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Integrated risk assessment of endocrine disruptors in the Uruguay River

School of Applied Sciences
Cranfield Water Science Institute

PhD Thesis
Academic Year: 2008 - 2013

Supervisors: Prof. Elise Cartmell
Dr Ana Soares
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This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

DEDICATION

This thesis is dedicated to my parents Adela and Olimpo, my children Derek and Alison, and the future generations.

IN MEMORY OF MY GRANDPARENTS ADELA AND VÍCTOR AND MY SISTER ROSE

LIST OF PUBLICATIONS AND CONFERENCE PRESENTATIONS

Peer review papers

I. **Míguez, D.**, Huertas, R., Carrara, V., Carnikian, A., Bouvier, E., Martinez, M.J., Keel, K, Pioda C., Darré, E., Pérez, R., Viera, S., Massa, E. (2012), Bioavailability, ecotoxicity, and geological characteristics of trace lead in sediments from two sites on Negro River, Uruguay, South America, *Environmental Geochemistry and Health* **34**(2),199-205.

II. **Míguez, D.**, Cartmell, E., Soares, A., Pollard, S. (2012), Tiered approach for exposure assessment of endocrine disruptors in the Uruguay River, In: *Proceedings of the Endocrine Disruptors 2012* Congress, Smithers, 14-15 February 2012, Munich, Germany.

III. Ferrari, G., Pérez, M.C., Dabezies, M., **Míguez, D.** & Saizar, C. (2011), Planktic cyanobacteria in the Lower Uruguay River, South America, *Fottea* **11**(1), 225–234.

IV. Cabrera, S., **Míguez, D.**, Irigaray, R. (2010), Estudio comparativo de la determinación del tamaño de partícula por dos métodos: tamizado en seco y difracción láser [Comparative study for the determination of two methods of particle size determination: dry sieving and laser diffraction], *Innotec* **5**, 23-28.

V. Keel, K, **Míguez, D.**, Soares, A., Parodi, A. (2010), Optimización de una técnica de medida de disrupción endocrina por medio de *Saccharomyces cereviceae* recombinantes [Optimisation of a measuring technique of endocrine disruption by means of recombinating Saccharomyces cereviceae], *Innotec* **5**, 34-37.

VI. **Míguez, D.**, Seoane, I, Carrara, V, Carnikian, A., Keel, K, Aizpun, A., Bouvier, E, Cartmell, E. (2010), Evaluación ecotoxicológica de sedimentos en una zona del Río Uruguay, con puntos finales indicadores de toxicidad aguda, sub-letal, crónica, reproductiva y teratogénica [Ecotoxicological evaluation of sediments in a zone of the Uruguay River, with endpoints indicative of acute, sub-lethal, chronic, reproductive and teratogenic toxicity], *Innotec* **5**, 3-10.

VII. Saizar, C., **Míguez, D.**, Dabezies, M., Texeira de Mello, F., Clemente, J., Ferrari, G., Boccardi, L., Tana, J. (2010), Línea de base para evaluar el impacto de una planta de celulosa en el Río Uruguay [Baseline to evaluate the impact of a pulp mil on the Uruguay River], *Innotec* **5**, 11-22.

VIII. **Míguez, D.** (2007), Implicancias del uso de nonoxinol en el Uruguay [Implications of the use of nonoxynol in Uruguay], *Innotec* **2**, 10-11.

IX. **Míguez, D.** (2007) Breve reseña sobre el Río Uruguay [Minireview on the Uruguay River], *Innotec* **2**, 7-9.

2. Posters

I. Keel, K., **Míguez, D.**, Parodi, A. (2012), Disrupción endocrina en *Pimephales promelas* expuestos a efluentes urbanos e industrial. [Endocrine disruption of Pimephales promelas exposed to urban and industrial effluents]. In: *XII Congresso Brasileiro de Ecotoxicologia* [*XII Brasilian Congress of Ecotoxicology, Society of Environmental Toxicology and Chemistry, SETAC*] Porto de Galinhas, Brazil.

II. Carnikian, A., **Míguez, D.**, Vizziano-Cantonnet, D. (2011), Histomorphological evaluation of *Pimephales promelas* male gonads after exposure to pulp mill and domestic discharges into the Uruguay River (Fray Bentos- Uruguay), In: *9th International Symposium on Reproductive Physiology of Fish*, Cochin, India.

III. Carrara, M. V., **Míguez, D.**, Dol, I. (2011), Comparación de dos métodos para la determinación de materia orgánica o carbono orgánico total en suelos y sedimentos [Comparison of wo methods for the determination of organic matter or total organic carbón in soils and sediments] In: *Segundo Encuentro Nacional de Ciencias Químicas, ENAQUI 2011*, Montevideo, Uruguay.

IV. **IV.** Irigaray, R., **Míguez, D.**, Fuentes, J. (2011), Evaluación de desempeño analítico de método de distribución de tamaño de partículas por difracción láser dinámica, comparación contra el método clásico de la pipeta para limo y arcilla [Evaluation of the analytical performance of the laser dynamic diffraction method of particle size distribution and comparison to the classical pipette

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V. Keel, K., **Míguez, D.**, Carnikian, A., Parodi, A. (2010) (2010), Desarrollo de los métodos QPCR y ELISA para determinar expresión del gen de vitelogenina en Pimephales promelas y su uso como biomarcador de disrupción endócrina en muestras de agua superficial de Uruguay. [Development of QPCR and ELISA methods to determine vitellogenin gene expression in *Pimephales promelas* and its use as biomarker of endocrine disruption in samples of surface water in Uruguay], In: *XIII Jornadas de Biociencias*, Piriápolis, Uruguay.

VI. **Míguez, D.**, Martínez, M.J., Dol, I. (2009), Desarrollo del método análisis por inyección en flujo para la determinación de fósforo total en sedimentos [Development of a flow injection analysis method for the determination of total phosphorus in sediments], In: *1er Congreso Nacional de Química Analítica, CUQA* [1st National Congress of Analytical Chemistry] Montevideo, Uruguay.

VII. Ferrari G., Cea J., **Míguez, D.**, Saizar C. (2009), Monitoreo de floraciones algales en el Río Negro, Uruguay [Monitoring of algal blooming in the Negro River, Uruguay]. In: *Cianobacterias: del conocimiento a la gestión* [Cyanobacteria: From knowledge to management] Montevideo, Uruguay.

VIII. **Míguez, D.**, Seoane, I. (2007), Aplicaciones de los bioensayos con *Daphnia magna* en evaluaciones ambientales [Applications of the *Daphnia magna* bioassays in environmental assessments]. In: *SETAC LA Congress*, Montevideo, Uruguay.

IX. **Míguez, D.**, Seoane, I, Carrara, V, Keel, K, Carnikian, A, Aizpún A, Bouvier, E, Cartmell E. (2010), Evaluación de los efectos de toxicidad crónica, de reproducción y de teratogenicidad en sedimentos del Río Uruguay por métodos ecotoxicológicos y químicos [Evaluation of the chronic toxicity, reproductive and teratogenicity effects in sediments from the Uruguay River by ecotoxicology and chemical methods] In: *XIII Jornadas de la Sociedad Uruguaya de Biociencias*, SUB, [XIII Workshop of the Uruguayan Society of Biosciences] Piriápolis, Uruguay.

X. **Míguez, D.**, Huertas R., Carrara, V, Carnikian, A, Bouvier, E, Martínez M.J., Keel, K, Pioda C., Darré E., Pérez R., Viera S., Massa, E. Bioavailability, ecotoxicity and geological characteristics of trace lead in sediments from two sites on Río Negro River, Uruguay. In: *3rd. Hemispheric Conference on Medical Geology*, 2009 Montevideo.

3. Presented conferences

I. **Míguez, D.** (2012), Tiered approach for exposure assessment of endocrine disruptors in the Uruguay River, *Endocrine Disruptors 2012*, Smithers, Munich, Germany.

II. **Míguez, D.** (2010), Evaluación de los efectos de toxicidad crónica, de reproducción y de teratogenicidad en sedimentos del Río Uruguay por métodos ecotoxicológicos y químicos [Evaluation of the chronic toxicity, reproductive and teratogenicity effects in sediments from the Uruguay River by ecotoxicology and chemical methods] In: *XIII Jornadas de la Sociedad Uruguaya de Biociencias, SUB*, Piriápolis, Uruguay.

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ABSTRACT

The potential reproductive, developmental, immunological, growth and carcinogenetic effects of endocrine disruptors in humans and wildlife is of global concern. Scarce prior risk analyses of these multiple stressors in river watersheds existed. Therefore, this thesis developed an integrated risk assessment of endocrine disruptors at a section of the Lower Uruguay River with industrial (a bleached Kraft pulp mill), domestic (cities) and agricultural (soy crops) sources. A preliminary risk assessment prioritised oestrogens and further compounds of concern in the watershed, notably nonylphenol, glyphosate, endosulfan, chlorophenols, dioxins and furans, polyaromatic hydrocarbons, polychlorinated byphenyls, rosin acids and phytosterols. Models predicted their multimedia distribution, and food web interactions, and then tested. A three-tiered exposure assessment first rated the river status with eutrophication risks using artificial neural networks, while growth effects evidenced in *Hyalella curvispina*. Then, river sampling sites were determined by hydrodynamic modelling, tracking pollutant transport by clustering and observing reproductive effects in *Ceriodapnia dubia*. Finally, target compounds were analysed and endocrine disruption studied from gene to population levels. Biomonitoring with *Astyanax fasciatus* wildfish found no intersex, but smaller testes downstream the pulp mill and lower condition factor near municipal discharges. Spinal malformations were observed exposing *Pimephales promelas* to sediment elutriates. When exposed to pulp mill effluent, egg production decreased by half. Anti-oestrogenic or androgenic effects were suggested by the toxicogenomic biomarkers *ESR1*, *ESR2*, *IGF-I* and *GHR*. The oestrogenicity of a stream receiving municipal wastewater was demonstrated by effects like estradiol in *ZP3*, *ESR1* and *IGF-I* expression, in agreement with the luciferase receptor-binding screen, and the occurrence of oestrogens and nonylphenol. Overall risks of endocrine disruptors were estimated with radar diagrams, pondering nonylphenol and endosulfan as of concern in the watershed.The risks of endocrine disruption to humans through fish and water ingestion were characterised as low, and from low to moderate to freshwater biota.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ANN	Artificial neural networks
AOX	Adsorbable organic halogens
AR	Androgen receptor
BCF	Bioconcentration factor
BKME	Bleached Kraft mill effluent
BFGS-1	Broyden-Fletcher-Goldfarb-Shanno (BFGS-1) algorithm
BMD	Benchmark dose
CARU	Comisión Administradora del Río Uruguay [Uruguay River Administrative Commission]
DEFRA	Department of Environment, Food and Rural Affairs
DINAGUA	Dirección Nacional de Agua [National Water Directorate]
DINAMA	Dirección Nacional de Medio Ambiente [National Environment Directorate]
DINARA	Dirección Nacional de Recursos Acuáticos [National Aquatic Resources Directorate]
DINAMIGE	Dirección Nacional de Minería y Geología [National Mining and Geology Directorate]
ED	Endocrine disruption
EDCs	Endocrine disrupting chemicals
EEQ	Oestradiol equivalent
EM	Expectation maximisation algorithm
EOX	Extractable organic chemicals
ER	Oestrogen receptor
GC-MS	Gas chromatography with mass spectrometry detection
ESR1	Oestrogen receptor 1
ESR2	Oestrogen receptor 2
GHF	Growth hormone factor
GSI	Gonadosomatic index
HQ	Hazard quotient
HPLC/MS-MS	High-performance liquid chromatography tandem mass spectrometry
HRGC/HRMS	High resolution gas chromatography coupled to high resolution mass spectrometry
IC50	Inhibitory concentration 50
IGF-I	Insulin-like growth factor 1
INACAL	Instituto Nacional de Calidad [National Institute of Quality]
ISO	International Organisation for Standardisation
K	Condition factor

LATU	Laboratorio Tecnológico del Uruguay [Technological Laboratory of Uruguay]
LC/ESI/MS/MS	Liquid chromatography coupled to electrospray ionization tandem mass spectrometry
LC50	Lethal concentration 50
LOD	Limit of detection
LOEC	Lowest effect concentration
MOA	Mechanism of action
MOE	Mode of exposure
MRLs	Minimal risk levels
ND	Not detectable
NOEC	No observed effect concentration
NP	Nonylphenol
OECD	Organisation for Economic Co-operation and Development
PAH	Polycyclic aromatic hydrocarbons
PBPK	Physiologically based pharmacokinetic
PCA	Principal component analysis
PCBs	Polychlorinated biphenyl
PEC	Predicted exposure concentration
PNEC	Predicted no effect concentration
POPs	Persistent organic pollutants
PRA	Preliminary risk assessment
QRA	Quantitative risk assessment
RA	Risk assessment
RA (%)	Relative activity (%)
RfD	Reference dose
TCDD	2,3,7,8-tetrachlorodibenzodioxin
TEF	Toxic equivalence factor of the toxicity of dioxins, furans and PCBs in terms of the most toxic form of dioxin, TCDD
TEQ	Toxicity equivalent
UDELAR	Universidad de la República, República Oriental del Uruguay [University of the Republic, Oriental Republic of Uruguay]
UF	Uncertainty factor
UKAS	United Kingdom Accreditation Service
USEPA	United States Environmental Protection Agency
USGS	United States Geological Survey
VTG	Vitellogenin
ZP3	*Zona pellucida* 3 gene

1 INTRODUCTION

The current methodology for human and environmental risk assessment of endocrine disruptors is still not fully harmonised (OECD 2012). Confounding factors such as the particular toxicokinetics characteristics of endocrine disrupting chemicals (EDCs), their different mechanisms of action and receptors, their multiple pathways and sources and ubiquity in nature, as they are present in nanograms per litre concentrations throughout the aqueous environment, are relevant. Remarkable issues are the preferential vulnerability of organisms to these xenobiotics at certain developmental stages, and that in some cases responses can be paradoxically higher at low-doses. Stressor concentrations below their individual thresholds can still combine with others giving rise to an observable effect.

Low concentrations of EDCs range from pg l^{-1} for dioxins and furans (Thuan et al. 2011), to ng l^{-1} for steroidal hormones in water and wastewater (Ying et al. 2009), and µg l^{-1} for alkylphenols (Céspedes et al. 2008). They globally occur in surface waters and characterise by low-dose effects (Kroes et al. 2004). Sheehan (2006) hypothesized that when EDCs are at these low exposure levels they do not elicit a dose-response threshold because of the endogenous hormones background effect. Mixture effects of EDCs are not easy to assess because of additive synergic, agonistic or antagonistic interactions (Kortenkamp 2007). This justified the use of a tiered approach that measured the concentrations of the EDCs, screened endocrine disruptive action with *in vitro* tests and confirmed the effects through a suite of *in vivo* assays. All these issues were part of the challenges during the development of this thesis.

1.1 Endocrine disrupting chemicals

Some of the released substances that may pollute the environment pertain to a group of chemicals collectively called EDCs for their ability to interfere with the normal functions of endocrine systems in higher organisms and affect homeostasis at all trophic hierarchy levels. According to Kavlock et al. (1996),

an EDC is defined as “an exogenous agent that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes”. This concept was extended to mixtures by the World Health Organisation (WHO 2002) and to effects not only on the organism but on its progeny, that could affect (sub)populations.

They may impair reproduction (Fowler et al. 2012), development (Sárria et al. 2011, Virtanen & Adamsson 2012), immunity (Utsuyama et al. 2002) or growth (Rodríguez et al. 2007), and they have been linked to increased risks of cancer of female reproductive organs (Ma 2009), breast (Okoh et al. 2011, Lee & Choi 2013) and prostate (Hu et al. 2012). A broad range of chemicals, including pesticides, pharmaceuticals, natural and synthetic oestrogens cosmetics and detergents, plasticisers, polychlorinated biphenyls, and others may be present in effluents and wastewater (Sarmah et al. 2006, Chang et al. 2009), and concomitantly as residues in water, sediment, soil and biota of the receiving media (Zhang et al. 2007, Ying et al. 2009). When detected in the environment as complex mixtures, EDCs may act additively even at low doses, exerting their actions below their active concentrations as single compounds, but they may also present synergistic or antagonistic modes of action.

1.1.1 Concern of endocrine disruption

The phenomenon referred to as endocrine disruption, was first realised with the appearance of uterine and vaginal/cervical cancers in young women whose mothers took the synthetic oestrogen diethylstilbestrol (DES) during pregnancy (Sassoon 2010). One of the reasons for the increasing global concern on the occurrence of EDCs in the environment is that exposures during early development, even at low doses, may seriously have an impact on postnatal development. Breast milk, contains balanced nutrition and immunity to new-borns and babies (Lawrence & Pane 2007) but it can also be a carrier of EDCs (Ulaszewska et al. 2011, Gebremichael et al. 2013, Guerranti et al. 2013, Lawrence 2013).

As argued by Faulk & Dolinoy (2011),"timing is everything" in the epigenetics determination of an organism affected by environmental exposure to EDCs either by gene transcription, translation and/or by post-translational modification of chromatin remodelling complexes. The protection of foetuses, new-borns babies and children from these exposures is in the global agenda (EU 2003 [COM/2003/0338], WHO 2011b, USEPA 2013). For the same reason, the current trend of investigation is to study the vulnerability of freshwater receptors to EDCs at early developmental stages (Crews et al. 2000, van Aerle et al. 2002, Sişman et al. 2007, Halder et al. 2010).

International research has reported the occurrence of EDCs in municipal wastewater (Jiang et al. 2005), as well as in some industrial effluents like tanneries, leather (Pothitou & Voutsa 2008) and pulp mills (Kostamo & Kukkonen 2003). As Jobling et al. (2006) states, the diverse effects of EDCs on the thyroid (Jugan et al. 2010), androgenic, oestrogenic, and corticosteroid systems of a wide range of animals, demands that research addresses the extent of the risk posed by EDCs to wildlife. However, the cause-effect relationship is still controversial in human beings but concern has risen due to the severity of probable harm that could affect individuals and populations, implying that regulatory decisions should be informed by risk assessments on this important topic (Fenner-Crisp 2000). Effects on development and reproduction of wildlife have been profusely documented (Colborn et al. 1993, Jobling & Tyler 2006). Living creatures potentially exposed to EDCs range from bacteria to mammals. Many reports demonstrate through laboratory experiments and field surveys that exposure of animals from the environmental release of EDCs exert reproductive or developmental effects on the individual and its offspring, affecting the viability of the species at a population level (Colborn & Smolen 1997). Several effects observed in wildlife are attributable to the exposure to chemicals (Jobling & Tyler 2006).

1.1.2 Anthropic activities as sources of endocrine disruptors and their effects

Human activities represent a myriad of sources possibly linked to the release of EDCs into the riverine environment. Rivers are of extreme importance as natural resources and vital as drinking water sources, for fisheries and other economic uses.

One of the most studied cases is the impact of pulp mill effluents on fish reproduction has been observed by international research (Larsson & Forlin 2002, Mc Master et al. 2006, Hewitt et al. 2008, Milestone et al. 2012). Pulp mill releases have been linked to effects on fish reproductive function and even producing anomalies varying from subtle functional changes, to permanent alterations including disturbed sex differentiation with the appearance of feminised or masculinised sex organs in exposed fish downstream the discharges into rivers. For instance, there is considerable evidence of reproductive and immunological effects in seals living in environments polluted with organochlorines, induction of VTG in fish near point sources pulp mills discharges. There are some cases of androgenic effects on aquatic organisms exposed to pulp mill effluents (Larsson & Forlin 2002). However, most findings refer to oestrogenic effects comprising delayed development of sex characteristics, de-masculinisation or feminisation of exposed male fish (e.g., ovipositor development), or masculinisation of female fish (tubercles and dorsal fin dot) (McMaster et al. 2006, Hewitt et al. 2008).

The occurrence of EDCs in rivers and receiving environments situated near sewage treatment plants also raises concerns of this type, challenging the removal efficacy by conventional treatment processes, and obviously even more in developing countries where usually the treatment is only primary treatment. Domestic wastewaters, as they are known to possess mixtures of EDCs, such as natural and synthetic steroidal hormones (Jobling et al. 2006). Substances frequently found in municipal wastewater are for example the non-ionic surfactant nonylphenol ethoxylate (NPE) and its degradation by-product

nonylphenol (NP) (Mills & Chichester 2005, Månsson et al. 2008)M, Koh et al. 2007, Soares et al. 2008). Nonylphenol ethoxylates (NPEs) are part of the alkylphenol ethoxylate group of non-ionic surfactants. NPEs are used as emulsifiers, dispersive agents, surfactants and/or wetting agents and are the primary source of inputs to the sea of NP and NPEs.

Agricultural runoff is yet another important input of EDCs into riverine ecosystems, resulting from the excessive use of persistent and/or toxic agrochemicals, as many pesticides (Lyons 1999, Grünfeld & Bonefeld-Jorgensen 2004), and herbicides have been reported as EDCs (Orton et al. 2009).

1.2 The Uruguay River research

Many human activities may pollute freshwater ecosystems, which in turn, may risk the welfare of human beings. In this regard, conducting an integrated risk assessment aims at integrating environmental effects in fish and other freshwater receptors and human effects and at examining the effects of EDC mixtures. The situation in South America concerning research on the occurrence, fate or effects of endocrine disruptors was negligible, and limited risk assessments had been completed to assess the pollution load and environmental and health effects of EDCs specifically in the Uruguay River. Moreover, there were scarce prior methods developed for effect- demonstration of endocrine disruption in the aquatic environment in the country. Therefore, the designed integrated risk assessment framework for mixtures of EDCs was developed and demonstrated at a section of the Uruguay River.

At the research area, municipal wastewaters are discharged after little or no treatment, non-point source pollution from agriculture is present, and a high-tonnage Kraft pulp mill is operating, discharging secondary treated final effluent into the river. The river serves as source water for drinking water production for Fray Bentos city and the intake is 3 km downstream from the pulp mill discharge pipe, which could imply a risk to human health. This is why it is necessary to

include an assessment for human health, as the river serves for drinking water production, and the population consumes fish caught from the river.

Examples of EDCs existing in municipal wastewaters are nonylphenol ethoxylates, as the detergent is still in use in Uruguay for industrial uses such as wool scouring and domestic applications (Míguez 2007b). Agrochemicals are extensively used in the soy fields and forestry in the area of study. For example, the main herbicide applied to transgenic soy fields in Uruguay and Argentina is glyphosate (Peruzzo et al. 2008, UNEP 2008). This molecule is not as persistent as other EDCs. Therefore, it was treated using a slightly different methodology than the others. Endosulfan and other persistent pesticides were considered, based on prior reports in fish tissues.

1.3 Why is risk assessment the topic of this research?

This risk assessment was intended to provide an objective outlook to assess the risks to human health and the environment at a river watershed with potential sources of EDCs to inform stakeholders including residents and the general community, academia, economically involved parties and those in charge of risk management decisions and policy actions.

1.3.1 Developing a risk assessment of endocrine disruptors from a global perspective

These issues have been included in international policies and research agendas (OECD 2003), recognising adverse effects provoked by EDCs on human health and the environment, as reflected by the conclusions of the Council of the European Union on the combined effects of chemicals (Council of the European Union 17820/09). In this sense, the European Environment and Health Strategy (COM 2004) emphatically stresses gaps in knowledge related to risk assessment methodologies, it includes foetuses, infants and children, and calls for the precautionary principle within the strategy for environmental contaminants. It aims at providing enough scientific evidence at the effect level (e.g. molecular, cellular, or tissue-related) to show the likelihood of health

impacts. The link between emissions of persistent organic pollutants (POPs) and their accumulation in ecosystems and foodstuffs is also recognised as a gap in research. In relation to this, some EDCs such as dioxins and furans or polyaromatic hydrocarbons are transported great distances through air pathways which therefore exceeds regional boundaries (Scheringer et al. 2009).

There are political implications for transboundary watercourses within shared basins, with human activities on both margins. Further, EDCs transports as residues in foodstuff. That is why a requirement of the EU legislation imposes strict demands on exporters to protect consumers. Therefore, the link between policy and research should be globally strengthened, and in particular in developing countries, representing the major providers of commodities. The situation in South America, including Uruguay, concerning research on the EDCs topic is still very scarce.

1.3.2 Challenges posed by endocrine disruptors to current risk assessment methodologies

Current methodologies for assessing human and wildlife health effects target at detecting effects rather than mechanisms, and may not adequately evaluate effects on the endocrine system. This is particularly true for exposures that occur during critical developmental periods when the endocrine system plays a key role in regulating essential physiological and morphological processes (USEPA 1996a). Endocrine disruptors represents a challenge to the traditional risk assessment paradigms which have traditionally dealt with associations between specific agents and a defined disease end-point, whereas the modulation of a communication system such as the endocrine system is a mechanism of toxicity rather than an adverse effect *per se*. Therefore, the methods are increasingly directed towards a mechanistic-based research.

Even though they have an emerging relevance to human and environmental health EDCs in many cases they lack a regulatory limit. They may be released to aquatic and soil as multiple stressors to all the environmental compartments,

generating potential adverse effects on receptors through different routes and pathways. Their actions may be chronic, long-term developmental or reproductive effects for the individual and/or its progeny (Caserta et al. 2008), eventually representing systemic risks at the population level, which may affect sustainability and biodiversity. Moreover, as de Voogt & van Hattum (2003) sustain, their multimedia transport, partitioning, and degradation pathways of interaction with target organisms, their toxicokinetics, and life-cycle aspects have to be considered to determine the final concentration in the target organs.

1.3.3 Why endeavour to integrate human beings and animals in one single assessment?

Endocrine disruptors are priority candidate substances for an integrated risk assessment as they could affect environmental species and human beings, the substance or its metabolites could bioaccumulate, while its mechanisms of action are still not completely elucidated (Bridges & Bridges 2004). Due to their ubiquity, it is necessary to include human beings as potential receptors and deal with risk assessment in an integrated manner. While there is scientific consensus that many chemicals are able to affect the endocrine system of animals and human beings arising from exposure to EDCs released into the environment, the debate still focuses on whether there is a risk for them at a population level, and when in mixtures at low doses.

As sustained by several authors (Suter et al. 2003, Sekizawa & Tanabe 2005, Vermeire et al. 2007), achieving integration is in theory possible in all risk assessment steps. Within the planning step, the conceptual model includes sources, pathways and effects both for humans and environmental receptors. Exposure assessment integrates sources, most of the pathways, fate, transport, bioaccumulation and biodegradation of substances is followed by a hazard analysis of common reported effects and mechanisms, biomarkers and bioindicators. Then, at the risk characterisation stage, the combined effects are evaluated through an exposure-response model based on the shared

mechanisms of action of humans and ecological receptors to integrate the assessment (WHO 2001, Vermeire et al. 2007).

Endocrine disruptors are fit for integration as they may potentially affect the environment and humans, provided bioaccumulation is expected in species used for human consumption. Integrated risk assessment can help understand impacts on human health and on ecosystems due to the extrapolation from animal experiments after defining mechanisms of action. So, in the risk characterisation phase the differences in mechanisms of action should be considered as a basis for inter-species extrapolation (Bridges & Bridges 2004). Epidemiology data, computer models and biomarkers are important tools in human health assessments to identify mixed effects (Robinson & MacDonell 2004.

1.3.4 The risk assessment approach used in this research

There were scarce prior experiences of risk assessments at a watershed level for multiple stressors, and few focused on the endocrine disruption endpoint, but in general only as single stressors. There was also a lack of a framework applying a holistic and systematic methodology for exposure assessment of application to river watersheds for both humans and animals. Therefore, this thesis aims at addressing the current areas of improvement for risk assessment methodologies, by designing a new framework for integrating humans and animals. To achieve integration, the mechanisms of action of the selected EDCs were taken into account to characterise the risks, and bioindicators and biomarkers of significance in humans and in animals were used.

The focus was a pulp mill and municipal wastewaters, but also on agricultural non-point-sources, identifying hazards based on a decision tree supported on a combined criteria set including the use in the watershed, societal demands, persistence, toxicity and mode of action. The special characteristics of EDCs concerning their occurrence in environmental mixtures of multiple stressors, released to air, aquatic and soil through different routes and pathways, to affect

the homeostasis of receptors, required a framework handling multimedia distribution and pathways and food-web processes. This is very important for interpretation of risks, and especially challenging at the low doses found in the environment. That was why experiments were designed to experimentally demonstrate bioavailability, fate and ecotoxicology and the food chain and sediment-water interfaces and processes, aided by predictions by fugacity models. Aside from fish, other freshwater species were studied as part of an ecosystem evaluation.

The current trend is diminishing the use of experimentation animals combining *in vitro*, *in silico*, *in vivo* and computational components within the testing strategy (Blaauboer & Andersen 2007). In line with this, a tiered exposure assessment was designed aiming at optimising the analytical effort to measure the occurrence of prioritised EDCs and their effects in freshwater biota while ensuring the maximum possible accuracy to inform risk management actions. Exposure and effects were evaluated in the laboratory, with the main point source discharges, and through field surveys ranging from gene to population levels. Fish reproduction and condition were included in the environmental component, as they are excellent laboratory models to test the input of toxicity of point-source discharges. They were employed both in field surveys and in laboratory exposure experiments.

The existence of fish biomarkers that are sensitiveto EDC (van der Oost et al. 2003) and their ecological importance in the food web in riverine ecosystems (Woodward & Hildrew 2002) makes fish suitable for surveillance biomonitoring programs. Therefore, it was of crucial importance to find a native species that could serve as a bioindicator for the Uruguay River. The fish *Astyanax fasciatus* is proposed as such species after two years and a half of biomonitoring comprising nearly one thousand specimens, demonstrating subtle effects at some sites near point-source discharges. This also resulted into a new biomonitoring of wildfish species to perform surveillance activities and further investigations related to endocrine disruption in a large river system.

Fish are relevant to the riverine ecosystem, but also as a link to assess the implications of environmental endocrine disruptors transferred by fish as one of the components of human diet. They are used as experimental models to understand basal reproductive mechanisms (Wester et al. 2004, Chianese et al. 2011). In this thesis, the reference fish species (*Pimephales promelas*) was employed to evaluate the responses upon exposure to effluents and wastewater, with a set of end-points ranging from functional, gene, structural and tissue levels (histopathology).

Interspecies extrapolation among fish and mammals can improve if aided by methods that take into account the shared mechanisms of action (Bridges 2003, Kortenkamp 2007, Ankley et al. 2009). Mechanistic aspects were taken into account to integrate humans and animals in one assessment through biomarkers and molecular biology screens, which also diminishing the need for experimentation animals, and decreased the uncertainties in intespecies extrapolation. One of the endpoints was vitellogenin, measured as the protein by immunochemical methods. As it is susceptible to cleavage, which may interfere with accurate quantification, the more preventive molecular biology techniques that measure gene transcripts by PCR were also used. The set of toxicogenomic biomarkers included the *vtg*, *ESR1*, *ESR2*, *GHR*, *IGF-I* and *ZP3* genes.

The classification of the mode of action was guided coupling receptor-binding methods with toxicogenomics, thus enhancing integration among humans and animal receptors, as molecular and gene receptor-binding methods are valid for both humans and animals.

The human health component was evaluated for the local population to the main industrial point sources that is the pulp mill, and in particular for the most sensitive receptors, including life-stages in the dose calculation. Potential impacts of environmental exposures of EDCs to children and other susceptible populations, such as pregnant/lactating women due to physiological changes during pregnancy and lactation and the lipophilicity of the chemicals that may transport from adipose tissue deposits and reach the foetus or the baby (Gochfeld 2007, Mead 2008).

The availability of exposure data at the population level was very limited, as birth defects in the male reproductive organs were not systematically reported in the country. However, a preliminary study was initiated with data obtained from the local and national hospitals, resulting in the inclusion of epidemiology data within the framework for further study (Table F-1).

Early life stages were also part of the environmental component, through developmental bioassays with neonate, embryo-larval and juvenile specimens.

Risk estimation was performed by calculation of hazard quotients, risk quotients, and by a probabilistic approach to determine the species sensitivity distribution of two pesticides. Risk characterisation was a complex matter but a new methodology to evaluate the significance of the risk and combine the risks of single hazards was developed.

1.4 Thesis outline

After exploring the background information, the focus was on the EDC problem and the risk assessment frameworks were examined in greater depth in the literature review in Chapter 2. This section begins by outlining some historical developments in risk assessment and risk management approaches, and the current trends in the field, the regulatory and policy requirements and the challenge imposed by special characteristics of the endocrine disruption hazard, with an introduction to a possible methodological approach. The review also examined environmental, human and societal aspects, with reference to international regulations and policies for the safe use of chemicals enforcing the integrated study of the hazards of multiple chemicals on humans and the environment throughout their life-cycles.

The remaining chapters are ordered so that they state the aims and objectives, describe the study area and introduce the conceptual model and the two-phased risk assessment. The prioritisation strategy, key to Phase I, or preliminary risk assessment, is explained in Chapter 6. The uncertainties of the derived decision rules are subjected to extensive scrutiny. Chapter 7 presents a novel tiered approach for the exposure and effects stages, which was experimentally demonstrated in this case at the Uruguay River for EDCs.

The remaining chapters estimate and characterise the risks. Particular interest is devoted to the combination of the multiple risks suggesting improvements across the quantitative risk assessment field with a potential for adoption to be of practical use to risk managers, policy makers and other stakeholders. A discussion was completed in Chapter 10, including further research, contributions to knowledge and risk management advice.

The conclusions drawn in Chapter 11 indicate that the proposed methods were able to demonstrate exposure and effects, and modes of action, derive doses and perform risk calculations, as a start-up of an iterative process, to contribute to diminish the complexity of this highly complex topic.

Eight appendices are provided at the end of this thesis. Appendix A provides supplementary information for the scenario setting with data on baseline water chemistry and climatic conditions and fish biodiversity. Appendix B presents the sampling sites and sampling methods, national regulations, as well as a relevant list of accredited tests and other tests used in this research, and quality control/quality assurance data. Appendix C reviews the glyphosate toxicity profile. Appendix D offers information on the models used, parameterisation data, artificial neural networks and clustering. Appendix E shows supplemental information on dose-response assessment. Appendix F includes preliminary data of cryptorchidism and hypospadias in the country. Appendix G has an evaluation of the sources of uncertainty and Appendix H, additional information on the rating of EDCs for the radar diagram for combined risk characterisation and Fermi solutions for vector combination.

2 LITERATURE REVIEW

2.1 Introduction

The aim of this review is to analyse risk assessment frameworks, and their suitability to assess the risks of both environmental and human health effects from complex mixtures of endocrine disrupting chemicals (EDCs). Matters that differentiate this topic from the assessment of other categories of chemicals are also presented, regarding particularities in mechanistic and toxicokinetics aspects, and applicable tools in all the risk assessment stages.

2.2 Risk assessment frameworks

The EU (2000) defines risk as “the probability and severity of an adverse effect/event occurring to man or the environment following exposure, under defined conditions, to a risk source(s)”.

Approaches used to perform a risk assessment of endocrine disruptors range from those considering either only the environmental or human health to those which attempt to achieve an integration of both aspects. The challenge is to seek the most suitable alternative that produces a risk assessment useful to decision makers in order to establish policies to manage the risks, to finally choose an integrated risk assessment framework, as human beings were considered within the environment, both as receptors and producers of substances with likelihood to produce harm.

2.2.1 The risk assessment paradigm

Risk assessment, in the concept of the United States National Academy of Science, referred to the characterisation of the potential adverse effects of human exposures to environmental hazards. The classical paradigm of human health risk assessment (NRC 1983) is composed of four steps: hazard identification, dose-response assessment, exposure assessment and risk

characterisation. It paradigm was modified in 1994 to include characterisation of each component (NRC 1994).

2.2.2 Human health risk assessment

“A human health risk assessment is the process to estimate the nature and probability of adverse health effects in humans who may be exposed to chemicals in contaminated environmental media, now or in the future” (USEPA 2012b).

2.2.3 Ecological risk assessment

Ecological risk assessment (ERA) is defined as “a process that evaluates the likelihood that adverse ecological effects may occur or are occurring as a result of exposure to one or more stressors” (USEPA1992). Stressor is “any physical, chemical, or biological entity that can cause an adverse effect”. It is composed of three phases: problem formulation, risk analysis, and risk characterisation. The relative importance of individual stressors and the total stress from multiple stressors is estimated (Novotny et al. 2005). Typically, a wide range of stressors affects a watershed, originated from sources including a wide variety of human activities and natural processes. Multiple stressors usually coexist in complex mixtures in the natural and built environment (NRC 1988, Otake et al. 2004, Rudel & Perovich 2009).

Chemical, biologic, radiologic, physical, and psychological stressors may be assessed as multiple-stressor with multiple-effects (Callahan & Sexton 2007), as they can act on receptors through multiple routes and pathways in low doses, at significant exposure levels, either in additive, synergistic or antagonistic ways (McKinlay et al. 2008). Physical stressors as for example, temperature, has been found to affect fish sex differentiation, may play a significant role in determining the biological response (Heugens et al. 2002, Penman & Piferrer 2008, Selim et al. 2009).

2.2.4 Watershed risk assessment

According to Novotny et al. (2005), risk assessments can be pattern-based or process-based. The first one uses statistical analysis to describe correlations between stressors and responses, while the second links cause to effect by experimental methods. The evaluation of risks can be done by probabilistic methods using fish and/or invertebrates and ecological considerations, such as the maximum species richness in pristine conditions. The joint probability over all stressors may give an estimation of multiple stressors. Mathematical modeling tools and the Geographical Information System software (GIS) are also applicable (Wickham & Wade 2002). Artificial neural networks (ANN) may be used to consider nonlinearity of effects, and cause/effect relationships may be integrated with Bayesian networks (Borsuk et al. 2004).

2.2.5 Cumulative risk assessment

One of the best suited approaches to assess the risk of multiple stressors is the cumulative risk assessment framework. This may include societal aspects with participatory involvement of stakeholders (Gentile & Harwell 2001, USEPA 2003), providing more reliability as all relevant sources, pathways, and routes of exposure are considered (Callahan & Sexton 2007), as well as potential toxicokinetics interactions (USEPA 2007a).

2.2.6 Integrated risk assessment

Harvey et al. (1995) argued that the traditional paradigm could be modified towards a more “holistic” approach, evolving to the integrated risk assessment paradigm, defined as “a science-based approach that combines the processes of risk estimation for humans, biota, and natural resources in one assessment” (WHO 2001, Suter et al. 2003, Vermeire et al. 2007).

Achieving integration should be possible in all risk assessment steps (Suter et al. 2003, Sekizawa & Tanabe 2005, Vermeire et al. 2007). Endocrine disruptors are fit for integration as they may potentially affect the environment and

humans, provided bioaccumulation is expected in species used for human consumption. Integrated risk assessment can help understand impacts on human health and on ecosystems after extrapolating from animal experiments based on mechanisms of action (Bridges & Bridges 2004). According to Briggs (2008), the traditional methodology might be used when the issue is straightforward, while comparative risk assessments are recommended if combined risk factors exist.

Assmuth & Hilden (2008) claim that integrating human health risks and eco-toxicological risks may be relevant for future policy development, and that the regulations on safety of chemicals and industrial operations should ideally include a multiplicity of stressors, compartments, geographical scales, and end-points. In this regard, the new European Union Regulation on chemicals and its safe use (REACH) (EC 1907/2006) enforces linking risks to human health and the environment for chemicals throughout their life cycle, and it takes into account mixture effects, combined exposure and cumulative effects. In United States, the Environmental Protection Agency, still discusses both topics separately but acknowledges the need for integration. In Japan, the law enforced in 1999, aims principally at protecting human health but also estimates effects on the environment of certain chemicals (Vermeire et al. 2007).

2.2.7 The application of a modified risk assessment framework to endocrine disruptor mixtures

Endocrine activity mediated by steroid hormone receptors is difficult to predict based solely on the actions of the individual chemicals found in a mixture, making it necessary to direct resources based on mechanistic understanding of multiple stressor effects (Lokke 2010).

The phased approach described by Menzie et al. (2007) can be used to combine effects in multiple stressors, which develops a conceptual model including all relevant stressors and their interactions, to then focus on those presumed with highest effects. As in the watershed RA, evaluation of single

effects considers combined effects through qualitative and/or quantitative methods. In an effects-based assessment, Bayesian networks may be used to represent cause-effects relationships and interactions among stressors (Hart et al. 2006). Stressors and their effects are then geographically located (for example through GIS software). In stressor-based assessments, the model represents how stressors affect receptors. Screening is done when stressors magnitudes are below thresholds. The additivity of doses is calculated through toxic equivalence or relative potency methods for chemicals considered toxicologically similar (Menzi et al. 2007).

2.2.8 Risk acceptability and regulatory and policy decisions

Risk assessments are tools for informing public health and environmental protection decisions (NRC 2009). The demands of stakeholders and the public concern regarding EDCs causing children developmental, reproductive or carcinogenic ailments result in restriction in the use or banning the production and marketing of certain substances. Examples of this are the banning in Europe of phthalates from babies and children toys and articles (Directive 2005/84/EC), the banning of nonylphenol that came into force in Europe in 2005 (Directive 2003/53/EC, Statutory Instrument of the Crown No. 1816, 2004). A recent example is the process towards banning bisphenol A (BPA) from baby started in Canada in 2008, but the controversy related to the complete banning continues (Health Canada 2010). In 2011 it was restricted in Europe (EU 2011), and banned from baby bottles and sipping cups by the US Food and Drug Administration (Federal Register 2012) in 2012. Notwithstanding, opposed interests such as plastic manufacturers, the media and consumers, have delayed a final decision for other packaging materials.

The community, non-governmental organisations (NGOs), health and environmental regulators, the manufacture industrial sector and drinking water companies will judge the acceptability of risk (DEFRA 2000).Therefore, the implementation of appropriate risk assessment methodologies is necessary to reach to regulatory decisions that ensure environment and human health

protection (Mason et al. 2007). In fact, this topic is present in the research agenda of many governmental and non-governmental funding agencies. Examples on-going projects are those funded by the European Commission Framework Programme (http://ec.europa.eu/research/endocrine/projects_ongoing_en.html), the USEPA (http://www.epa.gov/ncer/science/endocrine/researchproj.html) or the UK-Japan Joint Research on Endocrine Disrupters (http://www.uk-j.org/). Thus, research, legislation and stakeholder involvement are of key importance in environmental and human health protection.

2.3 Hazard identification: methods and opportunities for environmental and human health risk assessment integration

The International Programme on Chemical Safety (IPCS 2004) harmonised the definition of hazard as the “inherent property of an agent or situation having the potential to cause adverse effects when an organism, system, or (sub) population is exposed to that agent”. Hazard identification is a critical first step within any tier of a phased or tiered risk management framework (Pollard 2008).

2.3.1 Sources of endocrine disrupting chemicals

Possible sources of watercourses pollution with EDCs are wastewater sewage discharges, industrial effluents, or point and non-point source contamination of watercourses with agrochemicals such as herbicides or pesticides. Sewage discharges from cities contain residues of domestic products such excreta with residues of natural steroidal hormones and metabolites of the contraceptive pill as personal care products pharmaceuticals (Enick & Moore 2007), and detergents. Using passive samplers Alvarez et al. (2009) identified mixtures of emerging contaminants, several of which are EDCs downstream from wastewater discharges: fragrances, flame retardants, organochlorinated pesticides, and polycyclic aromatic hydrocarbons. Among the non-ionic surfactants part of detergent compositions, the alkylphenolic ethoxylates type persists in sewage sludge and river sediments. For example, nonylphenol (NP)

is a highly hydrophobic compound produced after biodegradation of nonylphenol ethoxylate that is also used in industrial and agricultural applications. Due to its toxicity and persistence, the use and production of NP have been banned in EU countries, and is strictly monitored in many other countries such as Canada and Japan (Soares et al. 2008, Chiu et al. 2010).

Pulp mills are among the industrial sectors associated with several endocrine disruption studies that link bleached Kraft pulp mill effluents (BKME) to both oestrogenic and androgenic effects on biota, depending on the process characteristics and wastewater treatment. The chlorination of organic matter with elemental chlorine used to produce dioxins and furans, which have been reduced by the current process using chlorine dioxide as bleaching agent, but this method still produces other halogenated organics. Various wood-extractive compounds from pulping, such as rosin acids and phytosterols found in pulp mills effluents are other potential EDCs (Lehtinen et al. 1998, Christianson-Heiska & Isomaa 2008, Hewitt et al. 2008). The main identified resin acids in pulp mill effluents are: pimaric, isopimaric, sandaracopimaric, palustric, dehydroabietic, abietic and neoabietic acid, monoterpenes, phenolics, fatty acids, sterols, guaiacyl–based lignin degradation products, diterpenoids, and dimethoxy stilbene (Ali & Sreekrishnan 2001, Belknap et al. 2006, Meriläinen & Oikari, 2008, Wartman et al. 2009).

Examples of other industry practices that might be sources of EDC release are wool scouring and mine sites that leach heavy metals and acid into nearby creeks and groundwater systems (Ying 2006).

Some representative examples of common sources of EDCs and typical environmental concentrations are summarised in Table 2-1.

Table 2-1 Sources of EDCs and typical environmental concentrations

Origin, use and occurrence	Source of environmental exposure	EDC group	Example molecule	Typical concentrations	Reference
Domestic and industrial (polycarbonate bottles)	Leaching from solid waste, sewage effluent	Polycarbonate	Bisphenol A	0.62 µg l^{-1}	Sánchez-Avila et al. 2009
Domestic, (contraceptive pills)	Sewage effluent	Pharmaceuticals	17α-ethynyl oestradiol	5.7 ng l^{-1}	Atkinson et al. 2012
Human and animal excreta	Sewage effluent	Natural steroid hormones	17β-oestradiol	5.0 ng l^{-1}	Koh et al. 2007
Domestic and industrial (laundry detergents, wool scouring processes)	Sewage sludge	Non-ionic surfactants	4-nonylphenol	829.3 mg kg^{-1}	González et al. 2010
Agricultural (soil fertilization)	Livestock waste	Male steroid hormones	Testosterone	10–1830 ng l^{-1}	Lange et al. 2002
Agricultural (dairy farming)	Streams contaminated by dairy cow excreta	Female steroid hormones	17β-oestradiol	0.04-3.6 ng l^{-1}	Matthiessen et al. 2006
Agricultural (weed and grass control in soybean crops)	Run-off	Herbicide	Glyphosate	0.1-0.7 mg l^{-1}	Peruzzo et al. 2008
Industrial (pulp and paper mills)	Contaminated fish	Resin acids	Pimaric acid	4-140 µg g^{-1}	Owens et al. 1994
Industrial (pulp and paper mills)	Industrial wastewater treatment plant	Chlorinated organics	2,4,6-trichlorophenol	1.5 µg l^{-1}	Owens et al. 1994
Industrial (pulp and paper mills)	Final stage secondary treatment	Phytosterols	β-sitosterol	58.42 µg l^{-1}	Landman et al. 2008

2.3.2 Nature of the hazard

Endocrine disrupting chemicals include a broad range of substances. The best known are the environmental oestrogens, alkyl phenols and the ethoxylates, the monomer in polycarbonate manufacture bisphenol A, some pesticides and chlorinated organic substances (Jobling & Tyler 2003). Some of the most prevalent and potent ones are natural animal hormones and plant oestrogens, but a myriad of synthetic compounds is able of causing ED. They may be evaluated as any other toxic chemical. However, the assessment has to deal with the fact that exposure of an organism to these substances may or may not affect it in its reproduction function and/or off-spring. This depends on the critical window of developmental programming (van aerle 2002, Savabieasfahani et al 2006).

Several reports on human developmental anomalies and reproductive impairments have raised international concern. It is thought adverse effects include endometriosis, reduced fertility and some types of cancers of breast and testis (Martin et al. 2007). The increase in testicular cancer incidence has been reported since 1950 in Europe (Bray et al. 2006), United States (Holmes et al. 2008) and New Zealand (Sarfati et al. 2011). Also, EDCs may cause prostate cancer according to epidemiologic evidence (Prins 2008, Hu et al. 2012) and studied with animal models, with exception of phystosterols which may have antitumorigenic effect (Parks et al. 2011). A heightened sensitivity of the prostate to EDCs is produced during the critical developmental window including *in utero* and neonatal time points and during puberty (Prins 2008). Even though controversies persist regarding possible effects of EDCs on male fertility, reduced sperm counts suggest the influence of environmental factors, above genetic predisposition (Eertmans et al. 2003).

More than 84000 synthetic chemicals are currently in commerce in the USA, as inventoried by Toxic Substance Control Act (TSCA) (USEPA 2010b). Of these, the Endocrine Disruptor Screening Program selected a priority list of about 15000 compounds to be screened for endocrine disruption and it currently

requires that pesticides are screened for their potential effects on the endocrine system (USEPA 2009c). The European Union established a priority list of substances for further evaluation and research on endocrine disruption, including mechanisms of action and models to estimate exposure (EC 1999, EC 2007).

2.3.3 Evidence of endocrine disruption in wildlife around the world, mixtures and their relation to sources

There are many reported impacts on wildlife reproduction and development in invertebrates, fish, reptiles, birds and mammals (Arukwe & Goksøyr 1998, Hood 2005, Iguchi & Katsu 2008). Several adverse endocrine effects in wildfish are shown on Table 2-2. Many examples exist in wildlife, such as the seals population decline in the Baltic (Kostamo et al. 2000, HELCOM 2010) and North Sea (NOAA 2009), the high levels of female egg yolk in male fish (Aravindakshan et al. 2004) or snail imposex and intersex around the world (Gooding et al. 2003).

Impacts on reproductive health may affect fish populations (Mills & Chichester 2005). Intersexuality of fish was observed in several investigations carried out in rivers around the world. Examples of this are Jobling et al. (2002) investigations in the UK that related abnormal reproductive female-like ducts, oocytes, and plasma VTG induction in exposed male fish to sewage discharges from cities, coupling field and laboratory experiments. Field studies with wild roach as a model fish confirmed the incidence and severity of intersex correlating with the predicted concentrations of the natural oestrogens (E1 and E2) and the synthetic contraceptive pill oestrogen (EE2) present (Jobling et al. 2006).

Not only municipal wastewaters could be the cause for intersex, as in experiments carried out in the Potomac River, US, researchers were able to link both agricultural inputs and municipal wastewater pollution to the high prevalence of intersex in fish (Blazer et al. 2007, Iwanowicz et al. 2009). Multiple sources of EDCs were demonstrated in various investigations at a

basin level as stormwater and other non-point source inputs of EDCs into the Minnesota lakes reflected the anthropogenic use of the land (Writer et al. 2010).

The oestrogenic activity of municipal wastewater correlates to demographics, as demonstrated by Brooks et al. (2003), who linked its variations to seasonal fluctuations in population at a university city in US , measuring the concentrations of EDCs and demonstrating the effects through *in vivo* and *in vitro* tests (fish exposure with VTG measurement and the yeast oestrogen screen). Mixed modes of action were found in secondary clarifier effluents by Stalter et al. (2011), as endocrine activities, expressed as equivalents: oestrogenicity (2.0- 2.8 ng l^{-1} oestradiol), anti-oestrogenicity(4- 22 mg l^{-1} 4-hydroxytamoxifen), androgenicity (1.9 - 2.0 ng l^{-1} testosterone), anti-androgenicity (302- 614 mg l^{-1} flutamide), and AhR agonistic activity (387- 741 ng l^{-1}β-naphthoflavone).

Pulp and paper mills may impact on air, water, and soil (Ahonen et al. 2006), and their effluents can generate ecotoxicity and alter the ecological status of a watercourse adding organic matter and nutrients causative of eutrophication, tannins and lignin that increase colour, and salts, that augment its conductivity (Karrash et al. 2006). Their toxicity has been demonstrated through fish surveys upstream and downstream from the discharge pipe. For instance, According to Munkittrick et al. (1994) and McMaster et al. 2006, after fish biomonitoring, concluded that chlorine bleaching is not the only causative agent and secondary treatment does not eliminate oestrogenic responses evidenced by decreased circulating levels of sex steroids, decreased gonadal size.

Most findings refer to increased VTG in male fish, a biomarker of oestrogenic activity; although because of technology changes, androgenic effects such as a biased male to female (Larsson & Forlin 2002) can also be seen. Sex ratios in zebrafish were affected by inducing VTG after exposed to ß- sitosterol (Nakari & Erkomaa 2003). Effluents affect reproduction in multiple fish species, evidenced by decreased gonad size, steroids and egg production, and anomalous secondary sex characteristics (Hewitt et al. 2008) (Table 2-2). The toxicity may

also be chronic, including endocrine disruption that may even provoke sex change in fish (Larsson & Forlin 2002). Effects on fish fecundity to pulp mill effluents vary with the EDCs concentration. Rickwood et al. (2006) observed stimulated egg production at 1% dilution in pair-breeding fathead minnow, while the undiluted sample reduced spawning.

Table 2-2 Effects of EDCs in wildlife evidenced through field studies

Animal	Effect	EDCs	Postulated mechanism or causative agent	Reference
Frog	High incidence of deformed frogs in Minnesota, United States	Multiple EDCs	Retinoid signaling pathways activation	Gardiner et al. 2003
Marine Gastropods	Masculinisation (imposex): accessory sex organs including sperm ducts, seminal vesicles, external sperm grooves, and penises	Low levels of tributyltin (TBT) (1ng l^{-1})	Aromatase inhibition, testosterone inhibition, neuroendocrine disorder or interaction with retinoid receptors	Novák et al. 2008
Wild roach (*Rutilus rutilus)*	Intersex, and high plasma VTG concentration	Multiple EDCs	Sewage effluent from wastewater treatment plant discharging into rivers	Jobling et al. 2006
Mosquitofish (*Gambusia affinis*)	Masculinisation (90% affected in number segments in the longest anal fin ray). Androgen-dependent gene expression by luciferase test	Kraft pulp mill effluent	Affinity for human androgen receptor (hAR)	Parks et al. 2001
Eastern Mosquitofish, (*Gambusia holbrooki*)	Androgenic activity measured in sediment by AR transcription assay with human receptor. Fish masculinisation	Paper mill effluent, river	Pine phytosteroids accumulate in sediments, they biodegrade, progesterone and androstenedione	Jenkins et al. 2003

2.3.4 Regulatory limits, societal demands and risk perception

Decision makers may face the difficulty that regulatory limits do not exist or lack harmonisation in the deriving criteria. Environmental concentrations of EDCs vary significantly among countries, and even though no mandatory limits exist for many very active EDCs compounds, such as oestrogens or bisphenol A, the detection limits for these may only be feasible by using very high technology analytics, such as mass spectrometry methods to achieve the low values mentioned as toxicologically relevant in the literature.

Public opinion significantly influences on policy-making. The focus for the risk management of herbicides is mainly on biological aspects, with less attention being paid to social concerns (Wyatt et al. 2011). One such example is glyphosate, a herbicide highly used in transgenic soy crops and forestry around the world, causing non-point source contamination, and localised exposures of soil and water biota near the spray area. Although it is relatively not persistent in biota, its safety is controversial because of its potential teratogenicity and carcinogenesis (Antoniou et al. 2012), justifying its inclusion in the scientific research agenda and a matter of discussion among industry, NGOs and the media (CEUTA 2006, Pazos 2008). Air-spray of this product in combination with endosulfan was taken to jury in Argentina after allegations of children illnesses and cancer (Forestal Web 2009, IPS 2013).

There are inconsistencies in regulatory limits. Again, in the case of glyphosate, no harmonisation exists. The limit set for drinking water is 0.7 mg l^{-1}, according to the US National Primary Drinking Water Regulations (USEPA 2010a), and 0.9 mg l^{-1} by WHO (WHO 2005). Although a risk assessment methodology was applied, it is based on human health for reproductive chronic toxicity, but no surface criteria exist for this EDC. Even if the drinking water guideline were used as a reference, the environmental concentrations reported by Peruzzo et al. (2008) as affecting biota could still be regarded as safe. On the other hand, the European Union Drinking Water Directive 98/83/EC would consider this limit

as unacceptable as the concentration of any pesticide in drinking water should not exceed 0.1 µg l^{-1}. However, this limit was derived without any reference to risk.

Another topic with high societal demands and political implications on both sides of the Uruguay River are the potential impacts of pulp mills and the EDCs coming from this source (International Court of Justice 2010).

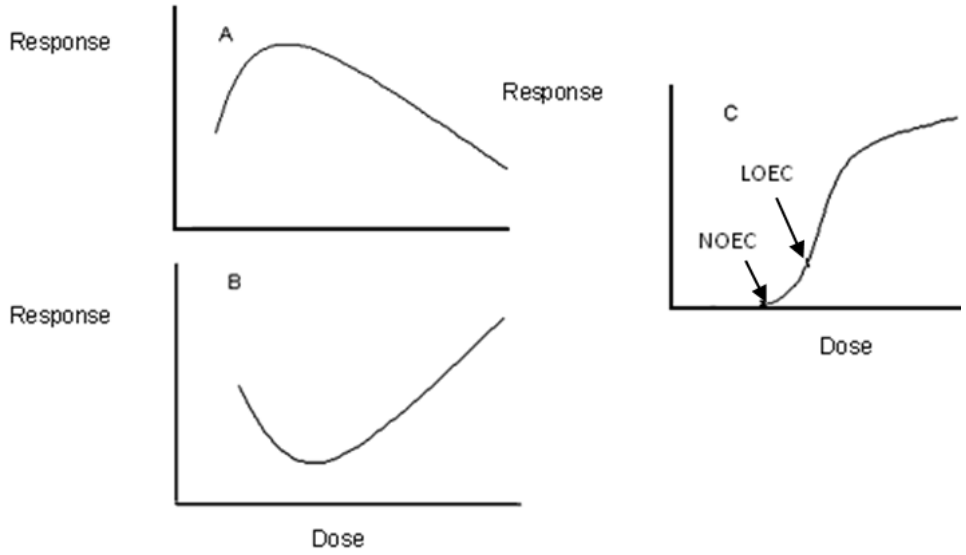
2.3.5 Uncertainty and confounding factors

Uncertainty refers to a lack of enough knowledge such as those arising from the analytical measurement, sampling and models and also from data gaps in the assessment, such as poor information on human exposures or the toxicity of a chemical (Williams & Paustenbach 2002). Some of uncertainty sources related to mechanisms of action, toxicokinetics and low-dose effects of EDCs are presented. The traditional toxicological paradigm that studies the exposure of adult individuals to high doses of chemicals resulting in mutagenesis, cancer, and death establishes clear causal relationships, traceable from exposure to the outcome of illness and death. The difficulty to proceed according to this concept in the case of EDCs is occasioned by their delayed response, after exposure to low doses during the embryonic stage of organ development (Crews et al. 2000).

Dose-dependent interactions are not always monophasic linear relationships. The dose–response relationship estimates safe exposures to chemicals, but the ‘‘pivotal question’’ (Daston 1993) is whether a threshold exists, as assumed for nongenotoxic chemicals. A dose–response model consistent with the data should be employed based in biological models, such as the Michaelis Menten model (Sheehan 2006), but this kinetic behaviour is not always easy to prove, as this equation applied to enzymes mechanisms, and steady-state conditions, meaning constant enzyme-substrate. This could be applicable for ER binding mechanisms but not necessarily for the not binding EDCs.

2.3.6 Hormesis and its connection to endocrine disruption

Hormesis has been defined as a biphasic dose–response phenomenon characterised by low-dose stimulation and a high-dose inhibition (Calabrese & Baldwin 2002, Calabrese 2008). The hypothesis that a paradoxal toxicity effect may appear at low-doses, showing an inverted U-shaped dose-response curve which is sometimes referred to as non-monotonic response, is still controversial according to some authors due to the uncertainties of the experimental design and of the testing methods (Mushak 2007). The patterns of J-shaped and inverted-U curves are shown in Figure 2-1.



A: hormetic curve with low-dose stimulatiory and high-dose inhibitory responses, B: hormetic curve with low-dose reduction and high-dose increase of adverse effects (J- or U-shaped curve), C: normal dose-response (Based on Kendall et al. 2001, Calabrese 2009, Hoffmann 2009).

Figure 2-1 Shapes of hormesis and normal dose-response curves

Even though the phenomenon of *hormesis* has been linked to growth stimulation of the organism (Stebbing 1997) or other apparent beneficial effects, other toxicity mechanisms different to those of higher concentrations might appear, probably connected to reproductive effects. Several chronic toxicity

tests carried out below the NOEL threshold level with various aquatic species have shown an increased number of offspring.

Several hypotheses on the genesis of *hormesis* consider the influence of nutrients, or detoxification processes on lowering the responses at higher concentrations. Hormesis may also be linked to chemical interactions, synergy or potentiation, especially in chronic experimental studies from yeasts to mammals. Chemicals that bind to hormonal receptors may perturb cell function even at very low doses (NRC 2009) and may induce an inverted U-shaped dose response. According to Calabrese (2008), these oestrogenic effects are clear examples of *hormesis*. The hypothesis that the mechanism of homodimerisation of steroid hormone receptors is responsible for the non-monotonic dose responses has been drawn (Zhang et al. 2008). The U-shaped dose-response curve might result because the binding to the ligand from monomeric to dimmers is non-linear before producing the gene expression of the hormone (Li et al. 2007).

The combined effect of chemicals may produce hormetic or non-hormetic effects, depending on the complexity of the interaction of the chemicals and the concentrations and characteristics of the organisms. Kefford et al. (2007), recommend considering the stimulatory effects when evaluating ecotoxicity tests at the individual level, but the interpretation of this effect is not clear at the community level. Some metals act as micronutrients in enzymatic, hormone systems and metalloprotein, essential for life (zinc, copper, iron, selenium). Then, it is can be supposed that some stimulatory effects such as growth *hormesis* is linked to biological adaptive responses at low level doses of toxicants, saturating the counter-inhibition capacity. Herbert Spencer anticipated this theory as early as in 1862, as antagonistic forces that enact to bring back equilibrium when disturbing forces work an excess of change in some direction.

Hormesis has been extensively seen in bioassays using reference invertebrate, such as cladocer crustaceans (Table 2-3).

Table 2-3 Hormetic effects in Cladocer crustacea

Species	Test	EDC or effluent	Experimental range/hormetic concentration	Hormetic effect	References
Daphnia carinata	21-d, 3 brood test	Chlorpyrifos	0.005 to 0.500 µg l^{-1}	Shortened time to the first brood in 2nd generation	Zalizniak et al. 2006
Daphnia magna	30-d	Fluoxetine	1 to 100 µg l^{-1}	Fecundity increase	Flaherty & Dodson 2005
Daphnia magna	25-d, LC50	Cadmium	0.5, 1.0 and 5.0 µg l^{-1}	Increase in neonates number and size	Bodar et al. 1988
Daphnia magna	Tolerance test, 2 brood	Mercury	Pre-exposure to 2.5 and 25 nM 1.5 to 15 nM	Increased weights and reproductive rates	Tsui et al. 2005
Ceriodaphnia dubia	7-d chronic toxicity test	Pulp mill effluent	At concentration < 40%	Reproduction stimulation	Middaugh et al. 1997

2.3.7 Influence of temperature on fish sex differentiation and on ecotoxicity

The influence of temperature on biological responses is a pertinent issue of investigation in view of global climate change (Brian et al. 2008). Environmental changes may also affect the sensitivity of organisms to chemicals, and toxicity in general increases with rising temperature (Heugens et al. 2002).

Fish vulnerability to thermal changes is influenced by EDCs. Investigations demonstrated that endosulfan decreased by 3 or 4ºC on exposure of several fish species to endosulfan or to chlorpyrifos (Patra et al. 2007).

Sex determination is the genetic or environmental process that establishes sex of an individual in a simple binary fate decision, whereas sex differentiation transforms an undifferentiated gonad initially ovary turns into an ovary or a testis, in a normal sex ratio of 1:1 (Penman & Piferrer 2008). In some fish, temperature triggers sex determination and differentiation, so, an increase may affect fish if produced during the most sensitive period of early development, yielding higher incidence of males than normal, or activating the brain-pituitary-gonadal axis of the reproductive cycle, augmenting E1 secretion and altering the expression of aromatase (*cyp19a*) (Penman & Piferrer 2008). The sensitivity to high temperatures has been observed in laboratory experiments, as for example with Medaka fish, *Oryzias latipe* (Selim et al. 2009). The influence of temperature on the VTG induction and gene expression of fathead minnow (*Pimephales promelas*) to a mixture of chemicals at 20 and 30ºC, was studied by Brian et al. (2008) demonstrating that VTG induction was faster at higher temperature, although of similar magnitude at the end of the two weeks exposure period.

2.3.8 Normal endocrine system function

The endocrine system comprises hormones of the hypothalamic–pituitary axis, glands and target organs. The hormones act on specialized cells groups in the anterior pituitary gland to stimulate or inhibit the secretion of other hormones.

Leydig cells in the testis secrete testosterone and insulin-like hormone 3, needed for testicular descent (Foresta et al. 2004). Testosterone is converted to dihydrotestosterone (DHT) in the prostate and external genitalia, which plays a role in differentiation of external genitalia, regulates testicular descent and development of external genitalia (Dušková & Pospíšilová 2011). On the other hand, oestrogens are produced primarily in the ovaries in females and testes in males, regulate the growth, development, and physiology of the reproductive system (Lee et al. 2012), and have mitogenic action in the uterus and breast (Zhu & Conney 1998).

2.3.9 Biosynthesis and mechanism of action of oestrogenic EDCs

There are genomic and non-genomic mechanisms of ED that may even coexist.

2.3.9.1 Genomic mechanisms of ED action

Direct binding of receptors producing the effect of such receptor (ostrogen or androgen-agonistics) (ER and AR-binding EDCs) by either:

i) Competitive binding to a receptor and causing the opposite action

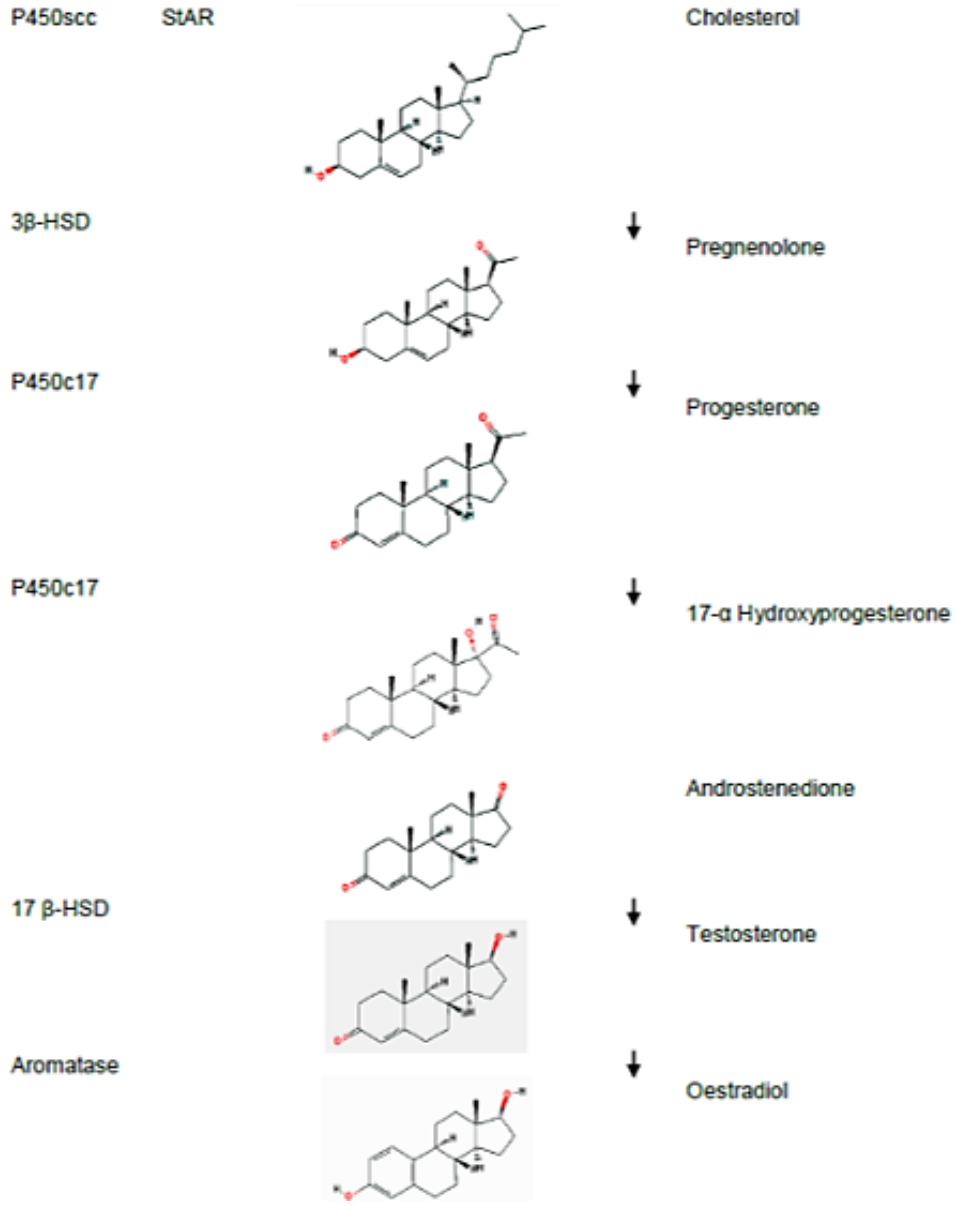
ii) Inhibition of the binding to the ostrogen and progesterone receptor

iii) Blockage of the thyroid receptor, involving the neuroendocrine system, with oestrogen-agonist action

In humans, the enzyme aromatase, present in the placenta of pregnant women, in the ovaries of premenopausal women and in peripheral adipose tissues of both sexes, converts testosterone into oestradiol (USEPA 2007b). Its normal blood concentration in ovulating women is 400 pg ml^{-1} (Medline Plus, Medical Encyclopedia, NIH).

Steroidal hormones are synthetised via cholesterol as precursor either, produced *de novo* or carried from plasma lipoproteins. To yield oestradiol one of the biosynthetical pathways is by methyl group oxidation of testosterone

mediated by aromatase to form a benzene ring (Figure 2-2) (Fisher 2004).



Cholesterol is transported inside the mitochondrial membrane by the steroidogenic acute regulatory protein (StAR), to be converted by cytochrome P450 side-chain-cleavage enzyme (P450scc) to pregnenolone. Within the smooth endoplasmic reticulum, the enzymes 3β-hydroxysteroid dehydrogenase (3-HSD), cytochrome P450 c17 (CYP 17), and 17β-hydroxysteroid dehydrogenase (17-HSD), participate in the biosynthetical pathways yielding testosterone. Designed from structures of PubChem Compound database, NCBI, National Institute of Health (http://www.ncbi.nlm.nih.gov/sites/entrez/)

Figure 2-2 Biosynthesis of steroidal hormones

In fish, induction of aromatase in the ovary also converts testosterone to oestrogen, stimulating the liver to secrete VTG, which is then transported in the bloodstream to the oocytes. The female secondary sex characteristics and negative feedback linkages to the hypothalamus and hypophysis are also induced by aromatase (Cyp19a1a). It also participates in testicular differentiation in both gonochoristic and hermaphrodite fish species. The masculinization of female fish may be mediated by the inhibition of Cyp19a1a enzymatic activity (Guiguen et al. 2009).

Several theories exist on how EDCs interact with receptors to trigger responses on the endocrine system causing either agonism or antagonism. In the case of the oestrogen receptors, the binding of the receptor to the membrane influences on proteins, while the cytosolic one binds to the genes of the chromosomes in the nucleus after binding with oestrogen, provoking RNA transcription. The most vulnerable period is the foetal period to the early postnatal developmental period when organs continue to undergo substantial development. The effect in general shows a latency before manifesting and it is likely for the organism being exposed organism to a mixture of EDCs. As seen under the *hormesis* section (2.5.5.), another characteristic is that in some cases a low dose effect could be more potent than at higher doses, following non-traditional dose-response curves.

Effects may be transmitted to subsequent generations through modifications of gene expression such as DNA methylation and histone acetylation.There are two oestrogen receptors, ERα and ERβ, structurally belonging to the oestrogen/thyroid hormone superfamily of nuclear receptors. ERβ is abundant in male urinary tract. Their functional domains are three: one with an amine group (A/B), the second with DNA binding groups (called C) and the D/E/F with ligand binding domains. After binding to the receptor, conformational changes occur and the gene transcription includes receptor dimerisation, interaction with DNA, participation of co-activators and formation of a pre-initiation complex (Nilson et al. 2001). Although ERs share high degree of amino acid homology,

one role of ERβ is to modulate ERα transcriptional activity, decreasing overall cellular sensitivity to estradiol (Hall & McDonnel 1999). The large nuclear receptor superfamily is involved in regulation embryonic development, maintenance of differentiated cellular phenotypes, metabolism and cell death. They comprise 24 among 48 nuclear receptors in the human genome with known ligands, the rest being 'orphan' receptors. It is fundamental to know that oestrogen signalling is as a balancing act between ERα and ERβ: ERα is often an activating factor, whereas ERβ suppresses the effects of ERα (Gronemeyer et al. 2004). These subtypes have recently been denominated *ESR1* and *ESR2*, respectively. In some fish species, there are two forms of the *ESR2* subtype, *ESR2b* and *ESR2a*, as Filby & Tyler (2005) demonstrated that all three *P. promelas* ERs express during early development. These differences might be important when extrapolating effects to humans.

2.3.9.2 Non-genomic mechanism of ED action

Cytochrome

Steroids are formed from cholesterol via a series of reactions, which involve the cytochrome P450 (CYP) isoforms. One of them, CYP 2C19 is the enzyme aromatase controls the formation of oestrogens (which contain an aromatic ring). Their expression occurs during the earliest foetal development and can be induced by DDT or inhibited by tributyl tin. The anti-androgenic effect of phthalates could be caused by interference with StAR, involved in the biosynthesis of testosterone (Fisher 2004).

Sulfotransferase enzymes

Oestrogens and their sulphonate esters are synthesised with the participation of sulphatases located on the surfaces of the target cells to release the free steroids, which can then enter the cell (Waring & Harris 2005). Sulfonation and desulfonation of steroid hormones modulates the activity and transport of steroid hormones in the bloodstream. The conjugation is mediated by sulfotransferases (SULT) that inactivate the hormone, while the sulfatase

removes the sulfonate group, activating them. Some EDCs (octylphenol, nonylphenol and natural flavonoids) inhibit SULT activity, increasing bioavailable oestrogens (Falany 1997, Fisher 2004, Waring & Harris 2005).

2.3.10 Hypothalamic-pituitary-gonadal disruptors

Some of the most recognised EDCs with MOA in the hypothalamic-pituitary-gonadal (HPG) axis according to Ankley et al. (2009) can be seen in Table 2-4 Hypothalamic-pituitary-gonadal disruptors

Table 2-4 Hypothalamic-pituitary-gonadal disruptors

EDC	Presumptive HPG target
Fipronil	GABA receptor antagonist
Fadrozole	CYP19 inhibitor
Vinclozolin	AR antagonist
Trenbolone	AR agonist
Ethinyl oestradiol	ER agonist

2.4 Exposure assessment

Exposure is defined as the “contact of an organism with a chemical or physical agent, quantified as the amount of chemical available at the exchange boundaries of the organism and available for absorption” (WHO-IPCS 2001).

Exposure assessment is the quantitative or qualitative evaluation of the contact and intake of the human body or animal with a chemical through the skin and openings. The exposure to the agent can be estimated by biological monitoring or measuring the environmental concentrations, using factors and modeling techniques (Paustenbach 2002). It links actual or expected exposure rates with the potential effects on the target species (humans, fishes, ecosystems).

The initiating factor is to establish whether there is a potential risk of exposure and harm and the sub-populations at risk. The increased concern regards environmental exposures to children, pregnant and lactating women (USEPA 2011).

According to USEPA (2011), risk to an individual or population can be represented as a continuum from the source through environmental exposure to dose to effect. The environmental stressor is released, transformed and transported via air, water, soil, dust, and diet. The pathways to become in contact with the chemical are inhalation, ingestion, or skin/eye contact. The individual's activity pattern and the concentration of the chemical determine the magnitude, frequency, and duration of the exposure. Finally, the internal dose is produced after absorption of the substance to generate an adverse effect upon interaction with the receptors or the target tissue

2.4.1 Ecosystems and human sub-populations potentially at risk of endocrine disruptive effects

Due to the ubiquity of EDCs and the widespread routes of exposure, most ecosystems and human populations are potentially at risk of endocrine disruption. Notwithstanding, under the scope of a risk assessment of EDCs the potentially most vulnerable risk subgroups are identified corresponding to maternal, foetal and early developmental stages as for reproductive function in both humans and animals, foetal life is most vulnerable because there are rapid structural and functional events (Diamanti-Kandarakis et al. 2009). The late embryonic and early postnatal period in mammals is a critical period for sexual differentiation (Gore 2008). The concern that either prenatal or childhood exposure to EDCs may be responsible for abnormalities in human sexual and reproductive health are still in the hypothetical ground. The existence of a global downward trend in sperm count is still inconclusive (Sharpe 2003, Eliasson 2010). However, many reports on exposure to high concentrations of recognised EDCs such as diethylstilbestrol (DES), certain polychlorinated biphenyls (PCBs), and dichlorodiphenyltrichloroethane (DDT) (Salazar-García

et al. 2004) demonstrate this fact. At low doses the question remains unanswered whether there could be a critical window where they could harm the foetal development (Hood 2005).

There is international consensus on identifying children as the most vulnerable group for EDCs exposure and harmful effects (Selevan et al. 2000), evidenced by statements endorsed by expert scientists in this topic (Prague Declaration on Endocrine Disruption 2005). The European Strategy for Environment and Health: Science, Children, Awareness, Legislation and Evaluation (SCALE), set as a priority agenda for the endocrine disruption in children. Exposure to insecticides and herbicides in agricultural practices has been linked to developmental or reproductive effects in wild animals and in human beings and children may get in contact with residues of pesticides through domestic exposure including the ingestion route of fruit or breast milk, suffering chronic effects (Goodman & Laverda 2002). Bretveld et al. (2008) associated the occupational exposure to pesticides to a prolonged time-to-pregnancy in male greenhouse workers exposed to pesticides before conception of their first pregnancy.

2.4.2 Conceptual model for exposure assessment

The *in vivo* pathways of lipophilic EDCs during pregnancy move with maternal fat, releasing bioaccumulated compounds to the blood. The liposoluble and persistent EDCs enter a woman's body through a number of different exposure routes (foodstuffs, environmental, occupational) throughout her life. After crossing the placental barrier and through the breast milk, the toxicant may reach the offspring (Mena & Milad 1998). This could explain paradoxically high levels of some EDCs found in early childhood (Fernández et al. 2007). As an example, nonylphenol can be found in human breast milk in concentrations ranging from 13 to 56 ng ml^{-1} with a mean value of 32 ng ml^{-1} (Ademollo et al. 2008). As a reference, the urinary excretion of NP may reach up to 1.57 µg l-1(1.39 µg g^{-1} creatinine) (Calafat et al. 2004).

One of the main sources of exposure to most chemicals is through the food chain. The bioconcentration of organics in beef, cow milk and vegetation correlates to the octanol-water partition coefficient (K_{ow}) to predict the bioaccumulation in the aquatic and terrestrial food chains (Travis & Arms 1988). Other sources of contamination, not coming from environmental inputs, like the exposure to plasticisers such as BPA or DEHP through canned food, baby bottles, or bottled water (Talsness et al. 2009) may be of higher relevance than those due to contaminated fish or tap water intakes.

Many models, such as the fugacity model, which allows predicting expected concentrations in six environmental compartments (water, air, soil, bottom and suspended sediment and fish) are based on the chemicals characteristics (MacKay et al. 1985). Cao et al. (2010), applying a fugacity model quantified the fate of oestrogens in environmental compartments and calculated the human equivalent dose from fish consumption as ten times higher than from drinking water consumption.

2.4.3 Biomarkers in biomonitoring and *in vivo* bioassays to assess exposure and effects of EDCs

Biomarkers link environmental exposures to quantitative estimates of human exposures to chemicals (U.S. EPA 2009c), and integrate human and animal receptors. Biomarkers are some of the most used tools to assess endocrine disruption. The IUPAC (Duffus et al. 2007) defined a biomarker as “an indicator signaling an event or condition in a biological system or sample and giving a measure of exposure, effect, or susceptibility”. “Such an indicator may be a measurable chemical, biochemical, physiological, behavioral or other alteration within an organism”. The adverse end-points of *in vitro* cellular test systems based on relevant biomarkers realiably estimate responses in humans and animals, through knowledge of the structural information (Blaauboer & Andersen 2007).

2.4.3.1 Vitellogenin

Vitellogenesis entails both VTG synthesis by the liver in response to endogenous E2 or exogenous oestrogenic chemicals, and uptake by growing oocytes where it is stored as yolk, the food reserve of developing embryos. Nicolas (1999) explained the oogenesis and vitellogenesis processes in fish. Briefly, VTG is synthetised in the liver, and its uptake by oocytes is triggered by temperature and/or photoperiod under gonadotropins control of the pituitary gland, regulated by the hypothalamic gonadotropin-releasing hormone. Oestradiol released into serum, bound to proteins or albumins, binds to ER, activates *vtg* transcription and enters hepatocytes by diffusion. Once in the oocytes, VTG incorporates into yolk platelets where it is proteolytically cleaved to lipovitellin and phosvitin under gonadotropin promotion. After oogenesis, gonadotropins releases into the bloodstream, induce oocyte growth, and ultimately, ovulation, stimulating follicle cells to synthesize oestrogens (primarily oestradiol). Although the VTG gene is normally silent, it can be induced if male fish are treated with oestrogens.

The use of VTG as biomarkers is recognised around the world, mainly to evaluate feminisation in male fish or other animals. The measurement can be done using blood plasma or liver extracts based on the sandwich enzyme-linked immunosorbent assay, specific for each fish species selected. This female phospholipoprotein synthetized in the liver precursor of egg yolk protein, non-detectable in normal male fish, can be induced when exposed to xenoestrogens (Jobling & Tyler 2003). It is used as an end-point in field biomonitoring and in laboratory experiments. Examples of the first are several Canadian studies finding evidence of higher than normal VTG, decreased gonad size and circulating steroids in fish living downstream from pulp mills and municipal sewage treatment plants (McMaster et al. 2006).

An increase of VTG in male fish as a biomarker of oestrogenicity in fish exposure assays based on USEPA or OECD protocols use several model fish such as Japanese medaka, fathead minnow or zebrafish (Panter et al.1998,

Gronen et al. 1999, Sohoni et al. 2001, Schwaiger et al. 2002, Holbech et al. 2006, Ankley et al. 2009, Kunz & Fent 2009). Additivity of effects in mixtures with fathead minnows (Brian et al. 2007), and binary mixtures with medaka (Sun et al. 2009), efficacy of treatment for reclaimed water from sewage with crucian carp (*Carassius carassius*) (An et al. 2008), are examples of its application. Nakari & Pessala (2005) applied it on isolated rainbow trout hepatocytes as an *in vitro* screening technique to demonstrate the oestrogenicity of flame retardants. Not always induction in male is the endpoint, as induction of VTG was observed in female fish in the case of glyphosate (An et al. 2008), and for resin acids, different results appeared in each one of the two generations tested (Christianson-Heiska et al. 2008). This makes the use of this biomarker not conclusive in some cases.

2.4.3.2 Choriogenin and *zona radiata* genes

Arukwe & Goksøyr (2003) reviewed the synthesis of eggshell proteins as a process starting by induction in the liver by E2, transport to the ovary, and uptake into maturing oocytes. Several of the encoding genes are present in mammals and well preserved among teleostean fish.

2.4.3.3 CYP1A

The induction of CYP1A activity, an enzyme composing the cytochrome P450 system participates in the synthesis of lipids and steroids. It can be measured by analysing the catalytic activity of ethoxyresorufin-*O*-deethylase (EROD). The binding to the arylhidrocarbon receptor (AhR) induces transcription of CYP1A, among other target genes, then, it can be used to assess exposure to aromatic hydrocarbons such as PCBs, dioxins and furans, PAHs (Song et al. 2006), wood sterols (Lehtinen et al. 1998, Jones et al. 2001, Goksøyr 2006). The responses of cytochrome P4501A1 in the liver were studied in the laboratory by exposing rainbow trout (*Oncorhynchus mykiss*) to river sediments.The EROD activity correlated to PAH concentrations which were determined as being within the range of 2000 to 7000 ng/g dry weight (Inzunza et al. 2006). The

extrapolation of results from animal studies of P450 induction to humans is a complex process that requires, ultimately, the direct proof from human studies (Ma & Lu 2007). The integration to human health is possible using this biomarker, as CYP enzymes are of critical importance in pharmaceuticals detoxification or production of toxic metabolite(s) (Dorne et al. 2007).

2.4.3.4 Retinoids

Retinoids coming from dietary sources of vitamin A signal and participate in biological processes, some of which related to the reproductive function. Carotenoids are precursors of vitamin A important in the feeding of embryo. This effect has been seen in fish (Landman et al. 2008) and in other animal species, like amphibians, as frog limb deformities were found to be linked to the retinoid system disruption (Novák et al. 2008). Oestradiol increases plasma retinol levels. Furthermore, as vitamin A participates in many developmental, reproductive, and immunological processes makes this biomarker suitable for studying the effects of dietary contaminants on marine mammal health (Mos et al. 2007).

2.4.3.5 Growth factors

Fish reproduction is mediated by steroidal hormones, temperature, season and food availability, but also by growth factors that participate in spermatogenesis and oocyte maturation. Insulin-like growth factors production in the liver is stimulated the growth hormone (GH) secreted in the anterior pituitary binds to the GH receptors (GHR) (Reinecke 2010, Reindl et al. 2011). The biomarkers growth hormone factor (*GHF*) and insuline-like growth factor-1 (*IGF-I*) are modernly used to determine reproductive endocrine disruption in fish as well as other adverse effects on homeostasis.

The growth hormone (GH) is modulated by steroids and regulates body growth and development (Leung et al. 2004). For example, disruption of the GH-IGF system by E2 is linked to depressed body of rainbow trout with concomitant decrease in hepatic *IGF-I* (Hanson 2012). The EDCs that reduce circulating GH

levels, such as NP and E2 may also diminish fish immunity and increase stress (Lerner et al. 2007). It has been suggested that E2 reduces hepatic sensitivity to GH and peripheral production of IGFs and that testosterone increases peripheral sensitivity to GH and IGF as well as increases peripheral production of IGFs (Norbeck & Sheridan 2011). The growth hormone (GH) and prolactin (PRL) from the pituitary gland and triiodothyronine (T3) and thyroxine (T4) from the thyroid glands enhance the effect of oestradiol (Sumpter & Jobling 1995).

2.4.3.6 Peroxisome proliferators-activated receptors

They pertain to the nuclear receptor family, acting as transcription factors in cellular differentiation, development, metabolism and tumorigenesis. From the mechanistic stand-point, some EDCs (organotins and phthalates) have been found to be agonist ligands for retinoid receptors and peroxisome proliferator-activated receptor γ which participate in adipogenesis and obesity (Grün & Blumberg 2006, Desvergne et al. 2009).

2.4.4 *In vitro* tests to assess the activity and toxicity of EDCs

In vitro methods are cost-effective and rapid alternatives to *in vivo* tests, useful as their adjuncts to assess the activity of potential EDCs or their mixtures. The need to limit the use of live animals has lately increased the development of *in vitro* methods, which are valuable tools provided interspecies extrapolation and *in vivo-in vitro* relationships are carefully established. The use of *in vitro* data in RA is relevant for both hazard identification and dose response assessment (Barton & Andersen 1998). Some of the assays that can be used as screening tools of oestrogenic activity are: yeast based assays (Routledge & Sumpter 1996, Gaido et al. 1997, García-Reyero et al. 2001), cell proliferation assays (binding, transfection and stably transfected cell lines assays (Takeuchi et al. 2008). They are good screening tools because of being rapid and fairly easy to apply and they may reduce the number of animals needed for exposure testing (Andersen & Krewski 2008). Table 2-5 summarises the most frequently used *in vitro* screens and tests to demonstrate oestrogenic effects.

Table 2-5 Most used *in vitro* tests methods to measure oestrogenicity

Name of method	Materials	Method	References
Yeast oestrogen screen (YES)	Yeast strain tranfected with human ER DNA sequences and a *lacZ* reporter gene (plasmid) encoding β-galactosidase enzyme.	Binding of EDC to ER triggers a colorimetric reaction with β-galactosidase, and the yellow substrate CPRG turns into a red dye, which is measured at 540 nm	Arnold et al. 1996 Routledge & Sumpter 1996
Transactivation assay ERCALUX®	Human breast adenocarcinoma cells (T47D)	Exposure of cells marked with luciferase. Reaction with EDC is measured as luciferase activity and correlated to calibration curve with E2	Boever et al. 2001
E-screen	MCF-7 cell culture	MCF-7 cells stimulate ER-dependent transcription and growth promotion of oestrogen-dependent cells in culture	Soto & Sonnenschein 1985
Competitive ER binding assay	Cell lines from uteri of ovariectomized Sprague-Dawley rats	Measurement of radioactivity to determine binding of ^{3}H-17 β-oestradiol in rat cytosolic recombinant ER	Blair et al. 2000

Methods utilising ER stable cell lines using T47D human breast cancer cells can be used for screening chemicals for oestrogenic and antioestrogenic activities. Several environmental oestrogens were tested using this method (T47D-KBluc cells assay). The lowest observed effect concentrations (LOEC) for genistein and 4-NP were 10 nM and 0.5 nM, respectively (Wilson et al. 2004). A promising method to detect anti-oestrogenicity uses MCF-7 cell line, which

possesses both ER subtypes, as well as the AhR pathway (Navas & Segner 2008).

The yeast oestrogen screen (YES) uses a genetically engineered strain transfected with human ER DNA sequences. After interacting with the oestrogenic substance there is a change in the conformation of the receptor. The dimmers of the ER are then located upstream of the *lacZ* reporter gene (encoding the enzyme b-galactosidase) present on a reporter plasmid. Incubation of these recombinant yeasts with EDCs triggers expression of β-galactosidase, which causes that of the reporter gene and this enzyme is secreted into the medium, metabolises the chromogenic substrate, chlorophenol red-b-D-galactopyranoside (CPRG), initially yellow, turning it into a red dye that can be measured by absorbance at 540 nm (Arnold et al. 1996, Routledge & Sumpter 1996).

Transactivation assays employ endogenous receptors, such as E-Screen and ERCALUX® assays, using MCF7 and T47D cells, respectively (Boever et al. 2001). They expose T47D human breast adenocarcinoma cells to the samples and the activity is calculated based on 17β-oestradiol (E2) calibration curve. The response is measured with a luminometer after adding luciferin substrate to each multiplate well. Oestrogenic potency is expressed as oestradiol equivalency (EEQ). It has been applied in the rivers Meuse and Rhine in the Netherlands along with fathead minnows exposure tests. The reference toxicant was 17α-ethinyl oestradiol (EE2) (Bogers et al. 2007).

Bioactive concentrations in human beings cannot easily be extrapolated from animal studies. To avoid using experimentation animals, screens with mammals cells (of rat or mice) rate the relative competitive ER binding activities of several EDCs taking DES as 100 %. As an example, the ER competitive-binding assay for NP with human cell lines characterises its bioactivity as -1.3 (LogRBA) (Kuiper et al. 1998) and -1.53 using rat cell lines from uteri of ovariectomised Sprague-Dawley rats (Blair et al. 2000). On Table C-3 in Appendix C, there are examples of relative ER binding activities found in databases for several EDCs

for the purpose of extrapolating toxicity data from rodents or other mammals to human beings. Figure B-9

2.4.5 Evaluation of effects with "–omics" technologies

The risk assessment of mixture effects is aided by the knowledge of mechanisms to diminish uncertainty and help to know if studies results can be extrapolated among species. Some of the technologies used are the following:

2.4.6 Genomics/transcriptomics

DNA array technologies allow thousands of genes to be surveyed in parallel, both for expression monitoring under various physiological conditions and in polymorphism analysis (Oberemm et al. 2005). An example of application of this methodology is a study conducted by Vizziano et al. (2008) using microarray analysis working with rainbow trout, *Oncorhynchus mykiss*. Fish were exposed to a natural non-aromatisable fish androgen (11β-hydroxyandrostenedione) and to an aromatase inhibitor (1,4,6-androstatriene-3,17-dione), in order to elucidate the steroid-induced masculinisation mechanism.

2.4.7 Proteomics, metabolomics and toxicogenetics

These methods serve to study respectively the proteins, metabolites and genetic variability of organisms exposed to toxicants. Other laboratory experiments with fish, such as those employed in the research by Ankley et al. (2009) incorporate to the VTG induction test, gene end-points measured via quantitative real-time polymerase chain reaction (PCR). The measurement of VTG is done using an ELISA immunochemical method. This technique can be applied to model fish (for example zebrafish, medaka, and fathead minnow), for which there is a publically available sequences and primers of the *vtg* gene can be easily obtained. Some ecologically relevant species of non-model fish can be used to evaluate the aquatic ecosystem exposed to EDCs applying a quantitative real-time polymerase chain reaction method with a *vtg* gene amplification (Biales et al. 2007).

2.4.8 Screening level site specific hazard identification using toxicogenomics methods

Toxicogenomics is “the application of genomic technologies (for example, genetics, genome sequence analysis, gene expression profiling, proteomics, metabolomics, and related approaches) to study the adverse effects of environmental and pharmaceutical chemicals on human health and the environment” (NRC 2007). Toxicogenomics data may provide a relatively inexpensive and quick test result that could be used to fill the gaps in general causation. The term ecotoxicogenomics is also used to refer to the integration of genomic-based science into ecotoxicology, to aid in minimising animal testing (Snape et al. 2004).

2.4.9 Periods of susceptibility

Disruption of endocrine function can impact greatly adult health, but exposures to EDCs during early life stages can have even more severe as during development. Exposures to toxicants during this critical period of programming can result in permanent abnormalities in endocrine function (WHO 2011b).

Pregnant and lactating women may also be a life stage of concern due to physiological changes during pregnancy and lactation and the characteristics of the chemicals. The consequences of exposures depend on the mechanism and type of action, the timing of exposure, and the dose of the chemical. Adverse effects can be manifest at birth (e.g. hypospadias and cryptorchidism in humans), in puberty (as delay or precocity), or in adulthood (e.g. infertility, alterations in accessory sex organs, disturbances in pregnancy maintenance, endometriosis, or premature reproductive senescence).

Testicular descent starts in mid-gestation until late gestation. This long span may explain the frequency of testicular maldescent cryptorchidism (2–9% of newborn). Male-type development is hormonally regulated, whereas female-type differentiation occurs in the absence of reproductive hormone action. Because the male phenotype depends on an induced pattern of gene

expression, the male foetus is susceptible to hormonal perturbations of the androgen signalling pathways. Likewise, genotypic females will be masculinised by exposure to sufficient amounts of androgens. An antiandrogen exposure would cause cryptorchidism if the exposure is late in pregnancy. Effects on puberty can be the result of earlier life stage exposure or exposure concurrent with the maturational process.

2.5 Dose-response assessment

An evaluation on the ways by which EDC are transformed and transported via air, water, soil, dust, and diet is done at this stage. The pathways for individuals to become in contact with the chemical are through inhalation, ingestion, or skin/eye contact. The individual's activity pattern, as well as the concentration of the chemical, determines the magnitude, frequency, and duration of the exposure. Then, the internal dose is produced after absorption, and finally an adverse effect may occur when the agent interacts with the receptors or the target tissue (USEPA 2012c).

Not every EDC follows the same dose-response curve, as monotonic and non-monotonic functions can be seen. The NOEL (no-observed-effect level (NOEL) is defined by IUPAC as the greatest concentration or amount of a substance, found by experiment or observation, that causes no alterations of morphology, functional capacity, growth, development, or life span of target organisms distinguishable from those observed in normal (control) organisms of the same species and strain under the same defined conditions of exposure. In Table 2-6, some examples of dose-response in aquatic invertebrates and fish are presented.

Table 2-6 Dose-response for endocrine disruption effects in freshwater organisms exposed to single EDCs

EDC chemical name	Taxonomic group	Species	Dose to produce effect (µg l^{-1})	Effect	Test conditions	Reference
Bisphenol A	Mollusk	*Marisa cornuaretis*	NOEC: 640	Developmental	12 weeks, juvenile snails	Forbes et al. 2007
Bisphenol A	Fish	*Brachydanio rerio*	EC50: 2.90	Embryo malformation, low hatchability	72 h exposure	Liu et al. 2007
Bisphenol A	Fish	*Pimephales promelas*	16 640 and 1280 640 1280	Altered spermatogenesis Growth inhibition and VTG induction in male fish Reduced hatchability in F1 generation Egg production inhibition	3 generation reproduction exposure test	Sohoni et al. 2001
Benzo-α-pirene (BaP) (PAH)	Fish	*Fundulus heteroclitus*	10	CYP19A1 expression decreased by about 50% in immature stage I oocytes	10 or 15-dexposure, *in situ* hybridization, several life-stages	Dong et al. 2008
Dehydroabietic acid (DHAA), resin acid	Fish	*Danio rerio*	50	Low plasma VTG in female in F0, high VTG and affected spermatogenesis in F1 males	2 generations, continuous	Christianson-Heiska et al. 2008

Table 2-6- *Continued*

EDC chemical name	Taxonomic group	Species	Dose to produce effect (µg l^{-1})	Effect	Test conditions	Reference
Glyphosate	Mollusk	*Pseudosuccinea columella*	1 mg l^{-1} 10 mg l^{-1}	Faster development of F3 embryos; hatching inhibition	3 generation continuous	Tate et al. 1997
4-Nonylphenol	Fish	*Rivulus marmoratus*	300 [1]	Testicular agenesis and oogenesis inhibition in 100 % fish	Static system, daily renewal	Tanaka & Grizzle 2002
4-Nonylphenol	Fish	*Oncorhynchus mykiss*	1 –10 10	High VTG in adult male fish plasma Low hatching rate	Intermittent exposure adult fish, 4 months until spawning	Schwaiger et al. 2002
4-Nonylphenol	Crustacean	*Ceriodaphnia dubia*	NOEC, reproduction: 1	Low hatching rate	7 days chronic exposure, static	Isidori et al. 2005
Oestrone	Fish	*Danio rerio*	LOEC: 14 ng l^{-1} 50 ng l^{-1}	VTG increase; higher female ratio	40 days fish sexual development test	Holbech et al. 2006
17β-Oestradiol	Fish	*Pimephales promelas*	100 ng l^{-1}	VTG increase; testicular growth inhibition	21 days male fish exposure	Panter et al. 1998
Phenanthrene, PAH	Fish	*Oryzias latipes*	NOEL: 100	Developmental, hatching	18 days, renewal	Rhodes et al. 2005

2.6 Effects testing: tiered methodology to demonstrate endocrine disruption

The usual methodologies are *in vivo*, such as the fish reproduction exposure assays and *in vitro* receptor binding bioassays, for androgens (Wartman et al. 2009) and oestrogens (Kunz et al. 2006). However, the latest international agreements on restricting animal experimentation tests for ethical and economic reasons (ICATM 2009) have destined *in vivo* methods to be applied as confirmatory tests, and whenever possible alternative methods should be used. Some of the most utilised tests relay on the use of fish as model experimental organism in various life-stages, as for example the 21 days reproduction fish test with fathead minnow (EPA/600/R-01/067).

The analysis of exposure and effects determines the EDCs concentration in environmental matrices (watercourses, ground water, drinking water, soil, sediment, air, biota), and the potential or actual effects. Several methods have been standardised to demonstrate dose-response relationships either by *in vivo*, *in vitro* tests or by combination of both (OECD 2012). High-throughput *in vitro* assays characterise dose-response relationships over a wide range of doses minimising the use of animals of experimentation (NRC 2007a, Andersen & Krewski 2008). This methodology may be incorporated in an IRA framework, to link human and ecological receptors through common toxicity mechanisms.

A scheme composed of tiers for the risk assessment of chemicals have been proposed based obtaining human exposure information, predicting exposure from bioavailability of the compound, patterns of use and production levels, undertaking a preliminary risk assessment to further apply testing with the minimum use of animals (Combs et al. 2003).

