Ecotoxicological risk of pollutants in Iberian rivers

Maja Kuzmanović

PhD Thesis

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Directors: Antoni Ginebreda Martí, Damia Barcelo Culleres

Environmental Chemistry Department, Institute of Environmental Assessment and Water Research-Spanish Council for Scientific Research (IDAEA-CSIC)

Catalan Institute for Water Research (ICRA)

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Maja Kuzmanović

Directors:		Tutor:
Dr. Antoni Ginebreda Martí	**Dr. Damia Barceló Culleres**	**Dr. Francisco Xavier Sánchez Vila**
Full Research Professor	Full Research Professor	Associate Professor
Environmental Chemistry IDAEA-CSIC	Environmental Chemistry IDAEA-CSIC Catalan Institute for Water Research ICRA	Civil and Environmental Engineering UPC

Barcelona, May 2017

CSIC

UNIVERSITAT POLITÈCNICA DE CATALUNYA BARCELONATECH

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Author:
Maja Kuzmanović

Supervised by:
Antoni Ginebreda Martí and Damia Barceló Culleres

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SUMMARY

More than one-third of Earth's available freshwater is used for anthropogenic purposes, which has led to its contamination by numerous chemical compounds. Their presence in the environment might have negative consequences for the environment and human health. In order to identify the compounds that might cause adverse effects in the ecosystems, ecotoxicological risk assessment is performed by comparing measured or predicted concentration of the compound in the environment with the threshold concentration of a certain effect. Due to the growing awareness of chemical risk in the environment, there is an increase of scientific literature on the subject. However, we are still gathering evidence of the effects in ecosystems caused by chemicals and identifying the main drivers of those changes.

The aim of this thesis is to contribute to the growing scientific knowledge on the ecotoxicological risk of chemical compounds in the freshwater environment. Four rivers of Iberian Peninsula were used as case studies in this thesis; namely Llobregat, Ebro, Júcar, and Guadalquivir. The main drivers of risk for ecosystems in those rivers were identified and the evidence of effects caused by chemical compounds was provided.

The thesis is divided into eight chapters. Chapter 1 introduces the context of the environmental problems related to chemicals and the main concepts of the current scientific approaches used to solve these problems. In the final part of Chapter 1, pollution of studied rivers is reviewed from the data gathered within the SCARCE project. The objectives and thesis structure are described in Chapter 2.

In Chapter 3, prioritization schemes for freshwater organic pollutants were reviewed. Occurrence and risk of selected important pollutants in Europe and North America were compared. Pesticides and pyrene were identified as main risk driving compounds. However, the risk contributing compounds varied between sites in Europe and North America indicating the need for inclusion of river basin specific pollutants in the risk assessment.

In Chapter 4, ranking index (RI), a new method for prioritization of pollutants is introduced. RI classifies the pollutants into three categories of concern on the basis of their ecotoxicological potential and distribution in the study area. First category (widespread acute risk) includes the compounds with acute risk at more than 50% of sampling sites, second category (widespread chronic risk or limited area acute risk) include compounds with either acute risk at several sampling sites or chronic risk at many sites, compounds with negligible risk are classified into third category (no risk). By applying the RI to the dataset of more than 200 pollutants measured in the surface water of studied rivers, we identified that pesticides (e.g. organophosphate insecticides) and industrial organic compounds (i.e. alkylphenols) were of highest concern in the area, based on their toxicity to freshwater invertebrates and to a lesser extent to fish and algae. Emerging contaminants, such as pharmaceuticals (e.g. sertraline or

losartan) or biocides (triclosan) were among the compounds classified in the second category of concern, due to their chronic risk, especially to algae.

In Chapter 5, the site-specific risk of organic chemicals mixtures and metals was performed using concentration addition model (CA). It was found that mixtures of organic compounds and metals, posed an acute risk at 42% and 45% of total 77 sampling sites, respectively. The chronic risk was present at all sampling sites. The major drivers of acute and chronic risk were pesticides and metals. But, pharmaceuticals, industrial compounds, and personal care were additional contributors to the chronic risk. From the legislation perspective, we demonstrate that the risk posed by the Water Framework Directive (WFD) priority pollutants (PP) was significant and those compounds were among the highest contributors to the risk. However, we found that banned pesticides and emerging contaminants significantly contributed to the risk for ecosystems of studied rivers. We used different macroinvertebrate based indicators to find the link between chemical pollution and ecosystems changes. The conventional biodiversity indexes (Shannon's and Margalef diversity indexes) were unsuccessful in showing the communities change in relation to pollution and were related to many environmental variables including e.g. temperature or percentage of urban land use. However, we were able to find a significant relationship between pesticides toxicity gradient and a decrease of SPEARindex ("Species at Risk"), the stressor-specific indicator for pesticide pollution.

In Chapter 6, we used the functional traits of macroinvertebrate communities to find the evidence of pesticides toxicity and urban-related multiple stressors in studied rivers. The hypothesis was that the trait composition of macroinvertebrate communities would reflect the strategies used to cope with the respective environmental stressors. To test this hypothesis comprehensive multivariate statistical analysis were performed. It was identified that multiple stressors (high metal pollution, nutrients, elevated temperature and flow alterations) were present at 50% of the sampling sites, mostly in urban areas. There was a significant difference between communities exposed to pesticides and those exposed to urban-related multiple stressors, but a much larger study would be necessary to exclude the influence of natural variation and give more support to our findings. At urban sites, communities' dominant traits were multivoltine indicating dominance of resilient taxa and deposit feeding, which could be associated with the taxa resistant to hydrological disturbances or presence of nutrients. In contrast, at pesticide impacted sites taxa with high levels of egg protection was dominant, indicating a higher risk for egg mortality at those sites, potentially due to pesticides. The functional diversity of assemblages at urban sites was low, suggesting the functional homogenization of assemblages in urban areas, which might increase the sensitivity of ecosystems to future stressors. The results and the main findings of the thesis are discussed in Chapter 7 and the general conclusions are given in Chapter 8.

RESUMEN

Más de un tercio del agua dulce disponible se utiliza con fines antropogénicos que conducen a su contaminación por numerosos productos químicos. Su presencia en el medio ambiente podría tener consecuencias negativas para el medio ambiente y la salud humana. Con el objetivo de identificar los compuestos que pueden causar efectos adversos en los ecosistemas, la evaluación del riesgo ecotoxicológico se realiza comparando la concentración medida o prevista del compuesto en el medio ambiente con la concentración límite de un cierto efecto. Debido a la conciencia del riesgo de compuestos químicos en el medio ambiente, hay un aumento de la literatura científica sobre el tema. Sin embargo, todavía estamos reuniendo pruebas de los efectos en los ecosistemas causados por los productos químicos y la identificación de los principales impulsores de esos cambios.

El objetivo de esta tesis es contribuir al conocimiento científico sobre el riesgo ecotoxicológico de los compuestos químicos en el medio acuático. Cuatro ríos de la Península Ibérica fueron utilizados como casos de estudio en esta tesis; El Llobregat, el Ebro, el Júcar y el Guadalquivir. Se identificaron los principales factores de riesgo para los ecosistemas en esos ríos y se proporcionó la evidencia de los efectos causados por los compuestos químicos.

La tesis se divide en ocho capítulos. El capítulo 1 presenta el contexto de los problemas ambientales relacionados con los productos químicos y los conceptos principales de los actuales enfoques científicos utilizados para resolver estos problemas. En la parte final del capítulo 1, se analiza la contaminación de los ríos estudiados a partir de los datos recogidos en el proyecto SCARCE. Los objetivos y la estructura de la tesis se describen en el capítulo 2.

En el Capítulo 3, se revisa los esquemas de priorización de los contaminantes orgánicos de agua dulce. Se compara la ocurrencia y el riesgo de determinados contaminantes importantes en Europa y América del Norte. Los plaguicidas y el pireno se identificaron como principales compuestos que conducen al riesgo. Sin embargo, los compuestos que contribuyen al riesgo variaron entre sitios en Europa y América del Norte, lo que indica la necesidad de incluir los contaminantes específicos de la cuenca en la evaluación del riesgo.

En el Capítulo 4, índice de clasificación (RI), se introduce un nuevo método para la priorización de contaminantes. RI clasifica los contaminantes en tres categorías de preocupación en base a su potencial ecotoxicológico y distribución en el área de estudio. La primera categoría (riesgo agudo generalizado) incluye los compuestos con riesgo agudo en más del 50% de los sitios de muestreo, la segunda categoría (riesgo crónico generalizado o riesgo agudo limitado) incluyen compuestos con riesgo agudo en varios sitios de muestreo o riesgo crónico en muchos sitios, Los compuestos con riesgo insignificante se clasifican en la tercera categoría (sin riesgo). Al aplicar el RI al conjunto de datos de más de 200 contaminantes medidos en el agua de superficie de los ríos estudiados, identificamos que los

pesticidas (por ejemplo, los insecticidas organofosforados) y los compuestos orgánicos industriales (es decir, los alquilfenoles) eran los más preocupantes en la zona, A invertebrados de agua dulce y en menor medida a peces y algas. Los contaminantes emergentes, como los productos farmacéuticos (por ejemplo, sertralina o losartán) o biocidas (triclosán) se encuentran entre los compuestos clasificados en la segunda categoría de preocupación, debido a su riesgo crónico, especialmente a las algas.

En el capítulo 5, el riesgo de mezclas de productos químicos orgánicos y metales se realizó utilizando el modelo de adición de concentración (AC). Se encontró que las mezclas de compuestos orgánicos y metales, plantearon un riesgo agudo en el 42% y el 45% de los 77 sitios de muestreo, respectivamente. El riesgo crónico estuvo presente en todos los sitios de muestreo. Los principales factores de riesgo agudo y crónico fueron los plaguicidas y los metales. Sin embargo, los productos farmacéuticos, los compuestos industriales y el cuidado personal contribuyeron adicionalmente al riesgo crónico. Desde el punto de vista de la legislación, demostramos que el riesgo planteado por los contaminantes prioritarios de la "Water Framework Directive" era significativo y que esos compuestos estaban entre los que más contribuyeron al riesgo. Sin embargo, encontramos que los plaguicidas prohibidos y los contaminantes emergentes contribuyeron significativamente al riesgo de los ecosistemas de los ríos estudiados. Utilizamos diferentes indicadores basados en macroinvertebrados para encontrar el vínculo entre la contaminación química y los cambios en los ecosistemas. Los índices convencionales de diversidad biológica (índices de diversidad de Shannon y Margalef) no tuvieron éxito al mostrar que las comunidades cambian en relación con la contaminación y estaban relacionadas con muchas variables ambientales que incluyen p. Temperatura o porcentaje de uso de la tierra urbana. Sin embargo, pudimos encontrar una relación significativa entre el gradiente de toxicidad de los pesticidas y una disminución de SPEARindex ("Species at Risk"), el indicador específico de estresor para la contaminación por plaguicidas.

En el capítulo 6 se utilizó la composición de rasgos ("traits") de las comunidades de macroinvertebrados para identificar los efectos de los plaguicidas y los múltiples factores de estrés asociados al uso urbano del territorio en diferentes lugares. La hipótesis propuesta fue que la composición de rasgos de los conjuntos de macroinvertebrados reflejaría las estrategias desarrolladas por los mismos para hacer frente a los respectivos factores de estrés ambiental. Para probar esta hipótesis se realizó un amplio análisis estadístico multivariante general, el cual puso de manifiesto que múltiples factores estresantes influyen en las asociaciones de macroinvertebrados acuáticos en el 50% de los puntos estudiados, principalmente en los situados en áreas urbanas. Se identificaron varios factores estresantes físicos y químicos (alta contaminación de metales, nutrientes, temperatura elevada y

alteraciones del flujo) como característicos de los sitios urbanos. Se encontró una relación estadísticamente significativa entre la composición de los rasgos y la exposición de los conjuntos de macroinvertebrados a factores estresantes ambientales. Los factores de estrés relacionados con la actividad urbana favorecen la selección de taxones principalmente univoltinos y que se alimentan de depósitos. Por el contrario, los sitios afectados por plaguicidas dan lugar a la selección de taxones con altos niveles de protección de los huevos (mejor supervivencia del huevo), lo que indica un riesgo potencialmente mayor de mortalidad de los mismos. Por otra parte, la diversidad de rasgos de los conjuntos de macroinvertebrados en los puntos situados en áreas urbanas fue baja en comparación con la observada en los sitios afectados por plaguicidas, lo que sugiere la homogeneización de dichos conjuntos en las zonas urbanas.

En el capítulo 7 se discuten los resultados y principales hallazgos de la tesis. Finalmente en el capítulo 8 se presentan las conclusiones generales.

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CHAPTER 1

INTRODUCTION

1.1 THE GLOBAL FRESHWATER

Water is one of the major natural resources on Earth. It is essential to sustain life and to provide habitat for numerous aquatic species. About 70 percent of the Earth's surface is covered by water. But, only less than 3 percent of water on Earth is freshwater and about 60 percent of that freshwater is contained in glaciers and permanent snow cover. Besides, the available freshwater is unevenly distributed throughout the world and water shortages are already present in some areas (UNESCO 2003). Growing human population and climate change will increase the scale of water availability problems in the near future. It is predicted that by 2025, most countries of Africa and West Asia will face severe water scarcity due to increasing population and water demands (Figure 1.1 UNEP, 2008).

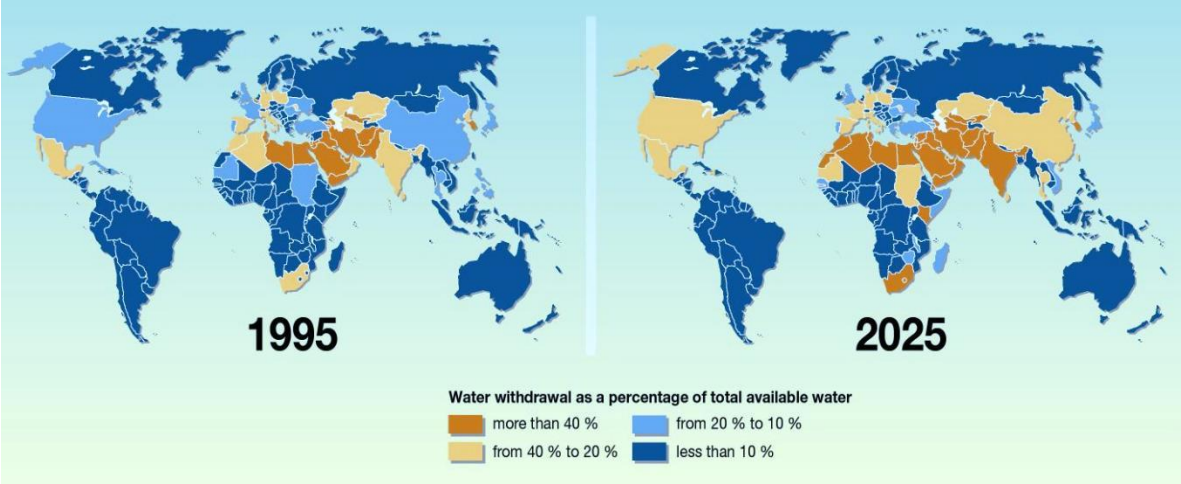


Figure. 1.1 Global water stress is increasing rapidly. It was estimated that more than 2.8 billion people in 48 countries will face water stress or scarcity by 2025, mostly in Africa and West Asia. The number of countries facing water stress or scarcity could rise to 54 by 2050, affecting about 40% of the projected global population (Gardner-Outlaw and Engelman 1997, UNEP 2008).

Despite the essential role of water for humans and ecosystems a great amount of freshwater is used in an unsustainable manner (UNESCO 2003) (Schwarzenbach, Escher et al. 2006). Water systems are widely transformed due to changes in land use, growing urbanization and industrialization and also engineering schemes like reservoirs or irrigation that are made to maximize human access to the water (Vörösmarty, Lettenmaier et al. 2004). More than one-third of available freshwater is used for anthropogenic purposes (i.e., agriculture, industry and domestic use) which can lead to its contamination by metals, nutrients and a variety of organic compounds (Schwarzenbach, Escher et al. 2006, Schwarzenbach, Egli et al. 2010). In fact, the availability of the freshwater resources is continuously decreasing due to the pollution caused by the agricultural runoffs and the disposal of insufficiently treated or untreated wastewater into natural waters. None the less this can have adverse effects on aquatic

ecosystems, but also the contamination can reach the groundwater, which might be used for human activities, including drinking (WWF, 2017).

Water quality degradation leads directly into environmental, social and economic problems. Water quality problems are common in both developing and developed countries (UNESCO 2003). Discharges of untreated wastewater can decrease aquatic biodiversity and elevate human health risks downstream. Since about 80% of today's sewage is discharged untreated this represents a worldwide problem, especially in the developing countries where proper wastewater treatment plants are not installed due to economic reasons, but also in developed countries since current wastewater treatment plants are not efficient enough to remove all the pollutants from the effluent (Eggen, Hollender et al. 2014) (Vörösmarty, Hoekstra et al. 2015). Furthermore, there is a growing concern about the adverse effects of emerging pollutants like pharmaceuticals, personal care products, pesticides and industrial chemicals, with still unknown long-term impacts on human health and ecosystems (UNESCO 2003).

The increasing degradation of surface and groundwater quality, with largely unknown long-term effects on aquatic life and on human health, could easily lead to environmental problems of great magnitude (Schwarzenbach, Escher et al. 2006). In the coming century, climate change and a growing imbalance among freshwater supply, consumption, and the population will alter the water cycle dramatically (Petrovic, Ginebreda et al. 2011). Therefore, the protection and sustainable management of freshwater sources are becoming of crucial importance. Undoubtedly, it is widely recognized that water quality degradation is one of the most serious ecological threats we face today. In the near future, the increasing pressure on water resources is expected due to demographic growth, urbanization and the effects related to climate change. Therefore, tackling the problem of global water pollution is one of the most important challenges of the present and future generations (UNESCO, 2003).

1.2 CHEMICAL POLLUTION OF FRESHWATER

According to the European Inventory of Existing Commercial Chemical Substances (EINECS), there are currently 100 000 commercially registered compounds in Europe and 30 000 of which are in daily use. Moreover, the increase in the chemical production is expected in the future. Many of these compounds eventually enter the natural freshwaters and may pose risk for aquatic ecosystems (Schwarzenbach, Escher et al. 2006). The problems related to macropollutants like nutrients or organic matter (occurring at µg/liter to mg/liter range) have been the subject of scientific studies for several decades but there is a lack of scientific knowledge regarding the presence and effects of micropollutants in natural ecosystems (Schwarzenbach, Escher et al. 2006). Current wastewater treatment plants have mainly been developed to remove macropollutansts such as organic matter, suspended solids or nutrients (Stamm, Räsänen et al. 2016). Micropollutants, including those referred to as emerging

contaminants, such as pharmaceuticals, personal care products, transformation products, household chemicals, some industrial chemicals or pesticides may not be eliminated in conventional wastewater treatment process and they leave treatment plants as a part of the effluent (Eggen, Hollender et al. 2014). Therefore, effluents may contain numerous chemical compounds in varying concentrations that were not previously considered in the pollution management. Besides, the diffuse sources of pollution such as runoff of pesticides from agricultural fields are widely recognized as one of the reasons for water quality degradation (Liess, Schäfer et al. 2008, Kattwinkel, Jan-Valentin et al. 2011). In fact, main sources of micropollutants in natural freshwaters are effluents of urban and industrial wastewater and surface runoff from agricultural fields or atmospheric deposition (Schwarzenbach, Escher et al. 2006) (Figure 1.2).

During the last decades, the occurrence of organic and inorganic micropollutants in the environment has attracted great interest and concern arisen about the possible undesirable effects of these compounds in the environment (Petrovic, Ginebreda et al. 2011). Micropollutants have been ubiquitously detected in freshwaters worldwide (e.g.,(Kolpin, Furlong et al. 2002, Loos, Carvalho et al. 2013, Busch, Schmidt et al. 2016, Ginebreda, Pérez et al. 2016, Kolpin, Glassmeyer et al. 2017). They occur in water bodies at very low concentrations mostly ranging from pg/liter to ng/liter (Ohe, Watanabe et al. 2004) but their levels are elevated above the natural background levels due to human activities (Stamm, Räsänen et al. 2016). Even though they are usually present in low concentrations, number and frequency of detections of micropollutants are increasing due to the improvement of analytical techniques (Brack, Altenburger et al. 2015). The concern for potential adverse effects of micropollutants in natural ecosystems is mostly due to the fact that many of these compounds are designed to be biologically active already at very low concentrations (e.g., pharmaceuticals, pesticides or biocides). Therefore, it is possible that similar or unexpected effects could be occurring in the environment on non-target species.

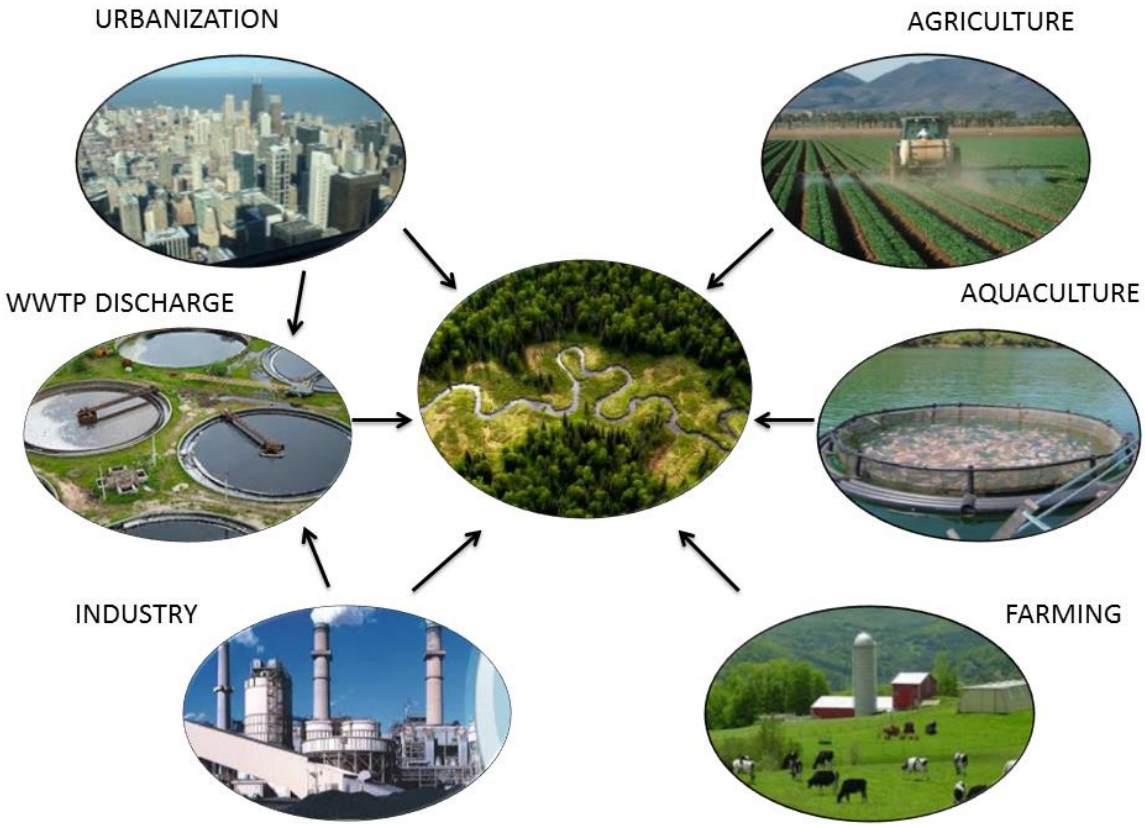


Figure 1.2 Sources of micropollutants in the freshwater

Besides, some of the micropollutants are very persistent in the environment (e.g., polybrominated diphenyl ethers used as flame retardants), while others are continuously released into the environment (e.g., pharmaceuticals and hormones). They can also potentially bioaccumulate and biomagnify through the food web or form toxic transformation products (Boxall, Sinclair et al. 2004, EPA 2014) (Kester, Bulduk et al. 2000, Sinclair and Boxall 2009). However, scientific knowledge and understanding on their effects including mixture effects, fate and accumulation is still limited, as well as the efforts on monitoring and regulating in freshwater and wastewater (Navarro-Ortega, Acuña et al. 2012). Micropollutants are a worldwide ecological issue, representing a potential threat to ecosystems and human health in both developing and developed countries due to the inadequate wastewater treatment (UNESCO 2003). Therefore, it is important to evaluate the risk of micropollutants and if necessary to include them into the monitoring and regulation programs (Boxall, Kolpin et al. 2003).

1.3 PRIORITIZATION OF POLLUTANTS

Because such a large number of chemicals released into the natural ecosystems there is a need to prioritize them according to their potential risk for aquatic species (von der Ohe, Dulio et al. 2011). However, it is impossible to conduct risk assessments for all the chemicals found in the environment. Besides, not all compounds that are present in the environment pose a significant threat to aquatic species. Different prioritization procedures are developed in order to direct the monitoring efforts towards the important compounds, to provide orientation to water managers and to contribute to the development of new regulations. These procedures are used to identify priority chemicals that, because of their importance, however, defined, should be examined with greater urgency and in preference to other chemicals (Troisi 2004). According to their importance as aquatic contaminants, several prioritization schemes were developed in recent years (Guillén, Ginebreda et al. 2012), Table1) based on different criteria.

Within the European Union (EU), the Water Framework Directive and its daughter directives the 2008/105/EC Environmental Quality Standards Directive, amended by the 2013/39/EU Directive are the main legislation for the protection and sustainable use of European freshwaters (Brack, Dulio et al. 2017). The aim of Water Framework Directive is to achieve good ecological and good chemical status of European surface waters by 2027. The good ecological status is evaluated on the basis of biological community descriptors, physic-chemical, and hydro-morphological quality elements. In order to evaluate good chemical status, the list of prioritized substances that are posing the highest threat to water quality has been identified by WFD. The prioritization was performed using combined monitoring-based and modelling-based priority setting that identified a list of priority substances that pose a significant risk to the EU aquatic environment and other hazardous substances from previous legislation (von der Ohe, Dulio et al. 2011). In addition, WFD requires identifying river basins specific pollutants (RBSPs) for different river basins.

The list includes contaminants which have been recognized as dangerous mainly on the basis of persistence, bioaccumulation and toxicity properties (PBT). In order to achieve good chemical status, water bodies must meet the Environmental Quality Standards (EQS) for the priority substances. EQS are regarded as protective thresholds to detect whether the environmental concentration of those substances pose a risk to aquatic species. Maximal acceptable concentration (MAC-EQS) and average annual concentration (AA-EQS) thresholds were established to account for the long term and short term effects, respectively. Furthermore, it is expected to update and review the list every 4 years. Moreover, EU Member States are obliged to identify pollutants of regional importance and provide EQS, monitoring schemes and regulatory measures for the deriving Environmental Quality Standards. That is, the Member States need to decide which are the candidate substances for further investigation and substances to be then declared as river basin specific pollutants (RBSPs) as

it was already done in Slovakia (Slobodnik, Mrafkova et al. 2012) and France (Botta, Dulio et al. 2012). However, in the evaluation of WFD in 2015, it was estimated that 47% of the water bodies failed to achieve good ecological status (EEA 2015) indicating more effort is needed.

1.4 ECOLOGICAL RISK ASSESSMENT OF MICROPOLLUTANTS

Risk assessment is the term used to describe the systematic procedure performed in order to assess the probability and severity of the potential adverse effects associated with an event (Shea and Thorsen 2012) (Figure 1.3). An ecological risk assessment (ERA) is defined as the process of evaluating the potential of the adverse effects in the environment as a result of exposure to one or more environmental stressors such as chemical pollutants, land change, disease, invasive species and climate change (USEPA 1992). Ecological risk assessment is used to provide a scientific basis for prioritizing problems that pose the greatest risk and to focus research efforts in areas of the greatest urgency (Shea and Thorsen 2012).

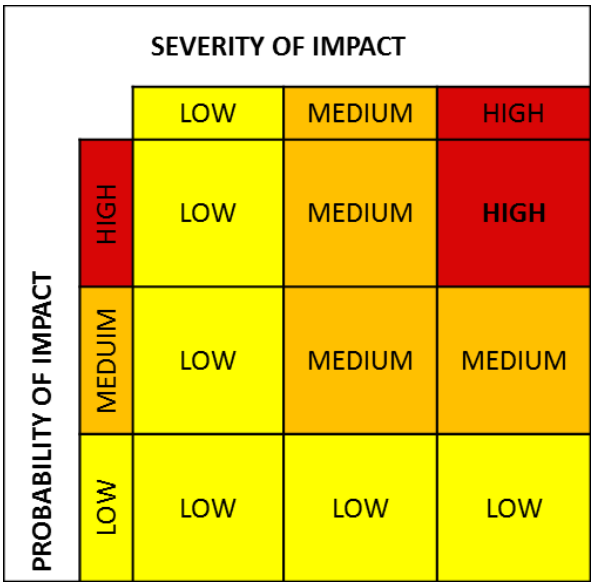


Figure 1.3 Simple risk assessment matrix visualizes the probability and severity of certain adverse event. It is used to prioritize and develop an effective strategy.

Chemical risk assessment generally follows a stepwise procedure of several tiered modules (Figure 1.4.) and provides a tool for evaluation and management of environmental pollution (Beyer, Petersen et al. 2014). It might be prospective i.e., assessing the effects that might occur in the environment due to predicted exposure to chemical or retrospective i.e., identifying the causal links between observed ecological effects and stressors in the environment (Calow and Forbes 2003). The general objective of chemical risk assessment is to protect the environment from adverse effects. An ongoing challenge and a great effort overall is needed to assess numerous chemicals and complex chemical mixtures while protecting many different species and diversity of ecosystems (Escher and Hermens 2002).

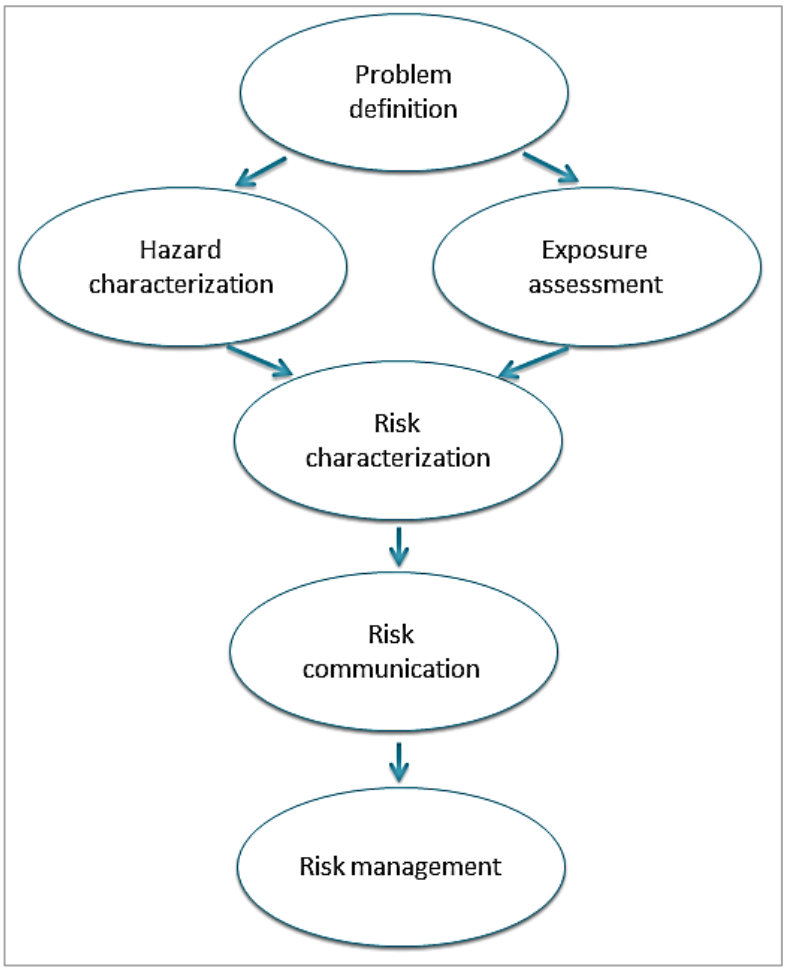


Figure 1.4 Chemical risk assessment procedure

ECOTOXICTY OF CHEMICALS

The basis for chemical risk assessment is found in ecotoxicology which assesses the dose-response curve of a certain chemical in standardized laboratory tests (Calow and Forbes 2003). Most commonly, the ecotoxicity of a given pollutant is determined by in vivo toxicity tests for standard test species (algae, invertebrates, and fish) each representing one trophic level (Rand 1995). The population of standard test species is environmentally exposed to different concentrations of chemical which provokes adverse effect or lethality on the certain percentage of individuals. The effect concentration express a percentile of individuals affected by a certain chemical (Figure 1.5.). Most commonly used a measure of acute toxicity is LC50, the concentration at which 50% of total test population suffers a lethal effect (Calow and Forbes 2003). Acute toxicity is defined as the effect caused by the short time exposure to the toxicant and chronic toxicity as a result of the long-term exposure to the toxicant (Leblanc 2004). The acute test provides the information of chemical concentration which, after short-term exposure to test species, provokes targeted endpoint effect, such as mortality (expressed as LC50) or sublethal effects such as immobility or growth stagnation (expressed as 50%

effect concentration, EC50). Chronic toxicity gives information of concentration of the chemical to which organism is exposed for the longer time or the whole lifetime of the organism and it is generally associated with sublethal effects (Leblanc 2004). Commonly used measures of chronic toxicity are no-observed-effect-concentration (NOEC) and lowest-observed-effect concentration (LOEC) that are showing the lowest concentrations of a chemical that provoke any observable effect on the test species after continuous, prolonged exposure (Leblanc 2004). Chronic toxicity data is still scarce for some group of compounds and sometimes can be derived from acute-to-chronic ratios (ACRs) (Ahlers, Riedhammer et al. 2006).

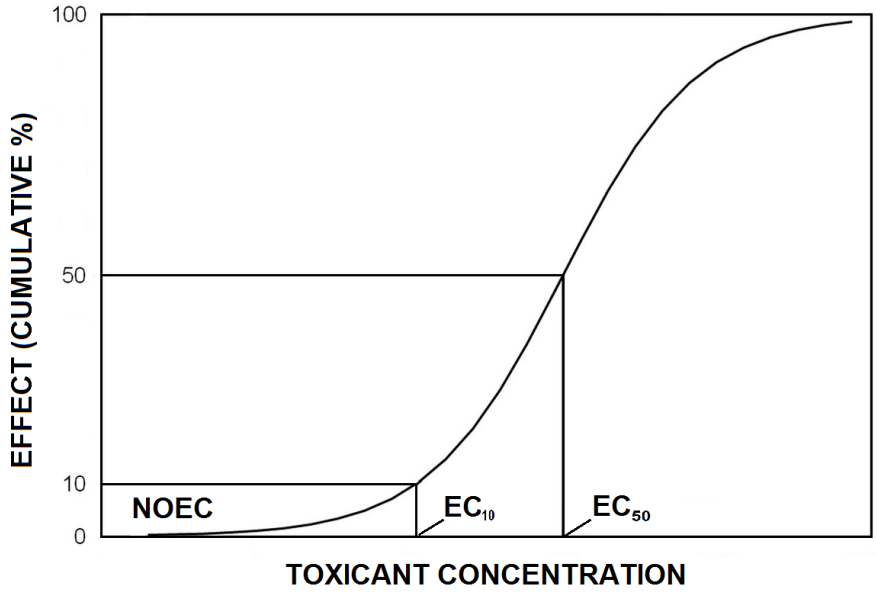


Figure 1.5 The cumulative percentage effects of a population or individuals when exposed to different concentrations of toxicant. NOEC-non-observed effect concentration, EC_{10} -10 % effect concentration and EC_{50}-50% effect concentration.

Besides, toxicity data can be estimated using different prediction models like QSARs (quantitative structure–activity relationship models) that are based on different physical-chemical properties such as octanol-water partitioning coefficient (Kow) or kinetic constant and are used to predict baseline toxicity. One of the commonly used tools for environmental toxicity assessment is EPA's ECOSAR™ tool. Also, read-across models were developed more recently that predict the toxicity of chemical based on structural similarity of compounds like atomic centered fragments (ACF) (Kühne, Ebert et al. 2009, Schüürmann, Ebert et al. 2011). Chemicals can have different modes of toxic action which can be broadly classified as specific and non-specific. By non-specific toxic action or baseline, toxicity chemical provokes a narcosis in an organism. Narcosis is the reference case because it is assumed to represent the minimal toxicity of a chemical (Rand 1995, Escher and Hermens 2002). It is caused by disturbance of normal functioning of cellular membranes (Schwarzenbach, Escher et al. 2006). Specific modes of toxic action cause different effects by altering the biological process by

binding to a specific molecule e.g., acetylcholinesterase (AChE) inhibitors that cause neurological malfunctions (Rand, 1995).

TIERED RISK ASSESSMENT

The very challenging issue of ecological risk assessment is extrapolating from the experimental results obtained from tests that included few test species in a simple laboratory setting to the effects in complex real ecological systems (Calow and Forbes 2003, Forbes and Calow 2013). There are several approaches that could be followed to account for the uncertainties resulting from this extrapolation. Depending on the adverse potential of the chemical in question, more than one step in the assessment may be performed leading to more realistic risk assessments.

The tiered procedure is the most common approach in environmental risk assessment to estimate thresholds of adverse effects which are the basis for the regulation of chemicals (Brock, Arts et al. 2006, Solomon 2008, Forbes and Calow 2013). A tier is defined as an assessment of exposure or effects resulting in an acceptable environmental concentration that can be used for regulatory purposes (Brock and Van Wijngaarden 2012). The lower tires of ERA are in general stricter, easier to perform and basis for higher tires, while higher tires are more realistic in reflecting the true environmental situation (Brock, Arts et al. 2006). The first tier is usually based on the acute data tests for standard test species and the application of an appropriate assessment factor (AF) in order to estimate predicted no-effects concentration (PNEC). An assessment factor is used to account for uncertainties in extrapolating effects from acute to chronic exposure, from the tested species to other potentially more sensitive species, and from the laboratory to field (Forbes and Calow 2013). If it is estimated in the first tier that chemical poses no risk or low risk to aquatic species, further tests are generally not necessary. However, if the first tier assessment shows potential risk, more sophisticated testing is required. Higher tiers may include e.g. the species sensitivity distribution (SSD), or additional tests like aquatic micro or mesocosm tests and food web or population models. SSD approach includes more species data (EC50 or NOEC) and estimates the concentration that potentially affects a certain percentage of species (usually 95%). The threshold concentration is considered to be protective for ecosystems and is used instead of a PNEC for assessing the risk (Calow and Forbes 2003). Even though SSD approach theoretically is more realistic than assessment factor approach, due to the general lack of data of species sensitivities it is difficult to get more sophisticated information than what is obtained by AF approach and it remains to be the approach most commonly used especially when large number of chemicals are considered (Forbes and Calow 2002). Failure at the highest tier means that intervention might be necessary (Forbes and Calow 2013). The aim of the tiered

approach is to minimize efforts of testing the chemicals that probably pose a low risk and to focus on the environmentally relevant chemicals.

The risk of a compound in a specific situation is assessed as the ratios of measured or predicted concentration and certain toxicity value (Equation 1.1). Hazard quotient (HQ) is the ratio of the potential exposure to a substance and the level at which no adverse effects are expected. It is calculated as a ratio of measured (MEC) or predicted environmental concentration (PEC) by appropriate predicted no-effect concentration (PNEC). Toxic unit (TU) (Sprague 1970) represents the ratio between the concentration of a component and its toxicological acute (e.g. LC_{50} or (EC_{50}) or chronic (e.g. long-term NOEC) value.

$$\mathrm{HQ} = \frac{\mathrm{MEC\ or\ PEC}}{\mathrm{PNEC}} \text{ ; } \mathrm{TU} = \frac{MEC\ or\ PEC}{EC50\ or\ NOEC} \qquad (1.1)$$

The values of hazard quotient higher than one in this type of risk assessment indicate the situation of the potential concert because the safe threshold value was exceeded.

Another subfield of ecotoxicology that is of increasing interest for risk assessment is the mixture toxicity of chemicals. That is because, in the environment, organisms are exposed to mixtures of many chemicals in different concentrations rather than to isolated chemicals which are affecting the organisms simultaneously (Schwarzenbach, Escher et al. 2006). Furthermore, the individual chemicals might be present at concentrations that are too low to cause adverse effects in the ecosystems, but additive or even synergistic effects in the mixture may enhance their effects (Schwarzenbach et al., 2006). The most frequently used concepts for mixture toxicity prediction are Concentration Addition (CA) and Independent Action (IA). CA is used for mixtures of chemicals with a similar mode of action, so the effects can be estimated directly from the sum of the concentrations of the mixture constituents (EuropeanCommission 2011). The concept can be mathematically expressed as following (Equation 1.2):

$$\sum_{i=1}^{n} \frac{c*_i}{ECx_i} = 1 \qquad (1.2)$$

For a mixture of n components, $c*_i$ is the concentration of the i^{th} compound which elicits x% total effect, ECx_i denotes the concentration of that substance which provokes x% effect if applied singly. Every fraction c_i/ECx_i also called toxic unit (TU) gives the concentration of a compound in the mixture scaled for its relative potency. If the sum of the toxic units equals 1 at a mixture concentration that provokes x% effect, the mixture behaves according to CA (Backhaus and Faust 2012).

IA assumes that the components of the mixture act on different subsystems (i.e., tissues, cells, molecular receptors) of an exposed organism but the resulting effect is the same. The expected mixture effect can be calculated according to the joint probability of statistically independent events as (Equation 3.3):

$$E(c_{mix}) = E(c_1 + \cdots + c_n) = \prod_{i=1}^{n} E(_{ci}) \quad (1.3)$$

$E(c_{mix})$ is the IA-expected overall effect (scaled to the range 0−1) of a mixture composed of n chemicals at a total concentration c_{mix}. $E(c_i)$ gives the effect of chemical i if applied singly in a concentration c_i which corresponds to the concentration of that component in the mixture. Both models are used to calculate mixture toxicity based on the toxicity and concentration of individual constituents of the mixture but they do not take into account possible synergistic effects (Backhaus and Faust 2012).

PRIORITY MIXTURES

The Water Framework Directive (EU 2000) and its daughter directives are the main legislation for the protection of European freshwaters (Brack, Dulio et al. 2017) with the new aim to achieve the good ecological and good chemical status of European water bodies by 2027. However, despite the efforts made by now, there is a general agreement that the goal of good ecological status will not be reached for the majority of European water bodies in the timeframe set by WFD and that the contribution of chemical contamination is still not fully understood (Altenburger, Ait-Aissa et al. 2015). Besides, due to the rising number of monitoring studies, it is becoming obvious that in the environment, the occurrence of the mixture of many chemicals rather than isolated chemicals seems to be the widespread scenario. Those compounds are impacting the biota simultaneously and therefore, the mixture effects should be of special interest to properly address the risk assessment of chemical contamination (Schwarzenbach, Escher et al. 2006). The EC recognized the challenge to solve the problem of chemical mixtures in the environment and their combined effects require scientific action (EC 2009). One of the first steps necessary to understand chemical mixtures in the environment is to identify the patterns of co-occurring compounds and relate them to land use or specific contamination sources and then the identified patterns can be used as the basis to identify the priority mixtures of potential ecotoxicological concern (Altenburger, Ait-Aissa et al. 2015). Furthermore, since usually, only a few compounds may explain most of the overall toxicity (Backhaus and Karlsson 2014) there is the opportunity to reduce the complexity and to focus on so-called drivers of mixture toxicity. EC suggests that the identification of priority mixtures should be a central task for future risk assessment (EC 2011).

1.5 IMPACTS OF MICROPOLLUTANTS IN FRESHWATER

The catastrophic effect of pollutants in freshwater can be very easy to observe due to a high level of mortally they cause. They are usually related to accidental spills and release of the extremely high concentration of contaminants in the environment (Liess and Beketov 2011).

Examples of this kind of accidents (e.g., pesticide spill from SANDOZ factory into Rhine river in 1986 (Van Urk, Kerkum et al. 1993)) are well documented and they attract a lot of general public attention (Liess and Beketov 2011, Stamm, Räsänen et al. 2016). Besides, adverse effects of the continuous input of pollutants in high concentrations are easily observable, such as e.g., eutrophication and subsequent fish kill as the result of untreated wastewater release (Stamm, Räsänen et al. 2016). However, effects of low levels of exposure to pollutants are difficult to identify. That is because many of the micropollutants generally occur in freshwater at sublethal concentrations and due to the fact that in such cases, multiple environmental influences shape the biological communities together with the pollutants (Liess, Schäfer et al. 2008) (Stamm, Räsänen et al. 2016). Nevertheless, low-level exposure may result in dramatic ecological effects (Liess and Beketov 2011). In the recent study by Malaj (Malaj, Von Der Ohe et al. 2014) it was assessed that organic chemicals are likely to cause acute and chronic effects in freshwaters across Europe. However, to establish the causal link between exposure to chemicals and effects observed in the environment is difficult because it is rare to find single stressor scenario in nature. Furthermore, the mechanism of change for many of the pollutants is still unknown (Schwarzenbach, Escher et al. 2006). Moreover, it is easier to observe the patterns of changes in ecosystems to than to understand the causes of those changes (Stamm, Räsänen et al. 2016).

MULTIPLE STRESSORS IN FRESHWATER

The various environmental pressures such as wastewater inputs or diffuse pollution can contribute to the introduction of one or more stressors in the environment (e.g. nutrients, sediment, micropollutants or increase of temperature). A stressor is defined a measurable variable that exceeds its natural levels and adversely affects individual taxa, community composition or ecosystem structure and functioning (Matthaei, Piggott et al. 2010). There is increasing evidence that multiple stressors are present in many freshwater habitats that might lead to unexpected effects on aquatic ecosystems (Nõges, Argillier et al. 2016). It was estimated that nearly 80% of the world's population is exposed to high levels of threat to water security and at 65% of freshwater habitats biodiversity was moderate to highly threaten by multiple stressors (Figure 1.6). These stressors include organic and inorganic pollution, excess input of nutrients, geomorphological alterations, hydrological stress, invasive species and pathogens (Vörösmarty, McIntyre et al. 2010). In the multiple stressors situation, it is very difficult to predict the biological or ecological responses, especially if the stressors interact (Townsend, Uhlmann et al. 2008). Stressors may have simple additive response i.e., they linearly add to each other or they can interact in different ways so they result in a larger (synergism) or smaller (antagonism) combined effect (Matthaei, Piggott et al. 2010). Stressor interactions represent a great challenge for ecosystem managers because their potential

combined effects should be jointly addressed in the management measures. Therefore, disentangling the effects of multiple stressors is of great importance to properly manage and protect freshwater biodiversity and ecosystem functioning (Feld, Segurado et al. 2016).

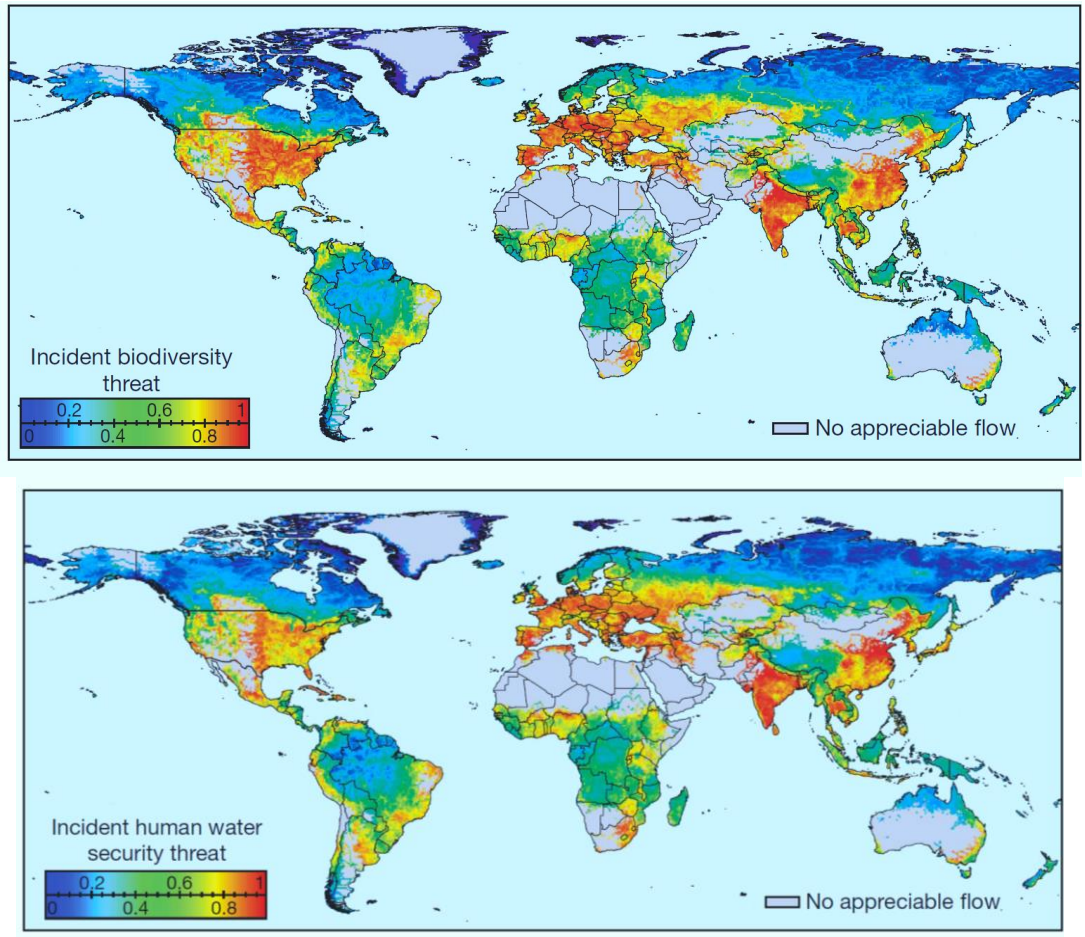


Figure 1.6 The global scale study performed by (Vörösmarty, McIntyre et al. 2010) shows impacts of multiple stressors on A) human water security and B) biodiversity. Two threat indices were produced using 23 geospatial drivers depicting environmental stressors (e.g., nutrients, pesticides or sediment loading) with known impacts on human water security and biodiversity. They found that nearly 80% of the world's population is exposed to high levels of threat to water security and at 65% of freshwater habitats biodiversity was moderate to highly threaten.

Biodiversity is the variety within and among life forms; it includes the variation of species, their functional traits, and genes. It can be measured as richness (i.e., the measure of the number of different biological units) or evenness (i.e., the measure of the proportion biological units present on a site). Ecosystem functions are biological, geochemical and physical processes that control the fluxes of energy, nutrients and organic matter through an environment. Their proper functioning is the basis for ecosystem services; the benefits that ecosystems provide to humanity (Cardinale et al., 2012). Consequences of the human-induced threats are already evident since it was observed that species, biological traits, and genes are eliminated at an

alarming rate (Cardinale et al., 2012). It is the question how will such losses of taxonomical and functional biodiversity alter the functioning of ecosystems and their ability to provide society with the goods and services needed (Cardinale et al., 2012). However, little is known beyond the described effects of single stressors on specific ecological endpoints and our understanding of the main causes for the losses of biodiversity still remains vague and protecting the world's freshwater resources requires diagnosing threats over a broad range of scales (Vörösmarty, McIntyre et al. 2010).

TRAIT BASED APPROACHES

To cope with the problem of multiple stressors it was proposed to use species traits (e.g., generation time, body size, body form, and dispersal ability) (Statzner et al., 2005; Tachet et al., 2010; Usseglio-Polatera et al., 2000) rather than conventionally used taxonomical approaches. Trait-based approaches are a powerful tool that can be used to link habitat characteristics to life history strategies (Southwood 1988). Traits may be used interpret changes in assemblages across environmental gradients and to improve the traditional freshwater biomonitoring (Dolédec and Statzner, 2008). According to the habitat template theory (Southwood, 1977) (Figure 1.7) the spatial and temporal characteristics of the habitat provide a framework against which species evolve their characteristic life-history strategies (i.e., a specific set of traits used to cope with environmental characteristics) (Poff, 1997; Townsend and Hildrew, 1994). Most stressors should affect only certain trait categories which make a trait-based approaches promising tool for biomonitoring (Statzner et al., 2001; Statzner et al., 2004; Statzner et al., 2005). Besides, some traits are thought to vary little across temporal and spatial scales, which makes them useful for large-scale studies (Statzner et al., 2001; Statzner et al., 2004; Statzner et al., 2005).

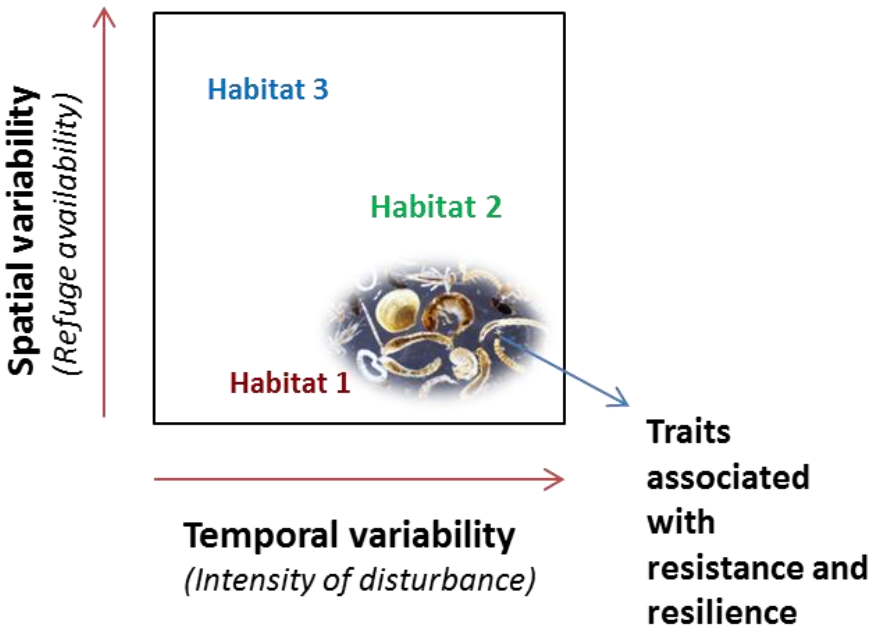


Figure 1.7 According to the habitat template theory (Southwood, 1977) the spatial and temporal variability of the habitat provides a framework against which species evolve their characteristic life-history strategies. Spatial variability could be seen as refuge availability and temporal variability as the intensity of disturbance Different combinations of spatial and temporal variability will result in different adaptations of species to specific environmental conditions. The habitats that provide the least spatial variability and the most of the disturbance would favor the most resistant species (Townsend and Hildrew, 1994).

