study on continental aquatic systems. Unnatural extinctions are most destructive in vital ecological processes: they have ripple effects and lead to ecosystem collapse, and consequently, to higher rates of extinction (Sodhi et al., 2009).

The evaluation of effects on aquatic organisms exposed to chemicals increases the understanding of risk of exposure. The use of modeling to cluster these findings in order to predict further effects of chemicals over the coming decades is a recent and rarely used method in the field of ecotoxicology. Another serious challenge in environmental toxicology

is evaluating the risk of exposure to complex mixtures of chemicals. In this sense, the modeling method discussed herein may contribute to the development of conservation policies, because it presents unequivocal arguments for the prioritization of regulatory actions by governments. Beketov and Liess (2012) point out that experimental ecotoxicology studies using small-scale model systems have clarified the mechanisms and propagation of effects through different levels of biological organization. However, attributing the loss of diversity to pollution is a delicate matter and requires studies on biodiversity and endangered species, which are scarce. Still, extrapolating the effects of toxicity from the laboratory scale to the spatial scale requires more collaborative research with other fields of science, such as chemistry and mathematics.

As this report shows, modeling is a promising tool. When combined with experimental studies, it encourages improvements to sewage treatment systems through the effective use of a water recycling system. As the current study demonstrates, water recycling treatments are clearly more efficient in removing contaminants than conventional treatment methods. These findings are relevant and can guide more effective public policies and conservation projects. Environmental impact analyses can include low-cost simulations and long-term-effect simulations.

Although *R. quelen* is not listed as a threatened species, the use of this species as a model in the current study still demonstrates the vulnerability fishes experience overall. The use of the modeling aids in the understanding of the real risk of exposure that will be experienced by future generations. These studies are vital for establishing policies on the presence of urban sewage in aquatic ecosystems. Further studies are needed and should include more sensitive native species or rare or endangered species such as *Astyanax sp*. (Characiformes); individual species characteristics can increase damage to a population, especially among endemic species or those with more restricted distribution areas.

According to Vitule and Abilhoa (2009), the risk of extinction has global projections, particularly in terms of its impact on biodiversity.

5. Conclusions and Future Directions

The experimental data showed that the pollutants contained in both treated and untreated sewage reduce the survival rates of *R. quelen* embryos and larvae of after 96 hours of exposure. The theoretical modeling results demonstrated that the negative impact on survival during the early life cycle stages can be propagated to other life cycle stages, thus decreasing population density and, in the most drastic cases (such as treatments that use chlorine), leading to local extinction.

The embryo-larval survival rate was found to be a very sensitive parameter when the population response was assessed, but more studies are required, especially studies *in situ* or experiments that reproduce natural conditions, in order to better understand the effect of contaminants on adult specimens when they are exposed to pollutants in water throughout the reproductive phase.

Ecotoxicological tests are a powerful water quality assessment tool. They use test organisms as biomarkers of toxic potential. When these sites are added to ecology-based models, they can contribute to our understanding effects of pollutants at the population level, the community level, and the ecosystem level. These tests can ultimately aid in the conservation of species and water resources.

Finally, the increased demand on water resources due to urbanization results in high water treatment costs due to the degradation of watersheds. Thus, the practice of water recycling can be considered an option that will reduce the discharge of pollutants into receiving water bodies and will thus conserve water resources for public supply. More and

higher-quality water will therefore be available for more important uses, which will improve both environmental protection and public health.

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Capítulo 3

Embryo toxicity assay in the fish species Rhamdia quelen (Teleostei, Heptaridae) to assess water quality in the Upper Iguaçu Basin (Paraná, Brazil)

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Embryo toxicity assay in the fish species *Rhamdia quelen* (Teleostei, Heptaridae) to assess water quality in the upper Iguaçu basin (Paraná, Brazil)

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Abstract

The Iguaçu River is the largest and one of the most important rivers in the Brazilian state of Paraná. The upper Iguaçu basin is represented by 32 affluent streams and small rivers, which are responsible for 60% of the water supply of the city of Curitiba and the surrounding region, collectively known as greater Curitiba. After crossing the large urban region, the

river is substantially polluted by domestic and industrial sewage. The Iguaçu River is classified as the second most polluted urban river in Brazil. Currently, few ecotoxicological studies have been performed on the Iguaçu River. The aim of the present study was to investigate the extent of water toxicity in the Iguacu River and the risk of exposure experienced by both the local biota and the human population. To perform this assessment, the survival effect and morphological deformities in embryos and larvae of *Rhamdia quelen* were evaluated. Additionally, a stochastic mode was used to assess the impact of human activities on population dynamics. The lethal and non-lethal effects were evaluated in all areas of the river that were studied, and a high level of pollution was found in the river. The results of chemical analysis in the water indicated point source and run-off pollution containing high levels PAHs and metals such as lead. The theoretical model showed that the pollutants present in water of the Iguaçu River may further reduce the population density. When associated with other effects described in earlier stages of research, this data may suggest a high risk of local extinction of fish species and may reflect the needs for studies that evaluate the real impact of human activities on the biota. Ideally, this research will contribute to the establishment of more complete conservation policies.

Keyboards

Iguaçu River, Water Quality, Biomarkers, Embryo Toxicology, *Rhamdia quelen*.

1. Introduction

According to the UNESCO (2015), the water crisis currently affects 40% of the world's population. In Brazil, the combination of demand and water quality degradation has resulted in a serious problem (Setti et al., 2001). A loss of biodiversity occurs when there are changes to the watercourses of rivers that traverse major cities (Shepp and Cummins, 1997).

This loss has consequences, such as loss of self-purification capacity and profound changes to the aquatic biota (Tundisi, 2011). According to the Millennium Ecosystem Assessment (2005), the loss of local biodiversity can have other consequences, such as the damages to ecosystem services.

According to the Brazilian National Sanitation Information System (SNIS, 2012), the average rate of sewage collection and treatment in the country is estimated to be 48.3%. Additionally, the management of water resources in Brazil is guided by laws based only on physical and chemical parameters, and these laws do not consider biological indicators that could assess environmental health and the risk of exposure experienced by humans.

According to Beketov and Liess (2012), a serious challenge in the field of ecotoxicology is identifying the causal relationships between exposure to contaminants and the ecological consequences in nature. However, many smaller-scale studies with microcosms and mesocosms have confirmed that many factors are relevant on large spatial scales. These factors include abiotic factors, such as chemicals (Norgaard and Cedergreen, 2010), and they significantly influence the strength of effects and the dynamics of recovery after contamination. Forbes et al. (2001) reported that a major criticism of standard ecotoxicological tests is the difficulty in translating the experimental results or effects to populations.

Fish embryo testing in ecotoxicological studies has been largely utilized as an accepted method in different models. Studies have been performed using the zebrafish (*Danio rerio*), the Japanese medaka (*Oryzias latipes*), and the fathead minnow (*Pimephales promelas*). According to Embry et al. (2010), the life cycle stages of fish (through the larval stage) may represent a way to establish definitive testing strategies and will provide another option for the acute testing of juvenile or adult fish. In Germany, fish embryo tests are

required for wastewater effluent testing as part of the guideline referred to as DIN 38415-6 (2001).

The Iguaçu River basin is the largest in Paraná State, with 70,800 km^2 divided into the upper, medium, and lower Iguaçu basin (Ingenito et al., 2004). It is formed by the confluence of the Irai and Atuba Rivers and is approximately 1,300 km in length (Vitule and Abilhoa, 2009). Data from the Brazilian Institute of Geography and Statistics (IBGE, 2008a; 2010b) point out that the Iguaçu River is one of the most polluted rivers in the country based on its low water quality index (WQI). This index is calculated based on treated and untreated sewage, inputs, and pollutant diffuse sources from intense urbanization and industrialization areas along the river. According to the Brazilian Department of Environment and Water Resources (SEMA, 2015), the Iguaçu River basin accounts for 28% of the state's water supply, and represents 80% of the human supply and 18% industrial usage. Finally, the Iguaçu River basin is of major economic and ecological importance due to its high diversity in and endemism of fish species, including endangered species, particularly at the headwaters (IPARDES, 2007).

Baumgartner et al. (2012) described 106 fish species in the Iguaçu basin. According to the same authors, Siluriformes is the most abundant order, with three species of the genus Rhamdia (*Rhamdia branneri* Haseman, 1911, *Rhamdia voulezi* Haseman, 1911, and *Rhamdia sp*). Thus, the current study considers the native Neotropical catfish *Rhamdia quelen* (Quoy and Gaimard, 1824), a species that is well known for its utilization in fish farms, its adaptability to laboratory tests, and its phylogenetic proximity to some endemic species from the Iguaçu River.

The subbasin of the upper Iguaçu River where the studied sites are concentrated is within a highly urbanized region surrounding the city of Curitiba, collectively known as greater Curitiba (the MRC, in Portuguese). Its human population has been reported to be

approximately 3.2 million inhabitants (IBGE, 2012b). Approximately 60% of this population is currently served by a sewage system, and 48% have access to both sewage collection and sewage treatment systems (Pegorini et al., 2003). These treatment systems are distributed among 27 wastewater treatment plants, or WWTPs (personal communication - SANEPAR). In addition, the industrial activities concentrated in the subbasins of the Barigui and Atuba Rivers contribute significantly to the pollution levels of the Iguaçu River. Porto (2007) described the irregular urbanization of areas containing wetlands and fountainheads as another important factor in the decline in the quality of the water reserved for public supply. These sources represent approximately 62% of the public supply for greater Curitiba (Andreoli et al., 1999; Abilhoa and Boscardin, 2004).

The aim of the present study was to evaluate the quality of the water from the Iguaçu River where it crosses the greater Curitiba region and the associated risk of exposure experienced by both the native biota and the surrounding human population. Thus, qualitative and quantitative analyses of most of the Persistent Organic Pollutants (POPs) and metals were performed using water from five sites along the upper Iguaçu River that are affected by urban activities. Additionally, biomarkers were applied to the early life cycle stages of *Rhamdia quelen* in order to identify the risk of exposure experienced by the local biota and also by human populations, including the theoretical model (Brito et al., 2015) in order to assess the relationship between toxic responses in the first life cycle stages and the species' transition into adulthood, and the impacts on the species' population dynamics.

2. Materials and Methods

2.1. Study Area

The upper Iguaçu River basin is located around greater Curitiba in the south of Brazil and consists of 30 main tributaries according to the Environmental Institute of Paraná (IAP, 2007) that represent 60% of the reservoirs used for water human supply. In the present study, five sites were selected that collectively represented approximately 40 km of the urban region surrounding the river. The criterion used to choose the studied sites was the most urbanized portion of the area of input from the main tributaries of the Iguaçu River (**Figure 1**).

