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The use of integrative approaches to characterize new mechanisms of toxicity of pollutants in the aquatic environment

PhD dissertation

(collection of published articles)

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Abbreviations and Acronyms

AChE	AcethylcholinEsterase
ACN	Acetonitrile
ACON	Aconitase
ACRID	Aacridone
ADREN	β2-adrenergic agonists to treat asthma
AhR-RYA	Aryl Hydrocarbon Receptor – Recombinant Yeast Assay
AM	Acquisition Mode
AMPHET	Amphetamine-like compounds and metabolites
ANGIO	Angiotensin II receptor antagonist
ANSAID	NSAID
ANTHEML	Anthelmintic veterinarian compounds
ANTIB	Antibiotics
ANTIHIST	Tricyclic Antihistamins
AOP	Adverse Outcome Pathway
AOPIATES	Opiates
APE	Alkylphenols
APYR	Antipyretic
ASTM	American Society for Testing Material
BAPeq	Benzo[a]pyrene equivalents
BBLCK	β-blockers
BDA	Bioassay-directed Analyses
bdl	Below Detection Limit
BPA	Bisphenol A
CAFF	Caffeine
CAS	Chemical Abstract Service
CAT	Catalase
CbE	CarboxylEsterase
cDNA	Complementary Deoxyribonucleic Acid
CDNB	1-chloro-2,4-dinitrobenzene
CE	Collision Energy
cEC	Contaminant of Emerging Concern
CIMET	Histamine H2-receptor antagonist
CLOPID	Thienopyridine class antiplatelet agent
COCAIN	Cocaine and metabolites
CT	Confirmation Transition
CV	Cone Voltage
d.w.	Dry Weight
DEXTH	Dexamethasone, anti-inflammatory glucocorticoid steroid
DIUREAS	Sulfonylureas
DIURET	Diuretics

DNA	Deoxyribonucleic Acid
DNAd	DNA damage
E2	Estrogenic compounds
E2Eq	Estradiol equivalents
EC	European Commission
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ECHA	European Chemicals Agency
EcR	Ecdysteroid Receptor
EDA	Effect-directed analyses
EDC	Endocrine Disruptors Compound
EDTA	Ethylenediaminetetraacetic Acid
EI	Electron Impact
ERA	Environmental risk assessment
ER-RYA	Estrogenic Receptor – Recombinant Yeast Assay
ESI	Electrospray Ionisation
ESI-	Negative Electrospray Ionization
ESI+	Positive Electrospray Ionization
EU	European Union
FAA	Fumarylacetoacetate
FED	Feeding inhibition rate
FIA	Flow Injection Analysis
FIBRAT	Fibrates
FZOLE	Azole fungicides
GABA	*gamma*-Aminobutyric acid
GC	Gas chromatography
GC-MS/MS	Gas Chromatography coupled to Tandem Mass Spectrometry
GST	Glutathione-S-Transferase
H	Shannon's Diversity Index
HAB	Harmful Algal Bloom
HPLC	High-Performance liquid chromatography
HSP70	Heat Shock Protein 70
HTRIAZ	Triazines
HTS	High-Throughput Screening
I	Phototactic Index
ICP-MS	Inductively Coupled Plasma Mass Spectroscopy
IDH	Isocytrate Dehydrogenase A
IDL	Instrumental Detection Limit
IOPROM	X-ray contrast media
KE	Key Event
L	Lower compartment
LC	Liquid Chromatography
LC-ESI-MS/MS	Liquid Chromatography–Electrospray Ionisation-Tandem Mass Spectrometry
LC-LC-MS/MS	Pre-concentration Liquid Chromatography-Tandem Mass Spectrometry

LC-MS	Liquid Chromatography coupled to Mass Spectrometry
LC-MS/MS	Liquid Chromatography coupled to Tandem Mass Spectrometry
LC–QTOF-MS	Liquid chromatography quadrupole time-of-flight mass spectrometry
LDH	Lactate Dehydrogenase
LOD	Limit of Detection
LoE	Lines of Evidence
LOQ	Limit of Quantification
LPO	Lipid Peroxidation
M	Medium compartment
m/z	Mass to charge ratio
MDL	Method Detection Limit
MeOH	Methanol
MIE	Molecular Initiating Event
MIH	Molt Inhibition Hormone
MoA	Mode of Action
mRNA	Messenger Ribonucleic Acid
MRP4	Multidrug Resistance Protein 4
MS/MS	Tandem Mass Spectrometry
MT2	Metallothioneins
NGS	Next Generation Sequencing
NI	Negative Ionization
NOEC	No Observable Effect Concentration
O2	Dissolved Oxygen
OA	Okadaic Acid
OECD	Organisation for Economic Co-operation and Development
OPIOIDS	Illicit drugs, opiods/opiates and metabolites
OPS	Organophosphorous and carbamate insecticides
PAHs	Polycyclic Aromatic Hydrocarbons
PARABEN	Parabens
PC1	Principal Component 1
PC2	Principal Component 2
PC3	Principal Component 3
PC4	Principal Component 4
PC5	Principal Component 5
PCA	Principal Component Analysis
PEC	Predicted Environmental Concentration
PFCs	Perfluorinated compounds
PFR	Phosphate Ester Flame Retardants
Pgp	P-glycoprotein
PI	Parental Ion
PLS	Partial Least Square Projections to Latent Structures regression
PNEC	Predicted No Effect Concentration
POTHER	Other pesticides

PP1	Protein Phosphatase type 1
PP2A	Protein Phosphatase type 2A
PPCP	Pharmaceutical and Personal Care Products
PrI	Precursor Ion
qPCR	Quantitative Polymerase Chain Reaction
QqQ	Triple Quadrupole
(Q)SAR	Quantitative structure-activity relationship
QT	Quatification Transition
r	Intrinsic rate of population growth
REACH	Registration, Evaluation, Authorization and Restriction of Chemical substances
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT	Retention Time
RXR	Retinoid X Receptor
RYA	Recombinant Yeast Assay
S	Species Richness
S/N	Signal to Noise
SD	Standard Deviation
SEROT	Psychiatric drugs acting on serotonin
SERT	Serotonin transporter
SPE	Solid Phase Extraction
SRM	Selected Reaction Monitoring
SS	Suspended Solids
SSRI	Selective Serotonin Reuptake Inhibitors
STATIN	Statins
STP	Sewage Treatment Plant
T	Temperature
TAMSUL	Selective α1 receptor antagonist
TDNP	Tablas de Daimiel National Park
TEQ	Toxic Equivalents
THg	Total Mercury
THIO	Thiolase
TIE	Toxicity Identification Evaluation
TRIAZOLES	Triazoles
U	Upper compartment
UGP	UDP-glucose Pyrophosphorylase
US-EPA	United States Environmental Protection Agency
UTM	Universal Transverse Mercator
VTG	Vitellogenin E
WARF	Warfarin
WFD	Water Framework Directive
WoE	Weight of Evidence
WWTP	Waste Water Treatment Plant

Summary

The last century showed our need and dependency on a full range of chemicals in Pharmaceutical and Personal Care Products (PPCP), industrial and/or agricultural applications, although the environmental and human hazards of most of them were not considered. Since the late 1970s, the EU started priority lists of chemicals of major concern and from the mid-1980s it was made compulsory to set up a comprehensive Environmental Risk Assessment (ERA) for all new commercialized chemicals. Among all natural systems, one of the most affected is the aquatic ecosystem, as it stands as the final destination for most anthropogenic contaminants. Its status represents a major concern considering that a good and appropriate water quality is fundamental for a sustainable development of human society and maintenance of the ecosystem biodiversity and stability. Some chemicals are highly persistent, some are applied repeatedly or continuously, or directly as mixtures, thus leading to a complex cocktail of very heterogeneous compounds that may provoke significant toxic effects. For this reason, there is a need to develop innovative integrated approaches for the monitoring and assessment of the quality of surface water bodies as well as the understanding of the underlying mechanisms of toxicity. Among the available techniques to assess effects, a combined use of chemical analyses and biological responses, e.g. biochemical, physiological and molecular, is a sound procedure for detecting the impact of anthropogenic contaminants in freshwater systems.

The overall goal of this thesis is the characterization of novel mechanisms of toxicity of contaminants in the aquatic ecosystem both in the field and in the laboratory. Field studies bearing different problematics were considered to specifically address three specific objectives, whereas a study conducted in the laboratory evaluating sublethal effects of pharmaceuticals at relevant environmental concentrations was pursued as fourth and final objective.

In chapter 2 (Rivetti et al., 2015a), a comparative study in three rivers of Spain showing different sources of anthropogenic pollution was performed, combining genetic, biochemical and individual biomarkers in *Daphnia magna*. Individuals

were transplanted across 12 sites from three Spanish river basins (Llobregat, Ebro, Jucar) and gene transcription, feeding rates and enzymatical responses in the field were assessed and compared with those obtained in re-constituted water treatments, spiked with organic extracts obtained from water samples collected at the same locations and sampling periods. Up to 166 trace contaminants were detected in water and classified by their mode of action into 45 groups that included metals, pharmaceuticals, pesticides, illicit drugs, and other industrial compounds. Biodiversity and species richness at the same locations were also evaluated. Both physico-chemical water parameters and transcription patterns of 13 genes encoding for general stress, metabolism and energy processes, molting and xenobiotic transporters corroborate phenotypic responses, differentiating sites within and across river basins. Principal Component Analyses (PCA) and Partial Least Square (PLS) regression analyses indicated that *in situ* responses of most genes, biomarkers and biodiversity indexes were affected by distinct environmental factors. Overall, this work allowed to test the usefulness of using transcriptomic responses of *D. magna* genes in the detection and identification of different types of environmental stressors in the field in transplanted organisms.

In chapter 3 (Rivetti et al., 2015b) and 4 (Rivetti et al., 2015c), it was developed a study of forensic ecotoxicology to unravel the major toxic components in a superfund site in Ebro River (Spain) during an unusual period of prolonged flushing flows. The study aimed to use a Toxicity Identificaton Evaluation (TIE) approach, using a combination of toxicity assays and chemical analytical/fractionation methods. Post-exposure feeding rates of *Daphnia magna* were used to assess toxic effects of whole, filtered and re-constituted water samples. Organochlorine content of suspended material was analyzed by solid phase extraction (SPE) and GC–MS/MS, whereas presence of mercury was also determined for all sites by use of an Advance Mercury Analyzer (AMA). Unexpectedly, observed toxic effects did not correspond to the analyzed contaminants and were greater upstream the superfund site than downstream. In order to test other potential sources of toxicity i.e. toxins produced by cyanobacteria present in upstream river reservoirs, a new liquid

chromatography tandem mass spectrometry (LC-MS/MS) method for fast determination of five toxins in suspended material and sediment samples was developed (chapter 3). The developed analytical method was successfully applied to analyze the presence of toxins in suspended solids and sediment from Ebro River (NE Spain) and Ebro Delta associated lagoons and both anatoxin-a and okadaic acid were detected but at different locations. The residue levels of the cyanotoxin anatoxin-a were correlated with observed feeding inhibition responses and confirmed in the lab using anatoxin-a produced by *Planktothrix agardhii*, a filamentous cyanobacteria.

In chapter 5 (Rivetti et al., 2017), a study of water quality in a Spanish natural reserve (Tabla de Daimiel, TDNP) and associated lagoons was implemented in order to analyze the potential risk of the constant input of micropollutants for the resident wildlife. We sampled 12 locations in TDNP and in the nearby Navaseca Pond during 2013, and performed a series of *in vivo* and *in vitro* bioassays, including *Daphnia magna* post-exposure feeding inhibition and recombinant yeast-based assays (RYA) for dioxin-like and estrogenic activities. Toxicity results were compared with the chemical analysis of PAHs and estrogenic compounds performed by GC-MS/MS and LC-MS/MS, respectively. Results showed a current good chemical status of TDNP, but threatened by both the inflow of wastewater treatment plants effluents from its river tributaries and by direct sewage discharges, as it occurs in the Navaseca Pond.

In chapter 6 (Rivetti et al., 2016) the hypothesis that different families of neuro-active pharmaceuticals may lead to similar phenotypic responses in *D. magna* was tested, focusing on alterations in reproduction and behavioral responses when exposed to low environmental relevant concentrations. Selected pharmaceuticals were widely prescribed compounds detected at considerable levels in the environment (ηg to few μg/L), namely carbamazepine, diazepam, propranolol. Fluoxetine was also included in behavioral assays. The three tested neuro-active pharmaceuticals were able to enhance reproduction at 1 ηg/L (propranolol), 0.1 μg/L (diazepam) and 1 μg/L (carbamazepine). Fluoxetine, carbamazepine and diazepam increased positive phototactic behavior at concentrations ranging from 1, 10 and 100 ηg/L, respectively.

Published papers presented in this thesis:

Rivetti, C., Campos, B., Faria, M., Català, N. D. C., Malik, A., Muñoz, I., Tauler R., Soares A., Osorio V., Pérez S., Gorga M., Petrovic M., Mastroianni N., López de Alda M., Masiá A., Campo J., Picó Y., Guasc H., Barceló D., Barata C. (2015a). Transcriptomic, biochemical and individual markers in transplanted Daphnia magna to characterize impacts in the field. Science of the Total Environment, 503, 200-212.

Rivetti, C., Gómez-Canela, C., Lacorte, S., & Barata, C. (2015b). Liquid chromatography coupled with tandem mass spectrometry to characterise trace levels of cyanobacteria and dinoflagellate toxins in suspended solids and sediments. Analytical and bioanalytical chemistry, 407(5), 1451-1462.

Rivetti, C., Gómez-Canela, C., Lacorte, S., Díez, S., Lázaro, W. L., & Barata, C. (2015c). Identification of compounds bound to suspended solids causing sub-lethal toxic effects in Daphnia magna. A field study on re-suspended particles during river floods in Ebro River. Aquatic Toxicology, 161, 41-50.

Rivetti, C., López-Perea, J. J., Laguna, C., Piña, B., Mateo, R., Eljarrat, E., Barceló, D., Barata, C. (2017). Integrated environmental risk assessment of chemical pollution in a Mediterranean floodplain by combining chemical and biological methods. Science of The Total Environment, 583, 248-256.

Rivetti, C., Campos, B., & Barata, C. (2016). Low environmental levels of neuro-active pharmaceuticals alter phototactic behaviour and reproduction in Daphnia magna. Aquatic Toxicology, 170, 289-296.

Resumen (castellano)

El pasado siglo demostró nuestra dependencia de un amplio abanico de sustancias químicas como fármacos, productos para la higiene personal (PPCP, en inglés), compuestos para la industria y la agricultura, cuya acumulación y riesgos potenciales para el medio ambiente y el ser humano no se consideraron durante bastantes años. A finales de los años 70, la Unión Europea (UE) comenzó a elaborar listas de aquellas sustancias químicas que, por sus características, resultaban altamente preocupantes y por tanto debían considerarse prioritarias. Fue mediados de los 80 cuando se legisló la obligatoriedad de efectuar una Evaluación de Riesgo Medioambiental (ERA, en inglés) exhaustiva de todos aquellos productos químicos que se comercializaran por vez primera. Entre todos los sistemas naturales, uno de los más afectados por la contaminación antropogénica es el ecosistema acuático, dado que es el destino final de muchos de los compuestos mencionados. Mantener dicho ecosistema en buen estado es de vital importancia ya que la calidad del agua es fundamental para el desarrollo sostenible de la sociedad y para mantener la estabilidad y biodiversidad de los ecosistemas. Algunas sustancias químicas son altamente persistentes, otras se aplican de forma repetitiva o continua y/o como mezclas. Todo esto conlleva la presencia en el medio acuático de un coctel complejo de compuestos muy heterogéneos, capaces de provocar graves efectos tóxicos. Por esta razón es necesario desarrollar estrategias innovadoras e integradas que permitan no sólo la monitorización y la evaluación de la calidad de las aguas superficiales sino también la comprensión de los mecanismos de toxicidad subyacentes. Dentro de las técnicas disponibles, combinar el análisis químico con el estudio de las respuestas biológicas (bioquímicas, fisiológicas y moleculares) de los organismos expuestos resulta altamente eficaz en la detección y estudio del impacto de los contaminantes antropogénicos en los sistemas de agua dulce.

El objetivo general de esta tesis es la caracterización de nuevos mecanismos de toxicidad de contaminantes presentes en los ecosistemas acuáticos, tanto en el campo como en el laboratorio. Las diferentes problemáticas que presentaron los estudios de campo se abordaron en tres de los objetivos

específicos, mientras que el estudio de los efectos subletales de fármacos a concentraciones relevantes desde el punto de vista medioambiental, se llevó a cabo en el laboratorio como cuarto y último objetivo.

En el Capítulo 2 (Rivetti et al., 2015a) se realizó un estudio comparativo de tres cuencas fluviales españolas, que presentaban contaminantes antropogénicos de distintas procedencias. Para ello se combinaron biomarcadores genéticos, bioquímicos e individuales de *Daphnia magna.* Los individuos fueron trasplantados en 12 puntos diferentes de las cuencas (Llobregat, Ebro, Júcar) y, tras su exposición, se evaluaron los cambios en transcripción génica, tasa de alimentación y varias respuestas enzimáticas. Los resultados obtenidos se compararon con los de individuos expuestos a muestras sintéticas, preparadas mediante la reconstitución en agua de los extractos orgánicos procedentes de muestras recogidas en el mismo lugar y período en el que se realizó la exposición. Por otro lado, el análisis químico reveló un total de 166 contaminantes traza en el agua muestreada, que se clasificaron en 45 grupos según su modo de acción, incluyendo: metales, fármacos, pesticidas, drogas ilegales y otros compuestos industriales. Además, se estudió la biodiversidad y abundancia de especies en las mismas localizaciones. Tanto los parámetros físico-químicos del agua como los patrones de trascripción de 13 genes diferentes que codifican por estrés general, procesos energéticos y metabolismo, muda y transporte de xenobióticos corroboraron las respuestas fenotípicas observadas, diferenciando tanto los puntos de exposición como las cuencas. El Análisis por Componentes Principales y regresión de Mínimos Cuadrados Parciales (PCA-PLS) indicó que las respuestas *in situ* de muchos genes y biomarcadores así como los índices de biodiversidad estaban afectados por distintos factores ambientales. En suma, con este trabajo se demuestra la utilidad de analizar variaciones en la respuesta transcriptómica de *D. magna* para detectar e identificar distintos factores de estrés ambiental en estudios de campo.

En el capítulo 3 (Rivetti et al., 2015b) y 4 (Rivetti et al., 2015c) se desarrolló un estudio de ecotoxicología forense para desenmascarar los componentes más tóxicos en una zona altamente contaminada del rio Ebro (España) durante un

inusual período de crecidas de dicho rio. En el estudio se propuso utilizar una Evaluación de la Identificación de Toxicidad (TIE, en inglés), mediante la combinación de ensayos de toxicidad y métodos químicos de análisis y fraccionamiento. Las muestras de agua adquiridas tras las crecidas, se filtraron para eliminar el material en suspensión y se conservó el residuo filtrado para su estudio. Los posibles efectos tóxicos del agua muestreada, del agua tras la filtración y de muestras sintéticas preparadas por reconstitucion del residuo filtrado, se evaluaron analizando los cambios en la tasa de alimentación de *Daphnia magna* tras exponerla a dichas muestras. Por otro lado se determinó el contenido en organoclorados del material en suspensión mediante extracción en fase sólida y análisis por GC-MS/MS, mientras que la presencia de mercurio se cuantificó mediante un Analizador Avanzado de Mercurio (AMA, en inglés). Los resultados indicaron que los efectos tóxicos observados no se debían a los contaminantes analizados y que eran mayores aguas arriba de la zona altamente contaminada. Dada la presencia de cianobacterias en los embalses aguas arriba del lugar de muestreo, se consideró la posibilidad de que la toxicidad se debiera a la presencia de toxinas producidas por dichas bacterias. Para evaluar dicha posibilidad, se desarrolló un nuevo método de LC-MS/MS dirigido a detectar la presencia de cinco toxinas en dos matrices: materia en suspensión y sedimentos (capítulo 3). Este método se aplicó con éxito en la detección de toxinas en los sólidos en suspensión y sedimentos del rio Ebro y de las lagunas de su delta, observándose la presencia de anatoxina-a y ácido okadáico en diferentes localizaciones. Los niveles de anatoxina-a se correlacionaron con la inhibición de la ingesta, y se confirmó en el laboratorio empleando anatoxina-a producida por *Planktothrix agardhii*, una cianobacteria filamentosa.

En el capítulo 5 (Rivetti et al., 2017) se implementó el estudio de calidad del agua en las Tablas de Daimiel (TDNP), una reserva natural española, y en sus lagunas asociadas con el fin de analizar el riesgo potencial en el que se encuentra la fauna silvestre residente en la zona, debido a la introducción constante de microcontaminantes por parte de los ríos que alimentan a TDNP. A lo largo de 2013 se tomaron muestras en 12 localizaciones diferentes, dentro

de TDNP y en el cercano Estanque de Navaseca. Estas muestras se utilizaron para llevar a cabo varias series de bioensayos *in vivo* e *in vitro,* incluyendo el estudio de la inhibición de la ingesta en *Daphnia magna* y la determinación de actividad estrogénica o tipo dioxina en levaduras recombinantes (ensayo RYA). Los resultados de toxicidad se compararon con el análisis químico de hidrocarburos aromáticos policíclicos (PAHs en inglés) y de compuestos estrógenicos, realizados mediante GC-MS/MS y LC-MS/MS respectivamente. Los resultados mostraron que TDNP se encuentra en buen estado químico pero está amenazado por el flujo procedente plantas de tratamiento de aguas residuales, que vierten en los ríos que alimentan a TDNP, y por vertido directo de aguas de alcantarillado, como sucede en el Estanque de Navaseca.

En el capítulo 6 (Rivetti et al.2016) se exploró la hipótesis de que la exposición de *D.magna* a fármacos neuroactivos de distintas familias y a concentraciones bajas, pero relevantes desde el punto de vista ambiental, podrían provocar respuestas fenotípicas como efectos sobre la reproducción y alteración del comportamiento. Se seleccionaron los siguientes fármacos: carbamacepina, diazepam, propanolol y fluoxetina (esta última incluida solo en ensayos de comportamiento), debido a que se recetan con frecuencia y que se han detectado en niveles considerables en el medioambiente (de ηg/L a pocos μg/L). Los tres primeros provocaron un aumento en la tasa de reproducción a concentraciones de 1ηg/L (propanolol), 0.1 μg/L (diazepam) y 1 μg/L (carbamacepina). Fluoxetina, carbamacepina y diazepam aumentaron el comportamiento fototáctico positivo en concentraciones desde 1, 10 y 100 ηg/L, respectivamente.

Chapter I.

General Introduction and thesis outline

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1.1 Background

1.1.1 We are a chemical society

Humankind has continually developed and progressed, and this advancement was never faster and more evident than in recent times. The last century showed our need and dependency on a full range of chemical products in order to achieve and keep our wellness and health. Indeed, we are surrounded at all moments by massive amounts (both in quantities and kinds) of natural and synthetic substances, many of which may threaten the environment. Nowadays, all substances are classified by a CAS Registry number that provide a unique, unambiguous identifier for chemicals and molecular structures. At the moment of writing this manuscript (December 2016), the CAS database contains more than 120 million registered chemicals (of which only 130 thousand are commercially available), both organic and inorganic, most of which are poorly tested or untested for potential health effects. Over the last century, we have been slowly introducing these chemicals in Pharmaceutical and Personal Care Products (PPCP), industrial and/or agricultural applications in order to improve processes, although the environmental and human hazards of most of them were not considered (Hartung, 2011). In the last decades society has been more aware on how we face problems with respect to our ecosystems protection and the scientific community has deeply focused on the evaluation of the toxicity of persistent and widely distributed chemical pollutants and their potential harm to the environment. Specific guidelines and protocols have been issued for those compounds considered of particular concern, aiming to a severe reduction of their usage and with the ultimate goal of their complete abolishment. Since then, in most industrialized countries comprehensive environmental legislation has been introduced in order to regulate the wide spectrum of different pollution sources and control release into the environment.

Nevertheless, there are still several classes of compounds e.g PPCPs, hormones and steroids, illicit drugs, flame retardants, engineered nanoparticles between others, not included in these priority lists and classical assessment and whose concern should be raised. These substances are referred to as “emerging contaminants”, although many of them have been used already for decades now.

1.1.2 Environmental Risk Assessment

Environmental risk assessment (ERA) is defined as ”the procedure by which the likely or actual adverse effects of pollutants and other anthropogenic activities on ecosystems and their components are estimated with a known degree of certainty using scientific methodologies (Namiesnik and Szefer, 2009)“. Conventional risk assessment generally aims at establishing a link between the effects induced by a chemical and the kind and extent of exposure expected or measured in the environment. Described in the Technical Guidance Document on Risk Assessment (EC, 2003), it is based on a risk quotient approach using the predicted environmental concentrations (PECs) and predicted no effect concentrations (PNECs). Since the late 1970s, the EU started priority lists of chemicals of major concern and since the mid 1980s it was made compulsory to set up a comprehensive ERA for all new commercialized chemicals. Indeed, risk is not an inherent property of a chemical toxicant but rather the product of its toxicity times the exposure received by a given organism, thus requiring deep investigation and application to case-studies scenarios (van Leeuwen and Vermeire, 2007). Risk assessment management can be divided into two different components: the scientifically oriented risk analysis, including hazard identification, effect assessment, exposure assessment and risk characterization; and the politically-oriented risk management, dealing with regulatory measures based on risk assessment (Walker et al., 2012). Those two components are intrinsically related to each other, unravelling the risk of a certain stressor (the former) and examining the possible solutions to the problem (the latter).

More recently, the EU also started the Registration, Evaluation, Authorization and Restriction of Chemical substances (REACH), which entered into force on 1

June 2007, in order to get environmental agencies and chemical industry to cooperate and with the final objective of merging the hazards for both human health and the environment. In fact, the overall aim of REACH is to improve the protection of human health and the environment through a better and earlier identification of the intrinsic toxicological properties of chemical substances. The REACH regulation places greater responsibility on industry to identify and manage the risks and to provide safety information of the substances. According to this European regulatory framework, standardized toxicity tests should be used for the assessment of the ecotoxicity hazard and estimate a value of PNEC (ECHA, 2008). Manufacturers and importers are then required to gather information on the properties of all chemical substances commercialized or used in intermediate processes, which will allow their safe handling and use, and to register the information in a central database (Bowman and Van Calster, 2007). The European Chemicals Agency (ECHA) has set three major deadlines for registration of chemicals, determined by tonnage manufactured and/or imported *per* year. Thus, all chemicals manufactured/imported within European Union (EU) at ≥ 1000 tonnes/year were required to be registered by December 1st 2010, ≥ 100 tonnes/year by June 1st 2013 and ≥ 1 tonne/year by June 1st 2018.

1.1.3 New challenges in Ecotoxicology

The term ecotoxicology was introduced in 1969 by René Truhaut (Truhaut, 1977), derived by the combination of ecology and toxicology. It was defined as "the branch of toxicology concerned with the study of toxic effects, caused by natural or synthetic pollutants, to the constituents of ecosystems, animal, vegetable and microbial, in an integral context" (Truhaut, 1977). The introduction of this new word reflected a growing concern about the harmful effects of chemicals (toxicology) on the environment and consequently on species other than humans (ecology). In other words, core mission of the discipline is to describe and unravel what happens to chemicals in the real world and to understand the mechanisms by which they disrupt normal biological performance, in order to develop appropriate measures or tools to prevent adverse outcomes. For this aim, main traditional areas of application of

ecotoxicology cover both laboratory exposures and field studies, including biomonitoring of environmental pollution in specific ecosystems, conduction of field trials or specific case-studies and ERA (Walker et al., 2012).

Currently, as result of all the above presented efforts, concentrations of many well-known pollutants have decreased, because new, less toxic and less persistent substances with low bio-accumulative potential have being developed and introduced. Nevertheless, ecosystems are still under threat.

Current challenges in ecotoxicology are (Eggen et al., 2004):

- Low concentrations of pollutants and long exposure times (chronic effects)
- Multiple effects by single pollutants
- Complex mixtures of pollutants
- Multiple natural stressors
- Ecosystem complexity

In order to answer these questions and face the ever increasing number of chemicals present in the environment, ecotoxicology has adapted accordingly to these new needs and innovative concepts have recently arisen.

1.1.4 Predictive ecotoxicology

Historically, ecotoxicology and ERA for chemicals have been largely based on toxicity data from whole animal testing with apical endpoints such as survival, growth and reproduction although this type of testing is slow, costly and time-consuming. At the same time, regulatory programs throughout the world are requiring data for an increasing number of chemicals and assessment scenarios, while cutting down on the use of animal testing. To meet this need, in these last years, efforts have been put in developing high-throughput screening (HTS) assays and new computational tools together with the use of alternative model animals and non-animal alternatives (including *in vitro* and *in silico* approaches) in order to develop simple and cost-effective approaches. Recent

advances in toxicological science, bioinformatics and systems biology have provided means to transform (eco)toxicology into a predictive science which utilize mechanistic, pathway-based data that have not typically been used in the past for ERA applications. This is aimed at identifying potential toxicants on the basis of an understanding of their mechanisms of biological action. Predictive ecotoxicology based on mechanistic data has become nowadays of key importance for ERA. In fact, a combination of new approaches and methods both in biology and chemistry is leading to a greater understanding of the mechanistic processes connecting chemical exposure and adverse outcome. These approaches should also be able to accelerate the screening and toxicity assessment for the ever increasing number of commercialized chemicals for their potential hazards to humans and ecosystems health (Garcia-Reyero, 2014). Following this trend, a major step has been the introduction of the concept of Mechanism of Action, defined as “a complete and detailed understanding of each and every step in the sequence of events that leads to a toxic outcome” (ECETOC, 2007). This concept should not be confused though with the similar expression Mode of Action (MoA). A MoA in fact describes more comprehensively “a common set of physiological and behavioral signs that characterize a type of adverse biological response to a specific chemical challenge, where the majority (but not all) of the biological steps are understood” (OECD, 2012). A novel tool with even broader potential in toxicology and ERA is the description of an Adverse Outcome Pathway (AOP). The AOP framework is a conceptual construct that provides a clear-cut mechanistic representation of the progression of critical toxicological events across different levels of biological organization, which lead to adverse outcomes relevant for ERA. In other words, it aims to describe a full cascade of biological events, beginning with a molecular initial event (MIE), progressing through a series of intermediate steps and key events (KE) at different biological levels to an observable adverse effect in a population (Ankley et al., 2010; Groh et al., 2015; Vinken, 2013). Indeed, a detailed mechanistic knowledge of observed effects would facilitate the development of alternative testing methods as well as help prioritize higher tiered toxicity testing (Wittwehr et al., 2017). As a consequence, this would improve our capacities of grouping

and read-across, thus allowing scientist to better extrapolate results and make inferences about the resultant toxic potential.

Nevertheless, in order to face the ever-increasing number of existing compounds, for which would be unthinkable to unravel all specific MoA or even more unlikely an AOP, the Read-Across hypothesis and Quantitative structure-activity relationship (Q)SAR were developed. Read-across is considered a non-testing method for filling data gaps which is based on an analogue or chemical category (Van Leeuwen et al., 2009). It must be scientifically supported and the inherent uncertainties must be addressed as uncertainty factors as often applied in weight-of evidence (WOE) studies (Burton et al., 2002). WOE refers to the interpretative methods of ERA. WOE approaches determine potential ecological impacts from chemicals or other stressors based on multiple lines of evidence (LOE). WOE studies include chemical and biological measurements, of which both laboratory and field components are considered of high importance (Chapman and Hollert, 2006; Linkov et al., 2009; Weed, 2005). Lastly, (Q)SARs are regression models and have been developed as valuable tools for predicting acute toxicity and classify toxicants, when little or no empirical data are available (Benigni et al., 2007; Kar and Roy, 2010). They can also be more generally applied to assign MoA to chemicals.

1.1.5 Aquatic ecosystem

Among all natural systems, the aquatic ecosystem is one of the most affected as it stands as the final destination for most anthropogenic contaminants and its sediments (either deposited or in suspension) as major sink, functioning as storage deposits (Rand, 1995; Schwarzenbach et al., 2006). A good and appropriate water quality is fundamental for a sustainable development of human society as well as for maintenance of the ecosystem biodiversity and stability. More specifically, freshwater ecosystems, including rivers and streams, wetlands, ponds and lakes, represent a major concern (Crook et al., 2015). Diversity in these ecosystems is often of high value, as they can face periods of isolation leading to development of distinct patterns of biodiversity and unique flora and fauna (Clements et al., 2012).

Nowadays, in many highly industrialized and agricultural areas, water contamination has become a high priority problem with the presence of mixtures of chemicals with potential toxic effects both on the ecosystems and human health (Ginebreda et al., 2014; Hering et al., 2015; Rosi-Marshall and Royer, 2012). In fact, most substances currently detected in rivers and lakes showed a high potential to exert noxious effects on aquatic species (Brodin et al., 2013; Ford and Fong, 2015; McNeil et al., 2016; Morrissey et al., 2015; Rosi-Marshall et al., 2015). As a response, priority lists of compounds showing hazard potential for the ecosystem have been built. Among them, one of the most relevant list is the “List of Priority substances” within the EU Water Framework Directive (WFD) - EU Directive 2000/60/EC (WFD, 2000). The inherent aim of the WFD is to protect and prevent deterioration of European waters and achieve a good chemical status of the water bodies, by “getting Europe’s waters cleaner and the citizen involved”, thus increasing awareness (Bjerregaard, 1998). Nevertheless, it implicitly relies on a good knowledge of the ecosystem functioning under specific environmental conditions, an ambitious assumption considering the complexity and heterogeneity of aquatic ecosystems (Allan et al., 2006; Martinez-Haro et al., 2015). This attitude is convenient, but on the other hand does not consider a great number of chemicals that are of emergent concern, namely used in personal care products, pharmaceutical compounds and other non-industrial, as the standard ERA evaluations do not cover specific sub-lethal effects that may exert impacts at different ecological levels. More recently, it was launched the EU Directive 2013/39/EU, that updates the previous water framework policy, highlighting the need to develop new water treatment technologies to deal with such problem (EU, 2013). Thus, the identification of ecological risks of environmental relevant pollutants to aquatic organisms is an essential point for environmental managers and policy makers to locally identify and alleviate chemical pressures in aquatic ecosystems. Classical WOE approaches implemented for ERA of aquatic ecosystem contamination include and combine information from three complementary and integrated LOEs: chemical analyses for the detection of pollution candidates; toxicity tests and biological responses at the individual scale used as fast and sensitive bio-indicators for toxicity; and community structure analyses to provide

evidence of toxicity effects in aquatic species at different levels of biological organization (Adams et al., 2000; Chapman and Smith, 2012; Santos et al., 2017; Vasseur and Cossu-Leguille, 2003). Providing early warning signals of toxicity effects on biota, specific biomarkers could be used as alternative to a third LOE, before the community level is seen affected (Damásio et al., 2010; Sanchez and Porcher, 2009).

1.1.6 Emerging contaminants

During the last decades analytical chemistry technologies underwent fast development, allowing the detection of chemicals down to trace levels as low as ηg/L or even pg/L and in complex environmental matrices e.g. water, sediments and wastewaters (Anumol et al., 2013; Burgess et al., 2015; Cristale et al., 2013; Gorga et al., 2013; Niu et al., 2014). Effective chromatographic separations coupled to high-resolution mass spectrometers have become common in the modern environmental research, increasing the awareness and understanding of the presence of classical and emerging contaminants in our ecosystems, as wel as their transformation and fate. This led to new lines of ecotoxicological research to highlight the complex ecological consequences that they may pose to biological systems (Noguera-Oviedo and Aga, 2016). Thanks to this increased sensitivity, we are now facing overwhelming evidences that "new" xenobiotic substances have been introduced and became nowadays ubiquitous in the aquatic environment. These substances, referred to as emerging contaminants or, better, contaminants of Emerging Concern (cECs), include a wide array of different compounds commonly derived from municipal, agricultural and industrial wastewater (Drewes and Shore, 2001; Michael et al., 2014; Noguera-Oviedo and Aga, 2016; Weissinger et al., 2016; Younos, 2005). cECs do not actually mean that they are new pollutants. Their release has most likely occurred for a long time, but may not have been detected until now, for which reason concerns have been raised much more recently (Sauvé and Desrosiers, 2014). Although low, these concentrations may be significant enough to induce toxic and synergistic effects on aquatic species due to their continual influx and/or persistence (Daughton and Ternes, 1999; Hernando et al., 2006; Isidori et al., 2009). Moreover, due to their continuous introduction into

the environment, they do not need to be persistent in order to compensate their transformation or removal rate. It is also now well documented that many cECs outflow the most widely used wastewater treatment process (Celiz et al., 2009; Martín et al., 2012; Nakada et al., 2006). However, due to their broad and increasing number and variety of characteristics, only few of these compounds have been so far toxicologically evaluated. As a result, the scientific community is becoming aware of the risks of cECs, and it is without surprise that these chemicals are now recollecting most of the resources for research, together with their inclusion within the list of priority chemicals for an in-depth ERA (Gavrilescu et al., 2015; Murray et al., 2010; Pereira et al., 2015; Stuart et al., 2012). The list of compounds recognized as cECs is very dynamic as it is the concept of “emerging”: chemicals that were considered emerging just a decade or two ago, nowadays might no longer be qualified, whereas new cECs (previously unknown molecules or for which environmental issues were not fully recognized earlier) may be now included. Current recognized cECs include a wide range of substances such as PPCPs, fragrances and synthetic musks, plasticizers, hormones and steroids, illicit drugs and drugs of abuse, flame retardants, engineered nanoparticles, chlorinated paraffines, perfluoroalkyl compounds, polar pesticides, food additives, algal toxins among others, bound to appear day by day (Gavrilescu et al., 2015; Lapworth et al., 2012; Ternes et al., 2015).

In general, most of the cECs present low acute toxicity, but their potential sub-lethal effects at low levels and long-term exposure and toxicological MoAs are mostly unknown or only known in humans (Claessens et al., 2013; Cristale et al., 2013; Drewes et al., 2005; Gibs et al., 2007; Hutchinson et al., 2013; Kinney et al., 2006; Noguera-Oviedo and Aga, 2016; Veldhoen et al., 2006; Woodling et al., 2006). In fact, for most cECs there is currently little information regarding their potential toxicological significance in ecosystems with special regard to the effects from long-term and low-level of environmental relevant exposures. Therefore, traditional toxicity test endpoints may not be sufficiently sensitive for cECs. Low-doses effects, non-monotonic dose responses and life-stage dependent effects cannot be often solved by traditional ERA approaches and

standard international guidelines, thus needing to be further explored and refined. As a matter of fact, a better understanding of the influence and effects of subtle toxicity on individual and population fitness would provide a broader integration of sub-lethal endpoints into the ERA frameworks (Groh et al., 2015). At this point, a more accurate description for cECs would be to define them as occurring chemicals or materials which have recently been detected or are suspected to be present in various ecosystems and whose properties are likely to significantly alter at some point the metabolism of a living being. Such compounds would remain “emerging” as long as there is a lack of information about the associated potential risks and adverse effects it may pose to human health and/or the environment and which are not yet subjected to regulatory criteria. This does not imply that all cECs will actually prove to have some toxic potential; the focus is the lack of ecotoxicological data and suitable environmental fate that prevents a proper evaluation of associated risks (Sauvé and Desrosiers, 2014).

1.1.6.1 Pharmaceuticals and neuro-active compounds

Pharmaceuticals are a large and diverse class of organic compounds used in the prevention and treatment of humans and animals diseases. In the last century, as a result of the rapid medical advances, a still increasing number of new medications and treatments have been developed, thus resulting in an increased consumption of drugs and their consequent release into wastewaters. Nowadays, more than 3000 different pharmaceuticals are available on the market, including analgesics, antibiotics, neuro-active compounds, lipid regulators, among others (Bottoni et al., 2010; Fent et al., 2006). Although their effects on human health have been investigated under every aspect, the full extent and complete consequences of their presence in the (aquatic) environment have not usually been sufficiently studied yet, for which reason they are considered cECs (Fabbri, 2015; Fent et al., 2006; Silva et al., 2015). The worldwide occurrence of psychiatric disorders led in the last decades to an increased use of neuro-active pharmaceuticals, i.e. antidepressants, anxiolytics and sedatives (Calisto and Esteves, 2009). According to OECD Health Statistics, between 2000 and 2012 the consumption of this class of compounds

has doubled on average in OECD countries (OECD, 2014). As a consequence of their extensive application, together with their tendency of persistence and accumulation, their present occurrence in the environment is in the range of ηg/L or µg/L (Calisto and Esteves, 2009). Although these levels are below the concentrations predicted to harm humans, as well as to cause acute toxicity to non-target organisms, their highly specific biological activity on the neuro-endocrine system makes them an important group of pharmaceuticals for evaluating ecotoxicological effects in aquatic non-target organisms (van der Ven et al., 2006). In fact, neuro-active pharmaceuticals are biologically active molecules, designed to exert specific effects on individuals receptors/pathway, characterized by a large window of pharmacological effect but low toxicity, thus making their effects on biota of physiological importance even though difficult to detect and evaluate (Bottoni et al., 2010). As a matter of fact, several authors already reported effects on invertebrates at very low concentrations (Calisto and Esteves, 2009; Campos et al., 2012a; Campos et al., 2012b; Fent et al., 2006; Ford and Fong, 2015).

1.1.6.2 Natural toxins

Natural toxins are a class of molecules produced by bacteria and eukaryotes that are poisonous, whose evolutionary function is to act as defensive agents against predation. They can be small molecules, peptides or larger proteins that are capable of causing harm when in contact with or after absorption by body tissues, affecting the normal physiology of an organism. Toxins can vary greatly in their toxicity, ranging from usually minor effects (such as a mosquito bite) to sudden death (such as poisoning by a neurotoxic toxin).

A well-known group of natural toxins causing acute toxicity are cyanotoxins, which are produced by cyanobacteria (bacteria also known as blue-green algae). Cyanobacteria are mostly found in lakes and oceans and have the ability to synthesize a great variety of secondary metabolites with various types of biochemical or biological activities, that can be extremely toxic (Bláha et al., 2009). Under optimal conditions (usually high eutrophication periods), cyanobacteria are able to reproduce exponentially and form algal blooms. During blooming the concentration of cyanotoxins produced can be high enough

to poison and even kill animals and/or humans. Cyanotoxins can also bio-accumulate in fish and shellfish, and cause important health problems to humans that consume them i.e. shellfish poisoning. Most common detected toxins include neurotoxins (e.g. anatoxin-a, saixitoxins), hepatotoxins (e.g. microcystins, nodularins, cylindrospermopsins), cytotoxins (e.g. lyngbyatoxin-a, cylindrospermopsins), and endotoxins (e.g. lipopolysaccharides) (Churro et al., 2012; Zanchett and Oliveira-Filho, 2013). Cyanotoxins can be produced by a wide variety of planktonic cyanobacteria. Some of the most commonly occurring toxic genera are *Microcystis*, *Anabaena*, and *Planktothrix* (Oscillatoria). Another important natural toxin is okadaic acid (OA), a strong phosphatase protein inhibitor produced by several species of dinoflagellates. It is known to accumulate in marine invertebrates, especially sponges and shellfish and to be one of the primary responsible for the diarrheic shellfish poisoning.

1.1.7 Aquatic toxicology

Aquatic toxicology is a multidisciplinary field, which integrates toxicology, ecology and environmental chemistry. The overall aim of the discipline is to increase our understanding of the impact of potentially toxic chemicals and study their effect on aquatic ecosystems. It also deals with the mechanisms of toxicity and the responses to toxic agents in aquatic environment at the community, species, tissue, cellular and molecular level (Campos et al., 2013; Campos et al., 2012a; Claessens et al., 2013; Cristale et al., 2013; Drewes et al., 2005; Gibs et al., 2007; Hutchinson et al., 2013; Jordão et al., 2016; Kinney et al., 2006; Veldhoen et al., 2006; Woodling et al., 2006).

Traditionally, this discipline has used toxicity tests to identify putatively harmful effects to organisms and ecosystems through the test of endpoints like mortality, reproduction and/or individual growth (Chevalier et al., 2015). As a matter of fact, current tests cover only a small set of laboratory organisms, and are often not sensitive enough, thus not able to unravel adverse effects of these compounds due to their experimental design (Noguera-Oviedo and Aga, 2016). Besides, most of the standardized ecotoxicological assays by organizations like Organization for Economic Co-operation and Development (OECD) and United States Environmental Protection Agency (US-EPA) focus on individual level

(apical) effects and do not provide information regarding toxic mechanisms and/or MoAs. New toxicity investigations are needed, using specific toxicity parameters that can lead to a more meaningful ERA (Chevalier et al., 2015). Overall, there is a general lack of chronic toxicity data of these emerging contaminants on non-target species and especially bio-active substances, i.e. pharmaceuticals and similar compounds, need more research about potential long-term ecotoxicological effects, particularly with respect to potential disturbances in hormonal homeostasis (endocrine disruption), reproductive outputs, immunological status, gene activation and/or silencing during long-term, low doses exposure. In fact, when evaluating the effects of PPCPs on non-target species, we should keep in mind that they are biologically active molecules, designed to target specific metabolic and molecular pathways in humans and/or animals. Under this perspective, they may or may not have similar effects in non-target species, as many targets are phylogenetically conserved (Gunnarsson et al., 2008). On the other hand, they can also have unexpected effects in other organisms due to biological and physiological differences that may alter the pharmacodynamics and/or pharmacokinetics. In-depth understanding of possible effects needs a mechanism-based approach focused on target molecules. This kind of approach should yield more meaningful results and deeper insights than the standard toxicity testing.

It is also important to stress that toxicity data are not always available for all potential affected species in a given environment (Cogliano, 2016; Wilson, 2006). Given this limitation, the overall objective of test organism selection is to choose models that are evocative of the major ecosystem components: aquatic algae and plants are representative of photosynthetic organisms, also called primary producers (Villain et al., 2016); invertebrate species such as scuds and water fleas, feeding on algae, decaying plant materials and bacteria, have a key position into the food web, being important sources of food for a variety of larger fish, birds and mammals (Baird and Burton, 2001; Miner et al., 2012); fish species are crucial for their great interest in the assessment of biological and biochemical responses to environmental contaminants as vertebrates (Powers, 1989).

1.1.8 Complex exposure scenarios

In aquatic ecosystems, organisms are normally exposed to complex mixtures of chemicals and it is relatively uncommon to find sites polluted with only one substance (Lydy and Austin, 2004; Villanueva et al., 2014; Walker, 2001). In fact, some chemicals are highly persistent, some are applied repeatedly or continuously, and others are directly applied as mixtures in order to increase efficiency or reduce costs, thus leading to a complex cocktail of very heterogeneous compounds, whose interactions are often unknown (Marking, 1977; Mayer, 1977). These mixtures may provoke toxic effects even when the individual stressors are present at concentrations lower than the No Observable Effect Concentration (NOEC) and their adverse effects are often underestimated (Brian et al., 2007; Kortenkamp, 2008). In fact, due to the large number of different chemical compounds present in environmental matrices, individual testing of each component may not be fully representative of the total mixture effect (Backhaus and Karlsson, 2014; González-Pleiter et al., 2013; Vasquez et al., 2014). For instance, contaminants with similar or different MoA can influence each other, thus resulting in an almost unlimited number of potential additive, synergistic or antagonistic combinations (Beyer et al., 2014).

In addition, natural factors i.e. physico-chemical variables such as temperature, pH, conductivity, may also act as stressors and increase the complexity of multiple stressor situations (Hering et al., 2015; Nõges et al., 2016). Furthermore, compounds that affect specific organisms or sensitive life-stages and compounds known to interact with highly conserved targets across *taxas* e.g. pharmaceuticals, may also represent a special concern (de Perre et al., 2016; Jager et al., 2016; Vasquez et al., 2014). Under this perspective, it is evident that there is no “one-size-fits-all” ecotoxicological bioassay that can be used to comprehensively assess the effects of complex mixtures of chemicals in the environment. Instead, a battery of bioassays need to be implemented to understand and describe important biological endpoints related to different physiological pathways (Noguera-Oviedo and Aga, 2016). Besides, given the ever increasing number of synthetic chemicals that are currently in use worldwide, it is logistically impossible to empirically assess the toxicity of each

of them to aquatic species and any possible combination in mixture. For this aim, robust predictive toxicity models are essential, in order to estimate their toxicity with acceptable precision (Altenburger et al., 2013; Lydy and Austin, 2004; Vighi et al., 2003). The accurate prediction of chemicals interactions in mixtures remains a priority topic in aquatic ecotoxicology, especially for when it results into synergistic toxicity (Cedergreen, 2014).

1.1.9 Integrative approach

In the frame of the WFD, an integrated approach is required for the monitoring and assessment of the quality of surface water bodies and the understanding of the underlying mechanisms of toxicity (Wernersson et al., 2015). Chemical analyses usually imply a prior knowledge and a choice about the type of substances to be monitored as it would be quite challenging to consider and quantify all substances that may be present in a specific ecosystem, for both technical and economic reasons. In the past, considerable high levels of compounds, but in limited number, were detected locally in the aquatic environment, producing acute and chronic effects on the communities and ecosystems, making it easier to target specific compounds of interest. Lately, instead, the situation has shifted: larger numbers of chemicals are being registered every year although detected at lower environmental concentrations and more geographically spread on a global scale. This raises the need of looking for more subtle, chronic, long-term effects of chemicals and their mixtures selecting bioassays, which can deliver the specificity and sensitivity required to detect possible adverse effects. The major examples come from exposures to a vast array of pharmaceuticals, personal care products and endocrine disruptors (Fabbri, 2015; Noguera-Oviedo and Aga, 2016; Vasquez et al., 2014). For a good assessment of the unexpected effects of chemicals in the field and/or in laboratory conditions, an integrative approach represents an innovative and relevant methodology, with special regard to the understanding of mechanisms of action and effects at the different biological organization levels (Fabbri, 2015; Schmitt-Jansen et al., 2008). Test strategies focused on responses across different biological organization levels, from genes to populations and/or multigenerational tests allow us to unravel new potential

MoA of emerging contaminants (Ankley et al., 2010; Barata et al., 2017; Brennan et al., 2006; Campos et al., 2016). The integrated test approach gives us the possibility to identify key-events and effects and then, uses this information to design novel tools for ERA and guide chemical analyses (Ankley et al., 2010). In fact, it aims to provide a deeper mechanistic insight into ERA and a stronger characterization of the MoA of substances. Among the available techniques to measure effects, an integrated use of chemical analyses and biological responses to pollutants, e.g. biochemical, physiological and molecular responses, is a sound procedure for detecting impact of anthropogenic contaminants in freshwater systems. Accordingly, an integrated chemical–biological approach is vitally important for the understanding and proper assessment of anthropogenic pressures and their effects on ecosystems. Such an approach is also necessary for prudent management, aiming at safeguarding the sustainable use of ecosystem goods and services. Thus, the main research focus is now increasingly directed to the development of integrated assessment of chemical pollution and new tools contributing to a more holistic ecosystem health assessment.

