Occurrence and effects of pharmaceuticals and personal care products: new contributions in predictive models, potential risks assessments and rankings of hazard

Doctoral Thesis

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DEPARTAMENTO DE INGENIERÍA QUÍMICA Y TECNOLOGÍA DEL MEDIO AMBIENTE

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Presentada por Sheyla Andrea Ortiz de García para optar al grado de doctor por la Universidad de Valladolid

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Rubén Irusta Mata
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SHEYLA ANDREA ORTIZ DE GARCÍA ha realizado bajo su dirección el trabajo "*Occurrence and effects of pharmaceuticals and personal care products: new contributions in predictive models, potential risks assessments and rankings of hazard*", en el Departamento de Ingeniería Química y Tecnología del Medio Ambiente de la Escuela de Ingenierías Industriales de la Universidad de Valladolid. Considerando que dicho trabajo reúne los requisitos para ser presentado como Tesis Doctoral expresan su conformidad con dicha presentación.

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Reunido el tribunal que ha juzgado la Tesis Doctoral titulada *"Occurrence and effects of pharmaceuticals and personal care products: new contributions in predictive models, potential risks assessments and rankings of hazard"* presentada por la Ingeniera Químico Sheyla Andrea Ortiz de García y en cumplimiento con lo establecido por el Real Decreto 99/2011 de 28 de enero de 2011 acuerda conceder por _____ la calificación de _____.

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1er Vocal 2º Vocal 3er Vocal

Un poco de ciencia aleja de Dios, pero mucha ciencia devuelve a Él

Louis Pasteur

He aquí mi secreto, que no puede ser más simple: sólo con el corazón se puede ver bien; lo esencial es invisible para los ojos

Antoine de Saint-Exupéry

A ILYWM...

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Resumen

Los productos activos farmacéuticos y de higiene personal (PPCPs por sus siglas en inglés) son un variado grupo de compuestos químicos que han sido encontrados en diferentes compartimientos del medio ambiente. Actualmente, es bien conocido que muchos PPCPs generan diversos efectos adversos en diferentes organismos de la cadena trófica en el medio ambiente, lo que ha originado una marcada preocupación por su presencia y destino en la naturaleza.

Miles de PPCPs (y sus metabolitos) ingresan al medio ambiente acuático diariamente mediante descargas puntuales y/o dispersas, ocasionando en muchos casos complejas interacciones que aumentan la problemática y generan cada día más interrogantes en el mundo científico. A pesar de los esfuerzos y avances llevados a cabo en los estudios de índole experimental, la gran cantidad de PPCPs y la carencia de datos en esta área del conocimiento, ha originado que las técnicas predictivas, sean cada vez más utilizadas, permitiendo un ahorro significativo de tiempo y dinero, a la vez que sustentan o complementan regulaciones, políticas y procesos de toma de decisión, mediante listas de compuestos de atención prioritaria.

Por ello, en la presente tesis se ha planteado el estudio de la ocurrencia y los efectos de algunos de los principales PPCPs en los ambientes acuáticos y estaciones depuradoras de aguas residuales (EDARs), generando nuevas contribuciones en el ámbito experimental, mediante el estudio de su ecotoxicidad sobre la bacteria bioluminiscente *Vibrio fischeri* y la biomasa procedente del tratamiento secundario de una EDAR, con el propósito de establecer modelos predictivos y evaluando su uso en la generación de índices de riesgos potenciales, clasificaciones de preocupación y listas de priorización.

Se ha estudiado una amplia variedad de PPCPs y metabolitos/productos de transformación. Estos PPCPs se seleccionaron en base a investigaciones previas de estudios de riesgo e impacto ambiental, datos recientes de consumo humano y su ocurrencia en ambientes acuáticos españoles. Adicionalmente, es necesario destacar que muchos de los PPCPs seleccionados coinciden con los PPCPs más comercializados y consumidos a nivel mundial.