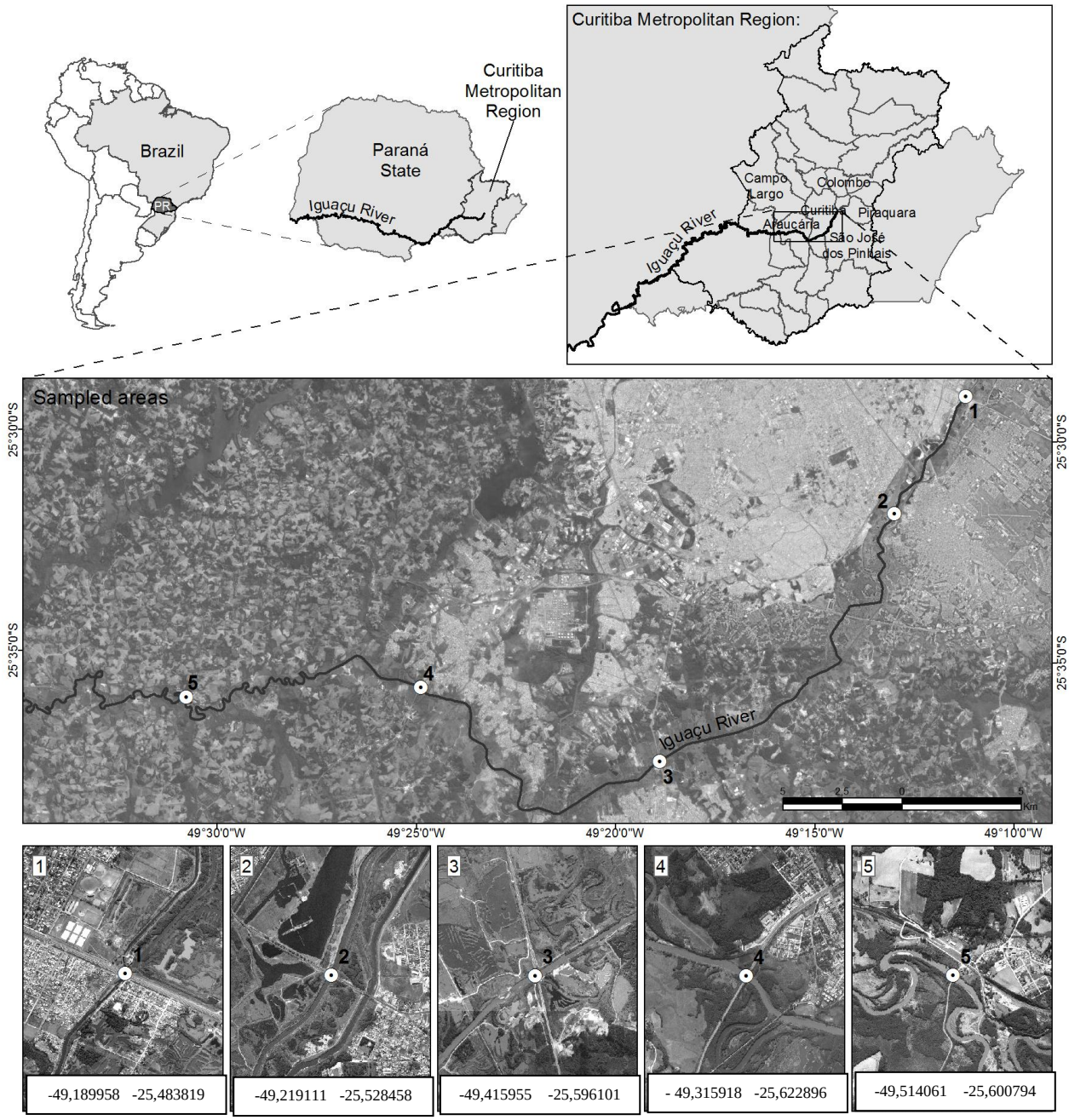


Figure 1. Location of the 5 studied Sites along the Upper Iguaçu River Basin in Metropolitan Region of Curitiba (MRC), Paraná State, Southern of Brazil. (S1) Headwaters, (S2) São José dos Pinhais, (S3) Fazenda Rio Grande, (S4)

Araucaria and (S5) Guajuvira.

As reflected in the data from IAP (2005), Site 1 is located at the formation of the Iguaçu River; Site 2 is characterized by the confluence of two major tributaries and

downstream a sewage treatment plant; Site 3 crosses the industrial area of Curitiba; Site 4 is located after the confluence with the Barigui River and is characterized by intense industrial activities, including an oil refinery; and Site 5 is located 15 km downstream from the industrial region of Araucaria and receives all of the effluent from the greater Curitiba region.

2.2. Water Sampling

The water samples were collected in December 2014 at a 30-cm depth of the water column. They were refrigerated and transported to the Cellular Toxicology Laboratory at the Federal University of Paraná. Under laboratory conditions, the samples were immediately filtered through a paper filter (12µm in porosity) to remove suspended solids. The test solutions were prepared after their 33% and 50% dilution in reconstituted water (0.0065 g/l CaCl2, 0.1335 g/l MgSO4, 0.0004 g/l KCl, and 0.0105 g/l NaHCO3) and were kept under refrigeration at 4ºC (ASTM, 1993).

2.3. Chemical Analysis

2.3.1. Organic Pollution Extraction Procedures

Water samples (1 L each) from the studied sites were stored in sterile amber vials and filtered using filter paper with a porosity of 0.50µm. The extraction procedures used were those described by Matter et al. (2004).

For solid phase extraction cartridges the water samples were conditioned with 10mL of hexane, 10mL of dichloromethane, and 10mL of methanol (high performance liquid chromatography). Next, 1000mL were passed through C18 cartridges (Cleanet C18-S SPE, Agilent Technologies) at a speed of 6-8 ml min^{-1}, dried under a nitrogen flow, and then eluted with 10mL of hexane, 10mL of a mixture of dichloromethane/hexane (1:1), 10mL of

a mixture of dichloromethane/ethyl acetate (1:1), 5mL dichloromethane, and 5mL of ethyl acetate. The resulting extract solution was placed in a rotary evaporator at 40°C and dissolved in 21mL of acetonitrile, with a total pre-concentration of 500 times.

2.3.2. Polycyclic Aromatic Hydrocarbon (PAH) Determination

After extraction, the organic extract was injected into a gas chromatograph (Agilent Technologies 7890) connected to a triple quadrupole mass detector (Agilent Technologies 7000) with a HP-5MS capillary column (30 m x 0.25 mm x 0:25 μm). The initial oven temperature was 70°C for 4 min, followed by an increase of 15°C min $^{-1}$ to 150°C, plus a posterior increase of 3°C min $^{-1}$ to 225°C. The temperature was then raised 5°C min^{-1} to 310°C, and it remained at 310°C for 10 minutes, for a total of61.33min of analysis. The injector temperature was set at 290°C, and injection was performed in pulsed splitless mode, with 1uL injected. The source temperature of the detector was 250°C.

For PAH quantification, an external calibration curve was used with concentrations of 0.01, 0.10, 0.50, 0.75, 1.00, 2.00, 5.00, and 10.00 μg mL^{-1}, which were prepared from a solution of 16 priority PAHs (Supelco and Sigma-Aldrich Co.) according to EPA protocols.

2.3.3. Determining Polybrominated Biphenyls (PBBs), Polychlorinated Biphenyls (PCBs), and Organochlorine Pesticides (OCs)

Quantities of PBBs, PCBs, and OCs in water was determined using an Agilent 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) connected to an Agilent mass spectrometer detector (Agilent 5975C inert MSD with Triple-Axis Detector). The gas chromatography column used was an HP-5 fused silica column (length 30m, ID 250μm, film thickness 0.25μm). The oven temperature was programmed to begin at 100ºC for 1 min, increasing by 5ºC min^{-1} to 140ºC (holding this temperature for 1 min), then

increasing by 1.5ºC min^{-1} to 250ºC (holding for 1 min), and finally increasing by 10ºC min^{-1} to 300ºC (holding for 10 min). The quantification of the analytes was based on a mixture of surrogate standards of PCB 103 (C-103N) and PCB 198 (C-198N) (both from AccuStandard, New Haven, CT, USA), which was added before sample extraction.

The organochlorine pollutants were identified through a comparison between the retention times based on the chromatographic peaks of the samples and those of external standard solutions using a known composition of 48 PCB congeners, 24 organochlorine pesticides, and 6 polybrominated biphenyls (**Table 2**).

Data acquisition was performed in selected ion monitoring (SIM) mode, and the Agilent Enhanced G1701 CA ChemStation was used to perform the measurements. PCBs, PBBs, and OCs were identified by matching the retention times to ion mass fragments from a standard mixture at different concentrations (1, 5, 10, 20, 80, 100, 150, and 200 pg μL^{-1}).

The quality assurance procedures included analyses of procedural blanks, surrogate recovery, and reference material (Wade and Cantillo, 1994). Procedural blanks were performed for each group of approximately nine samples using 20g of sodium sulphate anhydride heated to 450°C before extraction and analyzed in the same way as the samples were. The peaks found showed no external interference. The mean surrogate recoveries, which were based on the relationship with an internal standard (TCMX) added at the end of laboratory analyses, were found to be between 50 and 90% for PCB 103 and PCB 198.

Table 1. The persistent organic pollutants analyzed in the water samples from Upper Iguaçu River.

Polybrominated Biphenyls (PBDEs)	**Polychlorinated Biphenyls (PCBs)**				**Organochlorine Pesticides (OCPs)**	
BDE 28	PCB 8	PCB 77	PCB 132	PCB 177	Aldrin	op' - DDT
BDE 47	PCB 18	PCB 81	PCB 138	PCB 180	Dieldrin	op' - DDD
BDE 99	PCB 28	PCB 87	PCB 141	PCB 183	Eldrin	pp' - DDD
BDE 100	PCB 31	PCB 95	PCB 149	PCB 187	Isodrin	pp' - DDE
BDE 153	PCB 33	PCB 97	PCB 151	PCB 189	Endossulfan I	pp' - DDT

PCB 44	PCB 99	PCB 153	PCB 194	Endossulfan II	α-HCH
PCB 49	PCB 105	PCB 156	PCB 195	Heptachloro	β-HCH
PCB 52	PCB 110	PCB 157	PCB 201	Heptachloro Ep. A	γ-HCH
PCB 56/60	PCB 114	PCB 158	PCB 203	Heptachloro Ep. B	δ-HCH
PCB 66	PCB 118	PCB 167	PCB 206	Metoxichlor	α-chlordano
PCB 70	PCB 123	PCB 169	PCB 209	Mirex	γ-chlordano
PCB 74	PCB 126	PCB 170	PCB 177	HCB	Oxichlordan

2.3.4. Chemical Analysis of Metals

The water samples used for the chemical analysis of metals were kept in sterile, 1-L polyethylene bottles, fixed in nitric acid (HNO_3) 1:1, and kept under refrigeration at 4°C. The treatment used for to determine metal content was inductively coupled plasma-atomic emission spectrometry (ICP-OES), according to the protocol from the US EPA 6010C (EPA, 2007). The equipment used was an ES-710 Varian ICP-OES..