1.1.10 The use of biomarkers

In terms of ERA, it is clear that an integrated approach broadens the potential for the inclusion and use of a battery of biomarker determinations within the WFD. Biomarkers are functional measures of exposure to stressors expressed at the molecular, physiological or behavioral level (McCarty and Munkittrick, 1996). In other words, biomarkers are surrogate measures of biological responses within both field and laboratory studies. As a matter of fact, any alteration in molecular, cellular, biochemical, physiological or ecological processes may be considered and used as a biomarker. However, it should have biological significance, as it should be used only when it is possible to link its change to important and known biological processes, thus allowing inclusive results interpretation. Biomarkers were developed in response to the need for more subtle and sensitive indicators of sub-lethal effects to environmental stressors than traditional apical ecotoxicological bioassays as lethality, reproduction and/or growth impairment as endpoints. A combined approach

including both biomarkers and apical bioassays would add value and provide complementary evidence to be included in an integrated ERA approach together with chemical and ecological community measures (Hagger et al., 2006). Whereas traditional biomarkers focused mostly on organism physiology or biochemistry (i.e. feeding rate, behavior, enzymes activity, …), recent advances in molecular biology are extending the field to the molecular level (i.e. gene expression) (Jemec et al., 2010). It is important to remark that the effects produced by any contaminant at lower levels of biological organization, e.g. molecular or biochemical, generally occur earlier and faster than at higher levels (Van der Oost et al., 2003), e.g. organismal or community, and hence may offer a more sensitive early warning of toxicological risk within a community or ecosystem (Clements, 2000). However, until now, their overall relevance to provide unambiguous and ecologically significant information on the exposure/effects of toxicants has been under discussion (Amiard-Triquet et al., 2015; Forbes et al., 2006; Moore et al., 2004; Walker et al., 2012): in fact, although individual biomarkers play an important role in gaining relevant insights into the mechanisms of toxicity on whole-organism performance and are useful indicators of exposure, they seem not entirely reliable in providing useful predictions on relevant ecological effects (Forbes et al., 2006). Thus, it is important to highlight how, although all biomarkers provide useful knowledge on the exposure or effects, not all of them may be suitable for use in ERA (Hagger et al., 2006). Due to the complexity of the biological processes and multiple levels of organization, there is no single biomarker that can provide an unequivocal measure of toxicity or stress. The most comprehensive hazard evaluation is accomplished by the use of a battery of biomarkers targeting multiple endpoints at different levels of organization coupled with chemical analyses (Hagger et al., 2006). In the ERA framework, it is advisable to use a set of sensitive biomarkers in order to target short-term endpoints as well as longer-term more relevant effects and provide a WOE approach, establishing causal links between environmental stressor and biological/ecological outcomes.

1.1.11 Importance of field studies

A longstanding goal in ecotoxicology is the inclusion of environmental realism in the design and analysis of scientific studies. Since in real field situations aquatic organisms are being exposed to multiple chemical and environmental factors, each contributing to a final overall adverse effect, the use of a large set of biological responses may allow us to identify contaminants that might be especially hazardous. Similar approaches have already been successfully used in studies with invertebrates (Amiard et al., 2006; Brown et al., 2004; Damásio et al., 2010). Although aquatic animals are invariably exposed to a full set of anthropogenic and natural stressors, only a small number of contaminants may be responsible for the observed toxicity. The integrated use of a set of biomarker responses to pollutants is considered to be one of the best procedures to detect impacts of specific contaminants *in situ*. Therefore combining several biomarkers that can be related with exposure to specific stressors and toxicological responses should allow identifying and targeting toxic components within complex chemical mixtures. Previous results lend positive support to the use of bioassays in combination with biochemical responses and chemical analyses in order to assess effects and to identify toxic components within complex mixtures in the field, thus contributing to a more realistic assessment of ecological risks (Barata et al., 2007; Damásio et al., 2008). Nevertheless, specific tools are required to identify causes and elucidate links between exposure and effects.

Toxicity Identification Evaluation (TIE) is an integrated biological and chemical framework, which aims to identify toxic compounds in a complex environmental sample (water, soil, air, effluent), which cause a biological response (Wernersson et al., 2015). It was developed by the US-EPA first for aqueous samples and more recently also for sediment testing (EPA, 2007). TIE procedures often include three steps: characterization (phase I), identification (phase II) and confirmation (phase III). This strategy has grown increasingly popular since the 1990s, especially for pinpointing active substances present in complex mixtures displaying endocrine disrupting activities (Burnison et al., 2003). TIE procedures were mainly used in the past in connection with effluent

discharge regulations, but today they are also used in ERA as well as in remediation work. Several bioassay/effect-directed analyses (BDA/EDA) concepts are being developed based on the TIE procedures, such as receptor-based in vitro assays, to characterize biologically active contaminants present in sediment samples (Dindal et al., 2007; Hurst et al., 2004; Noguerol et al., 2006a; Noguerol et al., 2006b; Otte et al., 2008). As a precautionary remark, it is important to consider that whereas this kind of approach moves us toward a better understanding of causality of events, distinguishing from other confounding factors that may influence biological responses, it is still not possible to use this information as conclusive diagnostic proof of cause-effect relationships of individual substances (Barata et al., 2007; Burgess et al., 2013).

1.1.12 Gene-based ecotoxicological approaches

The connection of the genetic basis of variation in ecological important traits with its effects on population, community and ecosystem properties is nowadays among the most significant challenges in biology. During the last decade, advances in DNA sequencing and functional characterization of genomes have opened up a range of new possibilities and high-throughput molecular-based technologies. Their broad potential in the frame of ERA has been suggested repeatedly (Connon et al., 2012; Garcia-Reyero and Perkins, 2011; van Straalen and Feder, 2011; Villeneuve et al., 2011). The new term “Ecotoxicogenomics” was introduced in 2004 and defined as the integration of molecular-based studies (i.e. transcriptomics, proteomics, metabolomics and epigenomics, among others) into ecotoxicology studies (Kim et al., 2015; Snape et al., 2004). This newly born research area brings together the fields of molecular biology and ecotoxicology towards the study of chemical and physical interactions of pollutants and their effects, when released into the environment. Based on the knowledge that gene expression (transcription) can be deeply altered as a direct and/or indirect result of a contaminant exposure, it became of crucial importance to determine the changes of the transcriptome of an organism to completely understand the underlying MoA (Aardema and MacGregor, 2002; Piña et al., 2007). These molecular approaches can be used as suitable early warning systems and to provide powerful tools for high-

throughput screening of chemicals. In fact, gene expression is expected to be stress-specific and to respond quickly, from minutes to hours (López-Maury et al., 2008; Sørensen et al., 2005). Thus, the rapid development of toxicogenomics and associated high-throughput methods has greatly facilitated the in-depth characterization of molecular KE (Altenburger et al., 2012). In this regard, the first obvious advantage of using genomic information was the increase of the information that we could extract from one single experiment, being able to measure several gene expression shifts thus sorting and allowing better and more accurate conclusions. This would also allow to establish links between molecular biomarkers, as early signals and responses, to higher level responses, such as individual and population, in order to anticipate potential risks (Wernersson et al., 2015). Nowadays this new discipline is becoming a key tool for the assessment of environmental impacts of emerging pollutants, thanks to its potential to highlight toxicant specific gene expression patterns, namely defining new biomarkers that can be used to identify new and more accurate MoA (Piña and Barata, 2011). In fact, it offers a different approach to the ERA, and its application as a standardized procedure for risk assessment is presently being the object of discussion between scientists, regulators and policy-makers, weighting its advantages and disadvantages (Robbens et al., 2007).

A major step for our ability to monitor, study and understand the transcriptional patterns of genes transcription came with the development of new molecular techniques such as quantitative (Real-Time) Polymerase Chain Reaction (qPCR) and, more recently, microarrays and next generation sequencing (NGS) e.g RNAseq, that revolutionized the way how we were able to address genomic processes. Whereas qPCR analyses allow the monitoring of few genes at the time with high resolution in a quantitative way, techniques like microarrays and RNAseq offer opportunity for the screening of thousands of genes at once, providing a more complete picture of toxicologically significant events, although in a much more qualitative way (Martyniuk and Simmons, 2016; Newton et al., 2004; Wang et al., 2009).

Thanks to an integrative use of these techniques, we are able to study new potential genetic biomarkers, being every gene a potential biomarker of any given change: life-cycle changes, chemical changes, etc. In order to achieve this final goal of connecting genes, phenotypes, populations and ecosystems to improve our understanding of physiology, ecology and evolution, we need to apply modern genomic tools to model organisms known to have substantial and diverse ecological roles in natural environments (Miner et al., 2012). The ultimate purposes of integrating an OMICs-oriented approach into toxicity studies is to build toxicity pathways by obtaining a complex overview of stress-response profile and identifying key events leading to effects at high biological organization levels (apical). In addition, OMICs tools are of great importance in order to be able to study the effect of stressors at low concentrations i.e. NOEC levels, as the molecular endpoints are relatively more sensitive than conventional toxicological endpoints. It should be stressed that responses at the molecular level to a very low exposure level may not necessarily be the witness of an adverse outcome at the physiological level, but provide important information to unravel stress-induced signal transductions and the specific underlying MoA (Song et al., 2012).

1.1.13 Daphnia magna as a model organism

Daphnia magna, a freshwater micro-crustacean, is advocated as one of the best model organisms for ecotoxicology studies in freshwaters ecosystems and was used as test organism in all parts of this thesis. During the last decades, *Daphnia* has been successfully used as a model organism in a broad range of applications including evolution, ecology, toxicology and genomics (Lampert and Kinne, 2011; Lynch, 1984; McCauley et al., 1990; Shaw et al., 2008) with more than 200 years of data archived about its physiology, ecological role, development and evolution. More recently, *Daphnia* has slowly matured into a versatile genomic model thank to the work of the *Daphnia* Genomics Consortium, an international network of researchers whose joint efforts are committed to establish *Daphnia* as a premiere model system (Shaw et al., 2008; Stollewerk, 2010). This lead to the publication of the first *Daphnia* species genome for the related species *D. pulex (Colbourne et al., 2011)* and fully

assembled transcriptome for *D. magna* (Orsini et al., 2016). As a results, it has also been suggested as a primary model species for ecological genomics, transcriptomics and ecotoxicogenomics, fields that aim to understand how biotic and abiotic stressors, as well as evolutionary and life-history characteristics, affect gene expression and other biological and ecological changes (Colbourne et al., 2011; Ebert, 2011; Orsini et al., 2011; Orsini et al., 2016; Watanabe et al., 2008).

In this perspective, the freshwater zooplankter *Daphnia* offers a very powerful model with an exceptional potential. In fact, the combination of modern genomic tools together with well-documented community and ecosystem impacts makes *Daphnia* the ideal species for integrative investigation of mechanisms and MoAs that underlie responses to environmental changes (Miner et al., 2012; Shaw et al., 2008). Besides, they are internationally recognized as an indicator of environmental health and fitness and, consequently, as an important and widely used bioassay of aquatic toxicity to define regulatory limits. The use of this "super-model" has also recently been recommended by the NIH to be used in biomedical research.

The taxonomy classification of *Daphnia magna* Strauss is as follows (Boxshall, 2013): Animalia > Arthropoda > Branchiopoda > Phyllopoda > Diplostraca > Cladocera > Anomopoda > Daphniidae > *Daphnia* O.F. Muller, 1785 > *Daphnia magna* Straus, 1820.

D. magna (Fig. 1.1) is a brackish and freshwater organism, found in lakes and ponds all around the world and plays a key ecological role in the food web as a highly efficient grazer and a preferred prey item for fish and other invertebrate predators (Lampert and Kinne, 2011). Similarly to other crustaceans, its growth is not continuous, possible only by regular substitutions of its chitinous, non-elastic exoskeleton, a phenomenon known as molting (Ebert and Jacobs, 1991). *Daphnia* species are non-selective filter feeders, mainly grazing on algae, but able to retain and ingest without selection all the suspended particles that can be withheld by their filtering apparatus (>1um) (Gillis et al., 2005).

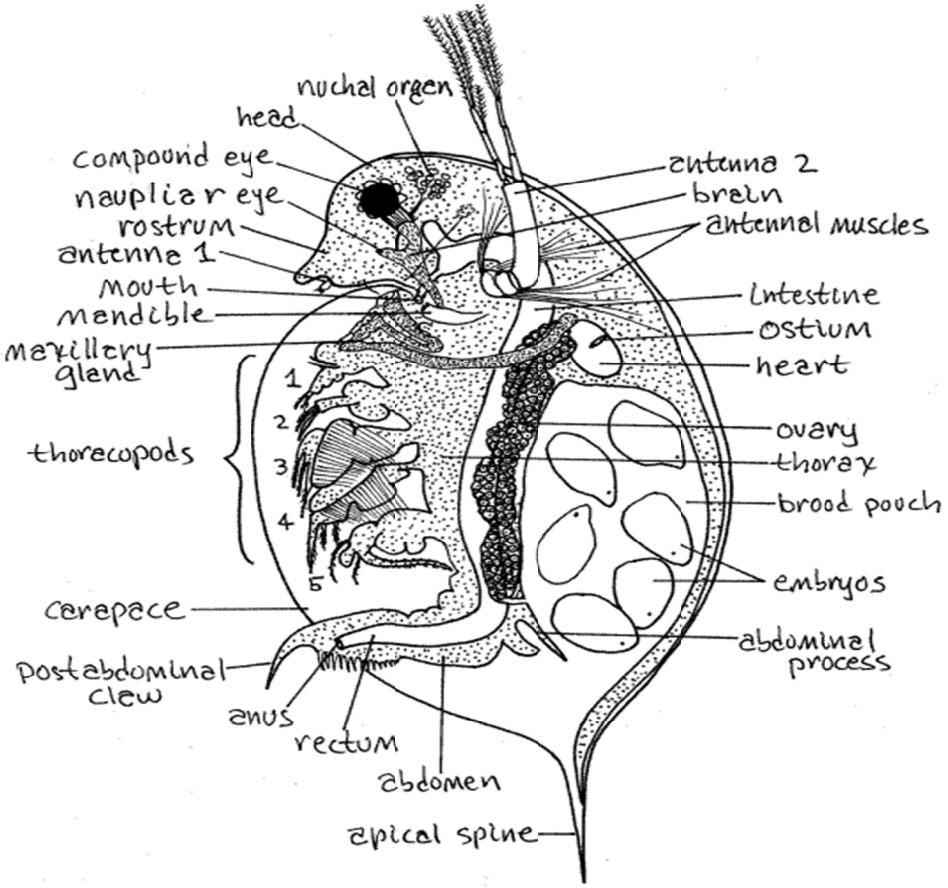

Figure 1.1 The functional anatomy of *Daphnia (From Richard Fox, Lander University)*

Another peculiar characteristic of *Daphnia* is its unusual life cycle (Fig. 1.2), which has been now studied for more than 150 years (Lubbock, 1857). In fact, most species are cyclic parthenogenic, able to produce two different types of eggs, in response to surrounding environmental cues. In presence of optimal environmental conditions (good water quality, low population density, non-limiting food conditions, absence of other stress factors, …) *Daphnia* reproduces through cyclical parthenogenesis (producing diploid eggs). During this phase, the population is composed exclusively by females, with the progeny showing a genetically identical background to the mother. Nevertheless, changes in the environmental conditions like food limitation, high population densities, short photoperiod or desiccation or chemical cues can instead activate sexual reproduction, leading to the production of haploid sexual eggs

(Kleiven et al., 1992; Zaffagnini, 1987). Likewise, sex determination is also environmentally driven and males showing sexual dimorphism are thus produced in response to suitable environmental prompts (Zaffagnini, 1987). Moreover, *Daphnia* also exhibits a wide range of remarkable polyphenisms, phenotypic body alterations including helmet and neck teeth formation, in response to predators, thus revealing its eco-plasticity and capacity of fast adaption to external stimuli (Weider and Pijanowska, 1993).

The attractiveness of *Daphnia* as an excellent candidate for ecotoxicological model organism is further enhanced by the fact that they are easy and inexpensive to maintain and have a rapid life cycle. Its lifecycle is ideally suited for experiments, due to its short reproduction time when compared to most eukaryotic model species: the reproductive maturity is reached within six to ten days and a reproductive batch occurs every three days hereafter (Ebert, 1992).

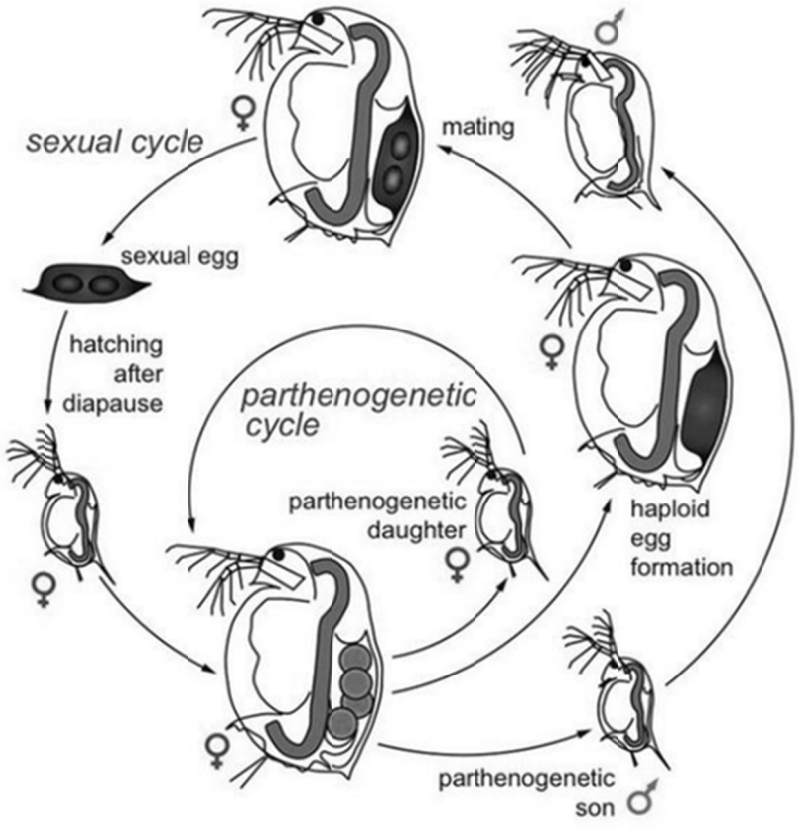

Figure 1.2 Life cycle of *Daphnia*, showing alternating parthenogenetic and sexual cycle (from Ebert, 1995)

Besides, through asexual parthenogenic reproduction, it is possible to produce genetically constant clonal lines, hereby reducing genetic variability, offering unparalleled opportunities of reproducibility. On the other hand, clonal lines with different genetic background can also be maintained to create experimental populations with controlled genetic variation and studying the genetic architecture underlying phenotypic variation in natural populations (Orsini et al., 2012).

1.2 Methodology and approaches

Due to the holistic nature and goals of this thesis, an extensive set of techniques was used for its successful accomplishment. In fact, in order to achieve full comprehensive assessment at different organization levels, e.g. ecological, physiological and molecular, the use of a wide selection of methods and skills belonging both to different fields of biology and chemistry were required. As already stated earlier, *Daphnia magna* was used as model organism throughout the progress of this work. Both *in vivo* and *in vitro* bioassays were included, together with molecular and chemical analyses in order to achieve a comprehensive integrative approach when dealing with a series of heterogeneous environmental clues.

1.2.1 In vivo bioassays

Daphnia magna standardized ecotoxicology tests endorsed by OECD, such as standard acute (immobilization test, 48 hours, according to OECD guideline 202) and chronic tests (reproduction test, 21 days, according to OECD guideline 211), were used to study the effects of emerging contaminants. Effects of other classes of pharmaceuticals on survival and reproduction of *D. magna* have been already evaluated in previous studies (Campos et al., 2012b; Dietrich et al., 2010; Dzialowski et al., 2006; Flaherty and Dodson, 2005). Comparing these two approaches, a reproduction test running over 21 days allows the simultaneous study of several sublethal endpoints i.e. molting, growth rate, time to reproductive maturity, number of offsprings, body sizes and others, thus

providing a more adequate and sensitive method to detect and unravel toxicity effects.

Other sublethal testing included previously validated feeding rate assessment assays and behavioral experiments, both used as powerful and effective methods to screen for sub-lethal toxicity, in the field and lab exposures, respectively (De Meester, 1991; McWilliam and Baird, 2002). In fact, to overcome current limitations on ERA procedures, a comprehensive evaluation often requires the development of specific bioassays able to detect these effects and to give insights into novel mechanisms of action (Bednarska et al., 2013). For this reason, it is paramount to highlight the importance of natural stressors inside ERA and to endorse the idea that ecotoxicological assessments should incorporate at least some, most representative natural environmental factors, including a range of biological and ecological circumstances (Bednarska et al., 2013), i.e. feeding patterns and locomotor migration in the water column. These may be difficult to be incorporated into standardized ecotoxicological testing, thus tailored-made approaches become of extreme importance.

1.2.2 In vitro bioassays

A second category of performed bioassays belongs instead to the *in vitro* testing, in which the use of specific tissues/cells is preferred rather than the live animal or plant (*in vivo*) in order to determine the biological activity of a substance. The main advantages of *in vitro* techniques are the increased sensitivity, specificity and reproducibility when compared to the more traditional *in vivo* testing. On the other hand, the results extrapolation on possible consequences for organisms is more difficult. Despite their intensive application in environmental research, their measurements in invertebrates have not been commonly applied yet in ERA or used for regulatory purposes (Jemec et al., 2010). *In vitro* techniques used in this thesis encompassed biochemical biomarkers and recombinant yeast assays (RYA).

Biochemical (or enzymatic) biomarkers of exposure are good indicators of toxicity including oxidative stress and neurotoxicity among others. At the base of

their functioning it lies the concept that exposure to several anthropogenic contaminants e.g. metals, redox cycling compounds, dioxins, may lead to oxidative stress and produce high levels of reactive oxygen species (ROS), thus leading to oxidative damages of the main cellular components, primarily lipids, proteins and DNA, and eventually to cell death (Halliwell and Gutteridge, 2007). Furthermore, they can result in an over-accumulation of the neurotransmitter acetylcholine, thus provoking neuromuscular paralysis and leading also to death. Among the many existing and validated biomarkers, the most widely used are antioxidant enzymes, important in the prevention of oxidative stress damages e.g. catalase (CAT) and glutathione-S-transferase (GST), lipid peroxidation (LPO) and DNA damage (DNAd), and enzymes related to neurotoxicity e.g. acethylcholinesterase (AChE) and carboxylesterase (CbE). Evaluation of the levels (activity) of these enzymes allows the identification of possible effects of the tested compound on the organisms. Their inherent capacity to detect toxic effects and causal mechanisms potentially responsible for effects at higher levels of organization made biochemical biomarkers one of the most promising tools for ecotoxicological applications in the last decades (Adams, 2002; Depledge and Fossi, 1994; Peakall and Walker, 1994). As a matter of fact, in the last decades they have been widely used as diagnostic tools in field studies (Barata et al., 2007; Damásio et al., 2010; Faria et al., 2009; Faria et al., 2010), but also in laboratory toxicity studies to test specific hypotheses concerning mechanisms of chemical impact (Forbes, 2000).

The second *in vitro* tool used in this compendium was the recombinant yeast assay (RYA) that allows to evaluate the ability of a given compound (or a mixture) to bind to a specific receptor and to elicit the physiological responses associated to this binding in *in vivo* conditions. This assay makes use of genetically modified yeast strains, an engineered yeast strain carrying at least two foreign genetic elements: the former allows the expression of a given vertebrate nuclear receptor; the latter, a reporter gene located under transcriptional control of the expressed nuclear receptor and whose expression is easy to quantify, is intended to allow monitoring of the activity of the expressed receptor e.g. an enzyme or a fluorescent protein (Fox et al., 2008).

Examples of application of RYA to environmental monitoring include the use of yeast strain harboring the receptor responding to presence of dioxin-like compounds (aryl hydrocarbon receptor, AhR-RYA) and estrogenic compounds (estrogen receptor, ER-RYA). This approach does not provide a precise chemical characterization of the ligand-receptor ligands, but their low cost, short time of execution and easiness of handling make them widely used when testing large numbers of samples or compounds (Barcelo and Hansen, 2008).

1.2.3 Molecular techniques

In order to widen the approach and get a deeper insight into the MoAs after field exposure, transcriptomic studies were also performed, including RNA extractions and cDNA synthesis, PCR and quantitative Real-Time PCR (qPCR). qPCR involves the study of specific gene expression (mRNA levels) characterized by a wide dynamic range, low quantification limits and the least biased results when compared to other methods such as microarrays and RNAseq (Dallas et al., 2005; Wang et al., 2009). It is specifically suitable when analyzing a restricted number of genes whose sequence is well known. Generally, environmental stress situations can be assessed at molecular level by choosing specific genetic biomarkers, representative of stress response and other specific metabolic pathways or linked to phenotypic endpoint of exposure, resulting in an exposure fingerprint, which provides information concerning the response of cells and organisms to changes in the external environment (Calzolai et al., 2007; Snell et al., 2003). Compared to conventional stress enzymatic biomarkers i.e. catalase, glutathione S-transferase and cholinesterase among others, gene expression analyses by qPCR are technically more demanding and costly but on the other hand more sensitive and represent a better tool to unravel toxicity mechanisms. Molecular biomarkers developed using this approach have the potential of providing early detection of environmental stress (being gene transcription the first affected step), inferring mechanisms of action and, overall, improving the monitoring of the environment (Calzolai et al., 2007).

1.2.4 Analytical chemistry

Chemical analyses performed according to the most advanced techniques of analytical chemistry were run in parallel to the bioassays in order to support results and provide additional information to explain results. This involved the use of several analytical tools, including both liquid and gas chromatography (LC and GC, respectively) coupled with mass spectrometers. Both techniques are analytical methods that combine the feature of chromatography separation together with mass spectrometry in order to identify substances (by their mass) within a complex sample, and are considered of extreme value in environmental chemistry, especially when dealing with forensic substance identification, thanks to their high specificity and low levels of detection.

The equipment is composed of two parts: the chromatographer that allows the separation of compounds thanks to their affinity to a stationary phase and the choice of the mobile phase that may be liquid (LC) or gaseous (GC); the mass spectrometer that ionizes and sorts the molecules based on their mass-to-charge ratio, thus measuring their mass weight (m/z). Among existing kinds of mass detector available, the triple quadrupole (TqD) composed of three consecutive linked quadrupole mass analyzers, allows for tandem mass spectrometry analyses (MS/MS). This involves multiple steps of mass spectrometry selection, with a step of fragmentation in between. The coupling of chromatography with mass spectrometry (LC- or GC-MS/MS) provides an additional performance enhancement in terms of resolution and sensitivity (in the order of ηg/L or even pg/L) and representes a major breakthrough in forensic environmental studies. Whereas GC-MS/MS is highly suitable for volatile compounds or molecule that can be easily vaporized, LC-MS/MS is usually the preferred choice allowing the identification and quantification of a wide battery of organic compounds down to ηg/L levels without time-consuming derivatization and with minimal sample cleanup (Bussy et al., 2016).

1.3 Study sites

Availability of water has been a major driver of socio-economic development in the entire Mediterranean basin, despite of its typical temporal variability. In these last decades, the intensification of climate change effects together with the increasing anthropogenic manipulation of hydrology has resulted in an even larger temporal variability. Thus, Mediterranean rivers and water bodies are undergoing severe alterations in their flow regime and stability. Mediterranean water bodies are also heavily impacted by extensive urban and industrial wastewater discharges and agricultural runoffs that often cannot be diluted by the natural flow of rivers. Under this perspective, different river basins from the Iberian Peninsula (Fig. 1.3) offer good examples of water bodies stressed by different anthropogenic pressures.

Figure 1.3 Geographical localization of the four study sites in the Iberian Peninsula: Llobregat (A), Ebro (B), Júcar (C) river basins and Tablas de Daimiel National Park (D) are shown.

1.3.1 Ebro River

The Ebro River (Fig. 1.3 B) is the largest and most important river in Spain, with 928 km in length, affected both by Atlantic and Mediterranean climate (http://www.chebro.es). At its end, it gives origin to the Ebro Delta, one of the largest wetland areas (320 km²) in the western Mediterranean region and a National Park since 1983 (Parc Natural del Delta de l'Ebre). Nowadays, the delta area is in intensive agricultural use mainly for rice, but also fruits and vegetables. Along its course, the Ebro River is largely regulated by several dams and channels, which have altered its hydrological and sedimentary regime and decreased its flow by approximately 30%. Agriculture and irrigation activities together with heavy industries concentrated close to the main cities in the basin have also deteriorated soil and water quality, making its water pollution a relevant issue. In particular, it is worth to mention the area of Flix in the lower part of the river course, where a chlor-alkali industry operated since the beginning of the 20th century and resulted in the accumulation of high amounts of heavily polluted sediments in the adjacent riverbed.

Major pollutants present at this site included high levels of organochlorides (hexachlorobenzene, DDEs–DDTs, polychlorostyrenes,…) and heavy metals (e.g. mercury, cadmium, nickel) and were carried downstream by the flow until its delta (Bosch et al., 2009; Fernandez et al., 1999; Soto et al., 2011).

1.3.2 Llobregat River

The Llobregat River (Fig. 1.3 A) is the second longest river in Catalonia (NE Spain), with a total length over 170 km. The river is heavily impacted in its lower course and water that was previously lost to the sea is now pumped upstream to increase the natural flow. This river is one of Barcelona's major drinking water resources (Catalonia, NE Spain). Though, it receives surface runoff from agricultural areas as well as extensive urban and industrial waste water discharges (Sabater et al., 2012). Consequently, waters have high concentration of pesticides, surfactants, pharmaceuticals and estrogenic compounds with important effects on the biological communities (Ginebreda et

al., 2010; González et al., 2012; Kuster et al., 2008; López-Serna et al., 2012; Muñoz et al., 2009; Sabater et al., 2012).

1.3.3 Jucar River

The Jucar River (Fig. 1.3 C) basin is located in the east of Spain, with a main stream length of approximately 500 km. The management of the system is very complex and presents considerable hydrologic variability, due to an intensive water use. The medium part of the basin is mainly characterized by agricultural and irrigation activities, whereas in the lower part of the river a great part of urbanized, industrial and agricultural pressures are present, decreasing substantially the water quality. The Jucar basin was designated as a European Pilot River Basin for the implementation of the WFD (Molina et al., 2011).

1.3.4 Tablas de Daimiel National Park

The Tablas de Daimiel National Park (TDNP, Fig. 1.3 D) is a floodplain wetland located in the Upper Guadiana Basin (central Spain) and represents one of the most important semiarid wetlands of the Mediterranean area. In 1980, the TDNP was declared by UNESCO one of the core areas of the Mancha Húmeda Biosphere Reserve, for its special relevance among European wetlands as an ecological refuge for many water birds and plant species. The wetland is the result of the mixture of inputs from two rivers, namely Gigüela and Guadiana, together with groundwater discharge from the West Mancha aquifer. The peripheral surface of the wetland is 1928 ha, but at present, the potentially flooded area is 1587 ha (Sánchez-Carrillo et al. 2010). Characterized by a semi-arid climate, it presents an irregular spatio-temporal rain distribution and high temperature in summer, thus making its water balance particularly fragile, showing water shortage considered a structural characteristic of the system (Sanchez-Ramos et al., 2016). Moreover, it is also subjected to intensive groundwater overexploitation since the late 1970s (Navarro et al., 2011) and water pollution due to population growth and agriculture and its associated industry development. Worth of mention is its proximity to a point-source pollution site due to the presence of a waste water treatment plant (WWTP).

1.4 Objectives

The overall goal of this thesis is the characterization of novel mechanisms of toxicity of contaminants present in the aquatic ecosystem both in the field and in the laboratory. Aquatic environments in the field are affected by complex mixtures of contaminants and other natural stressors making quite difficult to identify with a high degree of certainty toxic compounds. In this regard my approach was to use a large and diverse range of assays, biomarkers combined with multi-analytical mass spectrometry techniques. Conventional and behavioral assays were used in lab exposures to characterize the mechanisms of action of new emerging contaminants such as psychiatric drugs at realistic environmental concentrations. Field studies bearing different problematics were considered to specifically address three specific objectives, whereas a study conducted in the laboratory evaluating sublethal effects of neuro-active pharmaceuticals at relevant environmental concentrations was pursued as fourth and last objective.

- **Objective 1.** To test the usefulness of using transcriptomic responses of *D. magna* genes in detecting and identifying different types of environmental stressors in the field in transplanted organisms.

This objective was tested comparing transcriptomic responses of selected genes in transplanted animals across three river basins with detrimental physiological effects measured using already validated bioassays (feeding rates, biomarkers), corroborated by measurements through other validated bioassays. The study was conducted in three river basins affected by different anthropogenic pressures and climatic/flow conditions. The study is described in chapter 2 and it is entitled “Transcriptomic, biochemical and individual markers in transplanted *Daphnia magna* to characterize impacts in the field”.

- **Objective 2**. To characterize an unknown source of toxicity caused by small amounts of highly toxic bio-active compounds (cyanotoxins) bound to suspended particles in a highly contaminated site impacted by a chlor-alkali industry.

Testing this objective required the development of a new analytical method for a rare cyanotoxin “anatoxin-a” described in chapter 3 and entitled “Liquid chromatography coupled with tandem mass spectrometry to characterize trace levels of cyanobacteria and dinoflagellate toxins in suspended solids and sediments”, and to develop a test procedure to assess effects of toxic compounds bound to suspended material and able to distinguish specific effects of cyanotoxins from other potential causes of toxicity. The study is described in chapter 4 and entitled ”Identification of compounds bound to suspended solids causing sub-lethal toxic effects in *Daphnia magna*. A field study on re-suspended particles during river floods in Ebro River”.

- **Objective 3.** To combine chemical and toxicity assays (*both in vivo* and *in vitro*) to identify chemicals causing toxicity in the pristine mediterranean floodplain reserve (Tablas de Daimiel), which has a high Ecological value (RAMSAR site and UNESCO bird reserve).

This Natural reserve has been threatened in the past by an over-exploitation of its water sources and now the scarcity of water is amended from water coming from other river basins and water treated effluents. Thus, it is important to determine if water sources entering the flood plain of Tablas de Daimiel contain contaminants toxic to aquatic wild life. The study is described in chapter 5 and is entitled “Integrated environmental risk assessment of chemical pollution in a Mediterranean floodplain by combining chemical and biological methods”.

- **Objective 4.** To test the hypothesis that different families of neuro-active pharmaceuticals may lead to similar phenotypic responses in *D. magna*, such as enhance reproduction and alter behavioral responses, at low environmentally relevant concentrations.

This study is supported from a previous work conducted in our lab in which we found that fluoxetine, the active component of Prozac, enhanced reproduction in *Daphnia* and altered phototactic behavior in amphipods. The study is described in chapter 6 and entitled “Low environmental levels of neuro-active pharmaceuticals alter phototactic behavior and reproduction in *Daphnia magna*”.

Overall, this work illustrates, through three different field case studies and one laboratory-based study, the complementary role of classical bioassays, specific biomarkers and chemical analyses which, used in combination, provide major information to understand impacts of anthropogenic pressures with the potential to affect the aquatic environment. We discuss the inherent difficulties of field studies, where the interactions among chemicals and/or other confounding factors might occur, the limitations of this kind of approach and future improvements needed.

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Chapter II.

Transcriptomic, biochemical and individual markers in transplanted *Daphnia magna* to characterize impacts in the field

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Chapter II.

Transcriptomic, biochemical and individual markers in transplanted Daphnia magna to characterize impacts in the field[a]

2.1 Abstract

Daphnia magna individuals were transplanted across 12 sites from three Spanish river basins (Llobregat, Ebro, Jucar) showing different sources of pollution. Gene transcription, feeding and biochemical responses in the field were assessed and compared with those obtained in re-constituted water treatments spiked with organic eluates obtained from water samples collected at the same locations and sampling periods. Up to 166 trace contaminants were detected in water and classified by their mode of action into 45 groups that included metals, pharmaceuticals, pesticides, illicit drugs, and other industrial compounds. Physicochemical water parameters differentiated the three river basins with Llobregat having the highest levels of conductivity, metals and pharmaceuticals, followed by Ebro, whereas the Jucar river had the greatest levels of illicit drugs. *D. magna* grazing rates and cholinesterase activity responded similarly than the diversity of riparian benthic communities. Transcription patterns of 13 different genes encoding for general stress, metabolism and energy processes, molting and xenobiotic transporters corroborated phenotypic responses and differentiated sites within and across river basins. Principal Component analysis and Partial Least Square Projections to Latent Structures regression analyses indicated that measured *in situ* responses of most genes and biomarkers and that of benthic macroinvertebrate diversity indexes were affected by distinct environmental factors.

Conductivity, suspended solids and fungicides were negatively related with the diversity of macroinvertebrates cholinesterase, and feeding responses. Gene transcripts of heat shock protein and metallothionein were positively related with 11 classes of organic contaminants and 6 metals. Gene transcripts related with

signaling paths of molting and reproduction, sugar, protein and xenobiotic metabolism responded similarly in field and lab exposures and were related with high residue concentrations of analgesics, diuretics, psychiatric drugs, β blockers, illicit drugs, trizoles, bisphenol A, caffeine and pesticides. These results indicate that application of OMIC technologies in the field is a promising subject in water management.

Keywords: *Daphnia*, feeding, transcriptomics, gene, biomarker, *in situ*, benthic macroinvertebrate, river, water quality

2.2 Introduction

Identifying indicators of adverse change in ecological systems which can diagnose causal agents is a major challenge in environmental risk assessment (Baird and Burton, 2001). Traditionally, biomonitoring of fresh waters has been based on measures of community structure, focusing on biodiversity metrics (Rosenberg and Resh, 1993). Such diagnostic measures have been widely used to establish the ecological quality of water (Munné and Prat, 2009). Nevertheless, biodiversity metrics are based on the occurrence of species and hence they are mostly sensitive to dramatic changes such as reductions of individuals within species or even to species extinction. These features make biodiversity metrics unable to detect subtle changes of individual physiological responses. Furthermore, in many cases biodiversity metrics respond to other environmental factors than to trace contaminants (Baird and Burton, 2001).

The development of new bioassays with caged single species has allowed determining pollutant effects *in situ*. Key advantages of *in situ* bioassays over whole effluent toxicity tests and biological surveys of invertebrate communities include: a greater relevance to the natural situation, especially with respect to the contamination scenario; and their ability to detect effects more rapidly (hours to days) than resulting changes in community structure (months to years) measured during macroinvertebrate sampling (Maltby et al., 2002). More specifically, a set of *in situ* and cost effective bioassays based on feeding and biochemical responses of invertebrate species have permitted detecting lethal

and sublethal responses that are biologically linked with key ecological processes such as detritus processing and algal grazing rates, and of specific toxicological mechanisms (Barata et al., 2007; Burton Jr et al., 2005; Damásio et al., 2010; Maltby et al., 2002; Maltby et al., 2000; Schulz and Liess, 1999). Nevertheless such developments were still limited to few responses. The use of OMIC technologies may offer the possibility to extent those responses to many genes within individuals.

The water flea *Daphnia magna* is possibly the invertebrate species most used in toxicology and experimental ecology and together with its close relative *D. pulex* is used as a model for environmental genomics research (Piña and Barata, 2011). *D. pulex* genome has been fully sequenced and about 50% of its genome is annotated (Colbourne et al., 2011), thus that of its close relative *D. magna*, despite of being incomplete, may benefit from the former. Indeed several studies have identified gene markers within *D. magna* genome that respond to specific pollutants. These genes include general stress genes such as the heat shock protein 70 (HSP70) and metallothioneins (MT2), whose transcripts are induced by several metals (Ho, 2008; Poynton et al., 2007); genes involved in metabolic pathways, i.e. the metabolism of the sugars (UDP-glucose pyrophosphorylase, UGP), lipids (thiolase, THIO), amino acids (fumarylacetoacetate, FAA) and the Krebs cycle (isocytrate dehydrogenase (IDH), aconitase (ACON) (Campos et al., 2013); specific genes encoding key processes of growth and reproduction (vitellogenein, VTG; ecdysteroid receptor, EcR; retinoid X receptor, RXR and molt-inhibiting hormone, MIH) (Montagné et al., 2010; Tokishita et al., 2006; Wang and LeBlanc, 2009; Wang et al., 2007); and transporter genes from the multixenobiotic resistance mechanisms such as the P-glycoprotein (Pgp) and multidrug resistance protein 4 (MRP4) (Campos et al., 2014).

The Mediterranean basin is one of the world's regions most vulnerable to global change (Barceló and Sabater, 2010) and one of the "hot spots" for ongoing problems in water availability (Giorgi and Lionello, 2008). Here we tested the feasibility of using mRNA responses of *D. magna* genes in detecting and identify environmental stressors in the field having detrimental effects in river

biota. To do that, up to 19 responses in *D. magna* individuals transplanted in the field were used to evaluate the effects of up to 167 trace contaminants and five general physicochemical parameters in 12 sites belonging to three distinct Mediterranean rivers. The biodiversity of benthic macroinvertebrates were also considered to allow comparison of *D. magna* responses with those of the whole community. *D. magna* responses included that of post-exposure feeding rates, five enzymatic biomarkers (cholinesterase, carboxylesterease, lactate dehydrogenase, catalase, glutathione S transferase) and 13 genes (HSP70, MT2, ACON, IDH, UGP, FAA, THIO, VTG, EcR, MIH, RXR, PGP, MRP4). Secondly, the response of the studied genes together with that of post-exposure feeding rates was evaluated in *D. magna* individuals exposed in the lab to organic extracts of water samples collected at the studied sites. This allowed comparing the robustness and repeatability of the studied gene responses in detecting effects of organic contaminant residues. This study focused on three river basins Llobregat, Ebro and Jucar rivers (NE Spain).

2.3 Material and Methods

2.3.1 Study sites

Like most Mediterranean systems, Llobregat, Ebro and Jucar river basins natural resources have been greatly affected by human activities such as agriculture, urbanization, salinization by mining activities and an intensive water use for human consumption, which together have severely deteriorated the ecological status of the main rivers and tributaries since 1970s (Belenguer et al., 2014; Damásio et al., 2011; Damásio et al., 2010; Damásio et al., 2008; De Castro-Català et al., 2013; Fàbrega et al., 2013). The Llobregat River (northeast Spain) is 156.5 km in length, covers a catchment area of approximately 4,948 km^2, and its watershed is heavily populated (3,089,465 inhabitants in 1999). The Llobregat River is a paradigm of overexploited Mediterranean river with nearly 30% of its annual discharge used for drinking water. Moreover, the Llobregat River receives extensive urban and industrial wastewater discharges (137,000,000 m^3/year; 92% comes from the wastewater treatment plants) that cannot be diluted by its natural flow (0.68–6.5 m^3/s basal flow) (Muñoz et al.,

2009). The Ebro river is one of the largest river basins in Spain, 928 km in length and with a drainage basin of 85,550 km^2 and around 2,800,000 inhabitants living in the area. The most relevant economic activity in the region is basically agriculture (vineyards, cereals, fruit, corn, horticulture and rice production), but there are also some highly industrialized regions, mainly located in the northern-central part (Silva et al., 2011). The Júcar River is 497.5 km long drainage area of 21,600 km^2 and its mean annual flow is 10 m^3/s; it flows Eastern Spain, under a typical Mediterranean climate (Belenguer et al., 2014).

Deployment sites comprised four points along the Llobregat, Ebro and Jucar river systems, respectively (Fig. 2.1). The study stations were chosen as being examples of the different characteristics of the basins.

The Llobregat stations were L3 (Pont de Vilomara), L4 (Castellbell i el Vilar), L5 Abrera) and L7 (Sant Joan Despí). L3 site was located at the mid-section of the river, without much human impact. L4 was located after the junction with the Cardener river, characterized by its high conductivity and pollutants coming from Manresa sewage treatment plant (STP). L5 and L7 were located at the end of the mid and lower section of the river, downstream the STP of Monistrol de Montserrat and Barcelona, respectively. The Ebro study sites were all located in the upper-mid section of the river, an area with high water usage for agricultural purposes but also with an important human population settlement. E2 was located on the upper course at Miranda de Ebro. It is the first major city in the main stream. There is a large industrial area and the STP is less efficient than desired so the concentration of organic compounds in the river is high. E3, E4 and E5 sampling site received the influence of the wine field lixiviates from STP of Haro, Logroño and Tudela cities, respectively. Jucar Sampling stations were distributed along the full course of the river. The J2 site was located inside the city of Cuenca. Water at J4 site presented a strong eutrophic process and presence of filamentous algae along the reach was observed. J5 was just downstream of a small dam and J6 downstream the bypass of the irrigation channels that are used in the agricultural explorations further down.

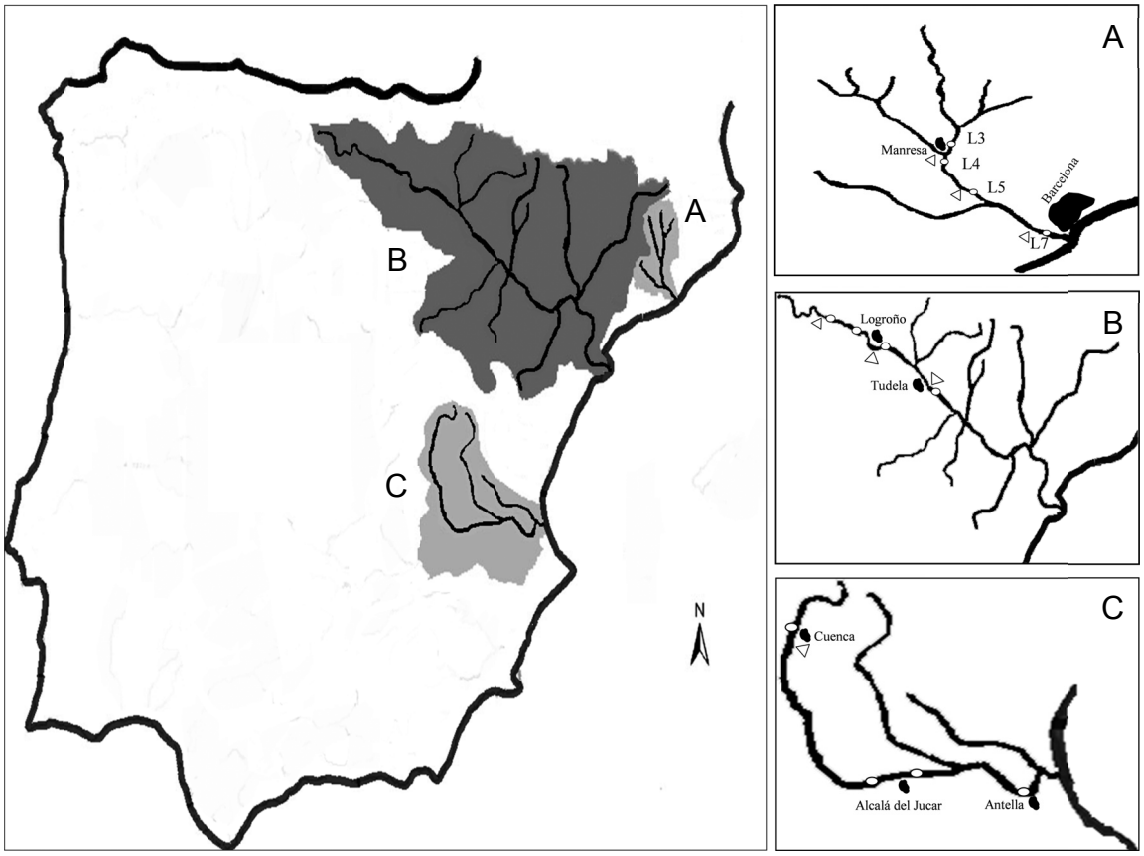

Figure 2.1 Study sites in Llobregat (A), Ebro (B) and Júcar (C) river basins (Iberian Peninsula). Site coordinates are in Table 2.1 Principal cities and sewage treatment plant effluents (Δ) are also depicted.

Table 2.1 Code, name and UTM coordinates for the studied sites.