En primer lugar se realizó un estudio con el fin de predecir la ocurrencia de 88 PPCPs, metabolitos y productos de transformación en medios acuáticos y EDARs, empleando un enfoque de balance de masa y validando los métodos propuestos con datos de concentraciones ambientales medidas (Capítulo 2). Se plantearon tres metodologías para estimar el consumo de los compuestos activos farmacéuticos en función de los datos estadísticos disponibles y se realizaron comparaciones en los casos de aquellos compuestos donde se estimó su consumo con más de una metodología. La comparativa entre las diferentes metodologías presentó resultados similares. La ocurrencia de estos PPCPs en ambientes acuáticos se estimó a partir de los datos de consumo y tomando en cuenta parámetros farmacocinéticos en humanos, automedicación y tratamiento en EDARs. Los analgésicos/antipiréticos (y sus metabolitos), antibióticos, medios de contraste de rayos X, los inhibidores de la bomba de protones y fragancias obtuvieron los valores más altos de ocurrencia. En un 60% de los casos donde se realizó la comparativa de los valores predichos con las concentraciones ambientales medidas se encontró concordancia.

Los modelos y metodologías propuestas en este estudio resultan una valiosa herramienta que puede ser utilizada en otros ámbitos geográficos, y los resultados generados constituyen datos de gran utilidad para estudios de riesgo o impacto ambiental.

Con el fin de evaluar predictivamente los principales efectos adversos de los PPCPs se planteó estimar el potencial de persistencia, bioacumulación y ecotoxicidad (índice PBT) en ambientes acuáticos utilizando software y metodologías actualizadas, basadas en modelos de relaciones cuantitativas de estructura-actividad ((Q)SAR, por sus siglas en inglés). Junto a estos tres efectos, se incorporó la ocurrencia (O) y se analizaron en conjunto los índices OPBT, generándose clasificaciones de preocupación y listas de priorización para estos compuestos (Capítulo 3).

El índice ambiental que presentó la mayor cantidad de compuestos en la categoría más alta de preocupación fue la persistencia. Analizando la lista de priorización según la evaluación del índice PBT y OPBT mediante la técnica de clasificación total, las hormonas, los antidepresivos (y sus metabolitos), los

reguladores de lípidos en la sangre y todos los compuestos de higiene personal fueron los principales PPCPs ubicados en la parte superior de la clasificación (mayor índice de preocupación). Los medios de contraste de rayos X, los inhibidores de la bomba de protones y algunos antibióticos (compuestos que presentaron alta ocurrencia) se incluyen dentro de los más peligrosos cuando se desarrolló la técnica de clasificación parcial OPBT.

En general, los estudios que involucran PPCPs son realizados tomando en consideración sólo los compuestos parentales. En esta investigación se demostró que una gran cantidad de metabolitos presentaron una puntuación de preocupación igual o superior que la de su compuesto parental, por lo tanto, y debido a la alta tasa de metabolización o transformación de muchas de estas sustancias, se hace necesario incluirlas en los estudios de riesgo/peligro ambiental y profundizar a nivel experimental sobre sus posibles efectos adversos en los diferentes organismos de la cadena trófica.

Recientemente, la Agencia Europea de Medicamentos (EMEA, por sus siglas en inglés) ha propuesto una serie de directrices con el fin de realizar evaluaciones de riesgo ambiental (ERAs) para medicamentos de uso humano. Estas ERAs se basan en valores de ecotoxicidad predictivos y/o experimentales, según la calidad de los datos disponibles. Las especies más utilizadas para la detección de ecotoxicidad en ambientes acuáticos son los peces, crustáceos y algas, pero no necesariamente son las especies más sensibles y tampoco reproducen los efectos causados en EDARs. Por ello, se planteó determinar la ecotoxicidad (en base a la concentración efectiva media, CE_{50}) de PPCPs sobre otro tipo de especie acuática, probablemente más sensible, las bacterias bioluminiscentes *Vibrio fischeri* (mediante el ensayo Microtox®). Para determinar el comportamiento de las EDARs, se evaluó el efecto de los PPCPs mediante ensayos respirométricos en la biomasa de reactores biológicos. A partir de estos resultados experimentales y de los valores predictivos ya estimados se desarrollaron dos propuestas de ERAs (Capítulo 4).