The use of multiple traits described by multiple trait categories, has been used elucidate the influence of different stressors e.g. heavy metal pollution and cargo ship traffic (Dolédec and Statzner 2008), eutrophication and fine sediment deposit (Dolédec, Phillips et al. 2006, Townsend, Uhlmann et al. 2008) and climate change and salinity (Townsend, Uhlmann et al. 2008). Besides, several trait-based metrics have been developed to target the specific stressors regardless the presence of multiple stressors in the field (Liess, Schäfer et al. 2008, Van den Brink, Alexander et al. 2011). To identify the effects of agricultural pesticides on macroinvertebrate communities the Species At Risk index (SPEARpesticides) was developed (Liess and von der Ohe 2005) and to identify the effects of general organic toxicants (SPEARorganic) (Beketov and Liess, 2008). There is an increasing number of studies relating the presence of pesticides and effects on sensitive species using SPEARindex (e.g. Liess and Von Der Ohe, 2005; Schäfer et al., 2007, (Munz, Burdon et al. 2017).

1.7. SCARCE AND NET-SCARCE PROJECTS

The research for this thesis was performed as the part of the Spanish Ministry of Economy and Competitiveness project SCARCE (Assessing and predicting effects on water quantity and quality in Iberian rivers caused by global change, Consolider-Ingenio 2010 CSD2009-00065, (Navarro-Ortega, Acuña et al. 2012) and the follow-up project NET-SCARCE (Consolider Research Network on the effects of water scarcity and global change on river systems, Redes de Excelencia CTM2015-69780-REDC). The SCARCE was a multidisciplinary project lasting from 2009 to 2014, and it aimed at describing, understanding and predicting the effects of global change on water quantity and quality in river Mediterranean river basins. NET-SCARCE is using the scientific heritage received from the SCARCE project and aims to consolidate the multi-disciplinary network established in its parent project in order to provide insight into the current challenges related to water scarcity under global change. The research under SCARCE project included a multidisciplinary team of scientists, from analytical chemists, hydrologists, engineers to ecotoxicologists and ecologists. They focused on collection and production of the data of anthropogenic influences on the water quality, water availability and ecosystems from different perspectives in order to provide the holistic picture of water problems and their impacts on ecosystems and society. Besides, the project actively involved the representatives of water authorities and stakeholders in order to improve the communication between scientists and water managers. In particular the SCARCE project had two main objectives, first was to perform the basic research in order to define the long-term patterns and mechanisms in the hydrology, water quality, habitat dynamics, and ecosystem structure and function of Mediterranean watersheds. The second objective was to relate the effects of key elements of global change such as climate change and human footprint on the freshwater ecosystem services. Finally, the overall SCARCE project was orientated to finalize, implement, and refine the River Basin Management Plans (RBMP) demanded by the EU Water Framework Directive. Four river basins situated in the Mediterranean part of the Iberian Peninsula were used as case studies in the SCARCE project and for this thesis: Ebro and Llobregat in the North-East, Júcar in the East and Guadalquivir in the South of the Peninsula (Figure 1.8). Samples of water, sediment and biota were collected at altogether 77 sampling sites along the Iberian. Peninsula, 14 sites in the Llobregat, 24 sites in the Ebro, 15 sites in the Júcar and 24 sites in the Guadalquivir.

Figure 1.8 Four river basins used as case studies.

PRESSURES IN IBERIAN RIVERS

The Mediterranean is one of the world's regions most vulnerable to global change (Barceló and Sabater 2010). The climate change models forecasted the increase of extreme weather and hydrological events in the near future (Petrovic, Ginebreda et al. 2011). Furthermore, a decrease of water availability in semiarid and arid areas and an increase of water temperature is expected (Calbó 2010). Low flow in summer and floods in autumn and winter are one of the natural characteristics of Mediterranean rivers (Gasith and Resh 1999) but these events will be more extreme in coming decades (UNESCO 2003). These events will increase pressured on natural water resources and cause the impacts on river ecosystems (Sabater and Tockner 2009) including the impact of micropollutants (Petrovic, Ginebreda et al. 2011). Due to a specific climate, land use and population density across the Iberian Peninsula, different pollution of these different pressures and impacts are present in each of basins used in this thesis. Studied river basins are situated in areas of multiple land use types, from natural forests and grasslands to agricultural lands and highly industrialized and urbanized areas (Figure 1.9).

The Llobregat is situated in the North East of Iberian Peninsula (Figure 1.8). It covers the area 4948 km2 and has the average discharge of 21 m3/s. The lower part of the basin (Figure 1.9) is subjected to strong anthropogenic pressures due to high levels of urbanization and industry mostly around the metropolitan area of Barcelona. It is very densely populated basin (Figure 1.10). In the middle part of the basin, most of the agricultural lands are situated. Urban and industrial wastewater are discharged in Llobregat, in addition to the surface run-off coming from salt mining (both natural and caused by human

activities) occurring in its middle basin. As a typical Mediterranean river, Llobregat is subjected to decreased flow in the summer periods as a consequence of Mediterranean climate (Gasith and Resh, 1999).

The Ebro basin is situated in North of the Peninsula (Figure 1.8). It covers the area of 85362 km2 and has the average discharge of 426m^3/s. The main pressures for water quality and quantity are coming from agricultural activities developed along the river basin and delta (Figure 1.9) and hydraulic infrastructures like dams and channels. The urban and industrial centers are scattered along the basin, mostly in the North East and central part. The population is densely concentrated in North West and around the delta (Figure 1.10).

The Júcar river basin is situated in the East of Iberian Peninsula (Figure 1.8) characterized by semi-arid climate. It covers the area of 21578km^2 and the average discharge of 49m^3/s. The most of the urban areas with dense population are located in the lower part of the basin (Figure 1.9 and Figure 1.10) while large agricultural areas are located in medium and lower parts (Figure 1.9).

The Guadalquivir river basin is located in the South of Iberian Peninsula (Figure 1.8). It covers the area of 57071km2 and has the average discharge of 164m^3/s. A large portion of the basin is devoted to agricultural use but also several large cities are located along the rivers such as Seville, Cordoba or Granada (Figure 1.9) where the most of the population is concentrated (Figure 1.10)

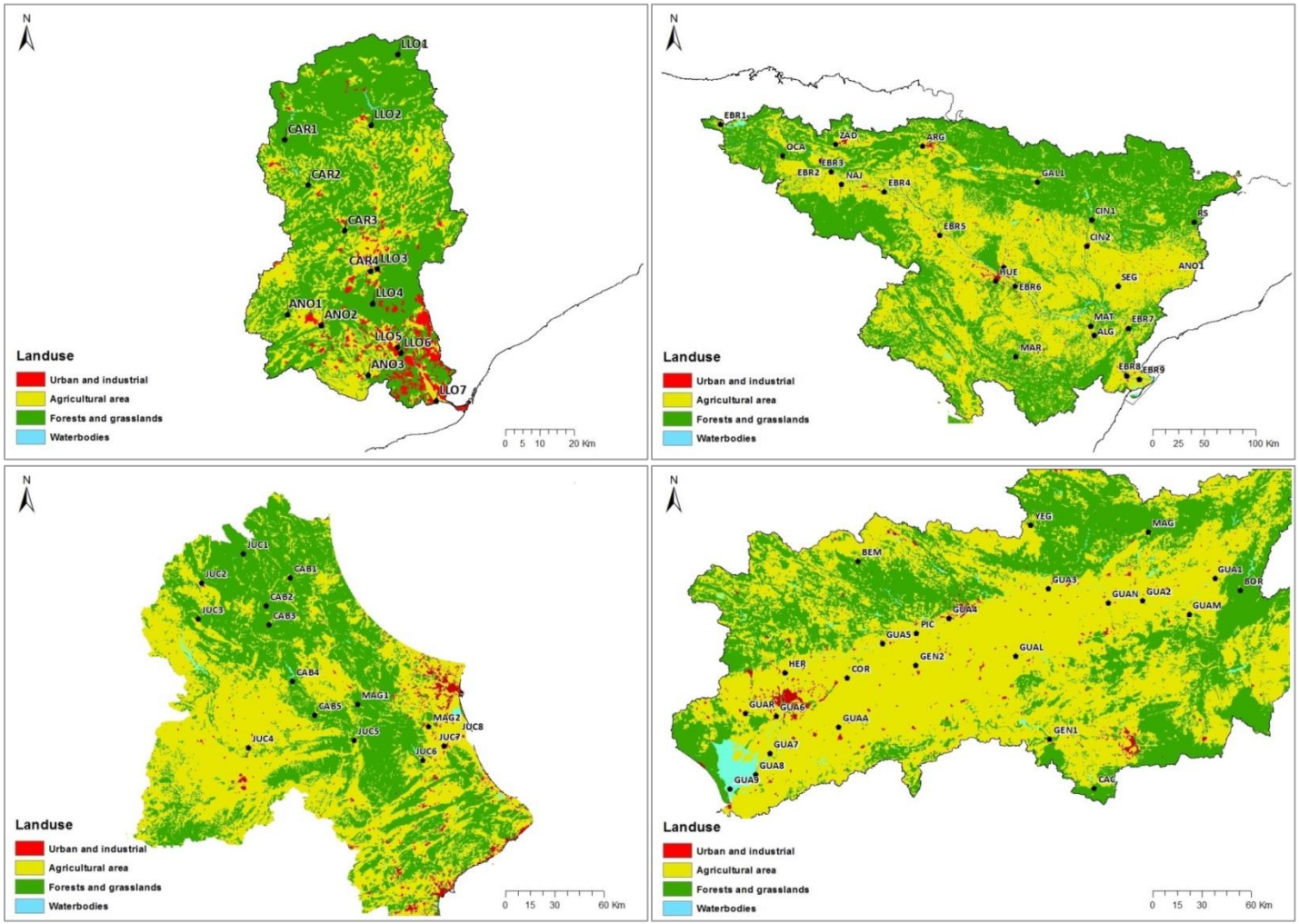


Figure 1.9 Land use types in studied basins with indicated sampling sites in A) Llobregat, B) Ebro, C) Júcar and D) Guadalquivir.

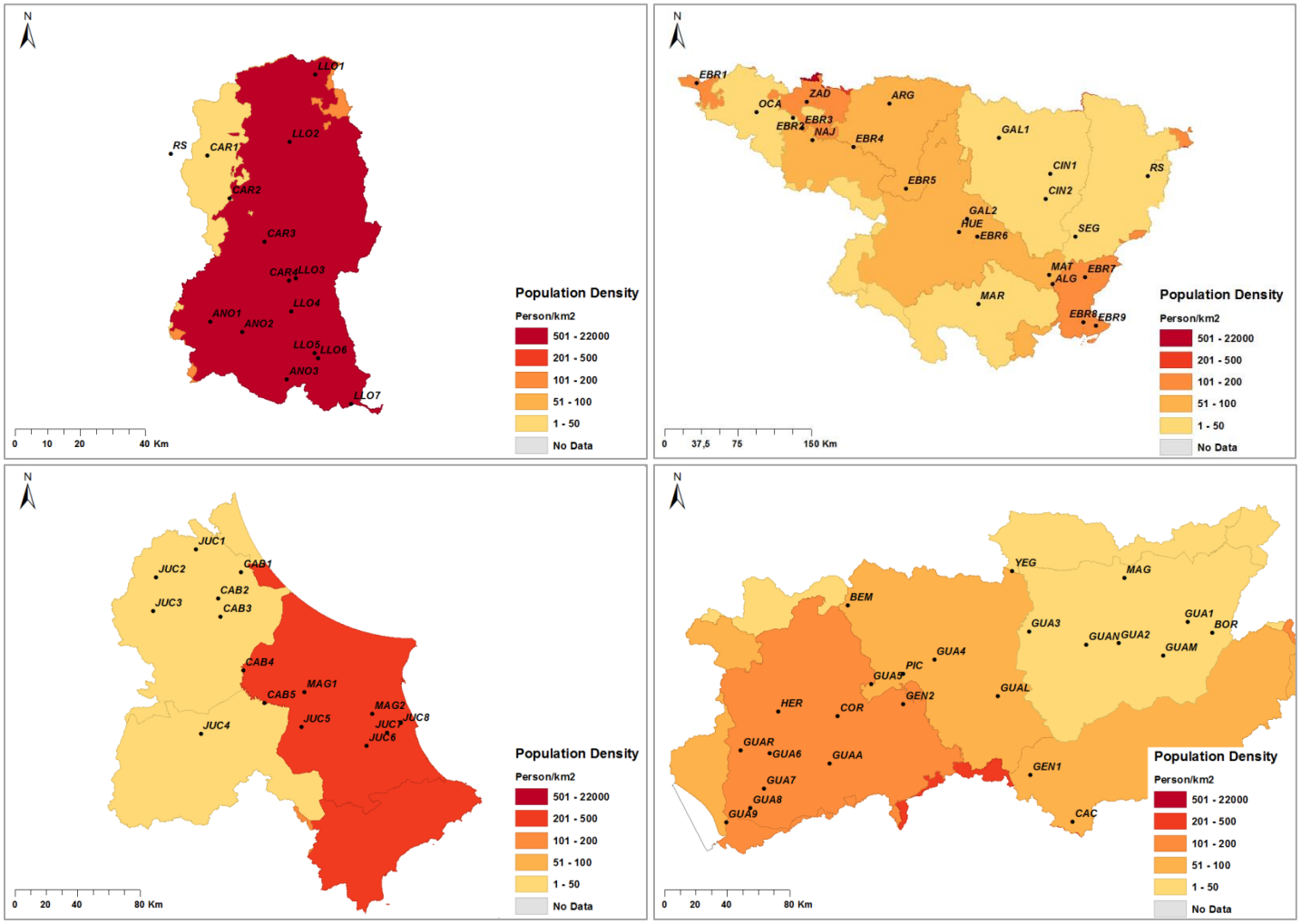


Figure 1.10 Population density in studied basins with indicated sampling sites in A) Llobregat, B) Ebro, C) Júcar and D) Guadalquivir.

This subsection summarizes the results of chemical measurements from SCARCE project which were used as the basis for this thesis; it was compiled from several published papers (Table 1.1) and SCARCE database. The number and concentrations of chemicals detected varied among the sampling sites and basins. The presence chemical mixtures were reflecting the dominant land use types upstream as different compounds were more abundant at the sites located in dominantly urban areas or agricultural areas (Figure 1.9 and 1.11). In general, more than 50 chemicals were detected in each sample. Of studied chemical groups, industrial organic compounds (IOC) were measured at highest concentrations at the majority of samples except in Júcar where pesticides were most abundant. A high number of pharmaceuticals were detected in all four rivers, as well as hormones, personal care products, and illicit drugs. Overall, Llobregat was the most contaminated river in terms of number and concentration of organic compounds. Several sites in Ebro, downstream of the urban areas were highly polluted by industrial chemicals, pharmaceuticals etc. Júcar pollution was dominated by pesticides. Overall Guadalquivir was the least contaminated river possibly reflecting the higher flow and dilution capacity of this river combined with relatively lower population density than in e.g. Llobregat basin.

Figure 1.11 Satellite images of different locations in of: A) Llobregat B) Ebro, C) Júcar D) Guadalquivir. Source: The world coordinate converter (http://twcc.fr

Table 1.1. Minimum and maximum number of individual chemicals (n=235) of each compound group detected in analyzed water.

Number of chemicals detected in sample	LLOBREGAT	EBRO	JÚCAR	GUADALQUIVIR	REFERENCE
PESTICIDES	6-11	6-17	6-17	8-15	(Masiá, Ibáñez et al. 2013)
INDUSTRIAL ORGANIC CHEMICALS	6-9	5-10	5-9	7-12	(Gorga, Insa et al. 2014)
PERFLOURINATED COMPOUNDS	0-10	0-8	1-9	3-9	(Masiá, Ibáñez et al. 2013)
PHARMACEUTICALS	10-55	9-60	35-40	9-35	*(Osorio, Proia et al. 2014)*
PERSONAL CARE PRODUCTS	0-10	0-7	3-7	2-8	(Gago-Ferrero, Mastroianni et al. 2013, Gorga, Insa et al. 2014)
HORMONES	1-3	0-5	0-3	0-4	(Gorga, Insa et al. 2014)
ILLICIT DRUGS	0-4	0-4	0-4	0-3	(Mastroianni, Postigo et al. 2013)

The pollution was considerably increasing downstream (Figure 1.12). It was particularly high in the lowest part of the basin (LLO6 6 and 7) surrounding Barcelona metropolitan area and in the Anoia tributary (Sites ANO1, 2 and 3) which is situated in the industrial area of Igualada. The compounds group measured at highest concentration at the majority of sampling sites was industrial organic compounds (IOCs, Figure 1.12, grey color). Of the compounds belonging to this group alkylphenols (octylphenol, nonylphenol, and related compounds) and anticorrosion agents as tolyltriazole and 1H-benzotriazole were the most relevant. The highest concentration IOCs (=10.5 µg/l) was measured at site LLOB7, the most downstream site of the basin. Pharmaceuticals were the second group in terms of concentration, especially around Barcelona in the lower part of the basin. Perflourinated compounds (PFCs) were abundant in Anoia tributary with concentration up 2.8µg/l measured at the site ANO2. The most abundant compounds of this group were PFBA and PFOS.

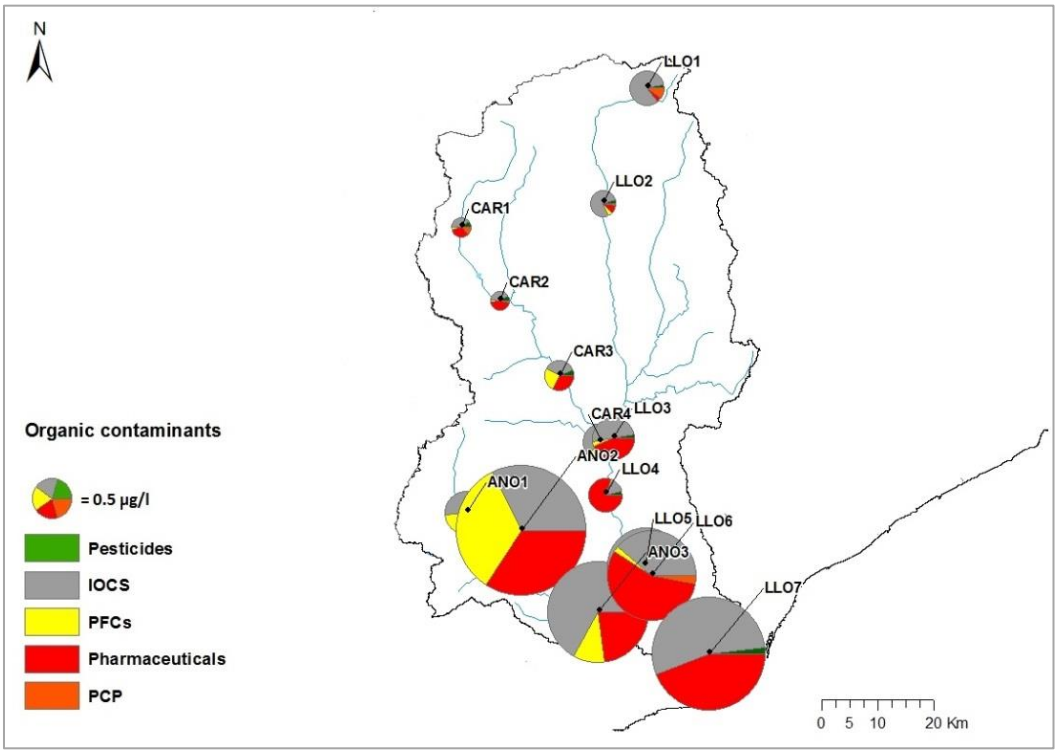


Figure 1.12 Pollution of Llobregat river water with organic chemicals

EBRO

In the Ebro, the highest concentration of organic contaminants (approximately 10µg/l) was measured at Zadorra site (Figure 1.13; ZAD) which is situated close to the wastewater treatment plant downstream of the city Vitoria in the Basque Country. As in the Llobregat, industrial organic chemicals were the major group of contaminants at almost all sampling sites. The second group corresponded to pharmaceuticals, which included the compounds belonging to different therapeutic classes. The maximum concentration of pharmaceuticals was measured at the Zadorra site as well. Compared to other studied basins, Ebro had a relatively higher number (Table 1.1) and concentrations of pharmaceuticals. The concentration levels of pesticides were relatively higher at sites in lower part of the basin and in the delta. At those dominantly agricultural sites including sites around delta (Ebro 8 and Ebro 9) pesticides were the major pollutants in terms of their concentration. However, compared to heavily contaminated sites like Zadorra or Arga the total concentration of all organic contaminants is relatively small (approx. 0.5 µg/l).

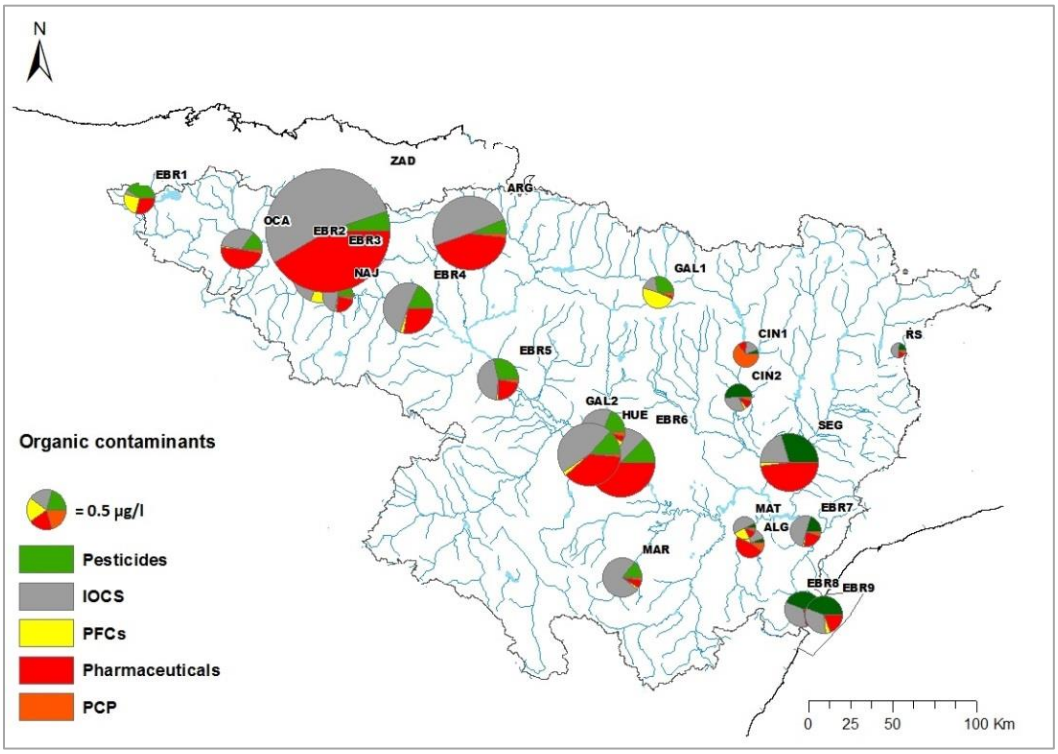


Figure 1.13 Pollution of Ebro basin

In the Júcar basin, pesticides (green color in Figure 1.14) were the main group of pollutants. Surprisingly, even at the sites located in dominantly natural land use (forests and grasslands) (Figure 1.9) without substantial areas of agricultural or urban land use upstream, pesticides were measured at concentrations that could possibly raise ecotoxicological concern. At most of the sampling sites in this basin, pesticides were dominant in terms of measured concentrations (Figure 1.14), except at MAG1 and MAG2 sites where industrial organic compounds were measured at highest concentration and at site JUC2 where perfluorinated compounds were dominant pollutants. MAG1 was the most polluted site in the river basin with a concentration of organic contaminants approximately 4µ/l. Compared to other river basins, pharmaceuticals and personal care products were measured at relatively lower concentrations.

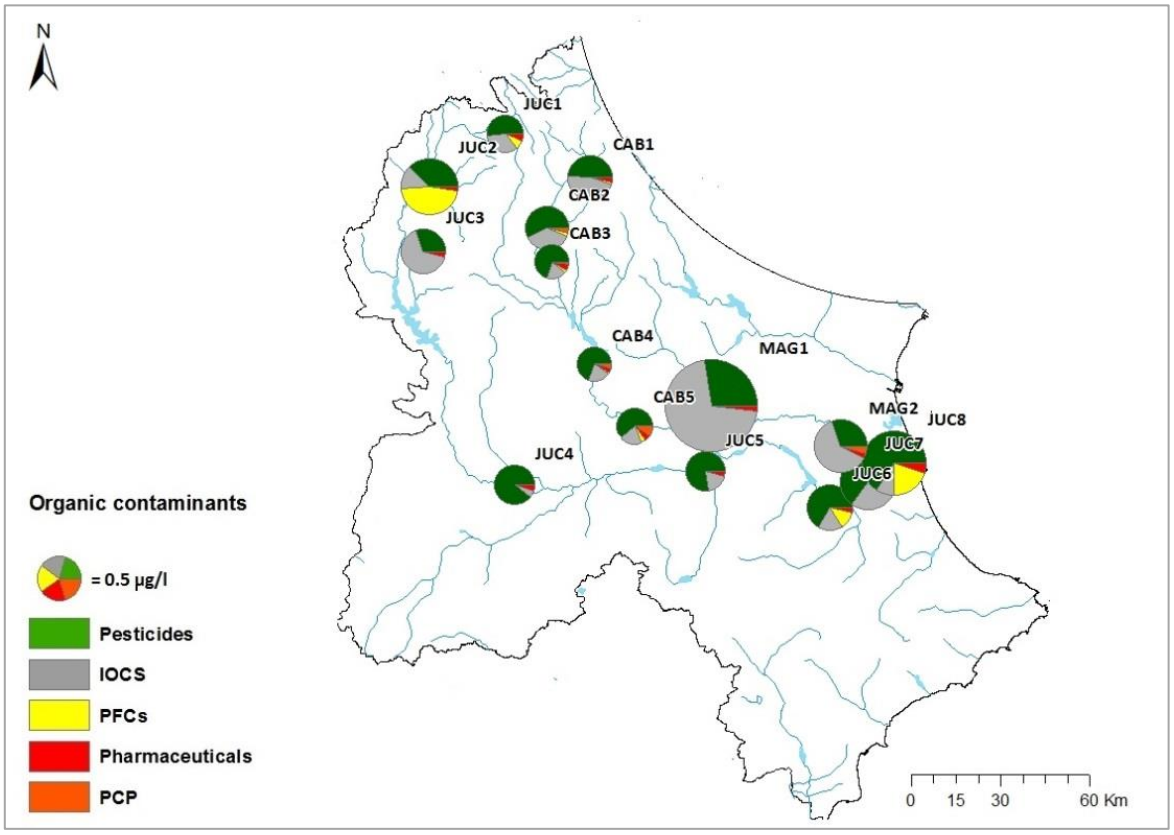


Figure 1.14 Pollution of river Júcar *with organic pollutants*

GUADALQUIVIR

Compared to other studied basins the Guadalquivir was the least contaminated. The main pollutants group in Guadalquivir was, like in Ebro and Llobregat, industrial organic compounds. The following group in terms of concentrations was perfluorinated compounds and at some sites pesticides or pharmaceuticals. Interestingly, personal care products (orange color in the Figure 1.15) were relatively in higher concentrations compared to other rivers and they were found in all samples (Table 1.1). But in comparison to the other studied basins, the Guadalquivir had a relative lower number of detected pharmaceuticals (Table 1.1). The pollution was slightly higher in the lower and middle part of the basin (Figure 1.15). But in general, the levels of pollution were similar along the main course of the river and smaller in the tributaries one exception of site GUAA, in the lower part of the basin which was the most polluted site in the river.

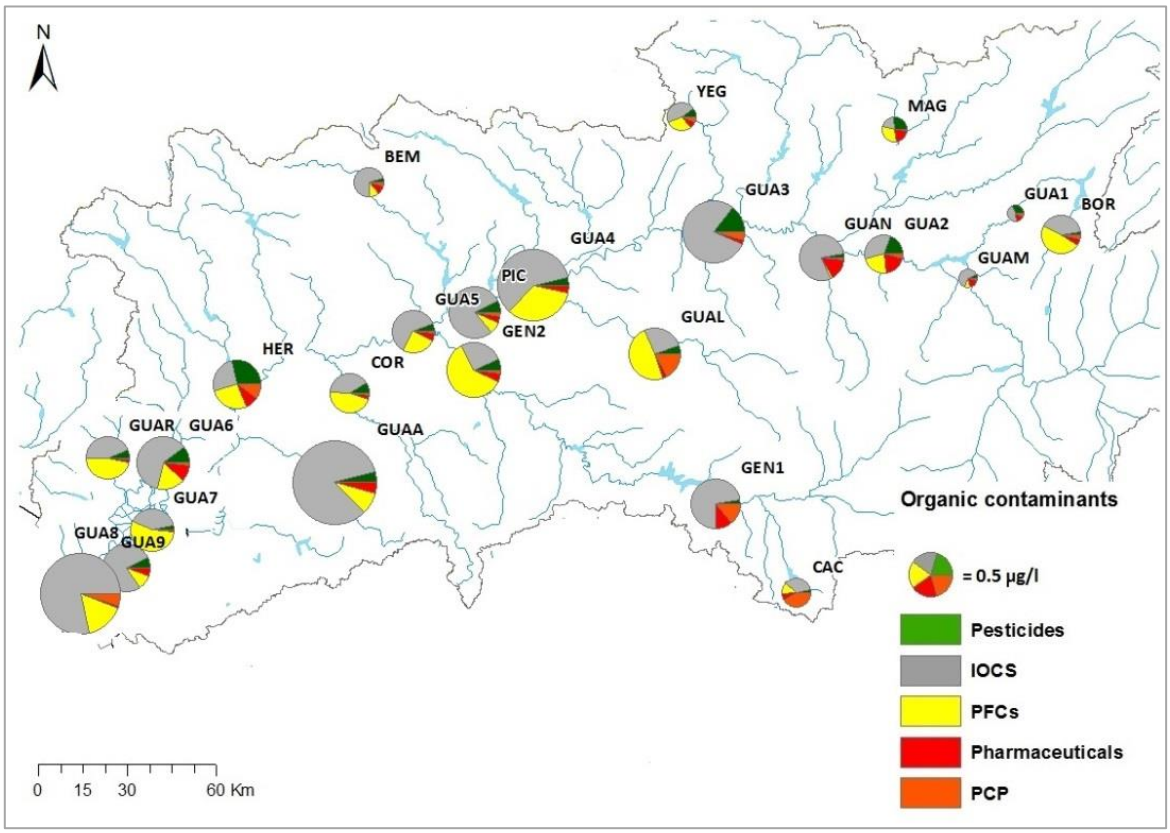


Figure 1.15 Organic pollution of Guadalquivir river basin

CHAPTER 2

OBJECTIVES AND THE STRUCTURE OF THE THESIS

OBJECTIVES

The general objectives of this thesis were to contribute to the growing scientific knowledge on the ecotoxicological risk of chemical compounds in the freshwater environment using four rivers of Iberian Peninsula as case studies; namely Llobregat, Ebro, Júcar, and Guadalquivir; to identify the main drivers of risk for ecosystems in those rivers and provide the evidence of effects caused by chemical compounds.

Several general objectives were addressed through the thesis:

- To characterize the level and the extent of pollution in studied Iberian river basins.
- To introduce the new method for prioritization of the pollutants according to their ecotoxicological risk.
- To prioritize chemical pollutants detected and determine the compounds of highest concern for each of studied river basins.
- To assess the level and spatial extent of the ecological risk posed by micropollutants and other stressors such as metals, nutrients or hydrological alterations in the studied basins.
- To use the local macroinvertebrate communities as a proxy for the general health of ecosystems; to identify effects of individual and multiple stressors on their taxonomical and functional elements.

The thesis is divided into eight chapters. Chapter 1 introduces the context of the environmental problems related to chemicals and the main concepts of the current scientific approaches used to solve these problems. In the final part of Chapter 1, pollution of studied rivers is reviewed from the data gathered within the SCARCE project. The objectives and thesis structure are described in Chapter 2. The schematic representation of the main structure and the objectives of the thesis are given in Figure 2.1.

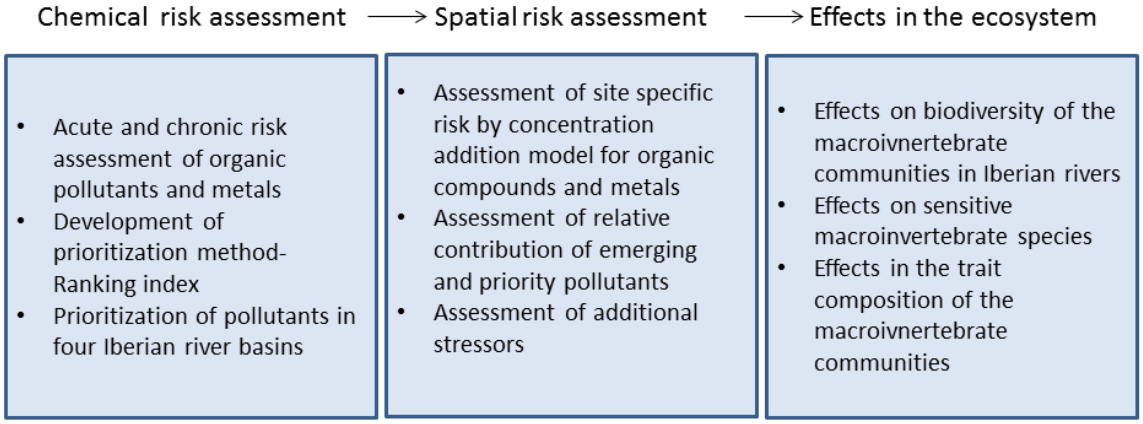


Figure 2.1 The schematic representation of the three main subjects of the thesis and the specific goals that were addressed to provide the information necessary to fulfill the main objectives of the thesis.

In Chapter 3, prioritization schemes for freshwater organic pollutants were reviewed. Occurrence and risk of selected important pollutants in Europe and North America were compared.

Specific goals of Chapter 3 were:

- To give the overview of prioritization schemes for freshwater pollutants.
- To compare the occurrence and risk of selected pollutants in Iberian rivers and rivers from different geographical areas.

In Chapter 4, ranking index (RI), a new method for prioritization of pollutants is introduced. The list of the compounds of highest concern for each river basin is provided.

Specific goals of Chapter 4 were:

- To develop a prioritization method based on the ecotoxicological potential of chemicals and their spatial relevance as a pollutant in a given area.

- To create the ecotoxicological database for 250 chemicals, including water exposure acute toxicity data for three standard test species; algae *Pseudokirchneriella subcapitata,* invertebrate *Daphnia magna,* and fish *Pimephales promelas* and other relevant species.
- To prioritize the chemicals according to their risk and the spatial relevance in each of the studied basins.

In Chapter 5, comprehensive risk assessment of organic chemicals mixtures and metals was performed using a concentration addition model (CA) and related to macroinvertebrate for general biodiversity and stressor-specific indicator for pesticide pollution.

Specific goals of Chapter 5 were:

- To assess the site-specific risk of organic pollution and metals.
- To create the risk maps for studied basins using ArcGis software.
- To assess the relative contribution of pollutants with different level of priority according to current European legislation.
- To identify the major drivers of the site-specific risk.
- To statistically analyze the relation between assessed chemical risk data and other stressors with taxonomical and trait-based macroinvertebrate community descriptors.

In Chapter 6, assessment of additional stressors was performed and the relationship among stressors and macroinvertebrate functional trait composition were investigated by extensive statistical modeling.

Specific goals of Chapter 6 were:

- To assess the presence of multiple stressors at sampling sites.
- To analyze the influence of different stressors to functional diversity and composition of macroinvertebrtate communities.

Results and the main findings of the thesis are discussed in Chapter 7 and the general conclusions are given in Chapter 8.

CHAPTER 3

RISK ASSESSMENT AND PRIORITIZATION OF POLLUTANTS IN CONTINENTAL MEDITERRANEAN WATERS BASED ON HAZARD QUOTIENTS

Based on the publication in Contributions to Science (2014)

Maja Kuzmanović, Antoni Ginebreda and Damia Barceló

3.1 INTRODUCTION

Chemical pollution is one of the major threats to aquatic systems nowadays (Schwarzenbach, Escher et al. 2006). In the European Union, there are more than 100,000 registered chemicals listed by EINECS (the European Inventory of Existing Commercial Chemical Substances) of which 30,000 to 70,000 may be considered of common industrial and/or domestic use. Depending on their physicochemical properties, amounts produced and mode of use many of these chemicals may enter the natural waters through sewage water discharge, surface runoff from agricultural fields, atmosphere deposition, accidental spills etc. Many of these compounds are not properly eliminated by conventional wastewater treatment plants and are continuously released into rivers as a part of the wastewater treatment effluent. The majority of compounds are present at low concentrations in the environment, but many of them may raise serious toxicological concerns (Schwarzenbach, Escher et al. 2006). Given the huge number of chemicals in the environment and existing time and budget constraints there is a need to prioritize chemicals in order to optimize monitoring efforts, as well as to provide appropriate and scientifically sound information to both legislators and water managers (von der Ohe, Dulio et al. 2011). Considering current legislation, in the European Union, the big upturn in aquatic environment protection was made by the introduction of the Water Framework Directive (WFD) that was established in 2000 and aims to achieve good ecological and good chemical status of European surface waters by the year of 2015. Using Combined Monitoring-based and Modeling-based Priority Setting Scheme WFD identifies a list of 33 priority substances that pose a significant risk to the EU aquatic environment (EU 2000) and 8 other hazardous substances from previous legislation. The lists of priority and hazardous substances include contaminants which have been long recognized as dangerous for the human health and environment. They are regulated because of their persistence, bioaccumulation and toxicity properties (PBT). In order to achieve good chemical status, water bodies must meet the Environmental Quality Standards (EQS)(EU 2000) i.e., to keep the levels of concentrations of these compounds below their EQS. Furthermore, it is expected to update and review the list of priority substances every 4 years. In this context, the European Commission has recently issued a proposal updating the list of priority substances by adding 15 new candidates. European Union Member States are obliged to identify pollutants of regional or local importance and provide EQS, monitoring schemes and regulatory measures for them. This means that the Member States need to decide which are the candidate substances for further

investigation are and which the substances to be then declared as river basin specific pollutants (Piha, Dulio et al. 2010). Due to specific biogeographical and socioeconomic conditions, different sets of compounds can be used, resulting in distinct pollution patterns. Due to a specific climate, agriculture, industry and population density of Mediterranean area and Iberian Peninsula as its representative, it is likely to expect distinct pollution of Mediterranean rivers compared to other geographical areas.

Furthermore, chemicals that are monitored on a regular basis are only a small fraction of all the chemicals present in the environment (Daughton and Ternes 1999). Many unregulated, emerging contaminants may have a significant impact on aquatic ecosystems and require special attention (Petrovic, Ginebreda et al. 2011). Examples of compounds classified as emerging contaminants are pharmaceuticals, personal care products, polar pesticides, natural toxins, biocides, perfluorinated compounds and nanomaterial (Petrovic, Ginebreda et al. 2011). Even though they are usually present at very low concentrations from pg/liter to ng/liter because of the improvement of analytical techniques, number and frequency of detections of emerging contaminants are increasing (Murray, Thomas et al. 2010). It is important to note that emerging environmental contaminants are not necessarily new chemicals, but the substances that have often been long time present in the environment but whose potentially adverse effects on human health and environment are only now being recognized (Piha, Dulio et al. 2010). Thus, it becomes clear that it is necessary to evaluate the risk of emerging contaminants and if some of them have potential to cause harmful effects to the ecosystem or human health to include them into the monitoring and regulation programs. Still, given a large number of chemical compounds in the environment, it is not possible to conduct risk assessments for all the chemicals. Moreover, not all the compounds present in the environment pose the significant risk to aquatic ecosystems or human health (von der Ohe, Dulio et al. 2011). This has led to the development of schemes for prioritizing compounds based on their potential risk in order to direct the monitoring efforts and regulation towards the important compounds. The assessment of whether or not a particular compound is a pollutant is based upon an understanding of its exposure i.e., its input, distribution and fate in a defined system, and of the effects that the compound has on organisms, including humans, due to its presence in the system (Schwarzenbach, Escher et al. 2006). A “priority” chemical is one that, because of its importance, however, defined, should be examined with greater urgency and in preference to other chemicals. One approach for identifying potentially dangerous compounds is a long-term screening of the environment

for a large set of chemicals together with an assessment of the potential toxicity of the observed concentrations, which can be done by use of measured or predicted effect concentrations for standard test species (von der Ohe, Dulio et al. 2011).

In this work, we review prioritization schemes that are focused on the risk of organic chemicals (including emerging contaminants) in a freshwater environment. We highlighted the compounds identified as important pollutants by multiple prioritization schemes. Furthermore, we collected the occurrence data for those compounds from two Mediterranean rivers (Ebro and Llobregat) and several rivers in North Europe and North America and conducted new prioritization exercise of those compounds in Mediterranean rivers and rivers from other geographical areas. Hazard quotients (HQ) were used to quantify risk and subsequently determine the rank associated to each pollutant. From the foregoing considerations, a) the aim of the present work was to give the overview of prioritization schemes that include emerging contaminants; b) to identify the compounds that are of specific concern in Mediterranean rivers and compare them to those important for other rivers worldwide.

3.2 OVERVIEW OF SELECTED PRIORITIZATION SCHEMES

Several schemes for prioritization of chemicals according to their importance as aquatic contaminants have been developed (Guillén, Ginebreda et al. 2012) and are summarized in Table 3.1. Majority of them are based on the criteria of persistence, bioaccumulation and toxicity of the chemical combined with a quantity of that chemical in the environment Common drawbacks to these schemes are those they use limited number of preselected chemicals and in most cases subjective judgment to make the decision for pre-selection of compounds or giving the specific weight to different criteria (Guillén, Ginebreda et al. 2012). In general, most of the prioritization schemes follow the same procedure. The first step is the pre-selection of the chemicals for the prioritization. For the selection of chemicals is important to identify the reasons for the prioritization. The pre-selection of chemicals may be done according to existing legislation and monitoring data or by identification of sources and pressures (Petrovic, Ginebreda et al. 2011). The second step includes the exposure and toxicity estimation. The exposure can be determined on the basis of monitoring data, i.e., environmental occurrence data (Johnson, Ternes et al. 2008). If monitoring data is not available, the exposure can be estimated in a predictive way by models. Models use the information about the chemical production quantity,

frequency of its release to the environment, the potential of its emission into the environment, emission data, its persistence in given system, the distance between the source and potentially endangered recipients, mechanisms of transport etc. (Boxall, Sinclair et al. 2004).

Table 3.1 Some of the proposed screening and prioritization procedures for different preselected contaminants, adaptation of (Guillén, Ginebreda et al. 2012)

Preselected compouns	Criteria	Results	Area	Reference
78 compounds of high concern	PBT properties estimated exposure levels	chlorphyros, ametryn, dichloufluanide, prometryn, chlorothalonil	EU	(Daginnus, Gottardo et al. 2010)
250 compounds measured in Swiss waters	exposure categorization	pentachlorophenol, PFOA, PFOS, azithromycin, ofloxacin, clarithromycin, erythromycin	Switzerland	(Götz, Stamm et al. 2010)
Data Assembled for the Environment Canada DSL*	persistence, bioaccumulation and the long range transport	[1,1′-biphenyl]-4,4′-diamine, N,N′-bis(2,4-dinitrophenyl)-3,3′-dimethoxy benzenamine, 1-naphthalenemethano mestranol, bisphenol A, 19-norethisterone	Canada	(Muir and Howard 2006)
Pharmaceuticals PCPs and endocrine disruptors measured in surface water	occurrence, treatment in WWTPs, ecological effects, human health effects	Demeclocycline Flumequine Tri(2-butoxyethyl) phosphate Methylbenzyldene camphor Estriol	USA	(Kumar and Xagoraraki 2010)
500 classical	frequency and	diazinon, azoxystrobin,	Germany	(von der

and emerging organic contaminants	extent of exceedence of PNEC	terbutylazine, heptachlor endosulfan, 4,4'-ddd, diuron, dEHP, irgarol dichlorvos, inorganic arsenic compounds, cobalt compounds, beryllium compounds, fenitrothion, disulfoton, parathion,	and Spain	Ohe, Dulio et al. 2011)
Chemicals of Japanese Pollutant Release and Transfer Register	human health environmental effects		Japan	(Lerche, Matsuzaki et al. 2004

** Environment Canada. Ecological Categorization of Substances on the Domestic Substances List (DSL); Government of Canada: Ottawa, ON, 2004.*

Considering prioritization in the perspective of ecosystems protection there is a need to identify the compounds of highest toxicity to which biological comminutes might be exposed to. For that purpose, it is crucial to have the information of compounds toxicity (Botta, Dulio et al. 2012). Toxicity of the chemical is usually determined by in vivo toxicity tests for standard test species such as algae, invertebrates, and fish so that different trophic levels could be covered. The concentration of the chemical that provokes harmful effect or lethality of test species is measured. The most common are the use of EC50 or LC50 as the indicator of acute toxicity. Acute toxicity tests measure the dose of a chemical that, after short-term exposure, provokes certain effect (mortality, immobility, growth stagnation, etc.) in the test species. On the other hand, chronic toxicity data refer to the dose of the chemical that provokes sub-lethal effect in the species after longer time exposure. Chronic exposure is especially important when considering chemicals that are present in the environment at low concentrations like emerging contaminants. Some of the chemicals that are present at low concentrations in the environment might be very persistent and might have been introduced into the environment continuously and may cause unexpected long term effects (Arnot, Mackay et al. 2006). However, chronic toxicity data is scarce for many compounds. Hence, predictive methodologies can be used to estimate toxicity data gaps. Determination of chemical toxicity can be estimated by QSARs (Sanderson, Johnson et al. 2003). The last step includes procedure or models for calculating the comparable risk of chemicals and final ranking or grouping the chemicals according to their risk. Furthermore, in the environment, organisms are exposed not to isolated chemical but to complex mixtures of many chemicals in different concentrations.

The individual components might be present at concentrations too low to raise concern but additive or even synergistic effects may occur that may result in higher toxicity of single compounds (Schwarzenbach, Escher et al. 2006). The most frequently used concepts for mixture ecotoxicity prediction are concentration addition (CA) and independent action (IA). Both models are used to calculate mixture toxicity based on the toxicity and concentration of individual constituents of the mixture and assume that all the components of the mixture affect the same endpoint. CA assumes that all compounds have similar modes of action, while IA assumes that components of the mixture affect different systems of the organism (Backhaus and Faust 2012). However, neither CA of IA takes into account possible synergistic and antagonistic effects of mixtures.

3.3 OCCURRENCE OF POLLUTANTS

For this comparative prioritization exercise, from the selected prioritization works, we selected 22 compounds that were multiple times proposed as important pollutants according to different prioritization criteria, as well as 15 new compounds of WFD list of proposed priority substances. The list of selected compounds contained both the classical and emerging contaminants. For Northern European and North American the mean and maximum measured environmental concentrations (MEC) of compounds in river water were collected from the literature for over 100 North European rivers from 27 European Countries and 139 rivers North America (Kumar and Xagoraraki 2010) (Kolpin, Furlong et al. 2002, Loos, Gawlik et al. 2009, von der Ohe, Dulio et al. 2011). For the Iberian rivers (Ebro and Llobregat) data was obtained from SCARCE-Consolider project database and literature (Claver, Ormad et al. 2006)

3.4 ECOTOXICITY

Toxicity data for standard test species were obtained from EPA's (Environmental Protection Agency) ECOTOX database and Footprint Pesticide Properties Database, or in the case of lack of test data were estimated by ECOSAR™. In the case of multiple data for the same compound, the lowest toxicity values were used. Collected data are summarized in Table 3.2. The QSARs from ECOSAR are used for aquatic toxicity prediction based on the similarity of structures to chemicals for which the aquatic toxicity measured data exists. Toxicity estimations are based on mathematical relationships between the K_{ow} values and the corresponding measured toxicity. Since 1981, the US EPA has successfully applied

QSARs to predict the aquatic toxicity of new industrial chemicals in the absence of test data (Sanderson, Johnson et al. 2003). However, it needs to be taken into account that the toxicity of those compounds with few data available can be underestimated which might lead to errors in this kind of comparative exercises.

Table 3.2 Acute toxicity of selected compounds collected from ECOSAR and test data from the literature:

Compound	CAS number	ECOSAR Acute toxicity- EC50 (mg/l)			TEST Acute toxicity- EC50 (mg/l)		
		Algae	Invertebrates	Fish	Algae	Invertebrates	Fish
Aclonifen	074070-46-5	1.075	1.815	1.852	0.47	1.2	0.67
Azithromycin	083905-01-5	1.874	3.023	18.822	1.971	3.066	19.827
Bifenox	042576-02-3	1.266	4.183	2.534	/	0.35	0.67
Bisphenol A	000080-05-7	1.331	5.237	1.284	2.7	7.75	4.6
Buprofezin	069327-76-0	273	1.525	2.172	2.1	0.42	0.33
Chlorothalonil	001897-45-6	6.503	4.624	6.982	0.007	0.028	0.008
Cyanazine	021725-46-2	0.121	30.167	44.869	0.2	42	4
Cybutryne	028159-98-0	0.025	3.682	2.123	0.001	5.3	0.75
Cypermethrin	052315-07-8	0.009	0.000835	0.00125	0.1	/	0.001
Diazinon	000333-41-5	1.372	0.00123	0.276	6.4	0.001	3.1
Dichlorvos	000062-73-7	2.01	0.03	14.811	5.8	/	0.1
Diclofenac	015307-	41.41	25.754	37.655	/	22.43	/

	86-5						
Dicofol	000115-32-2	0.1	0.053	0.05	0.075	0.2	0.124
Dieldrin	000060-57-1	0.18	0.055	0.214	0,1	0.25	0.001
Endrin	000072-20-8	0.18	0.055	0.054	0.18	0.004	/
Erythromycin	000114-07-8	6.369	8.617	46.882	0.02	113.07	/
Estrone	000053-16-7	8.74	2.184	3.834	8.74	2.184	/
Fenitrothion	000122-14-5	2.845	0.002	0.544	0.495	0.007	1.3
Heptachlor	000076-44-8	0.102	0.023	0.022	0.027	0.078	0.007
Heptachlor epoxide	001024-57-3	0.483	0.34	0.353	200	0.24	0.02
HBCDD	025637-99-4	0.024	0.004	0.004	/	0.0032	/
Imazalil	035554-44-0	0.121	0.594	0.656	0.87	3.1	1.48
Lindane	000058-89-9	2.761	1.565	2.238	2.5	0.516	0.022
Linuron	000330-55-2	0.144	3.61	12.442	0.016	0.12	3
Methidathion	000950-37-8	1.051	0.004	2.851	/	0.006	0.001
Methoxychlor	000072-43-5	0.348	0.115	0.144	0.6	0.001	0.052
Parathion - methyl	000298-00-0	5.967	0.004	1.087	3	0.007	2.7
PFOS	001763-23-1	32.647	16.916	23.664	/	37.36	/
Prochloraz	067747-	0.15	0.734	0.789	0.0055	4.3	1.5

Prometryn	09-5 007287- 19-6	0.034	5.606	3.973	0.002	9.7	2.9
Pyrene	000129- 00-0	0.656	0.287	0.386	0.015	0.004	/
Pyripoxyphen	095737- 68-1	0.392	0.136	0.172	0.15	0.4	0.27
Quinoxyfen	124495- 18-7	0.3	0.098	0.123	0.027	0.08	0.27
Terbutryn	000886- 50-0	0.033	5.336	3.701	0.002	7.1	0.82
Trichlorfon	000052- 68-6	0.11	0.041	19.951	10	/	0.7
Ethinylestradiol	000057- 63-6	3.671	0.98	1.296	0.84	/	/
Estradiol	000050- 28-2	4.299	1.129	1.578	4.299	2.87	/
2,4' DDD	000053- 19-0	0.232	0.019	0.087	0.232	/	/

3.5 HAZARD QUOTIENTS

Hazard quotients were calculated for three standard test species corresponding to three different trophic levels, as recommended by the WFD. HQ are defined as the ratio of predicted or measured environmental concentrations and their chronic toxicity, usually expressed as NOEC (non-observed effect concentrations) or PNEC values (Castiglioni, Fanelli et al. 2004, Bound and Voulvoulis 2006). When NOEC values are not available, EC50 or LC50 values from standard ecotoxicological tests can be used after correction by an assessment factor (EU 2000) intended to extrapolate from acute to chronic toxicity. For the calculation of HQ, we used a ratio of MEC (measured environmental concentrations) and estimated PNEC values from acute data EC50 divided by an assessment factor of 1000 as recommended by WFD (Equation 3.1).

$$\mathrm{HQi} = \frac{\mathrm{MECi}}{\mathrm{PNECi}};\ \mathrm{PNECi} = \frac{\mathrm{EC50i\ or\ LC50i}}{1000} \qquad (3.1)$$

By ranking the HQ we identify the most relevant pollutants for each trophic level and for Iberian rivers (with rivers Ebro and Llobregat rivers as representatives) and for North American and North European rivers.