Code	Name	UTM Coordinates
L3	Pont Viladomara	Zone 31 X: 405907 Y: 4617415
L4	Castellbell i el Vilar	Zone: 31 X: 403792 Y: 4607459
L5	Abrera	Zone: 31 X: 410078 Y: 4594291
L7	Sant Joan Despí	Zone: 31 X: 420247 Y: 4577928
E2	Miranda de Ebro	Zone: 30 X: 503672 Y: 4726140
E3	Haro	Zone: 30 X: 513141 Y:4715725
E4	Mendavia	Zone: 30 X: 565335 Y: 4696194
E5	El Bocal-Tudela	Zone: 30 X: 619147 Y: 4653811
J2	Cuenca	Zone: 30 X: 573092 Y: 4436231
J4	Quasiermas	Zone: 30 X: 601713 Y: 4336027
J5	Jalance	Zone: 30 X: 665927 Y: 4340496
J6	Zud de Antella	Zone: 30 X: 707741 Y: 4328283

2.3.2 Environmental measurements

A set of environmental variables were measured on each deployment occasion. Water physicochemical parameters including temperature (T; ^{o}C), pH, conductivity (μS/cm), dissolved oxygen (O_2, mg/l) and suspended solids (SS, mg/L) were obtained following (Damásio et al., 2008) procedures. Briefly, T, pH, conductivity and O_2 were measured *in situ* by using a WTW Multi 340i handheld meter, whereas total suspended solids were measured in the lab following ASTM Standard Methods (APHA-AWWA-WEF, 1995). Residue levels of eight metals in water and that of up to 158 organic contaminant residues were analysed. Organic contaminant residues were classify into 37 functional groups according to their mode of action or/and chemical structure. Further details of the established groups are in Table 2.2. Surface water samples (12) were taken from the studied sites. Duplicate water samples were collected in the middle of the current river with 2.5 L amber glass bottles. Within 48 h, water samples were vacuum filtered through 0.45 μm glass fiber filters and aliquoted into 1 L. The aliquots were extracted using OASIS HLB SPE cartridge (200 mg sorbent/6 mL cartridge, Waters) and eluted with different solvents according to the family of compounds that were to be analysed. Ilicit drugs were eluted with a gradient acetonitrile : water and analysed by isotope dilution on-line solid phase extraction-liquid chromatography-tandem mass spectrometry (SPE-LC-MS/MS) following the method described in (Postigo et al., 2008). Pharmaceuticals were eluted with methanol and the concentrations of the 73 compounds were determined using a multi-residue analytical method based on LC-MS/MS (Osorio et al., 2012). Most relevant environmental endocrine disruptors compounds (EDCs) and compounds suspected to be EDCs such as natural and synthetic estrogens and their conjugates, antimicrobials, parabens, bisphenol A, alkylphenolic compounds, benzotriazoles, and organophosphorus flame retardants were analysed using a fully automated approach in which water samples were directly injected into the chromatographic system and the target compounds were concentrated into the loading column. Thereafter, the analytes were transferred into the analytical column for subsequent detection by MS/MS (QqQ) (Gorga et al., 2013).

Table 2.2 Defined chemical classes according to the mode of action and chemical structure.

Chemical classes	Abreviations	Contaminants included
Metals		
As	As	
Co	Co	
Cu	Cu	
Fe	Fe	
Mn	Mn	
Ni	Ni	
Pb	Pb	
Zn	Zn	
Organic contaminants		
Analgesics: antipyretic	APYR	Phenazone, Propyphenazone
Analgesics, Opiates	AOPIATES	Oxycodone, Codeine
Analgesics: NSAID	ANSAID	Acetaminophen, Ibuprofen, Indomethacin, Diclofenac, Ketoprofen, Naproxen, Piroxicam, Meloxicam, Tenoxicam
Antibiotics	ANTIB	Erythromycin, Azithromycin, Clarithromycin, Tetracycline, Sulfamethoxazole, Trimethoprim, Metronidazole, 2-Hydroxy-Metronidazole, Ofloxacin, Ciprofloxacin, Cefalexin
Anticuagulant; Warfarin	WARF	
Anticuagulant; Aacridone	ACRID	
Lipid regulators, Fibrates	FIBRAT	Bezafibrate, Gemfibrozil
Lipid regulators, statins	STATIN	Pravastatin, Fluvastatin, Atorvastatin
Tricyclic antihistamins	ANTIHIST	Loratidine, Desloratidine
Antihistamins; Histamine H2-receptor antagonist	CIMET	Cimetidine
β blockers; treat hypertension	BBLCK	Atenolol, Sotalol, Metoprolol, Propanolol, Nadolol, carazolol
Angiotensin II receptor antagonist, treat hypertension	ANGIO	Irbesartan, Losartan, Valsartan
Diuretics; Sulfonylureas	DIUREAS	Torasemide, Glibenclamide
Diuretics	DIURET	Hidrochlorothiazide, Furosemide
Psychiatricc drugs acting on serotonin	SEROT	Fluoxetine, Norfluoxetine, Paroxetine, Sertraline, Citalopram, Venlafaxine, Trazodone
Psychiatric drugs acting on GABA type α receptors	GABA	Diazepam, Lorazepam, Alprazolam, Carbamazepine, Olanzapine

Table 2.2 (continuation)

Chemical classes	Abreviations	Contaminants included
β_2-adrenergic agonists to treat asthma	ADREN	Salbutamol, Xylazine, Azaperone
Anthelmintic veterinarian compounds	ANTHEML	Albendazol, Thiabendazole, Levamisol
Dexamethasone, anti-inflammatory glucocorticoid steroid	DEXTH	
X-ray contrast media	IOPROM	Iopromide
Selective α1 receptor antagonist	TAMSUL	Tamsulosin
Thienopyridine class antiplatelet agent	CLOPID	Clopidogrel
Azole fungicides	FZOLE	Azinphos methyl, Carbendazim, Imazalil, Prochloraz, Tebuconazole, Thiabendazole
Organophosphorous and carbamate insecticides	OPS	Chlorfenvinphos, Chlorpyriphos, Diazinon, Ethion, Fenthion, Malathion, Omethoate, Parathion-methyl, Methiocarb, Carbofuran, Fenthion
Herbicides; triazines	HTRIAZ	Deisopropylatrazine, Deethylatrazine, Simazine, Terbumeton, Terbumeton-deethyl, Terbuthylazine, Terbuthylazine-2-hydroxy, Terbutryn, Terbuthylazine-deethyl
Other pesticides	POTHER	Hexythiazox, Imidacloprid, Metolachlor, Pyriproxyfen
Illicit drugs, cocaine & metabolites (MB)	COCAIN	Benzoylecgonine, Cocaethylene, Cocaine
Illicit drugs, amphetamine-like compounds & MB	AMPHET	(±)-Amphetamine, (±)-MDMA, Ephedrine, (±)-Methamphetamine
Illicit drugs, opiods/opiates &MB	OPIOIDS	(±)-EDDP, (±)-Methadone, 6-acetylmorphine, Morphine
Triazoles; corrosion inhibitors	TRIAZOLES	1H-Benzotriazole, Tolytriazole
Parabens	PARABEN	Benzylparaben, Propylparaben, Ethylparaben, Methylparaben
Bisphenol A	BPA	
Caffeine	CAFF	
Estrogenic compounds; mainly estrone	E2	Estradiol, Estradiol 17-glucuronide, Estriol, Estrone, Estrone 3-glucuronide , Estrone 3-sulfate
Alkylphenols	APE	Nonylphenol, Nonylphenol diethoxylate, Nonylphenol monocarboxylate, Octylphenol , Octylphenol diethoxylate, Octylphenol monocarboxylate
Phosphate Ester Flame Retardants	PFR	Tris(2-chloroethyl) phosphate, Tris(butoxyethyl) phosphate, Tris(chloroisopropyl) phosphate
Perfluorinated compounds	PFCs	i,p-PFNS, L-PFBS, L-PFDS, L-PFHxS, L-PFOS, PFBA, PFDA , PFDoA, PFHxA, PFHxDA,PFOA , PFODA, PFTeDA , PFTrDA, PFUdA

Pesticides and PFCs were eluted using a mixture of dichloromethane : methanol (50:50) (v/v) and analysed by LC–MS/MS and LC–QTOF-MS (Masiá et al., 2013). Metal analyses dissolved in water were performed using 5 mL of filtered water (0.2 µm nylon membrane filters, Whatman) and acidified immediately with 1% of HNO_3 (65% suprapure, Merck). Analyses were done by inductively coupled plasma mass spectroscopy (ICP-MS 7500c Agilent Technologies, Inc., Wilmington, DE) (Bonet et al., 2013).

2.3.3 Biological condition

Invertebrate samples from sediment were randomly collected with a corer (24 cm^2 area, 5 replicates per site). Samples were sieved through a 500 µm mesh to separate invertebrates and fixed with 4% formaldehyde. The invertebrates were identified at species level and used to calculate species richness (S) and Shannon´s diversity index (Shannon and Weaver, 1963). More information is in (López-Doval et al., 2010)

2.3.4 Field and lab bioassays

2.3.4.1 Field exposures

In situ D. magna deployments were conducted as indicated by using the same test chambers and procedures of (Mc William and Baird, 2002) with only minor modifications that included 10 test chambers to allow collection of animals for gene and biomarker determination and to increase the number of replicates for post-exposure feeding rate measurements. Chambers were constructed from clear polyvinyl chloride cylindrical piping (13 cm long, 5 cm external diameter). Each chamber had two rectangular windows (7 x 3.5 cm) cut into either side of the cage, covered with 150 µm nylon mesh. Pipe ends were sealed with polypropylene caps. Groups of 4-5 chambers were placed inside a 13-mm^2 wire-mesh cylinder that was positioned in the stream perpendicular to flow. In each deployment, a lab control treatment with animals maintained in the lab and never exposed to the field was also included as a surrogate control. Deployments were conducted in 2011, on 15-16th September in Llobregat, 21-22nd September in Ebro and 5-6th October in Jucar. Within each period, deployments were conducted simultaneously in four locations that always

included at least a low polluted site. Briefly the procedure for the *in situ* bioassays was as follows. Juveniles were transported to field sites in groups of 10 in 175 glass jars filled with American Society for Testing Materials (ASTM) hard water (Mc William and Baird, 2002). At each site 10 chambers, each containing 10-20 individuals were placed inside a 13 mm^2 wire-mesh cylinder that was positioned in the stream perpendicular to flow.

2.3.4.2 Lab exposures to reconstituted water

A second exposure experiment was set in the lab to compare feeding and gene responses with those of the field and to test if responses observed in the field were related to organic contaminant pollution only. For that purpose we use the same water samples and extraction procedures as those reported for chemical analyses but without the addition of standards. Final eluates were then combined, evaporated to dryness with N_2 and re-suspended in 0.3 mL of acetone. Lab exposures were then conducted by exposing 50 animals in 2 L ASTM hard water dosed with 0.2 mL of the obtained acetone extract eluates. Exposures were conducted in 3 L glass bottles gently shaken (3 rpm) in an orbital incubator at 20ºC under darkness and lasted 24 h. Controls that were processed similarly as field samples, but using ASTM water only, were included in each trial. Each river was assayed simultaneously. After exposures animals were used for post-exposure feeding and gene responses as described below.

2.3.4.3 Post-exposure responses

After 24 h, animals were retrieved from chambers or lab exposure media. Twenty-five surviving animals of five chambers or collected from lab exposures were used to determine post-exposure feeding rates and the remaining individuals were pooled in Eppendorf's in groups of five, immediately frozen in liquid N_2 and kept at –80^oC until further gene analysis. From each of the remaining 5 chambers of field exposures, surviving animals were also pooled, frozen and used for biomarker determination.

Shortly after exposure (within 1 h) five surviving juveniles were placed into 60 mL screw-capped glass jars containing 50 mL of ASTM hard water, with *Chlorella vulgaris* (Beijerink, strain CCAP C211/12) at a concentration of 5 x 10^5

cells/mL, and allowed to feed for 4 h (Mc William and Baird, 2002). Three jars containing no animals were used to establish initial algal densities. Biomarker, gene and post-exposure feeding rates were also measured in animals maintained in the lab during the duration of the deployments and transported to the field sites to include as surrogate lab control. Post-exposure feeding experiments were conducted in darkness to avoid algal growth and under constant temperature conditions (20 $\pm$ 2ºC) provided by a thermostatised chamber. Individual feeding rates (cells animal^{-1} h^{-1}) were determined as the change in cell density during 4 h according to the method given by (Mc William and Baird, 2002). Cell density was estimated from absorbance measurements at λ = 650 nm in a dual-beam spectrophotometer (Uvikon 941) using standard calibration curves based on at least 20 data points, with an $r^2 > 0.98$.

2.3.4.4 Enzyme assays

Juveniles were homogenized at 4 ^{o}C in 1: 4 wet weight/buffer volume ratio in 100 mM phosphate buffer, pH 7.4 containing 100 mM KCl and 1 mM EDTA. Homogenates were centrifuged at 10 000 g for 10 min and the supernatants were immediately used as enzyme sources. Biochemical measurements were carried out on Uvikon 941 Plus dual-beam and Spectra-max Plus microplate reader spectrophotometers. Assays were run at least in duplicate. AChE was determined by a modification of the Ellman method adapted to mircroplate (Barata et al., 2004). Acetylcholinesterase activity was measured in the presence of 1 mM acetylthiocholine and 0.1 mM 5,5'-dithiobis-2-dinitrobenzoic acid (DTNB), and the increase of absorbance was measured at 405 nm. Catalase activity was measured by the decrease in absorbance at 240 nm due to H_2O_2 consumption (extinction coefficient 40 M-1 cm-1) according to (Aebi, 1974). The reaction volume was 1 mL and contained 50 mM phosphate buffer, pH 6.5, 50 mM H_2O_2 (Ni et al., 1990). Glutathione-S-transferase (GST) activity towards 1–chloro-2,4–dinitrobenzene (CDNB) was measured as described by (Habig et al., 1974). The reaction mixture contained 100 mM phosphate buffer (pH 7.5), 1 mM CDNB and 1 mM of reduced glutathione. The formation of S–2,4-dinitro-phenyl-glutathione conjugate was evaluated by monitoring the increase in absorbance at 340 nm. CbE activity was measured by the UV

method of Mastropaolo and Yourno (1981) in the presence of 0.25 mM α-naphtyl acetate, and the formation of naphthol monitored by the increase in absorbance at 235 nm (Barata et al., 2004). Lactate dehydrogenase (LDH) activity was determined according to Diamantino et al. (2001). Proteins were measured by the method of Bradford (1976) using serum albumin as standard.

2.3.4.5 Gene responses

Total RNA was isolated from the samples using Trizol reagent (Invitrogen, Carlsbad, USA) following the manufacturer's protocol and quantified in a NanoDrop D-1000 Spectrophotometer (NanoDrop Technologies, Delaware, DE). RNA quality was checked in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA). Quantities of 1 µg were retro-transcribed to cDNA using First Strand cDNA Synthesis Kit Roche®77 (Germany) and stored at -20ºC. Thirteen genes were selected for representation of different pathways/gene families: HSP70, MT2, ACON, IDH, UGP, FAA, THIO, VTG, EcR, MIH, RXR, PGP, MRP4. The gene glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was used as an internal control. For each of these genes primers were designed with Primer Quest (IDT Technologies, Coralville, IA) and are listed in Table 2.3. Aliquots of 10 ng were used to quantify specific transcripts in Lightcycler® 78 480 Real Time PCR System (Roche, Germany) using Lightcycler 480 SYBR Green I Master® (Roche, Germany). Relative abundance values of all genes were calculated from the second derivative of their respective amplification curve, Cp values calculated by technical triplicates. Cp values of target genes were compared to the corresponding reference genes.

2.3.5 Data analysis

Within each deployment date, post-exposure feeding rates, gene and enzymatic activity responses at the studied sites were converted to proportional responses relative to the surrogate lab controls. Proportional responses of all the studied 12 sites were then compared using one-way ANOVA, followed by post-hoc Tukey's multiple comparison test (Zar, 1996). Prior to analysis, data was log

transformed to meet ANOVA assumptions of normality and variance homoscedasticity.

Table 2.3 Primer pairs designed from available *D. magna* sequences for amplification of the selected genes.

	Primer sequence		
Genes	**forward**	**Reverse**	**Amplicon**
G3PDH	GACCATTACGCTGCTGAATACG	CCTTTGCTGACGCCGATAGG	100
ACON	AACTAACAACGGCACTGGCAC	CTTCTCCGTTAGCGCCTTTG	81
IDH	CTGTTTTCCGCGAACCTATCC	GACGACCAATGACAATGGGC	81
THIO	AGGCACACGCAATGTTTCC	TTTGGCCGTGCTAACGATG	81
FAA	ACTGGAACCCCACCTGGAGT	AACTTCGCATTCCACCACGT	81
UGP	GAATTGTGGCAAACCGCTTC	TTCCATCAACACCGCTCATC	81
HSP70	GACGTTGCTCCTCTGTCGCT	TGGGATAGTGGTGTTCCGCT	81
MT2	TGCGCTACTGGTGGTGAATG	CTTGCAGCAGGCGGACTT	81
VTG	GATTGCCAAAGATGCCGGT	TTCATCACCTCCTGCGAGC	100
EcR	GGGCAAGATGCTGAAGCTGT	AGCCGAAATGGCGTTACG	81
RXR	GTGTCGAGTGCAAGGACGAG	TTTTCCAGTTGGTTGAATGGG	100
MIH	GGCTTGCCTGAAAGTCTTGC	TTGCGTTAGCGGCCAATT	81
PGP	GTATCCAGTGCGGAAGTGGC	ACAGCGTATCGCTATTGCCC	100
MRP4	CCCGATCCCTTTACGTCGAT	GGTGGCGTCCTACATGAGTGT	100

To explore causal-effect relationships between the studied environmental variables and parameters and the biological responses, Principal Component Analysis (PCA) and Partial Least Square Projections to Latent Structures regression (PLS) methods were used (Damásio et al., 2008). PCA was used to investigate the existing relationships between samples and variables and to deduce how many independent sources (components) were needed to explain the observed (experimental) data variance. These included two PCA performed on biological responses obtained in field (18) and lab exposures (12) and one conducted on environmental variables (44).

PLS is a regression extension of PCA which was used to connect the information between biological responses (Y variables) and environmental (X) variables. PLS models can be expressed in terms of traditional regression coefficients, b, of the multilinear model (i.e. y = X b model), simplifying model interpretation for the general case where multiple latent variables are needed for a satisfactory data modeling and prediction. Information about the correlation

structure among variables and responses can be obtained using the VIP parameter. This parameter is a weighted sum of squares of PLS weights taking into account the amount of explained Y variance and it summarizes the information content of all latent and X variables (Wold et al., 1993). X variables having high VIP scores contributed greater to Y variance. We performed two PLS considering field and lab data. The latter analysis was limited to gene and post-exposure responses and organic contaminant residues.

Since variables were very different and they were not measured using the same scale units, the data was auto-scaled prior to analysis (each element was subtracted by its column mean and divided by the standard deviation of its column). The number of PCA and PLS components was finally selected according to cross validation leaving one out prediction errors criteria (Wold et al., 2001). PCA and PLS analyses were conducted using the Matlab 6.0 software (MathWorks, Natick, Massachusetts).

2.4 Results

2.4.1 Environmental water parameters

From the 158 organic residues measured in the water samples collected from the 12 sites, 22 were not detected and the remaining ones were grouped into 37 groups according to their mode of action or chemical structure. In some cases the groups depicted in Table 2.4 contained a single compound whereas in others the sum of many. The composition of each group and its full name is depicted in Table 2.2. In general, conductivity and suspended solids increase from upper to downstream reaches, being Llobregat the river having the highest levels. Water temperature was lower in Jucar sites. Measured oxygen levels in water and pH varied little across sites and were within ecological optimal values (Damásio et al., 2008). Selected contaminant groups varied dramatically across rivers and sites (Table 2.4). For most contaminant groups, the downstream site of Llobregat (L7) had the greatest values that on average were around 10 times higher than those of the rest of sites. As expected the PCA analysis performed for physico-chemical water parameters was dramatically influenced by site L7,

which alone explained 52.4% of data variance (Fig. 2.2 inlet graph). Without considering L7 and excluding four classes of contaminants that varied little across the remaining sites (warfarin, tricyclic antihistamins, tamsulosin, clopidogrel), the PCA resolved in five interpretable components that explained up to 79%of data variance (Table 2.5).

Sample scores obtained from each of the five principal components were significantly correlated with 31 out of the 44 environmental factors considered. The first component (PC1) explained up to 34.7% of data variance, separated the three rivers (Fig. 2.2) and its sample scores were significantly ($P<0.05$) correlated with measured suspended solids, four metals, eight pharmaceutical groups, two industrial chemical groups (triazoles and alkylphenols), opiod residues of illicit drugs, azole fungicides and dexamethasone. PC2 explained up to 14.8% of data variance, and mainly separated E2 from the rest due to its low levels of conductivity, Ni and bisphenol A. The third, fourth and fifth components explained 30% of data variance and their sample scores were significantly correlated with the values of 8 environmental factors (Table 2.5).

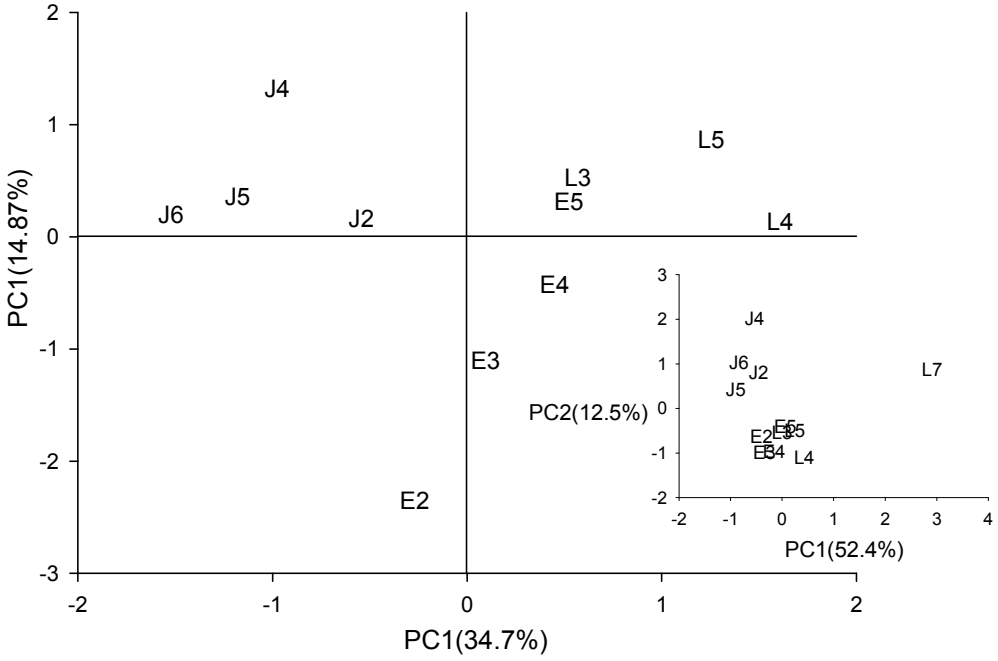

Figure 2.2 Representation of site scores for the Principal Component analyses performed on physico-chemical variables with (inlet graph) and without considering L7.

Table 2.4. Measured (Mean ± SD) physco-chemical water parameters across the studied river sites. Contaminant residues have been grouped in classes. See Table 2.2 for further explanation. Metal and organic contaminant levels are µg/L and ηg/L, respectively. The range (min-max) is also depicted.

Rivers	**Llobregat**				**Ebro**				**Jucar**			
Variables	**Mean**	**SD**	**Range**		**Mean**	**SD**	**Range**		**Mean**	**SD**	**Range**	
T (^{o}C)	21.9	1.7	19.9	23.9	20.8	1	19.9	21.8	17	3.5	14.6	22.2
O_2 (mg/l)	8.5	0.1	8.4	8.7	9.5	0.5	8.8	9.9	8.6	0.4	8.1	8.9
Cond (µS/cm)	1338.5	221.6	1105	1625	712.8	410.5	368	1272	751.5	181	530	972
pH	8.1	0.1	8	8.2	7.8	0.2	7.6	8.1	8.3	0.1	8.1	8.4
SS (mg/l)	42.2	11.6	32.1	55.4	22.6	30.3	3.4	67.8	10.7	6.1	3.1	17
As	3	1.8	1.7	5.7	1.4	0.1	1.3	1.5	1.1	0.3	0.8	1.5
Co	2	2.2	0.6	5.2	0.6	0.6	0.2	1.5	0.4	0.1	0.3	0.5
Cu	3.2	3.3	0.6	7.8	1.7	0.1	1.5	1.8	0.5	0.1	0.4	0.6
Fe	559.4	615.6	43.4	1273.5	47.2	43.8	14.9	110.9	67.7	72	15.2	169.6
Mn	92.6	124.4	8.5	277.6	8.3	3.4	5.9	13.2	3.6	3.5	1.1	8.6
Ni	6.3	8.4	1.4	18.9	1.2	0.4	0.7	1.5	1.4	0	1.4	1.4
Pb	1.1	1	0.2	2.2	0.6	0.4	0.2	1.2	0.2	0.2	0	0.5
Zn	15.4	12.5	3.8	32.2	4.9	2.2	2.2	7.6	4.4	2.1	2.9	7.4
APYR	1.3	0.2	1.2	1.6	1.2	0.1	1	1.3	0.7	0.4	0.4	1.2
AOPIATES	1.6	1	0.8	2.8	1.2	0.6	0.8	2.1	1.8	0.6	0.9	2.2
ANSAID	161.5	230.4	38.1	507	40.6	9.4	26.8	48	21.6	11.4	9.6	32.8
ANTIB	25.2	27.7	8.9	66.7	11.4	2.4	7.8	13	9.8	4.3	7.2	16.2
WARF	1	<0.1	1	1	1	<0.1	1	1	1	<0.1	1	1
ACRID	5.2	7.2	1.3	15.9	1.8	0.5	1.4	2.5	1.2	0	1.2	1.3
FIBRAT	83.1	127.4	13.7	274	10.1	5.5	4.5	17.5	3.1	3.1	0.2	7
STATIN	7.2	0.4	6.9	7.7	7.9	1.6	6.9	10.3	2.8	1.4	1.3	3.9
ANTIHIST	10.3	2.2	9.2	13.5	9.2	0.1	9.2	9.3	9.2	<0.1	9.2	9.2
CIMET	0.5	nd	<0.1	0.5	0.5	nd	<0.1	0.5	0.5	nd	<0.1	0.5

Table 2.4 continuation

Rivers	Llobregat				Ebro				Jucar			
Variables	**Mean**	**SD**	**Range**		**Mean**	**SD**	**Range**		**Mean**	**SD**	**Range**	
BBLCK	97.3	157.6	18.3	333.7	18.6	0.1	18.5	18.7	17.2	2.5	13.4	18.4
ANGIO	6.7	7.2	1.8	17.4	2.2	1.3	0.6	3.6	1	0.4	0.5	1.5
DIUREAS	1.7	1.2	0.9	3.5	1	0.1	<0.1	1.1	<0.1	nd	<0.1	<0.1
DIURET	125.4	196.8	20.1	420.4	5.1	2.9	1.5	8.4	1.1	0.6	0.6	1.7
SEROT	54.5	54.6	25.7	136.3	24.5	0.7	23.7	25.1	24.8	0.9	24.1	26
GABA	22.4	9.7	16.7	36.8	16.8	0.4	16.2	17.1	16.5	0.6	16.2	17.5
ADREN	1.4	0.5	0.7	2	0.7	0	0.7	0.8	1.4	0.4	0.8	1.6
ANTHEML	9.8	3.8	7.8	15.5	6.9	1	5.5	7.7	7.1	1	5.7	8.2
DEXTH	1.5	nd	<1	1.5	<1	nd	<1	<1	2.1	0.5	1.3	2.3
IOPROM	1.3	0.5	1.1	2	1.1	nd	<0.1	1.1	1.1	0.1	<0.1	1.1
TAMSUL	0.2	<0.1	0.2	0.2	0.2	<0.1	0.2	0.2	0.2	<0.1	0.2	0.2
CLOPID	5	2.4	3.7	8.6	3.6	0.1	3.6	3.7	3.5	0	3.5	3.5
FZOLE	132.7	nd	<0.6	132.7	8.7	6.6	<0.6	15.9	22.4	38.1	2	79.4
OPS	13	13.2	1.5	31.9	10.4	12	<0.6	18.8	11.9	5.7	5.6	17.7
HTRIAZ	30.5	51.2	<0.6	89.6	22.5	14.1	<0.6	38.6	17.4	4.7	<0.6	22.5
POTHER	19.7	25	4.8	57	3.9	4.4	<0.6	8.7	<0.6	nd	<0.6	<0.6
COCAIN	16.6	5.5	9.5	22.5	7.7	3.7	2.4	10.3	348.1	688	3.3	1380.1
AMPHET	40.3	70.1	2.9	145.4	6	4.7	1.5	12.2	5.2	9.3	0.3	19.1
OPIOIDS	15.3	24.2	1.5	51.5	1.6	0.7	1	2.6	0.2	0.1	0.1	0.3
TRIAZOLES	2405	3916.3	383.5	8279.1	595.7	332.6	215.5	1014.2	120.4	78.1	16.9	196.8
PARABEN	20.3	12.6	10.7	<0.5	6.8	5.6	1	12.3	30.7	37.6	2	81.4
BPA	80.8	22.3	51.5	103.5	37.9	52.8	1.7	114	62.8	14.5	54.5	84.4
CAFF	433.1	525.6	154.5	1220.9	210	122.4	51.7	340.9	506.5	758.3	109.6	1643.7
E2	4	2.7	1.5	6.3	1.4	0.6	0.9	2.1	1	0.5	0.6	1.5
APE	344.5	460	63.4	1032.1	224.9	232.5	39.6	529.4	34.9	32.4	5.1	79.9
PFR	431.3	624.5	99.8	1367.8	159.6	104.3	45.6	298.4	128.5	41.4	93.2	175.6
PFCs	32.4	35.5	5.1	80	30	17.7	9.5	48.2	33.7	45.5	7.3	101.9

Table 2.5 Results of the PCA performed on physico-chemical data depicted in Table 2.3. Site L7 and chemical groups WARF, ANTIHIST, TAMSUL, CLOPID have been removed from the analyses. % VAR, variance explained. Significant (P<0.05) Pearson correlations coefficients between sample PC scores and those of chemical classes are also depicted. * 0.05 < P < 0.01; ** P < 0.01.

	PC1	PC2	PC3	PC4	PC5
Eigenvalues	15.3	6.5	5.2	4.6	3.4
% VAR	34.7	14.8	11.9	10.5	7.6
Correlation					
SS	0.686*				
As	0.823**				
Cu	0.688*				
Mn	0.904**				
Zn	0.617*				
APYR	0.624*				
ANSAID	0.756**				
FIBRAT	0.908**				
ANGIO	0.746**				
DIUREAS	0.831**				
DIURET	0.930**				
SEROT	0.688*				
GABA	0.706*				
POTHER	0.765**				
OPIOIDS	0.956**				
APE	0.651*				
TRIAZOLES	0.763**				
DEXTH	-0.857**				
FZOLE	-0.644*				
COND		0.716*			
Ni		0.654*			
BPA		0.882**			
ACRID		-0.748**			
AMPHET			-0.604*		
CAFF			-0.658*		
ANTIB			-0.796**		
IOPROM				0.763**	
ANTHEML				0.643*	
PARABEN				-0.604*	
PFR				-0.695*	
T					0.702*

2.4.2 Biological responses

The mean percentage of animals recovered (dead and alive) from the chambers after field exposures were always higher than 95% and so mean survival rates. Except for carboxylesterase, the studied proportional responses in field transplanted *D. magna* varied significantly (P<0.05, ANOVA tests) across sites and rivers (Table 2.6). The PCA analysis performed on the remaining 18 significant (P<0.05) *D. magna* proportional responses and the Shannon's diversity index resolved into five interpretable components that explained 89.8% of data variance (Table 2.7). The sample scores of the five obtained components correlated significantly (P<0.05) with the mean proportional responses of the studied biological variables establishing six clusters. Post-exposure feeding and ChE proportional responses decreased in Llobregat and Ebro rivers from upstream to downstream reaches but not in Jucar and co-varied with the Shannon's diversity (Fig. 2.3 A). The rest of *D. magna* responses were clustered into five additional groups according to their variation across sites (Fig. 2.3 B-E). Interestingly proportional responses of gene markers, but those of the gene RXR, grouped differently from biomarkers.

From the 14 proportional biological responses determined for lab exposures only 12 varied significantly (P < 0.05, based on ANOVA) across sites (see Table 2.8). PCA conducted on those 12 variables identified three interpretable components explaining 84.1% of data variance (Table 2.7). PC1 explained most variance of data (58%) and its sample scores correlated significantly with mean proportional values of 9 out of the 11 genes considered. Sample scores of PC2 correlated with mean proportional responses of MRP4 and MT2 and proportional post-exposure feeding rates with PC3's sample scores. The resulting five interpretable clusters of laboratory *D. magna* responses are depicted in Fig. 2.4.

Bi-variate Pearson correlations between mean site proportional responses of *D. magna* individuals transplanted in the field or exposed in the lab to organic eluates of water samples collected at the studied sites showed significant (P<0.05) correlations for EcR, MIH, FAA, UGP, MRP4 (Table 2.9).

Table 2.6 Proportional biological responses (Mean SE; N =5) measured in *D. magna* individuals deployed at the studied sites. Different letters means significant (P<0.05) differences across sites following ANOVA and Tukey's multiple comparison tests. The number of species (S) and Shannon's diversity index (H) are also depicted.

Site	LLO3		LLO4		LLO5		LLO7		EBR2		EBR3		EBR4		EBR5		JUC2		JUC4		JUC5		JUC6	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
MT2	1,73	0,08 bc	1,80	0,26 bc	2,57	0,45 c	3,82	0,99 c	1,41	0,16 b	1,24	0,23 b	0,56	0,05 a	1,34	0,29 b	1,11	0,04 b	0,84	0,10 a	1,38	0,17 b	1,29	0,37 b
RXR	1,77	0,18 bc	1,77	0,17 bc	1,53	0,14 b	1,57	0,12 b	1,66	0,16 b	1,66	0,05 b	1,41	0,10 b	1,42	0,07 b	1,03	0,08 a	1,00	0,09 a	1,19	0,07 a	1,44	0,09 b
ECDY	0,77	0,14 a	0,77	0,10 a	0,89	0,16 a	0,93	0,05 a	1,24	0,12 ab	1,19	0,19 ab	0,71	0,15 a	0,88	0,11 a	1,12	0,08 a	0,99	0,26 a	1,31	0,13 b	1,35	0,16 b
VTG	0,63	0,06 bc	0,43	0,07 ab	0,20	0,05 a	0,16	0,02 a	0,25	0,03 ab	0,29	0,07 a	0,23	0,03 a	0,15	0,05 a	0,67	0,18 b	0,21	0,11 a	0,23	0,08 a	0,51	0,22 ab
MIH	0,71	0,08 a	0,95	0,16 ab	0,61	0,10 a	0,70	0,06 a	1,07	0,05 b	1,16	0,14 b	0,87	0,03 b	0,84	0,06 ab	0,93	0,07 ab	0,80	0,09 ab	0,95	0,09 ab	1,07	0,14 b
HSP70	1,49	0,17 bc	1,53	0,14 bc	1,29	0,12 ab	1,49	0,23 b	1,10	0,11 b	0,98	0,14 a	0,92	0,05 a	1,17	0,23 ab	0,89	0,03 a	0,86	0,09 a	1,05	0,12 ab	1,26	0,12 ab
ACON	1,27	0,07 bc	1,39	0,09 bc	1,11	0,12 a	1,00	0,01 a	0,98	0,05 ab	1,28	0,07 b	1,10	0,08 a	1,12	0,04 a	1,19	0,11 a	1,05	0,07 a	1,34	0,09 b	1,63	0,05 c
FAA	0,63	0,06 a	0,75	0,04 a	0,87	0,08 ab	0,91	0,08 b	1,00	0,02 ab	1,06	0,04 b	0,74	0,05 a	0,86	0,14 ab	0,96	0,07 b	0,98	0,19 b	1,21	0,14 c	1,32	0,15 c
IDH	0,99	0,07 ab	0,98	0,07 ab	0,72	0,06 a	0,70	0,04 a	0,85	0,06 b	0,91	0,04 ab	0,78	0,02 a	0,83	0,02 a	1,08	0,01 ab	0,97	0,03 ab	1,14	0,04 b	1,20	0,06 b
THIO	0,98	0,09 ab	0,78	0,03 ab	0,79	0,07 ab	0,83	0,04 ab	0,90	0,18 ab	0,82	0,07 ab	0,67	0,04 a	0,81	0,09 ab	0,87	0,11 ab	0,93	0,08 ab	1,07	0,11 b	1,13	0,09 b
UGP	0,85	0,05 a	0,91	0,06 ab	0,77	0,04 a	0,70	0,02 a	1,24	0,06 ab	1,17	0,12 b	1,08	0,04 ab	1,21	0,10 c	1,34	0,06 c	1,02	0,10 ab	1,24	0,05 c	1,12	0,11 b
PGP	0,79	0,03 a	0,66	0,08 a	0,91	0,16 a	1,39	0,08 b	1,20	0,22 b	1,22	0,23 b	0,51	0,10 a	1,20	0,35 b	0,88	0,08 a	1,09	0,24 ab	1,66	0,47 c	1,46	0,35 bc
MRP4	0,85	0,08 ab	0,99	0,05 ab	0,75	0,05 a	0,74	0,05 a	1,26	0,05 b	1,40	0,10 c	1,18	0,04 bc	1,49	0,15 d	0,97	0,05 b	0,96	0,09 ab	1,35	0,12 cd	1,34	0,23 cd
GST	1,07	0,05 b	1,06	0,03 b	0,96	0,04 ab	0,92	0,02 ab	1,05	0,08 b	0,92	0,06 ab	1,05	0,04 b	1,01	0,09 ab	0,88	0,05 a	0,89	0,06 a	0,95	0,05 ab	0,92	0,04 ab
LDH	1,09	0,05 b	1,05	0,04 ab	1,05	0,06 ab	1,00	0,06 ab	1,05	0,07 ab	0,96	0,07 ab	1,07	0,06 ab	1,18	0,11 b	0,87	0,06 a	0,87	0,07 a	0,96	0,06 ab	0,88	0,02 ab
ACHE	1,06	0,03 b	0,92	0,02 bc	1,00	0,04 bc	0,89	0,01 b	1,04	0,03 b	0,99	0,04 ab	0,96	0,03 ab	0,81	0,07 a	1,01	0,02 bc	1,03	0,07 b	1,08	0,03 b	0,92	0,05 b
CBE	0,87	0,03 a	0,98	0,05 a	0,86	0,05 a	0,83	0,06 a	0,97	0,09 a	0,96	0,10 a	0,98	0,11 a	0,80	0,08 a	0,99	0,11 a	0,97	0,15 a	0,87	0,10 a	0,71	0,11 a
CAT	0,99	0,04 ab	0,93	0,03 a	0,95	0,02 a	0,97	0,05 ab	1,07	0,07 b	0,95	0,05 a	0,92	0,06 a	1,12	0,05 b	0,79	0,05 a	0,79	0,05 a	0,97	0,07 ab	0,87	0,09 a
Feeding	0,77	0,02 cd	0,63	0,05 c	0,55	0,06 bc	0,43	0,02 a	0,83	0,05 c	0,86	0,07 c	0,62	0,06 c	0,40	0,06 a	0,31	0,06 a	0,50	0,10 b	0,58	0,07 b	0,49	0,05 a
S	19,00		7,00		8,00		3,00		10,00		7,00		4,00		5,00		8,00		10,00		14,00		1,00	
M	2,02		0,78		0,92		0,34		1,10		0,69		0,45		0,58		0,70		1,12		1,44		0,10	
H	2,61		1,71		1,67		1,04		1,88		1,33		1,17		1,13		0,93		1,92		1,79		0,30	

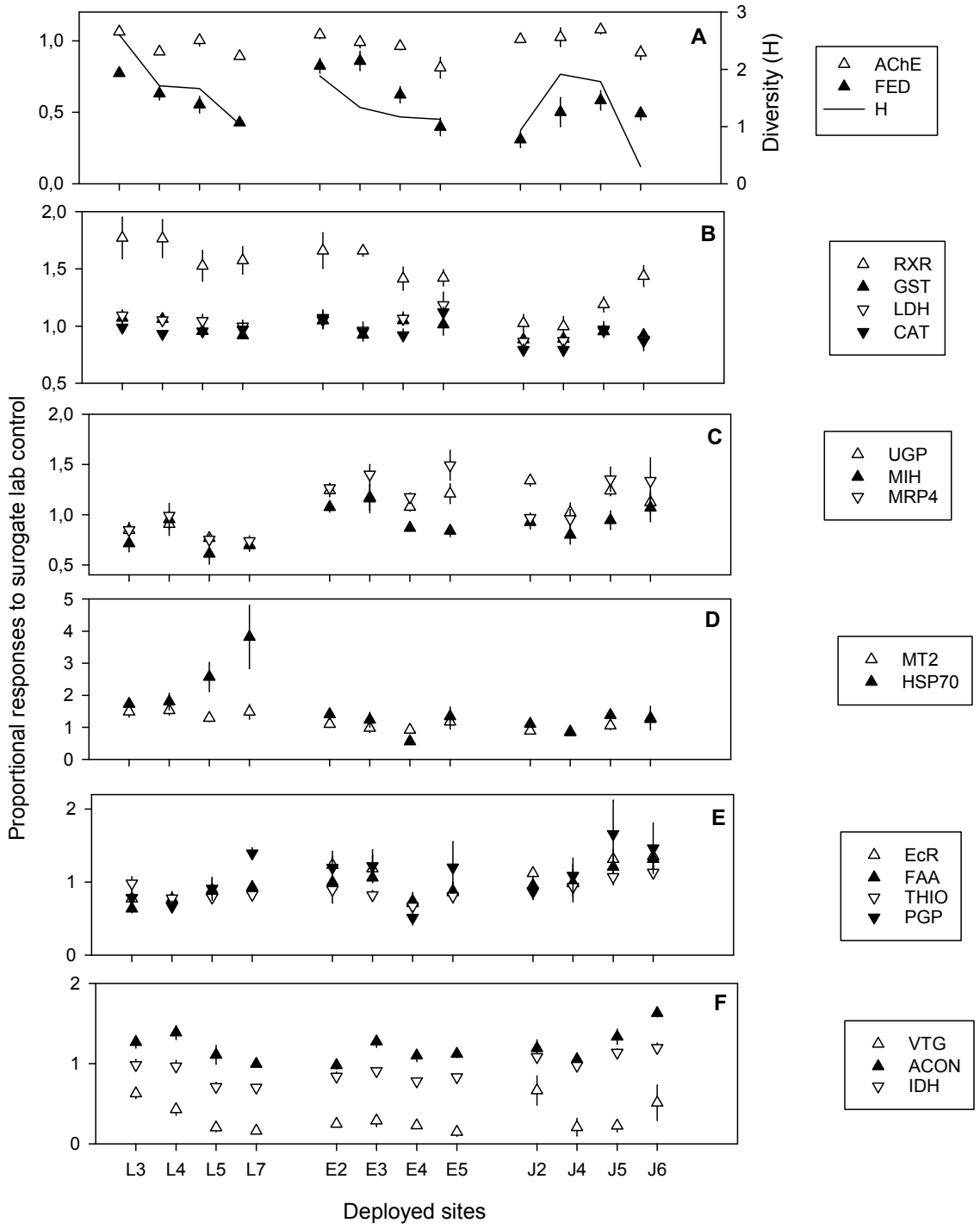

Figure 2.3 Proportional *D. magna* responses (Mean ± SE, N = 5) of individuals deployed across the studied sites. Responses have been grouped according to the clusters defined in previous Principal Component analyses. In Graph A the Shannon's diversity index H is also depicted. Each graph corresponds to a different cluster.

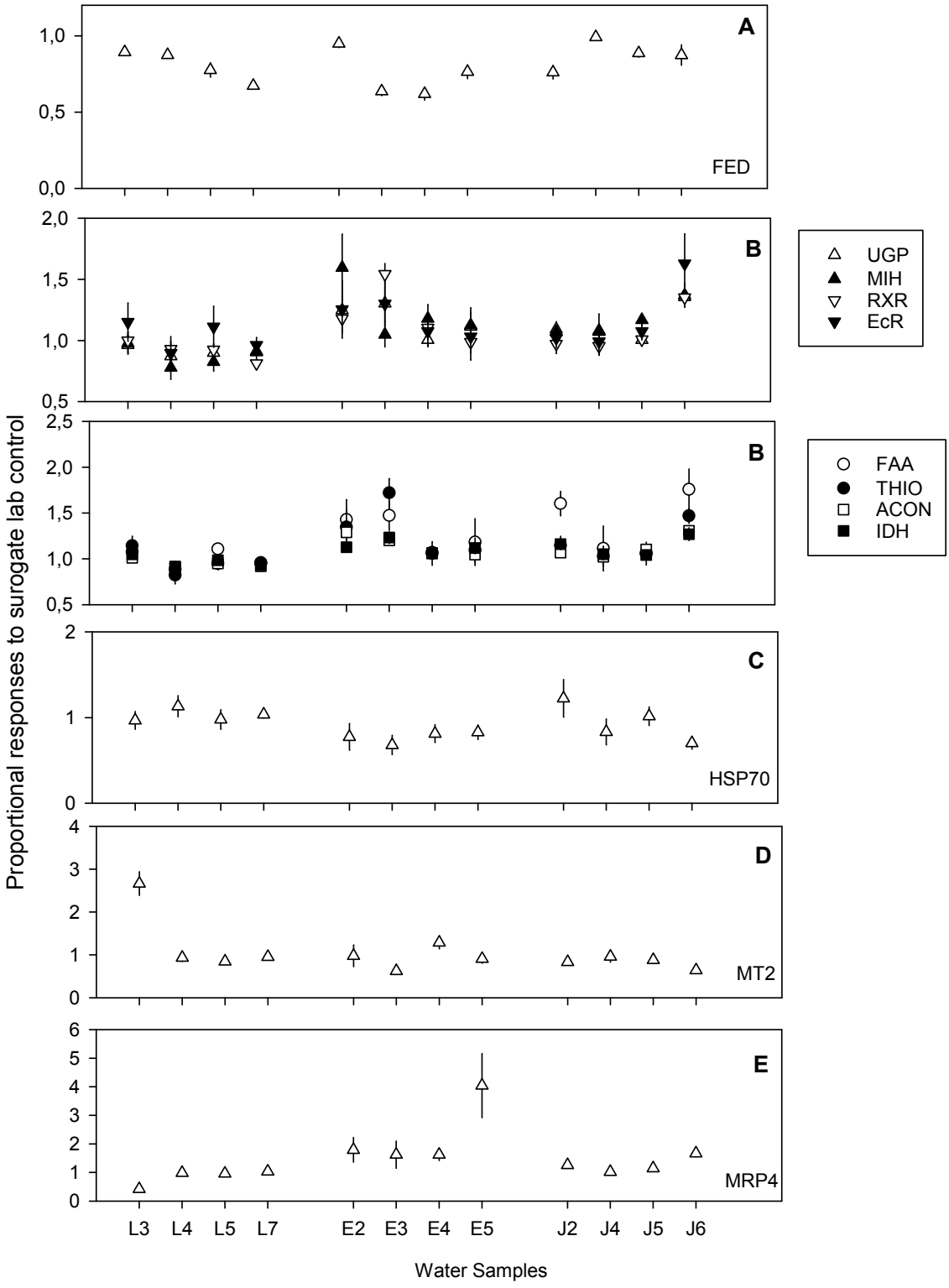

Figure 2.4 Proportional *D. magna* responses (Mean ± SE, N = 5) of individuals exposed to organic eluates obtained from water samples collected at the studied sites. Responses have been grouped according to the clusters defined in previous Principal Component analyses. Each cluster graph has a different letter. For clarity the cluster of graph B has been divided in two graphs.

Table 2.7 Results of Principal Component analyses performed on biological responses obtained in field and lab exposures. % VAR, variance explained. Significant (P<0.05) Pearson correlation coefficients between sample PC scores and those of biological responses are also depicted. * 0.05 < P < 0.01; ** P < 0.01.

	Field					Lab		
	PC1	PC2	PC3	PC4	PC5	PC1	PC2	PC3
Eigenvalues	6.5	3.3	2.8	2.5	1.9	6.9	1.9	1.3
% VAR	34.4	17.1	14.9	13.2	9.9	57.3	16.1	10.7
Correlation								
UGP	0.907**					0.909**		
MIH	0.811**					0.668*		
HSP70	-0.710**					-0.751**		
MRP4	0.879**						0.918**	
MT2	-0.858**						-0.751**	
RXR		0.886**				0.923**		
EcR			0.875**			0.916**		
FAA			0.865**			0.824**		
THIO			0.663*			0.968**		
PGP			0.968**					
VTG				0.832**				
ACON				0.863**		0.912**		
IDH				0.750**		0.915**		
GST		0.760**						
LDH		0.783**						
CAT		0.887**						
ACHE					0.931**			
FED					0.611*			0.923**
H					0.807**			

2.4.3 Relationships between environmental parameters and biological effects

PLS models performed to relate biological responses with environmental variables explained from 84 to 97% of the total variance of the former. In all analyses two PLS components were selected. PLS results for field variables are depicted in Table 2.10. For clarity we only depicted regression coefficients associated to VIP scores higher than 1. For interpretation purposes a cluster analysis was performed on regression coefficients, which differentiated eight clusters for biological and environmental variables, which are depicted in Table 2.10.

Table 2.8 Proportional biological responses (Mean SE; N =5) measured *in D. magna* individuals exposed in the lab to water organic extracts of water samples obtained at the studied sites. Different letters mean significant (P<0.05) differences across sites following ANOVA and Tukey's multiple comparison tests.

