Universidade do Estado do Rio de Janeiro
Linnaeus University

André Luís de Sá Salomão

Ocorrência e ecotoxicidade de desreguladores endócrinos químicos em ambientes aquáticos e em sistemas de tratamento de esgoto

Occurrence and ecotoxicity of endocrine disruptor chemicals in aquatic environment and sewage treatment systems

Kalmar
2014

André Luís de Sá Salomão

Ocorrência e ecotoxicidade de desreguladores endócrinos químicos em ambientes aquáticos e em sistemas de tratamento de esgoto

Occurrence and ecotoxicity of endocrine disruptor chemicals in aquatic environment and sewage treatment systems

Tese apresentada, como requisito parcial para obtenção do título de Doutor, ao Programa de Pós-Graduação em Meio Ambiente, da Universidade do Estado do Rio de Janeiro e do grau de Doctor, Ph.D. in Environmental Science, da Linnaeus University, em regime acordado de cotutela entre as duas Universidades.

Orientadora: Prof.ª Dra. Marcia Marques Gomes

Coorientador: Prof. Dr. William Hogland

Coorientadora: Prof.ª Dra.Daniele Maia Bila

Kalmar

2014

CATALOGAÇÃO NA FONTE
UERJ/ REDE SIRIUS / BIBLIOTECA CTC/A

S171 Salomão, André Luís de Sá
Ocorrência e ecotoxicidade de desreguladores endócrinos químicos em ambientes aquáticos e em sistemas de tratamento de esgoto = Occurrence and ecotoxicity of endocrine disruptor chemicals in aquatic environment and sewage treatment systems / André Luís de Sá Salomão – 2014.
99 f.
Oriendadora: Marcia Marques Gomes
Coorientador: William Hogland
Coorientadora: Daniele Maia Bila
Tese (doutorado) – Universidade do Estado do Rio de Janeiro em regime de cotutela com a *Linnaeus Universiy.*
1. Ecossistemas aquáticos – Tratamento – Teses. 2. Esgotos – Tratamento – Teses. 3. Hormonios – Peixes – Teses. I. Gomes, Marcia Marques. II. Hogland, William. III. Bila, Daniele Maia. IV. Universidade do Estado do Rio de Janeiro. V. Linnaeus University. VI. Título.
CDU 628.35

_____ _____

Assinatura Data

André Luís de Sá Salomão

Ocorrência e ecotoxicidade de desreguladores endócrinos químicos em ambientes aquáticos e em sistemas de tratamento de esgoto = ***Occurrence and ecotoxicity of endocrine disruptor chemicals in aquatic environment and sewage treatment systems***

Tese apresentada, como requisito parcial para obtenção do título de Doutor, ao Programa de Pós-Graduação em Meio Ambiente, da Universidade do Estado do Rio de Janeiro e do grau de Doctor, Ph.D. in Environmental Science da Linnaeus University, em regime acordado de cotutela entre as duas Universidades.

Aprovada em 23 de maio de 2014

Orientadora:

Prof.ª Dra. Marcia Marques Gomes (Orientadora)
Universidade do Estado do Rio de Janeiro - UERJ

Coorientadores:

Prof. Dr. William Hogland (Coorientador)
Linnaeus University - LNU, Suécia

Prof.ª Dra. Daniele Maia Bila (Coorientador)
Universidade do Estado do Rio de Janeiro - UERJ

Banca examinadora:

Prof. Dr. Paulo Sergio Martins de Carvalho (Oponente)
Universidade Federal de Pernambuco - UFPE

Prof.ª Dra. Catherine Legrand
Linnaeus University - LNU, Suécia

Prof. Dr. Per Larsson
Linnaeus University - LNU, Suécia

Dr. Pär Hallgren
Sweco Environment AB, Suécia

Prof.ª Dra. Lena Vought
Kristianstad University, Suécia

Kalmar

2014

DEDICATÓRIA

À minha amada esposa Julia e minha família

AGRADECIMENTOS

O Desenvolvimento desta Tese não seria possível sem a ajuda, suporte e orientação de várias pessoas e também dos “nossos amigos”; a todos vocês um muito obrigado especial!

Primeiro de tudo, eu gostaria de expressar minha mais profunda e calorosa gratidão a Marcia Marques, pela oportunidade de trabalhar com ela. Durante todo esse tempo tive o prazer de conviver e disfrutar de uma única e maravilhosa experiência. Pude aprender muito mais do que ciências e tive um crescimento tanto como pessoa como pesquisador. Por isso, posso dizer que tive mais que uma supervisora dedicada, eu tive grande amiga e até uma madrinha de casamento. Eu me considero um sortudo por ter você em minha vida. “Student by chance, friends by choice”. Obrigado por tudo!

Eu sou muito grato ao William Hogland, meu co-orientador, por seu apoio e orientação inspirador, que me mostrou que pequenas coisas e atitudes em nossas vidas pessoais podem refletir diretamente na maneira que nós trabalhamos (foco, concentração e liderança).

Marcia e William, obrigado pelos momentos inesquecíveis que passamos juntos, como em San Petersburg e no meu casamento, entre muitos outros.

Agradeço minha co-orientadora Daniele Bila, por todo carinho e atenção e por sempre estar disposta a me ajudar no que for preciso.

Meus amigos especiais Fabio e Michelle, muito obrigado pela amizade de vocês e toda ajuda e suporte que vocês nos deram durante esse ano na Suécia. Agradeço também ao Fabio pelos conselhos científicos e revisões na presente Tese.

Um obrigado especial aos meus amigos do grupo de pesquisa Bioprocess/LABIFI, UERJ, por todo suporte, ajuda e amizade e por me proporcionarem um ambiente tranquilo e prazeroso de trabalho: Rodrigo, Alexandre, Tadeu, Greyce, Raul, Gabi, Chiquinha, Douglas, Carmelita, Jorge, Sanye, Siomara, Maria Luiza, Lud, Graci, Antônia, Clooer, Barbara and also Prof. Odir Roque (*in memoriam*).

Um obrigado especial também aos amigos do grupo de pesquisa ESEG, Linnaeus University, por toda ajuda e amizade: Lee (querida amiga), Asim e Yahya (parceiros de sala); Amit e Eva (pelos conselhos e ajudas); Fabio; Charlotte

(agradáveis almoços juntos); Joacim (por toda ajuda e pela tradução do resumo para sueco), Henric e Henrik (por todo tipo de ajuda), Dr. Mait Kriipsalu, Marika, Kertu, Allar, Juris e Hadrien (conversas sempre muito agradáveis).

Aos professors e colegas da Linnaeus University-LNU (Dept. of Biology and Environmental Science) em especial: Prof. Edna Granéli, Andres, Sara, Christina, Ymke, Henrik, Sarfraz, Frederic, Laurent, Mireia, Eva, Olga, Devi, Nayani, Dao, Dandan, Xiaofen, Furong, Prof. Marie José, Daniel, Qing, Anna, Carina, e Ganna; e da Universidade do Estado do Rio de Janeiro-UERJ (PPGMA and PEAMB) em especial: Prof. Israel Felzenszwalb, Rafael, Lissandra, Leandro, Renata, Gustavo, Rodrigo, Ludmila, Piatã, Carlúcio, e Prof. Jayme Bastos. Obrigado por todo suporte a ajuda.

Gostaria de agradecer aos amigos brasileiros e suecos em Kalmar, amigos do Motionsligan 1979 e Kalmar tennisklubb pelos momentos agradáveis e prazerosos.

Ao apoio financeiro das agências de fomento CNPq (Processo Nº 556660/2009-9) e Ciência sem fronteiras (Processo SWE/CSF Nº 246145/2012-9) e Swedish Foundation of Research and High Education-STINT.

Esta Tese contou com a colaboração das empresas: Biofibra, com a fabricação e doação dos tanques para o Ecossistema Engenheirado; Rio Doce Piscicultura, com a doação dos specimens de peixe Nile tilapia; AB Gustaf Kähr, com o fornecimento do efluente proveniente de indústria de pisos de madeira, e também do grupo de pesquisa, Marine Plankton Group coordenado pela Profª. Edna Granéli (Marine Department at Linnaeus University) com a doação das duas espécies de algas (*P. subcapitata* and *D. subspicatus*) usadas nos Ensaios de Ecotoxicologia.

Minha querida e amada família, Avôs, Tios, Primos, Sobrinhos, Sogro e Sogras.

Minha amada esposa Julia, um muito obrigado por todo seu amor, cuidado e suporte durante toda nossa vida juntos e em especial durante os últimos meses de redação da Tese. Eu me considero um sortudo por ter você na minha vida.

Meus mais profundos e calorosos agradecimentos aos meus amados Pai Ricardo e Mãe Christina (por serem minha força motriz, e por todo suporte e força que vocês sempre me deram para que eu alcançasse meus objetivos), e meus irmãos Lipe, Lelê, Nona, Quinho, Babi e Didi por serem meus melhores amigos, amorosos, e que me apoiam e dão força o tempo todo.

The task is not so much to see what no one yet has seen, but to think what nobody yet has thought about that which everybody sees.

Arthur Schopenhauer

Crê em ti mesmo, age e verá os resultados. Quando te esforças, a vida também se esforça para te ajudar.

Francisco Cândido Xavier

RESUMO

SALOMÃO, A. L. S. **Ocorrência e Ecotoxicidade de Desreguladores Endócrinos Químicos em Ambientes Aquáticos e em Sistemas de Tratamento de Esgoto**. 2014. 99 p. Tese (Doutorado em Meio Ambiente) – Universidade do Estado do Rio de Janeiro, Rio de Janeiro; Linnaeus University, Kalmar, 2014.

A aplicabilidade de um método selecionado de medição indireta de vitelogenina (Vtg) em plasma sanguíneo de peixe, baseado na quantificação de fosfato álcali-lábil (alkali-labile phosphate-ALP) para acessar estrogenicidade em água, foi investigada na presente tese. O método foi originalmente desenvolvido para a espécie de peixe *Carassius carassius* (Carpa cruciana) e aplicado pela primeira vez na espécie *Oreochromis niloticus* (Tilápia do Nilo) no presente estudo. Com o objetivo de acessar a sensibilidade do método, em uma primeira etapa da investigação foram realizados estudos laboratoriais com soluções estoques de 17α-ethinylestradiol (EE2), 17β-estradiol (E2), e estrona (E1). Os efeitos destes hormônios foram investigados com base tanto na concentração quanto na carga, utilizando-se para tanto, unidades experimentais com volumes distintos (2 L e 130 L). Após a validação do método de ALP, a estrogenicidade foi avaliada nas seguintes águas contaminadas: (i) afluente e efluente de uma grande estação de tratamento de esgotos convencional (ETE) e de uma estação descentralizada de tratamento de esgoto de pequeno porte (Ecossistema Engenheirado-DEE); (ii) água superficial (SW) e água subterrânea (GW) coletadas em uma área de brejo contaminada com gasolina; (iii) água de uma lagoa urbana (LRF) da cidade do Rio de Janeiro, com alta densidade populacional e descarte clandestino de esgoto. Na segunda etapa foram analisados em microalgas os efeitos (outros que não disrupção endócrina) causados pelos hormônios EE2, E2 e E1. Os hormônios foram testados individualmente e em misturas, em culturas individuais e combinada (S+) das espécies de microalgas unicelulares *P. subcapitata* e *D. subspicatus*. Com base nos níveis de ALP para a espécie de peixe e no EC_{50} para as espécies de algas, os resultados mostraram que o EE2 e o E2 causaram disrupção endócrina superior e foram mais tóxicos do que o E1 para peixes e microalgas respectivamente. Quando em misturas (E+) de concentrações equivalentes (EE2:E2:E1), os estrogênios resultaram em efeito aditivo para as espécies *O. niloticus* e *P. subcapitata*, e menos que aditivo para *D. subspicatus* e cultivo misto de algas (S+). Culturas contendo ambas as espécies de algas (S+) por um longo período de exposição (96 h) resultaram na atenuação dos efeitos tóxicos causados pela exposição, tanto individual (EE2, E2 ou E1), quanto na mistura (E+) dos estrogênios, medidos em termos de EC_{50} (T_{0h} 0,07; 0,09; 0,18; e 0,06 μg mL^{-1}; e T_{96h} 1,29; 1,87; 5,58; e 4,61 μg mL^{-1}, respectivamente). O DEE apresentou uma maior eficiência na remoção dos disrutores endócrinos do que a ETE convencional. Foi detectada estrogenicidade em amostras da LRF, e de água SW e GW em área brejosa contaminada com gasolina. Os resultados dos ensaios sugerem que as interações (efeitos aditivos ou menos que aditivo) causadas pela mistura dos estrogênios assim como, as interações entre as espécies de algas afetaram o resultado final dos ensaios ecotoxicológicos. Um fator raramente abordado em estudos ecotoxicológicos que foi destacado na presente tese refere-se à importância de considerar não somente a concentração e a dosagem, mas também a carga aplicada e o volume das unidades experimentais. Devido à boa sensibilidade do *O. niloticus* quando exposto às concentrações relativamente baixas dos estrogênios, a combinação do método de ALP com os biomarcadores auxiliares (particularmente MN) pode ser um protocolo adequado para a detecção de estogenicidade e genotoxicidade respectivamente em diferentes ambiente aquáticos contaminados, como parte de um programa de monitoramento ambiental.

Palavras-chave: Desreguladores endócrinos. Alkali-labile phosphate. Hormônios estrogênicos. *Oreochromis niloticus*. Microalga unicellular verde. Efeito aditivo. Efeito menos que aditivo. Toxidade crônica.

ABSTRACT

SALOMÃO, A.L.S. **Occurrence and ecotoxicity of endocrine disruptor chemicals in aquatic environment and sewage treatment systems**. 2014. 99 p. Tese (Doutorado em Meio Ambiente) – Universidade do Estado do Rio de Janeiro, Rio de Janeiro; Linnaeus University, Kalmar, 2014.

The applicability of one selected method for indirect measurement of vitellogenin (Vtg) in fish plasma based on the quantification of alkali-labile phosphates (ALP method) to assess estrogenicity in water was investigated. The ALP method applied in this investigation was originally developed with *Carassius carassius* (Crucian carps). This thesis describes the first attempt of using this method with *Oreochromis niloticus* (Nile tilapia). In a first part of the investigation, laboratory studies were conducted with water spiked with 17α-ethinylestradiol (EE2), 17β-estradiol (E2), and estrone (E1) in order to assess the method sensitivity. The effects of these estrogens were investigated on the basis of both load and concentration, using experimental units with two different volumes (2 L and 130 L). After validation of the method, the estrogenicity of the following contaminated waters was assessed: (i) affluent and effluent of one large conventional municipal wastewater treatment plant (WWTP) and one small decentralized wastewater treatment plant (Decentralized Engineered Ecosystem-DEE); (ii) surface water (SW) and groundwater (GW) obtained from a gasoline-contaminated marshland; (iii) samples from a urban lagoon (LRF) located in Rio de Janeiro city with high density population and clandestine sewage discharge. An additional goal of the thesis was to assess the effect (other than endocrine disruption) caused by EE2, E2 and E1 to microalgae. Assays with single and mixed estrogens and single and combined cultures (S+) of the green microalgae *P. subcapitata* and *D. subspicatus* were carried out. The results have shown that EE2 and E2 were more estrogenic and toxic than E1 to the fishes and to the microalgae respectively. Mixed solutions of estrogens (E+) in equal proportions (EE2:E2:E1) resulted in additive effect on *O. niloticus* and *P. subcapitata* and less-than-additive effect on *D. subspicatus* and S+ measured as ALP (for fish) and EC_{50} (for microalgae). Combined cultivation of both algae species and longer exposure time (96 h) resulted in attenuation of the toxic effects caused by single (EE2, E2 or E1) and mixed (E+) estrogens according to EC_{50} (T_{0h} 0.07, 0.09, 0.18, and 0.06 µg mL^{-1}; and T_{96h} 1.29, 1.87, 5.58, and 4.61 µg mL^{-1}, respectively). The decentralized engineered ecosystem was more efficient than the conventional WWTP regarding estrogenicity removal from the final effluent. Estrogenicity was detected in some samples of the LRF and the SW and GW of the gasoline-contaminated marshland. Therefore, the investigations suggested that interactions (additive and less-than additive effect) take place when different estrogens are present in the water environment and interactions also occur between algae species, which affect the final toxicity. Additionally, the study highlighted the importance of taking into account not only concentration and dose regime but also the mass load and therefore, the volume used in the experimental units, which are rarely addressed in ecotoxicity assays. Considering the good sensitivity of *O. niloticus* exposed to relatively low concentrations of estrogens, the combination of the ALP method with auxiliary biomarkers (particularly MN) can be a suitable protocol for estrogenicity and genotoxicity detection in different contaminated waters as part of water environmental monitoring programs.

Keywords: Endocrine disruption. Alkali-labile phosphate. Estrogen hormones. *Oreochromis niloticus.* Unicellular green microalgae. Additive effect. Less-than additive effect. Chronic toxicity.

SVENSK SAMMANFATTNING

SALOMÃO, A.L.S. **Förekomst Och Ekotoxicitet Av Endokrinstörande Kemikalier I Vattenmiljön Och Avloppsvattensystem**. 2014. 99 p. Tese (Doutorado em Meio Ambiente) – Universidade do Estado do Rio de Janeiro, Rio de Janeiro; Linnaeus University, Kalmar, 2014.

Tillämpningen av en vald metod för indirekt mätning av vitellogenin (Vtg) i fiskplasma baserad på kvantifiering av alkali-labila fosfater (ALP-metoden) för att bedöma östrogenpåverkan i vatten undersöktes i denna studie. ALP-metoden användes i denna undersökning vilken i princip tar bort fria fosfater från fiskplasma, och som utvecklades ursprungligen med *Carassius carassius* (ruda). Denna avhandling beskriver det första försöket att använda denna metod med *Oreochromis niloticus* (niltilapia). I första delen av undersökningen genomfördes laboratoriestudier med vatten spikat med 17α - etinylestradiol (EE2), 17β - östradiol (E2) och östron (E1) i syfte att utvärdera metodkänsligheten. Effekterna av dessa östrogener undersöktes på grundval av både belastning och koncentration, med hjälp av experimentella enheter med två olika volymer (2 l och 130 l). Efter validering av metoden bedömdes östrogeniciteten av följande förorenade vatten: (i) tillflöde och utflöde i ett stort konventionellt kommunalt avloppsreningsverk (WWTP) och ett litet decentraliserat reningsverk (Engineered Ekosystem - DEE), (ii) ytvatten (SW) och grundvatten (GW) från en bensinförorenad träskmark, (iii) prover från en urban lagun (LRF) som ligger i en del av Rio de Janeiro med hög befolkningsdensitet och oidentifierad avloppstömning. Ytterligare ett mål med avhandlingen var att bedöma effekter (andra än endokrina störningar) som orsakas av EE2, E2 och E1 på mikroalger. Analyser med enskilda och blandade östrogener och enskilda och kombinerade kulturer (S+) av gröna mikroalgen *P. subcapitata* och *D. subspicatus* genomfördes. Resultaten visade att EE2 och E2 var mer östrogena och giftiga än E1 för fiskarna och på mikroalgerna. Blandade lösningar av östrogener (E+) i lika proportioner (EE2:E2:E1) resulterade i additiv effekt på *O. niloticus* och *P. subcapitata* och mindre än additiv effekt på *D. subspicatus* och S+ mätt som ALP (för fisk) och EC_{50} (för mikroalger). Kombinerad odling av de båda algarterna och längre exponeringstid (96 h) resulterade i minskning av de toxiska effekter som orsakas av enstaka (EE2, E2 eller E1) och blandade (E+) östrogen enligt EC_{50} (T_{0h} 0.07, 0.09, 0.18, and 0.06 µg mL^{-1}; and T_{96h} 1.29, 1.87, 5.58, and 4.61 µg mL^{-1}, respektive). Det decentraliserade konstgjorda ekosystemet var effektivare än det konventionella reningsverket med avseende på borttagning av östrogenet från utgående avloppsvatten. Östrogen upptäcktes i vissa prover från den urbana lagunen (LRF), ytvattnet (SW) och i grundvattnet (GW) i den bensinförorenade träskmarken. Undersökningarna tydde därför på att interaktioner (additiva och mindre än additiva effekter) äger rum när olika östrogener är närvarande i vattenmiljön och interaktioner sker också i olika algarter, vilket påverkar den slutliga toxiciteten. Studien betonade dessutom vikten av att ta hänsyn till inte bara koncentration och dosen utan också massbelastningen och därmed volymen som används i experimentella enheter, vilket sällan tas upp i ekotoxicitetsanalyser. Med tanke på känsligheten på *O. niloticus* när denna utsätts för relativt låga halter av östrogen, kan kombinationen av ALP-metoden med hjälp biomarkörer (särskilt micronucleus-MN) vara ett lämpligt protokoll för upptäckten av östrogenicitet och genotoxicitet i olika förorenade vatten som en del av övervakning av vattenmiljön.

Nyckelord: Endokrina störningar. Alkali-labila fosfater. Östrogenhormoner. *Oreochromis niloticus.* Encelliga gröna mikroalger. Additiv effekt. Mindre än additiv effekt. Kronisk förgiftning.

LISTA DE ABREVIAÇÕES E SIGLAS

WWTP	*Wastewater treatment plant*
DEE	*Decentralized Engineered Ecosystem*
LRF	*Rodrigo de Freitas lagoon*
GW	*Groundwater from a gasoline-contaminated marshland*
SW	*Superficial water from a gasoline-contaminated marshland*
ALP	*Alkali-labile phosphate*
Vtg	*Vitellogenin*
GSI	*Gonadosomatic index*
HSI	*Hepatosomatic index*
MN	*Micronucleus frequency*
EE2	*17α-ethinylestradiol*
E2	*17β-estradiol*
E1	*Estrone*
E+	*Estrogen mixture including EE2: E2: E1 (1:1:1)*
S+	*Combined algae culture (D. subspicatus and P. subcapitata)*
NOEC	*No-observed-effect concentration*
LOEC	*Lowest observed effect concentration*
EC_{50}	*Effect concentration that causes 50% reduction of algal growth*
GC-MS	*Gas Chromatography-Mass Spectrometry*

SUMÁRIO

INTRODUCTION

During recent years, the concern about the potential adverse effects caused by endocrine disruptors chemicals (EDC) and the consequent interference in the endocrine system of many species have been raised. The man-made chemicals (xenobiotics) have become a part of our every day life. Human daily exposure to natural and man-made EDC occurs via ingestion of food and water, inhalation of gases and particles in the air and also through skin. On top of the list the natural and synthetic estrogens and other hormones are placed as EDC. However, a wide range of substances are thought to cause endocrine disruption, including polychlorinated biphenyls, plasticizers such as bisphenol A, polychlorinated biphenyls, DDT, dioxin among many others. EDC are found in many products including pharmaceuticals and personal care products (PPCP), food, toys, plastic bottles, metal food cans, detergents, flame-retardants and pesticides.

Some of these chemical pollutants can affect the endocrine system and also interfere with the development processes of humans and wildlife species. Once in the environment, persistent EDC can be dispersed and carried by water and air to remote locations. These chemicals are also subjected to biological and environmental transformations that may form other EDC. The EDC can also be biomagnified through food webs to high levels reaching humans and other top predators.

Humans, domestic and wild species are continually and simultaneously exposed to a mixture of EDC. However, the efforts have been made mostly towards the establishment of links between single EDC and diseases. Risks associated to the exposure of humans and other species to mixtures of different EDC have been underestimated as much as interactions among contaminants and related effects. The current truly challenges are (1^{st}) to find out how to avoid that an increasing number of new man-made EDC reaches the aquatic environment and, (2^{nd}) once in the environment, to promote better understanding of the interactions and the severity of multiple effects caused on exposed organisms, as a tool to support future decisions regarding priority of investments and promotion of changes in the consumption patterns.

The present investigation is a contribution to the achievement of the second above-mentioned challenge. However, hundreds EDCs exist in nature. To detect their presence and to quantify them in the environment is an unattainable endless task due to technical,

economic and time constraints. A feasible and fast strategy to detect their presence in different compartments particularly in the water matrix (e.g. surface water, groundwater, sewage) is to use the very same effect they cause (endocrine disruption) as a biomarker and select a suitable bioindicator to do that. Many biomarkers in natural and genetically modified organisms have been used in the past years. Aquatic organisms are certainly among the most suitable ones to test the quality of water environments.

In the current thesis, the effects of single and combined natural and synthetic estrogens (so called truly EDC) were tested in spiked waters regarding their effects on Nile tilapia male fishes (species with a complex endocrine system) and green microalgae (species with no endocrine system susceptible to toxic effects caused by these hormones). Additionally, water environmental samples suspected of having EDC were tested regarding their effects on male fishes. The author also presents his own reflections and recommendations for upgrading experimental setups in the future considering not only the contaminant concentration but also load, volume of experimental unit and population density.

1. ENDOCRINE DISRUPTORS CHEMICALS

New man-made chemicals (xenobiotics) are continuously released as part of the industrial and economic development. These products include, medicines, disinfectants, contrast media, pharmaceuticals and personal care products, dyes and paints, laundry detergents, surfactants, pesticides, food preservatives and other additives, to name few (Fatta-Kassinos et al., 2010). Consequently, these xenobiotics are found in low concentrations (micropollutants) in the environment especially in aquatic ecosystems, water supplies, and wastewater treatment plants (WWTP).

Several emerging micropollutants have not been fully examined yet for their negative environmental effects. Compared with their parent compounds, even less is known about chemicals produced due to transformation in the environment. Many of these contaminants have been proved to act as endocrine disrupting chemicals (EDC) or endocrine modulators that disrupt the endocrine system of organisms, with the ability to work as agonists or antagonists to endogenous hormones (Hallgren et al., 2009; Matozzo and Marin, 2005; Matozzo et al., 2008; Soto et al., 1995).

Hormones are responsible for the regulation of the homeostasis, development processes, maintenance, reproduction and behavior of an organism (Harries et al., 1997; Kime et al., 1999; Kumar and Mohan, 2011; Matozzo et al., 2008; Pinto et al., 2008; Ra et al., 2011; Soto et al., 1995; Vogel, 2005).

The heterogeneous group of natural or synthetic (xenobiotic) EDC can modify levels and functions of hormone receptors, induce or inhibit signaling pathways, and mimic steroid hormones (estrogens and androgens) (Davis et al., 2009; Harries et al., 1997; Soto et al., 1995; Souza et al., 2013). These EDC act in different ways such as: binding to hormone receptors and interfering in the synthesis, secretion, storage, release or blocking, transport, metabolism, and action of the hormones in the blood (Kime et al., 1999; Kumar and Mohan, 2011; Matozzo et al., 2008). EDC can also promote adverse health effects on certain organisms and their descendants, such as infertility, sexual underdevelopment (by changing or inhibiting sexual behavior), attention deficit or hyperactivity, alteration of thyroid or adrenal cortical function, and are suspected to increase incidence of cancer and

birth defects (Cooper and Kavlock 1997; Damstra et al. 2002; Kavlock et al. 1996; Mander and Mitsch 2009).

EDC are detected mostly by analytical methods or biological responses. The most widely used technique to quantify EDC in environmental matrices is gas or liquid chromatography and mass spectrometry after sample preparation (Hallgren, 2009; Lindqvist et al., 2005; Petrovic et al., 2002; Ra et al., 2011; Stumpf et al., 1999; Ternes et al., 1999; Zorita et al., 2008). The biological responses are mostly measured by bioassays including: non-cellular, cellular, and whole-organism assays (Hallgren, 2009; Sumpter and Jobling, 1995; Yadav and Trivedi, 2009). Bioassays can also measure the combination of effects of the total estrogenic load equivalent, including effects caused by non-targeted compounds found in complex wastewaters, which have not been identified or quantified chemically (Clubbs and Brooks, 2007; Dietrich et al., 2010; Hallgren, 2009). Bioassays have several advantages over chemical analytical methods since they also assess additive, synergistic or antagonistic effects in a complex mixture of substances in environmental matrices (Hallgren, 2009; Silva et al., 2009).