Los resultados evidenciaron el siguiente orden de susceptibilidad global: *Vibrio fischeri* > algas > crustáceos > peces > biomasa de reactor biológico,

demostrando que para los PPCPs en estudio, la bacteria bioluminiscente (*Vibrio fischeri*) resulta ser la especie más sensible a los efectos adversos ocasionados por estos compuestos. Un 65.4% de los PPCPs en estudio fueron catalogados como “altamente tóxicos” o “perjudiciales para organismos acuáticos” en al menos dos pruebas de ecotoxicidad, según los criterios del Sistema Globalmente Armonizado de clasificación y etiquetado de productos químicos (SGA) de las Naciones Unidas. Los compuestos de higiene personal, antibióticos, inhibidor de la bomba de protones y un producto de transformación de un analgésico/antipirético presentaron algún tipo de riesgo en ambientes acuáticos y en la EDAR cuando se llevaron a cabo las ERAs.

En vista de los resultados ecotoxicológicos obtenidos para las bacterias *Vibrio fischeri* en la ERA, y debido a la escasa información que existe acerca de los efectos de los PPCPs en esta especie, se estudió la relación dosis-respuesta de estos compuestos sobre dichos microorganismos en diferentes rangos de concentración (a concentraciones cercanas a las existentes en los ambientes acuáticos y EDARs y en concentraciones alrededor de la CE_{50}). El estudio se llevó a cabo para las sustancias individuales y en una mezcla de ellas (Capítulo 5).

Un alto porcentaje de los PPCPs estudiados (90%) presentaron un buen ajuste estadístico en al menos uno de los tres modelos dosis-respuesta de regresión no lineal propuestos. El modelo de regresión no lineal de cuatro parámetros (sigmoidal de pendiente variable) fue el que mejor se ajustó en la mayoría de los casos. Utilizando el modelo con mejor ajuste para cada PPCP se calcularon las CE_{50}, CE_5 (en sustitución de la concentración mínima de efecto adverso observable o NOAEL, por sus siglas en inglés) y la CE_0 (en sustitución de la concentración sin efecto observable o NOEL, por sus siglas en inglés) los cuales representan parámetros ecotoxicológicos desconocidos para la mayoría de los PPCPs en estudio. Un 55% de los PPCPs mostraron un comportamiento hormético, es decir, estimulatorio a bajas concentraciones (concentraciones ambientales) e inhibitorio en dosis más altas (alrededor de la CE_{50}). Todos los compuestos que presentaron estimulación a bajas dosis sobre *Vibrio fischeri* evidencian narcosis (un modo de acción tóxica) a altas concentraciones, lo que permite inferir que existe cierta correlación entre estos dos fenómenos. Los

PPCPs que presentaron los efectos estimulatorios más altos fueron los analgésico/antiinflamatorios no esteroideos y los antiagregantes plaquetarios.

La mezcla de PPCPs a concentraciones por debajo de la CE_0 presentó un efecto estimulatorio más pronunciado que los observados en los compuestos individuales. Debido a la complejidad de la mezcla y a los posibles efectos sinérgicos y antagónicos presentes, los puntos dosis-respuesta obtenidos no fueron ajustados a los modelos utilizados tradicionalmente para mezclas. Por otra parte, cuando se aumentó el tiempo de exposición el efecto hormético disminuyó.

Las bacterias son microorganismos imprescindibles en la cadena alimenticia, por lo tanto, cualquier alteración o cambio que ocurra en esta especie afectará directa o indirectamente al resto de las especies en los diferentes niveles tróficos. De ahí la importancia de conocer la afectación que los PPCPs y muchos otros compuestos pueden ejercer sobre ellas.

Finalmente, y con el fin de aportar nuevos datos que permitan incluir mayor cantidad de PPCPs en estudios de análisis del ciclo de vida, se calcularon factores de caracterización (humanos y ecotoxicológicos) mediante la metodología USEtox^{TM}. Los factores de caracterización se utilizaron para elaborar una clasificación con puntuaciones de impacto utilizando la ocurrencia de PPCPs en ambientes acuáticos, aire y suelo, en España (Capítulo 6).