	LLO3		LLO4		LLO5		LLO7		EBR2		EBR3		EBR4		EBR5		JUC6		JUC5		JUC4		JUC2	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
MT2	2,66	0,27 c	0,93	0,09 ab	0,85	0,07 ab	0,95	0,06 ab	0,98	0,25 ab	0,63	0,05 a	1,29	0,15 b	0,91	0,10 ab	0,64	0,06 a	0,88	0,04 ab	0,96	0,13 ab	0,83	0,07 ab
RXR	1,00	0,05 a	0,93	0,06 a	0,92	0,05 a	0,81	0,03 a	1,18	0,12 b	1,54	0,09 a	1,10	0,10 ab	0,99	0,09 a	1,35	0,04 c	1,01	0,03 a	0,95	0,08 a	0,97	0,08 a
ECDY	1,15	0,16 ab	0,90	0,14 a	1,11	0,17 ab	0,96	0,03 a	1,25	0,23 ab	1,30	0,14 b	1,07	0,11 ab	1,03	0,19 ab	1,63	0,24 b	1,08	0,07 ab	0,99	0,11 ab	1,02	0,08 ab
VTG	1,92	0,70 a	2,64	0,79 a	0,46	0,08 a	1,25	0,37 a	1,38	0,43 a	0,50	0,07 a	1,35	0,57 a	0,93	0,26 a	2,17	1,14 a	1,27	0,31 a	0,99	0,29 a	0,99	0,14 a
MIH	0,98	0,09 a	0,78	0,10 a	0,83	0,08 a	0,91	0,11 a	1,60	0,27 b	1,05	0,10 a	1,18	0,11 ab	1,13	0,11 ab	1,37	0,10 b	1,17	0,05 ab	1,08	0,14 a	1,07	0,02 a
HSP70	0,97	0,10 ab	1,13	0,12 b	0,98	0,11 ab	1,04	0,04 ab	0,78	0,16 a	0,68	0,11 ab	0,81	0,10 ab	0,83	0,08 ab	0,70	0,07 a	1,02	0,11 ab	0,83	0,15 ab	1,22	0,22 b
ACON	1,01	0,03 a	0,90	0,07 a	0,95	0,05 a	0,92	0,03 a	1,29	0,08 b	1,20	0,02 b	1,06	0,05 ab	1,05	0,06 a	1,31	0,02 b	1,10	0,02 ab	1,02	0,06 a	1,07	0,03 ab
FAA	1,08	0,10 a	0,89	0,08 a	1,11	0,03 ab	0,96	0,04 a	1,43	0,22 bc	1,47	0,16 bc	1,06	0,13 a	1,18	0,26 ab	1,76	0,22 c	1,06	0,12 a	1,11	0,24 ab	1,60	0,13 c
IDH	1,05	0,04 ab	0,92	0,05 a	0,99	0,03 a	0,93	0,02 a	1,13	0,02 ab	1,23	0,07 b	1,07	0,05 ab	1,12	0,06 ab	1,27	0,07 b	1,04	0,02 ab	1,05	0,06 ab	1,16	0,06 ab
THIO	1,14	0,10 ab	0,82	0,10 a	0,95	0,07 a	0,95	0,03 a	1,35	0,19 b	1,72	0,16 c	1,07	0,07 ab	1,10	0,13 ab	1,47	0,08 bc	1,06	0,03 ab	1,03	0,11 ab	1,15	0,10 ab
UGP	0,96	0,07 b	0,87	0,07 a	0,90	0,04 a	0,90	0,02 a	1,25	0,13 b	1,30	0,18 b	1,01	0,06 b	1,11	0,16 b	1,36	0,07 b	1,01	0,02 b	1,07	0,07 b	1,09	0,06 b
PGP	1,30	0,31 a	0,74	0,16 a	1,18	0,38 a	1,84	0,83 a	1,39	0,18 a	1,82	0,49 a	0,71	0,09 a	1,25	0,19 a	0,96	0,18 a	0,86	0,10 a	0,94	0,09 a	0,96	0,11 a
MRP4	0,42	0,09 a	0,99	0,10 a	0,96	0,03 a	1,04	0,08 a	1,79	0,43 a	1,62	0,47 a	1,62	0,20 a	4,04	1,12 b	1,67	0,12 a	1,15	0,06 a	1,02	0,08 a	1,26	0,11 a
Feeding	0,89	0,02 c	0,87	0,02 c	0,78	0,05 b	0,67	0,02 a	0,95	0,03 d	0,64	0,03 a	0,62	0,04 a	0,76	0,05 b	0,87	0,07 c	0,89	0,03 c	0,99	0,02 d	0,76	0,04 b

Proportional responses of gene markers encoding for specific metabolic and molting processes (PGP, EcR, FAA, MIH, UGP, MRP4) were negatively related with up to 18 classes of contaminants. Proportional feeding rates, and those of GST, LDH, CAT and RXR were negatively and positively related with measured environmental factors. Up to 9 classes of organic contaminants, 6 metals and water temperature were positively related with responses of HSP70 and/or MT2. Nine environmental variables including (estrogenic compounds, antibiotics, β2-adrenergic agonists) were related positively with species diversity and proportional cholinesterase activity whereas fungicides, pyrine, analgesics, conductivity and suspended solids were negatively related with HSP70 and MT2. Environmental variables were grouped into three major and five minor clusters. One of the major clusters included pharmaceuticals (analgesics, lipid regulators, anticoagulants, sulfonylurea diuretics), opiod's illicit drugs, triazole and alkylphenol compounds, and covaried negatively with the response of 9 genes, and positively with that of three genes, feeding and three biomarkers. The cluster that included the metals As, Mn, Ni, diuretic, psychiatric drugs and anti-asthma compounds were positively related with the response of MT2 and HSP70 and negatively with that of MRP4, MIH, UGP.

Table 2.9 Pearson correlation coefficients obtained between *D. magna* responses obtained in the field and lab exposures considering either mean responses across sites or PLS regression coefficients estimated for organic pollutants. N, sample size .* P<0.05.

Responses	Mean responses per site	PLS regression coefficients
VTG	0.45	0.30
MT2	0.07	0.16
RXR	0.22	0.58*
EcR	0.61*	0.54*
MIH	0.64*	0.65*
HSP70	0.38	0.35*
ACON	0.30	0.22
FAA	0.62*	0.60*
IDH	0.47	0.07
THIO	0.27	-0.08
UGP	0.66*	0.45*
PGP	0.40	0.31
MRP4	0.69*	0.61*
FED	0.08	-0.07
N	12	33

The third major cluster included antibiotics, illicit drugs and caffeine that were negatively related with the response of most traits except with those of ACHE and H that covaried positively with them. From the remaining minor clusters and environmental variables those having greater coefficients were those of estrogenic compounds that were positively related with the response of VTG, ACHE, H; the cluster of fungicides and antipyrine analgesics that covaried negatively with the response of ACHE and H; opiate analgesics that covaried negatively with responses of CAT and H: and temperature, which was positively related with feeding and RXR responses.

From the 12 biological responses considered in lab exposures, PLS regression coefficients of 8 of them (RXR, EcR, MIH, HSP70, FAA, UGP, MRP4) were significantly ($P<0.05$, $N = 33$) and positively correlated with those of field responses (Table 2.9). This means that the relative response of eight genes to the studied organic contaminant classes was similar in field and lab exposures.

2.5 Discussion

This study aimed to identify biological active pollutants using *in situ D. magna* responses. It was implemented in three Mediterranean rivers differing in anthropogenic pressures and hence on pollution impacts. Indeed physicochemical water parameters such as water temperature, conductivity, suspended solids, five metals and 23 organic chemical functional classes differentiated the three rivers and within each river most and less polluted sites. Eighteen *D. magna* responses measured in individuals deployed in the field also differentiated the three rivers and sites. Biological responses clustered into six distinct groups. Post-exposure feeding rates and cholinesterase activity co-varied similarly with the diversity of macroinvertebrate communities from the studied sites.

Table 2.10 PLS regression coefficients having VIP scores higher than 1. Clustered biological and environmental variables are depicted in grey.

	VTG	THIO	PGP	EcR	IDH	ACON	FAA	MT2	HSP70	GST	LDH	CAT	FED	RXR	MRP4	MIH	UGP	ACHE	H
ANSAID		-	-	-0.04	-	-0.074	-						0.071	0.044					
ACRID		-0.07			-	-0.089				0.079		0.081	0.103	0.073					
STATIN					-0.09					0.137	0.138	0.167	0.142	0.136					
DIUREAS		-	-	-	-	-0.064	-		0.035	0.126	0.117	0.081		0.046		-	-		
OPIOIDS		-	-	-0.05	-		-	0.024	0.031	0.039	0.033			0.027		-	-		
TRIZOLES		-			-						0.068	0.064	0.074	0.072	0.086				
APE		-	-	-	-0.07			-											
FIBRAT		-	-	-	-		-	0.035			0.024				-0.069	-	-		
DEXTH		0.077	0.053	0.043	0.088	0.066	0.074			-0.084	-	-	-	-					
AOPIATES			-						-0.07	-0.116	-	-	-	-	-0.095				-
PARABEN		0.106	0.094		0.091	0.12			0.079									0.114	
POTHER			0.022	0.002			-	0.112	0.086	0.049	0.029			0.057	-0.049		-		0.089
As	-		-	-	-		-	0.103	0.042						-0.07	-	-		0.092
Mn			0.017	0.007			0.032	0.106	0.04	-0.04					-0.051	-	-		
Ni								0.052	0.049			-	-		-0.057	-	-0.06		
DIURET			-	-0.03			-	0.088	0.087		-			0.049	-0.061	-	-		
SEROT								0.14	0.06	-0.08	-0.06				-0.08	-0.05	-0.04		
ADREN		0.116						0.14	0.079						-0.099	-	-	0.131	0.113
Cond				-0.05				0.075	0.085				-			-	-	-0.105	-
IOPROM	0.096							0.081	0.066				-		-0.08				
GABA		-0.05	-0.04		-0.05			0.08		-0.10			-0.08	-0.06	-0.08	-0.07			
ANTIB	-		-	-	-	-0.142	-0.09						0.082		-0.09		-	0.132	0.153
BBLCK		-0.10		-0.06	-0.09	-0.18	-0.12		-0.08									0.13	0.19
COCAIN	-					-0.079						-		-					0.1
AMPHET	-		-	-	-	-0.111	-	-	-0.074					-		-			
CAFF	-					-0.077				-0.07		-		-					0.091
E2	0.226		-	-			-		0.071	0.101			0.093		-0.12	-	-	0.189	0.208
APYR	0.088										0.06			0.103				-0.129	-
FZOLE		0.08		0.073	0.072	0.157	0.121											-0.175	-0.21
CIMET	-																-		
ANGIO	-				-			0.064		-0.079							-		
Zn	0.121				0.083	0.088			0.057										
OPS	0.103	0.066			0.063														
SS				-			-				0.111	0.077	-			-		-0.149	-
Co				-			-		0.052		0.118	0.078	-			-		-0.13	
PFCs			0.091							0.1	0.117	0.145			0.073				0.123
Fe					0.083	0.091			0.05										
ANTHEML			-0.13	-0.12			-0.13						-0.11		-0.10	-0.10	-0.06		
T						0.077			0.074			0.084	0.111	0.151				-0.084	
HTRIAZ				0.07		-0.083										0.073	0.06		
PFR										-0.096									
Pb										0.074						0.07			
Cu		-0.05								0.078			0.053	0.071	0.074	0.082		-0.096	
BPA													-			-			

Previous studies performed in the Llobregat and Besós rivers using *D. magna* and field collected caddisfly larvae also found a good correlation between the ecological quality of riparian invertebrate communities, *D. magna* feeding rates and cholinesterase activity in both *D. magna* and caddisfly larvae (Damásio et al., 2011; Damásio et al., 2008). Thus *in situ D. magna* responses of feeding rates and cholinesterase activity can be considered good markers of ecological quality. Indeed post-exposure feeding rates have already been used in several studies to assess ecological quality of river biota (Barata et al., 2007; Damásio et al., 2011; Mc William and Baird, 2002; Puértolas et al., 2010). A second cluster included three biomarkers and the retinoic X receptor gene whose levels were inhibited towards downstream locations in Llobregat and Ebro River but increased in Jucar. Wang et al (2017) found that mRNA levels of the retinoic X receptor were high in reproductive females and were inhibited by insecticide terpenoids like pyriproxyfen. Thus, higher levels of RXR may indicate optimal conditions for Daphnia growth and reproduction and low levels increasing concentrations of insecticides. Enzymatic activities of CAT, GST and LDH followed similar response patterns than RXR thought less apparent. In the Llobregat river these enzymatic activities hardly varied across sites (values approached 1) but in downstream sites of the Ebro river increased, whereas in most sites of the Jucar river decreased. These results are consistent with previous studies that also reported no changes of CAT and GST activities across the same stations of Llobregat (Damásio et al., 2011). Inhibition or enhanced enzyme activities of CAT and GST in deployed in the field have been related to the presence of organophosporous pesticides, herbicides, alkylphenols, fungicides, metals and polycyclic aromatic hydrocarbons (Barata et al., 2007; Damásio et al., 2008). Another cluster was composed of genes encoding for specific responses such as sugar metabolism (UGP), molting (MIH) and xenobiotic transporter activity (MRP4). The responses of these genes varied across rivers and sites being down-regulated in Llobregat, up-regulated in Ebro and down and up-regulated in Jucar. There is reported evidence that UGP and MRP4 in *D. magna* are deregulated by psychiatric drugs and metals (Campos et al., 2014; Campos et al., 2013). A fourth cluster grouped genes codifying two stress proteins MT2 and HSP70 (Ho, 2008; Poynton et al., 2007). Metallothioneins like MT2 are involved in metal detoxification, binging to them and hence facilitating metal metabolism, whereas HSP70 protect other proteins

under stress (Asselman et al., 2013; Bond and Bradley, 1997; Haap and Köhler, 2009; Haap et al., 2008). Levels of mRNA of these two genes and specially those of MT2 were up-regulated in downstream sites of Llobregat that also had the highest levels of metals and of most other pollutants. A fifth cluster included genes having distinct functions such as those encoding for xenobiotic transporter proteins (PGP), those involve in the metabolism of amino acids (FAA) and lipids (THIO) and the gene encoding for the ecdysone receptor (EcR) (Campos et al., 2014; Campos et al., 2013; Kato et al., 2007). The response of these genes was up-regulated at the downstream sites of Llobregat and Jucar and down-regulated at E4 in Ebro river. Previous studies have reported that pharmaceuticals and pesticides deregulated those genes (Campos et al., 2014; Campos et al., 2013; Mu and Leblanc, 2004; Wang et al., 2011). A sixth cluster included vitellogenin and two genes from the Krebs cycle (ACON, IDH). mRNA levels of those genes varied between rivers and across sites as follows: within river basins mRNA gene levels decreased towards downstream sites in Llobregat, remained unchanged in Ebro and increased towards downstream reaches in Jucar. Note also that levels of VTG were always down-regulated relative to the surrogate lab control having the greatest levels of deregulation in Ebro. (Hannas et al., 2011) recently reported that the putative VTG gene in *D. magna* acts like a general stress gene that could be either up or down-regulated by many contaminants. There is also evidence that the transcripts of the two Krebs cycle genes (ACON, IDH) are deregulated by pollutants (Campos et al., 2013).

A further characterization of the relationships between measured responses and environmental factors was performed with the aid of the PLS excluding out L7. The most important relationships allowed to group biological responses into eight groups that were affected similarly by environmental parameters. These include cholinesterase and diversity responses that were affected negatively by conductivity, suspended solids in water, fungicides and antipyrine analgesics and positively by β-blockers, β2-adrenergic agonists and antibiotics. Residue levels of antipyrine analgesics and β2-adrenergic agonist were probably too low ≤2 ηg/L to have any effect on the measured responses and those of β-blockers hardly varied across most sites. In this study conductivity measured salinization that in Mediterranean rivers is

an important problem that deteriorates water quality (Damásio et al., 2011). High levels of suspended solids associated to anthropogenic impacts are also known to impair the ecological quality of rivers (Damásio et al., 2011). Fungicides are also known to reduce diversity of riparian communities directly impairing the physiology of aquatic organisms and indirectly by reducing fungus biomass and hence food for shredders (Maltby et al., 2009). There is evidence that low levels of antibiotics reduce the microbial load of invertebrates, promoting its growth and reproduction (Zalewski et al., 2011). Thus low levels of antibiotics could be beneficial for invertebrates and thus may increase diversity. Previous studies have reported that organophosphorous or carbamate insecticides inhibit cholinesterase acticities of *D. magna* at about 100 ηg/L (Barata et al., 2007; Barata et al., 2004). Therefore, measured organophosphorours and carbamate pesticides residue levels in water were probably too low (≤2 ηg/L) to have impaired cholinesterase activity in *Daphnia*. The cluster of the remaining biomarkers (CAT, GST, LDH) was related positively with a cluster of eight organic chemicals (i.e.analgesics, lipid regulators, diuretics, illicit drugs, trizoles) and negatively with up to 11. Excluding out pesticides, sublethal effects of organic chemical substances to *D. magna* rarely occur below µg/l (Constantine and Huggett, 2010; Yang et al., 2013). This means that from the above mentioned pollutant groups, residues of trizole compounds, NSAID analgesics and lipid regulators were the most likely to affect biological responses of deployed *D. magna* individuals. Nevertheless for these chemical groups effects on *D. magna* have been reported at the mg/L range (Heckmann et al., 2007; Seeland et al., 2012; Zurita et al., 2007).

The cluster of metallothionein and heat shock protein 70 was related positively with most studied environmental factors. This is expected to occur since these two proteins are known to be induced under stress acting as a detoxification mechanisms to metals (MT2) or protecting proteins (HSP70). (Asselman et al., 2013; Bond and Bradley, 1997; Haap and Köhler, 2009; Haap et al., 2008). Interestingly MT2 responses obtained in the field were not related with those observed in lab exposures to organic eluates neither their PLS regression coefficients. This means that responses of MT2 in the field were related to other factors than organic pollutants. Metals like As, Mn, Ni and conductivity were positively related with MT2,

which support recent findings reporting that mRNA levels of MT2 are inducible by several metals (Asselman et al., 2013). Genes encoding for key enzymes of the Krebs cycle such as IHD and ACON, VTG and those involved in the lipid (THIO) or xenobiotic metabolism (PGP) responded differently in the field and lab exposures. According to our experimental design biological responses to lab exposures should mimic those of the field for organic chemicals. This means that for those responses, the obtained PLS-relationships with measured organic residues have to be considered with caution since they can be associated to other sources of contamination than those measured in this study. This was the case for mRNA levels of vitellogenin in *D. magna* that was positively related with estrogenic steroids but not with other known estrogenic compounds such as alkylphenols, bisphenol A and triazoles (De Castro-Català et al., 2013). Furthermore, obtained PLS associations of vitellogenin in field exposures were not correlated with those obtained in lab exposures. In a previous study (De Castro-Català et al., 2013) reported a positive relationship between estrogenic compounds and the number of eggs per clutch in freshwater snails deployed to the same rivers and sites. Contrary to crustaceans like Daphnia (Hannas et al., 2011), gastropods respond to estrogenic compounds (Castro et al., 2007; Stange et al., 2012). Differences in the exposure scenario and/or variations in gene responses to environmental stressors may also explain the observed lack of relationships between field and lab exposures. During field assays animals were directly exposed to the river water flow and suspended matter and hence to a broader number of contaminants and other environmental factors than in lab exposures, which were conducted under static conditions and using organic extracts of filtered water samples. Genes encoding for general metabolic processes (IHD, ACON, THIO) are likely to be altered by several stressors apart from organic contaminants and that encoding for PGP is known to respond to many different organic and inorganic chemicals (Campos et al., 2014; Faria et al., 2011). Therefore, the higher complexity of field exposures may explain the observed discrepancies on gene responses between lab and field. This was clearly illustrated for feeding responses, which were more affected during field assays (Fig. 2.3 A, 2.4 A).

For genes related with molting and reproductive processes (MIH, EcR), sugar, protein and xenobiotic metabolism (UGP, FAA, MRP4), lab and field responses were similar and were negatively related with several pharmaceutical groups (diuretics, illicit drugs, lipid regulators-fibrates, psychiatric drugs, β2-adrenergic agonists), bisphenol A, estrogenic and anti-parasitic compounds, As, Mn, Ni, Co, conductivity and suspended solids. Few of the measured contaminants (trizoles, pesticides) were positively related with MRP4, MIH, FAA or/and UGP. Responses of the retinoic receptor RXR followed a distinct patern being positively and negatively related with the above mentioned and other pollutants like caffeine, analgesic opiates. An excess of suspended solids, salt and of metals like Ni, inhibit growth or/and reproduction in *D. magna* (Baillieul et al., 1996; Burton Jr et al., 2005; Diamond et al., 1992; Hoang et al., 2007; Pane et al., 2004; Robinson et al., 2010). Psychiatric drugs such as selective serotonine re-uptake inhibitors are known to disrupt signaling gene pathways of molting, reproduction, sugar and aminoacid metabolism at environmental relevant concentrations close to 1 µ/L (Campos et al., 2013). Despite that sublethal effects of fibrates, estrogenic steroids and bisphenol A occur in *D. magna* at mg/L (Brennan et al., 2006; Jeong et al., 2013; Zurita et al., 2007), in mixtures pharmaceuticals may trigger physiological responses at lower doses (Cleuvers, 2003). Many organic contaminants and metals can interact and act additively or synergically inhibiting xenobiotic transporter activity mediated by multidrug resistance proteins like MRP4 (Campos et al., 2014; Faria et al., 2011; Kurelec, 1997). In the present study many of the above mentioned pharmaceutical and industrial chemical groups occurred together and hence it should be feasible to establish a threshold biological effect at 100 ηg/L. Accordingly analgesics, diuretics, psychiatric drugs, β blockers, illicit drugs, trizoles, caffeine and measured pesticide levels should be considered of environmental concern.

2.6 Conclusions

In summary, our results led positive support to the use of sublethal specific gene responses in combination with *in situ Daphnia* feeding and biochemical responses to assess effects and identify environmentally detrimental factors within complex (multi-stressed) river systems in the field, thus contributing to a more realistic assessment

of ecological risks. *D. magna* responses of feeding and cholinesterase activity were able to assess the general ecological quality of the selected river sites and those of genes assessed specific effects of particular contaminant groups. Interestingly the contaminant groups that differentiated the studied sites (Table 2.5) did not follow those that were associated with specific biological response (Table 2.10). This means that risk assessment estimates based on chemical analyses have to be taken with caution (Fàbrega et al., 2013; Ginebreda et al., 2010). Furthermore, subdivision of chemical groups according to known mode of actions and the inclusion of lab exposures allowed to judge false positive relationships. The experimental procedures developed in this study indicate that in multi-stressed rivers biota is often chronically exposed to sublethal levels of contaminants and hence a large set of biological and chemical markers are needed to identify detrimental pollutants. Nevertheless, it is important to consider that, while the approach used in this study drives us closer towards the "*in situ* environmental hazard identification evaluation", issues arising from other confounding factors influencing *in situ Daphnia* responses still should be considered with caution in the interpretation of such findings as conclusive diagnostic proofs of individual factors causing effects.

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Chapter III.

Liquid chromatography coupled with tandem mass spectrometry analytical tools to characterize trace levels of cyanobacteria and dinoflagellate toxins in suspended solids and sediments

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Chapter III.

Liquid chromatography coupled with tandem mass spectrometry analytical tools to characterize trace levels of cyanobacteria and dinoflagellate toxins in suspended solids and sediments[a]

3.1 Abstract

Microcystins, anatoxins and okadaic acid are toxins produced by freshwater cyanobacteria and marine dinoflagellates. These toxins have been the responsible for the illness and death of biota and humans. To determine their presence in water during blooms, sensitive analytical methods are needed. In this study we have developed a new liquid chromatography tandem mass spectrometry (LC-MS/MS) method for fast multiresidue determination of five toxins in suspended material and sediment samples. For each target compound, two selected reaction monitoring (SRM) transitions were optimized. Chromatographic conditions were optimized considering that the compounds analyzed had different chemical structure and chromatographic behaviour. Using a Luna C18 column and specific SRM transitions, five phyco/phytotoxins were resolved. Method detection limits (MDL) for anatoxin-a, microcystins RR, LR, YR and okadaic acid were 7.1, 3.3, 81.7, 102.8 and 28.8 ng/g dry weight in sediment, respectively. The developed analytical method was successfully applied to analyze the presence of toxins in suspended solids and sediment from Ebro River (NE Spain) and Ebro Delta associated lagoons. Anatoxin-a was detected downstream of the Riba-Roja reservoir with levels ranging from 20 to 1120 ng/g d.w. of suspended solids. Okadaic acid was only detected in three samples collected in the Alfacs Bay (Ebro Delta, Spain) affected by *Dinophysis* blooms in 2012.

Keywords: cyanotoxins, okadaic acid, LC-MS/MS, sediment, Ebro

3.2 Introduction

The assessment of phyco and phytotoxins present in both fresh and marine water is an important issue in both environmental and human health (Chapela et al., 2008). Cyanobacteria play a fundamental role as primary producers. Though, under certain conditions they outbreak forming blooms that may compromise the use of water for drinking and recreational purposes, especially when bloom-forming species produce cyanotoxins (Ibelings and Havens, 2008). The most frequent and harmful cyanotoxins present in water are the hepatoxic microcystins and the neurotoxic anatoxins (Ibelings and Havens, 2008). In brackish and marine waters dinoflagellates and diatoms can produce phycotoxins that can enter into the food chain, accumulate in fish and shellfish and ultimate can affect humans. One of the most abundant phycotoxin is okadaic acid that is produced by dinoflagellates and produced diarrhoea, promotes tumor and apoptosis (Chapela et al., 2008). The molecular structures of these toxins (Fig. 3.1) reveal chemical differences that are important with respect to their simultaneous determination.

Preventive measures for recreational and consumer protection on harmful algal blooms (HABs) require powerful analytical methods. Liquid chromatography–electrospray ionisation-tandem mass spectrometry (LC-ESI-MS/MS) has been shown to represent a powerful and established method for the detection of several toxin groups (Cong et al., 2006; Chapela et al., 2008; Dörr et al., 2010; Gugger et al., 2005; Zhang et al., 2012). However, most methods developed until now, are focused to analyse phyco and phytotoxins in algae or water to detect bloom episodes or in food like shellfish to prevent consumers from being poisoned (Cong et al., 2006; Chapela et al., 2008; Dörr et al., 2010; Gugger et al., 2005; Zhang et al., 2012). Preventing blooming episodes of toxic cyanobacteria or algae in rivers, lakes or coastal areas, however, require the detection of toxic species or of their toxins in water before the occurrence of blooms. The presence of algae toxins in most cases is associated with the presence of their producers, thus they mostly occur in the suspended solid fraction inside algae cells (Aboal and Puig, 2005).

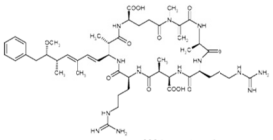
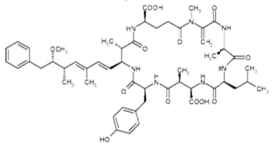
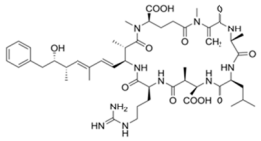
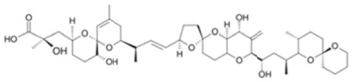
compound	CAS no.	molecular formula	water solubility mg L-1	Log P kow	structure
Microcystin RR	111755-37-4	$C_{49}H_{75}N_{13}O_{12}$	1038.20	> 1000.00	
Microcystin YR	101064-48-6	$C_{52}H_{72}N_{10}O_{13}$	1045.19	> 1000.00	
Microcystin LR	101043-37-6	$C_{49}H_{74}N_{10}O_{12}$	995.17	> 1000.00	
Anatoxin-a	64285-06-9	$C_{10}H_{15}NO$	165.23	14.00	
Okadaic acid	78111-17-8	$C_{44}H_{68}O_{13}$	805.00	insoluble	

Figure 3.1 Molecular formula and chemical properties of the studied toxins monitored using LC-MS/MS method. Log P, Kow was calculated using US EPA. (2012).

Nevertheless, in some cases toxins may be present in the water column associated to suspended matter and bottom sediments of water bodies. Hence the importance of sediments and suspended matter as a sink for the toxins may deserve more attention, including the possibility of toxins remobilisation and bioaccumulation (Schmidtkunz et al., 2009). This situation, for example, may occur during floods in arid or semi-arid climate regions where dams are used to regulate surface-water cycles, especially when water demand and its availability are imbalanced (Petrovic et al., 2011). In fact, in these locations toxins produced by cyanobacteria living in dams (Herry-Allani and Bouaïcha, 2013) may be easily remobilized downstream by sudden increases of river flow. With climate change, the impact by floods needs to be controlled, as an increase of extreme weather conditions, and subsequent extreme floods are predicted in certain regions (Ikeda et al., 2005; Kleinen and Petschel-Held, 2007).

Nowadays, LC-MS/MS is considered to be a well-accepted technique for the quantification determination of phytotoxins due to its efficient toxin separation, high selectivity, high sensitivity (lower limits of detection), and accurate and precise quantification (Chapela et al., 2008). However, phyco/phytotoxin characterization in sediments is still an emerging issue, often confounded by the complexity of the matrix. Little research has been performed to determine adsorbed toxins onto particles of sediment or suspended matter and the few existing studies are limited to analyses of microcystins (Schmidtkunz et al., 2009; Tsuji et al., 2001). One of the reasons explaining the scarcity of studies addressing cyanotoxins in sediment is that no suitable analytical method has been established yet. Matrix effects are one of the major drawbacks of analyzing cyanotoxins by liquid chromatography LC-MS/MS, especially when working in electrospray ionization mode (ESI), and may led to erroneous conclusions, resulting in the possible suppression or enhancement of analyte signal (Benijts et al., 2004). The use of internal standards closely matching targeted compounds enables the assessment of which matrix effects are influencing the quantification, but these internal standards are not currently available for most cyanotoxins (Chapela et al., 2008). Therefore, an extensive study to evaluate matrix effects should be included in the method validation, in order to ensure results reliability.

The aim of the present study is to develop a new fast analytical method based on solid liquid extraction and analysis by LC-ESI-MS/MS for simultaneous determination of toxins present in the suspended solids of water samples and sediments from Ebro River, the largest river of Spain, and Ebro Delta associated lagoons.

3.3 Experimental

3.3.1 Chemicals and materials

Pure analytical standards of 98–99% purity of anatoxin-a and okadaic acid were acquired from Santa Cruz Biotechnology (USA) and those of microcystins RR, LR, YR were obtained from Sigma–Aldrich (St. Louis, USA). Target compounds, molecular formula and the chemical structure are shown in Fig. 3.1 Methanol (MeOH), acetonitrile (ACN), and HPLC water (LiChrosolv grade) were supplied by

Merck (Darmstadt, Germany). In the preparation of the standards, an exhaustive control on handling procedures, storage conditions and safety rules has been followed, as specified by manufacturers.

3.3.2 Sample collection and preparation

Twenty-four water samples were collected from January 18th to June 22nd 2013 along the lower course of Ebro River between the Cinca River (CI), just before its junction with the Riba-Roja reservoir and the village of Mora d'Ebre, 50 km downstream (M; Fig. 3.2 A). Samples were collected at five different time points during a prolonged flash flow of 155 days (Fig. 3.2 C). Sampling sites included locations situated upstream the Riba-Roja dam (CI), at the Flix dam (RR, FR) and at downstream sites (MU, MD, A, M) (Fig. 3.2 A, B). In addition to the above water samples, twelve additional samples were considered. These included four sediment samples located between the meander and Ascó (sediment samples S5, S6; S7, S8, Fig. 3.2 A; (Bosch et al., 2009)); four samples collected at the Ebro's mouth (Sant Jaume d'Enveja, SJ) and at the end of three drainage channels of Ebro's Delta (D1, D2, D3) in July 2013 (Fig. 3.2 A). These drainage channels collect and transport the water from the south mid-delta rice fields into one of the sea lagoons (the Alfacs lagoon) (Barata et al., 2007). Finally, three samples were collected during February and March 2012 in Alfacs lagoon during a toxic algal bloom of Dinophysis. Sediment samples from Sitges coast (NE, Spain) that did not have detectable residue levels of the studied toxins were used for method development.

Total suspended solids of water samples were measured in the laboratory according to standard methods (Way, 2012). Once collected, suspended solids were separated from water by filtration in pre-weighed and pre-combusted (350ºC, 12h) 47 mm Whatman GF/F glass fiber filters within 24 hours from sampling. The filters were then frozen, lyophilized, weighted and frozen again. Sediment samples were also frozen, lyophilized, sieved <70 μm and frozen again. Freeze-thawing is a procedure that enhances lysing of cyanobacterial cells possibly present in the sediment, thus releasing intracellular toxins that would otherwise not be extractable (Barco et al., 2005). Suspended solid material on filters varied between 15 and 30 mg.

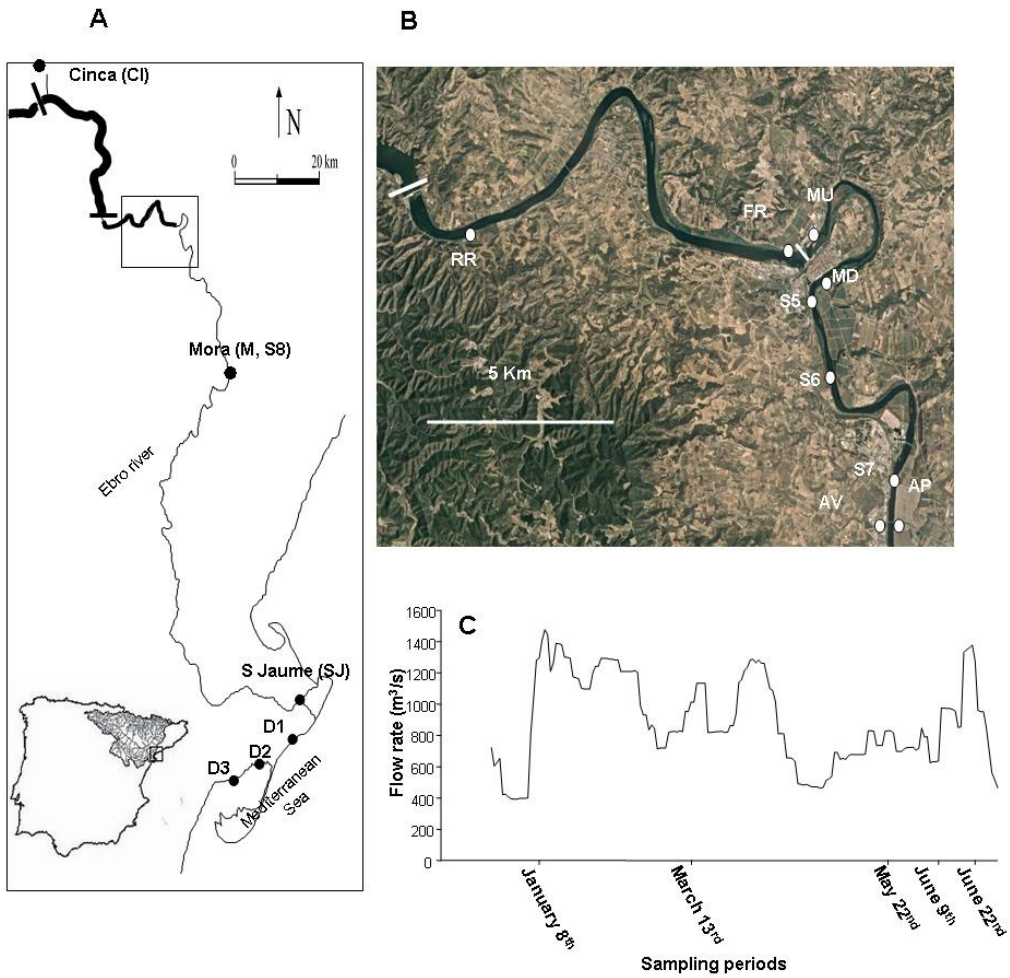


Figure 3.2 Studied sites within Ebro and Cinca Rivers (A, B) and water flow during the studies period (C). Bars indicate main dams. In (A) and (B) the location of sediment samples collected along the river (S5, S6, S7, S8) and water samples obtained in Sant Jaume d' Enveja (SJ) at the river mouth and at the drainage channels (D1, D2, D3) are also included.

Particle size and phytoplankton analyses peyformed with a Beckman Coulter LS Particle Size Analyzer and an inverted optical microscopy, respectively, showed that suspended solids was mainly composed of fine silt (75%; 4 μm < grain size < 62 μm) and clay (25%; grain size < 4 μm) with only few phytoplanktonic cells < 2 cells/mL (< 1 cells/mL of cyanobacteria). The sediment from Sitges used for method development also had the same grain distribution. The organic C and N content of suspended solids, analyzed by means of a Thermo Electron Flash 1112 elemental analyser (Thermo Scientific, UK), of suspended solids, varied little (4-5% of C, <0.05-0.25% of N). The organic content of C and N of the sediment samples from Sitges

was similar to those of suspended solids being 3.2% for C and 0.29% for N. Toxins from the filters were extracted according to (Barco et al., 2005) with some modifications. Filters with suspended solids were extracted with 5 mL of MeOH/water 80% by sonication three times for 10 min in ice and centrifuged at 8000 rpm for 5 min. The supernatants of the three extractions were then pooled, evaporated to almost dryness under light N_2 current, and reconstituted with 0.15 mL of MeOH in a chromatographic vial.

3.3.3 LC–MS/MS analysis

Toxins were measured using LC-MS/MS (TqDetector, Acquity Waters, USA) modifying a previous study that reported chromatographic conditions for separation of a wide range of toxins (Chapela et al., 2008). Separation was performed by using a Luna C18 (150 mm×2 mm ID, particle size 5 µm, Phenomenex, Torrance, USA) equipped with a Security Guard pre-column.

The mobile phase composition consisted of binary mixtures with 0.1% formic acid in ACN (A) and 0.1% formic acid in water (B). Gradient elution started at 5% A and 95% B, increased to 40% A in 5 min, 60% A in 10 min and reaching to 100% A in 20 min. Initial conditions to stabilize the system were attained in 5 min. Total run lasted 25 min. The system was operated at room temperature, the flow rate was set at 200 µL min^{-1} and 10 µL were injected. In the same run microcystins (RR, LR and YR) and anatoxin-a were measured under positive electrospray ionization mode (ESI+), whereas okadaic acid (OA) was detected using a negative electrospray ionization (ESI-) mode.

Flow injection analysis (FIA) was performed to determine the optimum cone voltage (between 1 and 90 V) to obtain the molecular ion with highest sensitivity and the optimum collision energies (between 5 and 80 eV) to obtain at least two intense fragment ions. Finally, acquisition was performed in SRM mode using two transitions from $[M+H]^+$ or $[M-H]^-$ precursor ion to daughter ions to identify each compound. The transitions used as well as the optimized cone voltages and collision energies are given in Table 3.1. The data were acquired and processed using the MassLynx v4.1 software package.

3.3.4 Quality assurance

Calibration was performed over a concentration range from 0.001 to 1 ng/µL, using seven calibration points. Quantification was performed by external standard calibration since labelled standards were not available for these compounds. Recovery studies were performed in triplicate directly in sediment samples or in re-constituted suspended solids produced by spiking analyte-free sediment from Sitges (15 mg, particle size < 70 µm) in pre-combusted (350ºC, 12h) 47 mm Whatman GF/F glass fibre filters. All the samples were spiked with 3.3 ng/g dry weight (d.w.) of microcystins, anatoxin-a and okadaic acid mixture. In addition, sediment matrix effect was estimated by comparing a spiked sediment and suspended solid-filter extract with a spiked solvent extract, using the same concentrations as in recovery studies Instrumental detection limits (IDLs) were determined using the lowest concentration standard detected that yielded a signal/noise (S/N) ratio equal to 3. Method detection limit (MDL) was calculated in the same way, using blank sediment samples spiked at the same concentration levels as for the recovery studies. Inter-assay variation was determined by measuring the same standard concentration (1 ng/µL) on three different days. Solvent blanks did not contain any of the investigated analytes, indicating no carryover effects in any of the LC–MS/MS runs.

Table 3.1 LC–MS/MS retention time (RT), acquisition mode (AM) and optimized parameters for the analysed toxins. PrI, Precursor Ion; CV, Cone voltage (V); QT, Quantification transition; CE, Collision energy (eV); CT, Confirmation transition.

Compound	RT (min)	AM	PrI (*m/z*)	CV (V)	QT (*m/z*)	CT (*m/z*)
					(CE, eV)	(CE, eV)
Anatoxin-a	1.92	ESI(+)	166 $[M+H]^+$	34	166>149 (18)	166>131 (18), 166>91 (20)
Microcystin-RR	6.53	ESI(+)	520 $[M+H]^+$	42	520>135 (36)	520>103 (48)
Microcystin-YR	7.47	ESI(+)	1046 $[M+H]^+$	74	1046>135 (59)	1046>107 (67)
Microcystin-LR	7.59	ESI(+)	996 $[M+H]^+$	45	996>135 (59)	996>213 (50)
Okadaic acid	17.30	ESI(-)	803 $[M-H]^-$	92	803>255 (41)	803>113 (48)

3.4 Results and discussion

3.4.1 Optimization of the ionization parameters and chromatographic separation

Optimization of the ionization source conditions was performed using LC-MS/MS in ESI(+) and ESI(-) mode acquisition. At 3 V extraction voltage and 3.5 kV capillary voltage, the protonated molecule in ESI(+) and the deprotonated molecule in ESI(-) (for okadaic acid) was formed as the base peak. Cone voltage (CV) was the major parameter influencing the intensity of signals, with compounds showing strong fragmentation and compounds forming very few ions. Optimum cone voltages were ranged from 34 to 74 V in ESI(+) and 92 V in ESI(-). Then, molecules were fragmented by optimisation of the collision energy (CE), and the two most abundant product ions of each compound were chosen for the SRM analysis to enhance selectivity and sensitivity. At this point, CE was optimized from 18 to 67 eV (ESI(+)) and 41 to 48 eV (ESI(-)). Table 3.1 shows the mass spectral information.

3.4.2 Mass spectral characterization

The mass spectra of target compounds are shown in Fig. 3.3. Anatoxin-a is a secondary, bicyclic amine alkaloid and cyanotoxin with acute toxicity. The ESI(+) spectrum of anatoxin-a showed the *m/z* 166 $[M+H]^+$ as base peak and the product ion spectra from the precursor yielded three intense fragment ions. At CE of 18 eV, the *m/z* 149 $[M-NH_3+H]^+$ was formed corresponding to the loss of an ammonia molecule (Fig. 3.3 B). At the same CE, the *m/z* 131 $[M-NH_3-H_2O+H]^+$ was formed corresponding to the loss of a water molecule respect to the fragment *m/z* 149; and at CE of 20 eV, the formation of cyclohepta-2,4,6-trien-1-ylium molecule (*m/z* 91 $[C_7H_7]^+$) was obtained. Sanchez et al. (Sanchez et al., 2014) analyzed anatoxin-a in *Anabaena sp*. cultures with LC-MS/MS obtaining the same fragmentation pathway in ESI(+) (*m/z* 149, *m/z* 131 and *m/z* 91). The SRM transition 166>149 was chosen for quantification and 166>131 and 166>91 was selected for confirmatory purposes (Table 3.1).

Microcystins are cyclic non ribosomal peptides produced by cyanobacteria (e.g. *Microcystis aeruginosa* and *Planktothrix sp*). They are cyanotoxins and can be very

toxic for plants and animals including humans. Their hepatotoxicity may cause serious damage to the liver. Microcystins can strongly inhibit protein phosphatases type 1 (PP1) and 2A (PP2A), and are linked to pansteatitis. Microcystin-RR showed the intense double-protonated molecule at m/z 520 $[M+2H]^{2+}$, resulting from the presence of two arginine residues that are both protonated. The MS/MS spectra from the precursor ion gave fragment ions at *m/z* 135 $[C_9H_{11}O]^+$ that corresponds to the loss of the 1-(2-methoxyethyl)benzene moiety, generated by alpha-cleavage at the methoxy group of the Adda beta-amino acid moiety (Namikoshi et al., 1992). At *m/z* 103 the obtained fragment ion $[135\text{-}CH_3OH]^+$ corresponds to the loss of MeOH from the 135 fragment ion (Fig. 3.3 A). (Kaloudis et al., 2013) analyzed mycrocystins in Lake Marathonas and also obtained the double-protonated molecule at *m/z* 519.8. They identified two fragment ions at *m/z* 135 (the same that in the present study) and at *m/z* 213 $[C_9H_{13}N_2O_4]^+$, characteristic of most microcystins but giving rise to a weaker signal in our analyses when compared to *m/z* 103. In this regard, the SRM transition 520>135 was chosen for quantification and 520>103 was selected for confirmatory purposes (Table 3.1). Mycrocystin-YR, with full scan LC-MS, produced the protonated molecule at *m/z* 1046 at 74 V of cone voltage (Table 3.1). MS/MS spectra from this precursor ion using 59 and 67 eV of CE, respectively, produced a fragment ion at *m/z* 135, characteristic of most mycrocystins and used herein as qualifier. The above mentioned ion at *m/z* 107 formed $[C_7H_7O]^+$ by cleavage of the *p*-cresol moiety (Fig. 3.3 A). Both 1046>135 and 1046>107 were used as a SRM transitions. Xu et al. (Xu et al., 2008) using LC-MS/MS reported also the protonated molecule of mycrocystin-YR at *m/z* 1045.8 $[M+H]^+$ and the formation of the two characteristic fragment ions at *m/z* 135 (the quantifier ion as in our study) and at *m/z* 213 using 63 and 55 eV of CE respectively, whereas we decided to use *m/z* 107 giving a better signal. Mycrocystin-LR is a naturally occurring toxin produced by cyanobacteria. It is considered the most toxic compound of this family. Mycrocystin-LR showed the intense protonated molecule at *m/z* 996 $[M+H]^+$. The MS/MS spectra from the precursor ion gave fragment ions at *m/z* 135 $[C_9H_{11}O]^+$ and at *m/z* 213 $[C_9H_{13}N_2O_4]^+$ which corresponds to the loss of a 4-(*N*-(1-carbamoylvinyl)-*N*-methylcarbamoyl)butanoic acid moiety (Fig. 3.3 A). The SRM transition 996>135 was chosen for quantification and 996>213 was selected for confirmatory purposes. (Xu et al., 2008) also obtained the protonated molecule at m/z 995.6 and the same

fragment ions at m/z 135.0 and m/z 213.3 using 55 eV of CE. Overall, in the present study, it was possible to detect and discriminate the microcystins using three different confirmation transitions, allowing their identification both with mass characterization and retention time.

Finally, the full scan ESI mass spectrum of okadaic acid at -92 V of CV was dominated by *m/z* 803 $[M-H]^-$. The MS/MS spectra of this precursor ion gave the fragment ions at *m/z* 255 $[C_{15}H_{27}O_3]^-$ and at *m/z* 113 $[C_6H_9O_2]^-$ using 41 and 48 eV of CE (Fig. 3.3 B). Both 803>255 and 803>113 were used as a SRM transitions. (Shen et al., 2013) analyzed okadaic acid and other marine toxins in shellfish. Using a declustering potential and a CE of -60 and -65 eV, respectively, the former authors obtained the deprotonated molecule at *m/z* 803.5 $[M-H]^-$ and the fragment ions at m/z 255.3 (the same as in our study) and at *m/z* 563.5.

3.4.3 Optimization of HPLC conditions

In order to achieve optimum resolution and compound detection, different mobile phase composition and gradients were tested using a Luna C18 column. Optimal HPLC conditions are already depicted in 3.3.3. Experimental – LC-MS/MS analysis. Under these conditions, no chromatographic coelutions occurred for any of the analysed compounds. With the conditions optimized, it was possible to determine anatoxin-a, three microcystins and okadaic acid in two ionization modes but within a single run. The ion chromatogram of a mix solution at 1 ng/μL containing all the target analytes using the Luna C18 column is shown in Fig. 3.4 A. In Fig. 3.4 B,C we depict the ion chromatograms of positive results of selected environmental samples.

3.4.4 Quality parameters and identification criteria

Sensitivity, linearity recoveries, precision and matrix effects were considered as criteria for the validation of the analytical methodology developed. Calibration curves were generated using linear regression analysis over the established range of concentrations. Good correlation coefficients (R^2>0.99) were obtained for all compounds with calibration curves ranking from 0.01 to 1 ng/μL for anatoxin-a and microcystin RR and from 0.005 to 1 ng/μL for the remaining compounds. IDL ranged from 0.01 (Anatoxin-a and mycrocystin RR) to 0.05 ng (mycrocystin LR, YR and okadaic acid). Inter-day precision ranges were from 10 to 25% at 1 ng/μL level, thus

indicating a robust method response. MDL for anatoxin-a, microcystins RR, LR, YR and okadaic acid were 7.1, 3.3, 81.7, 103 and 28.8 ng/g d.w. suspended solids/sediment, respectively. When referred to water volume the previous limits became 0.4, 0.2, 5.0, 6.2, 1.7 ng/L for the target compounds, respectively.

Percentage recoveries (Mean ± SD, N = 6) from the spiked sediments and suspended solids used for method development and were 76±12, 62±5, 81±6, 73±5, 93± 5 % for anatoxin-a, microcystins RR, LR, YR and okadaic acid, respectively. Reported recoveries and detection limits of the studied toxins are in most cases referred to units of water volume to meet criteria of the World Health Organization provisional guideline that for example establishes a limit for microcystin-LR of 1 µg/L. Nevertheless, several studies have reported detection levels of the studied toxins in biological tissues of fish and mollusks. In the EU the maximum value for okadaic acid in edible tissue is 160 ng/g (Chapela et al., 2008). In our study obtained analytical detection limits were within the lowest reported range for water (Al-Sammak et al., 2014; Chen et al., 2012; Ferranti et al., 2009; Li et al., 2011b; Shan et al., 2011; Wang et al., 2007; Yen et al., 2011; Zhang et al., 2004).