3.6 ENVIRONMENTAL OCCURRENCE OF SELECTED COMPOUNDS

The occurrence of selected compounds in water samples from Iberian Peninsula North Europe and North America are illustrated in Figures 3.1 and 3.2. Of the selected compounds PFOS was measured at highest concentrations in Iberian rivers. It is a compound that raises serious concern due its persistence, bioaccumulation potential and toxicity. It is a global pollutant, and it was measured in high concentrations in North American rivers as well (Figure 3.2). Pesticide imazalil was measured in high concentration in Iberian rivers, which might be the consequence of its extensive use in Mediterranean agriculture as citrus fungicide. Citrus fruits are one of the predominant cultures grown on the Mediterranean coast of Iberian Peninsula. Plasticizer bisphenol A was measured at high concentrations in the surroundings of Barcelona city, where large industrial areas are situated. Two pharmaceuticals were measured in high concentrations, anti-inflammatory diclofenac, and antibiotic azithromycin. The occurrence pharmaceuticals in such concentrations might be the consequence of their insufficient removal from effluent in wastewater treatment plants (Boxall, Rudd et al. 2012). High concentration of pharmaceutical erythromycin (1,71 µg/l) and hormone 17alpha-ethinylestradiol (0,831 µg/l) are measured in North American river waters (Kolpin, Furlong et al. 2002) indicating the same problem with insufficient wastewater treatment.

3.7 MEASURED VS. MODELED TOXICITY

Acute toxicity data of each compound for algae, invertebrates, and fish is presented in Table 3.2. In cases of lack of test data, toxicity was estimated by ECOSAR. Measured acute toxicity data was collected from literature and compared to those predicted by ECOSAR. The values of measured and modeled concentrations of selected compounds were in the same orders of magnitude and therefore for this risk assessment and prioritization purpose, both types of data were used.

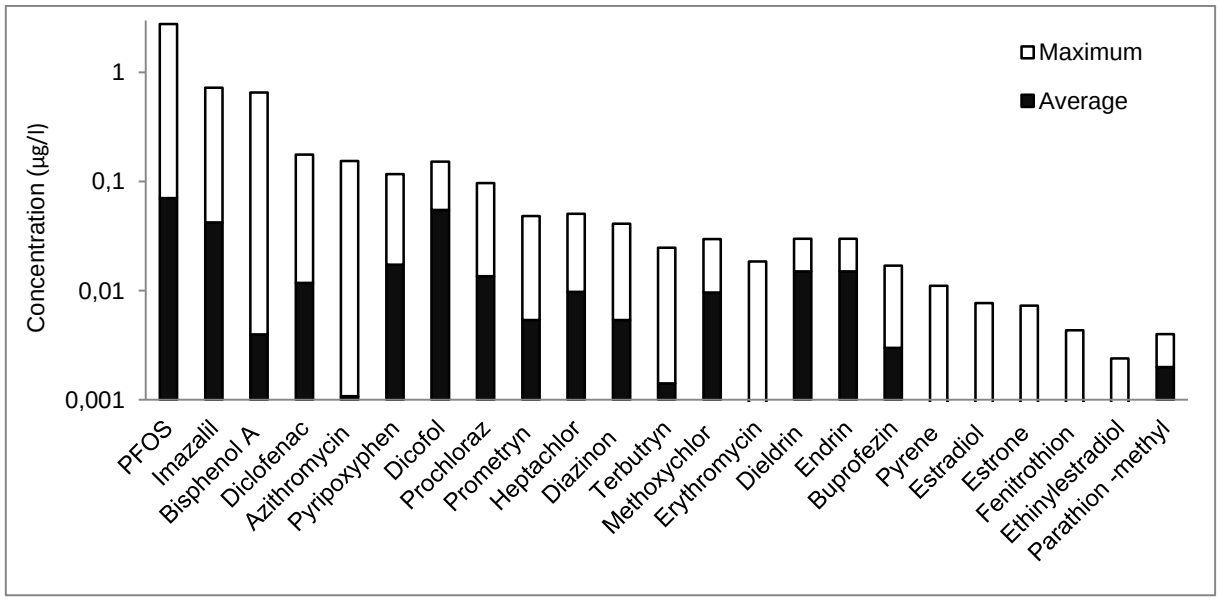


Figure 3.1 Occurrence of selected compounds in Iberian rivers.

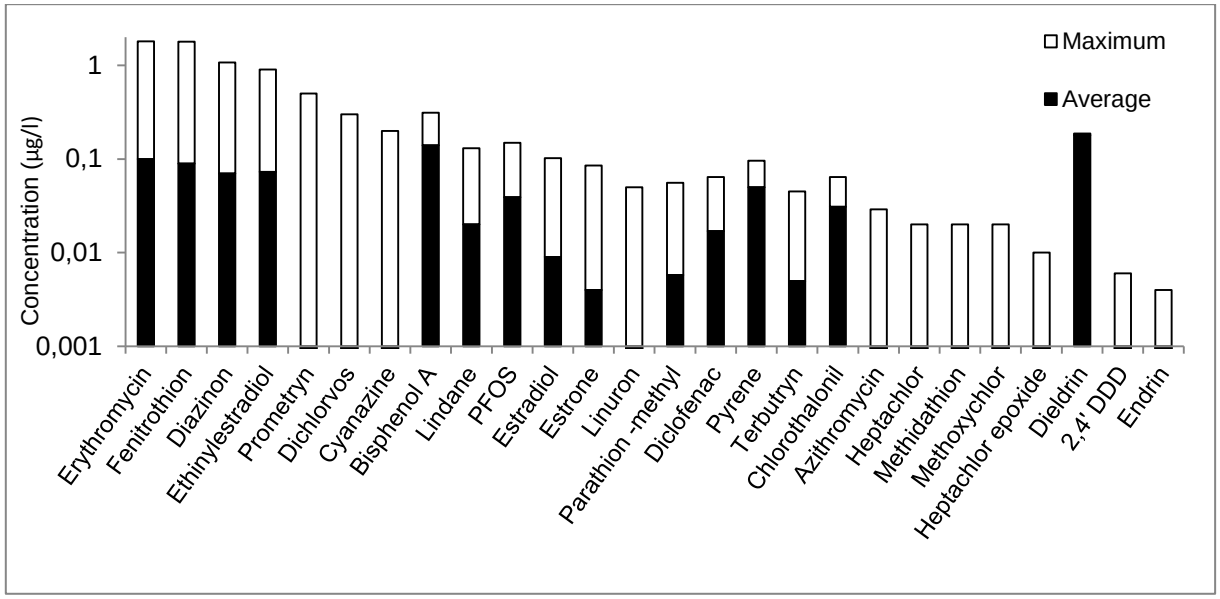


Figure 3.2 Occurrence of selected compounds in North American and North European rivers.

3.8 RISK BASED PRIORITIZATION

In general, HQ higher than 1 indicate a potential risk of a compound. We used predicted no-effect concentration (PNEC) by applying an assessment factor of 1000 to EC50 or LC50 acute toxicity data as recommended by the WFD. Before applying an assessment factor none of the compounds' HQ was higher than one, indicating no acute risk of single

compounds in studied rivers. It must be taken into consideration that assessment factor so high might lead to overestimation of risk for some of the compounds. On the other hand, some unexpected specific adverse effects (e.g., endocrine disruption), might be possible even at lower levels (Boxall, Rudd et al. 2012) but it was not covered by this risk assessment. In the case of lack of appropriate chronic toxicity data, the use of assessment factors is the generally accepted approach to deal with uncertainties (Backhaus and Karlsson 2014). Hazard quotients higher than 1, (in this case indicating potential chronic effects) had 22% of compounds for algae, 17% for invertebrates and 9% for fish in Iberian rivers (Table 3).

Comparing the risk expressed by HQ, the highest risk to algae, invertebrates, and fish is posed by pesticides, which are on the top of the ranking lists. In general, for Iberian rivers (Table 3.3), the compounds that pose the highest risk for algae were herbicides (prometryn and terbutryn), fungicide (prochloraz), and insecticides (heptachlor and dicofol). Some of the high concentrations compounds (e.g., bisphenol A or azithromycin) were not posing risk (HQ<1), indicating that risk is driven by above average toxic compounds and not by their abundance. Insecticides (diazinon, methoxychlor, endrin) and pyrene were the compounds of potential risk for invertebrates. Insecticides, such as dieldrin and heptachlor were the compounds of potential risk for fish. In North American and North European rivers, again pesticides were the most important pollutants. Insecticide methidathion, which is banned in Europe, was identified as posing the risk for North American rivers. Furthermore, pharmaceutical erythromycin showed a high risk for algae in North American rivers. Pharmaceuticals, in general, are a less toxic group of compounds, but in cases when they were present at high concentrations their risk was comparable to the risk of pesticides which indicates the need to include the emerging contaminants into risk assessment. Due to the lack of chronic toxicity data for them, it is difficult to assess the real risk of pharmaceuticals, especially because they are designed to be active in very low concentrations. Furthermore, estrone and estradiol are ranked very low at all lists indicating their relevance as pollutants are lower when compared to pesticides. However, they are known endocrine disruptors and they require more specific approaches to risk assessment.

Table 3.3 Ranked compounds according to HQ for algae, invertebrates and fish in Iberian rivers.

Rank	Compound	HQ Algae	Compound	HQ *Invertebrates*	Compound	HQ Fish
1	Prometryn	21.500	Diazinon	35.700	Dieldrin	15.000
2	Prochloraz	15.200	Methoxychlor	20.000	Heptachlor	5.8570
3	Terbutryn	11.700	Endrin	3.7500	Dicofol	0.7822
4	Heptachlor	1.5185	Pyrene	2.5750	Imazalil	0.4612
5	Dicofol	1.2933	Heptachlor	0.5256	Methoxychlor	0.3846
6	Erythromycin	0.9250	Fenitrothion	0.4900	Pyripoxyphen	0.3688
7	Imazalil	0.7847	Dicofol	0.4850	Endrin	0.2777
8	Pyrene	0.6866	Parathion-methyl	0.2857	Bisphenol A	0.1411
9	Pyripoxyphen	0.6640	Pyripoxyphen	0.2490	PFOS	0.1145
10	Bisphenol A	0.2405	Imazalil	0.2202	Prochloraz	0.0557
11	Dieldrin	0.1500	PFOS	0.1601	Buprofezin	0.0424
12	Endrin	0.0833	Bisphenol A	0.0838	Terbutryn	0.0285
13	PFOS	0.0829	Dieldrin	0.0600	Pyrene	0.0266
14	Azithromycin	0.0779	Azithromycin	0.0501	Prometryn	0.0148
15	Methoxychlor	0.0333	Buprofezin	0.0333	Diazinon	0.0115
16	Fenitrothion	0.0069	Prochloraz	0.0194	Azithromycin	0.0077
17	Buprofezin	0.0066	Diclofenac	0.0064	Estradiol	0.0049
18	Diazinon	0.0056	Prometryn	0.0044	Diclofenac	0.0044

19	Diclofenac	0.0039	Estrone	0.0033	Fenitrothion	0.0026
20	Ethinylestradiol	0.0026	Terbutryn	0.0033	Estrone	0.0019
21	Estradiol	0.0018	Estradiol	0.0026	Ethinylestradiol	0.0017
22	Estrone	0.0008	Ethinylestradiol	0.0022	Parathion-methyl	0.0007
23	Parathion-methyl	0.0006	Erythromycin	0.0002	Erythromycin	0.0004

For the North American and North Europe rivers, 31%, 35%, and 27 % of compounds for algae, invertebrates, and fish, respectively had HQ>1, indicating a potential risk (Table 3.4). Pharmaceutical erythromycin ranked second on the list for algae with very high HQ of 85. On the contrary, in Iberian rivers, its HQ was below 1, regarding it as no risk compound for these rivers. Different production volume and consumption of this pharmaceutical in the United States compared to Spain might be the reason for detection of higher concentrations of erythromycin in North American rivers (Kolpin, Furlong et al. 2002), which results in higher ranking according to its very high hazard quotient compared to Iberian rivers. Imazalil, which is found in high concentration in Iberian rivers, was not evaluated for these rivers since no data regarding its occurrence were available. Herbicide linuron and fungicide chlorothalonil are two potentially dangerous compounds for algae in North European and American rivers, however, data concerning their occurrence in Iberian rivers were lacking and therefore were not included in the evaluation for those rivers. The differences on the lists of ranked compounds are due to different occurrence patterns of compounds in those rivers. Such specificity is relevant and should be taken into account from the point of view of river basin management and implementation of the WFD.

Table 3.4 Ranked compounds according to HQ for algae, invertebrates and fish in North Europe and US rivers.

Rank	Compound	Algae	Compound	Invertebrates	Compound	Fish
1	Prometryn	250	Diazinon	1000.0	Methidathion	20.00
2	Erythromycin	85.00	Fenitrothion	242.85	Dieldrin	7.000
3	Terbutryn	20.00	Methoxychlor	20.00	Lindane	5.000
4	Chlorothalonil	4.714	Pyrene	11.50	Chlorothalonil	4.125
5	Fenitrothion	3.434	Dichlorvos	10.00	Dichlorvos	3.000
6	Linuron	3.125	Parathion - methyl	7.1429	Heptachlor	2.857
7	Pyrene	3.066	Methidathion	3.3333	Fenitrothion	1.307
8	Cyanazine	1.00	Chlorothalonil	1.1786	Ethinylestradiol	0.6412
9	Ethinylestradiol	0.9893	Endrin	1.0000	Heptachlor epoxide	0.5000
10	Heptachlor	0.7407	Ethinylestradiol	0.8480	Methoxychlor	0.3846
11	Diazinon	0.1563	Linuron	0.4167	Diazinon	0.3226
12	Dieldrin	0.0700	2,4' DDD	0.3158	Prometryn	0.1724
13	Bisphenol A	0.0637	Heptachlor	0.2564	Pyrene	0.1192
14	Dichlorvos	0.0517	Lindane	0.2132	Endrin	0.0741
15	Lindane	0.0440	Prometryn	0.0515	2,4' DDD	0.0690
16	Methoxychlor	0.0333	Heptachlor epoxide	0.0417	Estradiol	0.0589
17	2,4' DDD	0.0259	Estrone	0.0371	Cyanazine	0.0500

18	Endrin	0.0222	Estradiol	0.0324	Terbutryn	0.0488
19	Estradiol	0.0216	Dieldrin	0.0280	Bisphenol A	0.0374
20	Methidathion	0.0190	Bisphenol A	0.0222	Erythromycin	0.0363
21	Parathion - methyl	0.0167	Erythromycin	0.0150	Estrone	0.0211
22	Azithromycin	0.0147	Azithromycin	0.0095	Parathion - methyl	0.0185
23	Estrone	0.0093	PFOS	0.0065	Linuron	0.0167
24	PFOS	0.0034	Terbutryn	0.0056	PFOS	0.0046
25	Diclofenac	0.0011	Cyanazine	0.0048	Azithromycin	0.0015
26	Heptachlor epoxide	0.0001	Diclofenac	0.0018	Diclofenac	0.0012

3.9 CONCLUSIONS

In the present article, we selected the compounds that were proposed as important pollutants by different prioritization schemes and compared their occurrence and risk of in Mediterranean (Iberian) rivers with those in Northern European and American rivers. Homogenous experimental toxicity data for the same species, same test time and the same endpoint was not always available. Modeled toxicity data were used in these cases. The comparison of modeled and measured data showed that the levels of concentrations are in the same order of magnitude and therefore for this risk assessment and prioritization purpose both types of data can be used. Pesticides and pyrene were the highest risk compounds for Iberian rivers and North American and North European Rivers. Still, pharmaceuticals (i.e., erythromycin) posed a very high risk for algae indicating the need to include emerging contaminants in the risk assessment and river basin management. Compounds of highest potential for causing toxic effects for algae were mostly herbicides and fungicides (prometryn, prochloraz, terbutryn, heptachlor and dicofol). For invertebrates

compounds with potential risk were: diazinon, methoxychlor, endrin and pyrene, and for fish dieldrin and heptachlor. As expected, some differences were present between different geographical areas. For example, methidathion which is banned in Europe was a relevant pollutant in North America or pharmaceutical erythromycin which ranked second for algae with very high hazard quotient (HQ=85) in North American rivers, but it did not show risk for Iberian rivers. These differences highlight the need for river basin specific pollutants. Therefore, monitoring and regulation might be directed to different compounds depending on the area and its specific socio-economic activities.

CHAPTER 4

RISK ASSESSMENT BASED PRIORITIZATION OF 200 ORGANIC MICROPOLLUTANTS IN 4 IBERIAN RIVERS

Based on the publication in the Science of Total Environment Journal (2015)

Maja Kuzmanović, Antoni Ginebreda, Mira Petrović and Damia Barceló

4.1 INTRODUCTION

The use of chemicals by our technological society is continuously growing both in total amount as well as in the number of different substances among which organic chemicals play a major role. According to the European Inventory of Commercial Chemical Substances (EINECS) the number of substances commercially available in Europe is ca. 100,000 compounds and similar figures hold for the USA (Arnot, Mackay et al. 2006, Muir and Howard 2007). Depending on their intrinsic physical-chemical properties, volume and mode of use (Guillén, Ginebreda et al. 2012) many of these compounds may find their way into the aquatic environment either from the point or diffuse sources (Schwarzenbach, Escher et al. 2006) where they can affect freshwater ecosystems. In fact, chemical pollution is recognized as one of the major causes of their impairment (Vörösmarty, McIntyre et al. 2010). As regards chemical pollution, there are two aspects that seem of special concern: firstly, many of these pollutants are continuously and steadily released into the environment in low but increasingly measurable concentrations (Barceló and Petrovic 2007). Secondly, the long-term environmental and health effects of many of the compounds that are found in the environment are still unknown, especially because they occur in the in the form of complex chemical mixtures rather than alone.

Owing to the growing public awareness on the need of protecting both ecosystems and human health from the risks associated with chemical pollution, an increasingly important body of regulations has raised in the last years, especially in developed countries. In this context, the elaboration of lists of chemical substances based on a risk assessment procedure plays a major role. For instance, the Water Framework Directive which aims the achievement of good ecological and chemical status of European water bodies by the year of 2015. To achieve good chemical status water bodies must meet the Environmental Quality Standards (EQS) for 45 so-called priority substances (PS) and priority hazardous substances (PHS) from previous legislation. Furthermore, under the WFD, EU member states are obliged to set quality standards for river basin specific pollutants discharged in each water body and to take action to meet those quality standards by 2015. Advances in environmental analytical chemistry (Barceló and Petrovic 2007) have shown that regulated and monitored chemicals are only a small fraction of all the chemicals present in the environment (Petrovic, Ginebreda et al. 2011). Recently, attention has been directed to several families of contaminants (i.e., pharmaceuticals, personal care products, endocrine disrupting compounds, perfluorinated compounds, pesticides etc.) that are biologically active but still unregulated (Pal, Gin et al. 2010), collectively referred to as Emerging

Organic Contaminants (EOCs) (Kuster, López de Alda et al. 2008) and which have been detected in aquatic systems worldwide (Focazio, Kolpin et al. 2008); (Fromme, Küchler et al. 2002); (Kolpin, Furlong et al. 2002); (Leong, Benjamin Tan et al. 2007); (Loos, Gawlik et al. 2009); (Ternes 1998); (Silva, Jelic et al. 2011).

Due to the great number of chemical compounds potentially occurring in the environment there is a need to prioritize them for management optimization purposes (Daginnus, Gottardo et al. 2011, von der Ohe, Dulio et al. 2011). Therefore, identifying the chemicals of concern for a given river basin requires performing a suitable combination of monitoring and reliable assessment of risk. Risk assessment procedures consider both the potential hazard effect of a given substance and its exposure level (Daginnus, Gottardo et al. 2011), (Guillén, Ginebreda et al. 2012). While exposure can be obtained either from measurement (monitoring) or modeling, the hazard is derived from its intrinsic properties. Typically, these encompass persistence, bioaccumulation, and toxicity (referred to as PBT approach). However, in practice, due to the aforementioned continuous introduction in the environment of many compounds, persistence becomes less relevant (i.e., many pollutants are ubiquitous in the environment due to their continuous input). On the other hand, bioaccumulation and toxicity are often correlated. For that reason, many risk-assessment procedures are focused on ecotoxicity as a hazard measure, while persistence and bioaccumulation are disregarded. Since risk assessment depends both on the occurrence and effects of the compounds concerned, a reliable ecotoxicity based prioritization exercise should ideally fulfill the following requirements: (i) it should include ecotoxicity data corresponding to test organisms associated with different trophic levels, so that the effects on the real ecosystem are likely reflected and (ii) it should be based on comprehensive and on-site monitoring, so that exposure levels are representative of the catchment under study. We like to emphasize the latter point, since the (von der Ohe et al. 2011); (Vörösmarty, McIntyre et al. 2010); (Acuña, Datry et al. 2014). Whereas there are a number of prioritization exercises based on western and northern European and US rivers (Kumar and Xagoraraki 2010, Daginnus, Gottardo et al. 2011, Slobodnik, Mrafkova et al. 2012, Smital, Terzić et al. 2013), they are much less abundant for Mediterranean rivers and limited to regulated compounds (López-Doval, De Castro-Català et al. 2012) or are focused on certain families (Ginebreda, Muñoz et al. 2010, Vazquez-Roig, Andreu et al. 2012). In this context, the aims of this study were a) to perform an environmental risk assessment for ca. 200 organic micropollutants including both emerging and WFD priority contaminants monitored in four rivers located in the Mediterranean side of the Iberian

Peninsula, namely the Ebro, Llobregat, Júcar and Guadalquivir rivers; b) to prioritize them for each of the four river basins studied, taking into account their observed concentration levels together with their ecotoxicological potential. To this end, standard tests data corresponding to three organisms (algae, invertebrates, and fish) representative of different trophic levels were used, as recommended by the WFD.

The results of this work might add to the knowledge of river basin specific pollutants (RBSP) for Iberian river basins concerned as that is one of the requirements of WFD. As well as the relevant importance of emerging pollutants compared to regulated priority pollutants in terms ecological risk.

4.2 MATERIALS AND METHODS

4.2.1 STUDY AREA

Four Iberian river basins (Figure 4.1) were studied as representatives of Mediterranean streams. The main features of the studied rivers (Ebro, Llobregat, Jucar and Guadalquivir) are summarized in Table 4.1. Mediterranean rivers are characterized by low summer flow and large floods in autumn and winter seasons as a consequence of Mediterranean climate (Gasith and Resh 1999). In comparison to other regions of the world, the Mediterranean basin is one of the most vulnerable to climate changes (Barceló and Sabater 2010). In particular, Mediterranean rivers and streams are subjected to severe alterations in the flow regime because of a decreasing number of precipitation days and increase of heavy rain events. The resulting imbalance of available water during low flow periods (Acuña, Datry et al. 2014) and increasing anthropogenic pressures and demands for water lead to severe ecological and socio-economic problems. As a consequence, water scarcity and its

quality preservation are becoming an important issue in Mediterranean countries.

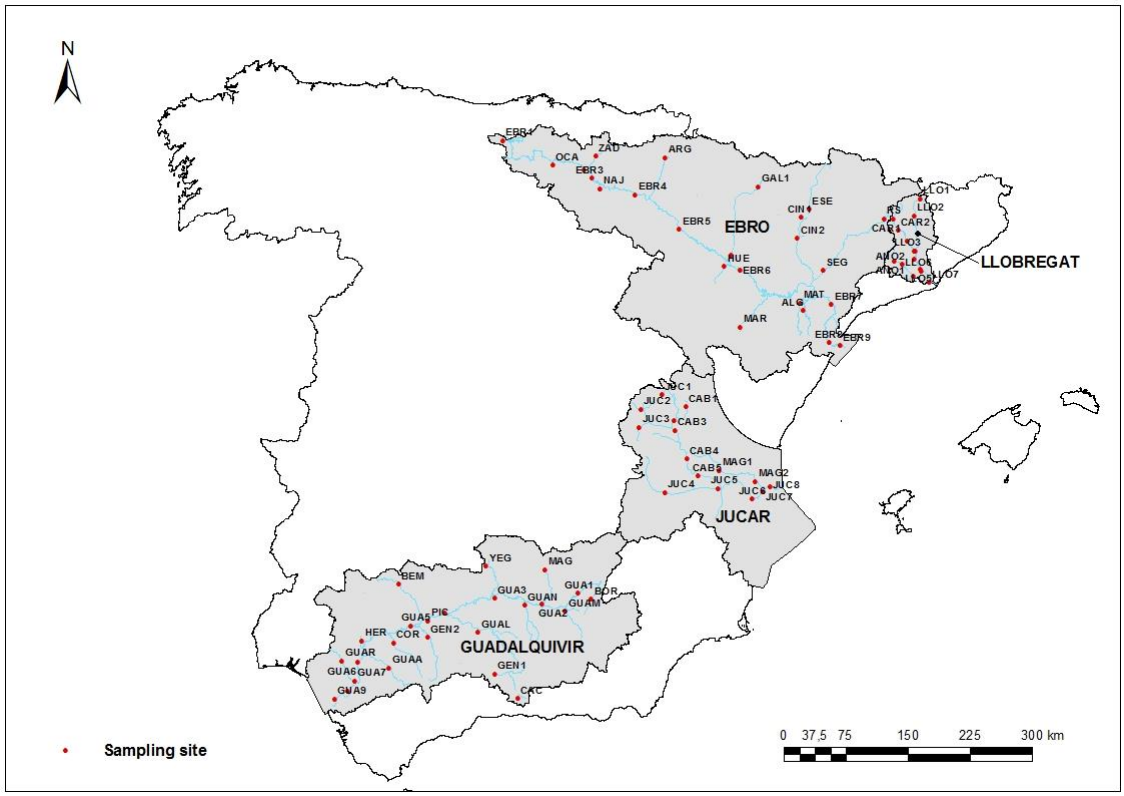


Figure 4.1 Iberian Peninsula and the four Mediterranean river basins studied. Red spots indicate the location of the sampling sites.

Llobregat is situated in North East of Spain. Because of its proximity to the Barcelona town and its metropolitan area, the lowest course of the river receives strong anthropogenic pressures. Urban and industrial wastewater are discharged in Llobregat, in addition to the surface run-off coming from salt mining (both natural and caused by human activities) occurring in its middle basin. Diffuse pollution from agriculture is also present. In spite of the severe pressures received, this river constitutes the major supply source of drinking water for Barcelona and surrounding cities (ca. 3 million inhabitants). Furthermore, as a typical Mediterranean river, it is regularly subjected to periodic floods and drought periods. Overall, this results in reduced dilution capacity during the periods of water scarcity and increasing the potential risk to the aquatic ecosystem.

The Ebro River is located in the North East of Spain and it is the largest river of the Iberian Peninsula in terms of flow discharge. It generates the Ebro Delta, a relevant wetland area (320 km²) in the western Mediterranean region with high ecologic value. The most important economic activity in the basin is agriculture (i.e., vineyards,

cereals, fruit, corn, horticulture along the river and rice production in the delta). In the last century of the river flow has decreased ca. 40% due to land use change (deforestation and increase of irrigation agriculture) as well as precipitation decrease. Though it is not densely inhabited (ca. 2.8 million inhabitants) almost half of the population is concentrated in the big cities. Industrialized regions are mainly situated in the North and central part around big cities of Zaragoza, Pamplona, and Lleida. The Ebro River receives urban contamination coming from the effluent discharges of many wastewater treatment plants (WWTPs).

The Júcar River is located in the East of Spain its population accounts approximately for 2.5 million inhabitants. Most of the agricultural and industrialized areas are located in the medium and lower parts of the river basin. Because of the semiarid climate of this region, the most important problems of this basin are associated with overexploitation of ground and surface water as well as a to water quality issues related to agriculture, urban and industrial pressures. The Júcar basin was designated as a European Pilot River Basin for the implementation of the WFD.

The Guadalquivir River is situated in South West of the Iberian Peninsula. It discharges into the Atlantic Ocean where it forms a navigable estuary. However, its hydrologic and biogeographic characteristics are typical of the Mediterranean region. Along the left side bank of the river estuary, it is located the Doñana National Park covering more than 500 km², Ramsar site, UNESCO Biosphere Reserve as well as a European Community Special Protection Area, considered one of the most important wildlife areas in SW Europe as a refuge for migratory birds.

The Guadalquivir River together with its tributaries serves as the main water source of the region including important cities such as Granada, Córdoba, and Seville. As a consequence of the high population, the river receives many inputs, from both natural and anthropogenic origin, that may cause deterioration of water quality. The lower Guadalquivir river basin is also impacted by reservoirs and dams and its regime is rather artificial. Furthermore, a large portion of the basin is devoted to agriculture especially the production of olives, Mediterranean fruits and rice in the lowest area which might result in water quality deterioration due to the usage of pesticides and fertilizers.

Table 4.1 Some characteristics of the four Mediterranean River basins studied

Basin	Catchment Area (km^2)	River Length (km)	Mean Annual Precipitation (mm)	Mean Discharge ($Hm^3\ y^{-1}$)	Population Density (inhab km^{-2})
Llobregat	4957	165	650	620	545
Ebro	85362	928	672	13408	34
Júcar	21578	512	448	810	207
Guadalquivir	57071	657	520	7230	69

4.2.2 SAMPLING

The prioritization exercise was based on river pollutants concentration data gathered within the Spanish research SCARCE-CONSOLIDER project (Navarro-Ortega, Acuña et al. 2012). Extensive monitoring of water, sediment, and biota from the four Iberian river basins was carried out in two monitoring campaigns (autumn 2010 and 2011). The autumn of 2010 was characterized by intense precipitation which resulted in the high flow of Iberian rivers, while autumn 2011 was dry and the river flows were low. Grab water samples were collected for chemical characterization at 77 selected locations in the Llobregat (15 sites), Ebro (23 sites), Júcar (15 sites) and Guadalquivir (24 sites) River Basins (Figure 4.1).

4.2.3 CHEMICAL ANALYSIS

Organic micropollutants were measured using previously published analytical techniques based on gas chromatography-tandem mass spectrometry and liquid chromatography-tandem and hybrid mass spectrometry. Water phase concentration data of 200 organic compounds belonging to different groups of priority and emerging contaminants: a) pesticides (49), b) pharmaceuticals and hormones (90) c) perfluorinated compounds (22) d) alkylphenols and other industrial organic compounds (14) e) drugs of abuse (8) and f) personal care products (19) were used for this study. Compounds below their limit of detection (LOD) were set equal to 0 for calculation purposes. List of measured compounds with their limits of detections and detection frequencies are available in Annex III as the Table AIII.1.

4.2.4 TOXIC UNITS

To assess the environmental risk of detected compounds from the ecotoxicological perspective the toxic unit (TU) approach (Sprague 1970) was used. TU is defined as the ratio of the compounds measured concentration (C_i) respect to a certain toxicity reference value. Typically EC50 or LC50 (effect or lethal concentration for 50% of individuals) values at 48 h or 96 h exposition time for standard test organisms are used (Equation 4.1). Here acute toxicity *in vivo* measured EC50 for algae, *Daphnia sp.* and fish were used. Toxicity data were collected form peer-reviewed literature and databases when available, mainly ECOTOX and Pesticides Properties Database. When more than one EC50 value was available, the lowest one for each test species was taken into consideration. Missing toxicity data were estimated by ECOSAR (Table AIII.2, Annex III).

$$TU_{i\ (algae,Daphnia\ sp.,fish)} = \frac{C_i}{C_i(ref)} \quad (4.1)$$

Where, TU_i is the toxic unit of compound *i* corresponding to a measured concentration C_i (µg/l) in water phase and $C_i(ref)$ (µg/l) is an ecotoxicity reference concentration for the same compound (EC50 for algae and *Daphnia sp*. and LC50 (µg/l) for fish respectively). The TU approach was recently recommended by the European for approximating the EQS (Environmental Quality Standards) for substances occurring in mixtures.

4.2.5 PRIORITIZATION APPROACH

For prioritization purposes, a 'ranking index' (RI) was developed which is a slight modification of prioritization approach developed by von der Ohe et al. (von der Ohe, Dulio et al. 2011). It is applicable to every compound in a certain area of study (here a river basin) and considers both the toxic units of the compound and their distribution in the area studied. To this end, six logTU ranges or classes were arbitrarily defined as specified in Table 4. 2, which cover the typical occurrence values found in environmental samples. Rank frequencies fx expressed as the fraction of sites (as a percentage) in the river basin where compound's logTU belongs to the specific rank class x are determined in the Equation 4.2:

$$f_x = \frac{n_x}{N_{total}}\ (\%) \quad (4.2)$$

Where n_x is a number of sites in the river basin falling in rank class x, N_{total} is the total number of sites per river. Sum of all the rank frequencies is equal to 100% as it covers all the sampling sites in the river basin. The compound's *ranking index* in the basin under study is defined by summing up the frequencies f_x multiplied by certain arbitrary weights w_x (Equation 4.3), (Table 4.2):

$$Ranking\ Index = \sum_{x=1}^{6} f_x \cdot w_x = (f_1 \times 1) + (f_2 \times 0.5) + (f_3 \times 0.25) + (f_4 \times 0.125) + (f_5 \times 0.0625) + (f_6 \times 0.0) \quad (4.3)$$

The *ranking index* is scaled from 0 to 100, where 100 means that compound's log transformed TU is higher than 0 in all sites in sampled river, and 0 that compound's log TU is not exceeding the value of -4 in any site. Log TU higher than 0 means that the concentration measured exceeds the EC50 value of the compound, which is the threshold for acute effects risk of standard test species concerned. The sixth rank was given the value 0 for those log TU that were less than -4 which stands for 1/10 000 of the EC50 value and it is expected not to cause short term or long term effects in the ecosystem in most of the cases (Liess and Von Der Ohe 2005, Beketov, Foit et al. 2009). Since ranking indexes are related to toxic units, they must be calculated for each test species (algae, *Daphnia* and fish).

Table 4.2 Definition of the six rank classes, their interval ranges and weights used in the calculation of the Rank Index.

Rank class *x*	Range *Log TU*	Weight w_x
1	> 0	1
2	<0,-1>	0.5
3	<-1,-2>	0.25
4	<-2,-3>	0.125
5	<-3,-4>	0.0625
6	<-4	0

4.3 RESULTS AND DISCUSSION

4.3.1 OCCURRENCE

Concentrations ranges of different classes of measured compounds (pharmaceuticals, hormones, personal care compounds, pesticides, perfluorinated compounds, industrial compounds and drugs of abuse) are given in Figure 4.2, for years 2010 and 2011 respectively. Among the different groups of compounds, industrial organic chemicals (IOC) were found in highest concentrations in all four studied rivers. The maximum concentration of IOCs was measured in Llobregat river, (Cmax IOC=10.5µg/l) close to the highly industrialized and urbanized area of Barcelona city (Figure 4.1, site LLOB7). This group of compounds included anticorrosion agents (tolyltriazole and 1H-benzotriazole) as well as alkylphenols (octylphenol, nonylphenol, and related nonylphenol monocarboxylate-NP1EC, nonylphenol diethoxylate-NP2EO as the most relevant. Perfluorinated compounds (PFCs) were measured in highest concentrations in Llobregat river basin as well. The maximum concentration of this group of compounds (Cmax PFCs=2.8µg/l) was measured at the site situated in the proximity of large industrial zone of Igualada surrounding the Barcelona city (Site ANO2, Figure 4.1). The perfluorobutanoic acid (PFBA) and perfluorooctanesulfonic acid (PFOS) were the most abundant compounds of this group.

The second group in terms of concentration range corresponded to pharmaceuticals, which encompass a great variety of compounds belonging to different therapeutic classes and are directly related to their use by the population living in the basin. The maximum concentration of pharmaceuticals (Cmax=3.9 µg/l) was measured in Ebro river at a site close to wastewater treatment plant Zadora, downstream of Vitoria city in Basque Country (Figure 4.1, site ZAD). A special case is that of caffeine that was found in concentrations up to 3.2 µg/l. As expected, highest concentrations of caffeine were found close to big cities like Zaragoza, Huesca, and Barcelona. It is likely due to inefficient removal of this compound from wastewater treatment plants (Zarrelli, DellaGreca et al. 2014). Comparatively illicit drugs (Cmax=1.3 µg/l in Júcar) and personal care compounds (Cmax=0.2 µg/l, in Guadalquivir) were detected at lower levels, being their use associated to the population as well. Generally, the Llobregat followed by some sites in the Ebro were the most contaminated river due to the corresponding urban and industrial pressure (Table 4.1). Pesticides were found in highest concentrations in the Júcar River (Cmax=1.23 µg/l) and it is the only of four rivers in which the pesticides are the main contributors to overall contamination as the result of agricultural pressures (Cmax=1.23 µg/l) When both monitoring campaigns 2010 and 2011 are compared, the concentrations of pesticides were mainly higher in the former one (µ2010=0.2µg/l, µ2011=0.05µ/l), even though that year was characterized by higher river flow. This was likely due to the run-off

effect of pesticides from surrounding agricultural fields caused by the intense precipitations. On the contrary, concentrations of industrial chemicals which are released into the environment from other types of sources (urban wastewater discharges, industrial wastewater etc.) were lower in 2010 possibly due to a dilution effect. However, the year 2010 was exceptionally wet for the Mediterranean area under study, and just the opposite scenario is rather the rule. Increasing water shortage is foreseen in the near future due to the simultaneous occurrence of low precipitation, high evaporation, land use changes and increasing water demand for irrigation and tourism(Hernández-Soriano, Mingorance et al. 2009). Overall this may result in a higher risk of chemical contamination of river water (Barceló and Sabater 2010).

4.3.2 RANKING INDEX

There is a general need for both acute and chronic toxicity data for many emerging compounds. Moreover, chronic toxicity data is of special interest for studied compounds like pharmaceuticals and personal care products. They are continuously released into the environment and have been detected in aquatic environments worldwide (Kolpin, Furlong et al. 2002, Scheurer, Sacher et al. 2009, Gago-Ferrero, Mastroianni et al. 2013, Carmona, Andreu et al. 2014). Changes in the ecosystem may result from their long time exposures even at low concentrations. Therefore, further research concerning their chronic toxicity is advocated (Minagh, Hernan et al. 2009, Ginebreda, Muñoz et al. 2010, Brausch and Rand 2011, Silva, Santos et al. 2011). Specific kinds of toxicity are not taken into account in this work and for most of the compounds; there is very few data available. However, it is important in the assessment of the potential risk of existing and new chemicals and should be included into risk assessment processes since some of the chemicals might have very specific modes of action and pose the risk to ecosystem beyond the acute toxicity. For some of the studied compounds, neither measured nor predicted (ECOSAR) toxicity data were available. In particular, only for 132, 136 and 135 of the 200 compounds were EC50 values data found for algae, Daphnia sp. and fish, respectively (Table AIII.2, Annex III). The rest of the compounds were excluded from further risk assessment (Table AIII.2, Annex III). The compounds whose RI was above 0% are summarized in Tables 4.3, 4.4 and 4.5 for algae, Daphnia and fish respectively. Compounds that are not included in those tables had RI of 0, that is, their log-transformed TUs were below -4 at all studied sites. According to RI pesticides were the group of

compounds posing the highest risk to ecosystems of studied rivers. In particular, insecticides were identified as most important for Daphnia sp. (chlorpyriphos, chlorfenvinphos, diazinon etc.) with RI up to 37% (Table 4.4).They are followed by alkylphenols including octylphenol, nonylphenol and related compounds. For fish, pesticides and alkylphenols were most important groups similar to Daphnia, however with the lower range of (RI (fish, max) =24%). The most important compounds for algae were herbicides and fungicides as well as several pharmaceuticals and personal care products (caffeine, sertraline, losartan, and triclosan) (Table 4.3).

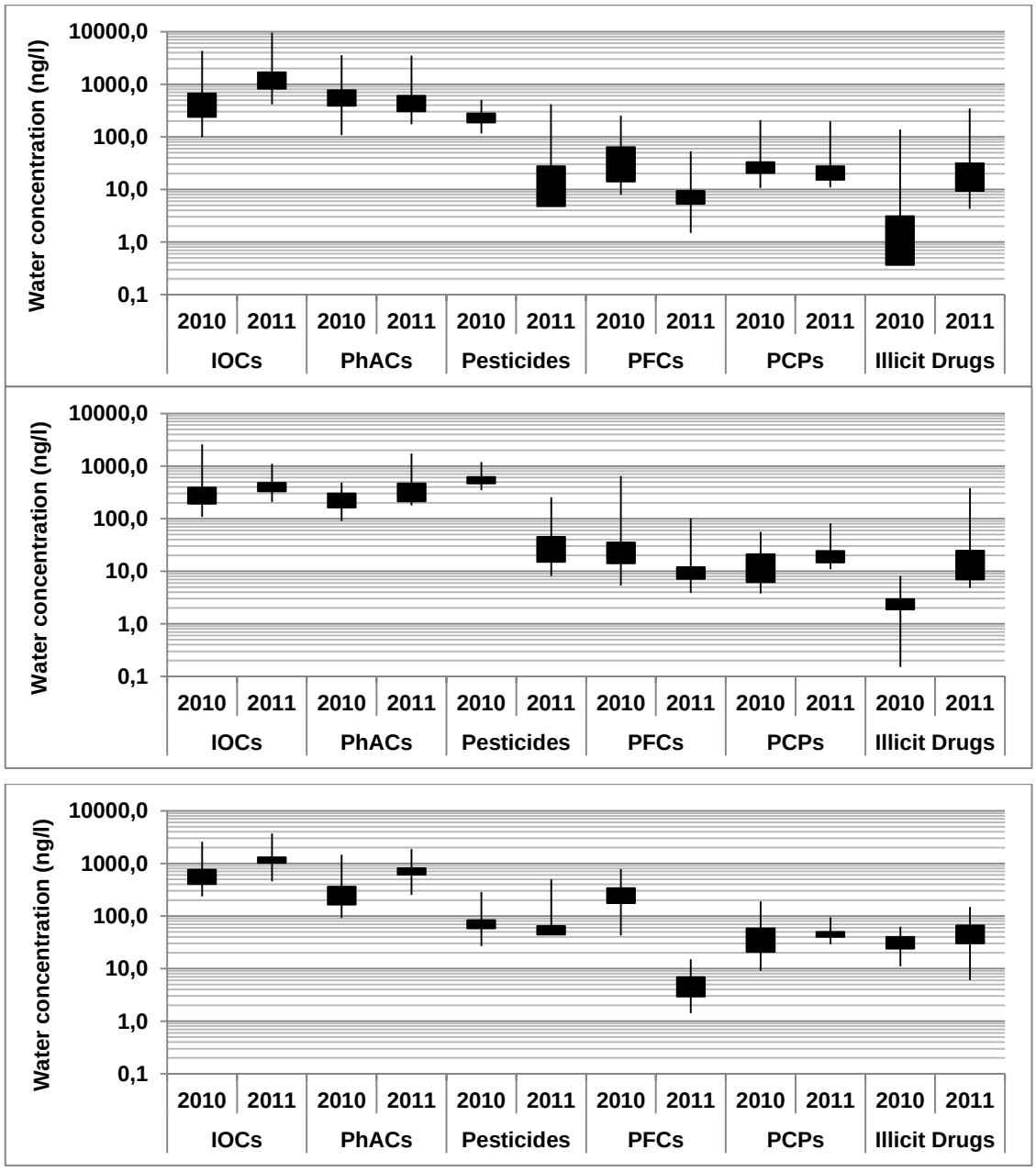


Figure 4.2 Concentration ranges by compound class in the four rivers studied in 2010 and 2011 (IOCs: industrial organic compounds; PhACs: Pharmaceuticals and hormones; PFCs: Perfluorinated compounds; PCPs: Personal Care Products). For each family, whiskers correspond to quartiles 100 and 25 and boxes to quartiles 75 and 50 (a) Llobregat;(b) Ebro;(c) Júcar and (d) Guadalquivir.

Pesticides were, due to their toxicity, the group posing the highest risk to all studied rivers even though they were not measured at highest concentrations compared to other groups of compounds. Therefore, the resulting RI was higher for Júcar compared to Llobregat which was the most contaminated river but mainly by urban and industrial related compounds. Since the compounds contributing mostly to the overall risk were pesticides it is expected to have the highest risk in the areas where they are used in abundance as it is the case for Júcar.

4.3.3 PRIORITIZATION

To select the most important compounds from the ecotoxicological point of view we have set a first arbitrary threshold of RI to 20 % because it suggests that the compounds logTUs were either in the range of -2 to -1 or were exceeding that range in high frequency. That is, they were in high toxic units at many sites in the river. This way both spatial relevance and the intensity of risk were taken into account. Compounds within this range (or higher) values of toxic units are suspected to cause acute effects on the most sensitive species in the ecosystem (Schäfer, Caquet et al. 2007) (Liess and Von Der Ohe 2005). A second threshold was set at RI of 10% which indicates that the logTU of the compound were either at many sites in the river in the range of -3 to -2 or were exceeding that range in several sites. The compounds found in low toxic units are associated with long-term effects in the ecosystem (Schäfer, Caquet et al. 2007). This scale is based on previous works comparing the effects caused by pesticides on aquatic macroinvertebrates with EC50 values for Daphnia sp. (Liess and Von Der Ohe 2005, Schäfer, Caquet et al. 2007, Beketov, Foit et al. 2009). However, it must be taken with precaution when applying to algae and fish since it has been observed in mesocosm studies (Brock 2000) that threshold for triggering changes is higher for these groups compared to invertebrates (Schäfer, Von Der Ohe et al. 2011). In this case, the sensitivity to measured organic chemicals in the four rivers to standard test species was ranked Daphnia sp. > fish > algae.

The compounds were ranked according to RI for each river, year and test species (Tables 4.3, 4.4 and 4.5). Ten compounds were selected as most important; i.e., those that exceeded the aforementioned RI threshold of 20% i.e. they were frequently found in high TU in the studied river. Those were chlorpyriphos and diazinon for all four river basins, chlorfenvinphos for the Ebro, Júcar and Guadalquivir, diclofenthion for Ebro and Júcar, prochloraz and ethion for Júcar and carbofuran, octylphenol and diuron for Llobregat. Most of them are pesticides and two alkylphenols (octylphenol and nonylphenol). Remarkably, five out of the ten chemicals selected in the present prioritization exercise were also classified as priority pollutants according to the WFD. They were namely insecticides chlorfenvinphos and chlorpyriphos, insecticide diuron and alkylphenols octylphenol and nonylphenol.

The compounds which are posing the highest risk to studied rivers were chlorfenvinphos for the Ebro (RIDaphnia=33%) and Júcar (RIDaphnia=37%) in 2010 and chlorpyriphos for Ebro (RIDaphnia =27%) in 2010, Llobregat (RIDaphnia =25%, 26%), and Júcar (RIDaphnia =35%, 25%) in 2010 and 2011 respectively (Table 4.6). Chlorpyriphos was among compounds posing the highest risk to fish as well. Their log transformed TUs distributed among 6 ranks are given in Figure 4.4 for the rivers where their RI was above 20%. Chlorfenvinphos and chlorpyriphos are organophosphorus compounds that were widely used as insecticides. In Mediterranean area, organophosphorus insecticides are widely used for the control of insect pests in many crops such as cereals, citrus, grapes, and olives (Hernández-Soriano, Mingorance et al. 2009, Belenguer, Martinez-Capel et al. 2014). They were both identified as priority pollutants according to WFD. Pesticides appear in peaks of concentrations so they might be overlooked if the monitoring campaigns are mistimed. Therefore the monitoring of these two compounds still requires special attention especially in Júcar River where they were measured at highest concentrations in the monitoring campaigns of this study.

4.3.4 INVERTEBREATES

Diazinon is an organophosphorus insecticide. It is used in agriculture to control insects on fruit, vegetable, nut and field crops. Diazinon is used in homes to control cockroaches, ants, and carpet beetles, and is in insecticidal pet collars. In the United States, it was banned for domestic uses in 2004. In Iberian Peninsula, it is used both for agricultural purposes (Belenguer, Martinez-Capel et al. 2014) and domestic pest control. The

threshold or RI 10 % was exceeded in all four rivers in 2010 that is might be expected to pose a risk to aquatic ecosystems in many sites in the rivers studied. It was selected as the compound with the highest priority rank in the work by von der Ohe et al. (von der Ohe, Dulio et al. 2011) for rivers Llobregat and Scheldt and one of the ten most important compounds for Japan in the prioritization work by Lerche et al. (Lerche, Matsuzaki et al. 2004).

Industrial organic chemicals nonylphenol and octylphenol result from the biodegradation of polyethoxylated alkyphenol surfactants. They are included in the list of priority substances in Water Framework Directive. Of the four studied rivers, the highest concentrations of these compounds and consequential risk were found in Llobregat River especially close to industrial areas surrounding Barcelona city.

Carbofuran is a broad spectrum carbamate pesticide that affects insects, mites, and nematodes on contact or after ingestion. It is used against soil and foliar pests of field, fruit, vegetable, and forest crops. It is exceeding the RI of 10 % in Llobregat for Daphnia sp. in 2010.

Table 4.3 Compounds with Risk Index respect algae equal or higher than 1 in the four rivers in 2010 and 2011.

Llobregat				Ebro				Júcar				Guadalquivir			
2010	RI %	2011	RI %	2010	RI %	2011	RI %	2010	RI %	2011	RI %	2010	RI %	2011	RI %
Diuron	13	Caffeine	7	Prochloraz	9	Caffeine	9	**Prochloraz**	**24**	Caffeine	8	Diuron	7	Caffeine	9
Caffeine	7	Sertraline	6	Caffeine	5	Sertraline	6	Pyriproxyphen	6	Sertraline	7	Caffeine	6	Nonylphenol	4
Triclosan	5	Diuron	5	Pyriproxyphen	5	Terbutrine	5	Caffeine	4	Diuron	2	Nonylphenol	4	Diuron	3
Isoproturon	4	Terbutrine	3	Triclosan	4	Diuron	3	Imazalil	4	Nonylphenol	1	Triclosan	3	Benzotriazole	1
Losartan	3	Triclosan	3	Losartan	2	Benzotriazole	2	Nonylphenol	3	Prochloraz	1	Atrazine	2	NP2EO	2
Nonylphenol	2	Simazine	2	Atrazine	2	Nonylphenol	2	Diclofenthion	3			Terbutrine	2	Prochloraz	2
NP1EC	2	Tolytriazol	2	Diuron	2	Triclosan	1	Atrazine	2			NP1EC	1	Simazine	2
Tolytriazol	2	Benzotriazole	1	Desethylatrazine	1	NP1EC	1	Triclosan	1			NP2EO	1	NP1EC	2
NP2EO	1	NP1EC	1	Isoproturon	1	Tolytriazol	1	NP1EC	1			Tolytriazol	1	Octylphenol	2
Terbutrine	1	Nonylphenol	1	NP1EC	1	Prochloraz	1	Desethylatrazine	1						
Erithromycin	1	Isoproturon	1	Simazine	1	Clarithromycin	1								
Clarithromycin	1	Atrazine	1	Tolytriazol	1	NP2EO	1								
Bisphenol A	1			Nonylphenol	1										
Prochloraz	1			Terbutrine	1										
Sertraline	1			Clarithromycin	1										
Losartan	1			Erithromycin	1										
Venlafaxine	1			Lorazepam	1										
Valsartan	1			NP2EO	1										
L-PFOS	1			Benzotriazole	1										
Lorazepam	1			Venlafaxine	1										

Table 4.4 Compounds with Risk Index respect Daphnia sp. equal or higher than in the four rivers in 2010 and 2011.

Llobregat				Ebro				Júcar				Guadalquivir			
2010	RI %	2011	RI %	2010	RI %	2011	RI %	2010	RI %	2011	RI %	2010	RI %	2011	RI %
Chlorpyriphos	**25**	**Chlorpyriphos**	**26**	**Chlorfenvinphos**	**33**	Chlorpyriphos	6	**Chlorfenvinphos**	**37**	**Chlorpyriphos**	**25**	Chlorpyriphos	24	Chlorpyriphos	6
Diazinon	13	Diazinon	12	**Chlorpyriphos**	**27**	Diazinon	4	**Chlorpyriphos**	**35**	**Ethion**	**23**	Diazinon	15	Nonylphenol	5
Carbofuran	12	NP1EC	4	**Diclofenthion**	**21**	Nonylphenol	3	**Diclofenthion**	**23**	Chlorfenvinphos	18	Chlorfenvinphos	14	Octylphenol	4
Octylphenol	12	Octylphenol	4	Diazinon	13	Octylpheno	2	Diazinon	15	Diazinon	5	Malathion	7	Diazinon	3
Azinphos Ethyl	9	Ethion	4	NP1EC	6	NP2EO	2	Ethion	12	Nonylphenol	2	NP2EO	5	NP2EO	2
Nonylphenol	6	NP1EC	4	Nonylphenol	2	NP1EC	1	Parathion-ethyl	9	Octylphenol	1	Ethion	4	NP1EC	2
NP1EC	6	Diuron	3	Parathion-Ethyl	2	Methiocarb	1	Octylphenol	6			Nonylphenol	3	Methiocarb	2
NP2EO	5	Nonylphenol	2	Octylphenol	1	Malathion	1	Pyriproxyphen	6			Octylphenol	2	Chlorfenvinphos	1
Malathion	4	Dimetoate	1	NP2EO	1	Chlorfenvinphos	1	Malathion	5			NP1EC	2	Ethion	1
Chlorfenvinphos	3	Chlorfenvinphos	1	Fenthion	1	Azinphos Ethyl	1	Nonylphenol	4			Diuron	1	Carbofuran	1
Methiocarb	2	Tolytriazol	1	Diuron	1	Azinphos Methyl	1	NP1EC	2					Diuron	1
Azinphos	2			Thiabendazole	1	Fenitrothion	1	NP2EO	2						
Fenitrothion	1			Losartan	1			Diclofenthion	1						
Sertraline	1			Venlafaxine	1			Imazalil	1						
Venlafaxine	1														

Table 4.5 Compounds with Risk Index respect fish equal or higher than 1 in the four rivers in 2010 and 2011.