The ecotoxicity assay is one example of bioassay that is widely applied in aquatic environmental monitoring and aquatic environmental risk assessment. Over the years, aquatic ecotoxicology became more than application of assays to assess the effects of specific substances on specific species. These assays are currently seen as multidisciplinary tools suitable to assess the effects of isolated or mixed substances not only in water but in complex aquatic environments, including different levels of the trophic chain, populations, communities and even the whole ecosystem (Angel et al., 2010; Ashauer et al., 2006; Costa et al., 2010; Per Hallgren et al., 2012). Therefore, the impact of disposed effluents in nature should be tested in different species of organisms, since each one may have a different sensibility (Silva et al., 2009).

One example of bioassay that is well-known and widely used to identify the presence of EDCs in aquatic environments is the detection of abnormal levels of vitellogenin (Vtg) in males and juvenile (immature) fishes (Davis et al., 2009; Hallgren et al., 2009; Kime et al., 1999; Larsson et al., 1999; Matozzo et al., 2008; Solé et al., 2001; Sumpter and Jobling, 1995; Verslycke et al., 2002). The indirect quantification of Vtg via the determination of alkali-labile phosphate (ALP) is widely used, and is an inexpensive

and rapid alternative, particularly useful for large-scale environmental monitoring programs using different species, in particular fish (Hallgren et al., 2009).

1.1. Overview of the thesis

The present thesis received financial support from four different sources as following:

(1) The construction, operation and monitoring of a decentralized Engineered Ecosystem (DEE) for decentralized treatment of sewage of small generators. The project originally funded in Rio de Janeiro by FAPERJ (E-26/110.446/2007) had as the final product an unconventional treatment plant, as described in **Paper I.** The inlet and outlet of this plant was included in the investigation of estrogenicity described in **Paper III**;

(2) The Swedish Foundation for International Cooperation in Research (STINT) financed through the exchange program between Linnaeus University (former University of Kalmar) and UERJ the training of the author in techniques and protocols for indirect measurement of vitellogenin in fish (at Kristianstad University, Sweden) later on implemented at UERJ, Brazil to develop research with Nile Tilapia (**Papers II** and **III**) and ecotoxicity assays using microalgae (at Linnaeus University, Sweden), later on implemented at UERJ, Brazil with the experiments that resulted in **Paper IV** and also applied at Linnaeus University, Sweden to develop experiments with industrial wastewater (**Paper V**). Both techniques were important for the monitoring studies conducted subsequently;

(3) The occurrence and the toxicity of endocrine disruptors in water bodies and sewage treatment systems in the State of Rio de Janeiro was the subject of the PhD project that gave the PhD scholarship (CNPq 556660/2009-9) enabling the implementation and application of the ALP method in laboratory assays with estrogenic hormones, as well

as in the monitoring of different areas for estrogenicity, as described in **Papers II, III and IV**;

(4) The *Science without Boarders Program*, from the National Board for Scientific and Technological Developmen-CNPq, Brazil gave the PhD scholarship (CNPq SWE/CSF 246145/2012-9), enabling the author to spend his fourth year of the PhD program at Linnaeus University, Sweden. Through the knowledge gained during the first three years and courses taken at LNU, a critical review highlighting relevant issues when planning an ecotoxicty assay was produced (**Paper VI**).

The laboratory work and data acquisition for **Papers I-IV**, were carried out at the Laboratory of Bioremediation and Phytotechnologies-LABIFI run by the BioProcess research group at Rio de Janeiro State University (UERJ), Brazil (period March 2010-February 2013). The laboratory assays and data acquisition for **Paper V** and the writing of **Papers II-VI** were carried out at the Laboratory run by the Environmental Science and Engineering Group (ESEG) Linnaeus University (LNU), Sweden (March 2013-May 2014).

In the present thesis, the investigation focused on three main areas related to the contamination of industrial and domestic wastewater treatment systems (inlet and outlet) and aquatic ecosystems, as follow:

(1) Decentralized wastewater treatment units associated to eco-technological approach (vegetated tanks) to remove organic load, nutrients and EDCs (estrogenicity) from domestic wastewater;

(2) Ecotoxicity assays with spiked waters with single and combined natural and synthetic estrogens to assess the effects on cultures of single and combined microalgae species (*P. subcapitata* and *D. subspicatus*) and one fish species (*O. niloticus*);

(3) Application of ecotoxicity assays with focus on four endpoints (endocrine disruption, gonadosomatic index-GSI, hepatosomatic index-HSI and genotoxicity as a micronucleus frequency) using *O. niloticus* as bioindicator on various contaminated water environments;

(4) Application of one ecotoxicity assay with focus on the endpoint growth inhibition to assess toxicity of effluent of wooden floor industry using one unicellular microalgae *P. subcapitata* as the bioindicator.

Based on lessons learned with the above-mentioned investigations and a literature survey, a critical review was produced, with focus on five relevant aspects that affect the results obtained with existing ecotoxicity assays. The five aspects addressed were: contaminant characteristics and its gateway and pathway; selected species (bioindicator) and biomarker; dose regime/dose mode; concentration vs. load; single vs. multiple exposure.

1.2. Aim and scope

The overall aim of the present thesis was to increase the knowledge about relevant issues regarding the evaluation of aquatic environmental contamination and monitoring programs, mostly related to chronic effects. To do that, the thesis focused on the capacity of wastewater treatment systems to remove organic load, nutrients and estrogenicity from wastewater; and also on the ecotoxicity assays to evaluate the estrogenicity of single and combined female hormones (natural and synthetic), as well as, to be used as a powerful tool in aquatic environmental monitoring.

Figure 1.1 shows the flow diagram of the present thesis. **Paper I** describe the processes that occur in a small and decentralized system (decentralized engineered ecosystem) designed to treat the domestic wastewater based on 11 months of monitoring and the contaminants removal efficiency. **Paper II, III, IV and V** assessed the endocrine disruption, gonads as well as liver alterations and genotoxic effects caused by single and combined synthetic and natural estrogens on fishes (**Paper II**) and the same effects caused by contaminated environmental samples with unknown contaminants and gasoline contaminated samples (**Paper III**). The toxic effects caused by single and combined synthetic and natural estrogens on two species of green microalgae are described in

Paper IV. A critical review with discussions about the main factors (including neglected ones) to be taken into account when establishing the experimental setup for an ecotoxicity assay based on the literature and the lessons learned with the experiments included in the present thesis are found **Paper VI**.

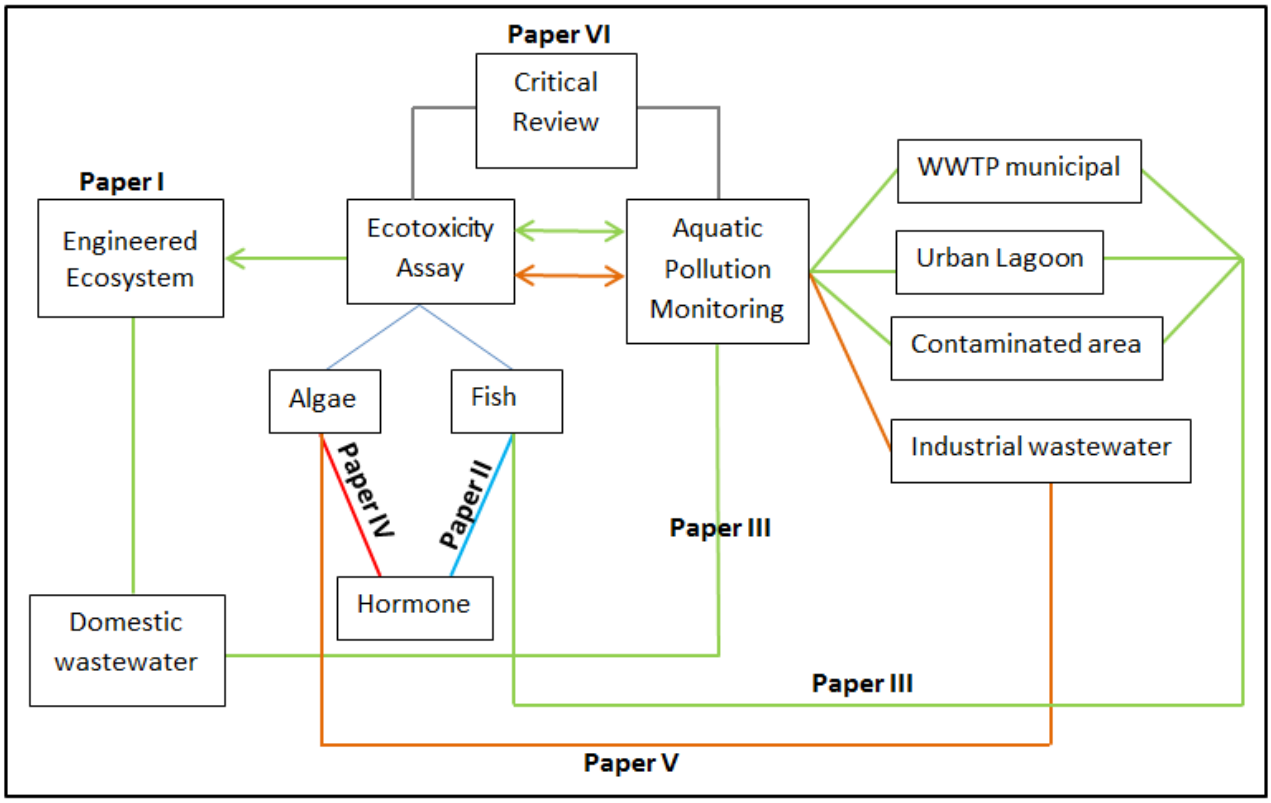


Fig. 1.1: *Schematic representation of this thesis. Paper I – Decentralized engineered ecosystem; Paper II – represented by the blue line; Paper III – represented by green lines; Paper IV – represented by red line; Paper V – represented by orange lines; Paper VI – gray lines.*

Decentralized engineered ecosystem

With the aim of assessing the treatment efficiency of an unconventional wastewater treatment system, the purpose of the investigation described in **Paper I** was to design, construct and test an on-site (decentralized) wastewater treatment multistage system named decentralized engineered ecosystem (DEE) due to the presence of vegetated tanks as final polishing for nutrients removal, suitable for small generator, including individual families, remote small hostels and resorts. The DEE was designed considering ecological engineering or ecotechnology principles such as: sustainable option for sewage treatment in regions with no sewerage system neither centralized WWTP; relatively low energy demand; no addition of chemicals for the treatment; high capacity to remove

organic matter and nutrients via microbiological processes and phytoremediation; removal of endocrine disruption present in the inlet; efficient enough to meet the threshold limits of the Brazilian and EU environmental regulations.

Estrogenicity detection by bioindicator in ecotoxicity assays

Ecotoxicity caused by natural and synthetic estrogens was assessed with three aquatic species belonging to two different trophic levels: two species of unicellular green microalgae (autotrophic level) *Pseudokirchneriella subcapitata* and *Desmodesmus subspicatus* (**Paper IV**), and one fish species, *Oreochromis niloticus* (heterotrophic level) (**Paper II**).

In the study presented in **Paper II**, the aim was to evaluate *O. niloticus* (Nile tilapia) as a potential bioindicator, measuring the alkali labile phosphate (ALP) levels in plasma of male fishes, as a biomarker that increases when vitellogenin increases after exposed of male fishes to estrogens or other EDC. The method applied was originally developed by Gagné and Blaise (2000) and then, improved by Hallgren et al. (2009) with Crucian Carp. In this study toxicity due to the presence of estrogens in water was assessed also by other biomarkers such as gonadosomatic index (GSI), hepatosomatic index (HSI) and frequency of micronucleus formation (MN) in erythrocytes. The experiment simulated intermittent discharges of single and combined estrogens in different concentrations in spiked water. An additional goal achieved was the reduction of the volume in experimental units (glass aquariums), which increased the feasibility of conducting more assays at the same time. A third goal was the comparison of the responses by the exposed fishes on the basis of both load and concentration, using experimental units with similar concentrations and different volumes (2 L and 130 L).

In **Paper IV**, the aim was to evaluate the effects of different concentrations of (single and mixed) natural and synthetic estrogens on algal growth and coenobium formation after the exposure of single and combined cultures of *Pseudokirchneriella subcapitata* and *Desmodesmus subspicatus*. The exposure of both species in the same culture (S+) aimed to assess the interspecific interactions in terms of the response to the presence of single and mixed estrogens, as expected to occur in contaminated aquatic environments.

Estrogenicity and toxicity detection in contaminated environmental samples

In **Paper III** the protocol by Hallgren et al (2009) applied to Nile tilapia was used in a variety of contaminated environmental samples including inlet and outlet of the wastewater

treated by the DEE described in Paper I; inlet and outlet of wastewater treated by a large conventional WWTP; water from an urban lagoon with clandestine discharge of sewage; surface water and groundwater samples from a marshland contaminated by gasoline (aged contamination). The ALP values found in the plasma of fishes exposed to these environmental samples were compared to the water (negative) control and to the lowest concentration that caused effect (LOEC) of the synthetic estrogen (17α-ethinylestradiol) used as positive control. This investigation also included the toxicity evaluation using morphological biomarkers such as hepatosomatic index (HSI) and gonadosomatic index (GSI), and genotoxicity evaluation using as biomarker the micronucleus frequency (MN).

The main objective of the **Paper V** was to compare growth response and the toxicity's magnitude of *Pseudokirchneriella subcapitata* exposed to the wastewater generated by cleaning and washing activities in a wooden floor industry before and after treatment by adsorption with activated carbon. The effect of pH, adjusting the pH of the industrial wastewater was also assessed by the algae growth response assay.

Understanding the relevance of well-studied and neglected factors in ecotoxicity assays

Paper VI presents a critical review limited to some important factors or variables to be taken into account when deciding or designing a ecotoxicity assay, which were: (i) contaminant characteristics and its gateway and pathway; (ii) selection of the bioindicator(s) and biomarker; (iii) dose regime/mode; (iv) the way the amount of contaminant to which the specimens are exposed is calculated; (v) single vs. multiple exposure. The review included a discussion about the way they affect the responses by exposed organisms and how the variable time affect them. The selection of these variables at the expenses of others was due to the surprisingly few publications that discuss the effects of two of them (iv and v) on the results or even mention them in the methodology description.

2. WATER CONTAMINATION

2.1. Wastewater treatment plants and EDC

In general, the main sources of water contamination are untreated and treated domestic and industrial wastewater and stormwater runoff from agricultural areas, continuously discharged livestock wastes and accidental chemical spills (Bejarano and Farr, 2013; Matozzo et al., 2008; McCahon and Pascoe, 1990). Among these sources, effluents from urban wastewater treatment plants (WWTP) have been considered as the main pathway for xenobiotics that enter the aquatic ecosystems (Ternes et al., 1999; Zorita et al., 2008).

According to Heberer (2002), municipal sewage can contain more than 80 compounds (in concentrations up to the level of μg/L), including estrogens and xenoestrogens among other endocrine disruptors. EDCs remain in the effluent because of the incomplete removal or conjugated forms of excreted (by urine and feces) natural human estrogens, pharmaceuticals, and estrogenic pharmaceuticals(Carballo et al., 2005; Desbrow et al., 1998; Matozzo et al., 2008; Solé et al., 2001; Ternes et al., 1999). Therefore, there is an increasing concern about the effects these compounds might have on humans and the environment. Pojana et al. (2004) have shown that the natural estrogen 17β-estradiol (E2) and the synthetic one 17α-ethinylestradiol (EE2) contributed to more than 97% of the total potential estrogenicity found in water bodies contaminated with sewage.

The low efficiency of WWTP regarding the removal of EDCs is basically due to physicochemical proprieties of these compounds (Carballo et al., 2005; Desbrow et al., 1998; Heberer, 2002; Matozzo et al., 2008; Solé et al., 2001; Song et al., 2009; Stumpf et al., 1999; Ternes et al., 1999; Ying et al., 2002). At the outflow of WWTPs with secondary treatment, the average concentration of estrogens ranges between 0.39–101 ng/L for estrone (E1); 0.2–64 ng/L for E2; and 0.59–42 ng/L for EE2 (Desbrow et al., 1998; Nakada et al., 2006; Servos et al., 2005; Song et al., 2009; Ying et al., 2002).

The presence of these estrogenic substances in WWTP outlets and their continuous release into aquatic environments have been associated with several biological responses by aquatic species (Dordio et al., 2010; Fent et al., 2006; Harries et al., 1999; Heberer, 2002; Purdom et al., 1994; Servos et al., 2005). The synthesis of vitellogenin increases and intersex is developed in exposed male fishes (Carballo et al., 2005; Harries et al., 1999; Servos et al., 2005; Solé et al., 2001).

Some advanced wastewater treatment technologies such as ozonation, photocatalysis, sorption by activated carbon, membrane filtration and membrane bioreactors can reduce the discharge of estrogens by WWTP. However, these technologies are expensive, require sophisticated operation and maintenance besides high costs and usually are not applied to treat municipal wastewater (Dordio et al., 2010; Gray and Sedlak, 2005; Song et al., 2009).

Constructed wetlands (CWs) have been considered in the literature as a promising polishing step to treat effluents from conventional wastewater treatment plants. CWs have shown some capacity to remove estrogens and some EDC (Dordio et al., 2010, 2009; Gray and Sedlak, 2005; Song et al., 2009) in a sustainable and low-cost manner (Song et al., 2009).

The removal efficiency of CWs has been attributed to the presence of algae and aquatic macrophytes in a continuous flow that accelerate and increase the efficiency for removal of E1, E2, and EE2, even when they are present in concentrations as low as ng/L (ppt) level (Gray and Sedlak, 2005; Shi et al., 2010). This would happen (according to the literature) initially through the quick sorption on algae and macrophyte roots (hydrophobic surfaces), followed by biodegradation and biotransformation carried out by microorganisms present in the wastewater and/or plant uptake (Song et al., 2009).

Macrophyte roots play an important role in plant–water–microorganism interactions in many ways (Brix, 1994). They provide microbial attachment sites (symbiosis), increase oxygen concentrations in the rhizosphere, and release exudates, such as carbohydrates and amino acids, that serve as nutrient sources for the growth and long-term survival of microorganisms. Nitrifying microorganisms, in particular, are capable of degrading organic pollutants (hydroxylation), which results in more hydrophilic compounds and provides a surface area for pollutant sorption, enabling further uptake and assimilation in plant tissues

(Alkorta and Garbisu, 2001; Karjalainen et al., 2001; Servos et al., 2005; Song et al., 2009).

The biodegradation of estrogens is likely to be more effective under aerobic than under anoxic or anaerobic conditions in a constructed wetland (Dordio et al., 2010; Song et al., 2009). The uptake rates of several hormones, herbicides, pharmaceuticals, and other EDCs by the root and rhizome of *Phragmites australis* were correlated with log K_{ow} values, in a range from 1-3 (Schröder et al., 2008). According to these observations, and considering the log K_{ow} values of E1 (3.4), E2 (3.1), and EE2 (3.9) (Feng et al., 2005) higher root densities would increase and allow a close contact with root surfaces and associated biological films facilitating biodegradation and possibly the uptake of estrogens by plants (Song et al., 2009).

2.2. Estrogenicity detection using fishes

The molecular structure of estradiol and the estrogen receptors are very similar in fish and mammals, which may explain the mimic action of the human natural hormone on fishes (Pakdel et al., 1990; Solé et al., 2001). The fish liver, particularly in females, contains high concentrations of estrogen receptors (Campbell et al., 1994) and the liver synthesizes large amounts of a heavily phosphorylated lipophosphoprotein named vitellogenin (Vtg) when stimulated by estrogen (Sumpter and Jobling, 1995). In this way, estrogens can interfere with the hypothalamic–pituitary–gonadal axis, disturbing the vitellogenesis in fish and changing the production of Vtg by liver cells, increasing blood levels of Vtg in females and also in males (Hallgren et al., 2009; Kime, 1998). This axis in fish shows close similarities to the mammalian homologue and has the important function of controlling reproduction, development and aging (Kime, 1998).

The Vtg synthesis is hormonally controlled by ovarian estradiol of female oviparous (and viviparous teleost) vertebrates and transported by the blood to the ovary. Once in the ovary, Vtg is taken up as vesicles and split into the yolk components that are stored in egg-yolk platelets inside the developing oocytes, which provide energy reserves for embryonic development (Hallgren, 2009). During the blood transport these components

(glyco-lipo-phospho-proteins) are embedded in a macromolecule called vitellogenin (Vtg) (Davis et al., 2009; Hallgren et al., 2009; Kime et al., 1999; Matozzo and Marin, 2005; Matozzo et al., 2008; Purdom et al., 1994; Rodas-Ortíz et al., 2008; Solé et al., 2001; Sumpter et al., 1996; Sumpter and Jobling, 1995). In maturing female fishes, Vtg is normally found at high levels in the plasma and it is required to promote the ovary growth that might contain thousands of yolky oocytes representing up to 25% of the female body weight (Davis et al., 2009; Sumpter and Jobling, 1995).

In males or juveniles (immature) fishes, the gene responsible for Vtg synthesis, although present, is not expressed under normal conditions (Kime et al., 1999; Matozzo et al., 2008; Solé et al., 2000; Sumpter et al., 1996). Vtg in males and juveniles (immature) fishes is a well-known protein biomarker for the presence of natural and synthetic estrogens as well as other EDC in aquatic environments. Vtg has been the focus of many previous investigations (Davis et al., 2009; Hallgren et al., 2009; Kime et al., 1999; Larsson et al., 1999; Matozzo and Marin, 2005; Matozzo et al., 2008; Rodas-Ortíz et al., 2008; Solé et al., 2001; Solé et al., 2000; Sumpter and Jobling, 1995; Verslycke et al., 2002).

The measurement of alkali-labile protein-bound phosphates or alkali-labile phosphates (ALP), found in high quantities in Vtg, has been recently developed as a method for indirect quantification of Vtg in fish, crustaceans, and bivalve mollusks (Blaise et al., 2002; Gagné et al., 2004; Gagné et al., 2005; Hallgren et al., 2012; Mandiki et al., 2005; Versonnen et al., 2004). This method is possible because the presence of ALP in fish plasma is specifically associated with Vtg (Verslycke et al., 2002). Indirect quantification of Vtg through ALP is considered an inexpensive and rapid alternative and also allows for large-scale environmental monitoring using different species, in particular fish (Hallgren et al., 2009). According to Verslycke et al. (2002) and Hallgren et al. (2009), from high to very high (0.73–0.99) correlation coefficients have been found between ALP and Vtg levels.

The original method by Gagné and Blaise (2000) based on ALP quantification for indirect measurement of Vtg in clams and mussels was modified by Hallgren et al. (2009) for ALP quantification in Crucian carp (*C. carassius*). The main improvement made by Halgren et al. (2009) was the removal of non-vitellogenin sources of phosphates such as phospholipids found in blood plasma and consequently, the reduction of yellowish color. The modified ALP method reduced the ALP detection limit down to 3.2 μg PO_4^{3-} per mL

plasma, which according to those authors (Hallgren et al., 2009) was six times lower than the original detection limit.

In addition to the evaluation of Vtg levels via ALP quantification the use of several strategies involving different biomarker to evaluate the induction of physiologic modifications of ecological relevance has become a common procedure (Hassanin et al., 2002; Bolognesi and Hayashi, 2011; Hallgren, 2009). The gonadosomatic (GSI) and hepatosomatic (HSI) indices are good examples of this combined approach, because both gonads and liver are intimately related to the reproduction process (Kime et al., 1999; Verslycke et al., 2002) and can help to interpret the results obtained with ALP.

The main detoxifying organ in all vertebrate is the liver, which is responsible for metabolizing, contributing to the excretion of toxic substances (Authman, 2011; Kime, 1998). The liver is also the site for deactivation of the gonads steroids and the target tissue (since it has estrogen receptors that specifically induce the synthesis of Vtg) (Kime, 1998). The fish liver is particularly rich in lipids, which facilitate the bioaccumulation of many organic pollutants (Kime, 1998) and therewith increases the risk of disrupting the activities of detoxifying enzymes and morphological damage to the liver (Kime, 1998). These enzymes are responsible for the oxidation, hydroxylation, deoxylation and methylation of toxic substances, prior to the final conjugation with glucuronic acid or sulphate (Ying et al., 2002). Because some of these enzymes play a major role in the deactivation of steroid hormones, when they are preferably used to metabolize a pollutant, it can result in slower deactivation of the bloodstream hormones resulting in a higher plasma concentration (Kime, 1998). According to Pereira et al. (1992; 1993) one of the morphological damages in the liver tissue is the formation of liver lesions or liver necrosis, and in the first case, there is an increase of hepatocytes. However, persisting the state of contamination or in cases of acute toxicity, the regeneration capacity of hepatocytes is lost followed by the substitution for the neoplastic or vacuolated cells, in a fibrosis process, resulting in the increase of volume or weight raising the HSI (Pereira et al., 1992; 1993). Therefore, it is important to evaluate the liver conditions in fishes exposed to contaminated waters.

Another effect caused by the contaminants is the alteration or impairing of Vtg production by the liver. This is likely to be also due to disturbances in the hypothalamic-pituitary-gonadal axis and the variation on the gonadotropin production altering the blood levels (Pereira et al., 1992 and 1993; Heiden et al., 2006; Laizer et al., 1996; Hwang et al.,

2000). This alteration will also affect the estrogen production by the gonads, which in turn, will affect the Vtg production by the liver (Heiden et al., 2006, Laizer et al., 1996, Pereira *et al.* 1992 and 1993). A secondary consequence would be the disturbance in the gonads development (and also the GSI), which may threat the development of the upcoming offspring.

Micronucleus formation is among the most applicable indicators used to identify genomic alterations (Bolognesi and Hayashi, 2011; Sponchiado et al., 2010). According to Bonassi et al. (2007) the micronucleus is the result of failure to repair lesions in DNA during cell division and chromosomal breakage (great damage). Another possibility is that chromosomes do not segregate properly during mitosis forming micronucleus (Bonassi et al., 2007).

According to Çavaş and Ergene-Gözükara (2005) and Özkan et al. (2011), fish are often used as sentinel organisms since they play an important role in the trophic web, and therefore, there is a high chance for accumulation of toxic substances through the trophic levels and the appearance of responses to even low concentrations of mutagens in the water.

Nile tilapia (*Oreochromis niloticus*) is the most cultivated cichlid in the world (De Silva et al., 2004). *O. niloticus* is obtained easily and has high adaptability to many different climate zones (e.g., tropical and subtropical regions). Several physiological characteristics of Nile tilapia (Bücker and Conceição, 2012; Davis et al., 2009) such as euryhaline characteristics, fast growth, and high tolerance to a large range of temperatures (8-42°C) and stressing factors make this species an excellent candidate for ecotoxicity tests, particularly when a large number of water samples are required (e.g., environmental monitoring programs). With the propose of monitoring or screening estrogenicity in water bodies, males of Nile tilapia have also an extra advantage that is the ALP baseline level in plasma of non-exposed *Oreochromis niloticus* (Nile tilapia) males which was proved in the present thesis to be low, as measured using the method improved by Hallgren et al. (2009). The ALP value in males (4.11 µg PO_4^{3-}/mL plasma, IQ_{25} = 3.38; IQ_{75} = 5.18) was lower than those of the other species (males) described in the literature. These results are presented in **Papers II** and **III** and will be discussed further in the present thesis.

2.3. Toxicity assays with green microalgae

In order to protect the water environment, numerous approaches have been applied for the development of cheaper and more effective methods, to reduce the amount of wastewater produced or, at least, to improve the quality of the treated effluent (Laohaprapanon et al., 2014, 2013). According to Lai et al. (2002) and Sijm et al. (1998), algae play an important role in the fate of organic compounds in the aquatic ecosystem and also, the attenuation of the estrogens effects may be related to degradation or uptake by algae in the water bodies, thereby acting as a vehicle for bioconcentration and subsequent biomagnification in higher trophic levels. Unicellular green algae have been part of wastewater treatment systems (**Paper I**), and increasing attention has recently been paid to the algae growth in wastewater as a sustainable way to produce biodiesel (Gressler et al. 2013; Park et al. 2011).