Los factores de caracterización para la ecotoxicidad en agua dulce resultaron ser más elevados que los de toxicidad humana (con una diferencia que va del orden de 10^3 hasta 10^{12}) lo que indica que la afectación de estos compuestos sobre la vidad acuática es mucho más relevante que sobre la salud humana. Las hormonas, antidepresivos, fragancias, antibióticos, bloqueadores de los receptores de la angiotensina y los reguladores de lípidos en la sangre destacaron en los niveles más altos de impacto en esta categorización.

A pesar de las limitaciones y diferencias intrínsecas de cada metodología, un grupo de PPCPs han destacado en las listas prioritarias de esta tesis: hormonas, antibióticos, inhibidor de la bomba de protones y productos de cuidado personal. Estos PPCPs pueden ser considerados compuestos prioritarios, los cuales

deberían ser sujetos a estudios más detallados de impacto ambiental y posiblemente a controles y regulaciones más estrictas.

Abstract

Pharmaceuticals and personal care products (PPCPs) are a varied group of chemicals compounds that have been found in different compartments of the environment. Many PPCPs generate varied adverse effects in different organisms throughout the food chain and the environment, generating marked concern due to their presence and fate in nature.

Thousands of PPCPs (and their metabolites) enter the aquatic environment daily through single and/or dispersed discharges, often resulting in complex interactions that increase the problem and generate more questions. Despite the efforts and advances of many experimental studies, the large number of PPCPs and the lack of data in this area of knowledge have resulted in predictive techniques becoming increasingly used, allowing a significant savings of time and money; these techniques have also resulted in the support of regulations, policies and decision-making processes that rely on lists of priority compounds.

Therefore, this thesis presents a study of the occurrence and effects of the main PPCPs in aquatic environments and wastewater treatment plants (WWTPs), generating new contributions to the experimental field by studying their ecotoxicity on the bioluminescent bacteria *Vibrio fischeri* and the biomass from the secondary treatment of a WWTP, with the purpose of establishing predictive models and evaluating their use for generating potential risk indexes, rankings of concern and priority.

A wide variety of PPCPs and metabolites/transformation products have been studied. These PPCPs were selected based on previous studies of risk assessment and environmental impact and recent data regarding human consumption and their occurrence in Spanish aquatic environments. Additionally, many of the selected PPCPs coincide with the PPCPs that are most commonly commercialized and consumed worldwide.

First, a study was conducted using an integral mass balance approach to predict the occurrence of 88 PPCPs/metabolites and transformation products in aquatic environments and WWTPs and to validate the proposed methods with the

measured environmental concentrations (Chapter 2). Three methodologies were proposed to estimate the consumption of pharmaceutically active compounds (PhACs) according to the available statistical data, and comparisons were made with these compounds, where consumption was estimated with more than one methodology. The comparison among the different methodologies presented similar results. Considering the consumption data, pharmacokinetic parameters in humans, self-medication and treatment in WWTPs, the occurrence levels of the evaluated PPCPs in aquatic environments were estimated. Analgesics/antipyretics (and their metabolites), antibiotics, X-ray contrast media, H_2 blockers and fragrances had the highest occurrence values in 60% of cases in which comparisons of predicted environmental concentrations and measured environmental concentrations were performed.

Despite the lack of data of measured environmental concentrations, the predicted values were consistent with the measured ones in 60% of cases.

The models and methodologies that were proposed in this study are a valuable tool that can be used in other geographical areas, and the results are useful data for risk or impact environmental assessments.

To predict the main adverse effects of PPCPs, it was proposed to estimate the persistence, bioaccumulation and ecotoxicity potential (PBT index) in aquatic environments and in WWTPs using software and updated methodologies based on Quantitative Structure-Activity relationships models ((Q)SARs). The occurrence (O) was added and analyzed jointly with the PBT index. Lists of rankings of concerns and priority were generated (Chapter 3).