2.4. Embryonic and Larval Toxicity Testing using *R. quelen*

Fertilized eggs from *Rhamdia quelen* were obtained from a local fish farm (www.pisciculturapanama.com.br) and transported to the Cellular Toxicology Laboratory at the Federal University of Paraná. The embryos were maintained at a temperature of 24ºC, and only viable eggs were selected for study using a stereoscope microscopy Olympus (× 0.67 to 6.7).

For each tested solution from each of the study sites, 96 eggs were individually transferred into the test wells of 96-well culture plates according to MacPhail et al. (2009) 8 hours post fertilization (hpf), a period which reflects the gastrula stage (Rodrigues-Galdino et al., 2010). Each well contained 200µl of tested solution prepared from the study sites samples (33% and 50% dilutions); for the control group, reconstituted water was used. The

experiment was conducted in triplicate, with 288 embryos per tested solution and for each exposure time (24, 48, 72 and 96 hpf), thus totaling 2,880 embryos per assay.

Every 24 hours, 100 µl (corresponding to 50% of the total volume) of the tested solutions from all of the 96 wells was changed to promote better levels of water oxygenation and ammonia removal. Hatching rates were determined 24 hpf. Larval survival was accepted at 96 hpf, and deformities were counted at 24, 48, 72, and 96 hpf. The embryos and larvae were incubated at a constant temperature of 24°C.

For hatching and survival rate analysis, the larvae were counted individually in 96-well plates using a Leica® DM/IL inverted light microscope. For the morphological evaluation, larvae were collected and immersed in Karnovisky's fixative solution (glutaraldehyde 2.5%, paraformaldehyde 2.5% in 0.1M sodium cacodylate buffer, 0.001 M $CaCl_2$, pH 7.2). After the fixative period (a minimum of 24 hours), samples were washed with a 0.1M sodium cacodylate buffer (2x) and then stored in 1-mL plastic Eppendorf® vials at 4°C until analysis. In order to determine toxic effects such as morphological alterations, damages, or deformities, the larvae were carefully transferred to microbiology well plates and photographed using a Sony® Cyber-Shot camera in an Olympus® light microscope. The deformities were classified into four categories according to Powers et al. (2011): (1) spine deformities; (2) fin deformities; (3) facial/cranial deformities; and (4) deformities of the thorax (**Table 2)**.

The Committee for the Ethics for Animal Experiments from the Biological Sciences Department of the Federal University of Parana (CEUA/BIO) certified that the procedures using animals in this study were in agreement with the Brazilian Guidelines for the Care and Use of Animals for Scientific and Teaching Purposes established by the Brazilian National Council for Control of Animal Experimentation (CONCEA), and that they are also within the international guidelines for animal experimentation.

Table 2. Category of deformities evaluated in larvae of *Rhamdia quelen* after exposure to water samples from studied Sites locate at Iguaçu River, according to Powers (2011) with modifications.

Categories	Deformities
(1) Spine	Kink in tail
	Kyphosis or lordosis
(2) Fins	Fins stunted or damaged
(3) Cranial	Oral deformities
	Eye swelling
	Absence of eyes
(4) Thorax	Cardiac edema
	Distension or body atrophy

2.5. Statistical Analyses

Survival rates and deformities were analyzed using two-way ANOVA after confirmation of normality. Kruskal-Wallis tests were used to analyze the differences between the control and each of the samples. In addition, one-way ANOVA was performed to verify differences among the five sampled areas in terms of the values the chemical concentrations obtained. Statistical tests were performed in Prism 5.0 software (GraphPad, San Diego, EUA) assuming a value of $p<0.05$ for statistical significance.

2.5.1. Principal Component Analysis (PCA)

The integration method sought to highlight associations among the variables measured in this study (both toxicity and contaminant levels) in water samples from Iguaçu River study sites that were diluted at two different concentrations (33% and 50%). Associations among the variables that indicate exposure (levels of metals and PAH) and effects (mortality and spine, cranial and thorax deformities) were assessed using factor analysis with principal component analysis as the extraction method (FA/PCA). The

variables were autoscaled (standardized) in order to be treated with equal importance. The variables selected for interpretation were those associated with the factors with a loading ≥ 0.50, a value that is more conservative than the loading cut-off recommended by Tabachnic and Fidell (1996). The relevance of the associations observed at each of the five sampling points (cases) was estimated by calculating the factor score from each case for the centroid of all cases for the original data. All of the statistical and multivariate analyses were performed using the Statistica software tool (Stat Soft, Inc. 2013; version 12). The differences between the survival curves of the control bioassay and each river water dilution (33% and 50%) were analyzed using the log-rank test (Mantel-Cox test) at a 5% significance level. The concentrations of Co, Cr, Ag and of PCBs were not included in the PCA, because these substances were not detected in the samples from any of the study sites located in the upper Iguaçu River.

2.6. Modeling Data

A theoretical model described by Brito et al. (2015) based on individual-based models (IBMs) was used to assess the relationship between exposure to toxicity in the early stages of *Rhamdia quelen* and the impacts on the population dynamics of the species. Thus, the lowest dilution (33%) and undiluted (100%) groups were considered, and individuals with deformities were considered to have reduced the survival rate. The model was built by classifying the fish life into three stages with their own probabilities of survival: probability of survival in phase A (embryo-larval), or S^A, which corresponded to the first 96 hours post fertilization; probability of survival in phase B (juvenile), or S^B, which corresponded with complete absorption of the yolk and 96 hpf to one year of age, but not reproductive; and probability of survival in phase C (adult), or S^C from one year of age until 21 years of age and reproductive.

3. Results

The distribution of the 16 PAHs and the total concentrations of PAHs (from 2.03 to 5.10 μg.L^{-1}) among the studied sites presented a large variation (**Table 3**). Site 5 was found to have the lowest concentration of PAHs in water, while sites S3 and S4 presented the highest values of these compounds. Naphthalene was the compound that presented the highest concentration (2.68 μg.L^{-1}) at site 3. Acenaphthylene, naphthalene, fluorene, and chrysene were found at all of the study sites, but benz[*a*]anthracene was identified at S1, S2, S3, and S4. Anthracene and phenanthrene were found only at S4. In general, compounds that have a lower molecular weight, or a weight between 2 and 3 aromatic rings (acenaphthylene, naphthalene, and phenanthrene), were found at higher concentrations at all of the study sites. An exception was benz[*a*]anthracene that was also found in high concentrations.

Because Brazil does not have established regulation that quantitatively determines the PAH values allowed in freshwater, is not established, the parameters provided by Resolution nº 357 of the Brazilian National Environment Council were used for comparison purposes (CONAMA, 2005). This agency described the concentration limits for certain PAHs, such as chrysene and benz[*a*]anthracene, and in the current study, these PAHs were found at values above legal limits at all of the study sites (>0.05μgL^{-1}) (**Table 3**).

Table 3. Detection of PHAs in studied Sites from Iguaçu River. The values were compared with Brazilian legislation (Resolution nº 357 of National Council of Environment). (S1) Headwaters; (S2) São José dos Pinhais; (S3) Fazenda Rio Grande; (S4) Araucaria; (S5) Guajuvira. The values are expressed in μgL^{-1}.

PAHs	S1	S2	S3	S4	S5	LOD	LOQ	Conama (μg/L)
Acenaphthene (Ace)	LOD	LOD	LOD	LOD	LOD	0.84	2.80	--
Acenaphthylene (Acy)	0.87	0.74	0.74	0.77	0.65	0.17	0.57	--
Naphthalene (Nap)	0.61	0.44	2.68	0.60	0.38	0.05	0.18	--

Fuorene (Flu)	0.48	0.48	0.51	0.53	0.46	0.11	0.38	--
Phenanthrene (Phe)	LOQ	LOQ	LOQ	0.89	LOQ	0.25	0.84	--
Anthracene (Ant)	LOQ	LOQ	LOQ	1.15	LOQ	0.30	1.01	--
Fuoranthene (Fluo)	LOD	LOD	LOD	LOD	LOD	0.81	2.71	--
Pyrene (Pyr)	LOD	LOD	LOD	LOD	LOD	1.71	5.70	--
Chrysene (Chry)	0.51	0.57	0.52	0.52	0.55	0.14	0.46	0.05
Benzo[a]anthracene (BaA)	0.62	0.70	0.64	0.64	LOQ	0.16	0.53	0.05
Benzo[k]fluoranthene	LOD	LOD	LOD	LOD	LOD	0.81	2.70	0.05
Benzo(b)fluorantene	LOQ	LOQ	LOQ	LOQ	LOQ	0.55	1.82	0.05
Indene [1,2,3-cd]pyrene	LOD	LOQ	LOD	LOD	LOD	0.66	2.19	0.05
Benzo (g,h and i)	LOD	LOD	LOD	LOD	LOD	5.21	17.35	0.05
Dibenzo[a,h]anthracene (DahA)	LOD	LOD	LOD	LOD	LOD	2.22	7.41	0.05
Benzo[a]pyrene (BaP)	LOD	LOD	LOD	LOD	LOD	0.87	2.91	0.05
∑ (HPAs)	3.09	2.93	5.08	5.10	2.03	--	--	--

LOD = Limit of detection; LOQ = Limit of quantification; (-) Not present levels defined by law.

The PBBs, PCBs, and OCs were found at values below the detection limit for analysis in water at all of the study sites. The analysis of metals (Cu, Mn, Zn, Fe, Co, Cr, Ag, Cd, and Pb) are shown in **Table 4**. Copper presented values higher than the legal limits established by Brazilian legislation at all sites except for S1, S2, and S3. Manganese and iron were also found above legal limits at all of the study sites on the Iguaçu River. Zinc was found at higher values only at S1, and lead was found at higher values at S4 and S5.

Table 4. Analysis of metals in the studied sites of Upper Iguaçu River. The results were compared with Brazilian legislation (Resolution nº 357 of National Council of Environment). The values are expressed in mgL^{-1}.

Sites	Cu	Mn	Zn	Fe	Co	Cr	Ag	Pb
S1	0,019	7,845	0,412	1,700	<	<	<	<
S2	0,013	4,543	0,144	1,410	<	<	<	<
S3	0,011	5,231	0,089	1,447	<	<	<	<
S4	0,009	4,869	0,043	1,327	<	<	<	0,016
S5	0,007	3,944	0,041	1,059	<	<	<	0,015
	0,009	0,10	0,18	0,30	0,05	0,05	0,01	0,010

* Method quantification limit (MQL). The value can only be in the form of trace, below the detection limit with values in ppb or ppt.