Unicellular green algae have been widely used in environmental studies and screening to detect toxicity in water and the species frequently used are *Desmodesmus subspicatus* and *Pseudokirchneriella subcapitata* among others (Cleuvers, 2004; Djomo et al., 2004; Kaczala et al., 2011; Lürling, 1998; Pavlić et al., 2005). There are many compelling reasons to use unicellular green algae as toxicity bioindicators in environmental monitoring programs. Among them, it is worth to mention that: (i) they are primary producers (transferring energy to the higher trophic levels) and therefore, changes in this primary trophic level can affect all higher levels of the food chain (high ecological relevance); (ii) they are frequently found in freshwaters, occupying an extensive range of habitats and withstand domestic sewage pollution; (iii) short lifecycles allow investigations about the toxic effects throughout several generations; (iv) they produce sensitive and quick response to environmental changes, showing adverse effects, and thereby allowing corrective regulatory and management actions to be taken before other undesirable impacts occur; (v) their cultivation is cost-effective, easy to implement and maintain in laboratories and they are widely used with well-established standard methods; (vi) they are part of mandatory sets of tests for notification of chemicals in the European Union states (Djomo et al., 2004; Kaczala et al., 2011; Lai et al., 2002; Pavlić et al., 2005; Silva et al., 2009; Trainor et al., 1976).

Algae are highly variable in their sensitivity to different groups of pharmaceuticals, surfactants and steroids estrogens (Cleuvers, 2004, 2003; Fent et al., 2006; Lai et al., 2002; Pavlić et al., 2005). According to Djomo et al. (2004), the toxicity of substances or compounds can be associated to intrinsic physicochemical properties of these compounds, such as the octanol-water portioning coefficient (log K_{ow}), volatility and aqueous solubility. This observation is in accordance with Cleuvers (2004) that established a correlation between the toxicity of substances with their log K_{ow} values as a baseline and reported that substances with higher log K_{ow} are expected to pose higher toxicity. Based on the EE2, E2 and E1 log K_{ow} values (3.9, 3.1 and 3.4, respectively) and their uptake by the algae, Lai et al. (2002) suggested that the bioconcentration of estrogens in the algae is due to an active binding mechanism such as binding to receptors or enzymes for biotransformation. Lai et al. (2002) also suggested that EE2 is more adsorbed by algae than the other estrogens. Therefore, it is highly relevant the investigation of the effects of contaminants found in aquatic environments and in wastewater (municipal and industrial) on the algal growth, which can be measured via *in vivo* chlorophyll fluorescence, as well as the morphological changes or phenotypic plasticity inducers.

The effects of some contaminants on the unicellular microalgae morphology are the expression of the phenotypic plasticity in response to contamination stress. This stress response (morphological changes or changes in growth and reproduction) in unicellular microalgae (e.g.: *D. subspicatus*) is well describe in the literature and can also be caused by environmental cues or anthropogenic disturbed ecosystems (Hessen and Donk, 1993; Lürling, 2006, 1998; Peña-Castro et al., 2004). According to Lürling (1998) more than 80% of the natural population (non-exposed) of *Scenedesmus acutus* is primarily unicellular. However, when exposed to certain contaminants, especially in the exponential growth phase, a rapidly formation of two-, four- and eight-celled coenobia is observed. Nevertheless, when the exposure is ceased a subsequent recovery of unicellular abundance can be observed in few days (Lürling, 1998; Peña-Castro et al., 2004). This morphological changes or coenobia formation in some cases may exceed the size of grazable particles and can reduce the grazing pressure, selecting the predator size (Hessen and Donk, 1993).

Another issue that should be taken into account is that in a real polluted water body, the algae and other aquatic species co-exist and are simultaneously exposed to several

contaminants. According to Drake (1991) small systems or cultures are very interesting way to explore competitive interactions and sequential effects among species (interspecific competition or interactions) when exposed or not to single or mixed contaminants, and can be key components of large systems. These features have not been explored enough by ecotoxicity assays.

3. METHODS

3.1. Decentralized engineered ecosystem (Paper I)

The system for sewage treatment was constructed in 2009, and still operating at the Center of Environmental Studies and Sustainable Development (CEADS), a research campus of the Rio de Janeiro State University (UERJ) in Ilha Grande, which means “big island” in Portuguese (23º18’ S, 44º19’ W), Angra dos Reis Municipality, Rio de Janeiro State, Brazil.

The system was dimensioned to treat 1248 L/day and 0.36 kg of BOD/day, with an average flow of 52 L/h, for a population of 12 residents (working as a hostel). The treatment units were constructed with glass fiber and the dimensions and operation parameters are shown in Fig. 3.1 and Table 3.1. The primary and secondary treatments units had the upflow design in order to improve the mass transfer between microorganisms and the wastewater to be treated, which in turn improves the removal of dissolved anaerobically biodegradable organic matter and accentuates oxidative processes.

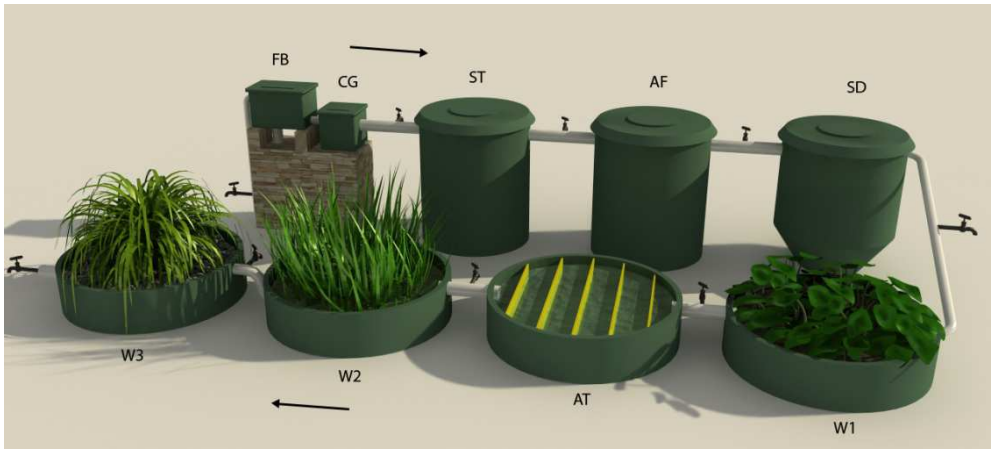


Fig. 3.1: *Graphical representation of the Decentralized Engineered Ecosystem. All units were constructed above ground level for didactic and demonstration purposes. FB: flow control box; GC: grease collector; ST: upflow septic tank; AF: aerated submersed filter; SD: secondary decanter; W1: surface flow; AT: algae tank; W2: vertical subsurface flow; W3: horizontal subsurface flow. (see Figure 1,* ***Paper I****).*

The Decentralized Engineered Ecosystem comprises of preliminary, primary, secondary and tertiary treatment. The sequence of treatment units included: Grease collector (GC); Upflow septic tank (ST); Upflow submersed aerated filter (AF); Secondary decanter (nowadays was replaced by Upflow biofilter filled with expanded clay) (SD); Surface flow wetland vegetated with *Eichhornia crassipes* (floating macrophyte) (W1); Chicane algae tank (AT); Vertical subsurface flow wetland vegetated with *Schoenoplectus sp*. (Cyperaceae) (W2); and Horizontal subsurface flow wetland vegetated with *Gramineae Panicum cf. racemosum* (Poaceae) (W3) (nowadays a sand filter is also found at the end). The aquatic macrophytes species were chosen according to characteristics such as fast growth rate and the role to be played in the treatment system.

The physical and chemical analyses were monitored from wastewater samples collected from the inlet and outlet of each tank every 15 days during 11 months. The biomass from the vegetated tanks was filtered and harvested (algae and macrophytes, respectively) every 15 days and dried in an oven for 48 h at 60 ºC or until a constant weight was reached. Fecal coliforms were analyzed according to the method 14053 Endo NPS (Sartorius Stedim) at three points: the inlet of the system; the outlet of the last wetland tank; and after the sand filter.

Table 3.1 Dimensioning and operational parameters for the DEE treatment units. *(Table 1,* **Paper I***)*

Unit	**Dimensioning parameters**	**Operational Parameters**
Population served	12 inhabitants or 16 guests/day in a hosting house	0.36 kg BOD/day; 1248 L/day; 52 L/h
Equalizing tank [a]	1.2 m × 1.8 m × 1.6 m; 3.5 m^3	–
FB[a]	0.6 m × 0.4 m × 0.4 m; 0.1 m^3	–
GC[a]	0.6 m × 0.4 m × 0.23 m; 0.05 m^3	HRT: 0 h
ST[b]	1.27 m; 1.15 m; 1.32 m^3	HRT: 25.4 h

AF[b]	1.17 m; 1.25 m; 1.35 m^3	SOL: 0.014 kg BOD/m^2/day; CSA: 200 m^2/m^3; AR: 53.25 m^3/day/ kg of BOD; HRT: 23.1 h
SD[b]	1.15 m; 1.15 m; 0.96 m^3	SLR: 1.2 m^3/m^2/day; HRT: 18.5 h
W1[b]	1.62 m; 0.33 m; 0.68 m^3	SLR: 0.6 m^3/m^2/day; MLR: 300 kg BOD/ha/day; HRT: 13.1 h
AT[b]	1.62 m; 0.27 m; 0.55 m^3	SLR: 0.6 m^3/m^2/day; MLR: 157.5 kg BOD/ha/day; HRT: 10.7 h
W2[b]	1.62 m; 0.27 m; 0.55 m^3	SLR: 0.6 m^3/m^2/day; Substrate depth: 0.25 m; BRA: 0.012 kg BOD/m^2/day; HRT: 4.3 h
W3[b]	1.62 m; 0.1 m; 0.21 m^3	SLR: 0.6 m^3/m^2/day; MLR: 99.3 kg BOD/ha/day; Substrate depth: 0.35 m; HRT: 2.4 h

Note: [a]Square tanks (width × length × depth); [b]cylindrical tanks (diameter; depth; volume); BOD: biochemical oxygen demand; HRT: hydraulic retention time; SOL: surface organic load; CSA: carrier surface area; AR: aeration rate; MLR: maximum load rate; SLR = surface loading rate; BRA: BOD rate applied.

3.2. Bioindicators applied (Papers II, III, IV and V)

Two levels of the food chain (primary producer and omnivorous), represented by 2 species of algae and one fish, were chosen for screening of estrogenicity and toxic effects in laboratory assays with spiked water (natural and synthetic estrogen), untreated and treated wastewater (municipal, domestic and industrial), environmental samples from two contaminated areas (one urban lagoon with clandestine discharge of sewage and one marshland with aged contamination by gasoline).

3.2.1. Nile tilapia (Papers II and III)

The Nile tilapia, *Oreochromis niloticus* (Perciformes, Cichlidae, with omnivorous feeding) was obtained at a fish farm in Brazil (Rio Doce Piscicultura), where the fishes received no hormone treatment during their cultivation. The fishes with standard length of 4-7 cm were cultivated in the Laboratory of Bioremediation and Phytotechnologies-LABIFI at UERJ, Rio de Janeiro, in 4 glass aquariums of 500 L each (not exceeding 2 g of fish/L) (Fig. 3.2). The aquariums were filled with water produced by filtration (25–0.5 μm) followed by double carbon-filtration of tap water (to remove chloride) and a biofilter with forced oxygen supplier. The animals were kept under the following conditions: water temperature 24 °C ± 2; pH 6.9 ± 0.2; dissolved oxygen (DO): 5.8 ± 0.3 mg/L; day/night periods: 14/10 h. One-fifth (20%) of the culture water was renewed manually once a week. Fishes were fed daily (approx. 3 percent of body biomass) with commercial trout pellets (Alcon Garden Basic Sticks, Camboriú, SC, Brazil) in amounts enough to guarantee that all food was eaten within 5 min. All the fish were fasted for 24 h before the experimental setup.

Fig. 3.2: *Oreochromis niloticus (Nile Tilapia) cultivation in 500 L aquarium with biofilter with forced oxygen supplier.*

3.2.2. Unicellular green microalgae (Papers IV and V)

Two unicellular freshwater microalgae species *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák (CCAP 278/4) (formerly *Selenastrum capricornutum* Printz) and *Desmodesmus subspicatus* (Chodat) E. Hegewald & A. Schmidt (SAG 86.81) (formerly *Scenedesmus subspicatus* Chodat) (Fig. 3.3) obtained at the Marine Department at

Linnaeus University, Sweden, were cultivated at LABIFI-UERJ in Rio de Janeiro, under static conditions in Jaworski's medium (Thompson et al., 1988). To avoid losses, the stock cultures were kept in incubators at two different locations and in triplicates for each algae species, following recommendations (Prof. Edna Granelli, pers. com.). The cultures were kept under cool white light with light intensity of 6,000 lux, with the light/dark cycle of 16/08 h, and temperature at 20 ± 2 °C (Kaczala et al., 2011). The stock was renewed monthly, and three generations were kept as a backup (Fig. 3.3).

Fig. 3.3: *Pseudokirchneriella subcapitata (left hand side), one of the two incubators with light and temperature control to algae cultivation (center), and Desmodesmus subspicatus (right hand side).*

3.3. Experimental setup with fishes

The experimental setup of all investigations with fishes is divided in 2 parts (**Paper II and III**).

The first part (**Paper II**) included the evaluation of *O. niloticus* as a potential bioindicator for ALP dosage by applying the method improved with Crucian carp by Hallgren *et al.*(2009) and others biomarkers such as: gonadosomatic index (GSI), hepatosomatic index (HSI) and frequency of micronucleus formation (MN) in erythrocytes. An additional goal established in this first part of the investigation was to assess estrogens load vs. concentration effects on the ALP levels of *O. niloticus*, using experimental units with two quite different volumes (2 L and 130 L) and different population densities.

The second part (**Paper III**) assessed the applicability of the modified method (**Paper II**) for determination of ALP plasma levels together with the other above-mentioned biomarkers (GSI, HSI, MN frequency), in order to test how versatile, resistant and sensitive

(to be consider a sentinel species) males of *O. niloticus* can be to detect endocrine disruption and geonotixicty in a large number of real contaminated samples.

In **Paper II**, two 15-day exposure experiments were conducted (Knudsen et al., 2011; Verslycke et al., 2002). In *Experiment 1*, fishes were exposed to EE2 in a 130 L aquarium (Fig. 3.4). In *Experiment 2*, fishes were exposed to E1, E2 and EE2 individually and in combination (1:1:1) in 2 L aquariums (Fig. 3.4). The fishes were kept at constant temperature (24 ± 2 °C), pH (6.9 ± 0.2), DO (5.2 ± 0.9 mg/L) and photoperiod (14/10 h). Both experiments adopted the intermittent pulse (intermittent dose regime) and the water was renewed every 5^{th} day (twice in 15 days) with new water containing the same initial concentration of estrogen. The length (7.4 ± 0.6 cm) and weight (14.2 ± 3.7 g) of the fishes exposed had no significant differences ($p < 0.05$). The correlation coefficient (r) between the length and weight of all fishes exposed was 0.97. Fishes were fed daily as described previously and fasted 12 h before blood sampling.

The *Experiment 1* (130 L glass aquariums of 55 × 55 × 43 cm), 12 specimens were exposed to each one of six concentrations of 17α-ethinylestradiol (0.016, 0.08, 0.4, 2.0, 10.0 and 50.0 μg/L). A negative water control (no estrogen or solvent added) and a solvent control (only solvent added) were also examined. This experiment used 17α-ethinylestradiol (EE2) because it is highly estrogenic potency active and causes physiological/biological effects. Additionally, there are homologies between EE2 receptors and enzymes in humans and fishes (Christen et al., 2010).

Experiment 2 (performed in 2 L glass aquariums 13 x 19-cm diameter x height) had only one male *O. niloticus* specimen per aquarium. The individual exposure was performed in five replicates for each estrogen (E1, E2, and EE2) and for the mixture (1:1:1) in each tested concentration. Based on the results obtained with *Experiment 1*, a narrower range of concentrations was applied in *Experiment 2* (0.012, 0.03, 0.12, 0.36 and 0.60 μg/L) which was compared with the negative control (no estrogen added). Due to technical limitations, specifically in the mixture of the estrogens, the final concentration 0.012 μg/L was substituted by 0.015 μg/L (0.005:0.005:0.005 μg/L of E1:E2:EE2). An additional objective achieved with Experiment 2 was to test small experimental units (2 L), which enable simultaneous analysis of many environmental samples in a monitoring program, for instance.

In order to detect the presence of endocrines disruptor chemicals (EDC) in environmental samples (**Paper III**), three more experiments (*Experiments 3, 4 and 5*) were carried out in 2 L aquarium in five replicates (Fig. 3.4), with 15 days of exposure (Knudsen et al., 2011; Verslycke et al., 2002), constant aeration, no filtration, and with the same conditions of fish feeding, photoperiod and temperature as describe in *Experiments 1* and *2*.

In each experiment (3, 4, 5) described below, fishes were exposed to waters from different environmental sources (without dilution) with quite distinct characteristics from each other.

In *Experiment 3*, fishes were exposed to inlet and outlet wastewater samples taken from: (i) a large WWTP with capacity to treat 400 L/s from a population of approximately 110,000 inhabitants. This WWTP had a pretreatment step with grating, sand, and grease collector, followed by an up flow anaerobic sludge blanket (UASB), an activated sludge aerated tank, and a secondary decanter. The characteristics of the WWTP inlet and outlet water were respectively: pH 7.2 and 7.5; DO 0.0 and 2.5 mg/L; and COD 466 and 79 mg/L and; (ii) a compact DEE for decentralized domestic wastewater treatment as describe in the **Paper I**. The characteristics of the DEE inlet and outlet water were, respectively: pH 6.9 and 7.2; DO 0.16 and 2.11 mg/L; and COD 554 and 76 mg/L.

In *Experiment 4*, The Rodrigo de Freitas Lagoon, which is located in the southern zone of the city of Rio de Janeiro, has a surface area of about 2 km^2 and is connected to the sea by one channel. Besides the lagoon surface area (which acts as an accumulation basin during heavy rainfalls) the drainage basin has 28 km^2 formed by the basins of three rivers that converge at one point in the lagoon. In the present study, three sampling points were selected: the point LRF1, located to the right side of the discharge point of the rivers (highest flow); point LRF2, located at the opposite side of the seawater inlet; and point LRF3, located on the opposite side of the river discharge point (1200 m away from it). The water characterization was: pH 8.1 ± 0.12; DO 5.5 ± 0.8 mg/L; salinity varying from 4 to 16 ‰ and water temperature 23 ± 0.5 °C.

In *Experiment 5*, Fishes were exposed to surface water and groundwater collected from a marshland contaminated area about 2 years after an accident that caused leakage of approximately 28,600 m^3 of gasoline. The investigation included marshland superficial water (SW) from 6 sampling points and groundwater (GW) from one well located

downstream the accident. The SW1-SW6 characteristics were: pH, 6.4 ± 0.7 (min 5.3 - max 7.6); water temperature during sampling 23 ± 0.5 °C; COD 601.0 ± 692.9 mg/L (min 173 mg/L; max 1987 mg/L). The groundwater GW1 characteristics were: pH, 6.7; water temperature during sampling 21°C; COD 147 mg/L.

In the experiments with environmental samples (*Experiments 3, 4 and 5*) above-described, fish specimens were exposed to undiluted water and they had the following standard length and weight: 7.4 ± 0.4 cm and 13.4 ± 3.1 g for WWTP and DEE (*Experiment 3*); 7.7 ± 0.6 cm and 16.1 ± 4.0 g for LRF (*Experiment 4*); 8.1 ± 0.4 cm and 19.5 ± 3.1 g for SW and GW (*Experiment 5*). The correlation coefficient (r) between the length and weight of all fishes was 0.97 ±0.01.

The controls for the experiments were: negative control (clean water obtained in the laboratory) and positive control (water spiked with EE2 with the amount of estrogen corresponding to the LOEC value (mass/mass of fishes) as previously established with the *Experiments 2.*

Fig. 3.4: *Experiment with twelve specimens of O. niloticus in 130 L aquariums and individual exposure in 2 L aquariums.*

3.3.1. Blood sampling from fishes

At the end of the exposure period in *Experiment 1 to 5* (**Paper II and III**) (15th day), blood samples (200–300 μl) were collected from the caudal vein (Fig. 3.5) using syringes (BD Ultra-Fine™ 50 U 12.7 mm × 0.33 mm needle) rinsed with heparin and transferred into ice-cooled vials. Plasma was collected after centrifugation (1000 × g, 10 min, 4 °C) in

cryogenic tubes and then snap frozen in liquid nitrogen and stored -80 °C (Hallgren et al., 2012; Hallgren et al., 2009) for further ALP level determination.

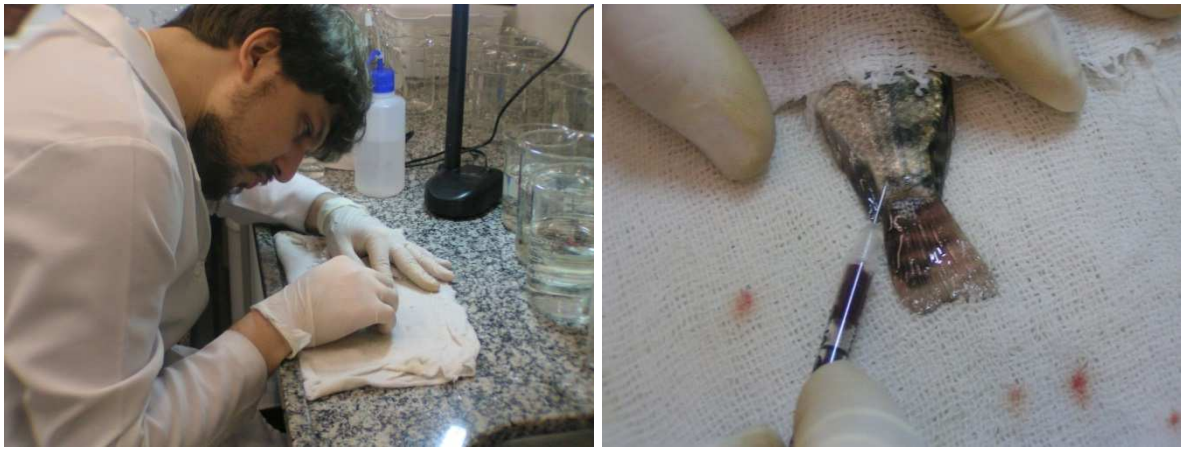


Fig. 3.5: *Blood collection from the caudal vein of O. niloticus with about 7 cm of standard length.*

3.3.2. Alkali-labile phosphate (ALP) levels in fishes plasma

To indirectly evaluate the vitellogenin (Vtg) levels in fish plasma, the biochemical determination of Alkali-labile phosphate (ALP) concentrations were performed based on the protocol developed by Gagné and Blaise (2000), for clams and mussels and modified by Hallgren *et al.* (2009), for application to Crucian carp (*Carassius carassius*). According to Hallgren et al. (2009), the ALP method was modified and improved with special emphasis on the removal of non-Vtg sources of phosphate, such as phospholipids found in blood plasma and removal of the yellowish color. Through these improvements, the modified ALP method had a detection limit as low as 3.2 µg PO_4^{3-} per mL plasma that was six times lower than other methods described in the literature. The levels of PO_4^{3-} were determined in the samples (96 plates wells) using a colorimetric measurement by reading the absorbance at 630 nm using a SpectraMax M3 Multimode Reader (Molecular Devices Inc.) (Fig. 3.6).

Fig. 3.6: *SpectraMax M3 Multimode Reader (Molecular Devices Inc.) used in a biochemical determinations of ALP concentrations in 96 plate wells by reading the absorbance at 630 nm. The columns A and B of the 96 plate wells were filled with the standard curve (1 to 9) and blank samples (10 to 12). The columns C to H (1 to 12) was filled in duplicate (C-D; E-F; G-H) with samples.*

According to the modified protocol, 100 µl of plasma was added to 54 µl of acetone and mixed by vortex and centrifuged at 5000 g for 5 min to precipitate Vtg plasma proteins. The supernatant was discarded and the pellet was washed twice with Tris-buffer (0.05M) [Tris (hydroxymethyl) aminomethane Base] and ethanol to remove the free phosphate. For alkaline treatment, 100 µl of 1 M NaOH was added to the pellets and the solution was incubated for 90 min at 70 ºC to allow hydrolysis of protein bound phosphates. The non-polar interferences were removed after the addition of 435 µL of 1-butanol (≥ 99 %). The results were expressed in µg of PO_4^{3-} per mL of plasma.

3.3.3. Morphological changes in fishes organs

After blood sampling to determine ALP levels, fishes were sacrificed by ethically severing their vertebrae (physical methods of euthanasia by decapitation), according to the American Veterinary Medical Association (AVMA, 2013).

Subsequently, the weight and length of the animals were registered and the liver and gonads were removed and weighed to calculate gonadosomatic (GSI) and hepatosomatic (HSI) indexes (Fig. 3.7). GSI or HSI were calculated as the mass of gonads or liver/body mass $\times$ 100 (Brink et al., 2012; Carballo et al., 2005; Hallgren et al., 2009).

The sex was confirmed by microscopically (400× magnification) histological examination of the gonads as described by Kinnberg *et al.* (2007). The ovaries were characterized by the presence of oocytes and their nucleus, and the testes by the presence of spermatids and spermatozoids (Fig. 3.8). In all experiments, only *O. niloticus* males were analyzed.

Fig. 3.7: *The red arrow indicates the gonads and the yellow arrow indicates the liver of O. niloticus. The gonads and the liver were removed and weighed to calculate the GSI and HSI.*

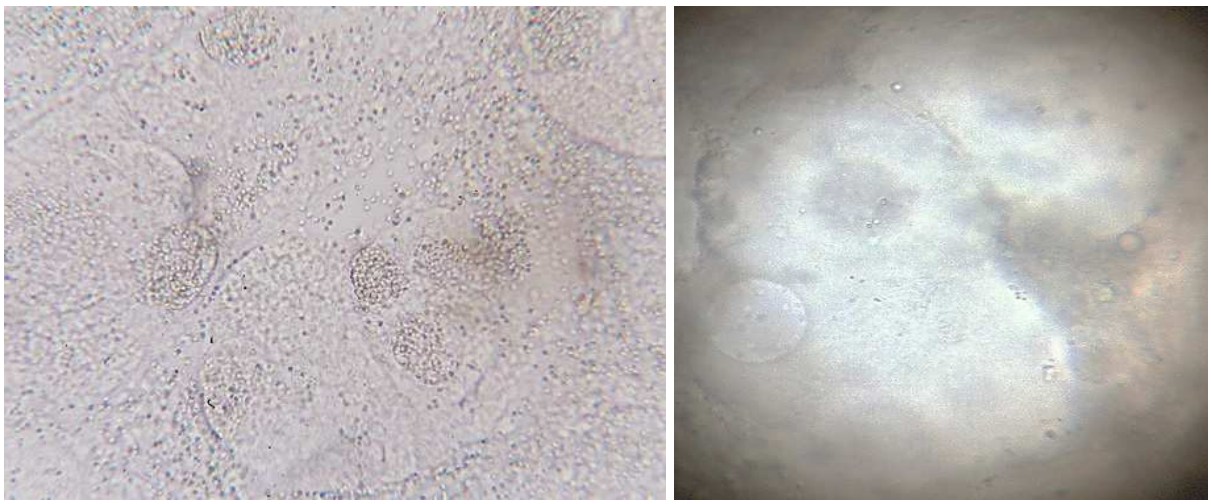


Fig. 3.8: *Histological images of the gonads of male and female of O. niloticus. In the left side, male gonads or testes represented by the spermatids and spermatozoids. In the right side, female gonads or ovaries characterized by the presence of oocytes and their nucleus.*

3.3.4. Genotoxicity assessment

The micronuclei (MN), used as genetic-damage indicators (Çavaş and Ergene-Gözükara, 2005) induced by contaminants in the environmental samples were counted in blood cells (erythrocytes) of exposed and not exposed Nile tilapia.

The slides were prepared by smearing one drop of blood on clean microscopic slides, left to air-dry at room temperature until completely dry, fixed in absolute methanol for 10 min, left to air-dry again at room temperature, and finally stained with 5 percent Giemsa for 15 min (Bücker and Conceição, 2012; Yadav and Trivedi, 2009).

A total of 1000 erythrocytes were examined for each specimen (five specimens per sample) under oil immersion at ×1000 magnification with a microscope (Carrasco et al., 1990). The frequencies of micronuclei (MN) were expressed per 1000 cells (‰). The slides were coded, randomized and scored by a single observer using a blind review (Rodriguez-Cea et al., 2003).