Llobregat				Ebro				Júcar				Guadalquivir			
2010	RI %	2011	RI %	2010	RI %	2011	RI %	2010	RI %	2011	RI %	2010	RI %	2011	RI %
Chlorpyriphos	13	Chlorpyriphos	9	Diclofenthion	20	Nonylphenol	3	Diclofenthion	24	Chlorpyriphos	13	Chlorpyriphos	12	Nonylphenol	4
NP1EC	7	NP1EC	1	Chlorpyriphos	12	Chlorpyriphos	2	Chlorpyriphos	19			Nonylphenol	4	Chlorpyriphos	4
Nonylphenol	6			Pyriproxyphen	5	NP2EO	1	Pyriproxyphen	6			NP1EC	3	NP1EC	2
NP2EO	5			NP1EC	2	NP1EC	1	Imazalil	5			NP2EO	1	NP2EO	1
Malathion	4			Imazalil	2			Nonylphenol	4			Malathion	1	Gemfibrozil	1
Gemfibrozil	4			NP2EO	1			NP1EC	2						
Bisphenol A	2			Gemfibrozil	1			Malathion	1						
L-PFOS	2			Nonylphenol	1			NP2EO	1						
Sulfamethaxole	1														

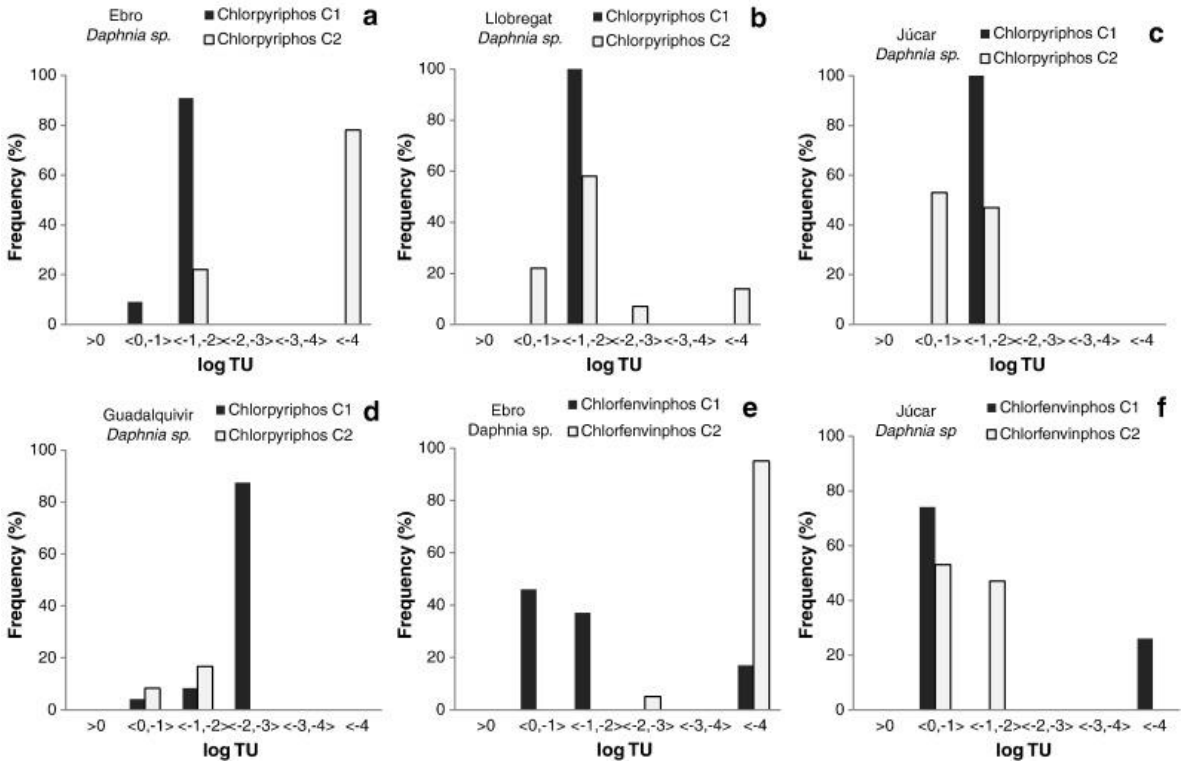


Figure 4.3 Ranges of logTU for most important compounds according to RI in two monitoring campaigns (C1-2010, C2-2011). Chlorpyriphos for Daphnia sp. in: a) Ebro b) Llobregat c) Júcar and d) Guadalquivir and Chlorfenvinphos: e) Ebro f) Júcar

4.3.5 ALGAE

Caffeine was one of the most important compounds in term of RI (algae). Even though it is not expected to cause acute effects in the ecosystem (RI below 20%) it is detected at 99% of the sites in concentrations up to 3.5µg/l. It is ubiquitous in surface waters and it has been proposed as a marker of the anthropogenic pressure on the environment (Buerge, Poiger et al. 2003, Zarrelli, DellaGreca et al. 2014). The EC50algae value for caffeine used was obtained from ECOSAR, therefore it should be taken with some caution before more, and preferably in vivo measured toxicity data will be available. In several studies it has been suggested that caffeine actually behaves as nutrient instead like toxicant for biofilm communities; low level of caffeine exposure (10µg/l) caused increased growth and cell volume of some constituents of biofilm (Lawrence, Swerhone et al. 2005). However, the same study showed the decrease of the biomass of other constituents of biofilm, as well as the more recent study by the same author (Lawrence, Zhu et al. 2012). It was found

that caffeine in combination with other pharmaceuticals like acetaminophen might have a synergistic effect (Fraker and Smith 2004). Acetaminophen is often detected in both surface waters and wastewater effluents worldwide (Kolpin, Furlong et al. 2002, Osorio, Marcé et al. 2012, Vazquez-Roig, Andreu et al. 2012).

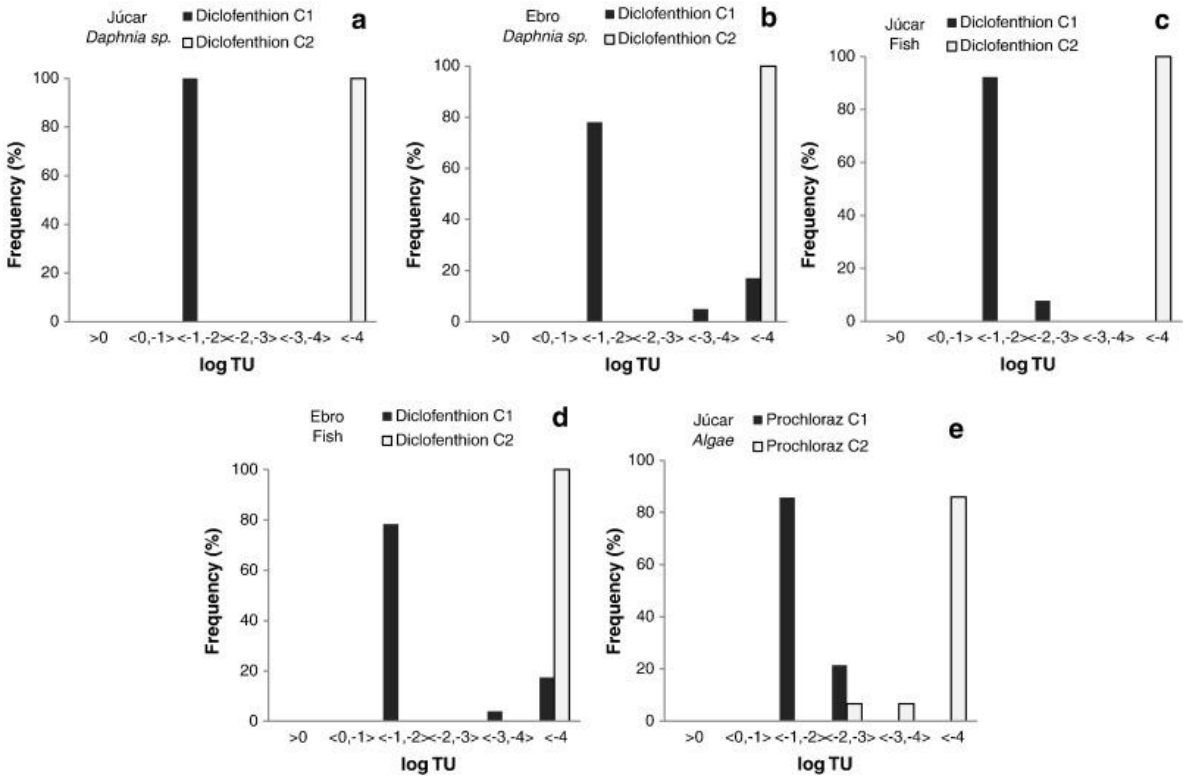


Figure 4.4 Ranges of logTU for most important compounds according to RI in two monitoring campaigns (C1-2010, C2-2011). Diclofenthion for Daphnia sp. a) Júcar and b) Ebro; for fish in: c) Júcar and d) Ebro and Prochloraz for algae in e) Júcar.

Diuron is herbicide that inhibits photosynthesis. It is used on a variety of fruit and nut crops, grains, cotton, corn, sugarcane, seed crops, coffee, hay etc. Diuron also has widespread use in non-agricultural applications e.g. along railway lines, roads; around commercial, industrial and farm buildings. It has some use as an algaecide in ornamental It was identified as an important pollutant by prioritization work by von der Ohe et al. (von der Ohe, Dulio et al. 2011) for rivers the Danube, Elbe, and Scheldt.

Prochloraz is an imidazole fungicide that is widely used in gardening and agriculture. It is used on wheat, barley, mushrooms, cherries, turf on golf courses, and in flower

production. In Iberian Peninsula, it is used mostly as a pesticide in rice, oat, wheat, potato, tomato, garlic and citrus cultivation. In the agricultural area of Ebro and in Júcar it was frequently found in high toxic units. Besides, it has been proven to cause endocrine disruption effects of certain species. Fish exposed to prochloraz showed a disturbance in male-female ratio and hormone levels (Kinnberg, Holbech et al. 2007, Ankley, Bencic et al. 2009) Also, feminization of the male rat offspring after perinatal exposure was observed (Vinggaard, Christiansen et al. 2005, Vinggaard, Hass et al. 2006). Prochloraz is exceeding the risk threshold in Jucar in 2010 (RI(algae)=24%) at 95 % of the sites it's log TU were in the range of -3 to -1 (Figure 4.5) which might result in chronic and acute effects in the ecosystem of that river.

4.3.6 FISH

Dichlofenthion is an organophosphorus insecticide. It has a high RI in the Ebro (RIDaphnia=21%, RIfish=20%) and Júcar (RIDaphnia=23%, RIfish=24%) in 2010. It is one of the most important compounds according to RI for these two rivers. Its log TUs were in the range from -2 to -1 in 2010 at the majority of the sites, while in 2011 they were mostly below -4 (Figure 4.5). The occurrence of dichlofenthion in the Júcar river has been related with usage in pest control for livestock and tomato grows (Belenguer, Martinez-Capel et al. 2014) characteristic for that area.

4.4 CONCLUSIONS

A general prioritization exercise has been done based on a Ranking Index. Although it has been applied to three common ecotoxicity indicators, it can be easily extended to any other in vivo or in vitro assay, providing there are data for all compounds. Generally, there is a lack of systematic ecotoxicity data for many compounds and the need of filling this gap is crucial for ecological risk assessment purposes. Emerging contaminants like pharmaceuticals were measured at many sites in studied rivers. They are not posing the risk of acute effects. However, since the long-term studies are generally lacking further research into their chronic toxicity is strongly advocated. Among the ten most important compounds for studied rivers, eight were pesticides, i.e., six insecticides, (chlorpyriphos, chlorfenvinphos, diazinon, dichlofenthion, ethion, and carbofuran); one fungicide (prochloraz) and one herbicide (diuron). The other two were the alkylphenols octylphenol and nonylphenol that result

from the degradation of polyethoxylated surfactants. Five out of those ten compounds were also included as priority pollutants according to WFD. Prioritization exercises should take into consideration the regional and local (i.e, climatologic, geo-physical and socio-economic) characteristics. This fully aligned with the WFD requirement of identifying specific pollutants discharged in any water-body. Specificity of Iberian Peninsula in terms of agriculture, climate, and precipitation needs to be taken into account when trying to identify rivers basin specific pollutants. The results of this work might lead to the conclusion that the intense precipitation played an important role into risk towards ecosystem as it triggered the runoff effect of pesticides from agricultural fields resulting in their relatively higher concentration in Júcar River and therefore the possibility of adverse effects in the ecosystem of that river. The prioritization scheme proposed here can be useful for regulatory purposes, as well as, for the implementation of the next River Basin Management Plans (RBPM) and Programmes of Measures (PM) required by the WFD.

CHAPTER 5

ECOTOXICOLOGICAL RISK ASSESSMENT OF CHEMICAL POLLUTION IN FOUR IBERIAN RIVER BASINS AND ITS RELATIONSHIP WITH THE AQUATIC MACROINVERTEBRATE COMMUNITY STATUS

Based on the publication in the Science of Total Environment Journal (2015)

Maja Kuzmanović, Julio C. López-Doval, Núria De Castro-Català, Helena Guasch, Mira Petrović, Isabel Muñoz, Antoni Ginebreda and Damià Barceló

5.1 INTRODUCTION

Aquatic ecosystems are impacted by a variety of stressors, including organic and inorganic stressors, excess input of nutrients, geomorphological alterations, land use changes, hydrological stress, invasive species and pathogens (Vörösmarty et al., 2010). As a consequence, the biodiversity decline is one of the greatest ecological problems threatening aquatic ecosystems (Beketov et al., 2013). However, little is known beyond the described effects of single stressors on specific ecological endpoints (Navarro-Ortega et al., 2015) and our understanding of the main causes for the losses of biodiversity still remains vague (Beketov, Kefford et al. 2013). Rivers are receiving numerous chemical compounds originated from anthropogenic activities on a daily basis. As a result, complex mixtures of potentially dangerous compounds are present in the aquatic environment. However, site-specific exposures can vary a lot and some sites are likely to be affected more than others due to local conditions and specific vulnerability characteristics (Brack, Altenburger et al. 2015). Thus, the characterization of the constituents of these mixtures and the identification of the compounds of the highest concern in different spatial frameworks is one of the key issues for the protection of natural ecosystems (Vörösmarty et al., 2010).

Besides a number of regulated pollutants which are known to exhibit adverse effects, there is a large number of chemicals currently in use that are not taken into account in the routine water quality monitoring (Barceló and Petrovic, 2007). These compounds are commonly referred to as emerging contaminants. They encompass a variety of substances used both in industry and households; such as pharmaceuticals, personal care products, hormones, industrial chemicals or their byproducts and the transformation products, all together having in common that their environmental allowed levels are not regulated. In the European Union, the Water Framework Directive (WFD) is the legislation concerning the chemical pollution which aims to achieve the good chemical status of water bodies by meeting the Environmental Quality Standards (EQS) for the 45 so-called priority substances (PS) and priority hazardous substances (PHS). In addition, under the WFD, the EU member states are obliged to set quality standards for river basin specific pollutants discharged in each water body and to take action to meet these quality standards as a part of ecological status. A question that remains open is to what extent priority pollutants represent chemical status in comparison with unregulated chemicals. Here we address this issue from the perspective of their associated ecotoxicological risk.

Another challenge for the scientist dealing with aquatic risk assessment is revealing the link between water pollution and biological community responses. Due to the presence of multiple stressors, their unknown joint effects and the complexity of the biological responses, it is very difficult to distinguish the influence of particular stressors on affected ecosystems. Moreover, in recent years, studies in ecology are increasingly emphasizing that biodiversity loss implies more than the mere loss of species (i. e. taxonomic diversity) (Feld, de Bello et al. 2014). Hence, the functional component of biodiversity should rather be addressed by using the concept of biological traits (e.g. generation time, body size) (Beketov and Liess 2008)(Feld et al., 2014). Commonly used taxonomic richness and diversity metrics (e.g. Shannon or Margalef diversity indexes) are dependent on both anthropogenic influences and natural longitudinal gradient of environmental factors in rivers as altitude, temperature, stream width, nutrition status and velocity (Minshall, Petersen et al. 1985, Paller, Specht et al. 2006, Beketov and Liess 2008) so they might not be able to characterize the toxicant-specific influence of ecosystems. To cope with this problem stressor-specific, traits based metric SPEAR index was developed for pesticides (Liess and Von Der Ohe 2005), general organic toxicants (e.g. petrochemicals, synthetic surfactants) (Beketov and Liess, 2008) and salinity (Schäfer, Von Der Ohe et al. 2011) which is poorly dependent on the natural longitudinal factors (Beketov and Liess, 2008).

In this context, our study is addressing the following objectives. First, to assess the area specific levels of the risk posed to aquatic ecosystems on the river basin level for more than 200 emerging and priority pollutants in four Iberian river basins using the toxic unit concept. Second, to evaluate whether the current list of WFD priority pollutants is enough to estimate the ecotoxicological risk in these basins or there are other compounds present that could be more or equally important in terms of risk. And third, to determine the potential relationship between the ecotoxicity associated with local mixtures of pollutants and aquatic macroinvertebrate biological community responses using four different metrics: Shannon and Margalef biodiversity indexes and SPEARpesticides and SPEARorganic.

To tackle these questions we used as case study four rivers of the Iberian Peninsula for which both biological and chemical data were previously gathered (Navarro-Ortega et al., 2012).

5.2.1 STUDY AREA

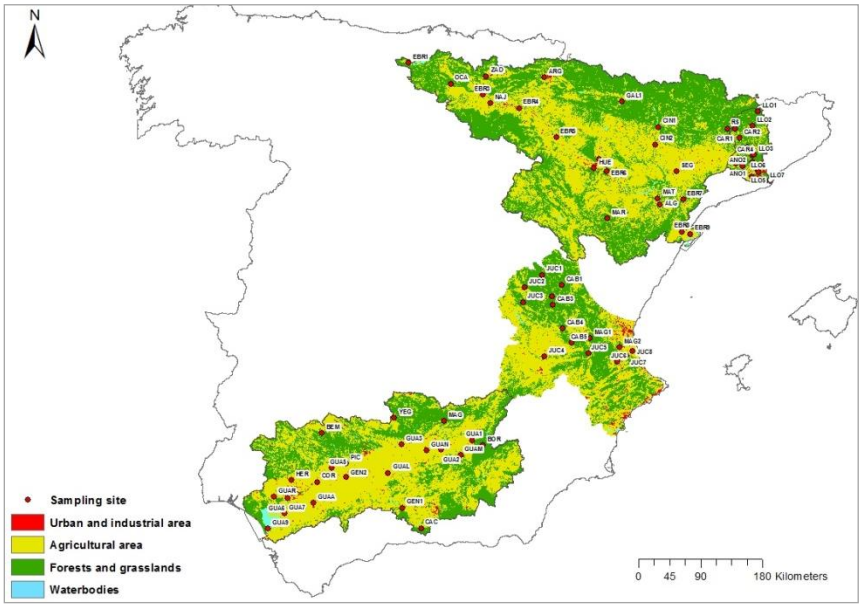


Figure 5.1 Studied river basins with the major land use types and the sampling sites indicated.

Four Iberian river basins (Figure 5.1) were studied as the representatives of Mediterranean rivers. A detailed description of the study area can be found elsewhere (Kuzmanović et al. 2015). The Llobregat is the river situated in the North East of Iberian Peninsula. The lower part of the basin is subjected to strong anthropogenic pressures due to the high proportion of the urban and industrial land use types in that area. In the middle part of the basin, most of the agricultural lands are situated. As a typical Mediterranean river, Llobregat is subjected to decreased flow in the summer periods as a consequence of Mediterranean climate (Gasith and Resh 1999). The Ebro is the large river situated in North of the Peninsula. The main pressures for water quality are coming from agriculture developed along the river basin. The urban and industrial centers are scattered in the basin, mostly in the North East and central part of the basin. The Júcar basin is situated in the East of Iberian Peninsula characterized by semi-arid climate. The most of the agricultural and urban areas are located in the medium and lower parts. Thus, these areas are receiving the most of the combined pressures together. The Guadalquivir basin, situated in the South of the Peninsula as a consequence of the high population, is subjected to strong anthropogenic pressures

that may cause deterioration of water quality. A large portion of the basin is devoted to agricultural use which might result in water quality deterioration due to the input of pesticides and fertilizers.

5.2.2 SAMPLING

The data used for this study were gathered within the Spanish research SCARCE-CONSOLIDER project (Navarro-Ortega et al., 2012). Extensive monitoring of water, sediment, and biota from the four Iberian river basins was carried out in two monitoring campaigns (autumn 2010 and 2011). Autumn of 2010 was characterized by intense precipitation, which resulted in a comparatively higher flow of Iberian rivers, while the autumn 2011 was dry and the river flows were low. Grab water samples were collected for chemical characterization at 77 selected locations in the Llobregat (15 sites), Ebro (23 sites), Júcar (15 sites) and Guadalquivir (24 sites) River Basins (Figure 5.1). Metals and biological data were measured at 19 sites: Llobregat (5 sites), Ebro (5 sites), Júcar (5 sites) and Guadalquivir (4 sites). Sites were selected in a gradient of pollution from sites presumably less polluted to downstream where pollution was accumulated. The major land use types in the catchments were calculated by simplifying the Corine land cover into three groups: urban, agricultural and natural (including forest and grasslands) by Arc Map 10.1 software.

5.2.3 MACROINVERTEBRATE SAMPLING

Five sediment samples were randomly collected at each site with a polyvinyl sand corer (24 cm^2 area). Samples were sieved through a 500-µm mesh and fixed with 4% formaldehyde. The invertebrates were sorted, counted and identified in the laboratory under a dissecting microscope (Leica Stereomicroscope). The identification was at the species level for almost all taxa – including Oligochaeta – with the exception of the Chironomids, which were identified at the genus level, and the Phylum Nematoda. Abundances were referred to the basis of sediment surface area (De Castro-Català et al., 2015).

To examine the biological status and link it with the chemical pollution three indexes were calculated: Shannon diversity index (H') (Shannon, 1949), Margalef diversity index (d) and Species at Risk (SPEAR) for general organic pollution $SPEAR_{organic}$ **(**Beketov and Liess, 2008) and pesticides $SPEAR_{pesticides}$ **(**Liess and Von Der Ohe, 2005)

(www.systemecology.eu/spear/spear-calculator/)**.** SPEAR is a species trait based index that links chemical quality and biological community composition. It provides an assessment of the magnitude of the ecological effects of pollution (Liess and Von Der Ohe, 2005). For the calculations, the species identified in sediment samples were used. When species was not present in the SPEAR database we selected the higher taxonomical order.

5.2.4 CHEMICAL ANALYSIS

Compounds were measured using previously published analytical techniques based on gas chromatography-tandem mass spectrometry and liquid chromatography-tandem and hybrid mass spectrometry (Table S1). Water phase concentration data of 200 compounds belonging to different groups of priority and emerging contaminants: a) pesticides (48), b) pharmaceuticals and hormones (90) c) perfluorinated compounds (21) d) alkylphenols and other industrial organic compounds (14) e) drugs of abuse (8) and f) personal care products (17) and g) metals (8) were used for this study. Compounds below their limit of detection (LOD) were excluded from the study. List of measured compounds and analytical methods used are available in Supporting Information (Table AIV.1 and AIV.2, Annex IV). Of 45 WFD priority pollutants, our dataset included seven pesticides, two industrial organic compounds and two metals (Table AIV.2, Annex IV). Metals concentrations were transformed to bioavailable fraction using biotic ligand model (BLM) (Di Toro et al., 2001). The final number of a number of chemicals that were used for risk assessment in this study (i. e. they were measured above their LOD is and their toxicity data was available) was 142.5.2.5 TOXICITY ASSESSMENT

The toxic unit (TU) approach (Sprague 1970) was used for the ecotoxicological risk assessment of measured concentrations of compounds (C_i). The TU of each compound was based on acute toxicity values i.e. EC_{50} (50% effective concentration) for reproduction and immobilization for algae and invertebrates respectively and LC_{50} (50% lethal concentration) for fish (Equation 5.1).

$$TU_{i\,(algae,invertebrates,fish)} = \frac{C_i}{EC50_i} \quad (5.1)$$

Where TUi is the toxic unit of a compound i; ci measured concentration (µg/L) of the compound in the water phase; EC50i or LC50i (µg/L) effective or lethal concentration of

50% of individuals when exposed to the substance concerned. The toxicity data of each chemical was collected for three standard test species (green algae pseudokirchneriella subcapitata, invertebrate Daphnia magna and fish pimephales promelas or oncorhynchus mykiss) from the literature and the databases when available, mainly ECOTOX (USEPA 2008)and Pesticides Properties Database (PAN 2015). Missing toxicity data were estimated by ECOSAR v.1.11. To determine site-specific toxic stress and compare it with biological quality, we used the classical concept of concentration addition (CA). It allows the prediction of the mixture toxicity from concentration and toxicity of constituents of the mixture (Backhaus and Faust, 2012) but without regarding possible synergistic and antagonistic effects between chemicals. Site specific toxic stress (TUsite) was calculated by summing all the individual TUi of each detected compound at all of the 77 studied sites. Since different effects in the ecosystem are expected from metals and organic compounds (López-Doval et al., 2012), toxic units for metals (TUmetals) and organic compounds (TUorganic) were calculated separately. Additionally, in order to find out how risk is allocated between regulated and unregulated compounds in our dataset, we grouped the compounds in the following manner. Firstly, we excluded the WFD priority pollutants from our dataset and examined which part of the total risk is allocated to "non-priority contaminants" (TUnon-priority) (Table AIV.2, Annex IV) by summing the toxic units of all the compounds detected in each sample except the WFD priority pollutants. Secondly, besides WFD priority pollutants, we excluded the other compounds regulated in the European Union (i.e. banned pesticides) (Table AIV.3, Annex IV). In that way, we examined the risk posed by the unregulated contaminants (TUunregulated) only. Finally, the site-specific risk was expressed as the logarithm of the mixture toxicity of metals, all the detected organic compounds, "non-priority compounds" and unregulated compounds (Equation 5.2):

$$\mathrm{TU}_{\mathrm{SITE(metals,organic,non\text{-}priority\ and\ unregulated)}} = \log \sum_{i=1}^{n} \mathrm{TU}_i \qquad (5.2)$$

Where, TU_i is the toxic unit of each of individual compound at the site. For convenience, along the present article TU associated with each site is expressed in log units. Having in mind the possible different modes of action of the studied compounds, there is a possible overestimation of risk. However, since the modes of action of many studied compounds are still unknown, we used the CA approach which is generally accepted as a first tier approach (Backhaus and Faust, 2012). Additionally, it was showed that the toxicity of the

mixture predicted by CA correlated with the SPEAR index (Schäfer et al., 2013) suggesting this is a valid approach for predicting the toxic stress for biological communities *in situ* (McKnight et al., 2015).

5.2.6 EFFECTS THRESHOLDS SELECTION

To determine the potential effects of chemical pollution on the biological communities in situ we used the effect thresholds as proposed by Malaj et al. (2014). The acute risk threshold was set at the TU ≥-1 (1/10 of EC50 or LC50) for all three test species since the acute effects in the ecosystem are generally expected at that level (Schäfer et al., 2011b; Schäfer et al., 2012; Van Wijngaarden et al., 2005). For the invertebrates, chronic risk threshold value of TU ≥ -3 (1/1000 of EC50) was used. Changes in communities have been observed above that threshold i.e., decrease of sensitive species and shift towards more resistant species assemblages (Beketov et al., 2013; Liess and Von Der Ohe, 2005; Schäfer et al., 2012). However, this threshold is based on the field studies of effects of pesticides on biological communities. Therefore, extrapolating this threshold to other groups of compounds could lead to over or underestimation of the risk for some of the compounds. Also, those studies used the maximum toxic unit (TUmax) in the sample, indicating the minimum estimated toxicity of the mixture as the toxicity of the most potent compound (Schäfer et al., 2013). In the case when the sum of toxic units is used to represent the mixture toxicity it should be noted that this is a bit more conservative approach but in line with the principle of screening-level risk assessments (McKnight et al., 2015). Due to the absence of studies relating pollution and long-term effects in communities, chronic risk thresholds for algae and fish were based on acute to the chronic ratio (Malaj et al., 2014). For algae, the acute to chronic factor 5 was used and for fish factor 10 (Heger, Jung et al. 1995, Länge, Hutchinson et al. 1998, Ahlers, Riedhammer et al. 2006).

5.2.7 STATISTICAL ANALYSIS

Analyses of variability and relations of toxic stress and biological indexes were performed by Principal Component Analysis (PCA) using Microsoft Excel XLSTAT statistical software. Toxic stress was characterized as the sum of TU (for invertebrates) per compound families, namely organic micropollutants and metals. Organic micropollutants were when necessary, grouped in several sub-classes, namely, pesticides, industrial organic chemicals (IOCs), pharmaceuticals, personal care products (PCPs) and perfluorinated

compounds (PFCs) (Table AIV.2, Annex IV). Linear regression and non-parametric correlations (Spearman correlation coefficient) were used to capture the relationships between toxic stress and changes in aquatic macroinvertebrate communities *in situ*.

5.3 RESULTS AND DISCUSSION

5.3.1 ECOTOXICOLOGICAL RISK ASSESSMENT

The toxic units (TUorganic) indicated that there was a risk of the acute effects in biological communities posed by organic compounds at 42% of the sampling sites (Figure AIV.1, Annex IV) and risk of chronic effects at all the studied sites (Figure 5.5A). Of the three test species used for risk assessment, invertebrates were the most sensitive group (Annex IV AIV2-4) due to the presence of highly toxic insecticides at many sampling sites. Considering the four studied rivers, the total number of sites with exceedance of the acute risk threshold was higher in 2010 (42% for invertebrates, 3 % for fish and none for algae), than in 2011 (20% for invertebrates and no exceedance for algae and fish). The highest number of sites exceeding the acute threshold was in the Ebro in 2010 (74% of sites) and in Júcar (67% and 60% in 2010 and 2011, respectively) (Figure 5.2) mostly due to the presence of insecticides chlorpyriphos, chlorfenvinphos, and ethion. On the contrary, in 2011 there was no exceedance of acute risk threshold in the Ebro due to relatively lower concentrations of those pesticides (Figure 5.4). In Llobregat and Guadalquivir there was exceedance of acute risk threshold at less than 25% of the sites (Figure 5.2). In 2011, the only area where the acute risk was increased compared to the previous year was in the lower part of the Llobregat basin (Figure 5.3).

Of all the organic compounds measured in water, the major contributors to the chemical risk were pesticides (Figure 5.4). The compounds responsible for acute risk in Llobregat were chlorpyriphos and azinphos ethyl and ethion. In Guadalquivir, there was an acute risk at only 4 sites in 2010 and 3 sites in 2011 (Figure 5.4) where high concentrations of chlorpyriphos, ethion and chlorfenvinphos were measured. In general, several pesticides were related with risk of acute effects (Figure 5.4) of which the most important were the insecticides chlorfenvinphos (29% of sites with acute risk exceedance in 2010) and chlorpyriphos (15% sites in 2010). They are both classified by WFD as priority compounds and were identified as the compounds of highest ecotoxicological concern in studied river basins (Kuzmanović et al., 2015). Whereas, in 2011 they were not present in water at such high concentrations and thus the resulting acute risk exceedance was evidently lower,

especially in the case of Ebro where chlorfenvinphos was detected only at one site in that year's sampling campaign (Figure AIV.5, Annex IV). The lower acute risk in 2011 might be an underestimation due to sampling in the dry period with the absence of precipitation which can trigger for the runoff effect of pesticides which were the most toxic compounds measured. Other pesticides not covered by WFD, but banned in the European Union (Table AIV.3, Annex IV) were also detected in water at high toxic units (e. g. ethion up to TU= -0.36 in the Júcar).

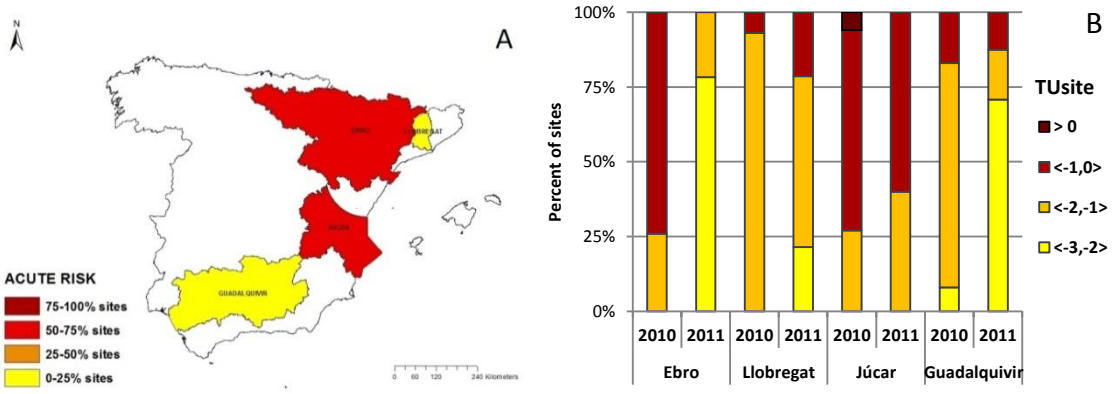


Figure 5.2 Percentage of sampling sites A) with acute risk exceedance and B) with TUsite (most sensitive test species) belonging to one of four toxic unit ranges for each of four river basins in 2010 and 2011.

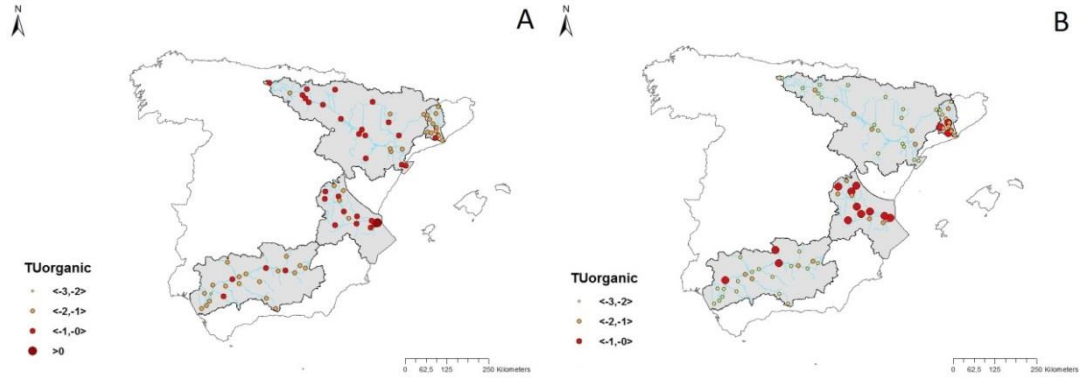


Figure 5.3 Toxic units (TUsite) (for the most sensitive test species) for organic compounds at 77 sampling sites in A) 2010 and B) 2011

5.3.2 CHRONIC EFFECTS RISK IN IBERIAN RIVERS

The chronic risk threshold was exceeded at all of the sampling sites (Figure 5.5A) for at least one of the test species. In 2011, the exceedance was the highest in the Júcar (all

sites), the Llobregat (80% of the sites), the Ebro (61% of the sites) and the Guadalquivir (55% of the sites) (Figures S2-4). While only pesticides and metals were responsible for acute risk, all measured compound groups except perfluorinated compounds exceeded the chronic risk threshold for at least one test species (Figure 5.4). Perfluorinated compounds were in low TU at all the sampling sites (Figure 5.4). Industrial organic compounds exceeded the chronic risk threshold at several sampling sites, mostly in the Guadalquivir (54%) and in the lower part of the Llobregat basin (50%). Of that group, the WFD priority compounds alkylphenols and their ethoxylate derivatives were the main contributors to toxic load among compounds detected. Personal care products exceeded algae chronic threshold (Figure 5.4) mostly due to triclosan that was detected around industrial and urban areas (lower part of the Llobregat and the Júcar basins, the northern part of the Ebro basin (Figure 5.1) Pharmaceuticals exceeded chronic risk threshold in the Llobregat basin in 2010 with the antidepressant sertraline as the compound mostly responsible for threshold exceedance.

However, in this study, we used acute toxicity data to assess the risk of both acute and chronic effects. Despite the fact that long-term chronic exposure to pollutants is a more realistic scenario (Eggen, Behra et al. 2004) there is a paucity of chronic toxicity data, especially for emerging contaminants. As stated by Calow and Forbes (Calow and Forbes 2003), there is uncertainty in extrapolating results from effects caused by short, high dose exposure to effects caused by long time exposures to low doses of chemicals. There are indications that chronic responses to some chemicals may be greater than expected from risk assessment procedures similar to the one we followed. The chemicals causing endocrine-disrupting effects at low environmental concentrations are the example for that, and it is reasonable to expect other types of specific chronic effects in the future caused by different compounds (Calow and Forbes, 2003).

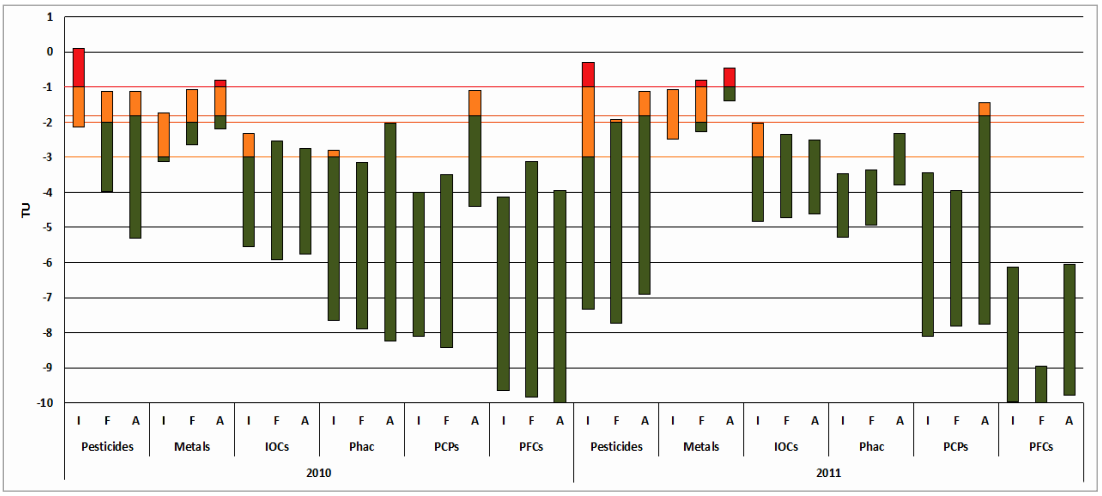


Figure 5.4 Minimum and maximum plot for TU summed for families of measured compounds at sampling sites (organic compounds, n=77; metals n=18) for algae (A), invertebrates (I) and fish (F) in 2010 and 2011. Red color indicate the exceedance of acute risk threshold for the species concerned. Orange indicate the exceedance of a chronic risk threshold (TU -3 for invertebrates., -2 for fish and -1.69 for algae). IOCs- industrial organics, Phac-pharmaceuticals, PCPs-personal care products, PFCs-perfluoralkyl compounds (list of all the compounds is available in Annex IV (Table AIV.1)

5.3.3 REGULATED VS. UNREGULATED CONTAMINANTS

The WFD priority contaminants list includes a limited number of priority and hazardous substances for chemical status regulation. However, the reality in the aquatic ecosystems is far more complex and those compounds that might be the most toxic are in fact just “the top of the iceberg”. There are numerous unregulated compounds present in the environment and their potential adverse effects should not be overlooked. Besides, some banned pesticides can still be found in the aquatic environment and pose the threat to biological communities. In this study, the “non-priority” contaminants ($TU_{non\text{-}priority}$) (i.e., those left when WFD priority compounds were excluded from the dataset) exceeded the chronic threshold at 98% of the studied sites (Figure 5.5B). However, the acute risk threshold was exceeded at six sites only. In any case, it is clear that we cannot exclude the risk for biological communities of studied rivers by regulating just WFD priority pollutants. Furthermore, when we excluded both the banned pesticides and the WFD priority pollutants from the dataset, the unregulated contaminants ($TU_{unregulated}$) exceeded the chronic risk threshold at 23% of sites. More precisely in Llobregat and Júcar (25-50% of sites) while in Ebro and Guadalquivir the exceedance of threshold happened at several

sites only (Figure 5.5C). In that group, the compounds responsible for chronic risk threshold exceedance were mainly unregulated pesticides, biocide triclosan and the antidepressant sertraline. Remarkably, some banned pesticides such as e.g. chlorfenvinphos or ethion have been found in the water at the levels high enough even to pose acute risk and even more of those that were posing a chronic risk (e.g. diclofenthion, parathion-ethyl etc.) The question remains, why banned pesticides are still found in water at such levels that pose threat to aquatic life. In some cases, European legislation bans the pesticides for agricultural purposes, but the product still can be used in urban settlements as biocide, thus could reach the rivers. In other cases the ban of the pesticides can be implied just for some types of the crops while it can be used for other crops. On the other hand, McKnight et al. (2015) found several pesticides in Danish streams that were not authorized for use in that country for long time periods. They related the presence of banned pesticides (mostly herbicides) in stream water with the groundwater input as one of the important pathways. Another possible source could be the remobilization of legacy pesticides from sediment. Obviously, both currently used and banned pesticides are still posing the risk for aquatic life in studied rivers and both should be considered for risk assessment purposes. Especially important would be to determine the sources of the banned pesticides. In general, the overall risk for aquatic ecosystems may often be dominated by a few components of the mixture (Kortenkamp and Faust 2010), which in this case were pesticides both classified as WFD priority pollutants and others (Kuzmanovic et al., 2015). However, the risk of chronic effects of less toxic compounds is still present. Therefore, the risk of adverse effects in biological communities of studied rivers cannot be excluded by setting environmental quality standards just for the WFD priority compounds. Rather, a variety of chemicals present in the environment should be taken into account for the proper risk characterization. Moreover, in this study, there is a possible underestimation of risk because other toxic compounds could be present in the river water but they have not been covered here. Also, the influence of the synergistic and antagonistic effects between chemicals on the overall risk was not considered by this study.

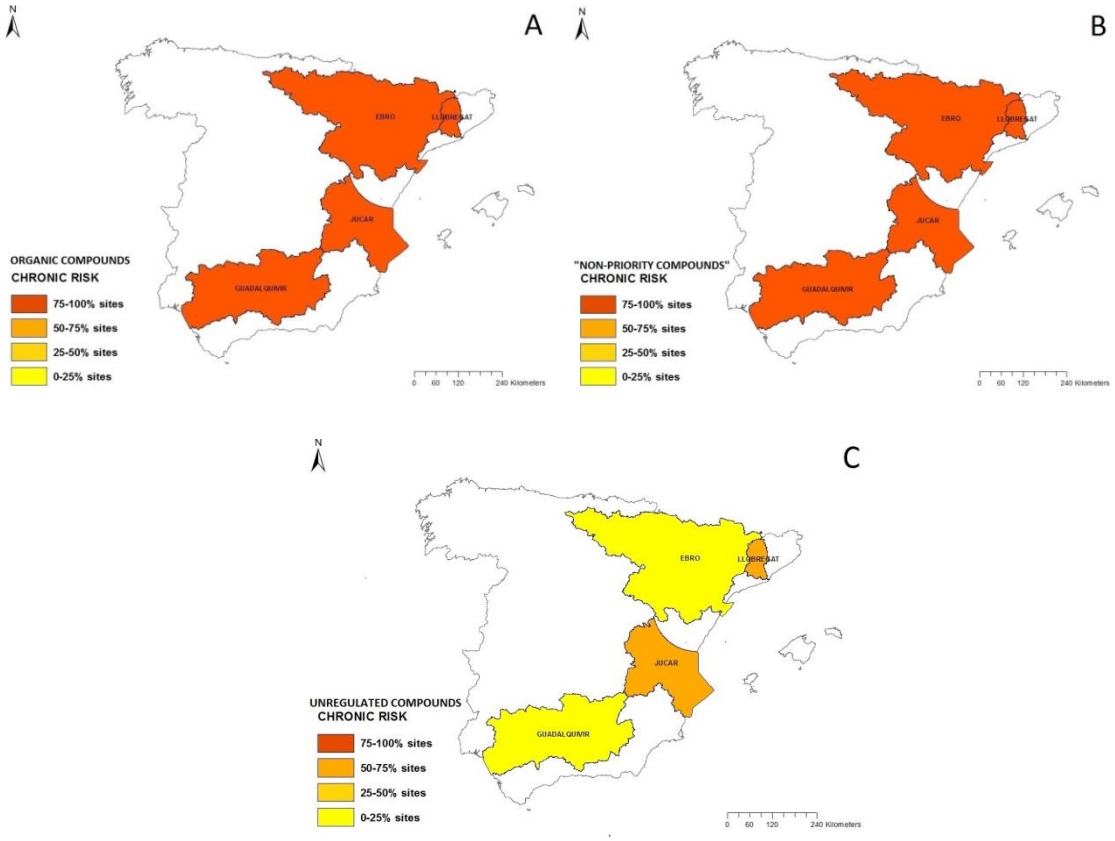


Figure 5.5 Chronic risk 2010 and 2011 -percentage of sites with exceedance of chronic risk threshold for at least one of three standard test species for A) organic compounds -the whole dataset, B) "non-priority" compounds and C) unregulated compounds

5.3.4 ECOTOXICOLOGICAL RISK –METALS VS. ORGANIC MICROPOLLUTANTS

Metals were measured in high toxic units at the majority of the sampling sites (n=18) (Figure AIV.6, Annex IV). While invertebrates were the most sensitive test species for organic chemicals, algae were the most sensitive species for metals (Figure 5.4). When compared the risk at sampling sites where both organic compounds and metals were measured (n=18), organic compounds risk was higher at the majority of the sites in 2010 (Figure AIV.6, Annex IV) due to the presence of highly toxic pesticides in water. This could be related to the hydrometeorological situation of that year characterized by intense precipitation that could have triggered runoff of pesticides from the surrounding agricultural fields. On the contrary, in 2011 metals risk was higher at the majority of sites in Ebro, Llobregat, and Guadalquivir due to higher concentrations of metals in water and the lower concentrations of some pesticides.

Among metals measured, copper and zinc contributed mostly to the overall toxicity. The acute risk threshold ($TU_{metals} \geq -1$) was exceeded at 11% of the sites in 2010 and at 44% of the sites in 2011. It was found in previous studies based on routine monitoring that metals (especially zinc and copper) were the most important compounds in terms of toxic units in the studied area, while organic chemicals monitored only slightly contributed to the risk (López-Doval et al., 2012). These findings should be taken with some caution since the number of organic micropollutants analyzed was limited. A study of Catalan river basins based on the species sensitivity distribution (Carafa et al., 2011) and routine monitoring data carried out by the local authorities found an increase of toxic risk associated with urban and industrial areas of the Llobregat river basin was likely attributable to metals, surfactants (e.g. nonylphenol) and the pesticide chlorpyrifos (Carafa et al., 2011). Again, this study only included a limited number of organic pollutants (mostly priority compounds).

5.3.5 BIOLOGICAL STATUS

Both diversity indexes (Shannon and Margalef) showed similar trends, decreasing downstream (Table 5.1) as a result of the reduction of the number and the abundance of species. The same trend was also observed in previous studies in the case of Llobregat river basin (López-Doval, Großschartner et al. 2010, Ginebreda, Kuzmanovic et al. 2014).

In addition to the general tendency to decrease downstream, low values of diversity were also found in some sites located relatively upstream (e.g. EBR2, JUC2 and GUA2). According to SPEAR index, biological status of most of the sampling sites was moderate to bad (Figure AIV.7, Annex IV). However, this general status should be taken with caution. SPEAR metric has been developed to evaluate the risk of the whole invertebrate community inhabiting all the habitats present in the river. In this study, we sampled only the sediment and only few species living in this habitat are actually classified “at risk” in the SPEAR metric. Most of the species found in our sediments are part of the family *Chironomidae* and the order *Oligochaeta*. The SPEAR determines both taxonomical groups as “not at risk” without distinction between species. Even though the described limitations, SPEAR index has been used previously to assess biological status of sediment community with satisfactory results (Wolfram, Höss et al. 2012). The invertebrates TU for the different compounds are suggesting several degrees of risk for biological communities and this could explain the community impairment observed with the biological indexes in all the sampling sites. Changes in the community structure due to priority and emerging pollutants have been described previously in Mediterranean rivers (Muñoz, López-Doval et

al. 2009, Ricart, Guasch et al. 2010, Brix, López-Doval et al. 2012), indicating the general biological impairment in relation to pollution.

Table 5.1 Biological descriptors for macroinvertebrates in sediment at the different sampling sites (EBR: Ebro; LLO: Llobregat; JUC: Jucar; GUA: Guadalquivir)

2010	**d**	**H'**	**SPEAR organic**	**SPEAR pesticides**	2011	**d**	**H'**	**SPEAR organic**	**SPEAR pesticides**
EBR1	2,08	3,29	-0,92	0	**2EBR1**	2,38	2,78	/	/
EBR2	0,32	1,25	-0,93	0	**2EBR2**	1,10	2,72	-0,74	0
EBR3	1,04	2,97	-0,61	0	**2EBR3**	0,69	1,92	-0,76	0
EBR4	/	/	/	/	**2EBR4**	0,45	1,69	-0,39	0
EBR5	0,50	1,92	-0,78	0	**2EBR5**	0,58	1,63	-0,47	22,47
LLO3	1,45	2,57	-0,61	8,13	**2LLO3**	2,02	3,76	-0,77	11,82
LLO4	0,57	2,16	-0,55	19,23	**2LLO4**	0,78	2,47	-0,56	24,99
LLO5	0,61	1,80	-0,83	0	**2LLO5**	0,92	2,40	-0,35	44,54
LLO6	0,17	0,81	-0,93	0	**2LLO6**	0,44	1,49	-0,61	0
LLO7	0,46	1,81	-0,64	22,27	**2LLO7**	0,34	1,50	-0,93	0
JUC1	3,06	3,73	-0,88	6,35	**2JUC1**	2,44	2,61	-1,09	10,52
JUC2	0,79	1,40	-0,92	0	**2JUC2**	0,70	1,35	-1,22	0
JUC4	1,57	3,24	-0,78	14,37	**2JUC4**	1,12	2,76	-0,69	0
JUC5	1,06	2,55	-0,85	19,02	**2JUC5**	1,44	2,58	-0,86	13,12
JUC6	0,34	1,50	-1,34	0	**2GUA2**	0,57	1,98	-0,74	0
GUA1	2,82	3,74	-0,71	0	**2GUA3**	1,34	3,30	-0,46	20,82
GUA4	0,72	2,28	-0,52	0	**2GUA4**	0,91	2,48	-0,62	0

d-Margalef richness index, H' -Shannon diversity index- SPEAR: Species at Risk index

5.3.6 RELATIONSHIP BETWEEN TOXIC STRESS AND BIOLOGICAL STATUS

The only statistically significant correlation (Spearman, $p< 0,05$) between the toxic stress of organic compounds and biological community descriptors was between SPEARorganic and TUorganic ($r=-0,490$) and TUpesticides ($r=-0,431$) (Table 5.2). Neither Shannon nor Margalef indexes were showing significant correlation with TUorganic (Table 5.2). Moreover, diversity indexes were not correlated with SPEARpesticides and SPEARorganic. It has been reported in several studies, that Shannon and similar biodiversity indexes were not suitable to identify the effects of pesticides at the community level (Ippolito et al., 2012) and are influenced by different natural and anthropogenic factors (Beketov and Liess, 2008). In this study, they were negatively correlated with metals (TUmetals) (Table 5.2). However, only Margalef index was significantly correlated with the metals toxic units TUmetals ($r=-0,515$) (Table 5.2). Metals toxic units were significantly and positively correlated with urban land use type, while Shannon and Margalef indexes were correlated negatively (Table 5.2). That is, we can relate the decrease of macroinvertebrate biodiversity to urban areas. Nevertheless, urban rivers are highly impacted by a variety of stressors and it is known that in some cases, more environmental stressors can interact with the toxicants (Liess et al., 2013). Besides chemical pollution, in urban rivers, there are often present habitat changes, temperature alterations and other stressors (Vörösmarty et al., 2010). Also, the natural gradient of environmental factors along the rivers is one of the most important sources of differences between biological communities (Beketov and Liess, 2008) and each site has its unique combination of natural factors (Schäfer et al., 2007) it should be taken into account when interpreting the macroinvertebrate biodiversity change along the river. The relation between biodiversity indexes and urban land use could be reflecting the response of the community to a variety of stressors present at the urban areas that are acting together along with the pollution.

Linear regression line between $SPEAR_{organic}$ and total organic stress at the site ($TU_{organic}$) was significant with $r^2= 0,235$ ($p>0,05$) and a relationship between $SPEAR_{pesticides}$ and $TU_{pesticides}$ with $r^2=0.104$ ($p>0,1$). Scatter plots show the relationship between losses of sensitive species with the increase of toxic stress of organic compound (Figure 5.6A) and pesticides (Figure 5.6B). All the sites were characterized by medium to high toxic stress (TU from-2,7 to 0) therefore the gradient of toxicity was relatively low and we could not

observe the communities composition in pollution free conditions (i.e., reference conditions).