The hatching rate of *R. quelen* embryos (24 hpf) exposed to water from all of the Iguaçu River study sites was higher at both dilutions (33% and 50%) when compared to the literature and control group (**Table 5**). The control presented the lowest hatching rate and also presented a statistically significant difference ($p < 0.05$) at 50% dilution at S3 and S4 (**Figure 2**).

Table 5. Percentage of hatching and survival of *R. quelen* larvae exposed to water from studied Sites of Upper Iguaçu River.

	Hatching (24 hpf)		Survival (96 hpf)	
Sites	Dilution 33%	Dilution 50%	Dilution 33%	Dilution 50%
1	99	86	38	56
2	98	98	55	49
3	89	99	50	52
4	95	99	49	56
5	98	93	41	42
Control	88	88	68	68
Cultivation systems	90[1]	90	60[2]	60

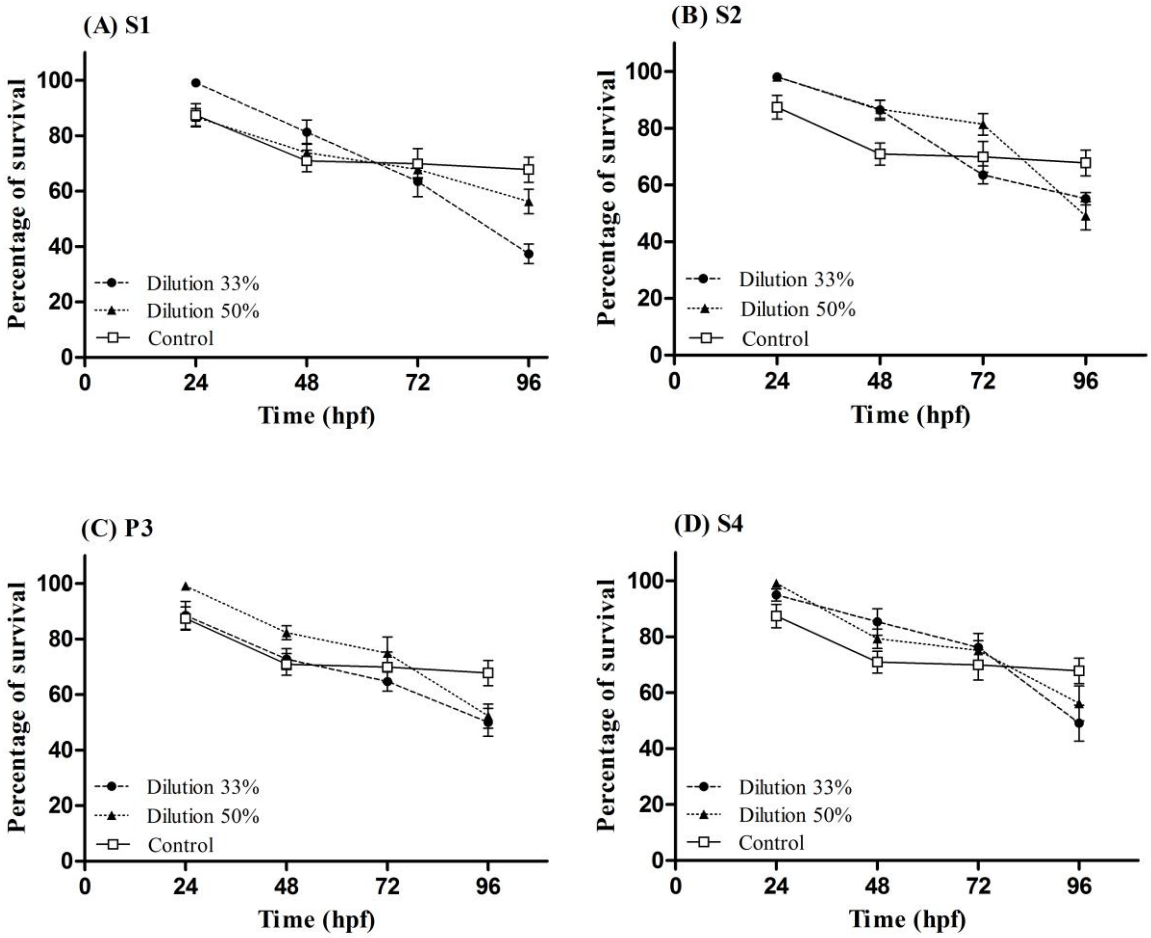
[1]According to Andreatta (1979) in artificial cultivation systems, the hatching rate of eggs can approach 90%.

[2] According to Ferreira et al., (2001) in artificial cultivation systems, the larvae survival can approach 60%.

The larval survival rate among specimens exposed to water from the Iguaçu River study sites decreased as exposure time increased at both dilutions. Mortality was found to be high at all of the sample sites when they were compared to the control, which presented a 68% survival rate. The difference in the larval survival rate between dilutions and the control was significant after 96 hpf, indicating that exposure time is a significant factor that affects mortality rates among exposed individuals (**Figure 3**). These differences were statistically significant between the control group and the 33% dilution group from S1 and S5; they were also statistically significant between the control and the 50% dilution group from S5.

The larval survival rate among specimens exposed to water from the Iguaçu River study sites decreased as a factor of exposure time and dilution concentration. The same result was observed when the mortality rate was compared to that of the control group; the difference in the larval survival rate between dilutions and control groups was found to be significant 96 hpf.

Though the survival rate was not found to be statistically significant among the studied areas, S1 and S4 presented the lowest survival rates at 33% dilution (38% and 41% respectively). Meanwhile, at 50% dilution, S5 presented the highest mortality rate (**Figure 2)**.



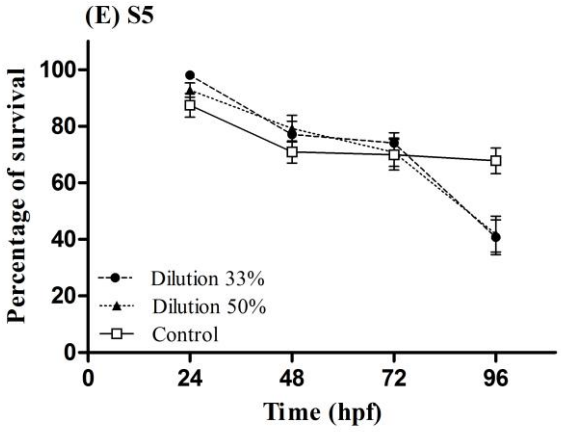


Figure 2. Survival rate of *R. quelen* exposed to water from studied Sites in the Iguaçu River at 24, 48, 72 and 96 hpf. (S1) Headwaters; (S2) São José dos Pinhais; (S3) Fazenda Rio Grande; (S4) Araucaria; (S5) Guajuvira.

The most common deformities were described as spinal, cranial, and thorax (**Figure 3**). However, the major deformities that were observed were spinal alterations, such as lordosis, kyphosis, and kinks in tails, all of which may affect the locomotion system. No significant differences in deformities were observed among the study sites at either of the tested dilutions ($p < 0.05$). Nonetheless, individuals exposed to water from S5 (33% dilution) presented the largest number of lesions. Among the thoracic deformities, cardiac edema was the most frequently, particularly among fish exposed to water at 50% dilution from S1, S2, and S3 (**Figure 4**).

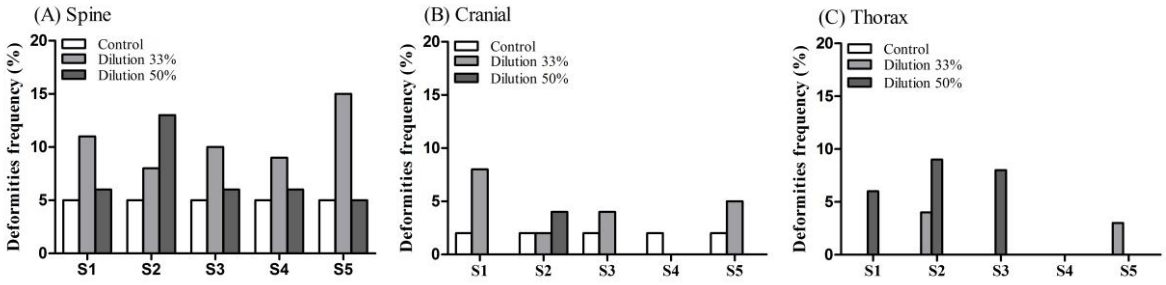


Figure 3. Deformities frequency in R. quelen larvae when exposed to water from the studied Sites in the Iguaçu River. (S1) Cabeceira; (S2) São José dos Pinhais; (S3) Fazenda Rio Grande; (S4) Araucaria; (S5) Guajuvira.