The MN observed were classified, according to the literature (Fenech et al., 2003; Carrasco et al., 1990) as: non-refractory particles, small nuclei with the same coloration as the nucleus cell and with a round or ovoid shape (Fig. 3.9).

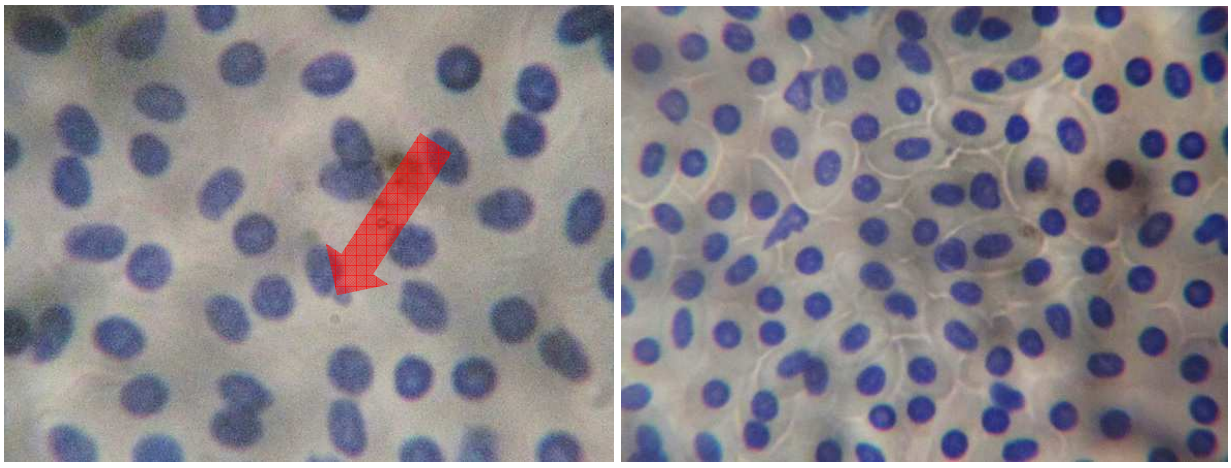


Fig. 3.9: *Blood cells (erythrocytes) slide of male Oreochromis niloticus, ×1000 magnification under oil immersion and stained with 5 percent Giemsa. The red arrow indicates the micronucleus (nuclear abnormalities) in response of genotoxicity effect.*

3.4. Algae inhibition growth test

This section is also divided in 2 parts (**Paper IV and V**). The first part (**Paper V**) was the application of the toxicity test with algae in industrial wastewater samples before and after the treatment with activated carbon and pH adjustment. This **Paper V** was part of the training and applicability of method conducted in the Linnaeus University laboratories, as a part of the exchange program between LNU and UERJ funded by the Swedish Foundation for International Cooperation in Research and Higher Education-STINT. The second part (**Paper IV**) was the evaluation of toxic effect by the presence of single and mixed estrogens and the interspecific interactions in response to the combination of two species of algae in the presence of single and mixed estrogens.

The algae toxicity tests (**Paper IV and V**) were performed based on the protocol established by Kaczala et al. (2011), Laohaprapanon et al. (2012) and OECD (2011), with duration of 96 h, under static conditional non-renewal technique. Four days before each experiments an initial growth culture was prepared for each specie individually (*D. subspicatus* in **Paper IV** and *P. subcapitata* in **Papers IV and V**) to ensure the exponential growth phase in the beginning of the test (Kaczala et al., 2011). A vertical laminar flow hood was used for manipulation of the cultures and samples.

In the **Paper V**, wastewater streams after cleaning/washing of machinery and indoor floors in a wooden floor industry were collected onsite, just after generation. Composite samples of these wastewaters were filtrated with 0.45 µm GF/C Whatman filter paper, prior to storage of samples at - 20 ºC for further used in batch sorption tests and toxicity assessment (Fig. 3.10). Two available commercial coal-based activated carbons (AC) were used for batch sorption study. Surface area of the activated carbon-AC_1 was 1,000 m^2/g (CAS number of 7440-44-0, 1.02184, Merck, Sweden) and the surface area of the activated carbon-AC_2 was 775 m^2/g (Highsorb 400, Norit).

A volume of 300 mL of wastewater was stirred with 150 g/L AC_1 and another 300 mL of wastewater was stirred with 240 g/L AC_2 in both cases in triplicate, under a constant agitation speed of 600 rpm. After 2 h of contact time, the wastewater samples were filtrated with 0.45 µm GF/C Whatman filter paper and characterized (soluble chemical oxygen demand, COD_s; dissolved organic carbon, DOC; phenol; formaldehyde; anionic and cationic surfactants). In the acute toxicity assay, the effects of four wastewater

samples were evaluated, including baseline wastewater (untreated-R), pH adjustment ($R_{7.5}$) and adsorption treated wastewater with AC_1 and AC_2.

In the **Paper V**, the test was carried out in 6 mL tubes filled with 2.5 mL of six concentrations series of each wastewater samples (100; 50; 12.5; 3.125; 0.78 and; 0.195%). All dilutions were tested in triplicates. Negative and positive controls using ultra-purified water and potassium dichromate (2 mg/L $K_2Cr_2O_7$) were conducted in parallel with the test samples. The test tubes were inoculated with 2.5 mL aliquots of stock culture of algae containing an initial density of 2×10^4 cells/mL. Therefore, the final cell density in each tube was 10^4 cells/mL (2.5 mL sample + 2.5 mL algae solution). The vials covered with Parafilm M were randomly arranged in racks (Fig. 3.10) (Kaczala et al., 2011). The assays were carried out under the same conditions used to keep algae cultures. After 96 h, growth responses of algae were measured based on chlorophyll concentration, which was extracted in 99% ethanol for 8 h from the 0 h and 96 h samples. The growth rate and the percentage of inhibition were calculated according to equations previously established by Laohaprapanon et al. (2012).

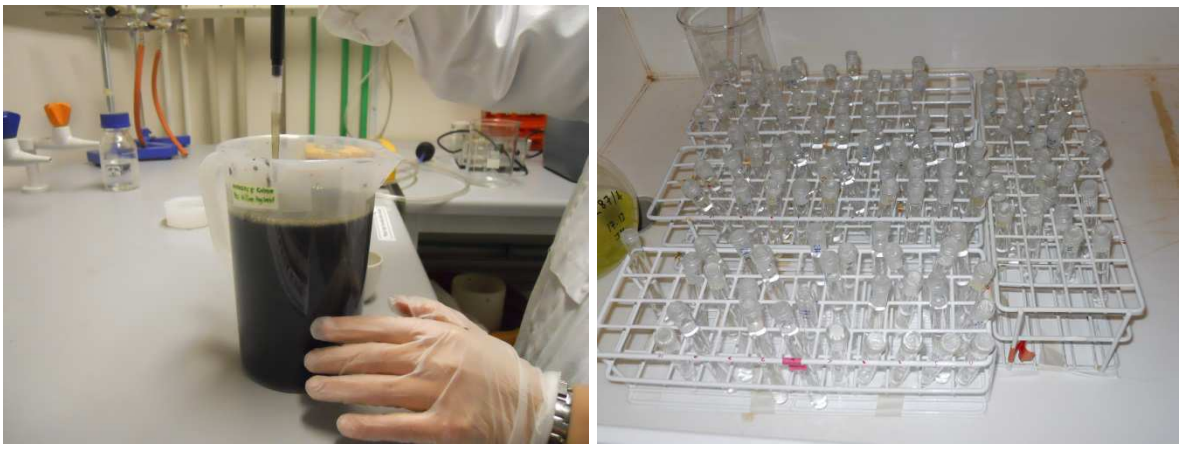


Fig. 3.10: *Wastewater generated by cleaning/washing of machinery and indoor floors in a wooden floor industry after filtration and before the sorption study. The vials covered with Parafilm M were randomly arranged in racks.*

In the **Paper IV**, algae toxicity tests were performed with spiked water (Milli-Q water) contaminated with two natural estrogenic hormones (E2 and E1), one synthetic estrogenic hormone (EE2) and the combination of them (E1:E2:EE2) in the proportion 1:1:1. The tests were carried out in 6 mL tubes (in triplicates) filled with 2 mL of spiked (Milli-Q) water and 2 mL of the stock culture of each alga to yield the following

concentrations: 0 (control), 0.01, 0.03, 0.1, 0.31, 1.0 and 3.2 µg/mL of estrogen. In order to choose the initial estrogens concentrations, the characteristics of the experimental design such as single initial dose and non-renewal conditions, as well as the available information about the half-life of the estrogens (Xu et al., 2012) were taken into consideration. The cell density was 10^4 cells/mL. The tests of the combined species (S+) followed the same procedure, but the initial cell density per species was 10^2 cells/mL to ensure the same final density of 10^4 cells/mL. A solvent control (acetone) at the highest concentration used and a positive control with potassium dichromate (VI) ($K_2Cr_2O_7$) were included. All tubes were randomly arranged in tube racks (Fig. 3.11) (Kaczala et al., 2011). The assays were carried out under the same conditions used to keep algae cultures. To avoid algae sedimentation, all tubes were hand-mixed twice per day during the test (Kaczala et al., 2011). Replicates were maintained in parallel for pH monitoring, to which glass electrodes could be introduced without interfering with the bioassays. Algal growth was measured via *in vivo* chlorophyll fluorescence and algal counting. *In vivo* chlorophyll fluorescence was measured three times for each tube using a SpectraMax M3 Multimode Reader (Molecular Devices Inc.) (Fig. 3.11) after 0, 24, 48, 72 and 96 h of exposure time (Kaczala et al., 2011). The average specific growth rate and the inhibition rate were based on chlorophyll fluorescence and calculated according to OECD (2011) and Kaczala et al. (2011).

Fig. 3.11: *Algae inhibition growth test in D. subspicatus and P. subcapitata (single and combined) exposed to hormones spiked water for 96 h. Vials randomly arranged in racks.*

After 96 h (**Paper IV**), approximately 2 mL of each sample were fixed in 1% of glutaraldehyde to further measure the algal density and morphology using a light microscope (100 x magnifications) and Neubauer's chamber. The morphology was only

examined for *D. subspicatus*, which is known to present a highly variable morphology (Kaur et al., 2012) that can range from a unicellular appearance (most common) to a two-celled coenobium, four-celled coenobium and even more than four-celled coenobium (Fig. 3.12) appearance when exposed to stressed conditions (Hessen and Donk, 1993; Lürling, 2006, 1998).

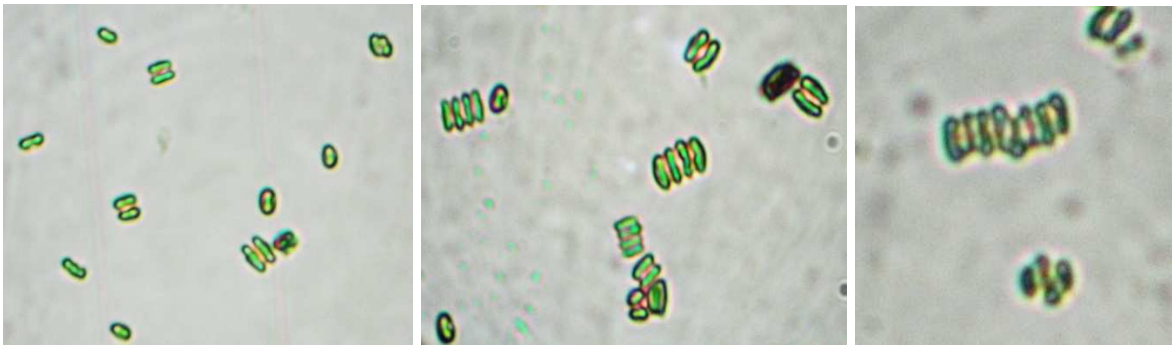


Fig. 3.12: *Single, two, four and eight coenobium formation of Desmodesmus subspicatus observed at the light microscope (400 x magnification).*

3.5. Physical and Chemical Analyses

The physical and chemical variables analyzed (**Papers I, II, III** and **IV**) according to the American Public Health Association (APHA 2005) were: pH; dissolved oxygen (DO); chemical oxygen demand (COD); temperature; nitrite (NO_2^-); nitrate (NO_3^-); ammonia (N-NH_4^+); and volatile suspended solids (VSS). Total phosphorus (TP) was analyzed (**Paper I**) according to the Hach method 1020 TNTplus™ 843 (approved by the United States Environmental Agency). In the **Paper V**, soluble chemical oxygen demand (COD_s), dissolved organic carbon (DOC), phenol, formaldehyde, anionic and cationic surfactants were analyzed using ready cuvette tests (Hach-Lange, Germany).

3.6. Hormones

Three estrogens were used in the experiments of the **Papers II and IV**: E1 [estrone (3-hydroxy-1,3,5(10)-estratrien-17-one), (≥99 percent, Sigma, CAS Number: 53-16-7)]; E2 [17β-estradiol (3,17β-dihydroxy-1,3,5(10)-estratriene) (≥98 percent, Sigma, CAS Number:

50-28-2)]; and EE2 [17α-ethinylestradiol (17α-ethynyl-1,3,5(10)-estratriene-3,17β-diol) (≥98 percent, Sigma, CAS Number 57-63-6)]. In the **Paper II** each estrogen was dissolved in ethanol (≥99 percent) and in **Paper IV** was dissolved in acetone (≥98 percent) and in all cases were individually added to spiked or stock solutions (Larsson et al., 1999).

3.7. Analysis by GC-MS

In order to compare nominal with measured (actual) concentrations, a preliminary study with three initial concentrations in the **Paper II** (0.016; 2.0 and 50 µg/L for E1, E2, and EE2) and in the **Paper IV** (5; 50 and 100 µg/L for E1, E2, EE2) in triplicates, following the procedure previously described (Hallgren et al., 2012; Souza et al., 2013). The differences in all cases were found to be below 6%. Based on this preliminary study, nominal concentrations were used in the next experiments.

The measurement of the actual concentration of each stock solution as well as the previous assays (500 mL) were performed using solid phase extraction (SPE) followed by gas chromatography-mass spectrometry (GC-MS) analysis (Fig. 3.13). The SPE were performed using Agilent Bond Elut C-18, 500 mg, 3 ml cartridges previously conditioned (Ternes et al., 1999). The samples, adjusted to pH 3.0, percolated at a flow rate of 10 mL/min (vacuum manifold) (Fig. 3.13). The samples were eluted in 2 x 2mL acetone; evaporated to dryness using a gentle stream of nitrogen; subjected to chemical derivatization (50µL of BSTFA) (Migowska et al., 2012); and analyzed by GC-MS. The estrogens recovery in the SPE was 80-85%. GC–MS analyses were performed using a 7890A Agilent GC coupled to 5975C triplo axis detector of MS and with a HP-5MS (Agilent Technologies) capillary column (30 m, 0.25 mm I.D., 0.25 µm film thickness). The oven temperature program was 40 °C for 1 min, from 40 °C to 80 °C at 20 °C/min, from 80 °C to 140 °C at 5 °C/min, from 140 °C to 200 °C at 100 °C/min and finally 1 min at 200 °C. Helium was the carrier gas at a constant flow rate of 0.93 mL/min. The injector temperature was 250 °C and the detector was 230 °C. Injections (1µL) were performed in splitless injector mode. Quantitative analysis was based on the peak areas of the quantitative ions recorded in SIM mode (342, 416, 425 m/z for E1, E2, EE2, respectively). Peak area ratios of samples were computed using Agilent Technology Chemstation

software. Calibration curves (0.005-50 µg/L) were generated using linear regression analysis with all values weighted equally. Detection limits and quantification limits were determined at a signal-to-noise ratio > 3 and in the concentration of 5.0 ± 0.1 ng/L for E1, E2 and EE2, respectively.

According to the laboratory quality control procedures, the calibration curve was valid for one month. With each set of analysis, a solvent blank and a 5.0 ng/L standard solution (lowest point of the calibration curve) were run in sequence to check possible background contamination, peak identification and quantification. The maximum standard deviation accepted was 15%. EE2 was used as internal standard for E2 and E1, and E2 was used for EE2 (Rose et al., 2002).

Fig. 3.13: *GC-MS Gas Chromatography (7890A Agilent) coupled to mass spectrometry (5975C triplo axis detector). Solid phase extraction (SPE) in an Agilent Bond Elut C-18, 500 mg, 3 ml cartridges.*

3.8. Statistical Analysis

GraphPad Prism (v. 6.03 for Windows, GraphPad Sofware, La Jolla California, USA) was used for the statistical analyses in the **Papers I**-**V**.

In **Paper I**, for comparison among means, one-way ANOVA, followed by a post-hoc Tukey's multiple comparison test was applied ($p < 0.05$). In **Paper II and III**, differences were tested for significance ($p < 0.05$) with Kruskal-Wallis followed by Dunn's multiple comparisons test and Mann-Whitney U test. In **Paper IV and V**, the means and standard

deviations (s.d.) were calculated from triplicate samples. The EC_{50} (concentration that causes 50% reduction of algal growth compared to the negative control) was calculated using non-linear regressions of the chlorophyll measurements. In **Papers II, IV and V**, the lowest observed effect concentration (LOEC) and the no-observed-effect concentration (NOEC) were considered as the lowest concentration with statistically significant differences and the highest concentration without statistically significant differences when compared to the control, respectively.

4. RESULTS AND DISCUSSION

4.1. Decentralized engineered ecosystem (DEE)

The decentralized engineered ecosystem (DEE) achieved high efficiency in the domestic effluent treatment with satisfactory transparency with absence of offensive odor and color. The final effluent had the following physicochemical characteristic: DO, 3.15 ± 0.65 mg/L; pH, 7.11 ± 0.24; VSS, 13.7 ± 4.2 mg/L; NO_2, 0.26 ± 0.16 mg/L; NO_3, 1.72 ± 0.56 mg/L; NH_4-N, 18.8 ± 9.3 mg/L; COD, 36.3 ± 12.7 mg/L and; TP, 14.0 ± 2.5 mg/L. The results of thermotolerant coliforms monitoring could be divided in two: before the sand filter was installed, the treated effluent was released with 2,563 ± 1,642 CFU/100 mL (colony forming units) (removal of 99.99%, compared with the raw wastewater $1.58 \times 10^8 \pm 4.56 \times 10^7$CFU/100 mL); and after the sand filter was installed, the removal was 99.999% and 403 ± 219 CFU/100 mL of thermotolerant coliforms remained in the final outlet. According to the Brazilian regulation (Conselho Nacional do Meio Ambiente (CONAMA), 2011), such efficiency, based on natural processes only, was considered good. As 80% of the samples were found to have below 500 CFU/100 mL and the threshold limit is 4,000 CFU/100 mL, the quality was compatible with primary contact uses. Most of the results achived in the final effuent were under the thesthold limmits according to the Brazilian environmental regulation (CONAMA 430 2011) and the EU Urban Wastewater Treatment Directive (UWTD) (91/271/EEC) (Frost, 2009). However, the total phosphorus (TP) was not sufficiently removed, a matter that requires further investigation and better design with the inclusion of a low cost and simple TP removal unit.

During the monitoring period of the DEE and according to Zimmels et al. (2006) and Solano et al. (2004), it was observed that some factor could interfere in the efficiency of the system and in the final concentrations of the effluent: flow rate, system design, climate, wastewater characteristics, and the macrophytes used. This efficiency achieved by each unit was due to the design and strategy adopted for each unit and the combination of them (Table 4.1).

In the septic tank (ST) the upflow was responsible for the improvement of the efficiency removal rates, converting the organic matter in to biogas, and reducing the amount of sludge retained in the bottom of the tank (Moussavi et al., 2010).

In the submerged aerated filter (AF), the upflow and the support media favored the retention of suspended unattached biomass, as well as, a high population density and the slow growth of the microorganisms (such as nitrifying bacteria), in the less turbulent sections of the filter reducing the need for sludge recycling (Cannon et al., 2000; Morgan-Sagastume and Noyola, 2008). Those factor allied with the DO concentrations > 3 mg/L and the low organic load rate (0.7 kg BOD/m^3/day) were the key points for the high efficiency of the nitrification process (Cannon et al., 2000; Morgan-Sagastume and Noyola, 2008; Zhang et al., 1994).

Table 4.1: Pollution removal (in %) achieved by each treatment unit (based on inflow and outflow to the specific unit). Contribution of each unit for the overall removal is shown in % within parentheses. In each column, values marked with the same letter are statistically similar; values marked with different letters are statistically different ($p < 0.05$; $n = 18$). *(Table 3*, **Paper I***).*

Unit	**Inflow–outflow removal in % (contribution to the overall removal in %)**					
	VSS	**NO_2**	**NO_3**	**NH_4-N**	**COD**	**TP**
ST	48.6 (48.6)^{abc}	32.4 (32.4)^{a}	4.3 (4.3)^{a}	−5.5 (–5.5)^{ac}	35.4 (35.4)^{abc}	27.3 (27.3)^{a}
AF	−13.9 (−7.1)^{d}	−2592 (−1753)^{b}	−2448 (–2342)^{b}	43.9 (49.4)^{b}	57.7 (37.3)^{d}	15 (10.9)^{ab}
SD	35.2 (62.1)^{abc}	93.5 (93.5)^{a}	64.4 (64.4)^{a}	19.1 (10.7)^{c}	23.8 (6.5)^{abc}	n.a.
T1 & T2[1]	**62.1 (62.1)**	**93.5 (93.5)***	**64.4 (64.4)***	**52.1 (54.6)**	**79.2 (79.2)**	**38.2 (38.2)**
W1	11.5 (4.4)^{ac}	–45.4 (–2.9)^{a}	88.2 (31.4)^{a}	5.6 (2.5)^{a}	40.5 (8.4)^{ad}	1.0 (0.6)^{c}
AT	62.9 (21.1)^{ac}	7.4 (0.7)^{a}	−11.5 (–0.5)^{a}	22.4 (9.6)^{ab}	21.1 (2.6)^{bc}	14.5 (8.8)^{bc}
W2	44.9 (5.6)^{ac}	14.8 (1.3)^{a}	17.3 (0.8)^{a}	8.3 (2.8)^{a}	25.1 (2.4)^{c}	10.2 (5.3)^{bc}
W3	−3.8 (−0.3)^{bd}	44.5 (3.3)^{a}	7.7 (0.3)^{a}	1.1 (0.4)^{ac}	7.4 (0.5)^{bc}	3.2 (1.5)^{c}
T3[2]	**81.2 (30.8)**	**36.4 (2.4)**	**90 (32.1)**	**33.6 (15.2)**	**67.5 (14.0)**	**26.4 (16.3)**
Total Removal	**92.9**	**95.9**	**96.4**	**69.8**	**93.2**	**54.5**

*Note: [1] Primary and secondary treatment together; [2] Tertiary treatment; * Denitrification process occurring in the SD, after the increment by the nitrification process, of 94.5% (NO_2) and 96% (NO_3) in AF; n.a. = not analyzed.*

In the secondary decanter (SD), even that was over dimensioned, made the major contribution to the denitrification process and promoted sedimentation of the VSS.

The vegetated tanks (macrophytes W1, W2 and W3 and algae AT) had smaller contribution on the overall treatment; however they promoted an important additional removal of nutrients (N and P). The efficiency of the vegetated tanks was due to the combination of different strategies such as the flow (superficial flow, chicaned flow, vertical and horizontal sub superficial flow) and the species of macrophytes (*Eichhornia crassipes*, *Schoenoplectus sp.* and *Panicum cf. racemosum*) and algae naturally present. These strategies also include the microorganism growth, biofilm formation, filtration, volatilization, and sedimentation, and an additional contribution by the plant biomass growth. Another key factor was the optimized biomass growth and consequently the nutrient uptake were assured by the fortnightly harvest of the macrophytes and algae, thus maintaining steady growth and eliminating the limiting factor of space, leading to further space optimization and seeking a greater biomass production within it. In the studied DEE, an overall surface area of 8.2 m^2 (algae and the vegetated tanks together) was responsible for an average biomass growth rate of 34.24 $g/m^2/day$. The estimation of nutrients and carbon uptake based on values obtained from the literature for the same species/taxonomic groups are presented in Table 4.2.

In terms of nutrient uptake, the DEE designed in a multistage approach performed better than some previously described constructed wetlands with the same macrophytes (Greenway, 2005; Kavanagh and Keller, 2007; Lin et al., 2002; Perbangkhem and Polprasert, 2010). The efficiency obtained by the DEE system studied might be explained by suitable environmental conditions (climate, solar radiation) and frequent biomass harvesting, which optimized the space and kept the macrophyte growth within an optimum rate. The DEE overall uptake rates considering algae and vegetated tanks together with a total surface area of 8.2 m^2 were 2.71 g N, 0.38 g P, and 19.43 g C $/m^2/day$.

Table 4.2: Mass balance (C, N, and P) in the vegetated tanks (n=13). *(Table 4*, **Paper I***).*

Species	Tank surface area (m^2)	Biomass growth rate ($g/m^2/day$)		C, N, and P macrophyte uptake according to the literature ([a, b, c, d, e]) (mg/g DW)	C, N, and P estimated removal by macrophytes in the DEE** ($g/m^2/day$)	Estimated removal by other processes occurring in the tanks ($g/m^2/day$)	Total removal of C, N, and P by the tanks of the DEE ($g/m^2/day$)
Eichhornia crassipes (Pontederiaceae)	2.06	12.10	**N**	42.8[a]	0.52	9.52	10.04
			P	6.5[b]	0.08	0.04	0.12
			C	425[c]	5.14	22.27	27.42
Algae*	2.06	13.53	**N**	150[c]	2.03	1.48	3.51
			P	20[c]	0.27	1.38	1.65
			C	800[d]	10.82	1.46	12.28
Schoenoplectus sp. (Cyperaceae)	2.06	4.36	**N**	14.6[e]	0.06	1.27	1.34
			P	2.6[e]	0.01	0.98	0.99
			C	396[e]	1.73	6.22	7.95
Panicum cf. racemosum (Poaceae)	2.06	4.25	**N**	23.7[e]	0.10	0.25	0.35
			P	5.7[e]	0.02	0.26	0.28
			C	410[e]	1.74	0.67	2.41
Total biomass	8.24	34.24	**N**	-	2.71	12.52	15.23
			P	-	0.38	2.66	3.04
			C	-	19.43	30.62	50.05

*Note: *Tank freely colonized by several algae species not identified in this study; [a]Wang et al. (2002); [b]Costa and Henry (2010); [c]Entwisle et al. (1997); [d]Estimation based on C:N:P (50:8:1); [e]Greenway and Woolley (1999); ** Contribution by each macrophyte in each tank based on the removal rates of the parameters (mg d^{-1}).*

4.2. Ecotoxicity assays

4.2.1. ALP levels in fish plasma as indication of estrogenicity in water

Based on the experiments *1* and *2* (**Paper II)** and *3 to 5* **(Paper III**), *O. niloticus* was considered a versatile and resistant sentinel, suitable for monitoring programs with the results described in this section.

The ALP baseline level in plasma of non-exposed *Oreochromis niloticus* (Nile tilapia) males measured with the method improved by Hallgren et al. (2009) was lower (median value = 4.11 µg PO_4^{3-}/mL plasma; IQ_{25} = 3.38; IQ_{75} = 5.18, **Paper II**) than those of other species (males) described in the literature. The ALP baseline levels informed in the literature for other fish species are the following: 14 ± 10 µg/mL for *Carassius carassius* (Hallgren et al., 2009); 70 µg/mL for *Oncorhynchus mykiss* (Verslycke et al., 2002); 10.8 µg/mL for *Acanthopagrus latus* (Naderi et al., 2012); 83 ± 20 µg/mL for *Perca fluviatilis* (Mandiki et al., 2005); 239 µg/mL for *Pimephales promelas* (Kramer et al., 1998) and 37 ±4 µg/mL for *Anguilla anguilla* (Versonnen et al., 2004).