2.7 Risk estimation

This phase is sometimes considered as the first stage of risk characterisation, but in some frameworks it constitutes a separate step (DEFRA 2000). At the risk estimation phase the likelihood of exposure is combined with an

assessment of effects for each toxicological endpoint, to identify the magnitude of risks.The assessment results are integrated linking exposure with effects to develop an estimation of the risk and the probability of adverse effects. As an example, the human exposure to bisphenol A was estimated with a factor of 500 on the NOAEL (10 for interspecies differences, 10 for inter-individual differences and 5 for the uncertainties of the database). Then, the overall NOAEL of 5 mg kg^{-1} body weight per day gave a temporary TDI of 0.01 mg kg^{-1} bw. Realistic worst-case estimates of consumer exposure via foodstuffs, ranged from 0.00048 mg kg^{-1} bw-day-1 for adults to 0.0016 mg kg^{-1} bw day^{-1} for infants,below this t-TDI of 0.01 mg kg^{-1} bw (EC 2002).

The measured or modeled environmental concentrations can be compared to the ADI (acceptable daily intake) to determine an accumulation effect through the TEF (toxic equivalent factor) and TEQ (toxic equivalent quotient). Further, another way of estimating risk is by comparing to the minimal risk level (MRL) that evaluates only chronic effects (EPA, 2000). Estimates of exposure concentration posing minimal risk to humans include adjustments to reflect human variability and extrapolation of data from laboratory animals to humans. Table 2-7 shows some examples of these estimations for some EDCs.

Table 2-7 Minimal Risk Levels

Name	**MRL (mg $kg^{-1}day^{-1}$)**	**Endpoint**
Di(2-ethylhexyl)phthalate	0.06	Reproduction
Hexachlorobenzene	0.00005	Developmental
Pentachlorophenol	0.001	Endocrinological
2,3,7,8-Tetrachlorodibenzo-p-dioxin	0.000001	Developmental

Source: Agency for Toxic Substances and Disease Registry (ATSDR) December 2008

For humans, the reference dose (RfD) is an estimate of a daily exposure to the human population (including sensitive subgroups) likely to be without an appreciable risk of deleterious effects during a lifetime. It is expressed in units of mg $kg^{-1}day^{-1}$ (USEPA 1993). It can be calculated by dividing the NOAEL or benchmark dose (Hays et al. 2007) by uncertainty factors to account for interspecies differences in response, intra-species variability and deficiencies in the database. The benchmark dose (BMD) approach fits a mathematical model incorporating the entire dose-response curve, with more certainty than the NOEC-LOEC approach and allows determining the dose even when responses are present at the first concentration tested. The oral RfD and risk units for several single compounds on human health by chronic exposure to ingestion of drinking water uses NOEL in some cases and LOAEL in others (Table 2-7, Table 2-8). The uncertainty factor (UF) accounts for intraspecies and interspecies variability based on rat toxicity data of the toxicity or other considerations.

Many limitations exist in obtaining complete information on tolerable doses for EDCs from current databases. For example, no reference doses exist for resin acids (isopimaric and others) or for sitosterol. A recommended oral RfD of 0.1 mg kg^{-1} day^{-1} has been established for 2,4,5-Trichlorophenol but it is not based on chronic or reproductive studies. The NOAEL for naphthalene for terminal body weight decrease in male rats determined as 71 mg kg^{-1} day^{-1} was then divided by an uncertainty factor (UF) of 3000 to arrive at a chronic RfD for naphthalene of 2x 10^{-2} mg kg^{-1} day^{-1}. In the case of PCBs, Aroclor 1016 had a RfD of 7x 10^{-5} mg kg^{-1} day^{-1}, UF= 100, endpoint reduced birth weight.

Very recently, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), has been reassessed, setting a RfD of 7x 10^{-10} mg kg^{-1}-day^{-1} bw, based on endocre disruption endpoints, and not only cancer decreased sperm count and motility in men exposed to TCDD as boys) (USEPA database Integrated Risk Information System, IRIS http://www.epa.gov/iris/subst/1024.htm, last revision 17/2/2012).

The World Health Organisation and the U.K. Food Standards Agency (FAO) Committee (JECFA) on dioxins set a NOAEL of 13 ng kg^{-1} (maternal body burden) to derive a RfD between 1-10 pg $kg^{-1}d^{-1}$ (TCDD TEQ) (Greene et al. 2003). The European Commission proposed a tolerable weekly intake of 2 pg-TEQ kg^{-1} bw, and a maximum level for fish and fishery products of 4 pg-TEQ g^{-1} wet weight for PCCD/Fs and 8 pg-TEQ g^{-1} wet weight for PCCD/Fs and dioxin-like PCBs (Commission Regulation (EC) 199/2006).

Table 2-8 Oral reference dose for human health risk assessment

EDC	**RfD (mg kg^{-1} day^{-1})**
Glyphosate	1×10^{-1}
Polyaromatic hydrocarbons (PAHs) (naphthalene)	2×10^{-2}
Polychlorinated biphenyls (PCBs) **Aroclor 1016** **Aroclor 1254**	 7×10^{-5} 2×10^{-5}
2,4,5-Trichlorophenol	1×10^{-1}
Endosulfan	6×10^{-3}
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	7×10^{-7}

Source: IRIS database (http://www.epa.gov/iris)

2.7.1 Risk quotients and hazard quotients approaches

Probabilistic and possibilistic logic approaches are found on the literature as statistical models for risk estimation. Even though they are useful to test for interactions of chemicals, one of the disadvantages of statistical methods is that mixture components have to be entirely known, which may not be the case for complex mixtures of environmental sources.

Risk management strategies can be viewed as a precautionary decision process based on the fuzzy logic decision-making process as adverse consequences of an event have uncertainties, which can be represented as

fuzzy sets (Cameron & Peloso 2005). Stochastic events can be dealt with by the probability theory, but it is not always easy to estimate the frequencies to calculate the probability distributions. In this case, the Bayesian approach is considered to be better suited, but the vague boundaries of ecosystems makes its application difficult (Jooste 2001).

The deterministic approach makes use of a risk quotient (RQ) calculated by dividing a point estimate of exposure by a point estimate of effects at a screening level estimation, and it is the ratio among PNEC/PEC (Environment Canada 2011).

The hazard quotient (HQ) is the ratio of an exposure level to a substance at a toxicity value selected for the risk assessment of that substance (e.g., LOAEL or NOAEL). The Hazard Index, is the sum of more than one hazard quotient for multiple substances and/or multiple exposure pathways. Risks of mixtures can be estimated provided the single compounds act through the same modes of action and exhibit additive responses. It is used at a screening level for human health assessment (USEPA 2012a, Health Canada 2004).

Finally, when evaluating populations, it is necessary to use epidemiology methods to estimate the exposure to multiple stressors and incorporate non-chemical stressors into cumulative risk assessments (Løkke et al. 2010).

Examples of models for aggregate and cumulative risks related to human exposure are SHEDS, Zartarian & Schultz (2009) and USEPA, HEDS (Human Exposure Database System) (USEPA 2009a). Probabilistic models calculate percentiles and their uncertainties for a target population applying Monte Carlo simulations to ponder population exposure (USEPA 2009b). An estimation of lognormal distributions of the tap water intake of children and adults to the overall population was done for public health assessments (Roseberry & Burmaster 1991).

2.8 Risk characterisation

This final step of the risk assessment process integrates data of the dose-response relationship of an agent with estimates of the degree of exposure in a population to characterise the likelihood and severity of health risk. It serves as an interface between risk assessment and risk management (Williams & Paustenbach 2002). This step has been defined by IPCS as: The qualitative and, wherever possible, quantitative determination, including attendant uncertainties, of the probability of occurrence of known and potential adverse effects of an agent in a given organism, system, or (sub)population, under defined exposure conditions (IPCS 2004).

The potential risk of a substance to the environment is commonly characterised by comparing the predicted environmental concentration (PEC) based on environmental exposure assessment with the predicted no effect concentration (PNEC) estimated from toxicity data.The risk and its uncertainties are characterised in light of how significant they might be, by reference to standards, including the combined multi-stressor, multiple effects aspects. For this task, in the case of mixtures, several approaches can be used to characterise the risk, but it still remains a research gap how to reflect more accurately the actual situation, and how to evaluate the risk of coexisting synergistic, antagonistic or additive substances as may exist in the environment. Moreover, even certain single EDCs may have both inhibitory and stimulating oestrogen-like actions depending on the target tissue, as explained by the theory of the selective receptor modulators (SERM) (Safe et al. 2002).

The weight-of-evidence (WOE) approaches can be used to perform integration of evidence of toxicity. These approaches have been developed in the fields of medical evidence, forensic science and radioactive waste management and they can be applied to environmental risk (Pollard et al. 2008), and to assess the risk of endocrine disruption in mixtures (Linkoy et al.2009).

The latest research aims to understand the link between environmental exposures and human health through biomarkers to estimate quantitatively human exposure to chemicals (USEPA 2009c). Endocrine disruptors are a good example, since endocrine activity mediated by receptors is difficult to predict only based on the actions of the chemicals found in a mixture. The final effect is not easy to predict where that coexist anti-oestrogen, oestrogenic and androgenic (Kortenkamp 2007) substances. In addition, the metabolites may differ in the action of the parent compounds (USEPA 2007a).

The importance of molecular biology studies to elucidate the mechanisms of action is clear. For example, the oestrogenicity can be investigated through the measurement of the affinity of a substance to bind to α-ER or ER-β receptors by applying methods such as the assay with recombinant yeasts and the system of ER-α gene MCF7 cells-based reporter. These techniques can also be applied to find additivity of mixtures. Gaido et al. (2003) have shown that chemicals in a mixture of EDCs can interact in additive or antagonistic ways and recommend developing risk assessment based on these multiple variables.

Toxicokinetics interactions refer to alterations in the absorption, distribution, metabolism or elimination of a toxic chemical (USEPA 2007). The special toxicokinetics characteristics of EDCs as discussed by Beronius et al. (2009) include non-monotonic dose response curves. Hence, any threshold can be assumed because endogenous hormones are already above the threshold of physiological, hampering extrapolation of high to low doses.

Data generated from epidemiology studies aided by computer modeling techniques such as quantitative-structure-activity-relationships (QSAR), can be used to develop cellular screens to compare responses to mixtures at the genetic and molecular levels, which can be supported by *in vivo* and *in vitro* tests based on mechanisms (Robinson & McDonnel 2004).

Current risk assessment methodologies do not establish how to assess the risk of mixtures, especially in the case when the stressors are at low concentrations, as in general found in the environment. This implies that their PNECs may underestimate risk if defined in terms of single substances. The challenge imposed to the traditional risk assessment paradigm of a threshold dose below which a substance will have no measurable effect when dealing with mixtures of EDCs makes relevant to devote efforts in developing a framework addressing these aspects. The following are methodologies useful to diminish the uncertainty of the risk assessment in this case.

2.8.1 Toxic Equivalency Factor approach

According to Safe (1998), the TEF approach for assessing the hazard of toxic chemical mixtures combines the contribution of each component and their relative potencies and can be represented through the following equation:

$$TEQ = \sum_{i} C_i \times TEF_i \quad \textbf{(2-1)}$$

Where

TEQ = toxic equivalent

Ci: concentration of each individual compound in the mixture

$TEFi$ = relative potencies

The toxic equivalency factor (TEF) approach has been applied for example for persistent halogenated aromatics AhR agonists.

2.8.2 Factorial analysis

Statistical designs can be used to study the interactions of more than three chemical compounds in a mixture, by full-factorial tests or by fractional factorial designs (Feron et al. 1998, Borgert et al. 2001, Charles et al. 2002, Sun et al. 2009).
The whole mixture approach

Mumtaz et al. (2010) argue that chemical mixture risk assessment of waste sites is often limited, incomplete, or inconclusive as data are not available for all mixture components. Although the doses of individual components can be joined in a hazard index (HI), the outcome of this calculation cannot be complete because of the lack of knowledge of all components in the mixture. Although the whole-mixture approach does not identify the causative agents for adverse effects, hazard can be characterised following exposure to the entire mixture (Groten et al. 2001).

2.9 Synthesis and thesis strategy

The special toxicokinetics characteristics of EDCs, their actions at low environmental doses, their occurrence as mixtures, multiple modes and mechanisms of action, multiple sources, pathways and receptors, their additive, synergic or antagonistic interactions, makes the topic challenging to attempt a risk assessment. After reviewing information on the different frameworks it became obvious that an integrated framework could be advantageous. However, as both humans and wildlife have to be evaluated based on knowledge of chemical properties, common mechanisms and toxicokinetics (Sekisawa & Tanabe 2005), data gaps were expected due to incomplete databases, which made it necessary to devote experimental time to derive dose-responses and describe effects to demonstrate the suitability of the framework. he two-phased structure was selected in order to first prioritise the EDCs and assess the main modes of action by receptor-binding screens. This also accounted for integration and extrapolation, as the application of

biochemical screens with human cell lines are indicative of human health and also responses of the receptors in animals. This method should be adopted, ideally in conjunction to epidemiology studies in the vulnerable sub-populations. As seen before, exposure and dose-response assessments could be done by multiple techniques, but the chosen one relied on the whole mixture approach to assess the effects of municipal wastewater inputs and pulp mill effluents, the two main identified point-sources in the watershed, and compare their effects to 17-β oestradiol at two concentration levels.

As predictions of endocrine disruption based only on chemical analysis or VTG induction are not accurate enough because they do not account for mixture effects, molecular interactions or complex modes of action, a suite of toxicogenomics biomarkers including the expression of the *vtg*, growth factors, *zona radiata* and oestrogen receptors 1 and 2 genes was used.

The risk estimation was completed after determining the effects and the dose through testing, based on the conceptual model showing the sources, stressors, pathways and receptors. In the case of a watershed level RA, bioavailability, bioaccumulation and biomagnification in the food chain had to be studied in all environmental compartments and pathways. Modelling techniques were chosen to evaluate the fate of the prioritised EDCs in air, sediment, water, soil and biota, coupling the mathematical approach with experimental studies.

The concentrations of the most probable EDCs in the matrixes of influence, such as drinking water, river water, sediment and fish, were analysed to enter into the databases and calculate the dose-response using models and computational tools. As no information of the toxicity of each EDCs at different exposure scenarios existed, species distribution studies were completed. Finally, as the risk assessment should provide a categorisation of the risk based on the severity and likelihood of the effects, this approach was taken in this thesis.

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3 AIMS AND OBJECTIVES

3.1 Aim

This research was conducted to develop an integrated risk assessment for human and environmental health to jointly combine the risks of multiple stressors in complex environmental mixtures, coming from multiple sources, acting through multiple pathways and causing reproductive and/or developmental impairments on aquatic animals and human beings, demonstrated at a reach and watershed of the Uruguay River.

3.2 Objectives

- To identify and evaluate multiple sources of hazards of EDCs by developing a comprehensive conceptual exposure model that integrates environmental and human health components

- To undertake a preliminary risk assessment to direct focused analytical effort

- To design a novel tiered analytical framework of increasing specificity to evaluate the environmental concentrations and effects to prioritise the risks of complex mixtures of EDCs on human and aquatic freshwater receptors

- To construct a matrix of likelihood, intensity and severity for risk estimation and to combine and characterise the risks using radar diagrams including uncertainties.

3.3 Research questions

- Do multiple chemical stressors in the Uruguay River impact on fish reproductive health?
- What is the dose of endocrine disruptors to which the human population of Fray Bentos is exposed to resulting from the use of the Uruguay River?

3.4 Research hypothesis

A combined effect of multiple substances from pulp mill effluents, wastewater discharges and agrochemicals is responsible for reproductive endocrine disruption effects on fish and human beings at the selected reach and watershed of the Uruguay River chosen for this research.

4 LOCATION, CATCHMENT AND SAMPLING

4.1 River description

In order to describe the sub-basin of the Uruguay River, a brief description of the geography of the La Plata River Basin (Figure 4-1), was completed, as the Uruguay River is one of the three large rivers systems composing it, with the other two, the Paraná and the Paraguay Rivers.

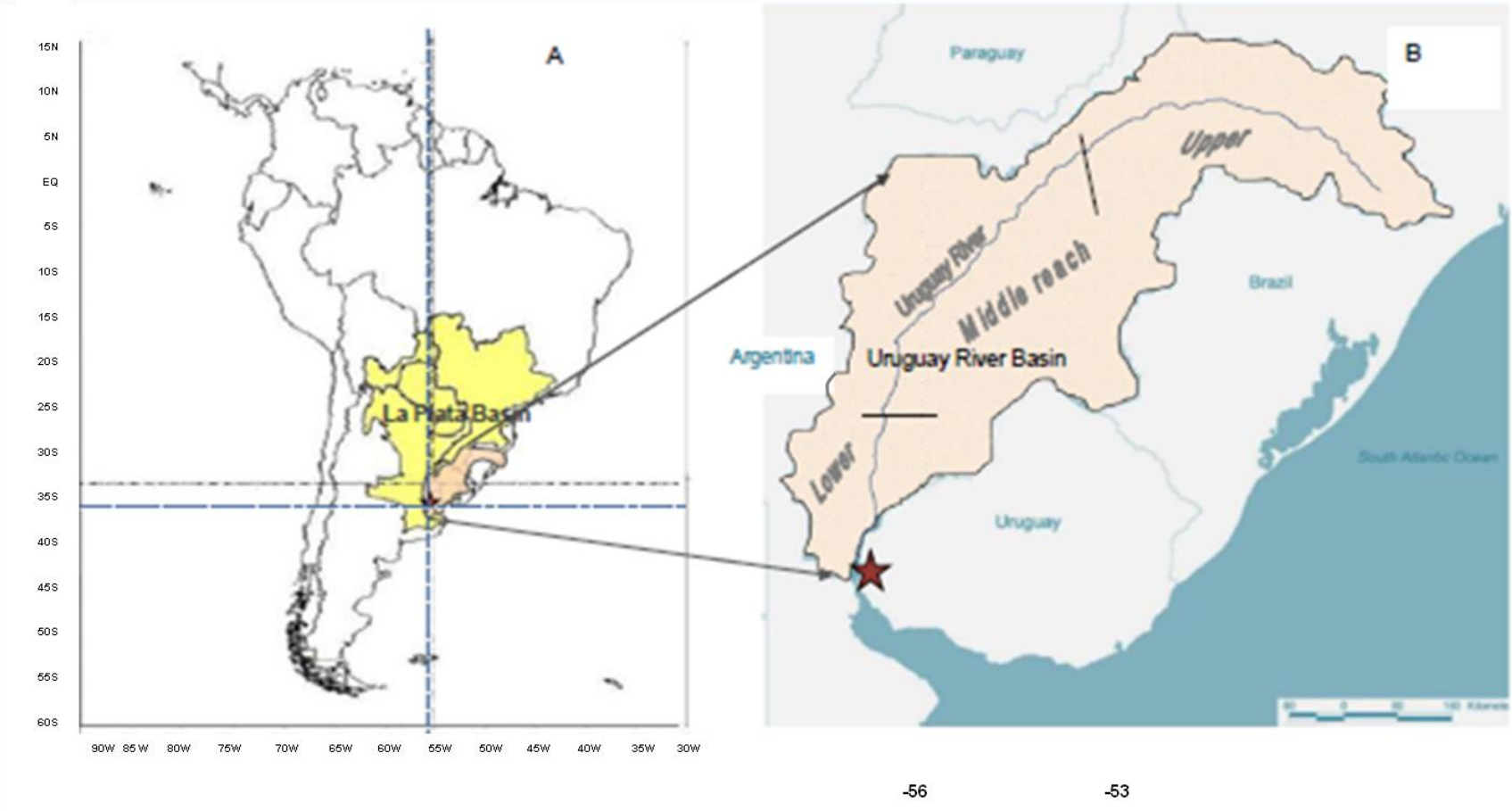
4.1.1 Geography

The Uruguay River basin has an approximate surface area of 339000 km^2. This transboundary international watercourse belongs to Argentina, Brazil and Uruguay. It rises at Serra do Mar (Brazil), extending to a length of 1800 km, finally draining into the La Plata Estuary. It runs through 32% Brazilian land, 38% between Argentina and Brazil and 30% between Argentina and Uruguay (CARU 1994). Its drainage basin lays within latitudes $28^o10'$ S and $37^o08'$ S (Zaniboni & Schulz 2003).

4.1.2 Topography, regions and dams

The topography of the Lower Uruguay River, starting at Salto Grande the slope is less than one centimetre per kilometre, and its bottom is sandy with some basaltic trains (CARU 1992). The river is composed of a series of rapids. The Yucumã waterfall divides Uruguay in Medium and Upper, while Salto Grande is the limit among the Medium and Low sections.

Between the cities of Colón (236 km) and Fray Bentos (102 km) many islands are within the stream. At the Gualeguaychú River drainage area, the islands disappear and the river widens from 8 to 12 km. A series of channels link the Paraná and the Uruguay Rivers, influenced by the speed and direction of La Plata River. At Nueva Palmira, the Uruguay River drains into the estuary.



A: La Plata Basin, B: approximate location of the three river-reaches (Upper Uruguay River, Middle Uruguay River and Lower Uruguay River)

Figure 4-1 The Uruguay River sub-basin within the La Plata River Basin

4.1.3 Affluent watercourses into the Uruguay River and navigability

The main tributary is the Negro River (Uruguay) which is 550 km in length and drains into the Uruguay River at a large delta of 5 km width at km 54. The Gualeguaychú River (Entre Ríos, Argentina) has a length of 120 km, with low margins with forests. The city Gualeguaychú (Argentina) is located 18 km before reaching the Uruguay River. The navigability is at 9 feet draught (CARU 1992), but there are plans to dredge the river to increase it to 23 feet (MRREE 2012).

4.1.4 Hydrology

The river Uruguay's hydrological system is composed of two different parts: the main river flow, letting more water volume through, and coastal zones where the water has less renewal and settles more (CARU 1994). Hydrological data exists since 1892, and its regime is irregular due to the rainfall. At its origin, 2000 mm yearly rainfall is typical and the range is among 1000 and 2000 mm (Zariboni & Shultz 2002). As other hydroelectric dams, Salto Grande provokes soil erosion, and biodiversity alterations in the basin (McAllister et al. 2001).

Winter and spring flows are in general high, with averages around 7000 $m^3 s^{-1}$. The low flow is during summer, reaching less than 2000 $m^3 s^{-1}$. Average flow is ca. 4500 $m^3 s^{-1}$. In April 1959, the maximum flow record was of 36000 $m^3 s^{-1}$ at Concordia Port (CARU 1994).

4.1.5 Orography and land cover

The map in Figure 4.2 shows the orography, and as it can be observed, no hills are present in that area of study. These images were extracted by working with the software Earth Explorer (http://earthexplorer.usgs.gov/) to observe the orography and the land cover of the research area.

At the Upper Uruguay River agriculture is based on soy, maize and beans. At the Medium and Low sub-basins, intensive cattle and soy and rice are

prevalent. *Eucalyptus* forests cover part of the Uruguayan side of the river margin. The population density is of 39 inhabitants per square kilometer, with 45% residing in rural areas (CARU 1994).

4.1.6 Geology and land use

The Uruguay River drains marine sedimentary rocks (Janiot & Molina 2001). It is the youngest in origin of the three rivers of the La Plata Basin, settling on top of sedimentary and volcanic rocks. Igneous deposits from the hills of Serra Geral, are of predominance and they cover the sedimentary rocks of Mesozoic and Neo-Paleozoic eras. The soil is in general loamy clay and shallow (CARU 1994. In the research area, there are several soil types: vertisol, mollic glyosol, haplic luvisols and phaeozem soils. Vertisols have montmorillonite clay. Mollic glyosols are more acidic than haplic luvisol, with pH values of 4.8, compared to 6.5 of the latter (Witkowska-Walczak 2003). Phaeozem soils are good for prairies, intensively leached in wet seasons, with dark, humous surface soils, and they are also used for the production of soybeans (FAO 2001) (Figure 4-4). The population density is 39 inhabitants per square kilometer, 45% in rural areas. Up-stream, agriculture is based on soy, maize and peas. In other parts of the basin, cattle breeding, soy and rice are the main activities. Only some fragments of wild woods still exist, and soil is covered mainly by pastures.

4.1.7 Site characterisation

The province of Río Negro has 53989 inhabitants (INE 2010). Fray Bentos is its main city, with 26654 inhabitants. The city of Fray Bentos has 26654 inhabitants according to the last national population survey done in 2004. It had a productive profile composed of agroindustries, services, commerce, tourism and forestry, with nearly no industrial activities in 2006 according to PACPYMES (2007). In November 2007, a pulp mill considered one of the biggest single line pulp mills ever built initiated operations in Fray Bentos outskirts, on the riverside.

4.1.8 Stress regime, contingencies and extreme events

There is a natural seasonal and inter-annual variability in precipitation, but some of the most relevant natural sources of stress include flooding (Appendix A), which in some years can reach enormous proportions, getting even to having to proceed to the evacuation of population (as in the year 2003 or by the end of 2009).
Natural forest fires are present especially during the summer season. Anthropogenic factors produced by the industrial sector include the modification of habitats because of constructions, increase of nutrient load and the generation of temperature and salinity gradients, use of the water source for commercial barges to carry cellulose and wood, use and release of toxic chemicals. Intentional fires of wetland vegetation occur, especially on the Argentinian side of the river. Seasonal changes may arise from tourism, recreation and fishing.

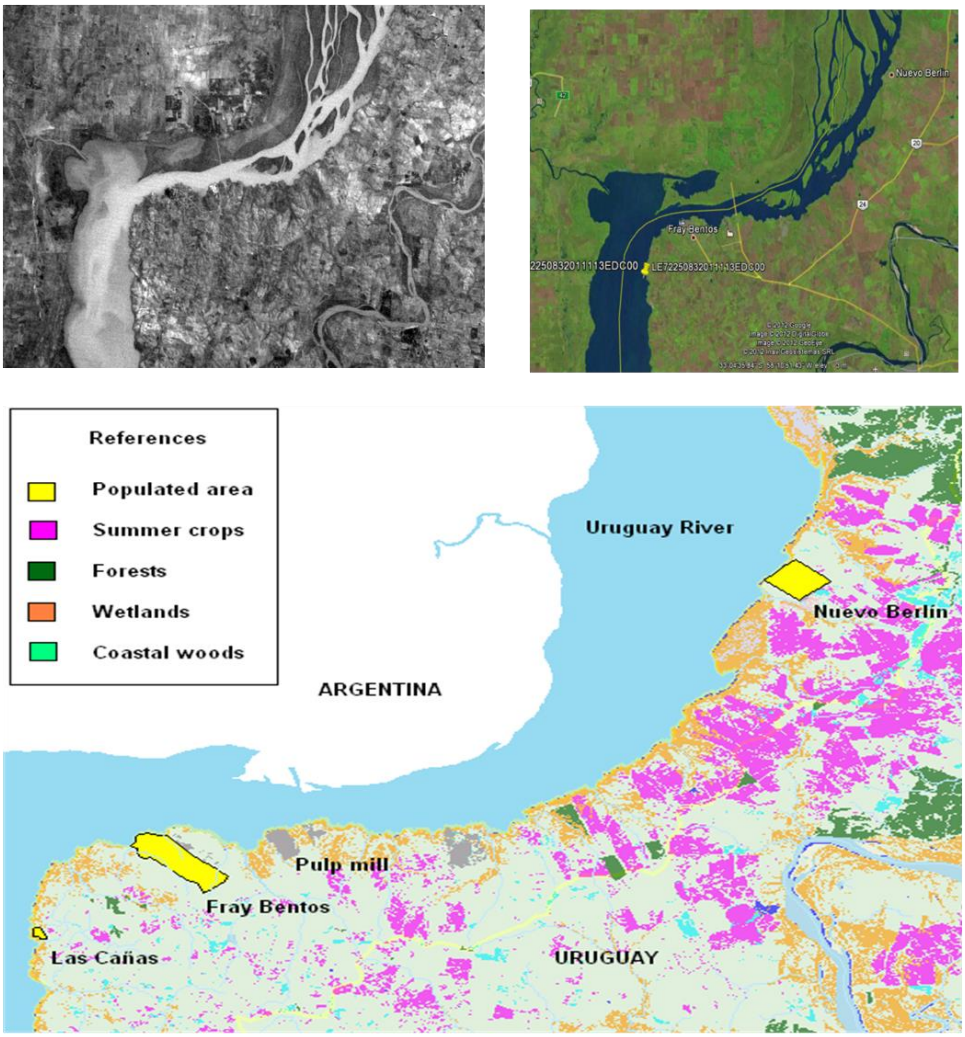
Global phenomena like climate change, may affect the temperature of the river, which in turn affects sexual differentiation. Fish can only survive among the upper and lower incipient lethal temperatures, the homeostatic resistance is exceeded, and the incipient lower lethal temperature for some temperate fish species is from 0-6°C (Panda 2009).

4.2 Catchment area under study

The landscape of the catchment areas under study consists mostly of level land.

4.2.1 Orography, landuse and landcover at the research area

In Figure 4-2, the land-cover as seen from the satellite at a section of the river where the research area was selected.



Satellite images processed with Landsat 7, Global Land Surveys (GLS2010) (parameters in Appendix B). Landuse map adapted from Ministry of Agriculture, RENARE (2009) GIS LANDSAT images http://www.mgap.gub.uy/renare/SIG/Sig_Informacion.htm

Figure 4-2 Satellite images of orography, landcover and landuse

The reaches and section of the drainage basin are delimited by the circumscript perimeter of a polygon around three buffer zones. Three buffer zones or 5 km perimeter were drawn around the relevant sites using a GIS tool (ArcGIS Explorer). The enclosing polygon area measures approximately 461square kilometres (Figure 4-3).

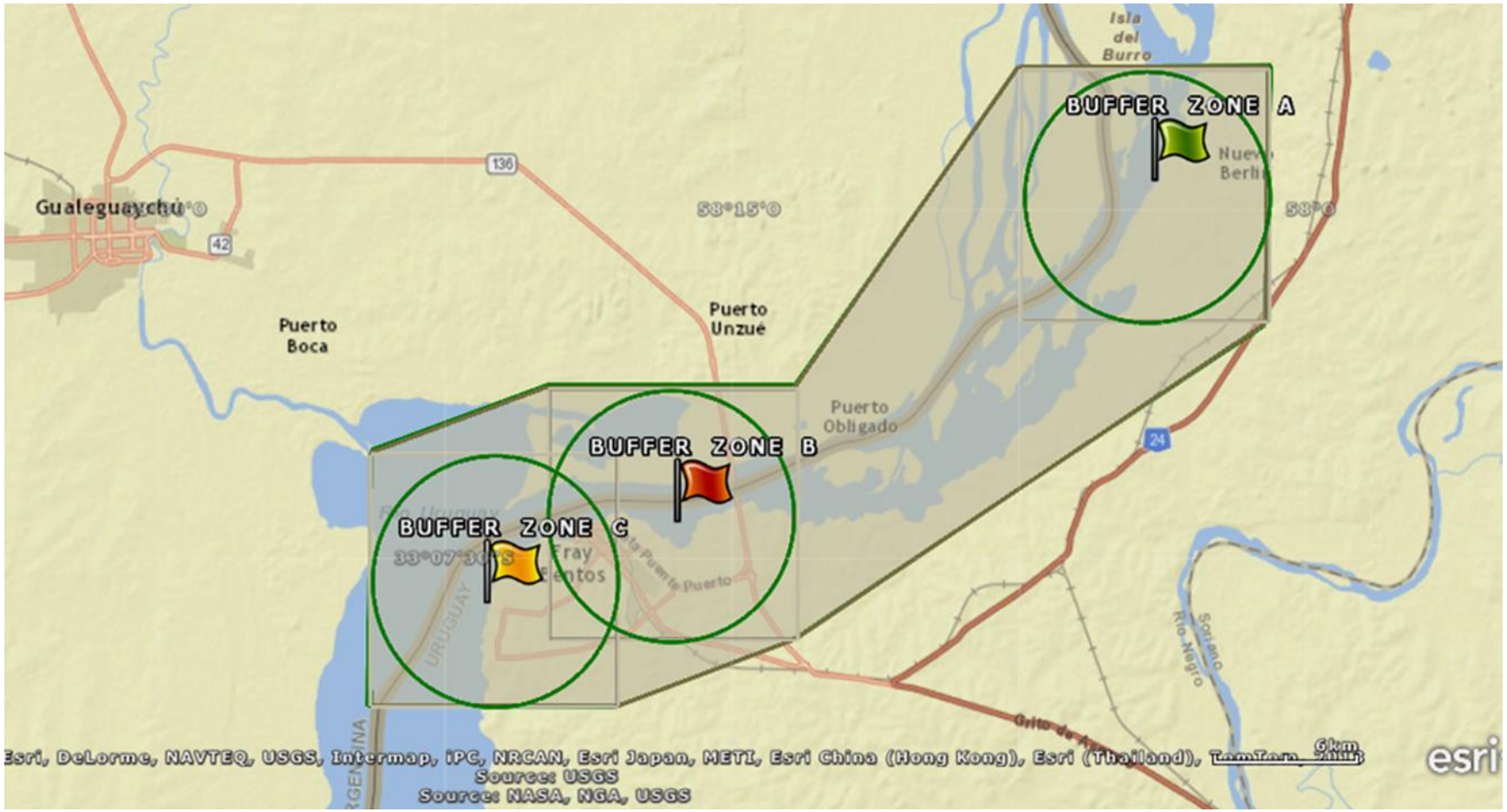


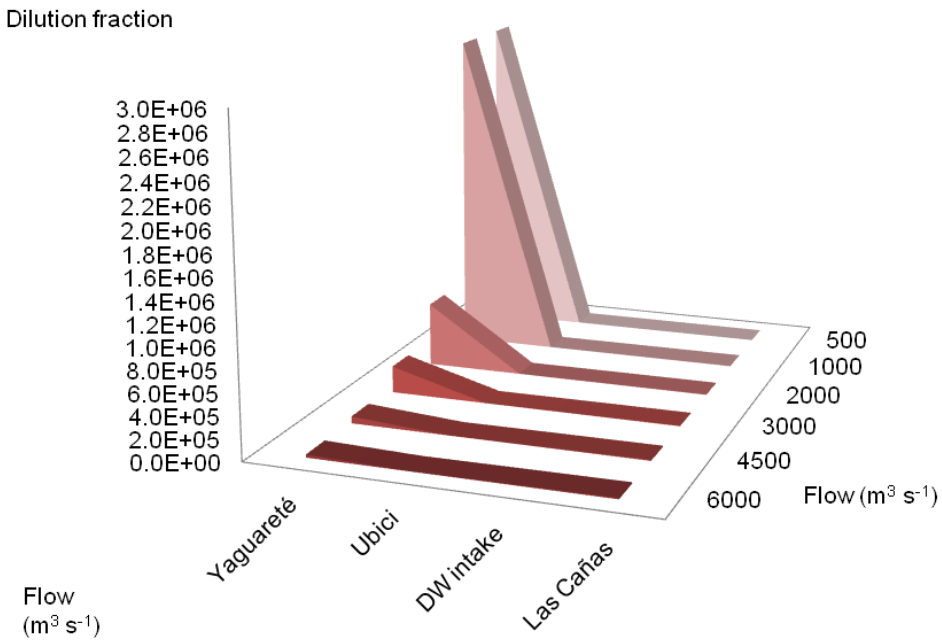
Figure 4-3 Catchment and buffer zones

4.2.2 Hydrodynamics model as a basis for the choice of sampling sites

The environmental impact assessment of the pulp mill includes a hydrodynamic model for the transport of contaminants from the pulp mill diffuser. The RMA-11 for finite element water quality hydrodynamic model, coupled with RMA-2 to integrate in the vertical dimension the tridimensional equations. RMA2 is a dynamic two-dimensional depth-averaged finite element hydrodynamic model for computing water surface elevations and horizontal velocity components for subcritical, free-surface flow, for far-field applications in unstratified water bodies. RMA2 uses the Reynolds form of the Navier-Stokes equations; Eddy viscosity coefficients are used to define turbulence characteristics (http://smig.usgs.gov/cgi-bin/SMIC/model_home_pages/model_home?selection=rma2).

The results of the hydrodynamics isoconcentration model for contaminant dispersion of the pulp mill effluent constituted the basis of the river water sampling sites selection, considering upstream and downstream sites, which is later described in Figure 4-6.

As it can be seen in Figure 4-4, the contaminant that would eventually be emitted from the diffusers of the pulp mill would be much more diluted at Yaguareté Bay than at the other sites, even when this beach is very near from the pulp mill. This site was included as a fish survey site (F2) to demonstrate the effects of the dilution.



	Yaguareté	Ubici	DW intake	Las Cañas
6000	2.8E+04	2.4E+03	7.5E+03	1.1E+04
4500	6.4E+04	1.4E+03	5.8E+03	8.7E+03
3000	2.5E+05	9.7E+02	4.0E+03	6.1E+03
2000	6.0E+05	6.4E+02	2.7E+03	4.1E+03
1000	3.0E+06	6.1E+02	2.3E+03	3.5E+03
500	3.0E+06	8.9E+02	4.0E+02	6.0E+02

DW intake: drinking water intake

Figure 4-4 Dilution factor from river flow model

The average flow (measured at Salto Grande dam) from 2005 to 2010 was 4293 $m^3 s^{-1}$. The flow was below average 33 times and 22 times above average (Figure 4-5).

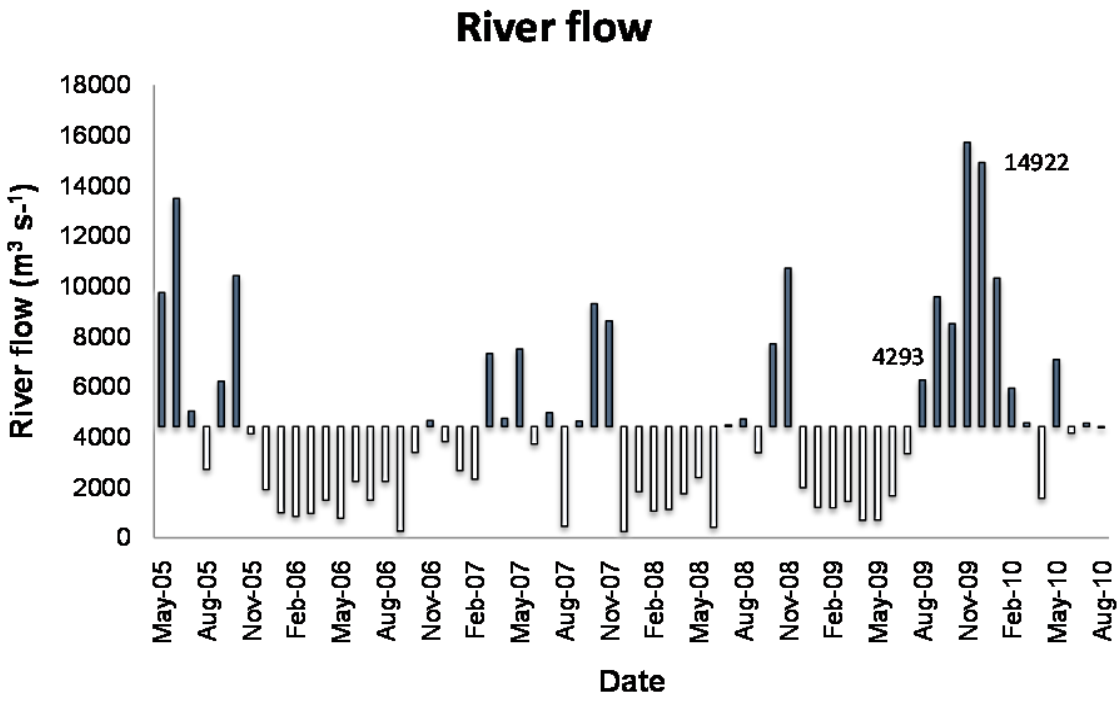


Figure 4-5 River flow variation

4.2.3 Sampling site locations

The location of the sampling sites for river water, streams, sediments and fish is presented in Table 2-1, and Figure 4-6. To locate the sites, the software ArcGis Explorer, ESRI, and Google Earth were used. Photographs of the sampling sites are in Appendix B.

Table 4-1 Master spreadsheet for the location of the sampling sites for river water, streams, sediments, soils and fish

		River water (R_i)/ Inland surface water (C_i)				Sediment (S_i)			Soil (L_i)			Fish (F_i)		
		Code	Name	Lat	Lon	Code	Lat	Lon	Code	Lat	Lon	Code	Lat	Lon
				S	W		S	W		S	W		S	W
Buffer zone	A	R1	Nuevo Berlín	33° 02' 2.6''	58° 07' 7.8''	S1	32°59'17"	58°05'0	L1	33°0'3.6	58°01'60	F1	32°58'15"	58°03'19"
									L2	33°02'70	58°4'21			
									L3	33°4'39	58°3'5.6			
	B	R2	Bridge	33° 05' 54.2''S	58° 14'10.9''				L4	33°8'9.0	58°16'1.0			
		R3	Fray Bentos	33° 06´ 28.6''S	58° 15'45.3''	S2	33°06'19.5"	58°15'59.6"	L5	33°6'59	58°17'29			
		R4	Yaguareté	33°07'03"	58°16'22"	S3	33°07'03"	58°16'22"				F2	33°07'09"	58°16'13"
		R5	Ubici	33°06'42"	58°17'07"	S4	33°06'42"	58°17'07"				F3	33°06'42"	58°17'07"
		R6	Drinking water intake	33º06´27.3"	58º17´47.1"									
		C1	Yaguareté stream	33º08´09"	58º16´01"	S5	33º08´09"	58º16´01"						
		C2	Fray bentos stream	33º07´16"	58º19´02	S6	33º07´16"	58º19´02						
	C	R7	Anglo Beach	33°07'10"	58°20'10"	S7	33°07'10"	58°20'10"						
		R8	Las Cañas	33° 09´ 52.6''	58° 21'38.3''	S8	33°09'52"	58°21'49"				F4	33°09'47"	58°21'34"
		R9	Gualeguaychú canal	33º06´40.6"	58º21´0.5"									

Lat: latitude; Lon: longitude; S: South; W: West

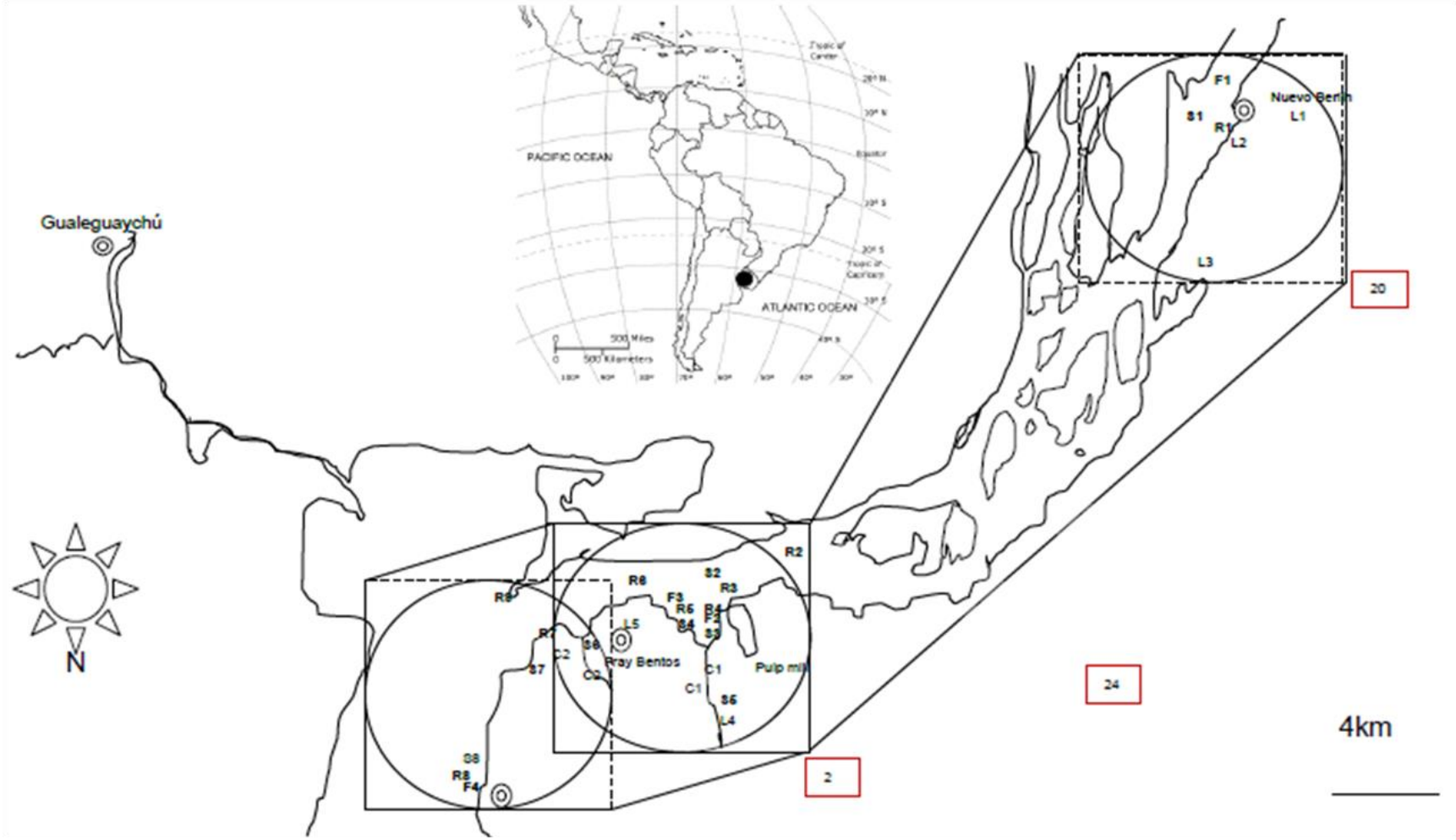


Figure 4-6 Map of the research area and sampling sites

4.2.4 Sampling and analysis scheme

The general sampling and analysis scheme is summarised on Table 4-2. The development of each new method is explained in more detailed in the exposure assessment chapter (Chapter 7 and Appendix B).

Table 4-2 Sampling and analysis scheme for waters, sediments, soils and biota

Matrix	Sampling frequency	Parameters
Drinking water	Yearly	Physico chemical Bioassays for screening
River water	Monthly Every two months Every six months Yearly	Physico chemical dioxins and furans Glyphosate Oestrogens
Stream water	Every six months	Chemical characterisation and exposure media for bioassays
Sediment	Every four months Yearly	Nutrients, particle size, EOX Bioassays
Soil	Every four months	Glyphosate Endosulfan
Municipal wastewater	Yearly	Chemical characterisation
Pulp mill effluent	Daily Monthly Every two months Yearly	Discharge parameters AOX, phenols dioxins and furans, sterols, rosin acids, chlorophenols Nonylphenol, oestrogens Exposure media for bioassays
Fish	Weekly	Biomonitoring endpoints
Mussels	Every six months	EOX
Snails	Punctual	EOX

4.2.5 Rationale for the choice of the sampling sites

The sampling sites shown on the map (Figure 2-1) and described in Table 4-1 were selected basing the choice on different reasons:

Four sampling sites were considered during the baseline monitoring studies carried out since 2005 until the pulp mill start-up by mid October 2007 and analyses was undertaken at the Technological Laboratory of Uruguay for the pulp mill to comply with DINAMA requirements. This monthly monitoring continues in order to verify compliance to national regulations (Decree 253/1979 and modifications, class 3; CARU Uruguay River regulation, Topic E3, Use 4) Appendix A. This information was used as background information on the river health and environmental status for the preliminary risk assessment for human health and the environment.

- The downstream sites were: downstream from the pulp mill of Fray Bentos (R3) and downstream from this city discharges and the pulp mill, at Las Cañas resort (R8).
- One site was set 1.5 km upstream near the international bridge José de San Martín between Uruguay and Argentina to evaluate reverse flow as a nearest reference conditions.
- The reference site was chosen near Nuevo Berlín village, 30 km upstream from the pulp mill (R1).

Apart from these sampling sites already part of the river surveillance monitoring (R1, R2, R3 and R8) two independent sampling points for river water were included in this research but only to measure selected compounds, markers of the pulp mill contaminants or EDCs (Tier 2, Chapter 7). The following additional sampling sites were selected:

- One sampling site (R6) set at a location in front of the river intake for drinking water production for Fray Bentos city, to allow a closer evaluation of possible human health risks.

- One sampling site (R9), located in the river at the dividing canal between Argentina and Uruguay (marked as Buoy No 90) was selected to estimate if there was an impact of Gualeguaychú city and industries set at the Argentinian ide with possible influence of the drainage of Gualeguaychú River.

- Samples were also extracted from Yaguareté Bay (R4), where the Yaguareté stream drains (C1) into the Uruguay River sampling was also carried out to evaluate the influence of both the pulp mill and agricultural non-point-sources, for being 1 km downstream from the plant, and near soy fields as the stream passes through several plots with such plantations.

- The nearest downstream site from the pulp mill is Ubici Beach (R5); thus, it was included as it has touristic activities and sport fishing. Anglo Beach (R7) served to evaluate the contamination from the sewage collection pipe into the river.

- The creek upstream this beach, Fray Bentos stream (C2) was analysed to evaluate the influence of municipal wastewater of city discharges of urban and suburban origins as well as the agrochemicals inputs from fields.

- The soil samples (L1-L3) were taken at buffer zone A, and L4 and L5 at buffer zone B.

4.2.6 Sampling methodology

Sampling was performed using UKAS accredited methods (Appendix B). Briefly, surface water was sampled according to the ISO 5667-6:2005 guide for sampling for physical and chemical assessment, consisting on the extraction of discrete samples by introducing the sampling bottle directly at sub-surface (about 25 cm below the water level) (Appendix B, Figure B-4)

Water was sampled from Las Cañas at the extreme section from the dock, while the others were collected by boat. Sampling, *in situ* measurements (of pH, conductivity and dissolved oxygen) and addition of preservative solutions and conditioning in cooling containers were done in each sampling campaign. The bottles were sent to the Fray Bentos laboratory or they were directly transferred to Montevideo to undergo further analyses. Preservatives added depend on the parameter under analysis. The type of containers and preservatives added are shown on Annex B. Pulp mill effluents were extracted from the final secondary treatment lagoon by means of an automatic refrigerated sampler that collected 24 hours composite samples. For the fish exposure test the samples were taken by sampling technicians with a pump that kept each fraction under refrigeration. The weekly composite samples were prepared by the Water and Chemicals Department at LATU Fray Bentos laboratory.

5 DESIGN OF THE INTEGRATED RISK ASSESSMENT FRAMEWORK AND PRELIMINARY RISK ASSESSMENT

An integrated risk assessment framework for human health and the environment risks toEDCs was developed for this research, considering multiple stressors and multiple sources: municipal wastewater, pulp mill effluents discharged into the receiving river, and agricultural non-point sources from soy fields and forestry. According to Menzi et al. (2007) an effect-based approach serves to study public health issues, by epidemiology data of conditions caused by multiple stressors. On the other hand, a stressor-based approach uses a prospective analysis for stressors influenced by environmental conditions, being then suitable for the environmental assessment component within an integrated framework. In this thesis, a combination of both effect- and stressor-based approaches was taken.

5.1 Structure of the two-phased framework

The two phased framework was composed of a preliminary risk assessment (PRA) and a refined stage, the quantitative risk assessment (QRA).

After the problem formulation stage, where the scenario is modelled explaining the activities that are more relevant in the geographical area under study, a conceptual model was created.

During Phase I, or the preliminary risk assessment (PRA) the first stage of the hazard identification was a decision model, to prioritise a list of EDCs of concern from a broader candidate list of possible compounds. The multimedia distribution of chemicals among environmental compartments was estimated with the multimedia fugacity model Level III, version 2.7, with inputs of the toxicity profiles of the EDCs. Then, an experimental screening of the main endocrine disruptive actions was completed in river water to conclude that selected oestrogenic and androgenic compounds were present, prior to Phase II commencement.

In Phase II, the exposure assessment design consisted on two parallel three-tiered structure, composed of chemical analysis and *in vivo* and *in vitro* bioassays of increasing specificity. The first stage studied the general health of the river, the second one evaluated the chronic and reproductive toxicity in biota and chemical markers of contaminant transport, and the third one more specific endocrine disruption end-points in fish through field surveys and laboratory experiments, and developmental studies in early life-stages.

The influence of bioavailability was taken into account by evaluating variations in ecotoxicity and sediment grain size at upstream and downstream sites from the pulp mill and municipal discharges. Food web processes were predicted by fugacity models, as well as experimetally, both under field and laboratory conditions, in representative planktic, benthic and pelagic species.

After this, the dose-response relationships were derived either from the aquatic exposure results based on measured concentrations in fish tissue and in drinking water, or estimated with databases, including the ingestion, dermal and inhalation routes, for all life stages ranging from babies to adult. The most critically exposed individual (pregnant and lactating women, babies and infants) of fishermen families were given special consideration.

The critical media and most sensitive ecological receptors were identified, to further develop a risk rating of target EDCs with software tools. Risk quotients were calculated to aquatic wildlife and/or to human health at relevant sites.

Species sensitivity distributions were modelled for typical and worse case scenarios to understand the severity of potential harm for environmental receptors at different taxa, employing the experimental exposure assessment results or literature data for the target compounds.

A risk estimation matrix was developed to evaluate the joint risks of the multiple stressors, both for human and environmental receptors. Finally, a quantitative risk characterisation of the overall risk was completed using radar diagrams,

and with this information, a set of recommendations for risk management was elaborated.

5.2 Planning and scoping of the risk assessment

The modelling tool for scoping was a decision tree, as recommended by Kroes et al. 2004 (Figure 5-1).

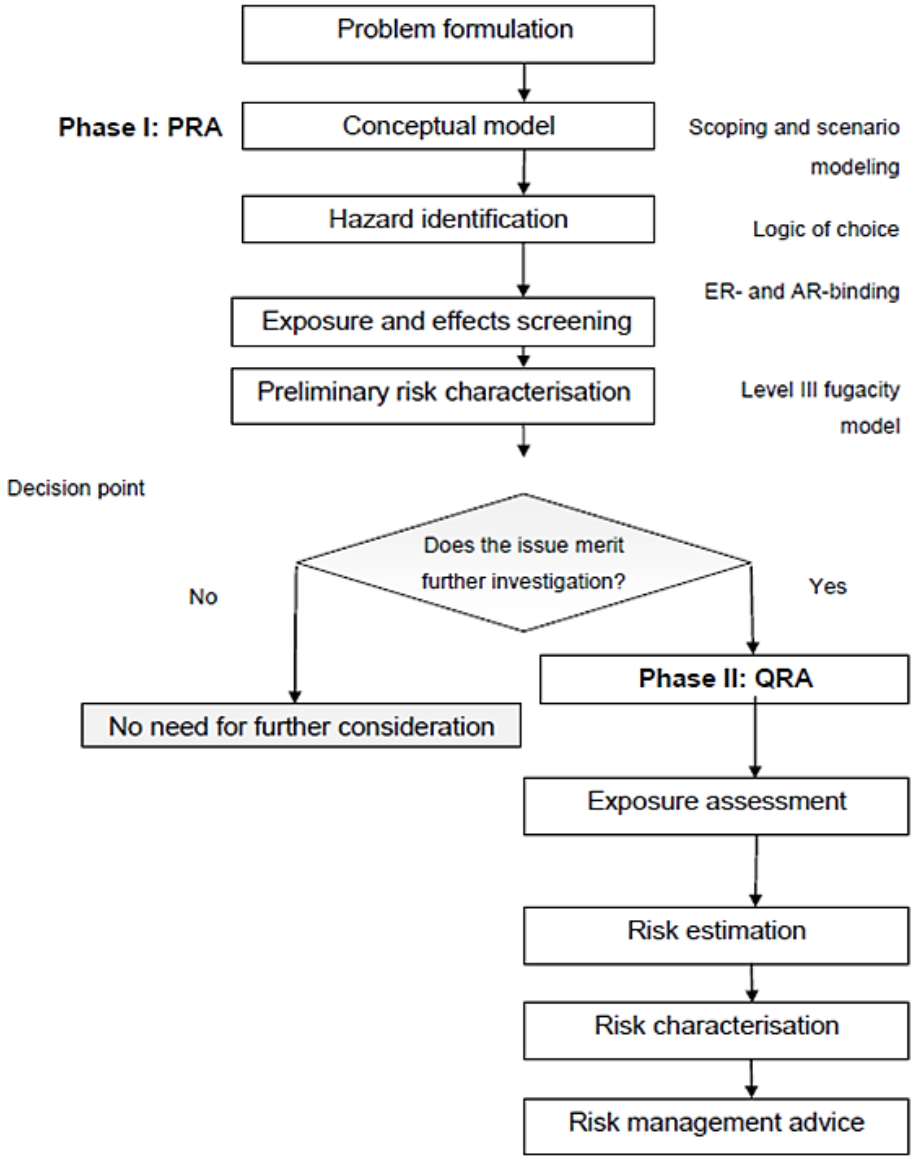


Figure 5-1 Schematic decision tree of the two phased integrated risk assessment framework

5.3 Phase I: Preliminary risk assessment (PRA)

The first phase of the risk assessment was a preliminary risk assessment for suspected EDCs in the river reach of the Lower Uruguay River. The paradigm is based partially on Kroes et al. (2004) in its structure, but using several approaches to estimate the dose according to the substance and mode of action and developing a strategy for risk prioritisation supported upon mechanistic principles. This aims at devoting analytical efforts proportionate to the risks, identifying key hazards and sensitive areas, before proceeding to a refined quantitative risk assessment. An estimation of the likelihood that endocrine disruption (ED) could be manifesting in receptors forms part of the assessment, to identify the need for other sampling sites sets the basis for the refined assessment design. The need for iteration was kept at a minimum to be more efficient, and reduce costs and time required to develop conclusions that will be useful for risk management.

5.3.1 Problem formulation

The context, scope and objectives of the assessment, the conceptual model for the system and an analysis plan were delineated as a basis of the problem formulation of the PRA, based on the concept of the integrated risk assessment (IRA) paradigm (Vermeire et al. 2007) (Figure 5-2).

First, the geographical scope of the research was established, a conceptual model was developed and then the environmental and human components were analysed following a plan to intend achieving the maximum possible integration.

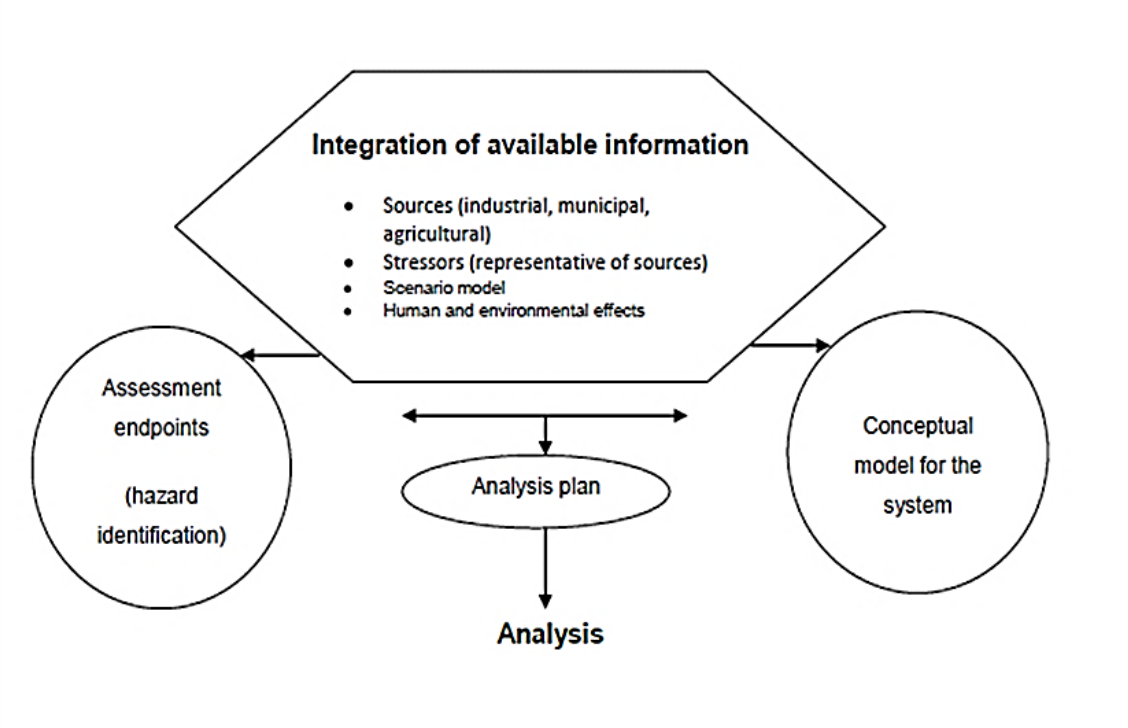


Figure 5-2 Problem formulation during preliminary risk assessment

5.3.2 Scope of the assessment

The scope focuses mainly on the aquatic environment and on human health due to the EDCs released by anthropogenic sources at a reach and watershed of the Uruguay River. The geographic area under investigation was defined for the boundaries and scope, establishing the framework of the study was at a section of the Uruguay River where multiple sources of EDCs can be evaluated (Figure 5-3).

The areas where eucalyptus forests are present on the Argentinian side were located on the map based on information from Brizuela et al. (2004); while on the Uruguayan side, they were based on maps by the Ministry of Agriculture.

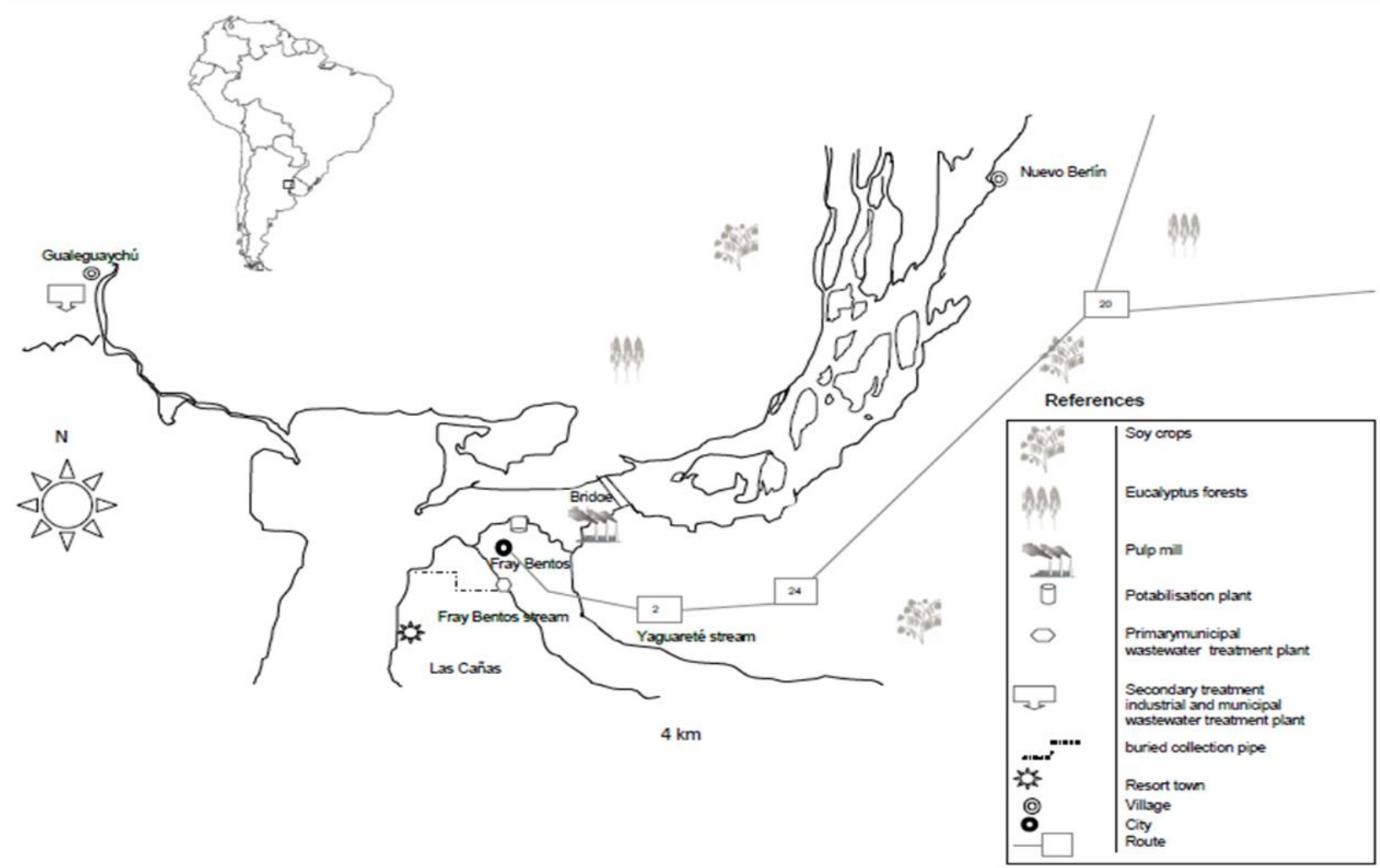


Figure 5-3 Geographical scope of the research and location of main sources

5.3.2.1 Ecosystem potentially at risk

At the section of the river selected, municipal wastewaters are discharged after little or no treatment, non-point source pollution from agriculture is present, a pulp mill is operating and there are industries that produce chlorate and other chemicals. The river serves as source water for drinking water production for Fray Bentos city and the intake is located 3 km downstream from the pulp mill discharge pipe. This could represent risks of contamination implying a potential risk to human health.

5.3.2.2 Preliminary assessment of the general river health

In Phase I, a characterisation of the general river health framed the scenario for further interpretation, and specific chemical analysis of EDCs. With a systemic concept of assessment, the initial evaluation of general risks to the river health includes human and environmental aspects. The goal of Phase I was to determine which hazards or risks required more in detail investigation and refined risk assessment, aiming at identifying possible key stressors or target compounds with ED activity. In this step of problem formulation, a description of the ecosystem potentially at risk and the relationship between assessment endpoints and measures of effect were presented.

Since 2005, monthly baseline studies were completed for physicochemical parameters, and are analysed continuosly after the pulp mill start-up, also including biological studies for plankton, benthic species, fish communities and fish tissue residue analysis. Most of the analysed physicochemical parameters complied with regulatory limits (Table A-1), with the exception of phosphorus that reached to concentrations in the order of 100 to up to 637 µg l^{-1}, well above the regulatory limit of 25 µg l^{-1}. Due to excessive nutrient concentrations, there were some algal blooms events during the summer. In January 2006, the river temperature at some locations rose to 32ºC, showing algal blooming. These algae insulated the water underneath and provoked an increase in pH levels to 9.2 by uptake of inorganic carbon. This high pH caused a dramatic increase in

the release of phosphorus from sediments or other sources, consequently increasing the algal growth, which in turn consumed the dissolved phosphorus (see graphs of physicochemical parameters during baseline studies and a photograph of algal blooming during January 2006 in Appendix A).

Metals median values were: Na 3.3 mg l^{-1}, K 1.5 mg l^{-1}, Fe 1.0 mg l^{-1} and below the detection and/or regulatory limits for the rest. The toxic ions cyanide and sulphide were found to be under the detection limit during the time lapse considered. In terms of organics, chlorinated pesticides were not detectable, and anionic surfactants showed only one data above detection limit, at the bridge location.

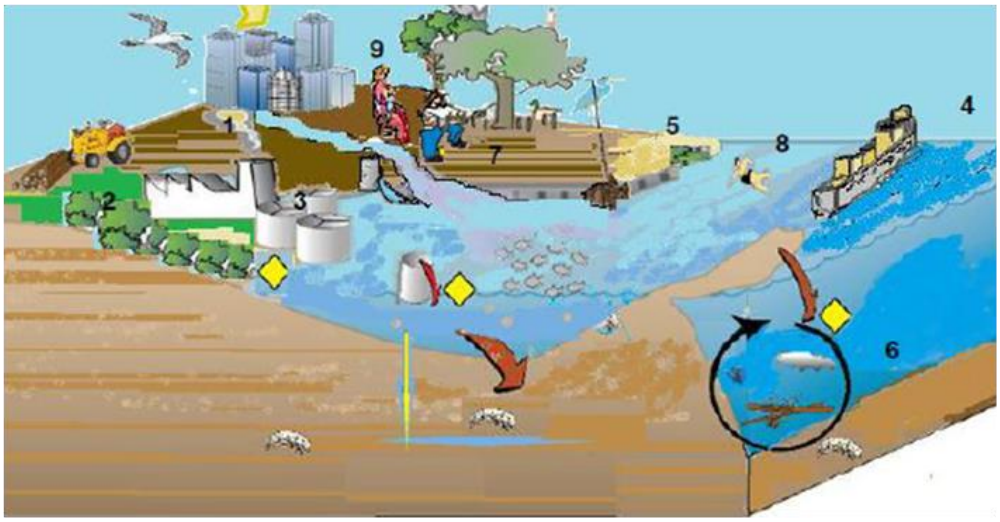
The unregulated parameter adsorbable organic halogens (AOX) was included as an indicator of chlorinated EDCs in water (PCBs, brominated and chlorinated pesticides and herbicides, chlorinated aromatic compounds and partly chlorinated humic substances, among others). The median concentration in the river was 8 µg l^{-1} expressed as Cl, or below quantitation (5 µg l^{-1}). However, a peak appeared in once at the end of 2005. Dioxins and furans were not detected, or they were in very low concentrations (1 pg l^{-1} median concentration, and maximum reported in March 2007, at the bridge (R2)) (Table A-1, Appendix A).The algal toxin microcystin-LR was above the 1 µg l^{-1} limit during the summer seasons, consistent with local eutrophic conditions.

Table 5-1 Organic contaminants in river water during baseline studies

	AOX (µg l^{-1})	**Chlorophenols (µg l^{-1})**	**Resin acids (µg l^{-1})**	**Phytosterols (µg l^{-1})**	**Dioxins and furans WHO-TEQ (pg l^{-1})**
Nuevo Berlín (R1)	8-11	0.089-0.104	27-224	ND-22	0.00-0.60
Fray Bentos (R3)	6-12	0.080-0.114	6-183	ND-2	0.00-0.89
Las Cañas (R8)	< 5-12	0.089-0.185	3-202	ND-8	0.00-0.96

5.3.2.3 Characterisation of the system

The boundaries of the system were establish to construct the conceptual model, identifying natural and anthropogenic stressors, sources, pathways, routes and the relationship among stressors, ecological factors and responses described. The sources and pathways were studied in more detail in Section 5.3.3. The main activities and uses of the river and the land in the watershed, possible sources and receptors in the area under study were identified as depicted in Figure 5-4. Fishing concentrates at Las Cañas, Nuevo Berlín and Ubici Beach, and swimming is mainly at Las Cañas during the summer season.



References: ◆ EDC

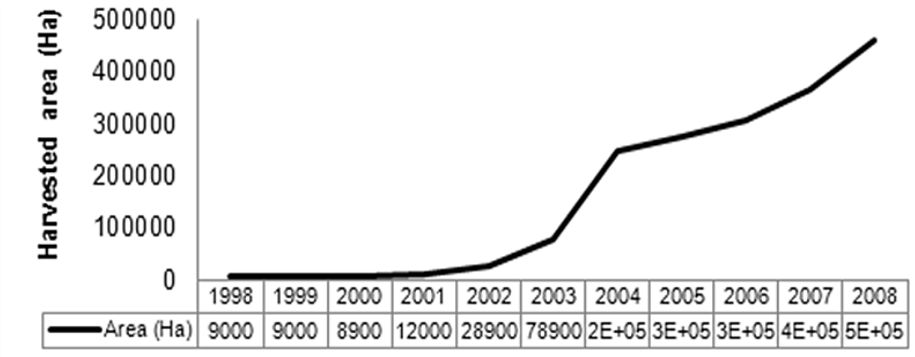
1. Municipal wastewater of Fray Bentos city pours into Fray Bentos stream that drains into the Uruguay River
2. Agricultural activities, forestry and soy crops, near streams leaching into the river
3. A pulp mill discharges effluent after secondary treatment
4. Barges carrying cellulose are the main transport by water
5. Wetland areas under protection
6. River with highly biodiverse ecosystem, with aquatic and sediment species, interrelated within the food web
7. Fishing activities
8. Swimming activities
9. Nursing mother, fishermen families and general population eating fish

Figure 5-4 Conceptual model for the system

For the last twenty years, studies conducted in North America, Scandinavia, and New Zealand have shown that pulp and paper mill effluents affect fish reproduction (Hewitt et al. 2008). This is why a high-tonnage production pulp mill that uses an ECF bleaching process located on the Uruguay River coast near Fray Bentos was included within the scope of this research.

Municipal wastewater, another probable source of environmental EDCs, such as oestrogens and nonylphenol and its ethoxylates, is discharged with only primary treatment from the city of Fray Bentos, Río Negro Province, Uruguay. On the Argentinian side, the secondary treatment plant collects wastewater and industrial discharges of Gualeguaychú city into a stream (Arroyo del Cura) an affluent of the Gualeguaychú River, may be another possible source of those compounds.

The land cover has been modified by human activities with soy plantations and eucalyptus forests Figure 5-5. In Uruguay, the land use has changed in the last years, becoming progressively more based on monocultures, mainly soybeans, and forestry, displacing natural woods. The most prevalent crops in Río Negro province is soy, with more than 5000 hectares concentrating in this area (Ministry of Agriculture 2004).



Graph designed using database FAOSTAT, © FAO Statistics Division 2010, Retrieved: 19/8/2010 http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor

Figure 5-5 Evolution of the territorial area harvested with soybeans in Uruguay (1998-2008)

The most used herbicide in these fields is glyphosate, a potential endocrine disruptor. Glyphosate (N- (phosphonomethyl)glycine) is a broad spectrum, non selective, post-emergent herbicide, widely used in agriculture (Araujo et al. 2003). Trangenic soy crops have been genetically engineered to be resistant to the herbicide ("Roundup Ready"), and this variety is practically 100% of the soy crops in Uruguay. Glyphosate is applied after the plant has emerged, but there could be an application before planting the seed and after this event (Bozzode Brum 2010). Glyphosate has the ability to adsorb to clay, leach to soil and percolates to groundwater (Vereecken 2005), and biodegradates to aminomethylphosphonic acid (AMPA) (Singh & Walker 2006). The toxicity profile for this compound is presented in Appendix C with data on physicochemical characteristics, biodegradability, fate and ecotoxicity, as well as a conceptual model showing the routes and pathways of contamination, which are different to the persistent EDCs.

5.3.3 Conceptual model

This human and environmental water risk assessment of reproductive endocrine disruption in a reach and watershed of the Uruguay River is designed considering multiple stressors and multiple sources of contamination were studied, identifying the pathways, receptors and effects. The most vulnerable population are new-born and lactating infants. The food chain is the main source of exposure, and in particular, fish consumption and drinking water are possible sources for the nursing mother and the pathway of distribution through the milk to the baby, but the direct intake of drinking water is important in the case of formula preparation. The environmental receptors are fish, crustacean and sediment dwelling organisms (Figure 5-6).

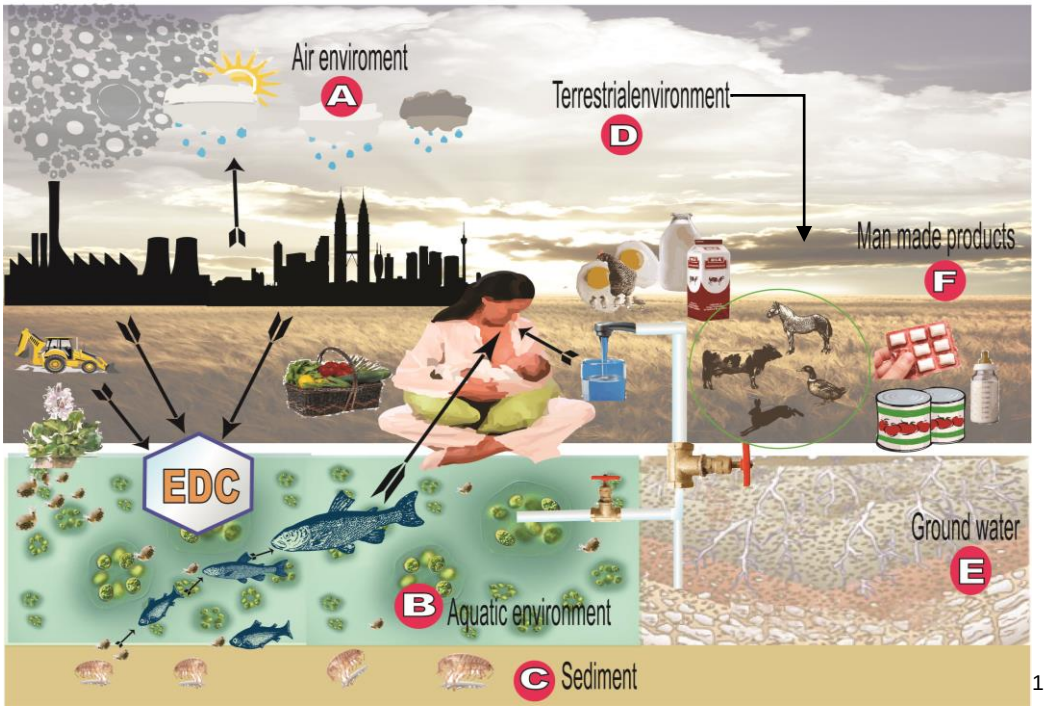


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Figure 5-6 Conceptual model describing the media and routes of exposure of human and environmental receptors to EDCs

Sediment-water interactions and the bioavailability of the toxicants adsorbed onto the particle of the sediment depend on its type and particle size, so they were included in the conceptual model.

The relationship of sources, routes, pathways and receptors is summarised in Tables 5-2 to 5-4. A more detailed study of the possible sources, stressors and pathways was done for the agricultural, industrial and domestic sources (D.1), with the aid of the software SCEM, USEPA.

1. Design concept by Diana Míguez, with Aldo Montoro´s help in graphics software

Table 5-2 Relationships between stressors, media, pathways, routes and receptors for agricultural sources

<table>
<tr><th>Source identification</th><th>Source mechanism</th><th>Stressors</th><th>Media</th><th>Routes</th><th colspan="2">Pathways</th><th>Receptors</th></tr>
<tr><td rowspan="2">Soy fields</td><td rowspan="2">Non-point/soil erosion</td><td rowspan="2">Glyphosate, endosulfan, other herbicides and pesticides</td><td rowspan="5">Air

Water

Soil and sediment

Biota</td><td>Air (spray application, wind and runoff)</td><td colspan="2">Inhalation (gaseous exchange)</td><td>Air and terrestrial animals and plants
Humans (agriculture workers, residents and tourists)</td></tr>
<tr><td>Surface Water</td><td rowspan="2">Ingestion of drinking water</td><td rowspan="3">Dermal (includes absorption by swimming or bathing, and uptake by plants)</td><td>Terrestrial animals, humans</td></tr>
<tr><td rowspan="3">Wetlands and woods</td><td rowspan="3">Natural and eventually intentional forest fires, intentional wetland fires</td><td rowspan="3">Dioxins and furans, PAHs</td><td>Groundwater</td><td rowspan="2">Aquatic animals (fish, invertebrates)</td></tr>
<tr><td>Soil</td><td>Non-dietary ingestion (dust)</td></tr>
<tr><td>Sediment</td><td colspan="2">Indirectly, through ingestion of fish and invertebrates contaminated by ingesting sediment</td><td rowspan="2">Humans (agriculture workers, residents and tourists)
Wildlife and pets</td></tr>
<tr><td>Food</td><td colspan="2">Ingestion of contaminated foodstuff (mainly fish, but also, breast milk, milk, eggs, poultry, beef, vegetables)</td></tr>
</table>

Table 5-3 Relationships between stressors, media, pathways, routes and receptors for municipal sources

Source mechanism	Stressors	Media	Routes	Pathways	Receptors
Source identification: cities, villages and towns(Gualeguaychú, Fray Bentos, Nuevo Berlín, Las Cañas)					
Non point/ solid waste burning, automobile exhausts	Dioxins and furans, PAHs	Air	Transport and dispersion Dissolution in water	Inhalation Ingestion	Air and terrestrial animals and plants; humans (agriculture workers, residents and tourists)
Solid waste	Plasticisers	Soil	Infiltration to groundwater Runoff to water and sediment	Ingestion, direct contact, inhalation of dust	
		Water	Swimming, bathing, direct ingestion, drinking water	Direct contact, ingestion, dermal	Depending on persistency of he EDC and the position of the organism in the food web
		Sediment	Sediment	Indirectly, through ingestion of fish and invertebrates contaminated by ingesting sediment	
Untreated or primary treated wastewater and sludge	Chlorinated organics, phenols, phytosterols	Biota	Food ingestion (mainly fish, but also, breast milk, milk, eggs, poultry, beef, vegetables) Aquatic animals and plants	Ingestion of foodstuff Aquatic food web processes	

Table 5-3- ***Continued***

Source mechanism	Stressors	Media	Routes	Pathways	Receptors
Source identification: cities, villages and towns(Gualeguaychú, Fray Bentos, Nuevo Berlín, Las Cañas)					
Non-food **consumer products (contraceptive pills, other pharmaceuticals and cosmetics)**	Steroidal hormones, spermicides (nonylphenol ethoxylate) Bisphenol A, Octylphenol, other plasticisers	Ingestion or injection of pharmaceuticals Dermal application(cosmetics, spermicides and certain drugs) Packaging and cooking materials transference		Dermal absorption; mucous adsorption, oral absorption; parenteral absorption	Humans (residents and tourists) Pets

Table 5-4 Relationships between stressors, media, pathways, routes and receptors for the main industrial source

Source identification	Source mechanism	Stressors (EDCs)	Media	Routes	Pathways	Receptors
Pulp mill **(Kraft process, chlorine dioxide bleach)**	Point/ effluent discharge	Chlorinated organics Phenols, phytosterols Resin acids, dioxins and furans	Air	Dispersion of combustion gases and fumes	Inhalation	Air and terrestrial animals and plants
	Sludge burial		Water	Food, water and sediments	Direct contact or ingestion	Terrestrial animals, humans, pets Aquatic animals (fish, invertebrates)
	Biomass combustion for energy production		Soil and sediment	Groundwater contamination	Ingestion of:dust,particles of sediment, drinking water	
		Dioxins and furans, PAHs, PCBs	Biota	Soil	Ingestion of contaminated soil or edible animals and plants	

5.3.4 Sources

Three main types of sources identified were: municipal, agricultural, and industrial.

5.3.4.1 Domestic sources

The residential sources are mainly domestic wastewater streams, and from hospital, restaurants, and small industrial discharges (milk farms, fruit preserves). As reviewed by Shareef et al. (2008) black water contains human excretions with steroid hormones. 17 α-ethynyl oestradiol (EE2) is of concern because it is a stable synthetic hormone which is commonly found in the formulation of oral contraceptives. The less stable natural hormone 17β-oestradiol (E2) and its metabolites oestrone (E1) and oestriol (E3) are also relevant as E2 is the principal endogenous steroid oestrogen in vertebrates, stimulating the growth and development of the female sex organs. Other natural steroid hormones are progesterone and testosterone.

The EDCs categories include pharmaceuticals (contraceptive pills), domestic and industrial detergent and biodegradation by-products (alkylphenols: 4-nonylphenol; OP and NP, and their ethoxylates; also used as spermicides in condoms), antimicrobial agents (e.g. triclosan, TCS), personal care products (musk fragrances, sunscreens), brominated flame retardants (PBDEs), persistent organic pollutants (POPs) (dioxins and furans, some pesticides, PAHs, PCBs), plasticisers (bisphenol A, octylphenols, phthalates).

Municipal wastewater in Fray Bentos is subject only to primary treatment to remove solids. The collection is underground and it ends at Anglo Beach (R7). The overflowing pours into Fray Bentos stream (C2), a watercourse that drains into the river after running through suburban dwellings and some soy fields and prairies.

Composite samples of crude municipal wastewater were collected on-site over 24 h, at the municipal wastewater primary treatment plant of Fray Bentos from the final discharge, by means of a pump that refrigerates extracted portions (brand Avalanche). The untreated sewage wastewater was toxic to crustacean and to luminescent bacteria, as well as to fish. Its oestrogenicity was within the typical expected values of 15 to 94 ng l^{-1} EEQ (Jugan et al. 2009) (Table 5-5).

Table 5-5 Crude final municipal wastewater characterisation

Parameter	**Concentration** (mg $l^{-1)}$
Sulphide (as S) (LOD: 0.1 mg l^{-1})	6.35
Ammonia (as N)	92.5
Oil and grease (LOD: 5 mg l^{-1})	14.5
AOX (as Cl) (LOD: 10 µg l^{-1})	38 µg l^{-1}- 1.73 mg l^{-1}
Phenols (as C_5H_6OH)	3.0- 205
Anionic surfactants (as LAS)	3.88
$cBOD_5$ (as O2)	285
COD (as O2)	658
Total Nitrogen	109
Total Phosphorus (as P)	35.2
Acute toxicity (*Pimephales promelas*) LC50, 96 h	35.4 % (Very toxic)
Acute toxicity (*Daphnia magna*) LC50,48 h)	5.66 % (Very toxic)
Acute toxicity (*Vibrio fischeri*)	15.8 % (Very toxic)
Oestrogen receptor-binding assay with ERCALUX® (as E2 equivalents)	34 ng l^{-1}
Androgen receptor-binding assay with ARCALUX (as dihydroxy testosterone equivalent)	73 ng l^{-1}

Levels of toxicity: according to Viana et al. (2001)

5.3.4.2 Agricultural non-point sources

Agricultural non-point sources comprise soy plantations and eucalyptus forest in the area that drain into affluent streams of the river.

5.3.4.3 Industrial sources

The main industrial source is the Fray Bentos pulp mill. The primary production processes of pulp mill and the effluent treatment plant were studied, as well as the potential hazards in normal and contingency operations. As shown on Table 2-1, the most prevalent substances coming from the pulping (rosin acids) and bleaching processes (chlorophenols) or rainwater deposition coming from incineration for biomass and black liquor burning as an energy source (dioxins and furans, polyaromatic hydrocarbons (PAHs). Most of the congeners of dioxins and furans, polychlorinated biphenyls (PCBs) and chlorophenols are also considered, although the bleaching process is intended to diminish the levels of chlorinated organic compounds.

Company reports that operations started in November 2007 with a mill capacity of 1 million tons per year of fully bleached eucalyptus pulp by the kraft process without elementary chlorine (ECF). The first stage is wood chipping, then cooking, followed by oxygen delignification and bleaching by chlorine dioxide (ECF). The pulp mill typically discharges 20- 25 m^3/air dried tones of cellulose (ADT) bleaching filtrates to be treated in the mill effluent treatment system. During the first year of operation, monitoring of the environmental impact for effluents according to the environmental permit for the Fray Bentos mill were analysed for compliance of the company to the environmental authorities by the Water and Chemicals Department, LATU. The results are presented on Table 5-6:

Table 5-6 Pulp mill effluent characterisation

pH	Conductivity (mS/m)	COD (as O_2) (mg l^{-1})	Colour (as Pt) (mg l^{-1})	SST (mg l^{-1})	BOD5 (as O_2) (mg l^{-1})
7.68	339	357	953	11.4	16.2
Ammonia (as N) (mg l^{-1})	**Nitrite (as N) (mg l^{-1})**	**Nitrate (as N) (mg l^{-1})**	**AOX (µg l^{-1})**	**Phenols (µg l^{-1})**	**Chlorate (µg l^{-1})**
0.04	0.10	0.13	727	4.9	82.3

Mean values

Saarela et al. (2008) described the eucalyptus pulp bleaching process. Briefly, the cooking process generates hexenuronic acids (HexAs) which should be removed for quality of the product by combined acid treatment (A-stage), hot chlorine dioxide or ozone stage. The designed total chlorine dioxide consumption in the bleaching process of the Fray Bentos mill is 20 kg active Cl/ADT.The weak black liquor from the fiberline is taken to the evaporation plant of nominal evaporation capacity 1100 tons water h^{-1}. Black liquor is concentrated to 80 % solids content to be fired in the recovery boiler. Pulp and paper mill effluent is treated to remove particulate, biochemical oxygen demand (BOD) and chemical oxygen demand (COD) produced in the manufacturing processes, discharging a large volume of water from the system. The design value for the effluent flow from the mill is 73 000 m^3d^{-1}.

The main part from the effluent comes from bleaching but there are also smaller effluents streams such as woodhandling, pulp drying, evaporation (secondary condensates) and causticizing in addition to the water taken from the cooling water circulation. The main equipment and stages comprise a coarse screening to remove bigger solid particles followed by a pre-treatment or primary clarification where the fiber is recovered in the primary clarifier to remove the solids (primarily fibres) from the high solids content stream. A pH adjustment and nutrient addition stage is done and the biological treatment with activated sludge takes place in two aeration basins (two units in parallel). There are two

units in parallel of 65 metres diameter each of secondary clarifiers, and an excess sludge disposal system. The treated effluent outfall to Uruguay River is through a set of diffuser pipes.

There are four measuring point for compliance routine monitoring, for river water quality, both upstream and downstream from the mill. Two different sampling stations, independent from these, were selected in this research to account for the water quality at the drinking water intake and to reflect the contamination from the Argentinian side of the river, at the river boundary in front of Gualeguaychú River.

The ambient air quality is routinely monitored at the measuring point in Fray Bentos city at a station operated by LATU and DINAMA. The maximum admissible for nitrous oxides, NO_x (NO_2) is 320 μg m^{-3} hourly average, SO_2 daily average of 125 μg m^{-3} (95% of time) and 365 μg m^{-3} (no more than once a year), and for PM_{10}, a daily average 150 μg m^{-3}. The standard for total reduced sulfur for 15 minutes average values of 3 μg m^{-3} (2% of the time, over a yearly base). This limit was exceeded in April 2008, but other possible sources due to smoke from widespread fires at Río Paraná of Argentina was extended over Fray Bentos vicinity.

The treated final pulp mill effluent is discharged to the Uruguay River through a submerged, multi-port diffuser at an average discharge rate of 0.80 m^3 s^{-1}, with an allowable monthly maximum load for BOD_5, chemical oxygen demand (COD), TSS, total phosphorus, total nitrogen and AOX acceptable. However, some fluctuations occurred, as BOD_5 exceeded the maximum concentration limits on four days in June 2009 and fecal coliform exceeded the on those dates and two days in December 2009. The long term average load exceeded the expected value for color. In 2009, the average AOX of the pulp mill effluent was 1.80 mg l^{-1} (0.72- 3.92 mg l^{-1}). The permit limit for this pulp mill is 6 mg l^{-1}. The most toxic dioxin and furan congeners 2,3,7,8-TCDD and 2,3,7,8-TCDF were below the 1 pg l^{-1} (as I-TEQ) level. The levels of fecal coliform in the effluent

exceeded the permit limit of 5000 UFC 100 ml^{-1} on two occasions in June 2009 and on two occasions in December 2009.

5.3.5 Routes of exposure

The routes of exposure for humans are drinking water and food ingestion, but fish is the main foodstuff taken into account in this study. The effects to human health may occur through the exposure to multiple contaminants, but the pathways are water and food, through the routes of drinking water and fish ingestion. The ingestion of pharmaceuticals may add uncertainty to the study, as their doses may be higher than the environmental ones.

5.3.5.1 Routes of human exposure

The main routes of human exposure to environmental chemicals are dust and vapor inhalation, dermal contact with contaminated soils or dusts, and ingestion of contaminated food, water, dust, or soil (Paustenbach 2000). In this thesis, for the route ingestion, drinking water, fish, and breastmilk, in the pertinent case, were considered, but dermal contact and inhalation of soil were taken into account. There is an indirect link between sediment- associated contaminants and human health, because of the role of sediment as a component within the food chain, and directly, through the inhalation of airborne particulates (Owens & Xu 2011). Pharmaceuticals, such as contraceptive pills and other hormones and compounds with endocrine disruptive effects were not evaluated, but their doses may be higher than the environmental ones (Appendix G for uncertainty assessment).

5.3.5.2 Agricultural runoff

Non-point sources such as those arising from agricultural activities may add endocrine disruptors by leaching from the soil. Microbial degradation is very important in soil, where microorganism consortia biodegrade organic compounds. In the case of the EDCs included in this research, the most important ones are agrochemicals used in crops in the area. The most prevalent

is glyphosate, used as herbicide in soy fields. Microbial degradation is relevant in soil, where microorganism consortia biodegrade organic compounds. In this research, among the agrochemicals used in crops in the area is glyphosate, used as herbicide in soy fields. It biodegradates in soil to aminomethylphosphonic acid (AMPA) and carbon dioxide but the phosphorus of soil competes for adsorption to clay (Prata et al. 2003). The commercial formulation of the herbicide glyphosate is toxic to human placental cells at concentrations lower than the usual in agricultural practices. Its aromatase activity disruption could be due not only to glyphosate but also to co-adjuvants (the surfactant nonylphenol or others), which enhance its bioavailability and/or bioaccumulation (Richard et al. 2005, Gasnier et al. 2009) (See C.1).

5.3.5.3 Groundwater

Groundwater in the research area is not the main drinking water supply, but it might be used as secondary source and for crop irrigation. The location of wells is shown in Figure B-8. However, due to the type of aquifer in the area that is not permeable to land and surface water contamination, the potential risk to artificial EDCs is considered low.

Geological contaminants, like metals, cannot be ruled out completely (Appendix G-1). In general, metals are below detection limits in most wells, but the concentrations of arsenic before potabilisation varies, and at some locations they could be slightly exceed the regulatory limit for drinking water of 0.02 mg l^{-1}, with a median concentration of 0.03 mg l^{-1} (UNIT 833:2008 drinking water standard). Chronic ingestion of arsenic has been linked to increased risk of cancer and reproductive effects. Its mechanism of endocrine disruption is an alterion of the steroid signaling at the level of receptor-mediated gene regulation for all five steroid receptors but it is postulated that it is also mediated by the retinoid system and thyroid hormone (Davey et al. 2008).

5.3.6 Pathways

The sediment-water interactions and the bioavailability of the toxicants adsorbed onto the particle of the sediment depend on its type and particle size, so they are included in the conceptual model. Sediment-water interactions and the bioavailability of the toxicants adsorbed onto the particle of the sediment depend on its type and particle size, so they were included in the conceptual model.

The environmental compartments are shown in Figure 5-8. In the terrestrial compartment, the study focused on human receptors, although the endocrine disruption end-points were extrapolated from databases with other species. To evaluate the aquatic compartment, the experimental work pointed at several target species: epibenthic invertebrate (*Hyalella curvispina*), planktonic invertebrate (*Daphnia magna*), piscivorous fish (*Astyanax fasciatus*) but that during juvenile stages is omnivorous, with a diet composed of (insects, seeds and fruits and leafy vegetal material, but also, grass, filamentous algae, sediments, mollusks, fish and nematodes), and omnivorous fish (*Pimephales promelas*).

Phytoplankton impacts were considered based on biodiversity and algal blooming in the area. A macrophyte (*Eichornia sp.*) that feeds macroinvertebrates (Poi de Neiff 2003) was studied for bioaccumulation.

Transport processes can be categorised into horizontal and vertical components, including volatilitsation, hydrolysis, photolysis, oxidation, sorption, desorption, and biodegradation. Development of experimental demonstrations includes determining concentrations in each of the compartments (Figure 5-7).

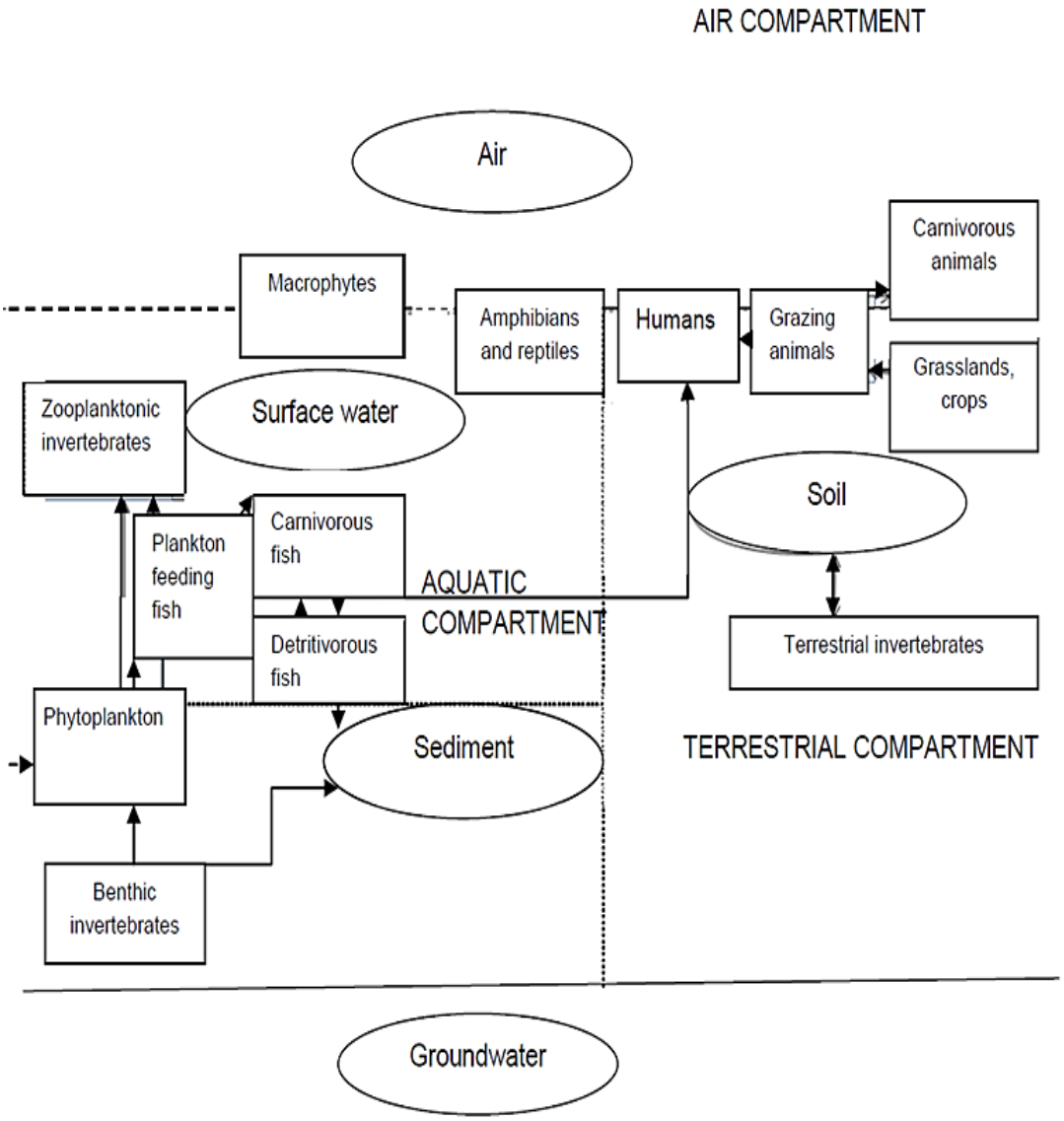


Figure 5-7 Environmental compartments and trophic chain interactions

Further, metabolic pathways until the production of an effect in the organism occur through a series of steps, namely the intake of the toxicant, its uptake into the cell, undergoing metabolism, where bioavailability is a key factor in the final effect, aside from the natural susceptibility of the receptor. This is represented in Figure 5-8.