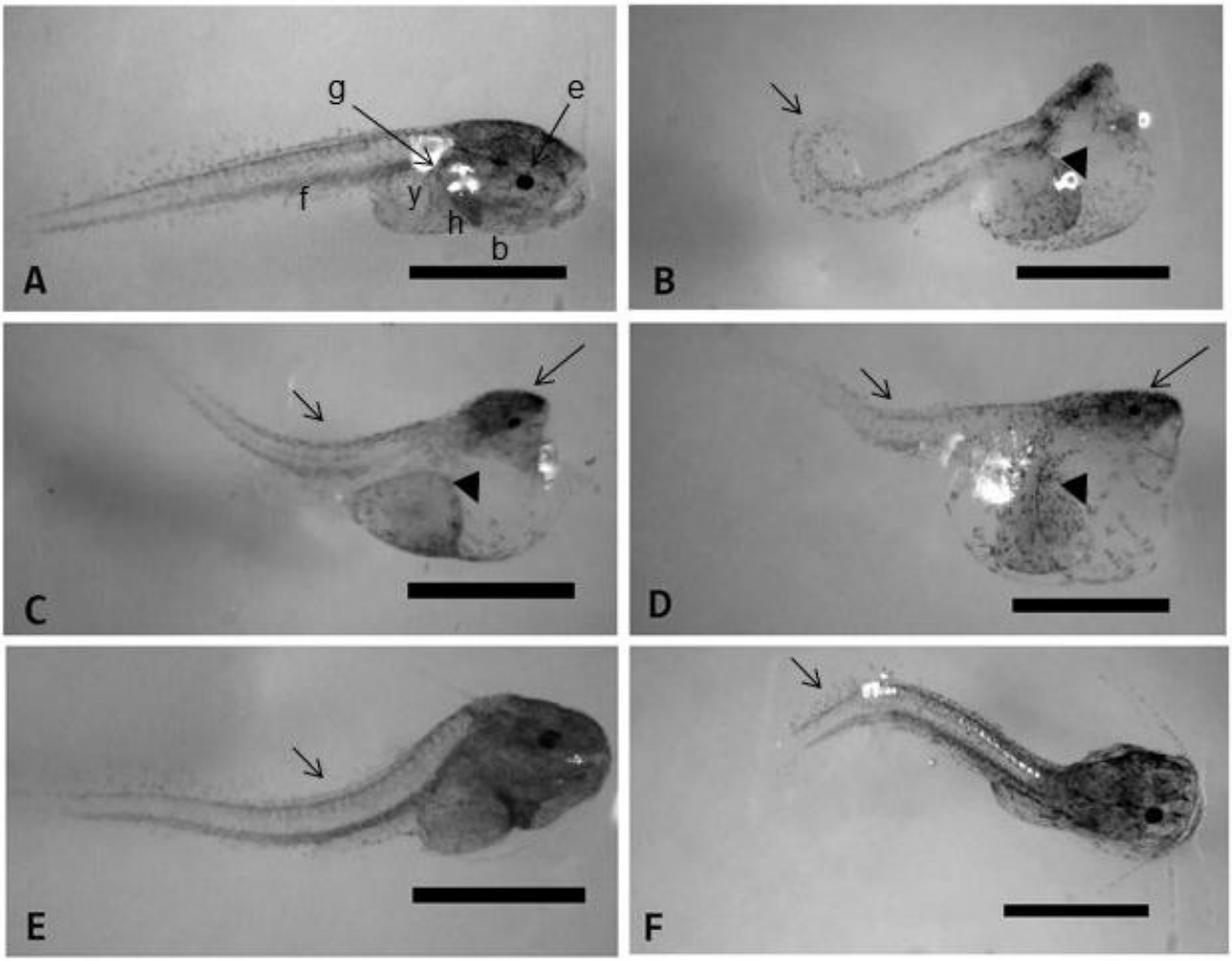


Figure 4. Deformities observed in larvae of *R. quelen* after 96 hpf of exposure to water from the studied Sites in the Iguaçu River 33% and 50% of dilutions. (A) Larvae with normal aspect and without alterations. e = eye, f = embryonic fin, g = gill arches, h = heart (pericardial cavity), y = yolk, b = barbells. (B, C, D) Cardiac edema (►) and malformation related to skeleton or muscle structure (arrows). (B) Showed a severe kink in tail. (C) Larvae with lordosis and body atrophy. (D) Cranial and vertebrate column damages. (E) Lordosis. (F) Damage in the vertebrate column and body atrophy. Scale: 1.0 mm.

The results of the principal component analysis (PCA) presented 3 factors (F1, F2, and F3). The first (F1) explained 36.1% of associations, while F2 explained 27.87% and F3 explained 25.89% (**Table 6**). According to the Kaiser criterion, only factors that explained more than 10%of the total variance were presented and discussed. The variables associated with a particular factor loading above 0.55 were considered relevant.

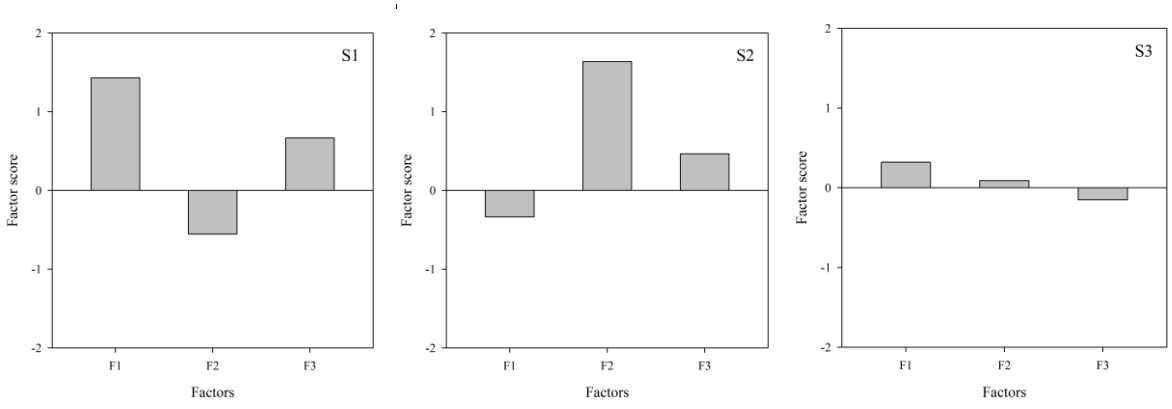
The concentration of metals (Cu, Fe, Mg, Zn) and the PAH acenaphthylene in the water were associated with F1 (positive loading values). The water from S1 and S3 were found to have higher scores for this factor, so these variables can be understood to be at higher concentrations at these points. Lead and chrysene concentrations in the water were still associated with F1 but with negative values, as were high mortality at 50% dilution and thoracic deformities at 33% dilution. The strongest associations were at S5 and S2.

Associated with F2 (positive loading values) were The PAHs chrysene and benz[*a*]anthracene were associated with F2 (positive loading values), as were the spinal, cranial, and thoracic deformities (50% dilution); the highest score was observed at S2. F2 (negative loading values) also presented a relationship with mortality and spinal deformities at 33% dilution; however, this relationship was not associated with pollutant concentrations. These associations were more frequently observed at S5 and S1.

F3 (positive value loadings) was associated with the larval mortality rate (50% dilution) and cranial and thoracic deformities (33% dilution). Variables associated positively with this factor were most relevant at S1, S2, and S5. Concentrations of PAHs such as fluorene, phenanthrene, anthracene, and lead were associated F3 with negative values and were most relevant at S4. The estimated factor scores from all of the study sites are presented in **Figure 5**, in which the prevalence of every component from each site is quantified and used to confirm the factor description.

Table 6. Sorted rotated factor loadings of 20 variables on the three principal factors of the five studied Sites from Upper Iguaçu River. Only variables with loadings > 0.55 (in bold text) were considered components of the factors.

		F1	F2	F3
	% variance	36,10	27,87	25,89
1	Cu	**0,89**	0,20	0,33
2	Fe	**0,97**	0,22	0,09
3	Mg	**0,95**	-0,21	0,19
4	Pb	**-0,56**	-0,47	**-0,59**
5	Zn	**0,83**	-0,05	0,46
6	Acenaphthylene	**0,95**	0,02	-0,08
7	Naphthalene	0,26	0,04	-0,14
8	Fluorene	0,29	0,15	**-0,91**
9	Phenanthrene	-0,06	-0,10	**-0,94**
10	Anthracene	-0,06	-0,10	**-0,94**
11	Chrysene	**-0,71**	0,51	0,43
12	Benz[a]anthracene	0,37	**0,89**	-0,26
13	Mortality 33%	0,25	**-0,86**	0,37
14	Spine 33%	-0,40	**-0,79**	0,45
15	Cranial 33%	0,45	-0,47	**0,76**
16	Thorax 33%	**-0,68**	0,46	0,51
17	Mortality 50%	**-0,84**	-0,09	0,53
18	Spine 50%	-0,08	**0,95**	0,20
19	Cranial 50%	-0,19	**0,91**	0,26
20	Thorax 50%	0,47	**0,70**	0,40



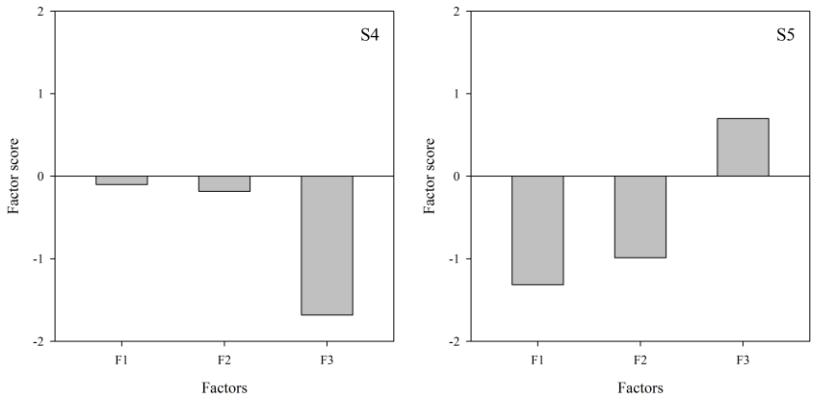


Figure 5. Estimated factor scores from each of five (S1 to S5) studied Sites from Upper Iguaçu River.

Experimental data revealed the intense impact of pollutants on embryos and larvae (stage A) of *R. quelen* specimens exposed to water from the upper Iguaçu River study sites. Thus, according to the theoretical model used, the impact on the early life cycle stages can interfere with the population integrity. **Figure 6** shows the evolution of this impact on the early life cycle stages of *Rhamdia quelen* over 200 years if the species' exposure to water from the upper Iguaçu River study sites continues. In a non-disturbed scenario in the absence of pollutants, the density increases to maximum density, where it oscillates around a fixed value that represents the population average. When all of the upper Iguaçu River study sites are considered, population density decreases relative to the control conditions.

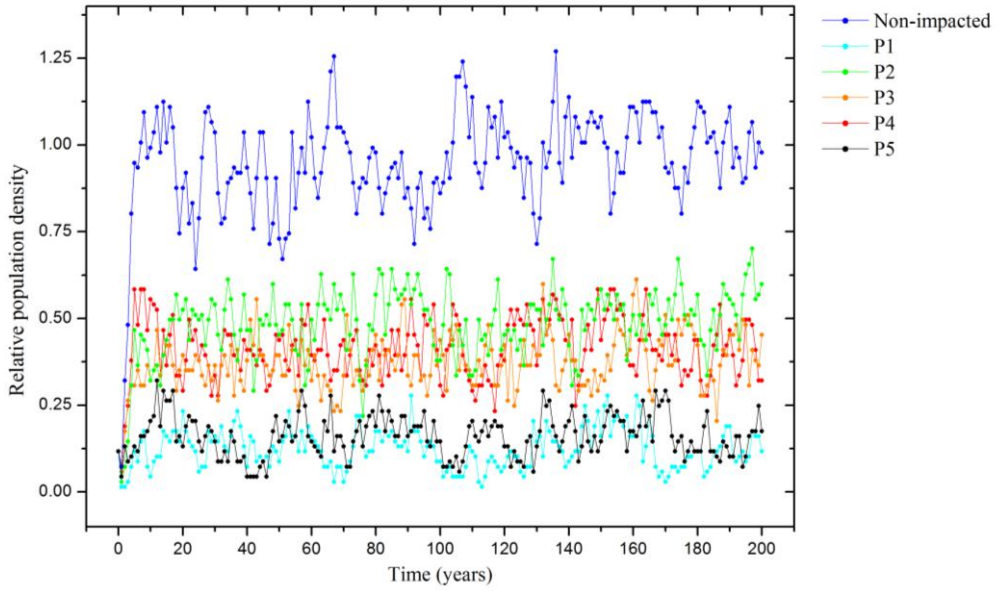


Figure 6. Example of temporal evolution of relative population size. The dark blue line shows the control situation where no pollutants are considered while the other lines represent the groups exposed to water from the studied Sites of the Upper Iguaçu river; S1 (light blue line) $S^A = 0.30$, $S^B = 0.38$ and $S^C = 0.31$; S2 (green line) $S^A = 0.48$, $S^B = 0.61$ and $S^C = 0.50$; S3 (orange line) $S^A = 0.43$, $S^B = 0.55$ and $S^C = 0.44$; S4 (red line) $S^A = 0.45$, $S^B = 0.57$ and $S^C = 0.46$ and S5 (black line) $S^A = 0.31$, $S^B = 0.39$ and $S^C = 0.32$. The population achieve its asymptotic behavior before 20 generations.