In *Experiment 1*, after 15 days of exposure in a 130-L aquarium, the ALP levels in males exposed to EE2 concentrations varying from 0.016 to 50 µg/L significantly increased from the median value of 6.75 µg of PO_4^{3-}/mL of plasma (IQ_{25} = 6.57; IQ_{75} = 8.03) to the median value of 1030 µg of PO_4^{3-}/mL of plasma (IQ_{25} = 942; IQ_{75} = 1096) respectively, and were significantly higher than the median value found in the control (4.44 µg of PO_4^{3-}/mL of plasma, IQ_{25} = 3.47; IQ_{75} = 5.91) (Fig. 4.1) (*Figure 1,* **Paper II**).

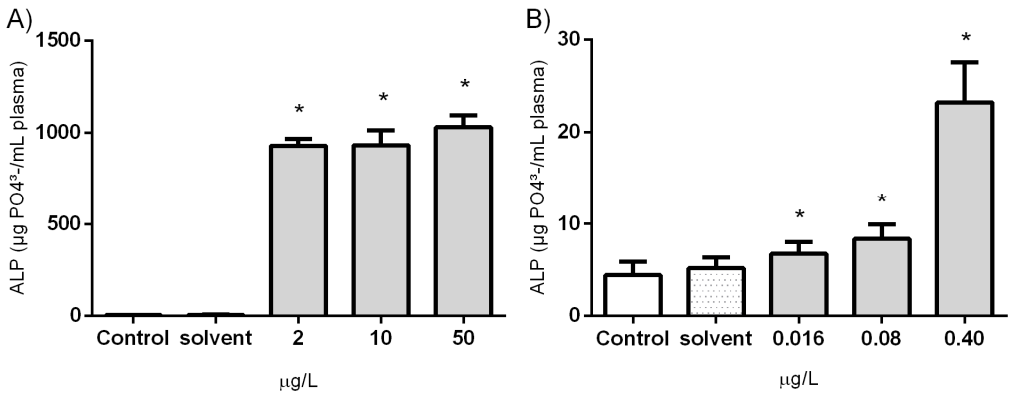


Fig. 4.1: *Alkali-labile phosphate (ALP) concentrations in the plasma of male O. niloticus after 15 days of exposure to control water and six concentrations of 17α-ethinylestradiol (EE2).*

In *Experiment 2*, after 15 days of exposure in 2-L aquarium, the ALP levels in the plasma of males exposed to 0.12 µg/L of EE2 (7.70 µg/mL, IQ_{25} = 5.84; IQ_{75} = 8.40), 0.12 µg/L of E2 (8.44 µg/mL, IQ_{25} = 8.26; IQ_{75} = 8.62) and 0.12 µg/L of E1:E2:EE2 (1:1:1) mixture (7.04 µg/mL, IQ_{25} = 6.39; IQ_{75} = 7.54); and 0.36 µg/L of E1 (7.74 µg/mL, IQ_{25} =

7.09; IQ_{75} = 8.25) were significantly higher than the control (4.11 µg PO_4^{3-}/mL plasma, IQ_{25} = 3.38; IQ_{75} = 5.18). However, no significant difference in the ALP level was found at lower concentrations than those for each estrogen and the combination of them compared with the control (Table 4.3) (*Figure 2*, **Paper II**).

An additive effect of the combined E1:E2:EE2 (1:1:1) on the ALP levels was observed due to the fact that most of concentrations of each individual estrogen had no significant difference in ALP levels in males compared in fishes exposed to the combination of them (1/3 concentration of each) at the same final concentration (Table 4.3). According to Thorpe et al. (2003) this interaction between contaminants must be taken into account because the ability of some chemicals to interact in an additive manner in some organisms at concentrations below their individual LOEC, emphasizes the need to consider the total load of these chemicals in water bodies.

The higher biologically potency of EE2 compared with E2 in terms of Vtg levels increase in plasma of male fishes was not confirmed as suggested by Thorpe et al. (2003). This result suggested that due to the similar mode of action (binding in the specific estrogen receptor) (Christen et al., 2010) those estrogens had similar potency in the tested fish species. Both estrogens (EE2 and E2) had three times higher potency than E1.

In all experiment the solvent control had no significant difference compared with the water control (F= 1.60; p = 0.25).

Table 4.3: ALP level (µg of PO_4^{3-}/mL of plasma) in *O. niloticus* males after 15 days of exposure with intermittent dose of the initial concentration in 2 L aquarium. Individual E1, E2, EE2 and combined (1:1:1) estrogens in different concentrations (µg/L), NOEC and LOEC values. (n = 5) *(Table 1*, **Paper II***).*

Estrogen	NOEC µg/L	LOEC µg/L	Control	0.012 µg/L	0.03 µg/L	0.12 µg/L	0.36 µg/L	0.60 µg/L
EE2	0.03	0.12	4.44	4.33^{aA}	4.99^{aA}	7.70^{aB}	8.40^{abB}	8.81^{aB}
E2	0.03	0.12		5.11^{aA}	5.35^{aA}	8.44^{aB}	8.86^{aB}	9.85^{aB}
E1	0.12	0.36		4.84^{aA}	5.04^{aA}	5.70^{bA}	7.74^{bB}	8.01^{bB}
Mixture	0.03	0.12		4.43^{aA}*	5.15^{aA}	7.04^{aB}	8.29^{abBC}	9.11^{aC}

*Note: NOEC: no observed effect concentration; LOEC: lowest observed effect concentration; *Due to operational limitations, the final total concentration of the mixture was 0.015 µg/L instead of 0.012 µg/L. Capital letter (A-D) compares rows (effect of each estrogen). Lower case letters (a, b) compares columns (effect of each concentration). Different letters means difference statistically significant (p < 0.05).*

Comparing the results of EE2 exposure of *Experiment 1 and 2*, in 130 L aquarium the lowest EE2 concentration that produced significantly higher ALP levels compared with the water control (LOEC) was 0.016 µg/L. However, in 2 L aquarium, the LOEC was found to be 0.12 µg/L of EE2. The LOEC in *Experiment 2* was therefore approximately 7.5 times higher than the LOEC observed in Experiment 1. To explain this result, the hypothesis was that the hormone load (mass of estrogen in each experimental unit) was the main independent variable or factor responsible for the alterations of ALP levels in the plasma of male fishes. The large differences in size between the aquarium used in *Experiment 1* (130 L) and in *Experiment 2* (2 L) resulted in large differences in terms of mass loads even for similar concentrations. Another factor to consider is the number of fishes per aquarium. Therefore, if the total load of EE2 in each experiment (including the water renewal) is divided by the number of exposed fishes (considering that they have similar weight) in terms of µg of EE2/fish a different view is uncovered (*Table 2*, **Paper II**). No significant differences were found between the ALP levels measured in fishes exposed to 0.016 µg/L (0.52 µg of EE2/fish) compared to 0.12 µg/L (0.72 µg of EE2/fish); and 0.08 µg/L (2.6 µg of EE2/fish) compared to 0.36 µg/L (2.2 µg of EE2/fish) (Fig 4.2 A and B). On the other hand, when the ALP levels in fishes exposed to similar concentrations of EE2 were compared (0.012 µg/L in 2 L and 0.016 µg/L in 130 L; 0.36 µg/L in 2 L and 0.40 µg/L in 130 L) significantly higher ALP levels were found in those fishes exposed to the higher loads (130 L aquarium) (Fig 4.2 C and D).

Issues related to the size of the experimental unit such as the aquarium volume is rarely addressed in the literature. The discussion about the relevance of load vs. concentration is back in **Paper VI**, where the authors highlight it as part of important factors to consider during the experimental design of ecotoxicology assays.

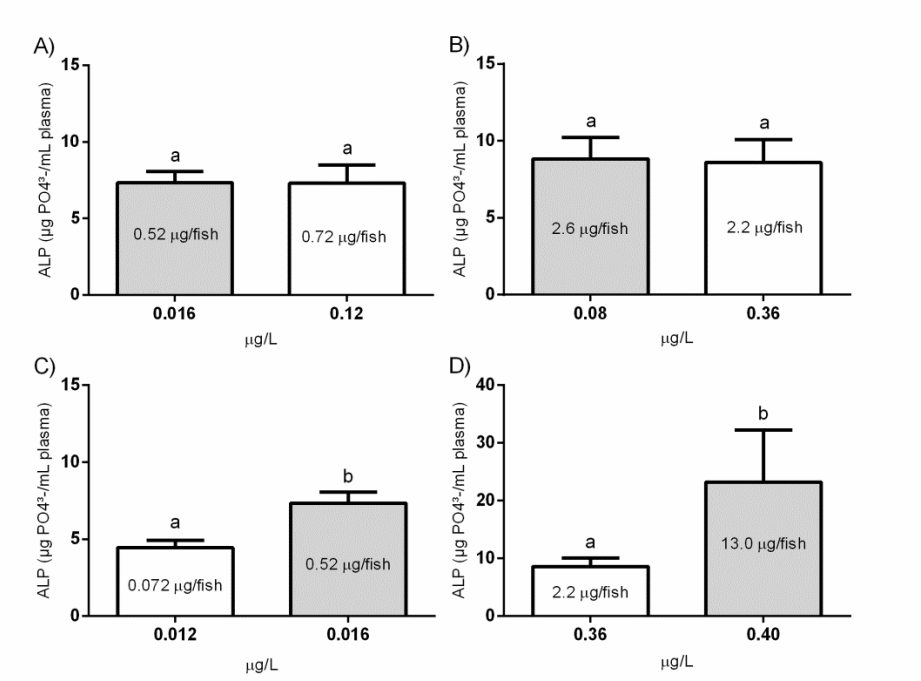


Fig. 4.2: *Alkali-labile phosphate (ALP) levels in* O. niloticus *males exposed to: (A and B) similar loads of EE2 (µg/fish) of EE2; (C and D) similar concentrations of EE2 (µg/L), but different loads, in 130 L aquarium (grey bars) and 2 L aquarium (white bars). Note: Different letters means difference statistically significant* ($p < 0.05$). (Figure 3, ***Paper II***).

After the application of the method modified with Crucian carp (Halgren et al, 2009) for determination of ALP plasma baseline levels in *O. niloticus* (**Paper II**) with determination of the lowest estrogen concentration (of single and combined, natural and synthetic estrogens) that produced significant increase of the ALP levels (LOEC), in *Experiments 3 to 5* (**Paper III**), the males of *O. niloticus* were exposed to environmental samples from largely distinct water environments. The results in terms of ALP levels were compared to the EE2 LOEC (0.12 µg/L) as a positive control as shown in *Experiment 2*, **Paper II** (Table 4.3 and Fig. 4.3). The synthetic EE2 was selected for comparisons based on the facts that: (i) it is more resistant than natural estrogens to biodegradation in natural water bodies (Jürgens et al., 2002); (ii) the EE2 half-life in water and sediments varies from 2 to 6 days (Ying et al., 2002), which fitted with the experimental design and planning.

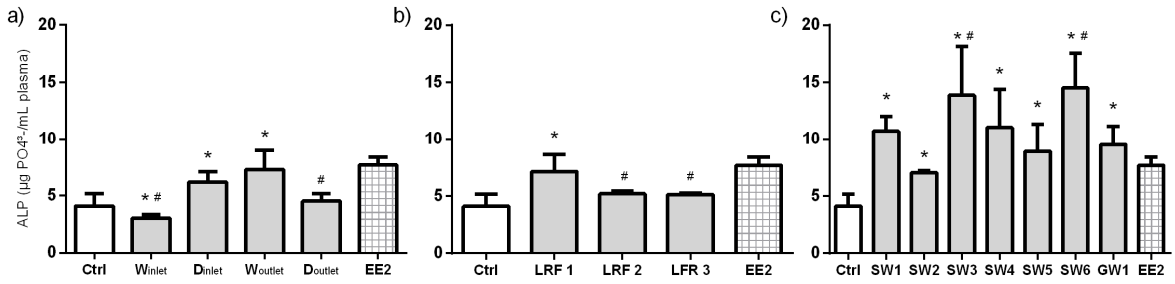


Fig. 4.3: *Alkali-labile phosphate (ALP) concentrations in the plasma of male O. niloticus after 15 days of exposure to control water; wastewater treatment plants; Decentralized Engineered Ecosystem; urban lagoon; contaminated site with gasoline samples; and 0.12 µg/L of 17α-ethinylestradiol (EE2).*

In *Experiment 3*, the ALP levels in plasma of fishes exposed to the WWTP inlet samples (Fig. 4.3) (*Figure 1*, **Paper III**) were significantly lower (median = 3.06 µg PO_4^{3-}/mL plasma, IQ_{25} = 2.76; IQ_{75} = 3.36) than the control (median = 4.11 µg PO_4^{3-}/mL plasma, IQ_{25} = 3.38; IQ_{75} = 5.18). One hypothesis not checked in the present study is that the presence of toxic contaminants impairs the liver functioning and depletes the ALP production. In contrast, the opposite occurred in fishes exposed to the outlet samples (7.29 µg PO_4^{3-}/mL plasma, IQ_{25} = 5.60; IQ_{75} = 8.98), and two hypothesis still to be tested were formulated to explain that: (i) occurrence of removal/reduction of the toxic substances during the treatment that cause the Vtg inhibition in the raw sewage; (ii) formation of new compounds (metabolites) during the treatment process, which act as endocrine disruptors (Fent et al., 2006; Heberer, 2002). No significant difference was found in ALP levels of fish exposed to the WWTP outlet and to 0.12 µg/L of EE2 (Fig. 4.2). In other words, the WWTP outlet had estrogenicity equivalent to the EE2 LOEC.

The ALP level in fishes plasma exposed to the inlet of the Decentralized Engineered Ecosystem (DEE) (median = 6.19 µg PO_4^{3-}/mL plasma, IQ_{25} = 5.75; IQ_{75} = 7.01) (Fig. 4.3) (*Figure 2,* **Paper III**) were significantly higher than that in the control (4.11 µg PO_4^{3-}/mL plasma, IQ_{25} = 3.38; IQ_{75} = 5.18). However, ALP in the outlet (median = 4.52 µg PO_4^{3-}/mL plasma, IQ_{25} = 4.28; IQ_{75} = 5.17) was similar to the control. The ALP of fishes exposed to the inlet was similar to the ALP of fishes exposed to the EE2 LOEC concentration (0.12 µg/L of EE2). In other words, the DDE inlet presented estrogenicity equivalent to the EE2 LOEC. On the other hand, the estrogenicity of the DEE outlet was significantly lower than

the EE2 LOEC (Fig. 4.2). These results suggested that the decentralized engineered ecosystem was able to reduce the endocrine disruption effect in the final effluent.

Large differences in the compositions of both sewages are likely to occur, since the DEE treat the domestic wastewater generated by 12 inhabitants and the WWTP treats the wastewater from 110,000 inhabitants in an urban area with several industrial activities.

There were also differences in treatment processes, as the municipal WWTP contained preliminary, primary, and secondary treatments, whereas the DEE comprised preliminary, primary, secondary and tertiary treatments, with the final steps formed by vegetated tanks.

Algae and aquatic macrophytes have been described as capable to remove estrogens from water (Gray and Sedlak, 2005; Shi et al., 2010), through sorption on alga biomass and macrophyte roots (hydrophobic surfaces), followed by biodegradation and biotransformation carried out by microorganisms and plant uptake (Song et al., 2009).

In the *Experiment 4*, males of Nile tilapia were exposed to water samples obtained from 3 sampling points in an urban lagoon (Rodrigo de Freitas lagoon, LRF) with wide variation of salinity (4 to 16‰). The ALP plasma concentrations increased significantly in fishes exposed to water samples obtained at LRF1 (median = 7.14 µg PO_4^{3-}/ mL plasma, IQ_{25} = 6.80; IQ_{75} = 8.62) compared to the control (median = 4.11 µg PO_4^{3-}/mL plasma, IQ_{25} = 3.38; IQ_{75} = 5.18). The ALP level at LRF1 was not significantly different from the EE2 ALP level (Fig. 4.3) (*Figure 3*, **Paper III**). The LRF1 sample was directly affected by the discharge of channeled rivers. Due to the densely occupied upper part of the drainage basin and the fact that many of these households have no connection to the sewerage network, it is reasonable to believe that the increase in ALP levels observed is associated with the presence of estrogens and/or xenoestrogens discharged into the river.

Regarding the other two sampling points LRF2 (located at the opposite side of the seawater inflow, median = 5.19 µg PO_4^{3-}/mL plasma, IQ_{25} = 5.05; IQ_{75} = 5.45) and LRF3 (located at the opposite side of the river discharge point, median = 5.14 µg PO_4^{3-}/mL plasma, IQ_{25} = 4.46; IQ_{75} = 5.25), no significant differences were found in the ALP levels compared with the control. The ALP levels in the LRF2 and LRF3 samples were significantly lower than the EE2.

The results illustrate the applicability of ALP level as a useful biomarker for detecting the presence of domestic sewage in water bodies. It is important to highlight that

the present investigation did not aim to conduct a complete environmental assessment of the urban lagoon, but just to evaluate the applicability of the modified ALP quantification method using *O. niloticus*, which is resistant to a wide range of salinity (4 to 16‰), among other adverse conditions.

In *Experiment 5*, males of Nile tilapia were exposed to surface water samples from a gasoline-contaminated marshland (SW) (lentic environment) and from a groundwater well (GW) in the same area. The chemical characterization of six-surface water (SW) and one groundwater (GW) sampling points is shown in Table 4.4. Significant increase was observed in the ALP levels in plasma of fishes exposed to all sampling points (Table 4.4) compared to the control and to the estrogen EE2 LOEC (Fig. 4.3) (*Figure 4*, **Paper III**). It was concluded that *O. niloticus* was particularly useful as bioindicator for estrogenicity when exposed to gasoline-contaminated waters samples (without dilution). Additionally, the ALP values in fishes exposed to these samples were significantly higher than those obtained from animals exposed to samples from the wastewater treatment plants (WWTP and DEE) and from the urban lagoon (LRF). The endocrine disruption effect and genetic responses of spiked water with diesel oil and gasoline has been previously described in the literature for the fish species *Anguilla anguilla* L. (Pacheco and Santos, 2001). However, to demonstrate this effect in highly contaminated real samples, resistant species such as *O. niloticus* are needed.

Table 4.4: Chemical characterization and biomarkers results of water of six marshland (SW1 to SW6) and one groundwater (GW1) sampling points from a gasoline-contaminated site. *(Table 1*, **Paper III***)*.

	SW1	**SW2**	**SW3**	**SW4**	**SW5**	**SW6**	**GW1**
BTEX (μg/L)	63.7	224.1	64.1	101.3	15.0	2.2	60104
TPH-GRO (μg/L)	1833	1854	391.6	735.4	150	150	322513
PAH (μg/L)	1.7	3.1	0.4	6.48	0.4	0.4	29.9
Cu (μg/L)	179	34	4.5	29	67	27	4.5
Cr (μg/L)	41.0	5.0	5.0	5.0	5.0	22.0	5.0
Fe (mg/L)	192.9	287	22.3	31.8	15.3	136	0.7
P (mg/L)	3.9	1.6	0.3	0.4	1.0	1.4	0.01
Mn (μg/L)	562	639	310	531	201	1310	28
Zn (μg/L)	633	284	35	150	192	176	84
Mg (mg/L)	3.4	1.4	1.2	1.5	1.5	3.2	0.3
ALP (μg/mL)	10.7	7.0	13.9	11.1	8.9	14.5	9.5

	(5.6; 12.0)	(6.9; 7.2)	(9.1; 18.1)	(6.5; 14.4)	(6.2; 11.3)	(10.3; 17.5)	(8.7; 11.2)
HSI (%)	0.42 (0.39; 0.55)	0.28 (0.23; 0.29)	0.49 (0.45; 0.50)	0.36 (0.26; 0.39)	0.33 (0.28; 0.35)	0.45 (0.18; 0.50)	0.37 (0.27; 0.42)
GSI (%)	0.16 (0.15; 0.25)	0.18 (0.09; 0.22)	0.18 (0.13; 0.24)	0.31 (0.1; 0.33)	0.14 (0.13; 0.22)	0.15 (0.10; 0.19)	0.22 (0.18; 0.23)
MN (‰)	8 (7; 9)	3 (2; 3.7)	3.5 (3; 4)	4 (2; 5)	6 (5; 7.5)	5 (2; 6.2)	4 (2; 5)

ALP=Alkali-Labile Phosphate median values (IQ_{25}; IQ_{75}); HSI=Hepatosomatic Index; GSI=Gonadosomatic Index; MN=Micronuclei frequency.

4.2.2. Nile Tilapia as a bioindicator of aquatic pollution: Auxiliary biomarkers

After the *Experiments 1* and *2* (**Paper II**) it was clear that the auxiliary tools used to the evaluation of morphological biomarkers such as hepatosomatic index (HSI, 0.12 ± 0.7% in non-exposed fishes) and gonadosomatic index (GSI, 0.56 ± 0.29% in non-exposed fishes), are less sensitive indicator for the presence of estrogens in small loads (≤ 3.6 μg/fish) than ALP levels in plasma. However they have brought useful information when fishes were exposed to higher loads of estrogens (≥ 13 μg/fish) (Fig. 4.4). Nevertheless, GSI and HSI are closely related to the endocrine disruption effects, due to the fact that when the gonads are affected, disturbances in the estrogen production by the ovary occur, which might in turn reduce Vtg production by the liver (Verslycke et al., 2002). It is also important to highlight the inverse relationship between the GSI and HSI (Harries et al., 1996).

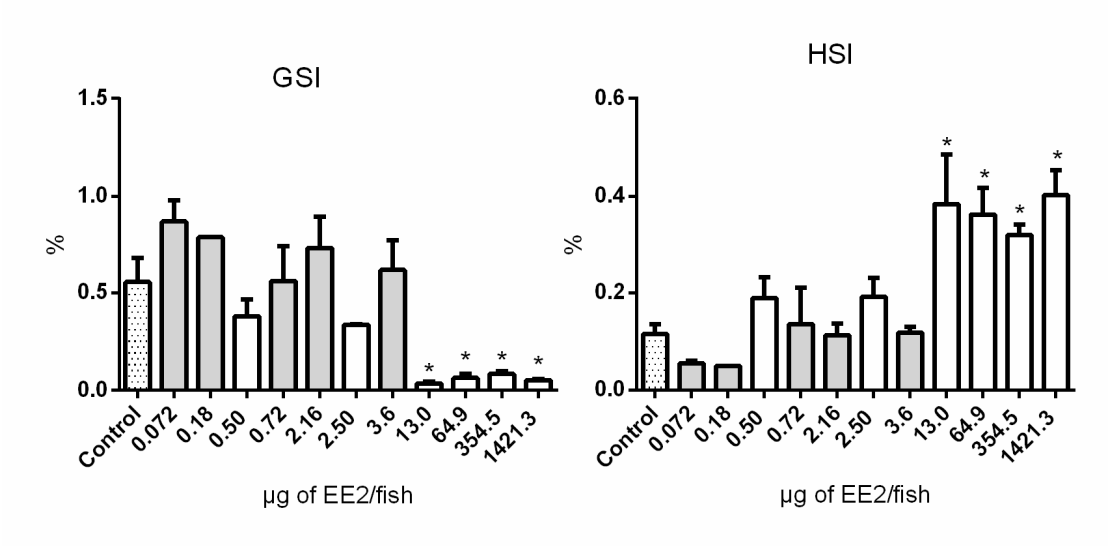


Fig. 4.4: *Morphological biomarkers hepatosomatic index (HSI) and gonadosomatic index (GSI) as an auxiliary biomarkers used to assess the effect of estrogens in water.*

In *Experiments 3, 4* and *5* (**Paper III**) fishes exposed to the water control samples (HSI median = 0.11 %, IQ_{25} = 0.07; IQ_{75} = 0.14; and GSI median = 0.56 %, IQ_{25} = 0.32; IQ_{75} = 0.58) were used to compare the results with the environmental samples (Fig. 4.5 and 4.6) (*Figures 3* and *4*, **Paper III**). The EE2 LOEC (0.12 µg/L of EE2, same applied in ALP level) was not applied in any case, due to the fact that no statistical differences were detected when compared to the water control (p = 0.64).

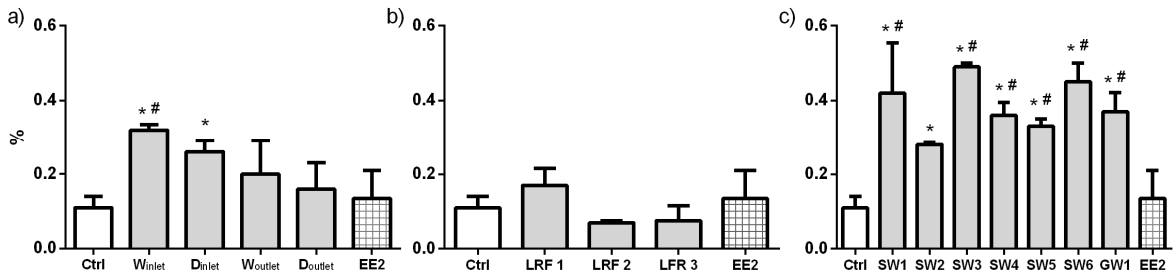
In *Experiment 3,* comparison with the control revealed a significant increase in the HSI of fishes exposed to both inlet samples (WWTP inlet median = 0.32 %, IQ_{25} = 0.30; IQ_{75} = 0.34; and DEE inlet median = 0.26 %, IQ_{25} = 0.24; IQ_{75} = 0.29). Fishes exposed to both WWTP and DEE outlet samples had their HSI not significantly different from the HSI of fishes exposed to the water control (WWTP outlet median = 0.20 %, IQ_{25} = 0.14; IQ_{75} = 0.29; and DEE outlet median = 0.16 %, IQ_{25} = 0.12; IQ_{75} = 0.23) (Fig. 4.5).

Fishes exposed to both inlet samples (WWTP inlet median = 0.22 %, IQ_{25} = 0.17; IQ_{75} = 0.27; and DEE inlet median = 0.23 %, IQ_{25} = 0.14; IQ_{75} = 0.28) had the gonads significantly less developed than those exposed to the water control. Similar to the results obtained for the HSI, the fishes exposed to both WWTP and DEE outlets had their GSI not significantly different from the GSI of fishes exposed to the water control (WWTP outlet median = 0.31 %, IQ_{25} = 0.28; IQ_{75} = 0.40; and DEE outlet median = 0.37 %, IQ_{25} = 0.34; IQ_{75} = 0.43) (Fig. 4.6).

In *Experiment 4,* fishes exposed to the water samples obtained at the LRF1, LRF2 and LRF3 sampling points had no significant differences when compared to the controls for both biomarkers HSI and GSI. The HSI observed were: LRF1 median = 0.17 %, IQ_{25} = 0.15, IQ_{75} = 0.21; LFR2 median = 0.07 %, IQ_{25} = 0.05, IQ_{75} = 0.08; and LRF3 median = 0.08 %, IQ_{25} = 0.05, IQ_{75} = 0.11. The GSI observed were: LRF1 median = 0.34 %, IQ_{25} = 0.16, IQ_{75} = 0.44; LRF2 median = 0.59 %, IQ_{25} = 0.33, IQ_{75} = 0.73; and LRF3 median = 0.75 %, IQ_{25} = 0.62, IQ_{75} = 0.89 (Figures 4.5 and 4.6).

In *Experiment 5*, water of all surface water and groundwater sampling points from the gasoline-contaminated marshland significantly increased the HSI (medians ranging from 0.18 to 0.55 %) (Fig. 4.5, Table 4.4) and decreased the GSI (medians ranging from 0.10 to 0.30 %) (Fig. 4.6, Table 4.4) in exposed fishes, compared to those exposed to the water control.

The positive control (EE2 LOEC equivalent to 0.12 µg/L of EE2), and two other contaminated samples (WWTP outlet and LRF1) had no effect on the HSI and the GSI indexes, but these same samples provoked detectable effects on the biomarker ALP. Because both organs (liver and gonads) are directly related to both toxicity in general and the endocrine disruption effects, it is recommended to check the inverse relation of HSI and GSI (Harries et al., 1996) in native (wild fishes) or in longer exposure time assays.