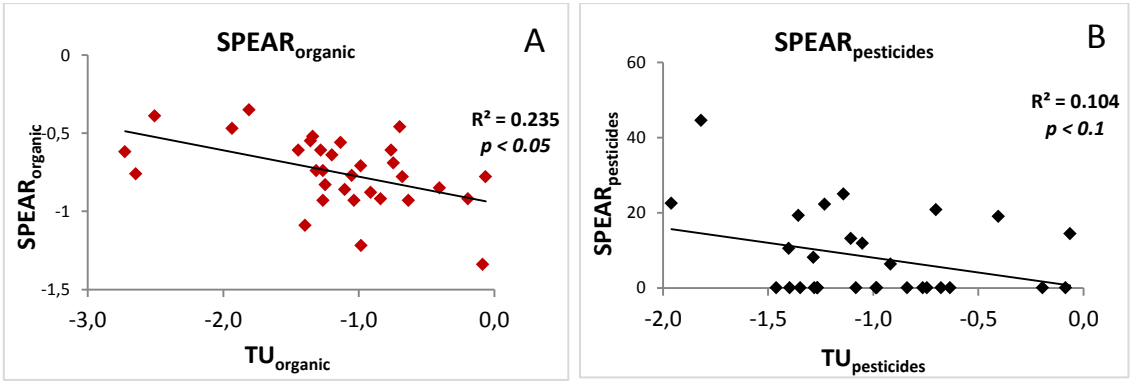


Figure 5.6 Relationship between invertebrate communities in situ and the toxic stress. A) Expressed as SPEARorganic and toxic units of organic compounds (TUorganic invertebrates). Linear regression is significant with r2= 0.235, p>0.05. B) Expressed as SPEARpesticides and toxic units of pesticides (TUpesticides, invertebrates). Linear regression is significant with r2= 0.104 at p>0.1.

Table 5. 2 Correlation matrix based on Spearman rank correlation test (in bold, p< 0,05)

Variables	**Urban**	**Agricultural**	**Natural**	**d**	**H'**	**SPEAR pesticides**	**SPEAR organic**	**TU_{metals}**	**TU_{IOC}**	**TU_{PCP}**	**TU pharmaceuticals**	**TU pesticides**	**TU organic**
Urban	1	-	-	-	-	-	-	-	-	-	-	-	-
Agricultural	0,134	1	-	-	-	-	-	-	-	-	-	-	-
Natural	-0,497	-0,817	1	-	-	-	-	-	-	-	-	-	-
d	-0,672	-0,068	0,375	1	-	-	-	-	-	-	-	-	-
H'	-0,436	0,134	0,140	0,883	1	-	-	-	-	-	-	-	-
SPEAR pesticides	0,120	0,014	0,028	0,232	0,269	1	-	-	-	-	-	-	-
SPEAR organic	0,339	0,337	-0,306	0,088	0,286	0,481	1	-	-	-	-	-	-
TU metals	0,600	0,010	-0,295	-0,515	-0,268	0,043	0,330	1	-	-	-	-	-
TU ioc	0,045	0,018	-0,063	0,004	-0,004	-0,117	-0,127	0,007	1	-	-	-	-
TU pcp	0,248	0,036	-0,151	-0,129	-0,092	0,061	0,105	0,210	-0,585	1	-	-	-
TU pharmaceuticals	0,490	-0,010	-0,243	-0,232	-0,151	0,344	0,303	0,492	-0,389	0,674	1	-	-
TU pesticides	-0,412	0,160	0,020	0,140	0,156	-0,229	-0,431	-0,405	0,323	-0,404	-0,606	1	-
TU organic	-0,394	-0,012	0,128	0,175	0,155	-0,073	-0,490	-0,459	/	/	/	/	1

Even though SPEARindex is designed to be a stressor-specific indicator it cannot be excluded that other stressors might have influenced the loss of sensitive species. This could be the case, especially since studied rivers are impacted by a multitude of anthropogenic stressors and some stressors are expected to cause similar changes in trait categories (Statzner and Bêche 2010, Rasmussen, McKnight et al. 2013). Besides, different co-occurring stressors (Liess and Beketov, 2011) and their complex relationships with biological communities (Liess et al., 2008) can mask the effects of single toxicant. Naturally, the use of $SPEAR_{pesticides}$ was showing the best results in agricultural streams where pesticides are the predominant stressors (Beketov et al., 2013; Schäfer et al., 2007). However, since only macroinvertebrates in the sediment were sampled in this study, the low values of $SPEAR_{pesticides}$ could be attributed to a relatively large proportion of tolerant species in that habitat (Wolfram, Höss et al. 2012, von der Ohe and Goedkoop 2013) and the starting bias in the data makes any conclusion difficult. However, $SPEAR_{organic}$ as a less specific indicator seems to be more suitable for the multi-chemical polluted rivers. In conclusion, when all four biological indexes used in this study are compared, the most suitable to relate changes in biological communities (i.e. decrease of sensitive species) to organic stress was the $SPEAR_{organic}$ indicator.

5.3.7 MULTIVARIATE ANALYSIS: BIOLOGICAL DESCRIPTORS AND TOXIC UNITS OF CHEMICAL GROUPS

A principle components analysis was performed, including variables representing the toxic stress of chemical families studied (i.e. the sum of toxic units of each group of chemicals), biological indexes and land uses expressed in percentage of agricultural, urban and natural respectively. The first two components were interpretable which explained 48% of the total variance (30.67% and 17.42% respectively).

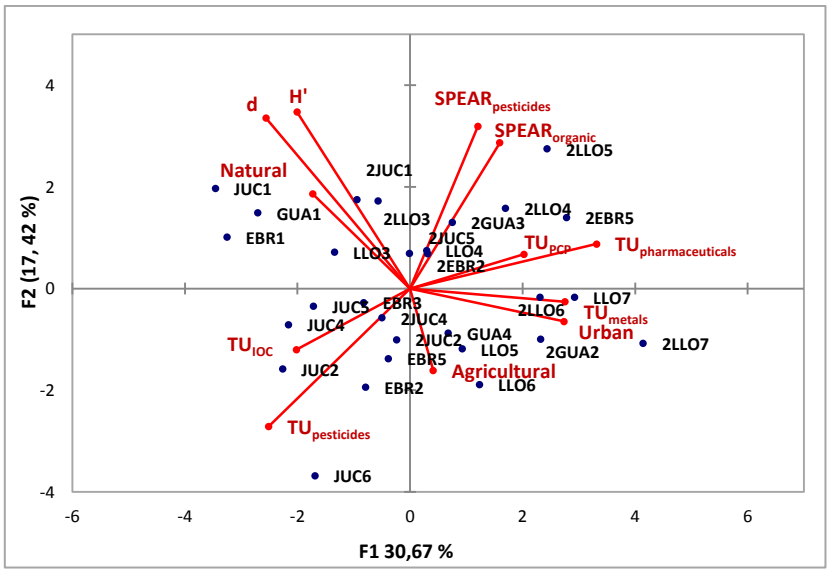


Figure 5.7 Biplot of the first two principal components. The first two components of the PCA explain 48% of the total variance (30.67% and 17.42%, respectively

The first component can be mainly related to pharmaceuticals, metals and personal care products that were grouped together and were related to urban land use type (Figure 5.7). On the other hand, pesticides, industrial organic compounds (IOC) and biodiversity contribute negatively. This component could be tentatively interpreted as representative of the stressors related to urban areas. Sites with higher diversity indexes coincide with the upstream areas and natural land use type (forests and grasslands). The second component roughly informs about biological quality with positive contributions of biological indexes (H', d, and SPEAR) and natural land use type, and negative contributions of industrial organic chemicals, pesticides, and agricultural land use type. The distribution of the sites is consistent with the above interpretation. Polluted sites subjected to high urban pressure such as those in the lower part of Llobregat (LLO6 and LLO7) are distributed along the first component. SPEARpesticides and SPEARorganic were negatively correlated with toxic stress of pesticides and industrial organic chemicals but were not reflecting the effect of urban origin (pharmaceuticals and PCPs). Therefore, we could assume pesticides and IOCs were those the compounds mostly influencing the decline of sensitive species.

5.4 CONCLUSIONS

In the present article, we have assessed the environmental risk associated with chemical pollution on the basis of their ecotoxicological properties. To that end, the toxic units approach based on three trophic levels (algae, invertebrates, and fish) was used and applied to four Iberian river basins. The spatial ecotoxicological risk was characterized using available occurrence concentration data of more than 200 organic chemicals and metals transformed into toxic units and subsequently aggregated using widely accepted mixture toxicity criteria (i.e., concentration addition). This methodology enabled to quantify and depict in risk maps both acute and chronic potential effects that can be of great value for water management purposes. Both organic micropollutants (particularly pesticides) and metals significantly contribute to acute ecotoxicological risk.

The used methodology also enabled to differentiate the respective contributions to environmental risk between regulated and unregulated compounds, thus showing that both categories of compounds need to be taken into account for proper risk assessment. Banned pesticides are still present in river water in high toxic units and could be causing acute and chronic effects in biological communities. The unregulated contaminants alone posed the chronic risk at 23% of the studied sites. These findings have obvious management implications, for instance in the design of adequate monitoring campaigns.

Chemical and ecological status of water ecosystems are key aspects of the WFD and both are explicitly considered. However, their interrelation is not always clear. Here we used ecotoxciological assessment as an explanatory “bridge” between both. The combined use of toxicity indexes, conventional diversity indexes, and traits-based indexes helped disentangle the relationships between macroinvertebrate communities and the different co-occurring stressors. Specifically, we found that the decline of aquatic macroinvertebrate sensitive species based on trait indexes (SPEAR) was correlated with the increase of organic load quantified in toxic units. Diversity indexes reflected in a general way the multiple stress conditions that the studied rivers were subjected to. These results were supported by multivariate statistical analysis in which both biological, land use and pollutants’ ecotoxicological risk variables were used to satisfactorily to explain the observed variability among sites. However, more work needs to be done in order to better understand the effects of co-occurring stressors in aquatic ecosystems. The appropriate combination of different community indicators and endpoints (e.g. behavior or functioning) will help to improve toxicological risk assessment of aquatic ecosystems.

CHAPTER 6

ENVIRONMENTAL STRESSORS AS A DRIVER OF THE TRAIT COMPOSITION OF BENTHIC MACROINVERTEBRATE ASSEMBLAGES IN POLLUTED IBERIAN RIVERS

Based on the publication in the Environmental Research Journal (2017)

Maja Kuzmanović, Sylvain Dolédec, Núria De Castro-Català, Antoni Ginebreda, Isabel Muñoz, Sergi Sabater and Damià Barceló

6.1 INTRODUCTION

River ecosystems are impacted by a variety of anthropogenic stressors (Vörösmarty, McIntyre et al. 2010) and changes in the taxonomic and functional diversity of local species are expected on the global scale (Olden, Poff et al. 2004). However, the successful quantification of the relationship between the occurrence of particular stressors and biological indicators across large geographical areas remains challenging. In addition, an increasing number of stressors are co-occurring and impact the biota simultaneously (Navarro-Ortega, Acuña et al. 2015). Therefore, it is of utmost importance to disentangle the effects of co-occurring stressors, in order to determine which stressor should be given priority in river basin management. The growing human population and resulting land use changes from natural to urban and agricultural have increased pressure on river ecosystems. Agriculture and urbanization are recognized as being amongst the main causes of stream impairment (Paul and Meyer 2001). Water and habitat quality are often degraded in the streams draining agricultural land (Allan 2004) due to the increased input of pesticides, sediments, and nutrients, as well as hydrological alterations due to water abstraction (Tilman, Cassman et al. 2002, Elbrecht, Beermann et al. 2016). Effects of pesticides on sensitive species have been observed in streams (e.g. Liess and Von Der Ohe, 2005; Schäfer et al., 2007) using trait-based SPEAR index. In a recent study by Malaj et al. (2014), the scale of the problem was revealed, since it was estimated that organic pollutants, among which pesticides were the major contributors to the risk, threaten the health of freshwater ecosystems across the whole of Europe. Furthermore, in streams draining urban land, consistent ecological degradation also occurs (Walsh, Roy et al. 2005). Increasing run-off from impervious surfaces (i.e., asphalt, concrete or stone), the input of storm water from piped drainage systems and wastewater discharges can cause drastic changes in urban streams (Paul and Meyer 2001). The symptoms generally associated with urbanization include “flashy” hydrograph, changes in channel morphology, high concentrations of metals, nutrients and organic toxicants and elevated water temperature. These modifications generally result in the decline of sensitive species (Wenger, Roy et al. 2009) and changes in ecosystem processes such as nutrient uptake (Paul and Meyer 2001).

Stream macroinvertebrates have long been used as indicators for water quality assessment (Rosenberg and Resh 1992). However, natural variability and confounding factors can mask the effect of a particular stressor (Schäfer, Caquet et al. 2007), especially over the large geographical area. To overcome this problem, more attention has been given to the use of the biological traits of taxa such as generation time, body size, body form and dispersal ability (Usseglio-Polatera, Bournaud et al. 2000, Statzner, Bady et al. 2005, Tachet, Richoux et al. 2010) These characteristics may be used to help interpret changes in assemblages across environmental gradients and to improve the robustness of traditional stream biomonitoring (Dolédec and Statzner 2008). According to the habitat template theory (Southwood 1977) the spatial and temporal characteristics of the habitat provide a framework against which species have evolved characteristic life-history strategies to maximize their fitness and survival (Townsend and Hildrew 1994, Poff 1997). Life-history strategies include different combinations of traits that represent the solution to a given ecological problem (Verberk et al., 2008). The use of multiple traits, described through multiple trait categories or states, has successfully discriminated between different stressors (Dolédec, Statzner et al. 1999, Dolédec and Statzner 2008, Mondy and Usseglio-Polatera 2013). Multiple-trait based approaches have shown promise for biomonitoring because most stressors should affect only certain trait categories (Statzner, Bis et al. 2001, Statzner, Dolédec et al. 2004, Statzner, Bady et al. 2005), which can be useful for discriminating among multiple stressors. Furthermore, unlike species composition, which changes along geographical and downstream gradients, some traits are thought to vary little across temporal and spatial scales, which makes them useful for large-scale studies (Statzner, Bis et al. 2001, Statzner, Dolédec et al. 2004, Statzner, Bady et al. 2005).

In this study, we used invertebrate traits to discriminate between the different types of human impacts in several basins of the Iberian Peninsula. We selected 16 sampling sites from four Mediterranean river basins with known human pressures (pesticides, multiple urban stressors and mixed). We further selected species traits that were thought to specifically respond to these stressors. The aim was to test the ability of multiple trait-based approaches to show that traits were not randomly distributed across assemblages in studied rivers and that different trait combinations responded to specific conditions in relation to the environment (urban vs. pesticide impacted).

6.2 MATERIALS AND METHODS

6.2.1 STUDY AREA

The study area included four river basins located across the Mediterranean part of the Iberian Peninsula: the Ebro and Llobregat in the North-East, Júcar in the East and Guadalquivir in the South of the Peninsula (Figure 1). A total of 16 sites were selected: four sites in the Ebro basin (coded E1, E2, E3 and E5), five sites in the Llobregat basin (L3, L4, L5, L6 and L7), five sites in the Júcar basin (J1, J2, J4, J5 and J6) and two sites in the Guadalquivir basin (G1 and G4). Each site receives a variety of diffuse and point source inputs depending on catchment land use (Figure AV.1, Table AV.1, in Annex V). Some of the sites are located in urban areas; the other sites are located in areas where a high risk of pesticide toxicity has previously been reported (López-Doval, De Castro-Català et al. 2012, De Castro-Català, Kuzmanovic et al. 2015, Kuzmanović, López-Doval et al. 2015). The data used in this study were gathered within the SCARCE-CONSOLIDER project (Navarro-Ortega, Acuña et al. 2012) in which the sampling for chemical and biological analyses was performed during the autumn of 2010.

Figure 6.1 Potential stressors at sampling sites including toxic units of pesticides ($TU_{pesticides}$), toxic units of metals (TU_{metals}), nutrients, low oxygen levels (lowO2), conductivity and temperature.

6.2.2 PHYSICAL AND CHEMICAL DATA

Organic pollutants were measured using analytical techniques based on gas chromatography-tandem mass spectrometry and liquid chromatography-tandem and hybrid mass spectrometry (Masiá, Ibáñez et al. 2013, Osorio, Proia et al. 2014). To assess the toxic risk at each sampling site, toxic units (TU) were calculated using the measured concentrations of the compound (MEC) and respective acute toxicity data (EC50) for *Daphnia sp*. The sums of toxic units for each of the compound families ($TU_{pesticides}$ and TU_{metals} in Table 6.1) were calculated as the risk estimate posed by different groups of toxicants. The major contributors to the pesticide toxicity risk were insecticides (e.g., chlorpyriphos or chlorfenvinphos) whereas copper was the main contributor to the metal toxicity risk. More details on measurements of the chemical compounds and risk assessment associated with our study can be found in Kuzmanović et al. (2016).

Other physical and chemical variables included average sediment particle size (Phimoy in Table 6.1) and variance (Phivar) at the Phi scale [range from -8 (boulder) to >10 (colloid)], flow variations (expressed as a 3-month coefficient of variation (CV) prior to sampling), average precipitation (3-month average), water temperature (T), dissolved oxygen (O_2), dissolved organic carbon (DOC), conductivity, nutrients (N-NO_3, and P-PO_4), percentage of organic matter in sediment (OM) and the altitude of sampling sites. The OM content, toxic units and nutrient data were log-transformed prior to analysis. The catchment land use types were estimated from Corine Land Cover (2006) using Arc Map 10.1 software and the variable that synthesized naturalness was calculated as the weighted mean of three categories (Urban, Agricultural, Natural) arbitrarily weighted by a coefficient of 1, 5 and 100, respectively (LU in Table 6.1; see Annex V). Further details on chemical and physical data measurements are available in Sabater et al. (2016).

Table 6.1 Mean and range (in parenthesis) of physical and chemical data (n=16).

Variable	Value
Altitude (m)	379 (5 - 1180)
LU (%)	40 (4.3 - 95)
CV (%)	45 (3 - 112)
Phivar (φ)	2.8 (0.5 - 5.3)
Phimoy (φ)	1.3 (-1 - 3.2)
O_2 (mg/L)	9.4 (5.9 - 11.2)
T (°C)	16 (6 - 28)
pH	8 (7.5 - 8.2)
Conductivity (µS/cm)	805 (162 - 1372)
DOC (mg C/L)	4.9 (2.1-10.2)
$N\text{-}NO_3$ (mg /L)	1.9 (0.1-9.2)
$N\text{-}NH_4$ (mg/L)	0.3 (0.1 - 1.2)
$P\text{-}PO_4$ (mg/L)	0.1 (0 - 0.6)
$TU_{pesticides}$	-0.8 (-1.4 - -0.1)
TU_{metals}	-1.7 (-3.3 - -0.7)
OM (%)	4.2 (1.1 - 13.2)
Precipitation (mm)	599 (100 - 1600)

6.2.3 SITE CLASSIFICATION

We determined which stressors were present at sampling sites (Figure 1) and according to the dominant stressor, sites were classified into three groups (pesticide impacted, urban and mixed). The pesticide impacted sites (E1, E5, J1, J2, J4, J5, J6, G1) were those where the acute risk was posed by pesticides (logTU>-1, (Kuzmanović et al., 2016; Figure AV.2, Annex V). Sites classified as urban (L3, L4, L5, L6, L7, and G4) were those impacted by other stressors (e.g., metals, nutrients, elevated temperature, low oxygen level, Figure 1) which were all highly correlated with urban land use (Table AV.2, Annex V). At urban sites, the risk of pesticide toxicity was below acute levels. Finally, two sites were classified as mixed (E2 and E3) because they were affected both by pesticides and multiple stressors related to urban land use. Whether an environmental variable can be considered a stressor was evaluated on the basis of thresholds derived from legislation or the literature (Table AV.3, Annex V).

6.2.4 MACROINVERTEBRATE SAMPLING

At each site, five sediment samples were randomly collected using a polyvinyl sand corer (24 cm^2 area). Each sample was sieved through a 500-µm mesh and fixed with 4% formaldehyde. Macroinvertebrates were sorted, counted and identified in the laboratory under a dissecting microscope (Leica Stereomicroscope). *Chironomidae* were identified at the genus level, while almost all other taxa were identified at the species level (list of taxa available in Annex V). Abundances were referred to on the basis of sediment surface area (De Castro-Català et al., 2015).

6.2.5 BIOLOGICAL TRAITS

Traits were derived from a European database compiled by Tachet et al. (Tachet et al., 2010) and completed for Mediterranean taxa by Bonada et al. (Bonada, Dolédec et al. 2007, Bonada and Dolédec 2011). In this database, the affinity of each taxon for a given trait state or category is quantified by a score with a value of 0 if there is no affinity of the taxon for the trait state or category, 1 if low affinity, 2 if medium affinity and ≥3 if strong affinity (i.e., fuzzy coding approach;(Chevenet, Doledec et al. 1994)). Trait information at the genus or species level was used when data was available, otherwise subfamily or family level information was used (for *Chironomidae, Oligochaetae* and *Enchytraeidae*, respectively). However, if taxonomic levels higher than species are used, the trait structure of assemblages is generally conserved (Dolédec, Olivier et al. 2000, Gayraud, Statzner et al. 2003).The initial trait table contained the affinities of the taxa, collected as described above, 39 trait categories distributed in 8 traits (Table AV.5, Annex V). Trait-affinity scores were further treated as frequency distributions, i.e., they were rescaled to sum to 1 for a given taxon and a given trait (e.g. Gayraud, Statzner et al. 2003, Schmera, Podani et al. 2015). We selected eight biological characteristics expected to respond to stressors in the studied basins. We selected the frequency affinities of taxa for small size (< 5 mm), short lifespan of adults (< 1 year), plurivoltinism (> 1 generation per year), and predation as separate traits. Egg protection was assessed by adding the frequency affinities of taxa for ovoviviparity, clutches in vegetation and terrestrial clutches. “Deposit feeding” combined the frequency affinities of taxa for absorption through tegument and deposit feeding *sensu stricto.* For each taxon, dispersal ability was assessed using an index similar to that proposed by Bonada et al., (Bonada, Dolédec et al. 2012), which uses the four dispersal categories (aquatic passive, aquatic active, aerial passive and aerial active) of the Tachet

et al. (2010) database. Aquatic passive was weighted by 1, aquatic active by 5, aerial passive by 10 and aerial active by 20. The dispersal ability of each taxon was thus computed as the weighted mean across the dispersal categories. Finally, we computed the food diversity ingested by each taxon as a Simpson index ($1 - \sum p_i^2$, with p_i as the proportion of a given food item).

6.2.6 DATA ANALYSIS

The relationship between macroinvertebrate traits and environmental variables was investigated by RLQ (Dolédec, Chessel et al. 1996) and fourth-corner analyses (Legendre, Galzin et al. 1997). RLQ is an extension of the co-inertia analysis (Chevenet, Doledec et al. 1994) that allows relating three tables: a trait table (named **Q**), an environmental table (**R**) and a species abundance table (**L**) that is used as the link between **Q** and **R**. RLQ provides the simultaneous ordination of species, their traits and the environmental variables. It has been successfully applied in ecological studies dealing with birds (Hausner, Yoccoz et al. 2003), beetles(Ribera, Dolédec et al. 2001), freshwater macroinvertebrates (Díaz, Alonso et al. 2008) and aquatic plants (Baattrup-Pedersen, Göthe et al. 2016). As recommended by authors, the three tables were analyzed separately prior to RLQ analysis. Correspondence analysis (CA) was performed on the abundance table and principal components analysis (PCA) on the trait and environmental tables, respectively. In brief, RLQ summarizes the multivariate structures by searching for the linear combinations of traits and environmental variables (describing stress) on which sites and taxa are projected, providing new site and taxa scores that are the most covariant. These new scores must be compared to those from the separate analyses of each table to assess how much of their variability is taken into account by the RLQ analysis and to evaluate the strength of the relationship between traits and stressors. The overall significance of this relationship was further assessed via a global Monte-Carlo test using 99999 random permutations of the table rows of **R** (sites; model 2;(Dray, Choler et al. 2014)) and of the table rows of **Q** (species; model 4; Dray et al., 2014). Fourth-corner analysis was used to find significant bivariate relationship between single trait and environmental variable. Furthermore, combination of RLQ and fourth-corner analysis was used to evaluate the significance of associations between traits and combination of environmental variables (identified by RLQ) and environmental variables and combination of traits (identified by RLQ, see Dray et al., 2014 for further details). The significance of relationship was assessed using Pearson r correlation coefficient (for details, see Dray et

al., 2014). In these two latter approaches, the false-discovery-rate adjustment method was used to correct *P*-values according to bias due to multiple-test comparisons. Statistical analyses and graphical outputs were computed with the ade4, vegan and corrplot packages implemented in the R software (Chessel, Dufour et al. 2004, Dray, Dufour et al. 2007, Core Team 2015, Oksanen, Blanchet et al. 2016).

6.3 RESULTS

6.3.1 SEPARATE ORDINATIONS

A correspondence analysis (CA) performed on the faunistic table yielded a first and second axis that explained 25.8% and 17.5% of the total variability, respectively. The best possible correlation between taxa and sites equaled 0.65 (square root of the first eigenvalue=0.42), suggesting a fairly good ordination of taxon composition. A chi-squared test further demonstrated the non-independence between sites and taxa ($P<0.001$). The first CA axis separated the Júcar river sites (J in Figure 6.2B) from the three other rivers. The second CA axis separated pesticide impacted sites from urban sites (Figure 2A). The Júcar had higher proportions of *Tanytarsus* sp., *Ephemera* sp. and *Potamopyrgus* sp., potentially reflecting coarser sand in the sediment than in the other rivers. The pesticide impacted sites of the Ebro and Guadalquivir rivers had more Oligochaetes (*Lumbriculus* sp., Enchytraeidae, *Limnodrilus* sp.) and chironomids (*Nanocladius* sp., *Stictochironomus* sp. and *Microspectra* sp.), which are commonly found in fine sediments. At the urban sites of the Llobregat (L in Figure 2B) and Guadalquivir (G in Figure 2B), *Cryptochironomus* sp., *Polypedilum* sp., *Limnodrilus* sp., *Micronecta* sp., *Potamothrix* sp. and *Caenis* sp. were more abundant.

A PCA performed on the environmental table yielded a first and second axis that explained 44.8% and 16.2% of the total variability, respectively. The first PCA axis separated the sites according to the stressors, i.e., urbanization (left side of the axis; Figure 3A) vs. pesticides (right side; Figure 3A). Two sites (E2 and E3) with a mixture of both types of stressors appeared between the above two groups (Figure 3A). Four basins differed in the stressor present (Figure 3B). In particular, sites in the Júcar basin were mainly affected by pesticides whereas sites in the Llobregat were mainly affected by urban stressors; the Ebro and Guadalquivir had sites with either one of the stressor types or a combination of both (Figure 3B). The first PCA axis thus opposed sites with mainly high pesticide toxicity ($TU_{pesticides}$, Table 6.2) and high dissolved oxygen concentration (O_2) to

sites with high values for temperature, dissolved organic carbon (DOC), nutrients (P-PO4, N-NO3), metals (TU_{metals}), precipitation and flow variation (CV) associated with urbanization (Table 6.2).

Table 6.2 PCA loadings for first two components

Variable	Comp1	Comp2
LU	0.3	-0.2
$N\text{-}NO_3$	-0.9	0.0
$P\text{-}PO_4$	-0.9	-0.3
O_2	0.7	0.1
DOC	-0.9	-0.1
Conductivity	-0.4	-0.7
Temperature	-0.9	-0.2
pH	0.3	0.3
$TU_{pesticides}$	0.7	-0.2
TU_{metals}	-0.9	0.2
OM	-0.3	0.9
Phimoy	-0.4	0.8
Phivar	-0.3	0.4
CV	-0.8	-0.0
Precipitation	-0.7	0.0

Finally, a PCA performed on the trait table yielded a first and second axis that explained 48.1% and 18.2% of the total variance, respectively. The taxa associated with the positive side of the first PCA axis included *Branchiura sp., Limnodrilus sp., Potamotrix sp., Lumbriculus sp*. and *Enchytraeidae,* which are prominently deposit feeders and plurivoltine taxa (Figure AV.1, see Annex V). The taxa associated with the negative side of the first PCA axis included all other taxa (e.g., *Dicranota sp., Caenis sp., Microspectra sp., Tanytarsus sp., Ephemera sp.*), which prominently protect their eggs, are short-lived and small-sized, and disperse easily (Figure AV.3, see Annex V). The second PCA axis was positively correlated with predation and food diversity.

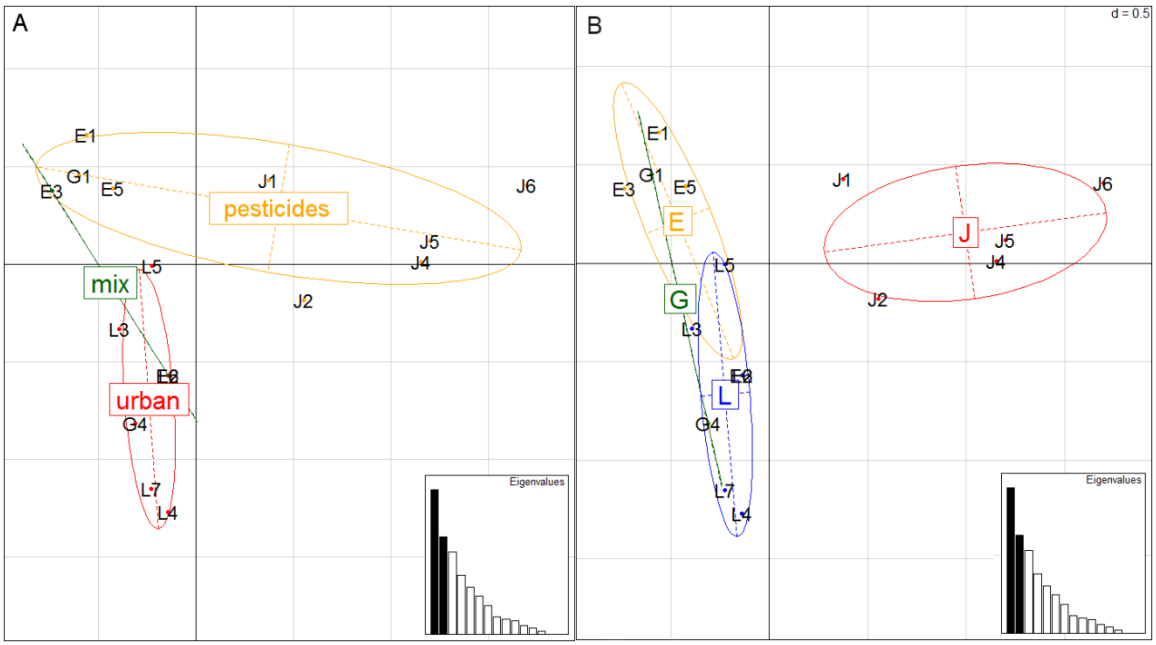


Figure 6.2 Results of a COA performed on fauna abundance showing the site ordination (A) grouped by dominant stressors and (B) by river basin (J-Júcar, E-Ebro, G-Guadalquivir, L-Llobregat). Inset represents the diagram of eigenvalues.

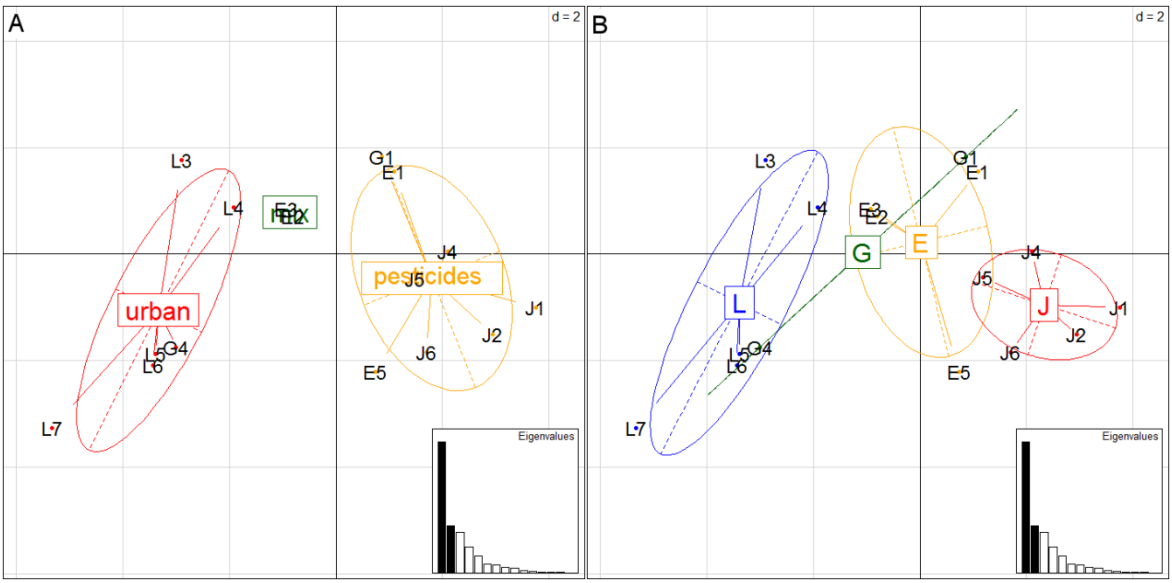


Figure 6.3 Results of a PCA performed on the environmental table showing sites (A) grouped by dominant stressors (B) by river basin (J-Júcar, E-Ebro, G-Guadalquivir, L-Llobregat). Inset represents the diagram of eigenvalues.

6.3.2 RLQ ANALYSIS

The relationship between the trait composition of macroinvertebrate assemblages and environmental stressors was globally significant (Model 2 simulated $P < 0.001$; Model 4 simulated $P < 0.025$). This relationship was mainly summarized by the first RLQ axis, which explained 85.6% of the total cross-variance between the traits and environment, whereas the second axis only contributed to 8.0% (Table 6.3). Because of this low value, the second axis will not be discussed further.

The first axis accounted for 97% of the variability of the environmental table and 70% of the variance of the trait table. In addition, the new set of site and species scores had a correlation of 0.33 along the first RLQ axis, which was 51% of the best possible correlation (i.e., obtained from the separate CA of the fauna abundance table; Table 6.3). In accordance with the high proportion of variability of the environmental table taken into account by RLQ (Table 6.3), the ordination of sites along the first RLQ axis was similar to that obtained from the separate PCA of environmental variables, which differentiated the urban from the pesticide impacted sites (Figure 6.4A). The first RLQ axis also partly incorporated differences in the sampling location (river basin) and the natural longitudinal variability of the sites (Figure 6.4B; explained variance=0.56; P<0.005), since most urban sites were situated along the lower parts of rivers, especially in Llobregat and pesticide impacted sites mainly along the upper and middle parts of the rivers. In contrast, unlike the separate CA of fauna in which sites in the Júcar basin were isolated from the other pesticides-impacted sites (Figure 6.2A), the first RLQ axis grouped together all the sites impacted by pesticides (Figures 6.4A, 6.4B and 6.4D), thus taking into account the variability of sites expressed along the second CA axis. Egg protection was the prominent trait of the taxa at pesticide impacted sites (Figures 6.4E and 6.4F). In fact, most of the traits dominated at those sites; whereas the prominent traits in assemblages at urban sites included plurivoltinism and deposit feeding, suggesting higher trait diversity at the pesticide impacted sites than at the urban sites (Figures 6.4A and 6.4E)

Table 6.3 Summary of the RLQ analysis

Total inertia: 2.226					
Eigenvalues:					
	Ax1	Ax2			
	1.907	0.179			
Projected inertia (%):					
	Ax1	Ax2			
	85.662	8.017			
Cumulative projected inertia (%):					
	Ax1	Ax2			
	85.660	93.680			
Eigenvalues decomposition:					
	eig	covariance	sdR	sdQ	correlation
eig1	1.907	1.381	2.550	1.649	0.328
eig2	0.179	0.422	1.290	1.514	0.216
Inertia & coinertia R:					
	inertia	max	ratio		
eig1	6.505	6.721	0.968		
eig1+2	8.169	9.145	0.893		
Inertia & coinertia Q:					
	inertia	max	ratio		
eig1	2.718	3.851	0.706		
eig1+2	5.011	5.308	0.944		
Correlation L:					
	correlation	max	ratio		
eig1	0.328	0.647	0.508		
eig2	0.216	0.531	0.407		

6.3.3 FOURTH-CORNER TEST

We were unable to find a significant bivariate trait–environmental variable association after applying the *P*-value adjustment. This result suggests that a combination of stressors, rather than a single stressor, was acting on a combination of traits. Therefore, we further investigated the relationship between individual traits and the first RLQ environmental axis (combination of environmental variables) and individual environmental variables and the first RLQ trait axis (combination of traits).

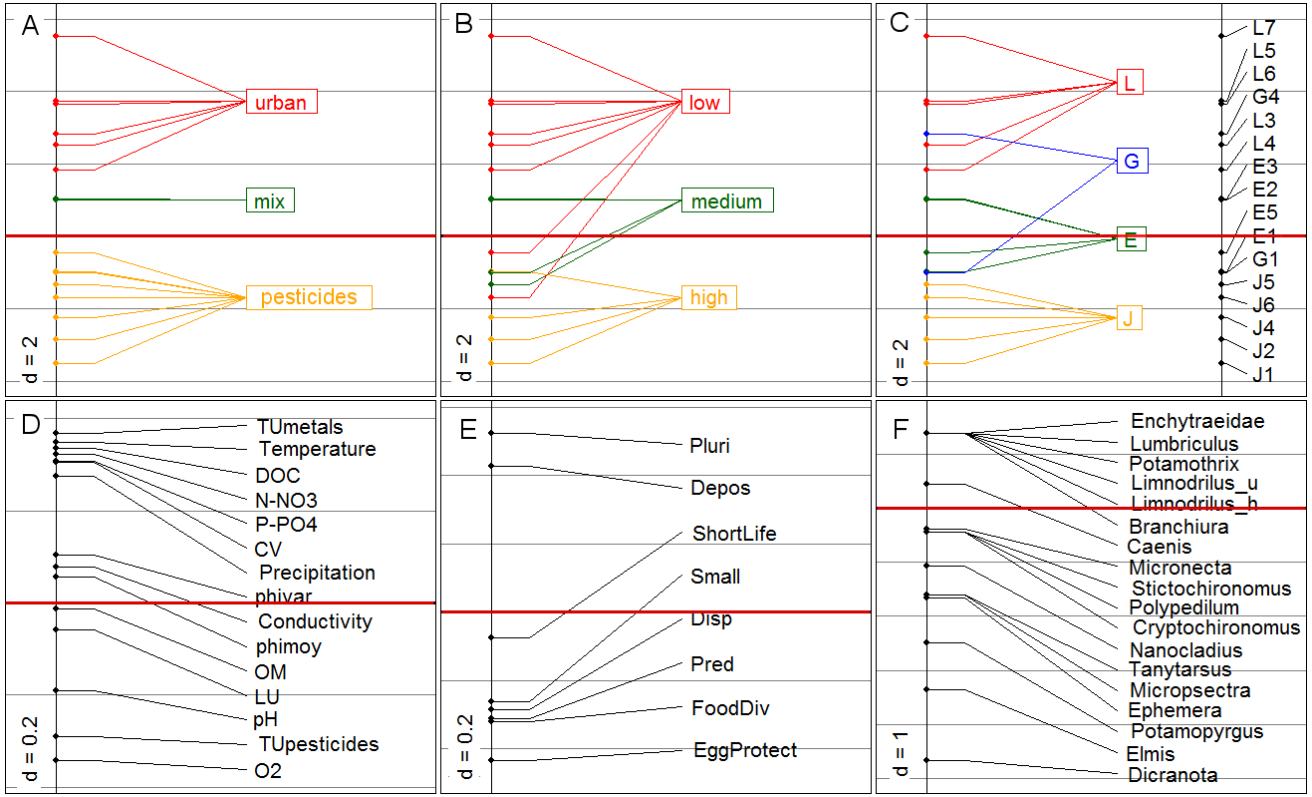


Figure 6.4 Results of the RLQ analysis that related taxa and their traits to the environmental variables. Sites grouped by (A) the dominant stressors, (B) the altitude (C) river basins (J-Júcar, E-Ebro, G-Guadalquivir, L- Llobregat), (D) environmental variables (phivar-sediment particle size variance, phimoy-average sediment particle size, LU- variable that synthesized naturalness, CV- flow variations) (E) traits (pluri-plurivoltinism, depos-deposit feeding, disp-dispersal ability, pred-predation, food div-food diversity) and (F) taxon scores along the first RLQ axis. The red horizontal line corresponds to zero at the first axis, it separates negative (up) from positive (down).

Plurivoltinism and deposit feeding were positively associated with the first RLQ environmental axis whereas egg protection was negatively associated with the first RLQ environmental axis (Figure 6.5A). The environmental variables significantly positively associated with the first RLQ trait axis were nutrients (N-NO3, P-PO4), DOC, temperature, TU_{metals}, CV, while oxygen contents (O_2) and $TU_{pesticides}$ were negatively related to the first RLQ trait axis (Figure 6.5B). In summary, there was a positive association between the environmental variables describing stress associated with urban sites (N-NO3, P-PO4, DOC, temperature and TU_{metals}) and plurivoltinism and deposit feeding whereas $TU_{pesticides}$ was associated with egg protection even if the oxygen content (O_2) was higher.

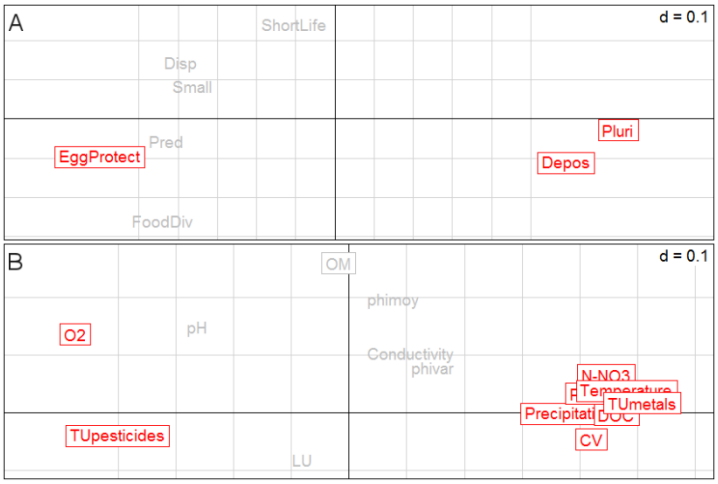


Figure 6.5 Significant relationships (P-adjusted <0.05 in red) represented along with the first-two RLQ axes (for readability) (A) between the RLQ environmental axes and individual traits and (B) between the RLQ trait axes and individual environmental variables (P-adjusted <0.1 in red). Non-significant relationships are labeled in light grey

6.4 DISCUSSION

Our analyses allowed us to assess the importance of different stressor (i.e., pesticides and multiple urban stressors) as drivers of the trait composition of macroinvertebrate assemblages of the selected rivers, confirming our first hypothesis that traits were not randomly distributed across assemblages. We observed that the gradients of urban stressors and pesticide toxicity pointed in opposite directions. Sites with more intense urbanization were less impacted by pesticides and sites with more pesticide pollution were less impacted by the various stressors associated with urbanization. The latter gradient was associated with altitude because lowland areas are generally more densely populated

in comparison to higher altitude areas, as observed elsewhere (e.g.,Dolédec, Phillips et al. 2006, Rico, Van den Brink et al. 2016)). The sites characterizing the extremes of this gradient differed in species assemblages and environmental conditions. Therefore, we expected such differences to be reflected in the life-history strategies of invertebrates (Verberk, Siepel et al. 2008). Indeed, as hypothesized (H2), the trait composition of assemblages at sites impacted by pesticides differed from that at urban sites, suggesting that different trait combinations respond to specific conditions in contrasting environments (urban vs. pesticide polluted). However, it is not excluded that pesticides are also affecting the trait composition at urban sites. But their concentration were at the sublethal levels so their influence at those sites is expected to be less pronounced compared to the sites where concentrations were high enough to cause acute effects.

The taxonomic composition differed greatly among river basins, in particular separating pesticide-impacted sites in the Júcar from the other sites in the other rivers impacted by the same stressor, suggesting that presence of similar stressors may not result in similar taxonomic composition. However, we observed a similar trait composition of macroinvertebrate assemblages at sites influenced by the same stressors in different rivers. For example, the trait composition of urban sites in the Llobregat (L3-7) (NE of Iberian Peninsula) was similar to that at the urban sites of the southernmost river, the Guadalquivir (G4). Besides, the trait composition of pesticide-impacted sites in the Eastern peninsula in the Júcar basin (J1-6) was similar to that observed at sites in the North-East in the Ebro (E1, E5) and in the South in the Guadalquivir (G1). Furthermore, sites including multiple urban stressors and pesticides (E2, E3) were positioned between these two extremes along the first RLQ axis, indicating their partial similarities to both urban and pesticide-impacted sites. The higher consistency of responses obtained from trait composition in comparison to taxonomic composition has previously been reported, and arises because traits are expressed in many species and trait composition can be compared among regions that differ in their taxonomic composition (Statzner, Bis et al. 2001, Horrigan and Baird 2008). However, given that first RLQ axis also partly incorporated differences in the sampling location (i.e., most of the urban sites were located in Llobregat and most of pesticide-impacted sites were located in Júcar) we cannot exclude completely the influence of sampling location and some unmeasured variables, especially hydromporhological alterations, to our results. Hydromprphological alterations might have the influence on the macroinvetebrate trait composition due to the simplification and changes of their habitat by e.g., water abstractions, flow regulations and

morphological alterations such as straightening and canalization. Besides, it would be ideal to include unimpacted or minimally impacted sites in this kind of study to have a reference sites for comparison with impacted sites. This would allow us to observe the deviations from the natural community composition in the presence of stressors. However, in our study even sites with high percentage of natural land were not free of stressors. This was surprising, since even the sites with very small areas of artificial land upstream had concentrations of pesticides at the ecologically relevant levels (e.g., J1 and L4).

Several studies have already described the influence of different types of stressors on the structure of benthic macroinvertebrate assemblages in Iberian rivers. These stressors have included pharmaceuticals (De Castro-Català et al., 2015), metals and pesticides (Kuzmanović et al., 2015b; López-Doval et al., 2012), as well as multiple co-occurring stressors (Sabater, Barceló et al. 2016). Here, we investigated the influence of different stressors on the functional trait structure of assemblages. Assemblages characterized by several species traits (e.g., predators or having small size and dispersal ability) dominated at pesticides-impacted sites. Egg protection was prominent, which indicate the higher risk for egg mortality possibly caused by high pesticide toxicity. In contrast, at urban sites species were mainly plurivoltine, which indicates resilience potential (Southwood 1977, Townsend and Hildrew 1994)). Plurivoltinism enable species to recover after disturbance events such as periodic exposure to toxicants or “flashy” hydrology (i.e. frequent larger flow events) that are characteristic of urban streams (Walsh et al., 2005), but also after natural disturbances such as high flow variability that are common in Mediterranean rivers (Bonada et al., 2007). The prominence of plurivoltine species, which increased with urbanization and flow irregularity, may suggest a confounding effect between stressors and natural flow variability. Moreover, as Mediterranean rivers have a naturally low flow during the summer and an associated lower dilution capacity, a higher exposure of species to toxicants can be expected (Petrovic, Ginebreda et al. 2011, Arenas-Sánchez, Rico et al. 2016). This interaction between stressors and natural factors may lead to more severe effects than in more temperate rivers. Moreover, deposit feeding was significantly related to urbanization, indicating a possible response to hydrological disturbances (Feio and Dolédec 2012) or nutrients, similarly to what was observed for the marine environment (Grall and Chauvaud 2002). Finally, urbanization was associated with a decrease in predator abundance. This could be related to heavy metal pollution due to higher exposure of the taxa from the “top of the food chain” to the metals due to the biomagnification (Dolédec and Statzner, 2008).

Since we were unable to find a single trait–environmental variable association we assumed that a combination of stressors was affecting the trait composition of assemblages, especially at urban sites where several stressors had significant effects on trait composition. Similarly, we found that several physical and chemical stressors were simultaneously influencing the invertebrate assemblages of the Iberian rivers and explaining the high proportion of taxonomic variability, indicating the shared effect of multiple stressors (Sabater et al., 2016). Finally, urbanization and its related stressors seem to have an important effect on the trait homogenization of assemblages since only two out of eight traits showed prominence at urban sites. Functional trait homogenization at the most impaired sites may be an expression of the loss of functional diversity due to the combined occurrence of habitat simplification and the presence of contaminants. This phenomenon may impair the functionality, stability, resilience and resistance of ecosystems by reducing species-specific responses to environmental changes (Olden, Poff et al. 2004).(Stachowicz, Fried et al. 2002) As a side effect, trait homogenization of assemblages within the whole region may increase its vulnerability to large-scale events such as climate change (Olden et al., 2004). Our study thus complements others that have recently attempted to assess the effect of different stressors on functional homogenization (Mondy and Usseglio-Polatera, 2014; Olden et al., 2004).

6.5 CONCLUSIONS

RLQ analyses coupled with fourth-corner permutation tests proved a powerful tool to reveal the difference in the trait composition of macroinvertebrate assemblages impacted by different types of stressors. Urbanization seems to have a somewhat stronger impact on trait composition than pesticides, since different life strategies were limited at urban sites which indicated trait homogenization. Our results suggested that macroinvertebrate assemblages at sites influenced by similar stressors in different rivers across the Iberian Peninsula may have a similar trait composition, despite their difference in taxonomic composition. This latter finding confirms that multiple trait-based approaches may usefully complement taxonomic approaches in large-scale studies. However, a larger study including more sampling sites and preferably minimally impacted sites would be necessary to exclude the potential influence of natural variability and give more support to our findings.

CHAPTER 7

GENERAL DISCUSSION

A Ranking index was developed and used to prioritize 200 organic micropollutants in four Iberian rivers (Chapter 4). The Ranking index is the prioritization method based on the toxic units (TU) of chemicals and it takes into account both intensity and spatial extent of a risk. It gives us the idea of the potential adverse effects of a compound and its relevance as a pollutant in the area of concern. Compounds were classified according to their RI in three categories of concern: first category (RI > 12.5) compounds posing acute risk at more than 50% of sampling sites, second (0<RI<12.5) compounds posing acute risk at several sampling sites or chronic risk at many sites) and third (RI<0) no risk. In the studied rivers, compounds that fall in the category of the highest concern (i.e., RI > 12.5) were pesticides and industrial organic compounds. These compounds were estimated to pose the risk of acute effects at more than 50% of the sampling sites in the studied river basins. Among them, two organophosphate insecticides (chlorpyriphos and chlorfenvinphos) were ranked highest. Pesticides like chlorpyriphos and chlorfenvinphos are the highly toxic and designed to be biologically active even at low concentrations. Because their high toxicity they are also classified as priority pollutants by the Water Framework Directive. When they are present in the environment they might cause effects on non-target species and since they were found in high toxic units in all studied basins, we might expect that they could cause a decrease of biodiversity or sensitive species in local biological communities which were further examined in Chapter 5.

Ecotoxicological risk assessment was performed for studied Iberian river basins (Chapter 5). The toxic units approach was used to assess the risk of individual compounds and the concentration addition model (CA) to assess the site-specific risk. The link between chemical pollution and aquatic macroinvertebrate communities in situ was examined by using stressor-specific indexes (SPEARorganic and SPEARpesticides) and general biodiversity indexes (Shannon and Margalef indexes). The results of the study suggested that organic chemicals posed the risk of acute effects at 42% of the sampling sites and the risk of chronic effects at all the sites. Metals posed the acute risk at 44% of the sites. The main drivers of risk were pesticides and metals. Several emerging contaminants (e.g. the antidepressant drug sertraline and the disinfectant triclosan) were contributing to the chronic effects risk. The thresholds for acute and chronic risk (acute risk TU> 01, chronic risk TU>-3) were derived from the literature (Van Wijngaarden, Brock et al. 2005, Schäfer, Von Der Ohe et al. 2012, Malaj, Von Der Ohe et al. 2014) .When comparing two sampling

campaigns (2010 and 2011), in 2010 the risk was dominated by organic pollution due to the presence of highly toxic pesticides, while in 2011 metals were the main contributors to risk. Compounds that are not regulated on the European level were posing the risk of chronic effects at 23% of the sites. The decline of macroinvertebrate taxa sensitive to organic contaminants expressed as the $SPEAR_{index}$ was correlated with the increase of toxic stress related to organic compounds. Biodiversity indexes were negatively correlated with the metals and the urban land use type in the catchment.

Finally, the trait composition of macroinvertebrate communities was used to identify the effects of pesticides and multiple stressors associated with urban land use at different sites of four studied rivers (Chapter 6). Several physical and chemical stressors (high metal pollution, nutrients, elevated temperature and flow alterations) affected the urban sites. The occurrence of multiple stressors influenced aquatic assemblages at 50% of the sites. We hypothesized that the trait composition of macroinvertebrate assemblages would reflect the strategies that the assemblages used to cope with the respective environmental stressors. We used RLQ (R-environmental data table, L-abundance of taxa table, Q-taxa traits table) and fourth corner analysis to address the relationship between stressors and the trait composition of benthic macroinvertebrates. We found a statistically significant relationship between the trait composition and the exposure of assemblages to environmental stressors. The first RLQ dimension, which explained most of the variability, clearly separated sites according to the stressors. Urban-related stressors selected taxa that were mainly plurivoltine and fed on deposits. In contrast, pesticide impacted sites selected taxa with high levels of egg protection (better egg survival), indicating a potentially higher risk for egg mortality. Moreover, the trait diversity of assemblages at urban sites was low compared to that observed in pesticide impacted sites, suggesting the homogenization of assemblages in urban areas.

The prioritization shown in Chapter 4 is performed for organic micropollutants only. However, when metals are included in the prioritization, they are identified among the most important pollutants in studied rivers (Table 7.1) (Kuzmanović et al. 2016). In fact, copper, nickel and zinc are identified as the three most important compounds for all rivers especially due to their toxicity to algae (Table 7.1). But, there is a potential problem with overestimation of toxicity of metals due to their site-specific bioavailability. This is an important consideration because the potential adverse effects are dependent on the bioavailability of the compounds in the given conditions. Therefore, their measured concentrations should be corrected to predict the site-specific bioavailable fraction in water samples by for example using the biotic ligand model (BLM) (Di Toro et al. 2001; Paquin et al. 2002; Paquin et al. 2000). The BLM predicts the level of metal binding at the biotic ligand (i.e., the site of action, e.g., fish gills), and this level of accumulation is related to a toxicological effect (Paquin et al. 2002). This approach requires additional data such as temperature, dissolved organic carbon, pH and water hardness which was available in our study. However, the model is developed for several metals only (i.e., we calculated bioavailable concentrations of copper, nickel, and zinc), so we could not apply it to other metals and their estimated risk (Table 7.1) should be taken with caution. Since the model was applicable to the most toxic metals, the calculation of site-specific risk by concentration addition model (Chapter 5) was possible by taking into account only those metals that were corrected by bioavailability. Doing so Cu, Zn, and Ni accounted for more than 95% of the total site-specific metals risk.