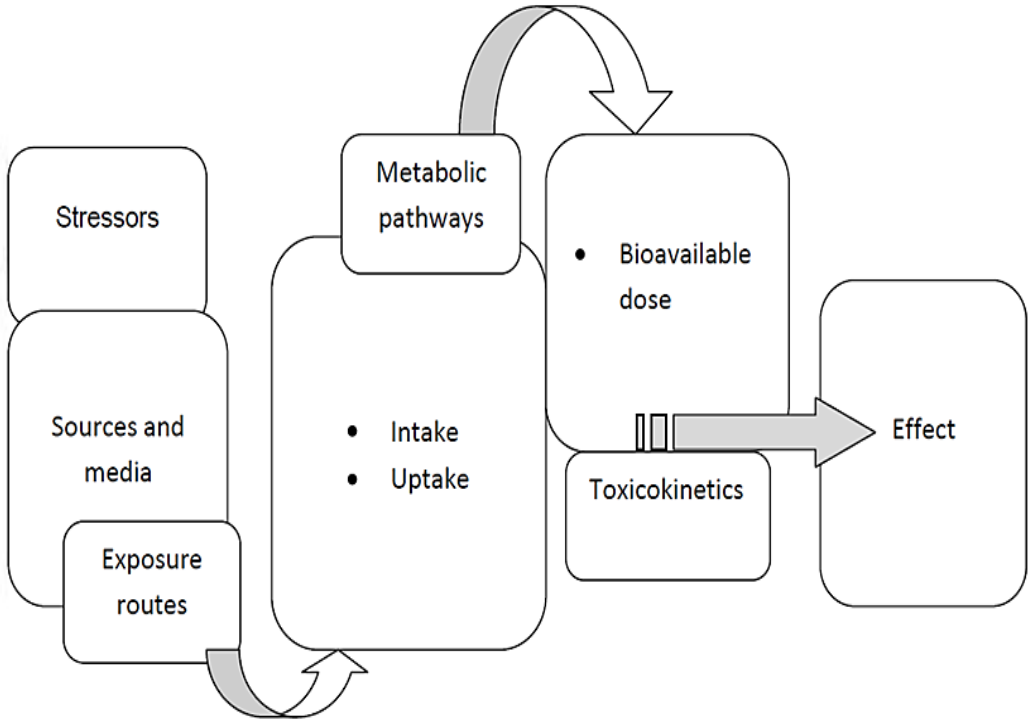
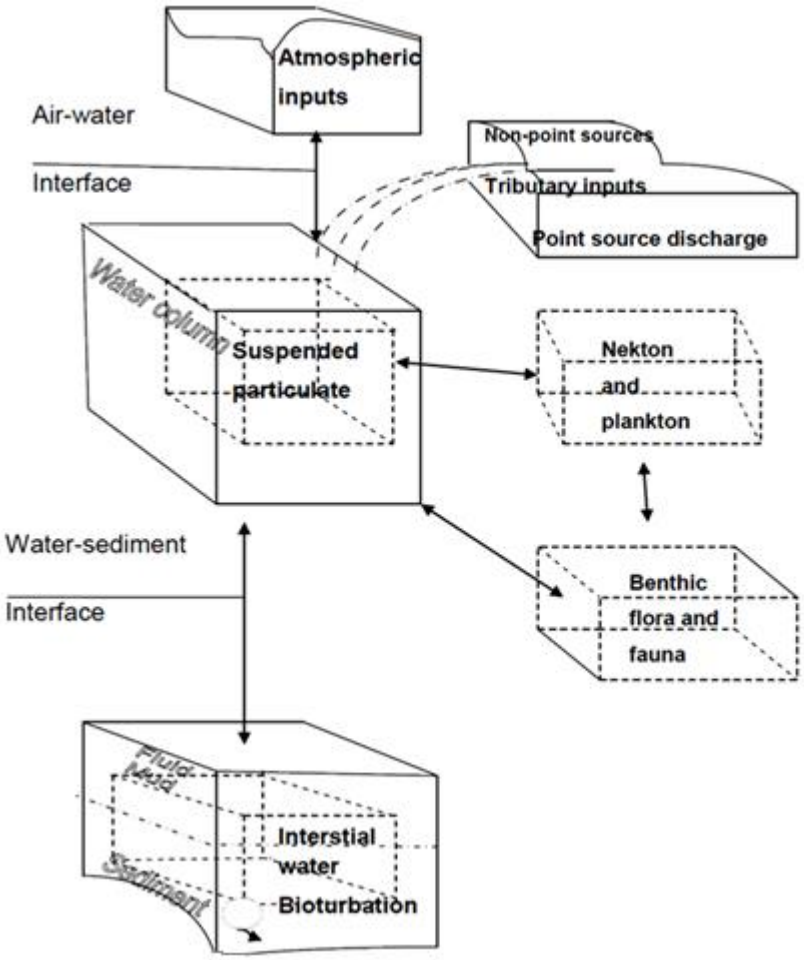


Figure 5-8 Metabolic pathways and exposure routes, from stressors to effects

5.3.6.1 Sediment

Benthic organisms live in contact with the sediment for all or parts of their life cycle. The partitioning to particulate matter suspended in water may settle and accumulate in sediments. The continuous fallout and re-deposition of atmospheric particulate matter to which lipophilic substances may be bound also increase exposure via this pathway. Processes among interfaces are represented in Figure 5-9.



Based on Bostater et al. (1978)

Figure 5-9 Processes of transport among interfaces and toxicological profile compartments

Exposure of aquatic biota to toxicants can be through several pathways: direct absorption by the organism or bioaccumulation and/or biomagnification through the food chain. The final environmental concentration will depend on the biodegradability extent (USEPA 2000b) and the existence of abiotic factors of degradation such as temperature and light. In the air compartment, the volatility of the compound, either alone or fixed to particulate matter influences on the amount of the EDC. Examples are the low molecular weight halogenated organics such as lindane, dioxins and furans and PAHs. Endocrine disruptors can dissolve, or be bound to sediment particles, they can transfer from sediment to the food chain in aquatic systems, bioconcentrate and biomagnify. Apart from POPs, alkyl ethoxylates and NPs have also been reported in sediments (Bennett et al.1998, Lye et al. 1999), and have oestrogenic (Chen et al. 2012b) and androgenic steroids (Ong et al. 2012). Human exposure via this route is low and restricted to consumption of bottom-feeding organisms.

The total dissolved solids concentration in the Uruguay River was an average of 75 mg l^{-1} (Table A-1). Erosion inputs to the river silicified limestone from the cliffs at the margins, or topsoil in agricultural areas.

Contact with the skin or inhalation were not regarded as primary routes although they may exist if the river is used for recreation. In the body, the absorption, distribution, metabolism and excretion differs according to each particular EDC.

5.3.6.2 Soil

As cited by WHO a number of potential EDCs (e.g., PCBs, dioxins, PBDEs) have been detected in soils and/or sewage sludge in different parts of the world. Farm animals may be exposed to contaminated soil through grazing and thus contribute to human exposure via this food chain pathway.

5.3.6.3 Dietary

Ingestion of EDCs and potential EDCs via food intake was generally considered as the major exposure route, both for humans and for most wildlife and may lead to bioaccumulation and biomagnification. The contribution of dietary exposure will vary as a function of dietary preferences, position in the food chain, and species and quantities consumed. Persistent, lipophilic organic pollutants bioaccumulate in species at the top levels of the food chain, and top predators, like fish-eating birds and marine mammals, bioaccumulate and biomagnify the persistent organic compounds (POPs). The metabolic pathways depend on the compound molecular structure and the individual variability.

Uruguay annual *per capita* beef consumption is estimated at 60 kg per year. Beef is primarily consumed in urban areas, while lamb is preferred in rural areas. Annual *per capita* chicken consumption is 18 kg per year. In July 2010, the Ministry of Livestock, Agriculture, and Fisheries of Uruguay (MGAP), regulated feedlot operations to comply with the proposed EU certification protocol for exports under the high-quality hormone free beef (not treated with hormones) (USDA 2010). Annual *per capita* lamb consumption is 6-7 kg per year, pork consumption is negligible, and fish is too expensive to compete with beef. However, at the area under study, fish is consumed mostly by the artisan fishermen families but also by the general population.

In the case of dioxins and furans, aside from accidental exposure, people are exposed primarily through high-fat food, such as dairy products, eggs, animal fats, and some fish (Kulkarni et al. 2008). Breastfeeding can be an important pathway. Information on levels concentrations of dioxins and furans in Uruguay is lacking, but as a reference, the mean PCDD/Fs level in human milk was 9.5 pgTEQ g^{-1} fat (based on data reviewed by Ulaszewska et al. 2011).

5.3.7 Receptors

Due to the ubiquitous characteristics of EDCs, any creature living can be a receptor. The focus in this research was on aquatic animals and human beings, but some consideration of aquatic plants was also made (Section 7.7.6.4).

5.3.7.1 Aquatic animals

The most likely sensitive species are invertebrate, fish, terrestrial animals, birds and human beings. For the scope of this research that focuses on aquatic toxicity, birds and terrestrial animals are not considered. The aquatic receptor organisms in the system of the Uruguay River have been identified through plankton, benthic and fish community studies during baseline monitoring (4.2.5), and through background information on the river ecosystem. According to partner researchers, fish populations and community structure was the same before the pulp mill operations at all three study areas. Muscle and bile analysis of different toxic pollutants show measurable concentrations but below internationally recommended levels.

5.3.7.2 Conceptual model of aquatic exposure routes

The environmental compartments potentially at risk in the area under study that were addressed during this investigation are described more succinctly in Figure 5-10, showing the most relevant pathways through which stressors may affect human and animal receptors.

This diagram served to plan the risk assessment of EDCs, considering a basic ecological approach, showing the most relevant pathways through which stressors may affect human and animal receptors, including the maternal-neonate cohort as the most vulnerable receptors.

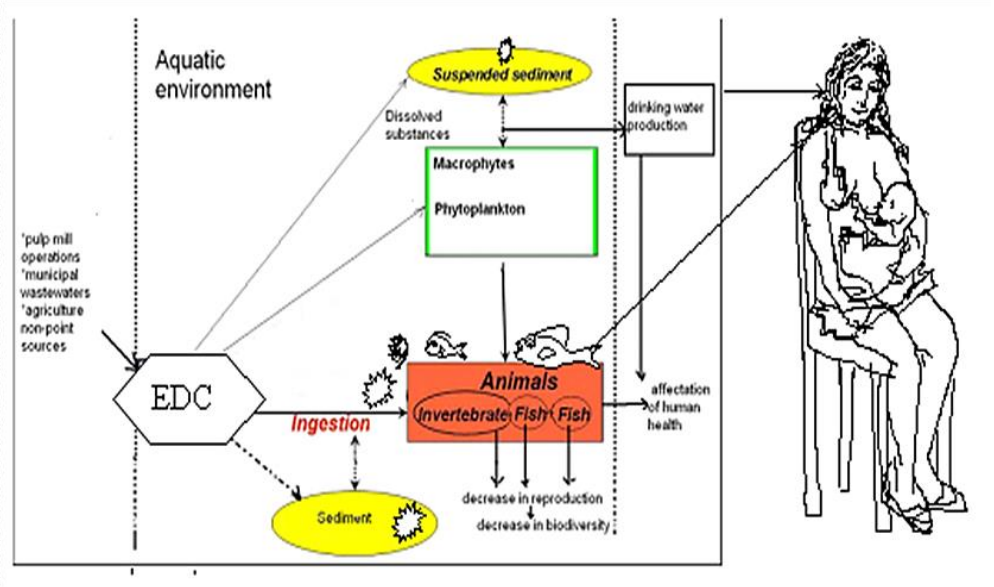


Figure 5-10 Conceptual model of aquatic exposure routes

5.3.7.3 Protection goals selected

The river is a transboundary resource and spawning area for maintenance of fish species. Due to the relative ease in characterisation and value, fish were the chosen protection goal as they can be quantified, are ecologically relevant, susceptible to the stressors, and have a high societal value. The protection of fish communities was relevant as the river is used for fishing, and fish are used for human consumption, but also as a visible representation of the ecosystem sustainability and vulnerability. Fish kills are reported by the press and in official communications upbringing great societal concern (Pazos 2008, Ríos et al. 2010, CARU 2010). The river health condition is evidenced by changes in the populations of aquatic organisms, visible as algal blooming events, producing microcystin LR and other toxins due to eutrophication for excess nutrients. We described this in prior research (Míguez 2007, Ferrari et al. 2009, Saizar et al.

2010, Ferrari et al. 2011). This situation might represent impacts to touristic activities, drinking water production and as causative of health issues and even linked to endocrine disruption (Rogers et al. 2011).

The Northern limit of the area under study has a protected wetland:Nuevo Berlín and Islas de Farrapos. National and international declarations of nature reserves reflect the importance of maintaining the pristine condition of ecosystems. In 2004, the Ramsar Conventions on wetlands designated "Esteros de Farrapos e Islas del Río Uruguay" as International Protected Wetland No. 1433. In November 2008, this site was declared a protected natural area, within the "Sistema Nacional de Äreas Protegidas " [National System of Protected Natural Areas], created by the Law 17234 of 22 February 2000 (Uruguay 2000). Its surface extends to 17496 ha. It is located in the Río Negro province, Uruguay, part of which pertains to the area under study. This site consists of alluvial areas and 24 islands, and vegetation, represented by several species including water hyacinths (*Eichhornia spp*) (RAMSAR 2004).

Fish species are similar all through its watercourse, with high biodiversity and productivity for a sub-tropical climate. More than 200 fish species have been identified, and more than 8 species 10^{-4} square kilometres exist (Appendix A, Figure A-3). After the exposure scenario was characterised, the study was restricted to potentially exposed fish species representative of the freshwater habitat of the Uruguay River.

The main trophic levels of fish are represented by detritivorous-iliophagus (mud-eating) fish such as sábalo (*Prochilodus lineatus*); omnivorous: boga (*Leporinus obtusidens*); piscivorous, big predators: “dorado” (*Salminus maxillosus*), “patí” (*Luciopimelodus patí*) and small predator fish: “mojarras” (*Astyanax fasciatus*) (Foti 1999). This latter fish species is a small, non-migratory fish chosen as bioindicator for biomonitor effects. Metabolic rates of small receptors are higher than those of large animals, resulting in a higher ingestion per body weight, which means an increased exposure potential. It is ecologically relevant for being one of the most prevalent species in the Uruguay River. Scarce prior

studies existed using this species, but it proved being a good bioindicator of reproduction in studies in Brazil (Shulz & Martins-Junior 2001) and its sensitivity to detect endocrine disruption from agricultural and domestic origin has been demonstrated in studies in the Grande River, Brazil (Prado et al. 2011). Although this species has not been used to monitor endocrine disruption in the Lower Uruguay River before, and never to detect impacts from pulp mill effluents, it has been used in field studies measuring hepatic porphyrines as biomarkers of contamination (Carrasco-Letelier et al. 2006).

The most likely sensitive species are invertebrate, fish, terrestrial animals, birds and human beings. For the scope of this research that focuses on aquatic toxicity, birds and terrestrial animals are not considered in the quantitative risk assessment. The aquatic receptor organisms in Uruguay River ecosystem were identified through plankton, benthic and fish community studies during the baseline monitoring. Fish populations and community structure was the same before the pulp mill operations at all three study areas. Muscle and bile analysis of different toxic pollutants show measurable concentrations but below internationally recommended levels (Saizar et al. 2010). However, reports on residues of chlorinated pesticides exist since early 90's, with historical data on fish tissue residues at Salto Grande dam, 600 km upstream the research area, for endosulfan I and II in the range of 0.028 to 0.072 µg g^{-1}, and 0.045 µg g^{-1} dieldrin and 0.075-0.198 endrin µg g^{-1} (Leites & Bellagamba 2001).

5.3.7.4 Human beings as receptors

Fishermen use small gill nets or canes to catch the fishes, while the commercial fishing companies that operate draft nets for the bigger species to be further processed as fish meal and oil at factories on the Argentinian side. Catfish are the most frequently caught species near Gualeguaychú River on the Argentinian side: sábalo and boga near Fray Bentos. Much of the produce is exported fresh and eviscerated while the rest is sold at local markets. In general, the artisan fishermen depend greatly on these resources to make a

living, and they dwell near the coastal zone. In 2004 a total of 220 fishermen were living on the Uruguayan side and approximately 104 on the Argentinian margin. It can be estimated that of 1596 tons caught from Fray Bentos to the River Plate but only 51 ton per month enter the internal market. The average fish consumption in the general population in Uruguay is 7 kg per year per person. A higher rate could be the case for this sub-population composed of fishermen and their families, estimated as an ingestion in a frequency of three times a week. These fish species are migratory, so it is not easy to find direct causal links to sources (CARU 2008), but non migratory and short distance migratory species such as *Pimelodus macculatus* (Figure B-26) are also consumed as there are found in the local market.

5.3.7.5 Critically exposed individuals

The most vulnerable sub-populations at risk were defined as pregnant/nursing women, foetuses and lactating children of fishermen families (2.4.1). The pulp mill workers could be exposed to dioxins and furans by a certain extent through the inhalation route. Studies carried out in Finland by Rosenberg et al. (1994) at a pulp mill that bleached wood with chlorine dioxide measured 2,3,7,8-substituted PCDDs/PCDFs in workplace air estimating a potential inhalation exposure at the bleaching plant and paper mill between 0.002 and 0.2 pg m^{-3}.

5.3.8 Candidate list of EDCs

The EDCs below the horizontal black line were excluded from the candidate list. As it was an iterative process, whenever evidence arose that merited inclusion they were studied at a screening level in Phase II. This was the case for thyroid disruptors, not the focus in this first assessment but widespread although with diffuse distribution and a low likelihood, taking into account the low population in the area under study (case of phthalates, bisphenol A). Tributyltin was excluded at this stage, although it could eventually be found as an antifoulant that might be used in the barges that carry cellulose pulp. Of all possible chlorophenols, 2,4,5-TCP was the only one included as it appeared in the pulp

mill effluent. Most of the EDCs were persistent, so, if they were not detected in river water during the monitoring activities, they could be included based on the high use in this area, and through sediment or fish tissue residues of fish caught in the river.

The reasons to exclude these substances from the list were several, but they were systematically sorted out through a decision process before the hazard identification stage.

Table 5-7 Primary list of EDCs with possible relevance in the river watershed under study

EDC	Uses	Main release processes	Up-take routes	Main endocrine disruptive effects/target organ	Occurrence in Uruguay River water (µg l^{-1})	References
Arsenic and its compounds	Unintentional, contaminant in fertilizers	Geology contamination of groundwater	Water	General reproductive and cancer	Below detection in surface water. Scarce events above regulatory limit in groundwater	Davey et al. 2008
Cadmium and its compounds	Unintentional Metal industry, batteries, plastics	Geology processes Domestic, industrial, agricultural	Air, dust, water, food	Abnormal sperm	*	ATSDR 2008
Chlorpyrifos	Insecticide	Agriculture: soy crops and others	Food, water	Testicular toxicity Fertility decrease in mammals	*	Joshi et al. 2007
Chlorophenols 2,4,5-trichlorophenol	Fungicide Unintentional	Agriculture Industrial: pulp mill effluent	Water, food	Oestrogenic	*	Owens et al. 1994
Dioxins and furans **Total dioxins and furans** **2,3,7,8-TCDD**	Unintentional	Incomplete combustion. Pulp and paper bleaching. Automobile exhaust, wood smoke	Air, food, water	Antioestrogenic Low sperm quality, decrease oestradiol	< 4- 31 pg l^{-1} < 0.3 pg l^{-1}	** Brunnberg et al. 2011 **
Endosulfan	Insecticide, rodenticide	Agriculture	Food, water	Neuroendocrine Developmental Antiandrogenic	ND (LD: 0.0005)	Stanley et al. 2009

Table 5-7- *Continued*

EDC	Uses	Main release processes	Up-take routes	Main endocrine disruptive effects/target organ	Occurrence in Uruguay River water (µg l^{-1})	References
Glyphosate	Herbicide	Agriculture in soy crops and forestry	Food, water	Mammalian placental cell	< 20	Richard et al. 2005
Nonylphenol	Precursor and biodegradation by-product of non ionic surfactants	Agriculture, domestic, industrial	Food, water	Oestrogenic	*	Soares et al. 2008
Polyaromatic hydrocarbons (PAHs)	Unintentional	Combustion Domestic, industrial, agricultural (wood preservation)	Air and dust transport water, food	Weakly oestrogenic or antioestrogenic	0.02-0.27	** Nicolas 1999
Polychlorinated biphenyls (PCBs)	Heat exchange fluids in electric transformers and capacitors	Industrial, domestic	Air, water, food	Thyroid, low spermatogenesis	*	** ATSDR 2001, Bansal & Zoeller 2008
Resin acids	Unintentional	Industrial: pulp mill effluents	Fish,water	Oestrogenic	< 10-115	** Ellis et al. 2003
Steroidal hormones	Unintentional	Domestic and agricultural Animal and human excreta	Fish, water	Oestrogenic Androgenic	*	

Table 5-7- *Continued*

EDC	Uses	Main release processes	Up-take routes	Main endocrine disruptive effects/target organ	Occurrence in Uruguay River water (µg l^{-1})	References
Sterols	Unintentional	Industrial: pulp mill effluents	Fish, water	Antiandrogenic Testes	50-100 µg l^{-1}	** MacLatchy &Van Der Kraak 1995
Synthetic steroidal hormones 17-α Ethynil oestradiol	Contraceptive pill	Domestic	Fish, water	Oestrogenic	*	Xu et al. 2008
2,4-D	Herbicide	Agriculture Domestic	Food, water	Developmental	ND (LD: 3)	
Cys-chlordane	Insecticides	Agriculture Domestic	Food, water	Oestrogenic	ND (LD 0.0005)	
Acetochlor	Herbicide	Agriculture	Food, water	Thyroid	-	Crump et al. 2002
Atrazine	Herbicide	Agriculture: soy and maize crops	Food, water	Pituitary	-	ATSDR 2003
Bisphenol A	Plasticiser	Domestic	Food, water	Feminisation	*	
DDT	Insecticide	Agriculture	Food, water		ND (LD 0.001)	

Table 5-7- *Continued*

EDC	Uses	Main release processes	Up-take routes	Main endocrine disruptive effects/target organ	Occurrence in Uruguay River water ($\mu g\ l^{-1}$)	References
Dicofol	Acaricide	Agriculture	Food, water	Egg thinning in birds		Wiemeyer et al. 2001
Dieldrin	Insecticide	Agriculture Domestic	Food, water	Hypothalamus	*	Martyniuk et al. 2012
Endrin	Insecticide, rodenticide	Agriculture	Food, water	Thyroid and adrenal	ND (LD: 0.0007)	ATSDR 1996
Fipronil	Acaracide, Insecticide	Agriculture	Food, water	Thyroid	*	Environment Australia Pesticide Project (315-379-9200), Leghait et al. 2009
Heptachlor	Insecticide	Agriculture	Food, water	Testes	ND (LD: 0.0004)	** Song et al. 2012
Lindane Gamma-HCH)	Insecticide	Agriculture Timber treatment	Food, water	Oestrogenic	ND (LD: 0.0005)	**
Methoxychlor	Insecticide	Agriculture	Food, water		ND (LD: 0.001)	**

Table 5-7- *Continued*

EDC	Uses	Main release processes	Up-take routes	Main endocrine disruptive effects/target organ	Occurrence in Uruguay River water ($\mu g\ l^{-1}$)	References
Mirex	Insecticide, fire retardant	Agriculture Domestic	Food, water		ND (LD: 0.001)	**
Phthalates	Plasticiser	Industrial, domestic	Food, water	Weakly oestrogenic	*	Lyche et al. 2009
Toxaphene	Insecticide	Agriculture	Fish, water, food	Oestrogenic, breast	*	Jørgensen et al. 1997
Trans-nonachlor	Insecticide	Agriculture		Thyroid and adrenal Testes	ND (LD:0.001)	Bondy et al. 2004, Cook et al. 2011
Tributyltin	Biocide	Antifouling in paints, wood, plasticsand insulants		Imposex in snails Androgenic	*	Gooding et al. 2003
Tributyltin compounds	Biocide	Wood preservation, antifouling in marine paints, in textiles and cooling towers	Fish, water	Pituitary and thyroid EDC	*	Adeeko et al. 2003
Vinclozolin	Fungicide	Agriculture used on rape, beans, peas, turf and apple blossom	Food, water	Antiandrogenic		Anway et al. 2006

ND: not detected; LD: limit of detection; * no prior data; ** LATU technical report; TDS: testicular dysgenesis syndrome

6 HAZARD IDENTIFICATION

6.1 Prioritisation strategy of EDCs in Phase I risk assessment

The architecture of the prioritisation model of EDCs for inclusion within Phase I of the risk assessment was composed of a series of sequential and conditional processes illustrated in a decision chart (Figure 6-1).

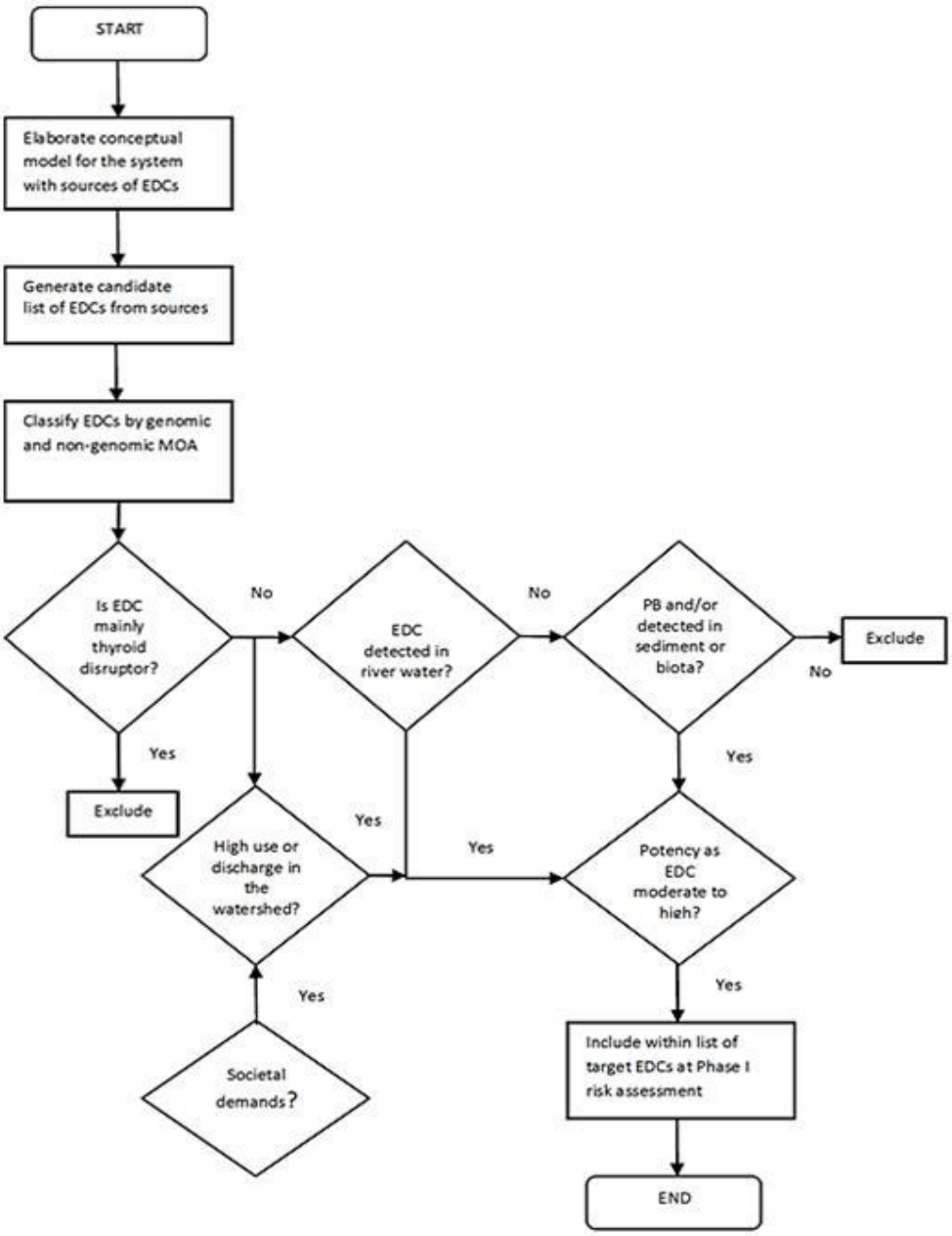


Figure 6-1 Decision chart of the prioritisation strategy

The criteria set is composed of seven components:

6.1.1 Relevant sources in the watershed

The initial candidate list contained prioritised EDCs derived from the sources within the geographical scope as described in the conceptual model of the system (Section 5.3.2.3).

6.1.2 Mechanism of action

Even though not all the mechanisms of ED are known, the most recognised were included in this research as one of the criteria and decision point for the hazard identification step of the RA. The EDCs were included in a classification system based on their principal mechanism of action, segregated initially into two categories: genomic (molecules with ligand binding activities) and non-genomic (other mechanistic pathways) (2.3.9). The EDCs that act mostly as thyroid disruptors were not included in the list.

6.1.3 Occurrence in river water

Historical data on river water monitoring carried out since 2005 considered EDCs above the limit of detection of each method. The detected EDCs were included in the list provided criterium No. 5 was met (potency of the compound is medium or high).

6.1.4 Persistence and bioaccumulation

The potential of bioaccumulation of an EDC depends on its lipophilicity and its metabolism within an organism (Feijtel et al. 1997). Even when some EDCs might not be detectable in water, they could still build-up inside sediment and biota. Bioaccumulated EDCs adsorb onto sediment particles and represent an exposure pathway for benthic biota. Eating contaminated shellfish or fish could in turn be an important human exposure pathway.

The employed criteria for P and B, respectively, were that degradation half-life in river water exceeds 40 days and/or 120 days in river sediment and BCF> 2000 for aquatic species (EN 16.3.2011). Intermediate categories were based on the ready biodegradation and ultimate degradation concepts (28 days). An additional component was log Kow, important to estimate bioaccumulation in compounds that lack an experimentally derived BCF. As Finizio et al. (1997) anticipated no reliable Kow values existed for every compound, and no harmonised BCF data, as some were derived by fish tissue analysis, and others by quantitative structure–activity relationships (QSAR) (Arnot & Gobas 2006, Bermúdez-Saldaña et al. 2005). Half-lives for the sediment compartment were assumed as 3-4 times longer than for water and soil if no other information was available (USEPA 2000a) (Table 6-1).

Table 6-1 Criteria for bioconcentration factor and persistency

Ranking	log Kow	P		BCF	Example	log Kow of EDC	References
		$t_{1/2}$ (days)					
		River water	River sediment				
Very low	<1	< 5	< 15	< 100	Glyphosate	-1.7	Finizio et al. 1997
Low	1- 2	5-10	16-30	100-2000	Glyphosate[2]	1.7	Finizio et al. 1997
Moderate	2-4	11-28	31-90	2000-5000	2,4,5-Trichlorophenol	3.72	Liu & Yu, 2005
High	4-6	29-40	91-120	> 5000	Nonylphenol	4.77	USEPA 2005
Very high	> 6	>40	> 120	< 100	Dioxins	6.64	Endicott & Cook 1994

BCF: bioconcentration factor; P: persistence; $t_{1/2}$: half life fate of the compound in the media water or sediment

[2] Different values log Kow (-1.7; 1.7) can be obtained depending on the pHbecause of glyphosate zwitterion characteristics (Finizio et al. 1997)

6.1.5 Potency

The potency of each of the selected EDCs concerns only to endocrine disruption activity (oestrogenity, androgenicity, anti-oestrogenity, anti-androgenicity) or developmental or reproductive toxicity. The database on potency was based on a review of the results of receptor binding activities methods and QSAR modeling. The potency ranking for ER-binding EDCs relied on that established in the Distributable Structure-Searchable Toxicity (DSSTox) Public Database Network, DSSTox NCTRER SDF (Blair et al. 2000, Fang et al. 2001, Branham et al. 2002, EDKB Database 2008, Ding et al. 2010).

6.1.6 Use patterns in the watershed

The EDCs that presented high usage in the watershed could be re-examined for inclusion depending on the compliance of any of the other criteria (from 3 to 6).

6.1.7 Legal and societal demands

Glyphosate was included due to the societal demands (Section 2.3.4), but also supported by its high use in the watershed, according to official data (Section 5.3.2.3). The study of chlorinated compounds and rosin acids released by the pulp mill was also justified by societal demands, and scientific information on the potential endocrine disruptive effects of this kind of industry on the river ecosystem receptors (Section 2.3.3). Legal issues backed up the inclusion of the persistent EDCs such as dioxins and furans within international treaties (Stockholm Convention 2008).

The processes were modelled, differentiating the seven components (D.2).

6.2 Preliminary list of EDCs

In the problem formulation stage, the contaminants of concern and their ED activities were identified using databases and reviewing publications. This effort to prioritise target chemicals will benefit further risk management action. Also, a toxicity profile was prepared for glyphosate (C.1) as this was not available from

the literature. Data on mechanism of action, acute, chronic and developmental toxicity for humans and aquatic animals, physicochemical characteristics and potential for bioaccumulation and biomagnifications were also gathered for the other target compounds.

6.3 Categories of prioritised EDCs and reasons for inclusion in the quantitative risk assessment

As reviewed in Chapter 2, the chemical categories that could pose endocrine disruptive activities is very broad, ranging from natural and synthetic steroidal hormones, pesticides, herbicides, biocides, pharmaceuticals, cosmetic compounds, surfactants, plasticisers, lignin derivatives, phytosterols, organochlorine compounds, etc..

6.3.1 Steroidal hormones

No previous data on the occurrence of these EDCs existed for the Uruguay River. Thus, due to their importance in terms of potency (Table 2-6) and probability of occurrence due to the existence of potential sources (such as untreated municipal wastewater, Section 5.3.3.1) the decision was to analyse them in samples from several sites in the river, as well as in the pulp mill effluent and in the stream that receives municipal wastewater (C2).

6.3.2 Pesticides and herbicides

The Uruguayan Decree 000/005 (DINAMA 2005) forbids these substances throughout their life cycle. Most products are imported and some applied for domestic use. The most prevalent use of pesticides is in agricultural crops and some horticulture and fruit production, and less intensively in livestock. In 2005, the herbicide import was 6726 tonnes, fungicide 1120 tonnes, insecticide 1238 tonnes and others, 481 tonnes, totaling 9566 tonnes. Within pesticides, the group with the largest increase is that of herbicides and within herbicides, glyphosate is the most used because of the growth in soy crops. Of the banned pesticides, toxaphene and hexachlorobenzene were never registered for agricultural use. The last imports of DDT are prior to1978.

A note on endosulfan: this compound was restricted to soy crops since 2007 (up to 0.5 kg active substance per hectare), and in 2011, endosulfan was banned in Uruguay (Decree 434/011).The air spray using airplanes is done sometimes over populated areas, not being careful about the wind direction (Moreira & Bianco 2005).

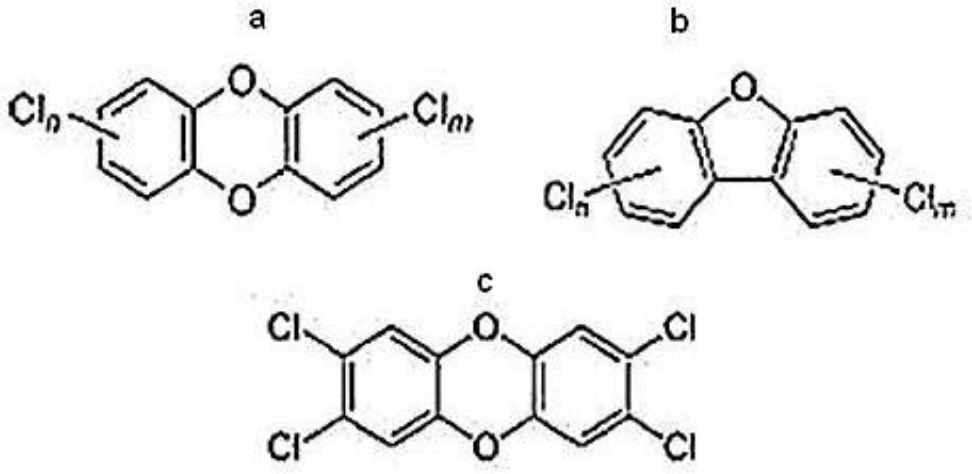
The main postulated mode of action of endosulfan is antiandrogenic, but also a developmental neurotoxic. *In vivo* studies in animals suggest that endosulfan may disrupt normal reproductive hormone levels in male animals, but that it is not an endocrine disrupter in females. Persistent depressed testicular testosterone was seen in male rats after intermediate duration oral exposures to endosulfan. Endosulfan I and II are neurotoxic, possibly through inhibition of the gamma-aminobutyric acid (GABA)-gated chloride channels, inhibiting fish touch response respectively at 2.2 µg l^{-1} and 23 µg l^{-1}. Tissue EC50, determined from the measured tissue concentrations, were 367ng g^{-1} for endosulfan I and 4552 ng g^{-1} for endosulfan sulphate (Stanley et al. 2009).

This chemical has not been detected in the Uruguay River water in any of the sampling areas (Nuevo Berlín, Bridge, Fray Bentos and Las Cañas). However, several species of trade fish from Uruguay River at Salto Grande, analysed from 1998 to 2001 were found to contain detectable concentrations of chlorinated pesticides, and the most frequent was endosulfan, which was quantified in 25% of the samples (0.02- 0.03 µg g^{-1} endosulfan I, and 0.05- 0.07 µg g^{-1} endosulfan II) (Leites & Bellagama 2001).

6.3.3 Dioxins and furans

These compounds were included due to their high persistence and air transport (Sinkkonen & Paasivirta 2000), high toxicity and probable sources of unintentional emissions (6.3.3.1).

Dioxins are polyhalogenated aromatic hydrocarbons with general structures as shown in Figure 6-2. The most toxic congener is 2,4,7,8-tetrachlorodibenzo dioxin.



a- Polychlorinated dibenzo-p-dioxins (PCDDs), derivatives of dibenzo-p-dioxin, 75 congeners; seven of them highly toxic

b- Polychlorinated dibenzofurans (PCDFs), derivatives of dibenzofuran, comprising 135 congeners (derivatives differing only in the number and location of chlorine atoms); ten of them with "dioxin-like" properties

c- 2,3,7,8-Tetrachlorodibenzo dioxin (2,3,7,8-TCDD), the most toxic congener

Figure 6-2 Molecular structures of polychlorinated aromatic compounds

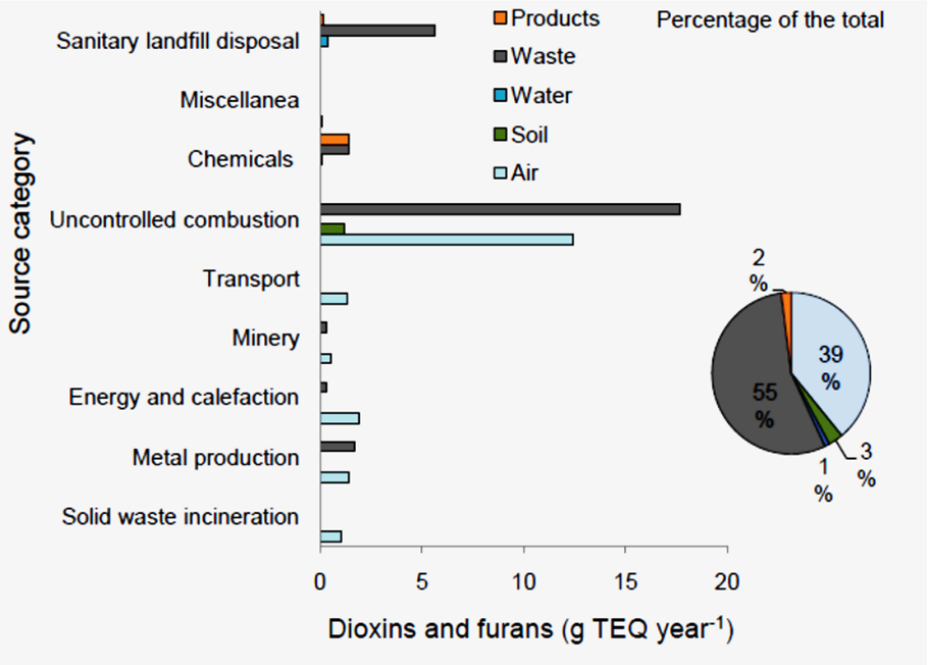
6.3.3.1 Sources of dioxins and furans in Uruguay

A classification according to the source distinguishes them in to four major categories: incineration, combustion, industrial and reservoir (Kulkarni et al. 2008).

The latest inventory of emissions carried out by the environmental authorities in Uruguay date from 2003. The chart below shows the dioxins and furans emissions per category of source, evidencing the highest participation of uncontrolled combustion to the total. In this inventory the pulp mill was not taken into account as it was not present yet, but the estimation was that it would have added 34 g TEQ per year to the total (considering an initial assumption of two pulp mills to be built on the margins of the Uruguay River) (Figure 6-3).

Total emissions were estimated at 49 g TEQ at country level, most due to uncontrolled combustion processes (66% of total). The installation of the pulp mill rose production of bleached kraft pulp from 30000 to about 1 million ton per year, which represents a potential release. An increase of about 30 g TEQ was estimated, representing an increase of nearly 70% with regards to total release for 2003; more than 80% (28 g TEQ) related to emissions of black liquor burning and 17% from bark waste burning (DINAMA 2006).

The identified sources by the environmental authority were: forest fires, burning domestic wastes in open air, incineration of hospital wastes. Burning wetland areas for deforestation at the Argentinian side of the watershed may at times influence the Uruguay margin, depending on the wind direction. They may also be formed in the production of cellulose paste and biomass burning. The lime production process line existing at the chemicals manufacture plant settled near the pulp mill is yet another possible release source. Chlorophenols may have high levels of dioxin impurities. For example, PCPs, banned in Europe in 1984, but still a dominant fungicide in wood preservation at sawmills contains hexa-, hepta- and octachlorinated PCDD/Fs (I-IxCDD/Fs, HpCDD/Fs and OCDD/F) (Assmuth & Vartiainen 1994).



Graph generated with data obtained from DINAMA (2006)

Figure 6-3 Sources of dioxins and furans in Uruguay

6.3.3.2 Process of dioxins formation by combustion

According to Stanmore (2004), dioxins form during combustion either by homogeneous pyrolysis between 500- 800°C, or by heterogeneous reaction at 200- 400°C with chlorine in solid phase or as atomic chlorine gas reacting on ash or soot particles, as for example during the process of biomass burning.

6.3.3.3 Toxicity of dioxins

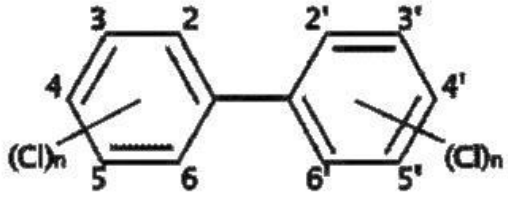
Developmental and reproductive toxicity have been observed upon exposure of animals to TCDD, such as skeletal craniofacial anomalies in zebra fish, impaired reproduction by inhibition of oestrogen biosynthesis and effects on the ovaries at tissue level (King-Heiden et al. 2012). The effects are mediated through binding to the aryl hydrocarbon (Ah) receptor. The lipid content of the animal influences their toxicity and bioconcentration. The severity of effects varies inversely to the animal fat, as the toxicant is stored in this fraction, directly proportional to the bioconcentration potential. Human toxicity ranges from several cancer types (Wolff & Toniolo 1995) to skin lesions (chloracne) and toxicity on the immune and neurological systems. The World Health Organisation set a tolerable daily intake of 1–4 pg day^{-1} kg^{-1} bw. The maximum limits for dioxin in food established by the EU range from 1 pg to 6 pg g^{-1} expressed as TEQ. For fish, the limit is 4 picograms per gram of fresh weight muscle meat.

The molecules 2,3,7,8-TCDD and 2,3,7,8-TCDF were selected because of its very high toxicity, while OCDD and OCDF because of the higher concentrations found, although their toxicities are 1000 less than the prior mentioned compounds. Octachlorodibenzo-*p*-dioxin (OCDD) is the most prevalent congener, but one of the least toxic, possibly due to its low solubility (74 pg l^{-1}) and very high lipid solubility (log K_{ow} 8.6) (Geyer et al. 2000). One possible source is pentachlorophenol (PCP), a compound used as wood preservative.

6.3.3. Polychlorinated biphenyls (PCBs)

Polychlorinated biphenyls (PCBs) are 209 congeners, which molecular structure is represented on Figure 6-4.

Twelve of them pose "dioxin-like" properties. Under certain conditions PCBs may form more toxic dibenzofurans through partial oxidation.



The possible chlorine atoms positions on the benzene rings are denoted by numbers assigned to the carbon atoms (http://en.wikipedia.org/wiki/Polychlorinated_biphenyl).

Figure 6-4 Molecular structure of polychlorinated biphenyls

The trade of PCBs demands their removal from equipments, or any stock of them, before 2025. The most relevant use is as dielectric oil in transformers. Of the estimated 42000 transformers operating in Uruguay, 95% belong to the electricity provider, UTE (DINAMA 2006), and the pulp mill has high voltage production to produce energy from biomass.

These compounds are found in fish tissue in the Uruguay River. According to Leites et al. (2011), 185 specimens analysed from 1998 to 2006 caught in the Uruguay River near the Salto Grande dam and several kilometers to the North, had concentrations of Aroclor 1016: 10 ng g^{-1}, Aroclor 1242: 8 ng g^{-1}, Aroclor 1254: 8 ng g^{-1} and Aroclor 1260: 7 ng g^{-1}.

6.3.4 Wood extractives and bleaching derivatives: phytosterols, resin acids and chlorinated phenols

Aquatic animals accumulate chlorinated organic compounds found in the pulp bleaching effluents, notably chlorophenols, chlorohydrocarbons, and chlorinated dibenzodioxins and furans (Pellinen et al. 1993, Ahonen et al. 2006, Hewitt et al. 2008). A mixture of complex substances, some of them unknown, probably coming from lignin degradation or bleaching may be causative agents. The

elemental chlorine free process (ECF) using chlorine dioxide from chlorate, is still able of forming halogenated organic substances (AOX) but with lesser risk or dioxins and furans formation (Hewitt et al. 2008).

6.3.4.1 Chlorophenols

Chlorophenols concentrations were very low in the pulp mill effluent (Figure 6-5). The only congener quantified in Fray Bentos pulp mill effluent from 2009-2010 several times during the analysis period, was 2,4,5-TCP, with mean concentration 0.19 µg l^{-1} (< 0.1- 1.3 µg l^{-1}). Therefore, this molecule was chosen as a model compound in this chemical category.
Another congener detected only twice in 2010 was 3,4,5-TCP, measured in concentrations of 0.24 and 0.29 µg l^{-1}.

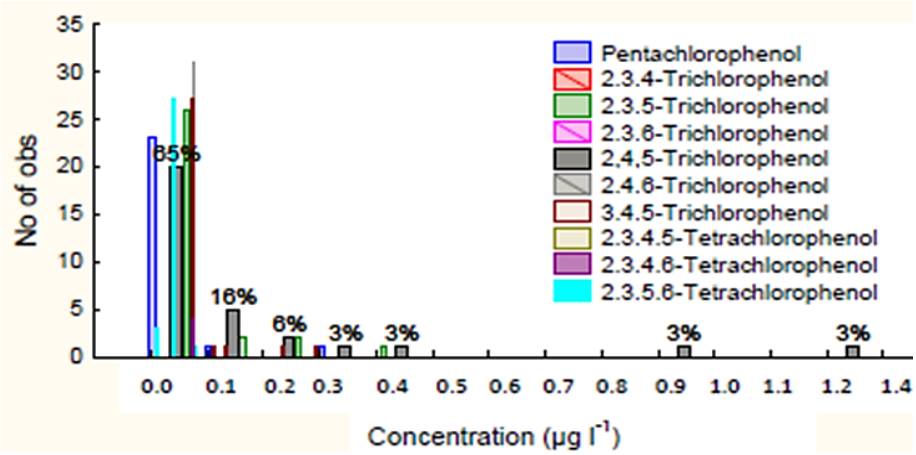


Figure 6-5 Frequency of detection and concentrations of chlorophenol congeners in pulp mill effluent

6.3.4.2 Resin acids

Resin acids are potential EDC, causing reproduction effects, through unknown mechanisms of action. Wood extractives are constituents of wood present in pulp and paper mill effluents, which may cause reproductive disturbances in fish. Of all the congeners, isopimaric acid is the most toxic (Peng & Roberts 2000) and less soluble than the rest (Volkman et al. 1993). Thus, the choice of isopimaric provides a more conservative estimate. Besides, the most prevalent

identified substances in the pulp mill effluent were pimaric acid (28 µg l^{-1}) and isopimaric acid (44 µg l^{-1}) in the period from the November 2007 to March 2009, with total resin acid concentration in the range from < 10 to 155 µg l^{-1} (Figure 6-6). Therefore, the study focused on isopimaric acid, as a model compound.

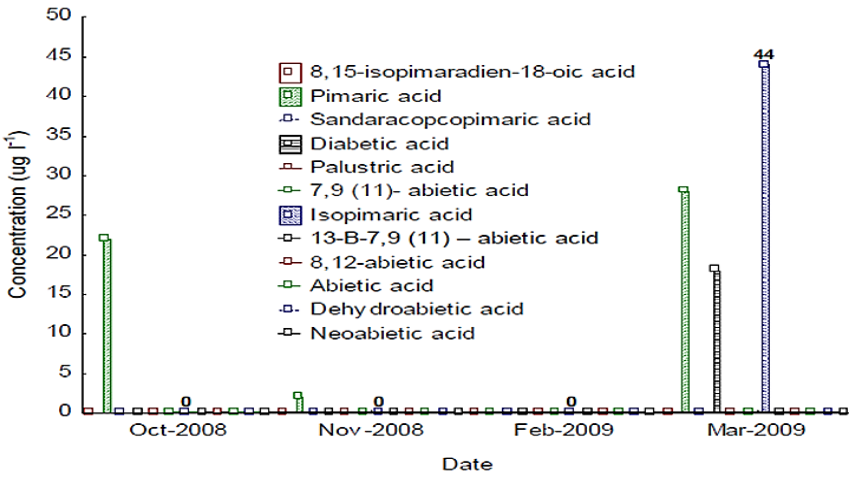


Figure 6-6 Resin acid composition in pulp mill effluent

6.3.4.3 Phytosterols

Plant sterols are found in foods such as oils, nuts and vegetables (Maguire et al. 2003), but also, many international investigations link these compounds to emissions of pulp mill effluents (See section 2.3.3). The most representative congener included in the refined RA was β-sitosterol. These compounds were below the limit of quantitation (50-100 µg l^{-1}) for all congeners, yet not undetectable.

6.3.5 Polycyclic aromatic hydrocarbons *(*PAHs*)*

The body of evidence supporting the carcinogenicity of PAHs to experimental animals and humans is extensive, but their weak oestrogenic and antioestrogenic actions have not been demonstrated in human populations (Santodonato 1997, Vondráček et al. 2002).

In this research, apart from diffuse pollution caused by traffic exhausts and home calefaction, two main probable sources of combustion by-products were identified: biomass burning at the pulp mill, and intentional wetland fires at the Argentinian side, and natural, unprovoked fires, at both margins of the river. The concentrations were in the range from not detectable to 0.28 µg l^{-1} (Figure 6-7).

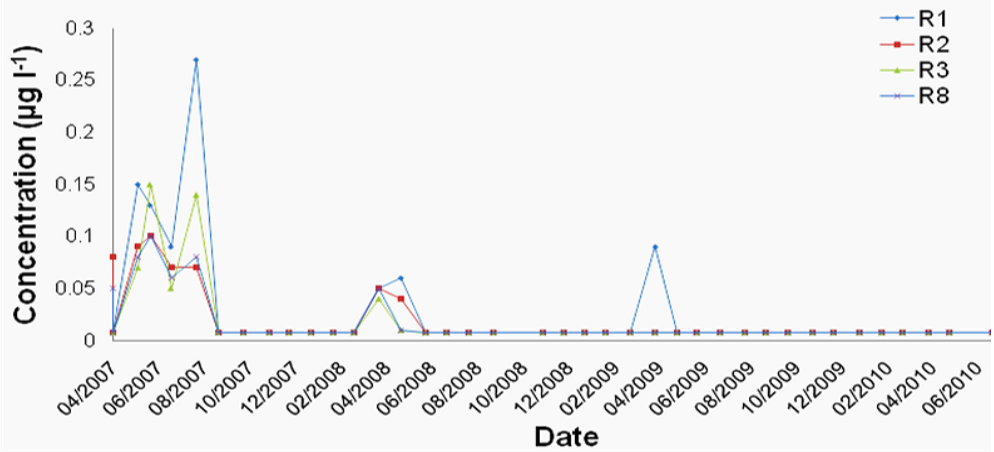


Figure 6-7 Polycyclic aromatic hydrocarbons in river water monthly trends from April 2007 to June 2010

Polycyclic aromatic hydrocarbons were also measured in sediments, presenting highly variable composition, but the most representative congeners were fluorene, naphthalene and phenanthrene (Figure 6-8).

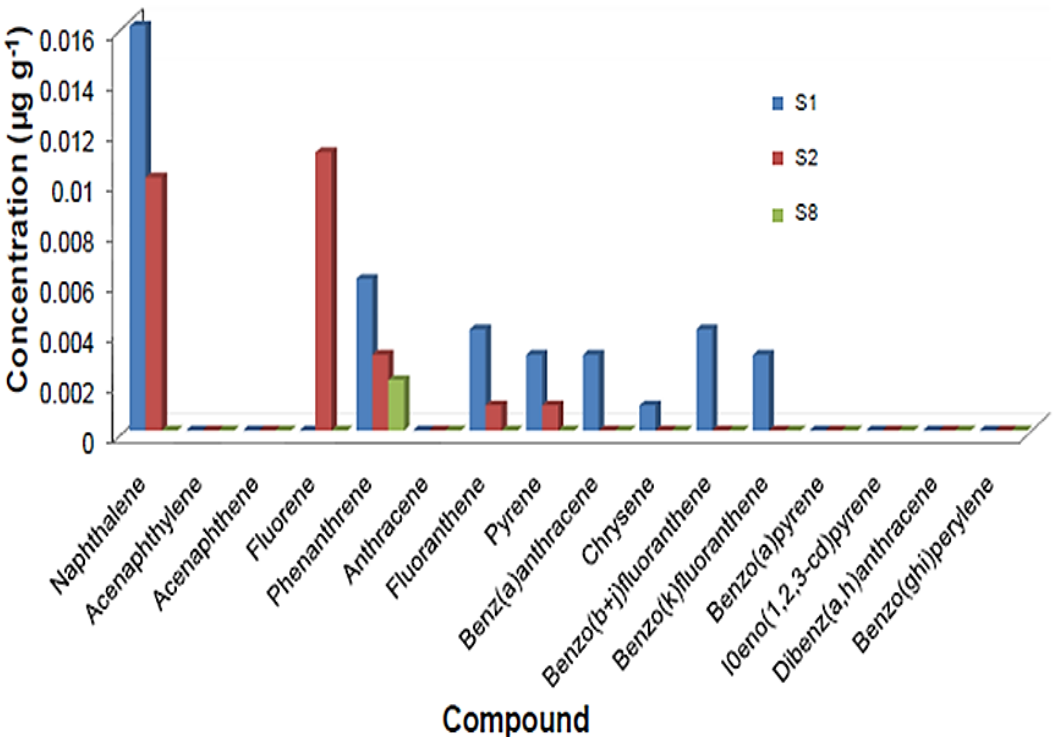


Figure 6-8 Polycyclic aromatic hydrocarbons in sediments

6.4 Decisions and list of prioritised endocrine disruptors

The decision rules were applied within the prioritisation process in Figure 6-1 reaching to the following list of EDCs to evaluate in the watershed under study (Table 6-2).

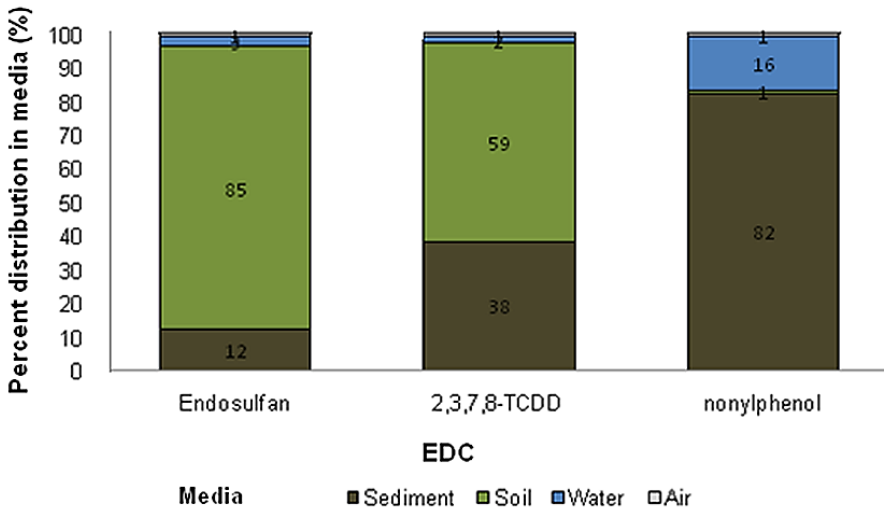
Table 6-2 Prioritised endocrine disruptors and summary of the rating criteria

Prioritised EDC	1st criterion	2nd criterion	Other criteria	Assessment of fate, bioaccumulation and toxicity
Glyphosate	High use in the watershed	Societal demands		Analysis in environmental matrixes
Dioxins	High persistence	High potency	Occurrence in river water	Multimedia modelling
Furans	High persistence	High potency	Occurrence in river water	Multimedia modelling
PCBs	High persistence	High bioaccumulation	Occurrence in biota	Multimedia modelling
PAHs	High persistence	High bioaccumulation	PAH	Multimedia modelling
2,4,5-trichlorophenol	Occurrence in pulp mill effluent	Not enough data on potency as EDC	Moderate persistence Moderate bioaccumulation	Exposure and effects experiment with spiked sediment Tissue residues
Nonylphenol	High use in the watershed	Moderate potency	Moderate persistence	Fish exposure to municipal wastewater
17-ß-oestradiol	Occurrence in river water	High potency		Fish exposure to municipal wastewater
Endosulfan	Potential EDC	Moderate persistence	Societal demands	Multimedia modelling
Isopimaric acid	Occurrence in pulp mill effluent	High persistence		Fish exposure to pulp mill effluent

6.4.1 Modelling of the fate and behaviour of endocrine disruptors

Exposure was evaluated considering the exposure pathways of the conceptual model, predicting the EDC concentration in the media where a particular receptor species inhabits. The theoretical media distributions, fate and behaviour of three target EDCs were estimated with a modified version of the fugacity-based model described by Paterson & MacKay (1989) (Figure 6-9). Level III model v. 2.7 provides predictions of steady-state, non-equilibrium concentrations and distributions of environmental contaminants to focus more attention on specific environmental compartments and site-specific conditions. Mackay et al. (2009) defines fugacity as the “escaping tendency” of a chemical in each of compartment.

The software predicted the relative distribution among media for three representative EDCs of probable occurrence: endosulfan because of agricultural use patterns in the area, nonylphenol, because of domestic use, and the most toxic dioxin congener, for the chlorinated compounds and air distribution pathways of chlorinated biomass burning.



Level III model v. 2.7 (CEMC)
http://www.trentu.ca/academic/aminss/envmodel/models/VBL3.html

Figure 6-9 Predicted media distribution of three persistent EDCs among sediment, soil, water and air

6.4.2 Multimedia processes for target endocrine disruptors

Equilibrium concentrations of EDCs were computed assuming that their emission to the environment is nearly constant. The loss processes are degrading reactions and advection. Each medium is assumed at different fugacities. Rates of intermedia transport are calculated using D values containing information on mass transfer coefficients, areas, deposition and resuspension rates, diffusion rates, and soil runoff rates. This model describes the EDC fate including the degradation and advection losses and intermedia transport processes (Figures 6-10 to Figure 6-12). The half- life times of 2,3,7,8-TCDD in cold weather is 200 hours in the air compartment, 4000 in water, and 900000 hours both in soil and in sediment (Sinkkonen & Paasivirta 2000). The predominant fate processes are suspension back into the air and through soil erosion, ultimately sinking in aquatic sediments, provoking bioaccumulation (Kulkarni et al. 2008).

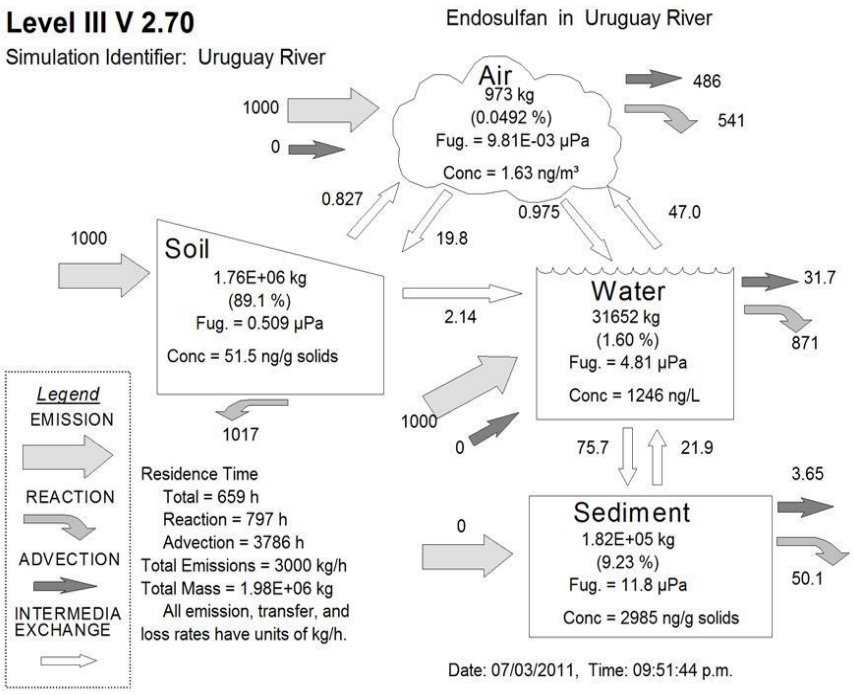


Figure 6-10 Simulated media processes por endosulfan

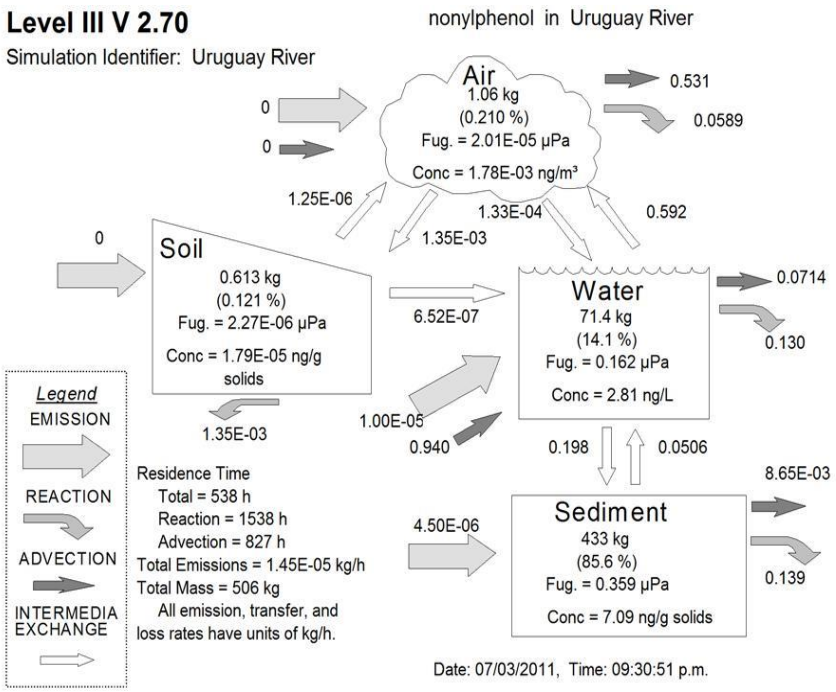


Figure 6-11 Simulated media processes por nonylphenol

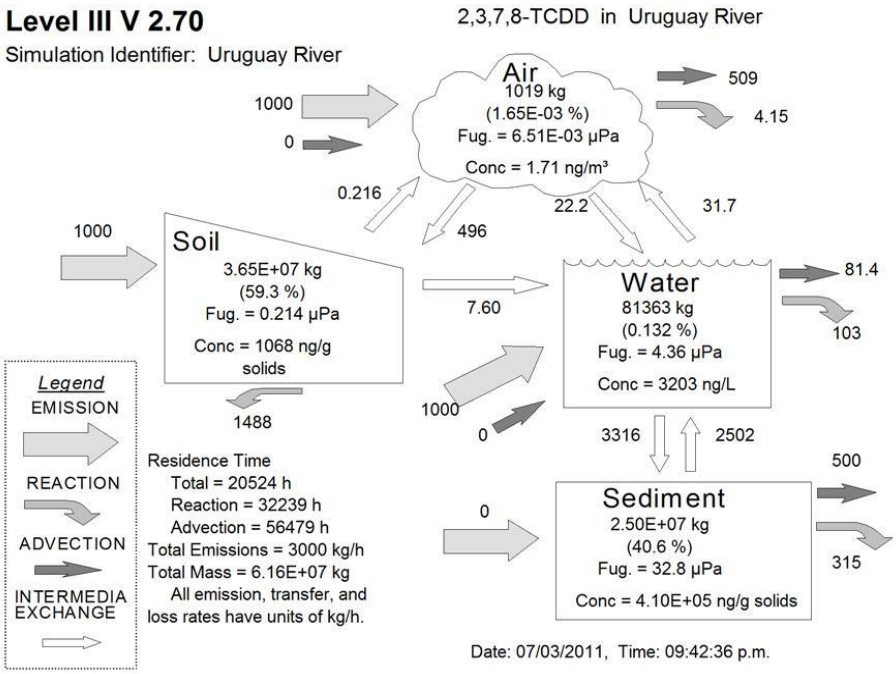


Figure 6-12 Simulated media processes por 2,3,7,8-TCDD

6.4.3 Exposure screening using ER- and AR-binding assays

A similar approach to the one proposed in this research at the screening level, was employed by Dutch researchers who developed an integrated RA in The Netherlands aquatic environment (Dick Vethaak et al. 2005). The effect based analysis aims at detecting ED effects due to mixtures of pollutants acting through similar mechanisms of toxic action, to produce a more reliable RA screening prior to relevant chemical analyses. By comparing the levels of xeno-oestrogens in the aquatic environment to effects thresholds found in the literature it is possible to develop a rapid appraisal to interpret the plausibility of local effects on fish due to EDCs.

The potency of various molecules relative to E2 were rated using the *in vitro* reporter assay ER-CALUX® (Oestrogen Receptor-mediated Chemical Activated LUciferase gene eXpression assay, Legler et al. 1999) (Table 6-3). The substance must be able to bind to the oestrogen receptor to be measurable by this method. This screening tool assesses effects both in wildlife as well as in humans as it is very sensitive and responsive for the oestrogen receptor binding. AR CALUX screen evaluated androgenic activity. Results for crude municipal wastewater were 34 ng l^{-1} EEQ and 73 ng l^{-1} DHT-AEQ (Table 5-5). As a comparison, the industrial effluent in the mentioned case had a median concentration of 0.267 ng l^{-1} EEQ, municipal wastewater: 0.09 ng l^{-1} EEQ and surface water: 0.021 ng l^{-1}EEQ.

6.4.4 Results of the exposure screening

This method was applied to screen the oestrogen activities of samples extracted from the river, from the main discharges (municipal and industrial) to the river and to drinking water and groundwater. The results are shown on Table 6-3.

Table 6-3 Oestrogen responses in surface water and main discharges to river

Date	Sample	ER CALUX® (ng l^{-1})
19/05/09	Drinking water intake in Uruguay River (R6)	ND
16/11/09		3.3
19/05/09	Buoy km 90, Gualeguaychú canal in Uruguay River (R9)	< 0.033
29/10/09	Fray Bentos stream receiving municipal wastewater, draining into Uruguay River (C2)	< 0.23
29/05/09		2.27
29/05/09	Pulp mill effluent, final discharge to river	0.096
08/12/09		ND
12/2009	Drinking water of Fray Bentos city taken at LATU, Unidad Fray Bentos Water laboratory	ND
12/2009	Groundwater at the pulp mill site	ND

ND: non-detectable; LD (ER CALUX): 0.024 ng l^{-1} as E2-EEQ

The androgenic activity in the same samples was evaluated using the AR CALUX test (Table 6-4).

Table 6-4 Androgen receptor binding in surface water and main discharges to river

Date	Sample	AR CALUX (ng l^{-1})
19/05/09	Water intake in Uruguay river (R6)	< 0.25
16/11/09		ND
19/05/09	Buoy km 90, Gualeguaychú canal in Uruguay river (R9)	ND
29/10/09	Fray Bentos stream receiving municipal wastewater drains into Uruguay River (C2)	ND
29/05/09		2.82
29/05/09	Pulp mill effluent, final discharge to river	< 0.47
08/12/09		< 0.89
12/2009	Drinking water of Fray Bentos city taken at Unidad Fray Bentos, LATU, laboratory	ND
12/2009	Groundwater at the pulp mill site	ND

ND: non-detectable; LD (AR CALUX): 0.69 ng l^{-1} as DHT AEQ

6.4.5 Discussion

The exposure screening performed using the ER CALUX test for oestrogenicity made it possible to determine if receptor-binders ligands existed in the main environmental matrices of consideration in the area. From these results one can see that even though drinking water and ground water have no detectable concentrations of EDCs, there remains a risk to human health if the potabilisation treatment process should fail, due to the appearance of some pulsed events of contamination in the river, especially after flooding (as during the spring 2009). The municipal discharge had higher oestrogen activity than the pulp mill effluent. However, this effluent was discharged at a flow of 0.8 m^3 s^{-1}.

6.5 Preliminary risk characterisation

Ecotoxicity testing is not necessary for very persistent and very bioaccumulating substances, because long-term effects from them can be anticipated (Gross et al. 2010). Therefore, no tests were designed for dioxins and furans, PCBs and PAHs with other mechanisms of action than binding to the oestrogen or androgen receptors. These compounds bind to the arylhydrocarbon receptor (AhR) with a potential for antioestrogenic activity (Navas & Segner 2006). A more refine analysis was done during Phase II considering concentrations before and after the pulp mill operations and tissue residues to assess doses for human health in a quantitative way. The PRA results are probable risks of oestrogenicity from urban wastewater especially after floods, due to oestrogenic activity screened by ERCALUX®. The pulp mill effluent also shows some oestrogenicity, but of a lesser extent. The decision was to proceed with the Quantitative Risk Assessment (Phase II) to further assess the risks of endocrine disruption and the possible causes to undertake risk management options.

6.5.1 Modelled risk quotients

The risk quotients (RQ) were calculated as the ratio between PEC to PNEC. An acceptable risk would be a RQ less than 1.Predicted media of concern for target EDCs are benthic invertebrates for nonylphenol and endosulfan and piscivorous fish for isopimaric acid and glyphosate, as estimated by Level III model (Figure 6-13).

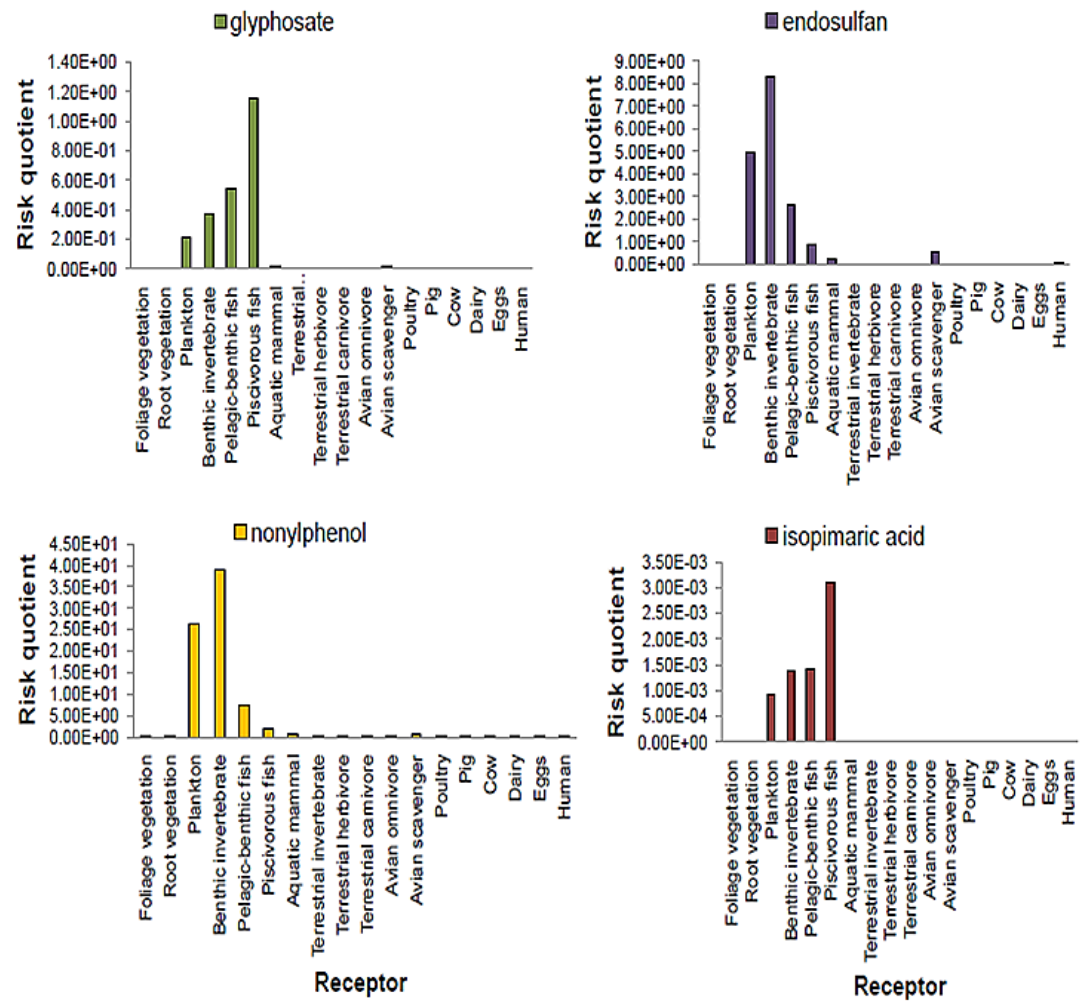


Figure 6-13 Risk quotients for receptors

6.6 Decision point

After the PRA, the decision to include other EDCs and to refine the study was taken to diminish uncertainties, as developed in Chapter 7.

7 EXPOSURE ASSESSMENT

This thesis proposes an alternative tiered approach for the exposure-effects assessment or dose-response assessment stages within an integrated risk framework of EDCs, supported upon mechanistic aspects of EDCs, combining *in vivo* bioassays and *in vitro* tests of significance in humans and in animals, to optimise the use of animals for experimentation. This methodology was suited to determine the dose-response and facilitated working in a logical order, with increasing specificity. The exposed or potentially populations were identified, linking effects assessment to exposure to calculate the doses of the prioritised EDCs.

7.1 Phase II. Quantitative risk assessment: general description

The design used in Phase II for exposure assessment is a tiered testing approach targeting at different mechanisms of action, multiple trophic levels and developmental stages keeping the iteration need for adding stressors and effects at a minimum, working with whole samples representing environmental complex mixtures and low doses.

One of the methodologies to assess the toxicity of complex environmental samples to account for synergistic or antagonistic interactions between the compounds inherently represented in the responses of the exposed organisms is the whole mixture approach (Kortenkamp et al. 2009). This method was proposed in Phase II, or refined risk assessment, as part of the aquatic exposure ecotoxicity testing of water and sediment fractions and in the tiered battery approach for exposure and effects using *in vivo* and *in vitro* bioassays and measuring the environmental concentrations of EDCs.

In the case of POPs with similar toxicity polychlorinated dioxins and furans (PCDD/F) and PAHs, the TEQ approach for component mixtures will be used for toxicity predictions for mixtures RA of ED. The approach also complies with the concepts of the one delineated by Teuschler et al. (2004).

As a possible risk for ED unrelated to only steroid hormones is found through the *in vitro* screen, other molecules could also be responsible of the effects. A deeper study of the sources, pathways and receptors was completed, and experimentally demonstrated. The emission sources and “compounds of potential concern” were identified as target chemicals to measure and were included in the list of prioritised EDCs (Table 6-2).

Information was not complete on toxicity in special concerning the ED endpoints to feed the posterior analysis stages. This was the case of 2,4,5-TCP, a potential stressor from the pulp mill effluent, with very scarce available information. The modeling indicated that this EDC concentrates partly in sediment (Section 6.4.1) Therefore, the experimental design included measuring bioavailability, exposure and toxicity from the sediment-water pathways in two potential scenarios: the natural conditions and the worse-case scenario by spiking sediments with a known concentration.

7.2 Tiered approach

All the species for experimentation for exposure assessment were obtained from cultures at the Water and Chemicals Department, Technological Laboratory of Uruguay. Images of the organisms used to perform the bioassays in our laboratory are presented in Appendix B.

In the experimental design, the analysis are set in parallel tiers of EDCs concentrations and toxic effects of increasing specificity to finally confirm endocrine disruption through the application of *in vivo* bioassays with specific biomarkers. This methodology was applied to assess the risks of EDCs to human and the environment at the watershed level in a reach of the Uruguay River, where domestic, industrial and agricultural potential sources of EDCs are present.

The lowest tier consists on an evaluation of the river health through *Daphnia magna* and *Pimephales promelas* acute toxicity tests and physicochemical analysis. The middle tier measures aggregate components, including adsorbable organic halogens (AOX) and phenols. Effects on invertebrate reproduction were assessed with the *Ceriodaphnia dubia* three brood chronic toxicity test and growth effects by the whole sediment *Hyalella curvispina* amphipod test. The highest tier is the most specific as the concentration of target EDCs ranging from steroid hormones to wood extractives, chlorophenols and NP are measured and effects demonstrated through *in vitro* tests, by the ER-and AR-binding activities (ERCALUX®, ARCALUX® and YES) in river water, drinking water and in municipal wastewater and industrial pulp mill effluents. The confirmatory test to be used is the 21 days *Pimephales promelas* fish reproduction test with end-points VTG, histology and gonadosomatic index (GSI) applied to wastewater and effluent.

The schematic description for the exposure and effects assessment of ED applied is shown in Figure 7-1:

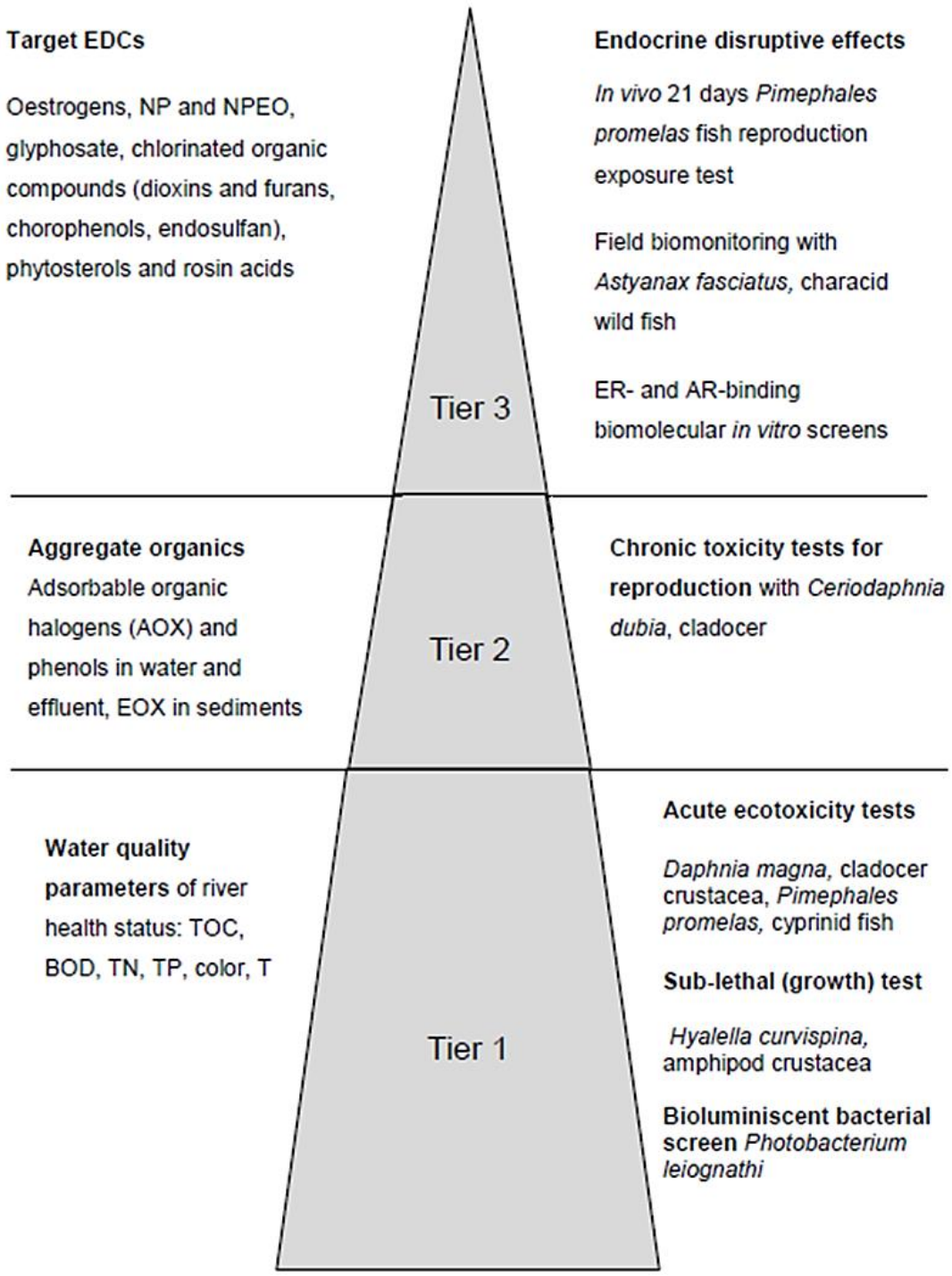


Figure 7-1 Tier methodology for Phase II exposure assessment

7.3 Tier 1

The European Water Framework Directive (WFD) classifies waters as “high”, “good”, “fair”, “poor” or “bad” *status* based on ecological, chemical and hydrological criteria (EU Directive 2000/60/EC, WFD, 1.2), differing for each river basin and strongly relying on the application of bioassays. Background levels should be specified for non-synthetic pollutants, and special provisions exist for specific synthetic pollutants (Directive 91/414/EC and Directive 98/8/EC on plant protection products and biocides, respectively).

The Uruguayan water quality standard (Decree 253/79, 1979) classifies water into four categories according to intended uses. The People´s Republic of China (2002) also rates waters suitable as drinking water source from 1 to 3, but with a total of 5 categories (Standard GB3838-2002). This latter set of criteria was used to rate water types based on our physicochemical analysis (Table 7-1).

Table 7-1 Water quality classification

Type	**Quality and possible uses**	PI	**COD**	**BOD**	**DO**	**TP**	**N**
		as O2				**as P**	**as N**
		(mg l^{-1})					
1	Source for drinking water, pristine	2	15	3	7.5	0.02	0.2
2	Source for drinking water and aquatic biota protection	4	15	3	6	0.1	0.5
3	Swimming, fisheries and aquaculture, drinking water source, crop and orchard irrigation	6	20	4	5	0.2	1.0
4	Industrial and recreation not for direct contact	10	30	6	3	0.3	1.5
5	Landscape	15	40	10	2	0.4	2.0

7.3.1 Physicochemical analysis

Physicochemical parameters chosen as markers of organic compounds with grading criteria were: permanganate index (PI), chemical oxygen demand

(COD), biochemical oxygen demand (BOD_7) and dissolved oxygen (DO). Data processing of chemicals was accomplished through the application of artificial neuron networks (ANN) as classifiers, to address the difficulties represented by possible local variability of this large river. The dataset of organic matter parameters (was trained in the Multi Layer Perceptron (MLP) ANN with the concentrations measured at R1as continuous input, and water types as categorical inputs. The exponential distribution was chosen to represent the dataset. In this condition, the train performance obtained was 100%. The resulting network name was 4-MLP16-3-3, and the training algorithm: Broyden-Fletcher-Goldfarb-Shanno (BFGS-1). The error function was calculated by the SOS method. The hidden and output activation nodes are assumed exponentially distributed.

Three-hidden layer unsupervised neural networks were constructed for computation of the river water nutrients total nitrogen (TN) and total phosphorus (MLP 8-3-3), trained with the BFGS-1 algorithm, error function entropy, hidden activation tanh, output activation softmax, training and testing performance of 100%. For prediction of time series the Kohonen learning algorithm was used (Kohonen & Honkela 2007).

Four sampling stations were monitored monthly from May 2005 to August 2010 to evaluate differences in water quality at a geographical scale. Data of river flow as well as physicochemical parameters (pH, water temperatures, color, suspended solids, NH4-N, NO2-N, NO3-N, dissolved oxygen (DO), chemical oxygen demand (COD), permanganate index, biochemical oxygen demand (BOD5), total phosphorus and phosphate concentration) were evaluated.

The mean water quality in all sites according to N classified as type 4, as the criteria was 75% percentile in the Gaussian curve, but data points sometimes exceeded this type for all of the sampling sites (Figure 7-2). In the case of TP, the distribution is not normal, but the classification was type 3, with some infrequent events exceeding as type 5 for R8.

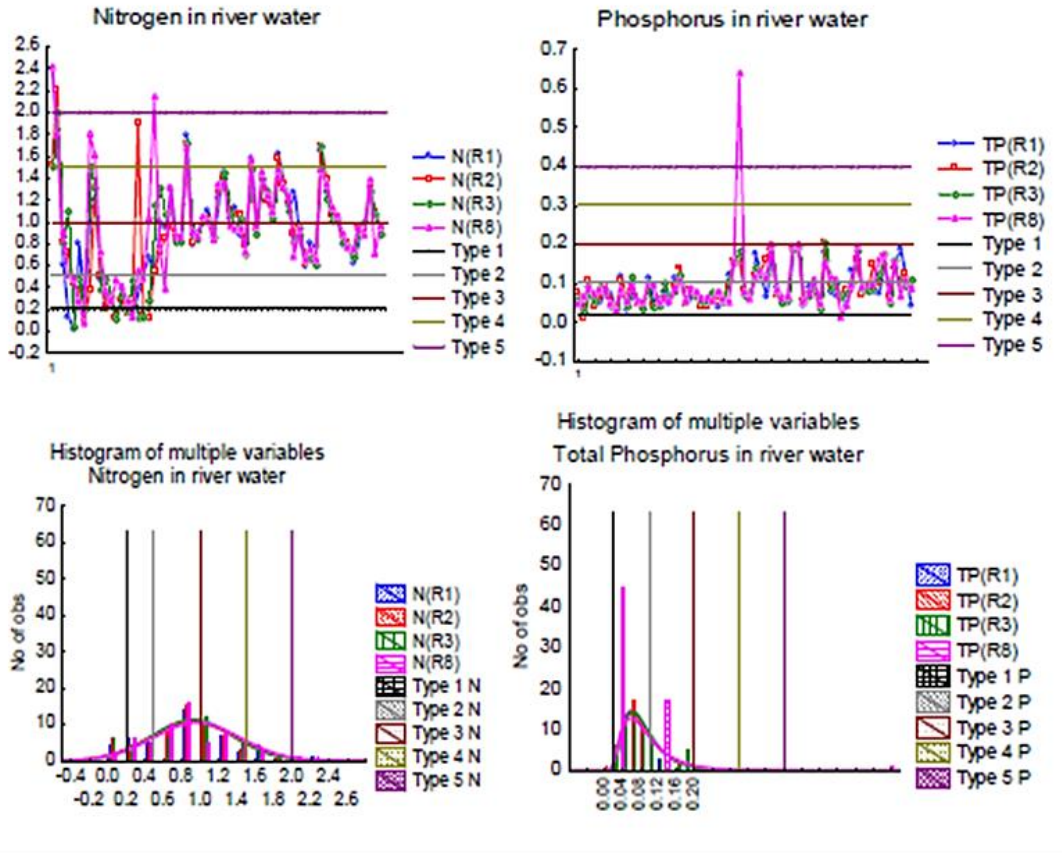


Figure 7-2 Nutrients in river water

The normality of the dataset was tested for N and TP. Total Phosphorus was not normal, but N complied with the criteria of the tests (pvalue>0.05) (K-S-p >0.20; Lilliefors-p >0.20). Background levels were set with the historical values of with analysis data points since 2005 (R1, R2, R3 and R8) for comparison against sites R4, R5, R6 and R7.

7.3.2 Aquatic toxicity tests

The potential exposure of organisms to chemicals can be studied by ecotoxicity methods that explore the individual differences in uptake, metabolism, and excretion, mechanism of action, habitats and ecosystem interrelationships of the biota (Persoone & Gilleitt 1990), recognising that the best way to represent ecosystem functions and toxicity at various trophic levels is through multi-species studies, after preliminary single species methods.

7.3.2.1 Acute toxicity test

The acute toxicity tests with *Daphnia magn*a (cladocer crustacean) were applied since 2005 monthly to river water, with results of LC50 >100% (not acutely toxic). Specimens were continuosly maintained in our laboratory since 1999. Both stock and experimental animals were kept in 2L glass beakers at 20ºC $\pm$ 1ºC on a (16 light: 8 dark) photoperiod with approximately 800 lux light intensity. All stock cultures were fed a diet of yeast and *Pseudokirchneriella subcapitata*, formerly known as *Selenastrum capricornutum* algae culture, grown in a Bold basal medium under sterile conditions. This test was also applied monthly since October 2007, to secondary treated pulp mill effluent, as well as the *Pimephales promelas* fish acute toxicity test, not revealing any acute toxicity in these matrixes by any of both bioassays.

7.3.3 Sediment toxicity tests

A set of bacterial screens and crustacean bioassays were performed.

7.3.3.1 Toxicity screening

A toxicity screening for metals and organic compounds was carried out with bioluminescent bacteria applied to the sediment elutriates. As described by Hastings & Nealson in 1979. *Photobacterium leiognathi* is a marine bioluminescent bacterium that lives in warm tropical waters in symbiosis with the pony fish.The ability to inhibit luminescence in the presence of toxic pollutants has been used to develop a commercial test kit (CheckLight®

ToxScreen III). It also discriminates between the presence of organic and metallic toxicants by the addition of two buffers that used in parallel enhance susceptibility to organic pollutants, and to heavy metals. The test was carried out in cuvettes, and luminescence measured using a luminometer after incubation (60 min at ambient temperature). The result was expressed as the percentage of incubation medium made up by the original water sample that produces 50% inhibition of luminescence. Any result where the threshold concentration of the original water sample is 50% or less of the total incubation medium is taken as toxicity (Roig et al. 2007).

The toxicity results in sediment elutriates, as IC50 after 30 minutes for metal toxicity and organics using this test kit were: at Nuevo Berlín (S1):19 and 37%, at Fray Bentos (S2): 18% and Las Cañas (S8): 35 and 30 %, respectively.

7.3.3.2 Sub-lethal toxicity: growth

The sub-lethal toxicity survival and growth test with the amphipod *Hyalella curvispina* was developed on whole sediment samples finding significant differences in the average specimen dry weight in the three tested samples (S1, S2 and S8) compared to the control (ANOVA, p=0.023, 95% confidence level). The differences in means were evaluated for each pair of data by the multiple ranges test, Fisher´s least significant difference (LSD) test, at 5% significance.

7.4 Tier 2

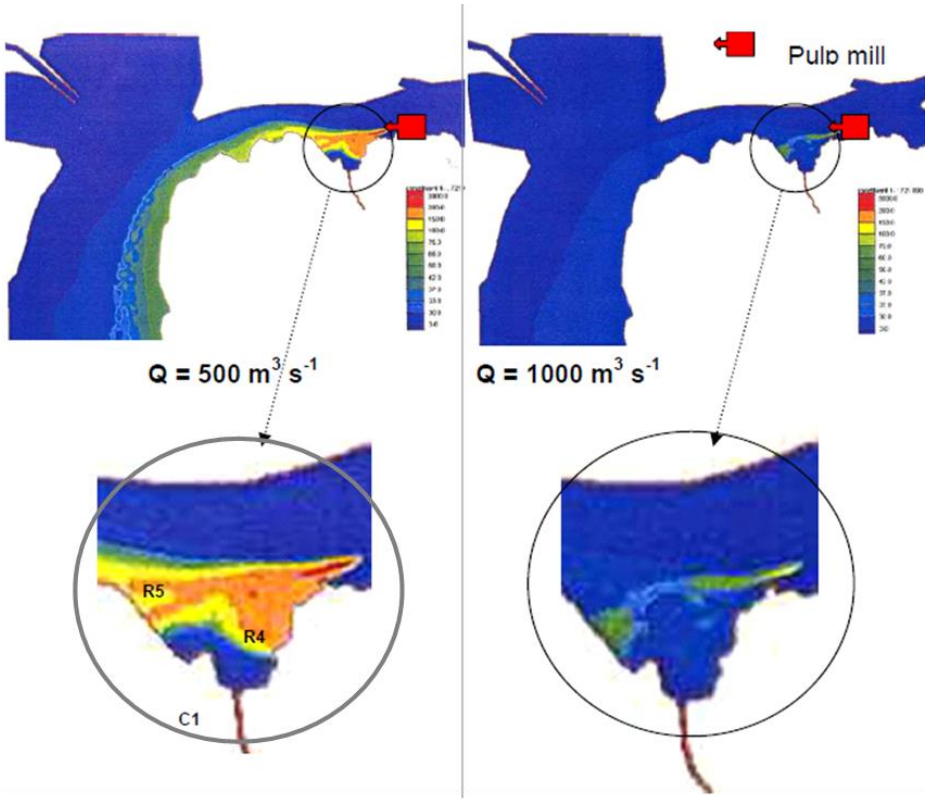
Both chemistry and ecotoxicity components were evaluated.

7.4.1 Chemistry of non- specific determinands markers of contamination

The chemical component aimed at evaluating the dispersion of chemical markers of chlorinated organic compounds and phenols, probably present in point sources by comparing the concentrations at upstream and downstream sites, focusing near the drinking water intake and the fishing sites for human

health and environmental protection. Ecotoxicity tests of survival, growth and reproduction end points with ecological relevance were included.

Different frequency and severity of exposure to the effluent outfall were expected even at locations very proximate to the plant based on the effluent plume isoconcentration model (Figure 7-3). The possibility of tracking it downstream the mill discharge by measuring conductivity variations as a surrogate of a tracer was explored, based on its high conductivity (339 $\pm$ 35 mS m^{-1}), that could modify local conductivity at near-field sites. Contaminant dilutions, at 500 and 1000 m^3 s^{-1} are approximately 2.7 x 10^6 in Yaguareté Bay (R4), while at Ubici Bay (R5), they are around 900 and 600 m^3 s^{-1} respectively. The low flow condition would be the worst-case scenario for the drinking water intake (R6) too, as the dilution decreases from around 2000 to 400 from 1000 to 500 m^3 s^{-1}. Specific sets of measurements were taken to assess differences. A site in the middle of the river stream (R2) was measured, at Yaguareté bay (R4) and Ubici Beach (R5). Another set was obtained at Yaguareté stream (C1). Readings of wind direction and wind velocity related to the sampling moment were used to evaluate if there could be influence of Yaguareté stream (C1) on R4 conductivity as it drained into the bay.



Redrawn after DINAMA (http://www.dinama.gub.uy, Evaluación de impacto ambiental, expediente 2004/14001/1/01177)

Figure 7-3 Iso-concentration maps at two flow conditions at R4 and R5

7.4.1.1 Comparison to background levels

Additional river water samples were extracted to test for phenols and AOX and sediments were dredged to analyse EOX and phenols. A dataset of 64 points of each parameter was analysed monthly from May 2005 to August 2010 (temperature, conductivity, pH, turbidity, AOX, and phenols) at four sampling sites. Non-detectable data were considered as zero and those below quantification limit converted to 75% of the limit. The conductivity trends at four sites in the Uruguay River show punctual differences near the pulp mill start-up with higher values at R2, R3 and R8 by comparison to R1. Box plots of mean values before and after October 2007 show a decrease in conductivity in general in every site after the time chosen for comparison (Tables 7-2 and Figure 7-4).

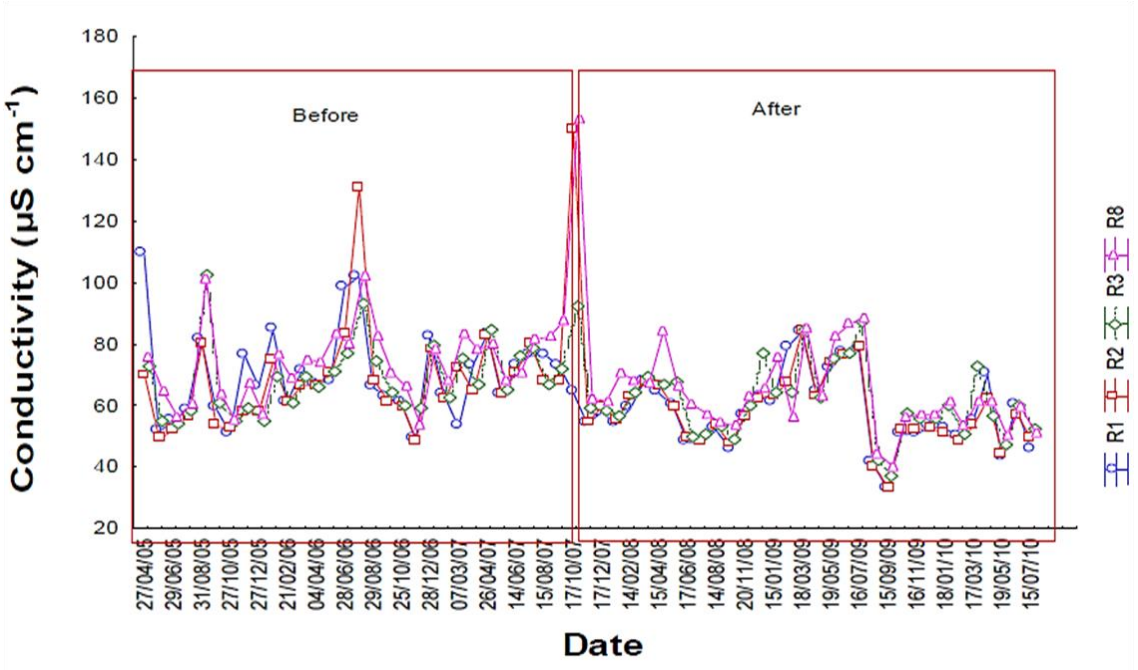


Figure 7-4 Mean conductivity variations before and after October 2007 at four sites in Uruguay River

To assess differences in more detail, specific sets of measurements were undertaken. A site in the middle of the river stream (R2) was measured, and at Yaguareté bay (R4) and Ubici Beach (R5). Another set was obtained at Yaguareté stream (C1). Readings of wind direction and wind velocity related to the sampling moment were used to evaluate if there could be influence of Yaguareté stream (C1) on R4 conductivity as it drained into the bay.

Replicate *in situ* measurements (from 2 to 30) of conductivity, temperature, pH and turbidity were performed at 0.5 and 1 m depths using a multiparameter probe YSI model 6600 V2 coupled to YSI MDS 650 datalogger.Additional samples were extracted to test for phenols and AOX and samples were dredged to analyse EOX and phenols in sediments. The differences in conductivity are evident among sites; R5 was the highest, during July campaign (Table 7-3).