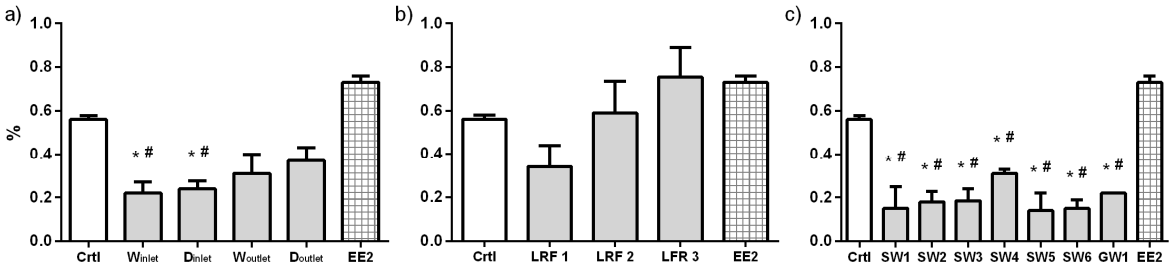


Fig. 4.5: *Hepatosomatic Index (HSI) (%) of male O. niloticus after 15 days of exposure to control water; wastewater treatment plants; decentralized engineered ecosystem; urban lagoon; contaminated site with gasoline samples; and 0.12 μg/L of 17α-ethinylestradiol (EE2).*

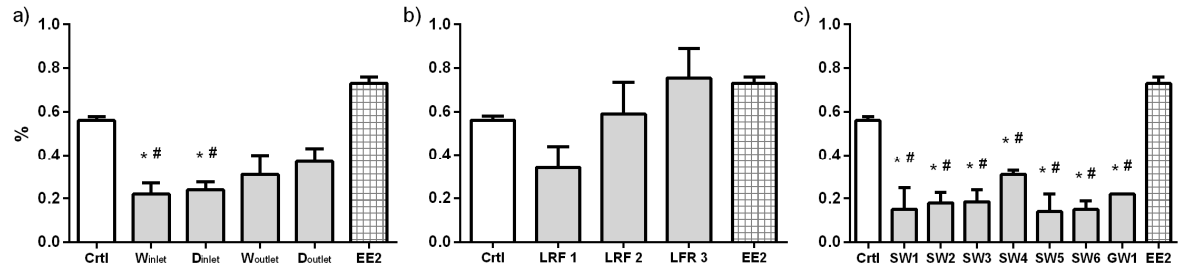


Fig. 4.6: *Gonadosomatic Index (GSI) (%) of male O. niloticus after 15 days of exposure to control water; wastewater treatment plants; decentralized engineered ecosystem; urban lagoon; contaminated site with gasoline samples; and 0.12 μg/L of 17α-ethinylestradiol (EE2).*

The micronucleus frequency (MN) proved to be a very sensitive biomarker of the presence of (individual or combined) natural or synthetic estrogens (*Experiment 2*, **Paper II**) in fishes exposed to loads as low as 0.07 µg/fish (0.012 µg/L) of all tested estrogens (E1, E2, EE2) as well as the combination of them (E+) compared to the levels in fishes exposed to the water control (0 ‰, $IQ_{25} = 0$; $IQ_{75} = 0$). In all tested concentrations (0.012 to 0.6 µg/L) the MN frequency varied from 2‰ to 6‰ for EE2; from 2‰ to 4‰ for E2; from 2‰ to 5‰ for E1; and the combination of estrogens the frequency was from 2‰ to 4‰. However, no significant difference was found among different estrogens at similar concentrations. Sponchiado et al. (2010) showed that under both acute and chronic exposure, estrogens can produce mutagenic and genotoxic effects on the blood cells of *O. niloticus*.

Most of the results obtained with Experiments 3 to 5 regarding MN frequency corroborated with the ALP results. MN frequency test proved to be a useful tool for assessment of genotoxicity in varied environmental samples with also estrogenicity action, using *O. niloticus* (Fig. 4.7).

In Experiment 3, the MN frequency in fishes exposed to the inlet (WWTP inlet median = 3 ‰, IQ_{25} = 3; IQ_{75} = 4.5; and DEE inlet median = 3 ‰, IQ_{25} = 2; IQ_{75} = 4) and in fishes exposed to the outlet (WWTP outlet median = 2 ‰, IQ_{25} = 2; IQ_{75} = 3.25) showed a significant increase in MN frequency compared with the water control (control, 0 ‰; IQ_{25} = 0; IQ_{75} = 0) (Fig. 4.7) (*Figures 2*, **Paper III**). No significant differences were found when these MN frequencies were compared to the MN frequency provoked in fishes exposed to EE2 LOEC (median = 3 ‰, IQ_{25} = 3; IQ_{75} = 4). Regarding fishes exposed to the DEE outlet samples (median = 1 ‰, IQ_{25} = 0; IQ_{75} = 1) no significant difference was found from those exposed to the water control.

In *Experiment 4*, the MN frequency in cells of fishes exposed to water control from all sampling points (LRF1 median = 1.5 ‰, IQ_{25} = 1; IQ_{75} = 2; LRF2 median = 2 ‰, IQ_{25} = 1; IQ_{75} = 2; and LRF3 median = 2 ‰, IQ_{25} = 1.25; IQ_{75} = 2) were significantly higher and significantly lower than the MN in cells of fishes exposed to the water control and to the EE2 LOEC, respectively. According to Jiraungkoorskul et al. (2007) and Rodriguez-Cea et al. (2003), changes in the MN frequency in blood cells of fishes is a good indicator of structural genomic changes to be used as bioassay for water bodies biomonitoring.

In Experiment 5, all water samples tested produced significantly higher MN frequency (medians from 2 ‰ to 9 ‰) compared to those of the water control (Fig. 4.7, Table 4.4). The MN frequency of fishes exposed to SW1 was even higher (SW1 median = 8 ‰, IQ_{25} = 7; IQ_{75} = 9) than those in the EE2 LOEC. In all other water samples, the MN frequency had no statistical difference from the MN frequency in fishes exposed to EE2 LOEC. The higher MN frequency observed in *O. niloticus* exposed to the marshland and groundwater samples, is partly explained by the naturally occurring high contents of metals in most of sampling points (due to the soil properties in the area) but is likely to be mostly related to the high concentrations of BTEX, THH-GRO and PAH in some samples, as the main root cause of nuclear abnormalities observed in cells of exposed fishes.

After *Experiments 1 to 5* it is possible to state that the combination of several biomarkers (ALP, GSI, HSI and MN) is a useful way to explore one bioindicator such as

fishes (Jiraungkoorskul et al., 2007), especially when the contaminants of interest have high liposolubility, high capacity to bioaccumulate in lipid structures and potential to generate effects at the cell nuclear level (Sponchiado et al., 2010). According to Sponchiado et al. (2010) accumulation increases the chances to generate effects in nuclear level.

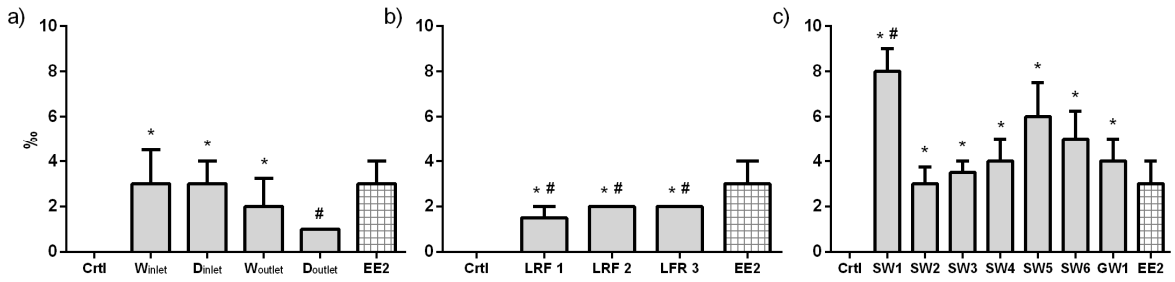


Fig. 4.7: *Micronuclei frequency (MN) (‰) in blood cells of male O. niloticus after 15 days of exposure to control water; wastewater treatment plants; decentralized Engineered Ecosystem; urban lagoon; contaminated site with gasoline samples; and 0.12 μg/L of 17α-ethinylestradiol (EE2).*

4.2.3. Unicellular microalgae as a bioindicator of toxicity after industrial wastewater treatment with activated carbon

Since the wastewater treatment using sorption (in batch mode) “per se” is not the main focus of the present thesis, the results are presented in a summarized form (more information is found in **Paper V**). Both activated carbons satisfactorily removed large part of the organic pollutants in the industrial wastewater (Table 4.5) (*Tables 1 and 2*, **Paper V**).

In toxicity assays, as expected, higher amounts of chemical substances measured as COD in the wastewater, corresponded to higher toxicity for the bioindicator. It is not yet clear which compound(s) in the wastewater were responsible for the toxicity observed, since the composition of this type of industrial wastewater is very complex. However, there are some evidences suggesting that not-measured organic constituents (such as formaldehyde, phenol and surfactants) found in the studied wastewater are potential inhibitors of algae growth (de Melo et al., 2013; Lürling, 2006; Tisler and Zagorc-Koncan,

1997; Torres et al., 2008). Surfactants can denature and bind proteins in the cell membrane and consequently affect the permeability to nutrients and chemicals (Torres et al., 2008) whereas formaldehyde is a well-known disinfectant.

Table 4.5: Untreated wastewater generated after cleaning/washing of surfaces and machinery in a wooden floor industry, and the removal rates after the batch sorption study with two types of activated carbon (AC_1 and AC_2).

Parameter	**Wastewater**	**Removal rates (%)**	
	(mg/L)	**AC_1**	**AC_2**
pH*	4.7	6.8	8.6
COD_s	8,790	77.8	79.6
DOC	5,096	75.1	76.5
Phenol	7.1	97.4	94.1
Formaldehyde	202	77.2	74.6
Anionic surfactants	1.6	93.1	93.1
Cationic surfactants	0.73	87.7	84.9

*Note: *all mean values are presented in mg/L, except pH.*

In the present study, at low wastewater concentrations, stimulatory effect was observed and wastewater with pH adjustment showed the highest stimulatory effect at concentrations of 0.78% and 0.019% v:v. However, after the wastewater treatment with adsorption and consequent removal of large portion of organic carbon sources from the wastewater, the stimulatory effect on algae growth was reduced. This means that in addition to sunlight, the algae can use external carbon source for cell energy. These properties are particularly relevant for bioremediation purposes (Subashchandrabose et al., 2013).

In addition to organic compounds in wastewater, the pH also plays an important role on algae growth. In such a complex industrial wastewater, changes in pH can affect different toxicity mechanisms to aquatic organisms, since the bioavailability of some pollutants (i.e. metal) are pH-dependent (Rai et al., 1994). As it is observed in the present study, when the wastewater pH was raised from 4.7 to 7.5 (Table 4.6), the EC_{50} increased from 0.85% to 4.0% (in other words, the toxicity was reduced to a large extent).

Table 4.6: EC_{50} and NOEC values of untreated wastewater without (R) and with ($R_{7.5}$) pH adjustment and wastewater treated with activated carbon AC_1 and AC_2; r^2 values (under parenthesis) obtained in toxicity assays with *P. subcapitata. (Tables 3*, **Paper V***).*

	R	**$R_{7.5}$**	**AC_1**	**AC_2**
$EC_{50,96h}$ (% v:v)	0.85	4.0	4.4	6.9
	(0.95)	(0.97)	(0.93)	(0.94)
$NOEC_{,96h}$ (% v:v)	0.049	0.78	1.56	1.56
	(0.88)	(0.86)	(0.90)	(0.89)

Carbon adsorption removed great amount of organics (about 75% of COD_s) from the studied wastewater but it was not efficient in removing toxicity, as suggested by the low value of EC_{50} (4.4% after AC_1 and 6.9% after AC_2 treatments). Moreover, the statistical analysis using one-way ANOVA (Table 4.6) shows that the EC_{50} of wastewater treated by ACs was not significantly different from the EC_{50} of the wastewater with adjusted pH ($p < 0.05$).

Regarding the NOEC value, pH adjustment increased NOEC significantly and treatment with AC_1 and AC_2 increased it even more. This result has confirmed that the pH adjustment was as good as the activated carbon adsorption in term of toxicity removal. However, a powerful and effective treatment of the cleaning wastewater generated by the wooden floor industry is required in order to improve the quality of the final effluent.

4.2.4. Single or combined unicellular microalgae species as a bioindicator of toxicity when exposed to estrogen hormones

Based on the EC_{50} values (Table 4.7) (*Tables 1,* **Paper IV**), natural and synthetic estrogens affected both *D. subspicatus* and *P. subcapitata*. The EE2 and E2 had similar toxicity effect, but were more toxic than E1 to both species. The EC_{50} of all tested estrogens increased with time, which suggested a time-response attenuation effect, as previously observed for *D. subspicatus* exposed to storm water runoff from wood storage areas (Kaczala et al., 2011) and water polluted with formaldehyde (López-Rodas et al., 2008). Lürling (1998) explained the gradual decrease in toxicity as an inactivation due to

adsorption and bioconcentration of the toxic compounds by the algae cells, which was also the explanation proposed by Shi et al. (2010) for the removal of E1, E2 and EE2 from wastewater by algae and duckweed. Sorption can be followed by bioconcentration of estrogens *via* an active transport or selective binding mechanism, such as binding to receptors or enzymes (Lai et al., 2002).

Algae are known to play an important role in the fate of organic compounds in aquatic ecosystems (Lai et al., 2002), and the attenuation of the toxic effects of estrogens may be related to degradation or uptake by the algae. After 96 h, the NOEC values had similar results when measured by fluorescence and algal counting. The NOEC values for *D. subspicatus* were: < 0.01 µg/mL (EE2 and E2); 0.10 µg/mL (E+) and; 0.31 µg/mL (E1). For *P. subcapitata* the NOEC values were: < 0.01 µg/mL (EE2 and E2); 0.10 µg/mL (E+) and; 0.10 µg/mL (E1).

When *D. subspicatus* was exposed to mixtures containing one third of each estrogen (E+) during 96 h, the effect on algal growth (based on EC_{50}) was less toxic than the individual effects caused by each estrogen (less-than-additive combined effect) (Table 4.7).

According to (Cleuvers, 2004) an additive effect was observed when *D. subspicatus* was exposed to anti-inflammatory drugs. However, when *P. subcapitata* was exposed to E+ for 96 h, the final effect on algal growth (based on EC_{50}) appeared to have a similar toxic effect, as suggested by Table 4.7. In this case when the *P. subcapitata* was exposed to the mixture of EE2, E2 and E1 (E+) in the same proportions, an additive effect was observed. Additionally, the NOEC results showed that toxic effects also occurred when E1, E2 and EE2 were applied as a mixture (E+) at concentrations below their individual NOEC, a phenomenon previously reported by Cleuvers (2004).

Table 4.7: EC_{50} mean value (min-max) in µg/mL of single E1, E2, EE2 and mixed (E+) estrogens (initial ratio 1:1:1) for individual *P. subcapitata* (PS) and *D. subspicatus* (DS) cultures and combined (S+) cultures (initial ratio 1:1) after exposure for 24, 48, 72 and 96 h. *(Table 1*, **Paper IV***).*

	$EC50_{24h}$			**$EC50_{48h}$**			**$EC50_{72h}$**			**$EC50_{96h}$**		
	PS	DS	S+	PS	DS	S+	PS	DS	S+	PS	DS	S+
EE2	0.01^{aA} (0.008-0.019)	0.04^{aB} (0.02-0.05)	0.07^{aC} (0.05-0.10)	0.32^{aA} (0.22-0.46)	0.11^{aB} (0.06-0.21)	0.60^{aC} (0.41-0.85)	0.46^{aA} (0.21-0.69)	0.48^{aA} (0.23-0.80)	1.02^{aB} (0.67-1.45)	0.80^{aA} (0.51-1.05)	0.73^{aA} (0.37-1.12)	1.29^{aB} (0.89-1.58)
E2	0.01^{aA} (0.007-0.012)	0.08^{aB} (0.06-0.10)	0.09^{bC} (0.07-0.21)	0.05^{bA} (0.01-0.09)	0.42^{aB} (0.31-0.55)	0.76^{aB} (0.48-1.13)	0.30^{aA} (0.12-0.67)	0.66^{aA} (0.58-0.75)	1.18^{aB} (0.45-1.82)	0.87^{aA} (0.60-1.37)	1.07^{aA} (0.8-1.49)	1.87^{aB} (0.53-2.8)

E1	0.65^{bA} (0.37-1.13)	2.02^{bB} (1.50-2.31)	0.18^{cC} (0.12-0.24)	1.28^{cA} (0.97-1.68)	2.40^{bB} (1.2-3.74)	2.03^{bB} (1.10-3.37)	3.74^{bA} (2.45-5.70)	3.53^{bA} (2.30-5.35)	3.35^{bA} (2.15-5.21)	4.39^{bA} (2.95-6.54)	7.24^{bB} (6.17-8.73)	5.58^{bC} (3.85-7.08)
E+	0.02^{aA} (0.018-0.028)	0.64^{cB} (0.22-1.19)	0.06^{aA} (0.04-0.08)	0.05^{bA} (0.04-0.07)	2.64^{bB} (1.75-3.75)	0.80^{aC} (0.53-1.21)	0.32^{aA} (0.22-0.47)	3.91^{bB} (2.39-4.49)	3.43^{bB} (2.39-4.19)	0.88^{aA} (0.46-1.36)	4.94^{cB} (3.05-6.01)	4.61^{cB} (3.69-5.75)

Note: Tukey's test: A, B, C - compares rows (effect of each estrogen on different algae populations (PS, DS, S+) and exposure time). a, b, c - compare columns (effect of each estrogen (EE2, E1, E1, E+) on the same algae population). Different letters indicate statistically significant differences ($p < 0.05$).

The frequencies of different morphologies observed in *D. subspicatus* growing in water control after 96 h were 96.1%, 2.5% and 1.4% for the unicellular, two-celled and four-celled coenobium morphology, respectively. Significant differences compared to control were observed in algae exposed to all concentrations (Fig. 4.8). Four-celled coenobium was more frequently observed in *D. subspicatus* exposed to EE2 (≥ 0.1 µg/mL). Two and four-celled coenobium morphologies were more frequent in algae exposed to E2 at higher concentrations (≥ 0.31 µg/mL). Changes in the morphology were significant but relatively less frequent in algae exposed to E1 (significantly less toxic) compared to those exposed to EE2 and E2. Regarding the exposure to the mixture E+, significant differences in the morphology of *D. subspicatus* were observed at all tested concentrations. These changes in the morphology were statistically similar to E1.

D. subspicatus has been described as presenting variable phenotypes or changes in growth and reproduction when exposed to environmental cues, as it seems to be the case for ecosystems disturbed by humans (Peña-Castro et al., 2004). Lürling (1998) highlighted that the population is primarily unicellular in the water control where more than 80% of the population has unicellular form. However, when exposed to certain substances (infochemicals), two-, four- and eight-celled coenobia were rapidly formed. Major morphological changes were observed in the exponential growth phase, followed by a subsequent recovery of unicellular abundance in a few days.

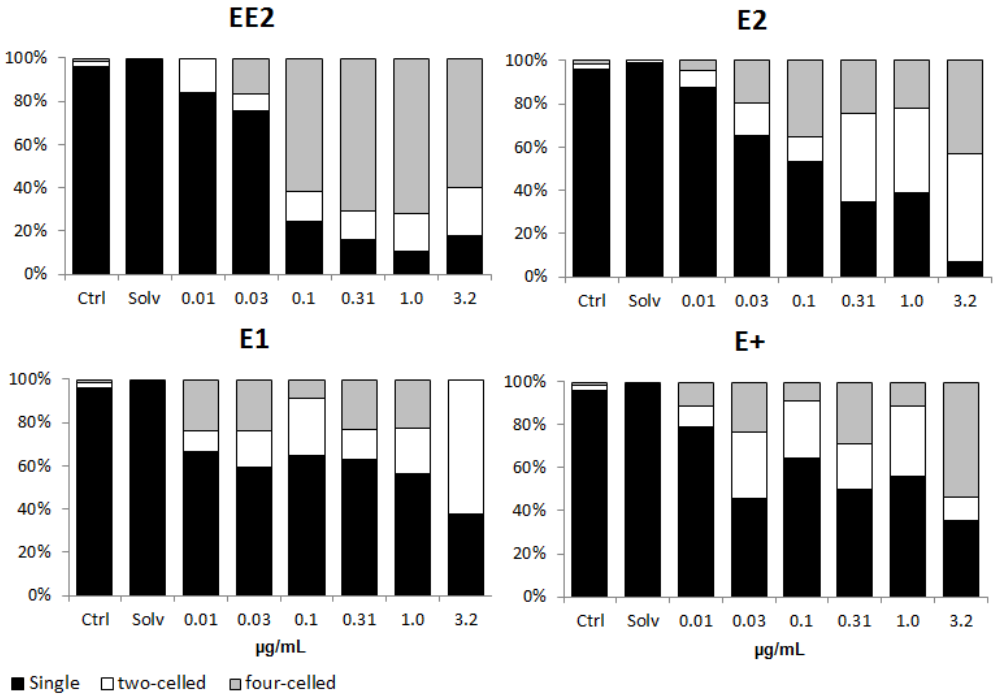


Fig. 4.8: *The prevalence of unicellular, two-celled coenobium and four-celled coenobium D. subspicatus morphologies after 96 h of exposure to the single estrogens: 17α-ethinylestradiol (EE2), 17β-estradiol (E2) and estrone (E1); and a mixture (E+) with an initial ratio of 1:1:1 at concentrations from 0.01 to 3.2 µg/mL* (Figure 1, **Paper IV**).

Similarly to the exposure of individual species to single and mixed (E+) estrogens, the effects in terms of EC_{50} on the combined algae species (S+) decreased progressively with time in all cases (Table 4.7). Additionally, the EE2 and E2 caused equivalent toxicity on S+. However, this toxicity was significantly higher (lower EC_{50}) than those caused by E1 and E+ on S+ cultures.

After 96 h, the NOEC measured for *S*+ by fluorescence and calculated from the algal counting reached the same values of 0.03 µg/mL (EE2) and 0.01 µg/mL (E2, E1 and E+) during the algal growth inhibition test.

After 96 h, in the control cultures of S+, the *D. subspicatus* represented 65% (in terms of density) of the entire population. In the presence of EE2 and E2, the prevalence of *D. subspicatus* in S+ cultures was even higher than in the control. According to Buchanan et al. (2013), lab-scale combined culture is a very interesting strategy to explore competitive interactions and sequential effects among species (interspecific competition or interactions).

The presence of *P. subcapitata* in the combined S+ culture did not induce coenobium formation by *D. subspicatus* in the control cultures (comparing the control cultures of Fig 4.8 and 4.9). However, individual at mixed estrogen treatments (E+) at all concentrations increased the frequency of coenobium formation by *D. subspicatus* in S+ (Fig. 4.9).

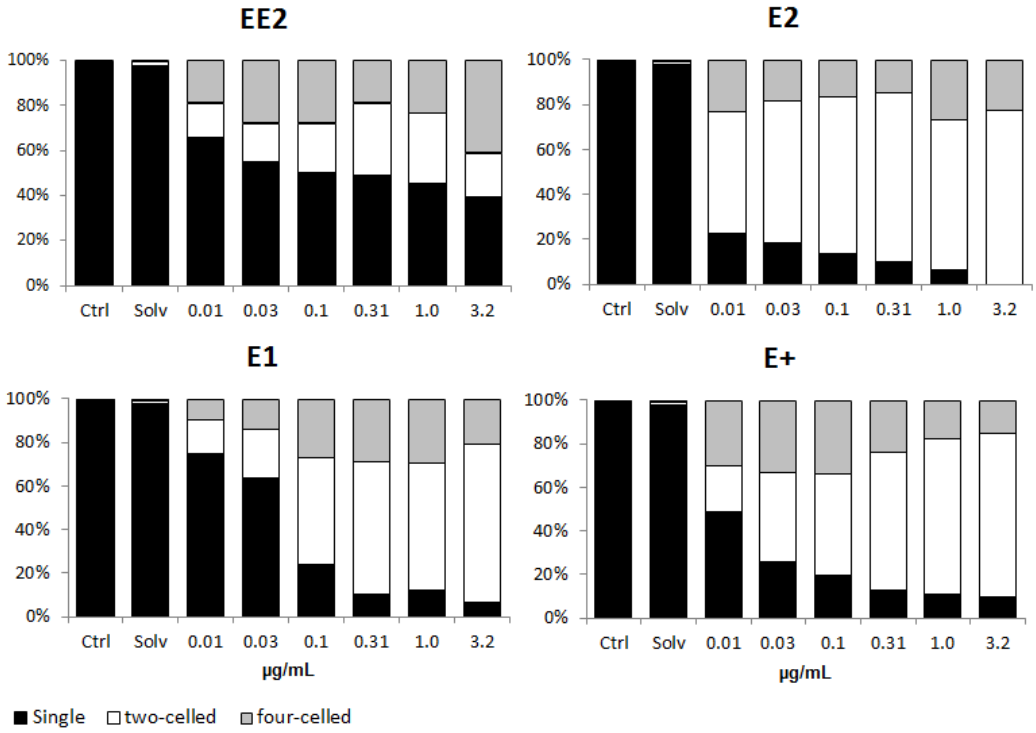


Fig. 4.9: *The prevalence of unicellular, two-celled coenobium and four-celled coenobium morphologies in the combined cultures of* D. subspicatus *and* P. subcapitata *after 96 h of exposure to the individual estrogens 17α-ethinylestradiol (EE2), 17β-estradiol (E2) and estrone (E1) and mixed estrogens (E+) at an initial ratio of 1:1:1 at concentrations that varied from 0.01 to 3.2 µg/mL.* (Figure 2, ***Paper IV***).

The morphology of *D. subspicatus* was most affected in S+ exposed to E2 at all concentrations, with the sum frequency of two-celled and four-celled coenobia ranging from 77 to 100% (Fig. 4.9). The exposure of S+ to mixed hormones (E+) also resulted in a high frequency of two-celled and four-celled coenobia (from 51 to 91%), followed by the exposure to E1 (25 to 93%) and then, to EE2 (34 to 61%).

After the toxicity assays presented in **Papers IV and V**, with *D. subspicatus* and *P. subcapitata* it was concluded that some aspects should be further investigated, such as

the effect of the attenuation occurring with time and the interspecific interactions observed in combined cultures, that brings up several issues and must be considered in laboratory assays included in environmental monitoring programs and also when algae are to be grown in wastewater treatment plants for the treatment itself and/or production of biodiesel.

4.3. Some relevant variables for ecotoxicity experiment design

Supporting the concept of ecotoxicology test but with a higher degree of realism regarding the environmental conditions, new perspectives are required. The response of a selected species to a range of concentrations of a certain contaminant is just the first approach. A number of physical, chemical and biological parameters not always considered actually affect considerably the organism's response (Diamond et al., 2006) (**Paper VI**).

Some important factors (among many others) to be taken into consideration when investigating toxicity effects in laboratory and field conditions are: *(1) Contaminant characteristics and its gateway and pathway; (2) Selected species (bioindicator) and its biomarkers; (3) Dose regime/dose mode; (4) Concentration vs. load; (5) Single vs. multiple exposure to contaminants; (6) single vs. multiple exposure of species.* Even though factors *(1), (2)* and *(3)* have been extensively discussed in aquatic ecotoxicology, they are addressed in the present discussion in order to keep factors *(4)*, *(5) and (6)* in perspective and to highlight the way they are all related. Factors *(4)*, *(5) and (6)* were selected in the present review at the expenses of other important ones, due to the surprisingly few publications that mention them in the methodology description and even less publications that discuss their contributions to the overall toxicity.

The multiple interactions between two or more factors can affect the organism response to a contaminant or mixture of contaminants is illustrated in Fig. 4.10.