Table 7.1 Compounds of highest concern for studied rivers are identified as the compounds that pose risk of acute effects at more than 50% of sampling sites in the river basin.

Compound	Llobregat			Ebro			Júcar			Guadalquivir		
	A	I	F	A	I	F	A	I	F	A	I	F
Cu	X	X	X	X	X	X	X	X	X	X	X	**X**
Zn	X			X	X	X	X			X		**X**
Ni	X	X	X	X			X			X		
Chlorpyriphos		X	X		X			X	X		X	
Fe	X		X	X			X			X		**X**
Chlorfenvinphos					X			X			X	
Diclofenthion					X	X		X	X			
Pb	X			X			X			X		
Diazinon		X			X			X			X	
Prochloraz							X					
Ethion								X				
Carbofuran		X										
OPs/NPs		X									X	
Diuron	X											
Mn	X											

Underlined are the priority pollutants identified by WFD. OPs/NPs-octylphenol and related compounds/ nonylphenol and related compounds. A-algae, I-invertebrates, F-fish.

EMERGING CONTAMINANTS

Many of the compounds were estimated to fall into the second category of concern identified by ranking index (0<RI<12.5, Table 7.2) (Kuzmanović, Ginebreda et al. 2016). This category included two types of chemicals i.e., highly toxic chemicals such as pesticides that were in high TU but only at several sites in the studied basins, therefore their broader spatial relevance as a pollutant was low. So, they were posing a risk of acute effects, but only in the very limited area of the basin. The second type included less toxic chemicals such as pharmaceuticals that were posing the risk of chronic effects at many sampling sites (Table 7.2). That is, they were posing the relatively lower risk, but at the large area of the basins. Pharmaceuticals such as sertraline or losartan are examples of

the compounds from this group, indicating their potential for causing chronic effects in the biota, especially algae (Table 7.2) which was also found in the North American rivers in Chapter 3. Besides, biocide triclosan was identified as a potential compound of concern for algae, similarly to what was recently found for Chinese Guangzhou river (Peng, Pan et al. 2017) and previously in Europe (von der Ohe, Schmitt-Jansen et al. 2012) and Brazil (Montagner, Jardim et al. 2014).

Table 7.2 Compounds with RI 0<RI<12.5; includes the highly toxic chemicals that are posing the high risk, but only at very limited area of the basins and the compounds that are posing relatively lower risk, but at the large area of the basins.

Compound	Llobregat			Ebro			Júcar			Guadalquivir		
	A	I	F	A	I	F	A	I	F	A	I	F
Sertraline	X	X		X			X					
Arsenic		X	X		X			X			X	
Triclosan	X			X			X			X		
Parathion-Ethyl					X			X				
Caffeine	X			X			X			X		
Terbutrine	X			X								
Isoproturon	X			X								
Losartan	X			X	X							
Imazalil				X		X	X	X	X			
Tolytriazol	X	X		X						X		
Simazine	X			X						X		
Atrazine				X			X			X		
Azinphos Ethyl		X			X						X	
Malathion		X	X		X			X	X		X	X
Azinphos Methyl		X			X							
Thiabendazole					X							
Methiocarb		X			X						X	
Venlafaxine	X	X		X	X							
Gemfibrozil			X									X

Underlined are the compounds identified as the priority pollutants by WFD. A-algae, I-invertebrates, F-fish.

Pharmaceuticals are of special interest as a chronic risk compounds due to their continuous release to the environment from WWTPs (Daughton and Ternes 1999, Petrovic, Ginebreda et al. 2011). But, for more accurate risk assessment of chronic effects chronic toxicity data should be used. Since many pharmaceuticals are emitted continuously into the environment, organisms will be exposed throughout their lifetime (Boxall, Rudd et al. 2012). However, chronic toxicity data for pharmaceuticals and other emerging contaminants is still very scarce (Von Der Ohe, De Deckere et al. 2009) and that is why we and many other authors e.g., (Von Der Ohe, De Deckere et al. 2009) (Malaj, Von Der Ohe et al. 2014) used the acute toxicity data to estimate the potential risk of these chemicals. In some cases, modeled data can be used to estimate the toxicity of chemicals such as those obtained from QSARs (quantitative structure activity relationships) (Altenburger, Walter et al. 2004) and read-across methods (Schüürmann, Ebert et al. 2011). In general, the lack of toxicity data still represents a great problem for accurate risk assessment and an obstacle to proper regulation of the compounds that might be ecotoxicologically relevant pollutants.

Ecosystems are generally exposed to a complex mixture of pharmaceuticals and other contaminants and their joint effects on the environment could be greater than predicted based on effects data for the single compounds (Backhaus and Karlsson 2014). Still, long-term combined toxicity of mixtures of chemicals is not taken into account for regulation purposes and there is a need to develop new approaches in order to assess this kind of risks (Boxall, Rudd et al. 2012). Since pharmaceuticals typically co-occur along with many other chemical and nonchemical stressors, their relative role in the environment needs to be compared with other stressors in specific environmental scenarios. In any case, there are indications of the adverse effects of pharmaceuticals and other micropollutants for ecosystems and human health if they reach water for human consumption. Therefore, efforts to minimize their occurrence and effects in the environment are logical and some steps have been taken already. In Switzerland currently there is a large governmental project of upgrading the WWTP Infrastructure by adding further steps to the conventional treatment process (ozonation and powder-activated carbon) (Eggen, Hollender et al. 2014) which was shown to remove a substantial amount of micropollutants from the effluent (Hollender, Zimmermann et al. 2009). However, is possible only in the developed countries where funding is available to support this kind of initiatives.

The ranking index prioritization (Chapter 4) was extended and applied for sediment contaminants (Kuzmanović, Ginebreda et al. 2016). For the sediment TUs calculation, first, it was necessary to calculate bioavailable pore water concentration and the sediment TUs were defined as the ratio of the estimated pore-water concentration of a contaminant and the water exposure based toxicity values. The pore water concentration estimates were done following the equilibrium partitioning approach (Di Toro, Zarba et al. 1991). It is generally thought that the pore water concentrations are better predictors of sediment toxicity to invertebrates compared to pesticides adsorbed to sediment particles (Rasmussen, Wiberg-Larsen et al. 2015). However, the uncertainty in this kind of sediment toxicity estimation exists because of other the possible exposure routes of sediment species to the toxicant which are not taken into account by the pore water exposure (e.g., ingestion of sediment) (Batley, Burton et al. 2002). Since the organic matter is assumed to be the major binding phase for non-ionic organic chemicals in sediments (Di Toro, Zarba et al. 1991) fraction of organic carbon in sediment (foc) and partitioning coefficient between organic carbon and water (Koc) were used to calculate the pore water concentration by means of Equation (7.1):

$$C_{PW} = \frac{C_s}{f_{OC} \times K_{OC}} \quad (7.1)$$

For the sediment, again the organophosphate insecticides chlorpyriphos and chlorfenvinphos were the most important pollutants (Table 7.3). In the study by (de Castro-Català, Kuzmanovic et al. 2016) of sediment ecotoxicity from the same four rivers organophosphate insecticides and metals were identified as the main contributors to the toxicity, as the evidence for this was found from combined approach of TUs, a battery of ecotoxicity bioassays and local invertebrate community description (de Castro-Català, Kuzmanovic et al. 2016). Since insecticides were found in higher concentrations in sediment than in water; their ranking index was higher as well (chlorpyriphos max RIsediment=80%; max RIwater=35%) indicating their role as sediment contaminant might be of even higher concern than in surface water. Many chemicals can physically and chemically bind to the sediments and persist in the environment for longer periods. Upon the change of conditions in the environment, they might become bioavailable and exert adverse effects on aquatic organisms (Zoppini, Ademollo et al. 2014). So, sediments may act both as a sink and as a source of pollution (Hollert, Dürr et al. 2000) and the

compounds such as some banned pesticides that are not in use today, may persist and accumulate in sediments (McKnight, Rasmussen et al. 2015). However, the contribution of the legacy contaminants is commonly an overlooked factor in the risk assessment.

Table 7.3 Compounds of highest concern in sediment of studied rivers are identified as the compounds that pose risk of acute effects at more than 50% of sampling sites in the river basin.

Rank	Compound	Llobregat			Ebro			Júcar			Guadalquivir		
		A	I	F	A	I	F	A	I	F	A	I	F
1	Chlorpyriphos		X	X		X	X		X	X		X	X
2	Chlorfenvinphos											X	
3	Nonylphenol	X	X	X								X	X
4	Diazinon		X						X			X	
5	Malathion								X				

Underlined are the compounds identified as the priority pollutants by WFD. A-algae, I-invertebrates, F-fish.

In a study from Denmark (Rasmussen, Wiberg-Larsen et al. 2015), the highest concentrations of legacy pesticides in sediments were found in the agricultural streams and their origin was related to past agricultural applications. There, the dominant legacy compounds included organochlorine pesticides, such as DDT and lindane, the organophosphate chlorpyrifos (which is authorized for use in Spain, but not in Denmark) and triazine herbicides such as terbuthylazine and simazine. Besides, legacy pesticides may enter the surface waters through several pathways including leaching from agricultural soils and landfills, groundwater inflow, atmospheric deposition or illegal private use (Aliyeva, Halsall et al. 2013) (McKnight, Rasmussen et al. 2015). In some cases, the banned pesticide continues to be used until stockpiles ran out or the ban does not apply to use some specific crops or situations. So in fact, it can still be legally available for some purposes. It is suggested (McKnight, Rasmussen et al. 2015) that legacy pesticides may generate a relatively constant exposure regime in surface waters and that chronic toxicity scenario is important in that case, again demonstrating the problem of the lack of chronic toxicity data in the literature.

In Chapter 5, we found that the banned pesticides significantly contribute to the risk in the surface water of studied rives. Several pesticides that are not authorized for use in Spain (e.g., chlorfenvinphos and ethion) have been measured in water at the levels that could be associated with acute effects and even more, compounds were measured at levels

associated with chronic risk (e.g. dichlofenthion, parathion-ethyl etc.). The findings of (McKnight, Rasmussen et al. 2015) and (Rasmussen, Wiberg-Larsen et al. 2015) and ours suggest that legacy and banned pesticides can still be highly significant contributors to the current risk of toxic effect in ecosystems and that neglecting those compounds in risk assessments may severely underestimate the real risk.

PRIORITY AND EMERGING POLLUTANTS RISK

In Chapter 5, we demonstrate that the risk to the ecosystems posed by the Water Framework Directive priority pollutants was significant and those compounds were among the highest contributors to the risk in studied rivers (Table 7.1). However, many other chemicals were associated with potential adverse effects. Those compounds include aforementioned banned pesticides, but also emerging contaminants such as pharmaceuticals (sertraline) or biocides (triclosan) (Table 7.2). Several other European studies come to similar conclusions. In the study of occurrence and toxicity of more than 300 organic pollutants in North Germany, it was found that most of the compounds responsible for potential acute effects in the ecosystems are not considered as priority pollutants by the WFD and that only 2 of 25 measured priority pollutants were found at the ecotoxicologically relevant levels (Schäfer, Von Der Ohe et al. 2011).

In Switzerland (Moschet, Wittmer et al. 2014) studied the occurrence of 249 compounds mainly pesticides and biocides and their transformation products. They found that chronic environmental quality standards were exceeded for 19 compounds in 78% of the water samples which would not be possible to observe by restricting the assessment to priority components only. So, they concluded that predicted mixture risk is significantly higher when comprehensive screening is performed compared to target screenings restricted to few pesticides such as WFD priority pollutants or a subset of the active ingredients applied in the highest quantities at the national level. This study demonstrates that the compounds not covered by WFD can significantly contribute to the risk for the aquatic ecosystems; similarly to our findings from Chapter 4 (Figure 7.1 and Figure 7.2).

In the study that compiled the monitoring data from continental 4,000 European sites (Malaj, Von Der Ohe et al. 2014), it was estimated that organic chemicals pose the risk of acute and chronic effects on sensitive fish, invertebrate, or algae species in 14% and 42% of the sites, respectively. This large-scale study indicates that, despite the development of scientific knowledge and the regularity efforts, organic pollution still represents a threat to European ecosystems health. Both chronic and acute risk increased with the higher

number of chemicals analyzed at each site. Interestingly, they found that the highest risk was in the French river basins. But they point out this is due to the fact that the most extensive monitoring was performed there and that ecotoxicologically relevant compounds were measured. Thus, they concluded that depending on the limitations of monitoring, the actual risk might be underestimated and for the more realistic risk assessment, the monitoring programs should be focused on the ecotoxicologically relevant chemicals. However, since there is evidence that some emerging chemicals (Slobodnik, Mrafkova et al. 2012, Slobodnik and Von Der Ohe 2015) other than those frequently monitored are likely to pose risk for ecosystems, they should be progressively identified and included in monitoring programs (Malaj, Von Der Ohe et al. 2014).

Furthermore, in the Chapters 3, 4 and 5 we demonstrate that emerging contaminants also contribute to the risk for aquatic ecosystems in Iberian rivers and also worldwide (Chapter 3). Figures 7.1 and 5.2 display the allocation of the acute and chronic risk, respectively, in the four studied rivers among the three groups of pollutants with different levels of priority, namely: emerging contaminants, WFD priority pollutants and the pesticides that are not authorized for use in Spain. Emerging contaminants alone were estimated to pose a risk of chronic effects at 23% of the sampling sites. The inclusion of banned pesticides increased that risk to 98% of the sites (Figure 7.2), and also an acute risk at 15% of the sites (Figure 7.1). The percentage of the sites with chronic risk posed by emerging pollutants was higher in Llobregat and Júcar (Figure 7.2), while the banned pesticides were substantial contributors to the chronic risk in all the studied basins Furthermore, by inclusion of WFD priority pollutants chronic risk was present at all the sampling sites and acute risk at 42% of the sites (Figure 7.1). Therefore, in the light of these findings we may conclude that the list of WFD priority pollutants includes the compounds that are among the most important contributors to the risk for ecosystems in the studied rivers, but that many other compounds can significantly contribute to this risk (both acute and chronic) and the priority pollutants list is not fully protective. Thus, the identification of the river basin specific pollutants should be or crucial importance for proper risk assessment.

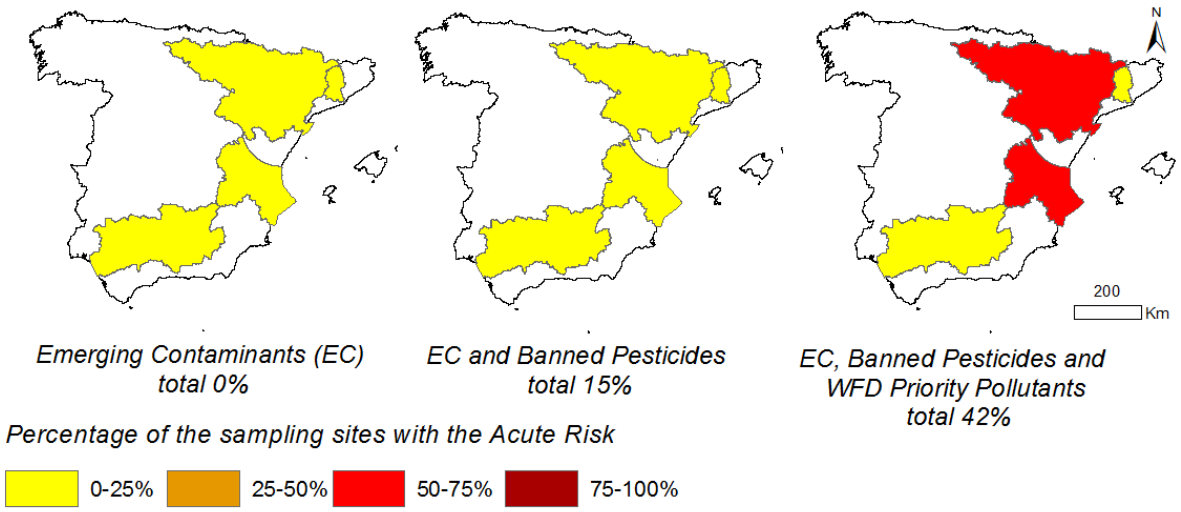


Figure 7.1 Allocation of the acute risk among the different groups of compounds according to their priority in the existing European legislation.

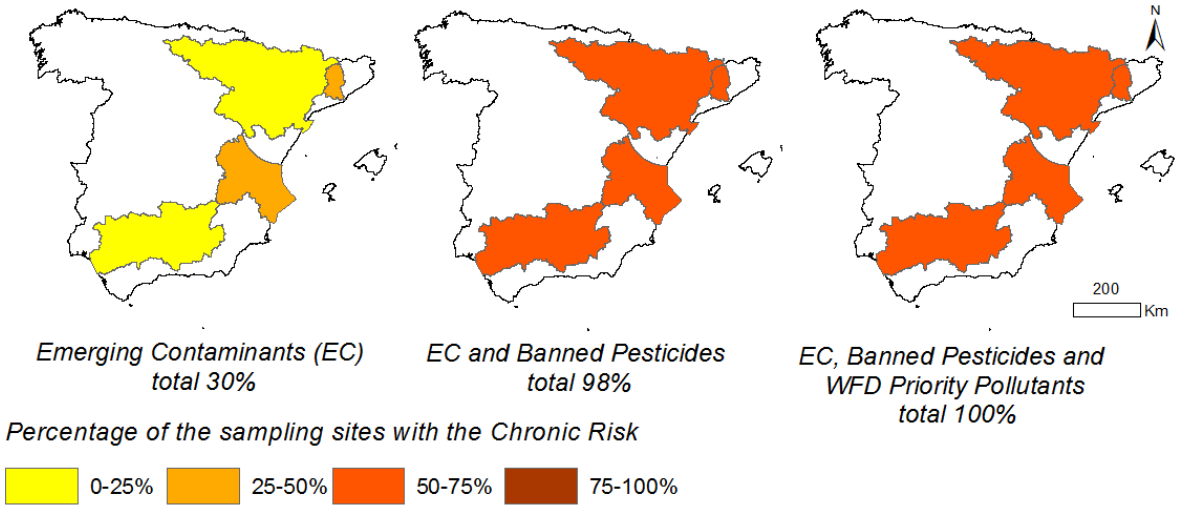


Figure 7.2 Allocation of the chronic risk among the different groups of compounds according to their priority in the existing European legislation.

MAIN DRIVERS OF RISK

The sites with highest pollution (Annex I) and (Chapter 5) the sites with the highest risk in the studied rivers were identified. The summary of these results is represented in Table 7.4, where the top 15 sites were ranked according to the levels of pollution and risk, respectively. The most polluted site (ANO2) was in the Llobregat, and this river basin was more polluted compared to other three basins (Figure 7.3). However, the pollution did not translate into risk, since the highest risk was in the Júcar basin (Figure 7.3). In fact, the

discrepancy between pollution and risk was quite remarkable. In the most polluted sites (Figure 7.4), the risk was quite low when compared to the sites with the highest risk (e.g., JUC8). And vice versa, the sites with the highest risk (all in the Júcar basin) (Figure 7.5) were not very polluted when compared to the heavily polluted sites in the Llobregat (Table 7.4). Only the site JUC8, which is the site with the highest risk, was among the top 15 most polluted sites (Table 7.4). When the basins are compared, the most polluted is Llobregat followed by Ebro and Júcar and the least polluted was Guadalquivir (Figure 7.3). On the other hand, the risk is the highest in Júcar, followed by the Ebro, and lower in the Llobregat and Guadalquivir (Figure 7.3). The fact that pollution does not necessarily translate into risk is an important point because it indicates that the risk is probably driven by the above average toxic substances (Munz, Burdon et al. 2017). Finding those relevant substances is of the greatest importance for the proper risk assessment and it might reduce the monitoring efforts in the future.

Table 7.4 Top 15 sites ranked according to the levels of organic pollution and by the risk.

Rank	Top sites by the Pollution	River	$C_{organic}$ (µg/l)	Top Sites by the Risk	River	$TU_{organic}$
1	ANO2	Llobregat	8,6	JUC8	Júcar	0.1
2	ZAD	Ebro	8,5	JUC7	Júcar	0.0
3	LLO7	Llobregat	6,5	JUC4	Júcar	-0.1
4	ANO3	Llobregat	5,1	JUC6	Júcar	-0.1
5	LLO6	Llobregat	4,0	CAB2	Júcar	-0.1
6	MAG1	Júcar	3,7	JUC2	Júcar	-0.2
7	GUAA	Guadalquivir	3,1	MAG2	Guadalquivir	-0.2
8	LLO5	Llobregat	3,1	MAG1	Guadalquivir	-0.3
9	ARG	Ebro	2,9	JUC3	Júcar	-0.4
10	EBR6	Ebro	2,5	JUC5	Júcar	-0.4
11	GUA9	Guadalquivir	2,3	CAB4	Júcar	-0.6
12	GUA4	Guadalquivir	2,2	HUE	Ebro	-0.6
13	HUE	Ebro	2,1	ARG	Ebro	-0.6
14	JUC8	Júcar	1,8	EBR2	Ebro	-0.6
15	SEG	Ebro	1,7	EBR4/ZAD	Ebro	-0.7

$C_{organic}$- sum of concentrations of all detected organic compounds, $TU_{organic}$-sum of toxic units of all detected organic compounds

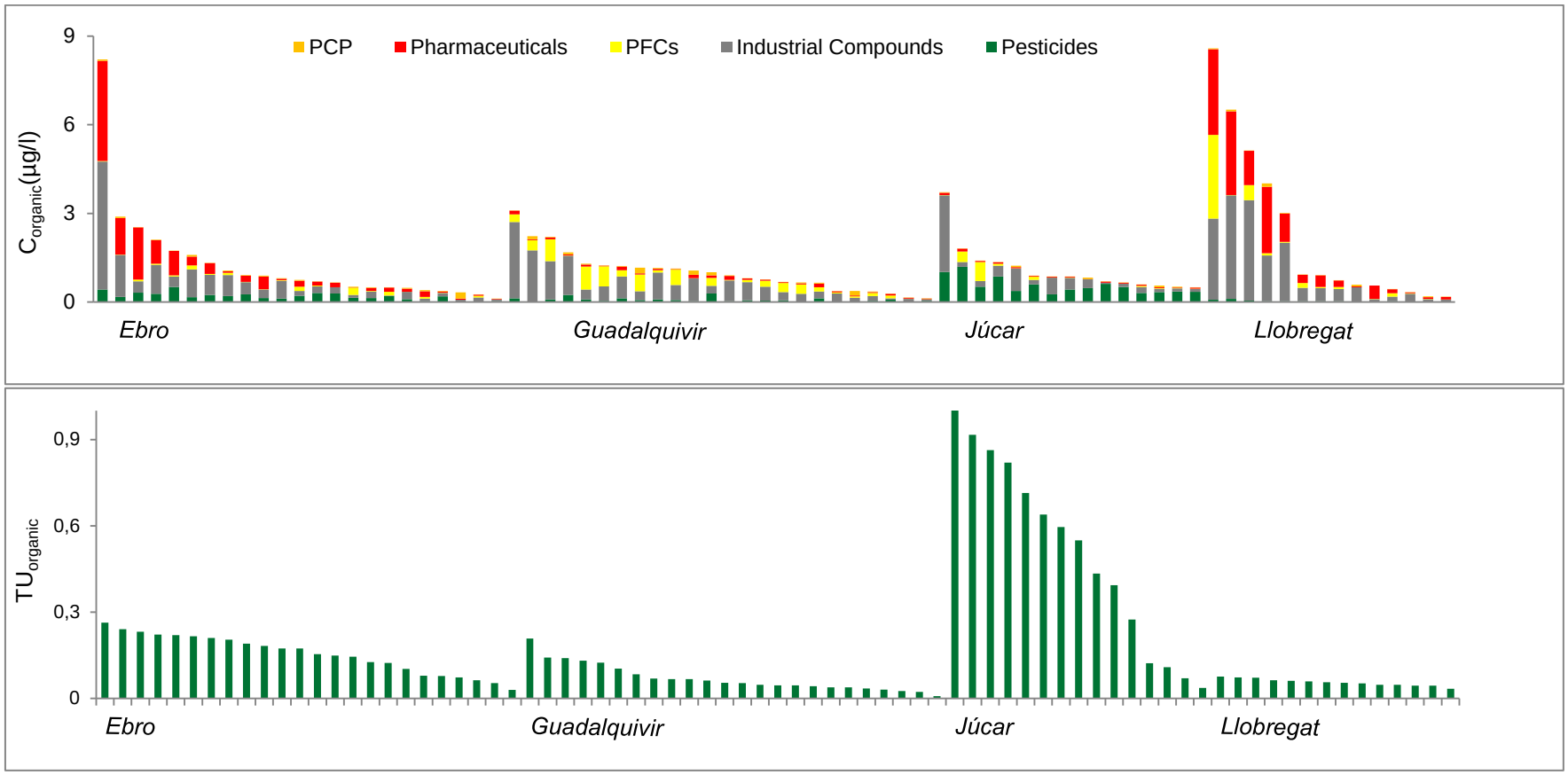
In Chapter 4 and 5 we found that, in general, just several compounds were the main contributors to the acute risk, similarly to the results that can be found in the recent literature (Backhaus and Karlsson 2014) (Malaj, Von Der Ohe et al. 2014, Munz, Burdon et

al. 2017). In Swiss rivers, a risk assessment of the wastewater-impacted streams in the low flow conditions was performed (Munz, Burdon et al. 2017). It was found that the pharmaceuticals and other household chemicals were dominant in the concentrations downstream of the WWTPs. However, the acute risk was mainly driven by pesticides, and generally, the majority of the risk was explained by few substances only (such as pesticides and diclofenac). The study of European basins (Malaj, Von Der Ohe et al. 2014) provides evidence that among the main contributors to the risk for algae were herbicides and insecticides for invertebrates and fish. From all the monitored chemicals, pesticides, tributyltin, polycyclic aromatic hydrocarbons, and brominated flame retardants were the major contributors to the risk and their presence was related to agricultural and urban areas upstream. These findings are important because they indicate that, in the future, monitoring efforts could be reduced if the typically co-occurring compounds are identified as the representatives of the mixture toxicity for specific land-uses or source types (Altenburger, Ait-Aissa et al. 2015). That is if the right combination of substances with the highest ecotoxicological potential are selected to identify the priority mixtures, as it was recommended by the European Commission (EC 2011).

From the Figures 7.4 and 7.5, it is clear that the main contributors to the risk in the studied Iberian rivers are pesticides (green color in the Figures). Moreover, the overall dominance of the pesticides risk is obvious because the risk is highest at those sites where their concentrations were higher (Figure 7.5). The compounds responsible for the majority of the risk were identified in Chapter 4 (e.g., chlorpyriphos chlorfenvinphos, dichlofenthion). Those chemicals are identified as the first category of concern chemicals by the ranking index (Table 7.1). Besides pesticides, metals were the groups of compounds that contributed to the acute risk (Chapter 5), but other compound groups contributed to the chronic risk. However, since we used acute toxicity data to evaluate the risk there are some considerations to be taken into account. Because some pharmaceuticals have high acute to chronic ratio their real risk might be underestimated (Munz, Burdon et al. 2017). Due to the lack of chronic toxicity data, it is difficult to assess the real risk of those compounds and some unexpected effects might be possible even at low concentrations (Calow and Forbes 2003).

Monitoring based on the grab samples (e.g., monthly or annually) might lead to the underestimation of real concentrations of some compounds and consequently the risk they pose to ecosystems (Schäfer, Kühn et al. 2016). Furthermore, some of the compounds

that are present in the environment at the concentrations below the limit of the detections (LOD) or the limits of quantification (LOQ) of the current analytical methods might be ecotoxicologically relevant. However, due to the limitations of the current analytical methods their risk might be non-detected (Malaj, Von Der Ohe et al. 2014) and development more sensitive analytical methods is necessary (Moschet, Wittmer et al. 2014).



Pollution	**Risk**
Llobregat>Ebro>Júcar>Guadalquivir	Júcar>Ebro>Llobregat>Guadalquivr

Figure 7.3 Pollution and risk levels at sampling sites in each of the studied rivers.

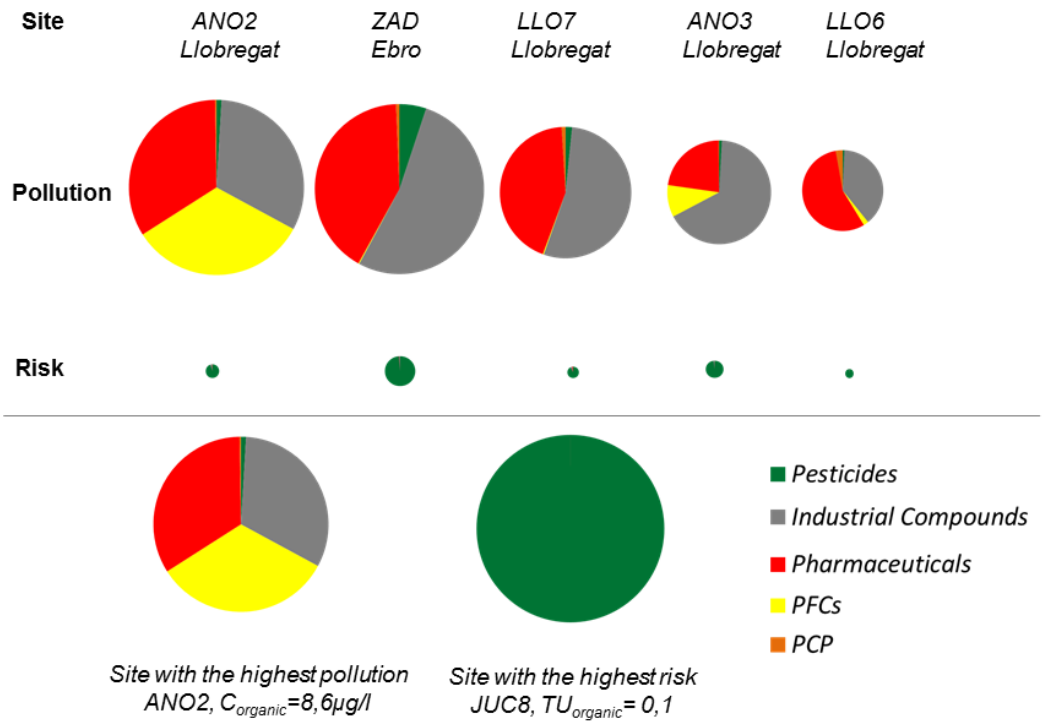


Figure 7.4 Top 5 sites according to the pollution are represented by the pie charts. The size of the pie represents the total measured concentration of organic pollutants relative to the site with the highest concentration of organic pollutants (ANO2). The pie slices in different colors represent the contribution of the each group of the pollutants to the total concentration. Risk relative to the site with the highest estimated risk (JUC8) is represented for each of these five sites. The pie slices in different colors represent the contribution of the each group of the total risk, and it is dominated by pesticides (green color).

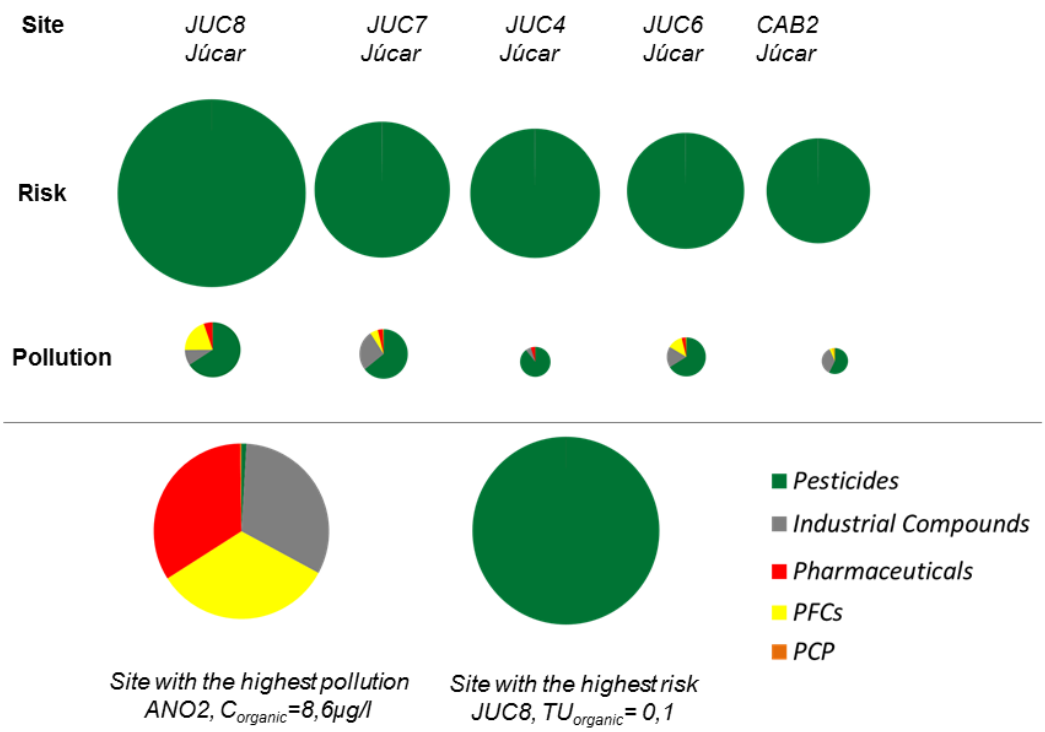


Figure 7.5 Top 5 sites according to the risk are represented by the pie charts. The size of the pie represents the total risk of organic pollutants relative to the site with the highest risk (JUC8). The pie slices in different colors represent the contribution of the each group of the total risk, and it is dominated by the pesticides (green color). The pollution of these sites is represented below, relative to the most polluted site (ANO2). The pie slices in different colors represent the contribution of the each group of the pollutants to the total concentration.

MULTIPLE STRESSORS

In Chapter 5, we show that organic pollutants posed the acute risk at 42% of the sampling sites and metals were estimated to pose the acute risk at 45% of the sites. In fact, metals were dominantly contributing to the risk for ecosystems at some sites (Figure 7.6). That is, their site-specific toxic units were higher than those of organic pollutants, especially in 2011, which was a dry year. The year 2010 had above average level of rainfall at the time of sampling, while 2011 was dry, as it is typical for a Mediterranean climate. The rainfall could trigger the pesticides runoff into rivers from the surrounding land, and thus increase the pesticides contents in the rivers and consequently the risk for the ecosystems. On the other hand, in the dry year, the river flow might be low which would reduce the dilution capacity of the river and result in the relative increase of the some pollutants that are continuously introduced into the rivers through wastewater treatment effluents or that are already present in the water (Petrovic, Ginebreda et al. 2011). In 2011, the risk at the majority of the sampling sites in all rivers was dominated by metals, except in Júcar where the number of sites with two types of risk was equally divided (Figure 7.6). The situation in 2010 was completely

different, and organic pollutants risk was dominant in all the studied river basins (Figure 7.6). This result puts the emphasis on the need for continuous monitoring of the pollutants since the “snapshot” information we get from the single monitoring campaign might not give us the necessary information to properly assess the risk for ecosystems.

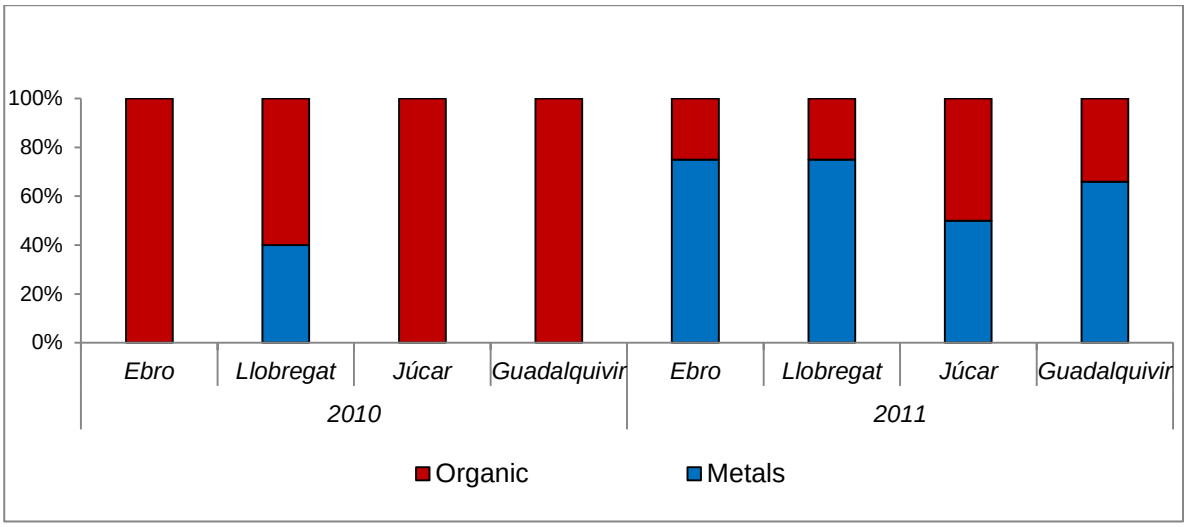


Figure 7.6 Percentage of the sampling sites with dominance of organic pollutant risk (red) and metals risk (blue). The sampling was performed in autumn of two consecutive years. The year 2010 had above average level of rainfall at that time, while 2011 was dry typically to Mediterranean climate.

The similar results were found in the study by (Carafa, Faggiano et al. 2011) for Catalan basins where metals and organic pollutants risk was assessed by multi-substance potentially affected fraction (msPAF) based on the species sensitivity distribution (SSD) (De Zwart and Posthuma 2005). They found that metals risk dominated at the majority of the sites. Furthermore, in the study that analyzed the Spanish monitoring programs carried out by water authorities and their suitability for ecotoxicological risk assessment in the same four Spanish basins as ours, high ecotoxicological risk was found in the majority of sampling points, to which metals were the main contributors to this risk (López-Doval, De Castro-Català et al. 2012). But on the contrary in the Swiss basins metals played only a minor role in the overall risk (Munz, Burdon et al. 2017) indicating again the importance to identify river basin specific pollutants. Furthermore, in order to confirm the feasibility of the predicted effects by the performed risk assessment, it is necessary to look for the expected effects in the ecosystems.

It is becoming increasingly obvious that most of the world’s rivers and streams are subjected to multiple stressors which can result in different effects in ecosystems (Vörösmarty, McIntyre et al. 2010). However, there is still not enough knowledge about the co-occurrence of stressors, about the prevalence, spatial patterns, interactions and effects (Schinegger, Trautwein et al. 2012). Besides, research and policies on chemicals are still not compliant

about the relative ecological effects of chemical mixtures and other stressors in the biological communities (Posthuma, Dyer et al. 2016). Furthermore, even though it is known that organic pollutants and other environmental stressors contribute to the reduction of aquatic biodiversity, current chemical risk assessment fails to protect biodiversity when multiple stressors concurrently affect organisms (Liess, Foit et al. 2016). In the study on multiple stressors influence od macroinvertebrates and biofilm we found that several physical and chemical stressors were simultaneously influencing the assemblages of the Iberian rivers and explaining the high proportion of taxonomic variability, indicating the shared effect of multiple stressors (Sabater, Barceló et al. 2016). Thus, the co-occurrence of stressors and the relative contribution of organic pollutants and other stressors to the risk should be evaluated (Schäfer, Kühn et al. 2016) and related to the effects in the aquatic systems (Posthuma, Dyer et al. 2016). By now, several studies have assessed the potential significance of different stressors on aquatic ecosystems but the evidence of the stressor-specific effects is still scarce. (Vörösmarty, McIntyre et al. 2010) evaluated that water pollution and stressors related to water resource development (i.e., resulting in habitat degradation) were the main threat to the worldwide ecosystems. The European study, (Schinegger, Trautwein et al. 2012) estimated that approximately 31% of all sites were affected by one, 29% by two, 28% by three and 12% by four pressure groups; only 21% were unaffected. In total, 47% of the sites were impacted by multiple stressors. They found that hydromorphological pressures were the most important in alpine regions and headwaters, whereas water quality and combined pressures prevailed in lowlands. In the recent review of the multiple stressors in freshwaters (Nõges, Argillier et al. 2016) identified that two or more co-occurring stressors is the most common scenario but just a few studies give the quantitative evidence of the multiple stressors effects. Besides, by now the ecologists were more focused on the stressors such as nutrients or hydrological degradation and leaving aside the effects of the pollutants. Ecotoxicologist are focused mainly on the laboratory-based tests to evaluate the effects chemicals on the single species which might not be realistic enough in the real-world ecosystems (Schäfer, Kühn et al. 2016). This is relevant issue especially for the mitigation measures, since focusing on the individual stressors might not be effective to reduce the risk and the integration of the ecological and ecotoxicological approaches can be useful to ensure the conservation of the biodiversity (Liess, Foit et al. 2016, Schäfer, Kühn et al. 2016). Furthermore, a combination of stressors does not necessarily result in additive effects, but rather synergism (larger combined effect) or antagonism (smaller combined effect) might appear. The combined effects have been identified in the case of nutrients and micropollutants, where antagonistic interaction reduces the toxic effects of the micropollutants in the presence of nutrients (Aristi, Casellas et al. 2016, Stamm, Räsänen et al. 2016). Overall, there is a need to understand such interactions

in order to be able to address the multiple stressors and to mitigate their effects in the ecosystems (Schäfer, Kühn et al. 2016).

In Chapters 5 and 6, we are discussing the estimated risk of organic pollutants, metals and other potential stressors to the effects on the macroinvertebrate biodiversity, sensitive species, and their functional traits. In Chapter 6, we hypothesized that the trait composition of macroinvertebrate assemblages would reflect the strategies used to cope with the respective environmental stressors. First, we evaluated the presence of the potential stressor at each of the sampling sites where both biological and chemical data was available (Figure 6.1, from Chapter 6). In the concurrence with the aforementioned multiple stressor studies, we found that multiple stressors were present at 50% of the sampling sites. However, we did not include e.g., hydromorphological disturbances due to the lack of the data. We mainly focused on chemical (toxicant and nutrients), physicochemical stressors (e.g., temperature or low oxygen level) and the land use data which was related to effects on the biodiversity and the sensitive species of the macroinvertebrates (Chapter 5) and their functional traits (Chapter 6). In our study potential additive or synergistic effects were not taken into account. In order to classify an environmental variable as a stressor, it is necessary to have a threshold value of the potential adverse effects in the ecosystem. For organic compounds, we used the threshold values that were reported from the literature (Van Wijngaarden, Brock et al. 2005, Schäfer, Von Der Ohe et al. 2012, Malaj, Von Der Ohe et al. 2014). We used the threshold value of 0.1 TU for the acute effect of organic compounds and metals as suggested by (Malaj, Von Der Ohe et al. 2014) and for other stressors, we used thresholds from the Spanish legislation (Real Decreto 817/2015 2015). However, for toxicants there are just several studies reporting the possible threshold values, thus more reliable cause-effect relationships in the field communities still need to be developed (Schäfer, Kühn et al. 2016).

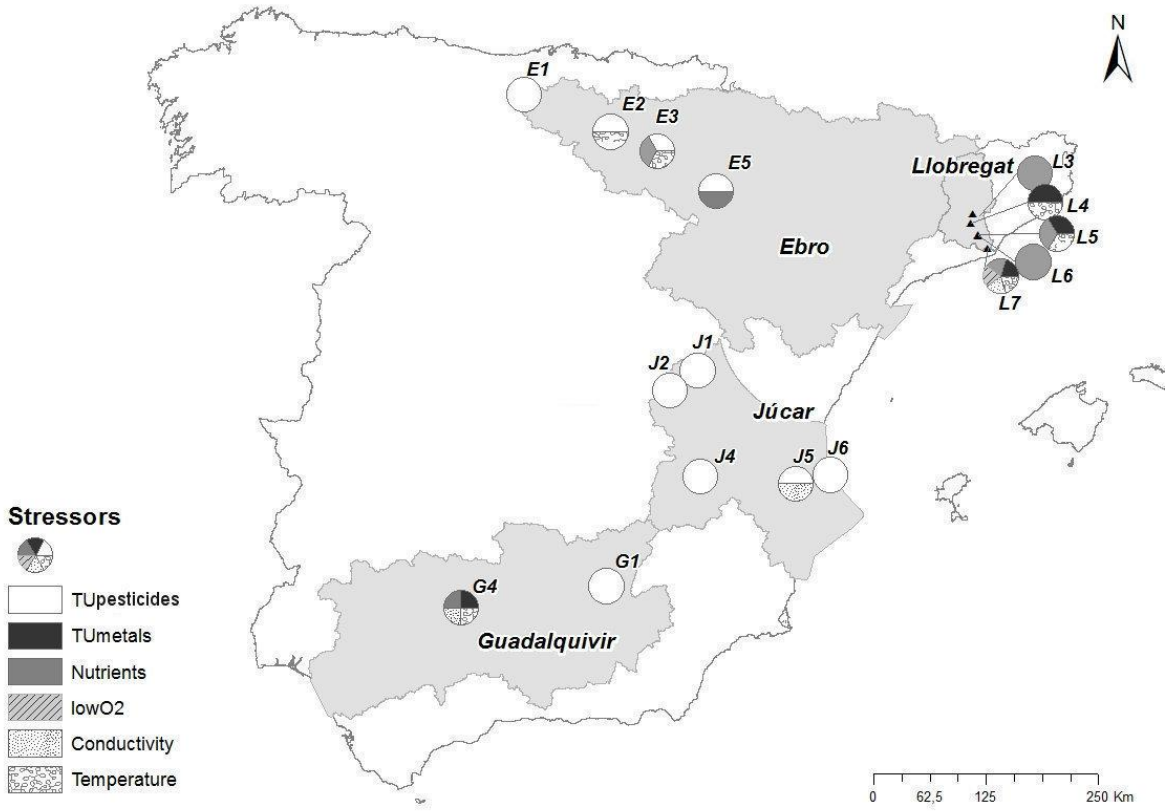


Figure 7.7 Potential stressors at sampling sites including toxic units of pesticides ($TU_{pesticides}$), toxic units of metals (TU_{metals}), nutrients, low oxygen levels ($lowO_2$), conductivity and temperature.

EFFECTS IN THE ECOSYSTEMS

To observe the effects of particular stressors in the ecosystems can be difficult due to the presence of multiple simultaneously acting stressors and natural variability of the biological communities (Menezes, Baird et al. 2010). Commonly used taxonomy-based approaches (such as e.g., Shannon-Wiener and Margalef biodiversity indexes) can be influenced by geographical and longitudinal gradient factors, so the whole community-based biodiversity metrics are not ideal indicators of anthropogenic effects on biodiversity at broad scales (Minshall, Petersen et al. 1985, Feld, Birk et al. 2016) (Beketov and Liess 2008). To overcome this problem, the use of biological traits (such as generation time, body size, body form and dispersal ability) (Statzner, Bady et al. 2005) and trait based indexes has been proposed. Multiple-trait based approaches have shown promise for biomonitoring because most of the stressors usually affect only certain trait categories (Statzner et al., 2001; Statzner et al., 2004; Statzner et al., 2005), which can be useful for discriminating among multiple stressors. The use of multiple traits and multiple trait categories, has been used to distinguish the influence of different stressors e.g. heavy metal pollution and cargo ship traffic (Dolédec and Statzner 2008), eutrophication and fine sediment deposit (Dolédec, Phillips et al. 2006, Townsend, Uhlmann et al. 2008) and climate change and salinity (Townsend,

Uhlmann et al. 2008). Furthermore, unlike species composition, which changes along geographical and downstream gradients, some traits are thought to vary little across temporal and spatial scales, which makes them useful for large-scale studies (Statzner, Hildrew et al. 2001, Statzner, Bady et al. 2005).

SPEAR index was developed as stressors specific indicator of organic pollution (Beketov and Liess 2008), in particular, pesticides (Liess and Von Der Ohe 2005) which are not dependent on the natural longitudinal gradients (Liess, Schäfer et al. 2008). It is developed on a trait-based concept and sensitive species are determined by a combination of specific traits including aspects of physiology, life cycle or behavior. In Chapter 5, we give the evidence of the effects of pesticides on the sensitive species classified by the SPEAR index (Liess and Von Der Ohe 2005). The risk assessed by toxic units was compared to macroinvertebrate data collected at the same sites. Results suggested that macroinvertebrate communities are impacted by organic pollutants; in particular, species defined as sensitive to organic pollutants decreased with increased toxic pressure, with pesticides as the main contributors to the toxicity (Figure 7.8). This finding is particularly interesting because it confirms that the influence of organic pollution is observable, even in the multiple stressors situation as it was the case in the rivers we studied. Similar results were found by (Munz, Burdon et al. 2017) where the pesticides were identified as the drivers of the risk in the wastewater treatment effluent. Despite the presence of other pollutants in high concentrations, they were able to link the predicted risk of pesticides to the effects in the local communities. In California (Chiu, Hunt et al. 2016) found that high pesticide toxicity in Sacramento river changed the macroinvertebrates communities and that the compositions trended toward taxa having higher resilience and resistance to pesticide exposure, based on SPEAR index. These changes could not be explained by the taxonomical approach, so the authors concluded that approaches such as SPEAR perform better than taxonomy-based approaches across large geographical scales and longitudinal gradients.

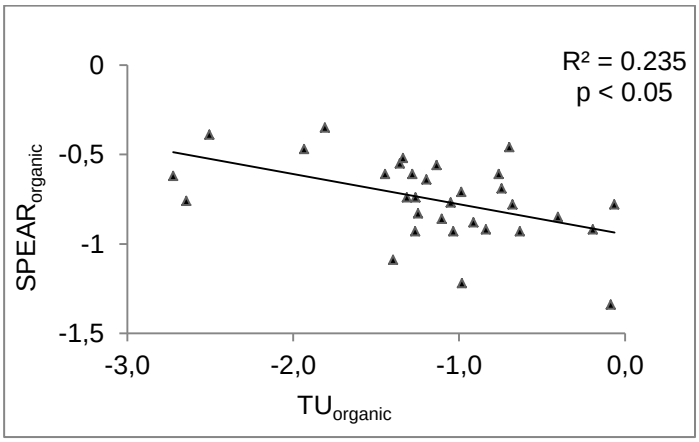


Figure 7.8 Decrease of SPEAR$_{index}$ with the increase of toxic pressure.

Furthermore, in the recent study from Switzerland (Baumgartner and Robinson 2017) found that taxonomic diversity was lowest at sites with the combination of stressors i.e., with morphological and water quality impairment which was present at agricultural sites. Sites impacted by urban settlements had low water quality but moderately impacted morphology. The SPEAR index mainly changed due to water quality degradation present at both agricultural and urban sites. But, macroinvertebrate diversity indices failed to detect anthropogenic stressors at urban sites, whereas the SPEAR pesticides index indicated poor water quality.

In our study we got similar results because the SPEAR index mainly changed due to water quality (Chapter 5), but, in our case urban sites seemed to be related to multiple stressors (Figures 7.9 and 7.10). At urban sites, the biodiversity measured by taxonomical biodiversity indexes was the lowest (Chapter 5). But, we were unable to differentiate between stressors, since there were many correlated variables (Figure 7.9).

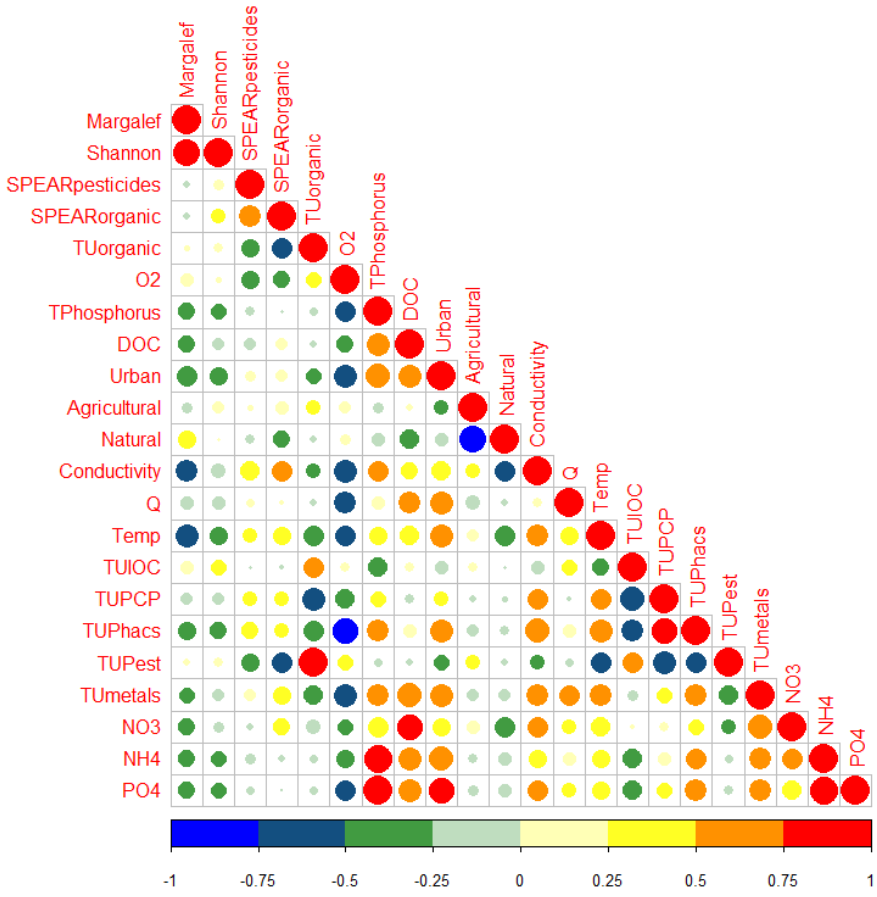


Figure 7.9 The correlation plot of the environmental variables and taxonomical (Shannon and Margalef indexes) and trait based indexes ($SPEAR_{organic}$ and $SPEAR_{pesticides}$). The strength of the correlation is given by the colors and the size of the circles. Warm colors represent positive correlation, and cold colors negative.