Table 7-2 In situ measurements at Yaguareté Bay and Ubici Beach

Site	Temperature (º C)	Conductivity (µS cm^{-1})	DO (mg l^{-1})	pH	Turbidity (FTU)
Date: 27/04/2011 Wind direction: 21º NNE Wind speed: 2.8 m s^{-1} Minor influence at the beach					
R5	21.40	76.40	---	7.54	24.46
R4	21.50	70.70	---	7.11	23.8
Date: 31/05/2011 Wind direction: 73º NE Wind speed: 0.2 m s^{-1} Minor influence at the beach					
R5	16.7	81.9	9.2	7.70	28.9
SD	0.045	0.25	0.047	0.100	1.127
R4	15.9	96.0	8.45	7.59	30.1
SD	0.004	0.0	0.116	0.048	4.137
R2	16.27	77.00	8.4	7.42	31.05
SD	0.007	0.000	0.177	0.023	0.9
C1	14.8	195	8.1	7.5	29.6
SD	0.055	0.38	0.24	0.012	4.172
Date: 21/06/2011 Wind direction: 110º ESE Wind speed 4.5 m s^{-1}Possibly at the bay					
R5	14.7	82.2	9.5	7.91	21.8
SD	0.028	0.4	0.015	0.040	0.84
R4	14.7	77.0	95.5	7.87	22.1
SD	0.014	0.0	1.4	0.015	0.55
R2	15.2	72.0	9.5	7.80	22.5
SD	0.29	0.000	0.011	0.032	0.8
C1	14.7	465	7.4	7.68	11.4
SD	0.313	0.0	0.039	0.056	0.545
Date: 26/07/2011 Wind direction: 245º WSW Wind speed m s^{-1} No influence					
R5	14.8	**130**	9.70	7.30	31.9
SD	0.008	53.1	0.000	0.031	1.13
R4	14.7	77.0	95.5	7.87	22.14
SD	0.014	0.000	1.4	0.015	0.554
R2	14.7	99.0	10.14	7.83	53.46
SD	0.009	0.000	0.370	0.191	30.7
C1	13.83	594	7.8	7.57	113.07
SD	0.016	0.000	0.042	0.029	0.531

Table 7-3 Median and range values of temperature, colour, turbidity, conductivity, pH, AOX, phenols at four sites of the Uruguay River

Site		R1	R2	R3	R8
Temperature (°C)	Median	19.8	19.9	19.7	20
	Range	(10.9, 32.5)	(11.2, 31.5)	(11.1, 30.7)	(10.7, 30.3)
Colour (FTU)	Median	70	70	70	70
	Range	(70,210)	(25,170)	(30,165)	(30, 160)
Conductivity (µS cm^{-1})	Median	67.5	67	68.3	74.2
	Range	(49.0, 109)	(48.0, 149)	(53.9, 103)	(53.0, 153)
pH	Median	7.59	7.59	7.51	7.60
	Range	(6.64, 8.77)	(6.74, 9.14)	(6.78, 8.98)	(6.59, 9.19)
Turbidity (FTU)	Median	21.5	22.0	21.0	20.5
	Range	(6.9, 47)	(7.1, 48)	(6.9, 42)	(7.2, 36)
AOX (as Cl)(µg l^{-1}) (LOD: 1 µg l^{-1})	Median	ND	ND	ND	ND
	Range	(0, 19)	(0, 25)	(0, 13)	(0, 11)
Phenols (as C_6H_5OH) (µg l^{-1}) (LOD: 1 µg l^{-1})	Median	< 1	< 1	< 1	< 1
	Range	(0, 6.2)	(0, 6.2)	(0, 4.9)	(0, 5.6)

ND: non-detectable; LOD: limit of detection

A relative increasing tendency is observed in the data points of colour and conductivity after October 2007, from R1 to R8 (Figure 7-5).

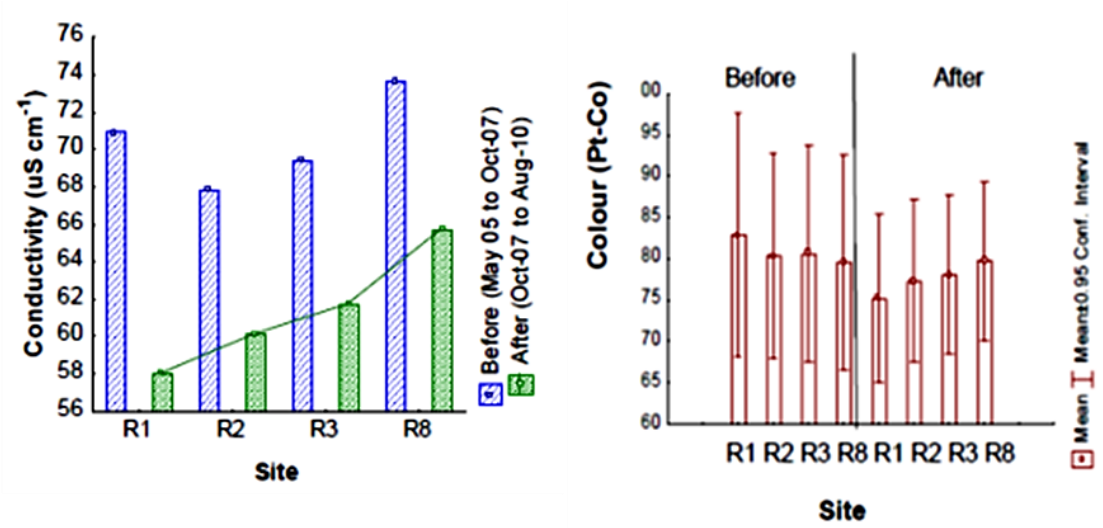


Figure 7-5 Conductivity and colour of river water before and after October 2007

An ANOVA test was performed for comparison of variances with the two tailed Levene´s and Bartlett´s tests resulting in identical variances ($p < 0.05$). The Kruskal-Wallis test was applied resulting that the samples came from different populations (p-value (two tailed) = 0.001), $p < 0.05$). X charts for conductivity fluctuations were then plot, with differences in the margins of variation at each site, but in the same order of magnitude. This allowed comparing the values prevalent at R4 and R5 to assess risks to aquatic biota and fish tainting at the nearest sites from the pulp mill, and at R6, to assess human health risks from drinking water. Minimum values of R6 conductivity lied within the reference site limits (R1) but the maximum was slightly over the limit (Figure 7-6).

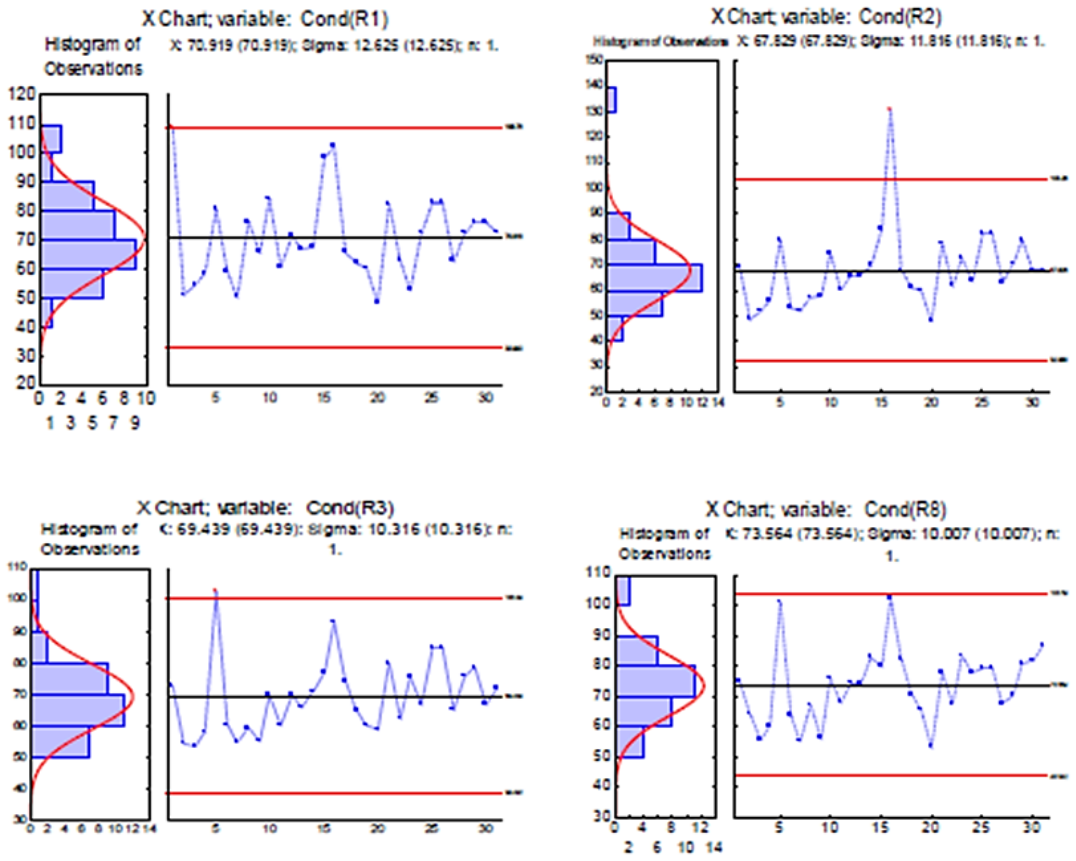


Figure 7-6 X charts with control limits of conductivity variations of river water from sites R1, R2, R3 and R8

Table 7-4 Conductivity variation

Site	Conductivity (µS cm^{-1})			
	Minimum	Maximum	Mean	Std. deviation
R1	33.0	108.8	64.5	29.1
R2	31.7	103.3	64.0	29.1
R3	33.6	100.4	65.6	26.8
R6	53.0	110.0	77.4	25.1
R8	33.9	103.6	69.6	28.0

The conductivity trends were also predicted with the simulation model (@Risk) using Latin Hypercube sampling, with 1000 iterations Monte Carlo random numbers. The best distribution was fit to each dataset (Figure 7-7).

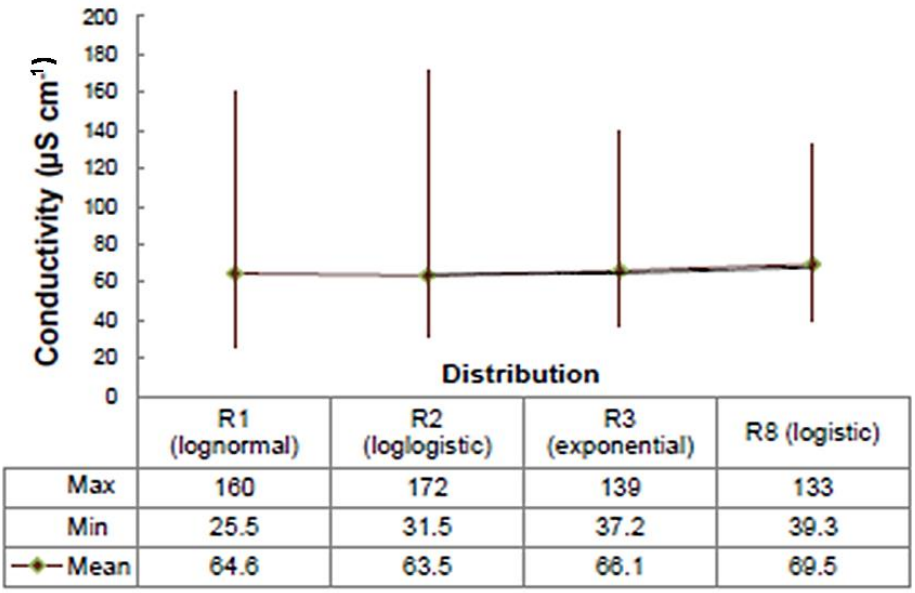


Figure 7-7 Conductivity trends modelled at four sites in the river (R1, R2, R3 and R8)

The Kohonen 1000 algorithm was used in the 1-SOFM 10-6 artificial neural network to evaluate differences in conductivity per site and date (Figure 7-8).

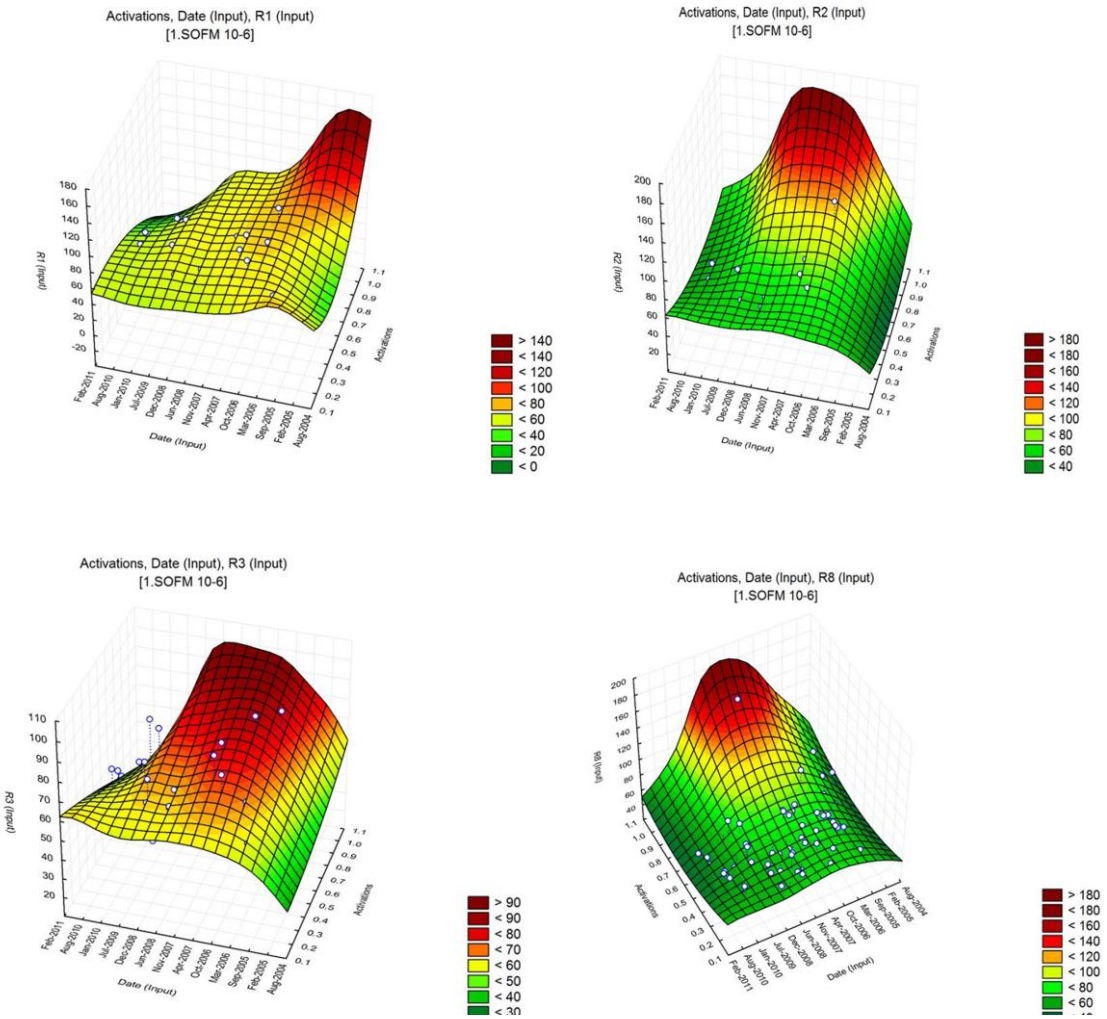


Figure 7-8 Artificial Neuron Network 3D plot of conductivity at four sites in the river

The correlation among factors was explored using multivariate factor analysis represented as 3D quadratic surface diagrams, revealing the highest responses among factors at sites R2 and R8 (Figure 7-9).

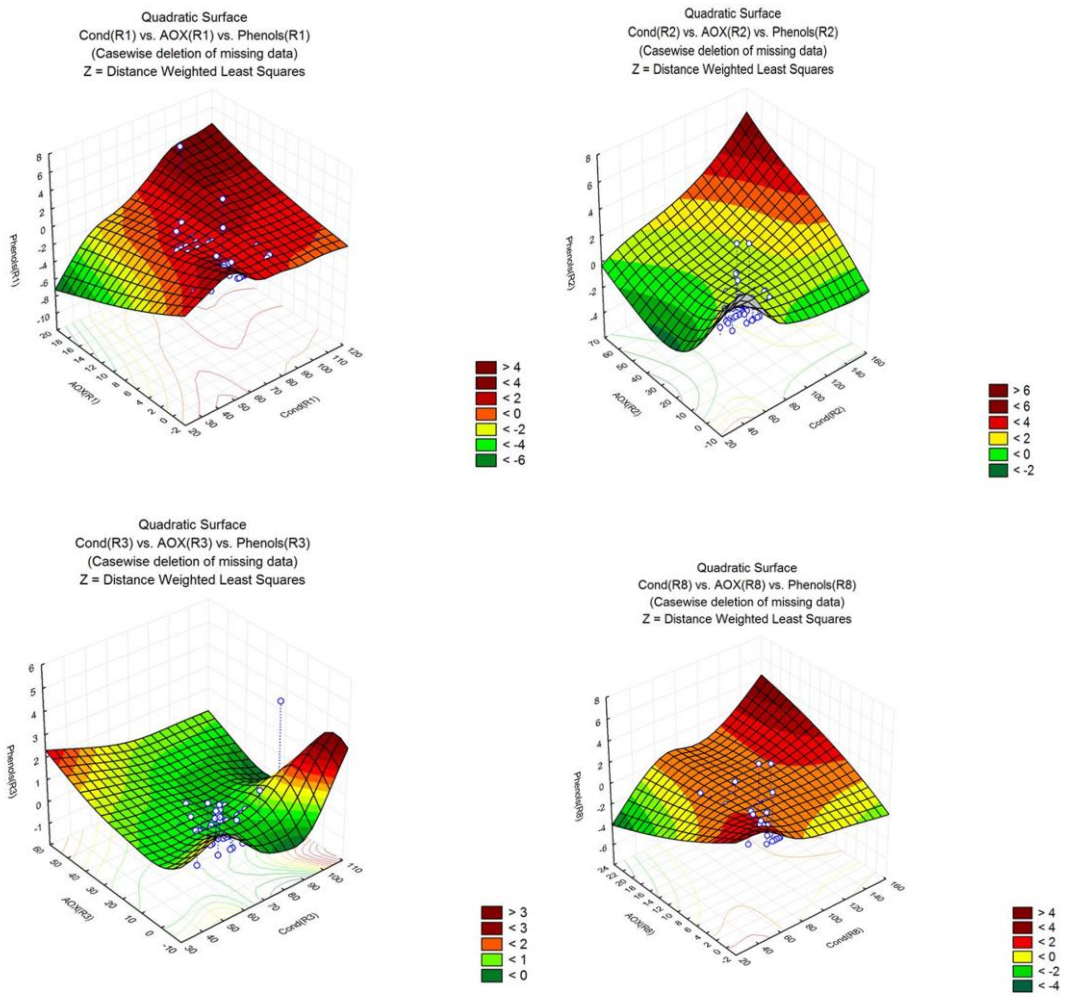
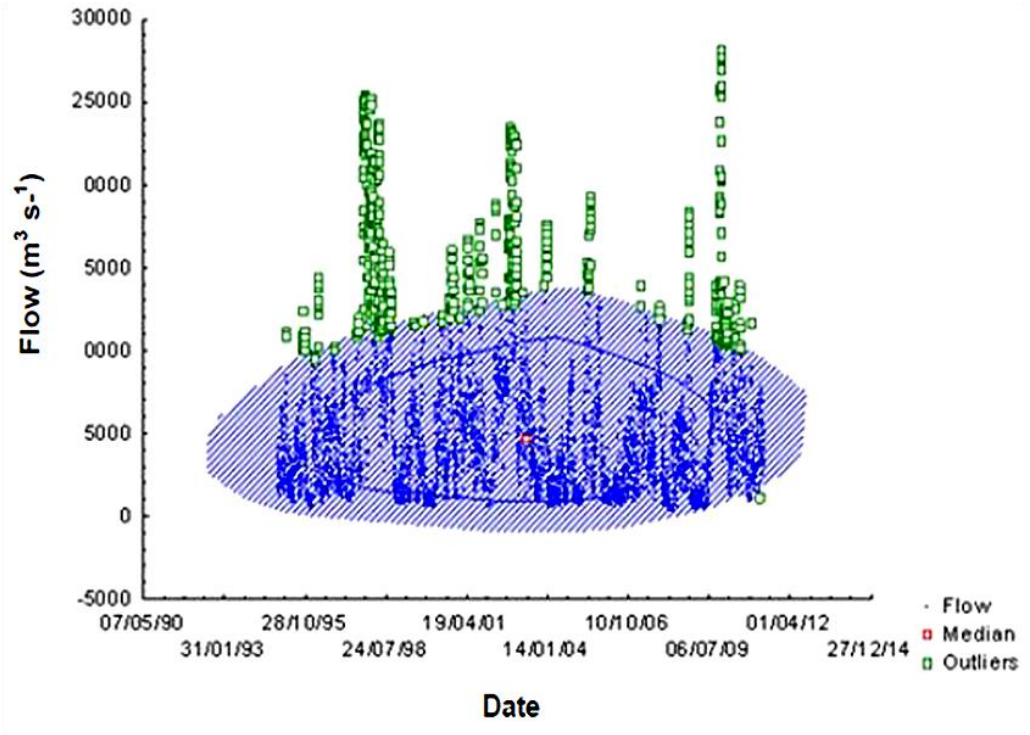


Figure 7-9 Correlation among AOX, phenols and conductivity at four sites

7.4.1.2 Flow regime and probability of flow conditions

Flow values were fit to the exponential-Weibull distribution, n = 6022, with @Risk. Flow: minimum: 306 m^3 s^{-1}; maximum: 22977 m^3 s^{-1}, mean: 4894 m^3 s^{-1}. Then, the hazard function for maximum likelihood was calculated to evaluate how often these conditions would be expected using STATISTICA ver. 8, resulting that 0.0056 is the likelihood of occurrence of 500 m^3 s^{-1} , while 0.0073 for 1000 m^3 s^{-1} flow (Figure 7-10).



Data granted by Dirección Nacional de Hidrografía, Salto Grande

Figure 7-10 River flow conditions from 1995-2011

As it can be observed the probability of a river flow condition of 4500 m^3 s^{-1} is 0.55, while for the low flow condition it is around 0.13. Under these two scenarios, the dilution differs among sites (Figure 7-11).

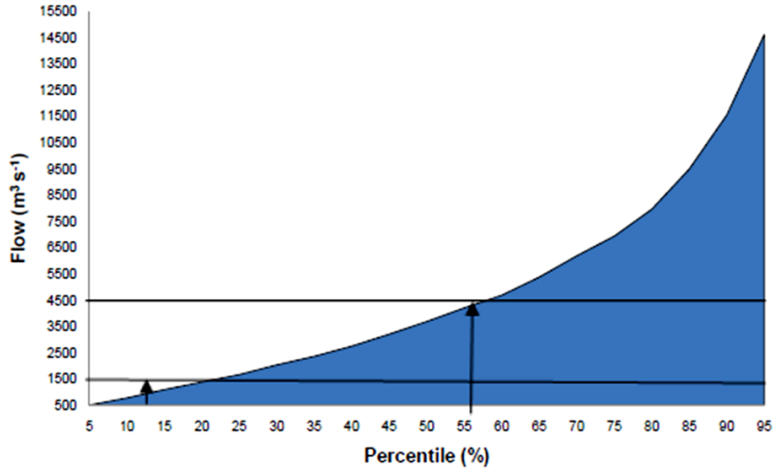
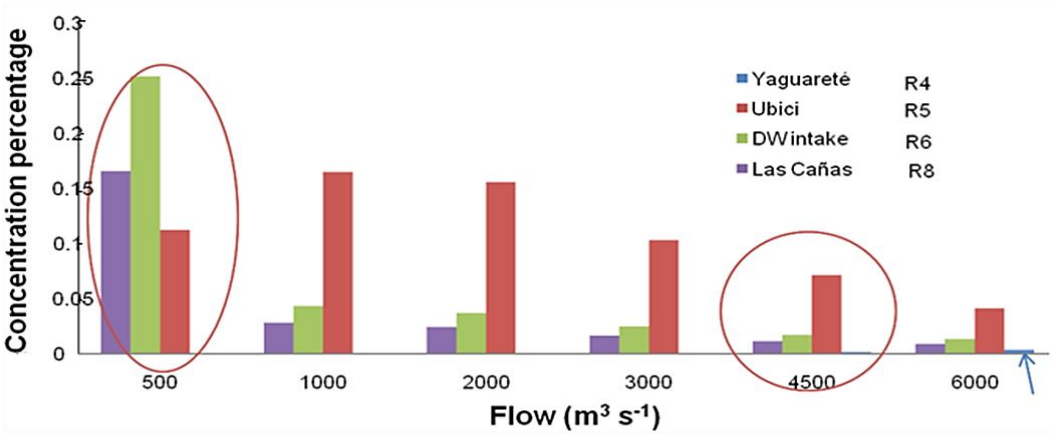


Figure 7-11 Weibull distribution of river flow

The probability of finding contamination from the water route is negligible for Yaguareté Bay (R2), with exception to air transport and contamination from Yaguareté Stream(C1) cannot be excluded. At Ubici Beach (R4) the concentrations are higher than at other sites at average flow, while at high flow they are higher at the drinking water intake (R6) (Figure 7-12).



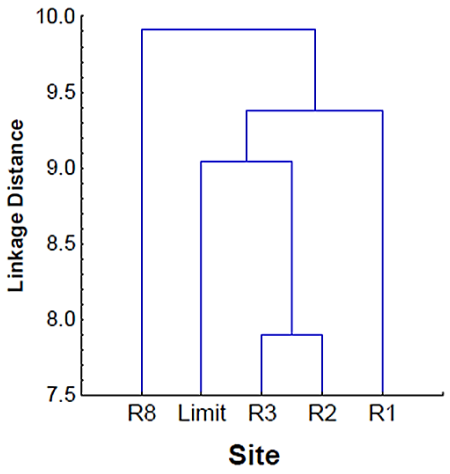
Plotted with data extracted from the environmental impact assessment, technical report

Figure 7-12 Scenarios of flow and dilutions of a tracer of initial concentration at the diffuser of 30 mg l^{-1}

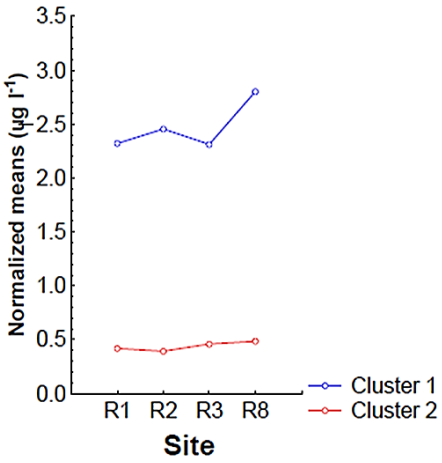
7.4.1.3 Cluster analysis of adsorbable organic halogens and phenols

The freshwater aquatic life guideline limit for phenols is 4 µg l^{-1} (CCME 1999), and the human health limit for drinking water adopted by DINAMA is 1 µg l^{-1}.

Clustering analysis with the EM (expectation maximisation) algorithm classified the probabilities distributions. Two concentration clusters were determined for phenols (Figure 7-13).



Tree diagram, five variables
Clustering with the EM algorithm
Single linkage
Euclidean distances



Graph of means for continuous variables

Figure 7-13 Cluster analysis of phenols with the EM algorithm

Sites were categorised in pair groups separated by Euclidean distances. Site R8 had the highest phenols concentrations since 2005.

The frequencies in phenols above guideline limits were different at the sites downstream the plant (Table 7-5).

Table 7-5 Frequency of phenols concentration exceeding the guideline limit

Site	Frequency of phenols concentration exceeding 1 µg l^{-1} limit (%) Before and after October 2007	
	before	after
R1	6.25	6.25
R2	4.69	12.5
R3	3.12	15.6
R8	9.45	14.1

Prediction bands, for a confidence level of 0.95, show that site R8 might exceed while R2 and R3 are at the limit for environmental protection. The limit for human health is exceeded in all cases but for R1 (Figure 7-14).

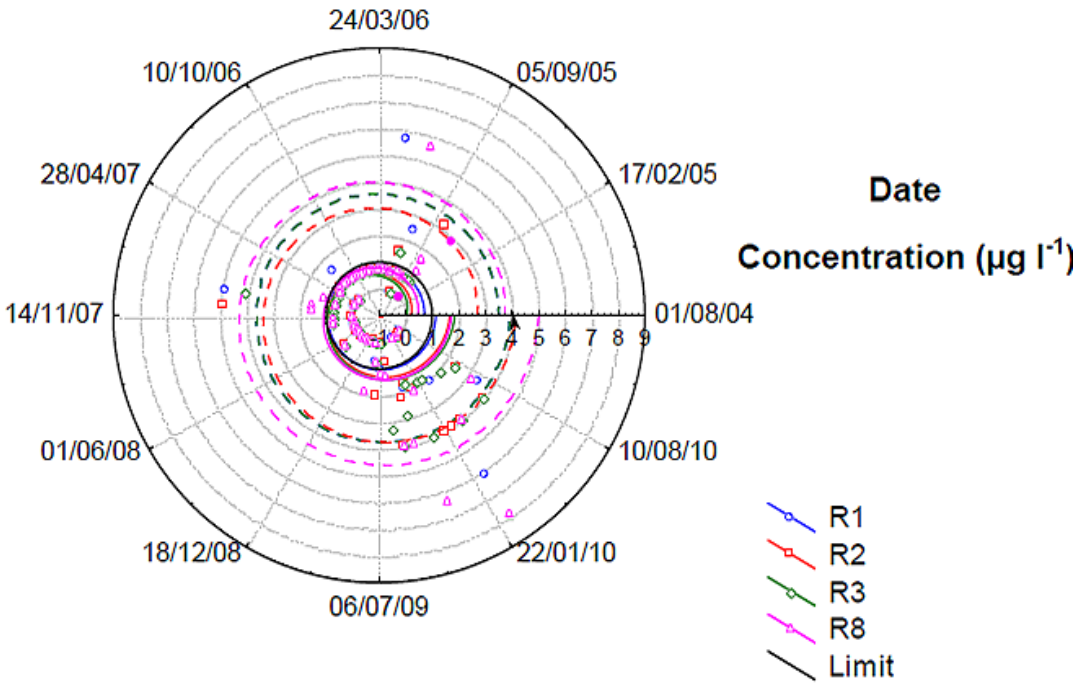


Figure 7-14 Variation of phenols since 2005

Phenols concentrations per site during all the period of analysis are shown in Figure 7-15. Their variation before and after the pulp mill excluding extreme events (from May 2008 to May 2009), for those sites with historical data show that at R5, phenols concentrations were above those measured at any of the other sites, even when compared with historical values.

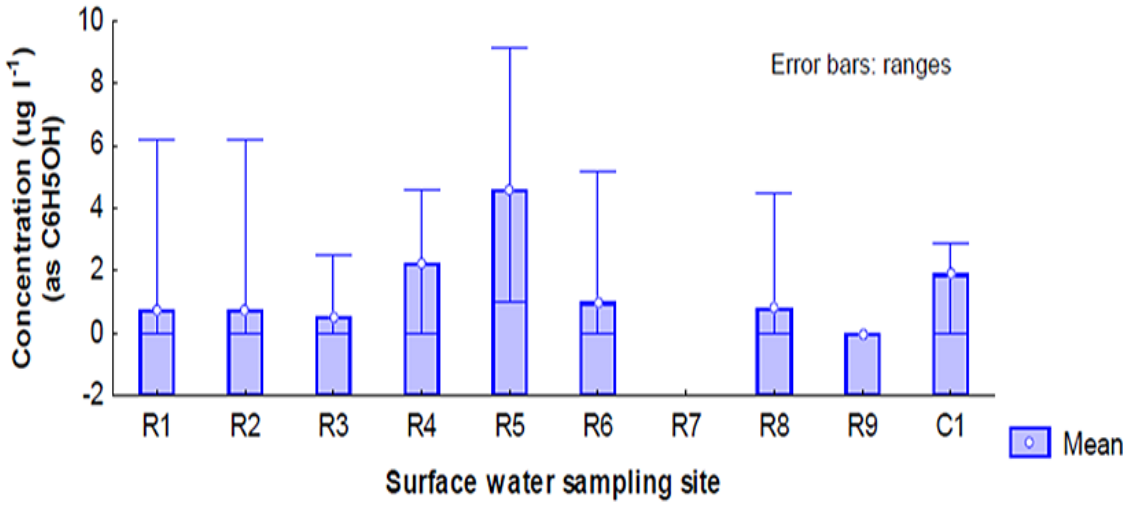


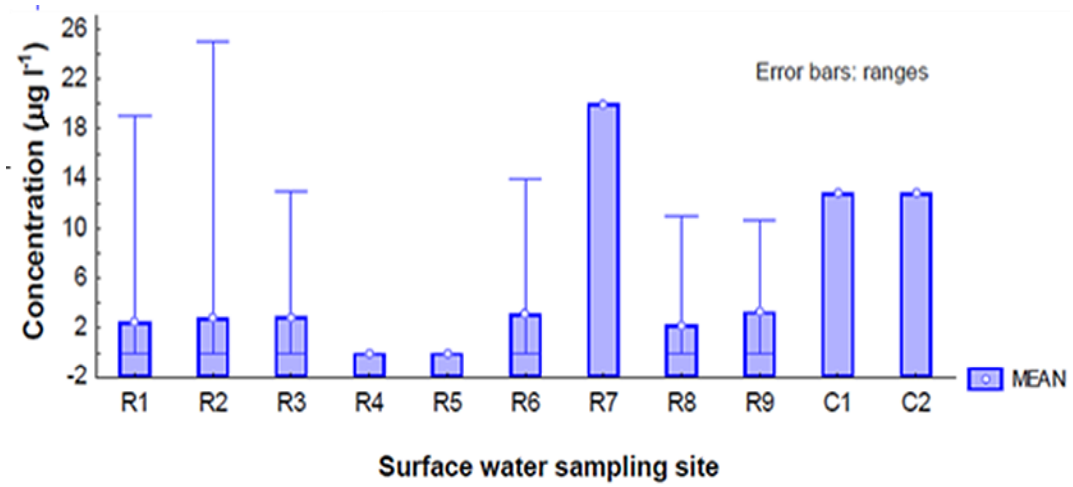
Figure 7-15 Phenols in river water

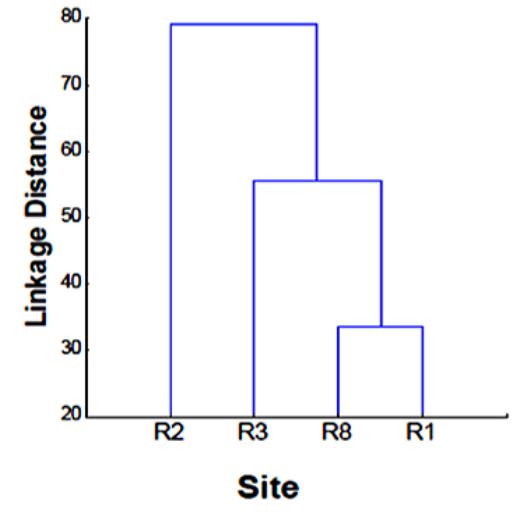
Adsorbable organic halides (AOX) are indicative of chlorinated organic compounds (PCBs, brominated and chlorinated pesticides and herbicides, chlorinated aromatic compounds and partly chlorinated humic substances, among others.

The limit of 20 µg l^{-1} was exceeded at Anglo Beach (R7) (maximum measured concentration: 27 µg l^{-1}), and also at R2 (Bridge), with a maximum of 22 µg l^{-1} (Figure 7-16).

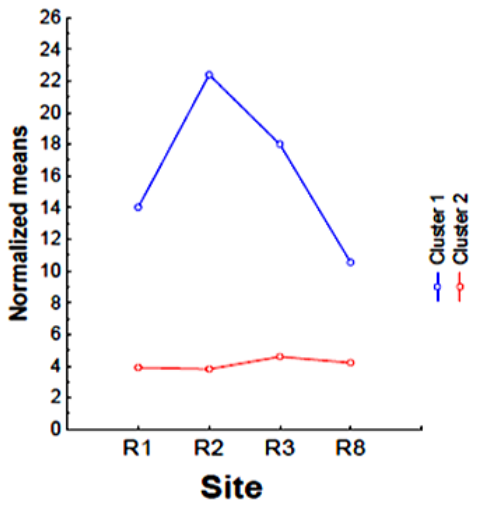
Figure 7-16 AOX in river and streams

The maximum value for cluster 1 corresponds to R2 (Figure 7-17).





Tree diagram, four variables
Unweighted pair-group average
Euclidean distances



Clustering with the EM algorithm

Figure 7-17 Cluster analysis of AOX with the EM algorithm

7.4.2 Ecotoxicity tests

The three brood *Ceriodaphnia dubia* chronic test with reproduction end-point was applied to effluents, elutriates, to the most probable site in the river according to the hydrodynamic study (Ubici, R4), and to Fray Bentos stream (C2) as it receives municipal wastewater. No acute nor chronic toxicity was evidenced in stream water or river water, but sediment elutriates showed chronic effects in reproduction at S1 and S8 (Table 7-6).

Table 7-6 *Ceriodaphnia dubia* three brood toxicity test results for river water, river sediment elutriates and stream water

Site	Chronic toxicity	Acute toxicity	LC50(48h)
	IC25 (%)	LC50(three brood end-point)	
S1	20.3 (12.5, 25.0)	> 50	> 50
S2	> 50	> 50	> 50
S8	27.0 (18.4, 38.0)	40.3 (29.7, 54.8)	> 50
R5	> 50	> 50	> 50
C2	> 50	> 50	> 50

LC: lethal concentration; IC: inhibitory concentration

A possible hormetic response was found in 1st brood after exposure to BKME as more neonates were produced than in the control (Table 7-7).

Table 7-7 *Ceriodaphnia dubia* chronic toxicity exposed to pulp mill effluent

Brood	No. exposed organisms		No. dead		Remaining adult	No. neonates	
	Control	Pulp mill effluent	Control	Pulp mill effluent		Control	Pulp mill effluent
1st	10	10	0	3	7	15	24
2nd	10	10	0	2	5	25	23
3rd	10	10	0	0	5	46	35
Total						86	82

7.5 Tier 3

Endocrine disruptors are characterised by enacting upon living organisms even at extremely low concentrations, affecting their homeostasis and reproductive functions. This implies that to quantify the occurrence of these analytes it is necessary to use highly sensitive analytical techniques. The estimation of the magnitude of possible effects on biota were performed using ecotoxicity tests with relevant species both in the field and in the laboratory.

7.5.1 Concentration of target EDCs

Many EDCs molecules may enter in the category of emerging contaminants, as there are no regulatory guidelines or limits. The multiplicity of matrices and low detection limits required represent a challenge for analytical chemists. The methods used to measure oestrogens are described in Appendix B.

1) Oestrogens

Oestrogens were measured in river water, wastewater and effluent by HPLC/MS-MS according to Koh et al. (2007) (Table 7-8).

Table 7-8 Oestrogens concentrations in river water, pulp mill effluent and stream water

Sample	Date	Concentration (ng l^{-1})				
		E1	E2	E3	EE2	E1-3S
R1	19/02/2009	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2
R2	19/02/2009	< 0.1	< 0.2	< 0.2	< 0.2	< 0.2
R3	19/02/2009	< 0.1	< 0.2	< 0.2	< 0.2	< 0.2
R8	19/02/2009	< 0.1	< 0.2	< 0.2	< 0.2	< 0.2
BKME	01/06/09-07/06/09	< 0.1	< 0.2	< 0.2	0.3	< 0.2
C2	27/02/2009	2.2	0.6	1.0	< 0.2	0.5

2) Alkylphenols and ethoxylates

Alkylphenols at Fray Bentos stream receiving municipal wastewater (7/01/2011) were higher than before: 4-p-nonylphenol (8.3 µg l^{-1}) and p-tert-octylphenol (12 µg l^{-1}) (Table 7-9).

Table 7-9 Alkylphenols and ethoxylates in river water, pulp mill effluent and stream water

Sample	NP & OP (µg l^{-1})	NPOE (µg l^{-1})	NP2OE (µg l^{-1})
MDL (µg l^{-1})	0.01	0.02	0.02
Drinking water intake (R6)19/03/09	0.36	ND	ND
Fray Bentos stream (C2)19/03/2009	1.7	ND	ND
Gualeguaychú canal (R9) 19/03/2009	0.24	0.24	ND
Pulp mill effluent 07/07/08-13/07/08	ND	ND	ND

NP & OP: nonylphenol and octylphenol; NPOE: mono-ethoxylated nonylphenol; NP2OE: di-ethoxylated nonylphenol

Nonylphenol and ethoxylates were also analysed in sediments (Table 7-10).

Table 7-10 Nonylphenol and ethoxylates in sediments

Sediment sample	NP (µg g^{-1})	NPOE (µg g^{-1})	NP2OE (µg g^{-1})
Fray Bentos stream (S5) 16/03/2011	7.7	6.0	0.67
Anglo Beach (S7) 25/05/2009	0.04	0.47	ND

ND not detected; LOD 0.02 µg g^{-1}

3) Glyphosate

A method by IC/conductivity detection with gradient separation was developed. The concentrations in river water for all sampling sites were not detectable. Then, after a more thorough study with a watershed focus, at three small creeks that drain into the Uruguay River after crossing soy fields (routes No. 2 and 24), but only a sample was under quantitation (< 22 µg l^{-1}), and the rest were not detectable. The sediment sample from Yaguareté Bay had the highest concentration (

Table **7-11**). Soil and sediment were analysed by a modified version of the IC method based on ISO 10304-1:2007, confirmed by a FMOCI derivatisation and HPLC-FLD method.

Table 7-11 Glyphosate and AMPA in sediments and soils

Sediment sample		**Glyphosate (µg g^{-1})**	**AMPA (µg g^{-1})**
Nuevo Berlín (S1)	11/2010	0.25	
Fray Bentos (S2)	11/2010	0.26	
Yaguareté (S3)	02/02/2011	< 0.2	
Ubici (S4)	02/02/2011	< 0.2	
Yaguareté stream (S5)	02/02/2011	0.63	0.77
Fray Bentos stream (S6)	21/06/2011	< 0.05	
Las Cañas (S8)	11/2010	0.26	
Soil, Nuevo Berlín	5/7/2011	0.14	0.32

4) Dioxins and furans

Median, maximum and minimum values for the four sites measured during baseline studies and surveillance monitoring of the river were: R1: 0.15 (<0.05, 0.24); R2: 0.16 (0.05, 0.31); R3: 0.19 (0.07, 0.27); R8: 0.14 (0.07, 0.24) pg l^{-1} (**Figure** 7-18).

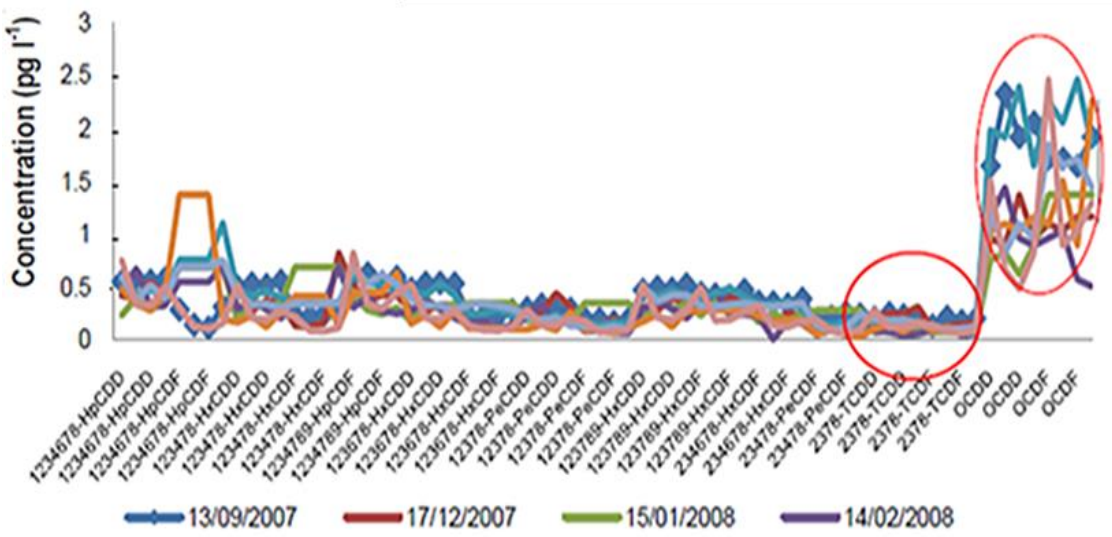


Figure 7-18 Dioxins and furans congeners in river water for sites R1, R2, R3 and R8

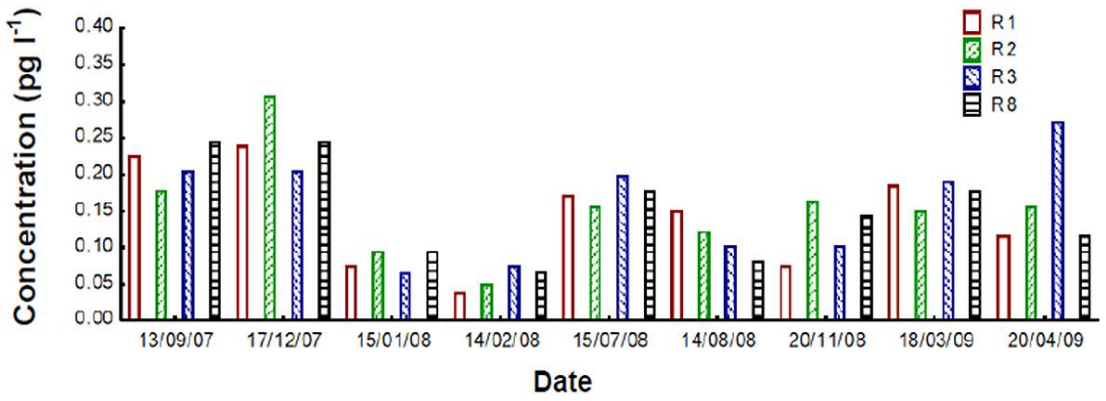


Figure 7-19 Concentrations of 2,3,7,8-TCDD in river water for sites R1, R2, R3 and R8

Just one peak in OCDD of 100 ng kg-1 dry weight, and total TCDD of 50 ng kg^{-1} dry weight, appeared in November 2009 in the analysed river sediments (Figure 7-20).

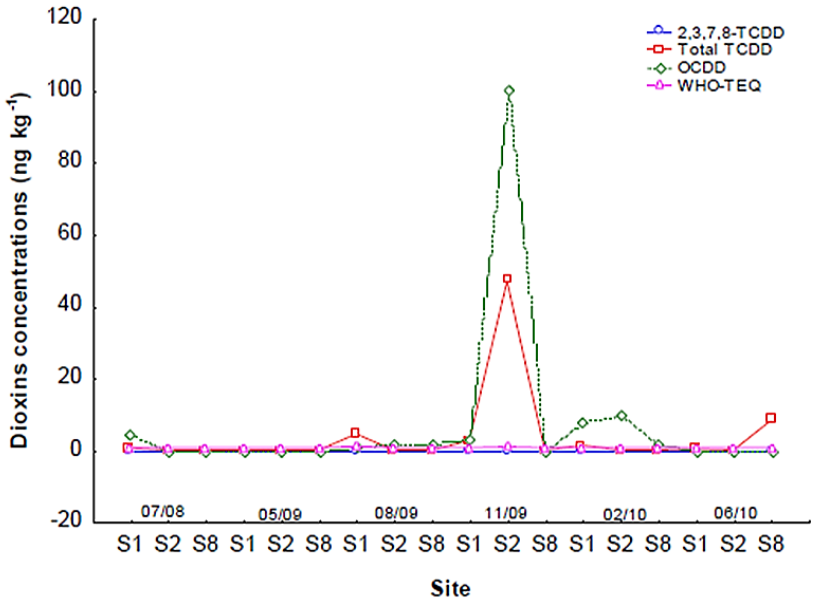


Figure 7-20 Dioxins in sediments for sites S1, S2 and S8

In the case of pulp mill effluents, the concentrations of 2,3,7,8-TCDD from April 09 to August 2010 (n=9) complied with the discharge permit (DINAMA R/DN/148/7) of 15 pg l^{-1}, with non detectable (LOD:1 pg l^{-1}) values and just one sample at 3.5 pg l^{-1} (April 2010). The OCDD congener was also not detectable (LOD: 4 pg l^{-1}). Total furans ranged from non- detectable to 0.27 pg l^{-1} TEQ.

The regulatory limit of dioxins and furans in fish is 4 pg g^{-1} WHO-PCDD/F-TEQ fresh weight. The concentrations of congeners and their variability in catfish muscle are also represented as radial plots (Figure 7-21) showing dates of consistently higher values from November 2006- April 2010.

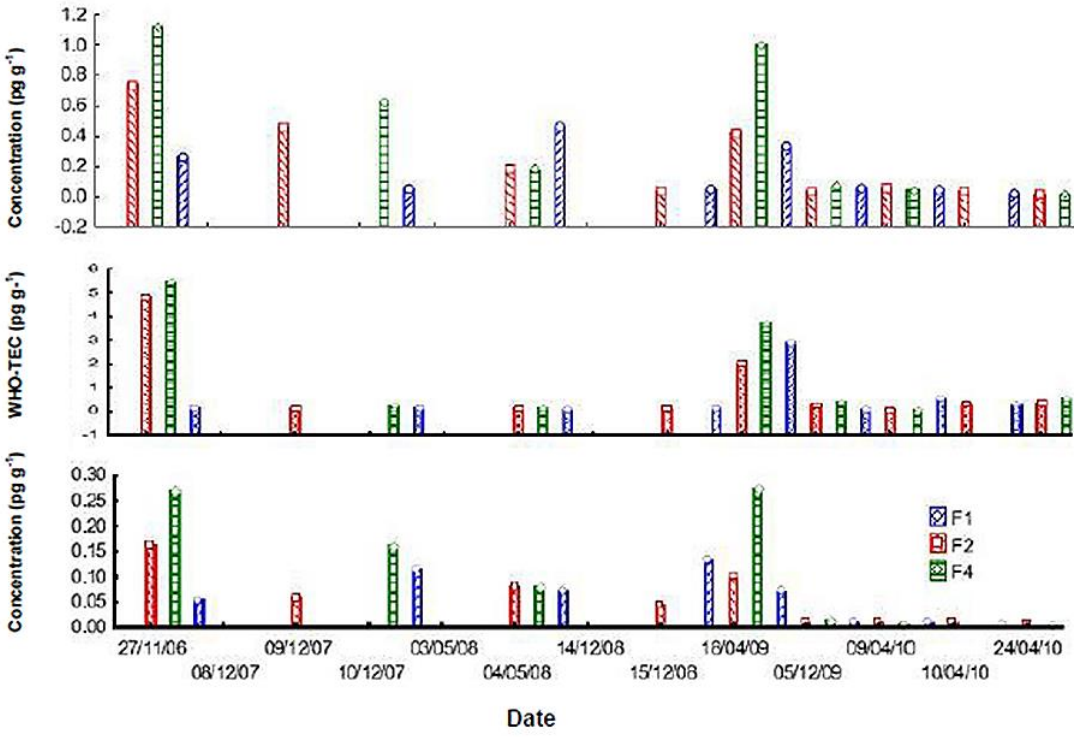
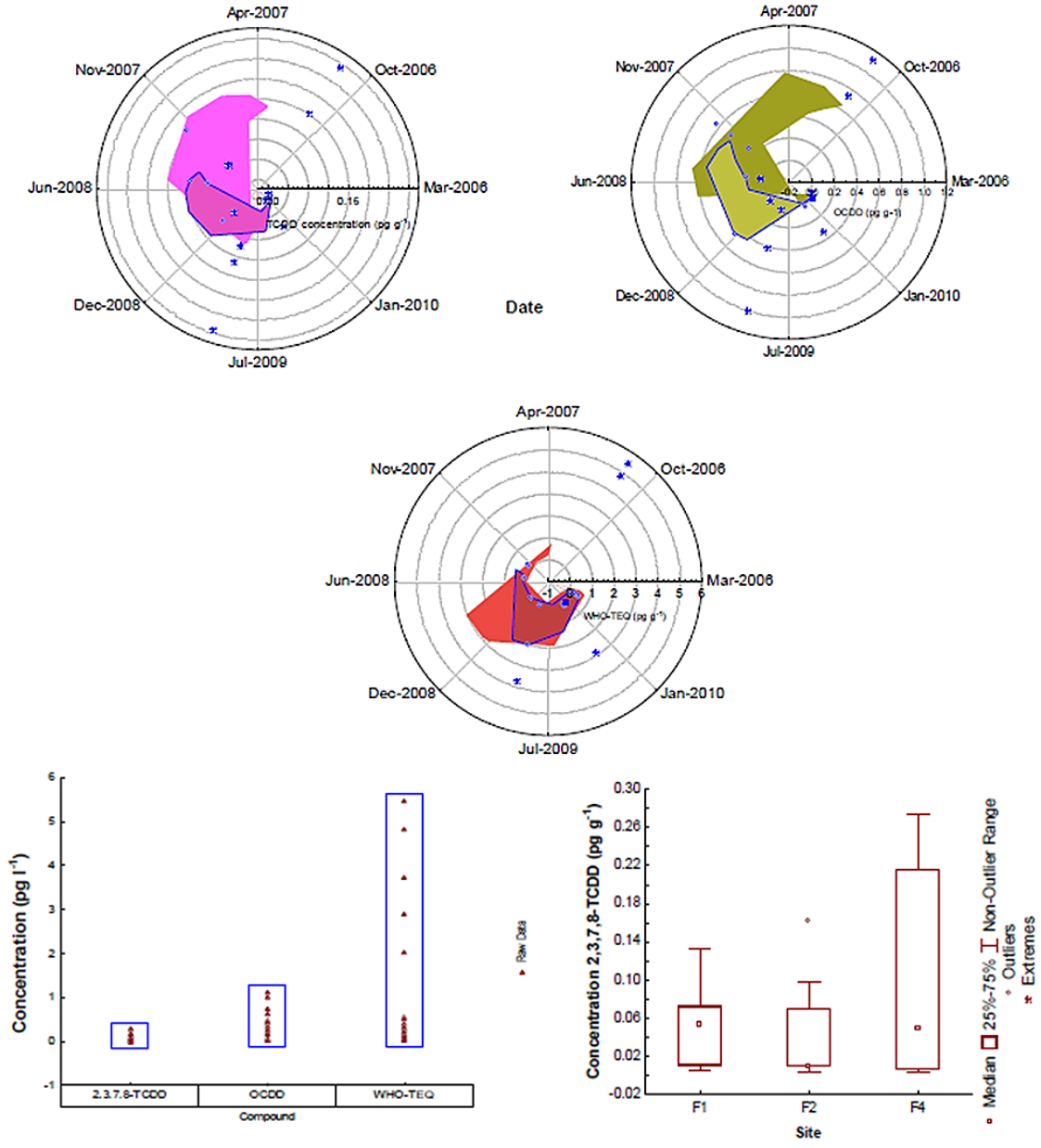


Figure 7-21 Temporal trend of dioxins residues in catfish muscle

Due to their very high toxicity and persistency, the food pathway for human beings was studied by analysing fish tissue residues. Catfish belonging to the species *Iheringichthys labrosus* (“bagre trompudo”) were caught at F1, F2 and F4 from November 2006 to April 2010. Median concentrations of total dioxins and congeners were 0.13 (0.04, 5.49) pg g^{-1}, expressed as WHO-TEQ, catfish muscle wet weight, while OCDD concentrations were 0.07 (0.0034, 0.07) pg g^{-1}.

The most toxic congener, 2,3,7,8-TCDD, was 0.07 (0.003, 0.28) pg g^{-1} (n = 29, median concentration) (Figure 7-22).



Radial plots: Red: total dioxins (WHO-TEQ); green: OCDD; purple: 2,3,7,8-TCDD
Bottom left: variability plot; right: box plots per site for 2,3,7,7-TCDD

Figure 7-22 Variability of dioxins in catfish muscle tissue

5) Polyaromatic hydrocarbons (PAHs)

The concentrations in river water were undetected for the most time since 2005, with scattered events of higher values especially at R1, during 2007 (Figure 7-23).

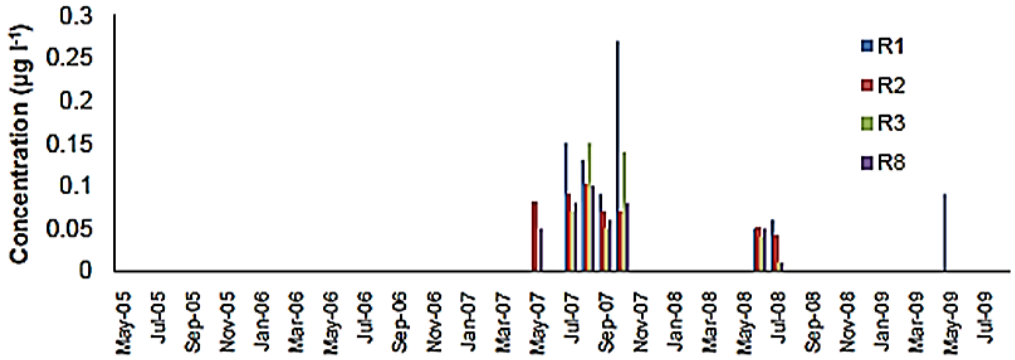


Figure 7-23 Polyaromatic hydrocarbons in river water

6) Polychlorinated byphenyls (PCBs)

The limit for dioxin-like PCBs is 8 pg g^{-1} WHO-PCDD/F-TEQ (EU Council Directive 1881/2006). The graph shows the catfish muscle residues of nor-ortho PCBs at sites F1, F2 and F4 (Figure 7-24).

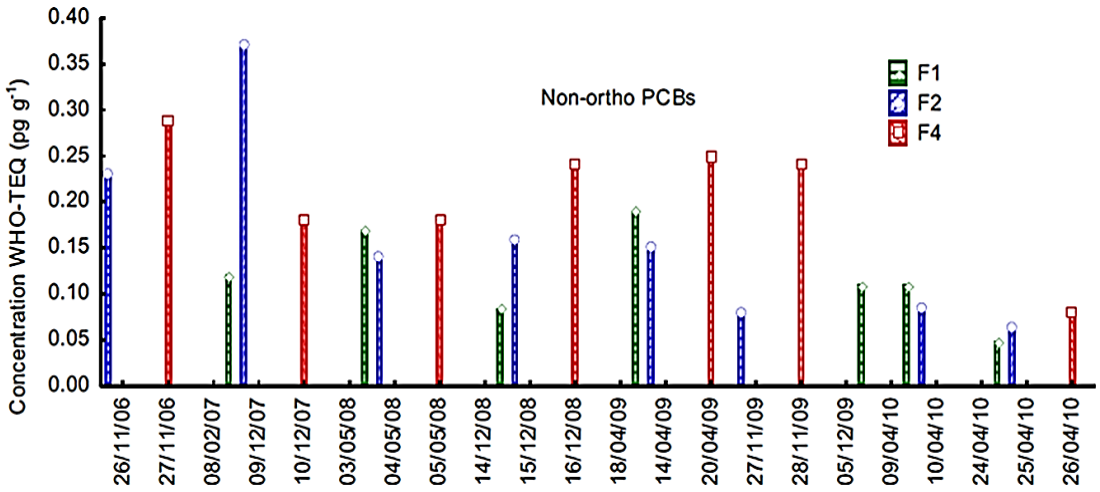


Figure 7-24 Muscle tissue residues of coplanar, non-*ortho*, dioxin-like PCBs (congeners 81, 77, 126, 169) in catfish

7) Chlorophenols

The most detected congener in BKME was 2,4,5-TCP (Figure 7-25). For this reason, the bioaccumulation and toxicity tests were performed using this reference toxicant.

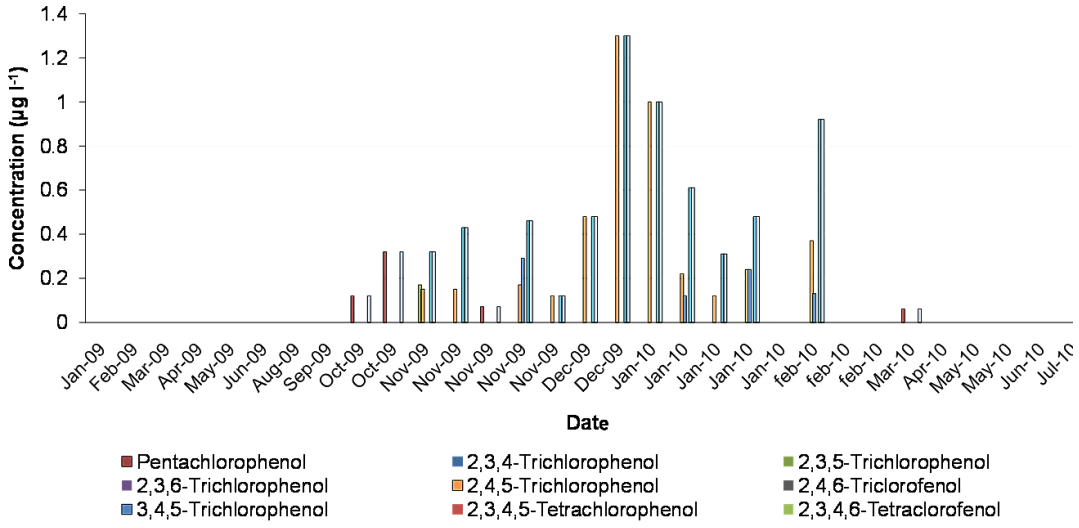


Figure 7-25 Chlorophenols in pulp mill effluent

8) Phthalates

Although not in the prioritised list, a prospective measurement of phthalates was carried out only at the most probable site finding that di-n-octyl phthalate (DNOP) was below quantitation limit at Fray Bentos stream (< 1 µg l^{-1}) (7/01/11). Di (2-ethylhexyl) phthalate (DEHP) was quantified as 2.7 µg l^{-1}.

7.5.2 Effects

Most of the methods were based on the mechanism of action of the EDC. For oestrogen receptor binding EDCs, the following techniques were applied:

7.5.2.1 *In vitro* screens

1) ERCALUX

With the results obtained in the exposure screening, the EEQ was evaluated both by an oestrogen equivalence factor-weighted addition of the measured individual oestrogens concentrations and by ERCALUX® method, using equation 7-1.

Oestrogenic Equivalent Concentration (EEQ)

$$EEQ = Ci \times TEFi \quad \textbf{(7-1)}$$

Where

$TEF_i = EC50_{(oestradiol)}/EC50_i$

Table 7-12 Oestrogen equivalents at the drinking water intake and Fray Bentos stream

Sample	Sum from oestrogen EEQ (ng l^{-1})	ERCALUX® as EEQ (ng l^{-1})
R6	0	ND-3.3
C2	2.8	2.27

ND: non-detectable; LOD: 0.024 ng l^{-1}

Uncertainties may arise from pulsatile characteristics of municipal wastewater discharges into C2, requiring further monitoring. Even so, it is expected that most of the oestrogenicity in this creek would be due to the presence of oestrogens (Table 7-8) with some influence of agricultural and solid waste runoff. In the case of the drinking water intake, the results varied from not

detectable to 3.3 ng l^{-1} in one occasion after the flood. This could be attributable not only to oestrogens but to other oestrogenic EDCs.

2) Yeast oestrogen screen (YES)

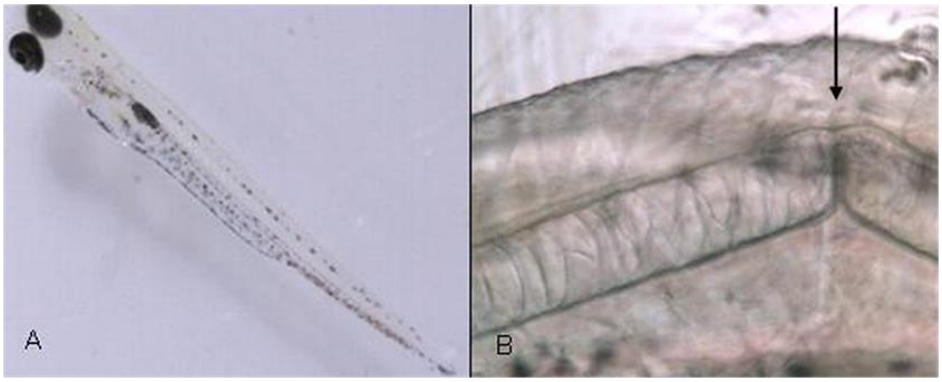
The yeast oestrogen screen (YES) assay is based on measuring the change in color from yellow to red of the chromogen chlorophenol red-β-D-galactopyranoside (CPRG) upon binding the receptor by an oestrogenic compound (Gaido et al. 1997). The *Sacchyromyces cerevisiae* strain has a DNA sequence of the human oestrogen receptor stably integrated and the lacz β-galactosidase enzyme of a yellow product that can be measured by absorbance at 420 nm. This *in vitro* assay was developed using a transformed yeast strain (*Saccharomyces cerevisiae* strain BJ3505).

Samples of river water, effluent, wastewater and tap water are measured by this method, but an improved detection limit could be achievable through the application of a Varian for RP- C18 solid- phase extraction disc filter to be able to load 10 litre of sample (Wenzel et al. 2003). The standard used was 17ß-oestradiol (E2) for a calibration curve in a concentration range between 1.2 ng l^{-1} and 7.5 mg l^{-1}. The sigmoid curves of dose - response obtained were fitted by the Hill function and r^2 were greater than 0.95. Limit of detection: 55 ng l^{-1} (for one litre of sample, concentrated through solid phase extraction). The calculated EC50 for ethynil oestradiol standard was 70.8 ± 13 ng l^{-1}.

7.5.2.2 *In vivo* tests

1) *Pimephales promelas* embryo-larval developmental test

Fish are subject to a broad variety of stressors because their homeostatic mechanisms are highly dependent on prevailing conditions in the aquatic surroundings (Harper & Wolf 2009). This makes them suitable models for evaluating endocrine-active compounds in the water column. To assess teratogenicity and larval malformations, the *Pimephales promelas* embryo larval development test was applied (USEPA 2000b). The fathead minnow (*Pimephales promelas*), a North American cyprinid fresh water fish is used for regulatory tests for the RA of chemicals (Gagnaire et al. 2009). Embryo larval stages are the most sensitive (Weis & Weis 1987), so, they are suitable to evaluate developmental toxicity. In this case, the exposure media were sediment elutriates. Lethality for Nuevo Berlín site was 21.5% while in Fray Bentos, 7.5%. In Nuevo Berlín no developmental anomalies were observed in the fish larvae. The sample elutriate from Fray Bentos had 3.3% spinal cord malformations (n=8) (Figure 7-26).



A: normal specimen exposed to control; B: spine malformation, in embryo- larvae exposed to elutriate of sediment S2

Figure 7-26 *P. promelas* larval spine malformations exposed to sediment elutriate

In this site, the number of malformations and lethality evidenced at the lowest concentration were higher (35%) than at higher concentrations (Figure 7-27).

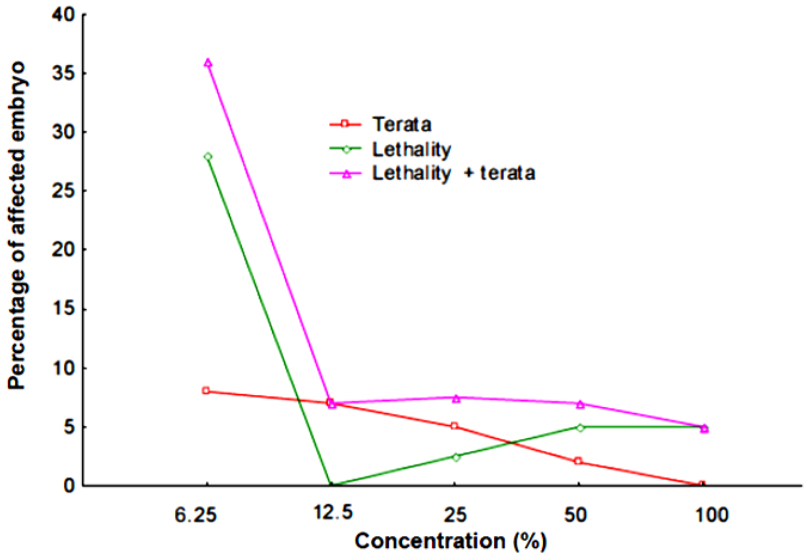


Figure 7-27 Dose-response curve for fish embryo larval lethality and developmental effects exposed to sediment elutriate

The distribution was not normal according to the Shapiro Wilks test. Then, the non parametric Kruskal Wallis test was applied ($\alpha = 0.05$) at the concentrations: control, 100, 50, 25, 12.5 and 6.25, with KW = 11.6 and $p = 0.04$, $p < 0.05$, meaning significant differences. No lethality or teratogenicity was found in Las Cañas (Míguez et al. 2010).

2) Short term Pimephales promelas fish reproduction test

The first two experiments were based on Kuntz & Fenz (2009), with non-spawning fish during 14 days. Then, four experiments were developed, exposing spawning fish during 21 days (EPA/600/R-01/067, USEPA 2002a, and OECD TG229), including biomolecular endpoints quantified by PCR. The development is detailed in Appendix B. Briefly, fish were exposed to BKME and to the stream water (C2), and the test validated with E2 as reference EDC at two concentration levels (150 and 450 ng l^{-1}). The description of the characteristics each of the six experiments is as follows:

Experiment No. 1

Pulp mill effluent, 14 days exposure period (end date: 28/05/2009)

After 7 days, the medium was changed to a composed sample of pulp mill final effluent representative of one month production. Fish were maintained under exposure during 14 days. Three replicate control tanks were run alongside each exposure. No spawning substrate was used. Measured end-points were VTG using ELISA method, gonadosomatic index.

Experiment No. 2

Municipal wastewater, 14 days exposure period (end date: 25/11/2009)

A pool of sexually differentiated both sex adult fish of 7 month and 15 days age was exposed under similar conditions than for protocol No. 1. The rest of the fish bodies were kept frozen for EDCs tissue residue analysis.

The following end-points were assessed under protocols Nos. 3 to 6: VTG using ELISA method, gonadosomatic index and gonadal developmental scoring, condition factor, oviposition (number of eggs attached to the substrate), number of nuptial tubercules and histology.

Experiments 1 and 2 were useful to develop the methodologies.

Experiment No. 3

Fray Bentos stream receiving municipal wastewater, 21 days exposure period (end date: 25/03/2010)

The exposed fish were sexually differentiated both sex adult fish of 7 month and 22 days age in similar exposure conditions as the prior experiences, but with a test duration of 21 days and spawning substrate consisting on a PVC tubes longitudinally cut in halves.

Experiment No. 4

Pulp mill effluent, 21 days exposure period (end 14/06/2010)

The exposed fish were sexually differentiated both sex adult fish of 8 month and 10 days age. Test duration: 21 days and spawning substrate.

Experiment No. 5

Standard toxicant: 17 ß oestradiol 150 ng l^{-1}, 21 days exposure period (end 7/10/2010)

Exposed fish were sexually differentiated both sex adult fish of 7 month and 10 days age.

Experiment No. 6

Standard toxicant: 17 ß oestradiol 450 ng l^{-1}, 21 days exposure period (end 21/12/2010).

Exposed fish were sexually differentiated both sex adult fish of 7 month age.

The characteristics of the samples for the exposure experiments are on Table B-3. None of the samples were acutely toxic.

End-points

The end-points were in the following hierarchical levels: functional, anatomical, tissue, molecular and gene. Tissue residues of target EDCs were analysed in one of the experiments, as biomarkers of exposure to domestic discharges.

a) Functional: egg production
b) Anatomic indexes, presence of ovipositor, nuptial tubercules
c) Tissue histopathology
d) Biomolecular: VTG protein expression
e) Toxicogenomics biomarkers (*vtg*, *ESR1*, *ESR2*, *GHR*, *IGF-I* and *ZP3* gene transcription)
f) Biomarkers of exposure: fish tissue residues

a) Functional

Egg production was measured daily as number of eggs spawned per female and then compared to the control (Figure 7-28). On day 7 the introduction of E2 diminished egg spawning to half in the 150 ng l^{-1} E2 experiment.

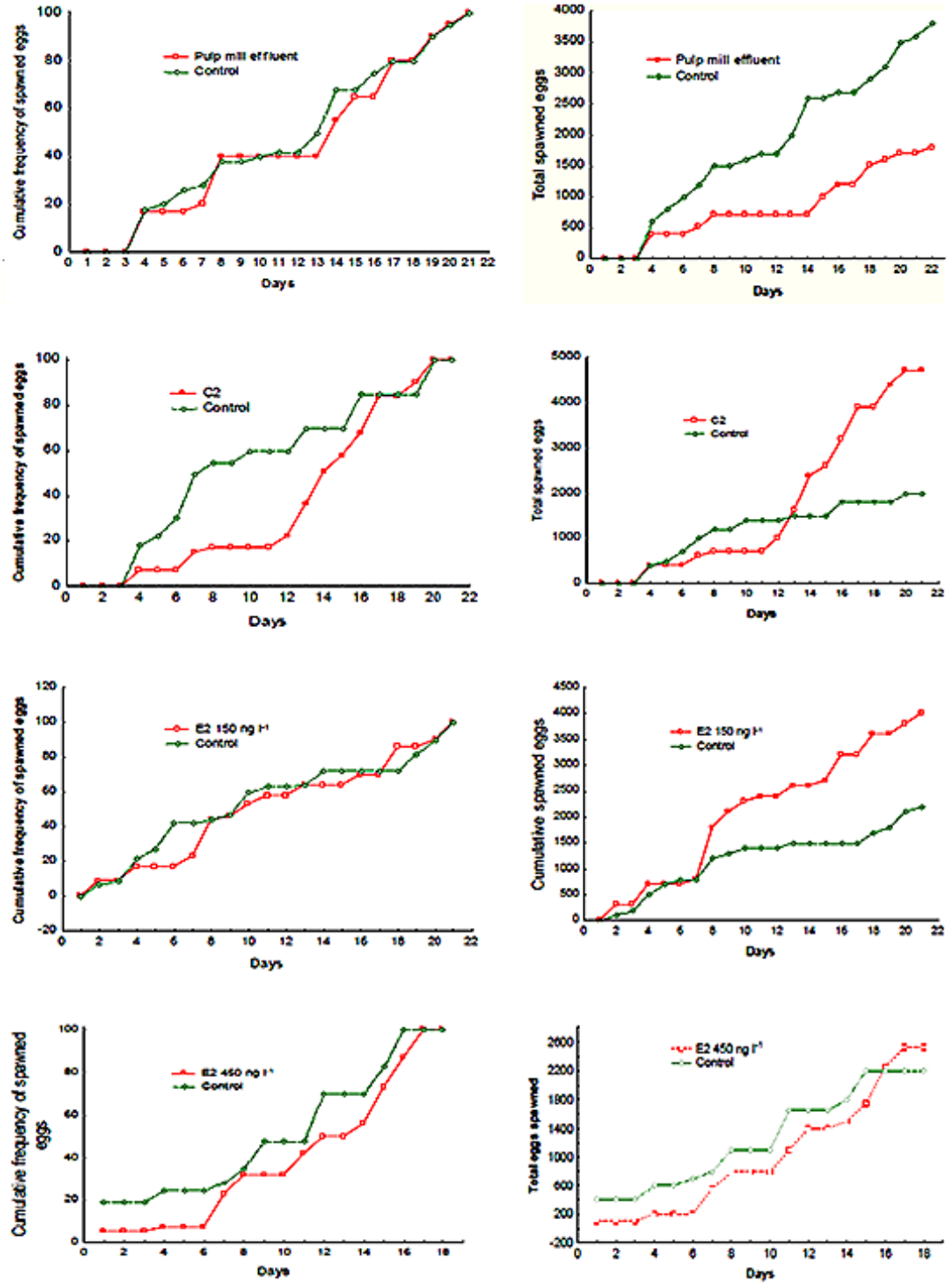


Figure 7-28 Egg production of *Pimephales promelas* exposed to pulp mill effluent, stream with wastewater contamination and two positive controls of E2 (at 150 and 450 ng l^{-1})

b) Anatomic

The gonadosomatic index (GSI) and condition factor (K) were determined. Nuptial tubercules, the specialised secondary sex characteristics found in the male fish, were counted, as an abnormal number or size of nuptial tubercules could imply effects of oestrogen receptor agonists, while if they appear in female fish, it could be due to androgenicing EDCs. A search for the presence of ovipositor in female fish and/or the abnormal appearance in male fish was carried out, but no abnormal structures were evident in any of the experiments.

c) Tissue: Gonad histomorphology

Histopathology analyses followed USEPA (2006) and Dietrich & Krieger (2010) guidelines. In fish, xenoestrogens can induce female proteins in males, and in some cases, the development of testis-ova (Genovese et al. 2011). Histomorphological alterations of the fathead minnow testis have been previously described for E2 (Miles-Richardson et al. 1999), 4-nonylphenol and nonylphenol ethoxylate (Wolf et al. 2004). Fish resposes after exposure to C2 and to BKME were compared to those provoked by E2. Higher concentrations of E2 (2780 ng l^{-1}) produce moderate alterations such as Sertoli cells hyperplasia and loss of germinal cells, degenerated spermatozoa and occasionally germ cell syncytia (Miles-Richarson et al. 1999), vacuolated cells and apoptotic body cells (Wolf et al. 2004), but these alterations were not observed in our experiments.

No evidences of intersex were found for the domestic and BKME neither for the E2 concentration applied (n = 16 female and n = 11 male). The only histological anomalies were a low frequency (less than 3%) of histiocytic cells, an increased number of spermatogonia in male fish exposed to E2 450 ng l^{-1}. These findings are currently being confirmed by quantitative analysis using an image processing software (Image ProPlus®). Ovaries were also evaluated, but qualitative histomorphology alterations were not observed in female fish exposed to neither treatment (Appendix B).

d) Biomolecular: VTG concentrations in liver homogenate, by ELISA method

Liver samples were subject to VTG determination applying a commercial ELISA kit (Biosense®). This test is specific of the *Pimephales promelas* species.

Sample	Male		Female	
	Control	Exposed	Control	Exposed
C2	0.015(0.005-0.155)	0.30 (0.24-0.37)	29 (20-57)	14 *(12-18)
BKME	0 (0-10)	0 (0.0-0.3)	20(19-37)	48 (18-51)
E2 150	3 (0-5)	9 (3-66)	40 (28-53)	23*(21-26)
E2 450	4 (1-8)	75 *(65-86)	27 (23-42)	14* (6-29)

e) Toxicogenomic biomarkers by the Polymerase Chain Reaction- Real Time (RT-PCR) method

The Quantitative Real Time PCR to determine the *vtg* gene transcription is regarded as an early warning system and as such, a more preventative method by comparison to VTG the measurement after its induction by EDCs. It is a sensitive and fast method for the detection of VTG transcripts because able to detect exposures of 5.0 ng l^{-1} 17α-ethinylestradiol (EE2) in shorter exposure periods than VTG expression (1-2 days) (Biales et al. 2007).

The results the toxicogenomics biomarkers are presented on Tables 7-13 to 7-16.

Table 7-13 *vtg* gene transcription by QRT-PCR

Sample	***vtg* PCR (Expression relative to 18S)**	
	Male	Female
C2	0.81(0.11-4.23)	0.38 (0.05-2.62)*
BKME	0.61 (0.14-2.99)	0.05* (0.0-1.95)
E2 150	13.6*(0.2-190)	3.2*(0.5-18.5)
E2 450	40.6*(13.4-125)	1.7*(0.6-5.3)

* Significant differences in measurements relative to the control taken as 1, at p value < 0.05.

Table 7-14 *ESR1* and *ESR2* genes by PCR

Sample	***ESR1***		***ESR2***	
	Male	Female	Male	Female
C2	2.25* (0.55-4.50)	1.78*(0.55-3.63)	1.04 (0.57-1.89)	0.39 (0.02-8.57)
BKME	0.80 (0.40-1.28)	0.26*(0.02-1.40)	2.13*(0.56-1.67)	2.90*(0.77-9.85)
E2 150	0.83 (0.30-2.06)	1.17 (0.367-4.72)	1.04 (0.10-8.31)	1.04 (0.10-8.31)
E2 450	2.33*(1.97-3.01)	0.86 (0.33-2.09)	0.59*(0.22-.27)	0.80 (0.30-2.01)

Table 7-15 Insuline-like growth factor and Growth hormone factor by PCR

Sample	***IGF-I***		***GRH***	
	Male	Female	Male	Female
C2	0.17*(0.05-0.48)	1.02(0.41-2.56)	1.56*(0.85-2.62)	0.84(0.013-6.61)
BKME	1.50*(0.64-3.56)	5.45*(1.53-57.3)	2.33*(1.16-4.53)	9.67*(2.56-37.0)
E2 150	0.52*(0.27-0.81)	0.64(0.12-2.10)	0.33*(0.02-4.96)	0.64(0.04-7.09)
E2 450	0.72*(0.54-0.87)	0.41*(0.16-0.99)	0.75*(0.42-1.47)	0.64(0.20-2.10)

Table 7-16 *Zona pellucida* gene by PCR

Sample	*ZP-3*	
	Male	Female
C2	6.07*(4.36-8.19)	4.65(0.63-50.4)
BKME	0.15*(0.09-0.23)	1.99(0.02-91.5)
E2 150	0.92(0.29-28.7)	0.68(0.00-8.22)
E2 450	1.44*(0.88-2.10)	126.2*(0.18-170)

f) Fish tissue residues of lab-exposed fish

In experiment No. 3 fish were exposed to Fray Bentos stream receiving municipal wastewater. Tissue residues were analysed for alkylphenols and ethoxylates. The concentration of 4-NP in fish muscle was determined as 17.5 ng g^{-1} and; 4-tert-octylphenol as 11.2 ng g^{-1}.

7.5.3 Field surveys

Field studies were applied to both environmental receptors: by fish biomonitoring, and human ecoepidemiology to assess current exposure and effects. Biomonitoring is “the systematic use of living organisms or their responses to determine the condition or changes of the environment” (cited by Li et al. 2010). Thus, it is fit to evaluate toxicity through multiple stressor interactions in a more realistic exposure scenario than laboratory tests alone (Crane et al. 2007), that focuses not only on individuals but in populations, and communities, including contamination as well as bioavailability in the analysis (Hanson 2009).

7.5.3.1 Fish biomonitoring with *Astyanax fasciatus*

Fish are at the top of the aquatic food web and are consumed by humans, making them an important tool to assess contamination, relatively easy to collect and identify to the species level. The characteristics of the reproductive maturation state of individuals may be extrapolated to populations. *Astyanax*

fasciatus (“mojarra”) is a small predator fish (Figure B-18, Appendix B). It is ecologically relevant and one of the most widespread species in the country watercourses. This wild fish representative of the Uruguay River aquatic ecosystems was chosen as a sentinel species, bioindicator of exposure to EDCs, because of its non migratory habits, relative abundance, and omnivorous feeding habits. Variations in morphometry and physiology indexes, tissue residues analysis of target EDCs as biomarkers of exposure were evaluated at four fishing sites.

Adult fish specimens of the chosen species were collected from three sites in the River Uruguay (Figure 7-29). The fishing locations were at:

- a reference site in buffer zone 1, near Nuevo Berlín village (F1), upstream from the pulp mill;
- two nearfield sites downstream the BKME outfall in buffer zone 2 (Yaguareté Bay (F2) and Ubici Beach (F3))
- one site located in buffer zone 3, mainly recipient of city discharges that could be considered a farfield downstream site from the pulp mill, artisan fishing site and touristic resort (Las Cañas (F4)).

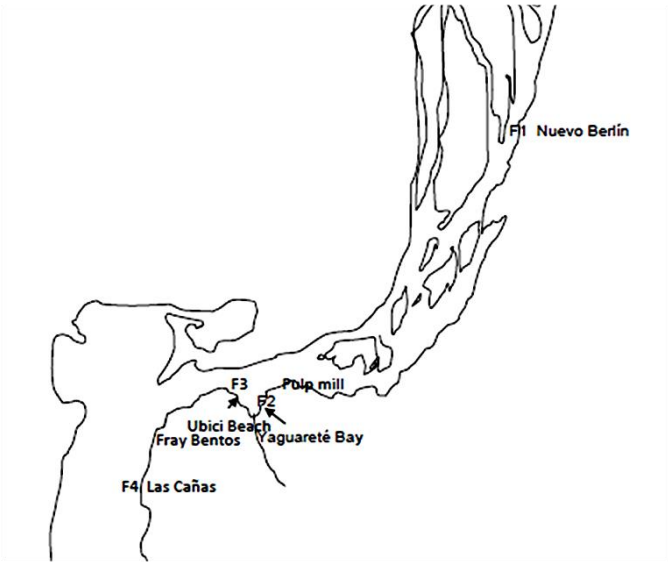


Figure 7-29 Fishing sites

The measured end-points evaluated variation in condition of this fish species by morphometric indices (5, 6 and 7), physiological indices (1, 2, and 4), biomarkers of bioaccumulation and exposure (7 and 8) and structural sub-cellular characteristics (9), comprising the following, per each site:

a) Sex ratio
b) Gonadal maturation stages scores
c) Length-weight relationship
d) Relative weight (Wr)
e) Condition factor (K)
f) Gonadosomatic index (GSI)
g) Liver retinol biomarker
h) Tissue residue for target EDCs
i) Gonads histopathology

7.5.3.1.1 Statistics

Frequentist and Bayesian approaches were used with STATISTICA v8 software (Levene's test, analysis of variance [ANOVA], Kruskal-Wallis, and analysis of covariance [ANCOVA]). The type I error was set at $\alpha = 0.05$. Data were assessed for homogeneity of residual variance, using Levene's test. When data were homogeneous, ANOVA was used for the analysis of variance. The Kruskal-Wallis was applied. In the case of GSI an analysis of covariance covariating with gonad stage was performed. The Wilcoxon matched pairs test was used to evaluate differences between pairs, followed by the *Post Hoc* tests Dunnet´s and Fisher LSD to determine among which pair of sites there were significant differences. For a power goal of 0.90 and type I error rate $\alpha = 0.05$, the minimum sample size was calculated to be 265 using the t-test.

A Bayesian classificator classified GMS. A supervised learning algorithm analyses the training data and produces an inferred function, which is called a classifier. The Naïve Bayes classification model determined the conditional frequency of developmental gonad stages per site. Naïve Bayesian classifiers

assume that the effect of an attribute value on a given class is independent of the values of the others (class conditional independence). Accuracy has been estimated in 98.59% in bioinformatics applications (Kumari et al. 2009). It is a generalization of the logistic regression, ands more accurate and faster than other methods (Elkan 1997). Naïve Bayes classification is based on the Bayes Theorem and the maximum *posteriori* hypothesis. The Bayes theorem states that:

$$P\ (h|D) = \frac{P\ (D|h)\ P\ (h))}{P\ (D)} \quad \textbf{(7-2)}$$

Where

$P\ (h)$ = Prior probability of hypothesis h- Prior

$P\ (D)$ = Prior probability of training data D-Evidence

$P\ (D|h)$ = Probability of D given h- Likelihood

$\mathrm{P}\ (\mathrm{h}|\mathrm{D})$ = Probability of h given D- Posterior probability

The posterior probability of class h_i can be calculated as:

$$P\ (h_i|D) = \frac{P\ (D|h_i) \times\ P\ (h_i))}{P\ (D)} = \frac{P\ (D|h_i) \times\ P\ (h_i)}{\sum_{i=1}^{K} P\ (D|h_i) \times\ P\ (h_i)} \quad \textbf{(7-3)}$$

Given the target value, the probability of observing the conjunction a_1, a_2.....a_n, equals the product of the probabilities for the individual attributes.

$$P\left(a_1, a_2 \ldots a_n \middle| V_j\right) = argmax\ v_j \in v\ \Pi P\left(ai \middle| v_j\right) \quad \textbf{(7-4)}$$

The number of distinct P ($a_i|v_j$) estimated from the training data is the number of distinct attribute values times the number of distinct target values:

$$v_{NB} = argmax\ v_j \in v\ \Pi P(ai|v_j) \quad \textbf{(7-5)}$$

a) Sex ratio

The sex ratio (number of males to number of female) evaluates possible endocrine effects at population level. The normal proportion is 1:1, but natural or anthropogenic factors may drift it towards male or female predominance. The calculated overall sex ratio was 65% female (491 specimens) and 35% male (247 specimens). The sex ratios distribution per site (Figure 7-30) for sites F1 to F3 is skewed toward female. The only site with a ratio approaching the normal 50:50 ratio is F4, with significant differences to the other sites (Wald statistic, $p<0.05$, binomial distribution, logit link function).

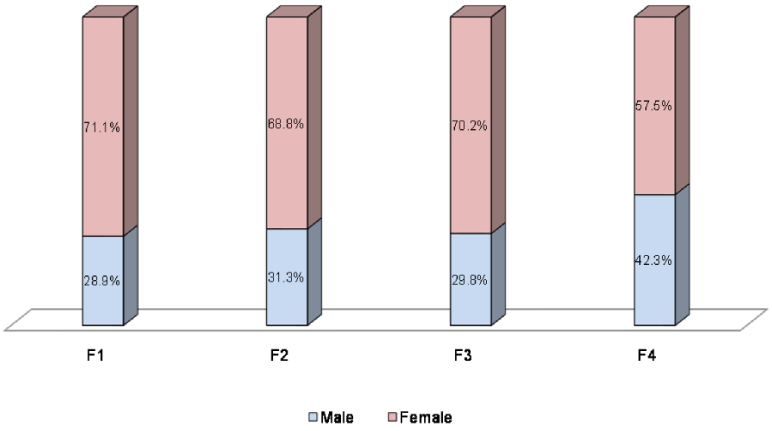


Figure 7-30 Sex ratio of *A. fasciatus,* categorised by site

b) Gonadal maturation stages scores

The gonadal maturation score (GMS) gives an idea of the sexual maturity of the fish. This species shows fractionated spawning, with peaks influenced by water temperature and rainfall (de Carvahlo et al. 2009). The gonad stage scale used by this author included only macroscopic characteristics (Appendix B).

A Naïve Bayes model was applied to length, weight and GMS to determine the conditional frequency of stages per each site, revealing geographical variations in distribution, with the least mature female specimens at site F2. In the case of male specimens, site F3 had mature specimens (Figure 7-31).

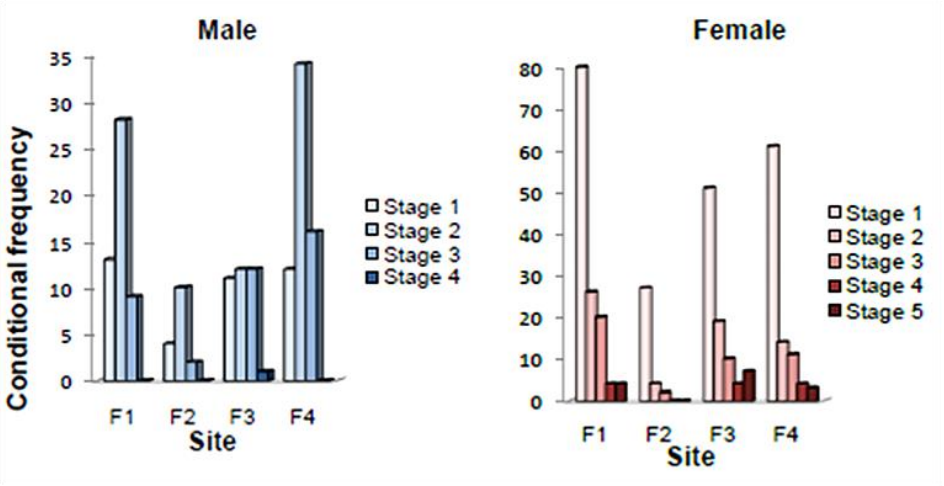


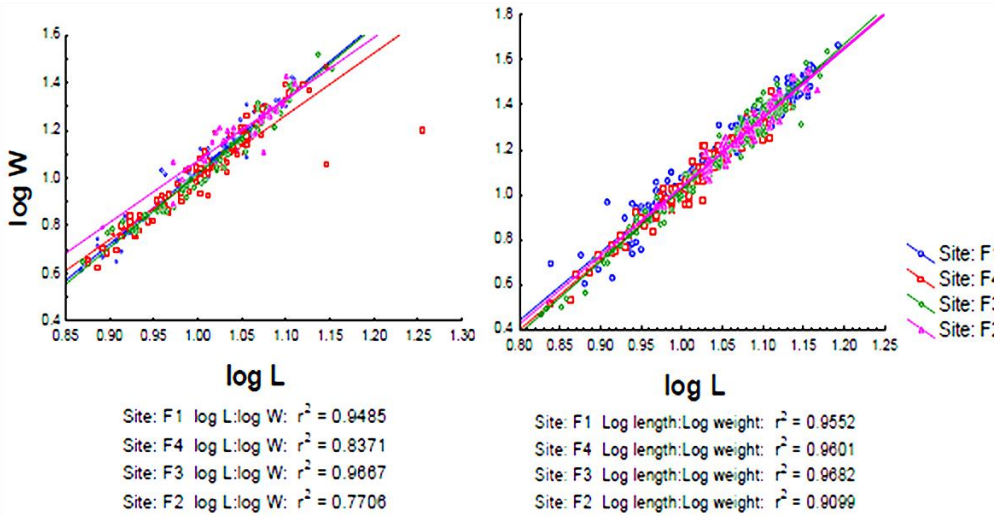
Figure 7-31 Gonadal maturity stages of A. fasciatus categorised by site and sex using the Naïve Bayes classificator

c) Weight-length relationships

According to Jobling (2008), the weight-length relationship is a measure of fish growth, connected to condition factor, a concept reflecting the fish well being and nutritional status or “fatness”, the quality and availability of food, sexual maturity, age and sex, and environmental factors, such as temperature, that may have an impact upon rates of development, growth and reproduction of fish.

The total length was measured using calipers from the anterior-most part of the fish to the tip of the longest caudal fin rays. Average length to weight relationship for female fish had a correlation coefficient r^2 =0.9072, and that for male fish, 0.6911.

The curve of log_{10}-transformed weight and length had better correlations. Figure 7-32 shows correlation coefficients for log L: log W at eachsite for both female and male fish**.**



Left: male; right: female

Figure 7-32 Log transformed weight - length relationships for *A. fasciatus*

d) Relative weight (Wr)

The Wr was calculated by the regression-line-percentile technique to develop standard weight (Ws) equations (Blackwell et al. 2000) (equation 7-7).

$$W_r = \left(\frac{W}{Ws}\right) \times 100 \qquad \textbf{(7-6)}$$

Where

W: observed weight

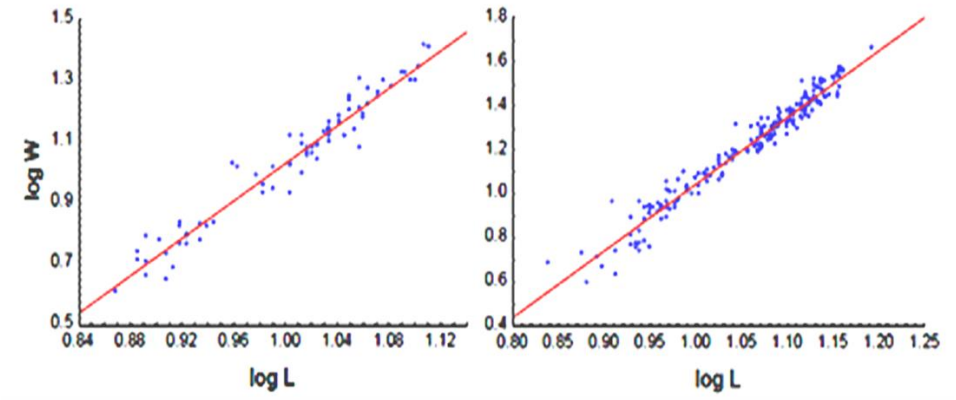
W_r: relative weight

W_s: standard weight, calculated as:

$$W_s = aL^b \quad \textbf{(7-7)}$$

Where L= length, a and b= parameters

Equations for female and male standard weight were derived for reference site F1, calculating equation 7-7 parameters as: Male: a= 0.00917; b= 3.0669; Female: a= 0.01033; b= 3.0318 (Figure 7-33).



Left: male; right: female, at reference site F1

Figure 7-33 Standard weight for male and female *A. fasciatus*

Even though significant differences were apparent for female fish during autumn and spring (Kruskal Wallis, $p < 0.05$), they were only 5% above or below normal. However, in spring, a decrease > 5% was evidenced for F3, while in summer, a 10% difference was found for female fish at F3 and F4. Winter male were plumper than normal at F4 (Figure 7-34).

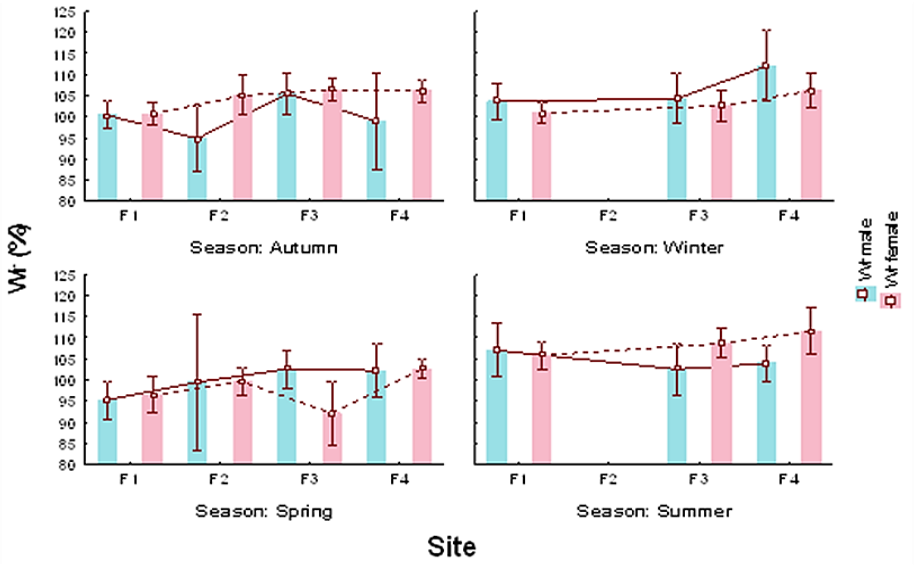


Figure 7-34 Mean plot of relative weight for female and male *A. fasciatus*

e) Condition factor

The condition factor (K) is an indicator of the overall health and well-being of the fish as it varies directly with nutrition, but it may also vary location to location within a species. The K index was calculated using equation 7.8, measuring gonad mass and total body mass measured in grams and length in centimetres.

$$K = \frac{W \times 100}{L^3} \qquad \textbf{(7-8)}$$

The Naïve Bayes classificator for weight, length and GMS results were that overall, during the whole sampling period, female fish were slightly smaller at F2 and F4, and male fish in less condition at F4 (Figure 7-35).

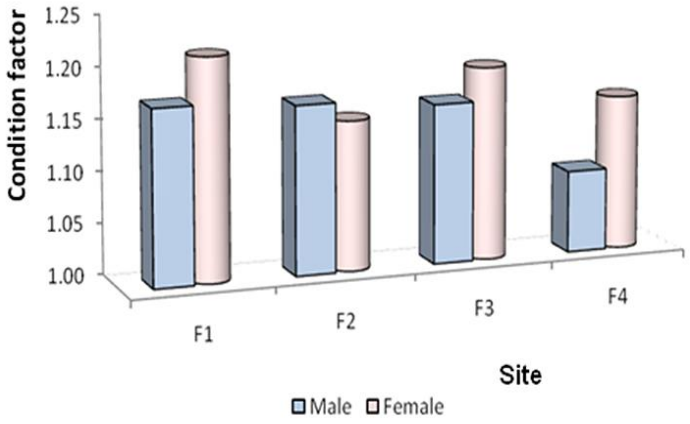
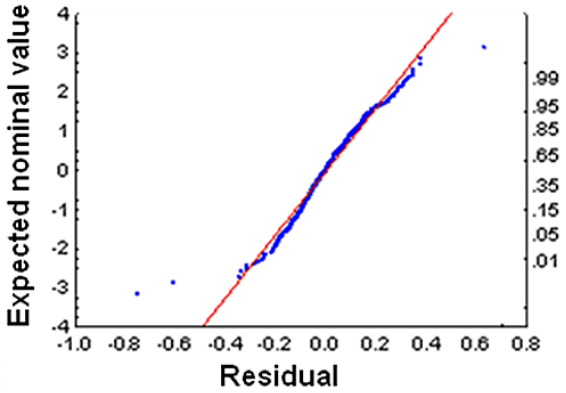


Figure 7-35 Bayesian classification of *A. fasciatus* condition factor

The ANOVA test of significance for condition factor revealed significant differences among sites ($p<0.05$). The Restricted Maximum Likelihood test (REML) compared the least square means with Type V decomposition. By frequentist statistics, the results are similar, but more marked differences are detected. Female fish show a descending trend going downstream with F4 (Las Cañas) the smallest K for each sex. In the case of male fish, the highest condition factor was calculated for site F2 (Yaguareté) (Figure 7-36).