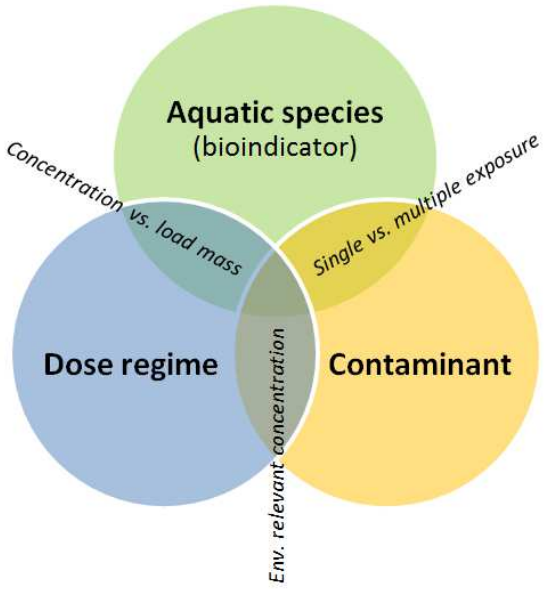


Fig 4.10: *Variables to consider when designing an ecotoxicity assay and their multiple interactions. The central point represents the interaction among all variables* (Figure 2, ***Paper VI***).

4.3.1. Contaminant's characteristics, gateway and pathway

The nature of the contaminant and its intrinsic characteristics (e.g.: physical and chemical properties, biodegradability) are often the reason why toxicity assays are carried out in the first place. However some properties related to the exposure condition, such as: source of contaminant and availability in the environment (single or in mixtures); routes travelled inside the organism, uptake rate; accumulation in the tissues (mechanisms of action); removal efficiency by the treatment systems (e.g.: wastewater treatment plants); and generated metabolites and by-products should be also consider in the toxicity assays (Angel et al., 2010; Ashauer et al., 2006; Landrum et al., 2012; McCahon and Pascoe, 1990; Reinert et al., 2002; Thorpe et al., 2003).

The way the contaminant enters the body of the aquatic bioindicator also influences the response (Ashauer et al., 2010; Canli and Atli, 2003; Cedergreen et al., 2005). The most commons ways are: *via* water (spiked or naturally contaminated) (Allert et al., 2013; Ashauer et al., 2010; Hassanin et al., 2002; Knudsen et al., 2011; Landrum et al., 2012; Ondarza et al., 2012; Salomão and Marques, 2014); *via* food or diet (spiked food or

exposed food) (Allert et al., 2013; Landrum et al., 2012; Lazartigues et al., 2013; Mandiki et al., 2005); and *via* intraperitoneal or intramuscular through injections in laboratory assays (Costa et al., 2010; Hallgren et al., 2009; Kumar, 2012; Ng et al., 2001; Purdom et al., 1994; Solé et al., 2000; Verslycke et al., 2002).

4.3.2. Selected bioindicator

In addition to the contaminant's physical and chemical characteristics, biological aspects, such as the intrinsic characteristics of the bioindicator are also important in determining the organism's response (Ashauer et al., 2006; Diamond et al., 2006; Landrum et al., 2012; Segner et al., 2003). During the process for selection of a bioindicator, the following should be considered: age or stage of development in the organism's life cycle (e.g.: eggs, embryos, juveniles, adults, the whole life length); sex status or rate and reproduction mode (sexual, asexual); seasoning events (e.g.: breeding periods, periods of greater or lesser activity, and molting cycle); eating habits (e.g.: primary producers, herbivorous, carnivorous, omnivorous, detritivorous, filterers or suspension-feeders); natural habitat conditions, life strategy and mobility (e.g.: benthic or pelagic, sessile or free-living species) among others (Costa et al., 2010; Per Hallgren et al., 2012; Reinert et al., 2002; Segner et al., 2003; Trudel and Rasmussen, 2006).

4.3.3. Dose regime

The dose regime (the mode the contaminant enters the aquatic system (or the experimental unit) is an important variable affecting the organism's response (Ashauer and Brown, 2013; Panter et al., 2000; Tennekes and Sánchez-Bayo, 2013; Zafar et al., 2011). The dose regime applied in most ecotoxicity assays are: (i) continuous (Amachree et al., 2013; Angel et al., 2010; Diamond et al., 2006; Thorpe et al., 2003; Vardy et al., 2011); (ii) intermittent or pulsed (Amachree et al., 2013; Angel et al., 2010; Ashauer et al., 2007;

Diamond et al., 2006; Per Hallgren et al., 2012; McCahon and Pascoe, 1990; Reinert et al., 2002; Souza et al., 2013); and (iii) episodic with a high initial peak or load simulating an accident (acute pulse of short duration) (Bejarano and Farr, 2013; Ondarza et al., 2012).

The selection of the most appropriate dose regime for a certain assay that intends to simulate the real conditions the contamination occurs should be decided on a case-by-case basis after considering a number of other variables (Landrum et al., 2012; Reinert et al., 2002) such as: (a) type of pollution source the assay simulates; (b) discharge frequency simulated; (c) water flow in the recipient water body (lentic or lotic) and; (d) hydrological dilution (Bejarano and Farr, 2013; Boxall et al., 2013; Handy, 1994; Reinert et al., 2002).

4.3.4. Concentration vs. load mass

Concentration remains the most widely used way to express the amount of contaminants in aquatic environments and environmental regulations still determine discharge threshold limits, based on concentrations in many countries (Diamond et al., 2006; Landrum et al., 2012). According to Landrum et al. (2012), in simulations of one dose regime the initial concentration of a certain contaminant provides an estimate of the initial maximum exposure. However, concentration is not always the most appropriate way to compare exposure (in laboratory and field scales) under different dynamic conditions.

In a real water environment, receiving water bodies can be lotic or lentic, having high or low flow, with different widths and depths, among other variables (Bejarano and Farr, 2013). In order to inform the mass of a contaminant in the aquatic environment, besides concentration, it is necessary to know the total water volume and also some bioindicator characteristics such as mobility, eating and breathing habits. Two experimental units with relevant differences in volumes can have the same concentration but the specimens will be exposed to different masses of contaminants per corporeal mass unit and therefore, the responses can be quite different too (Salomão and Marques, 2014) (**Paper II**).

Based on the literature, it is reasonable to consider that for bioindicators with certain features (e.g.: high mobility in the water mass, filtrating habits, organisms with gills like fishes), pollutant load mass might be the most appropriate way to assess toxicity. Based on the uptake rate, depuration rate, and also the maximum accumulation of each type of contaminant in the organism (critical body threshold) of each species load has been applied to interprets the toxic effects (Angel et al., 2010; Ashauer et al., 2007; Boxall et al., 2013; Diamond et al., 2006; Reinert et al., 2002; Salomão and Marques, 2014; Seim et al., 1984).

4.3.5. Single vs. multiple exposure

The exposure of aquatic organisms to a combination of contaminants (multiple exposure) is seldom addressed in toxicity assays (Dietrich et al., 2010; Heberer, 2002; Landrum et al., 2012; Lazartigues et al., 2013; Monosson, 2005; Thorpe et al., 2003) compared to the very large number of studies available with simple substances. The multi-exposure of aquatic organisms to combined contaminants may lead mainly to additive, synergistic or antagonistic effects and the results are likely to have more environmental relevance (Landrum et al., 2012; McCahon and Pascoe, 1990; Monosson, 2005; Thorpe et al., 2003). According to Thorpe *et al*. (2003) the fact that in nature one contaminant is rarely alone should be taken into account due to the ability some chemicals have for interactions in an additive or synergetic manner in some organisms at concentrations below their individual LOEC.

The presence of other species (multiple exposure of different species) in the aquatic environment (in the same or in different trophic levels or ecological niches) can also affect the processes and produce different ecological responses due to interspecific interactions, like competition and predation (Boxall et al., 2013; Hallgren et al., 2012; McCahon and Pascoe, 1990; Salomão et al., 2014; Segner et al., 2003), resulting also in predominance of some species (**Paper IV**) (Hallgren et al., 2012).

Finally, multiple exposure of more than one species exposed to more than one contaminant simultaneously is likely to be the most common situation in contaminated aquatic environments. In order to simulate properly these conditions, ecotoxicicity assays still have a long way to go.

CONCLUSIONS AND RECOMMENDATIONS

The decentralized engineered ecosystem (DEE) achieved satisfactory treatment and the final concentrations for all parameters (except for the total phosphorous) met the discharge threshold limits established by Brazilian and EU legislation (**Paper I**). The vegetated tanks (constructed wetlands) promoted an additional removal of nutrients (N and P). Based on the ALP levels measured in male fishes exposed to DEE outlet, the efficiency of the DEE regarding the removal of estrogenicity from the wastewater was higher than that one observed for a conventional large wastewater treatment plant WWTP (**Paper III)**. This superiority of the DEE over the WWTP might be attributed to the multistage and strategy design, the different types and frequency of biomass harvesting, but also to the different composition of the inflow in both systems.

Papers II, III, IV and V described as expected, the wide range of sensibility regarding the responses from algae and fishes species to the presence of estrogenic hormones, endocrine disruptors and toxic contaminants in the water.

In **Papers II and IV**, EE2 and E2 were found to be more toxic than E1 for two tested species of algae and one species of fish). As expected, *Oreochromis niloticus* was more sensitive than *D. subspicatus* and *P. subcapitata*. Based on the results the following decreasing sensitivity to estrogens was suggested: *O. niloticus*; *P. subcapitata*; *D. subspicatus*; and finally, the combination of both algae species (S+).

When combined in equal proportions (1:1:1) the studied estrogens (EE2:E2:E1) posed different effects on the tested species. An additive effect was observed on *O. niloticus* and on the green microalgae *P. subcapitata*, however less than additive effect was observed on the green microalgae *D. subspicatus*.

The combination of algae species and the intraspecific relationship, it was possible to conclude that: (1) *D. subspicatus* is a stronger competitor and prevailed over *P. subcapitata* in all situations where both were cultivated together (exposed or not to the contaminants); (2) the *P. subcapitata* did not affect the formation of coenobia by *D. subspicatus*; (3) the toxic effect was attenuated by the combination of them in the same culture and according to the time of exposure.

In **Papers III and V** the complex nature of the water contamination makes it difficult to draw robust conclusions and diagnoses about the environmental status of the studied

water environments. Further research is needed for clarification of how combinations of different species respond to complex mixtures of chemicals in water. In **Paper V** the industrial wastewater treatment with activated carbon or pH adjustment was not sufficient to eliminate toxicity from the cleaning wastewater generated by the wooden floor industry to the level required for discharge into recipient water bodies.

In **Paper III**, *O. niloticus* proved to be a good candidate species for environmental monitoring programs due to the high tolerance to different types of highly polluted waters (and levels of salinity), and also due to the low background (baseline) values for ALP in the plasma of males specimens, when compared to other fish species described in the literature. The combination of the applied ALP method (which removes free phosphates and yellowish color) with auxiliary biomarkers (particularly micronucleus) can form a potential protocol for respectively estrogenicity and genotoxicity detection in contaminated waters as part of water environmental monitoring programs.

The ecotoxicity studies carried out in the present thesis demonstrated the association among bioindicator, total mass applied, dose regime, contaminant type and the single or multiple exposures to single or multiple contaminants. The results obtained suggested that at least under some circumstances, total mass applied or pollutant load would be a more accurate approach for evaluation of the water quality status and also to interpret the results of toxicity tests. Additionally, risk assessments based on toxicity assays should take into account not only the total mass applied but also the frequency with which the contaminant of interest is discharged into the recipient water body (dose regime or mode); the number and size/age of organisms exposed (population density) and; the volume of the experimental units or the receiving water body.

Ecotoxicology tests to be developed in the future should be anchored in a more complete set of factors that influence the processes that determine the toxicity of a complex aquatic environment, both in terms of its chemical composition and the species that inhabit it.

REFERENCES

Alkorta, I., Garbisu, C., 2001. Phytoremediation of organic contaminants in soils. Bioresour. Technol. 79, 273–276.

Allert, a L., DiStefano, R.J., Fairchild, J.F., Schmitt, C.J., McKee, M.J., Girondo, J. a, Brumbaugh, W.G., May, T.W., 2013. Effects of historical lead-zinc mining on riffle-dwelling benthic fish and crayfish in the Big River of southeastern Missouri, USA. Ecotoxicology 22, 506–21. doi:10.1007/s10646-013-1043-3

Amachree, D., Moody, a J., Handy, R.D., 2013. Comparison of intermittent and continuous exposures to cadmium in the blue mussel, Mytilus edulis: accumulation and sub-lethal physiological effects. Ecotoxicol. Environ. Saf. 95, 19–26. doi:10.1016/j.ecoenv.2013.04.023

Angel, B.M., Simpson, S.L., Jolley, D.F., 2010. Toxicity to Melita plumulosa from intermittent and continuous exposures to dissolved copper. Environ. Toxicol. Chem. 29, 2823–30. doi:10.1002/etc.347

Ashauer, R., Boxall, A., Brown, C., 2006. Predicting effects on aquatic organisms from fluctuating or pulsed exposure to pesticides. Environ. Toxicol. Chem. 25, 1899–912.

Ashauer, R., Boxall, A.B. a, Brown, C.D., 2007. New ecotoxicological model to simulate survival of aquatic invertebrates after exposure to fluctuating and sequential pulses of pesticides. Environ. Sci. Technol. 41, 1480–6.

Ashauer, R., Brown, C.D., 2013. Highly time-variable exposure to chemicals--toward an assessment strategy. Integr. Environ. Assess. Manag. 9, e27–33. doi:10.1002/ieam.1421

Ashauer, R., Hintermeister, A., Caravatti, I., Kretschmann, A., Escher, B.I., 2010. Toxicokinetic and toxicodynamic modeling explains carry-over toxicity from exposure to diazinon by slow organism recovery. Environ. Sci. Technol. 44, 3963–3971. doi:10.1021/es903478b

AVMA, 2013. American Veterinary Medical Association - AVMA Guidelines for the Euthanasia of Animals□: 2013 Edition.

Bejarano, A.C., Farr, J.K., 2013. Development of short, acute exposure hazard estimates: A tool for assessing the effects of chemical spills in aquatic environments. Environ. Toxicol. Chem. 32, 1918–1927. doi:10.1002/etc.2255

Blaise, C., Gagné, F., Pellerin, J., Hansen, P.D., Trottier, S., 2002. Molluscan shellfish biomarker study of the Quebec, Canada, Saguenay Fjord with the soft-shell clam, Mya arenaria. Env. Toxicol 17, 170–186.

Bolognesi, C., Hayashi, M., 2011. Micronucleus assay in aquatic animals. Mutagenesis 26, 205–213. doi:10.1093/mutage/geq073

Bonassi, S., Znaor, A., Ceppi, M., Lando, C., Chang, W.P., Holland, N., Kirsch-Volders, M., Zeiger, E., Ban, S., Barale, R., Bigatti, M.P., Bolognesi, C., Cebulska-Wasilewska, A., Fabianova, E., Fucic, A., Hagmar, L., Joksic, G., Martelli, A., Migliore, L., Mirkova, E., Scarfi, M.R., Zijno, A., Norppa, H., Fenech, M., 2007. An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. Carcinogenesis 28, 625–31. doi:10.1093/carcin/bgl177

Boxall, A.B.A., Fogg, L.A., Ashauer, R., Bowles, T., Sinclair, C.J., Colyer, A., Brain, R.A., 2013. Effects of repeated pulsed herbicide exposures on the growth of aquatic macrophytes. Environ. Toxicol. Chem. 32, 193–200. doi:10.1002/etc.2040

Brink, K., van Vuren, J., Bornman, R., 2012. Responses of laboratory exposed catfish (Clarias gariepinus) to environmentally relevant concentrations of p,p'-DDT. Environ. Toxicol. Pharmacol. 34, 919–25. doi:10.1016/j.etap.2012.08.004

Brix, H., 1994. Functions of macrophytes in constructed wetlands. Water Sci. Technol. 29, 71–78.

Buchanan, J.J., Slater, F.R., Bai, X., Pratt, S., 2013. Algal growth and community structure in a mixed-culture system using coal seam gas water as the water source. Environ. Technol. 34, 695–701. doi:10.1080/09593330.2012.715676

Bücker, A., Conceição, M.B., 2012. Genotoxicity evaluation of tilapia (Oreochromis niloticus) exposed to waters from two sites of Itajaí-Açu River (SC, Brazil). J. Brazilian Soc. Ecotoxicol. 7, 51–56. doi:10.5132/jbse.2012.02.008

Campbell, P.M., Pottinger, T.G., Sumpter, J.P., 1994. Changes in the affinity of estrogen and androgen receptors accompanying changes in receptor abundance in brown and rainbow trout. Gen. Comp. Endocrinol. 94, 329–340.

Canli, M., Atli, G., 2003. The relationships between heavy metal (Cd, Cr, Cu, Fe, Pb, Zn) levels and the size of six Mediterranean fish species. Environ. Pollut. 121, 129–36.

Cannon, A.D., Gray, K.R., Biddlestone, A.J., Thayanithy, K., 2000. Pilot-Scale Development of a Bioreactor for the Treatment of Dairy Dirty Water. J. Agric. Eng. Res. 77, 327–334. doi:10.1006/jaer.2000.0595

Carballo, M., Aguayo, S., de la Torre, A., Muñoz, M.J., 2005. Plasma vitellogenin levels and gonadal morphology of wild carp (Cyprinus carpio L.) in a receiving rivers downstream of sewage treatment plants. Sci. Total Environ. 341, 71–9. doi:10.1016/j.scitotenv.2004.08.021

Carrasco, K.R., Tilbury, K.L., Myers, M.S., 1990. Assessment of the piscine micronucleus test as an in situ biological indicator of chemical contaminant effects. Can. J. Fish. Aquat. Sci. 47, 2123–2136. doi:10.1139/f90-237

Çavaş, T., Ergene-Gözükara, S., 2005. Induction of micronuclei and nuclear abnormalities in Oreochromis niloticus following exposure to petroleum refinery and chromium processing plant effluents. Aquat. Toxicol. 74, 264–71. doi:10.1016/j.aquatox.2005.06.001

Cedergreen, N., Andersen, L., Olesen, C.F., Spliid, H.H., Streibig, J.C., 2005. Does the effect of herbicide pulse exposure on aquatic plants depend on Kow or mode of action? Aquat. Toxicol. 71, 261–271. doi:10.1016/j.aquatox.2004.11.010

Christen, V., Hickmann, S., Rechenberg, B., Fent, K., 2010. Highly active human pharmaceuticals in aquatic systems: A concept for their identification based on their mode of action. Aquat. Toxicol. 96, 167–181. doi:10.1016/j.aquatox.2009.11.021

Cleuvers, M., 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicol. Lett. 142, 185–194. doi:10.1016/S0378-4274(03)00068-7

Cleuvers, M., 2004. Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. Ecotoxicol. Environ. Saf. 59, 309–15. doi:10.1016/S0147-6513(03)00141-6

Clubbs, R.L., Brooks, B.W., 2007. Daphnia magna responses to a vertebrate estrogen receptor agonist and an antagonist: a multigenerational study. Ecotoxicol. Environ. Saf. 67, 385–398. doi:10.1016/j.ecoenv.2007.01.009

Conselho Nacional do Meio Ambiente (CONAMA), 2011. Establishment of the conditions and standards for effluent discharge; supplements and amendment of

Resolution no. 357 (2005) of the National Environment Council (CONAMA). Brasilia, Brazil.

Cooper, R.L., Kavlock, R.J., 1997. Endocrine disruptors and reproductive development: A weight-of-evidence overview. J. Endocrinol. 152, 159–166.

Costa, D.D.M., Neto, F.F., Costa, M.D.M., Morais, R.N., Garcia, J.R.E., Esquivel, B.M., Ribeiro, C. a O., 2010. Vitellogenesis and other physiological responses induced by 17-beta-estradiol in males of freshwater fish Rhamdia quelen. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 151, 248–57. doi:10.1016/j.cbpc.2009.11.002

Costa, M.L.R., Henry, R., 2010. Phosphorus, nitrogen, and carbon contents of macrophytes in lakes lateral to a tropical river (Paranapanema River, São Paulo, Brazil). Acta Limnol. Bras. 22, 122–132. doi:10.4322/actalb.02202002

Damstra, T., Barlow, S., Bergman, A., Kavlock, R., Van Der Kraak, G., 2002. Global assessment of the state-of the-science of endocrine disruptors, in: Publication, No. WHO/PCS/EDC/02.2. World Health Organisation, Geneva.

Davis, L.K., Fox, B.K., Lim, C., Hiramatsu, N., Sullivan, C. V, Hirano, T., Grau, E.G., 2009. Induction of vitellogenin production in male tilapia (Oreochromis mossambicus) by commercial fish diets. Comp. Biochem. Physiol., Part A Mol. Integr. Physiol. 154, 249–54. doi:10.1016/j.cbpa.2009.06.009

De Melo, E.D., Mounteer, A.H., Leão, L.H.D.S., Bahia, R.C.B., Campos, I.M.F., 2013. Toxicity identification evaluation of cosmetics industry wastewater. J. Hazard. Mater. 244-245, 329–334. doi:10.1016/j.jhazmat.2012.11.051

De Silva, S.S., Subasinghe, R.P., Bartley, D.M., Lowther, A., 2004. Tilapias as alien aquatics in Asia and Pacific: a review. FAO Fish. Tech. Pap. 65.

Desbrow, C., Routledge, E.J., Brighty, G.C., Sumpter, J.P., Waldock, M., 1998. Identification of Estrogenic Chemicals in STW Effluent . 1 . Chemical Fractionation and in Vitro Biological Screening. Environ. Sci. Technol. 32, 1549–1558.

Diamond, J.M., Klaine, S.J., Butcher, J.B., 2006. Implications of pulsed chemical exposures for aquatic life criteria and wastewater permit limits. Environ. Sci. Technol. 40, 5132–8.

Dietrich, S., Ploessl, F., Bracher, F., Laforsch, C., 2010. Single and combined toxicity of pharmaceuticals at environmentally relevant concentrations in Daphnia magna--

a multigenerational study. Chemosphere 79, 60–6. doi:10.1016/j.chemosphere.2009.12.069

Djomo, J.E., Dauta, a, Ferrier, V., Narbonne, J.F., Monkiedje, a, Njine, T., Garrigues, P., 2004. Toxic effects of some major polyaromatic hydrocarbons found in crude oil and aquatic sediments on Scenedesmus subspicatus. Water Res. 38, 1817–21. doi:10.1016/j.watres.2003.10.023

Dordio, A., Carvalho, a J.P., Teixeira, D.M., Dias, C.B., Pinto, A.P., 2010. Removal of pharmaceuticals in microcosm constructed wetlands using Typha spp. and LECA. Bioresour. Technol. 101, 886–892. doi:10.1016/j.biortech.2009.09.001

Dordio, A. V., Estêvão Candeias, A.J., Pinto, A.P., Teixeira da Costa, C., Palace Carvalho, A.J., 2009. Preliminary media screening for application in the removal of clofibric acid, carbamazepine and ibuprofen by SSF-constructed wetlands. Ecol. Eng. 35, 290–302. doi:10.1016/j.ecoleng.2008.02.014

Entwisle, T.J., Sonneman, J.A., Lewis, S.H., 1997. Freshwater Algae in Australia. A Guide to Conspicuous Genera. Sainty & Associates, Sidney, Australia.

Fatta-Kassinos, D., Bester, K., Kümmerer, K. (Editors), 2010. Xenobiotics in the Urban Water Cycle Xenobiotics in the Urban Water Cycle - Mass Flows, Environmental Processes, Mitigation and Treatment Strategies, v. 16. ed. Springer.

Fenech, M., Chang, W.P., Kirsch-Volders, M., Holland, N., Bonassi, S., Zeiger, E., 2003. HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutat. Res. 534, 65–75.

Feng, X., Ding, S., Tu, J., Wu, F., Deng, N., 2005. Degradation of estrone in aqueous solution by photo-Fenton system. Sci. Total Environ. 345, 229–237. doi:10.1016/j.scitotenv.2004.11.008

Fent, K., Weston, A. a, Caminada, D., 2006. Ecotoxicology of human pharmaceuticals. Aquat. Toxicol. 76, 122–159. doi:10.1016/j.aquatox.2005.09.009

Frost, R.C., 2009. EU practice in setting wastewater emission limit values. Urban Wastewater Treatment Directive 91/271/EEC. Water Gov. West. EECCA Ctries. 1–29.

Gagné, F., Blaise, C., 2000. Organic alkali-labile phosphates in biological materials: A generic assay to detect vitellogenin in biological tissues. Env. Toxicol 15, 243–247.

Gagné, F., Blaise, C., Hellou, J., 2004. Endocrine disruption and health effects of caged mussels, Elliptio complanata, placed downstream from a primary-treated municipal effluent plume for 1 year. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 138, 33–44.

Gagné, F., Blaise, C., Pellerin, J., 2005. Altered exoskeleton composition and vitellogenesis in the crustacean Gammarus sp. collected at polluted sites in the Saguenay Fjord, Quebec, Canada. Env. Res 98, 89–99.

Gray, J.L., Sedlak, D.L., 2005. The fate of estrogenic hormones in an engineered treatment wetland with dense macrophytes. Water Environ. Res. 77, 24–31.

Greenway, M., 2005. The role of constructed wetlands in secondary effluent treatment and water reuse in subtropical and arid Australia. Ecol. Eng. 25, 501–509. doi:10.1016/j.ecoleng.2005.07.008

Greenway, M., Woolley, A., 1999. Constructed wetlands in Queensland: Performance efficiency and nutrient bioaccumulation. Ecol. Eng. 12, 39–55. doi:10.1016/S0925-8574(98)00053-6

Gressler, P.D., Bjerk, T.R., Schneider, R.D.C.S., Souza, M.P., Lobo, E.A., Zappe, A.L., Corbellini, V.A., Moraes, M.S.A., 2013. Cultivation of Desmodesmus subspicatus in a tubular photobioreactor for bioremediation and microalgae oil production. Environ. Technol. 1–11. doi:10.1080/09593330.2013.822523

Hallgren, P., 2009. Strategies for Monitoring of Endocrine Disrupting Chemicals in Aquatic Environment. Lund University.

Hallgren, P., Mårtensson, L., Mathiasson, L., 2009. Improved spectrophotometric vitellogenin determination via alkali-labile phosphate in fish plasma – a cost effective approach for assessment of endocrine disruption. Intern. J. Environ. Anal. Chem. 89, 1023–1042. doi:10.1080/03067310902795944

Hallgren, P., Mårtensson, L., Mathiasson, L., 2012. International Journal of Environmental A new spectrophotometric method for improved indirect measurement of low levels of vitellogenin using malachite green. Intern. J. Environ. Anal. Chem. 92, 894–908.

Hallgren, P., Sorita, Z., Berglund, O., Persson, A., 2012. Effects of 17α-ethinylestradiol on individual life-history parameters and estimated population growth rates of the freshwater gastropods Radix balthica and Bithynia tentaculata. Ecotoxicology 21, 803–810. doi:10.1007/s10646-011-0841-8

Handy, R.D., 1994. Intermittent exposure to aquatic pollutants□: assessment, toxicity and sublethal responses in fish and invertebrates. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 107, 171–184.

Harries, J.E., Janbakhsh, A., Jobling, S., Matthiessen, P., Sumpter, J.P., Tyler, C.R., 1999. Estrogenic potency of effluent from two sewage treatment works in the United Kindon. Environ. Toxicol. Chem. 18, 932–937.

Harries, J.E., Sheahan, D.A., Jobling, S., Matthiessen, P., Neall, P., Routledge, E.J., Rycroft, R., Sumpter, J.P., Tylor, T., 1996. A survery of estrogenic activity in United Kingdom inland waters. Environ. Toxicol. Chem. 15, 1993–2002.

Harries, J.E., Sheahan, D.A., Jobling, S., Matthiessen, P., Neall, P., Sumpter, J.P., Tylor, T., Zaman, N., 1997. Estrogenic activity in five United Kingdom Rivers detected by measurement of vitellogenesis in caged male trout. Environ. Toxicol. Chem. 16, 534–542.

Hassanin, A., Kuwahara, S., Nurhidayat, Tsukamoto, Y., Ogawa, K., Hiramatsu, K., Sasaki, F., 2002. Gonadosomatic index and testis morphology of common carp (Cyprinus carpio) in rivers contaminated with estrogenic chemicals. J. Vet. Med. Sci. 64, 921–926.

Heberer, T., 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol. Lett. 131, 5–17.

Hessen, D.O., Donk, E., 1993. Morphological changes in Scenedesmus induced by substances released from Daphnia. Arch. Hydrobiol. 127, 129–140.

Jiraungkoorskul, W., Kosai, P., Sahaphong, S., Kirtputra, P., Chawlab, J., Charucharoen, S., 2007. Evaluation of Micronucleus Test's Sensitivity in Freshwater Fish Species. Res J Env. Sci 1, 56–63.