The relation between biodiversity indexes and urban land use could be reflecting the response of the community to a variety of stressors present at those sites (Figure 7.9) (temperature change, increased salinity) that are acting together along with the pollution (metals, pharmaceuticals, nutrients) as it was concluded by (Sabater, Barceló et al. 2016). On the other hand, SPEAR index was significantly correlated only to pesticides toxic units, indicating the response of sensitive species to the gradient of the toxicity of the pesticides at studied sites. Contrary to other studies, (Burdon, Reyes et al. 2016, Munz, Burdon et al. 2017), we did not find the correlation of the agricultural land use and SPEARindex, due to the surprising fact the pesticides at toxicologically relevant concentrations were also present at sites with mostly natural land use (Annex I). There have been some evidence that responses of macroinvertebrates to other stressors can influence the detection of pesticide impacts on stream ecosystems in biomonitoring (Chiu, Hunt et al. 2016). In Denmark, for example, sedimentation and other habitat degradation precluded the detection of pesticide effects in agricultural streams (Rasmussen, Wiberg-Larsen et al. 2012). In our case, a significant relationship between pesticide risk and macroinvertebrate sensitivity to pesticides was observed despite the presence of other stressors. In the same way (Munz, Burdon et al. 2017) found that macroinvertebrates responded to pesticides in the wastewater effluents, despite the presence of other pollutants, suggesting that toxicity and not total concentrations are a key determinant of micropollutant impacts in mixed land use environments. These findings highlight the validity of approaches linking chemical data and risk predictions in detecting realized toxic effects that are observed in the field (Munz, Burdon et al. 2017).

As the taxonomical diversity, trait diversity was also the lowest at the urban sites (Chapter 6), indicating potential functional homogenization of assemblages in urban areas. This phenomenon may impair the functionality, stability, resilience and resistance of ecosystems by reducing species-specific responses to environmental changes (Stachowicz, Fried et al. 2002, Olden, Poff et al. 2004). As a side effect, trait homogenization of assemblages within the whole region may increase its vulnerability to large-scale events such as climate change (Olden, Poff et al. 2004). The taxonomic composition differed among river basins, suggesting potential natural differences in the assemblages along the geographical gradients of as suggested by (Minshall, Petersen et al. 1985). But, we found a statistically significant relationship between the trait composition and the combination of environmental stressors (Chapter 6). The stressors significantly related the to urban land were metals toxicity, increased temperature, nutrients, low levels of oxygen and also variable water flow and the traits related to these sites were plurivoltine and deposit feeding (Figure 7.10). Besides, the sites with more intense urbanization were less impacted by pesticides and vice versa, so the two opposing gradients were present (i.e., pesticides toxicity and urbanization). However, it is not excluded that pesticides are also affecting the trait composition at urban sites. But their

concentration was at the sublethal levels so their influence at those sites is expected to be less pronounced. The gradient of urbanization was partially associated with altitude because lowland areas are generally more densely populated in comparison to higher altitude areas, as observed elsewhere (Rico, Van den Brink et al. 2016). However, we observed a similar trait composition of macroinvertebrate assemblages at sites influenced by pesticides and urban stressors in different rivers (Figure 7.10). The higher consistency of traits responses compared to taxonomical responses to stressors that we observed is in the concurrence with the expectations. Since many taxa from different geographical locations can have the same traits, the responses of trait composition can be compared to the communities with different taxonomic composition (Dolédec, Phillips et al. 2006, Bonada, Dolédec et al. 2007). At pesticides impacted sites, egg protection was a prominent trait (Figure 7.10), which indicates the higher risk for egg mortality possibly caused by high pesticide toxicity. In contrast, at urban sites species were mainly plurivoltine, which indicates resilient taxa dominance (Southwood 1977). Plurivoltinism enables species to recover after disturbances such as periodic exposure to toxicants or extreme hydrological conditions. The prominence of plurivoltine species, which increased with urbanization and flow irregularity, may suggest a confounding effect between stressors and natural flow variability, also pointed out by (Sabater, Barceló et al. 2016). Moreover, deposit feeding trait may also indicate a possible response to hydrological disturbances (Feio and Dolédec 2012) and nutrients (Grall and Chauvaud 2002). Finally, urbanization was associated with a decrease in predator abundance which could be related to heavy metal pollution due to due to the biomagnification of metals through the food chain (Dolédec and Statzner 2008).

Since we were unable to find a single trait–environmental variable association we concluded that we have indications of which combination of stressors are affecting the trait composition of assemblages contrary to the expected that single trait would respond to the single stressor. The confirmation of the single stressor effects in multiple stressor situations seems to require larger study, including the larger number of sites and ideally inclusion of the reference sites for comparison with impacted sites. This would allow us to observe the deviations from the natural community composition in the presence of stressors. However, in our study, even sites with a high percentage of natural land were not free of stressors. This was surprising since even the sites with very small areas of artificial land upstream had concentrations of pesticides at the ecologically relevant levels. However this might be difficult in the rivers such as the ones we studied, as it is revealed from the recent studies, the presence of one or more stressors seem to be the rule rather than the exception (Nõges, Argillier et al. 2016) (Schäfer, Kühn et al. 2016). In conclusion, we found the different trait composition of macroinvertebrate communities in different environmental conditions and indications for the potentially responsible stressors; however a larger study including more

sampling sites and preferably minimally impacted sites would be necessary to exclude the potential influence of natural variability and give more support to our findings.

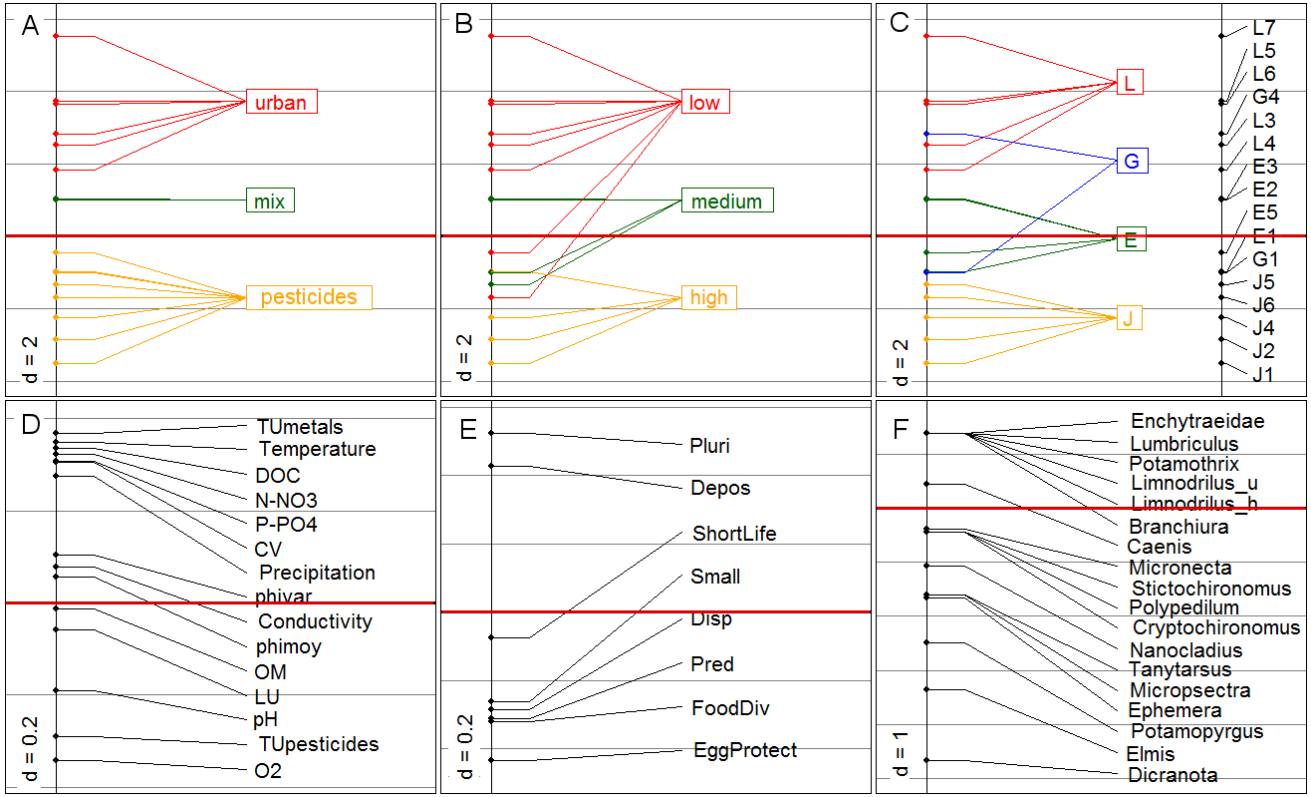


Figure 7.10 Results of the RLQ analysis that related taxa and their traits to the environmental variables. Sites grouped by (A) the dominant stressors, (B) the altitude (C) river basins (J-Júcar, E-Ebro, G-Guadalquivir, L- Llobregat), (D) environmental variables (phivar-sediment particle size variance, phimoy-average sediment particle size, LU- variable that synthesized naturalness, CV- flow variations) (E) traits (pluri-plurivoltinism, depos-deposit feeding, disp-dispersal ability, pred-predation, food div-food diversity) and (F) taxon scores along the first RLQ axis. The red horizontal line corresponds to zero at the first axis, it separates negative (up) from positive (down) score.

CHAPTER 8

CONCLUSIONS

Results obtained in this thesis, represented and discussed in the previous chapters have led to the following conclusions:

This thesis provides the comprehensive overview of ecotoxicological issues in the four Iberian rivers, namely: the Llobregat, the Ebro, the Júcar and the Guadalquivir.

PRIORITIZATION OF THE POLLUTANTS

- The prioritization method (ranking index-RI) was developed and used to prioritize more than 200 organic pollutants and metals in four Iberian rivers.
- Organic pollutants in the category of the highest concern (i.e. posing a widespread acute risk) were pesticides (chlorpyriphos, chlorfenvinphos, diazinon, dichlofenthion, ethion, carbofuran, prochloraz and diuron) and industrial organic compounds (octylphenol and nonylphenol).
- Metals in the category of highest concern were copper, nickel and zinc.
- Emerging contaminants such as pharmaceuticals (e.g. sertraline and losartan) and biocides (triclosan) were classified in the second category of concern (i.e. posing a widespread chronic risk).
- In general, the lack of toxicity data, especially chronic toxicity, represents a great problem for the accurate risk assessment.
- For the sediment, the organophosphate insecticides (chlorpyriphos and chlorfenvinphos) were the most important pollutants.
- The high ranking index indicated the relevance of sediment contamination in the studied basins.
- The contribution of the legacy contaminants in sediment should be taken into account for the accurate risk assessment.

SPATIAL RISK ASSESSMENT

- According to the pollution studied rivers can be ranked as follows: Llobregat>Ebro>Júcar>Guadalquivir.
- According to the ecotoxicological risk studied rivers can be ranked as follows: Júcar> Ebro>Llobregat> Guadalquivir.
- The pollution did not translate into risk; at the most polluted sites, risk was quite low when compared to the sites with the highest risk, and *vice versa*, the sites with the highest risk were not necessarily very polluted.

- Mixtures of organic compounds and metals, posed an acute risk at 42% and 45% of total 77 sampling sites, respectively.
- Chronic risk was present at all sampling sites.
- The major drivers of acute and chronic risk were pesticides and metals.
- Pharmaceuticals, industrial compounds and personal care produscts were additional contributors to the chronic risk.
- The site-specific risk was driven by the above average toxic compounds and not above average concentrations.
- The majority of the site-specific risk was driven by several compounds only, which varied across sites.
- The risk to the ecosystems posed by the WFD priority pollutants was significant and those compounds were among the highest contributors to the risk in studied rivers.
- Banned pesticides and emerging pollutants significantly contributed to the risk.
- The identification of the river basin specific pollutants should be of crucial importance for proper risk assessment.
- Due to the limitations of the current analytical methods the risk of low concentration chemicals might pass unnoticed and development of the development of the more sensitive analytical methods is necessary.

MULTIPLE STRESSORS AND EFFECTS IN THE ECOSYSTEMS

- Evidence of pesticide effects in macroinvertebrate communities was found by SPEARindex; the abundance of macroinvertebrate taxa sensitive to pesticides declined with the increase of pesticide toxicity.
- It was not possible to confirm the effects of chemical pollution on macroinvertebrates by the use of conventional taxonomical indexes (Shannon's and Margalef indexes) since they were correlated with several environmental variables.
- At urban areas, the taxonomical biodiversity of macroinvertebrates was the lowest.
- Multiple stressors were present at 50% of the sampling sites, mostly in urban areas.
- There was a statistically significant difference between communities exposed to pesticides and those exposed to urban-related multiple stressors, but a much

larger study would be necessary to exclude the influence of natural variation and give more support to our findings.

- At urban sites, communities' dominant traits were multivoltinism indicating dominance of resilient taxa and deposit feeding, which could be associated with the taxa resistant to hydrological disturbances or presence of nutrients.
- At pesticide impacted sites taxa with high levels of egg protection was dominant, indicating a higher risk for egg mortality at those sites, potentially due to pesticides.
- Functional biodiversity of macroinvetebrate communities was the lowest at urban sites, indicating potential functional homogenization of assemblages in urban areas.
- If functional homogenization of assemblages is present, communities at those sites may be more vulnerable to large-scale events such as climate change.
- Reliable cause-effect relationships in the field communities are lacking to develop meaningful thresholds for many stressors effects.

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ANNEX I

CONTAMINANTS OF EMERGING CONCERN IN MEDITERRANEAN WATERSHEDS

Maja Kuzmanović, Antoni Ginebreda, Mira Petrović and Damià Barceló

Published as a book chapter in The Handbook of Environmental Chemistry: Emerging Contaminants in River Ecosystems (2015)

ATTENTION ¡

Pages 168 to 186 of the thesis, containing the text mentioned above, are available at the editor's web
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ANNEX II

ASSESSMENT OF MULTI-CHEMICAL POLLUTION IN AQUATIC ECOSYSTEMS USING TOXIC UNITS: COMPOUND PRIORITIZATION, MIXTURE CHARACTERIZATION AND RELATIONSHIPS WITH BIOLOGICAL DESCRIPTORS

Antoni Ginebreda, Maja Kuzmanović, Helena Guasch, Miren López de Alda, Julio C. López-Doval, Isabel Muñoz, Marta Ricart, Anna M. Romaní, Sergi Sabater and Damià Barceló

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ANNEX III

SUPPLEMENTARY MATERIAL TO RISK ASSESSMENT BASED PRIORITIZATION OF 200 ORGANIC MICROPOLLUTANTS IN 4 IBERIAN RIVERS

Table AIII.1: List of measured compounds with their limits of detections and detection frequencies.

Compound	Compound class	Frequency of detection 2010	Frequency of detection 2011	Limit of detection (ng/L)	Reference
Acetaminophen	Pharmaceutical	36	41	0.04	SCARCE DB
Acridone	Pharmaceutical	56	71	0.03	SCARCE DB
Albendazol	Pharmaceutical	14	23	0.01	SCARCE DB
Alprazolam	Pharmaceutical	6	62	0.02	SCARCE DB
Amlodipine	Pharmaceutical	86	26	0.08	SCARCE DB
Atenolol	Pharmaceutical	64	69	0.02	SCARCE DB
Atorvastatin	Pharmaceutical	52	49	0.005	SCARCE DB
Azaperol	Pharmaceutical	0	0	0.32	SCARCE DB
Azaperone	Pharmaceutical	0	0	0.23	SCARCE DB
Azithromycin	Pharmaceutical	95	81	0.1	SCARCE DB
Bezafibrate	Pharmaceutical	34	50	0.02	SCARCE DB
Carazolol	Pharmaceutical	34	13	0.10	SCARCE DB
Carbamazepine	Pharmaceutical	44	64	0.01	SCARCE DB
Cefalexin	Pharmaceutical	4	3	0.2	SCARCE DB
Cimetidine	Pharmaceutical	49	8	0.1	SCARCE DB
Ciprofloxacin	Pharmaceutical	17	34	0.1	SCARCE DB
Citalopram	Pharmaceutical	45	66	0.02	SCARCE DB
Clarithromycin	Pharmaceutical	14	20	0.1	SCARCE DB
Clopidogrel	Pharmaceutical	66	71	0.01	SCARCE DB
Codeine	Pharmaceutical	70	69	0.02	SCARCE DB
Desloratidine	Pharmaceutical	24	16	0.04	SCARCE DB
Dexamethasone	Pharmaceutical	66	27	0.05	SCARCE DB
Diazepam	Pharmaceutical	18	61	0.05	SCARCE DB
Diclofenac	Pharmaceutical	41	63	0.6	SCARCE DB
Diltiazem	Pharmaceutical	74	48	0.02	SCARCE DB
Dimetridazole	Pharmaceutical	16	2	1.50	SCARCE DB
Enalapril	Pharmaceutical	13	2	0.47	SCARCE

					DB
Enalaprilat	Pharmaceutical	29	39	1.08	SCARCE DB
Erithromycin	Pharmaceutical	22	16	0.1	SCARCE DB
Famotidine	Pharmaceutical	0	4	0.1	SCARCE DB
Fluoxetine	Pharmaceutical	18	4	0.36	SCARCE DB
Fluvastatin	Pharmaceutical	17	15	0.03	SCARCE DB
Furosemide	Pharmaceutical	44	60	0.45	SCARCE DB
Gemfibrozil	Pharmaceutical	91	100	0.04	SCARCE DB
Glibenclamide	Pharmaceutical	3	1	0.60	SCARCE DB
Hidrochlorothiazide	Pharmaceutical	55	98	0.05	SCARCE DB
Hydrocodone	Pharmaceutical	3	18	0.6	SCARCE DB
Ibuprofen	Pharmaceutical	31	11	1.2	SCARCE DB
Indomethacine	Pharmaceutical	51	52	0.1	SCARCE DB
Iopromide	Pharmaceutical	61	32	0.18	SCARCE DB
Irbesartan	Pharmaceutical	64	81	0.02	SCARCE DB
Ketoprofen	Pharmaceutical	61	100	0.8	SCARCE DB
Levamisol	Pharmaceutical	70	59	0.01	SCARCE DB
Loratidine	Pharmaceutical	40	21	0.1	SCARCE DB
Lorazepam	Pharmaceutical	52	50	0.27	SCARCE DB
Losartan	Pharmaceutical	42	42	0.10	SCARCE DB
Meloxicam	Pharmaceutical	7	32	0.007	SCARCE DB
Metformin	Pharmaceutical	0	0	0.5	SCARCE DB
Metoprolol	Pharmaceutical	9	11	0.1	SCARCE DB
Metronidazole	Pharmaceutical	5	19	0.6	SCARCE DB
Metronidazole-Oh	Pharmaceutical	4	12	0.4	SCARCE DB
Nadolol	Pharmaceutical	12	3	0.06	SCARCE DB
Naproxen	Pharmaceutical	67	77	0.2	SCARCE DB
Norfluoxetine	Pharmaceutical	6	1	0.50	SCARCE DB
Ofloxacin	Pharmaceutical	14	6	0.04	SCARCE DB
Olanzapine	Pharmaceutical	4	5	0.04	SCARCE DB

Oxycodone	Pharmaceutical	40	43	0.1	SCARCE DB
Paroxetine	Pharmaceutical	58	35	0.16	SCARCE DB
Phenazone	Pharmaceutical	27	48	0.04	SCARCE DB
Piroxicam	Pharmaceutical	0	7	0.02	SCARCE DB
Pravastatin	Pharmaceutical	29	27	0.1	SCARCE DB
Propanolol	Pharmaceutical	18	25	0.04	SCARCE DB
Propyphenazone	Pharmaceutical	26	10	0.04	SCARCE DB
Ranitidine	Pharmaceutical	10	9	1.1	SCARCE DB
Ronidazole	Pharmaceutical	0	5	0.83	SCARCE DB
Salbutamol	Pharmaceutical	56	35	0.01	SCARCE DB
Sertraline	Pharmaceutical	3	5	0.63	SCARCE DB
Sotalol	Pharmaceutical	9	7	0.2	SCARCE DB
Sulfamethoxazole	Pharmaceutical	32	27	0.1	SCARCE DB
Tamsulosin	Pharmaceutical	29	5	0.02	SCARCE DB
Tenoxicam	Pharmaceutical	0	9	0.01	SCARCE DB
Tetracycline	Pharmaceutical	3	0	3.5	SCARCE DB
Torasemide	Pharmaceutical	34	48	0.02	SCARCE DB
Trazodone	Pharmaceutical	34	58	0.03	SCARCE DB
Trimethoprim	Pharmaceutical	27	91	0.1	SCARCE DB
Valsartan	Pharmaceutical	92	91	0.05	SCARCE DB
Venlafaxine	Pharmaceutical	49	79	0.02	SCARCE DB
Warfarin	Pharmaceutical	8	6	0.04	SCARCE DB
Xylazine	Pharmaceutical	4	9	0.03	SCARCE DB
Estradiol 17-glucuronide	Hormone	0	4	0.46	(Gorga, Petrovic et al. 2013)
Estriol	Hormone	3	4	0.17	(Gorga, Petrovic et al. 2013)
Estriol 16-glucuronide	Hormone	3	4	0.059	(Gorga, Petrovic et al. 2013)
Estriol 3-sulfate	Hormone	3	17	0.030	(Gorga,

					Petrovic et al. 2013)
Estrone	Hormone	64	56	0.050	(Gorga, Petrovic et al. 2013)
Estradiol	Hormone	86	8	0.037	(Gorga, Petrovic et al. 2013)
Estrone 3-glucuronide	Hormone	3	5	0.056	(Gorga, Petrovic et al. 2013)
Estrone 3-sulfate	Hormone	3	17	0.0038	(Gorga, Petrovic et al. 2013)
Ethinyl estradiol	Hormone	0	1	0.14	(Gorga, Petrovic et al. 2013)
Diethylstilbestrol	Hormone	1	1	0.043	(Gorga, Petrovic et al. 2013)
Caffeine	Stimulans	84	100	0.021	(Gorga, Petrovic et al. 2013)
Cocaine	Ilicit drug	63	96	0.02	SCARCE DB
Benzoylecgonine	Ilicit drug	81	94	0.02	SCARCE DB
LSD	Ilicit drug	0	0	0.32	SCARCE DB
Cannabidiol	Ilicit drug	0	0	2.27	SCARCE DB
Ephedrine	Ilicit drug	76	83	0.16	SCARCE DB
Methamphetamine	Ilicit drug	4	47	0.045	SCARCE DB
Lorazepam	Ilicit drug	12	34	1.01	SCARCE DB
Morphine	Ilicit drug	13	9	0.3	SCARCE DB
3-Hydroxycarbofuran	Pesticide	4	0	0.2	scarce db
Acethochlor	Pesticide	0	0	2	(Masiá, Ibáñez et al. 2013)
Alachlor	Pesticide	0	0	2	(Masiá, Ibáñez et al. 2013)
Atrazine	Pesticide	21	4	1.3	(Masiá, Ibáñez et al. 2013)
Azinphos ethyl	Pesticide	9	1	0.5	(Masiá,

					Ibáñez et al. 2013)
Azinphos methyl	Pesticide	4	1	0.5	(Masiá,
					Ibáñez et al. 2013)
Burpofezin	Pesticide	80	0	0.5	(Masiá,
					Ibáñez et al. 2013)
CARBENDAZIM	Pesticide	0	41	0.01	SCARCE
					DB
Carbofuran	Pesticide	21	3	0.2	(Masiá,
					Ibáñez et al. 2013)
Chlorfenvinphos	Pesticide	66	18	0.2	(Masiá,
					Ibáñez et al. 2013)
Chlorpyriphos	Pesticide	99	49	0.2	(Masiá,
					Ibáñez et al. 2013)
Deisopropylatrazine	Pesticide	28	1	2	(Masiá,
					Ibáñez et al. 2013)
Desethylatrazine	Pesticide	21	4	2	(Masiá,
					Ibáñez et al. 2013)
Diazinon	Pesticide	95	43	0.04	(Masiá,
					Ibáñez et al. 2013)
Diclofenthion	Pesticide	45	0	0.5	(Masiá,
					Ibáñez et al. 2013)
Dimetoate	Pesticide	28	0	1	(Masiá,
					Ibáñez et al. 2013)
Diuron	Pesticide	29	17	1	(Masiá,
					Ibáñez et al. 2013)
Ethion	Pesticide	8	22	0.5	(Masiá,
					Ibáñez et al. 2013)
Fenitrothion	Pesticide	1	1	2	(Masiá,
					Ibáñez et al. 2013)
Fenoxon	Pesticide	1	0	0.2	(Masiá,
					Ibáñez et al. 2013)
Fenthion	Pesticide	1	0	0.2	(Masiá,
					Ibáñez et al. 2013)
Fenthion Sulfone	Pesticide	3	1	0.2	(Masiá,
					Ibáñez et al. 2013)
Fenthion sulfoxide	Pesticide	1	0	0.2	(Masiá,
					Ibáñez et al. 2013)
Hexythiazox	Pesticide	78	11	0.2	(Masiá,
					Ibáñez et al. 2013)
Imazalil	Pesticide	62	33	0.3	(Masiá,

Imidacloprid	Pesticide	53	30	0.04	Ibáñez et al. 2013) (Masiá,
Isoproturon	Pesticide	16	8	0.3	Ibáñez et al. 2013) (Masiá,
Malathion	Pesticide	14	1	0.3	Ibáñez et al. 2013) (Masiá,
Methiocarb	Pesticide	4	8	0.3	Ibáñez et al. 2013) (Masiá,
Metoalachlor	Pesticide	5	12	0.3	Ibáñez et al. 2013) (Masiá,
Molinate	Pesticide	1	0	0.5	Ibáñez et al. 2013) (Masiá,
Ometoate	Pesticide	4	1	0.3	Ibáñez et al. 2013) (Masiá,
Parathion-ethyl	Pesticide	12	0	2	Ibáñez et al. 2013) (Masiá,
Parathion-methyl	Pesticide	0	0	2	Ibáñez et al. 2013) (Masiá,
Prochloraz	Pesticide	42	5	0.8	Ibáñez et al. 2013) (Masiá,
Propanil	Pesticide	0	0	0.3	Ibáñez et al. 2013) (Masiá,
Propazine	Pesticide	8	0	0.3	Ibáñez et al. 2013) (Masiá,
Pyriproxyphen	Pesticide	62	1	0.5	Ibáñez et al. 2013) (Masiá,
Simazine	Pesticide	4	8	2	Ibáñez et al. 2013) (Masiá,
Tebuconazole	Pesticide	/	13	0.13	SCARCE DB
Terbumeton	Pesticide	/	4	0.01	SCARCE DB
Terbumeton-Desethyl	Pesticide	/	14	0.13	SCARCE DB
Terbutilazine	Pesticide	/	22	0.4	SCARCE DB
Terbutilazine-2 Hidroxy	Pesticide	/	29	0.01	SCARCE DB
Terbutryn	Pesticide	8	20	0.5	[2]
TERBUTYLAZINE DEETHYL	Pesticide	/	29	0.4	SCARCE DB
THIABENDAZOLE	Pesticide	/	14	0.02	SCARCE DB

Tolclophos-methyl	Pesticide	14	1	0.5	[2]
1H-Benzotriazole	Industial organic	73	90	0.072	(Gorga, Petrovic et al. 2013)
Tolytriazol	Industrial organic	99	84	0.013	(Gorga, Petrovic et al. 2013)
Nonylphenol monoethoxylate	Industrial organic	0	0	62	(Gorga, Petrovic et al. 2013)
Octylphenol	Industrial organic	96	32	0.14	(Gorga, Petrovic et al. 2013)
Octylphenol diethoxylate	Industrial organic	96	73	0.011	(Gorga, Petrovic et al. 2013)
Octylphenol monocarboxylate	Industrial organic	0	1	0.065	(Gorga, Petrovic et al. 2013)
Octylphenol monoethoxylate	Industrial organic	0	0	17	(Gorga, Petrovic et al. 2013)
Tris(2-chloroethyl) phosphate	Industrial organic	100	97	0.034	(Gorga, Petrovic et al. 2013)
Tris(butoxyethyl) phosphate	Industrial organic	100	88	0.0024	(Gorga, Petrovic et al. 2013)
Tris(chloroisopropyl) phosphate	Industrial organic	100	100	0.0025	(Gorga, Petrovic et al. 2013)
Bisphenol A (BPA)	Industrial organic	68	88	0.11	(Gorga, Petrovic et al. 2013)
Nonylphenol (NP)	Industrial organic	91	42	0.013	(Gorga, Petrovic et al. 2013)
Nonylphenol diethoxylate	Industrial organic	94	96	0.013	(Gorga, Petrovic et al. 2013)
Nonylphenol monocarboxylate	Industrial organic	94	70	0.034	(Gorga, Petrovic et al. 2013)
L-PFOS	Perflourinated compound	26	77	0.004	SCARCE DB

PFBA	Perflourinated compound	77	52	0.04	SCARCE DB
PFOA	Perflourinated compound	52	43	0.04	SCARCE DB
PFNA	Perflourinated compound	14	18	0.4	SCARCE DB
PFDA	Perflourinated compound	13	40	0.04	SCARCE DB
PFUdA	Perflourinated compound	3	9	0.04	SCARCE DB
PFDoA	Perflourinated compound	0	13	0.8	SCARCE DB
L-PFBS	Perflourinated compound	4	52	0.02	SCARCE DB
L-PFDS	Perflourinated compound	0	14	0.004	SCARCE DB
i,p-PFNA	Perflourinated compound	14	19	0.4	SCARCE DB
I,pPFNS	Perflourinated compound	0	13	0.04	SCARCE DB
L-PFHpS	Perflourinated compound	0	3	0.04	SCARCE DB
L-PFHxS	Perflourinated compound	17	27	0.04	SCARCE DB
PFHpA	Perflourinated compound	25	5	0.4	SCARCE DB
PFHxA	Perflourinated compound	13	5	0.4	SCARCE DB
PFHxDA	Perflourinated compound	1	5	0.04	SCARCE DB
PFODA	Perflourinated compound	0	13	0.8	SCARCE DB
PFOSA	Perflourinated compound	0	0	0.2	SCARCE DB
PFPeA	Perflourinated compound	34	48	0.04	SCARCE DB
PFTeDA	Perflourinated compound	4	10	0.02	SCARCE DB
PFTrDA	Perflourinated compound	3	10	0.02	SCARCE DB
4-Methylbenzylidene camphor	Personal care product	18	48	3.5	(Gago-Ferrero, Mastroianni et al. 2013)
Benzophenone-3	Personal care product	14	43	0.7	(Gago-Ferrero, Mastroianni et al. 2013)
Ethylhexyl methoxycinnamate	Personal care product	9	14	0.72	SCARCE DB
Octocrylene	Personal care product	9	0	3	SCARCE DB
2,2'-Dihydroxy-4-methoxybenzophenone	Personal care product	0	0	1	(Gago-Ferrero, Mastroianni et al. 2013)

4,4'-Dihidroxybenzophenone	Personal care product	4	1	1.8	(Gago-Ferrero, Mastroianni et al. 2013)
4-Hydroxybenzophenone	Personal care product	4	5	1.1	(Gago-Ferrero, Mastroianni et al. 2013)
Benzophenone-1	Personal care product	0	22	1	(Gago-Ferrero, Mastroianni et al. 2013)
Benzophenone-2	Personal care product	16	0	1.2	(Gago-Ferrero, Mastroianni et al. 2013)
Ethyl 4-aminobenzoate	Personal care product	0	0	1.5	(Gago-Ferrero, Mastroianni et al. 2013)
Ethylhexyl dimethyl PABA	Personal care product	0	14	0.1	SCARCE DB
Ethylparaben	Personal care product	74	53	0.27	(Gorga, Petrovic et al. 2013)
Methylparaben	Personal care product	90	75	0.20	(Gorga, Petrovic et al. 2013)
Benzylparaben	Personal care product	30	40	0.031	(Gorga, Petrovic et al. 2013)
Propylparaben	Personal care product	99	94	0.021	(Gorga, Petrovic et al. 2013)
Triclorocaraban	Personal care product	0	7	0.036	(Gorga, Petrovic et al. 2013)
Triclosan	Personal care product	23	8	0.17	(Gorga, Petrovic et al. 2013)

SCARCE DB-Scarce Consolider project database

Table AIII.2. Toxicological data of studied compounds for algae, Daphnia and fish:

Compound	Compound class	EC50 algae (µg/l)	EC50 Daphnia (µg/l)	EC50 fish(µg/l)	Ref.
Acetaminophen	Pharmaceutical	134000	9200	378000	(Grung, Källqvist et al. 2008)
Acridone	Pharmaceutical	6738	3419	7817	E
Albendazol	Pharmaceutical	174	1225	2282	E
Alprazolam	Pharmaceutical	1064	2845	2499	E
Amlodipine	Pharmaceutical	6883	8479	4754	E
Amoxicilin	Pharmaceutical	/	/	/	/
Atenolol	Pharmaceutical	190000	205000	1096000	Ecotox
Atorvastatin	Pharmaceutical	/	/	/	/
Azaperol	Pharmaceutical	/	/	/	/
Azaperone	Pharmaceutical	833	1340	9743	E
Azithromycin	Pharmaceutical	1874	3070	1970	E
Bezafibrate	Pharmaceutical	18000	30000	6000	ECOTOX
Carazolol	Pharmaceutical	2660	60000	2500	(Sanderson, Johnson et al. 2003)
Carbamazepine	Pharmaceutical	85000	76300	35400	ECOTOX
Cefalexin	Pharmaceutical	/	/	/	/
Cimetidine	Pharmaceutical	787	379000	80402	E
Ciprofloxacin	Pharmaceutical	2970	60000	100000	
Citalopram	Pharmaceutical	360	652	4467	E
Clarithromycin	Pharmaceutical	46	3307	17364	
Clopidogrel	Pharmaceutical	/	/	/	/
Codeine	Pharmaceutical	1800	23000	16000	(Sanderson, Johnson et al. 2003)
Desloratidine	Pharmaceutical	26981	49307	75054	E
Dexamethasone	Pharmaceutical	983	21438	23910	E
Diazepam	Pharmaceutical	1249	3129	19307	E
Diclofenac	Pharmaceutical	14500	22000	532000	(Grung, Källqvist et al. 2008)
Diltiazem	Pharmaceutical	/	/	/	/
Dimetridazole	Pharmaceutical	350	4272	25695	E
Enalapril	Pharmaceutical	18695	46266	276429	E
Enalaprilat	Pharmaceutical	2523000	3690000	73000000	(Sanderson, Johnson et al. 2003)
Erithromycin	Pharmaceutical	20	30500	61500	VSDB
Famotidine	Pharmaceutical	478143	314690	3594432	E
Fluoxetine	Pharmaceutical	800	510	1700	E
Fluvastatin	Pharmaceutical	1350	5268	287	E
Furosemide	Pharmaceutical	19797	560033	521136	E
Gemfibrozil	Pharmaceutical	4000	4900	900	ECOTOX
Glibenclamide	Pharmaceutical	/	/	/	/
Hidrochlorothiazide	Pharmaceutical	/	/	/	/
Hydrocodone	Pharmaceutical	4239	5449	44844	E

Ibuprofen	Pharmaceutical	4000	34000	5000	ECOTOX
Indomethacine	Pharmaceutical	18000	26000	3900	(Sanderson, Johnson et al. 2003)
Iopromide	Pharmaceutical	370000000	7660000000	8650000000	(Sanderson, Johnson et al. 2003)
Irbesartan	Pharmaceutical	/	/	/	/
Ketoprofen	Pharmaceutical	164000	248000	32000	(Sanderson, Johnson et al. 2003)
Levamisol	Pharmaceutical	943	1394	175000	E
Loratidine	Pharmaceutical	62	100	115	E
Lorazepam	Pharmaceutical	1683	44712	49067	E
Losartan	Pharmaceutical	180	2100	2151	E
Meloxicam	Pharmaceutical	184	3994	1392	E
Metformin	Pharmaceutical	/	/	/	/
Metoprolol	Pharmaceutical	8305	9383	81557	E
Metronidazole	Pharmaceutical	40400	1000000	1060000	VSDB
Metronidazole-Oh	Pharmaceutical	/	/	/	/
Nadolol	Pharmaceutical	22538	22609	208809	E
Naproxen	Pharmaceutical	137944	121543	193337	E
Norfluoxetine	Pharmaceutical	/	/	/	/
Ofloxacin	Pharmaceutical	2444544	31750	19352000	E
Olanzapine	Pharmaceutical	52515	46786	458553	E
Oxycodone	Pharmaceutical	/	/	/	/
Paroxetine	Pharmaceutical	/	/	/	/
Phenazone	Pharmaceutical	1100	6700	3000	(Sanderson, Johnson et al. 2003)
Piroxicam	Pharmaceutical	289	768	4220	E
Pravastatin	Pharmaceutical	85494	8588	1800	E
Propanolol	Pharmaceutical	/	/	/	/
Propyphenazone	Pharmaceutical	1000	3500	9800	(Sanderson, Johnson et al. 2003)
Ranitidine	Pharmaceutical	66000	63000	1076000	(Sanderson, Johnson et al. 2003)
Ronidazole	Pharmaceutical	1080	19445	242023	E
Salbutamol	Pharmaceutical	/	/	/	/
Sertraline	Pharmaceutical	43	120	408	ECOTOX
Sotalol	Pharmaceutical	/	/	/	/
Sulfamethoxazole	Pharmaceutical	1900	25200	56200	(Grung, Källqvist et al. 2008)
Tamsulosin	Pharmaceutical	/	/	/	/
Tenoxicam	Pharmaceutical	/	/	/	/
Tetracycline	Pharmaceutical	6000	6000	220000	(Grung, Källqvist et al. 2008)
Torasemide	Pharmaceutical	/	/	/	/
Trazodone	Pharmaceutical	396	1567	1313	E
Trimethoprim	Pharmaceutical	16000	121000	795000	ECOTOX
Valsartan	Pharmaceutical	3865	44337	88094	E
Venlafaxine	Pharmaceutical	635	1062	7678	E

Warfarin	Pharmaceutical	/	/	/	/
Xylazine	Pharmaceutical	/	/	/	/
Estradiol 17-glucuronide	Hormone	/	/	/	/
Estriol	Hormone	22250	5235	12110	E
Estriol 16-glucuronide	Hormone	/	/	/	/
Estriol 3-sulfate	Hormone	/	/	/	/
Estrone	Hormone	8740	2184	3834	E
Estradiol	Hormone	4299	1129	1578	E
Estrone 3-glucuronide	Hormone	/	/	/	/
Estrone 3-sulfate	Hormone	/	/	/	/
Ethinyl estradiol	Hormone	2000	2500	1610	
Diethylstilbestrol	Hormone	330	180	97	(Sanderson, Johnson et al. 2003)
Caffeine	Stimulans	760	46000	46000	E
Cocaine	Ilicit drug	5482	5482	45092	
Benzoylecgonine	Ilicit drug	12041000	6805000	89593000	E
LSD	Ilicit drug	/	/	/	/
Cannabidiol	Ilicit drug	/	/	/	/
Ephedrine	Ilicit drug	26591	23805	232000	E
Methamphetamine	Ilicit drug	1967	2509	20511	E
Lorazepam	Ilicit drug	1683	44712	49008	E
Morphine	Ilicit drug	43555	32000	257000	E
3-Hydroxycarbofuran	Pesticide	16932	209	15680	E
Acethochlor	Pesticide	0,27	8600	360	PPDB
Alachlor	Pesticide	6	7700	6600	ECOTOX
Atrazine	Pesticide	9,5	35000	4500	ECOTOX
Azinphos ethyl	Pesticide	372	0,2	80	
Azinphos methyl	Pesticide	7150	1,1	20000	E
Burpofezin	Pesticide	330	420	2100	PPDB
CARBENDAZIM	Pesticide	/	/	/	/
Carbofuran	Pesticide	6500	9,4	180	PPDB
Chlorfenvinphos	Pesticide	1360	0,25	1100	PPDB
Chlorpyriphos	Pesticide	480	0,1	1,3	PPDB
Deisopropylatrazine	Pesticide	198	1348	38130	E
Desethylatrazine	Pesticide	2803	1259	68923	E
Diazinon	Pesticide	6400	1	3300	PPDB
Diclofenthion	Pesticide	420	1,1	1,25	PPDB
Dimetoate	Pesticide	30200	560	90400	PPDB
Diuron	Pesticide	2,4	270	6700	ECOTOX
Ethion	Pesticide	326	0,056	500	PPDB
Fenitrothion	Pesticide	1300	8,6	1300	PPDB
Fenoxon	Pesticide	1790	5,7	800	PPDB

Fenthion	Pesticide	/	/	/	/
Fenthion Sulfone	Pesticide	/	/	/	/
Fenthion sulfoxide	Pesticide	/	/	/	/
Hexythiazox	Pesticide	400	470	3200	PPDB
Imazalil	Pesticide	1480	3100	870	PPDB
Imidacloprid	Pesticide	10000	85000	211000	PPDB
Isoproturon	Pesticide	13	580	18000	PPDB
Malathion	Pesticide	13000	0,7	18	PPDB
Methiocarb	Pesticide	2200	8	650	PPDB
Metoalachlor	Pesticide	57100	23500	3900	PPDB
Molinate	Pesticide	500	14900	16000	PPDB
Ometoate	Pesticide	167500	22	9100	PPDB
Parathion-ethyl	Pesticide	500	2,5	1500	PPDB
Parathion-methyl	Pesticide	3000	7,3	2700	PPDB
Prochloraz	Pesticide	5,5	4300	1500	PPDB
Propanil	Pesticide	110	2390	5400	PPDB
Propazine	Pesticide	180	17700	17500	PPDB
Pyriproxyphen	Pesticide	150	400	270	PPDB
Simazine	Pesticide	40	1100	90000	PPDB
Tebuconazole	Pesticide	/	/	/	/
Terbumeton	Pesticide	/	/	/	/
Terbumeton-Desethyl	Pesticide	/	/	/	/
Terbutilazine	Pesticide	/	/	/	/
Terbutilazine-2 Hidroxy	Pesticide	/	/	/	/
Terbutryn	Pesticide	2,4	2060	1100	PPDB
TERBUTYLAZINE DEETHYL	Pesticide	/	/	/	/
THIABENDAZOLE	Pesticide	9000	810	550	PPDB
Tolclophos-methyl	Pesticide	780	/	690	PPDB
1H-Benzotriazole	Industial organic	5904	66766	28321	E
Tolytriazol	Industrial organic	3851	36053	16386	E
Nonylphenol monoethoxylate	Industrial organic	12200	12200	40000	PPDB
Octylphenol	Industrial organic	210	11	7200	PPDB
Octylphenol diethoxylate	Industrial organic	/	/	/	/
Octylphenol monocarboxylate	Industrial organic	/	/	/	/
Octylphenol monoethoxylate	Industrial organic	/	/	/	/
Tris(2-chloroethyl) phosphate	Industrial organic	38000	135300	90000	E
Tris(butoxyethyl) phosphate	Industrial organic	/	/	/	/
Tris(chloroisopropyl) phosphate	Industrial organic	47000	21315	31000	E
Bisphenol A (BPA)	Industrial organic	2700	7750	1284	(Sanderson, Johnson et al. 2003)
Nonylphenol (NP)	Industrial organic	197	140	170	ECOTOX

Nonylphenol diethoxylate	Industrial organic	555	211	274	E
Nonylphenol monocarboxylate	Industrial organic	2250	707	876	E
L-PFOS	Perflourinated compound	23640	37360	3640	
PFBA	Perflourinated compound	262150	177620	273920	
PFOA	Perflourinated compound	748098	207000	260820	
PFNA	Perflourinated compound	481632	92800	120640	
PFDA	Perflourinated compound	437414	77100	35980	
PFUdA	Perflourinated compound	318660	56400	33840	
PFDoA	Perflourinated compound	241916	73680	36840	
L-PFBS	Perflourinated compound	645000	1938000	502000	
L-PFDS	Perflourinated compound	/	4800	/	/
i,p-PFNA	Perflourinated compound	/	/	/	/
I,pPFNS	Perflourinated compound	/	/	/	/
L-PFHpS	Perflourinated compound	/	/	/	/
L-PFHxS	Perflourinated compound	/	/	/	/
PFHpA	Perflourinated compound	/	/	/	/
PFHxA	Perflourinated compound	/	/	/	/
PFHxDA	Perflourinated compound	/	/	/	/
PFODA	Perflourinated compound	/	/	/	/
PFOSA	Perflourinated compound	/	/	/	/
PFPeA	Perflourinated compound	/	/	/	/
PFTeDA	Perflourinated compound	/	/	/	/
PFTrDA	Perflourinated compound	/	/	/	/
4-Methylbenzylidene camphor	Personal care product	/	9900	560	(Fent, Kunz et al. 2010)
Benzophenone-3	Personal care product	/	1900	290	(Fent, Kunz et al. 2010)
Ethylhexyl methoxycinnamate	Personal care product	/	9870	620	(Fent, Kunz et al. 2010)
Octocrylene	Personal care product	/	/	/	/
2,2'-Dihydroxy-4-methoxybenzophenone	Personal care product	/	/	/	/
4,4'-Dihidroxybenzophenone	Personal care product	/	/	/	/
4-Hydroxybenzophenone	Personal care product	/	/	/	/
Benzophenone-1	Personal care product	/	/	/	/
Benzophenone-2	Personal care product	/	/	/	/
Ethyl 4-aminobenzoate	Personal care product	/	/	/	/

Ethylhexyl dimethyl PABA	Personal care product	/	/	/	/
Ethylparaben	Personal care product	20172	18700	34300	(Brausch and Rand 2011)
Methylparaben	Personal care product	18092	4600	20432	(Brausch and Rand 2011)
Benzylparaben	Personal care product	1735	4000	2300	(Brausch and Rand 2011)
Propylparaben	Personal care product	4407	2627	5643	(Brausch and Rand 2011)
Triclorocaraban	Personal care product	20	10	120	(Brausch and Rand 2011)
Triclosan	Personal care product	0,53	390	270	(Brausch and Rand 2011)

E-ECOSAR, VSDB-veterinary substances database, PPDB-pesticides properties database VSDB-VSDB: Veterinary Substances DataBase http://sitem.herts.ac.uk/aeru/vsdb/index.htm

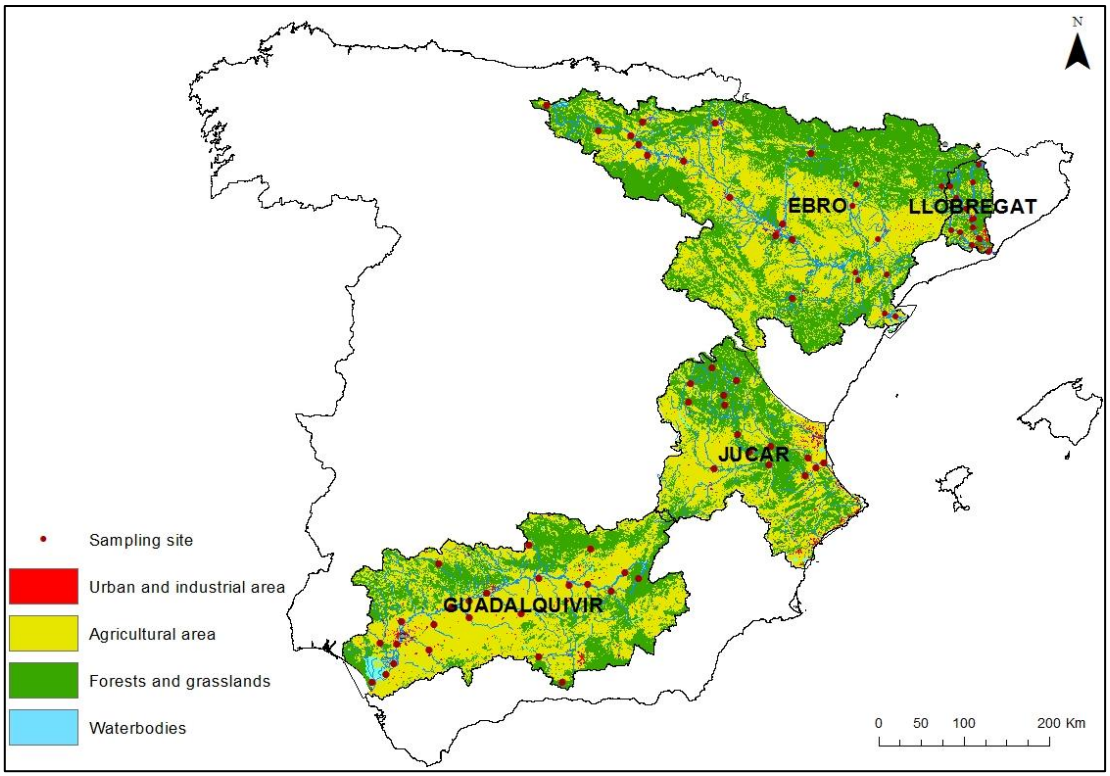

Figure AIII1. Map of study area with major types of landuse. Sampling sites are marked with red dots.