An statistics study was undertaken for all seasons and for autumn and spring as fish were caught during these seasons only at F2, but the diagram was the same as with the full set of data, but the current effect factor was 5.1347 and p= 0.0019, for n= 210. The statistics study was undertaken for all seasons and for autumn and spring as fish were caught during these seasons only at F2, but the diagram was the same as with the full set of data, but the current effect factor was 5.1347 and p= 0.0019, for n= 210.



Normal probability plot, raw residuals, dependent variable: condition factor

Figure 7-36 Normal probability plot for *A. fasciatus* condition factor

The pair-wise comparison by LSD test with simultaneous confidence intervals detected significant differences ($p < 0.05$) among F1 and F3 and F1 and F4 for female fish (n= 474). Further, removing outliers for a total n= 462, the differences were also apparent between F2 and F4.

In the case of male specimens the only pairs that were not significantly different were F1-F3 and F3-F4 (Figure 7-37).

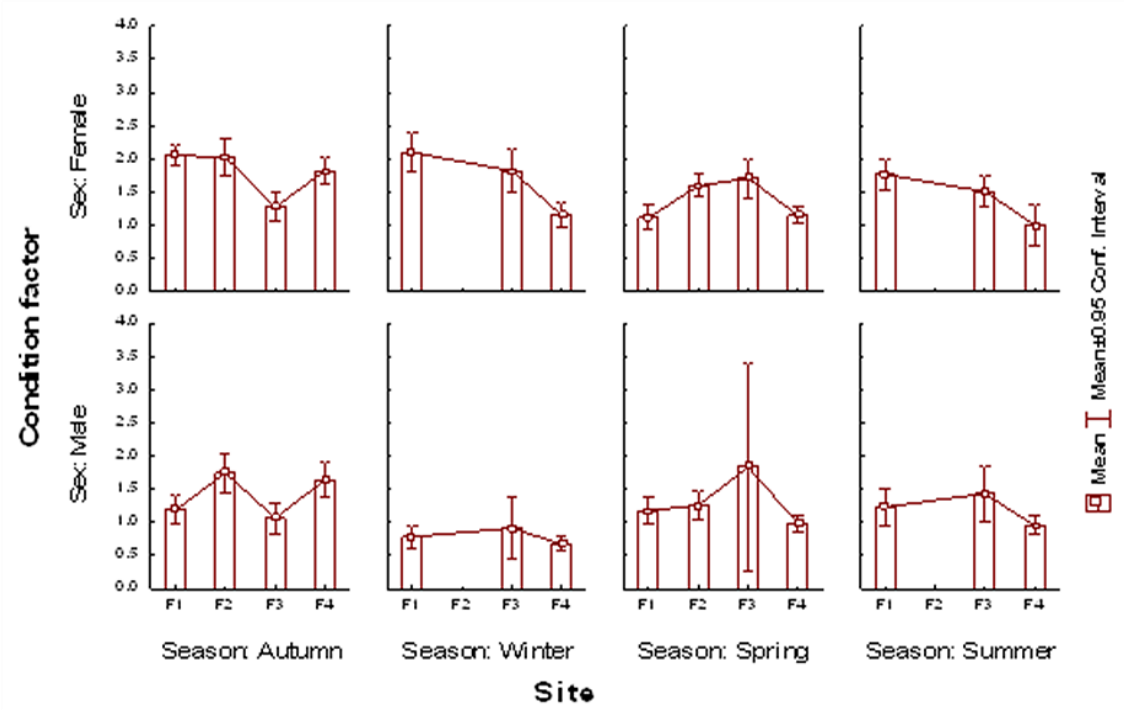


Figure 7-37 Comparison of condition factor for *A. fasciatus* categorised by site, sex and season

f) Gonadosomatic index (GSI)

This index provides information on the fish reproductive status reflecting the proportion of growth allocated to reproductive tissues in relation to somatic growth. As seasonal spawners, their gonad size changes related to gamete maturation stages. The GSI was calculated as follows:

$$GSI = \frac{gw \times 100}{W} \quad \textbf{(7-9)}$$

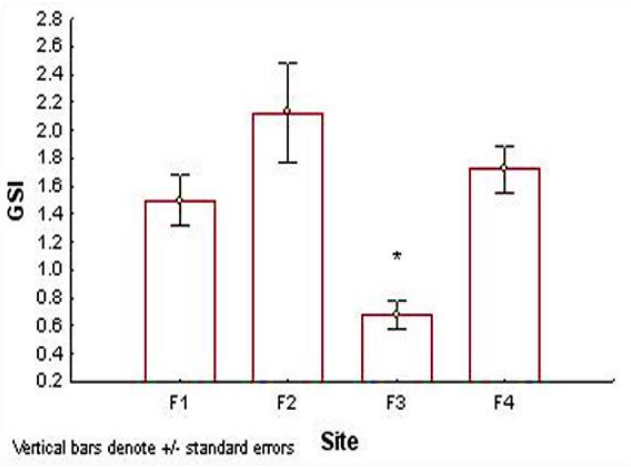
Where

GSI = gonadosomatic index

gw = gonad weight

W = total body weight

Significant differences for testes at stage 2 appeared in autumn 2010 (KW- H (3, 14) = 9.95, p= 0.019). The Wilcoxon matched pairs test suggested that differences existed between pairs. The Fisher LSD and Dunnet´s Post Hoc tests demonstrated significant differences (p<0.05) among sites F2 and F3. Hence, fish testes were smaller at Ubici Beach (F3) than at Yaguareté Bay (F2) at maturing gonadal stage (Figure 7-38).



* Significant differences

Figure 7-38 Gonadosomatic index of male *A. fasciatus*

The GSI for female fish were different among sites at stage 1 in autumn (n=104; F (3,104) = 4.5724, p =0.0048) and also during spring (F (3, 58) =2.8594, p= 0.0447). The differences applying the Dunnet Post Hoc test for p< 0.05 were between sites F2 and F3. This is not as highly relevant as fish were at an early developmental stage, not mature.

Spawning was evidenced by the highest gonad development scores consistent with the spring seasons. However, F4 showed a lower score than the others (Figure 7-39).

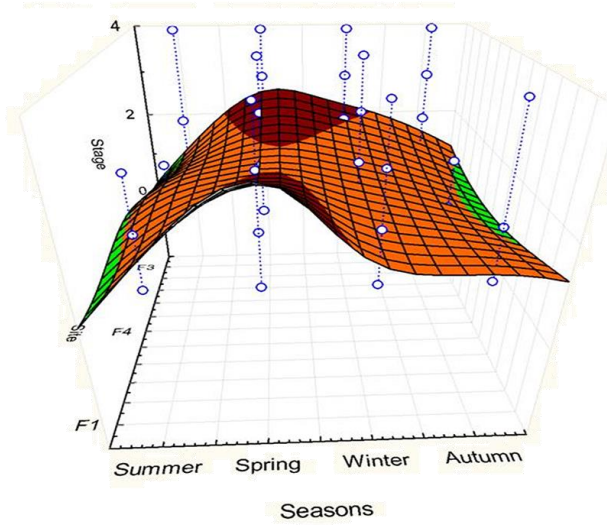


Figure 7-39 3D plot of ovaries development per season and site

Multivariate exploratory techniques represented as 3D plots show the ovaries maturity differences among sites (Figure 7-40).

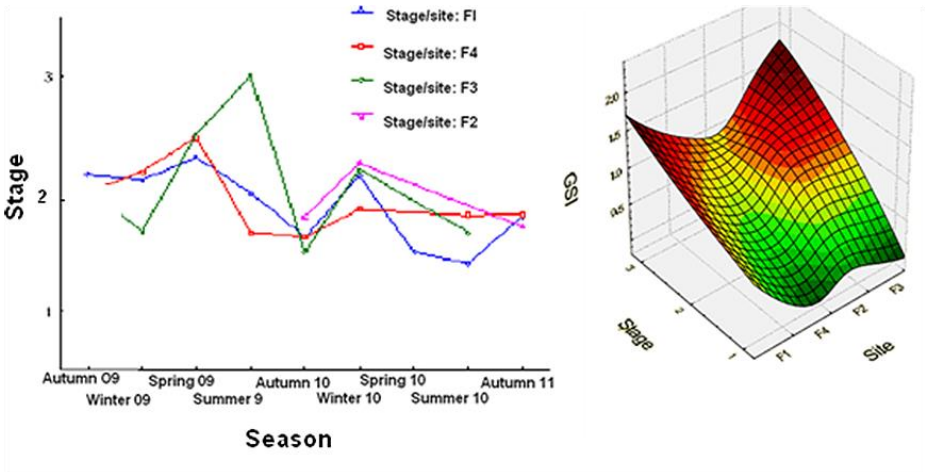


Figure 7-40 Seasonal variation of female fish gonad maturation scores

g) Liver retinol

Retinoids are vitally important regulators of homeostasis and normal embryo development (2.4.3.4). All trans-retinol level in fish liver is suggestive of a relative depletion in retinol levels at F2 when compared to F3 (Figure 7-41).

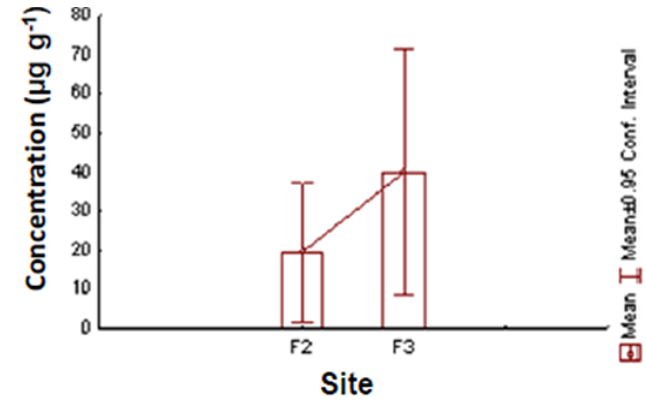


Figure 7-41 all *trans*-retinol in *A. fasciatus* liver at F2 and F3

h) Fish tissue residues

Fish tissue residues are biomarkers of exposure to EDCs and evidences of bioaccumulation, as body burdens relate to internal doses and effects. This approach has been recommended to evaluate exposure to persistent EDCs (Tillitt & Papoulias 2003). Some ligands of AhR and ER present in pulp mill effluents were found to accumulate in fish liver after exposure, and others to compete for AR and sex steroid binding protein (Hewitt et al. 2000).

Target EDCs concentrations were analysed in fish muscle. The EDCs biomarkers of exposure were: alkylphenols, endosulfan, β-sitosterol, and resin acids. Resin acids were < 3 mg kg^{-1} in all sites, and all the congeners were unquantifiable, with exception to PCP that was at the quantification limit (0.1 mg kg^{-1}). Alkylphenols, endosulfan, sitosterol, fish fat, sterols and cholesterol are represented in Figure 7-42 and Figure B-19.

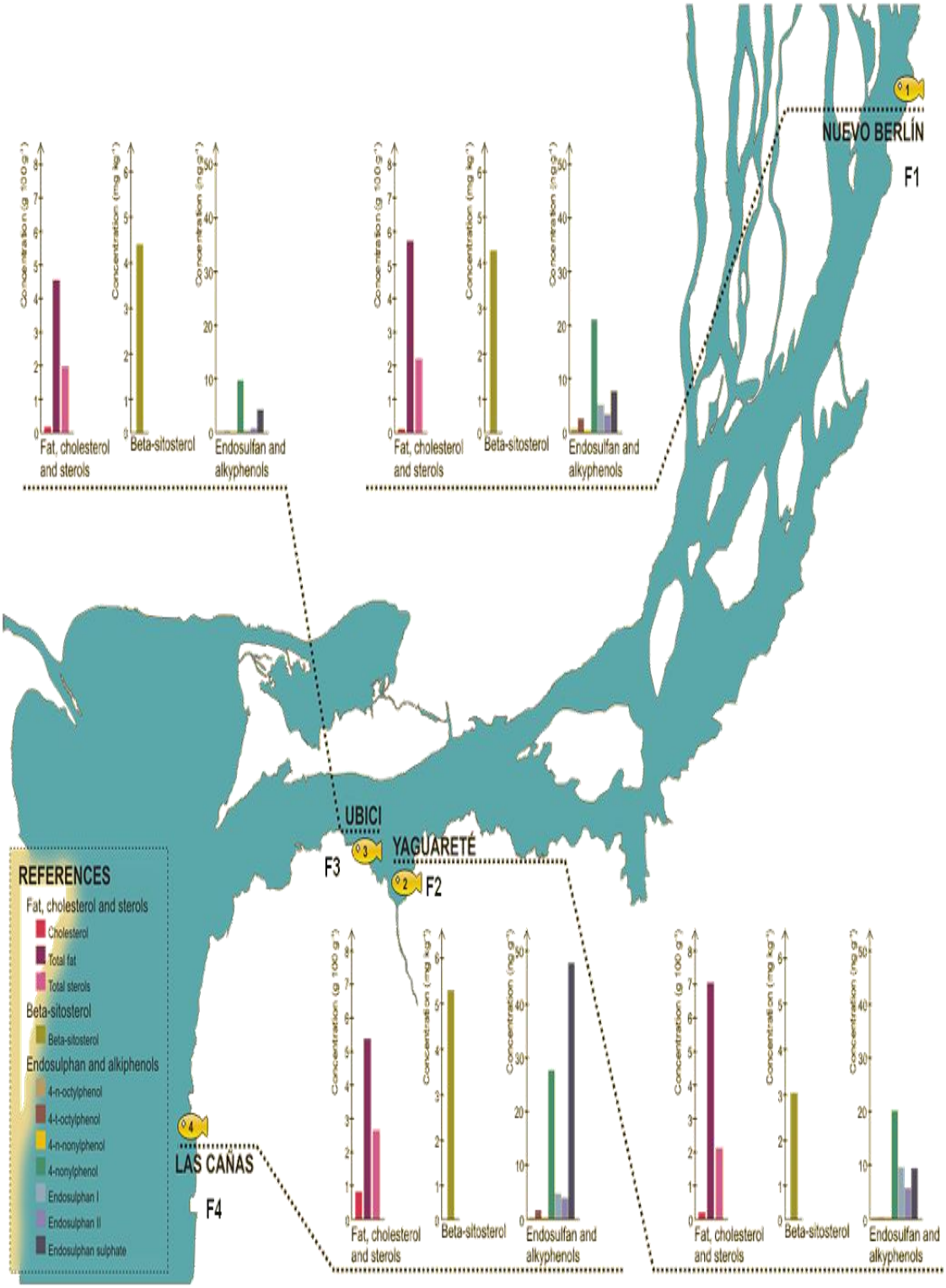


Illustration credits: Pablo Míguez, Javier Márquez & Diana Míguez, under Diana Míguez design and development

Figure 7-42 Geographical variations of fish tissue residues

i) Gonadal histopathology

The gonad histology analysis of *Astyanax fasciatus* was performed in fish caught at sites Nuevo Berlín (F1), Yaguareté (F2), Ubici Beach (F3) and Las Cañas resort (F4) in three campaigns, especially devoted to these activities (September 2009, March 2010, August 2011). Specimens were immediately dissected at the Water Department Fray Bentos. Gonads were severed and preserved in Bouin solution. The preparation of gonads was done by classical histology methods (dehidration, embedding and staining with hematoxylin and eosin). Sections of 4 µm were obtained at Histotecnología GL2 laboratory and observed under microscope with camera. This study was devoted to the investigation of intersex structures in the gonads. Intersex is the presence of both male and female gonadal tissues in one individual, of sporadic occurrence and a pathological condition in gonochoristic fish species (Dietrich & Krieger 2010). Additional parameters of the specimens for histology were also evaluated (Appendix B).

All the specimens presented normal characteristics according to their developmental stages. Normal ovaric tissue presented perinuclear, vitellogenic and atresic follicules. Normal testicular tissue presented seminiferous tubules with cysts at different developmental stages. No specimens of *A. fasciatus* had the gonad characteristics of intersex, presence of testis–ova or ova–testis.

7.6 Human health exposure assessment

Environmental epidemiology focuses on the effects of environmental exposures on health and disease in the population (Paddle & Harrington 2000, Brunekreef 2008). Neonatal malformations of the reproductive organs have been linked to ED. For example, pesticides and phthalates can express on abnormal anogenital distance in babies and infants (Thankamony et al. 2009). Although controversial (Martin et al. 2008), cryptorchidism has been hypothesized to be linked to *in utero* exposure to contraceptive pills. Similar effects as these

produced in humans have been reported in new-born male laboratory animal and wildlife species exposed to the EDCs (Gee et al. 2007).

Even though several studies support the idea of an increasing incidence of testicular cancer, controversy still remains on the sperm decline matter (Fisch et al. 2010), due to the high global variability and environmental, dietary and/or lifestyle factors (Safe 2005). Swan et al. (2000) reviewed the sperm count studies topic and ratified the previously reported trends in the period from 1934 to1996. Relying solely on classical epidemiology is not prompt enough to protect public health from illnesses with delayed onset such as those produced by EDCs as argued by Gochfeld (2003), and the inclusion of biomarkers is recommended.

7.6.1 Congenital anomalies of the male genitals

The worldwide yearly rate of major birth defects is about 6% of total births. A birth defect, or “congenital anomaly” is defined as an anatomical and/or functional defect resulting from disturbance of normal developmental processes. Major congenital malformations require medical or surgical care, minor need less medical intervention. In general, surveillance programmes focus on major malformations, thus, limited data are available on the incidence of minor malformations.

In the case of Uruguay the hypospadias rate is similar to Argentina, and some European countries (WHO 2003). A preliminary field study for the human health component was initiated by gathering epidemiology data of neonatal malformations of reproductive organs in especial, hypospadias and cryptorchidism of residents and critically exposed receptors at several life stages in the relevant cities and towns of the area under study. In the case of this research, data from the National Children Hospital Pereyra Rossell and at the local pediatric hospital are on surgically treated hypospadias and cryptorchidism (Appendix F).

Data on neonatal malformations are scarce in the country, but they preliminarily indicate that no cases of hypospadias or cryptorchidism deserving surgical treatment in Rio Negro province existed for patients born in the last 5 years (Appendix F).

7.6.2 Food web fate processes of target EDCs for aquatic receptors

Models based on fugacity for the calculation of bioaccumulation and magnification through the food web yielded EDCs concentrations in air, water, soil and sediment, but also predator-prey relationships. Avian and aquatic and terrestrial mammals were excluded from more detailed analysis. A simplified aquatic food web for the Uruguay River environment consists on: plankton, macrophytes, amphipods, bivalve molluscs, gastropods, detritivorous, omnivorous and piscivorous fishes. The fate, distribution and bioaccumulation of target EDCs and predator-prey relationships were estimated by parametrising the FOODWEB, CEMC model with physicochemical data to build a database (Appendix D).

The results for endosulfan, nonylphenol and 2,4,5-TCP were that piscivorous fish are predators of omnivorous fish with 75% relevance through this pathway , and through detritivorous fish, 25%. Omnivorus receive endosulfan through mussels (61%), detritivorous fish (37%) and plankton (2%). The main pathway for detritivorous fish is plankton, 3% through benthic and 1% macrophytes. For mussels, the only pathway considered of importance is plankton. For gastropods, the main pathway is also plankton, but 1% are macrophytes. This pattern was not similar for β-sitosterol, as for piscivorous fish detritivorous fish was the main pathway (86%) and 14% due to ingestion of omnivorous fish. To omnivorous fish, it was more proportionately distributed (18% omnivorous, 48% detritivorous and 34% plankton). The other taxa received the compound mainly through plankton.

7.6.2.1 Bioavailability assessment

Bioavailability analysis serves to reduce uncertainty to derive site-specific exposure estimates (NRC 2003). Its variations could determine different risks to aquatic organisms, and especially to benthic animals that dwell and feed in the sediment and may ingest contaminated particles (Figure 5-8).

The fate and behaviour of EDCs in the aquatic environment depends greatly on the physicochemical characteristics of the compound but also on the interactions with the sediment particles depending on their adsorption capacities. A site-specific characterisation of exposure is necessary as sediment composition may differ among sites (Kendal et al. 2001). The bioavailability and bioaccumulation potential were experimentally demonstrated, and predicted with fugacity models. Bioavailability was studied in prior experiences for the River Negro, a tributary of the River Uruguay, using a battery of bioassays (Míguez et al. 2012).

The particle size of sediments, partitioning of 2,4,5-TCP among the water and sediment phases, and ecotoxicity with a battery of bioassays in both phases were studied. Of the sediment fractions, clay and silt have the highest surface area and adsorption capacity, being able of adsorbing more toxicants than sand Direct transport of dissolved contaminants is the main exposure route for most benthic organisms, but also through ingestion of contaminated sediment particles. Desorption of contaminants from the particles or from the dissolved organic carbon are critical in determining the contamination through the sediment route. Organisms can be re-exposed after re-suspension in the water column, and, finally, bioaccumulation may lead to biomagnification through the trophic chain (Katagi 2006) (5.3.6.1).

The distribution among dissolved and suspended fractions was considered to predict bioaccumulation. This is inversely proportional to the octanol-water partitioning coefficient (log K_{ow}). Lipophilic substances tend to accumulate in the biomass and adsorb into sediment particles (Katagi 2006). In general, a

compound distributes in the organic matter if log K_{ow}> 1. In the case of chlorophenols log K_{ow}> 2. Therefore, they distribute in the sediment phase. Chlorophenols can contaminate water, soil and sediment, and may be found in bleached Kraft mill effluent (BKME) (ATSDR 1999). Extractable organic halogens (EOX) are composed by chlorophenols and other halogenated substances that can be extracted from the solid phase with ethyl acetate.

The compound 2,4,5-trichlorophenol (2,4,5-TCP) was chosen as model substance to estimate partitioning among water and sediment through the AOX/EOX ratio, as it was found in the BKME with higher frequency than other chlorophenols. In the period from January 2009 to July 2010, the frequency of detection of 2,4,5-TCP in monthly analysis reflected that 64.5% of the times the concentrations were almost undetectable, but they reached to concentrations up to 1.2-1.3 µg l^{-1} (at a 3.2% frequency), as shown on the lognormal histogram (Figure 7-43).

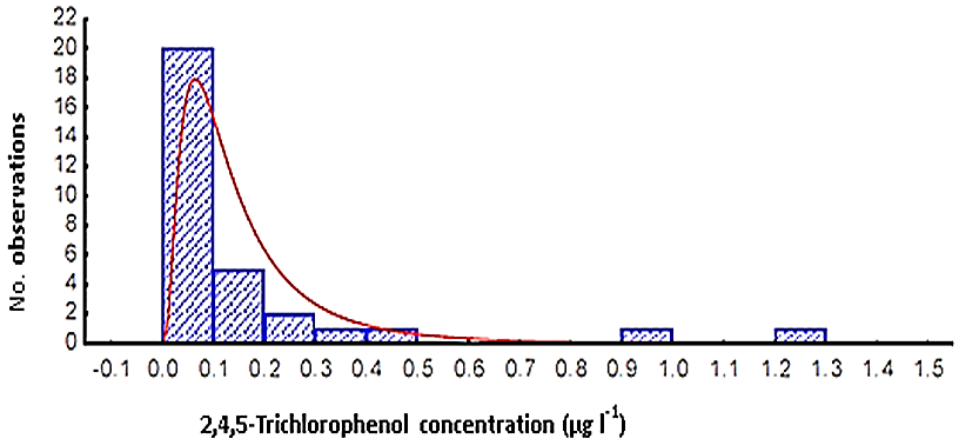


Figure 7-43 Lognormal frequency of 2,4,5-trichlorophenol in pulp mill effluent

Adsorbable organic halogens (AOX) were measured in the aqueous and EOX in the sediment fraction. Nutrients (nitrogen, phosphorus and organic matter) were analysed in this latter. The ecotoxicity was evaluated developing a bioassay with sediment dwelling organisms on whole sediment, and a battery of

bioassays with invertebrates and fish, on the water column in a site-specific manner. The following diagram (Figure 7-44) shows the experimental design:

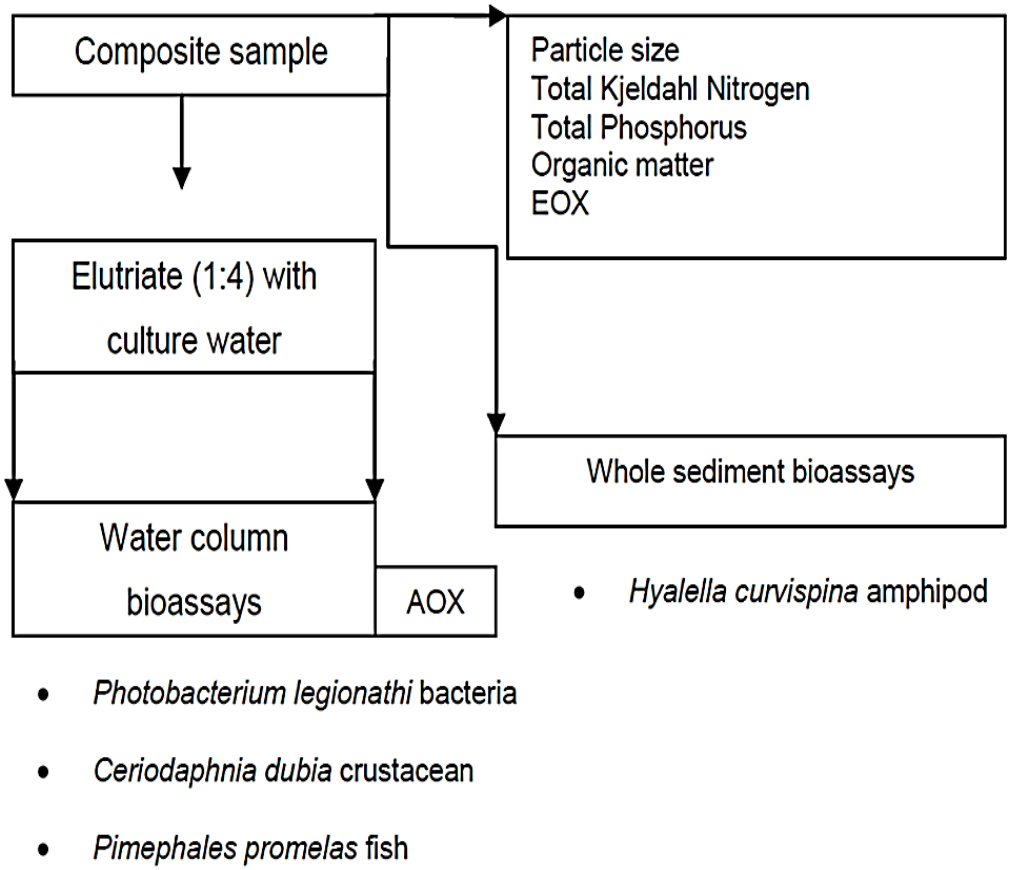


Figure 7-44 Experimental design for bioavailability evaluation of chlorinated organics

The method development is explained in Appendix B.

Nutrients concentrations are shown in Table 7-17. Organic matter has a similar concentration in all sites but phosphorus and nitrogen are higher in the S2 sample (2164 mg kg^{-1}, compared to 144 mg kg^{-1} for the control and 290 against 151 mg kg^{-1}, respectively). EOX and AOX were not detectable in any of the sites in the samples with no spiking.

Table 7-17 Nutrients and chlorinated organic compounds in sediments

Sample	Total Phosphorus (mg kg^{-1}) (as P)	Total Kjeldahl Nitrogen (mg kg^{-1}) (as N)	Organic matter (g 100 g^{-1})	EOX (as Cl) (mg kg^{-1}) (LOD 20 µg kg^{-1})	AOX (as Cl) (mg l^{-1}) (LOD 7 µg l^{-1})
S1	144	151	1.1	ND	ND
S2	2164	290	1.0	ND	ND
S8	457	116	0.6	ND	ND

ND: Not detectable; LOD: limit of detection

7.6.2.2 Ecotoxicity tests

These tests were part of the tiered approach but they were also employed to assess bioavailability. The testing conditions are summarised on Appendix B.

The results of IC50% with *P. legionathi*, in 15 and 30 minutes respectively, were: S1 19 % for metals toxicity and 37% for organics: S2, 18% and 2%, and S8 35 and 30% respectively. The IC50 (inhibition concentration 50%) is the threshold for toxicity. A 20% deviation from this level can be considered as an early warning (manual PCB Checklight).

The whole sediment *H. curvispina assay* found significant differences in dry weight per individual in the three analysed samples with regard to the control (ANOVA, p= 0.023, 95% confidence), and by Fisher´s least significant differences test at 5% significance.

The mortality rate for fish embryo at S1 was 21.5% and in S2, 7.5%. In S1 not malformations were found in any individual, however, S2 had 3.3 % malformations of the vertebral column (n = 8). The Shapiro-Wilks test proved

not normal distribution and Kruskal Wallis test demonstrated significant differences (KW = 11.6 and p= 0.04, p < 0.05). The number malformations and mortality at the lowest concentration were higher (35%) than at higher concentrations (Figure 7-26).

7.6.2.3 Bioavailability of a chlorinated model compound

The influence of the sediment composition on the bioavailability and toxicity of 2,4,5-TCP was further evaluated working with sediments of different textural classification (GRADISTAT v. 6) in unfiltered elutriates to also assess the influence of small particles in toxicity. In November 2010, sediments were dredged from coastal (S1) and deep areas (S2 and S8), and the responses were evaluated by a suite of bioassays at several hierarchical levels. The textural analysis of these sediments is presented on Table 7-18 and Appendix B. Assuming that particles of less than 8 microns are the suspended ones, as determined in the bioavailability experiment there could be an effect on the available EDC to the organisms related to the dissolved fraction. Therefore, the final dose would depend on the sediment characteristics.

Table 7-18 Particles and nutrients in sediment elutriates

Site	Particles < 8 µm (%)	Sand	Mud	Organic matter	Total phosphorus (mg kg^{-1})	Nitrogen (mg kg^{-1})
		(g 100 g^{-1})				
S1	0.0	100	0	0.1	47	22
S2	10.3	17.1	82.9	2.7	61	568
S8	4.0	59.5	40.4	0.8	140	192

Elutriates were again prepared by mixing 1.5 kg of each sediment with 6 litres ultrapure water, and dissolved each in 50 ml methanol. Volumes of 25 ml of 2,4,5-TCP stock solutions of were added to each to produce the theoretical

AOX concentrations in elutriates shown on Table 7-19, and AOX was measured in 10 ml aliquots of unfiltered elutriates.

Table 7-19 Theoretical and measured concentrations of 2,4,5-TCP in elutriates

Site	Theoretical AOX (mg l^{-1}) (as 2,4,5-TCP)	AOX measured in unfiltered elutriate (mg l^{-1}) (as 2,4,5-TCP)
S1	5.37	2.73
S2	4.98	3.36
S8	5.96	4.23

A battery of bioassays composed of *P. promelas* fish embryo and *Ceriodaphnia dubia* three brood assays, and the YES *in vitro* test were applied to evaluate embriotoxicity (teratogenicity and lethality), acute and chronic toxicity with endpoint reproduction, and ER-binding, respecitively, in unspiked and spiked sediments. These results (Table 7-20), were used to reconcile data produced in our laboratory with the literature, and to produce a site-specific characterisation of the risks of chlorinated phenols (Chapter 8) and to characterise the significance of this EDC at a population level (Chapter 9).

Table 7-20 Bioassays test results in unspiked and 2,4,5-TCP spiked sediments

Bioassays in unspiked sediments		Site		
		S1	**S2**	**S8**
***Pimephales promelas* (LC50%), acute**		>100	>100	>100
***Ceriodaphnia dubia* (LC50%, 48 h), acute**		>50	>50	>50
***Ceriodaphnia dubia* chronic toxicity test**	LC50(Three Brood Test)	>50	>50	>50
	IC25(Three Brood Test)	22.1	10.2	8.0
	IC50(Three Brood Test)	>50	24.8	22.9
	LOEC	25	12.5	6.2
	NOEC	12.5	6.2	3.1
YES (Yeast Oestrogen Screen) (as E2)	µg l^{-1}	ND	ND	ND
Bioassays in spiked sediments		**Site**		
		S1	**S2**	**S8**
***Pimephales promelas* (LC50%), acute**		14.1	15.6	< 6.25
LC_{50} , mg l^{-1} as 2,4,5-TCP		0.39	0.52	< 0.26
***Ceriodaphnia dubia* (LC_{50}, 48 h), acute**		29.3	10.1	35.4
LC_{50} / mg l^{-1} as 2,4,5-TCP		1.5	0.6298981	2.7794142
		0.63	1	2.8 7
***Ceriodaphnia dubia* chronic**	LC50 (Three Brood Test)	16.5	3.51	32.9
	IC25 (Three Brood Test)	3.28	< 3.12	3.26
	IC50 (Three Brood Test)	4.65	< 3.12	5.56
	LOEC	25	12.5	3.12
	NOEC	12.5	6.2	< 3.12
YES (Yeast Oestrogen Screen) (as E2)	µg l^{-1}	480	ND	ND

7.7 Bioaccumulation assessment

The bioaccumulation of organic molecules in fish tissue results not only from direct exposure through water and sediment, but also from indirect exposure through the dietary pathways. Sediment and surface water are the major pathways for transport and distribution of contaminants through the watershed and for direct exposure of biota. However, for very hydrophobic compounds,

exposure is not only dependent on the concentration of the EDC in the water and sediment pathways, but also to bioconcentration. In the case of hydrophobic EDCs sediment-bound EDCs concentrations provided less variable data and also tissue residues in biota (fish, mussels, snails and floating plants) to integrate data from the food web and multimedia distribution and calculate the dose to the receptors.

The concentration of halogenated organics was analysed in the organic matter of biota samples: floating macrophytes, fishes, snails and mussels.

7.7.1.1 EOX in gastropods

A specimen of *Pomacea sp.* snail (Figure B-25) was collected at Anglo Beach, where the collection pipe for the municipal wastewater discharges. Chlorinated organics were evaluated by analysing EOX. The measured concentration was as 1.4 µg kg^{-1}.

7.7.1.2 EOX in mollusks

Species analysed: *Corbicula sp..* The samples were taken at Ubici beach, near the pulp mill plant and transported frozen to the laboratorio at Montevideo. The method was based on Kostamo et al. (2000).The result was an average of 0.17 mg kg^{-1} lipid in one sample taken from Ubici beach.

7.7.1.3 EOX in floating plants

Floating plants are niches for river ecosystem habitats. Water hyacinths of the species *Eichhornia crassipes* float on the Uruguay River. Their stems and roots harbour nitrogen-fixing bacteria, vertebrates and invertebrates. Moorhens usually peck at water hyacinths and insects take nectar during flowering (Ruiz Téllez et al. 2008).Their application as tertiary treatment for Kraft mill effluents is promising, as organic matter removal efficiencies of 46–75% and 11–17% of total phenolic compounds have been achieved in experimental conditions (Lagos et al. 2009). Based on its nutrient removal capacity they has been proposed as a remediation measure for eutrophic lakes (Meerhoff et al. 2002).

An experiment was carried out by exposing *E. crassipes* to BKME, as described in Section B5-8 (Appendix B), demonstrating that chlorinated organics distributed in the plant and concentrated in the leaves. In parallel, nutrient removal was efficient. Nitrite was removed in 77%, nitrate, 99%, total phosphorus, 62% and soluble phosphorus, 94%.

7.7.1.4 Bioaccumulation of alkylphenols in gastropods

Alkylphenols were measured on a portion of the same snail specimen of the *genus Pomacea* collected at Anglo Beach, where the municipal collector pipe from the city of Fray Bentos pours the wastewater into the river. The concentrations were below quantitation limit (Table 7-21).

Table 7-21 Alkylphenols in gastropods

EDC	Concentration (µg kg^{-1})
4-n-octylphenol	< 0.5
4-tert-octylphenol	< 0.5
4-n-nonylphenol	< 0.5
4-nonylphenol	< 3.0

7.7.2 Fish tissue residues of EDCs in fish sold in the market

Fish tissue residues of prioritised EDCs were analysed in catfish bought at the local Fray Bentos open market. Spotted pimelodus or “bagre pintado” (*Pimelodus maculatus*) (taxonomic class: *Actinopterygii*, Order: Siluriforms) (Figure B-26) is an omnivore and detritus feeder fish species, a primary consumer and primary and secondary carnivore and pre-mineraliser that ingests organisms from planktonic, nektonic and benthic communities, plants, inorganic material, insect larvae and molluscs (9.6%). Its swimming speed is 5 lengths s^{-1} (Santos et al. 2008), implying that it is not long distances migratory.

Two sets of ten fish of the mentioned species were bought at the local market of Fray Bentos on 10th August 2010 and on 19th August 2010, respectively. Their weights, length and condition factor (K) were similar among groups. However, the hepatosomatic index (LSI) was significantly bigger (t- test, $p< 0.05$) in the case of the specimens bought on the 19th August. The most prominent EDCs found in fish tissues were 4-nonylphenol and endosulfan.

Table 7-22 Characteristics of *Pimelodus maculatus* specimens

Date	Weight	Length	K	LSI
10/08/2010	561.5 ± 91.99	27.9 ± 8.8	1.36 ± 0.42	1.21 ± 0.13
19/08/2010	564.5 ± 88.3	35.8 ± 2.22	1.24 ± 0.17	1.62 ± 0.40

Fish muscle residues were used to evaluate the dose via the dietary pathway. Fish liver residues were analysed to evaluate the effects on the fish health (Table 7-23).

Table 7-23 *Pimelodus maculatus* fish tissue residue

EDC	Muscle tissue residues (ng g^{-1})	Liver tissue residues (ng g^{-1})
4-n-Octylphenol	< 0.5	< 0.5
4-tert-Octylphenol	1.10	18.6
4-n-Nonylphenol	< 0.5	< 0.5
4-Nonylphenol	60.8	30.7
Endosulfan I	6.8	0.62
Endosulfan II	8.7	0.83
Endosulfan sulphate	170	22
Glyphosate	< 0.1 µg g^{-1}	< 0.1 µg g^{-1}
2,4,5-trichlorophenols	< 0.1 µg g^{-1}	< 0.1 µg g^{-1}
Resin acids	< 3 µg g^{-1}	29 µg g^{-1}

The OCDD, total dioxins and 2,3,7,8-TCDD in fish muscle were also studied (See Figure 7-21 and Figure 7-22) showing higher concentrations at F4 in all cases.

7.8 Summary of the exposure assessment results

Tier 1 rated water quality as type 3, concerning organic matter content, deeming it suitable for water abstraction. However, when nutrients were considered, the water quality fluctuates, reaching type 4 or worse, showing that the main hazard for river health is eutrophication. Sub-lethal growth effects in *Hyalella curvispina* were evidenced with whole sediments of Nuevo Berlín, Fray Bentos and Las Cañas.

Tier 2 determined that at Ubici Beach the phenols concentration was above that of the other sites and that there were events when the conductivity was much higher than the other sites, suggesting a probable influence of the effluent plume at this site. Sediment elutriates showed chronic effects to crustaceans at Las Cañas, but also at the upstream site, Nuevo Berlín.

In Tier 3, several EDCs were found to occur in the watershed, in especial in Fray Bentos stream that received municipal wastewater, and in particular nonylphenol, other alkylphenols and oestrogens. Fish exposure experiments with water of this stream produced biomarker responses consistent with oestrogenicity. Responses obtained after exposing fish to pulp mill effluent suggest androgenic or anti-oestrogenic activity, but the responsible agents are not clear. Agricultural inputs from non-point sources were mainly due to endosulfan, found in fish tissue, and glyphosate, measured in water and in streams sediment. The wildfish monitoring showed that at Ubici Beach, the beach downstream the pulp mill, the gonads were smaller than at the rest of the sites. The presence of low levels of phytosterols, resin acids, PAHs, PCBs, dioxins and furans are diffusely represented at a catchment level.

7.9 Exposure characterisation by principal component analysis

A global pondering on 19 variables were modelled in relation to the categorical variables Tiers 1a-b to 3a-b, by the principal components analysis (PCA), using the software STATISTICA (StatSoft, Inc. (2007), (data analysis software system), version 8.0. www.statsoft.com). A standardised biplot representing both variables and cases together in two dimensions and their relationships show that only Tiers 2a, 2b and 3a were found to be of influence. As 2a and 2b are on the same axis, there might be a correlation among them. Tier 2a is of more influence towards Tier 3a, meaning that chlorinated organics and phenols could be among the component EDCs (Figure 7-45).

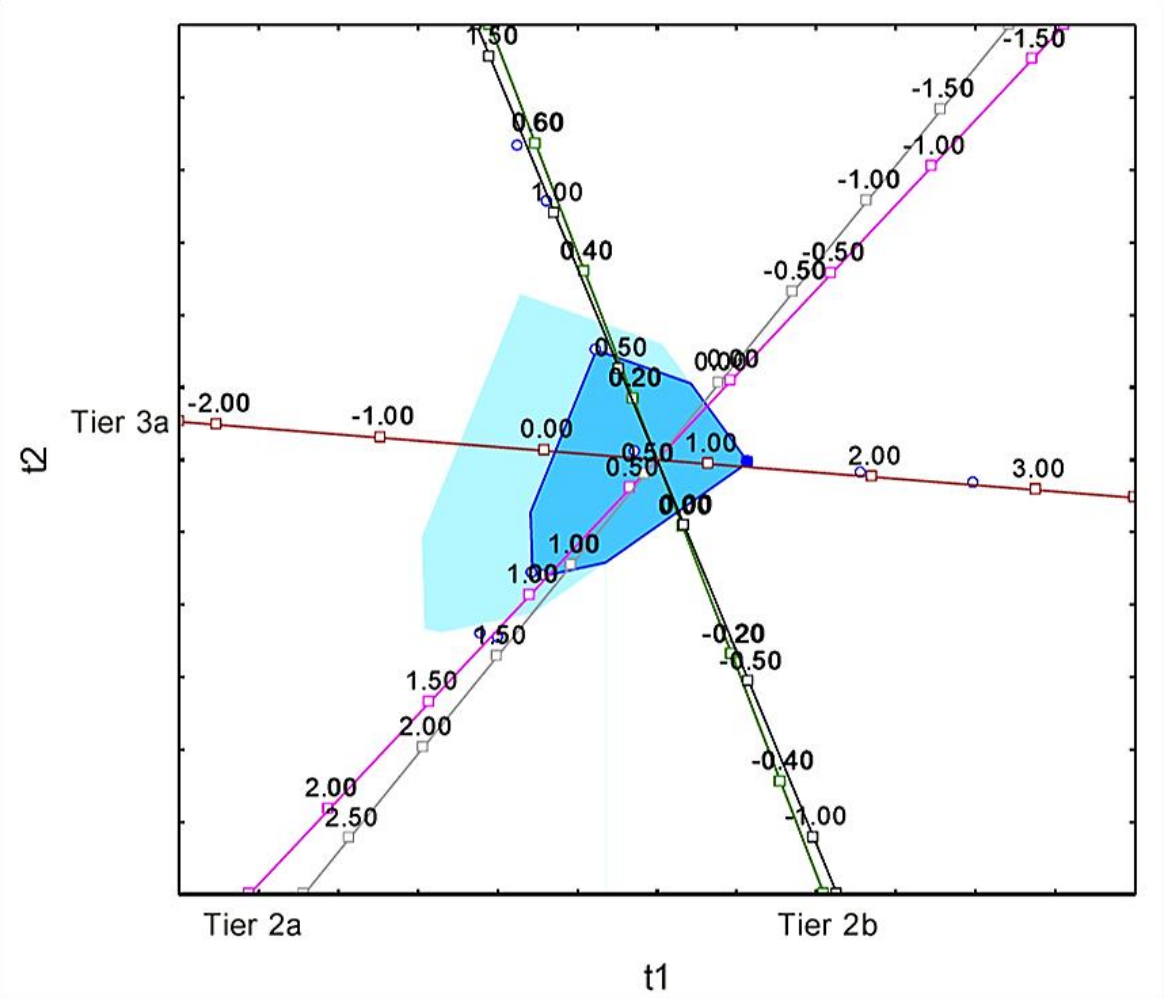
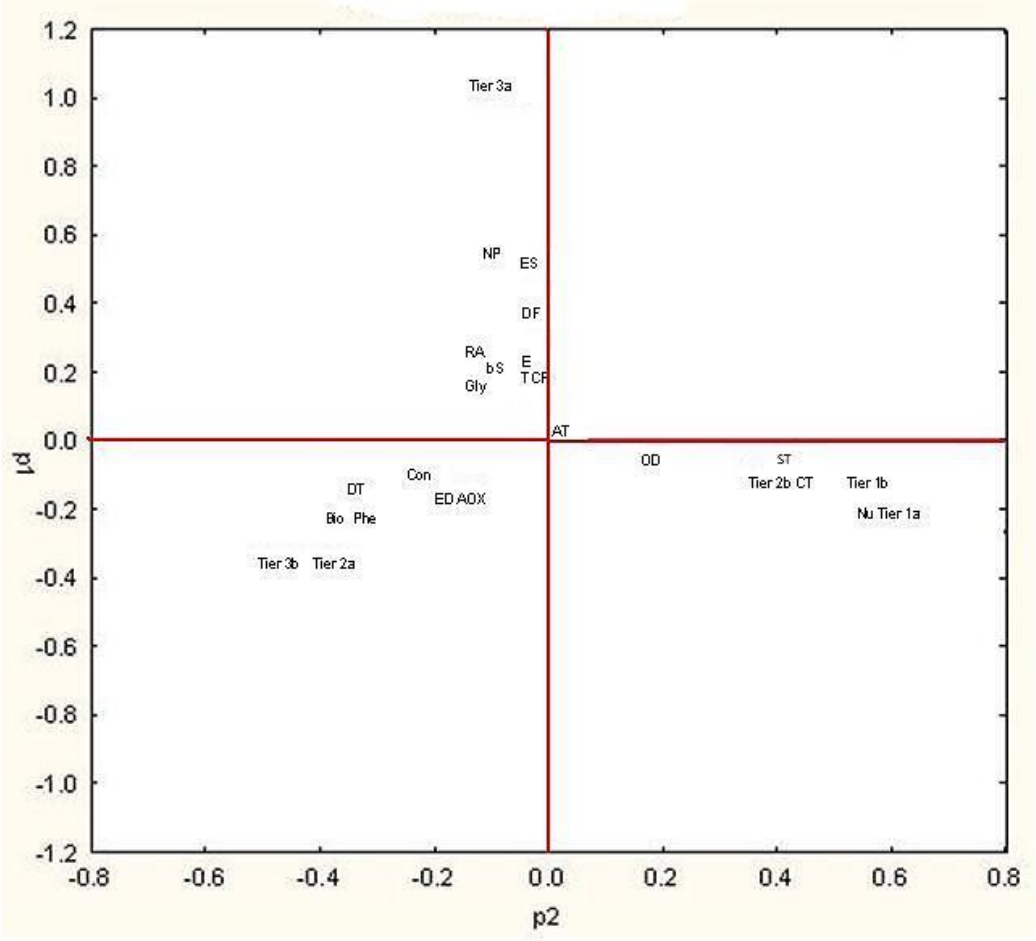


Figure 7-45 Influential tiers represented as PCA standaridised biplot

Then, a loading scatterplot (
Figure 7-46) showed that Tiers 3a correlates to EDCs; Tier 3b, to biomarkers responses and developmental toxicity, but also to components in Tier 2a, relating mainly to phenols. Tier 1a and 1b are connected to nutrients, but do not have direct influence on the EDCs. Nonylphenol and endosulfan are the most influential EDC. Chronic toxicity as well as sub-lethal toxicity are determined by Tier 2b, signifying that phenols could be a factor in the latter. Acute toxicity is not critical nor oxygen depletion in the watercourse.



OD: oxygen demand; DF: dioxins and furans; ST: sublethal toxicity; AT: acute toxicity; Nu: nutrients; Phe: phenols; ED: endocrine disruption; AOX: adsorbable organic halogens; Con: conductivity; DT: developmental toxicity; NP: nonylphenol: E: oestrogens; ES: endosulfan; Gly: glyphosate; bS: b-sitosterol; RA: resin acids; Bio: bioassays

Figure 7-46 Loading scatterplot of two components for the exposure assessment tiers and their variables

7.10 Dose-response assessment

The doses of EDCs to humans were calculated through the ingestion route for fish and drinking water. Further, the particle ingestion, inhalation and dermal pathways were considered for all the human life stages. Breast milk was included in the case of babies from 0-6 months. For environmental receptors, the NOEL/LOEL was calculated. However, as it is more suited to predict deterministic but not stochastic or probabilistic ED responses (Hirabayashi & Inoue 2011), and as down-modulation of receptors could provoke a lack of induction at higher doses (2.3.6), the benchmark dose method, that differentiates if a linear or a non linear curve slope exists seems more fitted. Therefore, the Benchmark Dose Software (BMDS) Version 2.1.1 (EPA/630/R-96/009) (Appendix E) was applied to calculate the VTG doses in fish exposure tests.

7.10.1 Dose calculation for fish consumption only

The following conservative estimate of intake solely of fish is presented, as Paustenbach (2002), page 802. The fraction of the original PCB concentration remaining after cooking is 50 %, giving a factor of 0.5. According to DINARA, the country average is 6.74 kg per year per person. Based on CARU – DINARA–INIDEP (2008), the average concentration of the total PCBs in fish muscle (sábalo, boga and tararira, species of commercial value) is 3.32 ng g^{-1}. The concentration was in the range <1 ng g^{-1} - 9.16 ng g^{-1} muscle wet weight. Even the highest value is well below the maximum allowable level according to FDA (2001) (2000 ng g^{-1}).

The dose of EDCs from fish consumption was calculated using equation 7-10:

$$Dose = \frac{Uf \times Fcook \times FC}{BW} \quad \textbf{(7-10)}$$

Where

Uf = fish consumption rate (kg d^{-1})

$Fcook$ =fraction of PCBs remaining after cooking (unitless)

FC = concentration of PCBs in fish (mg kg^{-1})

Table 7-24 Dose of EDCs from fish consumption

EDC	FC (mg kg^{-1})	Dose (mg kg^{-1} d^{-1})
2,4,5-trichlorophenol	< 0.1 x 10^{-3}	1.3 x 10^{-8}
4-n-Nonylphenol	< 0.5 x 10^{-3}	6.6 x 10^{-8}
4-n-Octylphenol	1.1 x 10^{-3}	1.5 x 10^{-7}
4-Nonylphenol	< 0.5 x 10^{-3}	6.6 x 10^{-8}
4-tert-Octylphenol	60.8 x 10^{-3}	8.0 x 10^{-6}
Endosulfan I	6.8 x 10^{-3}	9.0 x 10^{-7}
Endosulfan II	8.7 x 10^{-3}	1.1 x 10^{-6}
Endosulfan sulphate	170 x 10^{-3}	2.2 x 10^{-5}
Glyphosate	< 0.1 x 10^{-3}	1.3 x 10^{-8}
PCBs	3.3 x 10^{-3}	4.4 x 10^{-7}
Resin acids	< 3 x 10^{-3}	4.0 x 10^{-7}
β-sitosterol	5.5	7.3 x 10^{-4}

7.10.2 Dose from drinking water ingestion

The sensitive sub-population are pregnant (15-49 years) and lactating women, as many contaminants may be delivered to the foetus. In the case of women, the average water intake is 1147 ± 648 ml, while that for pregnant women is 1189 + 699 ml (3.7% higher than other non pregnant, non lactating women). For lactating women it implies 14.2% increase (1310 + 591 ml). At the preliminary level, 1.5 litres ingestion covers the differences for all adult ages (Technical Support Document for Exposure Assessment and Stochastic Analysis Sep.2000 (http://oehha.ca.gov/air/hot_spots/pdf/chap8.pdf)). The reference dose as Tolerable Daily Intake (TDI; mg kg^{-1} day^{-1}), was based on USEPA or Health Canada, from tables found in the website.

Table 7-25 Dose of endosulfan from drinking water and fish ingestion pathways

USEPA summary of dietary exposure and risk for endosulfan (2007)- Exposure and risk for drinking water only					
Population sub-group	Age (years)	Acute dietary exposure (µg kg^{-1} day^{-1})		Chronic dietary exposure	
		99.9th percentile	%aPAD	(µg kg^{-1})	%cPAD
Fray Bentos population	All	0.47	31	0.5	
infants	< 1	1.21	80	1.7	
Children	1–2	0.47	32	0.8	
Children	3–5	0.46	30	0.7	
Children	6–12	0.28	19	0.5	
Youth	13–19	0.31	20	0.4	
Adults	20–49	0.35	23	0.5	
Adults	≥50	0.24	16	0.5	
Females	13–49	0.33	22	0.5	
Exposure and risk for food only					
Fray Bentos population	All	0.11	7	0.004	0.6
infants	< 1	0.14	9	0.004	0.7
Children	1–2	0.24	16	0.013	2.1
Children	3–5	0.18	12	0.009	1.6
Children	6–12	0.13	9	0.003	1
Youth	13–19	0.085	6	0.003	0.6
Adults	20–49	0.09	6	0.003	0.4
Adults	≥50	yr	0.1	0.003	0.4
Females	13–49	yr	0.09	0.003	0.4
Exposure and risk for and drinking					
Fray Bentos population	population		0.48	0.007	1.1
All	infants <1	yr	1.21	0.015	2.4
Children	1–2	yr	0.53	0.017	2.9
Children	3–5	yr	0.55	0.014	2.3
Children	6–12	yr	0.3	0.009	1.5
Youth	13–19	yr	0.32	0.006	0.9
Adults	20–49	yr	0.35	0.006	0.9
Adults	≥50	yr	0.26	0.006	0.9
Females	13–49	yr	0.34	0.005	0.9

%aPAD: acute Population Adjusted Dose

% PAD = exposure (total dietary exposure)/PAD) x 100. The cPAD is equivalent to the chronic oral RfD value of 0.001mg kg^{-1} day^{-1}

7.10.3 Dose received by different human life stages for ingestion, inhalation and dermal exposure

A recalculation to approximate to the conceptual model (section 5.3) to include other pathways such as inhalation and dermal but considering only fish and drinking water (and breastmilk for lactating infants) as dietary routes produced the doses shown on Tables 7-38 and 7-39. Equations 7-11 to 7-14 were used to calculate the doses and 7-15 to combine them. A water rate ingestion of 1.5 litres covers the differences for all adult ages (Technical Support Document for Exposure Assessment and Stochastic Analysis September 2000 (http://oehha.ca.gov/air/hot_spots/pdf/chap8.pdf).

Parameters were similar to the Canadian, but fish ingestion rate was adjusted to the Uruguayan average one, with slight changes in body weights. For PCBs the food ingestion was the only pathway considered but life stages were taken into account, assuming twice a week to consider a population living more on fish than the rest of the average. The dose for toddlers results in 2.6 x 10^{-7} (HQ =0.0001); for children: 2.1 x 10^{-7} (HQ= 0.0010); for teens: 1.3 x 10^{-7} (HQ= 0.0010) and for adults: 1.2 x 10^{-7} (HQ= 0.0095), in the order of those calculated before (Table 7-26) with a different approach.

$$Dose_{si} = \frac{\left(C_s \times IR_s \times AF_{GIT} \times D_{hours} \times D_{days} \times D_{weeks}\right)}{BW \times 16 \times 365} \qquad \textbf{(7-11)}$$

Where $Dose_{si}$ = Dose of soil ingestion

Cs = Concentration (mg kg^{-1}) of contaminant in soils

IRs = Accidental soil ingestion rate for adult (kg day^{-1})

AFGIT = Absorption Factor for the gastrointestinal tract

D_{hours} = Hours per day with exposure (0 - 16)

D_{days} = Days in a week with exposure (0 - 7)

D_{weeks} = Weeks in a year with exposure (0 - 52)

bw = Body weight of receptor

$$Dose_{wi} = \frac{C_w \times IR_w \times AF_{GIT} \times D_{days} \times D_{weeks}}{BW \times 365} \quad \textbf{(7-12)}$$

$Dose_{wi}$ = Dose of water ingestion

IR_w = Drinking water ingestion rate (l day^{-1})

C_w = Concentration (mg kg^{-1}) of contaminant in soils

$$Dose_{fi} = \frac{C_{food} \times IR_{food} \times AF_{GIT} \times D_{days} \times D_{weeks}}{BW \times 365} \quad \textbf{(7-13)}$$

$Dose_{fi}$ = Dose of food ingestion

C_f = Concentration (mg kg^{-1}) of EDC in food

IR_{food} = Food ingestion rate (kg day^{-1})

$$Dose_{pi} = \frac{C_s \times P_{air} \times IR_a \times AF_{inh} \times D_{hours} \times D_{weeks}}{BW \times 365 \times 10\, e^{9}} \quad \textbf{(7-14)}$$

$Dose_{pi}$ = Dose of particle inhalation

C_s = Concentration of contaminant in soils

P_{air} = Concentration of particles in the air (µg m^{-3}) Used 0.76 µg m^3 for typical conditions as per USEPA (1992)

IRA = Inhalation rate (m^3 $hour^{-1}$)

AF_{inh} = Absorption Factor for the lungs

Table 7-26 Dose for endosulfan considering dermal, inhalatory and ingestion pathways

Age (years)	Dose (mg $kg^{-1}day^{-1}$) via pathway:					Total dose	Food ingestion
	Soil ingestion	Water ingestion	Particle inhalation	Food ingestion	Dermal contact		
Baby (0-6 months)	1.2×10^{-9}	0.0000080	9.4×10^{-8}	1.5×10^{-13}	2.7×10^{-7}	0.0000084	Breast milk
Toddler (7 month- 4)	3.1×10^{-9}	0.000015	1.5×10^{-7}	2.2×10^{-12}	0.0000067	0.000022	Fish only
Child (5-11)	3.9×10^{-10}	0.000010	1.2×10^{-7}	6.9×10^{-12}	0.0000046	0.000015	Fish only
Teen (12- 19)	4.2×10^{-10}	0.0000068	7.8×10^{-8}	3.7×10^{-12}	0.0000034	0.000010	Fish only
Adult (> 20)	1.8×10^{-10}	0.0000085	7.1×10^{-8}	3.4×10^{-12}	0.0000032	0.000012	Fish only

Software: Hazard Quotient Risk Calculation Toolkit http://www.popstoolkit.com/tools/HHRA/NonCarcinogen.aspx

Table 7-27 Dose and hazard quotient for nonylphenol considering dermal, inhalatory and ingestion pathways

Age (years)	**Dose (mg $kg^{-1}day^{-1}$) via pathway:**						**Total dose**	**Food ingestion**
	Soil ingestion	Water ingestion	Particle inhalation	Food ingestion	Dermal contact	**Hazard quotient**		
Baby (0-6 months)	7.8×10^{-12}	6.7×10^{-5}	1.7×10^{-14}	4.0×10^{-4}	8.0×10^{-10}	0.094	$4.7X\ 10^{-4}$	Breast milk
Toddler (7 month- 4)	3.2×10^{-11}	1.3×10^{-4}	7.4×10^{-14}	1.2×10^{-5}	4.3×10^{-9}	0.029	$1.4X\ 10^{-4}$	Fish only
Child (5-11)	3.9×10^{-12}	9.0×10^{-5}	5.7×10^{-14}	9.6×10^{-6}	7.3×10^{-10}	0.020	$9.9X\ 10^{-5}$	Fish only
Teen (12- 19)	2.1×10^{-12}	6.1×10^{-5}	3.4×10^{-14}	6.0×10^{-6}	5.4×10^{-10}	0.013	$6.4X\ 10^{-5}$	Fish only
Adult (> 20)	1.8×10^{-12}	7.7×10^{-5}	2.8×10^{-14}	5.4×10^{-6}	5.1×10^{-10}	0.016	$8.2X\ 10^{-5}$	Fish only

Software: Hazard Quotient Risk Calculation Toolkit http://www.popstoolkit.com/tools/HHRA/NonCarcinogen.aspx

8 RISK ESTIMATION

The magnitude of risk was determined by the hazard quotient (HQ) comparing the exposure point concentration to the health-based screening level. For environmental receptors, the approach taken depended on physicochemical, fate and toxicity characteristics of the EDCs, starting by a simple calculation of the ratio between the detected environmental concentration and the PNEC. This was not possible for all cases, as reported PNECs do not exist for every EDC. For target EDCs the mechanisms of action are not always equal. Therefore, only those EDCs with similar mechanism of actions can be combined by this approach.

8.1 Hazard quotient of EDCs via fish consumption

Based on the doses derived in Chapter 7, HQs were calculated to estimate human health risks through fish ingestion, drinking water intake and other routes, such as no-dietary routes through the dermal and inhalation pathways.

8.1.1 Dietary routes

To approximate to the problem, a simple calculation of HQ for PCBs through merely fish consumption by humans was completed. This was expressed as Equation 8-1:

$$HQ = \frac{Uf \times Fcook \times FC}{BW \times threshold} \quad \textbf{(8-1)}$$

Where

Uf=fish consumption rate (kg d^{-1})

$Fcook$ =fraction of PCBs remaining after cooking (unitless)

FC= concentration of PCBs in fish (mg kg^{-1})

$threshold$= highest dose that does not cause adverse effects in sensitive individuals (mg kg^{-1} d^{-1})

bw=body weight (kg)

To calculate the exposure by drinking water ingestion, the mean value varied according to the age: for infants of < 1 year old: 43.5 ml kg^{-1} d^{-1}; from 1-10 years old: 35.5 ml kg^{-1} d^{-1}; from 11-19 year old: 18.2 ml kg^{-1} d^{-1}, and from 20-64 years: 19.9 ml kg^{-1} d^{-1} (Selevan et al. 2000).

8.1.2 Reference doses for oral chronic ingestion and calculation of hazard quotients

The information to derive the HQs was based on the Integrated Risk Information System, IRIS database (http://www.epa.gov/iris) for chronic oral risk. High uncertainties currently exist because it is not always possible to get information on the endocrine disruption endpoint. The doses were calculated as in Table 7-24 . No RfDs for resin acids or for β-sitosterol existed on databases. Then, the same values as glyphosate and 2,4,5-TCP were assumed. For NP the value taken was 0.005 based on Nielsen et al. (2000).

Table 8-1 Hazard Quotient for EDCs for the general population from fish diet

EDC	Dose from fish ingestion only (mg kg^{-1} d^{-1})	RfD	HQ
2,4,5-trichlorophenol	1×10^{-8}	1×10^{-1}	1×10^{-7}
4-Nonylphenol	8×10^{-6}	5×10^{-3}	2×10^{-3}
Endosulfan (sum of α, β and sulphate)	2×10^{-5}	6×10^{-3}	2×10^{-4}
Glyphosate	1×10^{-8}	1×10^{-1}	1×10^{-7}
PCBs	4×10^{-7}	1×10^{-4}	3×10^{-3}
β-sitosterol	7×10^{-4}	1×10^{-1}*	7×10^{-3}
Resin acids	4×10^{-7}	5×10^{-3}*	8×10^{-5}

*assumed based on expert judgement and comparison to other compounds

In the IRIS database for mixed PCBs no information is reported on RfD but only for individual components with only data for Aroclor 1248, Aroclor 1016, Aroclor 1245. However, the TDI found in Health Canada is 0.00013 mg kg $^{-1}$ day^{-1}. In the case of nonylphenol and endosulfan a more in depth estimation was done taking into consideration the other non dietary pathways. For endosulfan a RfD of 6 x10^{-3} was used based on this database. These compounds were assessed by the TDI of dietary and non-dietary pathways.

8.1.3 Hazard quotients of nonylphenol and endosulfan through dietary and non-dietary routes

Absorption can take place especially by ingestion of contaminated food and 90% is of animal origin. Fish products, in general, represent only a small amount (about 10%) of diet, even if this percentage represents the main contamination route for humans. Another pathway is through skin absorption and inhalation. To assess all these, calculations of doses and HQs were done for two EDCs (nonylphenol and endosulfan) with the Hazard Quotient Risk Calculation Tool of the Persistent Organic Pollutants toolkit, Canadian POPs Trust Fund (http://www.popstoolkit.com/tools/HHRA/NonCarcinogen.aspx). The doses were calculated based on an ingestion rate on twice a week diet of fish.

For threshold contaminants, the Hazard Quotient (HQ) can be expressed as:

$$HQ = \frac{Dose\ rate}{RfD} = \frac{Exposure\ concentration}{RfC} = \frac{Estimated\ dose}{TDI} \qquad \textbf{(8-2)}$$

Table 8-2 Hazard Quotient for nonylphenol and endosulfanby life stage in exposed sub-population

Age (years)	Hazard Quotient	
	Nonylphenol	Endosulfan
Baby (0-6 months)	0.094	0.0014
Toddler (7 month- 4)	0.029	0.0036
Child (5-11)	0.020	0.0025
Teen (12- 19)	0.013	0.0017
Adult (> 20)	0.016	0.0020

The Reference Dose (RfD) was studied, after browsing databases, but even the most comprehensive ones (such as RAIS) did not have all pathways and EDCs, which precluded completing the task for every stressor. To further investigate the dose of nonylphenol in target organs a physiologically based pharmacokinetic (PBPK) model yielded, as expected, that the chemical concentrates in adipose tissue and in liver (Figure 8-1).

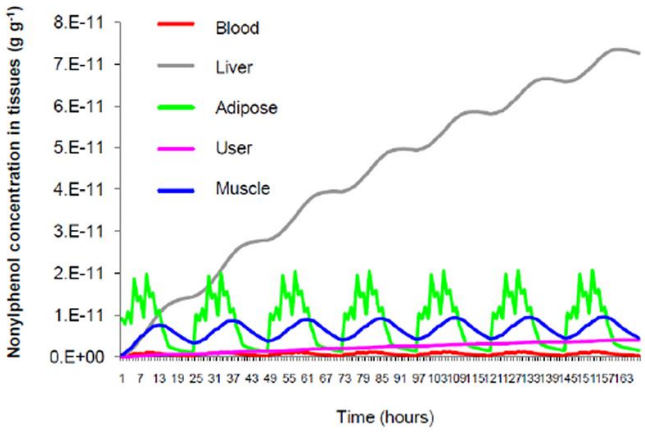


Figure 8-1 Nonylphenol metabolism modelled by PBPK multi-compartment model representing its fate, and distribution in blood, liver, muscle and adipose tissues

8.1.4 Risks of PAHs for the exposed human population

In river water total PAHs was 0.27 µg l^{-1}, in sediments 0.04 µg g^{-1} (6.3.5). As no information regarding levels in fish muscle was available, the average concentration 0.018 µg g^{-1} in Patrolecco et al. (2010) was assumed, and that in soil 0.3 µg g g^{-1} as the median concentration in Jones´ et al. (1989). The assumptions for sum PAHs in drinking water were 1 ng l^{-1}, the lower typical level mentioned in the latest guidelines WHO (2011a). The concentration in breast milk was estimated as the highest level reported by TOXNET database (2004) (http://toxnet.nlm.nih.gov/cgi-bin/sis/search/f?./temp/~EavYrU:2) as 0.03 µg kg^{-1}. As a reference, as the endpoint considered is not cancer, the TDI taken was the most stringent for non-carcinogenetic PAHs (0.04 mg kg^{-1} d^{-1}), based on TERA (2013) Database (http://www.tera.org/). The resulting HQ for adults and teens from all media was 0.004, while for children, toddlers and babies, 0.01.

8.1.5 Risks of dioxins and PCBs for the exposed human population

For adults, the evaluated median concentrations of 2,3,7,8-TCDD of 0.07 pg g^{-1} in fish, and soil concentration of 1 pg g^{-1}, produces an HQ for multimedia pathways of 0.06. However, in the worse-case scenario, if dioxins concentrations were constantly at the maximum quantified in fish muscle (5.5 pg g^{-1}), a HQ of 0.10 is obtained for the oral route in adults, but it would rise to a total HQ of 0.29 through all routes, meaning there would be a potential risk to the receptor, and could represent a matter of concern. No actual soil concentrations existed, so they were estimated as 10 pg g^{-1} that are equivalent to those reported in Australia by Müller et al. (2008).

Nursing infants can be exposed through breast milk, but no information was available to derive an accurate calculation based on the current situation. Therefore, ten, the mean concentration in several countries was taken instead as an indicative value: 9.5 pg g^{-1} WHO-TEQ per g of fat (Ulaszewska et al. 2011). If the daily intake of breast milk is 800 g (Mena & Milad 1998), and the

average fat content is 4%, then this would imply a dose of 0.3 pg day^{-1}. Assuming a body weight for the baby of 8 kg, it results in a dose of 0.038 pg kg^{-1} day^{-1}. The TDI can be assumed as 4 pg $kg^{-1}day^{-1}$, as for any other food. Therefore, the HQ was 0.0095.

In a similar way, the HQ was 0.0093 for babies receiving a mean concentration of PCBs of 9.3 pg g^{-1} WHO-TEQ per g of fat. For the maximum concentration of PCBs in catfish caught in the area under research of 0.38 pg g^{-1} the HQ for an adult was 0.0082.

8.2 Relative risk ranking for endocrine disruptors in the Uruguay River for ecological receptors

A screening of potential risk to ecological receptors estimated the risk for aquatic ecological receptors. The RAIDAR model version 1.00 (Arnot et al. 2006), identified the most critical medium and sensitive ecological receptor for selected EDCs. The estimated emission rates were estimated according to the use of the substance in the research area. The endpoint considered was not lethality but endocrine disruption manifested either as a VTG increase in fish or developmental or reproductive effects on crustacea.

The program was parametrised with physico chemical and ecotoxicity data (Appendix D). The medium of concern for endosulfan and NP are through sediment transport of contaminants, and benthic invertebrates are the probably most sensitive ecological receptors. In the cases of isopimaric acid and glyphosate, the information was very scarce but results suggest that piscivorous fish could be the most sensitive ecological receptors.

Nonylphenol is first in the risk ranking, second endosulfan, followed by glyphosate and isopimaric acid in this screening level assessment (Figure 8-2).

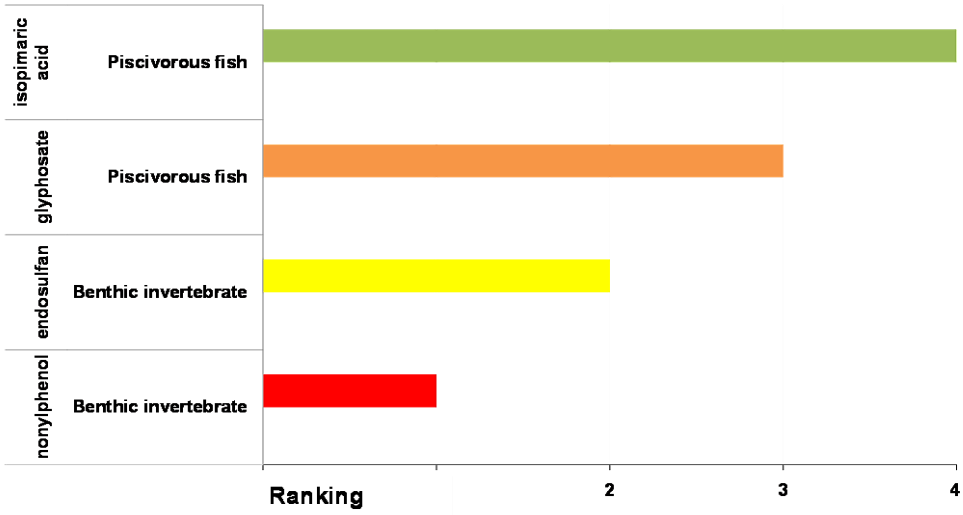


Figure 8-2 Relative risk ranking of target endocrine disruptors in the aquatic food web

8.3 Risk estimation for environmental receptors

Several approaches were taken depending on the EDCs physicochemical characteristics and occurrence in the watershed. In the cases if the PNEC existed in databases it was used to derive a risk quotient. On the other hand, when these values were not available, the hazard quotients were calculated

Endosulfan was evaluated according to the body burdens in fish based on its persistence; on the contrary, glyphosate was not, because of its rapid excretion and low bioaccumulation potential based on its low K_{ow} in the range of < -1.7 to 1.7 (Table 6-1).

8.3.1 Risk quotient of nonylphenol for environmental receptors

The PNEC reference for surface water was taken from the EU directive, while for sediments, the value selected by Fenner et al. (2002).

Risk Quotients (RQ) calculated for the case of nonylphenol for several key sites in the river (Table 9-3) demonstrate that concern exists in the case of the creek (Fray Bentos stream receiving municipal wastewater, C2 and S5).

The general criteria set in this case, was that if:

RQ < 1 Not immediate concern

RQ 1-10 Of concern, if inputs to the river increase

RQ 10- 100 Warning

RQ > 100 Immediate actions are required

A warning can be set for the concentration of nonylphenol in Fray Bentos stream sediment, because the concentration found in its waterswas of concern.

The river site source for water abstraction for drinking water treatment(R6) is at the limit of tolerance. One should hope that the treatment would reduce this further, but it is still of concern, moreover considering all the other possible stressors and the possibilities of ineffective treatment or works failure.At this point, also, there were events of detectable levels of oestrogenicity analysed by ERCALUX®, as reflected in the PRA (Table 6.4). Drinking water had no detectable levels of oestrogenicity by this method.

Table 8-3Screening level risk estimation for nonylphenol based on Risk Quotients

Site	PEC	PNEC	Risk Quotient
Source water (R6) (µg l^{-1})	0.36	0.33	1.1
Near to Gualeguaychú canal (R9) (µg l^{-1})	0.24	0.33	0.72
Fray Bentos stream (C2) (µg l^{-1})	1.69	0.33	5.12
Fray Bentos stream sediment (S5) (µg kg^{-1})	7680	27	18.2

PEC: predicted environmental concentration; PNEC: predicted no effect concentration

8.3.2 Hazard quotients for environmental receptors

Hazard quotients were ranked against the relative risks among sites to estimate the severity of effects. The HQ for single EDCs differentiated by site were calculated in water or sediment by dividing the PEC of EDC by toxicology criteria for probable-effects concentrations, that could be threshold like toxicity reference values (TRV) whenever available. Hazard quotients were developed for water and sediments of the Uruguay River watershed study area and also the concentrations of target EDCs were measured in fish tissues.

The criteria conditions for the evaluation of HQ were:

If the HQ < 0.1 no adverse effect is expected.

If 0.1 < HQ < 1, the hazard is low, but potential for adverse effects should be considered; and if 1.0 > HQ < 10, some adverse effect or moderate hazard is probable.

If HQ > 10, then high hazard can be anticipated

For the calculation of HQs, the preferable were sub-lethal and chronic toxicity results from laboratory experiments at several trophic levels to evaluate growth (*Hyalella curvispina*) and reproductive effects (*Ceriodaphnia dubia*) related to endocrine disruption and effects at population level, but also other reference species found in the literature.

8.3.2.1 Risks of glyphosate through sediments

As sediments are the sink for many contaminants and glyphosate concentrations were higher than in water, ranging from < 0.05 to a maximum of 0.63 µg g^{-1} (Yaguareté stream sediment, S5) the risk to benthic organisms was also assessed. However, there are many gaps in information on benthic toxicity to glyphosate to ascertain that sediment-dwelling organisms would not be harmed, and there are not many focusing on endocrine disruption. As a

reference, the toxicity to *Hyalella azteca* is 244 µg g^{-1} (Tsui & Chu 2004) producing an HQ of 0.002.

8.3.3 Risks of endosulfan

In the case of endosulfan, the concentrations in sediment, soil and water were not detectable. Then, the risk estimation was calculated considering the critical body burdens.

8.3.3.1 Risks calculated by body burdens of in fish

The Rand et al. (2010) study calculated a lethal body burden for all fish species analysed as 30.4 ng g^{-1}(90^{th} percentile). As tissue residues in aquatic organisms associate to biological responses, and lethality happens if fish reaches or exceeds the critical body residue, this approach evaluated the worse case scenario. Exposure is mostly acute and pulsatile based on the application according to these authors. The sum α, β and sulphate endosulfan concentrations in *A.fasciatus* small fish for F1: 16.6, F2: 26, F3: 5.9 and for F4: 59.2 ng g^{-1}. The quotient of these to the benchmark concentration, produces a HQ > 1 for F4, and near to one for F2. Their concentrations in *P.maculatus* catfish arerelevant as they were six times higher than the benchmark concentration (Table 7-21: total endosulfan 185.5 ng g^{-1}). These fish were bought in August 2010, when a major fish mortality event was occurring in the river. An indication, the lethal body burden in species like *Tilapia* is 4.6 ng g^{-1} (Kenneth & Willem 2010) implying a possible high risk for fish in the river at certain events possibly linked to the application of the pesticide.

9 RISK CHARACTERISATION

This step addresses the evaluation of the significance of the risk at several levels of depth depending on the stressor. How significant the risk is has to do with how many individuals in a population are affected, which can be assessed by a species sensitivity study, calculating the hazardous concentration with toxicity data of several organisms, and the population at risk at two plausible scenarios: a worse-case scenario and the analysed current conditions. For example, in the cases of point-sources, the hazard was evaluated based on the EDCs measured in the effluent or wastewater and the exposure and effects data used were the results of the exposure tests with fish.

9.1 Risks to freshwater receptors from exposure through water-sediment pathway and foodweb processes in river water sites

A summary of the results concerning ED endpoints from exposure and effects experiments (Chapter 7) to evaluate the sediment-water pathways and through biomonitoring with fish is given in Table 9-1. Reproductive effects were evidenced by the *Ceriodaphnia dubia* three brood tests in the sediment elutriate from Las Cañas (S8). Wildfish from this site (*Astyanax fasciatus*, F4) were in lower condition than the rest, considered as a growth sub-lethal effect. Developmental (*Pimephales promelas* embryo-larval test) and growth effects were observed in assays with Fray Bentos sediment elutriates (S2). In Ubici Beach (F3) the fish male gonads were smaller than at the rest of the sites. This anatomic variation could eventually produce diminished reproductive function. Sub-lethal growth effects in *Hyalella curvispina* were evidenced with whole sediments of Nuevo Berlín, Fray Bentos and Las Cañas. The sites where potential for hazards to aquatic life exist include Las Cañas (F4) and Ubici beaches (F3). No absence of fish is noticed in these sites, which are used for fishing. However, chronic effects on the fish might exist in the future.

Table 9-1 Endocrine disruptive endpoints in the river sites from the water-sediment pathway to freshwater receptors in river water and sediments

Effect	Site (and code of the media where effects were evidenced)				
	Buffer zone A	**Buffer zone B**			**Buffer zone C**
	Nuevo Berlín	Yaguareté	Ubici	Fray Bentos	Las Cañas
Developmental				S2 	
Reproductive			F3 		S8 
Sub-lethal (growth)	S1 			S2 	S8  F4 

Si: sediment; Fi: fish

9.2 Risks from agricultural sources

The risks of glyphosate and endosulfan were characterised with the aid of the Webfram model, with input of toxicity data taken from Egeis (2008), and the ones included in the reviewed information under toxicity profile (Appendix C).

9.2.1 Risk of glyphosate to environmental receptors

The acute and chronic toxicity of glyphosate were studied by species sensitivity distribution analysis including crustacean, fish and algae as aquatic environmental receptors (Figure 9-1). The difficulty arose from the scarcity of information on toxicity to receptors other than experiments on cellular toxicity.

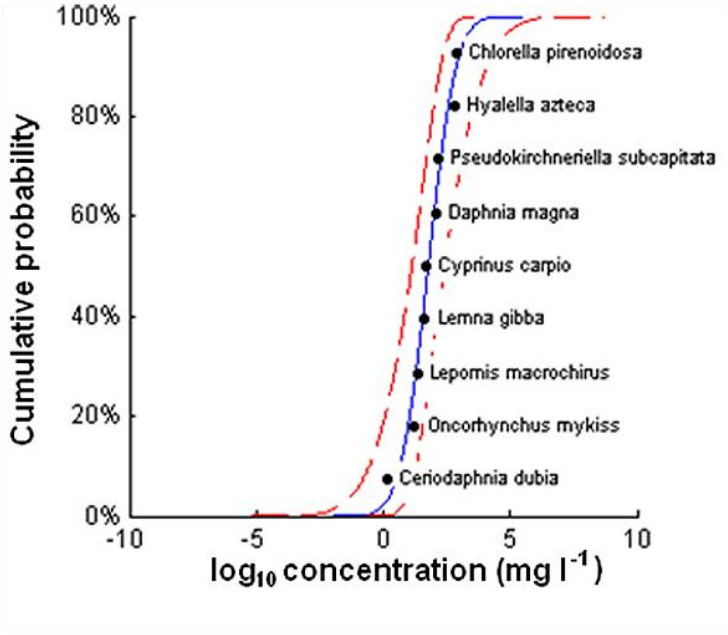
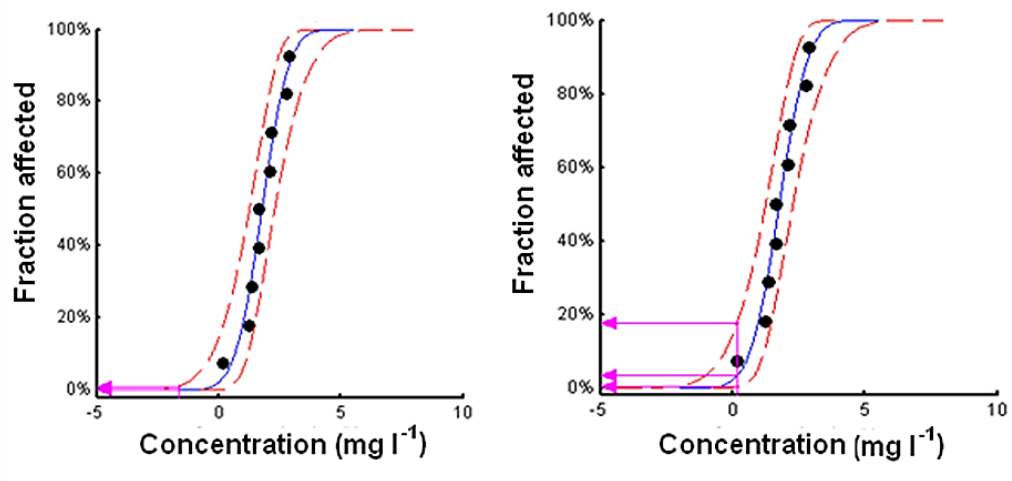


Figure 9-1Species sensitivity analysis for glyphosate acute exposure

9.2.1.1 Risk characterisation of acute exposure of glyphosate at two scenarios

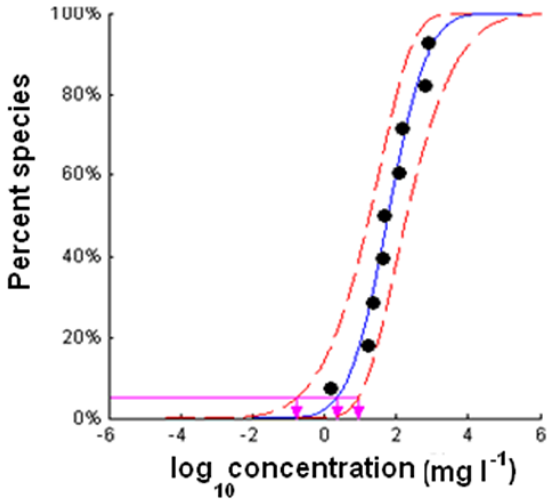
The exposure aquatic concentration was taken as the maximum water column concentration measured in the watershed (22 µg l^{-1}), to evaluate the fraction of affected species (FA) that would give an outlook to the significance of the risk. For this matter, two different scenarios were assumed: one, based on the measured concentrations, and the second one based on those quantified by Peruzzo et al. (2008) to be under a worse-case scenario as in these studies the glyphosate concentration ranged from 0.21-1.51 mgl^{-1} near soy fields.

In the first scenario, glyphosate had a low risk to environmental receptors, potentially affecting 2% of the species. However, in the second scenario glyphosate would enter within hazardous concentration limits of 2.2 (0.173-8.84) mg l^{-1} (Figure 9.2), affecting from 5 to 20% species.



Arrows show the % affected species at median, and 95th % concentrations.
Left: Concentration of 22 µg l^{-1}; right:concentration of 1.5 mg l^{-1}

Figure 9-2 Fraction of affected species by glyphosate at two exposure scenarios



Arrows show the log_{10} concentration at mean and 95th percentile concentrations

Figure 9-3 Hazardous concentration of glyphosate

Then, the same study was developed for the chronic exposure of glyphosate with toxicity data of algae, crustaceans and fish, as shown in Figure 9-4.

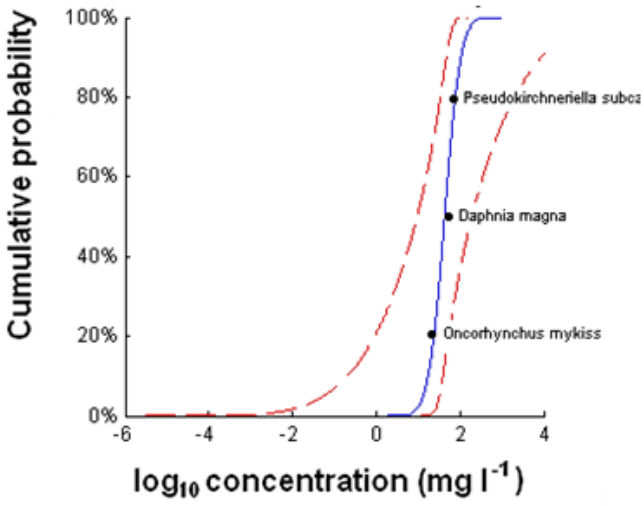


Figure 9-4 Species sensitivity analysis for glyphosate chronic exposure

In this case, the fraction of species affected was 0-0.3% for the scenario condition of 22 µg l^{-1}glyphosate.

9.2.2 Acute toxicity of endosulfan in baseline conditions

At an acute exposure, below quantitation level in river water, the percentage of species affected is 2%. The hazardous concentration values were 1.7 (0.8-2.3) µg l^{-1}.

The high risk event concentrations were not easy to detect due to the relative hydrophobicity of the EDC.

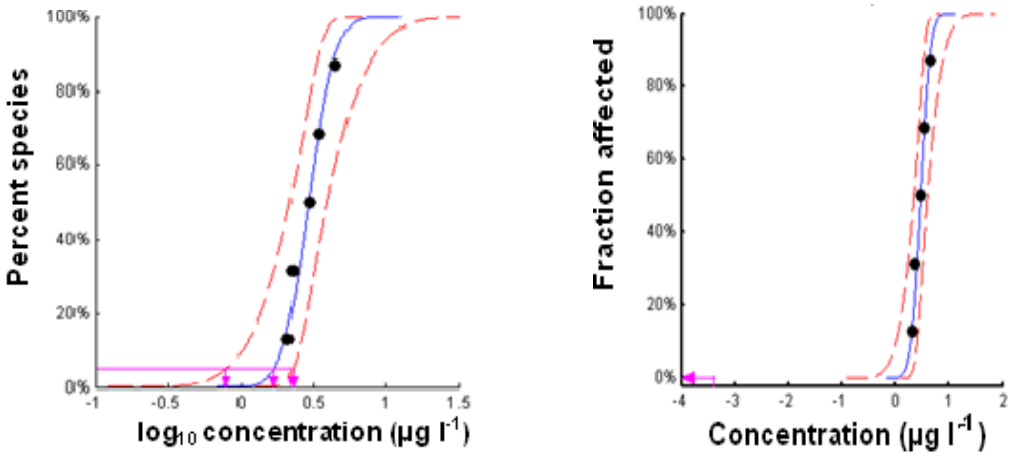


Figure 9-5 Species sensitivity analysis for endosulfan acute exposure

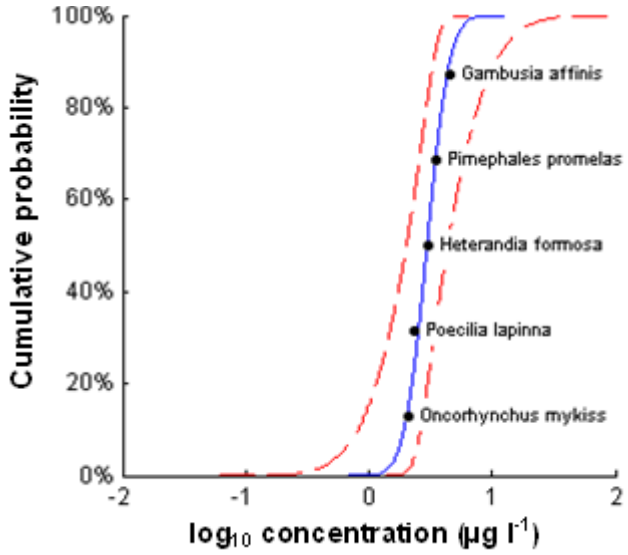


Figure 9-6 Hazardous concentration of endosulfan acute exposure and fraction of species affected

However, according to the fugacity models, as well as to investigations linking endosulfan toxicity to suspended particles, this factor was also characterised.

9.2.2.1 Risk of endosulfan in sediment and suspended particle- bound

No detectable concentrations of endosulfan, below 50 µg kg^{-1}, were consigned at any river sediment samples analysed (α + β) (Nuevo Berlín, Fray Bentos and Las Cañas) (n=5). A worse-case scenario would be occasioned by above average runoff from agricultural lands, although direct spraying would be another route. As a reference, Jergentz et al. (2004) reported concentrations of β-endosulfan up to 320 µg kg^{-1} in suspended-particle samples at a location in Argentinian territory. Taking these levels as a benchmark, and a report by the German Federal Environmental Agency (2004) that includes toxicity values for sediment-dwelling organisms, the Webfram software was used to evaluate the species sensitivity and the hazardous concentration. The reported LC50, 96 h for midges (*Chironomus tentans*) was determined as 20 µg $kg^{-1}$and NOEC < 6 µg kg^{-1}.

Data gaps exist regarding the toxicity to benthic species of endosulfan but the LC_{50}, 96 h to the *Polychaete Streblospio* benedic has been reported as 50 µg kg^{-1} (Chandler & Scott 1991). The hazardous concentration to these freshwater worms resulted in 6.94 (0-23.2) µg kg^{-1} for the baseline scenario (Figure 9-7).

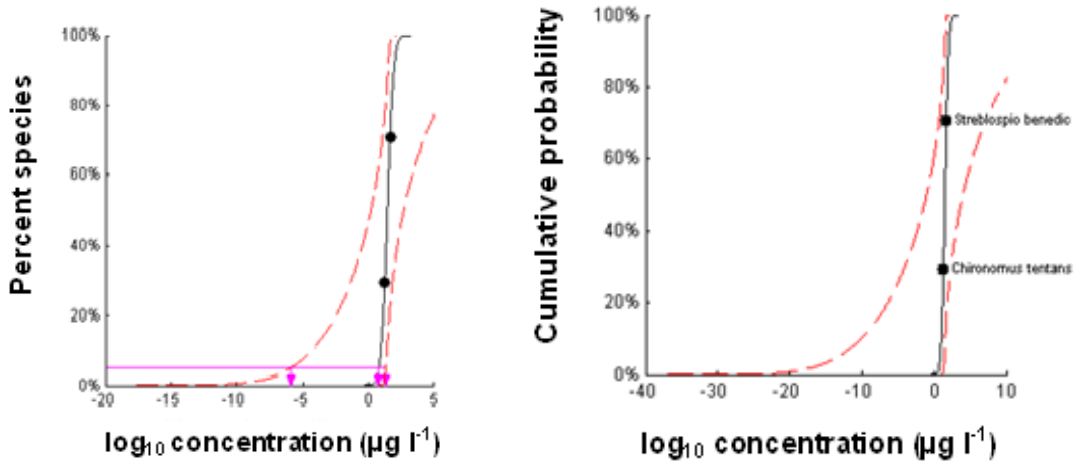


Figure 9-7Sensitivity analysis for benthic organisms of endosulfan and percentage of species affected

The fraction of affected species in this hypothetical worse-case scenario would range from 45.2 to 100% benthic species affected with a median value of 99.3%. The range is shown between the pink arrows in Figure 9-8.

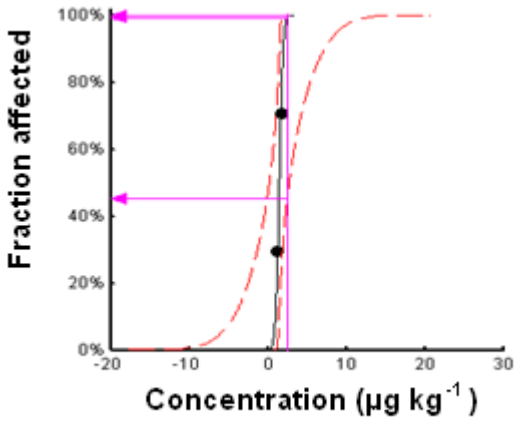


Figure 9-8 Fraction of species affected by endosulfan worse-case scenario

9.3 Risks to freshwater receptors from industrial sources (BKME)

The main hazards of multiple stressors in BKME are shown in Table 9-2, and effects produced by the whole sample inTable 9-3. Evidently, endocrine disruption exists, but the mode of action is unclear. Diminished gonad sizes in male fish downstream the pulp mill implies that antiandrogenic compounds could be present. ER- and AR-binding assays detected both oestrogenicity and androgenicity at low levels. Therefore, mixture it is very complex to derive a definite conclusion. However, taken alongside the biomarker results, it may be hypothesized that the increase in binding to *ESR2* receptor downmodulates the binding to *ESR1* receptor producing an antioestrogenic effect. Possible compounds are dioxins and furans, but no direct causal link can be postulated as the mixture is extremely complex and the frequency of detection of each compound very variable.

Table 9-2 Concentrations of endocrine disruptors in pulp mill effluent and main modes of action

EDC	Concentration	Main mode of action
β-Sitosterol	< 100 µg l^{-1}	Antiandrogenic
Pimaric acid	ND- 28 µg l^{-1}	Oestrogenic
Isopimaric acid	ND- 44 µg l^{-1}	Oestrogenic
2,4,5- Trichlorophenol	0.19 µg l^{-1} (<0.1-1.3)	Oestrogenic
2,3,7,8-TCDD	ND- 3.5 pg l^{-1}	Antioestrogenic
2,3,7,8-TCDF (as I-TEQ)	ND- 1.2 pg l^{-1}	Antioestrogenic

Table 9-3 Summary of effects in fish exposure assays to pulp mill effluent

End-point	♂	♀
VTG	=	=
vtg	=	↓
SR1	=	↓
ESR2	↑	↑
IGF-I	↑	↑
ZP3	↓	=
GHR	↑	↑
GSI	=	=
Egg production	-	↓
Nuptial tubercules	=	=
Histology	=	=

♂: male; ♀: female; ↑: statistically significant increase relative to the control group

↓: statistically significant decrease relative to the control group

9.4 Risks to freshwater receptors from municipal sources

As seen in Tables 9-4 and 9-5, the action in the case of wastewater contaminants is predominantly oestrogenic. Of the quantified target EDCs posing this activity are alkylphenols, phthalates, and oestrogens. The biomarkers results show that there are ER-binders. Egg production is accompanied by an increase in ZP3. There was no clear demonstration on effects on VTG on male gonads. The receptor binding assays showed a predominance of oestrogenic effects but androgenic activities also existed.

Not only ER-binders were found in the stream water. Phenols act through non-genomic as well as receptor binding mechanisms. For instance, BPA binds to extra-nuclear ERs, but it also affects thyroid function and androgen signalling (Alonso-Magdalena et al. 2012). These authors report a LOEC 0.1–1 pM (0.02-0.2 ng l^{-1}) for oestrogenic action in humans. The mode of action of phthalates has been hypothesized as antiandrogenic (Harris & Sumpter 2001), However, these EDCs provoke a decrease not only in testosterone but in oestradiol in fathead minnow by increased catabolism (Crago & Klaper 2012).

Table 9-4 Concentration of endocrine disruptors in Fray Bentos stream receiving municipal wastewater and main modes of action

EDC	Concentration	Main mode of action
Di-n-octyl phthalate (DNOP)	< 1 µg l^{-1}	Antiandrogenic
Di (2-ethylhexyl) phthalate (DEHP)	2.7 µg l^{-1}	Antiandrogenic
4-p-nonylphenol	8.3 µg l^{-1}	Oestrogenic
p-tert-octylphenol	12 µg l^{-1}	Oestrogenic
E1	2.2 ng l^{-1}	Oestrogenic
E2	0.6 ng l^{-1}	Oestrogenic
E3	1 ng l^{-1}	Oestrogenic
EE2	< 0.2 ng l^{-1}	Oestrogenic
E1-3S	0.5 ng l^{-1}	Oestrogenic
Bisphenol A	< 1 ng l^{-1}	Oestrogenic

Table 9-5 Summary of effects in fish exposure assays to Fray Bentos stream water receiving municipal wastewater

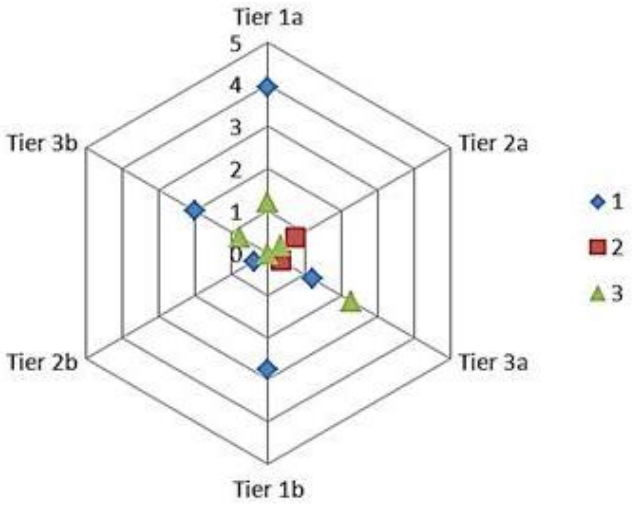
End-point	♂	♀
VTG	=	↓
vtg gene transcription	=	↓
ESR1	↑	↑
ESR2	=	=
IGF-I	↓	=
GHR	↑	=
ZP3	↑	↑
GSI	=	=
Egg production	--	↑
Nuptial tubercules	=	=
Histology	=	=

♂: male; ♀: female; ↑: statistically significant increase relative to the control group

↓: statistically significant decrease relative to the control group

9.5 Pondering of the exposure assessment results and combined risks by radar plots

To ponder the factors of influence in the tiered approach, a fast independent analysis was developed with the same 19 variables as for PCA (Column 1, Table D-5). Three components of influence were evaluated (Figure 9 9).



Component 1: General river health: 1a-1b highly ranked with possible sub-lethal toxicity. Tier 2a not highly influential in general river health. Tier 2b or chronic toxicity and hormesis possibly due to general contamination, eutrophic conditions by excess nutrients and/or algal toxins

Component 2: Contaminants markers of chlorinated EDCs and phenols (not para substituted): anthropic sources at a slight level of risk, not prone to be the highest responsible agents of the effects so far

Component 3: Endocrine disruption: caused mostly by the analysed EDCs (NP=ES>D&F>E=RA=bS), among others, not determined. General river health effects on ED, and markers of contamination pose a slight contribution. In the given conditions, chronic effects in the offspring have a slight risk of occurrence.

Figure 9-9 Radar plot for pondering tiers of exposure assessment

The joint risk representation for EDCs was based on radar diagrams. Based on the individual risks estimated before, the joint risk characterisation diagram is shown in Figure 9-10.