Besides, as the extraction of sediment can affect the ionization of target compounds, the matrix effect was calculated. Matrix-induced signal suppression or enhancement relative to the methanol spike (Mean ± SD, N =6) were 126.0±21.1, 126.5±15.1, 101.3±13.7, 96.0±5.8, 97.1± 5.8 % for anatoxin-a, microcystins RR, LR, YR and okadaic acid, respectively. Ion suppression results in a value less than 100%, whereas a value exceeding 100% suggests that there is ion enhancement. Obtained matrix signal responses relative to the solvent spike were within accepted limits (96-126%). Studies conducted in microcystins with more complex matrixes such as biological tissues reported ion suppression or enhancement of up to 50% (Karlsson et al., 2005). The few reported studies that have analysed microcystins or anatoxins in sediments or suspended solids did not provide quality assurance data to be compared with the obtained results (Chen et al., 2008; Klitzke et al., 2011; Rapala et al., 1994; Song et al., 2014).

A

A

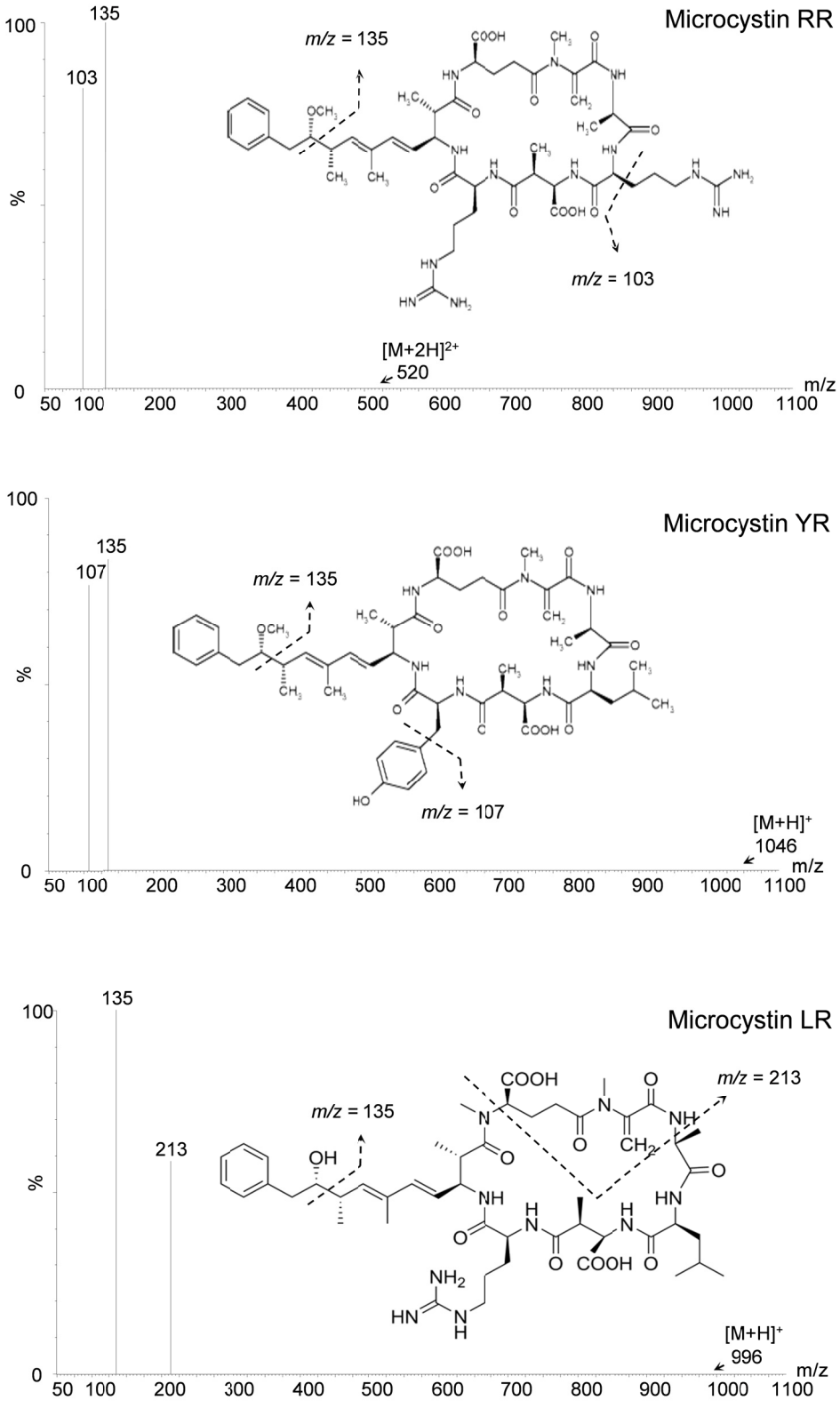


Figure 3.3 Mass spectra characterization of microcystins (A) and anatoxin-a and okadaic acid (B)

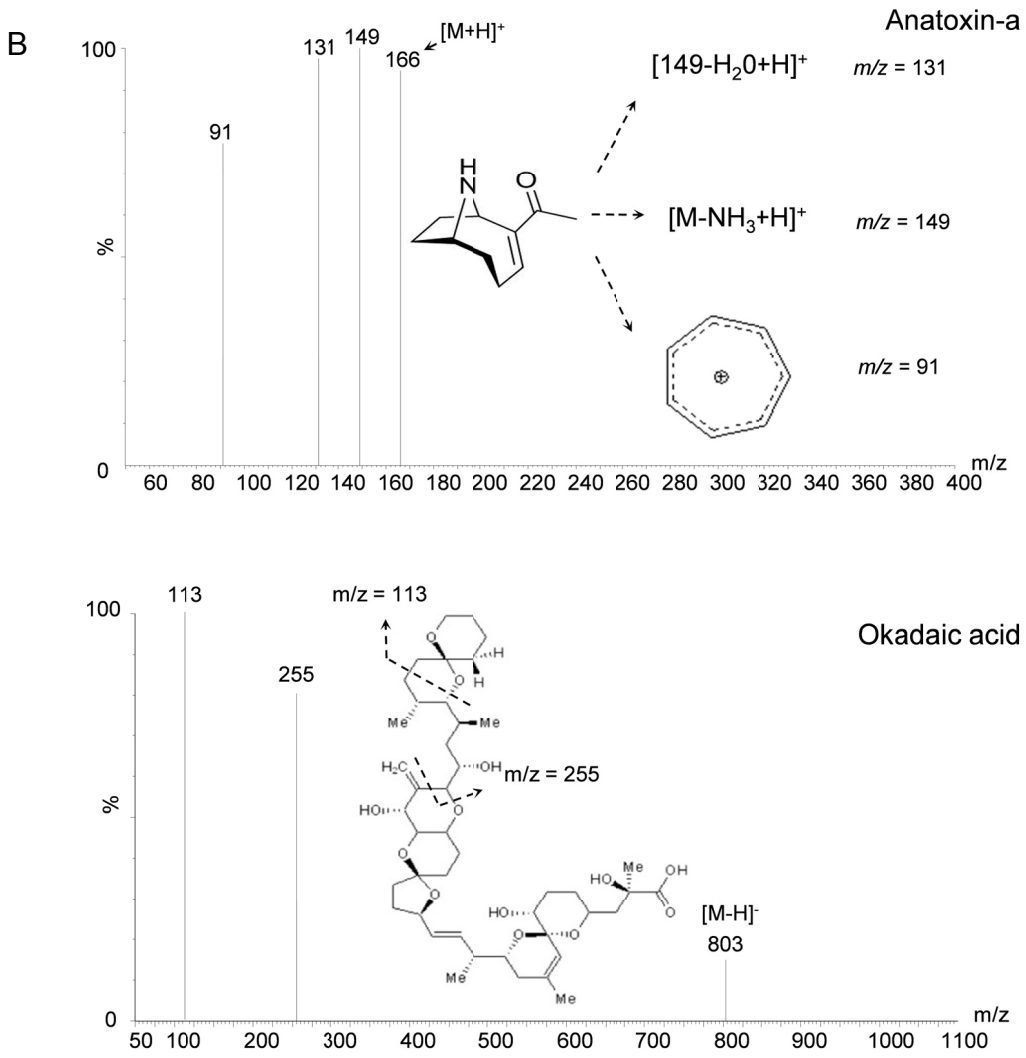


Figure 3.3 continuation

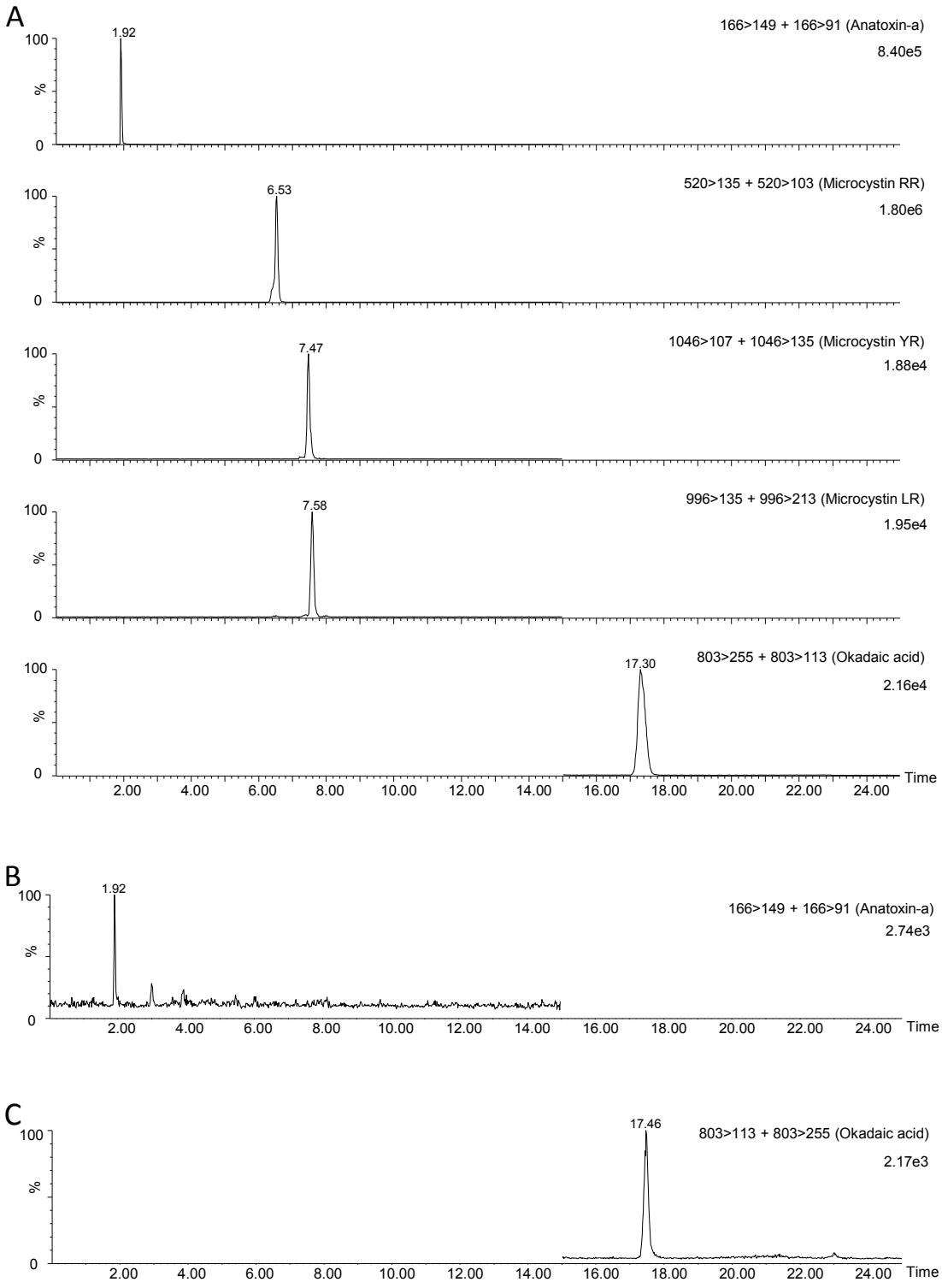


Figure 3.4 LC-MS/MS chromatogram of a standard solution at 1 ng/µL (A); and positive samples of anatoxin-a from FR June 22nd (B) and okadaic acid from March 22nd (C)

3.4.5 Environmental levels

During winter and spring 2013 an excess of rain and of accumulation and subsequent melting of snow in the Pyrenees made necessary to open the dams of Riba-Roja and Flix reservoirs. Opening the Riba-Roja reservoir dam dramatically increased the downstream water flow of Ebro River (Fig. 3.1 C) and consequently the amount of re-suspended particle matter (Table 3.2). The greatest levels of suspended solids measured in the study coincided with those periods of maximal water flow in January 8th and June 22nd.

From the five targeted toxins, only residues of anatoxin-a were detected in suspended solids from the Ebro's water samples downstream Riba-Roja reservoir with concentrations ranging from 20 to 1120 ng/g d.w. (Table 3.2). The highest levels were measured just downstream of Riba-Roja reservoir at RR when the dam was opened for the first time in January 28th, and the lowest in June 22nd at MU after 155 days of floods. When anatoxin-a residues in suspended mater were converted to levels per volume of filtrated water, concentrations ranged from 0.2-0.3 ng/L in June 9th to 6.8 ng/L at RR in January 28th. These levels are quite low when compared with those reported during cyanobacterial blooms that usually are within the µg/L or µg/g d.w. range (Santos et al., 2012). In our study the visual inspection of the suspended solids present in water samples under an inverted microscopy could detect only few cyanobacteria cells but instead fine clay material and bacteria associated to it (data not shown), thus indicating that residue levels of algae toxins should be low.

Of particular interest were water samples collected in January 28th that were enriched with anatoxin-a at RR and subsequent diluted downstream due to the increase of suspended solids, probably coming from mobilised bottom sediment during the flood. This means that measured anatoxin-a on suspended matter are likely to be related to sediment wastes coming from the Riba-Roja reservoir. Indeed, during floods, Ebro's reservoirs are partially emptied in order to erode the stored sediment, and evacuate them through the bottom outlets by using the water column pressure (Rovira and Ibàñez, 2007).

Table 3.2 Measured suspended solids (SS) and residue levels of anatoxin-a in the studied environmental samples. bdl: below detection limit. Units for sediment samples are in mg sediment dry weight (d.w.)

Date	Sites	SS (mg/L d.w.)	Anatoxin a Ng/g d.w.
Jan 28th	RR	4.8	1120.1
	FR	20.2	154.1
	MU	26.8	119.1
	MD	24.8	88.6
	A	30.1	133.5
	M	35.2	71.9
March13rd	RR	13.2	56.2
	MU	12.6	46.0
	A	12.6	86.3
May 22nd	RR	5.0	89.1
	FR	6.3	296.9
	MU	5.0	150.2
	MD	5.7	114.7
	A	6.1	79.8
	M	6.0	159.8
June 9th	CI	22.7	bdl
	RR	4.5	61.1
	A	5.8	36.5
June 22nd	RR	30.7	30.3
	FR	37.6	48.2
	MU	39.6	36.4
	MD	36.1	20.5
	A	34.6	35.8
	M	36.7	23.5
June 24th	SJ	4.78	bdl
	D1	18.6	bdl
	D2	22.0	bdl
	D3	19.5	bdl
Sediment	S5	14.7	bdl
	S6	16.3	bdl
	S7	13.5	bdl
	S8	15.1	bdl
	Sitges	17.5	bdl

The most common toxic cyanobacteria species found in Ebro's reservoirs are *Anabaena aphanizomenoides* and *Planktothrix cf. agardhii*, which are known to produce microcystins and anatoxins (de Hoyos et al., 2004; Quesada et al., 2004). It should be pointed out that despite our samplings were not carried out at the typical period for cyanobacteria blooming (from August to October), there is reported evidence that toxic cyanobacteria occurs along the year in Ebro's reservoirs (Quesada et al., 2004). There are also studies showing that toxic cyanobacteria of the genus *Anabaena* and *Planktothrix* can live in the bottom sediment of lakes even under heterotrophic conditions and winter (Li et al., 2011a; Mannan and Pakrasi, 1993; Savichtcheva et al., 2011; Toporowska et al., 2014). Microcystins, and specially anatoxin-a, are known to adsorb to fine clay material, accumulating in the sediment in which they can persist for long periods depending on the existing micro-flora (Chen et al., 2008; Chen et al., 2013; Kaminski et al., 2013; Klitzke et al., 2011; Rapala et al., 1994). The detection of anatoxin-a and not of microcystins in the analyzed suspended matter samples support the previous studies.

None of the targeted toxins were detected in sediment samples, neither in samples collected at the Ebro's River mouth located about 100 km downstream of Riba-Roja reservoir nor in water samples collected along Ebro's Delta drainage channels (Table 3.2 for anatoxin-a). These channels collect and drainage the water from the rice fields into the Ebro's associated bays (Barata et al., 2007). For the freshwater-associated phytotoxins, the previous results indicated that the detected anatoxin-a is not transported, at least at detectable levels, towards the river mouth or across the Delta throughout the net of channels used for rice production (Barata et al., 2007). Residue levels of okadaic acid, exclusively produced by marine dynoflagellates (Chapela et al., 2008), were only measured in three samples collected in 2012 during blooms of *Dinophysis spp.* in one of the Ebro's associated marine bays (Alfacs Bay) (Table 3.3). Levels of okadaic acid measured in suspended solids during the Dinophysis bloom were quite high ranking between 2405-9164 ng/g d.w., which corresponded to 6.7-50.4 ng/L (Table 3.3). There is ample reported information of periodical blooms of toxic dynoflagellates in Ebro's associated bays (Garcés et al., 1997; Garcés et al., 1999; Garibo et al., 2014; Loureiro et al., 2009; Quijano-Scheggia et al., 2008). Despite that marine water mix with river water in the

Ebro's mouth or in the studied Delta drainage channels (Barata et al., 2007; Gómez-Gutiérrez et al., 2011; Ibánez and Prat, 2003), we were unable to detect trace levels of okadaic acid in these locations. This is likely related to the fact that at the time of sampling there were no blooms of toxic dynoflagellates in that area.

Table 3.3 Measured suspended solids (SS) and residue levels of okadaic acid in the studied environmental samples. bdl: below detection limit. For clarity only positive samples are included.

Date	Sites	SS (mg/L)	Okadaic acid ng/g d.w.	Okadaic acid ηg/L
15th February 2012	Alfacs lagoon	2.8	2405.3	6.7
5th March 2012	Alfacs lagoon	4.6	4628.4	21.3
22nd March 2012	Alfacs lagoon	5.5	9164.1	50.4

3.5 Conclusions

Overall, there is a lack of data on the sedimentation and the fate of toxins in sediments and their possible remobilization into the water column. In this study we provide a reliable method to detect accurately marine and freshwater algae toxins in the suspended solid fraction of the water column and sediments. In fact, the proposed LC-ESI-MS/MS method proved to be a powerful tool for simultaneous extraction and determination of different classes of noxious toxins at trace levels. The extraction with methanol/water 80:20 (v/v) allows a good recovery for all studied toxins without carry-over of the matrix. The extracts are injected directly into the LC-ESI-MS/MS device and are well separated in a single 25 min chromatographic run and unambiguously detected by ESI-MS/MS. To our knowledge this is the first study analysing phytotoxins present in suspended solids mobilized during floods from reservoirs to downstream locations.

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Chapter IV.

Identification of compounds bound to suspended solids causing sub-lethal toxic effects in *Daphnia magna*. A field study on re-suspended particles during river floods in Ebro River

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Chapter IV.

Identification of compounds bound to suspended solids causing sub-lethal toxic effects in *Daphnia magna*. A field study on re-suspended particles during river floods in Ebro River[a]

4.1 Abstract

Identifying chemicals causing adverse effects in organisms present in water remains a challenge in environmental risk assessment. This study aimed to assess and identify toxic compounds bound to suspended solids re-suspended during a prolonged period of flushing flows in the lower part of Ebro River (NE, Spain). This area is contaminated with high amounts of organochlorine and mercury sediment wastes. Chemical characterization of suspended material was performed by solid phase extraction using a battery of non-polar and polar solvents and analyzed by GC–MS/MS and LC–MS/MS. Mercury content was also determined for all sites. Post-exposure feeding rates of *Daphnia magna* were used to assess toxic effects of whole and filtered water samples and of re-constituted laboratory water with re-suspended solid fractions. Organochlorine and mercury residues in the water samples increased from upstream to downstream locations. Conversely, toxic effects were greater at the upstream site than downstream of the superfund Flix reservoir. A further analysis of the suspended solid fraction identified a toxic component eluted within the 80:20 methanol : water fraction. Characterization of that toxic component fraction by LC–MS/MS identified the phytotoxin anatoxin-a, whose residue levels were correlated with observed feeding inhibition responses. Further feeding inhibition assays conducted in the lab using anatoxin-a produced from *Planktothrix agardhii*, a filamentous cyanobacteria, confirmed field results. This study provides evidence that in real field situation measured contaminant residues do not always agree with toxic effects.

Keywords: Toxicity identification evaluation, *Daphnia*, Feeding, Mercury, PCB, Cyanobacteria.

4.2 Introduction

The adsorption of chemicals onto sediment particles is an important process through which many contaminants are removed from the water column. Bottom sediments may act both as a sink and as a long-term source of toxicants (Burton, 2002). This dual role is particularly relevant during floods, in which pollutants stored in sediments may be easily remobilized by sudden increases of river flow and the consequent increase of toxicant concentrations. Nowadays, in fact, due to climate change, we are suffering an increase of severe weather conditions for certain regions, often characterized by an alternation of extreme events such as drought and flash floods, thus growing the awareness to the impacts caused by floods (Ikeda et al., 2005; Kleinen and Petschel-Held, 2007). Therefore, there is an increasing challenge among environmental toxicologists to identify substances within suspended particular matter having the potential to harm the biological communities (Stachel et al., 2005; Wölz et al., 2010).

Traditionally, identifying environmental contaminants has been performed using targeted chemical analysis following a prioritisation/ranking process. This approach is an effective way of analysing environmental samples, but has the drawback that certain unknown contaminants may be missed (e.g., components with low concentrations and high target toxicity, metabolites, transformation products or natural products). In recent years the development of effect-directed analysis (EDA) or toxicity identification procedures (TIE) have allowed to address the problem of unknowns using bioassays as diagnostic tools. Using these methods a complex environmental sample is extracted, fractionated and then analyzed using both biological assays and chemical analysis in order to link the presence of one or more compounds to their biological effects. Finally, the identified compounds need to be confirmed by testing uncontaminated samples spiked at concentrations measured in the obtained fractions. In these procedures, the selection of bioassays to be used in whole samples and their fractions are crucial. Therefore, there is a need to develop more ecologically relevant in vivo bioassays that can be used both for the whole

samples and fractions. This is most problematic when trying to assess toxicity of particle bound contaminants in suspended material in water, since the very few already existing bioassays have been only tested in sediments (Bosch et al., 2009; Phillips et al., 2009; Schmitt et al., 2011; Wölz et al., 2010). In this regard, particle-feeding organisms are of special interest since contaminated particles might end up in their gastrointestinal tract and exert toxic effects (Bosch et al., 2009). Recently, the use of cost effective sub-lethal *Daphnia magna* feeding tests combined with Toxicity Identification Evaluation (TIE) procedures have allowed the identification of water soluble and particle-bound compounds in sediments causing toxic effects (Bosch et al., 2009).

Likewise many other rivers located in arid or semi-arid climate regions, dams in the Ebro River (NE, Spain) are used to regulate surface-water cycles, especially when water demand and its availability are imbalanced (Petrovic et al., 2011). Flushing flows have been used typically to mitigate dam-induced impacts such as deterioration of riparian habitats (Gibbins et al., 2007) or reduction of sediment transport (Batalla and Vericat, 2009). As a counter effect, contaminants that are accumulated in sediments have been shown to be mobilized during these flood events (Kirchner et al., 2000; Quesada et al., 2014). This is especially problematic in Flix reservoir, where an organochlorine industry operates since the beginning of the 20th century. In fact, this long operational period, along with the construction of a dam next to the factory around 1960, resulted in the accumulation of high amounts of heavily polluted sediments in the adjacent riverbed (Bosch et al., 2009; Fernández et al., 1999; Grimalt, 2006). Major pollutants reported in these wastes include hexachlorobenzene (1900 ηg/g), polychlorobiphenyls (39,000 ηg/g), DDEs-DDTs (1300 ηg/g), polychlorostyrenes (360 ηg/g), polychloronaphthalenes (1100 ηg/g), mercury (49 μg/g), cadmium (2.3 μg/g), chromium (210 μg/g) and nickel (67 μg/g), mean values (Grimalt, 2006). Bosch et al. (2009) reported that mercury bound to waste sediment particles severely impaired grazing rates of filter feeders like *D. magna*. Furthermore, pollutants originated at Flix site are carried downstream by the Ebro River to its delta located 90 km away, where they can bio-accumulate and affect biota (Faria et al., 2010; Navarro et al., 2009; Pastor et al., 2004).

Nevertheless, there is no information of toxic effects of re-suspended industrial waste sediment material during flood events.

Another environmental problem associated to dams is the proliferation of noxious substances produced by cyanobacterial blooms (Agha et al., 2012; Herry-Allani and Bouaïcha, 2013). Several studies have reported the occurrence of toxic cyanobacteria species (e.g., *Anabaena, Planktothrix*), known to produce toxins such as microcystins and anatoxins in Ebro reservoirs at and downstream of Flix (de Hoyos et al., 2004; Quesada et al., 2004). The cyanobacteria mentioned above or/and their phytotoxins exert toxic effects to grazers like *Daphnia*, which are known to graze on them (Claska and Gilbert, 1998; Demott et al., 1991; Freitas et al., 2014).

This study aimed to use a TIE protocol implemented with in vivo *D. magna* feeding inhibition responses to evaluate and identify toxic compounds present in suspended solids during an unusual period of prolonged flushing flows along a contaminated area located at the low Ebro River Basin.

4.3 Material and methods

4.3.1 Water sampling

Thirty superficial water samples were collected from January 18th to June 22nd 2013 along the lower course of Ebro River (NE Spain) between its tributary, the Cinca river (CI) that ends at Riba-Roja reservoir, just before its junction with the reservoir and the village of Mora d'Ebre, which is located 50 km downstream (M; Fig. 4.1 A). Samples were collected at five different periods during a prolonged flash flow of 155 days (Fig. 4.1 C). Sampling sites included locations situated upstream the industrial sediment wastes (e.g., Cinca river, CI and Riba-Roja pier at Flix reservoir, RR), in front of the wildlife reserve located on the riverbank opposite to the factory (FR) and at the meander located immediately downstream from the dam (MU, MD) (Fig. 4.1 A and B). On June 22nd it was possible to take samples on the river bank opposite of MU at MF (Fig. 4.1 B). According to the river flow, MF site should have a greater contribution of mobilized industrial wastes than sites FR, MU and MD. Three locations further downstream were also sampled and included both river margins at Ascó village (Ascó pier, A; and Ascó village, AV) and at Mora village (M) (Fig. 4.1 A

and B). Part of the water from Flix reservoir is diverted through a bypass below the village of Flix to generate electrical power and it is released just downstream of MD. This means that a portion of the re-suspended sediment particles coming from the industrial sediment wastes, which are located at the left margin of Flix reservoir, may not reach MU, MF and MD. Conversely, water samples from Ascó and Mora should be fully enriched from re-suspended particles from contaminated Flix sediments.

In addition to the above water samples, three additional samples were included as negative controls. Two were obtained from elutriates of sediments obtained between the Meander and Ascó in a previous study (sediment samples S5, S6; (Bosch et al., 2009)). Elutriates were obtained after mixing 1 g of freeze dried and sieved (60 μm size) sediment in 1 L of ASTM hard water to obtain the fine particulate fraction. Another water sample was obtained outside the Ebro basin from the lagoons of Las Tablas de Daimiel National Park (TDNP), south-central Spain.

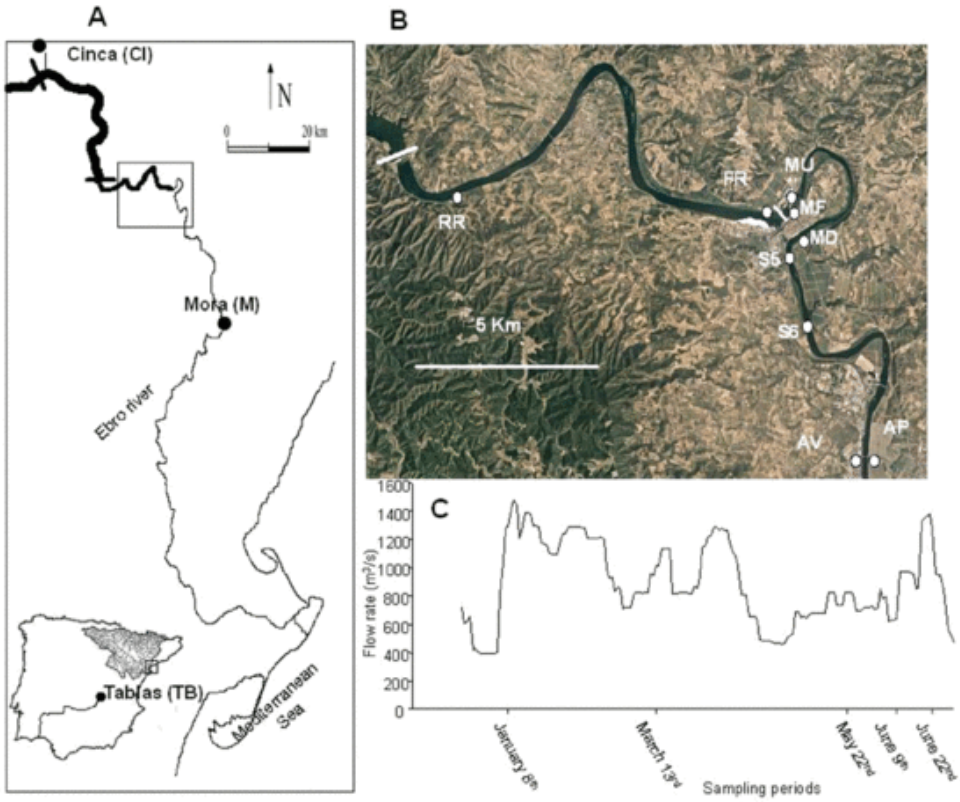

Figure 4.1 Studied sites within Ebro and Cinca rivers (A, B) and water flow (C) during the study period. Bars indicate main dams. In graph B the location of Flix industrial wastes is indicated.

4.3.2 Physico-chemical characterization of water samples

A set of environmental variables were measured on each sampling. Water physico-chemical parameters including temperature (T; °C), pH, conductivity (µS/cm), dissolved oxygen (O2, mg/L) and suspended solids (SS, mg/L) were obtained following (Damásio et al., 2008) procedures. Briefly, T, pH, conductivity and O2 were measured in situ by using a WTW Multi 340i handheld meter. Suspended solids were obtained after filtering 1 L of water sample through a Whatman grade GF/F glass fiber filter paper (0.7 µm pore size) previously washed with acetone, pre-combusted at 400°C and pre-weighted. Following filtering, the filter was freeze dried, weighed to assess suspended solids and then used to determine total mercury or organochlorine residues. Further characterizations of the suspended matter were conducted in water samples from January and May. Particle size analyses were performed using a Laser Diffraction particle size analyser (LS 13,320 MW, Beckam Coulter, Inc., USA), whereas carbon, nitrogen and hydrogen content was determined by Elemental Microanalyzer (A5) model Flash 1112 (Thermo Scientific, UK), performed by the modified Pregl-Dumas technique (dynamic flash combustion), using helium as carrier gas.

Analysis of total Hg in filters was performed following Carrasco et al. (2008) with minor modifications by means of the Advanced Mercury Analyzer AMA-254 (Altec, Prague, Czech Republic). In each run appropriate blanks (freeze dried filters previously loaded with 1 L of nanopure water) were used. The AMA instrument is based on catalytic combustion of sample, its pre-concentration by gold amalgamation, thermal desorption and atomic absorption spectrometry (AAS). Samples were homogenized, weighed and placed into the instrument, which is automatically introduced into the AMA. The entire analytical procedure was validated by analyzing total mercury in a certified reference sediment (MESS-3: 0.091 ± 0.009 mg/kg, National Research Council Canada, NRCC; Ottawa). Analyses of MESS-3 in the beginning and end of each set of samples (usually 10) ensured that the instrument remained calibrated during the analytical sequence. Detection and quantification limits were calculated from blank measurements giving values 0.2 and 0.7 ng Hg/g (d.w.).

Organochlorine residues in filters were extracted and analyzed as described with minor modifications (Sánchez-Avila et al., 2011). Prior to extraction, all the filters were spiked with a surrogate recovery standard (PCB congeners 65 and 209, Dr. Ehrenstorfer, GmbH, Augsburg, Germany). Washed and pre-combusted (400°C) filters were used as blanks and quality controls, spiked with the surrogate standards and the native solution containing all compounds to be analyzed. All filters were extracted in parallel with laboratory filter blanks by sonication (three times, 10 min each, with 1 min vortexing in between) using a mixture of 20 mL of hexane/dichloromethane (1:1, v/v), and centrifuged for 10 min at 3000 × g to remove filter leftovers. Then, the liquid extracts were cleaned up using Florisil (5 g) SPE cartridges, previously conditioned with hexane/dichloromethane (1:1, v/v). The sample extract was eluted using 2 × 15 mL of hexane/dichloromethane (1:1, v/v). The eluates were evaporated almost to dryness under nitrogen current and reconstituted in 100 µL of hexane. Hexachloro 1,3 butadiene (HCBu), hexachlorobenzene (HCB), α, β, γand δ hexachlorocyclohexanes (HCHs), heptachlor, α and β endosulfan, PCB congeners 28, 52, 101, 118, 138, 153 and 180, o,p′-DDE, p,p′-DDE, o,p′-DDD, p,p′-DDD, o,p′-DDT and p,p′-DDT were analyzed by gas chromatography coupled to tandem mass spectrometry using an Agilent 7890 A GC System (Agilent Technologies, Palo Alto, CA, USA) connected to a 7000 A triple quadrupole mass spectrometer (Agilent, USA). Calibration curves were determined for each compound to be quantified. The target compounds were positively identified by comparison of their retention times and two SRM transitions to the standard solutions. A Mass Hunter WorkStation Acquisition Software B.02.01 (Agilent Technologies) was used for data acquisition and automatic integration and quantification of the results. The accuracy of the method and detection limits were assessed according to the original protocol.

Phytotoxins residues of anatoxin-a and microcystins LR, RR and YR were extracted and analyzed in filter samples according to the method developed in (Rivetti et al. 2015). Analytical standards of microcystins RR ($C_{49}H_{75}N_{13}O_{12}$), LR ($C_{49}H_{74}N_{10}O_{12}$), YR ($C_{52}H_{72}N_{10}O_{13}$) were obtained from Sigma–Aldrich (St. Louis, USA) and that of anatoxin-a ($C_{10}H_{15}NO$) was purchased from Santa Cruz Biotechnology (USA). Briefly, filters with suspended solids were extracted with 5 mL of methanol : water

(80:20, v/v) by sonication for 10 min on ice and centrifuged at 8000 rpm for 5 min. This procedure was repeated three times. The supernatants of the three extractions were then pooled, evaporated to almost dryness under a gentle N2 current, and reconstituted with 0.15 mL of MeOH in a chromatography vial. Toxins were measured using LC–MS/MS (TqDetector, Acquity Waters, USA) (LC–MS/MS) with a Luna C18 column (150 mm × 2 mm ID, particle size 5 µm, Phenomenex, Torrance, USA). All chromatographic conditions followed the above cited method. Method detection limits (MDL) for anatoxin-a and microcystins RR, LR and YR were, respectively, 0.4, 0.2, 5.0, 6.2 ηg/L, when referred to water volume (Rivetti et al., 2015).

4.3.3 Phytoplankton determination

Aliquots (50 mL) of water samples collected in January and May were fixed with buffered formalin (4%) solution and stored at 4 °C to allow the determination of the phytoplankton. Samples were sedimented for at least 24 h and the entire surface of sedimentation was visually examined for taxonomic analysis of phytoplankton using Nikon Eclipse 90i (Nikon, Champigny sur Marne, France) microscope. Images were acquired with a Nikon Digital Sight DS-Ri1 camera and NIS Elements AR software (version 3.0) and saved as high resolution (3840 pixels × 3005 pixels) tagged image file format (TIFF). Algae identification was based on classic, specific and regional literature. Species relative density was determined following the method of Battarbee (1986).

4.3.4 Experimental animals

A single laboratory *D. magna* clone (clone F), which has been the subject of many investigations (Barata and Baird, 2000), was used for this study. Bulk cultures of 15 animals each were maintained in ASTM hard synthetic water as described by Barata et al. (2000). Animals were fed daily with *Chorella vulgaris* Beijerinck (106 cells/mL, corresponding to 3.6 g C/mL) (Barata and Baird, 1998). The culture medium was changed every other day, and neonates were removed within 24 h. From 200 to 250 neonates were then transferred to 4 L tanks and reared under the same conditions as their mothers until they reached their fourth instar (4–5 days at 20°C). At this stage groups of juveniles were used for feeding and toxicity studies.

4.3.5 Cyanobacteria cultures

Batch cultures of *Planktothrix agardhii* CCAP 1459/11 A (SAMS Research Services Ltd Scottish Marine Institute, Scotland, UK) were cultured in Jaworski's Medium (CCAP) at 20 ± 1°C under constant light intensity of 12 µmol m^{-2} s^{-1} (14 h light: 10 h dark) with gentle shaking to allow re-suspension. Cultures were concentrated by centrifugation, re-suspended in ASTM, stored at 4°C and used within 5 days for the confirmation phase within the TIE protocol. Cell densities of *P. agardhii* were reported as biovolume estimates following established procedures in Sun and Liu (2003).

4.3.6 TIE design

The TIE experimental design was focused to test the hypothesis that observed toxicity of water samples was associated with the particulate fraction and to identify putative toxic compounds (Table 4.1). To do that, feeding responses of *D. magna* were determined for all samples in unfiltered and filtered water samples using post-exposure feeding and feeding assays, respectively. According to our hypothesis, filtered water samples should not impair feeding due to the removal of suspended matter. Further TIE phase I–III assessment included testing that suspended solids were toxic, assessing that metals did not contribute to toxicity by pre-treating samples with EDTA, studying which organic fraction was toxic and identifying toxic compounds by LC–MS/MS. Finally the toxicity of identified compounds was confirmed using lab exposures to those compounds (Table 4.1). Confirmation that suspended solids were toxic was performed assessing post-exposure feeding effects of individuals exposed to ASTM hard water fortified with suspended solids extracted by means of filtration of water samples through a Whatman nylon filter (0.45 µm pore size). Post- exposure feeding responses to co-exposures of unfiltered water samples with 200 µM EDTA were performed to discard metallic mediated effects on feeding of suspended material (Bosch et al., 2009). Feeding responses to non-polar and polar solvent extracts of suspended solids attached to glass fiber filters were performed to assess the polar nature of the toxic organic fraction. Accordingly, suspended solids present in 1 L water samples collected in June 22nd at RR and filtered in a Whatman glass fiber filter (0,.7 µm pore size) were extracted using dichloromethane : hexane (1:1, v/v), dichloromethane :

methanol (1:1, v/v), methanol 100%, methanol : water (80:20 v/v), methanol : water (50:50, v/v). Following fractionation the toxicity of solvent extracts relative to that of water only exposures was determine. Within each run appropriate solvent controls with extraction of clean filters and ASTM controls were used. Finally, confirmation that particle bound anatoxin-a could account for the observed toxicity of suspended solids was further assessed by measuring post-exposure feeding responses of *D. magna* juveniles. Individuals were pre-exposed for 24 h to six concentrations of *P. agardhii* cells (0.2–8 × 10−3 mm3/L) containing measured residue levels of anatoxin-a, ranging from 0.1 to 4 ηg/L.

Table 4.1 Phases I, II and III toxicity identification manipulations and toxicity assays. [a] post-exposure feeding responses of ASTM water containing 1 g/L of fine (< 20 μm) sediment S5,S6 particles; [b] post-exposure feeding responses of ASTM water fortified with suspended solids extracted from water samples; [c] co-exposure of unfiltered samples from RR, AV with 0.1 mM EDTA; [d] feeding responses to solvent extracts of suspended solids from sampling sites RR, FR, MF, Mora; [f] exposures to pure algae cultures grown in the lab

T.I.E. procedures	Assays, analyses
Phase I. Toxicant characterization tests	
Unfiltered water samples: all samples Water samples from Tablas de Daimiel Water elutriates of samples S5, S6 [a].	Post-exposure feeding
Filtered water samples: all samples	Feeding
Phase II. Toxicant identification analyses	
Re-constituted lab samples with suspended solids Samples from 13rd March and May 22nd [b]	Post-exposure feeding
Unfiltered water samples from May 22nd and EDTA[c]	Post-exposure feeding
Solvent extraction fractions of suspended solids: non polar to polar; samples from June 22nd [d]	Feeding
Analysis of organochlorine, phytotoxins and total mercury in suspended material	Correlation analysis with post-exposure feeding responses
Phase III - confirmation	
Planktothrix agardhii cells [f]	Post-exposure feeding

4.3.7 Toxicity assays

Post-exposure feeding assays followed previously validated methods (Bosch et al., 2009). Four-day-old juveniles of *D. magna* were first pre-exposed in groups of 25 individuals to 1 L of unfiltered, re-constituted, water:sediment elutriates or water spiked with different concentrations of *P. agardhii* cells for 24 h in a rotary wheel (3 rpm). In each run laboratory controls were also included,exposing individuals to 1 L of ASTM hard water. After exposure, feeding rates were measured in groups of 5 individuals in 50 mL of ASTM hard water with 5×10^5 *Chlorella vulgaris* cells/mL. Incubations lasted 4 h and were performed in quintuplicate. Appropriated blanks (vessels with algae and no animals) were also included to account for algae growth. Direct feeding tests with filtered water samples or solvent extracts were assessed in 24 h toxicity tests following Barata et al. (2008). Groups of 5 juveniles were exposed to 100 mL of test concentrations in 120 mL borosilicate flasks in the presence of food. *C. vulgaris* was added at a concentration of 5×10^5 cells/mL (equivalent to 1.5 µg C/mL). Treatments consisted of ASTM hard water control, solvent controls when required and the studied water samples represented in five replicates. Each group of replicates consisted of five vessels with animals and one blank. Blanks were used to assure that initial algal concentrations did not increase significantly over the exposure period. Both direct feeding and post-exposure feeding experiments were conducted in the dark in order to avoid algal growth. Individual feeding rates (number of algal cells ingested per animal per hour) were determined as the change in cell density in 4 or 24 h according to the method described by Allen et al. (1995) and converted to proportional feeding rates relative to lab controls. Cell density was estimated from absorbance measurements at λ = 650 nm using standard calibration curves based on at least 20 data points ($r^2 > 0.98$).

4.3.8 Data analysis

Within each sampling date or TIE treatment, feeding responses were compared with laboratory control treatments using ANOVA followed by Dunnett's test. Feeding inhibition responses relative to controls were then compared across all sampling sites and periods using ANOVA followed by Tukey's multiple comparison tests. Feeding and proportional responses were log transformed to meet ANOVA assumptions of normality and variance homocedasticity (Zar, 198496). Relationships

between environmental factors and post-exposure feeding inhibition were assessed using Pearson correlation. Residue levels of anatoxin-a and suspended solids were further related with feeding inhibition responses using the Hill regression model of eq. 1.

$$E(\%inhibition) = \frac{100}{1+(EC_{50}/x)^{p}} \quad \text{(eq.1)}$$

where E is the effect in % of feeding inhibition, p is the shape parameter; EC50 is the concentration of a test compound causing 50% feeding inhibition of individuals, x is the concentration of the test compound. Regression parameters were estimated by the Least Square Method using the Levenberg–Marquardt algorithm. The standard error (SE) or 95% confidence intervals (CI) of each estimated parameter was then calculated from the standard deviation of the least square estimates (Zar 1984). Model accuracy was assessed by using the adjusted coefficient of determination (r^2) and by analyzing the residual distribution. Analyses were performed with the SPSS Statistics 17 package (SPSS inc., Chicago Ill).

4.4 Results

4.4.1 Physico-chemical characterization

Physico-chemical parameters of the studied sediment samples denoted substantial differences across sampling periods and sites (Table 4.2). Conductivity and oxygen levels decrease from January to June and were inversely related with water temperature (Pearson correlations of −0.65 and −0.75; respectively, $P < 0.05$, $N = 30$). Suspended solids present in the water column varied across sampling periods and sites, decreasing during the periods of lower water flow in May 22nd and June 9th and increasing toward the river downstream. Observed high levels of suspended solids in June 9th in the Cinca river (site CI) are related to the different flow regimen in comparison to that of the Ebro River, which is regulated by dams. Particle size and composition showed that suspended solids were mainly composed of fine silt (75%; 4 μm < grain size < 62 μm) and clay (25%; grain size <4 μm), which mean particle size distributions (mean ± SE,N = 6) decreasing from RR (16.1 ± 1.6 μm) toward M (12.9 ± 1.5 μm). Organic carbon varied little across

measured water samples (mean ± SE, N = 10) 4.8 ± 0.1%. Negative control samples (samples from TDNP and S5 and S6 from Ebro) had similar grain distribution as suspended solids in collected water samples, being mainly composed of fine silt (70–80%; 4 μm < grain size < 62 μm) and clay (20–30%; grain size <4 μm). The organic content of C and N of the sediment samples from negative controls were also similar to those of suspended solids, being 3.0–3.5% for C and 0.29% for N.

Total mercury measured in suspended matter and expressed in a water volume basis (ηg/L) correlated with that of suspended solids ($P < 0.05$; 0.56,N = 30), whereas organochlorine levels did not. Nevertheless, for both chemical groups, residue levels increased from upstream to lower reaches in most samplings. In June 9th the sample from Cinca river had the highest levels of suspended solids and of total Hg and in June 22nd the levels of total Hg were the highest at MF, which is located just downstream to the chlor-alkaly factory.

From the four phytotoxins analyzed (anatoxin-a and microcystins LR, RR and YR) in methanol:water (80:20, v/v) filter extracts, only anatoxin-a residues were detected in different amounts in all samples (Table 4.2). No phytotoxins were detected in the negative control samples.