Jürgens, M.D., Holthaus, K.I.E., Johnson, A.C., Smith, J.L., Hetheridge, M., Williams, R.J., 2002. The potential for estradiol and ethinylestradiol degradation in English rivers. Environ. Toxicol. Chem. 21, 480–488.

Kaczala, F., Salomon, P.S., Marques, M., Granéli, E., Hogland, W., 2011. Effects from log-yard stormwater runoff on the microalgae Scenedesmus subspicatus: intra-storm magnitude and variability. J. Hazard. Mater. 185, 732–739. doi:10.1016/j.jhazmat.2010.09.080

Karjalainen, H., Stefansdottir, G., Tuominen, L., Kairesalo, T., 2001. Do submersed plants enhance microbial activity in sediment? Aquat. Bot. 69, 1–13.

Kaur, S., Sarkar, M., Srivastava, R.B., Gogoi, H.K., Kalita, M.C., 2012. Fatty acid profiling and molecular characterization of some freshwater microalgae from India with potential for biodiesel production. N. Biotechnol. 29, 332–344. doi:10.1016/j.nbt.2011.10.009

Kavanagh, L.J., Keller, J., 2007. Engineered ecosystem for sustainable on-site wastewater treatment. Water Res. 41, 1823–1831. doi:10.1016/j.watres.2007.01.016

Kavlock, R.J., Daston, G.P., Derosa, C., Fenner-Crisp, P., Gray, L.E., Kaattari, S., Lucier, G., Luster, M., Mac, M.J., Maczka, C., Miller, R., Moore, J., Rolland, R., Scott, G., Sheehan, D.M., Sinks, T., Tilson, H.A., 1996. Research Needs for the Risk Assessment of Health and Environmental Effects of Endocrine Disruptors: A Report of the U.S. EPA-sponsored Workshop. Environ. Health Perspect. 104.

Kime, D.E., Nash, J.P., Scott, A.P., 1999. Vitellogenesis as a biomarker of reproductive disruption by xenobiotics. Aquaculture 177, 345–352. doi:10.1016/S0044-8486(99)00097-6

Kime, D. V, 1998. Endocrine Disrupton in Fish. Kluwer Academic Publishers, Norwell, Massachusetts.

Kinnberg, K., Holbech, H., Petersen, G.I., Bjerregaard, P., 2007. Effects of the fungicide prochloraz on the sexual development of zebrafish (Danio rerio). Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 145, 165–170. doi:10.1016/j.cbpc.2006.11.002

Knudsen, J.J.G., Holbech, H., Madsen, S.S., Bjerregaard, P., 2011. Uptake of 17β-estradiol and biomarker responses in brown trout (Salmo trutta) exposed to pulses. Environ. Pollut. 159, 3374–80. doi:10.1016/j.envpol.2011.08.039

Kramer, V.J., Miles-richardson, S., Pierens, S.L., Giesy, J.P., 1998. Reproductive impairment and induction of alkaline-labile phosphate , a biomarker of estrogen exposure , in fathead minnows (Pimephales promelas) exposed to waterborne 17B-estradiol. Aquat. Toxicol. 40, 335–360.

Kumar, A.K., Mohan, S.V., 2011. Endocrine disruptive synthetic estrogen (17α-ethynylestradiol) removal from aqueous phase through batch and column sorption studies: Mechanistic and kinetic analysis. Desalination 276, 66–74. doi:10.1016/j.desal.2011.03.022

Kumar, S.P., 2012. Micronucleus assay□: a sensitive indicator for aquatic pollution. Int. J. Res. Biosci. 1, 32–37.

Lai, K.M., Scrimshaw, M.D., Lester, J.N., 2002. Biotransformation and Bioconcentration of Steroid Estrogens by Chlorella vulgaris. Appl. Environ. Microbiol. 68, 859–864. doi:10.1128/AEM.68.2.859

Landrum, P.F., Chapman, P.M., Neff, J., Page, D.S., 2012. Influence of exposure and toxicokinetics on measures of aquatic toxicity for organic contaminants: A case study review. Integr. Environ. Assess. Manag. 9, 196–210. doi:10.1002/ieam.1388

Laohaprapanon, S., Kaczala, F., Salomon, P.S., Marques, M., Hogland, W., 2012. Wastewater generated during cleaning/washing procedures in a wood-floor industry: toxicity on the microalgae Desmodesmus subspicatus. Environ. Technol. 33, 2439–2446. doi:10.1080/09593330.2012.671853

Laohaprapanon, S., Marques, M., Kaczala, F., Hogland, W., 2013. Packed-Column of Granular Activated Carbons for Removal of Chemical Oxygen Demand from Industrial Wastewater. CLEAN - Soil, Air, Water 41, 244–250. doi:10.1002/clen.201100388

Laohaprapanon, S., Marquesa, M., Hogland, W., 2014. Anaerobic baffled reactor coupled with chemical precipitation for treatment and toxicity reduction of industrial wastewater. Environ. Technol. 35, 154–162. doi:10.1080/09593330.2013.821142

Larsson, D.G.J., Adolfsson-Erici, M., Parkkonen, J., Pettersson, M., Berg, A.H., Olsson, P.E., Förlin, L., 1999. Ethinyloestradiol — an undesired fish contraceptive? Aquat. Toxicol. 45, 91–97. doi:10.1016/S0166-445X(98)00112-X

Lazartigues, A., Thomas, M., Banas, D., Brun-Bellut, J., Cren-Olivé, C., Feidt, C., 2013. Accumulation and half-lives of 13 pesticides in muscle tissue of freshwater fishes through food exposure. Chemosphere 91, 530–5. doi:10.1016/j.chemosphere.2012.12.032

Lin, Y.-F., Jing, S.-R., Wang, T.-W., Lee, D.-Y., 2002. Effects of macrophytes and external carbon sources on nitrate removal from groundwater in constructed wetlands. Environ. Pollut. 119, 413–420.

Lindqvist, N., Tuhkanen, T., Kronberg, L., 2005. Occurrence of acidic pharmaceuticals in raw and treated sewages and in receiving waters. Water Res. 39, 2219–2228. doi:10.1016/j.watres.2005.04.003

López-Rodas, V., Perdigones, N., Marvá, F., Rouco, M., García-Cabrera, J.A., 2008. Adaptation of phytoplankton to novel residual materials of water pollution: an experimental model analysing the evolution of an experimental microalgal population

under formaldehyde contamination. Bull. Environ. Contam. Toxicol. 80, 158–62. doi:10.1007/s00128-007-9336-y

Lürling, M., 1998. Effect of grazing-associated infochemicals on growth and morphological development in Scenedesmus acutus (Chlorophyceae). J. Phycol. 34, 578–586.

Lürling, M., 2006. Effects of a surfactant (FFD-6) on Scenedesmus morphology and growth under different nutrient conditions. Chemosphere 62, 1351–1358. doi:10.1016/j.chemosphere.2005.07.031

Mander, U., Mitsch, W.J., 2009. Pollution control by wetlands. Ecol. Eng. 35, 153–158.

Mandiki, S.N., Babiak, I., Bopopi, J.M., Leprieur, F., Kestemont, P., 2005. Effects of sex steroids and their inhibitors on endocrine parameters and gender growth differences in Eurasian perch (Perca fluviatilis) juveniles. Steroids 70, 85–94.

Matozzo, V., Gagné, F., Marin, M.G., Ricciardi, F., Blaise, C., 2008. Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: a review. Environ. Int. 34, 531–45. doi:10.1016/j.envint.2007.09.008

Matozzo, V., Marin, M.G., 2005. Can 4-nonylphenol induce vitellogenin-like proteins in the clam Tapes philippinarum? Environ. Res. 97, 43–49. doi:10.1016/j.envres.2004.03.002

McCahon, C.P., Pascoe, D., 1990. Episodic pollution□: causes , toxicological effects and ecological significance. Funct. Ecol. 4, 375–383.

Migowska, N., Caban, M., Stepnowski, P., Kumirska, J., 2012. Simultaneous analysis of non-steroidal anti-inflammatory drugs and estrogenic hormones in water and wastewater samples using gas chromatography-mass spectrometry and gas chromatography with electron capture detection. Sci. Total Environ. 441, 77–88. doi:10.1016/j.scitotenv.2012.09.043

Monosson, E., 2005. Chemical Mixtures: Considering the Evolution of Toxicology and Chemical Assessment. Environ. Health Perspect. 113, 383–390. doi:10.1289/ehp.6987

Morgan-Sagastume, J.M., Noyola, A., 2008. Evaluation of an aerobic submerged filter packed with volcanic scoria. Bioresour. Technol. 99, 2528–2536. doi:10.1016/j.biortech.2007.04.068

Moussavi, G., Kazembeigi, F., Farzadkia, M., 2010. Performance of a pilot scale up-flow septic tank for on-site decentralized treatment of residential wastewater. Process Saf. Environ. Prot. 88, 47–52. doi:10.1016/j.psep.2009.10.001

Naderi, M., Safahieh, A., Dehghan Madise, S., Zolgharnin, H., Rajabzade Ghatrami, E., 2012. Induction of vitellogenin synthesis in immature male yellowfin seabream (Acanthopagrus latus) exposed to 4-nonylphenol and 17β-estradiol. Toxicol. Ind. Health 1–12. doi:10.1177/0748233712469646

Nakada, N., Tanishima, T., Shinohara, H., Kiri, K., Takada, H., 2006. Pharmaceutical chemicals and endocrine disrupters in municipal wastewater in Tokyo and their removal during activated sludge treatment. Water Res. 40, 3297–303. doi:10.1016/j.watres.2006.06.039

Ng, K.P., Datuin, J.P., Bern, H.A., 2001. Effects of estrogens in vitro and in vivo on cartilage growth in the tilapia (Oreochromis mossambicus). Gen. Comp. Endocrinol. 121, 295–304. doi:10.1006/gcen.2001.7598

OECD, 2011. Guidelines for the testing of chemical: Freshwater Alga and Cyanobacteria, Growth Inhibition Test. OECD 201. doi:10.1787/9789264069923-en

Ondarza, P.M., Gonzalez, M., Fillmann, G., Miglioranza, K.S.B., 2012. Increasing levels of persistent organic pollutants in rainbow trout (Oncorhynchus mykiss) following a mega-flooding episode in the Negro River basin, Argentinean Patagonia. Sci. Total Environ. 419, 233–239. doi:10.1016/j.scitotenv.2012.01.001

Özkan, F., Gündüz, S.G., Berköz, M., Hunt, A.Ö., 2011. Induction of micronuclei and other nuclear abnormalities in peripheral erythrocytes of Nile tilapia , Oreochromis niloticus , following exposure to sublethal cadmium doses. Turk J Zool 35, 585–592. doi:10.3906/zoo-0907-77

Pacheco, M., Santos, M. a, 2001. Biotransformation, endocrine, and genetic responses of Anguilla anguilla L. to petroleum distillate products and environmentally contaminated waters. Ecotoxicol. Environ. Saf. 49, 64–75. doi:10.1006/eesa.2000.2025

Pakdel, F., Le Gac, F., Goff, L.P., Valotaire, Y., 1990. Full length sequence an in vitro expression of rainbow trout estrogen receptor cDNA. Mol Cell Endocrinol 71, 195–204.

Panter, G.H., Thompson, R.S., Sumpter, J.P., 2000. Intermittent exposure of fish to estradiol. Environ. Sci. Technol. 34, 2756–2760. doi:10.1021/es991117u

Park, J.B.K., Craggs, R.J., Shilton, a N., 2011. Wastewater treatment high rate algal ponds for biofuel production. Bioresour. Technol. 102, 35–42. doi:10.1016/j.biortech.2010.06.158

Pavlić, Z., Vidaković-Cifrek, Z., Puntarić, D., 2005. Toxicity of surfactants to green microalgae Pseudokirchneriella subcapitata and Scenedesmus subspicatus and to marine diatoms Phaeodactylum tricornutum and Skeletonema costatum. Chemosphere 61, 1061–1068. doi:10.1016/j.chemosphere.2005.03.051

Peña-Castro, J.M., Martínez-Jerónimo, F., Esparza-García, F., Cañizares-Villanueva, R.O., 2004. Phenotypic plasticity in Scenedesmus incrassatulus (Chlorophyceae) in response to heavy metals stress. Chemosphere 57, 1629–1636. doi:10.1016/j.chemosphere.2004.06.041

Perbangkhem, T., Polprasert, C., 2010. Biomass production of papyrus (Cyperus papyrus) in constructed wetland treating low-strength domestic wastewater. Bioresour. Technol. 101, 833–835. doi:10.1016/j.biortech.2009.08.062

Petrovic, M., Solé, M., López de Alda, M.J., Barceló, D., 2002. Endocrine disruptors in sewage treatment plants, receiving river waters, and sediments: integration of chemical analysis and biological effects on feral carp. Environ. Toxicol. Chem. 21, 2146–56.

Pinto, R.M., Fernandes, E.S., Peters, V.M., Guerra, M. de O., 2008. Endocrine disruptors: methods description for in vivo evaluation of chemicals with estrogenic effects (Disruptores endócrinos□: descrição dos métodos para avaliação in vivo de produtos químicos com efeito estrogênico, In Portuguese). Bol. Cent. Biol. Reprod. 26, 23–28.

Pojana, G., Bonfà, A., Busetti, F., Collarin, A., Marcomini, A., 2004. Estrogenic potential of the Venice, Italy, lagoon waters. Estrogenic potential of the Venice, Italy, lagoon waters 23, 1874–1880.

Purdom, C.E., Hardiman, P.A., Bye, V.J., Eno, N.C., Tyler, C.R., Sumpter, J.P., 1994. Estrogenic Effects of Effluents from Sewage Treatment Works. Chem Ecol 8, 275–285.

Ra, J.-S., Lee, S.-H., Lee, J., Kim, H.Y., Lim, B.J., Kim, S.H., Kim, S.D., 2011. Occurrence of estrogenic chemicals in South Korean surface waters and municipal wastewaters. J. Environ. Monit. 13, 101–109. doi:10.1039/c0em00204f

Rai, P.K., Mallick, N., Rai, L.C., 1994. Effect of Cu and Ni on growth, mineral uptake, photosynthesis and enzyme activities of Chlorella vulgaris at different pH values. Biomed. Environ. Sci. 7, 56–67.

Rainbow, P.S., 2007. Trace metal bioaccumulation: models, metabolic availability and toxicity. Environ. Int. 33, 576–82. doi:10.1016/j.envint.2006.05.007

Reinert, K.H., Giddings, J.M., Judd, L., 2002. Effects analysis of time-varying or repeated exposures in aquatic ecological risk assessment of agrochemicals. Environ. Toxicol. Chem. 21, 1977–92.

Rodas-Ortíz, J.P., Ceja-Moreno, V., Chan-Cocom, M.E., Gold-Bouchot, G., 2008. Vitellogenin induction and increased plasma 17beta-estradiol concentrations in male Nile tilapia, Oreochromis niloticus, exposed to organochlorine pollutants and polycyclic aromatics hydrocarbons. Bull Env. Contam Toxicol 81, 543–547. doi:10.1007/s00128-008-9556-9

Rodriguez-Cea, A., Ayllon, F., Garcia-Vazquez, E., 2003. Micronucleus test in freshwater fish species: an evaluation of its sensitivity for application in field surveys. Ecotoxicol. Environ. Saf. 56, 442–448. doi:10.1016/S0147-6513(03)00073-3

Rose, J., Holbech, H., Lindholst, C., Nørum, U., Povlsen, A., Korsgaard, B., Bjerregaard, P., 2002. Vitellogenin induction by 17b-estradiol and 17a-ethinylestradiol in male zebrafish (Danio rerio). Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 131, 531–9.

Salomão, A.L. de S., Marques, M., 2014. Quantification of alkali-labile phosphate groups in the plasma of Oreochromis niloticus exposed to intermittent discharges of estrogens: Effect of concentrations vs. loads. Accepted.

Salomão, A.L. de S., Soroldoni, S., Marques, M., Hogland, W., Bila, D.M., 2014. Effects of single and mixed estrogens on single and combined cultures of D. subspicatus and P. subcapitata. Accepted.

Schröder, P., Daubner, D., Maier, H., Neustifter, J., Debus, R., 2008. Phytoremediation of organic xenobiotics - Glutathione dependent detoxification in Phragmites plants from European treatment sites. Bioresour. Technol. 99, 7183–7191. doi:10.1016/j.biortech.2007.12.081

Segner, H., Caroll, K., Fenske, M., Janssen, C.R., Maack, G., Pascoe, D., Sch, C., Vandenbergh, G.F., Watts, M., Wenzel, A., 2003. Identification of endocrine-disrupting

effects in aquatic vertebrates and invertebrates□: report from the European IDEA project. Ecotoxicol. Environ. Saf. 54, 302–314.

Seim, W.K., Curtis, L.R., Glenn, S.W., Chapman, G.A., 1984. Growth and Survival of Developing Steelhead Trout (Salmo gairdneri) Continuously or Intermittently Exposed to Copper. Can. J. Fish. Aquat. Sci. 41, 433–438.

Servos, M.R., Bennie, D.T., Burnison, B.K., Jurkovic, A., McInnis, R., Neheli, T., Schnell, A., Seto, P., Smyth, S., Ternes, T., 2005. Distribution of estrogens, 17beta-estradiol and estrone, in Canadian municipal wastewater treatment plants. Sci. Total Environ. 336, 155–170. doi:10.1016/j.scitotenv.2004.05.025

Shi, W., Wang, L., Rousseau, D.P.L., Lens, P.N.L., 2010. Removal of estrone, 17alpha-ethinylestradiol, and 17beta-estradiol in algae and duckweed-based wastewater treatment systems. Environ. Sci. Pollut. Res. Int. 17, 824–833.

Sijm, D.T.H.M., Broersen, K.W., Roode, D.F. de, Mayer, P., 1998. Bioconcentration kinetics of hydrophobic chemicals in different densities of Chlorella pyrenoidosa. Environ. Toxicol. Chem. 17, 1695–1704.

Silva, A., Figueiredo, S. a, Sales, M.G., Delerue-Matos, C., 2009. Ecotoxicity tests using the green algae Chlorella vulgaris--a useful tool in hazardous effluents management. J. Hazard. Mater. 167, 179–185. doi:10.1016/j.jhazmat.2008.12.102

Solano, M.., Soriano, P., Ciria, M.., 2004. Constructed Wetlands as a Sustainable Solution for Wastewater Treatment in Small Villages. Biosyst. Eng. 87, 109–118. doi:10.1016/j.biosystemseng.2003.10.005

Solé, M., Porte, C., Barceló, D., 2000. Vitellogenin Induction and Other Biochemical Responses in Carp, Cyprinus carpio , After Experimental Injection with 17α-Ethynylestradiol. Arch. Environ. Contam. Toxicol. 38, 494–500. doi:10.1007/s002449910065

Solé, M., Porte, C., Barceló, D., 2001. Analysis of the estrogenic activity of sewage treatment works and receiving waters using vitellogenin induction in fish as a biomarker. Trends Anal. Chem 20, 518–525.

Song, H.-L., Nakano, K., Taniguchi, T., Nomura, M., Nishimura, O., 2009. Estrogen removal from treated municipal effluent in small-scale constructed wetland with different depth. Bioresour. Technol. 100, 2945–2951. doi:10.1016/j.biortech.2009.01.045

Soto, A.M., Sonnenschein, C., Chung, K.L., Fernandez, M.F., Olea, N., Serrano, F.O., 1995. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ. Heal. Perspect. 103, 113–122.

Souza, M.S., Hallgren, P., Balseiro, E., Hansson, L.-A., 2013. Low concentrations, potential ecological consequences: synthetic estrogens alter life-history and demographic structures of aquatic invertebrates. Environ. Pollut. 178, 237–243. doi:10.1016/j.envpol.2013.03.038

Sponchiado, G., Lucena Reynaldo, E.M.F., Andrade, A.C.B., Vasconcelos, E.C., Adam, M.L., Oliveira, C.M.R., 2010. Genotoxic effects in erythrocytes of Oreochromis niloticus exposed to nanograms-per-liter concentration of 17β-estradiol (E2): An assessment using micronucleus test and comet assay. Water Air Soil Pollut 218, 353–360. doi:10.1007/s11270-010-0649-9

Stumpf, M., Ternes, T.A., Wilken, R.D., Rodrigues, S. V., Baumann, V., 1999. Polar Drug Residues in Sewage and Natural Waters in the State of Rio de Janeiro, Brazil. Sci. Total Environ. 225, 135–141.

Subashchandrabose, S.R., Ramakrishnan, B., Megharaj, M., Venkateswarlu, K., Naidu, R., 2013. Mixotrophic cyanobacteria and microalgae as distinctive biological agents for organic pollutant degradation. Environ. Int. 51, 59–72. doi:10.1016/j.envint.2012.10.007

Sumpter, J.P., Jobling, S., 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ. Heal. Perspect. 103, 173–178.

Sumpter, J.P., Jobling, S., Tyler, C.R., 1996. Oestrogenic substances in the aquatic environment and their potential impact on animals, particulary fish, in: Taylor, E. (Ed.), Toxicology of Aquatic Pollution. Cambridge University Press, New York, pp. 205–224.

Tennekes, H. a, Sánchez-Bayo, F., 2013. The molecular basis of simple relationships between exposure concentration and toxic effects with time. Toxicology 309, 39–51. doi:10.1016/j.tox.2013.04.007

Ternes, T.A., Stumpf, M., Mueller, J., Haberer, K., Wilken, R.D., Servos, M., 1999. Behavior and occurrence of estrogens in municipal sewage treatment plants-I. Investigations in Germany, Canada and Brazil. Sci. Total Environ. 225, 81–90.

Thompson, A.S., Rhodes, J.C., Pettman, I., 1988. Culture Collection of Algae and Protozoa. Catalogue of strains, 5th ed. Cumbria, U.K.

Thorpe, K.L., Cummings, R.I., Hutchinson, T.H., Scholze, M., Brighty, G., Sumpter, J.P., Tyler, C.R., 2003. Relative potencies and combination effects of steroidal estrogens in fish. Environ. Sci. Technol. 37, 1142–1149.

Tisler, T., Zagorc-Koncan, J., 1997. Comparative assessment of toxicity of phenol, formaldehyde, and industrial wastewater to aquatic organisms. Water. Air. Soil Pollut. 97, 315–322.

Torres, M. a, Barros, M.P., Campos, S.C.G., Pinto, E., Rajamani, S., Sayre, R.T., Colepicolo, P., 2008. Biochemical biomarkers in algae and marine pollution: a review. Ecotoxicol. Environ. Saf. 71, 1–15. doi:10.1016/j.ecoenv.2008.05.009

Trainor, F.R., Cain, J.R., Shubert, L.E., 1976. Morphology and nutrition of the colonial green alga Scenedesmus: 80 years later. Bot. Rev. 42, 5–25.

Trudel, M., Rasmussen, J.B., 2006. Bioenergetics and mercury dynamics in fish□: a modelling perspective. Can. J. Fish. Aquat. Sci. 63, 1890–1902. doi:10.1139/F06-081

Vardy, D.W., Tompsett, A.R., Sigurdson, J.L., Doering, J. a, Zhang, X., Giesy, J.P., Hecker, M., 2011. Effects of subchronic exposure of early life stages of white sturgeon (Acipenser transmontanus) to copper, cadmium, and zinc. Environ. Toxicol. Chem. 30, 2497–2505. doi:10.1002/etc.638

Verslycke, T., Vandenbergh, G.F., Versonnen, B., Arijs, K., Janssen, C.R., 2002. Induction of vitellogenesis in 17alpha-ethinylestradiol-exposed rainbow trout (Oncorhynchus mykiss): a method comparison. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 132, 483–492.

Versonnen, B.J., Goemans, G., Belpaire, C., Janssen, C.R., 2004. Vitellogenin content in European eel (Anguilla anguilla) in Flanders, Belgium. Environ. Pollut. 128, 363–371.

Vogel, J.M., 2005. Tunnel vision: The regulation of endocrine disruptors. Policy Sci 37, 277–303. doi:10.1007/s11077-005-1764-0

Wang, Q., Cui, Y., Dong, Y., 2002. Phytoremediation of Polluted Waters Potentials and Prospects of Wetland Plants. Acta Biotechnol. 22, 199–208.

Yadav, K.K., Trivedi, S.P., 2009. Sublethal exposure of heavy metals induces micronuclei in fish, Channa punctata. Chemosphere 77, 1495–500. doi:10.1016/j.chemosphere.2009.10.022

Ying, G.-G., Kookana, R.S., Ru, Y.-J., 2002. Occurrence and fate of hormone steroids in the environment. Env. Int 28, 545–551.

Zafar, M.I., Van Wijngaarden, R.P. a, Roessink, I., Van den Brink, P.J., 2011. Effects of time-variable exposure regimes of the insecticide chlorpyrifos on freshwater invertebrate communities in microcosms. Environ. Toxicol. Chem. 30, 1383–94. doi:10.1002/etc.509

Zhang, T.C., Fu, Y.C., Bishop, P.L., 1994. Competition in biofilms. Water Sci. Technol. 29, 263–270.

Zimmels, Y., Kirzhner, F., Malkovskaja, A., 2006. Application of Eichhornia crassipes and Pistia stratiotes for treatment of urban sewage in Israel. J. Environ. Manage. 81, 420–428. doi:10.1016/j.jenvman.2005.11.014

Zorita, S., Hallgren, P., Mathiasson, L., 2008. Steroid hormone determination in water using an environmentally friendly membrane based extraction technique. J Chromatogr A 1192, 1–8. doi:10.1016/j.chroma.2008.03.030

APÊNDICE A – Versão original publicada e Defendida na Linnaeus University

A presente Tese de Doutorado é o produto de um Convênio para cotutela internacional de Tese estabelecido entre a Universidade do Estado do Rio de Janeiro – UERJ, Brasil, representada por seu Magnífico Reitor Dr. Ricardo Vieiralves de Castro e a Linnaeus University – LNU, Suécia, representada pelo Vice Reitor Dr. Nils Nilsson.

Como previsto no Convênio, a Tese teve uma única defesa realizada em 23 de maio de 2014, na cidade de Kalmar na Suécia, no auditório da Linnaeus University e foi reconhecida pelas duas Universidades (em duas atas de defesa). A aprovação se deu seguindo as regras da Linnaeus University. A redação original foi publicada (ISBN: 978-91-87427-87-9) totalmente em inglês nos moldes da Linnaeus University (N° 173/2014), com o resumo em português, inglês e sueco. Entretanto a mesma foi formatada nos moldes da Universidade do Estado do Rio de Janeiro – UERJ, dando origem ao presente documento de Tese.

APÊNDICE B - Lista de publicações de autor da tese

I. **Salomão, A.L.S.**, Marques, M., Severo, R.G., Roque, O.C.C. (2012). Engineered ecosystem for on-site wastewater treatment in tropical areas. *Water Science and Technology* 66(10): 2131-2137.

II. **Salomão, A.L.S.**, Marques, M. (2014). Quantification of alkali-labile phosphate groups in the plasma of *Oreochromis niloticus* exposed to intermittent discharges of estrogens: Effect of concentrations *vs.* loads. *International Journal of Environmental Analytical Chemistry.* http://dx.doi.org/10.1080/03067319.2014.930845

III. **Salomão, A.L.S.**, Marques, M. Estrogenicity and genotoxicity detection in different contaminated waters using *Oreochromis niloticus* as bioindicator (*Accepted*).

IV. **Salomão, A.L.S.**, Soroldoni, S., Marques, M., Hogland, W., Bila, D.M. (2014). Effects of single and mixed estrogens on single and combined cultures of *D. subspicatus* and *P. subcapitata*. *Bulletin of Environmental Contamination and Toxicology* 93 (215–221) DOI 10.1007/s00128-014-1294-6

V. Laohaprapanon, S., **Salomão, A.L.S.**, Marques, M., Hogland, W. Toxicity evaluation of a wooden floor industrial wastewater treated with different activated carbons on the *Pseudokirchneriella subcapitata*. (*Submitted*).

VI. **Salomão, A.L.S.**, Marques, M. Relevant factors – some of them often neglected - when applying existing or developing new aquatic ecotoxicity assays. (*Manuscript*).