ANNEX IV

SUPPLEMENTARY MATERIAL TO ECOTOXICOLOGICAL RISK ASSESSMENT OF CHEMICAL POLLUTION IN FOUR IBERIAN RIVER BASINS AND ITS RELATIONSHIP WITH THE AQUATIC MACROINVERTEBRATE COMMUNITY STATUS

Table AIV.1: Analytical methods used for chemical measurements

Group of compounds	Method	Reference
Pesticides	Solid phase extraction(SPE) Liquid chromatography-tandem mass spectrometry(LC-MS/MS)	(Masiá, Ibáñez et al. 2013)
Pharmaceuticals	Solid phase extraction(SPE) Multi-residue analytical method based on LC-MS/MS	(Osorio, Proia et al. 2014)
Perflourinated compounds	Solid phase extraction(SPE) Liquid chromatography tandem mass spectrometry(LC-MS/MS)	(Masiá, Ibáñez et al. 2013)
Industrial organics	Dual column liquid chromatography / coupled to mass spectrometry (LC-LC-MS/MS	(Gorga, Petrovic et al. 2013, Gorga, Insa et al. 2015)
Ilicit drugs	Solid phase extraction(SPE) Multi-residue analytical method based on LC-MS/MS	(Mastroianni, Postigo et al. 2013)
UV filters	Solid phase extraction(SPE) Liquid chromatography-tandem mass spectrometry(LC-MS/MS)	(Gago-Ferrero, Mastroianni et al. 2013)

Table AIV.2: List of measured compounds

Compound	Compound class	Frequency of detection 2010	Frequency of detection 2011	Limit of detection (ng/L)	Reference
Acetaminophen	Pharmaceutical	36	41	0.04	SCARCE DB
Acridone	Pharmaceutical	56	71	0.03	SCARCE DB
Albendazol	Pharmaceutical	14	23	0.01	SCARCE DB
Alprazolam	Pharmaceutical	6	62	0.02	SCARCE DB
Amlodipine	Pharmaceutical	86	26	0.08	SCARCE DB
Atenolol	Pharmaceutical	64	69	0.02	SCARCE DB
Atorvastatin	Pharmaceutical	52	49	0.005	SCARCE DB
Azaperol	Pharmaceutical	0	0	0.32	SCARCE DB
Azaperone	Pharmaceutical	0	0	0.23	SCARCE DB
Azithromycin	Pharmaceutical	95	81	0.1	SCARCE DB
Bezafibrate	Pharmaceutical	34	50	0.02	SCARCE DB
Carazolol	Pharmaceutical	34	13	0.10	SCARCE DB
Carbamazepine	Pharmaceutical	44	64	0.01	SCARCE DB
Cefalexin	Pharmaceutical	4	3	0.2	SCARCE DB
Cimetidine	Pharmaceutical	49	8	0.1	SCARCE DB
Ciprofloxacin	Pharmaceutical	17	34	0.1	SCARCE DB
Citalopram	Pharmaceutical	45	66	0.02	SCARCE DB

Clarithromycin	Pharmaceutical	14	20	0.1	SCARCE DB
Clopidogrel	Pharmaceutical	66	71	0.01	SCARCE DB
Codeine	Pharmaceutical	70	69	0.02	SCARCE DB
Desloratidine	Pharmaceutical	24	16	0.04	SCARCE DB
Dexamethasone	Pharmaceutical	66	27	0.05	SCARCE DB
Diazepam	Pharmaceutical	18	61	0.05	SCARCE DB
Diclofenac	Pharmaceutical	41	63	0.6	SCARCE DB
Diltiazem	Pharmaceutical	74	48	0.02	SCARCE DB
Dimetridazole	Pharmaceutical	16	2	1.50	SCARCE DB
Enalapril	Pharmaceutical	13	2	0.47	SCARCE DB
Enalaprilat	Pharmaceutical	29	39	1.08	SCARCE DB
Erithromycin	Pharmaceutical	22	16	0.1	SCARCE DB
Famotidine	Pharmaceutical	0	4	0.1	SCARCE DB
Fluoxetine	Pharmaceutical	18	4	0.36	SCARCE DB
Fluvastatin	Pharmaceutical	17	15	0.03	SCARCE DB
Furosemide	Pharmaceutical	44	60	0.45	SCARCE DB
Gemfibrozil	Pharmaceutical	91	100	0.04	SCARCE DB
Glibenclamide	Pharmaceutical	3	1	0.60	SCARCE DB
Hidrochlorothiazide	Pharmaceutical	55	98	0.05	SCARCE DB
Hydrocodone	Pharmaceutical	3	18	0.6	SCARCE DB
Ibuprofen	Pharmaceutical	31	11	1.2	SCARCE DB
Indomethacine	Pharmaceutical	51	52	0.1	SCARCE DB
Iopromide	Pharmaceutical	61	32	0.18	SCARCE DB
Irbesartan	Pharmaceutical	64	81	0.02	SCARCE DB
Ketoprofen	Pharmaceutical	61	100	0.8	SCARCE DB
Levamisol	Pharmaceutical	70	59	0.01	SCARCE DB
Loratidine	Pharmaceutical	40	21	0.1	SCARCE DB
Lorazepam	Pharmaceutical	52	50	0.27	SCARCE DB
Losartan	Pharmaceutical	42	42	0.10	SCARCE DB
Meloxicam	Pharmaceutical	7	32	0.007	SCARCE DB
Metformin	Pharmaceutical	0	0	0.5	SCARCE DB
Metoprolol	Pharmaceutical	9	11	0.1	SCARCE DB
Metronidazole	Pharmaceutical	5	19	0.6	SCARCE DB
Metronidazole-Oh	Pharmaceutical	4	12	0.4	SCARCE DB
Nadolol	Pharmaceutical	12	3	0.06	SCARCE DB
Naproxen	Pharmaceutical	67	77	0.2	SCARCE DB
Norfluoxetine	Pharmaceutical	6	1	0.50	SCARCE DB
Ofloxacin	Pharmaceutical	14	6	0.04	SCARCE DB
Olanzapine	Pharmaceutical	4	5	0.04	SCARCE DB
Oxycodone	Pharmaceutical	40	43	0.1	SCARCE DB
Paroxetine	Pharmaceutical	58	35	0.16	SCARCE DB
Phenazone	Pharmaceutical	27	48	0.04	SCARCE DB
Piroxicam	Pharmaceutical	0	7	0.02	SCARCE DB

Pravastatin	Pharmaceutical	29	27	0.1	SCARCE DB
Propanolol	Pharmaceutical	18	25	0.04	SCARCE DB
Propyphenazone	Pharmaceutical	26	10	0.04	SCARCE DB
Ranitidine	Pharmaceutical	10	9	1.1	SCARCE DB
Ronidazole	Pharmaceutical	0	5	0.83	SCARCE DB
Salbutamol	Pharmaceutical	56	35	0.01	SCARCE DB
Sertraline	Pharmaceutical	3	5	0.63	SCARCE DB
Sotalol	Pharmaceutical	9	7	0.2	SCARCE DB
Sulfamethoxazole	Pharmaceutical	32	27	0.1	SCARCE DB
Tamsulosin	Pharmaceutical	29	5	0.02	SCARCE DB
Tenoxicam	Pharmaceutical	0	9	0.01	SCARCE DB
Tetracycline	Pharmaceutical	3	0	3.5	SCARCE DB
Torasemide	Pharmaceutical	34	48	0.02	SCARCE DB
Trazodone	Pharmaceutical	34	58	0.03	SCARCE DB
Trimethoprim	Pharmaceutical	27	91	0.1	SCARCE DB
Valsartan	Pharmaceutical	92	91	0.05	SCARCE DB
Venlafaxine	Pharmaceutical	49	79	0.02	SCARCE DB
Warfarin	Pharmaceutical	8	6	0.04	SCARCE DB
Xylazine	Pharmaceutical	4	9	0.03	SCARCE DB
Estradiol 17-glucuronide	Hormone	0	4	0.46	(Gorga, Petrovic et al. 2013)
Estriol	Hormone	3	4	0.17	(Gorga, Petrovic et al. 2013)
Estriol 16-glucuronide	Hormone	3	4	0.059	(Gorga, Petrovic et al. 2013)
Estriol 3-sulfate	Hormone	3	17	0.030	(Gorga, Petrovic et al. 2013)
Estrone	Hormone	64	56	0.050	(Gorga, Petrovic et al. 2013)
Estradiol	Hormone	86	8	0.037	(Gorga, Petrovic et al. 2013)
Estrone 3-glucuronide	Hormone	3	5	0.056	(Gorga, Petrovic et al. 2013)
Estrone 3-sulfate	Hormone	3	17	0.0038	(Gorga, Petrovic et al. 2013)
Ethinyl estradiol	Hormone	0	1	0.14	(Gorga, Petrovic et al. 2013)
Diethylstilbestrol	Hormone	1	1	0.043	(Gorga, Petrovic et al. 2013)
Caffeine	Stimulans	84	100	0.021	(Gorga, Petrovic et al. 2013)
Cocaine	Ilicit drug	63	96	0.02	SCARCE DB
Benzoylecgonine	Ilicit drug	81	94	0.02	SCARCE DB

LSD	Ilicit drug	0	0	0.32	SCARCE DB
Cannabidiol	Ilicit drug	0	0	2.27	SCARCE DB
Ephedrine	Ilicit drug	76	83	0.16	SCARCE DB
Methamphetamine	Ilicit drug	4	47	0.045	SCARCE DB
Lorazepam	Ilicit drug	12	34	1.01	SCARCE DB
Morphine	Ilicit drug	13	9	0.3	SCARCE DB
3-Hydroxycarbofuran	Pesticide	4	0	0.2	scarce db
Acethochlor	Pesticide	0	0	2	(Masiá, Ibáñez et al. 2013)
Alachlor	Pesticide	0	0	2	(Masiá, Ibáñez et al. 2013)
Atrazine	Pesticide	21	4	1.3	(Masiá, Ibáñez et al. 2013)
Azinphos ethyl	Pesticide	9	1	0.5	(Masiá, Ibáñez et al. 2013)
Azinphos methyl	Pesticide	4	1	0.5	(Masiá, Ibáñez et al. 2013)
Burpofezin	Pesticide	80	0	0.5	(Masiá, Ibáñez et al. 2013)
CARBENDAZIM	Pesticide	0	41	0.01	SCARCE DB
Carbofuran	Pesticide	21	3	0.2	(Masiá, Ibáñez et al. 2013)
Chlorfenvinphos	Pesticide	66	18	0.2	(Masiá, Ibáñez et al. 2013)
Chlorpyriphos	Pesticide	99	49	0.2	(Masiá, Ibáñez et al. 2013)
Deisopropylatrazine	Pesticide	28	1	2	(Masiá, Ibáñez et al. 2013)
Desethylatrazine	Pesticide	21	4	2	(Masiá, Ibáñez et al. 2013)
Diazinon	Pesticide	95	43	0.04	(Masiá, Ibáñez et al. 2013)
Diclofenthion	Pesticide	45	0	0.5	(Masiá, Ibáñez et al. 2013)
Dimetoate	Pesticide	28	0	1	(Masiá, Ibáñez et al. 2013)
Diuron	Pesticide	29	17	1	(Masiá, Ibáñez et al. 2013)
Ethion	Pesticide	8	22	0.5	(Masiá, Ibáñez et al. 2013)
Fenitrothion	Pesticide	1	1	2	(Masiá, Ibáñez et al. 2013)
Fenoxon	Pesticide	1	0	0.2	(Masiá,

Fenthion	Pesticide	1	0	0.2	Ibáñez et al. 2013) (Masiá, Ibáñez et al. 2013)
Fenthion Sulfone	Pesticide	3	1	0.2	(Masiá, Ibáñez et al. 2013)
Fenthion sulfoxide	Pesticide	1	0	0.2	(Masiá, Ibáñez et al. 2013)
Hexythiazox	Pesticide	78	11	0.2	(Masiá, Ibáñez et al. 2013)
Imazalil	Pesticide	62	33	0.3	(Masiá, Ibáñez et al. 2013)
Imidacloprid	Pesticide	53	30	0.04	(Masiá, Ibáñez et al. 2013)
Isoproturon	Pesticide	16	8	0.3	(Masiá, Ibáñez et al. 2013)
Malathion	Pesticide	14	1	0.3	(Masiá, Ibáñez et al. 2013)
Methiocarb	Pesticide	4	8	0.3	(Masiá, Ibáñez et al. 2013)
Metoalachlor	Pesticide	5	12	0.3	(Masiá, Ibáñez et al. 2013)
Molinate	Pesticide	1	0	0.5	(Masiá, Ibáñez et al. 2013)
Ometoate	Pesticide	4	1	0.3	(Masiá, Ibáñez et al. 2013)
Parathion-ethyl	Pesticide	12	0	2	(Masiá, Ibáñez et al. 2013)
Parathion-methyl	Pesticide	0	0	2	(Masiá, Ibáñez et al. 2013)
Prochloraz	Pesticide	42	5	0.8	(Masiá, Ibáñez et al. 2013)
Propanil	Pesticide	0	0	0.3	(Masiá, Ibáñez et al. 2013)
Propazine	Pesticide	8	0	0.3	(Masiá, Ibáñez et al. 2013)
Pyriproxyphen	Pesticide	62	1	0.5	(Masiá, Ibáñez et al. 2013)
Simazine	Pesticide	4	8	2	(Masiá, Ibáñez et al. 2013)
Tebuconazole	Pesticide	/	13	0.13	SCARCE DB
Terbumeton	Pesticide	/	4	0.01	SCARCE DB
Terbumeton-Desethyl	Pesticide	/	14	0.13	SCARCE DB
Terbutilazine	Pesticide	/	22	0.4	SCARCE DB
Terbutilazine-2	Pesticide	/	29	0.01	SCARCE DB

Hidroxy Terbutryn	Pesticide	8	20	0.5	SCARCE DB
Terbutylazine Deethyl	Pesticide	/	29	0.4	SCARCE DB
Thiabendazole	Pesticide	/	14	0.02	SCARCE DB
Tolclophos-methyl	Pesticide	14	1	0.5	SCARCE DB
1H-Benzotriazole	Industial organic	73	90	0.072	(Gorga, Petrovic et al. 2013)
Tolytriazol	Industrial organic	99	84	0.013	(Gorga, Petrovic et al. 2013)
Nonylphenol monoethoxylate	Industrial organic	0	0	62	(Gorga, Petrovic et al. 2013)
Octylphenol	Industrial organic	96	32	0.14	(Gorga, Petrovic et al. 2013)
Octylphenol diethoxylate	Industrial organic	96	73	0.011	(Gorga, Petrovic et al. 2013)
Octylphenol monocarboxylate	Industrial organic	0	1	0.065	(Gorga, Petrovic et al. 2013)
Octylphenol monoethoxylate	Industrial organic	0	0	17	(Gorga, Petrovic et al. 2013)
Tris(2-chloroethyl) phosphate	Industrial organi	100	97	0.034	(Gorga, Petrovic et al. 2013)
Tris(butoxyethyl) phosphate	Industrial organic	100	88	0.0024	(Gorga, Petrovic et al. 2013)
Tris(chloroisopropyl) phosphate	Industrial organic	100	100	0.0025	(Gorga, Petrovic et al. 2013)
Bisphenol A (BPA)	Industrial organic	68	88	0.11	(Gorga, Petrovic et al. 2013)
Nonylphenol (NP)	Industrial organic	91	42	0.013	(Gorga, Petrovic et al. 2013)
Nonylphenol diethoxylate	Industrial organic	94	96	0.013	(Gorga, Petrovic et al. 2013)
Nonylphenol monocarboxylate	Industrial organic	94	70	0.034	(Gorga, Petrovic et al. 2013)
L-PFOS	Perflourinated compound	26	77	0.004	SCARCE DB
PFBA	Perflourinated compound	77	52	0.04	SCARCE DB
PFOA	Perflourinated compound	52	43	0.04	SCARCE DB
PFNA	Perflourinated compound	14	18	0.4	SCARCE DB
PFDA	Perflourinated compound	13	40	0.04	SCARCE DB
PFUdA	Perflourinated compound	3	9	0.04	SCARCE DB
PFDoA	Perflourinated compound	0	13	0.8	SCARCE DB
L-PFBS	Perflourinated compound	4	52	0.02	SCARCE DB
L-PFDS	Perflourinated compound	0	14	0.004	SCARCE DB
i,p-PFNA	Perflourinated compound	14	19	0.4	SCARCE DB
I,pPFNS	Perflourinated compound	0	13	0.04	SCARCE DB

L-PFHpS	Perflourinated compound	0	3	0.04	SCARCE DB
L-PFHxS	Perflourinated compound	17	27	0.04	SCARCE DB
PFHpA	Perflourinated compound	25	5	0.4	SCARCE DB
PFHxA	Perflourinated compound	13	5	0.4	SCARCE DB
PFHxDA	Perflourinated compound	1	5	0.04	SCARCE DB
PFODA	Perflourinated compound	0	13	0.8	SCARCE DB
PFOSA	Perflourinated compound	0	0	0.2	SCARCE DB
PFPeA	Perflourinated compound	34	48	0.04	SCARCE DB
PFTeDA	Perflourinated compound	4	10	0.02	SCARCE DB
PFTrDA	Perflourinated compound	3	10	0.02	SCARCE DB
4-Methylbenzylidene camphor	Personal care product	18	48	3.5	(Gago-Ferrero, Mastroianni et al. 2013)
Benzophenone-3	Personal care product	14	43	0.7	(Gago-Ferrero, Mastroianni et al. 2013)
Ethylhexyl methoxycinnamate	Personal care product	9	14	0.72	SCARCE DB
Octocrylene	Personal care product	9	0	3	SCARCE DB
2,2'-Dihydroxy-4-methoxybenzophenone	Personal care product	0	0	1	(Gago-Ferrero, Mastroianni et al. 2013)
4,4'-Dihidroxybenzophenone	Personal care product	4	1	1.8	(Gago-Ferrero, Mastroianni et al. 2013)
4-Hydroxybenzophenone	Personal care product	4	5	1.1	(Gago-Ferrero, Mastroianni et al. 2013)
Benzophenone-1	Personal care product	0	22	1	(Gago-Ferrero, Mastroianni et al. 2013)
Benzophenone-2	Personal care product	16	0	1.2	(Gago-Ferrero, Mastroianni et al. 2013)
Ethyl 4-aminobenzoate	Personal care product	0	0	1.5	(Gago-Ferrero, Mastroianni et al. 2013)
Ethylhexyl dimethyl	Personal care product	0	14	0.1	SCARCE DB
Ethylparaben	Personal care product	74	53	0.27	(Gorga, Petrovic et al. 2013)
Methylparaben	Personal care product	90	75	0.20	(Gorga, Petrovic et

					al. 2013)
Benzylparaben	Personal care product	30	40	0.031	(Gorga, Petrovic et al. 2013)
Propylparaben	Personal care product	99	94	0.021	(Gorga, Petrovic et al. 2013)
Triclorocaraban	Personal care product	0	7	0.036	(Gorga, Petrovic et al. 2013)
Triclosan	Personal care product	23	8	0.17	(Gorga, Petrovic et al. 2013)
Iron	Metals	100	100		SCARCE DB
Copper	Metals	74	95		SCARCE DB
Manganese	Metals	100	100		SCARCE DB
Nickel	Metals	95	100		SCARCE DB
Cobalt	Metals	47	100		SCARCE DB
Zinc	Metals	68	100		SCARCE DB
Lead	Metals	1	100		SCARCE DB
Arsenic	Metals	1	100		SCARCE DB

In red- the WFD priority pollutants, in blue- compounds on the Watch list of substances for Union-wide monitoring as set out in Article 8b of Directive 2008/105/EC

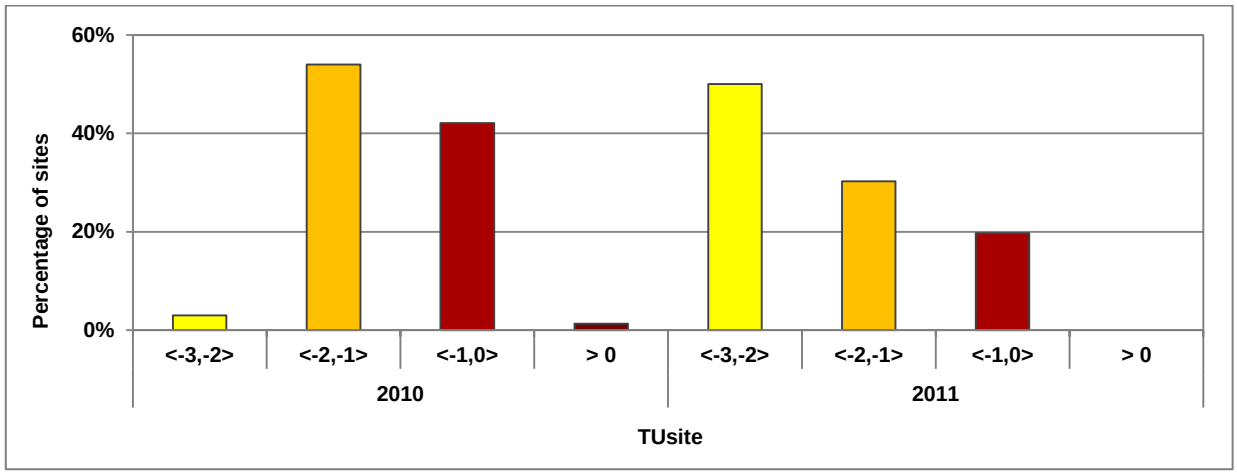

Figure AIV.1 Percentage of sites of all four river basins with TUsite (the most sensitive test species) belonging to one of four toxic unit ranges in 2010 and 2011. In red color are the toxic units associated with the acute effects (TU≥-1).

Table AIV.3: Regulation status of pesticides used in this study
http://ec.europa.eu/sanco_pesticides/public

Compound	Type	Status under Reg. (EC) No 1107/2009	**Legislation**
3-Hydroxycarbofuran	Metabolite	-	-
Acethochlor	Herbicide	-	-
Alachlor	Herbicide	Not approved	**WFD** 06/966/EC
Atrazine	Herbicide	Not approved	**WFD** 06/966/EC
Azinphos ethyl	Insecticide	Not approved	95/276/EC
Azinphos methyl	Insecticide	Not approved	Reg. 1335/2005
Buprofezin	Insecticide	**Approved**	2011/6/EUReg. (EU) No 540/2011 (2008/771/EC)
Carbendazim	Insecticide	Not approved	Reg. (EU) No 540/2011Reg. (EU) No 542/2011 (2006/135/EC,2010/70/EC,2011/58/EU)
Carbofuran	Insecticide	Not approved	2007/416
Chlorfenvinphos	Insecticide	Not approved	**WFD** 2002/2076
Chlorpyriphos	Insecticide	**Approved**	**WFD** 05/72/EC Reg. (EU) No 540/2011Reg. (EU) No 762/2013
Deisopropylatrazine	Metabolite	-	-
Desethylatrazine	Metabolite	-	-
Diazinon	Insecticide	Not approved	2007/393
Diclofenthion	Insecticide/ Nematicide	Not approved	2002/2076
Dimethoate	Insecticide	**Approved**	07/25/EC Reg. (EU) No 540/2011
Diuron	Herbicide	**Approved**	**WFD** 08/91/EC Reg. (EU) No 540/2011
Ethion	Insecticide	Not approved	2002/2076
Fenitrothion	Insecticide	Not approved	2007/379
Fenoxon	Metabolite	-	-
Fenthion	Insecticide	Not approved	04/140/EC
Fenthion Sulfone	Metabolite	-	-
Fenthion sulfoxide	Metabolite	-	-
Hexythiazox	Insecticide	**Approved**	2011/46/EU Reg.(EU) No 540/2011
Imazalil	Fungicide	**Approved**	Reg. (EU) No 705/2011 (1997/73/EC, 2007/21/EC, 2010/57/EU Reg. (EU) No 540/2011)
Imidacloprid	Insecticid	**Approved**	Reg. (EU) No 485/2013Reg. (EU) No 540/2011

	e		(2008/116/EC,2010/21/EU)
Isoproturon	Herbicide	**Approved**	**WFD** 02/18/EC Reg. (EU) No 540/2011
Malathion	Insecticide	**Approved**	2010/17/EU Reg. (EU) No 540/2011
Methiocarb	Insecticide	**Approved**	07/5/EC Reg. (EU) No 187/2014Reg. (EU) No 540/2011
Metolachlor	Herbicide	Not approved	2002/2076
Molinate	Herbicide	Not approved	03/81/EC Reg. (EU) No 540/2011
Ometoate	Insecticide	Not approved	2002/2076
Parathion-ethyl	Insecticide	Not approved	03/166/EC
Parathion-methyl	Insecticide	Not approved	03/166/EC
Prochloraz	Fungicide	**Approved**	Reg. (EU) No 1143/2011 (2008/934)
Propanil	Herbicide	Not approved	Reg. (EU) No 1078/2011 (2008/769)
Propazine	Herbicide	Not approved	2002/2076
Pyriproxyphen	Insecticide	**Approved**	2010/39/EU Reg. (EU) No 540/2011
Simazine	Herbicide	Not approved	**WFD** 04/247/EC
Terbuconazole	Fungicide	**Approved**	2008/125Reg. (EU) No 540/2011Reg. (EU) No 921/2014

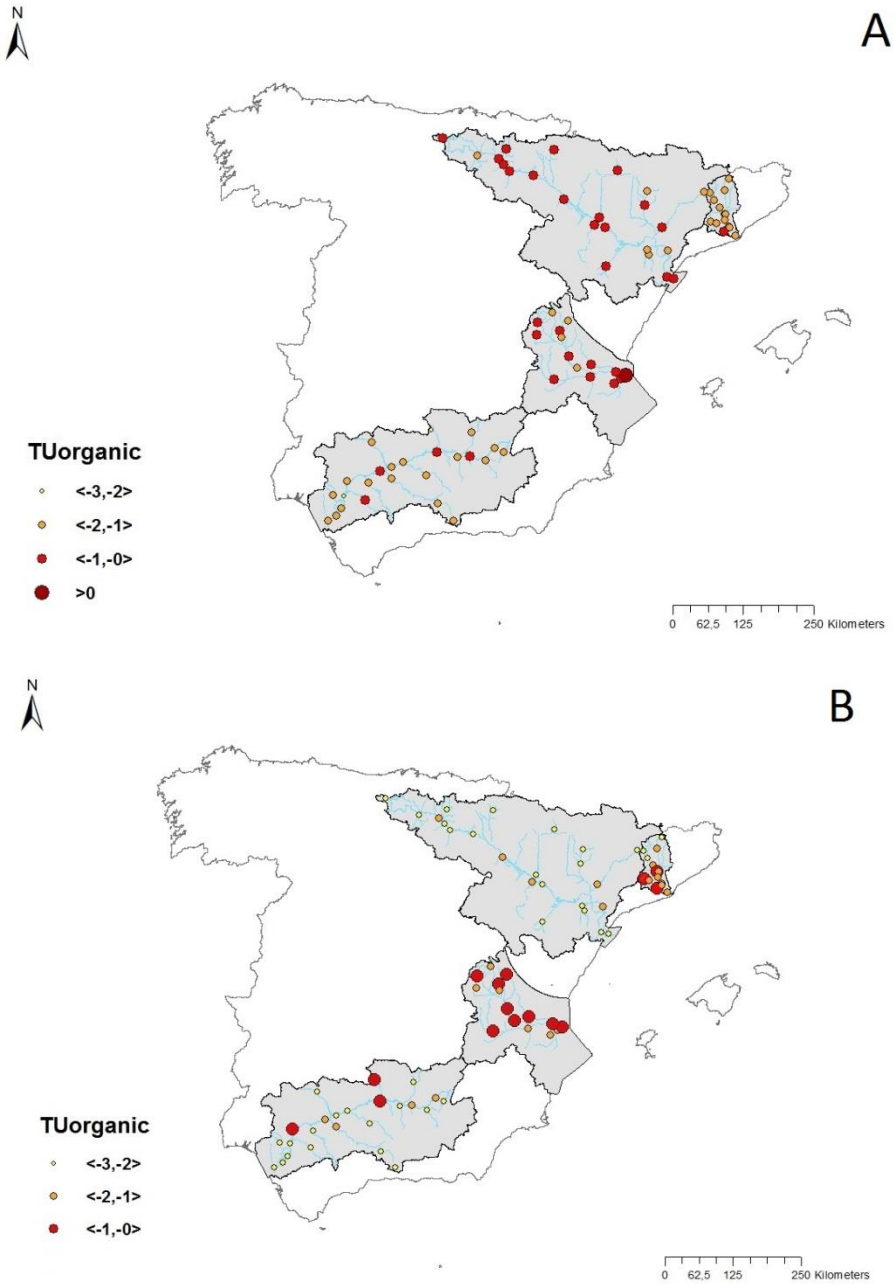

Figure AIV.2 Sites specific risk in 2010 (a) and 2011(b) - Invertebrates

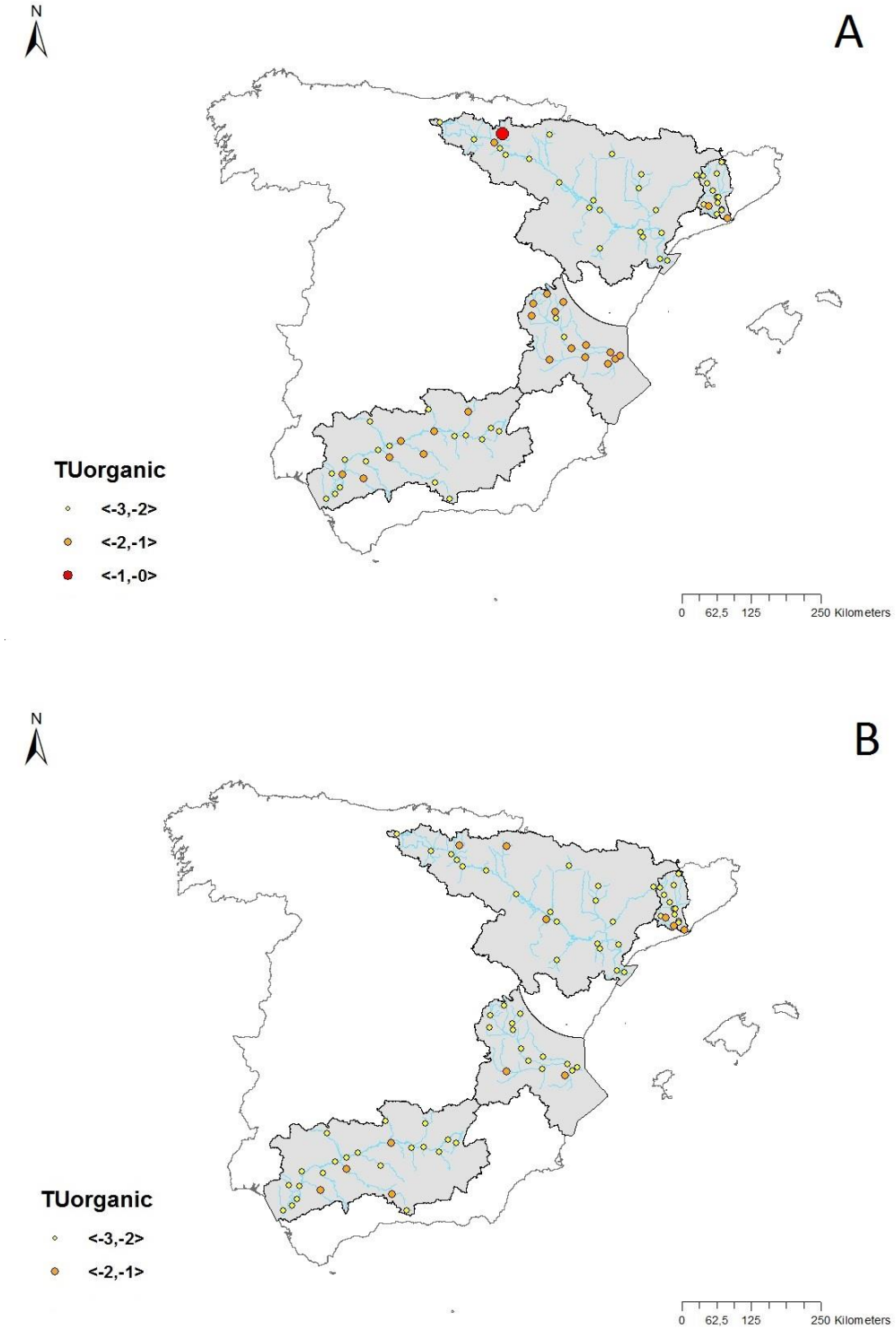

Figure AIV.3 Sites specific risk in 2010 (a) and 2011(b) - Algae

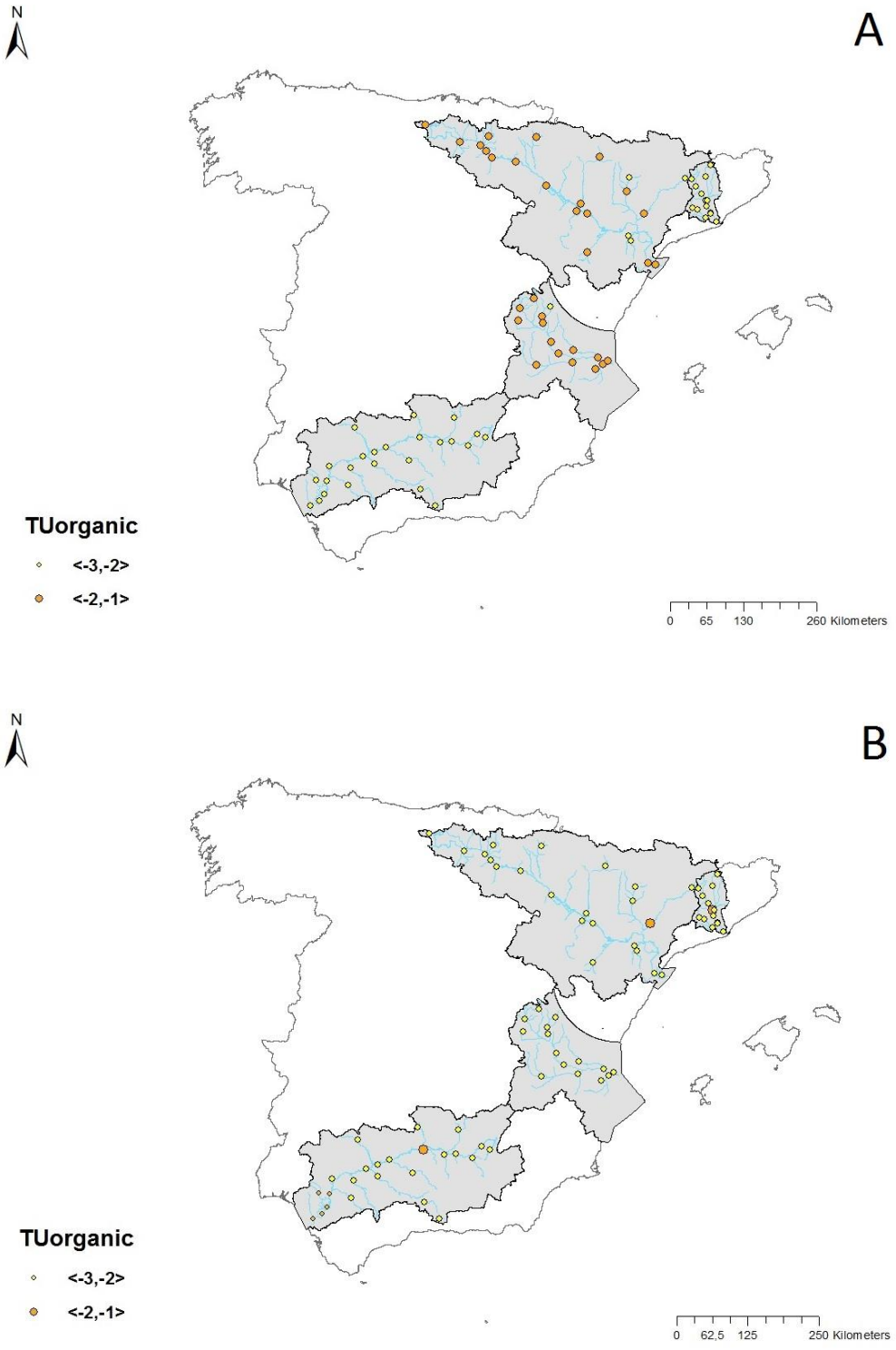

Figure AIV.4 Sites specific risk in 2010 (a) and 2011(b) - Fish

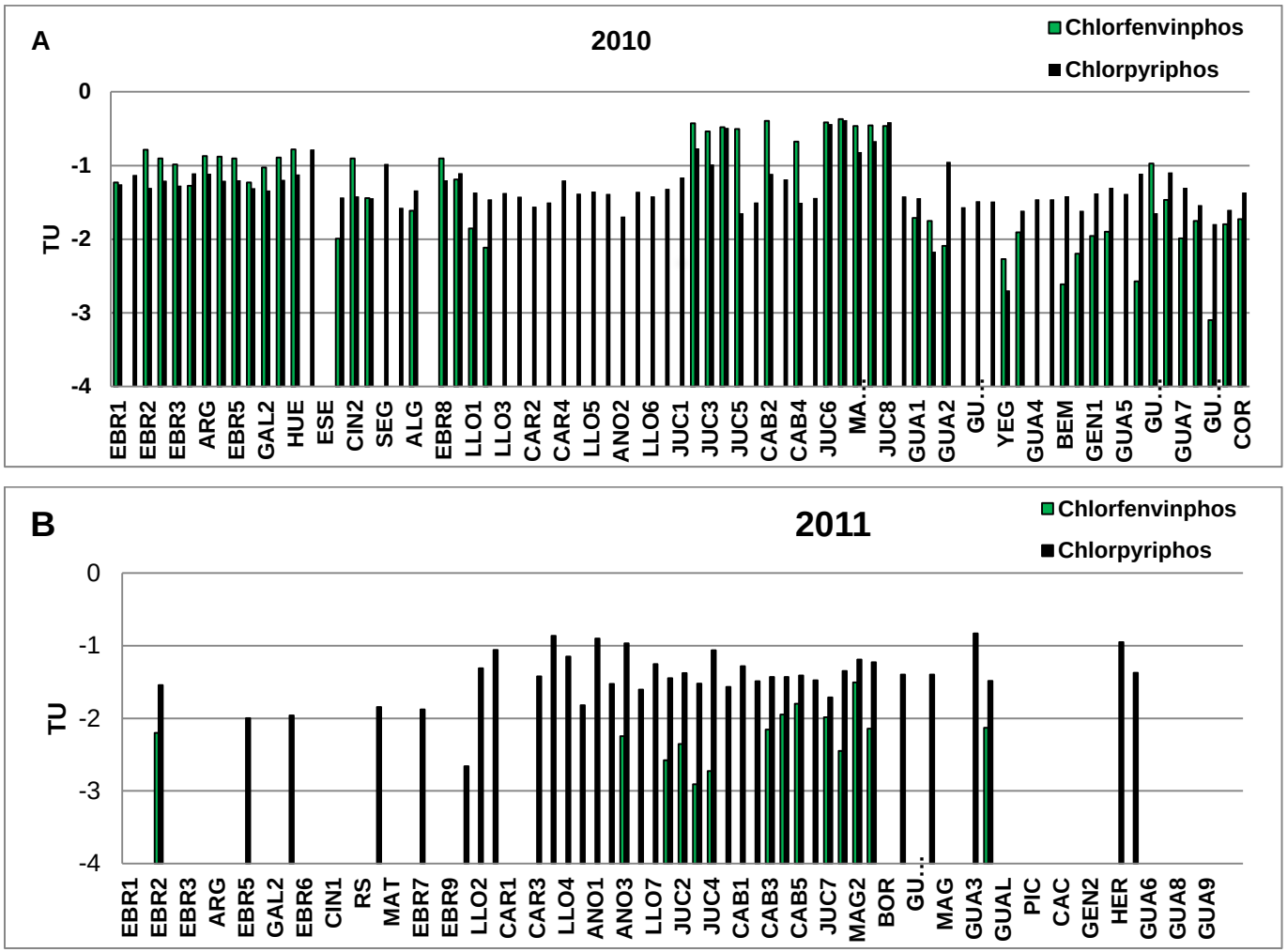

Figure AIV.5. Toxic units of insecticides chlorfenvinphos and chlorpyripos in A) 2010 and B) 2011

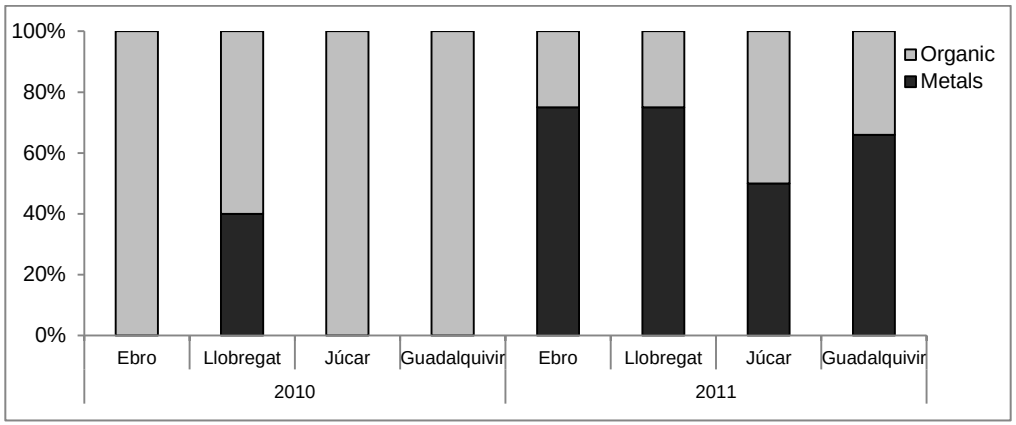

Figure AIV.6. Proportion of sites with risk dominated by metals (black) and organics (grey) in 2010 and 2011 in each of four studied river basis.

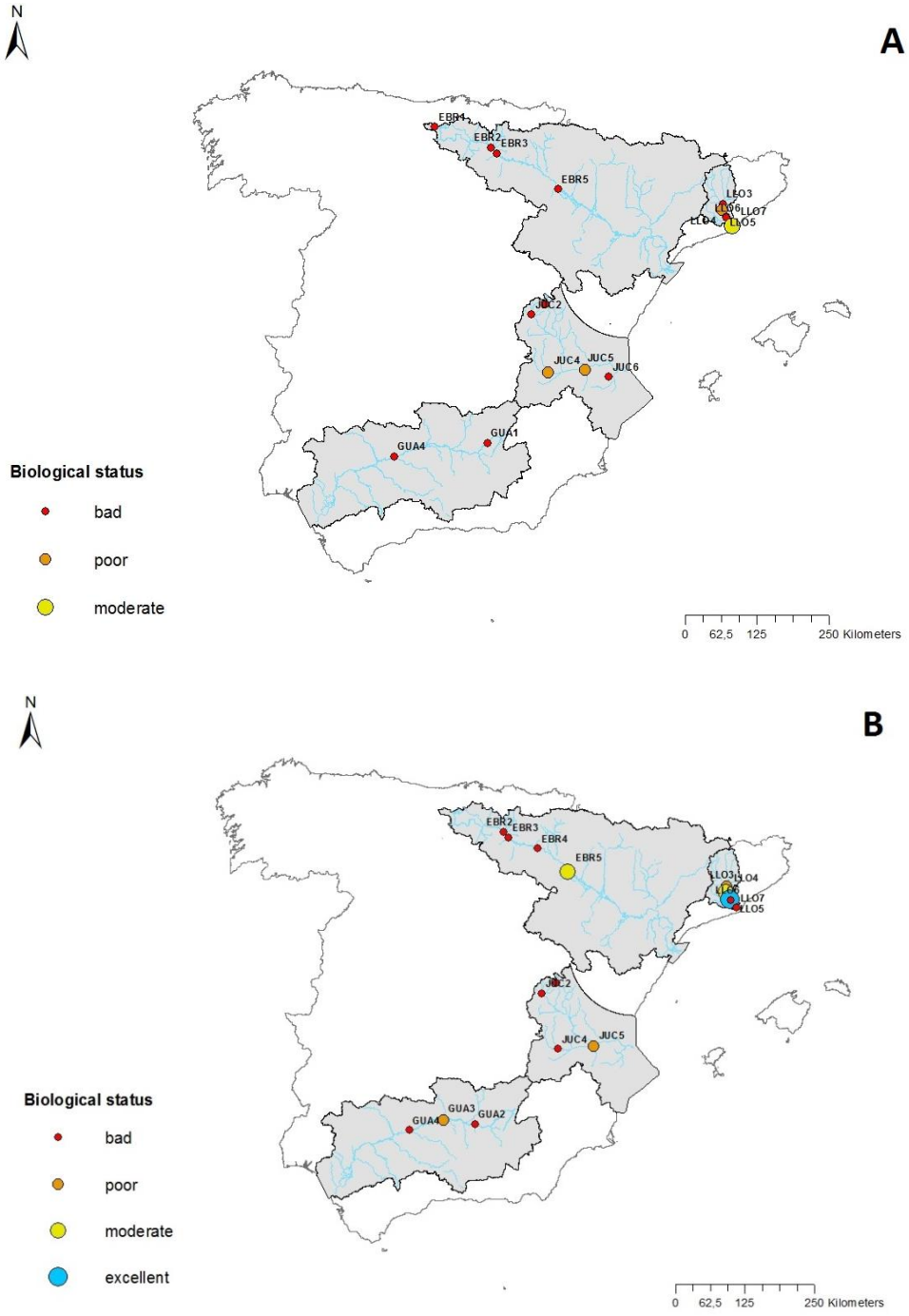

Figure AIV.7-Biological status- based on SPEAR corresponding to 2010 (a) and 2011 (b)

ANNEX V

SUPPLEMENTARY MATERIAL TO ENVIRONMENTAL STRESSORS AS A DRIVER OF THE TRAIT COMPOSITION OF BENTHIC MACROINVERTEBRATE ASSEMBLAGES IN POLLUTED IBERIAN RIVERS

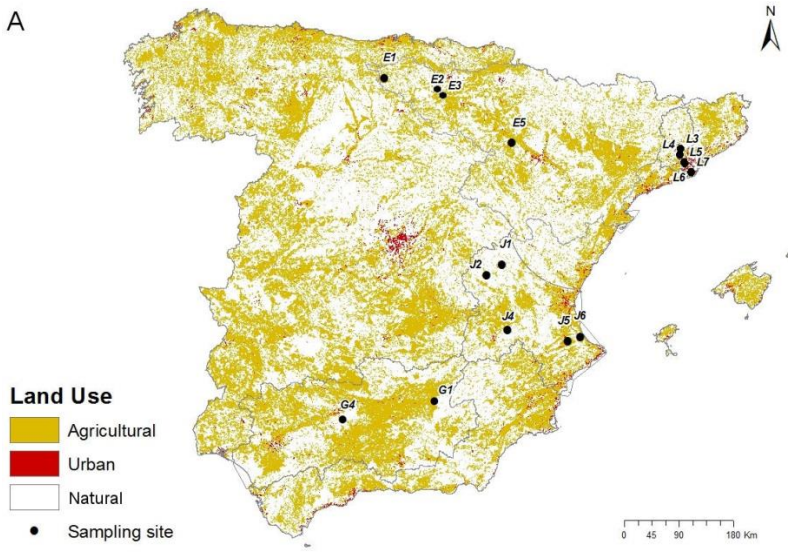

Figure AV1. Locations of the sampling sites on the land use map of Spain; agricultural areas appear in yellow, urban areas in red and natural types of land use in white.

Table AV.1. Land Use (LU) variable synthesizing the naturalness in the sub-basin was calculated and the weighted mean (weights 1, 5, 100) of urban agricultural and natural, land use percentages respectively, in the sub-basin.

	Urban	Agricultural	Natural
	%	%	%
E1	5	70	25
E2	10	85	5
E3	6	45	54
E5	5	90	6
L3	15	45	40
L4	10	10	80
L5	25	25	50
L6	25	25	50
L7	50	30	20
J1	0.1	5.5	94
J2	5	20	75
J4	1	90	9
J5	0.6	70	29
J6	2	38	60
G1	1	90	9
G4	40	55	5

Sites classification criteria

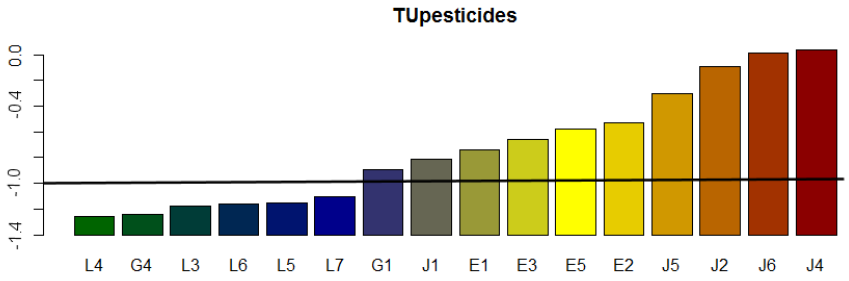

Figure AV.2. Values of $TU_{pesticides}$ at sampling sites. The sites with log TU >-1 (log) were considered to be impacted by pesticides.

Table AV.2. Correlation matrix of environmental data and land use types (adopted from (Kuzmanović, López-Doval et al. 2015)).

	Urban	Agricultural	Natural	N-NO3	P-PO4	O2	DOC	Conductivity	Temperature	pH	TUpesticides	TUmetals	OM	phimoy	phivar	CV
Urban	1	-0.29	-0.25	0.77	0.91	-0.55	0.81	0.62	0.75	-0.36	-0.63	0.67	-0.33	-0.11	-0.14	0.71
Agricultural	-0.29	1	-0.86	0.01	-0.23	0.15	-0.3	-0.31	-0.16	-0.35	0.36	0	0.2	0.29	0.13	-0.45
Natural	-0.25	-0.86	1	-0.42	-0.24	0.12	-0.13	-0.03	-0.22	0.54	-0.02	-0.35	-0.02	-0.23	-0.05	0.08
N-NO3	0.77	0.01	-0.42	1	0.72	-0.35	0.77	0.5	0.76	-0.2	-0.61	0.84	-0.16	0.06	-0.15	0.56
P-PO4	0.91	-0.23	-0.24	0.72	1	-0.65	0.76	0.53	0.82	-0.49	-0.55	0.68	-0.17	0.07	0.13	0.64
O2	-0.55	0.15	0.12	-0.35	-0.65	1	-0.53	-0.08	-0.68	0.53	0.41	-0.67	0.02	-0.1	-0.21	-0.61
DOC	0.81	-0.3	-0.13	0.77	0.76	-0.53	1	0.41	0.62	-0.16	-0.71	0.7	-0.2	-0.06	-0.09	0.81
Conductivity	0.62	-0.31	-0.03	0.5	0.53	-0.08	0.41	1	0.53	-0.01	-0.14	0.19	-0.38	-0.41	-0.39	0.42
Temperature	0.75	-0.16	-0.22	0.76	0.82	-0.68	0.62	0.53	1	-0.42	-0.43	0.77	-0.15	0.14	-0.03	0.51
pH	-0.36	-0.35	0.54	-0.2	-0.49	0.53	-0.16	-0.01	-0.42	1	-0.01	-0.32	0.19	-0.16	-0.32	-0.03
TUpesticides	-0.63	0.36	-0.02	-0.61	-0.55	0.41	-0.71	-0.14	-0.43	-0.01	1	-0.77	-0.14	-0.08	-0.13	-0.69
TUmetals	0.67	0	-0.35	0.84	0.68	-0.67	0.7	0.19	0.77	-0.32	-0.77	1	0.11	0.25	0.13	0.62
OM	-0.33	0.2	-0.02	-0.16	-0.17	0.02	-0.2	-0.38	-0.15	0.19	-0.14	0.11	1	0.71	0.63	-0.02
phimoy	-0.11	0.29	-0.23	0.06	0.07	-0.1	-0.06	-0.41	0.14	-0.16	-0.08	0.25	0.71	1	0.39	-0.15
phivar	-0.14	0.13	-0.05	-0.15	0.13	-0.21	-0.09	-0.39	-0.03	-0.32	-0.13	0.13	0.63	0.39	1	0.01
CV	0.71	-0.45	0.08	0.56	0.64	-0.61	0.81	0.42	0.51	-0.03	-0.69	0.62	-0.02	-0.15	0.01	1

Table AV.3: Thresholds for environmental variables to be considered a stressor.

Variable	Threshold value	Rationale
Pesticides (toxic unit)	0.1	Possible acute effects (Van Wijngaarden, Brock et al. 2005, Schäfer, Von Der Ohe et al. 2012, Malaj, Von Der Ohe et al. 2014)
Metals (toxic unit)	0.1	Possible acute effects (Malaj, Von Der Ohe et al. 2014)
Nitrates ($N\text{-}NO_3$ mg/l)	2.3	(RealDecreto817/2015 2015)
Phosphates ($P\text{-}PO_4$ mg/l)	0.14	(RealDecreto817/2015 2015)
Dissolved oxygen (mg/l)	5	(RealDecreto817/2015 2015)
Conductivity (µS/cm)	1200	(RealDecreto817/2015 2015)
Temperature (°C)	>20	Potential effects on sensitive species

Table AV.4. List of taxa used in this study

Caenis luctuosa
Ephemera danica
Elmis sp.
Dicranota sp.
Micronecta sp.
Potamopyrgus antipodarum
Cryptochironomus sp.
Polypedilum sp.
Stictochironomus sp.
Micropsectra sp.
Nanocladius sp.
Tanytarsus sp.
Branchiura sowerbyi

Limnodrilus hoffmeisteri
Limnodrilus udekemianus
Potamothrix hammoniensis
Lumbriculus variegatus
F. Enchytraeidae

Table AV.5. List of traits and trait modalities used in this study

Trait	All trait modalities	Code	Traits Used	Trait code
Max size	<5	less_than5	Small size (size <5mm)	Small
	5-10	bet_5_10		
	10-10	bet_10_20		
	20-40	bet_20_40		
	>40	more_40		
Life cycle duration	≤ 1 year	less_1y	Short life cycle (less than 1 year)	ShortLife
	> 1 year	more_1y		
Potential number of reproduction cycles per year	<1	less_1	Plurivoltinism (>1)	Pluri
	1	equal_1		
	>1	more_1		
Reproduction	Ovoviviparity	ovoviviparity	Egg protection (ovoviviparity+clutches)	EggProtect
	Free eggs	free_eggs		
	Cemented eggs	cemented_eggs		
	Cemented clutches	clutches_cemented		
	Free clutches	clutches_free		
	Clutches in vegetation	clutches_in_vegetation		
	Terrestrial clutches	clutches_terrestrial		
	Asexual	asexual		
Dipsersal	Aquatic passive	aquatic_passive	Dispersal index(Bonada, Dolédec et al. 2012)	Disp
	Aquatic active	aquatic_active		
	Aerial passive	aerial_passive		
	Aerial active	aerial_active		
Feeding habit	Absorber	abs_teguments	Predator (predator)	Pred
	Deposit feeder	depos_feed		
	Schreeder	schreeder		
	Scraper	scraper		
	Filter feeder	filter_feeder	Deposit feeder (absorption through tegument+ deposit	Depos
	Piecer	piecer		
	Predator	predator		

	Parasite	parasite	feeder)	
Food	Fine sediment + microrganisms	fine_sed_microorg	Food diversity (Simpson index-all food trait modalities)	FoodDiv
	Detritus < 1 mm	detritus_less_1		
	Plant detritus ≥ 1 mm	plant_detritus		
	Living microphytes	microphytes		
	Living macrophytes	macrophytes		
	Dead animal > 1 mm	dead_animal		
	Living microinvertebrates	microinvertebrates		
	Living macroinvertebrates	macroinvertebrates		
	Vertebrates	vertebrates		

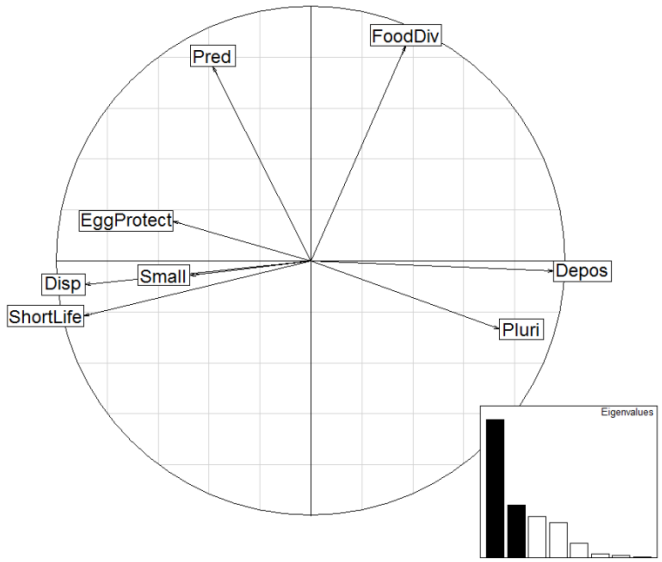

Figure AV.3. Result of a PCA performed on the taxa-by-traits matrix showing the trait associations. Inset represents the diagram of eigenvalues.

ANNEX VI

CURRICULUM VITAE

PUBLICATIONS

RESEARCH ARTICLES

1. **Kuzmanović M.,** Dolédec S., De Castro Català N., Ginebreda A., Muñoz I., Sabater S. and Barceló D.

> **Effects of land-use related stressors on the trait composition of benthic macroinvertebrate assemblages in Iberian rivers**- accepted in Environmental Research Journal.

2. Sabater S., Barceló D., De Castro Català N., Ginebreda A., **Kuzmanović M**., Pico Y., Ponsati L., Tornés E. and Muñoz I.

> **Shared effects of organic microcontaminants and environmental stressors on biofilms and invertebrates in impaired rivers**
> Environmental Pollution, 210, pp. 303–314 (2016)

3. De Castro Català N., **Kuzmanović M.,** Roig N., Sierra J., Ginebreda A., Barceló D., Pérez S., Petrović M., Picó Y., Schuhmacher M. and Muñoz I.

> **Ecotoxicity of sediments in rivers: Invertebrate community, toxicity bioassays and the toxic unit approach as complementary assessment tools**
> Science of Total Environment, 540, pp. 297-306 (2015)

4. **Kuzmanović M.,** Lopez-Doval J., De Castro Català N., Petrović M., Munoz I., Guasch H., Ginebreda A., and Barceló D.

> **Ecotoxicological risk assessment of chemical pollution in four Iberian river basins and its relationship with the aquatic macroinvertebrate community status**
> Science of Total Environment 540, pp. 324-333 (2015)

5. Banjac Z., Ginebreda A., **Kuzmanović M**., Marcé R., Nadal M. and Barceló D.

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