When we consider the impact of exposure to Iguaçu River water only on phase A, a major decline in the number of individuals in embryo-larval stages can be observed (less than 50% survival in all areas), but an impact on population density does not occur (**Figure 7**; S^B or S^C are at maximum values). Even when we consider only individuals exposed to water from S1 and S2, which had the lowest survival rate (S^A = 0.30 and 0.31), the population tends to reach maximum density in adulthood.

When we consider the impact that embryo and larval exposure has on the juvenile stage (phase B), the propagation of effects is low; the population is still quite resistant to disturbances (blue area in **Figure 7A**). For most S^A and S^B combinations, population density is similar to population density in the absence of pollutants. Only when survival values for

both stages are very low are a decline in population density and risk of local extinction observed.

The scenario represents the most important results of modeling: the impact of exposure to water from the Iguaçu River study sites are also considered into adulthood (phase C). The effect of pollutants on the embryo-larval stage is equivalent to the effect on adults. The exposure to water from the five study sites was found to have a strong effect on the size of the population (red area of **Figure 7B**). S2 presented the highest embryo and larval survival rate; but population density still remained close to 30% (intersection lines), a result which indicates that the impact propagates strongly when the adults stage is considered.

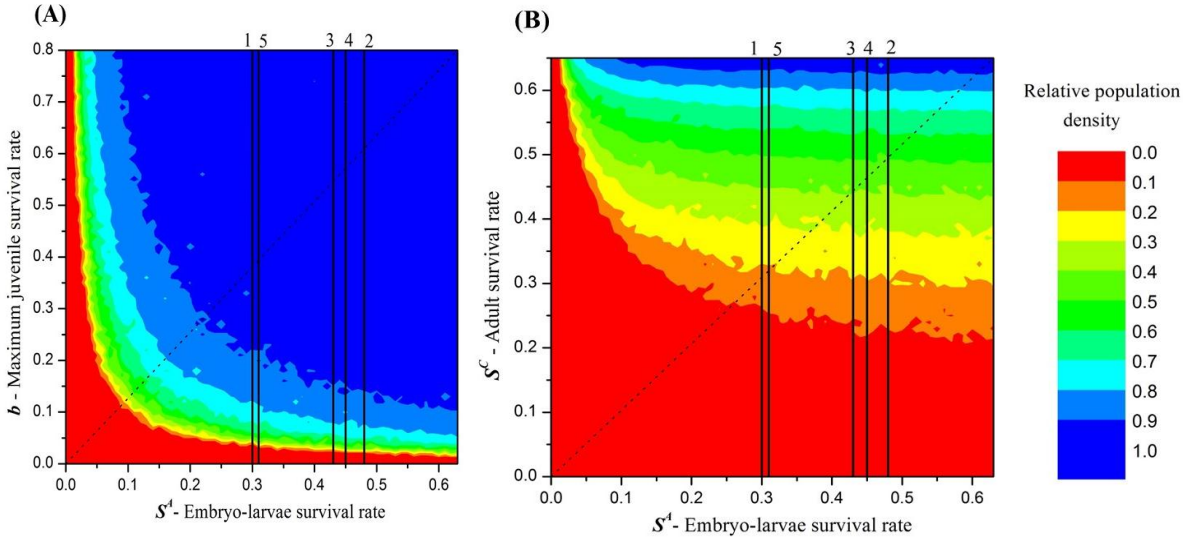


Figure 7. Phase diagram of *R. quelen* population dynamic as function of larval, juveniles and adult survival rate. The colors from red to blue color indicates extinction to highest density in absence of environmental perturbations. **(A)** Shows the population impact when the perturbation occurs during the embryo-larvae and juvenile stages while **(B)** shows the population impact when the perturbation occurs during embryo-larvae and adult stages. The vertical lines indicate the larval survival rate obtained in each studied Site of the Iguaçu River. The dot line shows a hypothetical situation where the juveniles and adults are proportionally impacted as the embryo-larvae, and the lines intersection correspond to the described for the Figure 6.

4. Discussion

The absence of a pollution gradient for the presence of contaminants within the studied area of the Iguaçu River is explained by multivariate sources, but the presence of toxic metals and some organic pollutants in the water confirms the impact of urban and industrial activities on the river and the occurrence of run-off pollution. All of the study sites located in the Upper Iguaçu River are highly impacted by sewage from industrial, urban, and agriculture activities. Site 1, which is located after the confluence of the Irai and Barigui Rivers, is impacted by urban sewage and by the largest sewage treatment plant in the region (the Atuba Sul WWTP), which releases approximately 1.120 $L.s^{-1}$ of treated effluent within the Iguaçu River (Franco, 2010). According to IAP (2005), the Irai and Barigui tributaries are historically classified as highly polluted. The mortality rates observed among embryos and larvae after their exposure to water from Site 1 at 33% dilution corroborates this data. It is important to note, however, that the second-highest mortality rate was found among specimens exposed to water from Site 5, which was more significantly impacted by PAHs than by metals. This data reflects the high complexity of the impact of these chemicals on the river.

Total PAH concentration in the water from the study sites confirms that there is a constant release of this class of pollutants into the water; the values found reflect the impact of urban activities on the decreased water quality of the Iguaçu River. The most highly represented compounds were those of the pyrogenic class, including as acenaphthylene, and naphthalene. According to Zhang (2004), these PAHs can be generated by the pyrolysis of wood or by coal burning, but Blanchard et al. (2004) described that the hydrolysis of organic matter originating from municipal sewage after wastewater treatment contributes to the increased levels of low-weight PAHs. Studies by SUDERSHA (1997) and COMEC (1999), both of which are water quality regulatory agencies in Paraná State, indicate the fact that

raw sewage dumped into the Iguaçu River is a source of PAHs. This data is logical, given with the presence of the 27 WWTPs distributed along the Iguaçu River and its tributaries (SANEPAR, 2015). In addition, emissions from human activities, incineration by-products from industrial and municipal waste, oil spills and oil derivatives and, in particular, the combustion of biomass and fossil fuels (Baird and Cann, 2008), all strongly contribute to PAH concentrations in water.

The PAHs with a high molecular weight, such as chrysene and benz[*a*]anthracene, were also quite abundant and over legal limits established by Brazilian legislation at all of the study sites except Site 5. According to Soclo et al. (2000), these compounds are most frequently associated with fossil fuel burning; a correlation which would explain their occurrence at sites with high automotive vehicle traffic. Benz[*a*]anthracene represented 23-43% of the total amount of PAHs identified in the water from the Iguaçu River study sites. The occurrence of this compound is of great concern due to the known carcinogenic potential ascribed to the molecule (WHO, 1998). Additionally, the fact that the highest concentrations of PAHs were found in water from Sites 3 and 4 reflects the negative participation of the two major industrial areas of greater Curitiba, which include a large oil refinery.

Guo et al. (2009) report that, once they are released in the water, most PAHs spread as diffuse pollution and are deposited in the sediment because of their hydrophobic characteristics. This behavior would explain the lower level of these compounds at Site 5. This data is corroborated by Leite et al. (2011) who reported that the PAHs were found to be more concentrated in the sediment of Iguaçu River and particularly in the Barigui River in the city of Araucaria (which corresponds to Site 4). Still, the ubiquity of PAHs in the samples studied indicates a wide distribution of these compounds in all environmental compartments. Their high persistence and the tendency toward PAH bioaccumulation (Neff,

1979) reflect the fact that these compounds are an important class of pollutants in the Iguaçu River and that the local biota and human population are therefore at risk of exposure. According to the United States government, PAHs require a set of regulations to protect people from the possible health effects, and laws have been established to limit people's eating, drinking, or breathing of PAHs (ATSDR, 1995).

The amounts of polybrominated biphenyls (PBBs), polychlorinated biphenyls (PCBs), and organochlorine pesticides (OCs) were found in the current study at values below detection limits (0.5 to 0.1 $\mu g.L^{-1}$). A large number of these contaminants represent a class of persistent organic pollutants that are of global concern, and they remain in most environmental matrices (water, sediments, soil, and air) (Breivik et al., 2002), and also in sewage, even after treatment (Yao et al., 2014). The high lipophilicity of these compounds may explain the absence of their detection in the water matrix, where filtration could prevent the detection of molecules associated with organic matter or even with microbiota. Leite et al. (2011) described a similar situation, particularly around Sites 2 and 4, though below the concentration effect threshold (TEC). Thus, we cannot rule out the possibility that low concentrations of these compounds increase the risk of exposure among the biota of the Iguaçu River, a possibility which would also explain some of the results of this study.

The metal concentration observed in the headwaters around S1 could be explained by the release of treated sewage into this location, which is particularly common in the Atuba River. This release into the Atuba River negatively affects the quality of the Iguaçu water. According to the IAP (2005), this area has historically presented sewage pollution, with high levels of toxic metals (Pb, Ni, and Cr). Site 3 is close to an intense industrial area where only 68% of sewage is treated and where only 82% of households are served by a sewer system. This region of the river is also impacted by pollutants from an inactivate landfill and from the leachate of an old treatment plant. The detection of lead and other metals in water from

Sites 4 and 5 is explained by the proximity of a high concentration of factories, including an oil refinery as well as the proximity of a very intense flow of automotive vehicles on the many highways nearby. This region was seriously affected by an oil spill in 2000, in which approximately four million liters of oil were spilled into the Iguaçu River along a 50-km extension (Katsumiti et al., 2013).