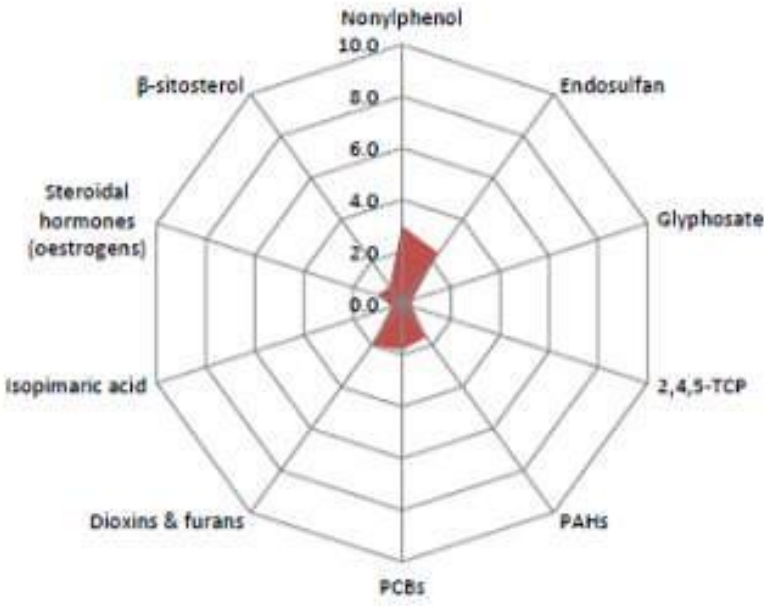


Figure 9-10 Radar diagram of joint risk characterisation

It became evident that nonylphenol and endosulfan were the most relevant EDCs in the watershed. In the case of NP, this is because of its occurrence in the watershed, as it was found in river water, in the Fray Bentos stream, in its sediment and in fish tissue, but also because of the PEC/PNEC higher than 1, especially in the case of the stream. The $PNEC_{water}$, confirmed by ECB (2013) is 0.33 µg l^{-1} for water, while $PNEC_{sediment}$ is 0.039 µg g^{-1} (Table 9-6). In the case of endosulfan, this is based on the severity of potential effects, and the concentrations measured in fish (8.3.1).

Table 9-6 Nonylphenol in river water, stream water receiving municipal wastewater, industrial effluent, stream sediment and wild fish

NP concentrations	Industrial (µg l^{-1})	River water (µg l^{-1})	River sediment (µg g^{-1})	Stream water receiving municipal wastewater		Fish (ng g^{-1})
				(C2) water (µg l^{-1})	sediment (S6) (µg g^{-1})	
Experimental	< 0.01	0.069-0.36	0.037	1.7-8.3	7.68	7.9-28
Typical (Lintelman 2003**)**	142-330	< 0.01-644	< 0.0015-72	< 0.02-69	< 0.0015-72	-
PNEC	-	0.33	0.039	0.033	0.039	-
PEC/PNEC	-	2.1-10.9	0.95	5.2-25.2	197	-

To characterise the combined risks, the EDCs were grouped together according to their similarities in modes of action, taking care not to exclude chemicals contributing to joint effects, but yet keeping them as a manageable number of prioritised EDCs. This issue was treated not only as a “whole mixture approach” by exposing aquatic organisms to effluent and wastewater but also through a “component based” approach, based on biota residues and the environmental matrix residues of EDCs. As seen in Chapter 2, by the whole mixture approach the toxic effects of all compounds present are accounted for, and the synergistic or antagonistic interactions through the observed responses of the exposed organisms. However, as the exposure situation in the environment is highly dynamic, this was combined with “component based” methodologies, to diminish the uncertainties.

9.5.1 Statistical analysis of frequency and severity

From a statistical point of view, the risk, or significance of the occurrence of an event has two components: frequency of the event and severity of the event. Frequency is the number of events within a given time period, represented for example by the Poisson distribution. The Fermi expanded solution was used to

estimate each distribution (based on Peleg et al. 2007). More information on the distributions calculated by the expanded Fermi solution are in Appendix G.

9.6 Causality assessment

If multiple stressors exist, causality relationships are not easily inferred whenever cumulative or synergistic effects are expected (Culp et al. 2009), but the epidemiological criteria to determine causality include the following:

9.6.1 Specificity

In the case of biomonitoring there are several molecules that could be responsible of the effects. Laboratory testing was required to explore the expected responses.The reference EDC had specific toxic responses, similar to the municipal wastewater laden stream.

9.6.2 Strength of association

The strength of association criterion refers to precision of the association cause-effect. There could be correlation but poor correlations with contaminants in water or sediment. The body burdens could be the best piece of information obtain at the moment to infer certain possible associations with the effects, but the laboratory tests showed correlations by comparison to the responses of the control with E2.

9.6.3 Time order

Time order refers to the necessity that the cause precedes the effect in time. The historical contamination can be studied by the water and sediment contamination at each site. No historical epidemiology data exist on the effects for humans. Ecoepidemiology data show fish mortality events, but they could be attributable to many other factors including climate change. However, fish caught at the area before a major event (August 2010), presented high concentration of the target EDCs.

9.6.4 Consistency on replication

This criterion concerns to whether the association has repeatedly been observed by different researchers, times and places, species, and using study designs. This is not the case, as this is a very new research, but several research teams are currently working on this subject, networking with our team. International examples that support many of the findings as mentioned in the literature review.

9.6.5 Coherence

The final criterion concerns with coherence. It comprises several parts relating to whether the new cause–effect data conflict with the natural history and biology of the disease. There are plausible sources and pathways of these EDCs into the Uruguay River, and extensive experimental and chemical analytical data to show the fate and distribution of these compounds and routes of exposure of organisms in the aquatic food chains. The biota analysis reveals that chlorinated organic compounds exist at several trophic levels: aquatic plants, mussels, snails and fish, as predicted by the foodweb model. Possible causal links in wildlife are shown in Table 9-7. The same is true for NP, found in environmental matrixes such as water, sediment and fish tissues. Even though human beings are most likely exposed to mixtures of EDCs, hypospadias, cryptorchidism, and other effects were still not conclusive as related to them.

Table 9-7 Causality assessment

No.	Postulate	Exposure	Effect/ biomarker response	
			BKME	**C2**
1	Toxicity regularly associated with exposure to the toxicant and any contributory causal factors	Wildlife exposed because of measurable EDCs in river water and sediment and as tissue residues	After exposure to BKME, antioestrogenic or androgenic effects were evidenced; Diminished gonad size in fish downstream the pulp mill outfall	Fish tissue residues of mixtures indicative of a possible link to sources Lower condition and less mature fish downstream city discharges
2	Indicators of exposure to the toxicant must be found in the affected organisms	Measurable NP and oestrogensat C2 Not clear responsible agent (D&F, resin acids, unknown)	Anti-oestrogenic or androgenic postulated because of biomarkers responses	NP in *A. fasciatus* downstream from the city but also at other locations
3	Toxicity when organisms are exposed to the toxicant under controlled conditions	NP in reference fish exposed to C2 (21 d *P. promelas* assay) BKME	VTG increase in males, and other biomarkers, most likely oestrogenic Possibly anti-oestrogenic	After exposure to C2, oestrogenic effects
4	Same indicators of exposure and effects identified both in controlled exposures as in the field	NP in wild fish and in reference fish exposed to C2 BKME has CP, resin acids and other EDCs found in biota in the field	Smaller gonads in the field; GSI of fish in the lab	Oestrogenic responses in the field: not clear, but clear in reference fish

9.7 Overall risk characterisation

The severity and likelihood of possible harms were rated and the overall risk estimated using a matrix.The severity of the impact of the risk event was assessed on a scale from 1 to 10, where 1 and 10 represent the minimum and maximum possible impact of an occurrence of a risk. The likelihood of occurrence was also assessed on a scale from 1 to 10, where 1 represents a very low probability of the risk event actually occurring while 10 represents a very high probability of occurrence. The severity of consequences to both environmental and human receptors are ponderd as on Tables 9-7:

Table 9-8 Severity levels for receptors

Level	Environmental receptors	Human beings
10	Catastrophic loss of species or fish kill events of high frequency, lethality in bioassays rated as toxic	Catastrophic loss in life expectancy, increase in newborn death or stillbirth
9	Serious effects on fish productivity or richness in fish species at population level, lethality in bioassays rated as moderately toxic	Serious decrease in fertility rate, or increase in reproductive cancer rate
8	Intersex or other severe tissue anomaly of the gonads	Increase in the hypospadia rates
7	Developmental effects above normal or high chronic toxicity in bioassays	Early menarche
6	Reproductive effects in chronic tests, egg production diminished or dramatically augmented, functional changes in gonads	Higher rates of endometriosis
5	Gonadal changes in size	Increased cryptorchidism rates
4	Moderate effects in chronic toxicity in one or slight effects in more than one species, no lethality in bioassays	Growth effects
3	Changes in biomarkers or category of slightly toxic in any test-	Moderate effects as changes in anogenital distances in newborns
2	Changes in growth in one or more species, no lethality	Slight effects
1	Not acute, chronic or developmental toxicity	No effects

In the case of environmental receptors, no intersex, but effects in egg production and gonad size changes and low frequency developmental effects in fish embryo (6, 5, 7). Therefore, 7 per the likelihood, 4 (low frequency, possible), gives a moderate grade. In the case of humans, there are many uncertainties, but, estimating the risk from the likely received dose, it would be

low. The overall risk rate was then determined by applying the following matrix (Table 9-9):

Table 9-9 Likelihood and severity matrix

Rating			Severity				
			1-2	**2-4**	**4-7**	**7-9**	**9-10**
Likelihood	Almost certain	**1-2**	Medium	Medium	High	High	Extreme
	Likely	**2-4**	Low	Medium	High	High	Extreme
	Possible	**4-7**	Low	Medium	Medium	High	Extreme
	Unlikely	**7-9**	Low	Low	Medium	Medium	High
	Rare	**9-10**	Low	Low	Medium	Medium	High
Overall risk level			**1-2**	**2-4**	**4-7**	**7-9**	**9-10**
(L X S)/10			Insignificant	Minor	**Moderate**	Major	Catastrophic

Freshwater wildlife; Human beings

10 DISCUSSION

Most of the known chemicals with ED activity are of synthetic origin (2.3.2). In some cases, their transport can exceed regional boundaries, calling for global solutions, and a stronger link between policy and research.

10.1 Issues and values to protect through an integrated approach

"Water is life" is the goal of the United Nations for 2005-2015. This requires action to counteract that two out of three people will probably be lacking sufficient freshwater by 2025 (WHO 2012). The increasing awareness of the risks to water quality and quantity is driving water governance to reconcile economic and social demands with environmental sustainability (Akech 2012). Although water scarcity in Uruguay is not an issue, the protection of source water from contamination is important to minimise health risks.

10.1.1 Why pursue integration?

Integration in RA is crucial, as human well-being depends on ecosystem services (Maltby & Acreman (2011), as those provided by freshwater ecosystems. Even when the major goal would be protecting drinking water and fish for humans, ethical and sustainability precepts makes it necessary to include environmental receptors. Based on this concept, society, industrial stakeholders, and authorities should work synergistically to enable efficient mitigation or reduction measures.

10.1.2 Can we improve risk assessment by integrating humans and aquatic animals in the case of endocrine disruptors?

Methodologically, it is not easy to integrate both humans and wildlife in a unique assessment because of inherent differences in habitats and biologic complexity. Notwithstanding, the ERA paradigm has shifted towards integration (Suter et al. 2003). This thesis relied on testing based on mechanistic considerations to extrapolate observable effects to humans targeting endocrine disruption at

several levels. Moreover, the used biochemical screens express oestrogen and androgen human receptors. Figure 10-1 shows the areas where integration was achieved, and other potential areas for further integration:

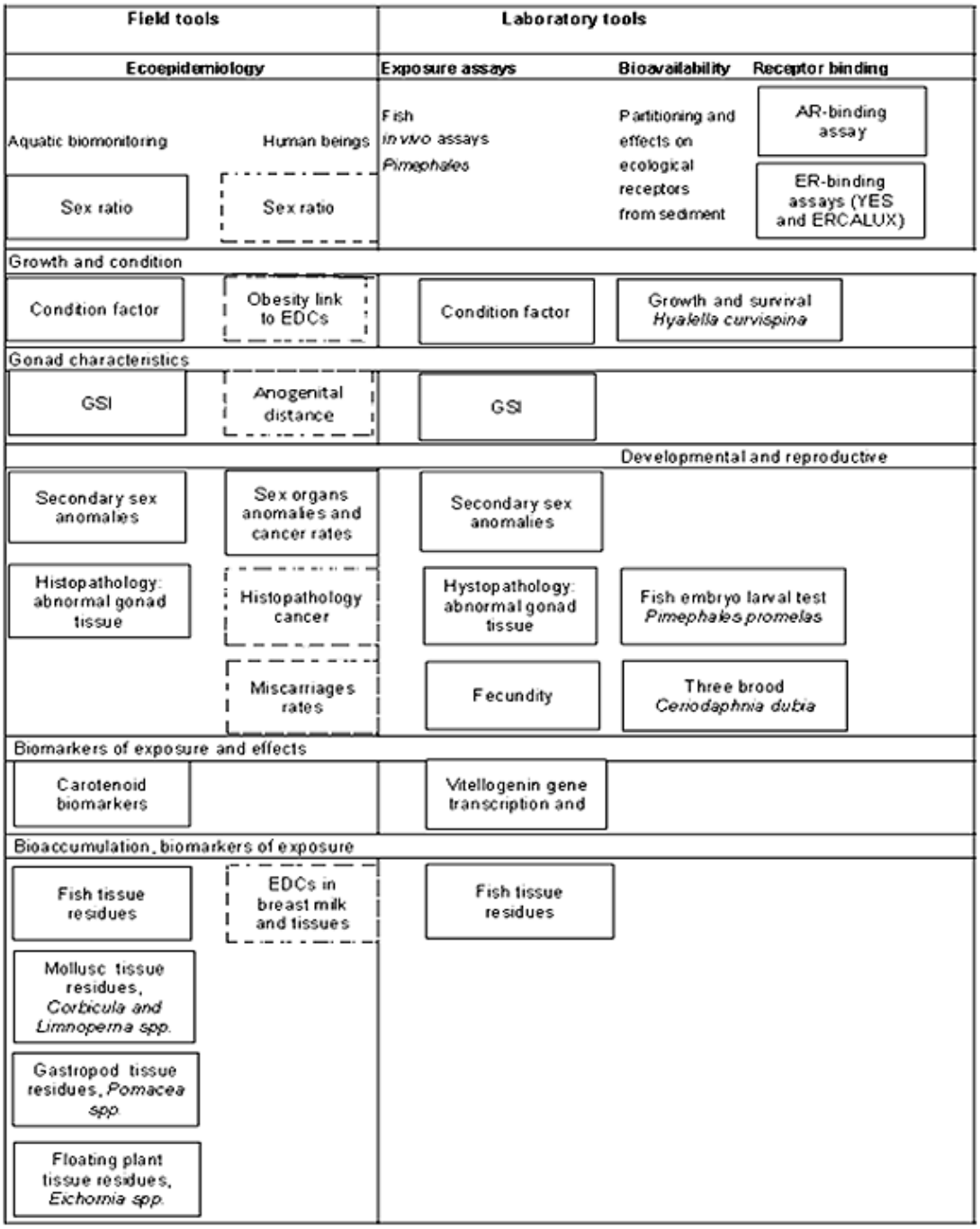


Figure 10-1 Tools for integration in exposure assessment

10.2 Hazard identification and prioritisation system

Only a few compounds known to cause adverse effects on endocrine system were on the priority list of the EU (Halme et al. 2010). As a response, the prioritisation stage provided a general methodology aimed to be of global application to aid to achieve this task.

Prioritisation sorted out chemicals based on a low exposure and/or effects potentials, in any environmental compartment of relevance in the watershed reaching to a prioritised list of hazards with a risk vision. This information was subsequently combined with physicochemical and mechanistic criteria. This conformed a “logic-of-choice” flow-chart to prioritise candidate substances subject to the refined RA stage. Although undetected in water, some of highly hydrophobic compounds were reconsidered based on their bioaccumulative potential, and high usage in the watershed, which made it probable that they existed in the sediment or biota. This decision path proved right as endosulfan was found in fish but not in water, as also predicted by the fugacity model for distribution among environmental compartments.

10.2.1 Preliminary Risk Assessment: receptor-binding assays and modelling

Effects-strategies were concerned initially not with causative agents, but with observable outcomes (effects), through the application of screening assays to determine biologic endpoints of interest (oestrogenicity, anti-oestrogeniciy, androgenicity and anti-androgenicity) and then gain preliminary information regarding those effects, as part of the PRA.

The simplest test that reliably points to an effect with few false negatives (3%), should be applied, which made ERCALUX® and ARCALUX (van der Burg et al. 2010) a good option based on their good sensitivity, in the order of pg l^{-1}. As androgenicity and oestrogenicity in wastewater is mainly due to receptor binding mechanisms, this screening was suitable, also based on prior successful use of

this method by Schriks et al. (2009) to evaluate oestrogen and androgen binding activities in the River Rhine. In this thesis, results indicated that it was of merit to study ED in the watershed at a refined level, as R-binders existed. As EDCs with non-genomic mechanisms of action would not be identified by these methods (2.10.3.2), Phase II also included other mechanisms of action and quantitatively determined the likelihood and severity of ED.

10.3 Quantitative Risk Assessment: tiered approach for exposure assessment and the calculation of doses

The goal of Phase II was to demonstrate successively: the general health of the river, the fate and distribution of the main point sources discharges, and exposure and effects with acceptable sensitivity to determine the magnitude of adverse responses and modes of endocrine disruptive action. The link from source to stressors existing in the water, sediment, and soil, to the fish tissue and to effects was explored.

The current trend is to move from vertebrate to *in vitro* and *in vivo* tests and computer simulations (Inglis 2007, Vermeire et al. 2007). This thesis is in line with this, as mammals were not used, but the minimum possible vertebrates. Other tiered approaches still employ mammals (USEPA 2009d). Not many examples of tiered approaches existed for IRA, and not any to assess endocrine disruption at the catchment level. The starting point was the general river health because nutrient imbalances could jeopardize the life of biota due to oxygen depletion, caused by excessive algal growth. Taking all this into consideration, a holistic approach to the topic of EDCs at the watershed level was relevant.

The designed tiered approach coupled analysing selected EDCs in environmental compartments and biological receptors with an effects-based approach, to diagnose the current situation in a geographically referenced fashion. The hierarchical sequence of tests or endpoints balanced the uncertainties with resources, and explored modes of toxicity action of the water-

sediment phases. Comparative studies, including general toxicity, target-organ, teratogenicity, and reproduction tests contrasted the toxicity potential at different sites, simultaneously, to achieve comparisons in one experiment.

10.3.1 Tier 1: water classification and the risk of eutrophic conditions related to endocrine disruption

Tier 1 rated the water as with certain eutrophication risks. It has been hypothesized that cyanobacteria causes not only cancer but endocrine effects (Rogers et al. 2011). In large rivers the N: P relationship could approximate to Redfield´s ratio (16:16:1) as they tend to eutrophic conditions (Justić et al. 1995). In the Uruguay River, the mean is 544:13:1, reflecting the incidence of suspended sediments.

The EU Water Framework Directive (WFD) requires controlling pollutants in water, including EDCs. However, the guideline to establish reference baseline conditions are not provided (European Parliament 2000), nor to determine deviations from reference conditions. In this thesis, a novel method, using artificial neural networks trained with historical data established background levels to then evaluate other strategically set sampling locations such as the drinking water intake and the dividing canal of this transboundary watercourse in reference to those. Therefore, the lower tier set the scenario in terms of the general river health, crucial to achieve more accuracy in interpretation and less confounding in the following stages. The only precedent work is the use of ANN to model river water quality data in India (Singh et al. 2009) and in China (Li et al. 2010), but the one used in this thesis trained the networks with established limits to compare background levels to posterior data at other locations in the river.

The identification of eutrophication as a major problem in the river in Tier 1 is consistent with prior investigations (Míguez et al. 2007, Saizar et al. 2010), as well as through the new methodology using artificial neural networks.

Eutrophication affects the general aquatic ecosystem health but it has been hypothesized that components of *Microcystis* sp. algae could possible up-regulate *vtg* in fish larvae (Rogers et al. 2011). In addition, microcystins bioaccumulate in embryos (Pavagadhi et al. 2013) probably provoking compensatory growth effects in fish larvae (El Ghazali et al. 2009).

Although the matrixes under study were not acutely toxic, sub-lethal growth effects in sensitive benthic organisms (*H. curvispina*) was observed from the sediment pathway. Prior experiences with this native amphipod demonstrated sub-lethal toxicity with river whole sediments (Míguez et al. 2012).

10.3.2 Tier 2: tracking the transport of contaminants indicative of endocrine disruptors

"In rivers, the water that you touch is the last of what has passed and the first of that which comes; so with present time" — (Leonardo da Vinci)

This concept was used to study environmental conditions upstream and downstream the relevant sites based on the flow and the isoconcentration model. The transport of contaminants was tracked by clustering two chemical markers of the pulp mill effluent. This task proved difficult due to variable climatic conditions that change the volume of the river, but some remarkable differences were observed, especially in phenols (Figure 7-15). The near-field and the far-field sites from the pulp mill and the city (R5 and R8) exceed in phenols with a frequency of exceedance higher after the pulp mill start-up than before. The tracking of the plume was completed with conductivity as a tool. The confounding factor was the high conductivity of Yaguareté Stream. During the measurements, the wind had little influence, and one of the values was higher than normal at R5 (Table 7-2).

The study of source water was identified as an important factor for the accurate estimation of risk, which appeared affected after flooding. Time series analysis of phenols and AOX in raw water showed benefits as an early warning system

within regular monitoring to discriminate peaks from random fluctuations. The flooding event occurred in November 2009 might also have had an impact on the conductivity and colour variations in the river water (Figure A-1).

The main source of AOX is the pulp mill that may have mean concentrations of 727 µg l^{-1}(Table 5-6) with an effluent flow of 0.8 $m^3 s^{-1}$. Mussel species from the immediately downstream beach (F3) contained quantifiable levels of EOX (7.7.1.2). Notwithstanding, in areas downstream from the sewage outfall (Anglo Beach) (was within the typical expected values of 15 to 94 ng l-1 EEQ (Jugan et al. 2009) (Table 2-1) the municipal wastewater concentrations, were in the range 0.38-1.73 mg l^{-1}. Both upstream activities could be of influence, as observed by the EOX present in snail tissue (7.7.1.1).

Regarding effects, a slight chronic toxicity and biostimulation (*hormesis*) evidenced by higher number of neonates in remaining adult organisms than in the control (Table 7-6) resulted when this test was applied to pulp mill effluents. These findings correlate with prior research experiments with this species and pulp mill (Middaugh et al. 1997).

10.3.3 Tier 3: biomarkers and whole organism endpoints to assess exposure in the laboratory and in the field

The relationship among biomarkers and whole organism endpoints was examined by Bosker et al. (2010), determining that egg related to E2, changes in female VTG levels, and female gonad size. As mentioned (2.4.3, Ankley et al. 2008) and according to Kime et al. (1999) abnormally low VTG production in females may indicate malfunctions in the reproductive endocrine system and inhibit ovarian growth. No induction in male fish was observed at a significant level, but augmented levels in *ESR1*, ZP3 and GHR were found after exposure to municipal discharges. On the other hand, the BKME provoked an increment in the expression of the *ESR2* gene and a concomitant decrease in *ESR1* in females, as well as augmented growth factors in both sexes.

10.3.3.1 The use of *Astyanax fasciatus* as sentinel fish to biomonitor EDCs in South American rivers

Few prior experiences existed on the use of *Astyanax fasciatus* as a sentinel species. Namely, a Brazilian investigation of its reproductive biology in a reservoir system (Shulz & Martins-Junior 2000, de Carvalho et al. 2009), and more recently, the demonstration of its sensitivity to detect feminisation, intersex and contamination by xenoestrogens downstream municipal wastewater discharges (Prado et al. 2011). In Uruguay, it was used in other watersheds as bioindicator of contamination by the measurement of hepatic porphyrines (Carrasco-Lettelier et al. 2006).

10.3.3.2 Fish are exposed to mixtures of endocrine disrupting compounds in the Uruguay River

The hypothesis that fish living the Uruguay River are immersed in a mixture of EDCs is substantiated by the occurrence of the prioritised EDCs in tissue residues of fish and other biota (Figure 7-42 and Section 7.7). These multiple stressors were probably released from combined inputs ranging from large industrial activities to municipal and agriculture point and non-point sources.

Persistent EDCs bioaccumulate, so, exposure can be estimated by measuring tissue residues. However, for less persistent EDCs, such as glyphosate, exposure must be measured in all environmental compartments. Tillitt & Papoulias (2003) consider it difficult to predict the potential toxicity through tissue residues only, making it necessary to use biological monitoring of health effects in fish and wildlife species.

Tissue data and biomarker responses provided evidences of exposure to EDCs. For instance, endosulfan seems to be widespread in the Uruguay River, concentrating near agricultural areas, near streams that receive agricultural runoff (for the site F2 is near Yaguareté stream that is near soy fields). Downstream from the pulp mill, fish tissue residues of β-sitosterol are slightly higher than background level (taken as F1), while Yaguareté site (F2) away

from direct influence of the plant according to the isoconcentration model represented in Figure 7-3, and diluted by the stream water, has the lowest values. The farthest downstream site (F4) had the highest level of EDCs in fish tissue (Figure 7-42).

Endosulfan concentrations increased near streams with agricultural influence (F2 receives Yaguareté stream, F4 is downstream from the city, but also receives the Fray Bentos stream influence). The concentration of 4-nonylphenol is higher in F4 as it receives the influence of the city discharge pipe and the resort town. At F1 and F2 an impact of agriculture could be the reason why there are quantifiable levels of this compound. Other alkylphenols are lower than this. Their origin may be from residues or solid waste like plastics.

The lowest fish tissue residues appeared in specimens from Yaguareté Bay (F2), consistent with the hydrodinamics study. Alkylphenol residues were measured after exposure to F2 with municipal wastewater as well as in wild fish caught in the river. It is remarkable the occurrence of NP in waters, sediments and fish in the watershed. Additional observations are that cholesterol levels are higher where β-sitosterol is lower in fish tissue, possibly because this compound lowers circulating and gonadal concentrations of cholesterol (Sharpe et al. 2007), but also other EDCs could have this action. It can be postulated that the origin could be from the pulp mill and natural decay of forest and vegetation material. Other possible responsible chemicals are resin acids which are found at higher concentrations in liver than in muscle (Table 7-23).

As reviewed by Novák et al. (2008) environmental pollutants may cause a decrease, but in the case of some chlorinated pesticides an increase in hepatic retinol levels, while persistent organochlorinated organics carried by air transport may deplete hepatic retinol (Xu et al. 2002). Other EDCs, such as polybrominated organics (Chen et al. 2012a) also increase retinol in the liver. Unbleached wood extractive substances are suggested of being able to diminish retinol levels (Alsop et al. 2003). Reduced ovary pigmentation due to

carotenoid depletion and diminished biosynthetic capacity of sex steroids was observed in the fish in a pulp mill-impacted site (Landman et al. 2008). The evidence up to the moment is still not enough to derive causality links for differences in retinol among sites (Figure 7-41), but the extent of them so far, is remarkable. Excess retinol produces vertebral deformities in fish (Haga et al. 2011), an effect evidenced in the exposure experiment with fish embryo (Figure 7-27).

Fish bought in the local market had concentrations of endosulfan I + II of 0.016 µg g^{-1}.This agrees with those reported by Ríos et al. (2010) in the range from 0.011 to 0.038 µg g^{-1} for fish of this species caught in Esteros de Farrapos and Nuevo Berlín.

10.3.3.3 Fish experience endocrine disruptive effects in the laboratory and in the field

The interpretation of effects endpoints for the sentinel wild fish was challenging, as baseline levels relative to the life history of the chosen bioindicator species did not exist. Changes in sex ratios or intersex gonads could affect populations, and as said before, it cannot be attributable to any particular stressor, as fish masculinisation downstream Kraft pulp mills outfalls (Larsson & Förlin 2002), and feminisation (Parrott et al. 2004), have been reported, depending on the pulp mill effluent treatments or cellulose production processes.

In field studies intersex was not found, possibly because EDCs were not in high enough concentrations as to produce these effects or the frequency of this effect was not detectable, which in Brazilian streams had incidences from 1.9-9.8% (Pereira de Sá et al. 2008).

Other effects found were related to sex ratio (2.3.3).The reasons why the sex is female-biased at sampling sites F1, F2 and F3 are not easy to identify. There are cases of masculinisation of fish downstream from Kraft pulp mill effluents (Larsson & Förlin 2002). On the other hand, sex ratios could also be skewed toward females (Parrott el al. 2004), depending on the pulp mill effluent

treatments or cellulose production processes. In Brazilian investigations, municipal wastewater has been linked to these effects. Even when the normal (1:1) ratio can be expected for *Astyanax*, in some cases a predominance of female fishes was observed (de Alcântara Santos & Costa Novaes 2008, de Carvalho et al. 2009). According to Achione-Nzeh (2010) higher female proportion would be a reproductive strategy to protect them from predatory fishes, or a decrease in males appears with increasing age (Teubner et al. 2011), but this could also be related to stressors.

Smaller fish and in worse condition were found at F4 than in other sites. This suggests that contaminants from the municipal wastewater and upstream activities could have had an impact on the general fish health.

As represented in Figure 7-38, male fish had smaller gonads at F3 than at F2. According to the isoconcentration model, Ubici Beach (F3) has higher probability of receiving the pulp mill effluent than at F2 (Figure 7-3). This finding is in correlation with Munkittrick et al. (1994), who pioneered in describing this effect in relation to BKME outfalls. Female fish were less mature at F4.

The severe flooding event occurring in November 2009 (Appendix A-3) could have exerted impacts on the fish community. According to de Carvahlo et al (2009), this species shows fractionated spawning and reproduction throughout the year, with peaks influenced by water temperature and rainfall. Nevertheless, not much information exists on this issue in subtropical latitudes; but freshwater temperate fishes generally spawn in spring and early summer, with photoperiod, temperature and seasonal rainfall among other factors, important in regulating their reproductive cycles (FAO 1981).

In the laboratory, experiments with non-spawning fathead minnow with 14 days duration, according to Kunz & Fent (2009) found very variable VTG levels possibly because fish were younger than in the USEPA protocol making concentrations too low to measure them in whole fish homogenate. Spawning substrates were used in the following experiments to generate higher

concentrations, finding that fecundity of fish diminished after exposure to BKME, an effect that was described by Parrott et al. (2006) in relation to this type of effluent, and associated to depressed steroid and VTG production by Ankley et al. (2008). A possible correlation of VTG to fecundity has also been described (Miller et al. 2007, Jensen et al. 2007). In contrast, egg production doubled in fish exposed to the stream receiving municipal wastewater by comparison to the control, as also observed by Höger et al. (2006) but when exposing rainbow trout to diluted sewage.

10.3.3.4 Toxicogenomics as an approach to diminish uncertainties

"Oestrogenicity" can mean affinity to the oestrogen receptor, the ability to activate expression of oestrogen-dependent genes, or stimulation of cell proliferation of ER-competent cells. Therefore, as binding to the nuclear receptor is one of the main mechanisms of action many analytical probes determine it (either oestrogenic or androgenic). We found effects in fish from exposure to BKME that could be attributed to the interplay among *ESR1* and *ESR2*.

Interpreting biomarkers responses is challenging for mixtures as other pollutants may modify metabolic enzymes and transporter pumps of EDCs (Celander 2011). On the other hand, even when measuring *vtg t*ranscripts by quantitative real-time polymerase chain reaction (QPCR) assay can detect exposures of 5.0 ngl^{-1} EE2 (Biales et al. 2007), Ankley et al. (2010) recommend that gene expression of *vtg* be studied in the context of alterations in the reproductive capacity of fish, as the gene may or may not express. The strategy taken in this thesis was not only measuring *vtg*, but as part of a suite of biomarkers to increase the bulk of evidences.

10.3.3.5 Occurrence, fate, partitioning, biomagnification, foodweb processes and toxicity of EDCs and complex mixtures

After reviewing and trying several exposure models during this thesis, it was recognised that complete software models for exposure and toxicity were lacking for many EDCs. However, the approach used included the use of models based on fugacity for the calculation of bioaccumulation and magnification through the food web to predict the fate, transport, bioavailability and bioaccumulation to guide further search of EDCs in water, sediment, soil or biota. The multimedia model showed that chlorinated phenols as well as nonylphenol and endosulfan had similar predator-prey relationships, with fish higher in the trophic chain influenced by the contamination in other fish, and mussels. On the other hand, the main pathway in the foodweb for β-sitosterol is through plankton up to omnivorous fish. This result is consistent with Martins et al. (2011) as this terrigenous sterol reaches sediments and associates with phytoplankton.

Bioaccumulation, partitioning and toxicokinetics were studied both experimentally (analysing the tissue distribution of a chlorophenolic model EDC in a floating plant), and in field conditions (by studying the distribution of several target EDCs in fish liver and muscle). The preferential tissue distribution for macrophytes was in the leaves, while in fish, mainly in liver. As an example, resin acids were not quantifiable in muscle, but high liver, implying that, as muscle is the edible part, the risks to humans are negligible, but they could pose a risk to fish based on its metabolic pathway. The metabolism was also modelled, by the PBPK model to find that nonylphenol bioconcentrates in liver and in fat tissue (Figure 8-1), and endosulfan in slowly perfused tissues, such as fat, skin, and muscle. This agrees with Table 7-23, meaning that endosulfan poses a higher risk to humans than to fish, as the internal dose is much higher in muscle than in liver.

Environmental concentrations of alkylphenols are postulated to be linked mainly to urban sources in the research area. However, agricultural sources may influence as nonylphenol ethoxylates are used as part of glyphosate formulations, supported by the finding that at Nuevo Berlín, a village with a small population, fish tissue also had residues of NP.

The measured concentrations of oestrogens in the Uruguay River were low; mostly under the detection limits (0.2 ng l^{-1}), but the highest ones belonged to the Fray Bentos stream receiving municipal wastewater (2.2 ng l^{-1} E1; 0.6 ng l^{-1} E2). Typical concentrations of oestrogens in municipal wastewater range from: oestrone 5–20 ng l^{-1}, 17β- oestradiol 1–10 ng l^{-1} (Burkhardt-Holm 2010).

Although, the extrapolation of the set PNEC limits for European settings to the Uruguay River has to be done with care, the PNEC >1 (for NP suggests that measures to reduce the discharge of anthropogenic substances to urban areas and better agricultural practices are necessary to achieve good water status.

10.3.3.6 Bioavailability, partitioning and ecotoxicity through the sediment-water pathways and multimedia model predictions

An experiment was designed to explore the bioavailability of 2,4,5-TCP, a chlorinated EDC found in pulp mill effluents with a frequency of at a 3.2%. The through the AOX/EOX ratio demonstrated that this compound partitions between phases. It also concluded that the bioaccumulation and severity of the risk to aquatic biota from exposure to this EDC is a function of particle size. As expected, dissolved substances had more impact on animals living in the water column, but also the particle-bound EDCs seemed to affect both early development stages and sediment-dwelling biota as a secondary route. Laboratory analysis also showed that chlorinated organics were actually accumulating in snails, mussels and macrophytes. Bivalves accumulate chemicals to much higher concentrations than those in ambient water (NRC 1991, Galloway 2006), and in fact showed measurable concentrations of chlorinated organics (10.3.2).

One of the most prevalent compounds found in both sources resulted to be alkylphenols, which had been *a priori* prioritised since they are used in large quantities and emissions of these compounds were likely to end up in urban as well as agricultural runoffs. This was proved as alkylphenols investigated in waters, biota and sediments were detected and measure in all this matrixes. Nonylphenol, a ubiquitous emerging contaminant was shown to have fluctuating concentrations in surface water, with significant concentrations in sediments and in fish.

Agricultural and urban runoffs are significant pathways of nutrients input into the water column and the sediment. One finding was that the nutrients concentration in S2 was higher than in the control site, and that the clay and silt fractions varied according to sites, influencing on toxicity.

Regarding effects, the *in vitro* screening with *P. leiognathi* (Ulitzur et al. 2002, Broers & Lappalainen 2004), detected metal and organic toxicity in all areas, but more especially at site S2.The results of the bioassays with *C. dubia* showed toxic effects on reproduction in S1 and S8. The growth of *H. curvispina* was significantly lower than that to the control after exposure to these sediments, possibly due to ingestion of contaminated sediment particles. For this bioassay, the growth of organisms was significantly lower with regard to the control in all samples.

A borderline effect for the development endpoint in fish embryos was observed at S2 site, consisting on spinal cord anomalies of a frequency still near what is considered normal (4%). The curve dose-response observed for the appearance of vertebral malformations follows a non-monotonic behaviour. Signs of toxicity and teratogenicity evidenced by organisms that thrive on the sediment, as well as by species that live in the water column, indicate that the toxicity routes in the analysed sediments of the Uruguay River would not be solely from dissolved pollutants but also through suspended particles.

10.4 Risk estimation, the magnitude of risks and data gaps

Individuals are not at risk from the consequences of a hazard if they are not exposed to it. The critical factor linking exposure of vulnerable organs or processes to risk is the quantity of bioavailable toxicant. A sub-population is vulnerable if it is more likely to be adversely affected by a stressor than the general population. Vulnerability reflects how much a system is likely to experience harm due to exposure to a hazard. The extent of the impact depends on the system sensitivity to respond to stressors, not only on the potency or frequency of the hazard (Fäussel 2006, Ippolito et al. 2010).The susceptibility of the sub-population to the EDCs also faces an increased likelihood of sustaining an adverse effect depending on a life state (e.g., pregnant, foetus, infant). Therefore, risk is the likelihood that a threat arising from a hazard turns into harm, or consequence, if the resistance of the receptor is defeated, depending on its vulnerability (See Appendix G).

10.4.1 Severity of effects and species sensitivities

The severity of the hazard was described by a dose–response relationship from several exposure tests at a range of doses, for example in the case of YES, and exposure tests. The *in vivo* assays revealed that some taxa are more sensitive to EDCs than others, with the most sensitive organisms tested crustacean and early developmental stages of fish. Models were used to predict that the media of concern were benthic invertebrates for nonylphenol and endosulfan, and piscivorous fish for isopimaric acid and glyphosate. In this case, the scarcity of data for the two latter mentioned ones increased the uncertainty. The model did not predict that sediment was the medium of concern for glyphosate, but this compound was found in sediment (

Table **7-11**). This could be due to its zwitterion characteristics and the particularities to the binding to particles (Khoury et al. 2010).

10.4.2 Doses to humans

Calculation of human intakes from measured contamination of foodstuff is not straightforward as concentrations in sampled animals can differ substantially after preparation and cooking (Humphrey 1976, cited by NAS 1991). This aspect is in the calculation by Paustenbach as an empiric factor to account for cooking. The POPs calculator includes other relevant pathways as dermal and inhalation pathways. There exist data gaps in databases, especially for systemic end-points, as most are for acute exposures, still lacking specific doses for any of the pathologies or conditions related to ED or they only deal with oral doses.

10.5 Risk characterisation in the midst of complexity

In the risk characterisation stage, the pairing strategy combined information from the literature and computer databases and of EDCs determined in mixtures. Biomarkers provided a linkage between field and laboratory data. The measurement of VTG gave an insight to oestrogenicity of reference fish exposed to the main source point discharges. A limitation was the lack of a historical database for the test to considering the natural variability. Watanabe et al. (2007) stressed the importance of determining what can be expected as normal, in order to be able to decide if a response was biologically significant or not.

10.5.1 Multiple mechanisms of action

Multiple mechanisms of action can coexist (Hutchinson et al. 2006). For instance, NP can act an oestrogen or androgen antagonist and alter gonadotrophin synthesis and secretion. Sumpter & Jobling (1995) state enhanced oestrogenicity could occur when fish are exposed simultaneously to various oestrogenic EDCs (as is likely in rivers receiving effluent). The question is what happens with the same mixture that possesses androgenic EDCs from municipal sources, as well as anti-oestrogenic and anti-androgenic compounds, and if effects would be counteracted or not. Considering the multiplicity of

possible mechanisms of action including oestrogeniciy, androgenicity, anti-oestrogenicity and antiandrogenicity, mediated or not by nuclear receptor binding (Janošek et al. 2006), this matter is less simple than hitherto thought. Other mechanisms of action could be mediated by retinoic acid, as exemplified by studies with frog embryos after treatment with low doses of glyphosate (Paganelli et al. 2010).

10.5.2 Lag time and extrapolation to humans

Irreversible or long lasting effects, or affecting future generations are the most worrying effects of EDCs, but as people may migrate to other areas of the country or abroad, effects could be left unidentified. Fishermen, the differentially exposed sub-population, live and work near a source of pollution, rely on this type of food to survive, and get potentially higher levels of these pollutant than the general population. However, at this point the evaluation of adverse manifestations in the critically exposed sub-group to the recent sources, such as the pulp mill is not possible because the outcomes may take decades to emerge (long latency).

10.5.3 Methodological approaches to evaluate mixtures and their uncertainties

Summing up the toxicities of each component in complex mixtures and assessing their interactions could be overwhelming and impractical (NRC 1988). Then, the strategy was to use the “whole mixture approach” to include any other EDCs that could exist in the point source discharges (Chapter 7), as only some of the constituents were known.

10.5.4 Additivity of responses

Similar acting EDCs have additive responses (Kortenkamp & Altenburger 1999, Heneweer et al. 2005, Zhu et al. 2008), but the mixture effects of different classes of EDCs can be puzzling. A classical example of antagonism is the inhibitory effects of AhR-agonists on some E2-induced responses by down-

regulating ER expression (Safe 2003). For example, EDCs mixtures structurally diverse that act as agonist in some tissues and antagonist in others may have SERM (Safe et al. 2002). In this case, after binding the ligand to oestrogen the receptor conformation changes occurr, and nuclear factors for gene expression are recruited thereby selectively inhibiting or stimulating oestrogen-like action in tissues, as for example the antioestrogenic drug tamoxifen (Gaido et al. 2003). In this research, all of these modalities were possible, justifying the application of the whole mixture approach.

10.5.5 The whole mixture approach

Many complex mixtures are heterogeneous, varying from source to source, the conditions governing their formation (NRC 1988). This variability was seen after studying the effluent chemical composition. The final outcome of exposure to a complex mixture is uncertain, even when extensive chemical analysis was done. The inclusion of a battery of bioassays to compare all the effects on biota simultaneously in one experiment was the strategy employed in sediment elutriates and effluents. Although individual effects of single agents were not evaluated, target EDCs were measured in the mixture. Particularly challenging were pulp mill effluents, as their components may be oestrogenic or androgenic and act as agonists or antagonists, depending on the effluent concentrations (Orrego 2010).

Some of the observed biologic effects, as vertebral malformations or *hormesis* are not easily reproducible with different samples of the mixture. This implies more sampling and analysis, to increment statistical significance. Some of the mixtures were evidenced as fish tissue residues of variable composition and as chlorinated organics, extracted from BKME and concentrated in the leaves of the floating plant *E.crassipes*. Apart from pulp mill discharges, compounds of this type were also found downstream the city in other biota and sediment.

10.6 Causality in aquatic systems

Koch's postulates were modified by for use in ecoepidemiology causation by Suter (1990, 1993, 1998). Fredricks & Relman (1996) reformulated the postulates to fit virology. When Suter´s postulates for toxic agents were contrasted against some of the thesis findings to explore plausible causality links, several conclusions arose, as presented in Section 9.6, some with low and some with high uncertainties.

As Maltbly (2006) sustains, distinguishing among "contaminated" and "un-contaminated" sites is not enough to infer causality, and controlled experiments should be used. In this thesis both laboratory and field studies were developed. This approach was valid, as for example, NP was present in fish tissue after exposure to municipal wastewater and also in fish downstream from the city, implying a highly probable causal link to domestic use of the surfactant precursor, confirmed by the persistence of the ethoxylate in the stream sediment. Causality inference is not always as straightforward, as the some effects could be multiple, and upstream concentrations of NP were found, probably linked to agricultural use.

10.6.1 Developmental effects from the sediment-water pathway

Weis & Weis (1987) state that the skeletal system is highly sensitive to teratogens, and if in frequency above normal, the anomalies could indicate endocrine disruption (Villeneuve et al. 2005), as oestrogenic receptors are also present in bone cells and EDCs may interrupt the ossification control (Warner & Jenkins 2007). Spinal deformities were observed after exposing embryo to sediments elutriates of Fray Bentos (S2) (Figure 7-26). This has been observed near pulp mill effluent outfalls (Bengtsson 1998, Svanberg et al. 1996), and after exposure to chlorinated compounds or whole effluent (Bengtsson et al. 1988, Hardig et al. 1988). In addition, herbicides such as glyphosate (Kelly et al. 2010), metals, such as cadmium (Lugowska 2007, Table 2-3), or microcystin LR (Oberemm et al.1997) have been linked to severe skeletal deformities.

Microcystin-LR was measured in the river associated to algal blooms in concentrations above the drinking water guidelines limit of 1 µg l^{-1}. Hence, the causal effect from cyanobacteria cannot either be ruled out as causative of this noxious effect.

10.7 Uncertainty assessment

Both stressor-based and effects-based studies were carried out to determine geographically linked sources-to-effects related to multiple stressor mixtures. Evaluating the impacts from upstream activities is not straightforward for non-point source releases from contaminated soil run-off, or air transport. Also, as Jobling et al. (2003) pointed out, the time lag between exposure and biological response, the bioaccumulation in fish tissue not manifesting in the individual but in its progeny, and the variability of concentrations determining seasonal variations in responses are additional sources of uncertainty. More on this topic, is presented below, and in Table G-1.

10.7.1.1 Site-specific conditions affecting organism susceptibility

Matrix variations among sites of the river based on different organic matter and particle size were evaluated at environmental doses with a model EDC and organisms of several trophic levels, to diminish uncertainty, and to derive chronic toxicity data, which cannot be inferred from acute toxicity data.

Differences in sediment composition greatly influenced the species vulnerability as it increased the bioavailability of the toxicants, as demonstrated by the bioavailability and sediment toxicity experiment (7.6.2.1).There differences in vulnerability in some areas of the river. This is important in terms of risk management, to determine “hot spots” where risks are more likely than at other areas. The differences between gonadosomatic index and condition factor of fish from one site to the demonstrated the influence of sources, fate and mixture combinations.

10.7.1.2 Differences in organism sensitivities related to life-stages

In humans, there is no real un-exposed population to ED, but the most sensitive sub-populations are foetuses, infants and pregnant women, which were considered in dose calculation. As long- term effects depend on the window of highest sensitivity of the exposed receptor, in general early life-stages, in animals (van Aerle et al. 2002) as well as in humans (Selevan et al. 2000), then embryo to juvenile stages should be the most sensitive for this category of toxicant. Reproductive life stages are also more prone to alterations due to ED. Therefore, the choice of tests with embryo, neonate and reproductive stages accounted for this, and their sensitivity to toxicants in sediment elutriates was demonstrated.

10.7.1.3 Interactions and toxicokinetics of endocrine disruption

According to Brian et al. (2005) oestrogenic EDCs act in an additive manner, which can drive to erroneous conclusions of considering an absence of risk as at low concentrations single agents may not produce a certain effect which after reaching a threshold when acting as a mixture could be expressed. However, similar modes of action are not always additive implying that regulatory limits are not easy to apply. For instance, dioxins and furans can be antagonistic with other anti-oestrogenic compounds, such as PCP (Zhao et al. 2006), but they are synergistic with PCBs (Bannister & Safe 1987).

The toxicological paradigm that NOAEL responses observed at high doses can be extrapolated at low doses does not always apply to EDCs. According to Hirabayashi& Inoue (2011), this concept is suited for deterministic but not stochastic or probabilistic biological responses. The theory of *hormesis* relates to low doses causing higher responses than high doses (Figure 2-1), and, in fact, we observed at the lowest doses fish larval spine malformations, and increased neonates in the three brood test with crustaceans.

The existence or not of threshold for many EDCs and low-dose *hormesis* constitutea source of uncertainty (2.3.6).Even when this toxicokinetics behaviour at low-doses continues being controversial (Rhomberg & Goodman 2012, Vandenberg et al. 2012), it was observed in this thesis. Evidences of non monotonic behavior for the assessment point of dose-response curves were found after exposure to sediment elutriates as fish embryo larval teratogenicity (Figure 7-27) and *hormesis* evidenced after exposing *C. dubia* to BKMEs (Table 7-7).

10.7.1.4 Inter-species extrapolation

Extrapolation between levels of biological organisation was addressed through an experimental design based on mechanisms of action and biochemical receptors, and between species extrapolation though a battery of organisms representative of the food chain. Water and fish ingestion are common pathways, and biomagnification affects both in the same way. The fish *Astyanax fasciatus* was the diagnostic tool to unravel potential effects at the population level. Yet, extrapolation of their responses to those of fathead minnows in the laboratory is not devoid of uncertainties. Their sensitivity varies also in the Northern Hemisphere within lab and field species and within each group (Lange et al. 2012). The variability among taxa was noticed with a battery of bioassays and also by sensitivity distribution analysis (9.2).

10.7.1.5 Analytical uncertainties in trace analysis and biomolecular methods

Even with the uncanny sensitivities achieved by modern analytical methods, unthinkable years ago, the very low environmental concentrations able to exert ED still represent a challenge. In trace analysis, the analytical uncertainties detect-and no-detect, type I and type II are important. Measurement implies uncertainty owing to natural variability and intrinsic faults in accuracy, precision and recovery of analytical methods. Compounds detected at very low concentrations in river water were dioxins and furans, and chlorinated

pesticides were below limits of detection of chromatography methods, but some persistent EDCs such as endosulfan were detected in biota.

10.7.1.6 Methods to assess receptor binding

The evaluation of the analytical “fit-for-purpose” concept deemed ERCALUX® and ARCALUX as suitable to screen ED at the PRA because of their low rate of false negatives (10.2.1) and high sensitivity (LOD 24 pg l^{-1} 17ß-estradiol-EEQ for ERCALUX®), but YES was only applied to test effluents and wastewater, with lesser stringent requirements. This screen indicated non-detectable oestrogenic activity (LOD 55 ng l^{-1} E2) in effluent and wastewater samples. However, ER-CALUX® results suggest that the toxic response in YES could be underestimating oestrogenic activity or diminishing the response if anti-oestrogenic compounds existed, or cytotoxic compounds were present.

10.7.2 Vitellogenin as a biomarker

Even though this biomarker is one of the most recognised, surprisingly, androgens may also take part in VTG synthesis. Andersen et al. (2006) exposed adult male zebra fish in short-term experiments to methyltestosterone at low concentrations (4.5 ng l^{-1}), observing that VTG increased significantly. The non-aromatisable dihydrotestosterone (DHT) AR agonist induces antioestrogenic responses through direct and/or indirect modulation of VTG, steroid hormone and total cytochrome P450 levels. Alterations in metabolism mediated by AR binding may be responsible for the VTG and E2 decreases by DHT (Shilling & Williams 2000).

10.7.2.1 Prioritisation

In absence of a complete computational tool, a decision tree was designed, which, as any decision, is not completely devoid of uncertainties. Additional information on these and other uncertainty sources is in Table G-1 (based on Danston et al. 2003). Recommended tools according to literature and those used in this thesis were compared to evaluate the adequacy of the framework

and its application. For instance, metals were within regulatory limits, with exception of arsenic in few water wells, but current information regarding cadmium is scarce. Although not specifically prioritised, they can play a role in ED (10.7.2.4). Also, thyroid disruptors were initially considered for the QRA but in a prospective study in the stream their concentrations were detectable, which will merit more refined studies.

10.7.2.2 Sample variability and reproducibility

There was a high variability in pulp mill effluent composition in terms of chlorophenols, resin acids and sterols. For example, abietic acid was until 2010 when it was found in concentrations up to 55 µg l^{-1}. Fray Bentos stream composition also varies greatly, influenced by rainfall, unsteady inputs of municipal wastewater, leaching of pesticides and herbicides. Variability was also observed in effects, from receptor binding screens to fish exposure experiments, as sampling can be only a snapshot of a possible situation. Also, even when clear *in vivo* effects in biomarkers and egg production appeared when exposing fish to undiluted BKME, actual effects in the river might be local and not seen in the whole of the river, as it was a full strength sample. The reproducibility of vertebral malformations or *hormesis* is not easy to obtain due to the low frequency of effects and natural sample variability.

10.7.2.3 Database scarcity

In the case of human health, trade-offs among prospective and retrospective studies were taken, as many years of follow up in human health was unfeasible, and illness could manifest many years after exposure. The current information on neonatal malformations was yet not complete enough to derive this relationship. The paucity of pre-existing data made it difficult to achieve a complete assignment of sensitivities based on the species level in the ecological component, but a rather broad range of taxa were considered (Chapter 9).

10.7.2.4 Concurrent mechanisms of endocrine disruption

Thyroid may coexist with oestrogen and androgenic disruption for pentachlorophenol, 4-nonylphenol, bisphenol A, and phthalates (Ishihara et. 2003). They were not specifically included as they were sorted them out in the prioritisation stage, but some of them entered the list because of their other MOAs. As presented on 2.3.9.2, endocrine disruptors may act through genomic or act non-genomic mechanisms. Chlorophenols are low oestrogen binders but they act via inhibition of oestrogen sulfotransferase (Harris et al. 2005). Hydroxylated metabolites of PCBs, bind to the thyroid hormone transport protein, competitively inhibiting thyroxine but they also inhibit SULT1E1 (Waring & Harris 2005). Bisphenol A and butyl benzyl phthalate (Li & Gramatica 2010) and alkylphenols (Xu et al. 2005) are both oestrogenic and anti-androgenic.

Metals could coparticipate in ED within environmental mixtures. Both types of toxicity were found with *P. leiognathi screen* in sediment elutriates (7.3.3.1). Arsenic, cadmium, lead and mercury are potential EDCs (Moore et al. 1997, Sokol 2002). As Davey et al. (2008) mention, arsenic modifies the gene expression of retinoid and thyroid receptors provoking developmental effects and teratogenicity. Cadmium binds both to ER and ARs, affects steroid synthesis, testicular, placental tissues and embryo, and may increase the risk of breast cancer (Takiguchi &Yoshihara 2006). Lead elicits toxicity on sperm morphology, affects growth and reproduction of earthworms (Zheng & Canyang 2009) and follicular steroidogenesis in fish (Chaube et al. 2010). Mercury impacts on spermatogenesis in taxa ranging from fish to primates (Xinqiang et al. 2000) decreases testosterone levels, and increases thyroxine plasma levels at occupational exposures (Iavicoli et al. 2009).

10.7.2.5 Joint effects

The question on how to predict actions in the case of complex mixtures still not fully answered. Mixtures posing all possible effects oestrogenicity, anti-oestrogenicity, androgenicity and anti-androgenicity, were found in this research

and were also described by Stalter et al. (2011). This fact stresses the huge complexity of the mixtures issue, as the actions that would actually be expressed in the animal could either be feminisation or masculinisation, when exposed to this cocktail of EDCs, depending on their concentrations, potencies and individual susceptibility, among other factors.

Comprehending that the bulk of uncertainties is high, a representation of the joint effect was performed as the sum vector (Figure 10-2) with probabilities of effects of each MOA (oestrogenic, androgenic, anti-oestrogenic and anti-androgenic) estimated based on the expanded Fermi solution (Appendix H).

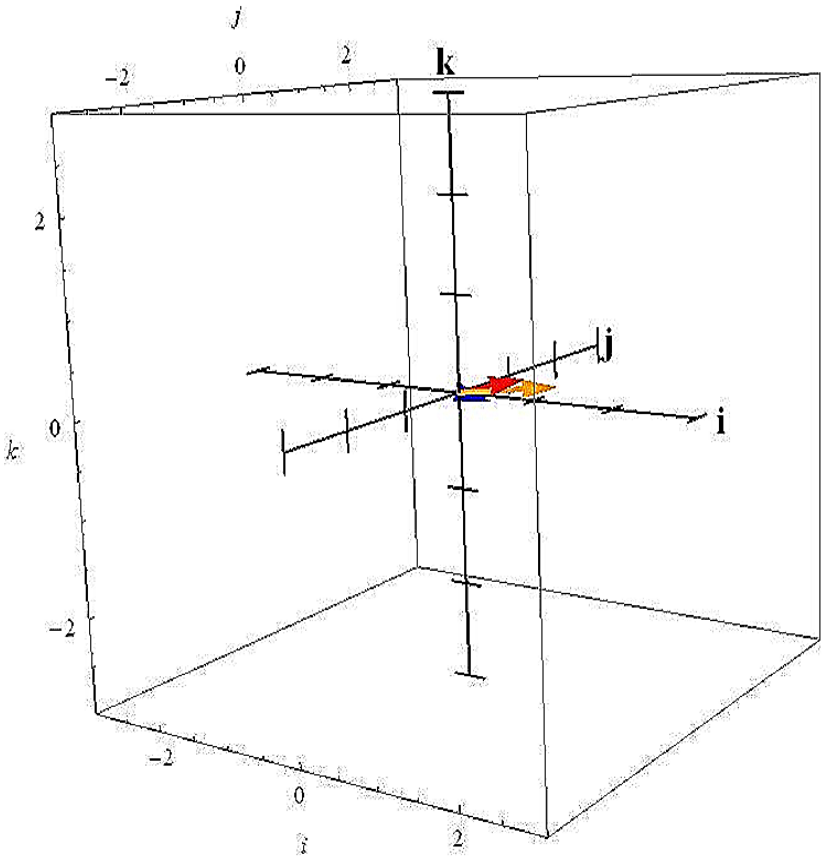


Figure 10-2 Proposed joint evaluation of oestrogenic, anti-oestrogenic, anti-androgenic and androgenic risks as vectors

10.8 Risk management advice

Even though in the case of EDCs at the current state of the science uncertainties still remain, especially on causality, objective and sound information was provided within the existing limited resources that allows to prioritise risks.

10.8.1.1 Risk management approaches

At the macro dimension, this RA aims to be an input to strategic risk management. This should include economic, legal, social, cultural and political values (Richter & Laster 2004, Prpich et al. 2011), under the Pressure-State-Response concept (Pollard et al. 2004).

10.8.1.2 Risk acceptability criteria to guide risk management decisions

Risk assessment is one of the inputs in acceptable decision processes (Pate-Cornell 1994). Let´s consider the legal terminology that relates to risks categories and risk acceptability criteria, where risk can be categorised according to Suter (1995). A *de minimis* risk could be ignored in subsequent assessments or decisions, an indeterminate one is not clearly significant, and *de manifestis* risk should be transferred to remediation or control. In Table 10-1 the cases when action should be taken are shown.

The recommended path (COM 2007) is to apply the precautionary approach when HQ or RQ > 1. However, to allocate resources in an economically feasible manner, yet achieving the maximum possible environmental and health protection, risks should be prioritised. In this thesis, some of the sites complied with *de manifestis* conditions, especially from nonylphenol (8.3.1) and endosulfan (8.3.3), drawing towards pertinent risk management actions in limiting the use of these EDCs.

Table 10-1 Risk acceptability criteria

<table>
<tr><th colspan="2">Criteria for de manifestis and de minimis risks</th><th rowspan="2">Reference</th></tr>
<tr><th>Environmental</th><th>Human health risks</th></tr>
<tr><td rowspan="4">Threatened or endangered species, wetlands, and ecological components, or < 20% reduction in abundance or production of a population.</td><td>Excess cancer risk $\geq 10^{-4}$</td><td rowspan="2">Suter et al. 1995</td></tr>
<tr><td>de manifestis: HQ $\geq$ 1 for individual contaminant or combined exposures of similar toxicological effects</td></tr>
<tr><td>annual probability of chronic disease > 10^{-3} yr^{-1}</td><td rowspan="2">Pate-Cornell 1994</td></tr>
<tr><td>de minimis risk threshold, 10^{-7}</td></tr>
<tr><td>de minimis risk threshold: HQ or RQ $\geq$ 1 any EDC
From grade 4 to 10
Table 9-9</td><td>From grade 4 to 10
Table 9-9</td><td>This thesis</td></tr>
</table>

10.8.1.3 River basins as the unit towards an effective Integrated Water Resources Management

Most transboundary watercourses agreements establish joint organisations as essential mechanisms for water “governance”, based on river basins to institute effective integrated water management. At the macro scale, this approach should be used in this river, as this watershed is part of La Plata Basin.

Existing paradigms in the case of transboundary water regimes should be counteracted to include all possible impact factors at both margins of the river. Even though there is a marked trend towards river basin management at the national level (DINASA 2010), international co-operation in the Uruguay River

Basin should accomplish a refined assessment, with participation of all stakeholders, within the existing legal framework.

10.8.1.4 Regulatory and technological issues

It is advisable to study banning alkylphenols and ethoxylates, in especial NP, and also enforce further restrictions on persistent EDCs like endosulfan and other herbicides and pesticides, and include them within regulations.

Study introducing technological changes, such as better agricultural practices, tertiary treatment (reverse osmose or constructed wetlands with floating plants) and/or increment closed-loop systems in the pulp mill and treating sewage before dumping it into the river. Municipal wastewater should be more effectively managed as not discharging them into the river without proper treatment.

Agricultural management practices geared towards reducing nutrient leaching must be fostered to diminish diffuse pollution, including industrial effluents and municipal wastewater. This requires energetic policy and managerial actions with an urgent need to diminish nitrogen and phosphorus inputs in the river, as well as other pollutants. In this sense, a move towards a pro-active process to achieve an improvement in water quality with cooperation of both countries and stakeholder involvement is required.

10.8.1.5 Implications of the risk assessment results to surveillance monitoring schemes

Water quality surveillance monitoring should continue but incorporating the additional “hot-spots” sites as determined in this research (Ubici Beach and the drinking water intake) focusing on the most prevalent compounds, such as NP and endosulfan in all matrixes, and especially in fish tissue, including dioxins and furans due to their high toxicity and ubiquitous nature. The incorporation of *A. fasciatus* as a sentinel fish species is proposed, based on its sensitivity demonstrated in this research, on its abundance, feeding habits and ecological

relevance. The sediment-water pathway resulted of critical importance (7.6.2.2). This should be included in surveillance including biossays and trace analysis of EDCs and metals in sediments, and take this into account in the event of dredging.

10.9 Where to allocate efforts and resources? Further research

It is advisable to iteratively refine this assessment, reconsidering for inclusion some of the EDCs if there are reasons of merit to do so, and continue evaluating other EDCs in fish, study in more detail the influence of metals.

Continue by developing a river vulnerability assessment, including the key components: stress, adaptation and cooperation (resolution of transboundary conflicts), as UNEP (2009).

Address the scarcity of hazard information for developmental, reproductive, and neurological effects, as recognised by Williams & Paustenbach (2002), and detected during the development of this thesis. Pursueing epidemiology studies of cancer, reproductive affections and neonatal malformations, after completing a national database. Definitely, the development of better software tools is also a research gap for this geographical area, and tools to study the air dispersion pathways of dioxins and furans to quantify emissions, to diminish assumptions in modelling. A modeling tool with the relevant endpoints for ED is necessary, as most models have parameters of acute toxicity only.

From the experimental point of view, there should be efforts in designing assessment methods for combined modes of action to explore cause-effect relationships and other mechanisms of action, such as the retinoid system and peroxisome transcriptional factors, brain aromatase and histology analysis at a quantitative level. Full cycle experiments or multigenerational studies, and other biochemical screens can be valuable, as well as other effects monitoring technologies, as for example caged fish or mussel systems near the main discharges or passive adsorption membranes to determine a fingerprint of possible EDCs present, and track the pulp mill effluent plume with remote

sensors and models. Also, examine the possibility of differentiating groups of toxicants and performing a toxicity identification evaluation (TIE). Measure EDCs for which there are no data in sediments, such as resin acids.

The biomolecular methods should advance in South America, through the development of vtg measurement for wildfish; in this case, for example, *Cychlasoma* sp., for with there are available primers and it is fit to evaluate streams. In the long run, elucidate the genes for *Astyanax fasciatus*, a valuable sentinel species for South America rivers.

For human health, analyse the concentrations of EDCs in breast milk. Further research is needed to demonstrate effects and carry out birth defect registries and epidemiology studies designed to track delayed effects of environmental exposures (Solomon & Schettler 2000). In Uruguay the registry started being mandatory, but gathering data will take some years to be significant for an epidemiological study.

Further work should be done to demonstrate the links of EDCs as a factor increasing the vulnerability and decreased immunity of fish to sudden weather changes. This field is very relevant to the geographical area under study, but also as global phenomena linked to vulnerability and sustainability (See Appendix G).

Finally, finding the solutions to this issue includes more research not only on preventive clean technologies, but on EDCs removal methods from sewage and effluents.

10.10 Contributions

An integrated risk assessment of EDCs in a multiple stressor scenario had not yet been attempted before. In summary, human and ecological receptors were integrated to demonstrate endocrine disruption at molecular, tissue, organismal and population levels in a large river watershed. This was achieved by coupling *in vivo* and *in vitro* laboratory experiments with modelling, data mining and field

studies, including a suite of biomarkers, with a mechanistic view to diminish uncertainties.

This new integrated risk assessment methodology of application to any river watershed addressed some critical research needs posed by the EDCs and mixtures topic, optimising resources and developing tools for risk assessment and results for decision makers. The exposure assessment demonstrated areas where fish and other aquatic animals would be at higher risk of experiencing more effects from multiple stressors (“hot spots”). The battery of bioassays as well as the species distribution studies provided with an ecosytemic outlook. The knowledge-base on the state of the environment in terms of EDCs was incremented for this area of the Uruguay River, to bring up evidences for effective risk management considering global, regional and local phenomena and physical and chemical stressors within complex environmental mixtures.

In more detail, some of the contributions of this thesis are:

- Designed a novel two-phased integrated framework to assess the risk of ED from complex environmental mixtures for human health and the environment, of application to any river basin
- Developed a system to prioritise EDCs of concern that underwent a refined risk assessment, through a criteria set that took into account the stakeholders, chemical aspects and mechanisms of action
- Established a better knowledge-base of the exposure to EDCs in the Uruguayan environment and enhanced the scientific knowledge on priority EDCs from a global perspective
- Designed and developed methodological strategies to address effects, causative agents, and predictions, through an innovative analytical tiered approach for the demonstration of ED and other toxicity endpoints, while optimising resources and animals of experimentation use

- Created a system to objectively assign a rating to the river *status* using artificial neural networks, of potential application to any stream, beyond any classical evaluation of regulatory compliance
- Processed water quality monitoring data, derived baseline limits for the studied section of the Uruguay River by the aforementioned methodology
- Explored the use of conductivity and chlorinated organics and phenols to track the effluent plume in the river and demonstrated the influence of local effects consistent with the hydrodinamics model
- Used *Astyanax fasciatus* wildfish as bioindicator species to biomonitor the exposure of EDCs for the first time in the Lower Uruguay River
- Introduced a fish reference species, *Pimephales promelas*, for bioassays and endocrine disruption not existing in the country before
- Lead and participated in a multidisciplinary team with international networking that developed a suite of *in vitro* and *in vivo* methods for ecotoxicity testing and ED not existing in Uruguay and never applied before to evaluate this river
- Advanced the standard fish reproduction test to include refined end-points at molecular, gene, tissue and organism levels with quantitative real time PCR, a suite of biomarkers of oestrogenicity and antioestrogenicity and proposed the use of quantitative histology methods to evaluate subtle gonadal changes, more sensitive and accurate than the classic ones
- Proved the sensitivity of early life-stages as a tool to detect developmental effects from low-doses of environmental stressors
- Identified the appearance of the non-monotonic toxicokinetic behaviour as part of the experimental findings
- Constructed a toxicity profile for glyphosate and gathered information on several other EDCs

- Proposed the initiation of epidemiology studies on birth defects in sex organs birth due to environmental contaminants in the country
- Evaluated differences in bioavailability of contaminants taking into account the sediment characteristics and applied a suite of ecotoxicity tests not yet attempted at this extent before in the region
- Used modeling tools, data mining, clustering, algorithms and Bayes statistics, as a new approach to evaluate data for exposure assessment and risk estimation
- Evaluated concentrations of steroidal hormones and NP for the first time in this watershed, and recognised that more effective environmental management of municipal discharges is needed as their occurrence in environmental compartments was linked mainly to urban sources
- Proposed and collaborated in the development of methods using strains of bacteria to screens for toxicity and oestrogenicity for the first time in Uruguay
- Proposed and evaluated receptor binding assays for oestrogenicity and androgenicity for the first time in Uruguay
- Derived doses for humans and aquatic receptors through fish ingestion and water intake and several routes and pathways
- Introduced the calculation of species sensitivity studies as a tool to evaluate the ecosystem effects
- Evaluated the effects on biota at environmental doses of stressors for developmental, reproductive and toxic responses, linking hydro-geochemistry of the water-sediment phases and bioaccumulation for a target EDC assessing risks in a site-specific way

- Generated knowledge on the state of the environment in terms of ED in the Uruguay River considering global, regional and local phenomena and physical and chemical stressors
- Worked at expert committees to justify the inclusion of nonylphenol and glyphosate as part of the future water regulations
- Collaborated in answering if Kraft chlorine bleached pulp process generates impacts from discharges of EDCs in the river, and if these are estrogenic or androgenic, or if there is a mixture of stressors from several sources aside from industrial
- Applied multivariate analysis to characterise the meaning of the tiered exposure assessment
- Created a new rated matrix to estimate the risks with categories for severity and likelihood of effects for both humans and animals
- Proposed a new way to combine the risks of mixed effects of ED from pulp mill effluents, municipal wastewaters and agricultural inputs into the river using Cobweb diagrams
- Proposed an innovative approach based on the Fermi solution and vectors operations in 3D for the combination of risks from EDCs of different modes of action.

11 CONCLUSIONS

This thesis reached to the following conclusions:

The preliminary risk assessment concluded that:

- Receptor-binders ligands of oestrogenicity and androgenicity exist in the main environmental matrices of consideration in the research area, but not in drinking water or in groundwater. However, the Uruguay River as source water at the abstraction point had detectable concentrations of oestrogen binding compounds (16/11/2009, EEQ 3.3 ng l^{-1}, as E2) in instances after flooding.

The quantitative risk assessment concluded that:

- The river *status* is acceptable as classified by artificial neural networks and background data, but there is a risk of eutrophication that may be accompanied by outbreaks of mycrocystin-LR that has also been linked to endocrine disruption
- The effluent plume reaches with higher frequency to Ubici Beach and this can be observed in the concentrations of phenols in the area, as well as the effects evidenced in fish. The concentrations of phenols were also found in higher concentrations at the drinking water intake point after flooding events.
- The designed tiered approach proved being suitable to determine effects in wildlife with a combined strategy consisting of *in vitro* screens and *in vivo* tests, laboratory exposure tests and fish surveys.

Fish are exposed to a mixture of EDCs from agricultural, domestic and industrial sources

Fish in the Uruguay River are exposed to multiple EDCs, demonstrated by the occurrence of several of the prioritised suspect molecules in the tissues of specimens living in the river as well as in reference fish exposed in the

laboratory to whole samples of pulp mill effluent and the stream water contaminated by municipal wastewater.

Fish show endocrine effects from exposure to EDCs:

- Low severity effects on the endocrine system were demonstrated in juvenile fish exposed to environmental complex mixtures, as no tissue affectations of significance appeared. However, smaller gonad sizes were observed in fish downstream the pulp mill.
- Mixtures of EDCs originated from the city discharges, the pulp mill, and agriculture impact on fish reproductive health and general condition.
- Early life-stages are the most affected as developmental effects expressed as spinal deformities appeared in a borderline frequency above what is considered normal.
- The sediment-water phases are important pathways to the toxicity in the Uruguay River
- A battery of bioassays confirmed the evidence at several biological hierarchical levels. Benthic biota and fish embryo are affected by toxicants present in the smaller size particles of sediments.

In laboratory experiments the presence of EDCs in municipal wastewater and in pulp mill effluents were accompanied by effects:

- The kraft pulp mill effluent produced a marked decrease (half than normal) in *Pimephales promelas* egg production. The postulated modes of action are anti-oestrogenic or androgenic based on biomarkers results. In the case of municipal wastewater inputs, although no feminised fish appeared, the effect was oestrogenic as demonstrated by toxicogenomic biomarkers, as *ESR1* gene expression was augmented alike the treatment to E2. Also, oestrogenicity can be demonstrated based on the results of the yeast screen, the ER-binding tests and the effects on fish, and stressors found in fish tissue and sediment (NP) and in stream water (NP, phthalates, oestrogens). In the case of the undiluted final Kraft pulp mill effluent, an

anti-oestrogenic or androgenic MOA can be postulated because *ESR1* decreased and *ESR2* increased.

- The oestrogenicity of municipal wastewater inputs into Fray Bentos stream, affluent of the River Uruguay was demonstrated by an increase in egg production and the results of toxicogenomics biomarkers.

Identification of "hot spots" for risk management:

- Geographic sites with relative higher risk for either the environment or human health were determined. At Las Cañas resort, fish had less mature ovaries than other sites and there were less female to male specimens than in other sites, while the condition factor of female fish was lower. At Ubici Beach, fish testes were smaller than at the upstream site. A trend in lower retinols was suggested Yaguareté Bay, that could be due to diffuse pollution from agriculture and air transport of EDCs. The drinking water intake received traces of phenols after flooding. Fray Bentos stream, affluent of the Uruguay River is one of the most affected from municipal wastewater, based on chemical analysis and *in vivo* and *in vitro* assays.

Humans are exposed to EDCs but the current risks are low:

- The risk of exposure of humans to EDCs from a fish diet, and multiple pathways is low, considering the individual hazard quotients calculated for the main EDCs. However, the chance of additive actions should not be overlooked. This is especially true for children within the critically exposed groups. No endocrine disruptive effects were evident during the time period of the analysis with the existing information.

Low-dose effects

- Explored in this research at the very low concentrations found in actual environmental matrixes

Identification of priority EDCs for the watershed

- The highest priority EDCs are endosulfan, nonylphenol, 2,4,5-trichlorophenol, dioxins and furans, and with a lesser relevance, isopimaric acid and β-sitosterol.

Toxicokinetics of EDCs

- Non-monotonic dose-response curves were observed in the case of developmental effects in fish embryo and reproductive effects in *Ceriodaphnia dubia* crustacean.

Integration of human and environmental receptors

- The classical paradigm of four steps of RA for the case of EDCs was modified with an integrated approach. By applying the proposed framework it was possible to integrate both humans and animals receptors into the same study to demonstrate endocrine disruption to aquatic animals and to human beings of complex environmental mixtures applied to a model riverine environment with a watershed vision.

Finally, this thesis is hoped to effectively inform risk managers, decision makers and stakeholders concerning the risks of endocrine disruption to keep on demonstrating that sustainable development is possible for the advance of Humanity.

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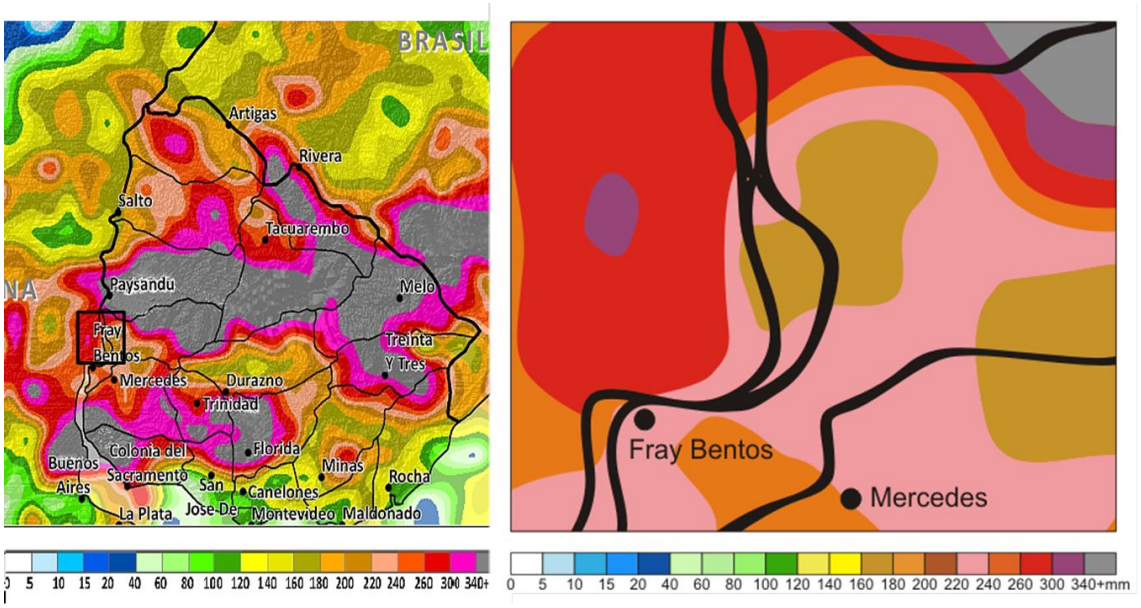
APPENDICES

Appendix A Extreme events, water quality at baseline conditions, and fish biodiversity

A.1 Flooding event at the Uruguay River and research area

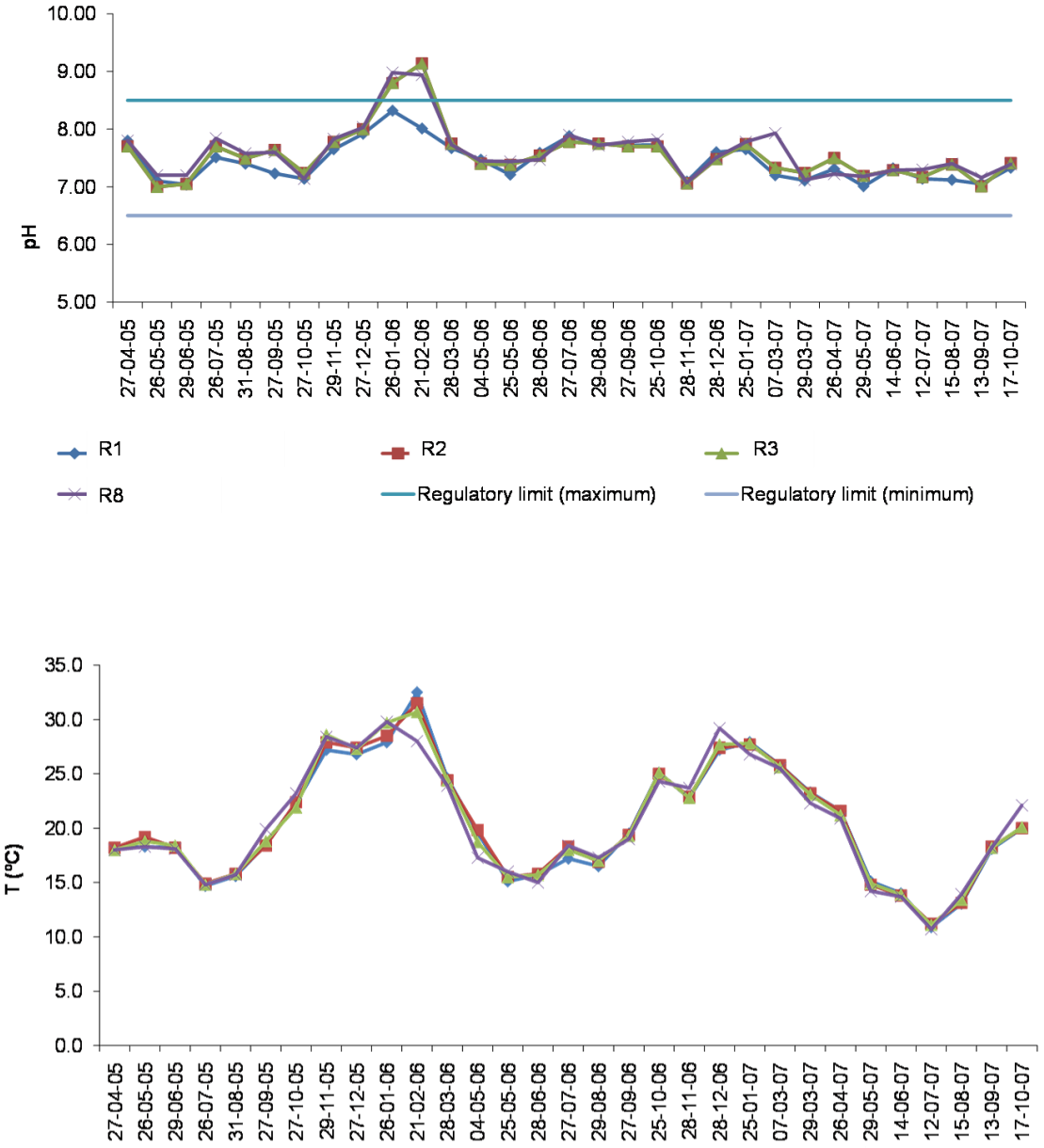


Pablo Míguez, Diana Míguez

GEOSERVIR/CATHALAC/NASA/USAID/ Detail: zoomed simplified image: research area, rain fall in mm

Figure A-1 Flooding event during November 2009 in the Lower Uruguay River and schematic detail of the research area

A.2 Baseline conditions



Upper graph: pH; lower: temperature

Figure A-2 Trends in pH and temperature during baseline studies

Figure A-3 Algal blooming event during baseline studies

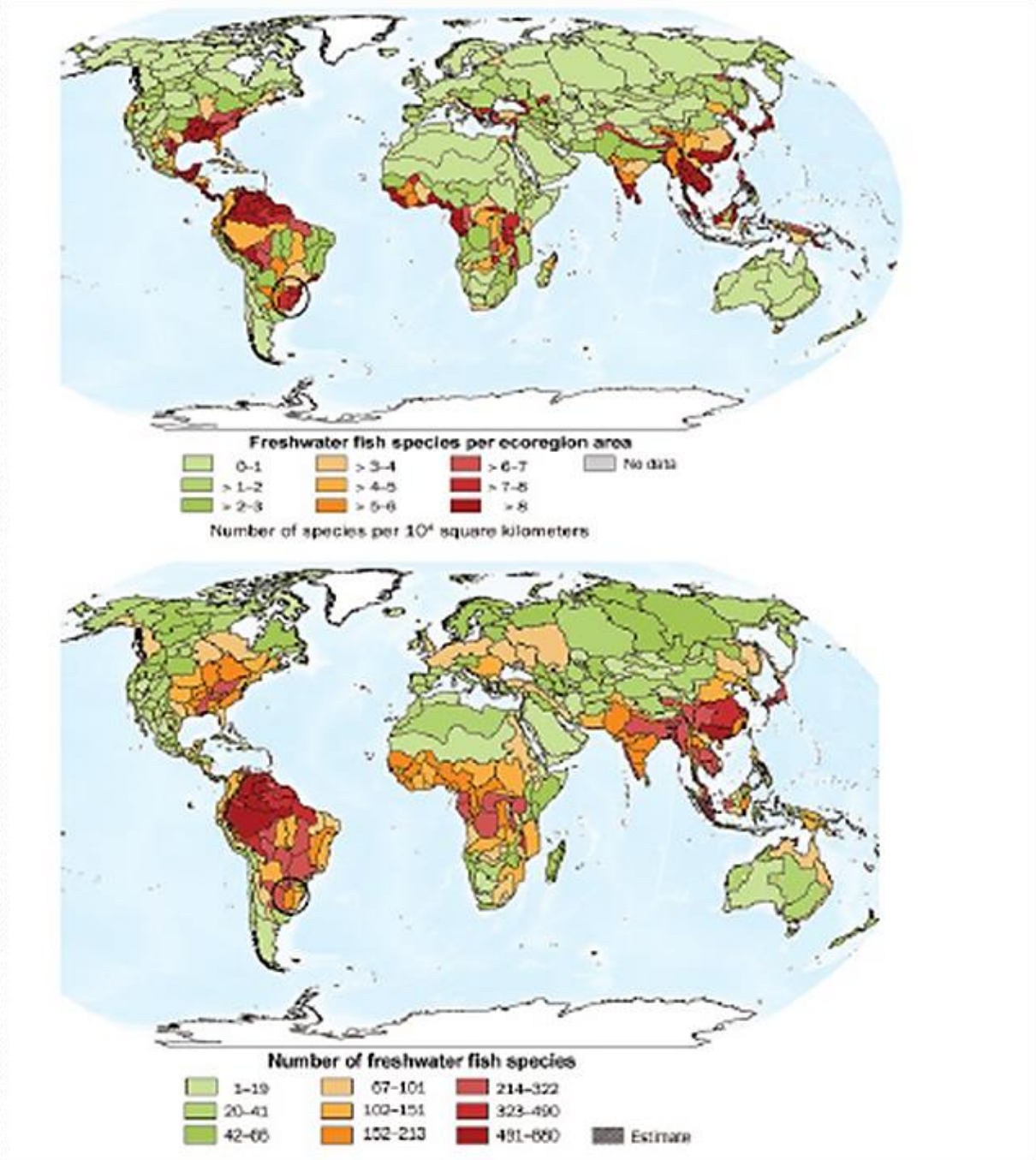
Table A-1 Baseline physicochemical analysis of river water

Parameter	Units	Nuevo Berlín (R1)		Bridge (R2)		Pulp mill (R3)		Las Cañas (R8)		Regulatory/ Permit limits
		Median	Range	Median	Range	Median	Range	Median	Range	
Temperature	ºC	10.9	21.1-32.5	21.3	11.2-31.5	21.4	11.1-30.7	21.3	10.7-29.8	30
Dissolved oxygen	mg l^{-1}	6.8	8.6-10.3	8.6	7.2-10.8	8.6	7.4-10.4	8.7	7.4-10.5	> 5
Conductivity	µS cm^{-1}	69.0	49-102	69	48.0-131	69	53.9-103	75	53.0-153	
pH	pH	7.5	7.0-8.3	7.6	7.0-9.1	7.6	7.0-9.0	7.6	7.0-9.2	6.5-8.5
Color (as Pt)	mg l^{-1}	82	30-210	80	30-170	81	30-160	80	30-160	
Turbidity	FNU	20.0	9.0-57.0	20.9	9.3-51.0	19.2	9.4-62.0	21.5	8.5-66.0	50
Temperature	ºC	10.9	21.1-32.5	21.3	11.2-31.5	21.4	11.1-30.7	21.3	10.7-29.8	30
Total suspended solids	mg l^{-1}	12	<5-28.5	11	<5-32.5	9	<5-23.2	14	<5-60.3	
Total dissolved solids	mg l^{-1}	72.8	32-145	74.6	30-158	74.1	35-116	77.7	30-128	
TOC (as C)	mg l^{-1}	3	1.5-5.8	3	1.5-5.9	3	0.6-5.8	3	0.8-15.7	
BOD_7	mg l^{-1}	1	<0.5-4.8	2	<0.5-5.2	2	<0.5-5.7	2	<0.5-15.5	5
Total nitrogen (as N)	mg l^{-1}	1	0.22-2.40	1	0.07-2.20	1	0.04-2.00	1	0.07-2.40	
Total Phosphorus (as P)	µg l^{-1}	77	25.9-121	80	29.3-197	78	24.9-138	89	26.7-637	25

Table A-1- ***Continued***

Parameter	Units	Nuevo Berlín (R1)		Bridge (R2)		Pulp mill (R3)		Las Cañas (R8)		Regulatory / Permit limits
		Median	Range	Median	Range	Median	Range	Median	Range	
Nitrate	mg l^{-1}	0.57	0.12-1.41	0.58	0.05-1.40	0.58	0.07-1.42	0.57	0.04-1.31	10
Nitrite	µg l^{-1}	7.7	0.0-44.8	7.0	0.0-40.0	7.5	0.0-31.3	5.7	0.0-32.3	
Ammonia	µg l^{-1}	67.6	ND-260	104	ND-540	76.4	ND-320.0	88.1	ND-360.0	20
Dissolved phosphorus	µg l^{-1}	39.6	8.0-102	33.9	4.6-116	33.5	6.9-82.0	41.2	5.5-114.0	
Chloride	mg l^{-1}	2.07	0.98-4.94	2.09	1.09-9.04	2.02	1.07-6.15	2.26	1.11-5.63	
Sulphate (as SO_4)	mg l^{-1}	1.59	0.94-4.09	1.66	0.91-6.83	1.62	0.92-3.10	1.82	0.91-3.22	
Reactive silica	mg l^{-1}	15.4	11.5-17.7	15.2	8.4-17.9	15.3	7.2-17.7	15.2	4.0-17.3	
Iron	mg l^{-1}	1.4	0.4-4.5	1.3	0.4-4.5	1.3	0.4-3.9	1.4	0.5-3.5	
Potassium	mg l^{-1}	1.6	1.1-2.8	1.6	1.1-2.8	1.6	0.7-4.2	1.7	1.2-3.6	
Sodium	mg l^{-1}	3.1	1.1-2.8	3.2	1.7-5.7	3.2	1.9-5.7	3.7	2.0-6.0	
Microcystin-LR	µg l^{-1}	0.41	<0.03-0.79	7.17	0.16-14.1	0.46	0.45-0.46	0.45	0.24-0.66	1

A.3 Fish richness and biodiversity



Abell et al. 2008

Figure A-4 Freshwater fish species in the region under research

Appendix B Materials and methods for sampling, testing and analysis

B.1 Sampling sites

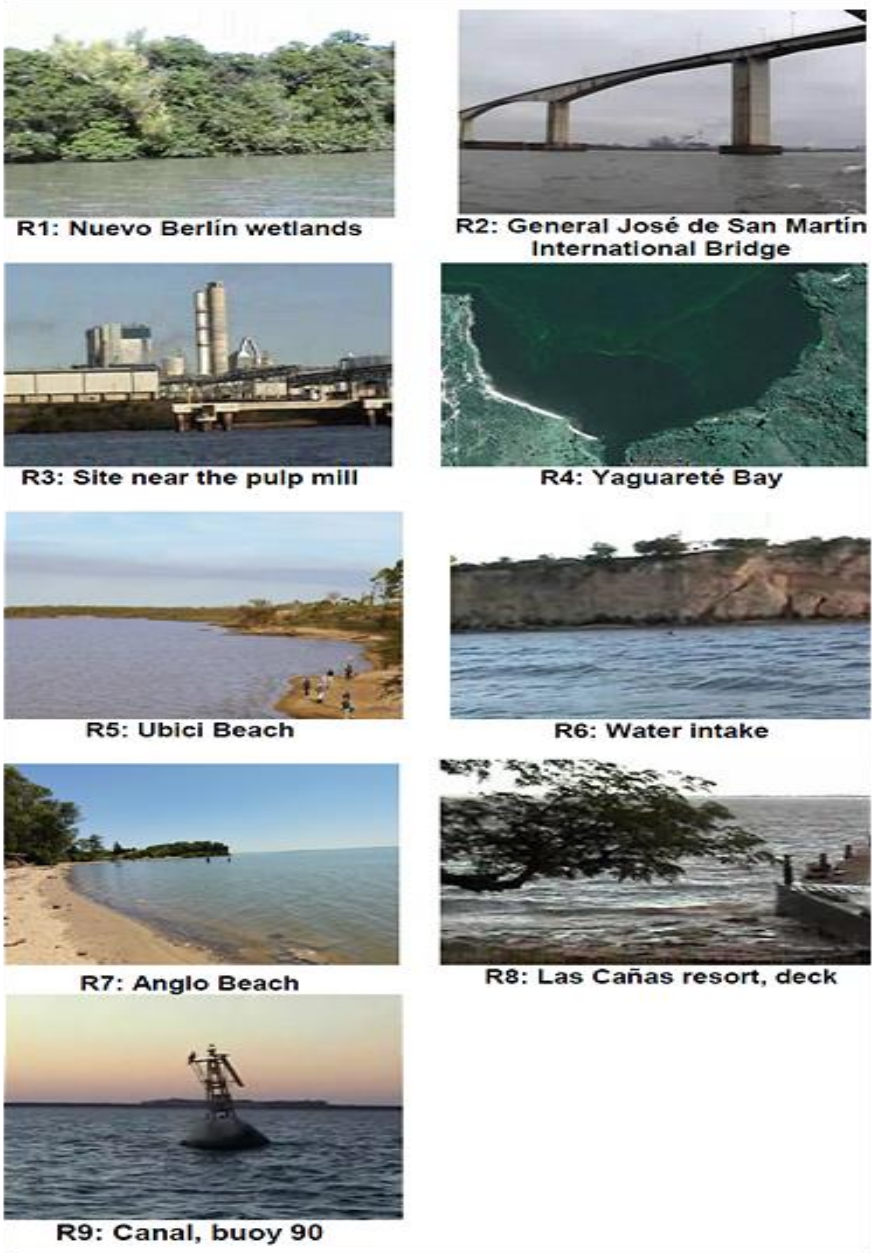


Figure B-1 River water sampling sites

C1: Yaguarete stream

C2: Fray Bentos stream at wastewater discharge

Fray Bentos stream at the suburban area

Figure B-2 Streams sampling sites

F1: Nuevo Berlín

F4: Las Cañas

The location of sampling sites was done with ArcGis Explorer

Figure B-3 Nuevo Berlín and Las Cañas fishing sites

Above (from left to right): ship used during sampling and *in situ* measurements; direct sampling; addition of preservatives procedure

Below (from left to right): Ponar dredge sampler; sediment sample; automatic refrigerated effluent sampler

Figure B-4 Photograph of river water, sediment and effluent sampling

Figure B-5 Satellite view of the pulp mill effluent showing effluent treatment and diffusers

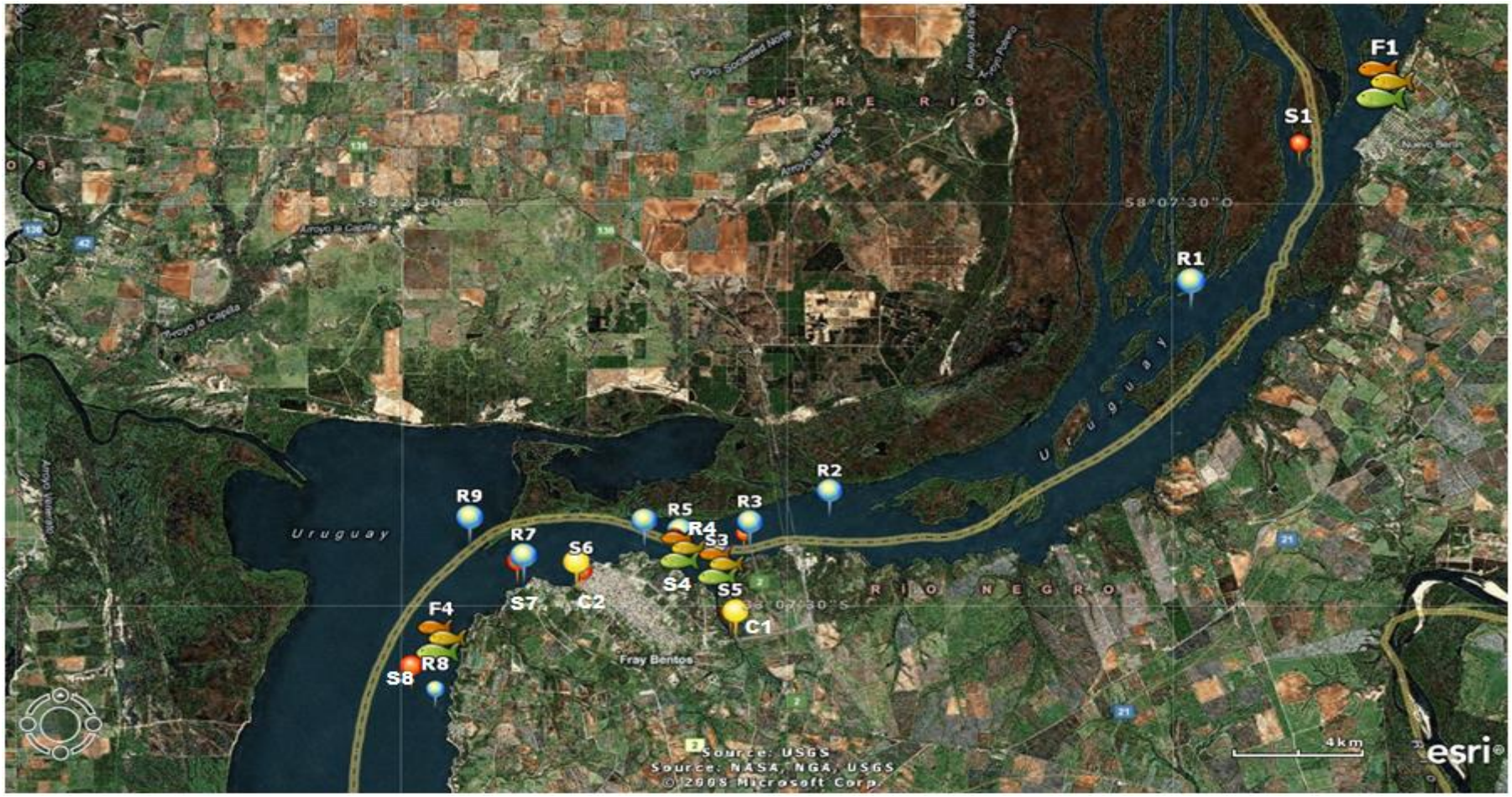


Figure B-6 Satellite view of the location of sampling sites for water, sediment and fish

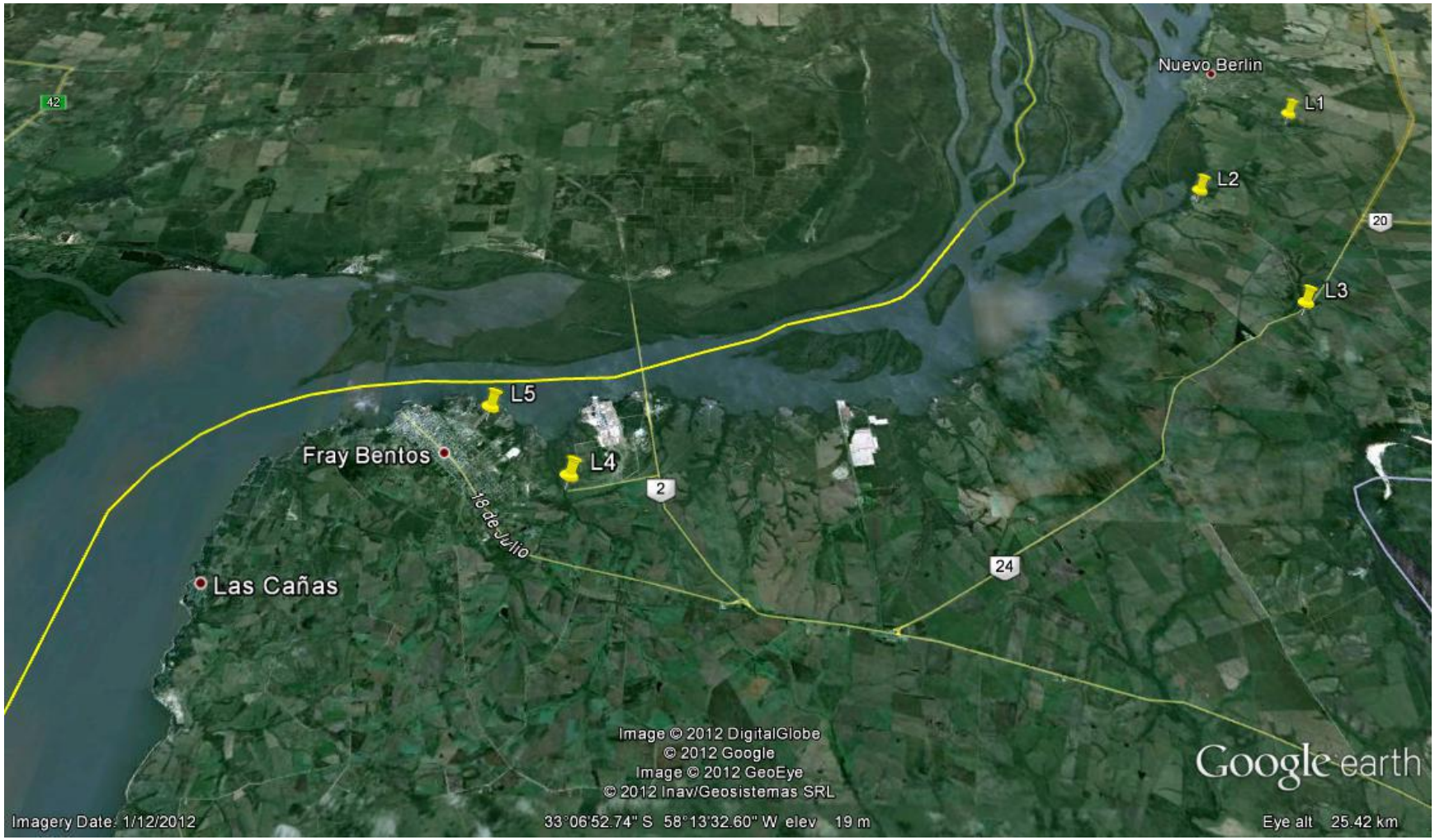


Figure B-7 Satellite view of the location of soil sampling sites

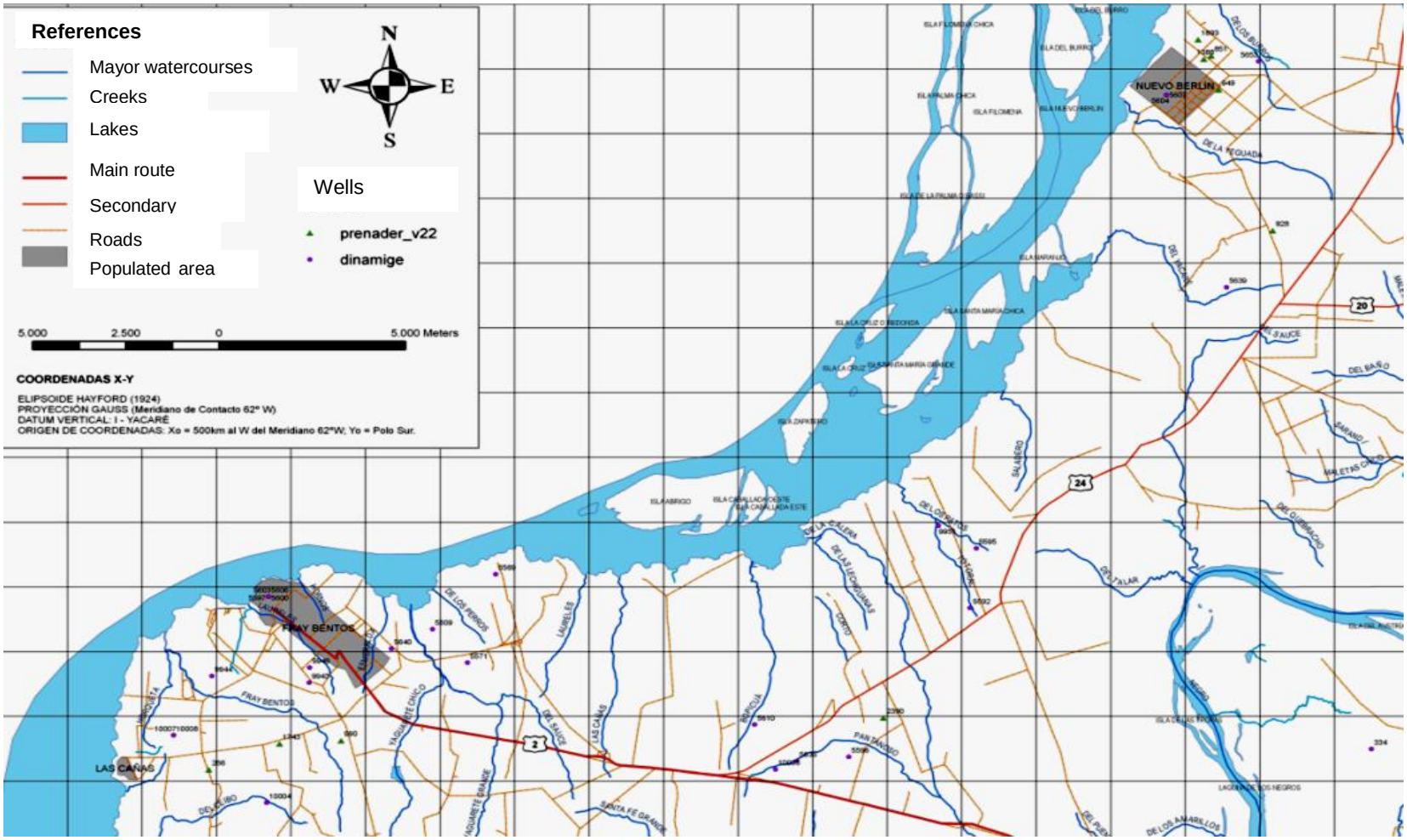


Figure B-8 Location of groundwater wells

Table B-1 Containers, required volumes, preservatives and procedures used in sampling and conditioning of samples

Analysis	Container	Volume (ml)	Preservative	On field
Cyanide	P or G, previously rinsed with ultrapure water and then with the sample	1000	2 ml NaOH 6 N	Adjust pH≥12 (with pH paper indicator)
Phenols	Amber G. Do not rinse previously with the simple because the analytes adhere to the container walls. Teflon or aluminum foil lined P lid	1000	1 g anhydrous $CuSO_4$	Adjust to pH <2 with H_3PO_4.
Chemical Oxygen Demand	P or G	125	2.5 ml H_2SO_4 4 M	Adjust pH between 1 and 2
AOX	Amber P or G, without air at the top G preferable for concentrations AOX < 50 µg l^{-1} as Cl	1000	10 ml Na_2SO_3 1 M	Once full, add 6 ml HNO_3 with Pasteur pipette to pH 1-2
Oil and grease	G, previously washed with hexane	1000	5 ml de HCl (1+1)	Adjust pH between 1 and 2
Soluble Phosphorus	Amber G	125	----------	Filtrate on the field through 0,45 µm membrane
Total Phosphorus	Amber G	125	H_2SO_4	Adjust pH between 1 and 2
BOD	G, special closure up to the headspace	300	----------	----------

G: glass bottle; P:plastic bottle

Table B1- ***Continued***

Analysis	Container	Volume (ml)	Preservative	On field
Dioxins	P, rinsed with ethanol	1000	----------	----------
Metals	P , first use	50	----------	Preserved in the laboratory
Hardness	P or G	250	HNO_3 or H_2SO_4	Adjust pH1-2
Chlorophyl	P or G , amber color	1000	----------	Filtrate through 0,45 µm glass fiber filter, keep filter protected from light, and freeze
Sulfide	P or G no air chamber	1000	Zinc acetate 2N solution	Add 4 drops zinc acetate 2N per each 100 ml
Pesticides	P (PET) or G, up to the headspace	2000	----------	----------
Ammonia	P or G	250	H_2SO_4	Filtrate and then adjust pH between 1 and 2
TOC	P or G	100	H_2SO_4	Adjust el pH between 1 and 2, unless organic volatiles are suspected
Chromium (VI)	P or G rinsed with HNO_3 (1+1)	250	----------	----------
Kjeldah Total Nitrogen	P or G	250	H_2SO_4	Adjust pH between and 2

B.2 Accredited methods

Table B-2 Excerpt of the schedule of ISO 17025 accreditation granted by UKAS to LATU for water, wastewater and sediments, sampling and testing

UKAS TESTING 1893 Accredited to ISO/IEC 17025:2005	**Schedule of Accreditation** issued by **United Kingdom Accreditation Service** 21 - 47 High Street, Feltham, Middlesex, TW13 4UN, UK **Technological Laboratory of Uruguay** **Issue No:** 040 **Issue date:** 09 February 2011
Testing performed by the Organisation at the locations specified	

Materials/Products tested	Type of test/Properties measured/Range of measurement	Standard specifications/ Equipment/Techniques used	Location Code
WATERS and EFFLUENTS	Microbiological Tests Enumeration:		
Potable, including mineral water, fresh surface and groundwater	Total aerobic colony count	PEC.MIC.018 based on Standard methods for the examination of water and waste water APHA, 21st Edition, 2005, Item 9215 A and B	Lab - MIC
Potable, including mineral water, fresh surface and groundwater and wastewater effluents	Coliforms Thermotolerant coliforms *Escherichia coli (presumptive)*	PEC.MIC.030 incorporating ISO 9308-2:1990 using MPN technique	Lab - MIC
Potable, including mineral water	Total coliforms *Escherichia coli (presumptive)*	PEC.MIC.016 using Endo Agar based on Standard methods for the examination of water and waste water APHA, 21st Edition, 2005, Item 9222B using membrane filtration technique	Lab - MIC

UKAS TESTING 1893 Accredited to ISO/IEC 17025:2005	**Schedule of Accreditation** **issued by** **United Kingdom Accreditation Service** 21 - 47 High Street, Feltham, Middlesex, TW13 4UN, UK **Technological Laboratory of Uruguay** **Issue No:** 040 **Issue date:** 09 February 2011

Testing performed by the Organisation at the locations specified

Materials/Products tested	Type of test/Properties measured/Range of measurement	Standard specifications/ Equipment/Techniques used	Location Code
WATERS and EFFLUENTS (cont'd)	Microbiological Tests (cont'd) Enumeration: (cont'd)		
Sea water, fresh surface waters and effluents	Total coliforms Thermotolerant (faecal) coliforms	PEC.MIC.016 using mFC Agar based on Standard methods for the examination of water and waste water APHA, 21st Edition, 2005, Item 9222D using membrane filtration technique	Lab - MIC
Potable, including bottled and mineral waters, and groundwater , including boreholes and wells	Isolation and enumeration of *Pseudomonas aeruginosa*	PEC.MIC.034 based on Standard methods for the examination of water and waste water APHA, 21st Edition, 2005 Method 9213	Lab - MIC
WATERS	Microbiological Tests		
Potable waters, including mineral water	Total aerobic colony count	PEC.MIC.018 based on Standard methods for the examination of water and waste water APHA, 21st Edition, 2005, Item 9215 A and B	Lab - MICFB
Potable, including mineral water, fresh surface and groundwater and wastewater effluents	Coliforms Thermotolerant coliforms *Escherichia coli (presumptive)*	PEC.MIC.030 incorporating ISO 9308-2:1990 using MPN technique	Lab - MICFB
Potable, including mineral water	Total coliforms *Escherichia coli (presumptive)*	PEC.MIC.016 using Endo Agar based on Standard methods for the examination of water and waste water APHA, 21st Edition, 2005, Item 9222B using membrane filtration technique	Lab - MICFB

UKAS TESTING 1893 Accredited to ISO/IEC 17025:2005	**Schedule of Accreditation** issued by **United Kingdom Accreditation Service** 21 - 47 High Street, Feltham, Middlesex, TW13 4UN, UK **Technological Laboratory of Uruguay** **Issue No:** 040 **Issue date:** 09 February 2011

Testing performed by the Organisation at the locations specified

Materials/Products tested	Type of test/Properties measured/Range of measurement	Standard specifications/ Equipment/Techniques used	Location Code
WATERS (cont'd)	Microbiological Tests (cont'd)		
Sea water and ground water	Total coliforms Thermotolerant (faecal) coliforms	PEC.MIC.016 using mFC Agar based on Standard methods for the examination of water and waste water APHA, 21st Edition, 2005, Item 9222D using membrane filtration technique	Lab - MICFB
Potable, including bottled and mineral waters, and groundwater , including boreholes and wells	Isolation and enumeration of *Pseudomonas aeruginosa*	PEC.MIC.034 based on Standard methods for the examination of water and waste water APHA, 21st Edition, 2005, Method 9213	Lab - MICFB
WATERS	Biological Tests		
Freshwaters	Biomass determination, identification and enumeration of benthic invertebrates to family level	PEC.MAM.200 based on USA EPA/620/R-95/008, 1995 Environmental Monitoring Assessment programme Laboratory Manual - Volume 1: Biological and Physical Analysis	Lab - MAM
WATERS and SEDIMENTS	Sampling		
Surface river waters	Collection of samples for biological examination (plankton)	PRD.MUA.007 qualitative and quantitative using a variety of sampling equipment as described in ITR.MUA 200 and 201 based on Standard Methods for the Examination of Water and Wastewater - APHA 21st Edition, 2005, Part 10200	Site (Environmental - MAM/MAMFB)

B.3 Development of analytical methods

B.3.1 Satellite images

The Global Land Surveys (GLS2010) developed by the NASA and the USGS with core acquisition dates of 2009-2010 with data of the satellites Landsat 7 ETM+ and Landsat 5 TM. The records on the search carried out are as follows:

Acquisition Date: 2011/04/23
Entity ID: LE72250832011113EDC00
WRS Path: 225WRS Row: 83
Northwest Corner Coordinates: 32°13'38.89"S, 59°08'00.64"W
Northeast Corner Coordinates: 32°31'17.58"S, 57°06'21.13"W
Southwest Corner Coordinates: 33°49'33.67"S, 59°35'32.57"W
Southeast Corner Coordinates: 34°07'32.48"S, 57°31'36.19"W
Satellite Number: Landsat7
Scene Size: 234036825
Zone Number: 21
Sun Elevation: 34.2442954
Sun Azimuth: 40.6461739
Orientation: NUP
Product Type: L1T
Resampling Technique: CC
Datum: WGS84
Gap Fill Percent: 98
Gap Fill Acquisition Date: (2011-05-09)

B.3.2 Tier 3: Analyses of concentrations of target EDCs

1) Oestrogens

Target analytes selected were the natural oestrogens: estrone (E1); 17β-oestradiol (E2); oestriol (E3); sulphate conjugate of oestrone (E1-3S)) and the synthetic contraceptive oestrogen (17α-ethinyl oestradiol). These compounds were identified and measured in river water, sewage wastewater and pulp mill effluent, after filtration, solid phase extraction (RP-C18), gel permeation and LC/MS/MS by Koh et al. (2007) method, at Cranfield University. The solid-phase adsorption stage was done at the laboratory of the Water and Wastewater department of the LATU (LATU), Montevideo. The reagents used were: methanol (MeO), ACS pure, Merck. Ultrapure water, organics-free was obtained using a Simplicity® Plus equipment, Millipore Corp.