Visual inspection of the water samples under microscope evidenced the presence of fine clay material, aggregated organic matter and bacterial growth associated. The presence of algae was limited to few cells (mean ± SE, N = 12) 1.8 ± 0.1 cells/mL, from which cyanobacteria were only detected at concentrations <1 cell/mL in upstream locations of RR and FR. Identified taxa included Bacillariophyceae (*Melosira varians C., Fragilaria sp.*), Chlorophyceae (*Oedogonium sp., Ulothrix sp.*) and Cyanophyceae (*Aphanothece halophytica, Merismopedia sp.*). Images of the samples are presented in Fig. 4.2

Table 4.2 Measured physico-chemical water parameters of samples. Conductivity, oxygen levels, temperature, suspended solids, total mercury and organochlorine residue levels in water are reported as μS/cm, mg/L, ^{o}C, mg/L, ηg/L and ηg/L, respectively. Abbreviations are explained in the text. LOD, below detection limit; empty cells are missing values; TD, Tablas de Daimiel water sample. [a] Physicochemical values are those of ASTM hard water and laboratory control conditions; [b] THg values in ηg/L obtained from Bosch et al. (2009)

Date	Sites	pH	Cond	O2	T	SS	THg	Ana	DDTs	HCH	HCL	PCBs	OCL
Jan 28th	RR	7.9	1013	11.2	8.1	4.8	1.4	6.8	0.66	11.88	0.03	0.86	2.51
	FR	8.5	1002	10.6	8.1	20.2	3.8	3.5	1.06	4.36	0.04	0.48	5.98
	MU	8.7	994	11.2	8.8	26.8	5.5	3.1	0.84	4.27	0.07	0.3	5.48
	MD	8.1	1013	10.9	8	24.8	5.9	2.2	2.26	7.16	0.02	1.05	10.5
	AP	8.6	1014	9.8	8.6	29.8	11.7		9.17	4.68	0.01	2.33	16.18
	AV	8.5	1010	9.1	9.7	30.1	12.4	3.8	4.22	5.39	0.03	0.68	10.32
	M	8.4	1013	9.9	8.7	35.2	24.5	2.4	20.54	6.39	2.47	2.25	31.65
March 13 rd	RR	8.2	705	13	8.9	13.2	2.2	1.2	0.09	0.69	0.02	0.05	1.18
	FR	7.8	684	11.8	9.8	13.9	2.7		0.59	1.24	0.05	0.2	2.07
	MU	8.1	703	11.6	9.6	12.6	2.7	1.0	1.11	1.87	0.06	0.39	3.43
	MD	8.1	705	13.2	9.2	12.5	2.6		1.77	1.61	0.04	0.37	3.78
	AP	8.4	704	13.4	9.1	12.8	3.3		2.68	2.03	0.13	0.75	5.59
	AV	8.2	708	13.2	11.1	12.6	2.6	1.1	3.1	1.17	LOD	0.48	4.76
	M	8.2	707	13.2	9.9	12.5	4.0		2.78	12.07	0.12	0.63	15.61
May 22nd	RR	7.5	746	10.6	15.2	5.0	1.2	0.5					
	FR	7.5	738	9.6	15.5	6.3	1.5	1.9					
	MU	7.6	744	10.1	15	5.0	1.2	0.7					
	MD	7.6	743	9.5	15.3	5.7	1.6	0.7					
	AV	7.6	756	10.2	16.1	6.1	3.1	0.5					
	M	7.7	750	10.9	16.1	6.0	2.4	1.0					
June 9th	CI	6.8	727	6.2	18.7	22.7	6.9	0.2					
	RR	8.4	837	8.9	20	4.5	1.1	0.3					
	AV	8	857	6.0	17.2	5.8	2.6	0.3					
June 22nd	RR	7.4	603	8.0	18.3	30.7	3.0	0.9					
	FR	7.2	591	7.9	18.5	37.6	3.6	1.8					
	MF	7.4	610	7.6	18.6	35.6	18.1	1.0					
	MU	7.4	494	7.7	18.5	39.6	3.5	1.4					
	MD	7.4	585	8.0	18.5	36.1	3.9	0.7					
	AV	7.4	590	8.3	20.2	34.6	4.3	0.9					
	M	7.5	583	8.4	19.2	36.7	5.1	0.5					
Negative	S5	8.1	540	9.7	20	1000	2700	LOD	9150.6	2.5		5701.1	14854.2
Controls [a]	S6	8.1	540	9.7	20	1000	1300	LOD	826.1	3.3		297.7	1127.1
	TD	8.1	540	9.7	20	15	LOD	LOD					

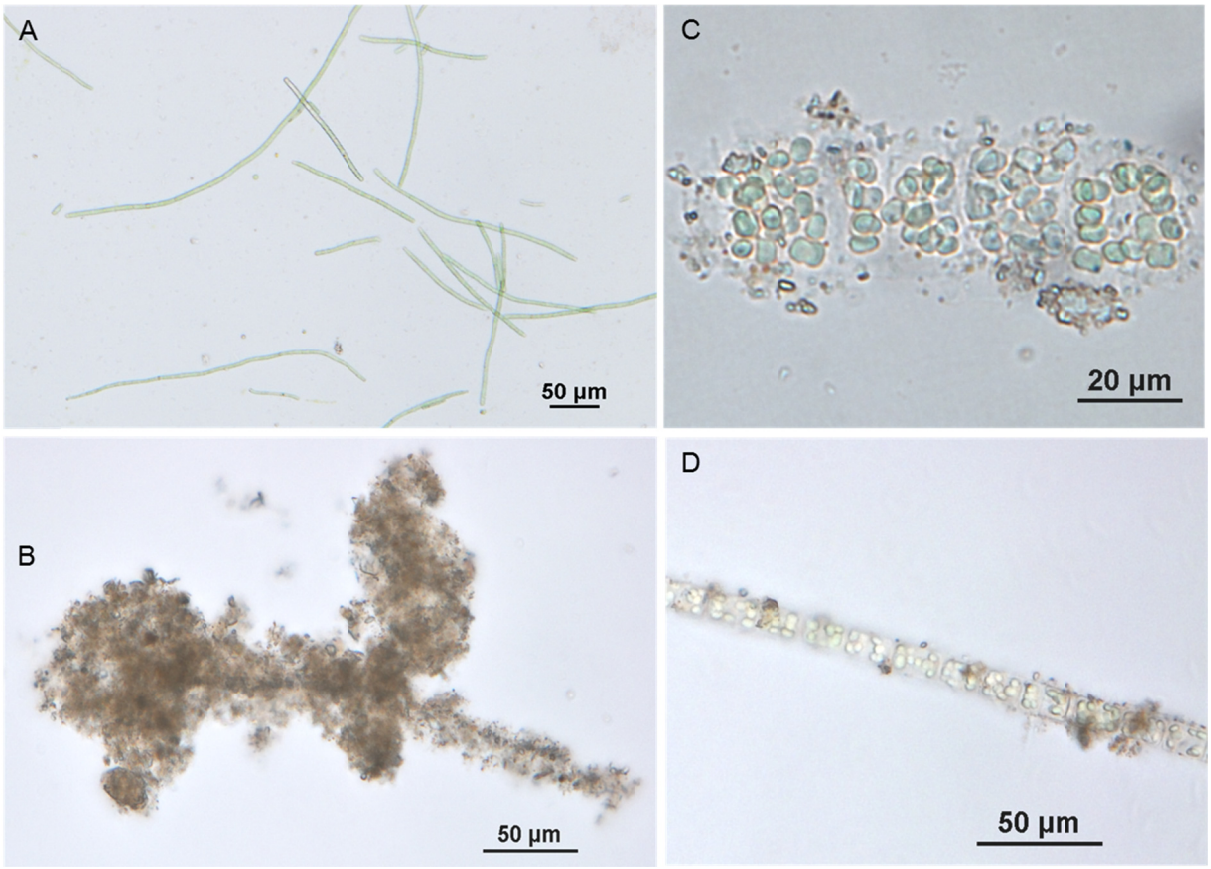

Figure 4.2 Images of phytoplanktonic species detected in water samples together with that of *Planktothrix agardhii* lab cultures (A). (B) Organic matter; (C) Cyanobacteria (*Merismopedia sp.*); (D) Chlorophyceae (*Ulothrix sp.*)

4.4.2 Toxicological responses

Across all tests, feeding rates of lab controls varied little being within (mean ± SE, N = 5) 5.21 ± 0.28 and 5.44 ± 0.16 × 10^5 cells/individual/h. For the sake of clarity, feeding rates are depicted as inhibition responses with respect to lab controls. Except for samplings of June 9th, post-exposure feeding rates of *D. magna* juveniles exposed to unfiltered water samples collected along the studied sites were significantly lower ($P < 0.05$) than those of lab controls (Table 4.3). Conversely, feeding rates of individuals exposed to filtered water samples were not different ($P < 0.05$, based on Dunnett's tests) than those of lab controls (Table 4.3).

The amount of re-suspended solids in 1 L water samples recovered from nylon filters were (mean ± SE, N = 6–7) 42.5 ± 2.7% and 50.7 ± 2.1% those measured directly in field collected water samples. Post-exposure feeding responses in

ASTM hard water fortified with suspended solids recovered from nylon filters were about 50% of those observed in whole water samples and in all cases were significantly ($P < 0.05$) inhibited relative to controls (Table 4.3).

Feeding responses of negative controls included two treatments of ASTM hard water fortified with 1 g/L of fine sediment samples from the same study area collected years before (S5, S6: (Bosch et al., 2009) and a water sample collected in the lagoons of Las Tablas de Daimiel National Park. In all three cases feeding responses were not inhibited (Table 4.3).

A further TIE assessment was performed using EDTA and different solvent extractions to assess the relative contribution of metallic, non-polar and polar contaminants in the measured toxicity of suspended solids. Results, as shown in Fig. 4.3, indicated no changes in feeding inhibition between intact water samples and those fortified with 200 µM of EDTA.

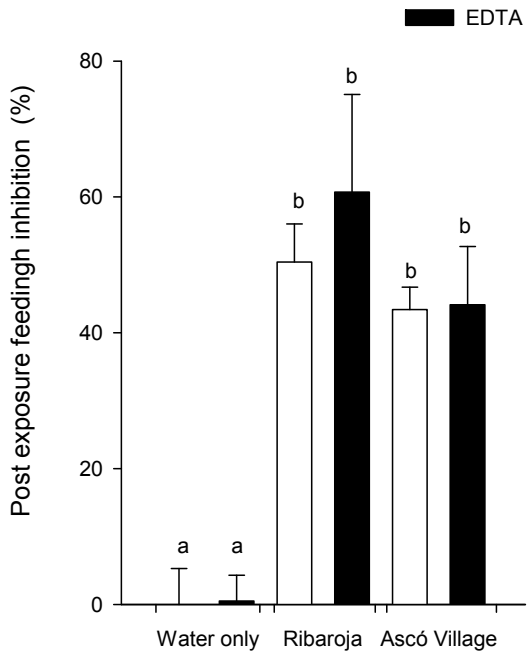

Figure 4.3 Post-exposure feeding inhibition responses (mean ± SE, N = 5) of *D. magna* individuals exposed to selected water samples from May 22nd with and without 200 µM of EDTA. Significant ($P < 0.05$) differences across samples following ANOVA and Tukey's test are indicated by different letters.

Table 4.3 Feeding inhibition (%) (Mean, SE, N = 5) relative to lab control treatments of unfiltered, filtered and re-constituted water samples across the studied periods and sites. * indicates significantly (P < 0.05) different feeding rates following ANOVA and Dunnett's tests. PE, post-exposure feeding.

		Unfiltered PE feeding			**Filtered Feeding**			**Reconstituted PE feeding**		
Periods	**Sites**	**Mean**	**SE**		**Mean**	**SE**		**Mean**	**SE**	
Jan 28th	RR	55.3	3.1	*	-8.3	0.8				
	FR	78.4	5.1	*	-9.1	1.2				
	MU	68.8	2.4	*	-15.4	0.4				
	MD	67.3	5.4	*	-11.2	1.3				
	AP	64.6	6.1	*	-6.2	0.5				
	AV	61.5	4.4	*	-6.8	3.0				
	M	50.3	5.5	*	-6.6	1.4				
March 13 rd	RR	55.0	3.6	*	2.1	1.0		20.4	4.1	*
	FR	57.1	1.7	*	1.8	1.6		31.4	3.7	*
	MU	64.7	0.9	*	-5.5	1.3		42.3	2.9	*
	MD	68.9	2.7	*	-2.1	.6		32.8	3.2	*
	AP	50.1	2.6	*	0.9	.5		24.8	1.5	*
	AV	58.1	3.7	*	2.5	.6		31.4	3.9	*
	M	60.9	3.9	*	1.8	2.0		38.7	1.4	*
May 22 nd	RR	54.8	3.1	*	-5.8	.6		33.8	3.7	*
	FR	48.5	4.2	*	0.6	.9		28.8	1.3	*
	MU	56.4	4.2	*	-9.1	.8		20.8	2.5	*
	MD	54.5	3.8	*	-12.0	1.2		29.2	4.1	*
	AV	47.9	3.0	*	2.3	2.1		21.1	1.0	*
	M	51.8	3.1	*	-10.0	1.4		20.4	1.8	*
June 9 th	CI	<0.1	4.2		1.7	4.2				
	RR	9.6	2.9		4.0	4.0				
	AV	10.5	2.6		-1.1	2.7				
June 22 nd	RR	78.4	3.4	*	-2.7	0.4		43.1	1.5	*
	FR	72.7	3.0	*	-11.0	0.2		39.1	1.0	*
	MF	81.5	0.4	*	-9.9	0.2		45.6	1.1	*
	MU	67.0	1.1	*	-8.6	0.4		33.9	1.2	*
	MD	75.3	2.1	*	-9.7	0.4		42.3	0.4	*
	AV	71.1	3.1	*	-12.6	0.9		39.2	0.9	*
	M	71.1	1.3	*	-10.7	0.4		41.5	0.6	*
Negative controls	S5	-2.7	5.7							
	S6	-3.6	5.3							
	Tablas Daimiel	4.9	2.3							

Results depicted in Fig. 4.4 A indicated that the extraction of filters with methanol : water (80:20, v/v) gave the highest toxicity. Toxic recoveries in these extracts of suspended solids filtered in Whatman fiber glass filters varied between 31.8 and 53.6% of those measured in intact water samples (Fig. 4.4 B).

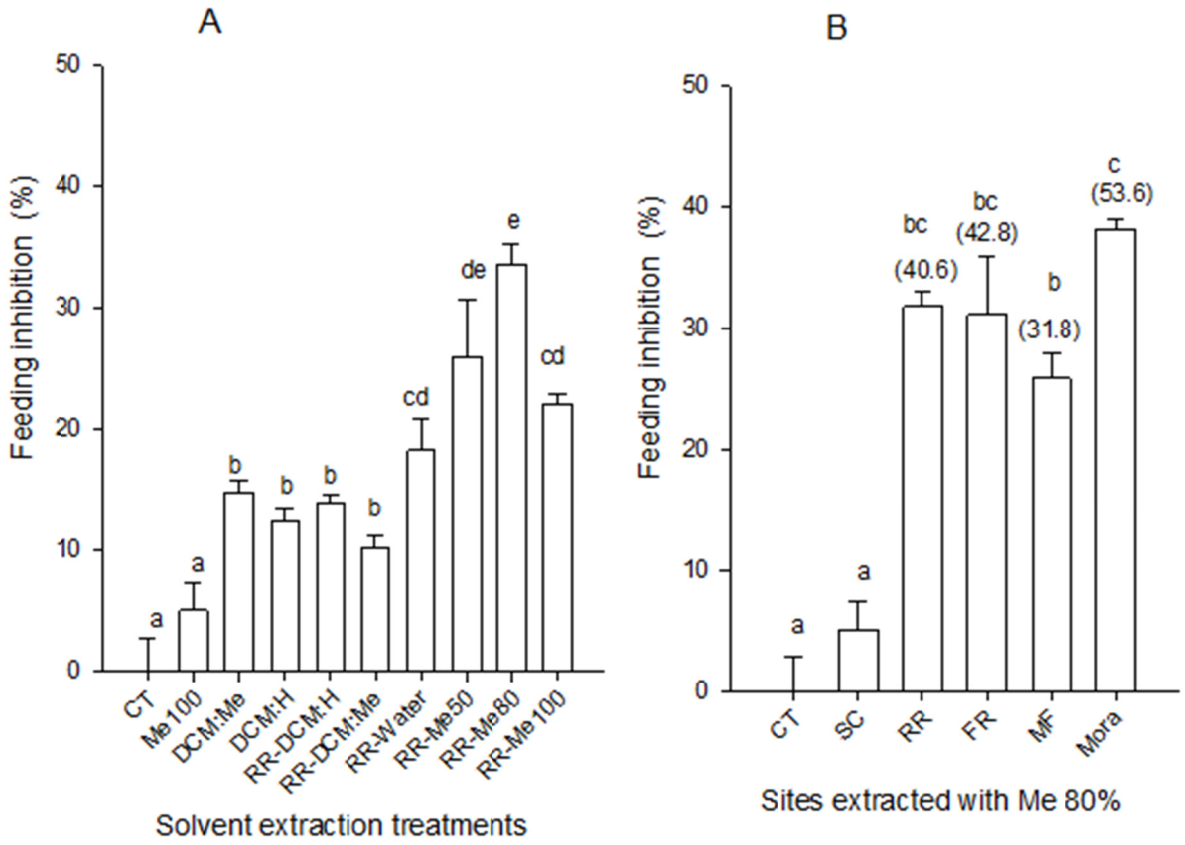

Figure 4.4 Feeding inhibition responses (mean ± SE, N = 5) of *D. magna* individuals exposed to solvent extracts of glass fiber filters preloaded with 1 L of suspended material from water samples collected in June 22nd. Responses to different solvent extracts of samples coming from Riba-Roja and those of methanol : water (80:20, v/v) extracts from different locations are depicted in graph A and B, respectively. In graph B values above bars indicate % feeding recovery from intact samples. Significant ($P < 0.05$) differences across treatments/samples following ANOVA and Tukey's test are indicated by different letters. Treatments in graph A are non solvent controls (CT), solvent controls of 100% methanol (Me100), solvent controls dichloromethane : methanol 1:1, v/v (DCM:Me); those preceeded by RR are referred to filter solvent extracts from Riba-Roja; methanol: water combinations are indicated as 50, 80 and 100%. In graph B, SC refers to methanol 80% solvent control.

Correlation analyses between physico-chemical parameters and feeding inhibition responses only gave significant ($P < 0.05$) relationships between feeding and levels of suspended solids ($\delta = 0.49$, $N = 30$) and of anatoxin-a residues ($\delta = 0.65$, $N = 29$). Regression fits depicted in Fig. 4.5 indicated that feeding inhibition versus anatoxin-a levels could be accurately predicted by the Hill models ($P < 0.05$, r2 = 0.66, $N = 29$, Fig. 4.5 A) having an EC50 ± SE of 0.64 ± 0.11 ηg/L of anatoxin-a. Suspended solids versus feeding inhibition responses had a quite poor fit to the Hill model ($P < 0.05$, $r^2 = 0.23$, $N = 30$, Fig. 4.5 B). Confirmation TIE phase III assays using post-exposure feeding inhibition responses of *D. magna* individuals exposed to *P. agardhii* ranging from 0.24 × 10−3 to 8 × 10−3 mm^3/L evidenced a strong feeding inhibition. Chemical analyses of cyanotoxins present in the tested concentrations of *P. agardhii* cells only detected residue levels of anatoxin-a. Feeding inhibition responses plotted against measured residue levels of anatoxin-a produced by *P. agardhii* showed a good fit with the Hill equation ($P < 0.05$, $r^2 = 0.893$, $N = 23$) having an EC50 ± SE of 0.14 ± 0.01 ηg/L of anatoxin-a (Fig. 4.5 C).

4.5 Discussion

During winter and spring 2013, an excess of rain combined with high amounts of accumulated snow and consequent melting waters from the Pyrenees, made it necessary to open the dams of Riba-Roja and Flix reservoirs. The opening of the Riba-Roja reservoir dam dramatically increased the water flow of Ebro River downstream (Fig. 4.1 C) and consequently the amount of re-suspended particle matter as well as mercury and organochlorine residues associated with it (Table 4.2). The greatest levels of suspended solids measured in the study coincided with those periods of maximal water flow, respectively, January 8th and June 22nd. However, residue levels of total Hg associated to particle matter were greater in January than in June, probably due to the prolonged wash of the riverbed and banks (Quesada et al., 2014). In all samplings, contaminant residue levels increased from up- to downstream locations and were especially high in the water samples collected just downstream of the contaminated wastes of Flix at MF in June 22nd. These results indicated that there was a continuous mobilization and enrichment of contaminants associated with sediment particles coming from Flix industrial wastes toward the downstream

location of Mora (Quesada et al., 2014). The contribution of known historical contaminant outputs coming from industrial activities located upstream in Cinca River (Fig. 4.1 A), may explain the measured high levels of organochlorine and mercury residues at RR and CI (de la Cal et al., 2008; Eljarrat et al., 2008; Lavado et al., 2006; Raldúa et al., 1997).

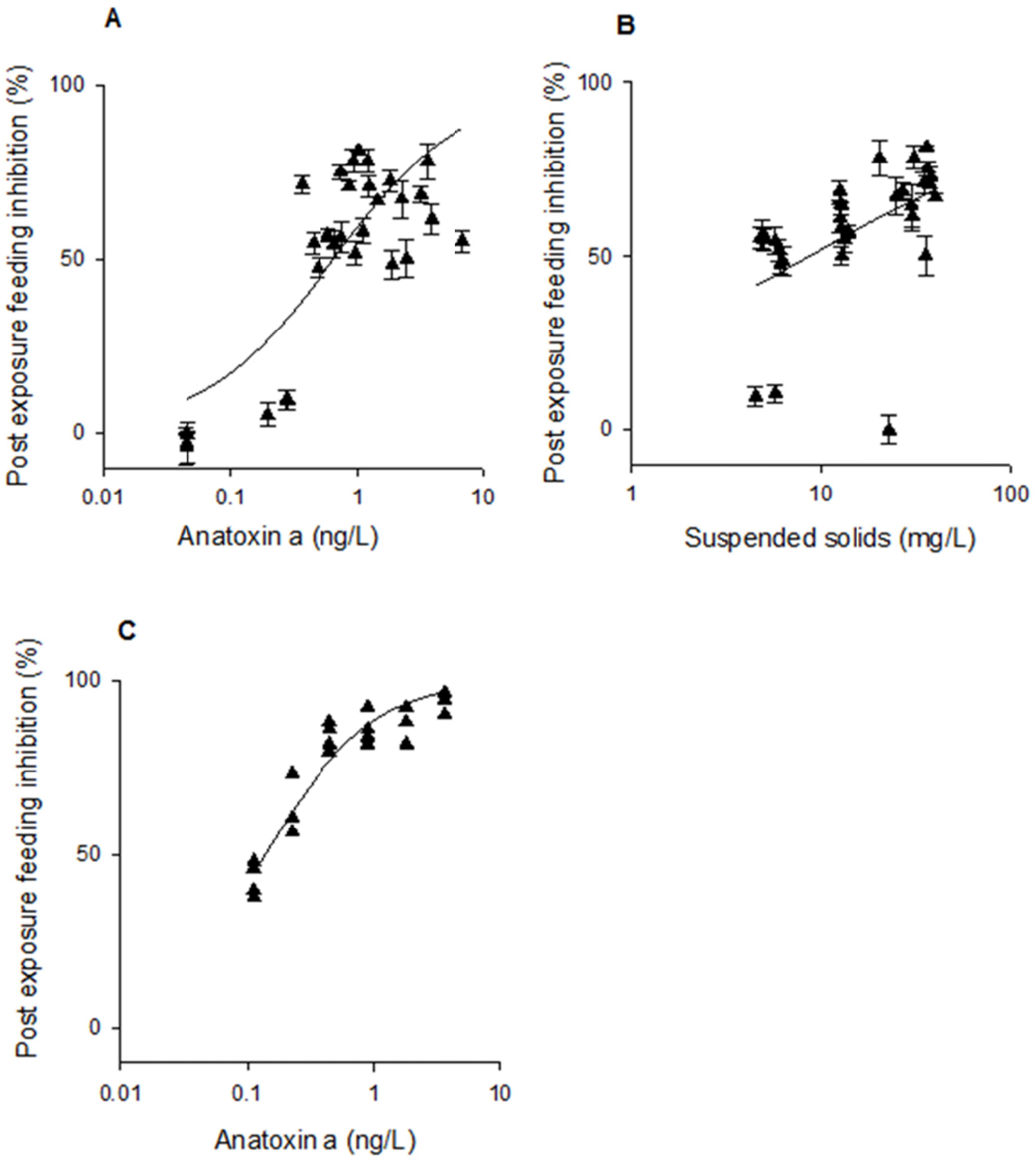

Figure 4.5 Post-exposure feeding inhibition vs levels of anatoxin-a (A), suspended solids (B) or anatoxin a levels of *Planktothrix agardhii* (C) relationships. Lines are regression fits to the Hill model. Errors bars in graphs A and B are SE of feeding values (N = 5).

Thus the results reported in this study for metallic and organochlorine residue levels associated to suspended solids agree with and support previous findings showing that industrial wastes of Flix are being mobilized toward downstream locations during flushing floods (Quesada et al., 2014). Results obtained in toxicity assays conducted in unfiltered and filtered samples and in re-constituted ASTM water with suspended solids indicated that toxic effects were associated exclusively to suspended matter. There were strong inhibitory effects of *D. magna* feeding rates across most water samples with little variation between upstream and downstream sites, the exception being those water samples collected in June 9th that showed no toxicity. Unexpectedly toxic effects were unrelated to measured contaminants (i.e., organochlorine and total mercury). Two independent pieces of evidence corroborated the previous results: (1) treatment of water samples with EDTA did not diminished toxicity, which indicates that neither measured mercury nor other metals contributed to the observed toxicity; (2) water elutriates obtained spiking 1 g of sediments from sediments S5 and S6 into 1 L of ASTM were not toxic to *D. magna* juveniles, despite containing higher levels of mercury and organochlorine residues than the water samples collected across the studied sites and periods. In a previous study it was shown that treatment with EDTA was able to diminish most of the observed effects of metals bound to sediment wastes from Flix (Bosch et al., 2009). In the same study elutriates of sediments S5 and S6 were also non-toxic to *D. magna*. Thus, our results indicated that despite of being re-suspended, the sediment wastes of Flix had a negligible contribution to observed toxicity of suspended solids.

Of particular interest were water samples collected at RR, which despite of being upstream of the contaminated wastes of Flix, were as toxic as those collected downstream. There is no industry and little agricultural activity around RR, and this location has been considered as a reference site in several previous works (Benejam et al., 2010; Bosch et al., 2009; Carrasco et al., 2008; Faria et al., 2010; Navarro et al., 2009). This means that measured toxic effects on suspended matter are likely to be related to wastes coming from the Riba-Roja reservoir. Indeed the ‘flushing flood' method used in the lower Ebro River region consists in partially emptying the reservoir in order to erode the stored sediment and evacuate them through the bottom outlets by using the water column pressure (Rovira and Ibàñez, 2007). Reported evidences of sources of toxicity in the Ebro's reservoirs such as Riba-Roja are limited to organochlorine

and mercury wastes coming from industrial activities located upstream (de la Cal et al., 2008; Eljarrat et al., 2008; Faria et al., 2010; Lavado et al., 2006; Navarro et al., 2009; Raldúa et al., 1997) and toxic cyanobacteria (de Hoyos et al., 2004; Quesada et al., 2004). The most common toxic cyanobacteria genus found in Ebro's reservoirs are *Anabaena* and *Planktothrix*, which can produce microcystins and anatoxins (de Hoyos et al., 2004; Quesada et al., 2004). In our study, the visual inspection of the suspended solids present in water samples under microscope detected only very few colonies of Cyanobacteria, e.g. *Merismopedia* sp. and *Aphanotece* sp. in upstream locations, with no reported evidence of species producing anatoxins. The previous findings, however, did not discard the occurrence and presence of toxic cyanobacteria species around the area. Indeed in October 2014 we have detected *Planktothrix sp*. communities growing in the periphyton at MU (unpublished data). The analysis of the toxic fraction of suspended matter extracts, however, evidenced the presence of residues of anatoxin-a that correlated quite well with observed feeding inhibition responses. It should be pointed out that despite our samplings were not carried out at the typical period for cyanobacterial blooming (from August to October), there is reported evidence that toxic cyanobacteria occur all year long in Ebro's reservoirs (Quesada et al., 2004). There is also described evidence that toxic cyanobacteria of the genus *Anabaena* and *Planktothrix* can live in the bottom sediment of lakes even under heterotrophic conditions and survive through winter (Li et al., 2011; Mannan and Pakrasi, 1993; Savichtcheva et al., 2011; Toporowska et al., 2014). Microcystins and especially anatoxin-a are known to adsorb to fine clay material, accumulating in the sediment, in which they can persist for long periods depending on the existing microflora (Chen et al., 2008; Chen et al., 2013; Kaminski et al., 2013; Klitzke et al., 2011; Rapala et al., 1994). The detection of anatoxin-a and not of any microcystins in the analyzed suspended matter samples rich in clay particles support the previous studies.

Confirmation TIE III assays using lab exposures against naturally produced anatoxin-a by cyanobacteria of species *P. agardhii* confirmed the results obtained in field samples. Post-exposure feeding inhibition assays performed with cells of *P. agardhii* re-suspended in ASTM showed significant detrimental effects at ηg/L of anatoxin-a. Feeding response were inhibited at lower amounts of anatoxin-a when *D. magna* were exposed to pure cultures of *P. agardhii* compared to field collected water samples. This is an expected result provided

that, in the lab, individuals were exposed to pure cultures of cyanobacteria that are known to produce many different bioactive molecules that may act synergistically (Ibelings and Havens, 2008). It is also important to consider that the highest concentrations of P. agardhii tested in the lab ($8 \times 10{-}3$ mm^3/L) were within the lowest range reported for natural populations of *Planktothrix* occurring in oligotrophic lakes (Ostermaier et al., 2012). Thus, our results showed that low or almost undetectable concentrations of *P. agardhii* cells were highly toxic to *D. magna*.

4.6 Conclusions

In summary, results reported in this study provided evidence that post-exposure feeding responses of *D. magna* can be used to identify toxic components present in suspended material using TIE approaches. Results indicated that non-targeted pollutants present in suspended matter, such as cyanotoxins, affected feeding rates. This is the first evidence showing that the release and re-suspension of bottom sediments from reservoirs during flushing floods may be detrimental to grazers living downstream the river due to the presence of cyanobacteria toxins.

4.7 Acknowledgements

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Chapter V.

Integrated environmental risk assessment of chemical pollution in a Mediterranean floodplain by combining chemical and biological methods

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Chapter V.

Integrated environmental risk assessment of chemical pollution in a Mediterranean floodplain by combining chemical and biological methods[a]

5.1 Abstract

The Tablas de Daimiel National Park (TDNP) is a unique floodplain ecosystem in central Spain, serving as permanent resting and breeding areas for many waterbird species. In the last decades, this biodiversity hotspot has been severely endangered by poorly treated wastewater discharges from upstream urban communities arriving through its two major contributors, the Cigüela and Guadiana rivers. In this work, we analyzed the potential risk of this constant input of micropollutants (estrogens, dioxin-like compounds and other endocrine disruptors) for the resident wildlife. We sampled 12 locations in TDNP and in the nearby Navaseca Pond during 2013, and performed a series of *in vivo* and *in vitro* bioassays, including *Daphnia magna* post-exposure feeding inhibition and recombinant yeast-based assays for dioxin-like and estrogenic activities. These results were then compared with the chemical composition of the samples, analyzed by GC-MS/MS and LC-MS/MS, and evaluated according to their toxic potential as toxic equivalents or TEQ. The Navaseca Pond, heavily impacted by wastewater from the town of Daimiel, showed the highest levels of toxic compounds, estrogenic activity, and *Daphnia* toxicity. Conversely, the less impacted TDNP sites showed low residue levels of contaminants, low estrogenicity and dioxin-like activity and negligible toxicity. The results indicates that the current good chemical status of TDNP is menaced by both the inflow of wastewater treatment plants effluents from Guadiana and Cigüela rivers into TDNP tributaries and, as it occurs in the Navaseca Pond, by direct sewage discharges.

Keywords: contaminant, endocrine disruption, freshwater, UNESCO Biosphere Reserve, Ramsar site.

5.2 Introduction

River floodplains provide human society with many important ecosystem services (Postel and Carpenter, 1997), but their ecological status is determined by several complex parameters, including fluvial dynamics and groundwater and man-made processes (Alvarez-Cobelas et al., 2001). In recent times, intensive agriculture combined with groundwater extraction for irrigation, and overall decreases in surface and groundwater quality have appeared as major threats for river floodplains (Tockner and Stanford, 2002). As a consequence, most floodplains are functionally extinct nowadays in North America and Europe, including the Mediterranean (Brinson and Malvárez, 2002; Tockner and Stanford, 2002). In Spain, floodplains present the worst conservation situation among all wetlands, as more than 79% of the surface they possessed in the 19th century has been lost by draining for cultivation (Casado et al., 1992). A worse-case scenario of a severely impacted Mediterranean floodplain are Tablas de Daimiel National Park (TDNP), which is located at the center of Spain, and it is considered a Biosphere reserve by UNESCO, a Ramsar site and refuge for migratory birds and aquatic plants. Its many valuable features come from the structure of submerged vegetation communities, dominated by a mosaic of *Cladium mariscus* sawgrass-emergent stands and open water habitats ("tablas") (Cirujano et al., 1996), and of extensive stonewort (Charophyceae) communities. The latter plant community is extremely important for the survival of many migratory and resident waterbirds (Cirujano et al., 1996). The waterbird community in TDNP has been famous since at least the Middle Ages, being cited in the ancient hunting literature sources (Coronado et al., 1974). Almost 200 bird species have been recorded in this wetland and its surrounding terrestrial habitats; this figure includes practically every inland waterbird species known in southern Europe. Registers of ichthyofaunal diversity in TDNP from the 16th Century onwards reflected a richness of species that remained so at the turn of the 20th Century. However, recent invasive events of freshwater fish, such as the common carp (*Cyprinus carpio*), are negatively affecting aquatic vegetation, both directly by consuming macrophytes, and indirectly by changing water quality (Laguna et al., 2016).

The wetlands of TDNP are the result of the mixture of inputs from Cigüela and Guadiana rivers, together with groundwater discharge from the West Mancha aquifer (Alvarez-Cobelas et al., 2001; Berzas et al., 2000). The reduction of the drainage area and an overexploitation of groundwater for irrigation purposes lead to the near desiccation of TDNP and, finally, to the ignition of a smoldering peat fire inside the TDNP in August 2009. This fire posed an enormous risk for both the physical structure supporting the ecosystem and the quality of groundwater beneath it (Moreno et al., 2011), especially considering that fires are an important source of pollution by polycyclic aromatic hydrocarbons (PAHs) for Mediterranean rivers (Vila-Escale et al., 2007). A TDNP Hydric Regeneration Plan was implemented to stop or at least mitigate this environmental degradation. The plan includes the artificial recharge of the wetlands by pumping groundwater from nearby wells and, occasionally, by diverting water from the Tajo River to the Cigüela River (Berzas et al., 2000).

TDNP also suffers from water pollution associated with human population growth and the subsequent agriculture and industry developments (Berzas et al., 2000; Sanchez-Ramos et al., 2016). Pollution sources include dispersed-source pollution from surrounding agriculture and industrial activities, and point-source pollution from wastewater treatment plants (WWTP) discharging treated or untreated effluents into the Cigüela and Guadiana rivers (Sanchez-Ramos et al., 2016). One of such contaminated sites is the Navaseca Pond, which is located at the Guadiana River basin and that receives wastewater effluents from the town of Daimiel, both treated and untreated, particularly during heavy rain episodes. Besides the town of Daimiel, Villarrubia de los Ojos town discharges wastewater effluents to Cigüela River from its wastewater treatment plant. Another nearby town, Fuente el Fresno, has been discharging untreated effluents into Cañada Lobosa stream until 2013 when its wastewater treatment plant started to operate. This means that there is potential risk of contaminants to cause detrimental effects on living biota in the TDNP. In recent years, the populations of herbivorous waterfowl have shown a marked decrease, potentially linked to the deterioration of submerged macrophyte stands by introduced fish species, such as the common carp *Cyprinus carpio* (Laguna et al., 2016). There is, however, the possibility that some noxious contaminants

are affecting birds directly or indirectly being toxic to aquatic invertebrates and plants, which are food sources for aquatic birds. The aim of the present study is to characterize noxious organic contaminants in TDNP and their main water sources, by sampling 14 locations in the wetland of TDNP and its nearby Navaseca Pond during 2013. We determined residue levels of up to 31 different organic compounds including estrogens, antimicrobials, preservatives, plasticizers, alkylphenols, anticorrosives and flame retardants, that are often found in treated wastewater effluents, some of them with known estrogenic activity to vertebrates (Gorga et al., 2013). Additionally, PAHs present in suspended solids of water samples were determined, since some of them are known to have dioxin-like activity (Misaki et al., 2007). Chemical determinations were complemented with measurements of total estrogenicity and dioxin-like activity using in vitro recombinant yeast assays (RYAs) (Bosch et al., 2009; Céspedes et al., 2004; Noguerol et al., 2006a) and of general toxicity to aquatic invertebrates using post-exposure *Daphnia magna* feeding toxicity tests (Bosch et al., 2009; Rivetti et al., 2015b). Combining chemical and toxicity assays will allow identifying chemicals causing toxic effects, which may help the implementation of future remediation strategies in the TDNP.

5.3 Material and methods

5.3.1 Study area

TDNP is situated in central Spain at the SW corner of the Mancha Húmeda Biosphere Reserve (MAB program, UNESCO), within the province of Ciudad Real (39º 08' 17" N, 3º 41' 50" S, Fig. 5.1 A, B). The Park covers an area of 3030 ha, of which almost 2000 ha consists of a fluctuating Mediterranean floodplain, which is fed by water from the Guadiana and Cigüela rivers and the underlying aquifer (Fig. 5.1C). This region has a semiarid continental Mediterranean climate, with an extremely irregular rainfall regime, the average annual rainfall being between 400 and 500 mm, and having an average temperature of 14°C (Alvarez-Cobelas et al., 2001; Cirujano et al., 1996).

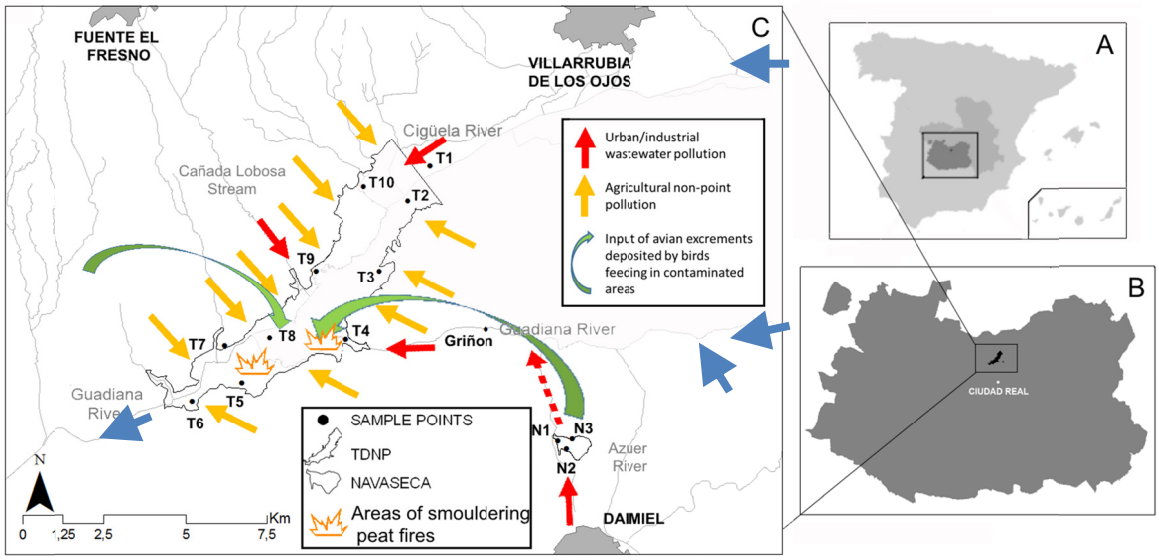

Figure 5.1 Putative pollutant inputs for Tablas de Daimiel (TDNP). Geographical location of TDNP within Spain (A) and Castilla La Mancha region (B). Detailed map of TDNP and its surroundings, including Navaseca Pond and the location of the experimental stations (C). Yellow and red arrows indicate potential contaminant inputs from diffuse agricultural sources and from urban/industrial discharges, respectively. Green arrows indicate potential inputs of pollutants linked to faecal depositions of colonial waterbirds feeding at high trophic levels in polluted surrounding areas. Flow direction of water courses are indicated by blue arrows.

5.3.2 Experimental design and water sampling

Organic contaminants from both diffuse (e.g. historical smoldering peat fires) and point (WWTP) pollution sources were analysed in ten experimental stations sampled after the main rain season in May 2013 throughout TDNP, and representing different hydrogeological areas (Fig. 5.1 C). In TDNP, the stations T1, T2, T3, T9 and T10 are mostly influenced by the Cigüela River, as they are located upstream the join with Guadiana River, whereas stations T4, T5, T6, T7 and T8 are downstream that join and have the influence of Guadiana River too (Fig. 5.1 C). Stations T4-8 may also be affected by diffuse pollution of PAHs coming from historical smoldering peat fires (Moreno et al., 2011). To study seasonal variation, the ten stations were also sampled during the main dry season in July 2013. Unfortunately, station T8 was dry and samples from station T3 were accidentally lost. Furthermore, July sampling included an

additional station from the Guadiana River (Griñon station), and three stations at the Navaseca Pond, one at the discharging point of the Daimiel WWTP (N1), and two more sites at both extremes of the pond (N2, N3, Fig. 5.1 C). Fig. 5.1 C depicts different sources of diffuse pollution influencing the TDNP, coming from agricultural activities, from Villarubia de los Ojos WWTP discharges through the Cigüela River, from Fuente el Fresno through Cañada Lobosa stream, and from contaminated bird faeces (Sanchez-Ramos et al., 2016). Approximately 4 L of water were collected per site, stored in 1 L amber glass bottles, and transported to the laboratory under cooled conditions (4ºC).

5.3.3 Sample processing and physico-chemical analyses

Upon reception, samples were filtered through glass fiber Whatman GF/C filters (GE Healthcare Ltd.) previously washed with acetone, pre-combusted at 400ºC and pre-weighted. Following filtering, filter residues were freeze dried, weighed to assess total suspended particles (mg/L) and then stored at -20ºC until their use for chemical and dioxin-like activity determinations. Additional water physico-chemical parameters, including pH, conductivity (mS/cm), dissolved oxygen (O_2, mg/L), were measured using a WTW Multi 340i handheld meter.

Estrogenic compounds and other chemicals commonly found in WWTP effluents were analyzed in filtered water samples using a fully automated method, based on column switching using EQuanTM columns for an integrated sample pre-concentration and liquid chromatography coupled to tandem mass spectrometry (LC–LC–MS/MS) (Gorga et al., 2013). Up to 31 different chemicals were selected, including both recognized and suspected environmental endocrine disruptors (EDCs), such as natural and synthetic estrogens and their conjugates, antimicrobials, parabens, bisphenol A, alkylphenolic compounds, benzotriazoles, and organophosphorus flame retardants. Analytical conditions were fully described in the previous study (Gorga et al., 2013). In brief, the Thermo Scientific EQuanTM system for online sample pre-concentration and analysis consisted of a triple quadrupole (QqQ) MS with an electrospray ionization source (ESI), two LC quaternary pumps (Finnigan Surveyor L-Pump) and two LC columns, one for pre-concentration of the sample and the second for the analytical separation. The injection volume

was set at 5 mL. Spiked samples with a solution of surrogate standards were directly injected into the chromatographic system and the target compounds were pre-concentrated into the loading column by a stream of mobile phase (aqueous : organic solvent (98:2, v/v)). Thereafter, analytes were transferred from pre-concentration column to the analytical column using the same mobile phases than previous step through both columns. Chromatographic separation of compounds detected under negative ionization (NI) conditions was performed under gradient elution condition using water (A) and methanol (B). The initial condition was 50% B, then the gradient was linearly increased to 70% B in 2 min, increased to 100% B in 6 min and kept isocratic for 6 min. Compounds detected under positive ionization (PI) conditions were separated using the same gradient program using solvent system containing water–methanol both phases with 20 mM of ammonium formiate and 0.1% of acetic acid. Detection was carried out using a mass spectrometer TSQ Vantatge, equipped with an ESI turbo spray interface. The operating parameters were as follows for NI and PI, respectively: spray voltage 2500/3000 V, sheath gas pressure 40/40 (N2), auxiliary gas pressure 20/20 (N_2), ion sweep gas pressure 0.5/0.5 (N_2) and transfer tube temperature 270/300ºC. The precursor and product ions of individual target compounds were obtained by tuning after direct injection of 1 ppm. The optimized MS/MS parameters for SRM analysis of the analytes are given in (Gorga et al., 2013). Natural and synthetic estrogens and conjugates, antimicrobials/ disinfectants, preservatives, BPA, and the alkylphenolic compounds (OP, NP, OP1EC and NP1EC) were detected under NI conditions as $[M-H]^-$. Diagnostic ions used for the analysis of anticorrosives, organophosphorus flame retardants compounds and the chemical biomarker caffeine, in PI mode were those corresponding to $[M+H]^+$, while for the alkylphenolic compounds (OP1EO, OP2EO, NP1EO and NP2EO) the adduct precursor ion $[M+NH_3]^+$ was analysed. Identification and confirmation of the analytes were based on retention time for all monitored transitions (± 1 s) and a ratio between the two monitored transitions within 15% of the theoretical value (calculated upon standards). Internal standard quantification was performed using deuterated compounds. The instrumental parameters showed good linearity in the concentration range studied (2.5–3000 ηg/L) for all compounds (r^2>0.99). Intra-day variation was below 8.0%. Solvent and matrix blank

samples were also analyzed and no carry-over effect was detected throughout the analyses. Mean recoveries (±SD) in water for the detected compounds were 77(±15)%. Limits of detection calculated at a signal-to-noise ratio of 3 are depicted in Table 5.1, and ranged between 0.01 to 0.27 ηg/L.

Levels of PAHs dissolved in water and adsorbed to suspended particles were obtained following previous procedures (Martinez et al., 2004; Rivetti et al., 2015a). For extracting dissolved PAHs, 10% (v/v) of methanol was added to 1 L of filtered water and the solution was mixed. The internal/surrogate standards composed of five deuterated PAHs were added at this stage at a concentration of 0.5 µg/L. Filtered water sample pre-concentration (500 mL) was performed by SPE using HLB (6 mL, 200 mg) cartridges and a Baker vacuum system (J.T. Baker, The Netherlands). The SPE cartridges were subsequently conditioned (at a flow rate of 1 mL/min) with 10 mL of hexane, followed by 10 mL of dichloromethane, 10 mL of methanol and 15 mL of water. Cartridges were eluted, at the same flow rate, with 10 mL of dichloromethane: hexane (1:1, v/v), then eluents evaporated with a gentle stream of nitrogen, reconstituted to a final volume of 100 µL with hexane. PAHs from suspended solids retained in freeze dried filter were extracted as follows. Filters were broken in small pieces and then inserted in a glass tube with 30 mL hexane : dichloromethane (1:1, v/v) and placed in the ultrasonic bath for 10 min. The surrogate/internal standard was added at this stage at the same concentration as for water samples. Afterwards, the solution was centrifuged during 5 min at 2500 rpm. Extraction steps were repeated three times. The sonicated extracts were evaporated at room temperature under nitrogen and reconstituted in 100 µL of hexane. Samples were analysed by a GC System (Carlo Erba GC 8000) coupled to a quadrupole mass spectrometer (Fisons MD 800).

Table 5.1 Contaminant residue levels (ηg/L) and general physico-chemical parameters of water samples collected from TDNP and Navaseca Reservoir in May (M) and July (J). Abbreviations are described in Gorga et al. (2013) and Martinez et al. (2003). D, lower than limit of detection (LOD).

	Cigüela site									Guadiana site										Navaseca		LOD (ng/L)
ng/L	T1-M	T2-M	3-M	T9-M	T10-M	T1-J	T2-J	T9-J	T10-J	T4-M	T5-M	T6-M	T7-M	T8-M	Griñon-J	T4-J	T5-J	T6-J	T7-J	N1-J	N2-J	N3-J	
Estrogens																							
Estriol	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	0.7	D	D	0.17
Estrone	D	D	D	D	D	D	D	3.7	D	D	D	D	D	D	D	D	2.8	D	D	6.739	D	D	0.05
Antimicrobials																							
Triclosan	D	D	D	D	25.5	D	D	D	D	D	D	D	D	D	D	D	D	D	D	39.1	D	1.1	0.03
Preservatives																							
Methylparaben	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	0.02
Ethylparaben	D	D	D	D	D	D	1.2	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	0.27
Propylparaben	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	1.8	1.5	0.02
Plasticizer																							
Bisphenol A	6.3	6.8	10.5	6.7	4.1	D	4.5	3.2	8.5	34.3	17.4	16.2	8.8	7.7	D	1.3	0.7	D	D	44.5	6.7	3	0.12
Alkylphenols																							
Nonylphenol	D	D	D	D	26	30.1	D	D	D	D	D	D	D	D	D	21.8	D	D	D	D	D	D	0.14
Octylphenol	0.5	D	D	1	1	D	0.8	D	0.6	D	0.5	0.1	0.4	0.3	D	D	D	D	D	3.2	0.3	0.1	0.14
Nonylphenol monocarboxylate	5.2	D	D	D	D	D	D	D	D	2	D	D	D	D	D	D	D	D	D	208.6	185	197	0.03
Nonylphenol diethoxylate	21.4	19	24.3	8.9	25.9	D	15.3	D	11.9	18.8	33.8	4.5	20.6	19.6	D	D	19.1	D	D	56.6	19.3	12.7	0.01
Anticorrosives																							
1H-Benzotriazole	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	59.7	44.1	43.7	0.07
Tolytriazol	20.2	9.9	D	D	11.2	44.3	20.3	4.4	D	2.5	D	2.5	D	D	D	9.7	D	D	4	307.2	294.5	265.5	0.01
Organophosphorous flame retardants																							
Tris(butoxyethyl)phosphate	D	D	D	D	D	D	D	D	D	D	D	D	D	27.6	D	D	D	D	D	82.8	64.5	62.2	0.05
Tris(chloroisopropyl)phospate	59.7	52.8	10.4	24.9	47.2	155.5	152.2	64.2	49.4	18.3	5.8	40.5	36.7	71.9	D	18	20.8	32.2	29	535.5	961.7	963.8	0.06
Tris(2-chloroethyl)phosphate	D	D	D	D	D	D	D	17	D	D	D	D	D	D	D	D	D	D	D	486.5	452.9	561.6	0.03
PAHs																							
Naphtalene	0.1	0.3	0.1	0.1	0.1	2.1	0.1	0.1	0.7	0.2	0.2	0.1	0.4	0.4	0.2	0.3	0.1	D	0.2	1.4	0.6	0.9	0.02
Acenaphtylene	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	0.4	1.7	0.5	0.9	0.12
Acenaphtene	0.2	0.3	D	0.3	D	0.3	0.3	0.6	D	0.3	D	D	D	1.5	D	0.5	0.7	D	0.7	2.1	1.7	1.7	0.08
Fluorene	0.3	0.5	0.1	0.5	0.7	1.1	0.3	0.2	0.6	0.5	0.1	D	0.2	0.7	0.3	0.7	0.9	0.3	1.2	3.2	2.7	2.8	0.03
Phenantrene	0.4	0.9	0.1	0.9	2	0.9	0.5	0.3	0.1	1.2	D	D	D	1.4	1.1	0.9	1.1	1	1.2	1.8	1.4	1.4	0.02
Antracene	D	D	D	D	0.1	D	D	D	D	D	D	D	D	D	D	D	D	D	D	0.1	D	D	0.02
Fluorantene	0.1	D	0.4	0.6	0.4	0.6	1	D	0.3	0.4	0.3	0.2	0.3	0.6	0.4	0.4	0.4	0.9	0.5	D	0.1	D	0.01
Pyrene	0.8	D	2.8	3.7	2.3	3.8	4.7	D	2.4	2.5	1.7	1.8	2	3.6	2.4	2.3	2.2	4.2	3.3	D	D	D	0.01
Benzo[**a**]anthracene	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	0.01
Crysene	D	D	0.1	D	0.1	0.1	0.1	D	D	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	D	0.1	0.01
Benzo[*b*]fluoranthene	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	0.02
Benzo[*k*]fluoranthene	D	D	D	D	0.1	D	D	D	D	D	D	D	0.1	D	D	D	D	D	D	D	D	D	0.01
Benzo[*a*]pyrene	D	D	D	D	D	D	D	D	D	D	D	D	D	0.1	D	D	D	D	D	D	D	D	0.02
Indeno[1,2,3-*cd*]pyrene	D	D	D	D	D	D	D	D	D	D	D	D	D	0.1	D	D	D	D	D	D	D	D	0.02
Benzo[*a,h*]anthracene	D	D	0.1	D	D	D	0.4	D	D	D	D	D	D	0.1	0.1	0.1	D	0.1	0.1	D	D	D	0.01
Dibenzo[*ghi*]perylene	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	0.01
Chemical biomarker																							
Caffeine	16.6	3.6	2.8	1	3.7	D	D	1.5	D	0.5	D	6.4	0.7	38.6	189	101.6	D	0.4	D	262.3	57.5	53.8	0.02
pH	7.89	7.98	7.94	7.95	7.8	7.6	7.64	7.92	7.92	7.82	7.91	7.8	8.02	8	7.9	7.8	8	7.83	7.75	7.9	7.87	8.15	

The system was operated in electron impact mode (EI, 70 eV). The separation was achieved with a 30 m × 0.25 mm i.d. DB-5 column (J&W Scientific, Folsom, CA, USA) coated with 5% diphenyl–polydimethylsiloxane (film thickness 0.25_m). The oven temperature was programmed from 60ºC (holding time 1 min) to 175ºC at 6ºC/min (holding time 4 min) to 235ºC at 3ºC/min and finally to 300ºC at 8ºC/min, keeping the final temperature for 5 min. Injection was performed in splitless mode, keeping the split valve closed for 48 s. Helium was the carrier gas (50 cm/s). Injector, transfer line and ion source temperatures were 280, 250 and 200ºC, respectively. Peak detection and integration were carried out using Masslab software. For increased sensitivity and specificity, quantification was performed in time scheduled selected ion monitoring (SIM) using three ions for each of the 16 PAHs analyzed. Five deuterated PAHs were used, one in each elution window. Internal standard quantification was performed for all target compounds using the respective deuterated compound present within each chromatographic window. Intra- and inter-day variability of the method was between 0.5 and 6% for most compounds, up to 13% for fluoranthene. Mean recoveries (±SD) in water and suspended solids for the 16 analysed PAHs were 75 (±15)% and 72 (±14)%, respectively. Limits of detection calculated at a signal-to-noise ratio of 3 are depicted in Table 5.1 and ranged between 0.01 to 0.12 ηg/L.