The higher hatching rates that occurred at both of the dilutions concentrations studied show that the chemical conditions of the Iguaçu River do not interfere with the initial stage of the species' development. However, the experimental design used does not allow for further discussion; only the eggs were exposed, which is an example of periodic exposure. A more complex study exposing the previous generation is necessary for an evaluation of the effects of Iguaçu water on egg development and, consequently, on the hatching rate. When the larvae were exposed after hatching until 96 hpf, the effects of chemicals present in the water from Iguaçu River were eminent. A significant mortality rate was observed as a dependent variable, a result which shows that the pollutants present in the river can decrease the survival rate of *Rhamdia quelen* larvae. These conditions were observed at all of the study sites, a finding which suggests that the differences in chemical concentration among the sites did not significantly interfere with this parameter. A decreased survival rate among fishes in the embryo–larval stages has been reported by others and has been explained by the fishes' high sensibility to chemical pollutants (Kendall et al., 2001). According to the same authors, this kind of approach has been successfully adopted in the laboratory on a small scale in order to reproduce field conditions. Thus, the current study used chemical analysis and modeling as a promising tool for assessing the quality of the water from the Iguaçu River.

Skeleton deformities such as lordosis, scoliosis, and kinks in tails were the most frequently observed morphological effects on larvae exposed to water from the Iguaçu River

(Site 1) at 33% dilution and on those from Site 2 at a higher dilution (50%).This site in particular experiences a greater impacted from metals, but not from PAHs. In general, the sites with higher concentrations of metals in the water presented lower survive rates 96 hpf, and the concentrations exceeded those established by Brazilian law. The results therefore suggest that larval survival depends on toxic metal concentration in water, particularly when the metals are cooper, zinc, and manganese. This result is in accordance with the findings described by Adema-Hannes and Shenker (2008) when *Coryphaena hippurus* embryos were exposed to copper. Additionally, the same authors identified the development of edema and of skeletal and yolk sac deformities, while Debruyn et al., (2007) described an increase in the occurrence and severity of multiple skeletal deformities such as lordosis and scoliosis in *Coregonus clupeaformis* embryos and larvae exposed to PAHs. Though most organic pollutants were found under detection limits in the current analysis, the effects of these pollutants and of others that were not investigated cannot be ruled out in the explanation of the deformities seen among *R. quelen* larvae.

The cranial abnormalities found in larvae exposed to 30% diluted water from Sites S1 and S3 are consistent with high levels of metals and of PAHs, respectively. This kind of parameter was reported by Debruyn et al. (2007) in larvae exposed to pesticides. Thorax injuries were more frequent in larvae exposed to more highly diluted samples (50%) from sites that experienced a greater impacted from metals (Sites 1, 2, and 3), especially in the case of the cardiac edema. Similar results were described by Carls and Rice (1988) among larvae were exposed to PAHs. In the current study, Sites 1, 2, and 3 presented the highest benz[*a*]anthracene concentrations, a finding which suggests that this kind of pollutant could be associated with this deformity.

The FA/PCA revealed relationships between the variables associated with the same axis (factor). When the variables from Site 1 were analyzed separately, a higher mortality

rate, high concentrations of metals, and a relatively high amount of PAHs (3.09) were observed. These findings were corroborated by the FA/PCA and the factor scores for S1, since the analysis revealed that metals (Cu, Fe, Mg and Zn) and acenaphthylene in F1 were associated with toxicity responses in F2 (mortality and spinal deformities) and in F3 (mortality and cranial deformities).Although these variables were not associated with the same factor, these results suggest that the low quality of water at this site is correlated with mortality and with thorax deformities.

Site 2 revealed an important association between toxicity responses and some individual PAHs. F2 was the factor with the highest score, and the highest frequencies of deformities (spinal, cranial, and thoracic deformities) were associated with high levels of benz[*a*]anthracene. Though F1 and F3 presented lower scores, they were still found to have relevant toxicity endpoints - cranial deformities in F3, and thorax deformities and mortality in F1 – the latter of which were found to be associated with relatively high chrysene levels. Site 3 also had a positive score for F1 (which, as explained before, reflected metals and acenaphthylene to be associated with toxicity responses) and moderate toxicity responses associated with benz[*a*]anthracene (as shown by the low positive score of F2). It is important to consider that, though naphthalene was not found to be associated with toxicity repsonse in the FA/PCA, it was found at the highest levels at Site 3.

Site 4 showed high of relevance of variables associated with F3 with negative loadings, including lead, fluorene, phenanthrene, and anthracene. F2 was also found to be moderately relevant (with a lower score) in terms of spinal deformities and mortality at 33% dilution at Site 4.

Site 5 presented negative scores for F1, which represents high frequencies of thorax deformities and mortality at 50% dilution associated with lead and chrysene. The negative score for F2 and the positive score for F3 reinforce the toxicity at this site, since higher

mortality (at 33% dilution), deformities in the spine (both in F2), and cranial deformities (in F3) were all found to be associated. Mortality and frequency of the lesions are therefore thought to be associated with the pollutant concentration, a finding which suggests that the toxic responses observed in individuals may share a cause-and-effect relationship with the pollutants measured. Thus, it can be concluded that heavy metals and organic pollutants generally result in early embryonic mortality and embryonic malformations. It is difficult to identify the substances responsible for physiological or behavioral disorders, though injuries can be linked to exposure to environmental stressors (Westernhagen et al., 1988), as well as to the temperature and physical-chemical parameters of the water (Prochazka, 2009).

In summary, FA/PCA results suggest that, at S1, metals (Cu, Fe, Mg and Zn) and PAHs (Acenaphthylene) can be found at higher levels and that, though they were not associated with the same factor, mortality and deformities (spine and cranial) were also relevant. At S2, several toxicity endpoints (mortality and deformities of the spine, cranium, and thorax) at both sample dilutions (33% and 50%) were relevant; these responses were associated with some PAHs (chrysene and benz[*a*]anthracene). This situation indicates that such compounds may have contributed to the toxicity levels observed. However, it is important to note that the concentrations of these compounds in the water varied only slightly among the sampling sites, a finding which indicates that other contaminants could also be responsible for this toxicity. S3 and S4 presented weak relationships between the measured contaminants and toxicity; at S3, metals presented moderate relevance and at S4, lead and some PAHs (fluorene, phenanthrene, and anthracene) were important variables. Finally, at S5, lead and chrysene were associated with mortality (50% dilution) and, even when the samples were diluted at 33%, these contaminants were associated with thorax deformities. At S5, mortality and spine deformity were also relevant.

Individual-based models (IBMs) have been applied with increasing frequency to environmental toxicology studies (Grimm, 1999), mainly due to their practical functionality in conservation studies. Thus, the modeling was applied to ecology in the present work to evaluate the impact of pollutants on catfish larvae in order to make projections about future damages to populations.

An important challenge in ecotoxicology and even in aquatic toxicology studies is to apply the data to real conditions. The use of modeling tools is therefore scarce despite the support they offer to environmental management and conservation studies. The current modeling results are an additional and essential parameter for reiterating the evidence of the observed impacts of chemicals on the Iguaçu River and the consequent decrease in water quality. When the models were applied to the scenarios, pollutants were found to put limited pressure on phase A but more pressure on phase B. In other words, the effects of chemicals present in the Iguaçu River on embryo-larvae and juvenile populations of *R. quelen* is not as evident in terms of potential extinction as when the effects on the population are considered. Another study conducted in 2014 (unpublished results) revealed that the exposure of larvae to the same Iguaçu water without dilution resulted in a 100% mortality rate in all of the samples from the sites used in the current study. The early mortality rates observed in embryos clearly reflect the low quality of the water from the Iguaçu River. The current data, which is corroborated by other studies on Iguaçu River water quality (Machado et al., 2014a; Machado et al., 2014b; Gallotta and Christensen, 2012), demonstrated that the presence of chemicals due to human activities potentially endangers the native fauna due to the negative effects on health, survival, and reproductive success.

When the scenario in which the pollutant pressure affects the juvenile phase (B) was analyzed, a higher mortality rate was observed than that of the juveniles in the absence of pollutants. However, it is thought that the population will be able to recover the balance over

the years in this scenario. Meanwhile, when the propagation effect of pollutants on adult *R. quelen* specimens was analyzed, individual oscillations were found to be quite sensitive. When specimens were exposed to water from Site 2, a higher larval survival rate was observed, though the population density was found to be below 50%. This results differs from those of Site 3 and Site 4 — populations exposed to those pollution conditions were found to have a density below 40%. At S1 and S5, population density fell below 10%.

According to Lande (1993), smaller populations are more sensitive to stochastic events. This idea likely explains the extinction predicted in the models that used populations exposed to water from Sites 1 and 5. Thus, both internal mechanisms (demographic stochasticity) or external mechanisms (environmental stochasticity) could be the cause (Pimm et al., 1988). Although stochasticity may indicate the probability of the size of a given population (Renshaw, 1991), the modeling allows us to discern between different stochastic processes, such as the persistence or extinction of a population. This finding is important, since individual variability may be crucial for the regulation and persistence of a studied population (Uchmanski, 1999). Additionally, this parameter can be used in the evaluation of future scenarios by measuring the risk of exposure and broadening the set of ecotoxicological tests in order to achieve a better understanding of the presence of chemicals in aquatic environments.

Sewage discharge regulation in Brazil follows CONAMA resolution Nº357. However, as described by McKean (1980), many other substances that are present in sewage as a result of urban or even industrial activities are not covered under the current legislation. Water quality monitoring based only on physical and chemical parameters and some biological aspects results in an incomplete diagnosis and may obscure the real conditions and water quality (Lied and Rodrigues, 2011).

The upper Iguaçu River was classified as Class 2 by Brazilian legislation, which means the water can be used for human supply after adequate treatment and protection of the aquatic biota. The present study points to at least two problems: (I) it is urgent that a monitoring program be developed to evaluate the current risk of exposure experienced by the biota and the human populations along the upper Iguaçu River through parameters other than those established to Brazilian legislation, and (II) the country needs to consider using other biological parameters in order to investigate water quality. The current classification of upper Iguaçu River is not in accordance with the quality described by Machado et al. (2013) or by Porto (2007); according to the intensity of sewage release in the river and its tributaries, these researchers declare that the real classification should be Class 4 or worse.

Many studies have reported that the current treatment is not satisfactory. Nieto (2000) described that 66.7% of the effluent from industrial activities still presents risk of exposure after treatment. The same situation was described by Knie (1998) in a study on the treatment of leachate from the largest landfill in Curitiba, located downstream from S3, and also by and Hamada (2008) in a study on household and industrial waste. In general, these studies showed the inefficiency or only partial success of the sewage treatment methods that are currently in use in the region.

The ichthyofauna of the upper Iguaçu River consists of 47 species, nine of which are considered endemic (Abilhoa, 2005). According to Vitule and Abilhoa (2009), urbanization is the most important cause of the decreasing diversity among fish populations. This kind of pollution is able to reduce the ecological parameters necessary for maintaining species richness, thus favoring less sensitive species and potentially increasing the risk of extinction of more sensitive species. This region of the upper Iguaçu River headwaters is indicated as a priority area for conservation (Heringer and Montenegro, 2000) due to the fragility of the ecosystem and the eminent risk of degradation caused by the urban activities concentrated

along the rivers. According to Ingenito et al. (2004) and Vitule and Abilhoa (2009), the absence of data limits both the establishment of policies that would protect the icthyofauna and the development of conservation strategies. The present data is consistent with the findings in the studies described above. The important findings of the current study highlight points that could be strategic for environmental management policies.