Solid-phase extraction procedure: For the adsorption procedure stock spiking solutions of 10 ml each of the five deuterated standards of 1 mg ml-1 in methanol/water (10:90) were prepared. Two hundred milliliters of a mixed solution containing 0.1 µg ml^{-1} of each standard was prepared after diluting the stock spiking solutions. Each sample was filtered through a glass microfiber filter Whatman 934-AH. One portion of the filtered sample was spiked with 150 µl of the mixed solution per liter of sample. The preconditioning of Sep-Pak® solid phase extraction (SPE) cartridge (500 mg, 6cm3) cartridge passing through, by means of a vacuum filtering manifold, a mixture composed of 5 ml of methanol and 5 ml of ultrapure water organic free. Not letting the silica dry out, 500 ml of both spiked and un-spiked samples were drained through each cartridge cartridges keeping the flow rate constant between 10 ml min-1 under vacuum. The cartridges were rinsed using 6 ml of ultrapure water and air was forced through to dry them for an hour, and then kept frozen. The cartridges were packed and kept frozen at -20˚C until transportation and instrumental analysis at Cranfield University, where analytes were desorbed and analysed according to the following procedure:

The natural and synthetic oestrogens: estrone (E1); 17β-oestradiol (E2); oestriol (E3); sulphate conjugate of estrone (E1-3S); and 17α-ethinyl oestradiol (EE2) were determined by Koh et al. (2007) method. All steroid oestrogens were determined in the dissolved and adsorbed phases in all samples. The analytical methodology has been described elsewhere (Koh et al*.* 2007). In summary, sewage samples were filtered through GF/C filters (VWR International, Leicestershire, UK) prior to solid phase extraction (SPE). The oestrogens on the dissolved phase were extracted and analysed (Koh et al. 2007). The adsorbed and sludge samples were solvent extracted and then subjected to clean-up via silica SPE cartridge 500 mg per 3cc (Waters Ltd, Hertfordshire, UK). This purified sample was then further processed via gel permeation chromatography, anion-exchange and then quantified by LC/MS/MS (Koh et al. 2007). 117

Eluted extracts were evaporated to dryness using a rotary evaporator, reconstituted and transferred to autosampler vials prior to the analysis using LC/MS/MS. Solids phases were stored on filter papers for posterior analysis.

Reagents and chemicals: ethylacetate (EtOAc), acetonitrile (ACN), dichloromethane (DCM) and methanol (MeOH) provided by Rathburn, UK. Triethylamine, ammonium acetate and acetic acid were purchased from Sigma-Aldrich Dorset.

All oestrogen standards (>98% chemical purity) were purchased from Sigma Aldrich (Dorset, UK). Deuterated (*d3/4/5*) labelled internal standards of estrone-2,4,16,16-*d4* (E1-*d*4), 17β-oestradiol-2,4,16,16,17-*d5* (E2-*d*5), estriol-2,4,17-*d3* (E3-*d*3), 17α-ethynylestradiol-2,4,16,16-*d4* (EE2-*d*4) and sodium estrone-2,4,16,16-*d4* sulfate (E1-3S-*d*4) were obtained from C/D/N Isotopes (QMX Laboratories, Essex, UK) with >98% chemical purity. Stock solutions were prepared in methanol.

Analyses were determined using LC/ESI/MS/MS using an HPLC (Waters Alliance HPLC system 2695) coupled to a Waters Quattro Premier XE mass spectrometer fitted with a Z-Spray ESI source (Micromass, UK).

Pharmaceuticals were separated on a Gemini C18 column (3µm particle size, 100mm x 2mm i.d., Phenomenex, UK). The mass spectrometer was operated in the negative electrospray ionisation (ESI–) or positive electrospray mode (ESI+) using multiple reaction monitoring (MRM). Instrument control, data acquisition and data processing were performed with TargetLynx software (Waters, UK). Nitrogen used as the nebulizer gas and Argon as the collision gas. The conditions for detection by the mass spectrometer were: capillary voltage, 0.80 kV in positive mode and -2.5kV in negative mode; extractor lens at 3.0V; RF lens at 0.0 V in 118 positive mode and 0.1V in negative mode; multiplier voltage, 666V; desolvation gas flow, 1000 l h^{-1}; cone gas flow at 50 l h^{-1}; desolvation temperature at 350°C and source temperature at 120°C. The MRM dwell times were 0.05 seconds. Tuning of parent and daughter ions was by direct injection of 10 µg ml^{-1} single standards at 50 µl min^{-1}.

2) Alkylphenols and ethoxylates

The extraction of the compounds was done by shaking the samples with a mixture of dichloromethane: methanol and performing a liquid-liquid extraction. The identification, resolution and quantification were done by GC-MS, using selected ion monitoring (SIM) with a HRGC/HRMS (EPA Method 8270).

3) Glyphosate

The chromatographic system used was a Dionex ICS - 2500 Ion Chromatography System, Dionex Corp, Sunnyvale CA, USA, composed of the following parts: pump, and autosampler, detector module with conductivity detector, conductivity suppression module, and temperature control module.

The columns used were Dionex Ion Pac AS19-HC, 2 x 250 mm (analytical) and Dionex Ion Pac AG19-HC. The eluent was 45 mM potassium hydroxide, at a flow of 0.25 ml min^{-1}.

High purity Helium was used for degassing. The injection volume was 100 µl and detection was done using an electrochemical/conductivity detector. The

elution was carried out using a potassiumhydroxide solution by means of a pump (GS50). The ion exchange columns used were Dionex IONPAC® AS19-HC (2x250 mm, P/N 062886), with a Dionex IONPAC® AG19-HC (2x50 mm, P/N 062888) pre-column. The software was Chromeleon® 6.8 SR6 Chromatography Data System. LOD= 10 µg l^{-1} and a LOQ= 20 µg l^{-1}. Samples were filtered through regenerated cellulose membrane syringe filters (Minisart® RC25, Sartorius, 0.45 µm), and directly poured into the autosampler vials. A calibration curve was prepared in the range of 20 to 500 µg l^{-1} using a glyphosate standard (Institute of Organic Industrial Chemistry, Warsaw, Certified Analytical Standard, 98.7± 0.1 % m m^{-1} pure) in ultrapure organic-free water (Simplicity Plus®, MilliPore Inc.).

4) Endosulfan

The method used was a GC-MS technique based on ISO 6468. Endosulfan concentrations were not detectable (LOD 0.0004 µg g^{-1}) in river water, nor the soil samples analysed at Nuevo Berlín (LOD 0.001 µg g^{-1}), as well as the sediment samples from Ubici Beach (R5), Yaguareté Bay (R4) and the Bridge site in the river (R2).

5) Dioxins and furans

The method entrains a solvent extraction and solid phase adsorption. High resolution GC-MS was used to detect and quantify the compounds using as reference 13C-labeled internal standards. The LOD for individual PCDD/PCDF compounds were 0.05-1.9 pg l^{-1} (THL, the National Public Health Institute of Finland).

6) PAHs

Samples were analysed after hexane extraction by GC-MS, SIM. LOQ= 0.01 µg l^{-1}.

7) PCBs in water

PCB congeners were extracted from water samples with hexane and analysed by GC-MS, SIM-technique. Quantification was carried out using internal standard. As results the concentrations of 15 congeners were reported. Total amount of PCBs were calculated according to ISO-EN 12766-2. The determination limits for a congener and total amount of PCB are 0.05 µg l^{-1} and 0.25 µg l^{-1}, respectively.

8) Resin acids (isopimaric acid and others)

The following method, based on was used both for the determination of phytosterols and resin acids in river water samples. After stirring the samples, 4.0 ml are drawn into a test tube and 2 ml of a cholesterol internal standard dissolved in MTBE (methyl tertiary butyl ether) are added after adjusting pH to 3.5. The test tube is vigorously shaken for 1 minute, centrifuged at 300G for 5 minutes, and the MTBE layer on top carefully withdrawn, repeating this operation twice. The combined MTBE extracts are evaporated to dryness with pure nitrogen gas stream. The sample is then silylated using 80µl of BSTFA and 40µl of TMCS at + 70 ºC for 60 minutes. The samples are analysed using gas chromatography flame ionization detector (GC-FID) and confirmed by GC-MS if necessary (based on Örså and Holmbom, 1994 and Bergelin et al. 2003).

9) Phytosterols (β-Sitosterol and others)

The response factors are determined for campesterol, campestanol, ß-sitosterol and sitostanol using a commercial product with a known amount of these compounds. For the other compounds the response factor of ß-sitosterol is used. Water samples were spiked to determine the detection limit. The lowest level is 10 µg l^{-1} ß-sitosterol.

10) Chlorophenols (2,4,5-Trichlorophenol and other congeners)

Basic samples are acetylated and extracted with hexane and analysed GC-MS, SIM-technique. Quantification is carried out using internal standard. LOQ = 0.5 µg l^{-1}.

B.3.3 *In vitro* screens

1) YES

The yeast culture is continuosly kept in glycerol at -70ºC for subsequent uses. The reconstitution stage was done by allowing the culture to grow in selective media plates 2X. The seed was transferred to 5 ml Gold medium,containing yeast nitrogen base without amino acids as described by Gaido et al. (1997) and let grow over night. The culture was then diluted to an OD660 nm of 0.03 and a volume of 100 µl of copper sulfate pentahydrated 10 mM (AR ACS, Mallinckrodt Chemical, United States) was added per 20 ml yeast solution. A volume of 5 ml of the culture was added to each 50 ml tube, adding 5 µl ethanol; 2 tubes per sample, adding 5 µl of each sample extract and a tube for oestradiol standard (5 µl of the corresponding standard). Serial dilutions of a stock solution of 1.89 g l^{-1} (certified reference material, DrEhrenstorfer, Augsburg, Germany) in absolute ethanol. The concentrations were from 1.75 ng l^{-1} to 7.5 µg l^{-1}.

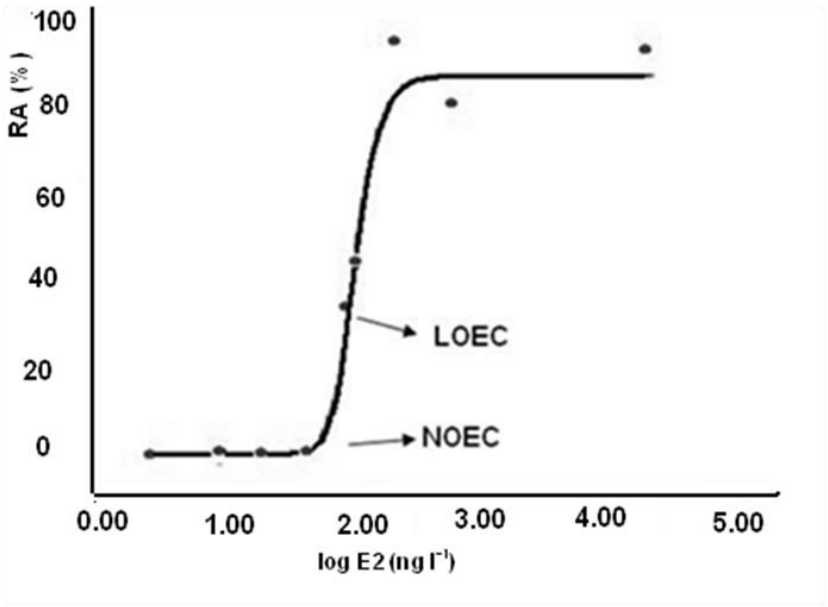
After incubating for 18 hours at 30ºC and 300 rpm, it was diluted to OD 0.25 (660 nm). Aliquots of 5ml were dispensed in each 50 ml tubes containing sample and dilutions of the E2 stock solution (diluted 1:1000), used for the calibration curve. Then, 5µl of standard solutions and samples were added in each tube. The tubes were incubated for 18h at 30C, with shaking. The broth was diluted 1:10 using growth medium and absorbance was measured at 660 nm. The samples were diluted to an absorbance of 0.25 in Eppendorf tubes. Then, 100 µl of diluted broth aliquots were pipetted in the 96 well plates in triplicates. The β-galactosidase activity was measured using a Yeast β-

Galactosidase Assay Kit (Pierce, Rockford, IL, USA) following the instructions of the manufacturer. The aliquots of the yeast were kept frozen at - 80ºC in cryovials with sterile glycerol 30 % solution and equal portion of culturing medium. A volume of 70 µl of each of the tubes in three replicates in an ELISA plate, 70 µl working solution (Y-PER reagent mixed 1:y1 with β-galactosidase assay buffer) and absorbance was measured at 660 nm with a multiplate reader (Multiskan EX de Thermo Scientific, Shanghai, China). The chronometer was used to count the time and when a yellow color appeared absorbance was read at 420 nm (Yeast β Galactosidase Assay Kit Instructions Microplate Plate Assay Protocol, non-stopped).

The water used for the preparation of solutions was ultrapure and previously sterilized by filtering through 0.2 µm (cellulose nitrate filters, Santorius stedim, Goettingen, Alemania). All the glassware was previously sterilized and working in sterile environment.

The samples were extracted from the creek receiving municipal wastewater (C2) and pulp mill secondary effluent. A volume of 1 litre of sample was brought to pH 2.0 with H_2SO_4 (95-97 % pure, Merck, Darmstad, Alemania) and filtered through 0.45 µm (glass microfiber filters, Macherey Nagel, Deutshan, Switzerland). The reverse phase extraction was done through prepared columns (Sep-Pak® Vac 6cc, 500mg, Waters, Ireland) using a filtering equipment easy-Prep (Whatman). The column was previously added with 5 ml anhydrous methanol (Mallinckrodt Chemical, United States) and 5 ml water. After the extraction the column was centrifuged for 10 minutes at 2000 g and dried under Nitrogen stream. Elution was carried out with 5 ml acetone (pure, Merck, Darmstad, Germany), then dried under Nitrogen stream and reconstituted in 1 ml absolute ethanol (Mallinckrodt Chemicals, United States). It was kept at 4ºC until testing (for a maximum of 14 days).

The relative activities were used for statistics and the dose-response sigmoid adjusted by the Hill function (inrate Hill-n/Michaelis-Menten/line/quadratic/lag-ase/monomolecular) (Figure 7-29).



RA(%) Relative activity

Figure B-9 Dose-response curve for the binding activity to oestrogens of recombinant yeast exposed to E2

B.3.4 *In vivo* assays

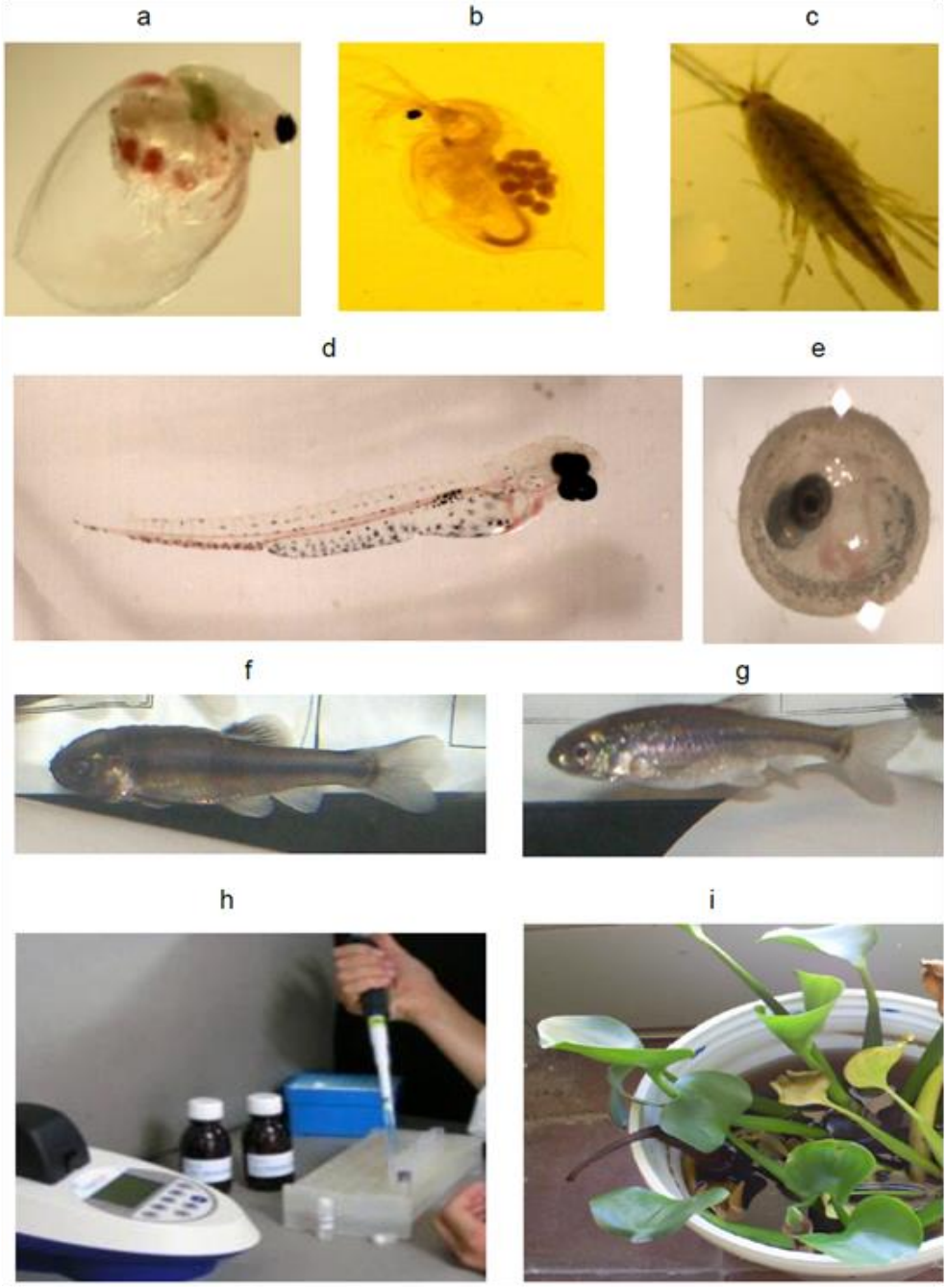


Figure B-10 Photos of the specimens used in ecotoxicity tests

a: *Ceriodaphnia dubia,* cladocer crustacean; b: *Daphnia magna,* cladocer crustacean; c: *Hyalella curvispina,* amphipod crustacean; *Pimephales promelas* teleost *Cyprinid* fish: d: larvae, e: embryo, f: male adult, g: female adult; h: CheckLight® test kit with *Photobacterium leiognathi,* bioluminescent bacteria; i: *Eichornia crassipes,* freshwater aquatic macrophyte plant.

1) Three brood *Ceriodaphnia dubia* reproduction test

The *Ceriodaphnia dubi*a culture was held in 1 l glass beakers containing hard medium with hardness equal to 250 mg l^{-1} expressed as $CaCO_3$. Temperature was maintained at 25 ± 2 ºC under continuous illumination (ranging from 300 to 450 lux). The medium was renewed and organisms fed with a 10 ml suspension of the green algae species, *Pseudokircheneriella subcapitata* (4 x 10^8 cells ml^{-1}), trout chow flakes (5 g l^{-1}) and yeast (5 g l^{-1}), three times a week, under static regime, measuring dissolved oxygen and pH in each sample both at the start and at the end of testing.

The test was run according to Environment Canada (2007), with test duration of 7 days as USEPA (2002b) on young daphnids (<24 h old). One organism, in ten replicates, was exposed to seven concentrations (two- d dilutions) in glass beakers with 20 ml of an appropriate concentration of single compound in the ISO hard medium, incubated at 25ºC with a 16:8 h light: dark cycle (500 lux). Daphnids were fed daily, organisms monitored for survival, and number of released neonates of each brood per day.

The comparison of the number of offspring at the end of the test in the sample batch and the control allowed calculating IC25, the concentration which gave rise to a 50% population growth inhibition. The results were analysed using ICPin
http://water.epa.gov/scitech/methods/cwa/wet/upload/2007_07_10_methods_wet_disk1_ctmapg-l.pdf) and ToxCal software programs. The test was run in sediment elutriates (1:4), and also in one sample of pulp mill effluent at 100% dilution. The test was done by exposing 10 organisms per beaker and couting neonates produced during three broods in 96 h.

2) 21 days fathead minnow (*Pimephales promelas*) exposure test

The images (Figure B-10) depict several activities during method development.

A: aquaria with rearing fish B: detail of a rearing pair of fishes at the aquarium with spawning substrate; C: stock aquarium; D: exposure vessels with experiment; E: weighing fish; F: dissecting and keeping gonads for histology and molecular biology; G: a detail of the excision procedure; H: preparation for molecular biology

Figure B-11 21 days fathead minnow *(Pimephales promelas)* fish exposure test

The fish specimens were obtained from the U.S. Environmental Protection Agency (Environmental Monitoring Systems Laboratory, Cincinnati, U.S.A.), and are maintained in continuous culturing at the aquaculture facilities of the Ecotoxicology Area of the Water and Chemicals Department, Technological Laboratory of Uruguay since August 2007. The conditions of the fish rearing are based on USEPA (1988). These fish, and their subsequent offspring, were used to conduct 5 exposure studies.

The laboratory area devoted to aquaculture has 45 m^2 surface, equipped with plumbing facilities. Air conditioning keeps a controlled environment (25± 2ºC) and lighting is provided by mercury tubes protected with plastic covers and photoperiod timer to ensure 16:8 hours (light:darkness). The culturing water is produced by filtering tap water through a filtration system (TGI; Topway Global Inc., California, USA, model TGI-525) composed of filters of different sizes, activated carbon and a UV light module in series (TGI, Topway Global Inc., California, USA, model UV-LS2A-304J). A 200 liter aquarium was used for fish stock culture rearing, with a Resun 4000 mechanical and activated carbon filtering system with pump delivering 500 l h^{-1} flow. The culturing method was based on USEPA (1988, 2002). The temperature of the aquaria was controled as well as pH, conductivity and disolved oxygen. Once a week, a third part of the total volume of the stock aquaria was replenished with fresh culture water.

After sampling, the sample was kept under refrigeration at 4ºC. A volume of 8 liters of sample was partially renewed in a proportion of 90% of the sample every 48 hours, providing semi-static conditions. During exposure, the fish were fed three times a day (early morning: live *Artemia* spp. nauplii, and the following two, at noon and early afternoon Tetramin® fish food flakes). The photoperiod was maintained at 16 hours light: 8 hours dark by the same system as for the fish culture. A pool of sexually differentiated both sex adult fish of 7 month and 20 days age was continuously maintained in a 200 liter glass stock aquarium with a re-circulating pump (Resun HF- 2002, China; 500 l h^{-1}). Fish were acclimated to the experimental conditions in 10 liters tanks. In each case, control and exposed groups, containing two males and four females (7 months

old) were exposed in 10L aquaria (n=36) with spawning substrates for 21 days on a semi-static flow with renewal of the testing solution every 48h. The samples were: surface water receiving municipal wastewater obtained from Fray Bentos stream in March 2010, and final pulp mill effluent collected in May 2010 by means of a pump brand Avalanche. By this procedure, 200 litres were extracted in refrigeration to replenish the media in a partial renewal regime. For validation purposes and derivation of dose-response curves, the refence toxicant used was E2 at two levels of concentration (150 and 450 ng l^{-l}).

Table B-3 Characteristics of the pulp mill effluent and stream water receiving municipal wastewater for exposure assessment

Parameter concentration (mg $l^{-1)}$)	**BKME**	**C2**
Sulphide (as S) (LOD: 0.1 mg l^{-1})	ND	
Ammonia (as N)	0.030	
Oil and grease (LOD: 5 mg l^{-1})	ND	
AOX (as Cl) (LOD: 10 µg l^{-1})	1.43-2.46	ND
Phenols (as C_5H_6OH)	0.001-0.005	
Anionic surfactants (as LAS)	0.047-0.060	
$cBOD_5$ (as O2)	18.0	
COD (as O2)	194 mg l^{-1}	12
Total Nitrogen	0.79-1.49 mg l^{-1}	
Total Phosphorus (as P)	1.12 mg l^{-1}	0.5
Acute toxicity (*Pimephales promelas*) LC50, 96 h	> 100 %	>100%
Acute toxicity (*Daphnia magna*) LC50,48 h)	> 100 %	
Acute toxicity (*Vibrio fischeri*)	> 100 %	

> 100 %: Non acutely toxic to the test organism (Viana et al. 2001)

On the 21st day fish were weighed using a Shimadzu (TX323L, Japan) balance to the 0.001 g and measured using a caliber (Mitutoyo, Japan), sacrificed with a cervical incision and dissected under a binocular magnifier (Olympus VM, Japan). Gonads were excised and then sectioned by scalpel in hemi-gonads and one of the halves weighted, and the other half stored immediately in liquid Nitrogen and then transferred to a -80ºC freezer for posterior molecular analysis. Those devoted to histology were fixed in Bouin´s fixative for 24 h, and then dyed with Hematoxylin and Eosin stain.

The sections were then examined for abnormal findings such an increase in spermatogonias, and signs of testis degeneration as cell apoptosis or vacuolization. The other hemi-gonad was sectioned again transversally in two parts, fixing one part in Bouin´s fluid for histology and preserving the remaining in PAF (paraformaldehyde, Fluka). After a waiting period of three hours they were transferred to methanol absolute (Mallinkrod Baker Inc., Phillipsburg, NJ, USA), for future *in situ* hybridization analysis to test for aromatase concentration. Some of the livers were excised and deproteinised and some kept in liquid Nitrogen to analyse *vtg* gene transcription by RT-PCR (based on Biales et al. 2007).

Table B-4 Egg production of *P. promelas* in 21 days exposure test

Sample		Total No. eggs
Control	Pulp mill effluent	3838
Exposed		1767
Control	C2	2116
Exposed		4005
Control	E2 150 ng l^{-1}	2116
Exposed		4005
Control	E2 450 ng l^{-1}	2208
Exposed		2575

Table B-5 Gonadosomatic index, condition factor and nuptial tubercules in exposed and control *P. promelas* fish

GSI				
Sample	**Female**		**Male**	
	Control	**Exposed**	**Control**	**Exposed**
C2	11 (10-15)	9 (7-12)	1.2 (1.0-1.6)	1.4 (1.2-5.7)
Pulp mill effluent	11 (10-15)	14 (9-18)	1.6 (1.3-1.8)	1.5 (1.4-1.5)
E2 150 ng l^{-1}	12 (10-15)	10 (8-16)	1.4 (1.3-1.5)	1.0 (0.8-1.6)
E2 450 ng l^{-1}	16 (13-17)	12 (10-13)	1.0 (0.7-1.4)	1.3 (1.2-1.9)
K				
C2	0.45 (0.4-0.56)	1.36* (1.21-1.6)	2.45 (1.64-4.32)	3.04 (2.50-3.69)
Pulp mill effluent	1.29 (1.23-2.37)	1.17 (0.69-1.49)	5.53 (5.23-7.55)	4.455 (3.24-5.435)
E2 150 ng^{-1}	3.16 (2.60-4.21)	0.765 * (0.64-1.06)	9.35 (6.19-13.29)	2.98 * (2.53-3.88)
E2 450 ng l^{-1}	0.81 (0.6-1.0)	0.835 (0.715-1.08)	4.24 (3.35-5.30)	4.61 (4.53-4.89)

*Significant differences

Table B-6 Nuptial tubercules in exposed and control *P. promelas* fish

Nuptial tubercules	**Score**			
	C2	**Pulp mill effluent**	**E2 (150 ng l^{-1})**	**E2 (450 ng l^{-1})**
Control	42±4	35±2	36±3	35±3
Exposed	44±2	31±3	32±5	37±3

VTG concentrations in liver homogenate by ELISA method

Working with protein liver extracts the expression of VTG was measured using the sandwich ELISA method (Biosense® Fathead minnow VTG ELISA kit)**.** The sandwich ELISA method is based on the specific binding between antibodies

and VTG (Vtg) to measure Vtg specifically in *Pimephales promelas* (fathead minnow). The specific Capture antibody is coating microplate wells. The binding of this to Vtg in the sample, and another detecting antibody, labelled with the enzyme horseradish peroxidase (HRP), create a sandwich of Vtg and antibody. The enzyme activity is determined by adding a substrate giving a colored product, with intensity directly proportional to the amount of Vtg present. The protocol was according to instructions of the Biosense kit (http://www.biosense.com/docs/FHM(2005.1).pdf).

QT-PCR

Briefly, the total liver RNA was extracted using Trizon reagent, Invitrogen. The real time-PCR was done using Sybr green as fluorophorus in a thermocycler at Molecular Biology Unit at Institute Pasteur, Montevideo. Total liver RNA was extracted with Trizon reagent, Invitrogen. The real time-PCR was measured using Sybr green as fluorophorus in a thermocycler at the Molecular Biology Unit at Institute Pasteur, Montevideo.

A mix of the following reagents: oligo dT, dNTP (dNTP set PCR grade and random hexamer primer (Quiagen, Maryland, USA) was prepared with water, adding to each reaction tube the necessary sample quantity to produce 2 µg RNA and they are heated for 65ºC in water bath (Gemmy industrial corporation, model YCM-04M,Taiwan) for 5 minutes. Immediately after, the mixture was rapidly introduced in an ice bath and then swiftly centrifuged for a spin (to the maximum and then turned off) to homogenise and bring the bottom liquid phase up.

A volume of 4 µl 5X first stran buffer, 2 µl 0.1 M DTT and 1 µl RNAse inhibitor (Cat. MO3075, Biolabs, England) were added per sample, again centrifuged with Reverse transcriptase reagent (Cat. 28025-021, Invitrogen, already including DTT, 5x Buffer, M-MLV).Then, after incubating at 37ºC for 2 minutes 1 µl of MuLV retrotrasncriptase, it was homogeneised by means of a pipette, twirling with the micropipette tip, and immediately introduced in ice. The

preparation was inserted in the RT PCR thermocycler (Corbett Life Science Rotor Gen 6000 Thermocycler, Australia), operating in sequential cycles for 10 minutes at 25ºC, 50 minutes at 37ºC and 15 minutes at 70ºC.

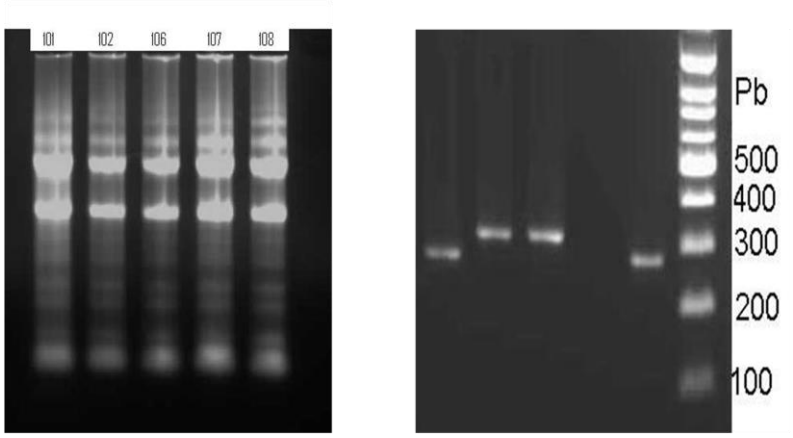
The first step consisted on the RNA extraction. The liver tissue was homogenised in 10 to 20 volumes of TRI Reagent solution (Applied Biosystems, Cat No.AM9738) (1 ml per 50 mg tissue), not exceeding 10 % of the total volume, and then incubated for 5 minutes at room temperature. The sample was centrifuged for 10 minutes at 12000 g (Thermo Electron Corp. MicroCL 17) at 4ºC. Then, the liquid phase containing RNA was transferred to a clean 1.5 ml Epperdorf tube and the rest formed a dry pellet where the extracellular material, membranes and DNA remain. A volume of 200 µl de chloroform was added per each ml of TRI reagent used. The mixture was homogenized and incubated during 5 to 15 minutes at room temperature and centrifuged at 12000 g for 10-15 minutes at 4ºC. After this process, the non colored upper layer was transferred to a clean tube.

The RNA was extracted with syringes 22Gx1 (Misawa, Tokyo, Japón). The RNA remained in the aqueous phase, while DNA and proteins in the organic phase and, between phases. After adding 500 µl isopropanol per ml of TRI reagent, the contents of the tube were mixed by vortex for 5 to 10 seconds and incubated at room temperature for 5 to 10 minutes. The overlaying phase was discarded after centrifuging at 12000 g for 10-15 minutes at a temperature range of 4-25ºC. The RNA precipitated as a white pellet in the bottom or side of the tube. A volume of 1 ml ethanol 75% (made up from absolute ethanol, Synth, Brazil) per ml of TRI reagent utilized was added and the pellets were washed, centrifuged at 7500 g for 5 minutes at 4-25ºC, and if the precipitate floats or no compact pellet was formed, the centrifuged speed was increased to 12000 g for 5 minutes. The ethanol was carefully removed by means of a plastic micropipette tip without leaving traces, and, if necessary, centrifuged again. The RNA was dried for 3 to 5 minutes with a tungsten lamp. The RNA was dissolved in buffer or RNAse free water (distilled water, DNase, RNase free (molecular

biology grade), Gibco, Invitrogen Corp.). This was resuspended in 30 or 40 µl RNAse free water. If necessary, to achieve resuspension, was aided by incubation at 55-60 ºC to complete dissolution. The preparation was kept at 4ºC in refrigerator for immediate use, or maintained at -70ºC for long storage times.

The standard operating procedure for Quantitative “Real time” PCR (QPCR) VTG protocol using HS DyNamo® SYBR Green kit was followed. Real-time PCR was performed by combining 3 µl water, 5 µl PCR mix (Quantimix Easy SYG, kit Biotools B&M Labs, S.A., Madrid, Spain) and 0.5 µl specific primer per sample (Biosearch tecnologies, sequence Genbank Access No. AF130354: TGACAAGCCAACAGCAAGAG; TTAGCCGCCATAGGAATGTG). The sample dilution was 1/100 in Eppendorf tube of 0.2 ml. A volume of 1 µl of sample was introduced inside each tube. Two tubes were prepared per sample for VTG amplification and 2 tubes per sample for gen 18S (“house keeping”) amplification, in duplicate, prepared by mixing 18S primer pair with increasing amounts of 18S Competimer (classic II18S, Cat. No. AM1716, Ambion).

The agarose gels are shown in Figure B-12, with the RNA profile.

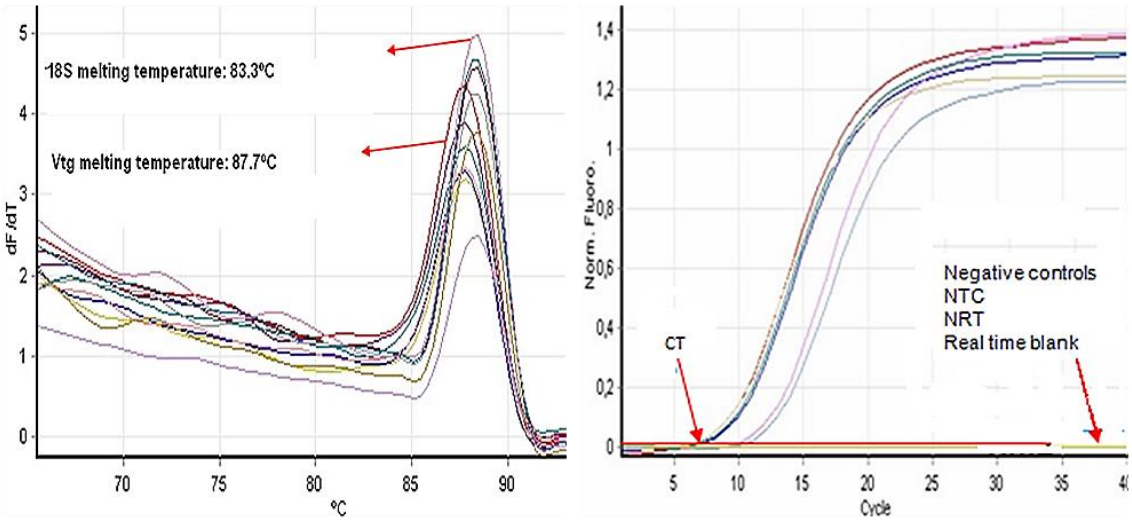


Left: Extracted RNA, showing characteristic RNA profile; Right: 40 cycle products PCR runs.
Lanes 1 and 5: samples of cDNA of female fish with Vtg primers (279 pairs of bases).
Lanes 2 and 3: cDNA female fish samples with primers for 18S (324 pairs of bases).
Lane 5: blank. Lane 7: molecular weight marker.

Figure B-12 Agarose electrophoresis gels of the RT-PCR method for VTG measurement

The calibration curve was drawn for 18S and Vtg. The criteria for melting temperature are shown and the negative controls carried out with blanks. The efficiency of the process measured as variation of the slope was 0.986 for Vtg and 0.975 for 18S. A calibration curve was drawn for each, obtaining the following relationships for (18S): $y = -3.382x + 3.2$; $r^2 = 0.998$, and for Vtg: $y = -3.355x + 1.99$; $r^2 = 0.996$.

The checks of the conditions were done by verifying the melting temperature and the negative controls run (Figure B-13).



Left: Melting temperatures (desnaturalization of 50 % strands) should be equal for all the cDNA samples to amplify VTG and equal in every sample to amplify 18S); Right: Negative controls used in RT-PCR were: NTC (not template control) without amplification mold; NTR (nontranslated region) without the MuLV enzyme: Blanks were run in each batch. All blanks had CTs higher than 30 cycles.

Figure B-13 Melting temperature and negative controls of RT-PCR

The diagram in Figure B-14 shows that the primers used are at different exons, therefore, only mRNA is amplified, not genomic DNA. The blue line represents the genomic DNA, the red ones the exons and the green ones the amplified fragment (in this latter, the used primers are depicted).

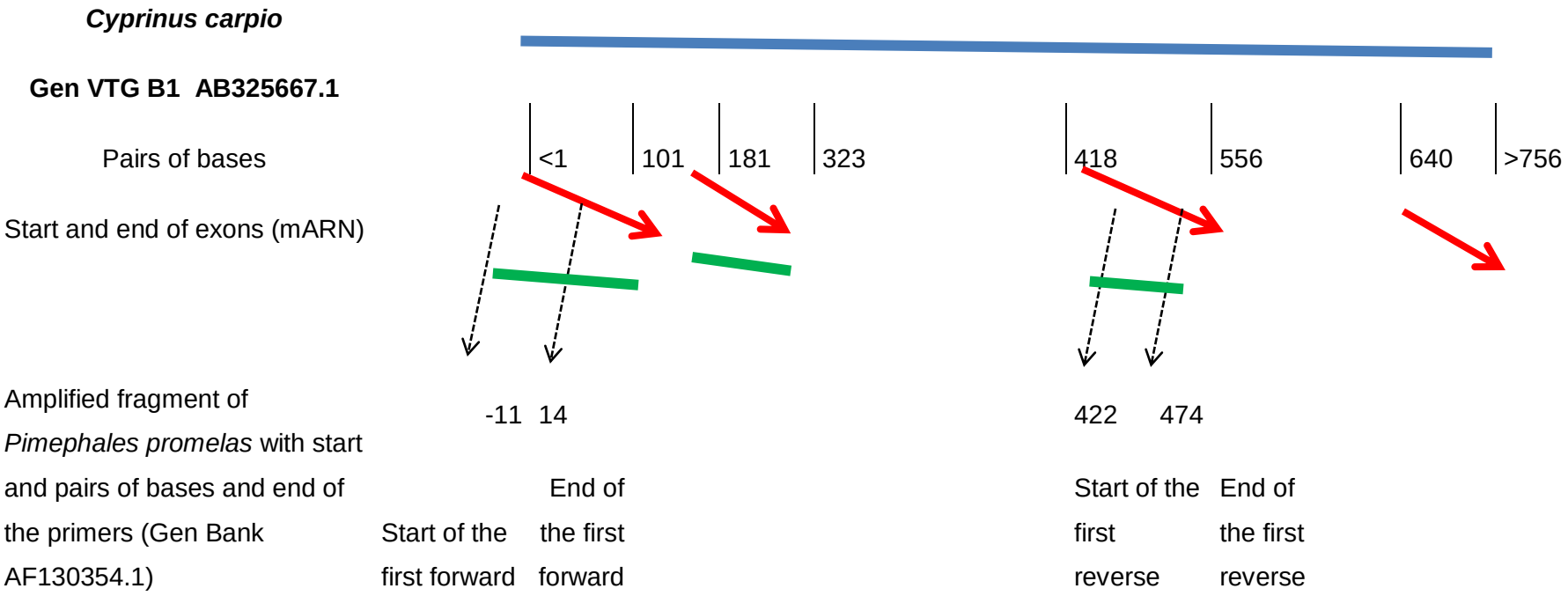


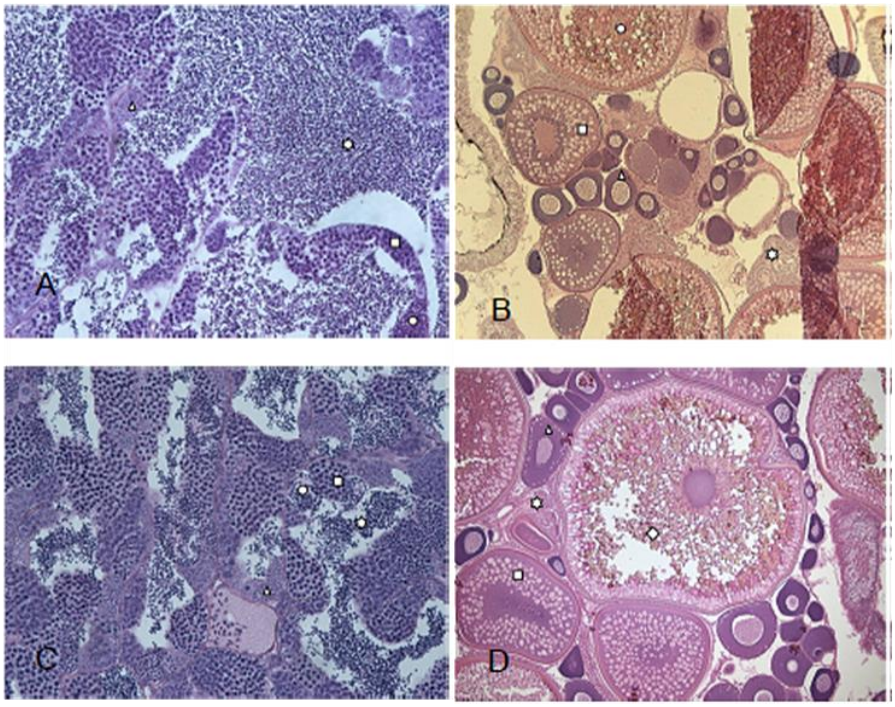
Figure B-14 mRNA amplification

Histopathology analysis

Table B-7 Additional parameters evaluated during histological analysis

Parameter			Sex			
			F1	F2	F3	F4
Factor	Date	Sex				
n	Sep.09	Any	7	0	3	6
	Mar.10	Any	2	0	0	0
	Aug.11	Any	9	10	8	0
	Total	Any	18	10	11	6
Sex	Sep.09	Female	3	0	3	4
		Male	4	0	0	2
	Mar.10	Female	2	0	0	0
		Male	0	0	0	0
	Aug.11	Female	5	7	6	0
		Male	4	3	2	0
Stage	Sep.09	Female	2(I), 1(V)	0	2(I), 1(II)	1(II), 3(III)
		Male	3 (II), 1(I)	0	0	1(III), 1(IV)
	Mar.10	Female	2(V)	0	0	0
		Male	0	0	0	0
	Aug.11	Female	2(I),2(II),1(III)	3(II),3(III),1(IV)	6(II)	0
		Male	1(II),3(III)	1(III),2 (IV)	2(III)	0

n: number of specimens; Female: (I) Resting; (II) Initial ripe; (III) Advanced ripe/mature; (IV) Partially spent; (V) Totally spent. Male: (I) Resting; (II) Initial ripe; (III) Advanced ripe/ mature; (IV) Partially spent; (V) Totally spent. After de Carvalho et al. (2009)



A) Specimen 79, M, CG, 40 X HE; B) Specimen 86, F, CG, 10 X HE; C) Specimen 103, M, EG, 40 X HE; D) Specimen 100, F, EG, 10 X HE; Date: March 2010

Figure B-15 Histopathology results for fish exposure test to stream water receiving municipal wastewater

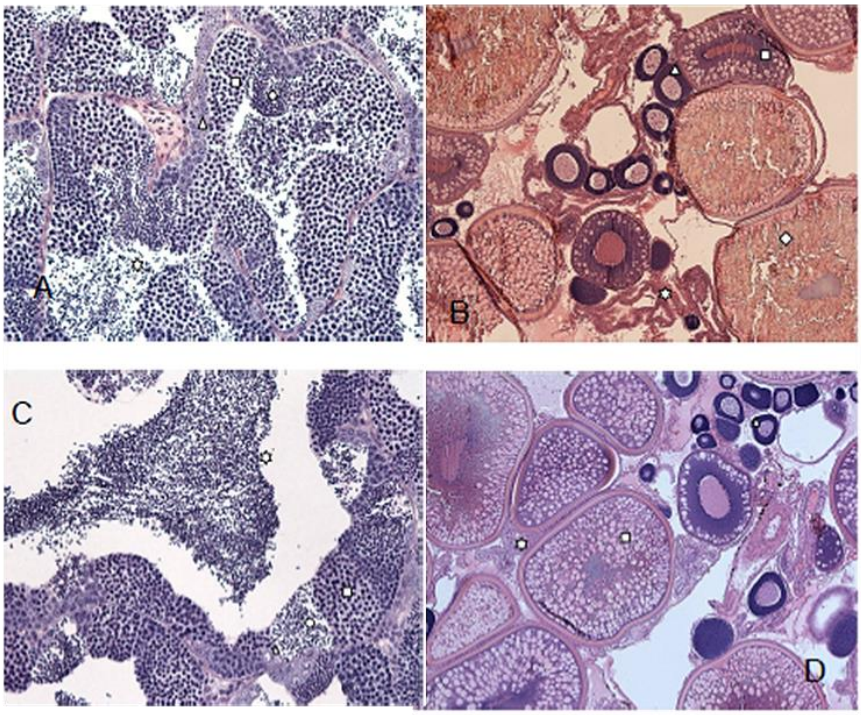
Keys to items in the histology pictures

Female

- Perinuclear oocytes
- Cortical alveolar oocytes
- Early vitellogenic oocyte
- Late vitellogenic oocyte
- Mature oocyte
- Atretic oocyte

Male

- Spermatogonia
- Spermatocytes
- Spermatids
- Spermatozoa



A) Specimen 103, M, CG, 40 X HE; B) Specimen 110, F, CG, 10 X HE; C) Specimen 115, M, EG, 40 X HE; D) Specimen 117, F, EG, 10 X HE; Date: June 2010

Figure B-16 Histopathology results for fish exposure test to pulp mill effluent

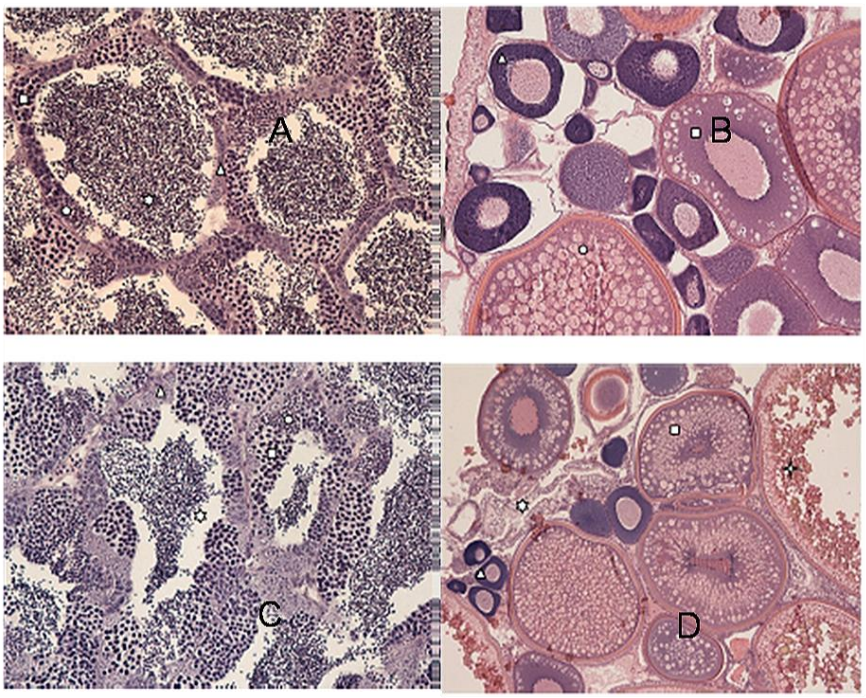
Keys to items in the histology pictures

Female

- Perinuclear oocytes
- Cortical alveolar oocytes
- Early vitellogenic oocyte
- Late vitellogenic oocyte
- Mature oocyte
- Atretic oocyte

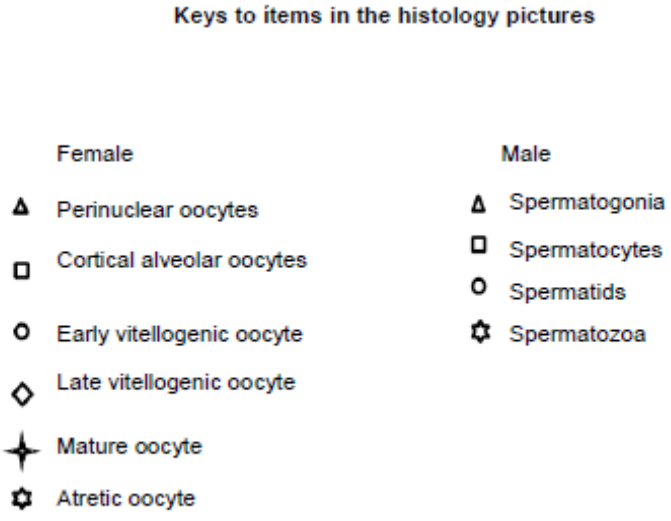
Male

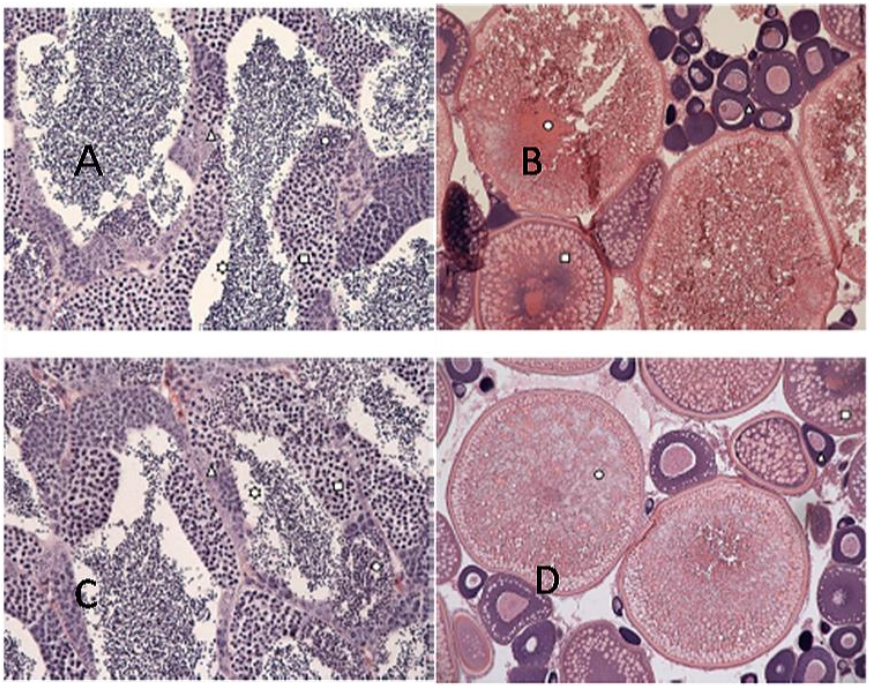
- Spermatogonia
- Spermatocytes
- Spermatids
- Spermatozoa



A) Specimen 173, M, CG, 40 X HE; B) Specimen 180, F, CG, 10 X HE; C) Specimen 154, M, EG, 40 X HE; D) Specimen 146, F, EG, 10 X HE; Date: October 2010

Figure B-17 Histopathology results for fish exposure test to 17-β estradiol 150 ng l^{-1}





A) Specimen 187, M, CG, 40 X HE; B) Specimen 183, F, CG, 10 X HE; C) Specimen 203, M, EG, 40 X HE; D) Specimen 204, F, EG, 10 X HE; Date: December 2010

Figure B-18 Histopathology results for fish exposure test to 17-β oestradiol 450 ng l^{-1}

Keys to items in the histology pictures

Female	Male
Perinuclear oocytes	Spermatogonia
Cortical alveolar oocytes	Spermatocytes
Early vitellogenic oocyte	Spermatids
Late vitellogenic oocyte	Spermatozoa
Mature oocyte	
Atretic oocyte	

Fish tissue residues

Samples were sent frozen to an accredited European laboratory (245 g muscle and 92 grams of liver) for tissue residue analysis. Briefly, the fundamental analysis steps for biota samples were as follows: An addition of d17-Octylphenol, 13C6-Nonylphenol is used as marked internal standard substances (spike), extraction of the homogenised sample material by way of acetonitrile. Then, a liquid/liquid-extraction by way of n-hexane is undertaken followed by derivatisation with BSTFA. A clean-up of the extract is performed by way of column chromatography (SiO2). The detection and quantification is done by means of gas chromatography coupled with mass spectrometry (GC/MS) via the internal standards.

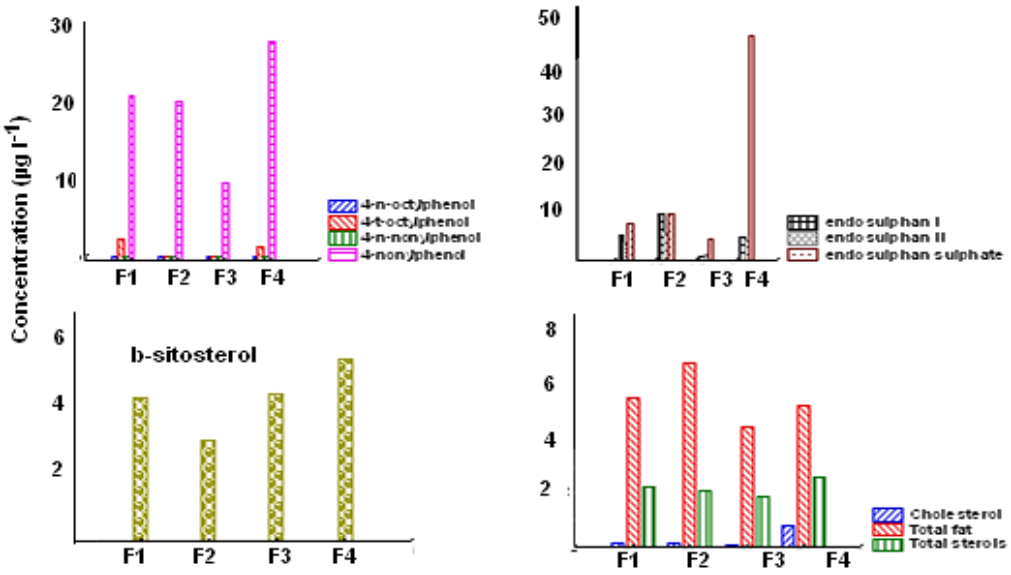


Figure B-19 Fish tissue residues

5) Fish biomonitoring with *Astyanax fasciatus*

After developing the protocols and studying the taxonomy, a total of 944 fish specimens of “mojarra” (*Astyanax fasciatus*) were captured from 2009 winter to autumn 2011 by expert fishermen using gills or nets at four sites in the river from autumn 2009 to autumn 2011 at frequencies of approximately 10 specimens per site and per week. After being caught, specimens were sent to Fray Bentos laboratory. Some fish were analysed for morphometric parameters, while others were frozen and sent to the Water and Chemicals Department, Technological Laboratory of Uruguay, Montevideo for further analysis.

Fish were each assigned a number and sampling site code and photographs taken as a record. Fish were measured with a calibrated ruler to determine total length (from the point of the snout to the end of the tail).

Total mass and gonad mass were measured in grams to the milligram division by means of an electronic analytical balance (Shimadzu (TX323L, Japan). They were sexed after necropsy and examination of gonads. Gonadal maturation stages and spawning type determined through macro- and microscopic analysis, and by variation in the gonadosomatic index (GSI), calculated as a percentage of total body weight. After dissection, the gonads were visually characterised and weighed to the 0.01 g.

Banded astyanax *Actinopterygii* (ray-finned fishes) Characiformes (Characins) > *Characidae* (Characins) http://www.fishbase.org/summary/Astyanax-fasciatus.html (“Mojarra”) *Astyanax sp. aff. fasciatus*. Boulenger, 1887 (Teixeira de Mello et al. 2011)

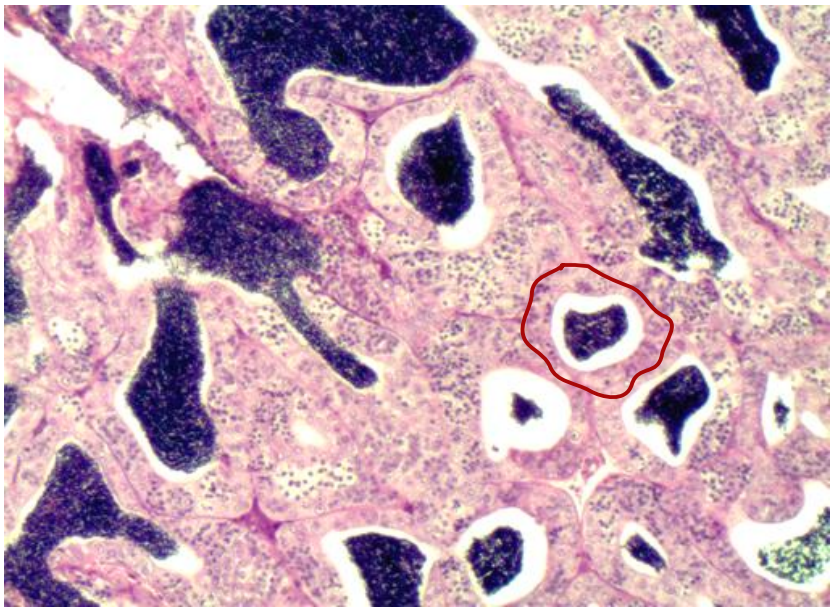
Figure B-20 *Astyanax fasciatus*

For histopathology studies the fishes caught in four of the sampling campaigns were fixed in situ using Bouins´s fixation fluid and after 24 hours, they were preserved in 70% ethanol for storage and labeled to undergo histology procedures.

Table B-8 Gonadal maturity scores scale for female and male fish

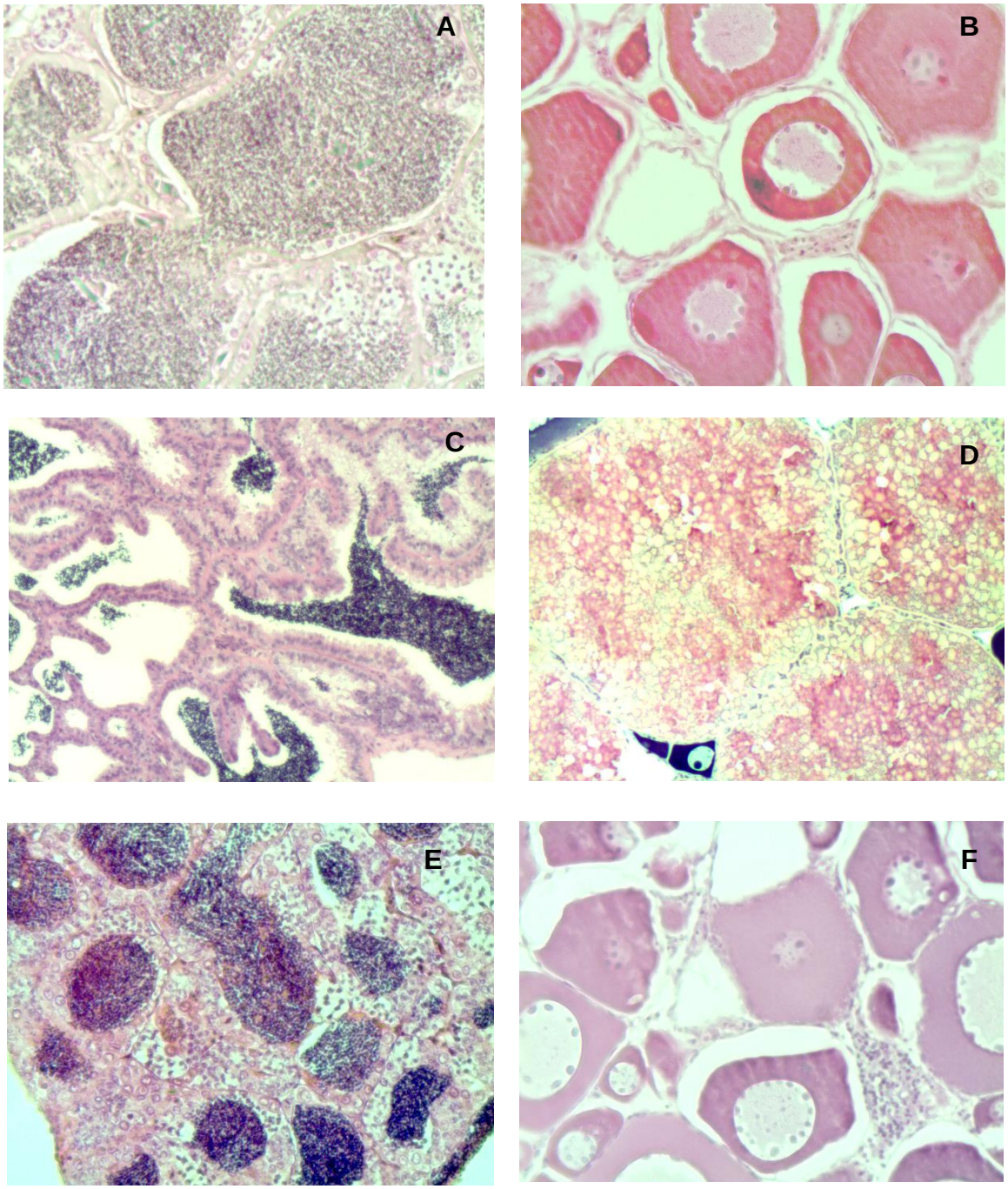
Score	Key	Description	
		Ovaries	**Testes**
1	At rest	Translucent, thin, surrounded by fat	Thin, transparent, small, surrounded by fat
2	Initial maturation	Consistent and voluminous, Surrounded by fatty masses	Bigger, whitish, surrounded by fatty masses
3	Advanced maturation/mature	Consistent, almost at the highest volume, yellowish, surrounded by fat Occupying the biggest fraction of the cellomatic cavity Visible oocytes	Occupying great part of the celomatic cavity, milky white, surrounded by fatty masses
4	Partially emptied	Flaccid, hemorrhagic, areas with few visible oocytes, few fatty deposits	Flaccid, milky white, with transparent areas, surrounded by little amount of fat
5	Totally emptied	Very flaccid, hemorrhagic, no visible presence of oocytes, very small quantity of fat	Maximum volume, low fat content.

Typical histology of testes and ovaries, respectively, observed in fish caught at the reference site (F1).



Male *A. fasciatus*. Nuevo Berlín, September 2009
In blue: spermatozoids; Red line: Spermatocyst. Developmental stage: II

Figure B-21 *Astyanax fasciatus* testes histological characterisation at the reference site (F1)



A and B: testes and ovaries, respectively, at F2; C and D: testes and ovaries, respectively, at F3; E and F: testes and ovaries, respectively, at F4

Figure B-22 *A. fasciatus* gonadal histological characterisation

B.3.5 Toxicity in river sediments through the water-sediment pathways and bioavailability, bioaccumulation and toxicity of a chlorinated organic

The concentration of halogenated organics was analysed in the organic matter of biota samples: floating macrophytes, fishes, snails and mussels. Organic matter was extracted using a SOXTEC apparatus, Foss Tecator, Sweden using 40 ml of a mixture of cyclohexane: isopropanol (1:1). A portion of 10 ml of the mixture was evaporated under a Nitrogen stream. Finally, the extract was redissolved in 0.5 ml cyclohexane and in each case volumes of 25 µl were injected into the TOX-100, Total Organic Halogen Analyser, Mitsubishi Chemical Corporation.

Reagents

Isopropylic alcohol: Mallinckrodt Chemicals Lote H08B01 (ACS) 99.5% pure

Cyclohexane: Mallinckrodt Chemicals Lote 4878KVVB 100% pure

Acetone: Merck KGaA 99.8% pure

Bottom sediments samples were extracted with a Petit Ponar grab (Appendix B) from transects of the River in three areas: Nuevo Berlin (S1), Fray Bentos (S2) (site close to the city and immediately downstream to the pulp mill) and Las Cañas (S8) (tourist city, downstream from S2). The portions of each central transect samples were mixed together to prepare composed representative samples of each sampling area. Then, an aliquot of each was placed in a 10 litres stainless steel container and mixed mechanically with a paddle agitator (Janke & Kunkel model RW20, IKA-WERK, Staufen, Germany) with deionised water filtered through charcoal and treated with UV light, in 1:4 proportion, using the protocol for elutriate preparation (USEPA 1998).

The sample of S8 was spiked with a solution prepared by weighing 0.3827 g 2,4,5-Trichlorophenol in 200 ml methanol. The concentration of AOX resulting was 1.03 g l^{-1}, as Cl. A sub-sample of 1503 g of composite sediment was mixed with 6 litres of water and 50.0 ml of the solution of 2,4,5-Trichlorophenol. The

size of the suspended particles was analysed by laser diffraction (ASTM UOP856-07). Dissolved AOX was determined after filtering elutriates through a 0.45 µm glass microfibre filter. The EOX was determined in the deposited residue on the filter and 54 and 125 ml were taken for replicate analysis.

1) Physical testing of river sediments by sieving and decanting and by laser diffraction techniques

Particle size was measured by sieving and decanting (FAO 1970) (Figure B-23).

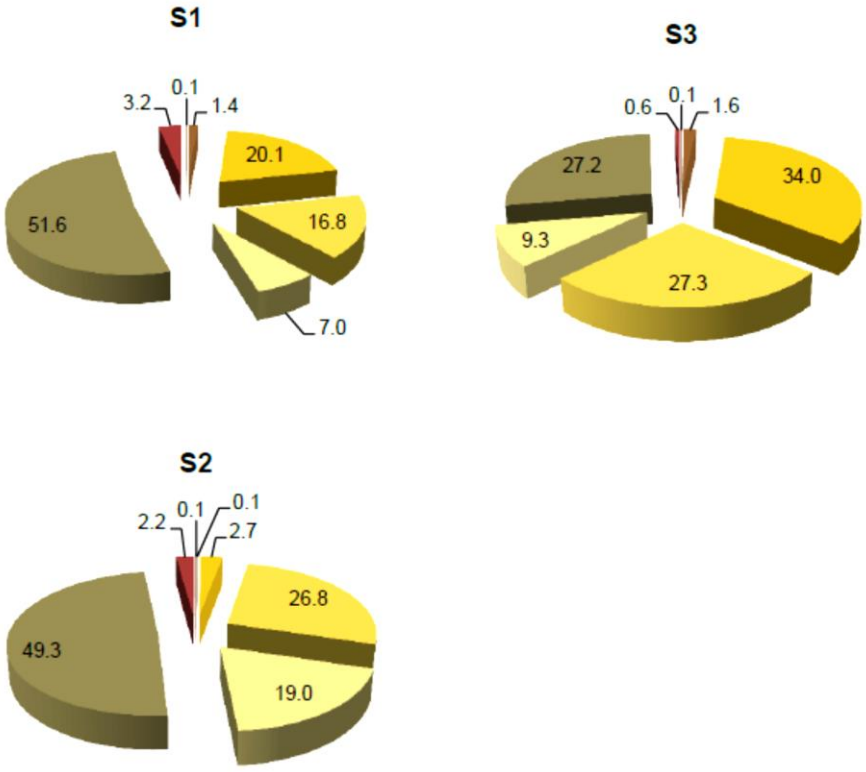


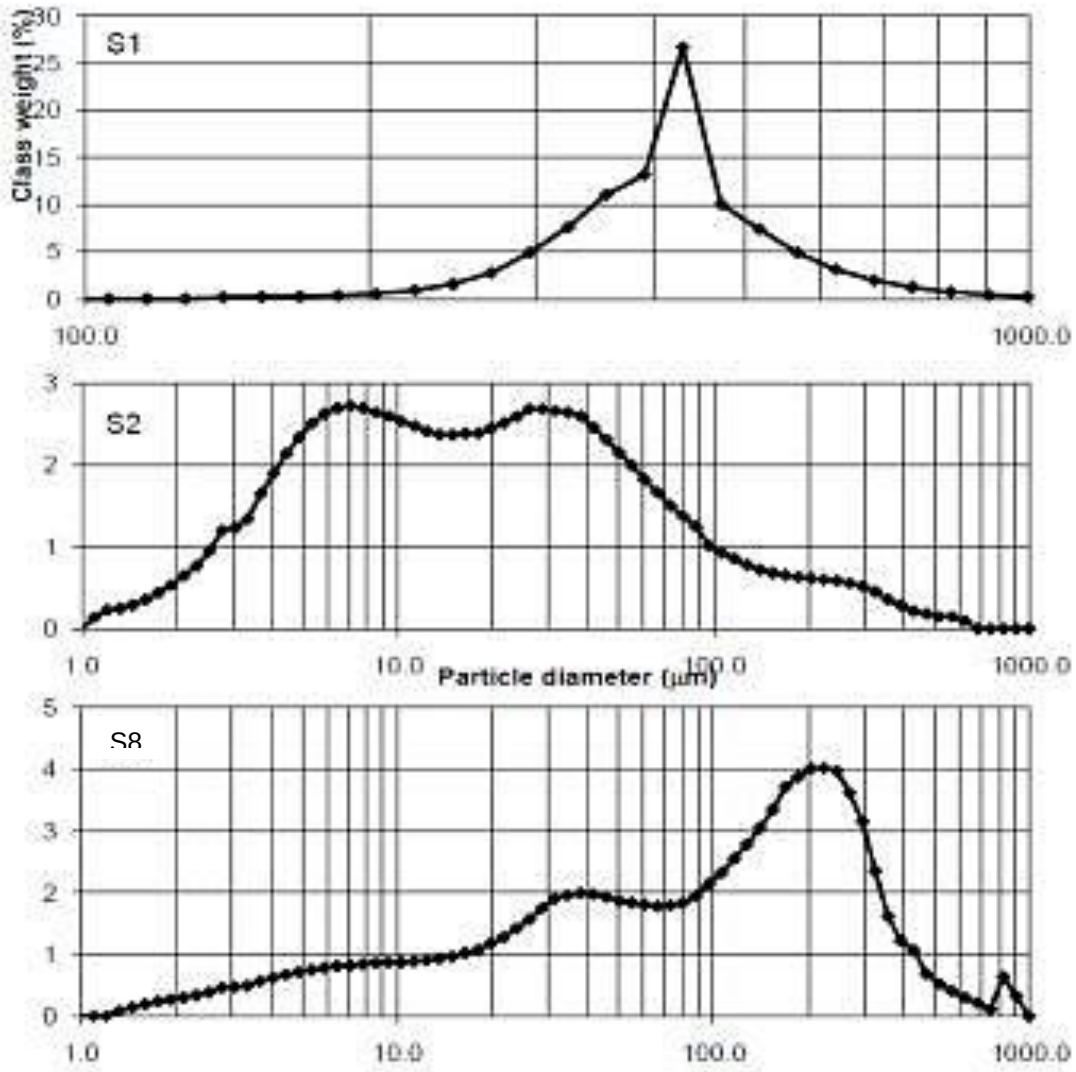
Figure B-23 Textural analysis of river sediments S1, S2 and S8

Sediment particle size was measured by laser diffraction method (UOP, 2007) using a tri-laser Microtrac model S3500 (Microtrac Inc., Florida, United States)

equipment. Textural analysis resulted in different classifications for each of the three locations (Table B-9).

Table B-9 Textural analysis of three sediment samples

Texture of the sediment		**Site**		
		Nuevo Berlín (S1) coastal	**Fray Bentos (S2)**	**Las Cañas (S8)**
Gravel		0.0	0.0	0.0
Sand		100	17.1	59.5
Mud		0.0	82.9	40.4
Textural group		Sand	Sandy mud	Muddy sand
Sediment name		Sand	Very fine sandy coarse silt	Very coarse silty fine sand
Particle size distribution				
Name of the sediment	**Particle size**	**Nuevo Berlín (S1) coastal**	**Fray Bentos (S2)**	**Las Cañas (S8)**
Very coarse sand	2 mm	0.1	0.0	0.0
Coarse sand	1	18.8	0.4	2.4
Medium sand	500 µm	77.6	2.8	14.9
Fine sand	250	3.5	4.9	25.8
Name of the sediment	**Particle size**	**Nuevo Berlín (S1) coastal**	**Fray Bentos (S2)**	**Las Cañas (S8)**
Very fine sand	125	0.0	9.1	15.4
Very coarse silt	63	0.0	17.2	14.2
Coarse silt	31	0.0	18.8	10.1
Medium silt	16	0.0	18.5	6.7
Fine silt	8	0.0	18.1	5.5
Very fine silt	4	0.0	8.2	3.2
Clay	2	0.0	2.1	0.8



Size scale according to Uden (1914) and Wentworth (1922) modified by GRADISTAT.

Figure B-24 Particle size distribution of river sediment at three sites

2) Chemical analysis of chlorinated organics as adsorbable and extractable fractions

The EOX of sediments were analysed by ultrasound extraction of 1 g per wet sample with ethyl acetate > 99.5 % pure (Mallinckrodt Chemicals, Xalostoc, Mexico) (USEPA 1996b). An aliquot of 25 µl of the extract was directly placed in the pyrolysis furnace using a stream of O2/Ar (150 – 200 ml min^{-1}) and halide hydrogen (HX) was determined by microcoulometry using a combustion/

coulombimetry equipment (Mitsubishi TOX 100 thing Instrument, Norwood, United States). The AOX of aqueous samples were determined after adsorption of the solution charcoal column flow-through system previous to the combustion/coulombimetry (standard ISO 9562: 2004). The standard for calibration of AOX and EOX was 2,4,5-trichlorophenol 98% pure (Aldrich, France). The total phosphorus concentra wottion was analysed by manual digestion followed by flow injection analysis method (FIA) (Míguez et al. 2009) using a QuikChem 8500 (Lachat Instruments, Loveland, United States) equipment.The limit of detection (LOD) was 6 mg kg^{-1}.

Total Kjeldahl nitrogen was determined by digesting 1g of the air-dried sediment sample by the classical method based on ISO 1984 adapted to Kjeldhal tubes using a Gerhardt digesting block, at 410°C. The solution was then titrated with HCl 0.01 N green bromocresol and methyl red mixed indicator, turning from the greenish to grayish violet at the endpoint. LOD = 0.7 mg kg^{-1}. The content of organic matter in the sediment was measured in muffle furnace, at 400 °C (Soil Survey Laboratory Methods Manual 2004).

As shown in Figure B-24, S1 and S2 samples had higher silt and clay content than S8 (55.2, 51.5, 27.8 g 100 g^{-1}, respectively).

3) Bioassays with crustaceans and fish embryo in sediment elutriates and with epibenthic amphipods in whole sediment

A battery of bioassays composed of *P. promelas* fish embryo and *Ceriodaphnia dubia* three brood assays, and the YES *in vitro* test evaluated embriotoxicity (teratogenicity and lethality), acute and chronic toxicity with endpoint reproduction, and ER-binding, respecitively, in unspiked and spiked sediments. Briefly, in the case of the embryo toxicity test, the experimental conditions for were: 240 embryos per 200 ml volume sample of elutriate, semi-static regime with partial renewal every 24 h, to assess the teratogenicity endpoint after 168

hours of exposure based on USEPA Test Method 1001.0. The photoperiod was held at a 16:8 (light: darkness).

Table B-10 Bioassays conditions

Condition	**Species name**			
	Phosphobacterium legiognathi	***Hyalella curvispina***	***Ceriodaphnia dubia***	***Pimephales promelas***
Stage	Bacterial culture	Juvenile (7 - 14 days)	Neonate (<24 h)	Embryo
Nº organisms	Bacterial culture	10 per aquaria	1 neonate per beaker 10 per dilution	240
Sample volume	5 ml	100 ml	15 ml	70 ml
Sample type	Elutriate	Whole sediment	Elutriate	Elutriate
Regime	Static	Static	Semi-static, daily renewal	Semi-static, daily renewal
Nº replicates	2	4	10	4
Dilutions (%)	80, 40, 20, 10, 5, 2.5	Logarithmic	Geometric (from 50%)	Geometric
End-point	Luminiscence measurement	Survival and growth	Survival and growth	Teratogenicity Lethality
Temperature (ºC)	30 ±2	23±2	23±2	20±2
Photoperiod (light:darkness, hours)	No	16:8	16:8	16:8
Aireation	No	Yes	No	No
References	PCB- Checklight Instruction Manual	USEPA 600/R-99/064 Test Method 100.1, 2000	USEPA 821-R-02-013 Test Method 1002.0 2002	USEPA-821-R-02-013.Test Method 1001.0

The dilution water was obtained by filtration of water through a filter system consisting of a mechanical filter of 5 µm, activated carbon from 20µm and 5µm and UV light (first, first-525, Brea-California, United States) producing water of the following characteristics: total hardness (mg l^{-1}, as CaCO3) > 25, dissolved oxygen (mg l^{-1}) > 60%, pH 7-8. Photos of larvae were taken with a binocular Microscope Olympus SZ61. The samples were taken at Ubici beach, near the pulp mill plant and transported frozen to the laboratorio at Montevideo. The method was based on Kostamo et al. (2000).The result was an average of 0.17 mg kg^{-1} lipid in one sample taken from Ubici beach.

B.3.6 Bioaccumulation of chlorinated organics in molluscs and gastropods determined as EOX

The frozen samples of *Corbicula sp. were* thawed and shelled. The initial weight was recorded and the mussels were minced using a high speed Ultra-Turrax® tissue homogeniser. The homogenised sample was separated into three portions, and put into Falcon plastic tubes previously rinsed with isopropanol. A volume of 5.0 ml of 2,4,5-trichlorophenol (0.1914 g in 100 ml of methanol) was added to the sample number 3 for a recovery check. A 100 ml portion of a solution of Celite (10 g l^{-1}) was passed through a Whatman No. 41 paper filter with the aid of vacuum. Then, each sample solution was filtered through the filter prepared with Celite as mentioned before.

The filters and their contents were set inside Soxtec® cellulose thimbles Ø 33mm. After assembling the automatic fat extractor equipment Soxtec, Tecator®, the samples were extracted with volumes of 40 ml of a (1:1) mixture of isopropanol:cyclohexane contained in aluminum crucibles. The working parameters were: 175ºC, 1 hour, boiling position, and 30 minutes, rinsing position. The solvent was evaporated for five minutes in the equipment and the crucibles were dried in an oven during 15 minutes at 105ºC. After cooling down, the crucibles were weighed to obtain the lipid content. The extract was reconstituted with 10 ml cyclohexane and washed 3 times with diluted hydrochloric acid to pH 2. The organic phase was dried through sodium sulfate

and evaporated using a Rapid Vap, Labconco® equipment, under a N_2 stream. The dried samples were redissolved in 0.5 ml of cyclohexane. Volumes of 5 µl were injected in the boat to enter the pyrolisis oven of a combustion/coulombimetric equipment (Mitsubishi, COSA model TOX-100/ABC). The combustion/carrier gas mixture was an O_2/Ar stream (150:200 ml/min). The halogen acid gas was finally measured in the coulombimetric cell and read in the computer converted as mass according to the current that passed through it.

Figure B-25 *Pomacea spp.* snail collected at Anglo Beach and image of the sewage collection pipe at the same beach

Alkylphenols were measured on a portion of the same snail specimen of the genus *Pomacea* collected at Anglo Beach, where the municipal collector pipe from the city of Fray Bentos pours the wastewater into the river.

B.3.7 Bioaccumulation of endocrine disruptors in market fish

Alkylphenols were quantified by GC/MS, endosulfan and endosulfan sulphate by HRMS, glyphosate by HPLC-FLD, and chlorophenols and resin acids by GC-MSD.

Figure B-26 *Pimelodus maculatus* fish species

Bioaccumulation and distribution in plant tissues of chlorinated organics from pulp mill effluent in water hyacinth floating plant

An experiment was carried out by exposing *E. crassipes* to pulp mill effluent. The effluent composition was characterised in terms of nutrient content (ammonia, nitrites, nitrates, total and soluble phosphorus), pH, electrical conductivity, color, BOD_5 and AOX.

Reagents

Isopropylic alcohol: Mallinckrodt Chemicals Lote H08B01 (ACS) 99.5% pure

Cyclohexane: Mallinckrodt Chemicals Lote 4878KVVB 100% pure

Acetone: Merck KGaA 99.8 % pure

Effluent samples were obtained from the Kraft mill near the city of Fray Bentos, Río Negro, Uruguay from the secondary treatment plant. A composite sample was produced by mixing samples of the final effluent after secondary treatment representative of the production of winter 2009. In November 2009, duplicate specimens of *E. crassipes* were exposed to the effluent. The regimen was in

batch, in 20 litres polyethylene vessels containing five litres each of the effluent sample. The exposure length was 31 days, with partial renovation, under natural light and at room temperature. Losses in culture volume due to evaporation and to absorption through the plant roots were counteracted by addition of deionized water to the original level every other day. Collection of E. crassipes was done at an upstream site (latitude: 33° 5'35.31"S; longitude: 58°10'43.05"W). The unexposed specimen was sampled in 2006, before the pulp mill start-up.

After the exposure period the plants were harvested, and then minced using a mortar and liquid nitrogen. Their lipophilic fraction was extracted using 40 ml of a mixture of cyclohexane: acetone (1:1) by a semi-automatic liquid-liquid extractor (Soxtec System HT 1043, Tecator, Sweden).The residues remaining in each crucible were weighed, reconstituted with the solvent mixture and evaporated under a Nitrogen stream.

The evaporated residues were then dissolved with 0.5 ml cyclohexane and 15 µl injected to finally measure EOX by the combustion-coulombimetry method, using a Mitsubishi-COSA equipment, model TOX-100. The EOX were analysed in the root, stem and leaves fractions. The total halogens (TX) were also determined. The resulting blend was weighed and then directly burnt in the boat of the TOX-100, to determine total halogens (TX) by combustion/coulombimetry.

The distribution pathway of chlorinated organic compounds existing in the pulp mill effluent was evidenced by an increase in concentration from the root to the stem and to the leaves (Figure B-27).

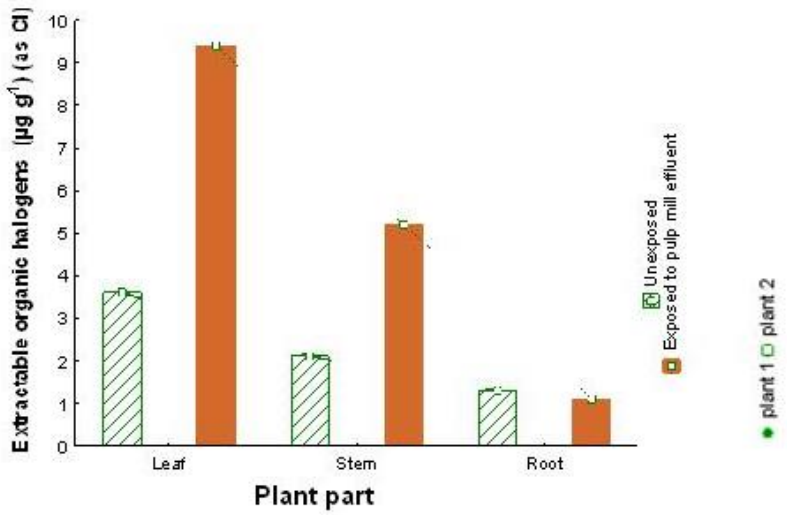


Figure B-27 Bioaccumulation of organohalogens in *Eichornia Crassipes* floating plant in stem, root and leaves

Colour was used as a monitoring item during the test. The behaviour of colour variation was similar in both of the experiences. One of the reasons that could explain the growth inhibition after about 20 days of exposure is the high salinity, as suggested by Sooknah & Wilkie (2004).

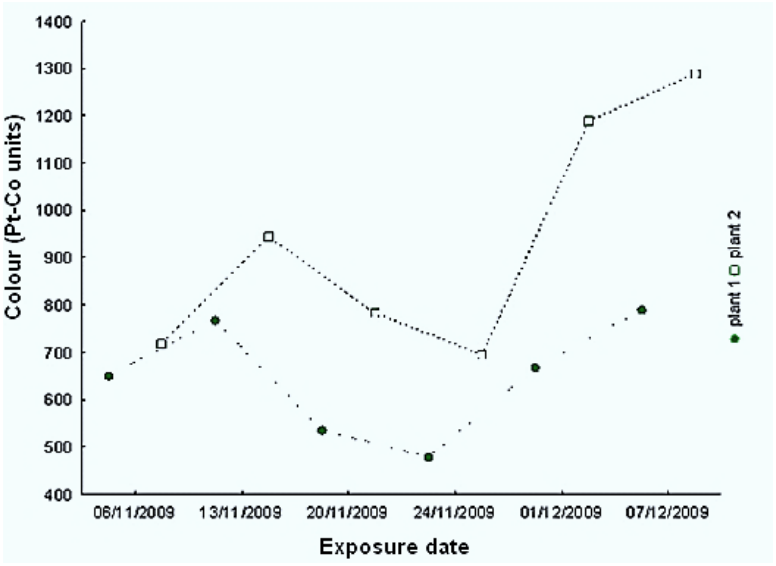


Figure B-28 Colour variation in effluent after exposing *E. crassipes*

The mean initial plant mass was 497 ± 311 and the final plant mass was 277 + 256 g. The BOD_5 of the composite effluent samples was 29.0 mg l^{-1}, ammonia concentration was 0.563 mg l^{-1} and AOX, as Cl, 1.84 mg l^{-1}.

In this experimental setting, the plants nutrient uptake was evidenced by the decrease in phosphorus and nitrate.

Table B-11 Nutrient, acidity and salinity uptake of *E. Crassipes* exposed to pulp mill effluent

Parameter	Pre-exposure value	Final value
Conductivity (µS cm^{-1})	1326	5300
pH	7.18	7.80
Nitrite (as N) (mg l^{-1})	0.31	0.072
Nitrate (as N) (mg l^{-1})	1.34	0.016
Total phosphorus (as P) (mg l^{-1})	1.47	0.564
Soluble phosphorus (as P) (mg l^{-1})	1.47	0.095

B.4 Quality Control/Quality Assurance

The following are some of the quality control charts currently used for the verification of the performance of the methods.