5.3.4 Dioxin-like and estrogenic activity

Two recombinant yeast assays (RYAs) were used to quantify the presence of estrogens (ER-RYA) and dioxin-like activity (AhR-RYA) in water samples as described elsewhere (Céspedes et al., 2004; Noguerol et al., 2006a). Preliminary tests indicated that most estrogenic activity was present in filtered water whereas dioxin-like activity was found in both filtered water and suspended solid filters (i.e. filters). Filtered water sample pre-concentration was performed following the same protocol as for the PAH analyses eluting with 10 mL of either dichloromethane : hexane (1:1, v/v) or dichloromethane : acetone (1:1, v/v), for dioxin-like and estrogenic activity, respectively. Final extracts for measuring estrogenic activity were then evaporated with a gentle stream of N_2, and reconstituted to a final volume of 0.5 mL methanol. Dioxin-like activities

were determined in composite samples including 0.25 mL of methanol reconstituted filtered water sample extracts and 0.25 mL of methanol reconstituted dichloromethane: hexane 1:1 extracts from filter samples. No surrogate standards were added to the extracts prepared for the RYA assays.

The ER-RYA was performed using the yeast strain BY4741 (MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0) from EUROSCARF (Frankfurt, Germany) transformed with plasmids pH5HE0 (hER) and pVitBX2 (ERE-LacZ) (Noguerol et al., 2006b). For the AhR-RYA we used the YCM4 yeast strain (Miller, 1997), harboring a chromosomally integrated construct that co-expresses the hAHR and ARNT genes under the Gal1-10 promoter and the pDRE23-Z (XRE5-CYC1-LacZ) (Noguerol et al., 2006b). RYA tests were performed in 96-well polypropylene microtiter plates (NUNC, Roskilde, Denmark) as described by Noguerol et al. (2006b). β-Galactosidase activity was measured by fluorescence in a Synergy 2 spectrofluorometer (BioTec, USA) at 355 nm excitation and 460 nm emission wavelengths, using 4-methylumbelliferone β-D-galactopyranoside (MuGal, Sigma Aldrich Chemical, Germany) as a fluorogenic substrate (Noguerol et al., 2006b). β-Galactosidase activity values were calculated as the rate of increase in time in the arbitrary units of fluorescence, by means of standard linear regression models (Noguerol et al., 2006b). Samples were tested in duplicate. Each plate included positive (1 mM ß-naphthoflavone for dioxin-like, 10 nM estradiol for estrogenic), negative (5% of vehicle, methanol), and inhibitory (sample extract plus either 1 mM β-naphthoflavone or 10 nM estradiol) control (Noguerol et al., 2006b).

Estrogenic or dioxin-like activities were expressed as estradiol equivalents (E2Eq., ER-RYA) or benzo[a]pyrene (B[a]Pyr) equivalents (BaPeq, AhR-RYA), calculated as the EC50 of each sample divided by the calculated EC50 for estradiol (74 ηg/L) or for B[a]Pyr (280 µg/L), respectively (Noguerol et al., 2006b), after consideration of the volume of water corresponding to each mL of extract tested.

Measured ER-RYA activities (E2eq) were compared with predicted ones from measured chemical residues of natural estrogens, alkylphenolic compounds,

triazoles and bisphenol A using the conversion factors provided by Céspedes et al. (2004)). Similarly measured AhR-RYA activities (BaPeq) were compared with predicted ones from measured PAH residues following Misaki et al. (2007).

5.3.5 Feeding toxicity tests

A single laboratory *D. magna* clone (clone F), which has been the subject of many investigations (Barata and Baird, 2000), was selected for this study. Bulk cultures of 15 animals each were maintained in ASTM hard synthetic water as described elsewhere (Barata and Baird, 2000). Animals were fed daily with *Chlorella vulgaris* Beijerinck (10^6 cells/mL, corresponding to 3.6 g C/mL). The culture medium was changed every other day, and neonates were removed within 24 h. Between 200 to 250 neonates were then transferred to 4 L tanks and reared under the same conditions as their mothers until they reached their fourth instar (4–5 days at 20 ºC). At this stage groups of juveniles were used for post-exposure feeding toxicity studies.

Post-exposure feeding assays followed previously validated methods (Bosch et al., 2009; Rivetti et al., 2015b). Four-day-old juveniles of *D. magna* were first pre-exposed in groups of 25 individuals to 1 L of unfiltered water for 24 h in a rotary wheel (3 rpm). In each run laboratory controls were also included, exposing individuals to 1 L of ASTM hard water. After exposure, feeding rates were measured in groups of 5 individuals in 50 mL of ASTM hard water with 5×10^5 *Chlorella vulgaris* cells/mL. Incubations lasted 4 h and were performed in quintuplicate. Appropriated blanks (vessels with algae and no animals) were also included to account for algae growth. Feeding experiments were conducted in the dark in order to avoid algal growth. Individual feeding rates (number of algal cells ingested per animal per hour) were determined as the change in cell density in 4 h and converted to proportional feeding inhibition relative to lab controls. Cell density was estimated from absorbance measurements at 650 nm using standard calibration curves based on at least 20 data points (r^2> 0.98).

5.3.6 Data Analyses

Measurements of organic contaminant levels and endocrine activity in water samples were not replicated and hence could not be compared individually.

Instead spatial and temporal differences in the measured responses of TDNP areas belonging to the influence of Cigüela and Guadiana rivers and Navaseca Pond in May and July were compared using one way ANOVA. To reduce the number of environmental variables and avoid an excessive occurrence of undetected contaminant levels across stations (i.e. zero values), the 35 contaminants detected in the studied water samples were grouped into nine chemical classes: triazoles, PAHs, organophosphorous flame retardants, alkylphenols, estrogens, parabens, triclosan, caffeine and bisphenol A. One way ANOVA followed by Tukey's post-hoc tests were used to analyse feeding inhibition responses, as unbalanced samples across sites and seasons did not allowed to perform a full two way ANOVA.

Principal Component Analysis (PCA) was used to explore variability patterns and co-correlations between the studied environmental variables (Jolliffe, 2002). The initial model included the nine chemical classes mentioned above plus four physico-chemical ones (pH, oxygen concentration, conductivity and suspended solids). Non detected values were set to half the detection limit. Since variables were very different and they were not measured using the same scale units, data was auto-scaled and normalized prior to analysis. pH and conductivity were excluded from the final model as they fail in the Kaiser-Meyer-Olkin test of adequacy of variables for PCA (Jolliffe, 2002). Dioxin-like and estrogenic activities were compared both to pollutant concentrations and to PCA sample scores by linear regression analyses.

5.4 Results

5.4.1 Environmental variables

Measured PAH levels in filtered water were three fold lower than those measured in suspended solids and both fractions varied similarly across the studied samples. For the sake of clarity both fractions were considered together. Ten of the twelve studied physico-chemical variables showed significant differences (ANOVAs, $P<0.05$) across TDNP influenced areas, Navaseca Pond, and /or seasons. Nine of those variables (all but oxygen levels)

are shown in Figure 5.2, the complete set is shown in Table 5.1. Oxygen levels in Navaseca Pond were lower (Mean ± SE, 7.2 ± 0.5 mg/L) than those of the rest of samples, which approached saturation values (9-10 mg/L) (Table 5.1). Navaseca Pond had the greatest levels of triazoles, PAHs, flame retardants, alkylphenols, parabens and total suspended solids (SS) (Fig 5.2). TDNP sites from Guadiana side and Navaseca Pond had the highest levels of caffeine and BPA. TDNP sites from Guadiana side and Navaseca Pond had the highest levels of conductivity (Fig. 5.2). The highest levels for many pollution indicators corresponded to summer samples, including flame retardants and parabens concentrations, conductivity for TDPN Cigüela samples, and caffeine levels for TDPN Guadiana samples. Conversely, levels of BPA in TDPN samples influenced by the Guadiana River were higher in May than in July (Fig. 5.2).

5.4.2 Biological effects

ER-RYA and AhR-RYA assays indicated low estrogenic and dioxin-like activities across the studied water samples (Fig. 5.3). Measured total estrogenic activity was linearly related with predicted estrogenicity values obtained from chemically measured estrogenic compound concentration (r^2 = 0.75, Fig. 5.3 A). Measured total dioxin-like activity also showed a significant linear relationship with predicted values ($P < 0.05$, r^2 = 0.26), but, in this case, the observed values were about one order of magnitude higher than the ones predicted from the observed PAH concentrations (PAHs, Fig. 5.3 B).

There were significant differences in feeding inhibition rates among the studied samples and seasons ($P<0.05$; $F_{21,89}$ = 50.3). Navaseca Pond samples were the most toxic ones, inhibiting feeding almost completely (76-90%, Fig. 5.4). Moderate levels of feeding inhibition (18-37%) were observed in water samples from TDNP sites T1, T2, T10 and T5 in May and low levels (0-13%) in the rest of samples (Fig. 5.4).

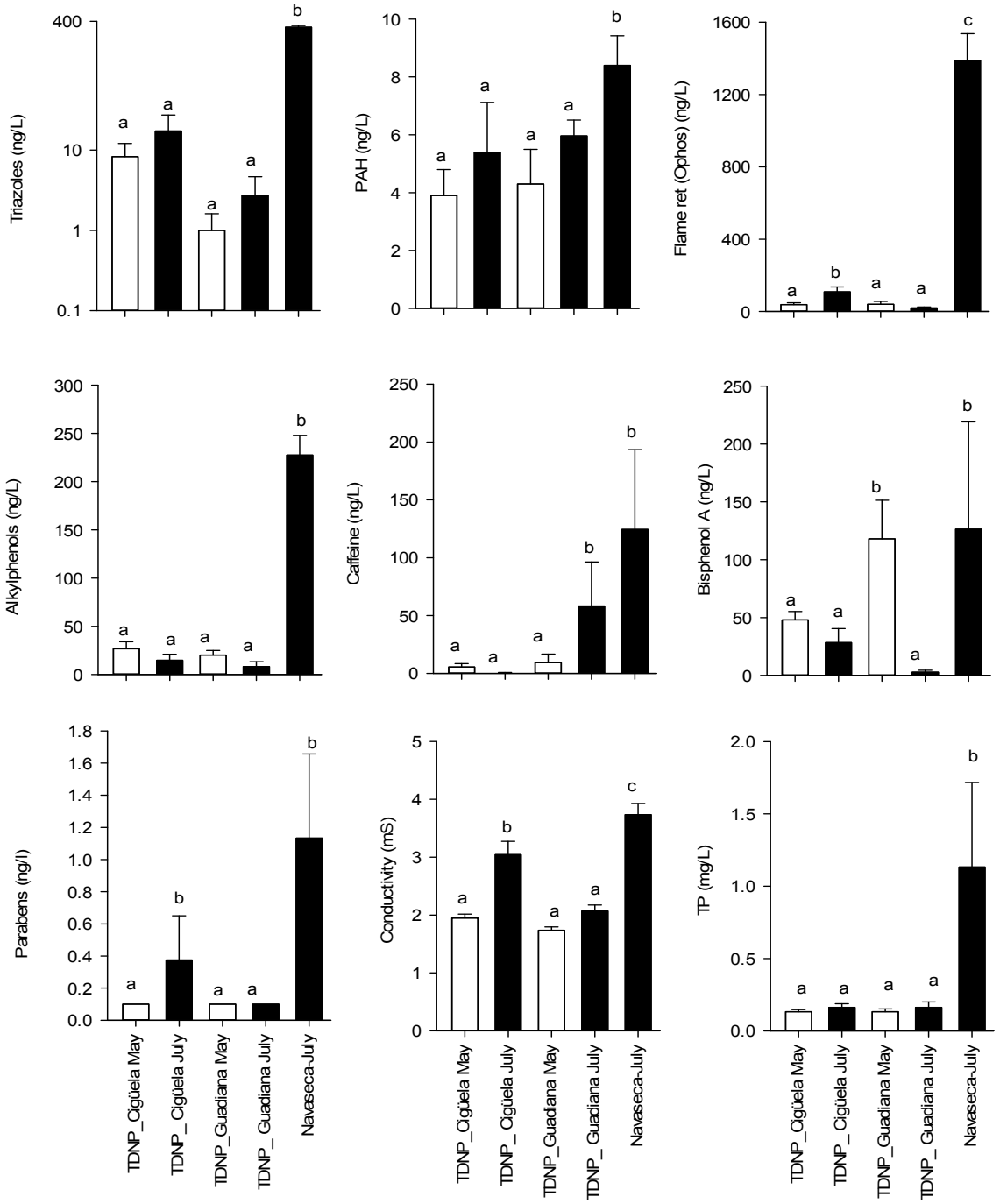

Figure 5.2 Mean ±SE (N = 3-5) of selected chemical groups and physico-chemical responses measured in water samples taken across Tablas de Daimiel National Park (TDNP) in the areas influenced by Cigüela and Guadiana rivers and in Navaseca Pond. Different letters indicated significant (P<0.05) differences among sites following ANOVA and Tukey's post-hoc test.

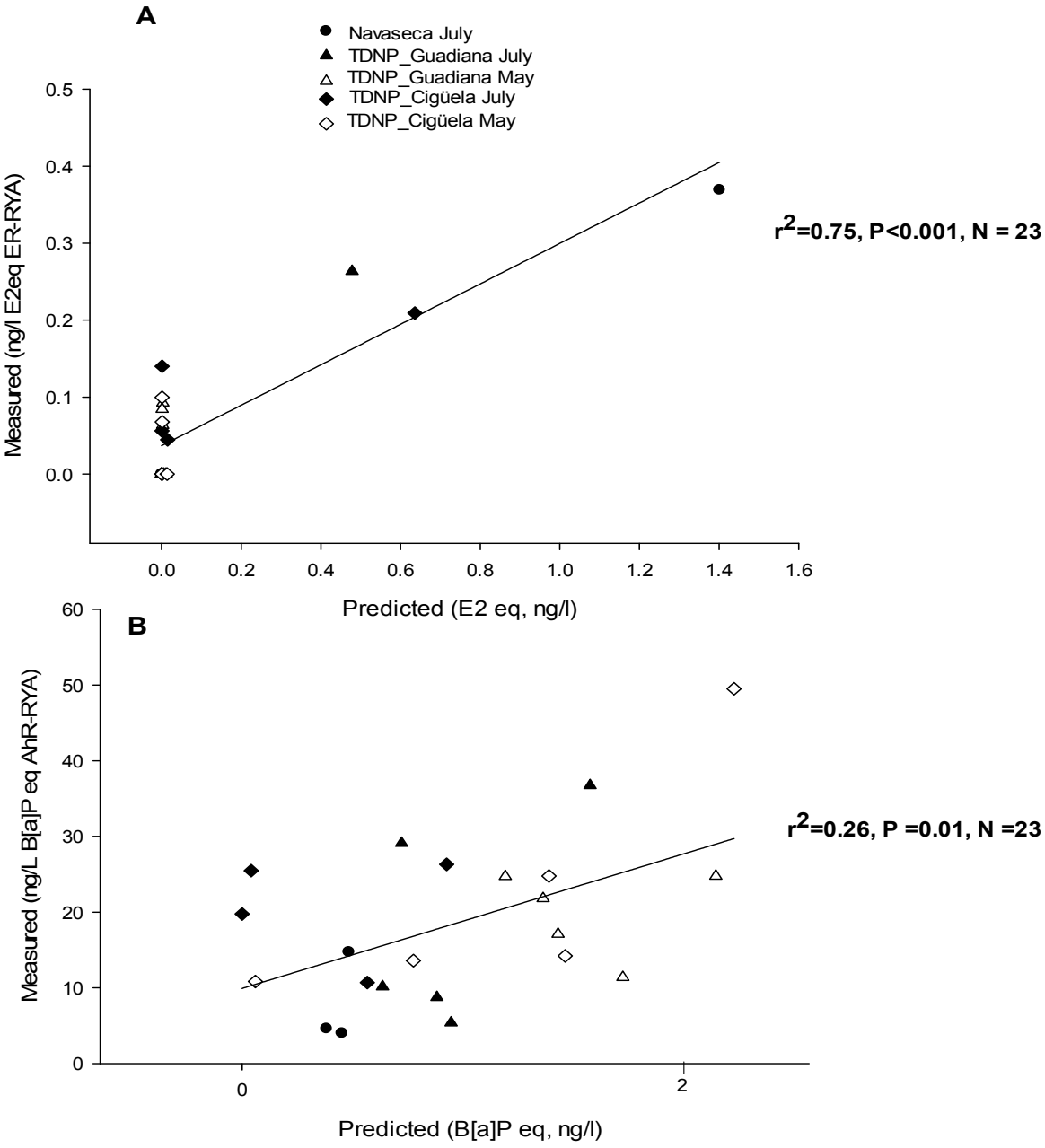

Figure 5.3 Predicted versus measured estrogenicity (A) and dioxin-like (B) effects of the studied water samples. Fitted regression lines and r^2 are also depicted.

5.4.3 Associations between environmental and biological variables

PCA performed on measured environmental factors defined two interpretable components that explained 78.5 % of data variance. Bi-plots of the first two principal components are depicted in Fig. 5.5 A. PC1 was determined by positive loadings of all variables having the greatest scores for triazoles, alkylphenols and TP. Accordingly, PC1 separated TDNP sites from Navaseca Pond ones. PC2 was defined by positive loadings of parabens and negative ones of BPA, estrogens and triclosan. PC2 differentiated Navaseca Pond 1 from the rest of sites. PC1 and PC2 scores were linearly related with feeding inhibition (r^2 = 0.70, P < 0.05) and estrogenic activity (RYA-E2 eq,; r^2= 0. 34, P<0.05), respectively (Fig. 5.5 B,C).

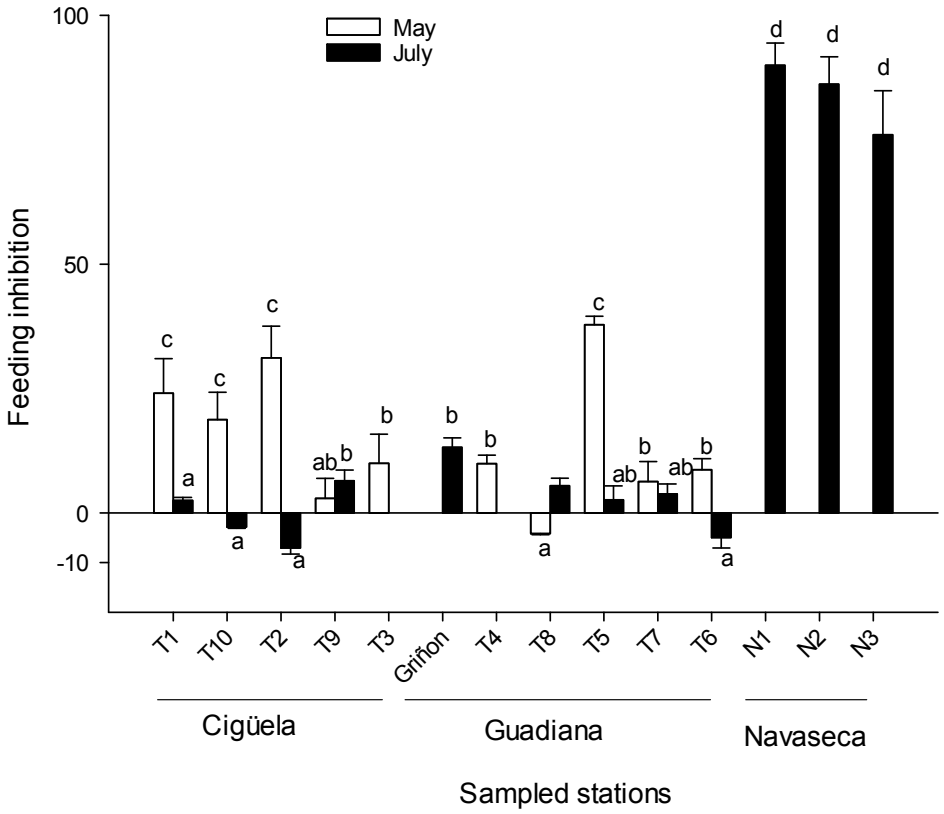

Figure 5.4 Feeding inhibition responses relative to control treatments across the studied water samples. Different letters indicated significant (P<0.05) differences following ANOVA and Tukey's post hoc test.

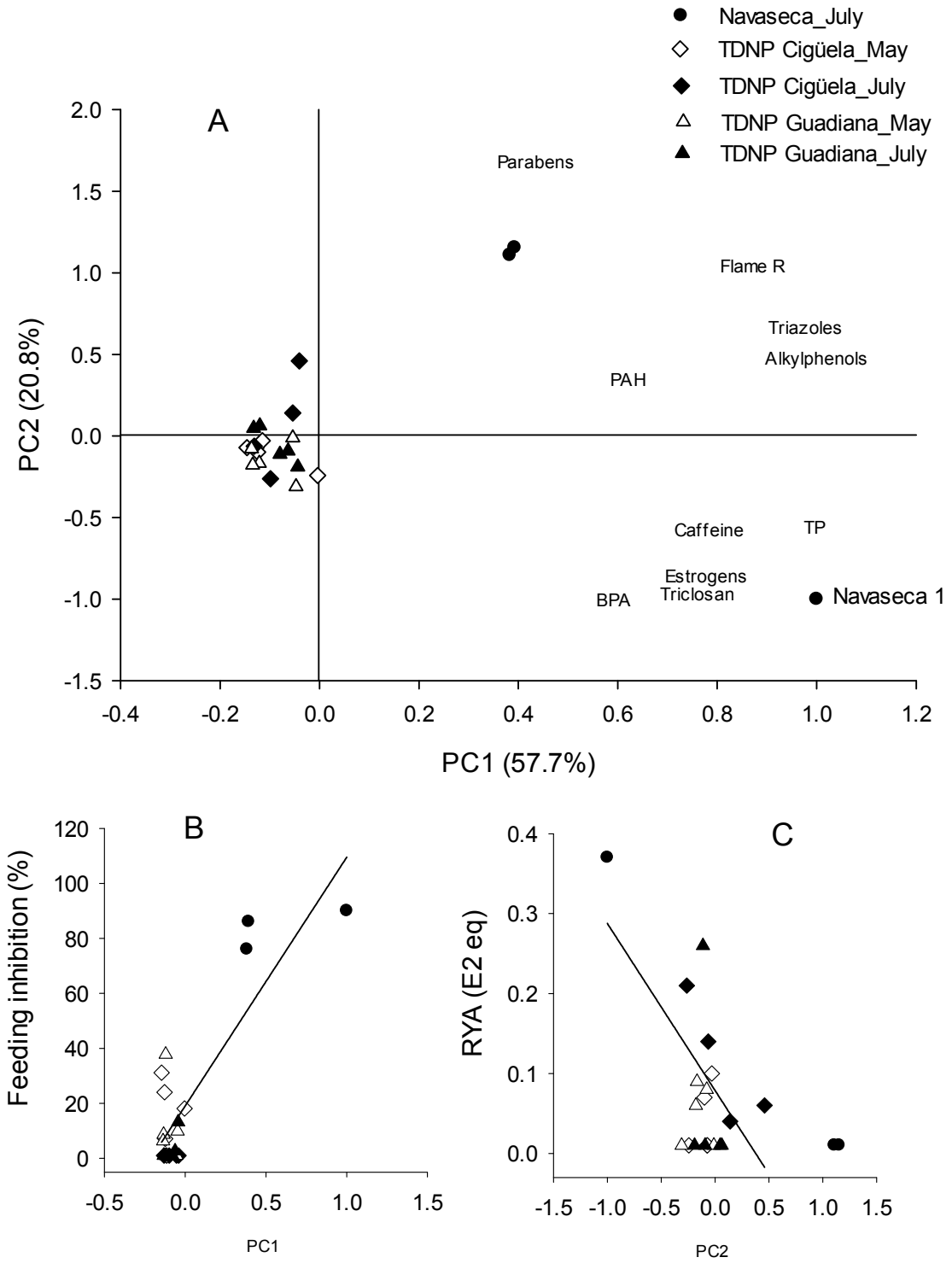

Figure 5.5 Bi-plot of variable loadings and sampling scores of the first two Principal Components (A) of the analysis performed with selected chemical groups and physico-chemical variables measured across the studied samples from TDNP and Navaseca Pond. Graphs B and C show Bi-plots of PC1, PC2 sample scores against feeding inhibition and estrogenic activity, respectively. Lines in graphs B and C are fitted linear regression curves.

5.5 Discussion

Most measured contaminant levels across most TDNP sampling sites were lower than those reported for surface waters of several rivers across Spain (Gorga et al., 2013). Residue levels of total PAHs in water in TDNP sites varied between 4-6 ηg/L, which are quite low compared to those measured in water from Mediterranean creeks just after forest fires (Vila-Escale et al., 2007). These low amounts of PAHs in TDNP water samples indicate that this wetland is probably no longer affected by PAHs coming from smoldering peat fires that took place years ago, when TDNP suffered a severe drought period (Moreno et al., 2011). There was no consistent pattern of pollution across sites of TDNP influenced by Cigüela and Guadiana Rivers, neither in May nor in July.For example, residues of BPA and caffeine were higher in May and July, respectively, in sites influenced by the Guadiana River; and those of parabens and organophosphorous flame retardants were higher in July in sites influenced by Cigüela River. Surface waters subjected to the Mediterranean semi-arid climate regime typically show higher levels of WWTP-related pollutants in summer, as there is less water and hence less dilution of pollution coming from effluent discharges (Collado et al., 2014). Navaseca Pond may release its contaminated waters into Guadiana River, but this only occurs sporadically when the pond and the WWTP are overloaded during heavy rains. Nevertheless, it is important to note that TDNP sites influenced by the Cigüela River receive more constantly pollutants from WWTP effluents of the Villarubia de los Ojos town (Fig. 5.1 C). This is therefore consistent with the high measured residue levels of flame retardants and parabens in TDNP sites influenced by the Cigüela River sampled during the July campaign.

PCA analyses identified two sources of pollutants mainly associated with Navaseca Pond: PC1 was defined by high loads of residue levels of some compounds usually found in WWTP effluents, like organophosphorate flame retardants, anticorrosive triazoles, and alkylphenols, which were from 5 to 10 times higher in Navaseca Pond than in TDNP. These results are in line with the reported differences in concentrations of these substances between surface waters and WWTP effluents (Gorga et al., 2013). This supports also the

premise that Navaseca Pond receives poorly treated sewage effluents from WWTP of Daimiel town. The second PC2 component was defined by other compounds such as estrogens, disinfectants, preservatives, BPA and caffeine, which were still higher in Navaseca Pond than in TDNP, although their levels were within the lowest range detected in WWTP effluents (Gorga et al., 2013). Our data on chemical residue levels, thus, indicated that the impact of pollutants from Navaseca Pond and/or from other WWTP effluents on TDNP during our sampling period was not as important as predicted by the study of Sanchez-Ramos et al. (2016)). This previous study, however, only analyzed physico-chemical parameters and not particular contaminants, and it only included sampling stations close to waste water effluent discharges rather than inside TDNP.

Measured maximal levels of estrogenic and dioxin-like activities across the studied samples were 0.4 ηg/L E2eq and 50 ηg/L BaPeq, respectively. Reported EC50 for ER-RYA and AhR-RYA assays are calculated as 74 ηg/L E2eq and 280 µg/L BaPeq, respectively (Céspedes et al., 2004; Olivares et al., 2013). This means that in this study measured endocrine disruption activities across samples were quite low, which are in line with low measured residue levels of estrogenic compounds and PAHs. Indeed in surface waters receiving discharges from WWTP, ER-RYA activities as high as 8 ηg/L E2eq have been reported (Céspedes et al., 2005). For dioxin-like activity, the values obtained using AhR-RYA were 10 fold higher than those predicted from analysed PAH residues. This observed excess between measured and predicted dioxin-like activities are common across studies and are due to the existence of other compounds aside PAHs that also are able to activate the AhR receptor (Mesquita et al., 2014). Compounds such as polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated/ polybrominated biphenyls (PCBs/PBBs), and dioxins can also produce dioxin-like activity (Olivares et al., 2011). Nevertheless, the above mentioned organochlorine/brominated compounds are mostly related to the combustion of industrial wastes and/or associated to industrial activities, which are absent or of minor importance around TDNP (Berzas et al., 2000; Sanchez-Ramos et al.,

2016). It is also interesting to point out that in this study dioxin-like activity was not correlated with any of the two components of the PCA, which means that this disruptive activity was not related to any identified source of pollution. It is then likely that the excess of dioxin-like activity was at least partially coming from naturally occurring humic materials and/or other natural substances (Janošek et al., 2007).

The studied samples of TDNP did not or did only marginally (0-17%) impair the grazing rates of *D. magna*. Conversely, water samples from Navaseca Pond were quite toxic to *D. magna,* reducing feeding rates by more than 80%. Feeding inhibition response in *D. magna* is a cost-effective and sensitive endpoint that has been tested against natural toxins and many pollutants from industrial, domestic and agriculture origin (Barata et al., 2008; Rivetti et al., 2015b). Feeding inhibition is also an ecological response since feeding impairment in *D. magna,* as well as in most organisms, is directly related to reduced food acquisition and hence to detrimental effects on growth, reproduction and survival, which ultimately translated into reduced population growth rates (Barata and Baird, 2000). The post-exposure feeding assay used in this study allowed to assess the toxicity of both dissolved and particle bound contaminants (Rivetti et al., 2015b). *D. magna* grazing rates are especially sensitive to natural cyano-toxins, followed by pesticides, and less sensitive to industrial/domestic contaminants (Barata et al., 2008; Barata et al., 2007; Rivetti et al., 2015b). From all measured chemical residues, nonylphenol and BPA were the most toxic, impairing *D. magna* feeding rates at around 0.1 and 15 mg/L, respectively (Jordão et al., 2016). Thus it is unlikely that measured residue levels at Navaseca Pond, which were at most in the low µg/L range, were toxic to *D. magna*. This means that measured toxicity in Navaseca Pond probably came from non-tested compounds. There are reported studies indicating that pesticides that are often found at high amounts in WWTP effluents and/or cyanobacteria toxins that occur naturally in Spanish surface waters can severely inhibit *D. magna* feeding rates (Damásio et al., 2008; Rivetti et al., 2015b) . As there is no reported information of pesticide residue

levels and of cyanotoxins in TDNP, future research should thus be focused on characterizing the toxic compounds present in that floodplain.

5.6 Conclusions

In this present study, the combination of endocrine disruption and general toxicity assays with targeted chemical analyses allowed to detect potential sources of contamination in TDNP. Measured pollutant levels and related endocrine disrupting activity and toxicity were low in samples from TDNP, but considerably higher for the Navaseca Pond samples, especially regarding *D. magna* toxicity. This pond is highly impacted by treated wastewater effluents from Daimiel, and its close proximity to the Guadiana River constitutes an imminent risk of contamination for TDNP. Furthermore, many waterbirds use the contaminated euthrophic Navaseca Pond as a feeding ground. Therefore there is also a real risk of those birds inhabiting TDNP to be exposed to the contaminants present in Navaseca Pond when they visit that pond. Future action plans for TDNP should therefore improve the water quality of Navaseca Pond by adapting Daimiel WWTP capacity to the actual volumes of sewage water emissions from the town of Daimiel, with special attention to the management of large inputs during heavy rain periods.

Treated sewage water is in many semiarid countries, such as Spain, an important contribution to maintain a minimum ecological flow in some rivers. In the particular case of the wetlands of the Biosphere Reserve of “La Mancha Húmeda”, the treated sewage maintains some of the wetlands of international importance and it has been also evaluated as a potential resource to flood TDNP during drought periods (Navarro et al., 2011). The present work contributes to evaluate the chemical risk concomitant with sewage water from urban and industrial sources that adds to biotic risks associated with eutrophication and the dispersion of pathogens (Anza et al., 2014). The use of treated sewage water for “closed” wetlands, in contrast with rivers, must take into account the overload with some persistent chemicals that can affect in the long term the health of the wildlife and the ecosystem in general.

5.7 Acknowledgments

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Chapter VI.

Low environmental levels of neuro-active pharmaceuticals alter phototactic behavior and reproduction in *Daphnia magna*

[a] Rivetti, C., Campos, B., & Barata, C. (2016). Low environmental levels of neuro-active pharmaceuticals alter phototactic behavior and reproduction in Daphnia magna. Aquatic Toxicology, 170, 289-296

Chapter VI.

Low environmental levels of neuro-active pharmaceuticals alter phototactic behavior and reproduction in *Daphnia magna*[a]

6.1 Abstract

Assessing the risks of emerging contaminants such as pharmaceuticals in the environment requires an understanding of their exposure regime and their effects at environmentally relevant concentrations across species. *Daphnia magna* represents an excellent invertebrate model species to study the mode of action of emerging pollutants, allowing the assessment of effects at different biological levels. The present study aims to test the hypothesis that different families of neuro-active pharmaceuticals at low environmentally relevant concentrations may lead to similar phenotypic responses in *D. magna*. Phenotypic traits included reproduction and behavioral responses. Selected pharmaceuticals were carbamazepine, diazepam and propranolol, three widely prescribed compounds, already detected at considerable levels in the environment (ng to few µg/L). Fluoxetine was also included in behavioral assays. The three tested neuro-active pharmaceuticals were able to enhance reproduction at 1 ηg/L of propranolol, 0.1 µg/L of diazepam and 1 µg/L of carbamazepine. Fluoxetine, carbamazepine and diazepam increased positive phototactic behavior at concentrations ranging from 1, 10 and 100 ηg/L, respectively. Reported responses were non-monotonic, which means that eco-toxicity testing of pharmaceuticals need to assess effects at the ηg/L range.

Keywords: population; crustacean; beta-blockers; diazepam; carbamazepine; neuroendocrine

6.2 Introduction

Assessing the risks of long-term exposure to low doses of human prescribed pharmaceuticals is an identified research need (Fent et al., 2006). Treated and untreated wastewater effluents are the main route that brings human pharmaceuticals and/or their metabolites to water. Consequently, pharmaceuticals are continuously released into the environment and thus their negative effects are independent from their persistence in the environment (Fent et al., 2006; Petrović et al., 2003). In surface waters concentrations of measured human pharmaceuticals are often in the ηg/L range. However, targeted ecotoxicological studies using environmental relevant concentrations and focusing on subtle environmental effects are scarce. Recently several studies have reported that very low concentrations of antidepressants and anxiolytic drugs alter the behavior of fish, molluscs and crustaceans (Brodin et al., 2013; Fong and Ford, 2014; Ford and Fong, 2015). Human targets of antidepressants, anxiolytic and neuropathic drugs such as selective serotonin re-uptake inhibitors (SSRI), drugs blocking voltage-gated sodium channels and/or GABA agonists and certain antihypertensive compounds are highly conserved across vertebrates and 61% of them are also found in the invertebrate crustacean *Daphnia* (Gunnarsson et al., 2008). Therefore, neuro-active drugs may also affect aquatic invertebrates. It is important to note, that several neuro-active compounds are designed to affect neurotransmitters (serotonin, dopamine, epinephrine, *gamma*-aminobutyric acid-GABA), which regulate many physiological and behavioural processes (Fong and Ford, 2014; Ford and Fong, 2015). There is also an increased number of studies showing that effects of antidepressants at low concentrations do not follow a monotonic response (Fong and Ford, 2014; Ford and Fong, 2015). This behavior is common among endocrine and neuro-active compounds that at low concentrations act specifically on their target sites, whereas at high concentrations became toxic and hence impair survival, growth and/or reproduction irrespectively of its primary mode of action (Vandenberg et al., 2012). This means that there is an urgent need to measure subtle but

consistent effects of human pharmaceuticals at low concentration levels in non-target organisms.

The crustacean and aquatic ecotoxicological model organism *Daphnia magna* share with vertebrates several of the neurotransmitters that are targeted by antidepressant and other neuro-active drugs. These include the presence of serotonin, dopamine, epinephrine and GABA receptor signaling pathways (Campbell et al., 2004; Campos et al., 2013b; Ehrenström and Berglind, 1988; McCoole et al., 2012a; McCoole et al., 2012b; Weiss et al., 2012). There is also evidence that the SSRI fluoxetine, carbamazepine and propranolol increase offspring production at 10, 1 and 50 µg/L, respectively (Campos et al., 2012a; Lürling et al., 2006; Stanley et al., 2006). In amphipods the SSRIs fluoxetine and sertraline altered phototaxis and swimming behavior at quite low concentrations ranging from 1 to 100 ηg/L (Bossus et al., 2014; Guler and Ford, 2010). In *D. magna* negative phototactic behavior is directly linked to diel vertical migration along the water column, which prevents *Daphnia* to be preyed upon fish during daylight (Cousyn et al., 2001; De Meester, 1993). Thus, this response is an ecologically relevant trait. The aim of the present study is to determine changes in phototactic behavior and reproduction in *D. magna* individuals exposed to four widely prescribed neuro-active drugs using low environmental concentrations ranging from high ηg/L to low µg/L. The studied compounds included the anti-depressant SSRI fluoxetine, the anxiolytic diazepam, the neuropathic and anti-epilepsy drug carbamazepine and the antihypertensive compound propranolol. Selective serotonin reuptake inhibitors (SSRIs) act by blocking the re-uptake of serotonin in the nerve synapses. This effect is used worldwide to treat clinical depression in humans (Rang et al., 1995), with the consequences that these compounds are nowadays widespread in the environment. Surveys in US have reported levels of 12-540 ηg/L of fluoxetine, the active ingredient of Prozac, in surface waters and effluents (Kolpin et al., 2002) and total concentrations of SSRIs in aquatic systems were measured in the range of 840 ηg/L to 3.2 µg/L (Metcalfe et al., 2010; Vasskog et al., 2008). Diazepam, first marketed as Valium, is widely used to treat anxiety. Diazepam enhances the effect of the neurotransmitter GABA by binding to the

benzodiazepine site on the $GABA_A$ receptor (via the constituent chlorine atom) leading to central nervous system depression (Riss et al., 2008). Concentrations of diazepam ranging from 4 to 40 ηg/L have been found in Spanish urban rivers (Valcárcel et al., 2012). Carbamazepine is a medication used primarily in the treatment of epilepsy and neuropathic pain. It stabilizes the inactivated state of voltage-gated sodium channels, making fewer of these channels available to subsequently open. This leaves the affected cells less excitable until the drug dissociates (Ambrósio et al., 2002). Carbamazepine is also a GABA receptor agonist since it potentiates GABA receptors made up of alpha1, beta2, and gamma2 subunits (Ambrósio et al., 2002). Carbamazepine is fairly persistent in water and hence can be found at concentrations ranging from 1 to up to 3000 ηg/L in rivers receiving waste water treatment effluents (Muñoz et al., 2009; Tixier et al., 2003). Propranolol is a nonselective beta blocker widely prescribed to treat high blood pressure and a number of heart dysrhythmias. It blocks the action of epinephrine and norepinephrine on both β_1- and β_2-adrenergic receptors (Wisler et al., 2007). Propranolol is also quite persistent in water and can be found at 10-60 ηg/L in surface water (Bendz et al., 2005; Muñoz et al., 2009).

6.3 Methods

6.3.1 Chemicals

Fluoxetine hydrochloride (CAS-No 56296-78-7; analytical standard, purity 100%), diazepam (CAS-No 439-14-5; analytical standard, purity 99%), carbamazepine (CAS-No 298-46-4; analytical standard, purity 99%) and propranolol hydrochloride (CAS-No 318-98-9; analytical standard, purity 99%) were purchased from Sigma-Aldrich (USA/Netherlands). All other chemicals were analytical grade and were obtained from Merck (Germany).

6.3.2 Experimental animals

A single *D. magna* clone F, extensively characterized in previous studies (Barata and Baird, 2000) was used for all assays. Individual or bulk cultures of 10 animals/L were maintained in ASTM hard synthetic water (ASTM, 1994) as it

has been describes previously (Barata and Baird, 2000). Individual or bulk cultures were fed daily with *Chorella vulgaris* Beijerinck ($5x10^5$ cells/mL, corresponding to 1.8 μg C/mL; (Barata and Baird, 2000). The culture medium was changed every other day, and neonates were removed within 24 h. Photoperiod was set to 14 h light: 10 h dark cycle and temperature at 20 ± 1 °C.

6.3.3 Reproduction tests

Reproduction tests followed established OECD guidelines with only minor modifications (Barata and Baird, 2000). Effects of fluoxetine on reproduction responses of *D. magna* have been already studied in previous studies (Campos et al., 2013b; Campos et al., 2012b), thus reproductive responses of this compound were not tested. Two independent experiments were performed. In the first one, neonates (< 24 h old) were exposed until their fourth brood (approx. 21-23 days at 20°C) to 0.01, 0.1, 1, 10 and 100 μg/L of diazepam, carbamazepine and propranolol. The previous concentration range allowed to define lowest effect concentrations for all compounds but propranolol since this compound already affected measured responses at 0.01 μg/L. Therefore a second experiment was conducted to test lower concentrations of propranolol: 0.1, 1, 10, 100 and 1000 ηg/L. Animals were exposed individually to the tested chemicals in 100 mL of ASTM hard water at the food ration of 5 x 10^5 cells/mL of *C. vulgaris*. The same concentration of ethanol 50 μL/L) was used in all treatments as a carrier solvent and a solvent treatment was also included. Each treatment was replicated 10 times. The test medium was changed every other day. For each individual its survival, age at first reproduction and brood size were monitored. The intrinsic rate of population growth (r) was computed iteratively from the Lotka (Lotka, 1922) equation (eq. 1) using the measured age, specific survival and fecundity rates:

$$\sum_{x=0}^{\infty} e^{-rx} l_x \qquad m_x = 1 \qquad \text{(eq. 1)}$$

Where l_x is the proportion of the females surviving to age x (days) and m_x is the number of juveniles produced *per* surviving female between the ages x and

x+1. The age at birth was set to 0 days and survival probability (*l*) to 1 since mortality was absent in most treatments (in 16 out of 23) and when occurred it was low (10%) and related to handling rather than to toxic effects.

6.3.4 Phototactic Behavior

Changes in phototactic behavior were quantified by determining the mean phototactic response of 5 individuals in the presence and absence of the tested chemical concentration. Tested concentrations were selected from previously conducted reproduction assays. Behavioral assays were replicated four times. Three different type of behavioral experiments were conducted: with 8-day-old adults exposed during their entire life (experiment 1); with 8-day-old adults exposed for 48 h, from 6 until day 8 (experiment 2); with 48-h-old juveniles exposed during their entire life (experiment 3). In experiments 2 and 3 performed with carbamazepine, diazepam and propranolol exposure concentrations were limited to those that in experiment 1 had the highest effects. Exposures were performed in groups of 5 individuals in 500 mL of test media for adults or 100 mL of test media for juveniles. Ethanol (< 50 µL/L) was used as a carrier solvent and a solvent treatment was also included. Exposures were performed as described above. After reaching the desired age, adults or juveniles were used to determine phototactic behavior. The experimental design and setup to measure phototactic behavior followed previous studies (Cousyn et al., 2001; De Meester, 1991, 1993). In short, the experimental set-up consisted of a small glass column (25 cm height, 5 cm internal cross-section), placed in a darkened box, and illuminated from above with a 50 W compact fluorescent light source (OSRAM; Germany). Light intensity at the water surface was about 500 Wm^{-2}.The bottom of the column was covered by black pebbles, so that light reflection was minimized. The column was externally divided in an upper compartment of 3 cm height, a lower compartment of 3 cm height, and a middle compartment of 10 cm height, and placed in a constant temperature (20 ± 2°C) room. In each experiment and replicate 5 individuals were followed. The experimental column was also filled with ASTM hard water alone or with ASTM hard water spiked with the desired test concentration. Once placed in the experimental column, the animals were given 5 minutes of adaptation to the

water column, followed by 5 minutes in darkness, after which the light source was lit. At 1 min intervals, the positions of the test animals were recorded. A percentage positively phototactic behavior can be assigned to the number of animals in the upper compartment, while a percentage negatively phototactic behavior can be defined for the lower 3 cm of the column. The phototactic index (I) is defined as (U - L) / (U + M + L), in which U, M, and L are the number of animal-observations in the upper, middle, and lower compartment, respectively. The phototactic index (I) was averaged over the last ten minutes of the experiment to minimize the influence of the initial light reaction.

6.3.5 Chemical analyses

Stability of each compound during the tests was confirmed using solid-phase extraction and liquid chromatography-tandem mass spectrometry. From reproduction and behavioral tests duplicated water samples of freshly made and old (48 hours) test solutions were collected and pre-concentrated using Oasis HLB SPE cartridges (200 mg), conditioned with 10 mL of methanol followed by 10 mL of water. 500 mL of ASTM water were pre-concentrated at a flow rate of 10 mL/min and eluted with 2 x 5 mL of methanol. The eluate was then reduced under nitrogen to almost dryness and reconstituted in 500 µL of methanol. All compounds were measured using LC-ESI-MS/MS (TqDetector, Acquity Waters, USA) following a previous study reporting an analytical method for simultaneous identification of a wide range of pharmaceuticals with minor changes (López-Serna et al., 2011). Separation was performed by using a Luna C18 (150 mm×2 mm ID, particle size 5 µm, Phenomenex, Torrance, USA) equipped with a SecurityGuard pre-column. The mobile phase composition consisted of binary mixtures with 0.1% formic acid in ACN (A) and 0.1% formic acid in water (B). The gradient of elution started at 5% A , then increased to 40% A in 5 min, 60% A in 10 min, reaching 100% A in 20 min and then return to initial conditions within 5 min. The system was operated at room temperature, the flow rate was set at 200 µL min^{-1} and 10 µL were injected. Fluoxetine, carbamazepine, diazepam and propranolol were analyzed under positive electrospray ionization mode (ESI+). Acquisition was performed in SRM mode using two transitions from $[M+H]^+$ precursor ion to daughter ions to identify each compound. The

transitions used as well as the cone voltages and collision energies were in accordance with the above mentioned work (López-Serna et al., 2011). Quantification was based on external calibration standard 8 point curves (range between 0.5-1000 μg/L). Limits of detection and quantification (LOD,LOQ) defined as the minimum detectable amount of analyte with a signal-to-noise ratio of 3:1 and 10:1, respectively, were 1.35, 4.52 ηg/L for fluoxetine; 0.15, 0.52 ηg/L for diazepam; 0.07, 0.021 ηg/L for carbamazepine and 0.02, 0.06 for propranolol. The data were acquired and processed using the MassLynx v4.1 software package.

6.3.6 Data analyses

Effects of the studied chemical treatments on reproduction and population growth rates responses relative to non-exposed controls were assessed using parametric one way ANOVA followed by Dunnet's post hoc tests. Age at first reproduction and behavioral responses were compared using non parametric ANOVA (Kruskal- Wallis) analyses and Wilcoxon and Wilcox's post hoc tests since these variables did not meet the ANOVA assumptions of normality and/or variance homoscedasticity (Zar, 1996).

6.4 Results

6.4.1 Chemical analyses

Measured residue levels of the tested concentrations in freshly prepared solutions (Table 6.1, 0 h) were pretty close to nominal values being in 17 out of 21 cases within 20% of nominal ones and having the max deviation of 33%. In all treatments but one (0.001 ηg/L of propranolol) measured concentrations of old test solutions were within 20% of freshly prepared ones (Table 6.1, 48 h). For the sake of clarity hereafter we will refer to nominal values.

6.4.2 Life-History effects

Mortality was observed in only 7 of the 23 treatments, that never exceeded 10% (one out of ten individuals used per treatment). Replicates that did not survive until the end of the exposure period were removed from statistical analyses. In

the first and second experiments the tested pharmaceuticals affected significantly ($P<0.05$) total offspring production of exposed females ($F_{16,147}$ = 2.8, $F_{6,60}$ = 8.7) enhancing reproduction relative to both solvent and non-solvent controls at concentrations as low as 0.01 µg/L of propranolol, 0.1 µg/L of diazepam and 1 µg/L of carbamazepine (Fig. 6.1 A).

Table 6.1 Nominal and measured (Mean ±SD) concentrations (µg/L) of the tested chemicals in freshly prepared (0 h) and old (48 h) test solutions.