5. Conclusions and Future Directions

The five sampled areas of the upper Iguaçu River (S4 and S4 in particular) were found to have copper, manganese, zinc, iron, and lead concentrations above the legal limits established by Brazilian law. The water was also found to have high concentrations of PAHs — particularly acenaphthylene, Naphthalene, Fuorene, Chrysene and Benz[a]anthracene — at all of the sites. This finding is indicative of contamination by both treated and untreated domestic sewage, industrial waste, and diffuse sources of pollution, such as the drainage that originates in the surrounding urban areas.

The highest mortality rate was observed at S1, where the Iguaçu River is formed. This finding means that the water quality of the tributaries is highly compromised. Thus, the sampled areas did not reflect a contamination gradient, a factor which is reflected in the high mortality rate of embryos and larvae at all 5 sites. This mortality rate also indicates that the water quality does not meet the parameters required for environmental and human health safety.

The Iguaçu River is extremely important, particularly because the subbasin of the river (the area under study) includes most of the water supply sources in use by the human populations in the region. This is therefore a very critical situation, and urgent urban planning and environmental recovery measures are needed to prioritize the watersheds used for the local water supply.

Densely populated areas and the large cities that they surround have a variety of impacts on water quality. This impact is reflected in the irreversible effects on local rivers, which include the extinction of local species and high degrees of contamination that prevent the water's use for human supply. The disappearance of species recorded in the main channel of the river presents only a small part of the problem; the main warning signs are the loss of biodiversity and limitations to the services that the water source can provide, since the headwaters of the Iguaçu River are a strategic hotspot for the drinking water supply used by the greater Curitiba area.

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4. CONSIDERAÇÕES FINAIS

Os estágios iniciais de *Rhamdia quelen* mostraram-se excelentes bioindicadores para avaliação de efeitos de contaminantes presentes nos efluentes de estações de tratamento (convencional e tecnologia de reuso) e da qualidade da água do rio Iguaçu, estando, portanto, esse estudo em concordância com a implementação de normas internacionais para utilização de embriões de peixe para testes de águas residuais

A tecnologia de reuso como tratamento adicional dado ao esgoto tratado oriundo da ETE Atuba Sul mostrou-se muito eficiente na remoção dos potenciais contaminantes, tendo em vista que a mortalidade dos embriões e larvas de *R. quelen* expostos durante 96 hpf a esse tratamento, apresentou-se muito semelhante a situação de controle, ou seja, na ausência de poluentes.

A etapa final do tratamento de reuso inclui cloro como um agente de esterilização contra os agentes patogênicos, causando uma elevada taxa de mortalidade nos embriões e larvas de *R. quelen*. No entanto, de acordo com a Resolução do Conama 357/05, a desinfecção é um passo obrigatório no tratamento convencional para lançamento de efluentes em rios classe 2, o que deve ser revisto, através do uso de agentes esterilizantes menos prejudiciais à biota.

O tratamento de reuso promoveria um retorno de água mais adequado para o ambiente, o que em longo prazo traria impactos positivos sobre a saúde pública, a economia local e nacional e a qualidade dos ecossistemas aquáticos. No entanto, a implementação de tecnologia capaz de produção em grande escala de água potável exigiria mudanças na legislação, investimentos financeiros e em pesquisa, além da implementação de testes para investigação de riscos de exposição. Sendo assim, face à crescente demanda pela água tratada e potável, o reuso torna-se um componente importante no planejamento,

desenvolvimento e utilização dos recursos hídricos, representando um potencial emergente que visa à racionalização do uso considerado um bem finito e dotado de valor econômico.

A aplicação da modelagem teórica demostrou que o impacto negativo causado pelos poluentes na taxa de sobrevivência das fases iniciais de *R. quelen* pode ser propagado para as outras fases do ciclo de vida da espécie, causando uma diminuição na densidade populacional e em casos mais drásticos, pode aumentar as chances de extinções locais devido a mecanismos internos (estocasticidade demográfica) ou externos (estocasticidade ambiental).

A modelagem aplicada à Ecotoxicologia pode contribuir para a elucidação de mecanismos ecológicos, baseados em sistemas experimentais de pequena escala e propagação de seus efeitos através de diferentes níveis de organização biológica, além de contribuir para elaboração de políticas que visem a conservação das espécies e dos recursos hídricos, e priorizar ações regulatórias mais restritivas para tratamento e lançamento de efluentes.

A exposição de *R. quelen*, uma espécie nativa e com ocorrência na área avaliada, corrobora com os estudos já realizados na região, indicando um alto índice de degradação, determinado pelos efeitos letais (mortalidade) e sub-letais (deformidades) nos embriões e larvas expostos.

Não foi possível observar um gradiente de contaminação ao longo dos 5 pontos amostrados no rio Iguaçu, visto que há uma concentração de metais e HPAs em todas os pontos estudados, especialmente naqueles localizados na área de cabeceiras do rio Iguaçu, formado pelo rio Atuba e Iraí. Isto indica que esses afluentes encontram-se extremamente degradados, sendo a ETE Atuba Sul uma importante fonte de contaminantes favorecendo o estado atual em que se encontra o rio Iguaçu.

Estudar as alterações apresentadas por uma espécie nativa em laboratório, reproduzindo as condições ambientais, é importante para a compreensão dos efeitos da contaminação em ecossistemas tropicais e subtropicais, além da relevância ecológica da região avaliada, que se caracteriza por um grande endemismo da ictiofauna.

Embora a bacia do Alto Rio Iguaçu seja a principal mantenedora do abastecimento público da cidade de Curitiba e região metropolitana, não existe uma rotina de monitoramento da água, através de testes ecotoxicológicos, colocando em evidência a necessidade de uma maior geração de dados sobre a toxicidade em peixes refletindo a qualidade da água.

Finalmente, é de extrema importância que a gestão dos recursos hídricos no Brasil deixe de ser pautada apenas em parâmetros físico-químicos para monitoramento e avaliação da qualidade da água, principalmente, no que tange aos recursos hídricos destinados ao abastecimento público, devido ao risco ao risco de exposição para populações humanas e a saúde ambiental.

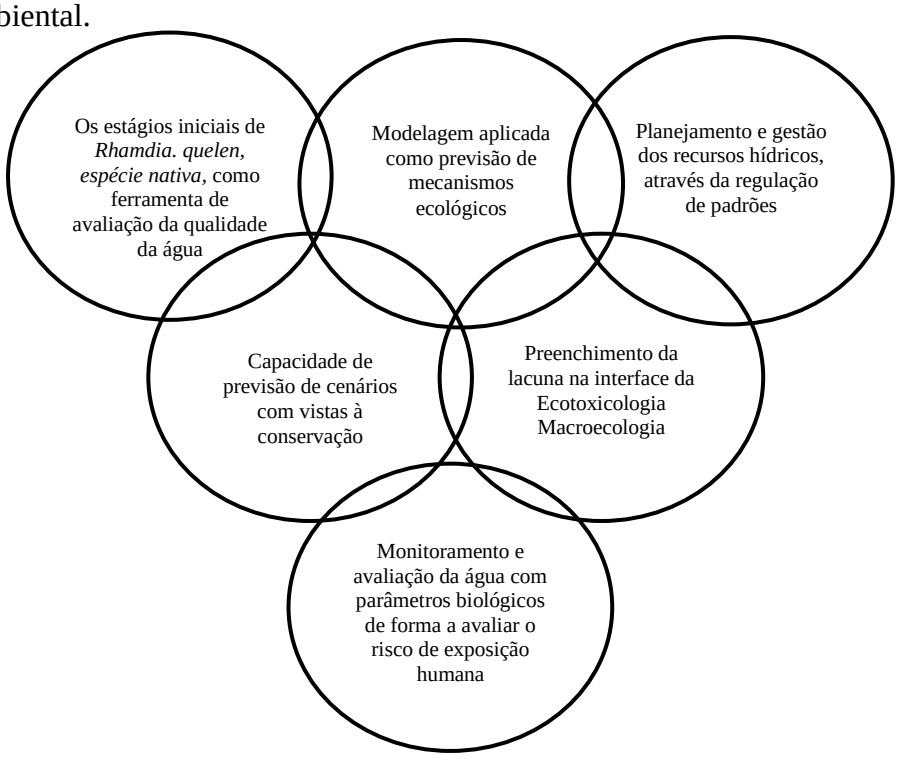

Figura 31. Síntese das conclusões e intersecções com as áreas avaliadas no estudo.

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6. ANEXOS

ANEXO 1 – CERTIFICADO DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS DO SETOR DE CIÊNCIAS BIOLÓGICAS DA UNIVERSIDADE FEDERAL DO PARANÁ (CEUA/BIO – UFPR)

Ministério da Educação
UNIVERSIDADE FEDERAL DO PARANÁ
Setor de Ciências Biológicas
Comissão de Ética no Uso de Animais
(CEUA)

Nº 872

CERTIFICADO

A Comissão de Ética no Uso de Animais do Setor de Ciências Biológicas da Universidade Federal do Paraná (CEUA/BIO – UFPR), instituída pela Resolução Nº 86/11 do Conselho de Ensino Pesquisa e Extensão (CEPE), de 22 de dezembro de 2011, **CERTIFICA** que os procedimentos utilizando animais no projeto de pesquisa abaixo especificado estão de acordo com a Diretriz Brasileira para o Cuidado e a Utilização de Animais para fins Científicos e Didáticos (DBCA) estabelecidas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e com as normas internacionais para a experimentação animal.

STATEMENT

The Ethics Committee for Animal Use from the Biological Sciences Section of the Federal University of Paraná (CEUA/BIO – UFPR), established by the Resolution Nº 86/11 of the Teaching Research and Extension Council (CEPE) on December 22nd 2011, **CERTIFIES** that the procedures using animals in the research project specified below are in agreement with the Brazilian Guidelines for Care and Use of Animals for Scientific and Teaching purposes established by the National Council for Control of Animal Experimentation (CONCEA) and with the international guidelines for animal experimentation.

PROCESSO/PROCESS: 23075.063869/2015-81

APROVADO/APPROVAL: 08/04/2015 – R.O. 03/2015

TÍTULO: Estudo integrado de avalição da qualidade da água da bacia do Alto Iguaçu e o potencial risco de exposição para a ictiofauna

TITLE: Assessment study integrated of water quality in the Upper Iguaçu basin and the potential risk of exposure to the fish fauna

AUTORES/AUTHORS: Ciro Alberto de Oliveira Ribeiro, Izabella Andrade Brito

DEPARTAMENTO/DEPARTMENT: Biologia Celular

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