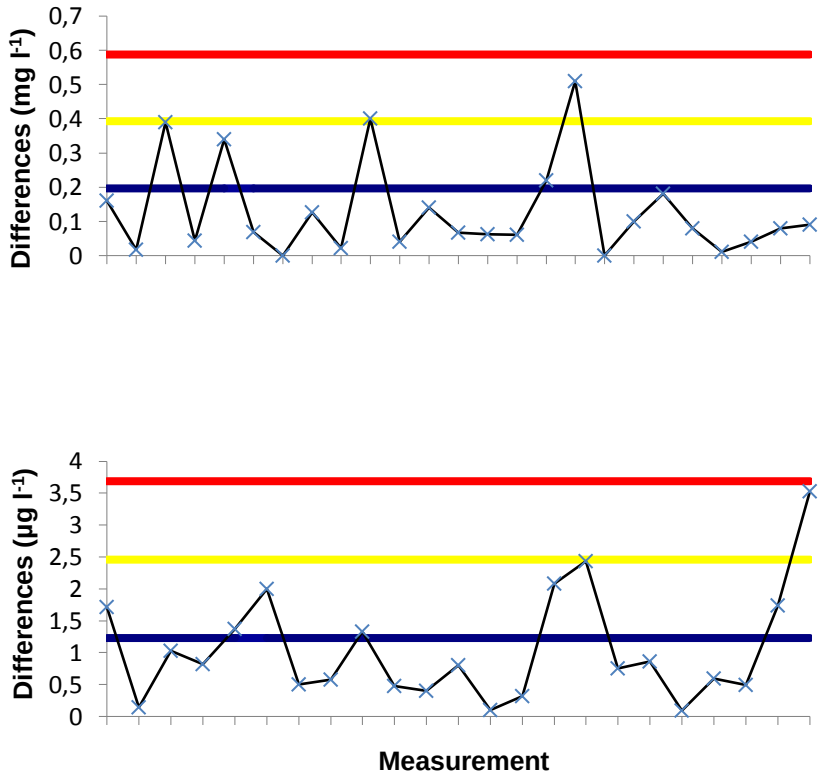


Figure B-29 Precision control charts for adsorbable organic halogens (AOX)

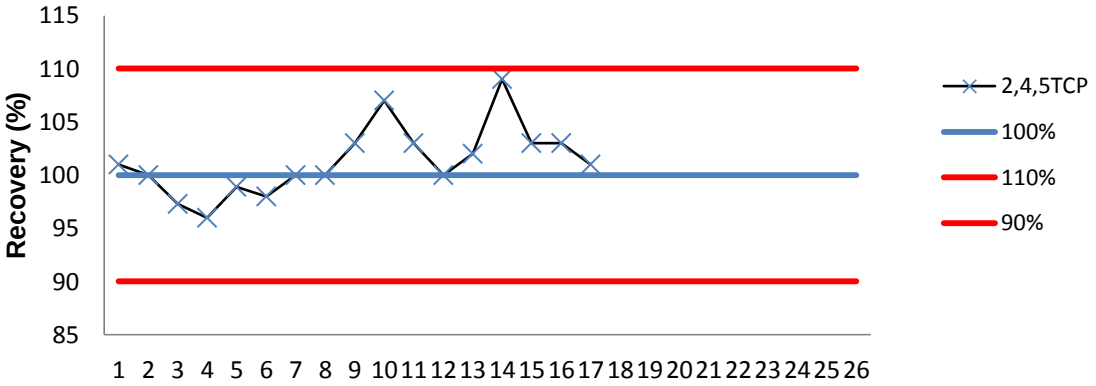


Figure B-30 Recovery check of 2,4,5-trichlorophenol for adsorbable organic halogens method

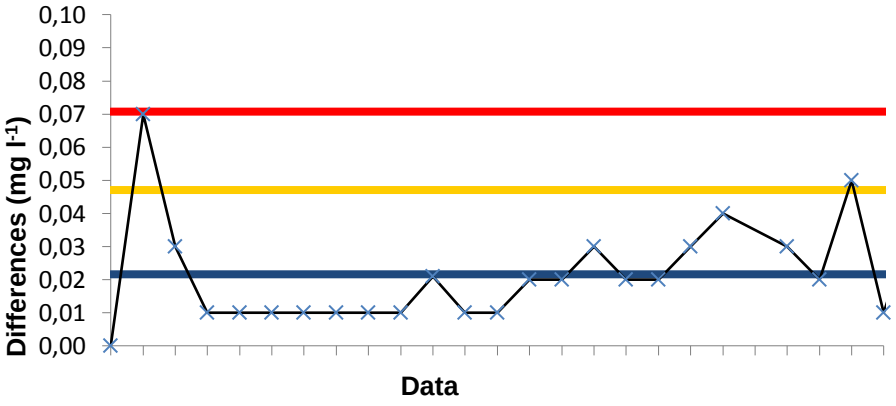


Figure B-31 Precision dispersion chart for total nitrogen

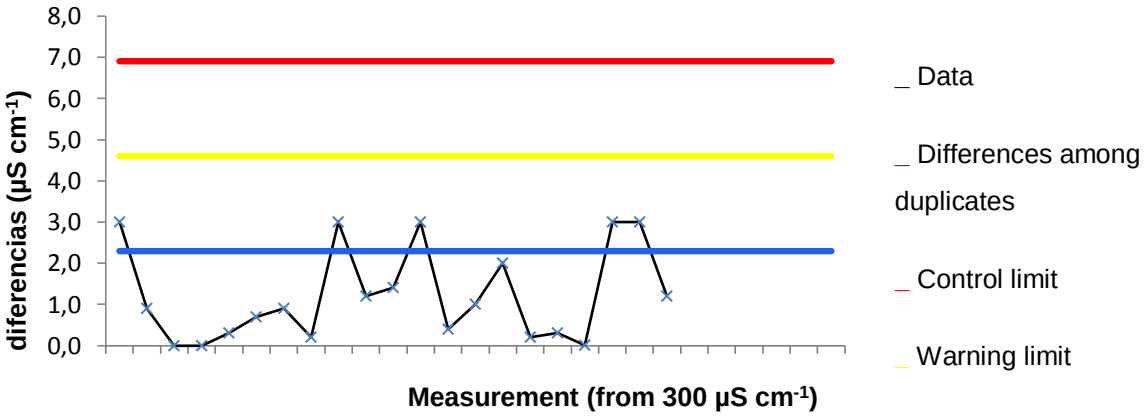


Figure B-32 Precision control charts for conductivity

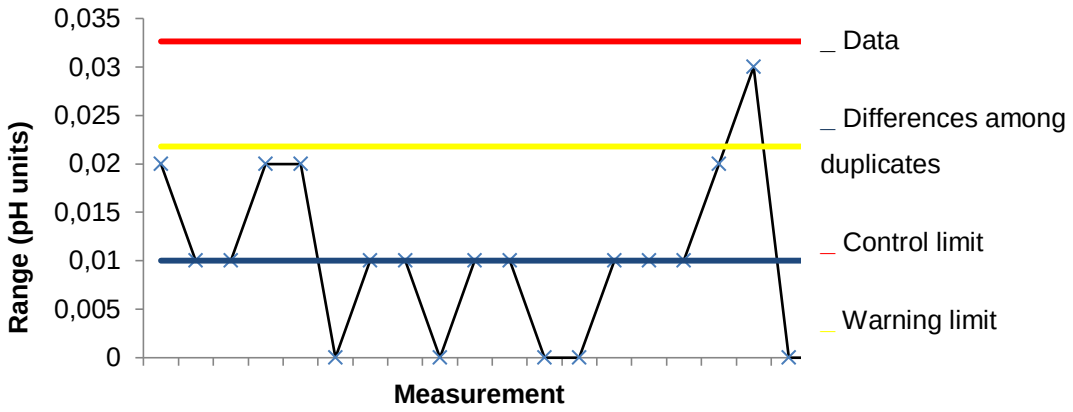


Figure B-33 Precision control chart for pH

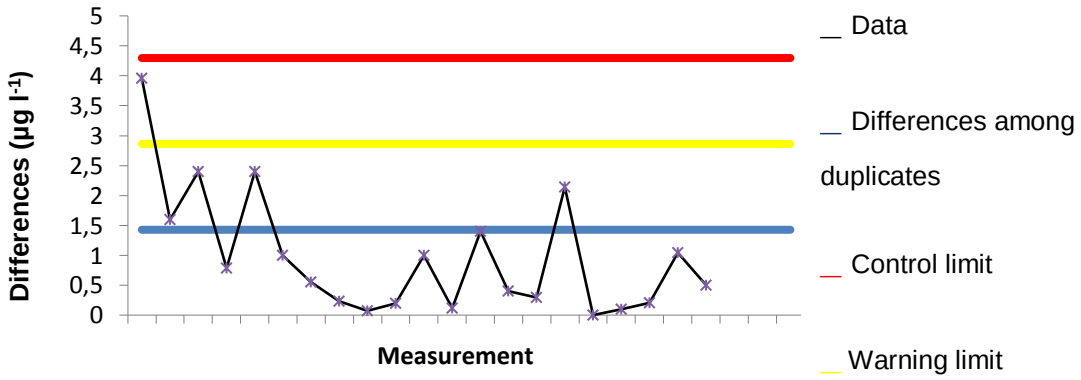


Figure B-34 Precision control chart for phenols

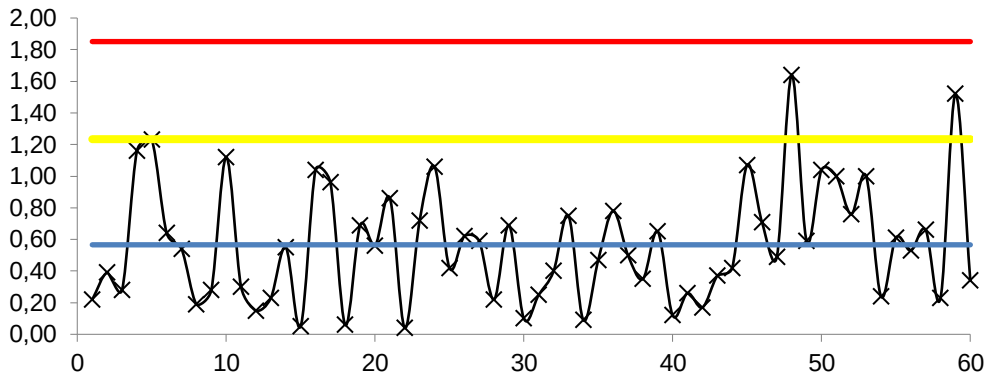
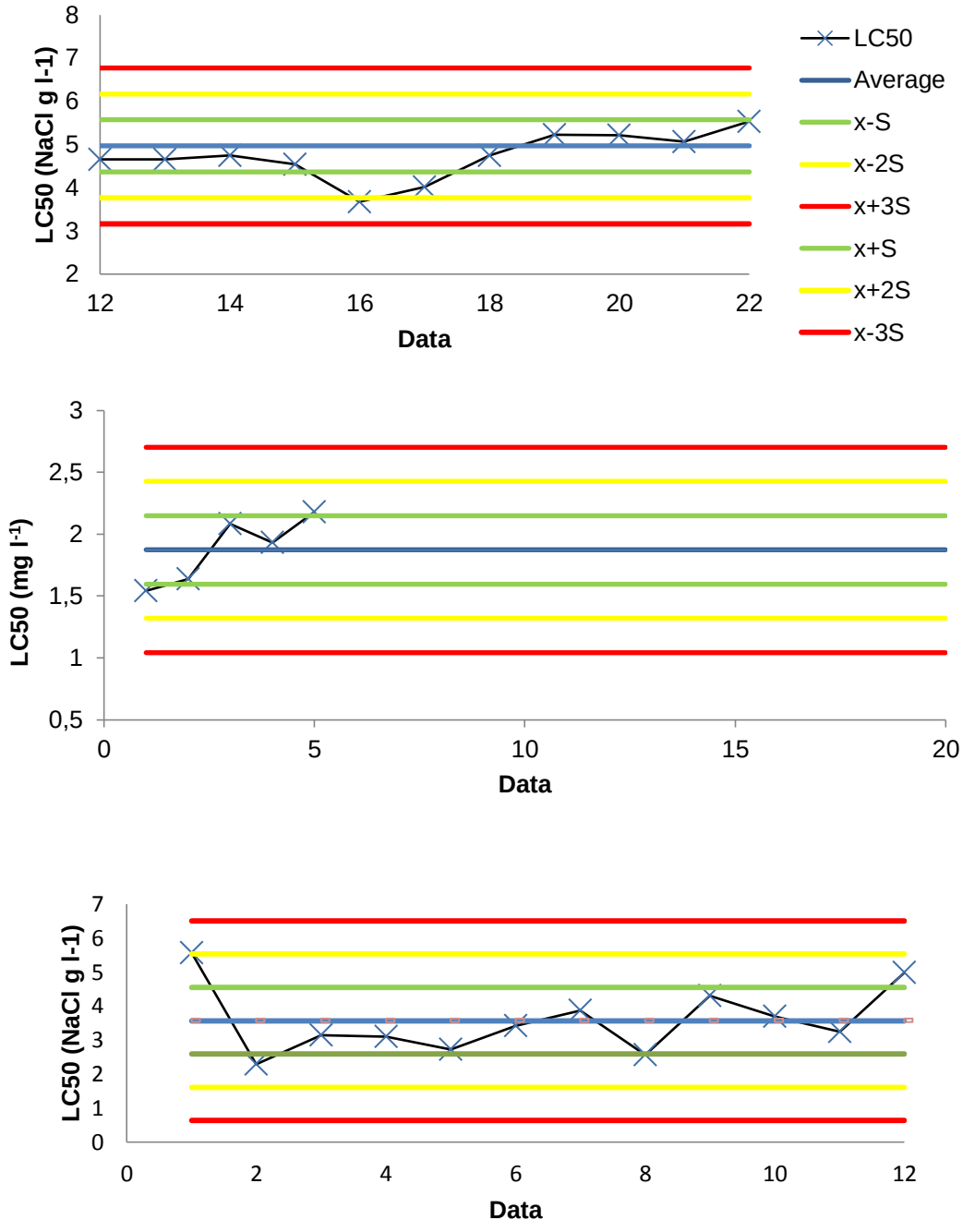


Figure B-35 Precision dispersion chart for CT of vtg (triplicates in the order of analysis, following ISO 8258:1991Shewhart control charts)



A: *Daphnia magna*; B: *Pimephales promelas* larval test; C: *Ceriodaphnia dubia*

Figure B-36 Accuracy check for LC50 of bioassays with reference toxicant

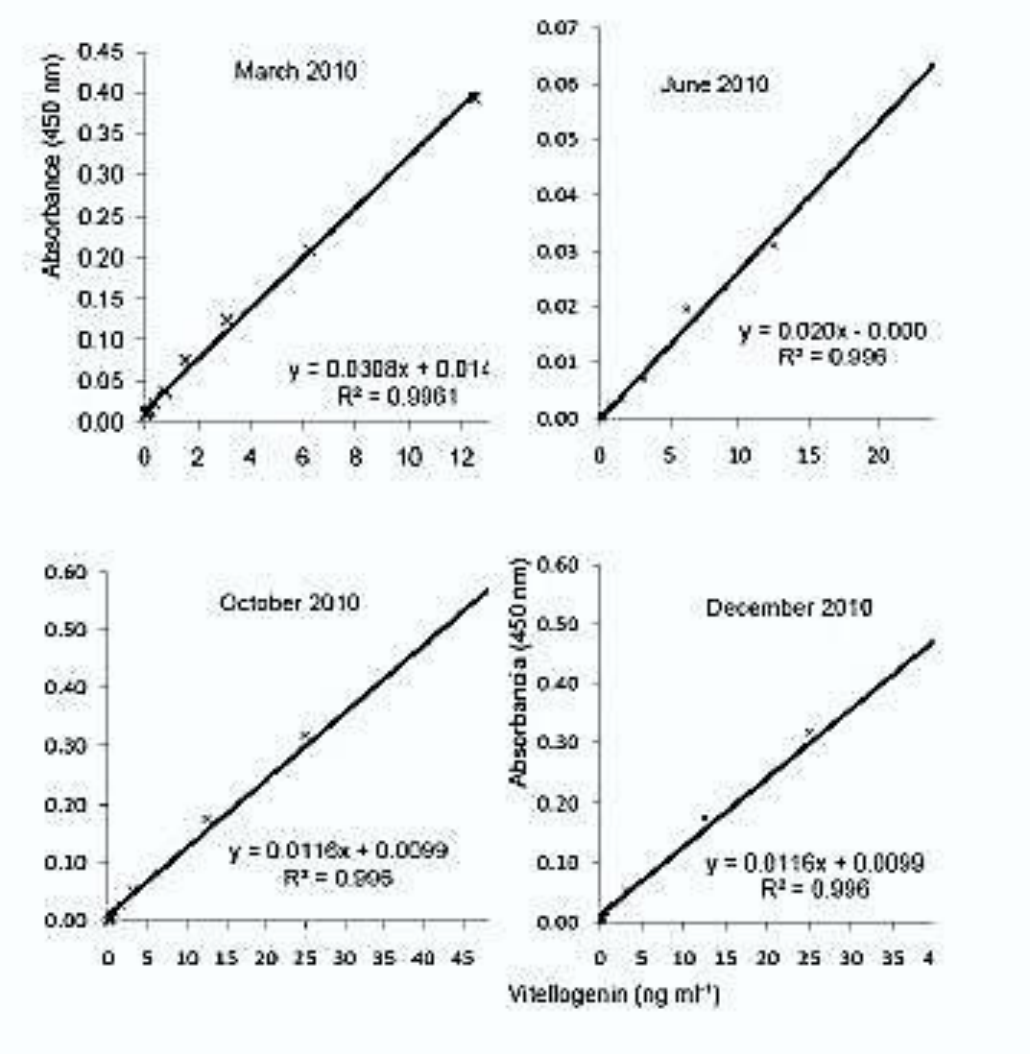


Figure B-37 Slope control of vitellogenin by immunochemical method (ELISA)

Appendix C Toxicity profiles and binding activities of selected endocrine disruptors

C.1 Glyphosate toxicity profile and conceptual model for assessment

The especial characteristics of glyphosate in terms of its lower persistency and modes of application, and the relative scarcity of toxicity data, in particular for endocrine disruption end-points, justified gathering information on this EDC and developing a conceptual model for its study.

Table C-1 Toxicity profile of glyphosate

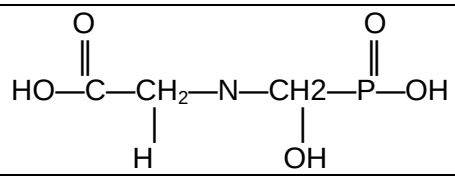
Parameters	Characteristics	References
Molecular formula	$C_3H_8NO_5P$	Herbicide Handbook of the Weed Science Society of America, 4th ed., 1979; WHO 2005
Structural formula		
Molecular weight	169.1	
CAS No.	1071-83-6 1066-51-9	
IUPAC name	*N*-(phosphonomethyl)glycine	
Salts	Isopropylamine (CAS 38641-94-0); potassium (CAS 39600-42-5), and ammonium (CAS 40465-66-5)	
Physicochemical properties	Weak organic acid	
Vapour pressure	$<10^{-5}$ Pa at 25 °C (negligible)	
Melting point	185 °C (decomposes at 199 °C)	
Log *n*-octanol/water partition coefficient	-2.8	
Water solubility	10.1 g l^{-1} at 20°C	

Table C-1- ***Continued***

Parameters	Characteristics	References
Specific gravity	1.70 gcm^{-3}	Idem ant., WHO 2005
Major uses	Broad-spectrum post-emergence herbicide in agriculture and forestry	
Uruguay use	Imports in 2008:12400 ton per year. 70% of the total herbicides. Glyphosate isopropilamide is the most used compound.	MGAP-DGSA (2008)
Mechanism of action as herbicide	Inhibition of 5-enolpyruvylshikimate-3-phosphate synthase blocks the synthesis of aromatic amino acids	Cedergreen et al. 2007
Environmental fate and biodegradation		
Microbial biodegradation	Soil, aquatic sediment and water. Primary biodegradation product AMPA in aerobic conditions. Mineralization to carbon dioxide. Mineralization by indigenous soil micro-organisms in gravel: 11 to 32% after 31 days of incubation at 30º C.	WHO 2005 Strange-Hansen et al. 2005
Half-lives for biodegradation in water	Between 12 h and 7 weeks	CCME 1989
Chemical stability	Stable in water, not photochemically degradable	FAO/WHO, 1986
Groundwater contamination potential	Run-off or leaching from terrestrial applications It may percolate to groundwater	WHO 2005 Vereecken 2005
Bioaccumulation		
Bioaccumulation	Low bioaccumulation because of high water solubility. Residues in fish, crustaceans and mollusks (0.2-0.7 mg kg^{-1} fish muscle)	WHO 2005 FAO/WHO 1986
Environmental concentrations		
Air	Occupational: silvicultural spraying <1.3 µg m^{-3}; highest concentration: 15.7 µg m^{-3} Highest estimated exposure (dermal and	IPCS1994

	inhalation): 8000 µg h^{-1}, about 40 µg kg^{-1} bwd (8-h working day for a 60-kg adult)	
Water	Concentration in waters near soy plantations: 0.10- 0.70 mg l^{-1}. Concentration in sediments and soils: 0.5-5.0 mg kg^{-1}	Peruzzo et al. 2008
Food	Mean residue levels in cereals after pre-harvest application: 0.2-4.8 mg kg^{-1}, remaining in flour 0.16 mg kg^{-1}. Fish exposed to water 10 mg l^{-1} for 14 days: 0.2–0.7 mg kg^{-1}, decreases after exposure to glyphosate-free water In animal livers: 0.12 mg kg^{-1}; found in bone; not detected in muscle or milk	FAO/WHO 1986
Main routes of human exposure	Inhalation and dermal exposure in occupational settings. Consumption of water and food for general population	WHO 2005
Acute exposure routes	42 % of cases occupational, 37% accidental and the rest intentional, in a study from 1997-2002, in Uruguay	Burger and Fernández 2004
Routes of aquatic exposure	Major source of exposure: food, as it adsorbs to particulate matter and degrades in the aquatic environment	CCME 1989
Toxicokinetics		
Ingestion and adsorption	Gastrointestinal adsorption: incomplete in rodents and goats: approximately 30% dose	WHO 2005
Distribution	Highest concentration: bones	
Biotransformation	Very low, unchanged in urine and foeces	
Elimination	Very low, through exhaled air. AMPA metabolite (0.2–0.3%) moderately absorbed (around 20%). Excretion via urine, with < 0.1% dose expired as carbon dioxide. Biliary excretion: 5-8%. Excretion in milk (goats): minor (concentration $\leq$ 0.1 mg kg^{-1} whole milk at 120 mg kg^{-1} dose)	IPCS 1994, FAO/WHO1987

Table C-1- *Continued*

Parameters	Characteristics	References
Effects using *in vitro* and *in vivo* bioassays		
Acute toxicity	Very low by oral and dermal routes LD50s: 1950 to >5000 mg kg^{-1} bw for mice, rats and goats *Ceriodaphnia dubia; Hyalella azteca*: LC50: 1.5–415 mg l^{-1} depending on the commercial preparation. LC50s: 2.59 mg l^{-1} in crayfish (*Orconectes nais*; 96-h) to 5600 mg l^{-1} in midge larvae (*Chironomus riparius*; 48-h) Acute toxicity in mammals, oral, dermal: LD50 > 5000 mg kg^{-1}	IPCS1994 Tsui & Chu 2004 USEPA 2008
Sub-lethal toxicity	Oxidative stress in fish tissues. Superoxide dismutase activities reduced by 51–68% in brain, 58–67% in liver and 33–53% in kidney of treated fish	Luschak et al. 2009
Ecological impacts		
Phytotoxicity	Acutely toxic to non-target plants including aquatic plants and algae Moderately toxic increasing with higher temperatures and combined with surfactants in preparations (nonylphenol or polyethoxylated tallowamine) May inhibit the growth of natural soil microbes	Carlisle &Trevors 1988 Howe et al. 2004 Hendricks 1992
Developmental toxicity	It affects amphibian metamorphosis and causes gonadal abnormalities	Howe et al. 2004
Sediment toxicity	Sediment–porewater partitioning increases with sediment organic carbon. Major exposure route: via pore water.: *Hyalella azteca*: 244 mg kg^{-1}-340 mg kg^{-1}	Tsui & Chu 2004

Table C-1- *Continued*

Parameters	Characteristics	References
Chronic toxicity	Rats: NOAEL:175-1000 mg kg^{-1} bw per day	IRDC 1980; IPCS 1994
Long-term exposure and carcinogenicity	Incidence of interstitial cell tumors in testes increased (0/50, 3/50, 1/50 and 6/50 vs. historical control range 3–7%)	De Roos et al. 2005
Reproductive and developmental toxicity	Effects in rabbits: decreased body weight, gastrointestinal and renal affections, decreased sperm concentration, fructose and osmolality Effects on spermatogenesis and/or indirectly via hypothalamic-pituitary-testis axis NOAEL maternal in the rabbit developmental toxicity: 175 mg kg^{-1} bwd Some agricultural workers using glyphosate have pregnancy problems, but its mechanism of action in mammals is questioned LD50 with embryonic cells: 0.3%. The embryonic cells appear to be 2–4 times more sensitive than the placental ones. At lower doses, from 0.01% (with 210 lM glyphosate) in 24 h, it is an aromatase disruptor in human placenta and embryonic cells	Youssef et al. 1995 Walsh et al. 2000 Richard et al. 2005 Benachour et al. 2007
Mechanism of toxicity	Disruption of hormone signaling, because thyroid hormone receptor bmRNA transcript levels elevate by exposure to formulations containing glyphosate and POEA Toxic to human placental JEG3 cells Disrupts aromatase activity and mRNA levels and interacts with the active site of the enzyme Steroidogenesis inhibited through disruption of acute regulatory (staR) protein expression	IPCS 1994 Richard et al. 2005 Walsh et al. 2000
Mutagenicity and related end-points	No mutagenic effect in a range of genotoxicity assays *in vitro* and *in vivo*	WHO 2005

Table C-1- ***Continued***

Parameters	Characteristics	References
Effects on humans		
Acute	Intoxications with technical glyphosate herbicide formulation have been reported (erosion of the gastrointestinal tract)	Burger & Fernández 2004
Drinking water guideline level	The EU limit for any pesticide in drinking water is 0.1 mg l^{-1}	
Health-based value for human exposure	0.9 mg l^{-1} (based on ADI of 0.3 mg kg^{-1} bw, for a 60 kg adult consuming 2 litres of drinking water per day, and allocating 10% of the ADI to drinking water) Toxic effects enhanced by coadjuvants. HQ calculated as the (expected environmental concentration) EEC divided by the LC_{10}. If HQ < 0.1 then, acceptable for valued ecosystem components such as fish. Subpopulations for estimates of exposure are applicator adults, and children. If MOEs > 100, then low potential for acute toxicity MOEs: 3370-5420 mg kg^{-1}in children and adults, respectively	WHO 2005

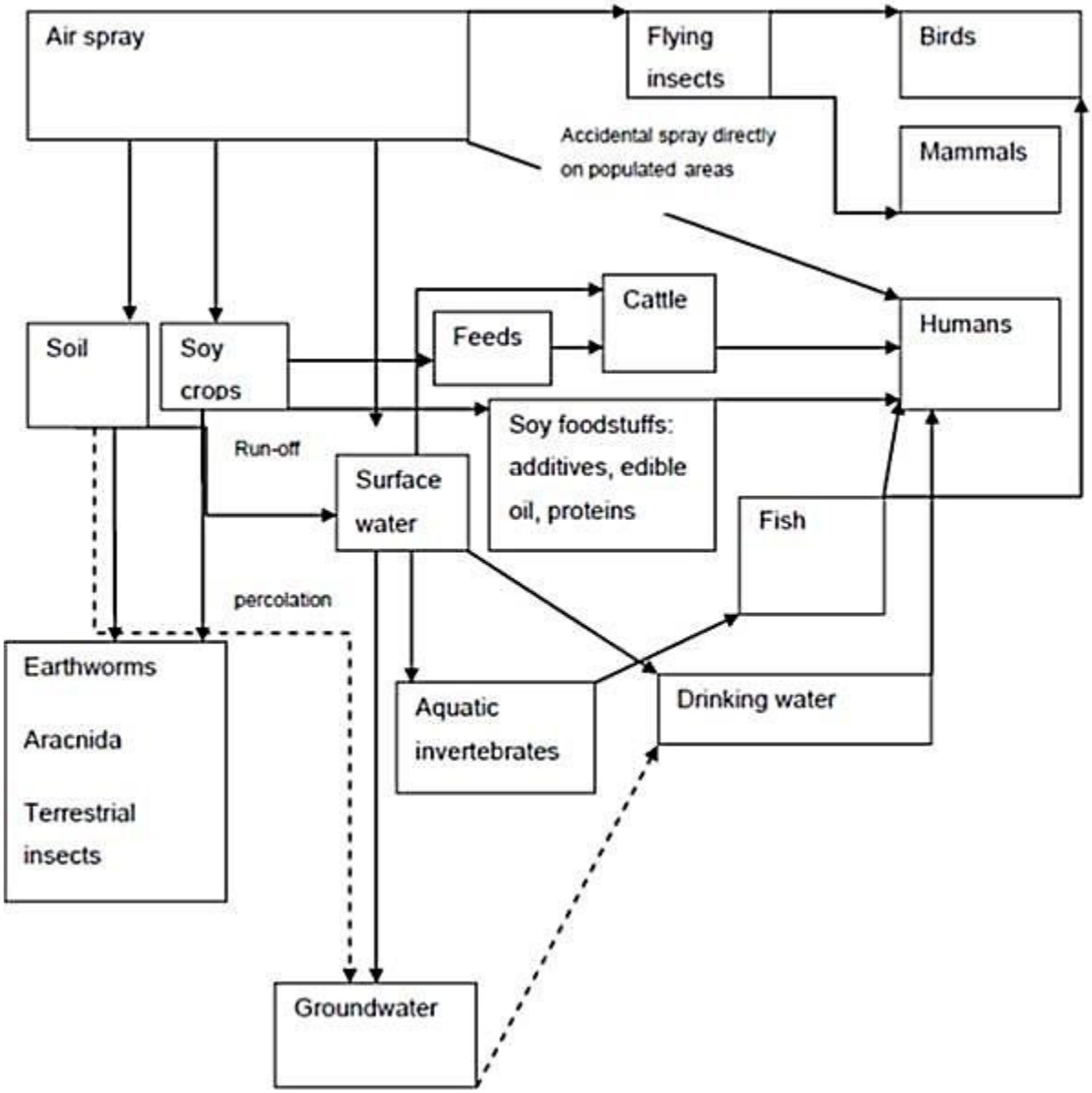


Figure C-1 Glyphosate conceptual model

Table C-2 WHO-TEF values for dioxins, furans and PCBs

Congener	WHO-TEF values (2005)
2,3,7,8-TCDD	1
1,2,3,7,8-PeCDD	1
1,2,3,4,7,8-HxCDD	0.1
1,2,3,6,7,8-HxCDD	0.1
1,2,3,7,8,9-HxCDD	0.1
1,2,3,4,6,7,8-HpCDD	0.01
OCDD	0.0003
2,3,7,8-TCDF	0.1
1,2,3,7,8-PeCDF	0.03
2,3,4,7,8-PeCDF	0.3
1,2,3,4,7,8-HxCDF	0.1
1,2,3,6,7,8-HxCDF	0.1
1,2,3,7,8,9-HxCDF	0.1
2,3,4,6,7,8-HxCDF	0.1
1,2,3,4,6,7,8-HpCDF	0.01
1,2,3,4,7,8,9-HpCDF	0.01
OCDF	0.0003
Non-ortho-substituted PCBs	
3,3´,4,4´-tetraCB (PCB77)	0.00003
3,4,4´,5-tetraCB (PCB81)	0.00003
3,3´,4,4´,5-pentaCB (PCB126)	0.00003
3,3´,4,4´,5,5´-hexaCB (PCB169)	0.00003
Mono-ortho-substituted PCBs	
2,3,3´,4,4´-pentaCB (PCB105)	0.00003
2,3,4,4´,5-pentaCB (PCB114)	0.00003
2,3´,4,4´,5-pentaCB (PCB118)	0.00003
2´,3,4,4´,5-pentaCB (PCB123)	0.00003
2,3,3´,4,4´,5-hexaCB (PCB156)	0.00003
2,3,3´,4,4´,5´-hexaCB (PCB157)	0.00003
2,3´,4,4´,5,5´-hexaCB (PCB167)	0.00003
2,3,3´,4,4´,5,5´-heptaCB (PCB189)	0.00003

WHO 2005 Re-evaluation of human and mammalian toxic equivalency factors (TEFs)
(http://www.who.int/foodsafety/chem/tef_update/en/index.html

Table C-3 Activities of selected EDCs that act as ER-binders

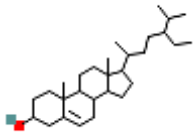
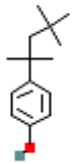
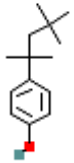
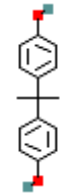
EDC name	Molecular structure	CAS No.	MW	Log RBA	Species	References
17b-Oestradiol (E2)		50-28-2	272.39	2	Human	Kuiper et al. 1997
4-t-Octylphenol		140-66-9	206.33	-0.7	Human	Bolger et al.1998
4-t-Octylphenol		140-66-9	206.33	-1.82	Rat	Blair et al. 2000
Bisphenol A		80-05-7	228.29	-1.30	Rat	Sippl 2002

Table C-3- *Continued*

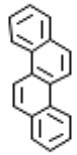
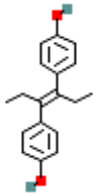
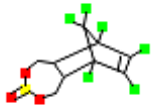
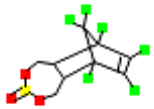
EDC name	Molecular structure	CAS No.	MW	Log RBA	Species	References
Chrysene		218-01-9	228.29	-10000	Rat	Blair et al. 2000
Diethylstilbestrol (DES)		56-53-1	268.35	2.46	Mouse	Sadler et al. 1998
Endosulfan, technical grade		115-29-7	406.93	-10000	Rat	Blair et al. 2000
Endosulfan, technical grade		115-29-7	406.93	-100	Human	Kuiper et al. 1998

Table C-3- *Continued*

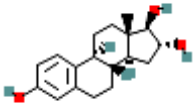
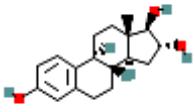
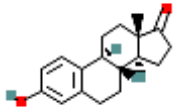
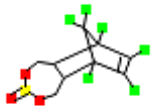
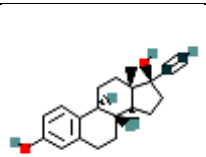
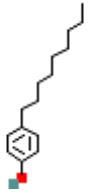
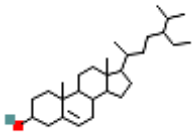
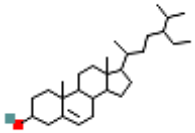
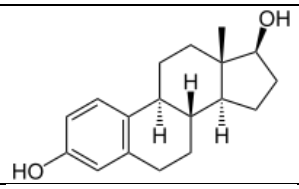
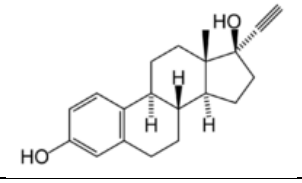
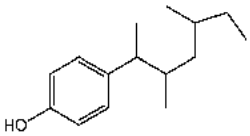
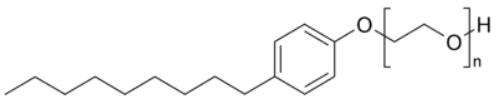
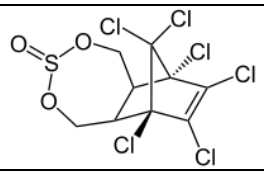
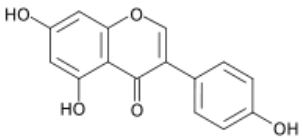
EDC name	Molecular structure	CAS No.	MW	Log RBA	Species	References
Oestriol		50-27-1	288.39	1.27	Mouse	Waller et al. 1996
Oestriol		50-27-1	288.39	1.15	Human	Kuiper et al. 1997
Oestrone		53-16-7	270.37	1.57	Human	Kuiper et al. 1997
Endosulfan, technical grade		115-29-7	406.93	-100	Human	Kuiper et al. 1998

Table C-3- *Continued*

EDC name	Molecular structure	CAS No.	MW	Log RBA	Species	References
Ethynyl oestradiol		57-63-6	296.41	2.28	Rat	Blair et al. 2000
Nonylphenol		25154-52-3	220.35	-1.05	Human	Kuiper et al. 1998
β-Sitosterol		83-46-5	414.71	-1000	Human	Kuiper et al. 1997
β-Sitosterol		83-46-5	414.71	-10000	Rat	Blair et al. 2000

Source: EDKB Database 2008 and posterior modifications

Table C-4 Oestrogen potency of selected EDCs relative to oestradiol in ER-CALUX® assay

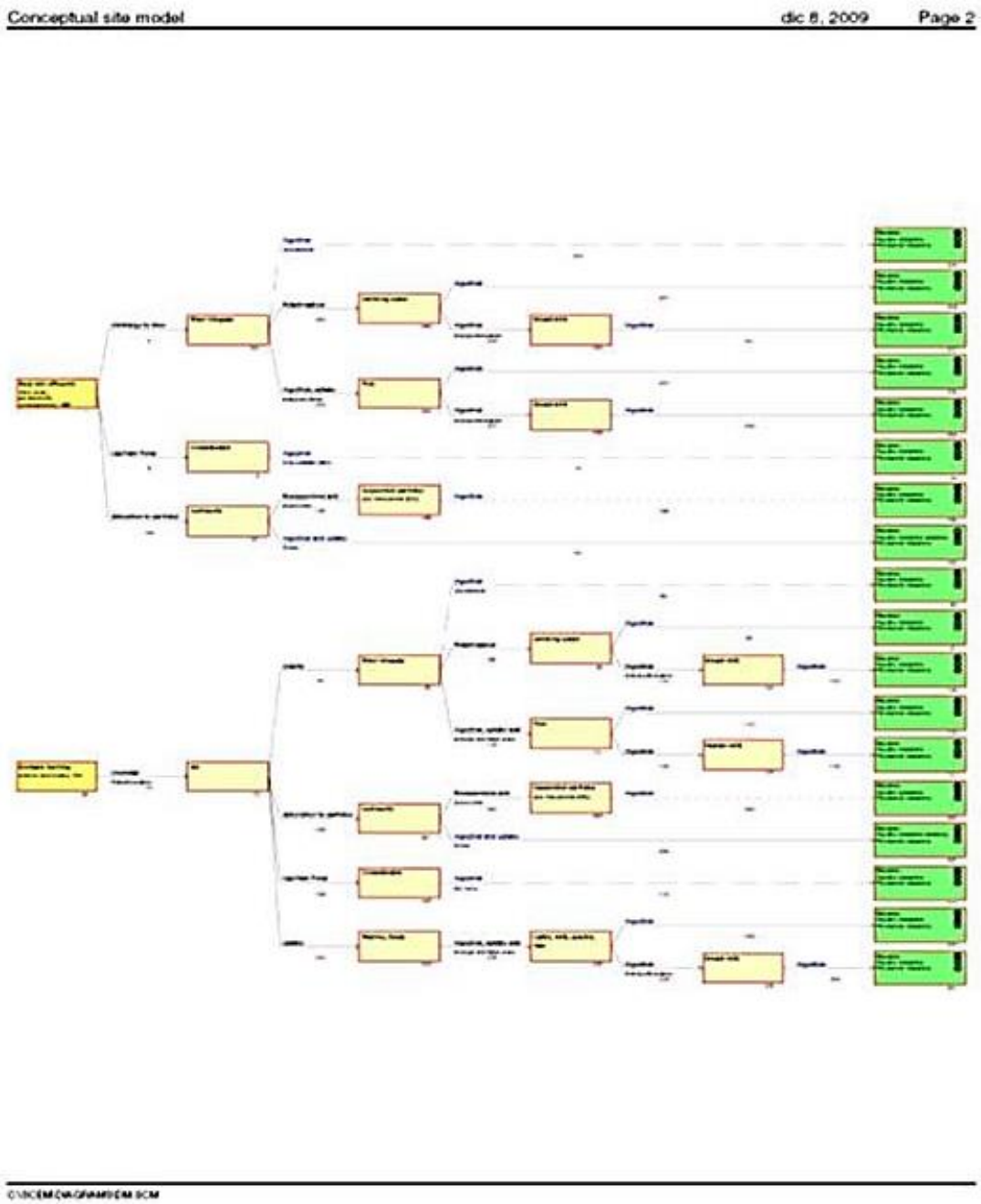
EDC	Structure	EEF
17-ß oestradiol (E2)		1
Ethinyl oestradiol		1.2
4-nonylphenol (NP)		2.3×10^{-5}
NP1EO		3.8×10^{-6}
NP2EO		1.1×10^{-6}
Endosulfan		1.9×10^{-6}
Phytosterol (Genistein)		6.0×10^{-5}

EEF: oestradiol equivalence factor for EDCs in the ER-CALUX bioassay

Based on Dick Vethaak et al. 2005

Appendix D Mathematical, fugacity, foodweb models and data mining

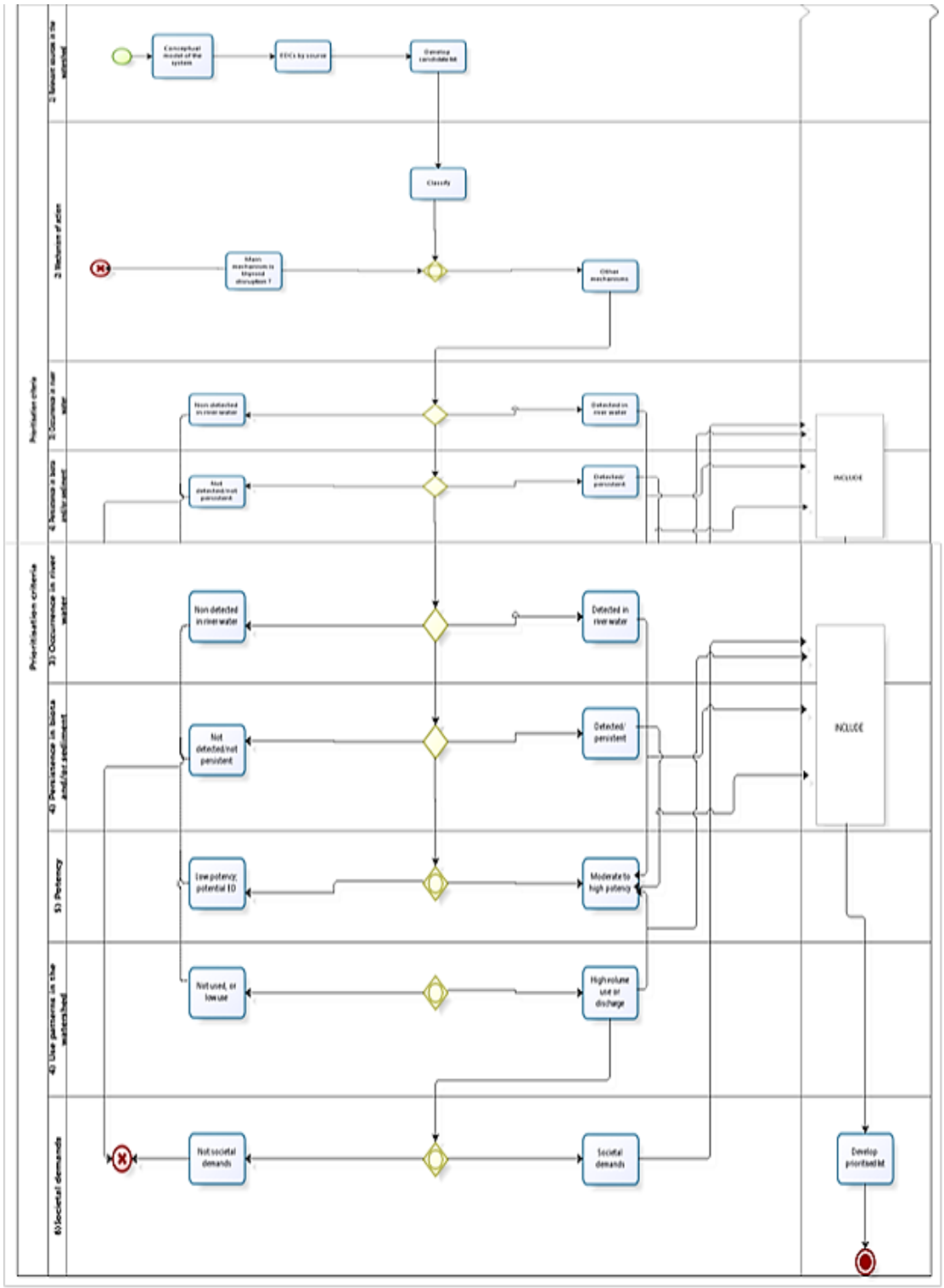
D.1 Conceptual site model construction



D.2

Figure D-1 Conceptual site model for pulp mill source

Model for the logic of choice process of endocrine disruptors



D.3 Relative ranking for EDCs

Table D-1 Level II and Level III risk for nonylphenol, isopimaric acid, endosulfan and glyphosate

Risk based on objective endpoints				
Level II				
CAS	25154	5835267	115297	1071836
Chemical Name	nonylphenol	isopimaric acid	endosulfan	glyphosate
Estimated Emission (Ea) t y^{-1}	0.18	0.01	1.0 E-01	1.0E-01
Critical Emission (Ec) t y^{-1}	1102957	NA	100	NA
RAF (Ea/Ec)	1.6E-07	NA	1.0E-03	NA
Medium of Concern	Sediment	NA	Sediment	NA
Relative Rank	2	NA	1	NA
Persistence (Reaction) days	0.78	0.12	8.3	7.7E-03
Persistence (Reaction) hours	18.6	2.8	199	0.18
Long-Range Transport in Air (La) km	540	85.2	47.1	4.3
Level III				
Critical Emission (Ec) t/y	29.7	NA	1.2	NA
RAF (Ea/Ec)	6.1E-03	NA	8.21E-02	NA
Risk ID Bin	D	NA	C	NA
Medium of Concern	Sediment	NA	Sediment	NA
Relative Rank	2	NA	1	NA

Persistence (Reaction) days	10.4	1.7	13.5	6.3
Persistence (Reaction) hours	250	40.1	323	150.3
Long-Range Transport in Air (La) km	NA	NA	NA	NA
Risk based on effects endpoints				
Level II				
CAS	25154	5835267	115297	1071836
Chemical Name	nonylphenol	isopimaric acid	endosulfan	glyphosate
Estimated Emission (Ea) t/y	0.18	0.01	1.00E-01	1.00E-01
Critical Emission (Ec) t/y	4.2E+07	1.5E+23	433561	3.98E+09
RAF (Ea/Ec)	4.3E-09	6.8E-26	2.3E-07	2.51E-11
Persistence (Reaction) days	0.78	0.12	8.3	7.7E-03
Persistence (Reaction) hours	18.6	2.8	199	0.18
Long-range Transport in Air (La) km	540	85.2	47.1	4.3
Level III				
Critical Emission (Ec) t/y	226	2805103	1054	7617
RAF (Ea/Ec)	8.0E-04	3.6E-09	9.5E-05	1.3E-05
Medium of Concern	Benthic invertebrate	Piscivorous fish	Benthic invertebrate	Piscivorous fish
Relative Rank	1	4	2	3

D.4 RAIDAR parameters

Table D-2 RAIDAR parameters for nonylphenol

Nonylphenol		**References**
CAS Number	25154-52-3	
Molecular weight	220.4	USEPA 2005
Degradation/metabolism half life (h)		
Air	12.5	
Water	391.2	
Sediment	2160	
Soil	314.4	Chang et al. 2007
Biota	312	USEPA(2005)
Humans	3 h elimination in blood, but it persists in fat	Müller 1998
log K_{ow}	4.77	Brix et al. 2001
Henry´s law constant	$4x10^{-5}$ atm-m^3 mol^{-1}	
K air-water	2.00173371	
Vapour pressure (Pa)	1.30E-03 Pa	
pKa	10.28	
Estimated emission rate		
0.18 t y^{-1}		
Estimated: 0.20% concentration in low foam detergents, 1 kg/home, 2 g/month, 30000 inhabitants, 7500 homes		
Ecotoxicity		
Soil	*Eisenia foetida* reproduction 13.7 mg kg^{-1}	Leschber 2006
Sediment	*Neomysis integer* 0.01 µg l^{-1}	Ghekiere et al. 2006
	Potamypyrgus antipodarum 10 µg kg^{-1}	Duft et al. 2003
Water	*Pimephales promelas* 7.4 µg l^{-1}	Miles-Richardson et al. 1999
BCF (*P. promelas*)	100.4	USEPA(2005)

D.5 Food web relationships

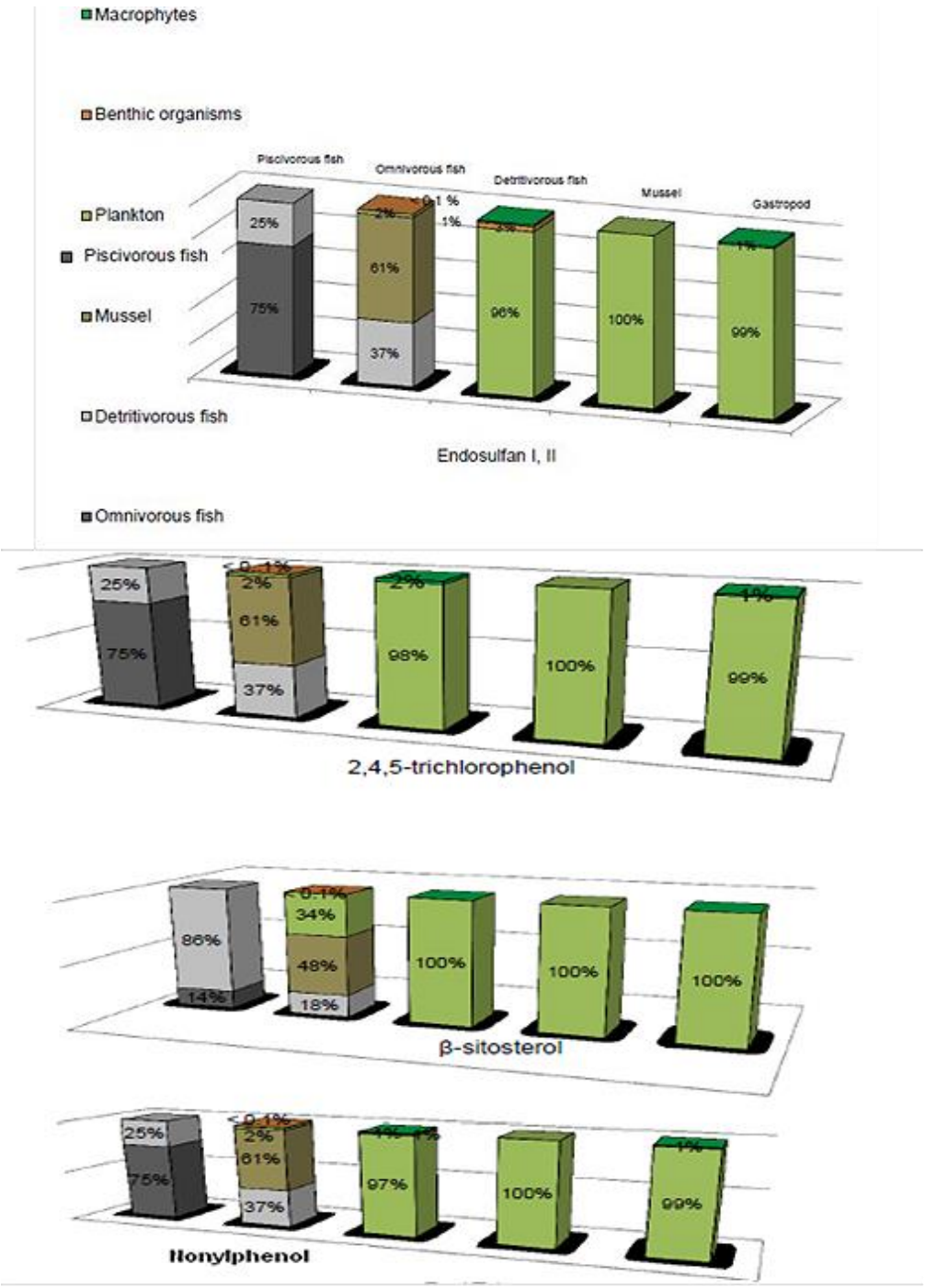


Figure D-2 Distribution of target EDCs through the aquatic food web showing predator-prey relationships

D.6 Artificial neuron networks

Table D-3 Predictions spreadsheet for river water quality at four sites of the river trained and tested in artificial neuron networks for non spefic organic determinands

	DO				PI				COD				BOD			
	R1	R2	R3	R8	R1	R2	R3	R8	R1	R2	R3	R8	R1	R2	R3	R8
Minimum (Train)	8.9	8.8	8.6	8.7	3.6	4.0	4.3	4.0	0.75	0.75	8.2	8.2	1.3	1.1	1.2	1.2
Maximum (Train)	9.8	9.7	9.6	9.6	6.0	5.7	5.6	5.4	19.0	19.0	19.0	19.0	3.6	5.2	5.7	15.7
Mean (Train)	9.3	9.24	9.14	9.28	4.5	4.6	4.8	4.53	6.83	6.83	11.8	11.8	2.13	3.20	3.60	6.43
Standard deviation (Train)	0.44	0.44	0.50	0.53	1.29	0.93	0.72	0.76	10.5	10.5	6.21	6.21	1.27	2.05	2.26	8.05
Minimum (Test)	8.31	8.14	8.34	8.05	4.00	3.90	3.70	3.90	0.751	0.751	8.25	8.25	1.40	1.80	1.30	1.40
Maximum (Test)	8.71	8.46	8.36	8.45	5.20	4.60	5.00	5.30	0.751	0.751	8.25	8.25	4.80	4.40	4.80	2.80
Mean (Test)	8.51	8.30	8.35	8.25	4.60	4.25	4.35	4.60	0.751	0.751	8.25	8.25	3.10	3.10	3.05	2.10
Standard deviation (Test)	0.28	0.23	0.01	0.28	0.85	0.50	0.92	0.99	0.0	0.0	0.0	0.0	2.40	1.84	2.47	0.99
Minimum (Overall)	8.3	8.1	8.3	8.0	3.6	3.9	3.7	3.9	0.75	0.75	8.2	8.2	1.3	1.1	1.2	1.2
Maximum (Overall)	9.8	9.7	9.6	9.6	6.0	5.7	5.6	5.4	19.0	19.0	19.0	19.0	4.8	5.2	5.7	15.7
Mean (Overall)	9.0	8.9	8.8	8.9	4.6	4.5	4.6	4.6	4.4	4.4	10.4	10.4	2.5	3.2	3.4	4.7
Standard deviation (Overall)	0.56	0.62	0.56	0.69	1.00	0.73	0.72	0.73	8.2	8.2	4.8	4.8	1.6	1.7	2.0	6.2

Table D-4 Sensitivity analysis of artificial neural networks for river water classification based on oxygen demand

	Type concentration limits (mg l^{-1})			Confidence levels					Global sensitivity analysis	
	2	**4**	**5**		**Sample**	**Type**				
PI	4	10	15			**2**	**4**	**5**	**Parameter**	**4.MLP 16-3-3**
COD	15	30	40	1	Test	0.34	0.31	0.35	DO(R1)	6
BOD	3	6	10	2	Train	0.56	0.21	0.23	PI(R)	1.29
DO	6	3	2	3	Test	0.29	0.35	0.36	COD(R1)	1.51
PI(R1)	6.0	4.0	3.6	4	Train	0.21	0.58	0.21	BOD(R1)	1.97
COD(R1)	19.0	0.75	0.75	5	Train	0.21	0.23	0.56	PI	2.72
BOD(R1)	1.5	3.6	1.3	Total		3.00	1.00	1.00	COD	2.70
Output	2	2	2	Correct		1.00	1.00	1.00	BOD	2.96
Residuals	Correct	Incorrect	Incorrect	Incorrect		2.00	0.00	0.00	DO	2.63
Type - Confidence levels	0.33	0.33	0.33	Correct (%)		33.0	100.0	100.0		
				Incorrect (%)		66.0	0.00	0.00		

	2	4	5
DO(R2)	8.78	9.66	9.27
DO(R3)	8.63	9.62	9.16
DO(R8)	8.67	9.63	9.54
PI(R2)	5.70	4.20	4.00
PI(R3)	5.60	4.30	4.40
PI(R8)	5.40	4.00	4.20
COD(R2)	19.0	0.75	0.75
COD(R3)	19.0	8.25	8.25
COD(R8)	19.0	8.25	8.25
BOD(R2)	5.20	3.30	1.10
BOD(R3)	5.70	3.90	1.20
BOD(R8)	15.7	2.40	1.20

Samples: Test, train						
		1	2	3	4	5
Sample		Test	Train	Test	Train	Train
N(R1)	Input	2.4	1.7	0.6	0.13	0.01
TP(R1)	Input	0.074	0.026	0.088	0.045	0.049
N	Input	0.5	0.5	1.5	1.5	2
TP	Input	0.1	0.1	0.1	0.1	0.1
Type	Target	1	2	3	4	5
Type - Output	3. MLP 8-3-3	4	2	5	4	5
Type - Residuals	3. MLP 8-3-3		Correct		Correct	Correct
Type		2	4	5		
N(R1)	Input	1.7	0.13	0.01		
TP(R1)	Input	0.026	0.045	0.049		
N	Input	0.5	1.5	2		
TP	Input	0.1	0.1	0.1		
Type	Target	2	4	5		
Type - Output	1. MLP 8-3-3	2	4	5		
Type - Residuals	1. MLP 8-3-3	Correct	Correct	Correct		
Type - Confidence levels	1. MLP 8-3-3	1	1	1		
N(R2)		2.2	0.71	0.45		

N(R3)		2	1.1	0.04
N(R8)		1.8	0.51	0.47
TP(R2)		0.011	0.041	0.059
TP(R3)		0.025	0.051	0.088
TP(R8)		0.066	0.074	0.082
Predictions spreadsheet for type, samples: test				
Type		2	4	5
Type - Target	2	4		5
Type - Output - 1. MLP 8-3-3	2	4		5
Type - Residuals - 1. MLP8-3-3	Correct	Correct		Correct
Type - Confidence levels –	1. MLP 8-3-3	1	1	1
Predictions spreadsheet network: 1.SOFM 5-1 Samples: Train. **Training algorithm Kohonen**		Type - Input		
Position		(1, 1)	(1, 1)	(1, 1)
Activations		1.15	0.83	1.02
N(R1)		1.7	0.13	0.01
N(R2)		2.2	0.71	0.45
N(R3)		2	1.1	0.04
N(R8)		1.8	0.51	0.47
TP(R1)		0.026	0.045	0.049

TP(R2)	0.011	0.041	0.059
TP(R3)	0.025	0.051	0.088
TP(R8)	0.066	0.074	0.082
Index	1		
Net. name	SOFM 5-1		
Training error	1.03		
Test error	1.02		
Training algorithm: Kohonen 1000			

D.7 Clustering of AOX and phenols in river water by the EM algorithm

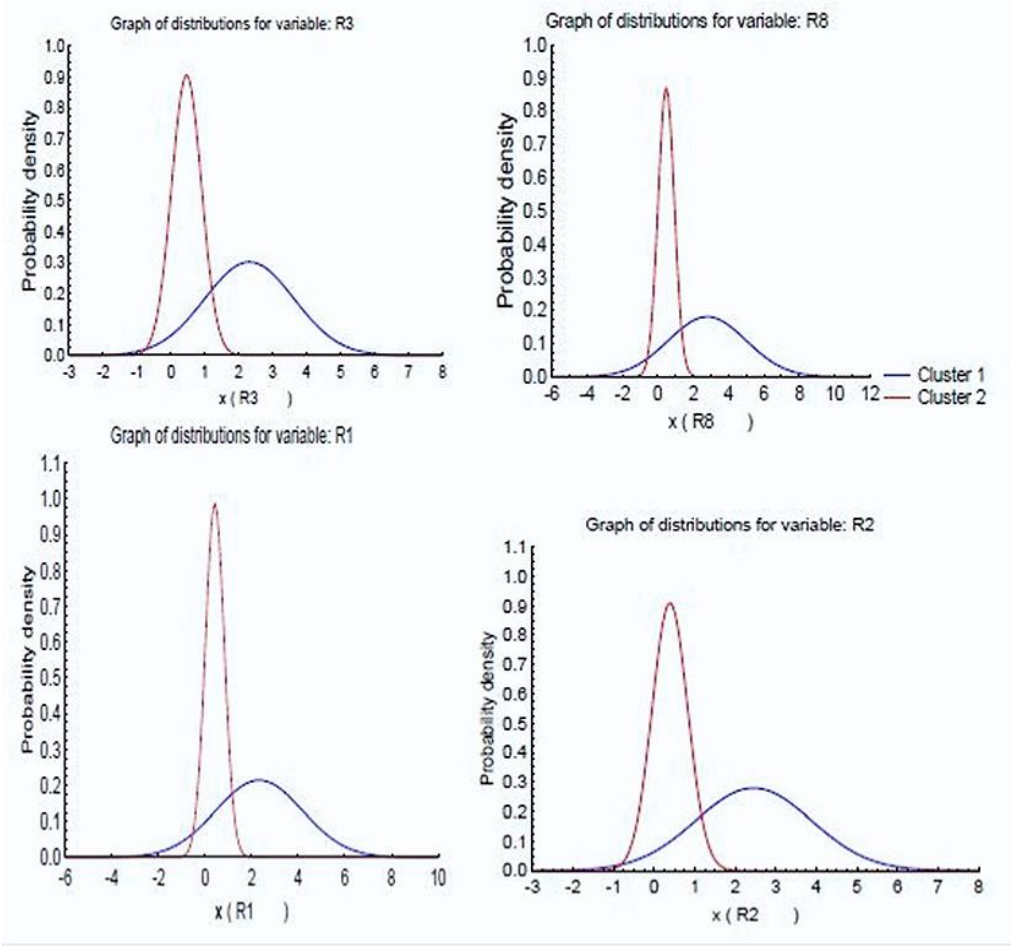


Figure D-3 Distributions for phenols clusters

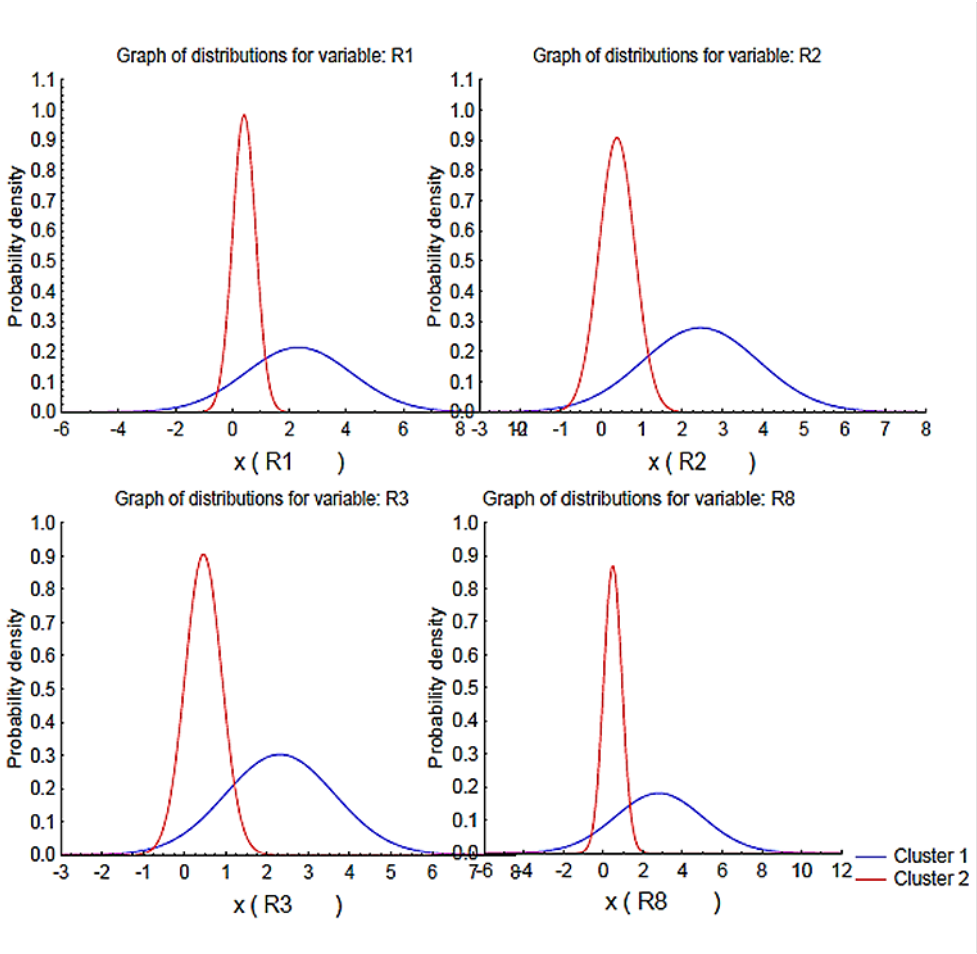


Figure D-4 Distributions for AOX clusters

D.8 Principal component analysis, variables construction

Table D-5 PCA components and variables

Variable description	Variable number	Category value	Component 1	Component 2
Tier 1a	2		-0.235801	0.5611
Tier 2a	3		-0.39625	-0.417127
Tier 3a	4		0.963258	-0.075687
Tier 1b	5		-0.169667	0.435555
Tier 2b	6		-0.169667	0.435555
Tier 3b	7		-0.400104	-0.486345
Nut	1	102	-0.213185	0.520683
OD	1	103	-0.09358	0.182479
Phenols	1	104	-0.264875	-0.290345
Conductivity	1	105	-0.187842	-0.189104
AOX	1	106	-0.187842	-0.189104
NP	1	107	0.482901	-0.042709
Estrogens	1	108	0.138449	-0.005318
Endosulfan	1	109	0.482901	-0.042709
Glyphosate	1	110	0.138449	-0.005318
TCP	1	111	0.138449	-0.005318
Rosin acids	1	112	0.138449	-0.005318
D&F	1	113	0.310675	-0.024014
Sitosterol	1	114	0.138449	-0.005318
Acute toxicity	1	115	-0.033777	0.013378
Sublethal toxicity	1	116	-0.169667	0.435555
Chronic toxicity	1	117	-0.169667	0.435555
Developmental toxicity	1	118	-0.238886	-0.295325
Endocrine disruption effects (diminished gonad size, skewed sex ratio)	1	119	-0.170516	-0.192424
Biomarkers of endocrine disruption (anti-oestrogenicity and oestrogenicity)	1	120	-0.238886	-0.295325

Appendix E Dose-response assessment

In the present integrated framework, the dose-response assessment approaches were bound to the specific characteristics of the experimental datasets. The main dose-response calculations within the environmental component dealt with fish exposure tests end-points of whole mixtures (pulp mill effluent and municipal wastewater). In this case, there were differences in the applicable models, as some of them were describes better by dichotomous functions while others by continuous functions. The limitation of applying the calibration curve done using a single chemical (E2) to whole mixtures is that interaction among antagonistic, synergistic or additive substances might exist. The benchmark dose (BMD) approach using the software BMDS 2.1.2 modeled the dose-response for VTG (VTG) of *Pimephales promelas* fish in the exposure experiment using several statistical models.

Responses were processed as continuous data, with BMR expressed as a change in the mean from control values, where the benchmark is one standard deviation of the control mean, including the uncertainty based on a Bayesian posterior. The effective dose (ED) of E2 for the VTG induction endpoint was estimated at two dose levels: 150 and 450 ng l^{-1}. The best fitting model was chosen based on the nearness of the dose-response curve graph and the tabulated scaled residuals. The polynomial function, but also the power model produced BMDs and BMDLs of similar magnitude (Figure 12-27). The adequacy of the power model signified small scaled residuals, as well as in the best proximity of observed and estimated data points near the BMD. The calculated BMD was of 252 ng l^{-1}.

Table E-1 Dose-response for VTG induction in male fish

Dose ($ng\ l^{-1}$)	No. of observations	Response (VTG induction in male fish liver) ($ng\ g^{-1}$)
0	12	0
0	12	3
0	12	5
0	8	1
0	8	4
0	8	8
150	12	3
150	12	9
150	12	66
450	8	65
450	8	75
450	8	86

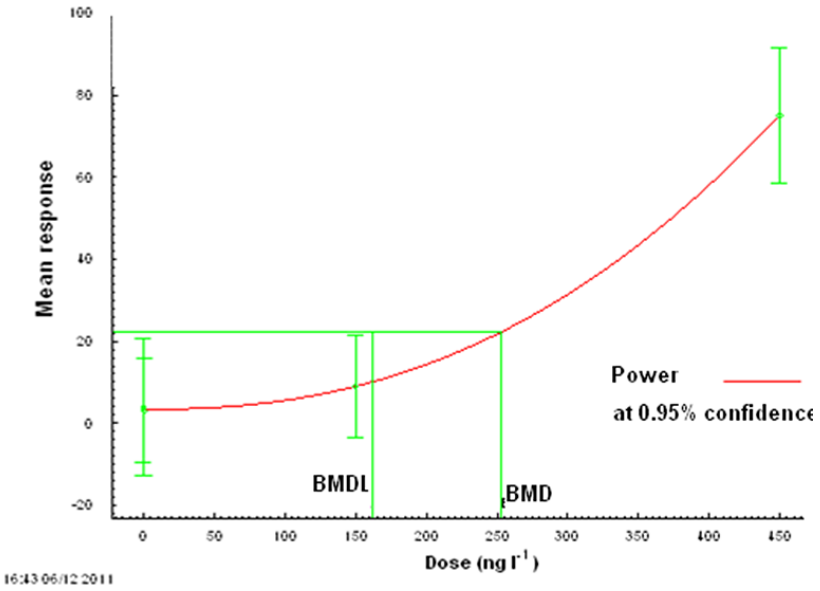


Figure E-1 Power model for benchmark dose of vitellogenin induction in male fish

Table E-2 Endosulfan parameters for dose calculation

Endosulfan		**References**
CAS Number	115-29-7	
Molecular weight	406.9	PAN Pesticides Database – Ch http://www.pesticideinfo.org/Detail_Chemical.jsp?Rec_Id
Water solubility	0.32 mg l^{-1} @ 22º C	Silva and Beauvais 2010
Degradation/metabolism half life (h)		
Air	1.23 h	ATSDR 2011
Water	24	UNEP (2007)
Sediment	105 d	UNEP (2007)
Soil	50 d	E X T O X N E T. Extension Toxicology Network Pesticide Information Profiles http://extoxnet.orst.edu/pips/endosulf.htm
Biota	34 h	ATSDR 2011
log K_{ow}	4.7	Burns et al. 2008
Vapour pressure (Pa)	1200 mPa @ 80 C	EXTOXNET http://extoxnet.orst.edu/pips/endosulf.htm
Ecotoxicity		
Sediment	Midge Based on freshv sediment toxici in porewater (NOAEC=0.35 µ	ECOTOX database, test number Aquatic Test No.733596

Table E-3 Resin acids parameters for dose calculation

Rosin acids (isopimaric acid)		**References**
CAS Number	65997-06-0	Environment Canada 2011
Molecular weight	302.5	
Water solubility	1.18 mg l^{-1}	Peng & Roberts 2000
Degradation/metabolism half life (d)		
Air		
Water	more than 182 d	
Sediment	365 d	
Soil	182 d	
Biota	4 d	
Humans		
log K_{oc}	3.2	
Vapour pressure (Pa)	3.24E-04	
Ecotoxicity		
Endocrine disruption	0.15 mg l^{-1}	http://ecb.jrc.ec.europa.eu/documents/PBT_EVALUATION/PBT_sum080_CAS_61790-51-0.pdf
BCF	180	
pKa	6.44	

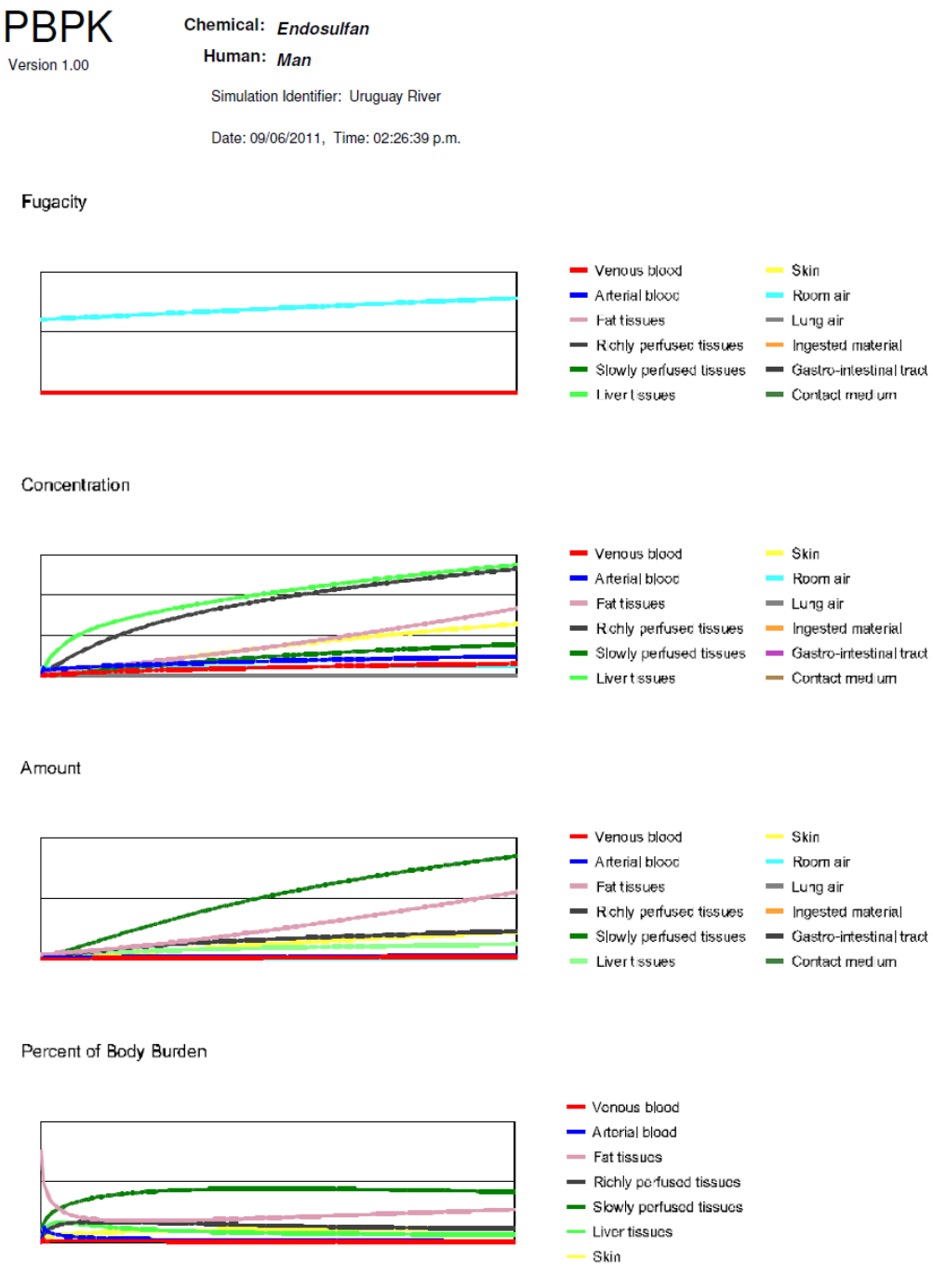
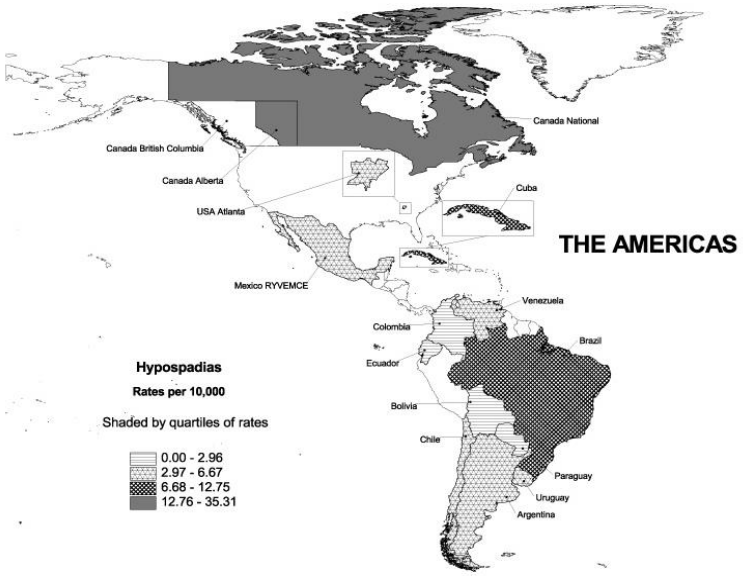


Figure E-2 PBPK model for endosulfan

Appendix F Ecoepidemiology tools to detect endocrine disruption: Preliminary data

The map below shows the reported incidence of hypospadias in Uruguay (Figure F-1), in the range from 2.97-6.67 in 10000 similar than Argentina.



Source: WHO (2003) International Clearinghouse for Birth Defects Monitoring Systems. International Centre for Birth Defects, World Atlas for Birth Defects / International Centre for Birth Defects of the International Clearinghouse for Birth Defects Monitoring Systems, 2nd ed. (http://www.prenatal.tv/lecturas/world%20atlas%20of%20birth%20defects.pdf)

Figure F-1 The Americas incidence rate of hypospadias

Historical data of hypospadias in Uruguay, Chile and Argentina, for the period from 1967-1970 was 5.6 per 10000 (Castilla et al. 1974). In this work, official data were gathered from 2006 to 2009. Surgeries performed at Fray Bentos (Río Negro Department) hospital were done on older patients: 12 year (05/06/2007); 15 years (13/04/2010); 15 years (22/03/2011);10 years (07/10/2011); 6 years (04/05/2007). Table F-1 shows the country situation during 2009 regarding cryptorchidism and hypospadias cases. Patients were operated on at the National Children Hospital, Pereyra Rossel. No records from Río Negro Department, where Fray Bentos is settled, were registered.

Table F-1 Cryptorchidism and hypospadias cases subject to surgery

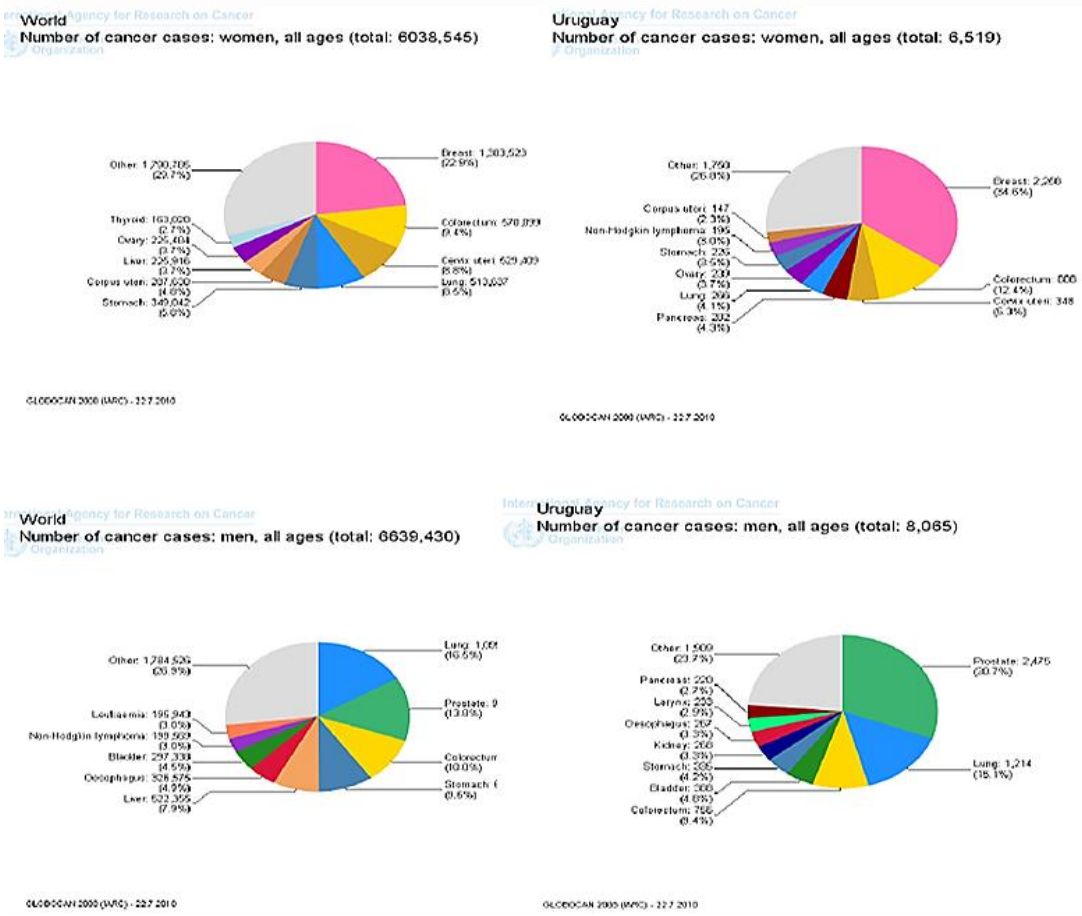
Date	Age	Location	Surgical diagnosis
13/10/2009	5	Rocha	Left cryptorchidism
04/06/2009	3	Montevideo	Right cryptorchidism
22/09/2009	10	Montevideo	Left hydrocele
02/06/2009	4	Canelones	Right cryptorchidism
11/06/2009	1	Montevideo	Right cryptorchidism
29/01/2009	11	Montevideo	Right cryptorchidism
28/10/2009	6	Montevideo	Left cryptorchidism
17/11/2009	10	San jose	Right cryptorchidism
13/03/2009	14	Montevideo	Right cryptorchidism
22/09/2009	8	Montevideo	Right cryptorchidism
29/01/2009	2	Montevideo	Testicular atrophia and epididimus malformation
22/10/2009	11	Rocha	Left cryptorchidism
24/03/2009	2	Montevideo	Left cryptorchidism
18/06/2009	5	Montevideo	Right cryptorchidism
11/06/2009	6	San jose	Left cryptorchidism
05/05/2009	8	Montevideo	Cryptorchidism
05/11/2009	14	Montevideo	Right cryptorchidism
17/03/2009	9	Montevideo	Right cryptorchidism
30/06/2009	9	Artigas	Cryptorchidism
17/02/2009	15	Montevideo	Left cryptorchidism
12/05/2009	10	Montevideo	Bilateral cryptorchidism
16/06/2009	3	Montevideo	Left cryptorchidism
15/10/2009	7	Canelones	Right cryptorchidism
26/05/2009	2	Paysandu	Right cryptorchidism
27/10/2009	12	Montevideo	Right cryptorchidism
02/06/2009	5	Canelones	Left cryptorchidism
31/03/2009	6	Montevideo	Right cryptorchidism
23/06/2009	5	Canelones	Left cryptorchidism
19/03/2009	7	Montevideo	Right cryptorchidism
03/11/2009	12	San jose	Right cryptorchidism
22/09/2009	10	Montevideo	Right cryptorchidism
17/11/2009	3	Montevideo	Left cryptorchidism
22/09/2009	7	Montevideo	Right cryptorchidism
28/07/2009	1	Canelones	Left cryptorchidism

Date	Age	Location	Surgical diagnosis
16/06/2009	2	Treinta y Tres	Right cryptorchidism
17/03/2009	3	Canelones	Bilateral cryptorchidism
26/02/2009	8	Montevideo	Right cryptorchidism
23/06/2009	12	Canelones	Right cryptorchidism
02/04/2009	2	Montevideo	Left cryptorchidism
10/03/2009	11	Montevideo	Right cryptorchidism, severe
05/11/2009	5	Montevideo	Right cryptorchidism
12/05/2009	12	Canelones	Bilateral cryptorchidism
08/10/2009	7	Montevideo	Bilateral cryptorchidism
14/10/2009	11	Montevideo	Cryptorchidism
26/03/2009	11	Montevideo	Left cryptorchidism
31/03/2009	9	Canelones	Right cryptorchidism
11/08/2009	5	Montevideo	Right testicular agenesis
09/06/2009	7	San jose	Testicular agenesis
17/03/2009	3	Montevideo	Left cryptorchidism
09/06/2009	10	Rivera	Bilateral cryptorchidism
24/11/2009	2	San jose	Left cryptorchidism
19/03/2009	5	Montevideo	Left cryptorchidism
28/05/2009	1	Canelones	Right cryptorchidism
14/04/2009	13	Canelones	Right cryptorchidism
28/10/2009	4	Montevideo	Right cryptorchidism
18/02/2009	9	Montevideo	Hypospadia
19/08/2009	2	Rocha	Hypospadia
30/09/2009	9	Lavalleja	Hypospadia
21/10/2009	3	Maldonado	Hypospadia
16/12/2009	6	Canelones	Hypospadia
07/10/2009	8	Colonia	Hypospadia, counter clock-wise rotation
17/03/2009	9	Montevideo	Right cryptorchidism
30/06/2009	9	Artigas	Cryptorchidism
17/02/2009	15	Montevideo	Left cryptorchidism
12/05/2009	10	Montevideo	Bilateral cryptorchidism
16/06/2009	3	Montevideo	Left cryptorchidism
15/10/2009	7	Canelones	Right cryptorchidism
26/05/2009	2	Paysandu	Right cryptorchidism
27/10/2009	12	Montevideo	Right cryptorchidism
02/06/2009	5	Canelones	Left cryptorchidism

Date	Age	Location	Surgical diagnosis
31/03/2009	6	Montevideo	Right cryptorchidism
23/06/2009	5	Canelones	Left cryptorchidism
19/03/2009	7	Montevideo	Right cryptorchidism
03/11/2009	12	San jose	Right cryptorchidism
22/09/2009	10	Montevideo	Right cryptorchidism
17/11/2009	3	Montevideo	Left cryptorchidism
22/09/2009	7	Montevideo	Right cryptorchidism
28/07/2009	1	Canelones	Left cryptorchidism
16/06/2009	2	Treinta y Tres	Right cryptorchidism
17/03/2009	3	Canelones	Bilateral cryptorchidism
26/02/2009	8	Montevideo	Right cryptorchidism
23/06/2009	12	Canelones	Right cryptorchidism
02/04/2009	2	Montevideo	Left cryptorchidism
10/03/2009	11	Montevideo	Right cryptorchidism severe
05/11/2009	5	Montevideo	Right cryptorchidism
12/05/2009	12	Canelones	Bilateral cryptorchidism
08/10/2009	7	Montevideo	Bilateral cryptorchidism
14/10/2009	11	Montevideo	Cryptorchidism
26/03/2009	11	Montevideo	Left cryptorchidism
31/03/2009	9	Canelones	Right cryptorchidism
11/08/2009	5	Montevideo	Right testicular agenesis
09/06/2009	7	San jose	Testicular agenesis
17/03/2009	3	Montevideo	Left cryptorchidism
09/06/2009	10	Rivera	Bilateral cryptorchidism
24/11/2009	2	San jose	Left cryptorchidism
19/03/2009	5	Montevideo	Left cryptorchidism
28/05/2009	1	Canelones	Right cryptorchidism
14/04/2009	13	Canelones	Right cryptorchidism
28/10/2009	4	Montevideo	Right cryptorchidism
18/02/2009	9	Montevideo	Hypospadia
19/08/2009	2	Rocha	Hypospadia
30/09/2009	9	Lavalleja	Hypospadia
21/10/2009	3	Maldonado	Hypospadia
16/12/2009	6	Canelones	Hypospadia
07/10/2009	8	Colonia	Hypospadia, counter clock wise rotation

F.1 Cancer of the reproductive organs

Even though no direct causal link can be assigned exclusively to EDCs, cancer of reproductive organs is among the associated effects. Therefore, an overview of the situation in Uruguay was done assessing international databases. The WHO (2008) epidemiology database on cancer incidence (GLOBOCAN 2008), was accessed. Elevated cancer levels are observed in all the country by comparison to world rates regarding breast (34.6% compared to 22.9 % globally) and prostate cancer types (30.7% compared to 13.8 globally). The charts below show this clearly (Figures 2-4and 2-5). The pink areas show breast cancer and green areas prostate cancer rates, respectively.



Source: World Health Organisation (2008) GLOBOCAN, Cancer Incidence and Mortality Worldwide in 2008 International Agency for Research in Cancer (http://globocan.iarc.fr/)

Figure F-2 Relative incidence of breast and prostate cancer in Uruguay in relation to other cancer types and compared with the World incidences

Appendix G Uncertainty assessment

Table G-1 Uncertainty sources

Effects in populations	
Uncertainty sources	**Limitations and uncertainties**
Cause-effect relationships not completely clear	Uncertainty in database on observed effects and potential etiologies At risk population is small (only 24000 inh), so, the rate of malformations could be too low to detect Statistical power; fishing gear; analytical uncertainties
Evidences: reproductive and developmental effects in fish, as for example diminished gonad weight near pulp mill discharges; effects in benthic biota and crustaceans	
Chemical classes, potencies and mode of action	
List of recognised EDCs comprising chlorinated organic compounds, polyaromatic hydrocarbons, steroids, BPA, some herbicides, NP	Lack of exhaustiveness of the EDC list (pharmaceuticals and cosmetics, etc.) Latency of developmental effects may be undetected until sexual maturity
Tools for prioritisation of chemicals	
Biocomputational tools Rate of false negatives	No predictive QSAR programs for all the compounds. Methods should have low rate of false negatives and false positives
Dose-response characteristics in the low-dose region	
Non monotonic inverted U-shaped dose-response curves reproducibility not tested at a sufficient statistical robustness. Sample variability Organism sensitivity	This effect may be a very low frequency one that may appear when exposing organisms to a sample that may change in composition in time.

Table G- 1 (*Continued*)

Methods to evaluate ED	
Non-monotonic dose responses Assumption of monotonic curves for NOAEL derivation not always the case Intrinsic analytical uncertainty	Some hormonal systems (thyroid) may not be adequately represented Not all possible EDCs and mixtures are elucidated
Extrapolation	
Difficulty to extrapolate across species and from *in vitro* to *in vivo* assays Difficulty to extrapolate effects at high exposure levels to those with non-monotonic dose-responses	Inconsistent information from different sources with different values for toxicity Benchmark dose: not possible for every chemical. Scarce information on the ED endpoint in databases No mammals included
Effects of exposure to multiple EDCs	
Application of TEQ and TEF developed for mixtures of AhR ligands	Not easy to apply to oestrogens, anti-androgens, or other EDC-mediated modes of action
Exposure to mixtures of EDC working through a common mechanism	
Additivity for oestrogenic EDCs postulated	Not database for relative potencies and lack of sufficient evidence on additivity Should be demonstrated but not easy to predict with different MOE interactions
Degree of human and wildlife exposure to EDCs	
Potential EDCs without complete data to be able to assess exposure	No pharmaceuticals other than steroidal hormones are included
Major sources and environmental fates of EDCs	
Sewage treatment processes Fungicides and herbicides Pulp mill effluents Human diet potential sources of EDCs (phytoestrogens) Phthalates, bisphenol A plasticisers	Fungicides not included Herbicides: only glyphosate Other pesticides are used in the area Unknown substances Scarce data of phthalates and bisphenol included because of cost reasons and prioritisation

Table G-2 Recommended tools and adequacy of the framework

Recommended tools	Tools used within the framework
Effects in populations	
Ecoepidemiology approach to determine causation	Screening-level ecopidemiology for human health risk component Fish survey for biomonitoring for environmental risk component
Chemical classes, potencies and mode of action	
Screening battery representing critically sensitive life stages and all mechanisms, composed from receptor binding assays to animal studies with rats, frogs and fish to test for more substances Prenatal development tests at most sensitive life stages	Tiered testing of both chemicals and effects Battery of bioassays with *in vitro* screens from receptor binding assays to *in vivo* crustacean and fish Embryo-larval fish tests; use of epidemiology data of neonate human beings
Tools for prioritisation of chemicals to evaluate	
Receptor binding *in vitro* assays	ER CALUX; AR CALUX
Dose-response characteristics in the low-dose region	
End points included in newer testing guidelines for reproductive toxicity If effects are below the presumed NOAEL, the design of safety studies should include a wider range of dose levels	Thyroid system disruptores were not included Hormesis and non monotonic dose-response curves observed with embryo-larval tests, crustacean reproduction and amphipod growth sub-lethal tests Exposure assessment with sediment elutriates at low environmental doses and with real environmental samples at wide range of dilutions
Evaluation of effects of exposure to multiple EDCs	
TEQ used for dioxins mixtures	TEQ for oestrogens relative to oestradiol TEQ for dioxins

Table G-2 (*Continued*)

Recommended tools	Tools used within the framework
Extrapolation	
Mechanistic approaches at the molecular level (e.g., gene expression) Weight of evidence principle using tiered battery of tests to contemplate most mechanisms of action	PCR gene expression of VTG in exposed fish Recombinant yeast expressing human ER ER- and AR-binding assays Battery of tests from gene, molecular, tissue, functional and to population in several biological trophic levels
Effects of exposure to multiple EDCs	
TEQ for dioxins and furans. Some prior experiences on oestrogens	TEQ for oestrogens relative to oestradiol and for dioxins and furans
Degree of human and wildlife exposure to EDCs	
In the case of this research resin acids and glyphosate are potential EDCs	Human receptors included in the conceptual model as part of environmental receptors
Major sources and environmental fates of EDCs	
Study from literature review	After literature review (Chapter 2), scenario and sources (Chapter 5): Major sewage contaminants with ED: NP and ethoxylates, steroids Pulp mill effluent main EDCs (chlorophenols, phytosterols and resin acids) dioxins and furans, PCBs and PAHs also included. Agriculture chemicals
Exposure to mixtures of EDC working through a common mechanism	
Several approaches, such as tests with mixtures or whole sample testing	Whole sample testing, tiered approach Cob-web diagrams for combination of risks

G.1 Vulnerability, high-risk events, and uncertainty on the causes of fish mortality

One of the most obvious high risk events that are included in a vulnerability analysis as they could reach to catastrophic dimensions are fish mortality events. The most frequent cause is reduced oxygen in the water, which in turn may be due to factors such as drought, algae bloom, overpopulation, or a sustained increase in water temperature, but also to sudden temperature drops, infectious diseases or toxicity. Fish mortality events may be the first visible signs of environmental stress and may have a direct impact on other uses of the water such as for water abstraction. Several cases of fish mortality were evidenced in the last years in the Uruguay River. Several fish kills events were reported in locations on both margins of the river during 2010 (19/07 Ñandubaysal (Gualeguaychú - Argentina) 300 fishes; 21/07 La Concordia: 10000 fishes; 21/07 Las Brisas: 800 fishes; 28/07 Puerto Luis 100 fishes; 29/07 Federación: 1500; 5/08 Bridge: 300; 10/08: Ñandubaysal: 12000. Some fishes were found dead in streams in the basin in several occasions and the causative agent was linked to endosulfan (Piazza et al. 2011) and other pesticides (Pazos 2008, Ríos et al. 2010). In some of these events, residue levels in fish tissue exceeded permitted levels tenfold

Even when natural catastrophes are not assignable to humans in most cases, as some global phenomena like climate change, for example, could have an indirect human influence. In this regard, a major fish kill, of about 23000 specimens, most of them juvenile, of several species occurred in August 2010. The official report linked climate change to this event (cold temperatures, 6-7°C, near the minimum tolerable temperature for warm climate fish) (CARU 2010). However, EDCs could have also played a role, as, in this thesis, tissue residues of several EDCs were measured in fish caught just before this event. A decrease of hepcidin by oestradiol, an antimicrobial peptide and iron-regulatory enzyme also found in mammals as iron modulator has been postulated as responsible of a decrease in immunity and homeostasis of fish (Robertson et al. 2009, Robertson 2011).

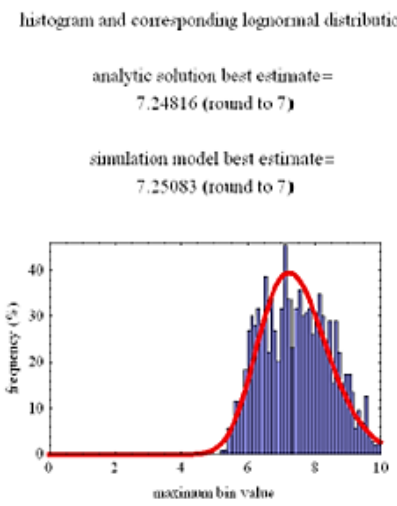
Appendix H Additional information on the development of the radar diagram and Fermi solution

Table H-1 Rating of endocrine disrupting chemicals

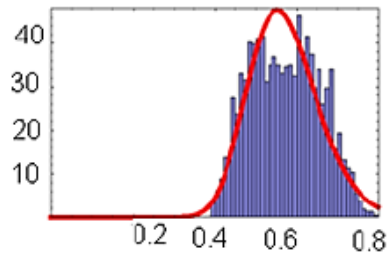
EDC		Main action	Likelihood	Frequency of detection	Impact severity	Factor		
Nonylphenol	+	Oestrogenic	1	1	8.0	8.0	0.8	0.8
Endosulfan	+	Anti-androgenic	0.3	0.5	9.0	1.4	0.14	0.14
Glyphosate	+	Anti androgenic	0.2	0.5	7.0	0.7	0.07	
2,4,5-TCP	+	Oestrogenic	0.5	0.2	7.0	0.7	0.07	
PAHs	-	Anti-oestrogenic, AhR mediated	0.5	0.1	8.0	0.4	0.04	0.04
PCBs	-	Anti-oestrogenic, AhR mediated	0.2	0.2	9.0	0.4	0.04	0.04
Dioxins & furans	-	Anti-oestrogenic, AhR mediated	0.2	0.1	10.0	0.2	0.02	0.02
Isopimaric acid	+	Oestrogenic	0.5	0.2	5.0	0.5	0.05	
β-Sitosterol	+	Anti-androgenic	0.8	0.4	5.0	1.6	0.16	0.16
RF= | P x F x I|								

Keys	Overall risk
Impact:	Very low risk
0 to 10	0 to 1
Likelihood:	Low risk 1
0 to 1	to 2
Frequency: times/total, base 1	Medium risk
	2 to 4
	High risk
	4 to 6
	Very high risk
	6 to 8
	Extreme 8 to 10

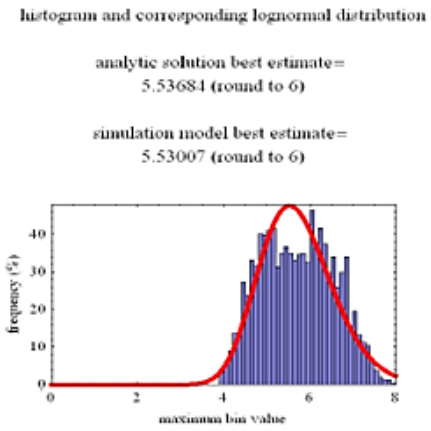
H.1 Fermi solution histograms and lognormal distribution for endocrine disruption



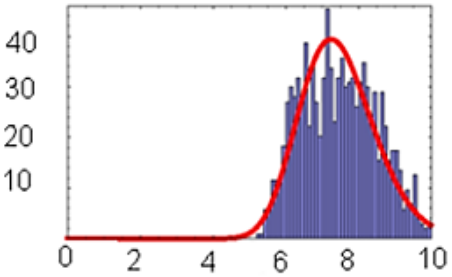
Oestrogenic



Anti-androgenic



Androgenic



Anti-oestrogenic

Expanded Fermi Solution for Risk Assessment, Wolfram Demostration Project (http://demonstrations.wolfram.com/ExpandedFermiSolutionForRiskAssessment/)

Figure H-1 Lognormal distributions and Fermi solutions for endocrine disruptive modes of action