Chemical	Nominal		Measured (0 h)		Measured (48 h)	
		N	Mean	SD	Mean	SD
Fluoxetine	0.01	4	0.011	0.0009	0.009	0.002
	0.1	4	0.128	0.005	0.114	0.01
	1	4	1.330	0.059	1.139	0.076
	10	4	12.065	0.148	10.419	0.123
	100	4	104.734	4.180	93.513	2.328
Carbamazepine	0.01	4	0.011	0.0005	0.010	0.0004
	0.1	4	0.114	0.005	0.102	0.004
	1	4	0.9	0.062	0.838	0.060
	10	4	10.0	0.468	8.354	0.502
	100	4	100.0	10.277	89.413	4.798
Diazepam	0.01	4	0.013	0.0004	0.011	0.002
	0.1	4	0.117	0.0009	0.103	0.008
	1	4	1.06	0.015	0.958	0.032
	10	4	10	0.105	9.030	0.099
	100	4	100	6.520	93.171	0.383
Propranolol	0.001	4	0.0013	0.0005	0.001	0.0003
	0.01	4	0.011	0.0090	0.010	0.008
	0.1	4	0.113	0.0008	0.108	0.012
	1	4	1.242	0.079	1.135	0.125
	10	4	11.497	0.159	10.723	0.531
	100	4	115.600	2.543	107.518	2.059

In the second experiment, which was limited to test lower propranolol concentrations, effects occurred at 1 ηg/L (Fig. 6.1 B). Concentration effects of reproduction were non-monotonic, increasing at intermediate concentrations and decreasing at higher concentrations. Bi-plots of population growth rate responses with age at first reproduction are depicted in Fig. 6.2. Population growth rate responses, were either unchanged or negatively affected ($P<0.05$) by the tested pharmaceuticals in the first ($F_{16,147}=0.91$, Fig. 6.2 A) and second experiment ($F_{6,56} = 3.7$; Fig. 6.2 B), respectively. Negative effects on population growth rate effects were related to the fact that the studied pharmaceuticals increased significantly ($P<0.05$) age at first reproduction of exposed females (Kruskal-Wallis tests and df for first and second experiments were 27.2, df = 16 and 16.2, df = 6, respectively), and that this trait was strongly and negatively correlated with population growth rates (Fig 6.2 A, B). It is also worth noting that total offspring production was positively ($P<0.05$) correlated with population growth rate in the first experiment ($r = 0.34$, $N = 164$, $P<0.05$) but not in the second one ($r = 0.19$, $N = 65$).

6.4.3 Behavioural effects

Behavioural responses performed on adults and juveniles are reported in Fig. 6.3. The more negative the index the greater negative phototactic behavior. Fluoxetine showed the greatest effects on adults exposed during 8 days diminishing significantly ($P < 0.05$) negative phototactic behavior at 1 ηg/L. Carbamazepine decreased negative phototactic behavior at 10 and 100 ηg/L in adults exposed for just 48 h or during their entire life, respectively. Diazepam decreased the phototactic behavior of adults exposed only for 48 h at 0.1 µg/L. Propranolol did not affected significantly ($P < 0.05$) the phototactic behavior of exposed adults. Juveniles showed a lower negative phototactic behavior and only those exposed to carbamazepine had their negative phototactic behavior significantly ($P < 0.05$) diminished relative to unexposed ones at 100 ηg/L. Likewise occurred for reproduction responses, behavioral responses were also non-monotonic.

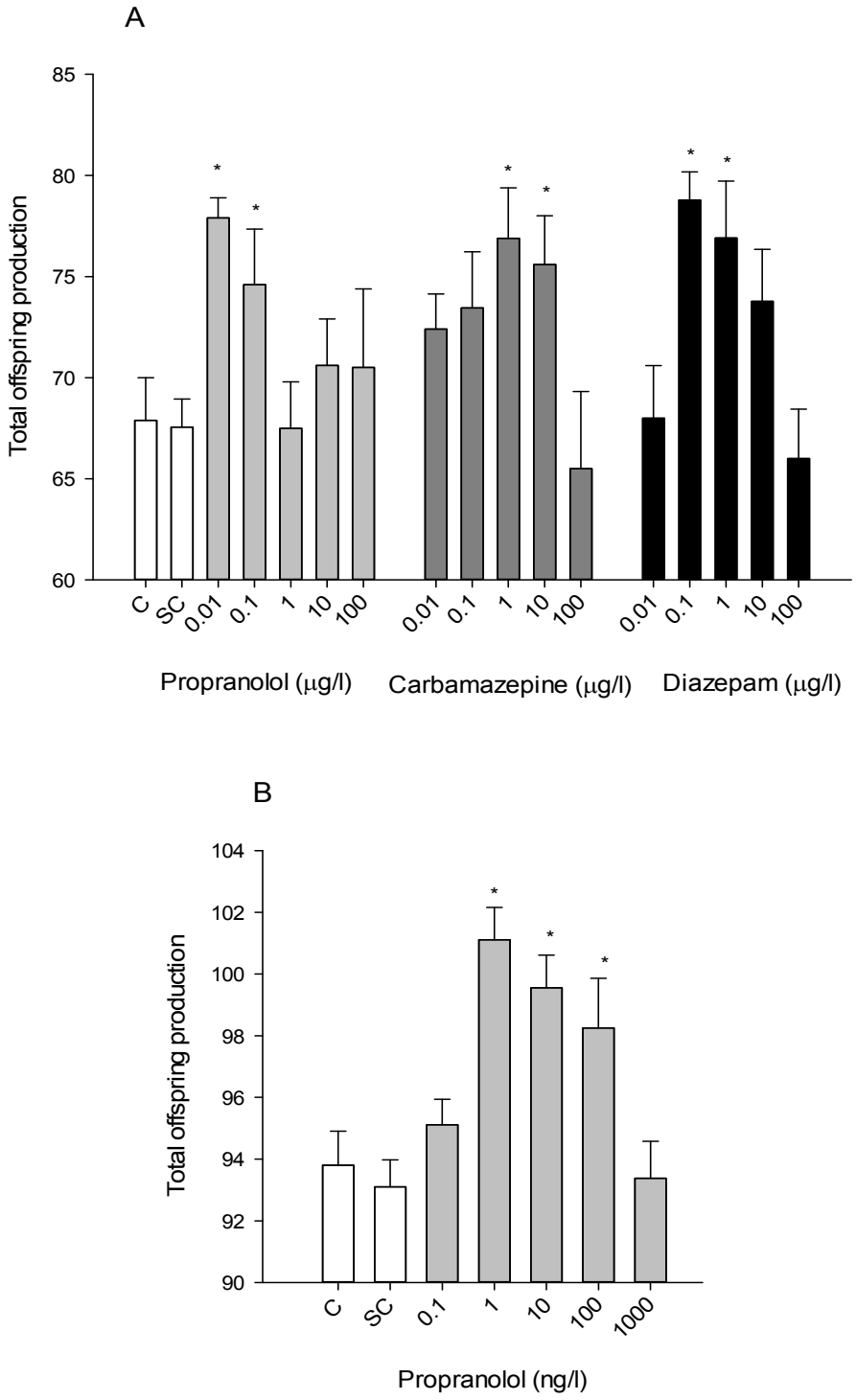

Figure 6.1 Cumulative total offspring production (Mean ± SE, N=10) of *D. magna* individuals exposed to the tested pharmaceuticals during 21 days in the first (A) and second (B) experiments. * indicated significant (P<0.05) differences following ANOVA and Dunnett's post hoc tests. C, SC are control and solvent controls, respectively.

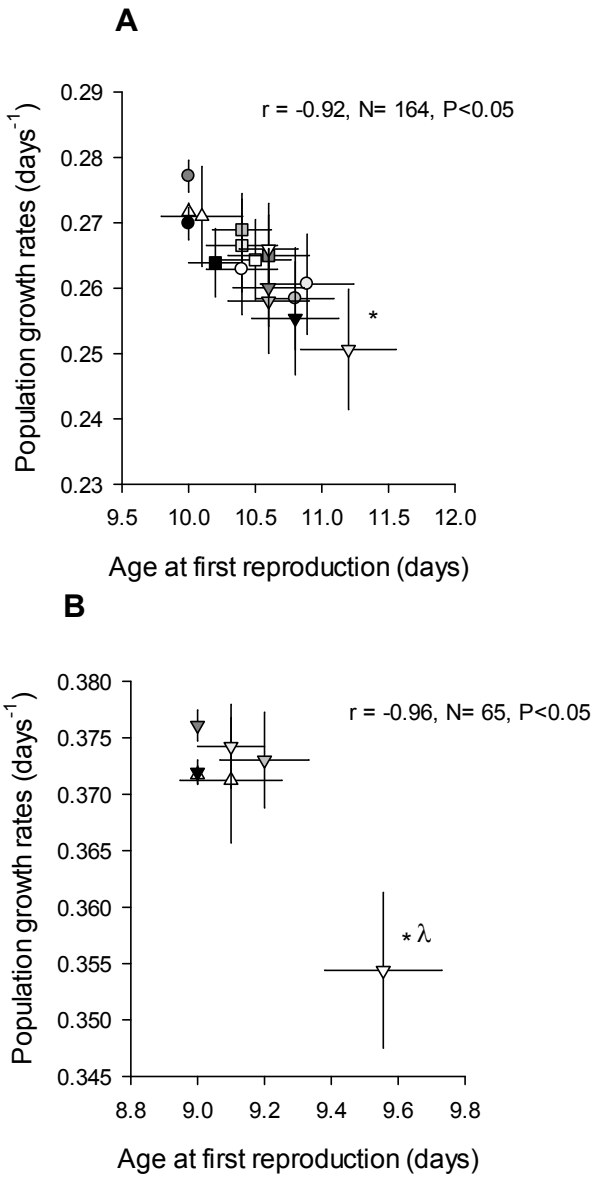

Figure 6.2 Population growth rate vs age at first reproduction (Mean ±SE, N=10) bi-plots of *D. magna* individuals exposed to the tested pharmaceuticals during 21 days in the first (A) and second (B) experiments. Upright triangles, inverse triangles, squares and circles correspond to controls (unexposed and solvent controls), propranolol, carbamazepine and diazepam treatments, respectively. Darker symbols represent increasing concentrations. In each graph correlation coefficients are depicted. *,λ represent significant (P<0.05) treatment effects relative to controls of age at first reproduction and population growth rates, respectively, following ANOVA/Kruskal-Wallis and post-hoc Dunnett's/Wilcoxon and Wilcox's tests.

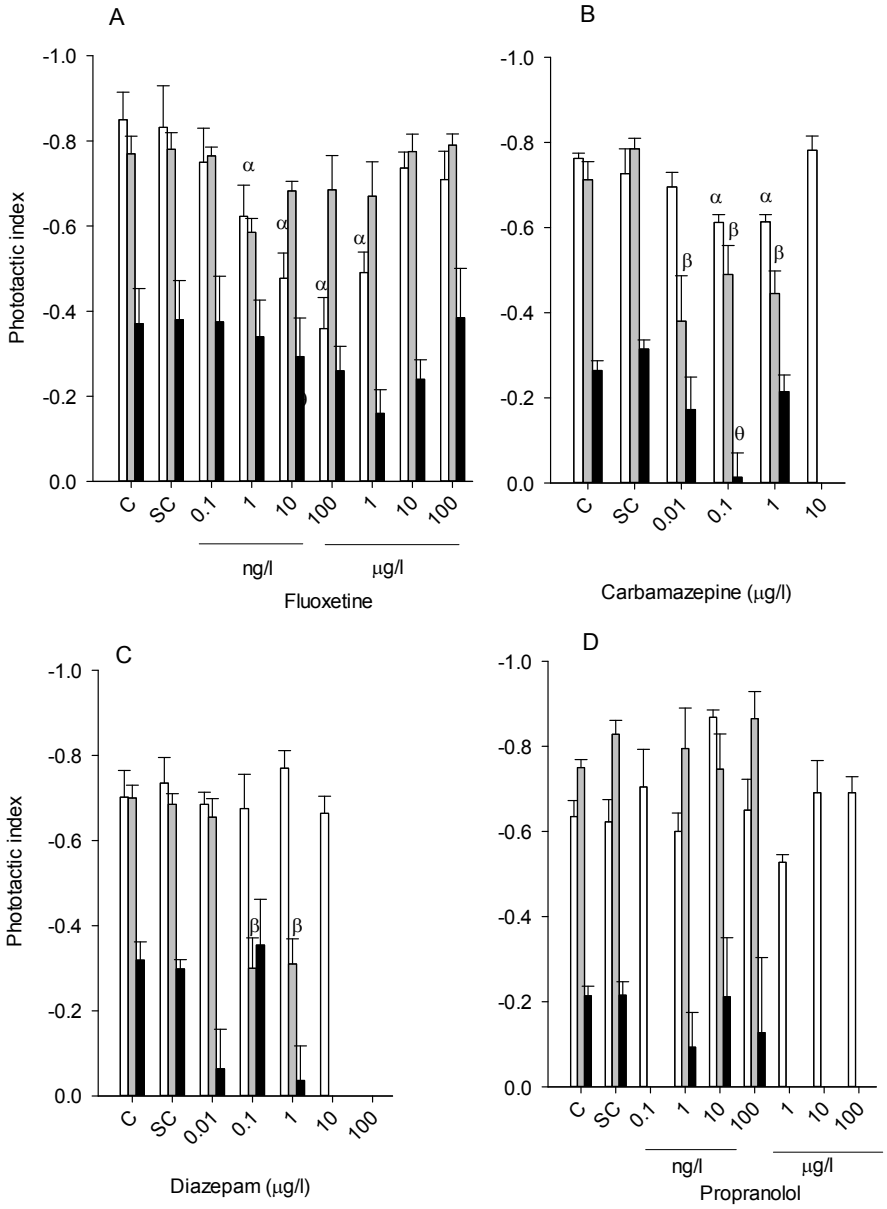

Figure 6.3 Phototactic index (Mean ± SE, N=4) of *D. magna* juveniles and adults exposed to fluoxetine (A), carbamazepine (B), diazepam (C) and propranolol (D). Results from experiments performed on 8 day old adults exposed during their entire life or 2 days and those of juveniles exposed for 48 h are depicted in white, grey and black bars, respectively. α, β and θ identify those treatments significantly ($P<0.05$) different than solvent controls in adults exposed for 8 and 2 days and juveniles, respectively, following Kruskal-Wallis and Wilcoxon and Wilcox's post hoc tests. C, SC are control and solvent controls, respectively.

6.5 Discussion

Propranolol, diazepam and carbamazepine enhanced offspring produced at environmental relevant concentrations of 1 ηg/L, 0.1 µg/L and 1 µg/L, respectively. Several studies have reported previously that fluoxetine enhanced offspring production across a concentration rage of 10-80 µg/L (Campos et al., 2012b; Flaherty and Dodson, 2005). Lürling et al. (2006) and Wolfe et al. (2015) also found that carbamazepine and fluoxetine enhanced offspring production at 1-2.3 and 1 µg/L, respectively. Observed effects, however, were non-monotonic and occurred always within one or two orders of magnitude. All tested pharmaceuticals and fluoxetine are neuro-active compounds since targeted neurotransmitters (serotonin, GABA, epinephrine and norepinephrine) and/or their signaling pathways. Neurotransmitters control neuroendocrine organs that regulate most physiological and behavioral processes in crustaceans (Christie, 2011; Fong and Ford, 2014).

Recently, it was hypothesized that increased levels of synaptic serotonin made available by SSRI treatment increased post-synaptic neuronal activity in *D. magna*, which changes the perception of the food environment and switches life-history responses towards those normally found only at highest levels of food available: *D. magna* females reproduce earlier and produce more but smaller offspring (Campos et al., 2012a; Campos et al., 2012b). Experimental evidence confirmed this hypothesis only in part such that SSRIs affected offspring production at limited but not at high food rations. The phenotype associated with SSRI exposure, i.e. increased offspring production, was reverted when animals were co-exposed to SSRI and the serotonin-receptor antagonist cyproheptadine (Campos et al., 2012a). It was also shown that SSRIs de-regulate genes related to serotonin signaling pathways in *D. magna* (Campos et al., 2013a). Putative 5-$HT_{1,7}$ receptors and serotonin transporter (SERT) gene homologues have also been found in the *D. magna* close relative *D. pulex* genome (McCoole et al., 2012a). The above mentioned data, however, do not demonstrate that SSRI act similarly than humans. Indeed in the zebra mussel (*Dreissena polymorpha*) it was reported that SSRIs can act as ligands of 5-HT receptors (Fong et al., 2003) inducing spawning. In the nematode

Caenorhabditis elegans effects of the SSRI fluoxetine on egg laying behavior also occurs in transgenic organisms lacking serotonin transporters and related receptors (Dempsey et al., 2005). This means that in invertebrates SSRIs can affect serotonin transporters, serotonin and other receptors. In crustaceans serotonin regulates neurosecretory organs that release neuro-hormones that control reproduction, growth, maturation, immune function, metabolism, behavior and color physiology (Fong and Ford, 2014). The GABA signaling pathway has been related to the expression of predatory induced life-history defenses in *Daphnia* like growth, the timing and reproductive output (Weiss et al., 2012). GABA has also been detected in the neuroendocrine pericardian organ in crabs (Christie, 2011). β-blockers affect *Daphnia* heart beat similarly than in humans (Villegas-Navarro et al., 2003). Thus it is likely that the tested pharmaceuticals affected reproduction altering the neuroendocrine system in *Daphnia*.

Mechanisms for non-monotonic response of endocrine active compounds are well known and include cytotoxicity, cell and tissue-specific receptors and cofactors, receptor selectivity, receptor down-regulation and desensitization, receptor competition, and endocrine negative feedback loops (Vandenberg et al., 2012). Previous studies reported that in *Daphnia* carbamazepine decreased population growth rates at 200 µg/L, and propranolol and fluoxetine impaired reproduction at 110 µg/L and 125 µg/L, respectively (Dzialowski et al., 2006; Hansen et al., 2008; Lürling et al., 2006). These reported inhibitory concentrations are quite close to the upper tested range (100 µg/L). There is also reported evidence that in other related species such as *Ceriodaphnia*, sertraline, which is also a SSRI, delayed reproduction, inhibited growth and reproduction at 4.8 µg/L in second generation exposed individuals. Thus observed non-monotonic response can be associated to general toxicity mechanisms occurring at high concentrations. Nevertheless, caution has to be paid when comparing toxicity across studies since factors such as pH and enantionespecificity can affect the toxicity of the studied pharmaceuticals (Boström and Berglund, 2015; Stanley et al., 2007; Stanley et al., 2006)

Most pharmaceutical treatments also delayed reproduction although only propranolol did so significantly. In growing populations the timing of reproduction (i.e. age at first reproduction) and offspring production contributed linearly and logarithmically to population growth rates, respectively (Barata et al., 2002). This means that the tested compounds, despite of enhancing reproduction, also delayed reproduction and hence affected negative on population growth rates. As a result population growth rates decreased in most pharmaceutical treatments although only in the second experiment were significantly affected by propranolol.

Behavioral responses of *D. magna* adults and juveniles exposed to fluoxetine, carbamazepine and diazepam were affected at similar concentrations than those observed for reproduction and were also non-monotonic. All three chemicals decreased negative phototactic behavior, which means that made exposed individuals more attracted to light. Observed behavioral changes to light thus are similar to those reported in the amphipod *Echinogammarus marinus* (Guler and Ford, 2010). Adults exposed for just 48 h to carbamazepine and diazepam showed greater responses than those exposed during their entire life. For fluoxetine, however, despite that adults exposed during short and long periods had similar response patterns, only the latter group had their behavior significantly affected. Fluoxetine was the compound having the strongest effect on phototactic behavior, followed by carbamazepine and diazepam. Negative phototactic behavior of adult females decreased from 1 ηg/L to 1 μg/L of fluoxetine, from 0.01 to 1 μg/L of carbamazepine and from 0.1 to 1 μg/L of diazepam Despite that *D. magna* juveniles had lower negative phototactic behavior, they responded similarly to the tested chemicals than adults. There is reported evidence showing that antidepressant action of fluoxetine in humans occurs after long exposures of about a month (Pérez et al., 2001), but recent studies indicated that effects of antidepressants can be detected within hours when more sensitive responses are measured (Schaefer et al., 2014). This is not the case for carbamazepine or diazepam that act shortly after being ingested (Besser, 1967; Post, 1988). In the amphipod *Echinogammarus*

marinus behavioral effects of fluoxetine were obtained after exposures of one week or longer.

Our results, thus, agree with most of previous studies and indicate that carbamazepine and diazepam affected phenotypic responses (i.e. behavior) shortly after exposures, whereas those of fluoxetine required at least one week. Likewise reproduction, behavioral effects were non-monotonic. Guler and Ford (2010) found that behavioral responses were non-monotonic and that fluoxetine decreased negative phototactic behavior in the amphipod *E. marinus* having the greatest effects at 100 ηg/L. Carbamazepine, on the other hand, only affected geotaxis behavior of *E. marinus*, the position of the animals in the water column without a light stimulus, at 10 µg/L. Locomotion and ventilation behavioral responses of *Gammarus pulex* to fluoxetine, carbamazepine and ibuprofen showed a dual response (De Lange et al., 2006): at low concentrations (1-100 ηg/L) the three pharmaceuticals increased ventilation, whereas at high concentrations increase locomotion. Neurotransmitter receptors are subject to ligand-induced desensitization, that is, they can become unresponsive upon prolonged exposure to their neurotransmitter (Nicosia et al., 2003; Yamauchi et al., 2006). Thus, desensitisation could explain the reduced behavior effect at higher concentrations and at longer exposures. Reported effects on phototaxis behaviour in *D. magna* have also been reported for other chemicals. The antibiotics lincomycin (5 mg/L) and bacitracin (10 mg/L) decreased phototaxis but aminosidine (10 mg/L) increased it (di Delupis et al., 1992). There are also reported results showing that cadmium and naphthalene decreased phototaxis in *D. magna*, but at concentrations close to those impairing survival (60 µg/L Cadmium and 1 mg/L naphtahalene). Thus when studying changes on behavior it is important to differentiate between specific and general toxic effects. In our study, concentrations of the tested pharmaceuticals causing changes in behavior were too low to be considered toxic, thus observed effects were likely to be related to a specific neurological response. Despite that the studied pharmaceuticals affected reproduction and behavior at similar concentrations, existing experimental evidence is too limited to hypothesize that both responses may share a common mechanisms of action. In crustaceans and in particular in

Daphnia, serotonin together with dopamine and other ammines regulate behavior responses to light (Christie and McCoole, 2012; Ehrenström and Berglind, 1988; Fong and Ford, 2014; Strauss and Dircksen, 2010). If fluoxetine increases serotonin activity in *D. magna* (Campos et al., 2012a), then it is likely that by doing so also alters its response to light stimulus. The main target of carbamazepine in humans is blocking voltage dependent sodium channels (Ambrósio et al., 2002), but there is reported information indicating that carbamazepine also causes increases in extracellular serotonin levels (Dailey et al., 1997). There is also evidence that in mammals carbamazepine activates dopamine receptors (Montezinho et al., 2007). Accordingly, carbamazepine may also act like fluoxetine increasing serotonin/dopamine activity and hence altering behavioral responses to light. There is reported information showing that diazepam decreases anxiolytic behavior in fish and increases locomotion activity in decapod crustaceans, probably acting on GABA receptors (Bencan et al., 2009; Snyder and Peeke, 2001). Diazepam is also known to reduce expressed anti-predatory life-history behavior in *Daphnia* interacting with GABA (Weiss et al., 2012). Negative phototactic behavior is an adaptive anti-predatory behavior (Cousyn et al., 2001) and hence could be also regulated by GABA and be affected by diazepam. Our results for diazepam, thus, agree with the previous argument. Propranolol not only binds to β- adrenergic receptors but also to 5-HT_1 receptors in humans acting as an antagonist (Tierney, 2001). There is, however, no reported information of behavioral effects of β-blockers in *Daphnia* or in other crustacean species. In crickets α-adrenergic receptor antagonist but not β-blockers alter aggressive behavior to intruders (Rillich et al., 2011).

Reduced phototactive behavior of animals exposed to fluoxetine, carbamazepine and diazepam is maladaptive since it would increase the chance of adults to be preyed upon fish in surface waters during daylight. Fish predation in *Daphnia* is size dependent, being stronger in larger individuals (Barata et al., 2001). Consequently we should expect less marked negative phototaxis in juveniles as it was observed in this study.

6.6 Conclusions

In summary, the four tested neuro-active pharmaceuticals were able to enhance reproduction and, except propranolol, also to alter phototactic behavior in a maladaptive manner. The studied pharmaceuticals enhanced reproduction at a cost of delaying first reproduction and hence negatively affected population growth rates. Altered phototactic behavior is always maladaptive in *Daphnia* populations living in lakes with fish. Differential responses occurred at concentrations ranging from 1 ηg/L to 1 µg/L, which are close to measured residue levels of these pharmaceuticals in surface waters. Reported responses were non-monotonic and indicate that at low levels these compounds act specifically but at high concentrations act unspecifically and become toxic. This means that environmental risk assessment toxicity testing of pharmaceuticals need to focus in assessing specific physiological effects that for neuro-active pharmaceuticals may occur at the ηg/L range. This is the case for anti-depressants (Fong and Ford, 2014; Ford and Fong, 2015).

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Chapter VII.

General Discussion and Conclusions

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7.1 General Discussion

During the last decades, the release of a wide array of chemicals, including the ones of emerging concern, has been threatening the health and balance of ecosystems (Bottoni et al., 2010; Ginebreda et al., 2014; Kummerer, 2010). In this context, elucidating potential adverse effects of chemicals, excluding confounding factors e.g. abiotic, geographical and additional stressors among others, constitutes a new pressing challenge for the scientific community (Floury et al., 2017; Navarro-Ortega et al., 2015). All this increases the inherent difficulties of field studies, in which potential interactions among different chemicals and their interplay with environmental factors make the identification of sublethal toxic triggers extremely challenging (Amiard-Triquet, 2015; Hooper et al., 2013; Laetz et al., 2015).

The ultimate objective of ERA is to assess the ongoing status of a specific ecosystem and provide sufficient information for decision-making with the final purpose of protecting the environment from unwanted effects of chemicals (Forbes and Galic, 2016). New upcoming regulations, such as the European chemicals legislation (REACH) and the WFD, brought more attention towards the possible negative effects in ecosystems and increased the demands on ERA (Hernando et al., 2011; Wilks et al., 2015). Nowadays, we are facing the massive task of assessing the risk for a continuously increasing number of compounds and complex chemical mixtures while protecting the integrity and diversity of ecosystems.

While assessing the risk for human health concerns only one species, ERA should ideally consider a multitude of species, with their diversity in morphology, physiology, and ecological peculiarities (Breitholtz et al., 2006). In practice this is not possible, an issue which may have strong consequences for the relevance of the process. The choice of a selected number of representative

key species for testing is critical and interspecies extrapolations of effects (seen in a few test species, and often only under experimental conditions) to the entire ecosystem are paramount (Breitholtz et al., 2006; Walker et al., 2012). Among the available test organisms, *D. magna* represents a good ecotoxicological model due to its key ecological role in freshwater ecosystems as a grazer of algae and prey for many fish and invertebrate predators (Lampert and Kinne, 2011). *D. magna* is also easy to culture, it has a short life-cycle and in the lab it often reproduces parthenogenetically, so that it can be maintained as genetically homogeneous clonal lines (Ebert, 1992). The physiology and ecology of the *D. magna* and related species is well known and its genome has been fully sequenced and mostly annotated, thus having a great potential for mechanistic applications (Colbourne et al., 2011; Orsini et al., 2016; Shaw et al., 2008). This confidence is supported by the always increasing number of papers using *Daphnia* species as their model organism.

In order to expand the scientific basis of ERA, another crucial issue to be addressed is to unravel mechanisms or MoA of chemical pollutants (Breitholtz et al., 2006; Escher and Hermens, 2002). Understanding what happens at the molecular level when a chemical enters an organism and then how these effects propagate and translate into an adverse effect (apical endpoint) is of key importance for a more causal comprehension of toxic effects (Ankley et al., 2007; Ankley et al., 2010; Forbes and Galic, 2016). The increasing use of sensitive biomarkers and endpoints together with advanced molecular techniques in ecotoxicology would help to reveal new MoAs for individual substances and finally improve future ERA. By all means, the selection of specific endpoints and test organisms is a challenging task. Under this view and in order to meet the objectives of environmental protection, it is extremely important the continuous development of new methods and approaches which include scientifically relevant combinations of test methods, species, and endpoints. Integration of different endpoints and bioassays coupled with chemical analyses stays paramount (Bednarska et al., 2013; Hagger et al., 2006; Hernando et al., 2011; Santos et al., 2017; Wilks et al., 2015).

The work presented in this thesis covers different approaches of biomonitoring (through three field-case studies and one study in laboratory), different bioassays and contaminant types, thus allowing important conclusions under an ERA perspective and at the same time defining important holistic methodological approaches in order to improve it. Overall, the use of sensitive endpoints as physiological biomarkers of exposure e.g. feeding inhibition test and behavioral assay, *in vitro* approaches and molecular techniques provides us with deeper information than traditional responses such as death or reproduction which are generally unsuitable when dealing with environmental relevant concentration (usually low, in the range of ηg/L) and their consequences.

In chapter 2 of this monography, a comparative study of three Spanish rivers (Ebro, Llobregat, Jucar) was performed with the combined use of molecular (gene expression), physiological (biochemical responses) and individual (feeding inhibition rate) markers in *Daphnia magna* and these results were related to over 150 trace contaminants detected in the waters. The main aim of this study was to validate the use of molecular biomarkers in field studies. In the past decade McWilliam and Baird (2002) developed a cost effective post-exposure feeding field assay in *Daphnia*, which evaluates detrimental effects on grazing rates of previously exposed organisms in the field. The assay was proven to be robust in detecting effects of metals, pyrethroids and other pesticides and insensitive to confounding environmental factors such as changes of flow rates, water temperatures and presence of suspended solids (Mc William and Baird, 2002). Moreover, in previous studies, effects on feeding rates were complemented with effects measured in selected enzymatic biomarkers, thus improving the value of the post-exposure feeding *Daphnia magna* bioassay (Barata et al., 2007; Damasio et al., 2011; Damasio et al., 2008; Puertolas et al., 2011). The response of antioxidant, phase II detoxification and B-esterase enzyme activities (AChE, CbE) were also quite robust, showing low variability across clean sites, thus allowing to differentially evaluate detrimental effects in river sites affected by complex waste water effluents and pesticides. Nevertheless, with the exception B-esterases, the used

biomarkers and individual response were quite unspecific, thus preventing the identification of specific effects of particular contaminants. In chapter 2, the previous methodology was further improved by including the transcription pattern of 13 different genes encoding for general stress, metabolism and energy processes, molting and xenobiotic transporters. Daphnids were exposed directly in the field using cages deployed in the river water to increase the similarity to the natural scenario; additionally, individuals were also exposed in the laboratory to re-constituted water spiked with organic eluates extracted from water samples obtained concurrently at the same sites. The latter lab-reconstituted water procedure allowed to test if the response of the selected genes was specific of measured contaminants or in response to other factors in the field. To the best of our knowledge, this was the first work presenting this kind of comparison between field and laboratory exposure using water samples collected simultaneously at the same sampling site. The chosen biomarkers allowed the differentiation of the three river basins, heterogeneous for location and sources of pollution. Multivariate analyses indicated that measured *in situ* responses of most genes, of biomarkers and those of benthic macroinvertebrate diversity indexes were affected by distinct environmental factors. Conductivity, suspended solids and fungicides were negatively related with the diversity of macroinvertebrates, cholinesterase and feeding responses, which supports previous findings (Damasio et al., 2008). Gene transcripts of heat shock protein and metallothionein were positively related with 11 classes of organic contaminants and 6 metals. About 8 of the 14 genes considered responded similarly in field and lab exposures and were related with high residue concentrations of pharmaceuticals, triazoles, bisphenol A, caffeine and pesticides. These genes were related with signaling pathways of molting and reproduction, sugar, protein and xenobiotic metabolism. Overall, these results indicate that the application of molecular-based technologies in the field is a promising subject in water management. Nevertheless, consistency of gene responses was lower when compared to biochemical biomarkers, probably due to the high sensitive and early response of gene expression to any stimulus. A deeper knowledge in the *Daphnia* genome and gene function will allow in future to better define MoAs in response to contaminant and identify fingerprint genes

to be used as stress biomarkers through response to specific chemicals and/or scenarios of exposure.

In chapters 3 and 4, a study of forensic ecotoxicology was developed to unravel the major toxic components in a superfund site in Ebro River (Spain), using a combined approach of sublethal toxicity assays and chemical analytical/fractionation methods. Following a Toxicity Identification Evaluation (TIE) approach it was possible the identification of water soluble and particle-bound compounds in suspended solids causing important toxic effects. Nowadays the use of untargeted chemical analyses of environmental samples is still of little value due to its complexity, poor sensitivity and high costs, thus the use of specific bioassays as diagnostic tools can guide the identification of unknown toxic compounds that would have been missed by the traditional targeted chemical analysis. This is often accomplished by using TIE approaches, but these methods often suffer from the use of toxicity assays with poor specificity and sensitivity, thus being unable to detect effects of highly active substances that are present in very low amounts. The sampling of the presented study was performed during an event of extreme high water in an area highly contaminated by a chlor-alkali factory, which for over 50 years has been releasing organochloride residues and heavy metals that accumulated at high amounts in sediments nearby the factory wastewater outlet (Bosch et al., 2009). Targeted chemical analyses of known compounds revealed that under high water flow conditions organochlorine compounds and mercury present in the sediment were re-suspended and hence increased dramatically in sampling sites downstream the chlor-alkali factory. However, toxicity of water was higher upstream the factory, so it was unrelated with analyzed organochloride and metal residues in suspended material. Uncoupled chemical and toxicity data is a frequent result that prevents many studies to be published, although it may also lead to alternative hypotheses such as the iceberg effect (Tang et al., 2013). The previous hypothesis states that toxicity in the field is caused in most cases by many more compounds than those identified by using bio-analytical ecotoxicity tools. Indeed our laboratory has been facing this problem for years.

This means that upstream of the chlor-alkali industry there is an additional contaminant source, not yet identified.

Ebro River is subject of high regulations by many dams that are used as reservoirs of water and for electric power production (Petrovic et al., 2011). Several studies have reported the occurrence of toxic cyanobacteria species (e.g. *Anabaena*, *Planktothrix*) known to produce toxins such as microcystins and anatoxins upstream and in Flix reservoirs as well as in locations downstream (Hoyos et al., 2004; Quesada et al., 2004). Reported cyanobacteria occurrence, however, was limited to late spring and summer months when algal blooms of these species are more likely to occur. During periods of high water flow in Ebro River (when the accumulated ice/snow in mountains melt and/or during periods of heavy rain), dams are opened and hence reservoir waters from the bottom are mobilized and released into the river. This was the case of the upstream sampling site, which is located just after a big reservoir (Riba-Roja). In this regard, it was hypothesized that specific cyanotoxins mobilized from Riba-Roja reservoir were causing most of the observed toxic effects. It is important to take into consideration that our hypothesis faced three arguments against it: an unappropriated sampling time (winter, early spring), which is unusual to detect cyanobacteria, the potential occurrence of rare cyanotoxins such as anatoxin-a and the fact that there was unclear evidence that cyanobacteria cells were present in water samples. Thus, to test this hypothesis, it was necessary to develop a robust TIE procedure adapted to the chemical characteristics of known cyanotoxins, which are polar and water soluble, and are often present inside cyanobacteria cells, only being released to the water column during algae blooms, which was not the case.

Bioassays included:

- post-exposure *D. magna* feeding rate assay, which consists in exposing juveniles for 24 h to unfiltered water samples in a rotary wheel and then the assessment of feeding inhibition effects. This assay was previously proven to be quite robust in detecting toxic effects associated to suspended solids (Bosch et al., 2009);

- confirmation assays, in which animals were exposed to re-constituted lab water with suspended solids obtained in the field, which allowed to corroborate that the toxicity due only to suspended material;
- negative control assays, in which animals were exposed to filtered water samples, thus showing that there was no toxic components dissolved in water;
- feeding assays conducted with organic extracts of suspended solids obtained with distinct combinations of water, methanol and dichloromethane : methanol. This last step allowed the confirmation that toxic compounds present in suspended solids had an hydrophilic nature which resembled that of cyanotoxins.

Furthermore, the study was performed during several months using an exhaustive sampling campaign to demonstrate that the observed “toxic phenomena” were reproducible during periods of high water flow, when dams were open, but not during periods of low water flow. For this aim, it was also necessary to develop appropriate LC-MS analytical tools to be able to analyze selected cyanotoxins in the suspended solid water fraction. A new detection method by LC-MS/MS for three specific families of toxins was developed, which included three mycrocystins, anatoxin-a and okadaic acid (chapter 3). Several chromatography methods are available nowadays to detect these toxins in waters, but little effort has been put in their determination when bound to particles in sediments or suspended matters. Besides, the identification and quantification of the toxins present in environmental samples encountered the added inherent difficulty of the lack of (deuterated) commercial standards combined with the existence of many different isoforms of the same toxin. The new developed method allowed the identification of anatoxin-a residues bound to the suspended matter of the sampled river water, causing the observed toxicity. Results were then successfully verified by performing further bioassays and chemical analyses using a lab culture of an algal strain (*Plankthothrix agardhii*) able to produce anatoxin-a. Lab assays allowed fulfilling the last step of confirmation required by the TIE phase III approach. TIE approaches are powerful available procedures for performing environmental diagnostics on

water samples and whole sediments to detect anthropogenic contaminants that cause toxic effects, though using different strategies (Burgess et al., 2013). In this work. the TIE procedure on whole-organism (*in vivo*) toxicity testing allowed identifying with high certainty the compound responsible for the observed toxicity. Whereas there are several examples of successful application of TIE approach on waterbodies and sediments for toxicity evaluation (Burgess et al., 2000; Norberg-King et al., 1991; Werner et al., 2000), this study represents the first one that used a TIE approach to identify biological active cyanotoxins in real samples.

Another important ecotoxicological procedure to identify toxic components in complex environmental samples is the Effect Direct Analysis (EDA) that may or may not be included in a TIE approach. It is based on the use of specific assays (in many cases *in vitro* ones), sensitive to specific chemical families. This approach was used in chapter 5, where an integrated study of chemical analyses and biological indicators provided an overall insight into the water quality of Tablas de Daimiel National Park (TDNP), a lagoon of high ecological value for aquatic wildlife. The study was part of a wider biomonitoring program held during the last five years in the area, aimed to assess the overall chemical impact on the ecosystem, especially with regard to effects on resident birds and fishes. This work differs from the previous ones in the characteristics of geographical sampling location, bioassays used (a combination of *in vivo* and *in vitro* testing) and chemical analyses performed (including both GC-MS/MS and LC-MS/MS). A successful application of this kind of approach in environmental studies has been used for a fast screening of unknown pollutants in the environment and to improve the analysis of joint effects of mixtures (König et al., 2017). *In vitro* testing, such as Recombinant Yeast Assays (RYA) for detection of dioxin-like activity and estrogenicity, is a powerful methodology as alternative to animal (*in vivo*) methods, being more stable (precise), less time-consuming and generally less expensive (Beyer et al., 2014; Mazzeo et al., 2016; Mesquita et al., 2014; Puy-Azurmendi et al., 2014). Besides, the use of appropriate *in vitro* systems allows studying early cellular responses and predicting toxicity effects *in vivo* (Blaauboer et al., 2012). In the last decades,

different approaches have been used in order to provide a successful ecological water status assessment. The use of bioassays/biomarkers (when compared to studies at the community level) are especially useful as early warning systems of toxicity and also to explore the causes of ecological impairment, thus allowing to better understand the causal relationships of observed effects (Damásio et al., 2007; Martinez-Haro et al., 2015; Prat et al., 2013). Main water sources to TDNP floodplain come from two rivers (Guadiana and Cigüela River). Both rivers receive effluents from waste water treatment plants (WWTP) of nearby urban nuclei. This means that there is a high potential for strong estrogenic compounds present in WWTP effluents to contaminate the floodplain and affect aquatic vertebrate biota living on it. Besides, nearby the lagoon there is also a wastewater drainage pond (Navaseca), which is used as a feeding ground by many species of aquatic birds living in the area, thus representing a threat for the wildlife inhabiting TDNP. Furthermore, the reduction of the drainage area and an overexploitation of groundwater for irrigation purposes led in the past decades to the near desiccation of TDNP and, finally, to the ignition of a smoldering peat fire inside the TDNP in August 2009. This fire posed an enormous risk for both the physical structure supporting the ecosystem and the quality of groundwater beneath it (Moreno et al., 2011). Moreover, fires are an important source of pollution by polycyclic aromatic hydrocarbons (PAHs) for Mediterranean rivers (Vila-Escale et al., 2007). Following this, a TDNP Hydric Regeneration Plan was implemented to stop (or at least mitigate) this environmental degradation. Nevertheless, it is possible that today PAHs or related compounds having dioxin-like activity may still be present in TDNP and affect aquatic biota living there. As a consequence, considering the above mentioned sources of pollution, the study of chapter 5 focused on the chemical and biological characterization of estrogenic and dioxin-like compounds typically found in WWTP effluents and surface waters affected by forest fires (PAHs). In addition, we use feeding rate responses of *D. magna* as a generalized measure of sublethal toxicity. Results indicated that the water from TDNP was relatively clean, showing low residue levels of most analyzed pollutants and low estrogenic and dioxin-like activities. Conversely, water samples from Navaseca pond had elevated levels of estrogenic compounds as well as associated

estrogenic activity and inhibited *D. magna* feeding rates. Unfortunately, potential contaminants inhibiting *D. magna* feeding rates such as some pesticides and cyanotoxins were not analyzed. In conclusion the study presented in Chapter 5 indicates that TDNP, despite of having a good ecological water quality, is threatened by nearby waste water drainage ponds like Navaseca, which may act as a pollution source of contamination. Future remediation strategies should focus on improving the water quality of these associated ponds.

In chapter 6, a laboratory-based assessment of the potentially detrimental consequences of neuro-active pharmaceuticals in *D. magna* is presented. Concentrations used were in the ηg/L and μg/L range to simulate those encountered normally in the environment. Their effects on aquatic biota may be difficult to evaluate as they are bioactive molecules specifically designed to have very low toxicity, but to exert a precise biological effect through specific receptors/pathways (Bottoni et al., 2010; Daughton and Ternes, 1999; Fent et al., 2006). It is important to note that several neuro-active compounds are designed to affect neurotransmitters (serotonin, dopamine, epinephrine, *gamma*-aminobutyric acid-GABA), which regulate many physiological and behavioral processes (Fong and Ford, 2014; Ford and Fong, 2015). Recently, several studies have reported that very low concentrations of antidepressants and anxiolytic drugs alter the behavior of fish, mollusks and crustaceans (Brodin et al., 2013; Fong and Ford, 2014; Ford and Fong, 2015). Therefore, neuro-active drugs may also affect aquatic invertebrates. This is probably related to the fact that human targets of antidepressants, anxiolytic and neuropathic drugs such as selective serotonin re-uptake inhibitors (SSRIs), drugs blocking voltage-gated sodium channels and/or GABA agonists as well as certain antihypertensive compounds are highly conserved across vertebrates and 61% of them are also found in the invertebrate crustacean *Daphnia* (Gunnarsson et al., 2008). Phototactic behavior, either negative or positive, is a response common to most organisms (Haney, 1988; Ringelberg, 1964). Many organisms escape from light since they would be more visible to predators (Cousyn et al., 2001; De Meester and Cousyn, 1997; Lampert, 1989; Ringelberg, 1999). Recent studies indicated that psychiatric drugs, such as SSRIs, affect the

phototactic behavior of amphipods (Bossus et al., 2014; Guler and Ford, 2010). In daphnids, the vertical migration in the water column in response to light exposure provides a sensitive approach to assess potential sublethal effects that would not be assessed by more traditional testing (De Meester, 1991, 1993; Michels et al., 1999). *Daphnia* diel migration represents an important evolutionary achievement of protection behavior against predators, where a normal pattern includes a nocturnal ascent (for feeding closer to the surface) and a morning descent (for preservation purposes) (Lampert, 1989; Loose, 1993), thus any change affecting the vertical movement is of extreme importance under an ecological perspective. Previously, it was also reported in our laboratory that SSRIs were able to enhance reproduction in *Daphnia* under low food conditions since the mentioned compounds increased the levels of serotonin in the brain as they did in humans (Campos et al., 2012b; Campos et al., 2016). Several other traits are also affected by SSRIs exposure. Exposed organisms showed increased levels of aerobic metabolism, matured earlier and produced more although smaller offspring under low food conditions (Campos et al., 2012a). This switch in the reproductive strategy was proven to be detrimental under food depletion, when the production of few but larger offspring is ecologically more beneficial since offspring size increase their tolerance to starvation, while reducing the time to sexual maturity. In this chapter, the aim was to test the premise that other neuro-active drugs could have similar effects as SSRIs and in addition they could alter phototactic behavior. The substances tested included the SSRI fluoxetine, the anxiolytic diazepam, the neuropathic and anti-epileptic drug carbamazepine and the antihypertensive propranolol. SSRIs act by blocking the re-uptake of serotonin in the nerve synapses (Spinks and Spinks, 2002). Diazepam enhances the effect of the neurotransmitter GABA by binding to the benzodiazepine site on the $GABA_A$ receptor, leading to central nervous system depression (Riss et al., 2008). Carbamazepine is also a GABA receptor agonist since it potentiates GABA receptors (Ambrósio et al., 2002). Propranolol is a nonselective beta-blocker that blocks the action of epinephrine and norepinephrine on adrenergic receptors (Wisler et al., 2007). Results indicated that all tested drugs were able to increased offspring production at the ηg/L or low μg/L range, while

psychiatric, anxiolytic and anti-epilepsy drugs also decreased the response of *D. magna* individuals to light at these concentrations. These results suggest that these drugs produced similar effects in *Daphnia* despite having a different primary MoA, at least in humans. In fact, observed effects on reproduction and on behavior indicate that the tested compounds probably share a molecular target or pathway, most likely at the central nervous system, which regulates multiple physiological processes. This was the case of fluoxetine that by increasing serotonin activity changed the perception of food in *Daphnia,* increasing reproduction rates and aerobic metabolism (Campos et al., 2013; Campos et al., 2012a). Further work is needed to elucidate the mechanisms of action of the remaining three pharmaceuticals. For instance, there is evidence in humans that carbamazepine and fluoxetine de-regulated the brain arachidonic acid pathway (Bazinet, 2009), which in *Daphnia* is involved in the regulation of reproduction (Ginjupalli et al., 2015). It is also likely that the lack of a hemato-encefalic barrier in *Daphnia* allows these drugs to act in an unspecific way on neuronal receptors, thus affecting common regulatory pathways. Indeed unpublished work conducted later in our laboratory, led to the creation of serotonin-depleted *Daphnia* clones, using CRISPR/Cas9-mediated targeted mutagenesis (Nakanishi et al., 2014). Exposure of these knock-down animals to the SSRI fluoxetine showed enhanced reproduction (unpublished), as it was previouslt observed in the “wild-type” clone. This indicates that SSRIs may interact directly with serotonin or other neuronal post-synaptic receptors. We are also undergoing the development of a LC-MS/MS analytical method in order to measure neurotransmitter levels in *Daphnia* (e.g. serotonin, dopamine, epinephrine, norepinephrine, gamma-aminobutyric acid- GABA) which may help to elucidate more exhaustively the MoA of these drugs in the near future.

7.2 Conclusions

Overall, this thesis aimed to prove the importance and need of a complementary role of classical bioassays, specific biomarkers, molecular-based techniques and chemical analyses. When used in combination, all these approaches provide significant information to understand potential impacts of

anthropogenic pressures on aquatic biota. Along seven chapters, we discuss the inherent difficulties of field studies (where the interactions among chemicals and/or other confounding factors might occur) compared to laboratory-based methodologies, the limitations of this kind of approaches and future improvements needed.

The following conclusions were achieved:

1. The combination of individual, biochemical and gene transcription biomarkers in *D. magna* field assays coupled with chemical analyses of contaminant residues allowed a better assessment of causative effects of contaminants across three river basins impacted by different anthropogenic pressures.

2. Gene transcription responses have the advantage over biochemical responses to be less sample demanding and allow testing many signaling pathways at the same time. However, molecular responses are more variable and hence further work is required to assess their natural variability and specificity.

3. The use of *in situ* field exposures in parallel with lab exposures in reconstituted water with contaminant organic extracts is a robust method in order to confirm if observed effects in the field are caused by the correspective analyzed contaminants.

4. A new analytical method to simultaneously measure mycrocystins, anatoxin-a and okadaic acid levels bound to suspended particles within the water column and sediments was developed and applied to environmental samples from the Ebro River and Delta, revealing the presence of anatoxin-a and okadaic acid at different sites.

5. The combination of analytical methods to detect trace contaminants with the use of sensitive *D. magna* feeding and post-exposure feeding assays

toghether with an appropriated TIE approach allowed to identify an unknown source of toxicity present in the particle water fraction.

6. The cyanotoxin anatoxin-a (produced by the cyanobacteria *Planktothrix)* bound to river particles was correlated with the toxicity effects measured in the low Ebro River during an unusual period of high water flow.

7. The use of a combination of effect-directed bio-analytical assays, including recombinant yeast assays for both human estrogen and aryl receptor, *Daphnia* feeding inhibition assays and chemical analyses of estrogenic compounds and polycyclic aromatic hydrocarbons, allowed to characterize the ecological quality of water from Las Tablas de Daimiel floodplain and its associated ponds.

8. Water from las Tablas de Daimiel floodplain has low levels of contaminants and of related estrogenic and dioxin-like activity whereas that of the associated pond Navaseca was toxic to *Daphnia* and it highly contaminated by estrogenic compounds.

9. Neuro-active pharmaceuticals targeting different neurological pathways in humans were able to disrupt in a similar way the reproduction of *Daphnia*, enhancing offspring production at environmental relevant concentrations (ηg/L). Some of them including psychiatric, anxiolytic and anti-epileptic drugs, were also able to alter *Daphnia* phototactic behavior.

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