The environmental index that had the greatest number of compounds in the highest category of concern was persistence. Hormones, antidepressants (and their metabolites), blood lipid regulators and all personal care products under study were located in the top of the PBT and OPBT total rankings of concern (highest indexes of concern). X-ray contrast media, H_2 blockers and some antibiotics (compounds that showed high occurrence) were included as the most dangerous when an OPBT partial ranking of concern was developed.

In general, studies involving PPCPs have only considered the parent compounds. This investigation showed that a large number of metabolites had a concern score that was equal to or greater than that of their parent compounds; therefore, due to the high metabolization or transformation rate of many of these substances, their inclusion in environmental risk/hazard assessments is necessary to improve the experimental understanding of their adverse effects on the different organisms of the trophic chain.

Recently, the European Medicines Agency (EMEA) has generated guidelines to conduct environmental risk assessments (ERAs) for medicinal products for human use. These ERAs are based on predictive and/or experimental ecotoxicity values, depending on the quality of available data. The species that are most used for the detection of ecotoxicity in aquatic environments are fish, crustaceans and algae, but these species are not necessarily the most sensitive and do not reproduce the effects caused in WWTPs. Therefore, the ecotoxicity determination (half of the maximal effective concentration, EC_{50}) of PPCPs was determined in other aquatic species that were likely to be more sensitive using the bioluminescent bacterium *Vibrio fischeri* (by Microtox® assay). To determine the behavior of WWTPs, the effect of PPCPs was evaluated by respirometric assays on the biomass of biological reactors. From these experimental results and the predictive values already estimated, two ERAs were developed (Chapter 4).

The results showed the following order of overall susceptibility: *Vibrio fischeri* > Algae > Crustaceans > Fish > biomass of the biological reactor, indicating that for the PPCPs under study, bioluminescent bacteria (*Vibrio fischeri*) are the most sensitive species to the adverse effects that are caused by these compounds. A total of 65.4% of the PPCPs in this study were classified as "highly toxic" or "harmful to aquatic organisms" in at least two ecotoxicity tests according to the criteria of the Globally Harmonized System of Classification and Labeling of Chemicals of the United Nations. The personal care products, antibiotics, H_2 blockers, and a degradation product of an analgesic/antipyretic presented some type of risk in aquatic environments and in the WWTPs where ERAs were carried out.

In view of the ecotoxicological results for *Vibrio fischeri* bacteria in the ERA and due to the limited information of the effects of PPCPs on this species, the dose-responses of these compounds in the selected microorganisms over different concentration ranges were studied (at concentrations close to those found in aquatic environments and WWTPs and at concentrations near the EC_{50}). The study was conducted for the individual substances and for their mixture (Chapter 5).

A high percentage of PPCPs (90%) presented a good dose-response statistical fit in at least one of the three proposed non-linear regression models. The four-parameter non-linear regression model (sigmoidal variable slope) was the best fit in most cases. Using the model with best fit for each PPCP, the EC_{50}, EC_5 (used instead of the lowest observed adverse effect level (LOAEL)) and EC_0 (used instead of no observed adverse effect level (NOAEL)) were calculated, which are ecotoxicological parameters that were not previously estimated for most of the PPCPs under study. A total of 55% of the PPCPs showed hormetic behavior, stimulation at low concentrations and inhibition at higher doses. All of the compounds that showed stimulation at low concentrations exhibit narcosis (a mode of toxic action) at high concentrations. This behavior allows the inference that there is some correlation between these two phenomena. The PPCPs that had the strongest stimulatory effects were analgesic/non-steroidal anti-inflammatory drugs and a platelet aggregation inhibitor.

PPCP mixtures at concentrations below the EC_0 presented a more pronounced stimulatory effect than did those that were observed for the individual compounds. Due to the complexity of the mixtures and the potential synergistic and antagonistic effects, the dose-response data were not adjusted to the traditional models that were used for mixtures. Moreover, when the exposure time increased, the hormetic effect decreased.

Bacteria are essential microorganisms in the food chain; therefore, any alteration or change to these species will directly affect other species at different trophic levels. Hence, it is important to know the effects that PPCPs and many other compounds can exert.

Finally, to provide new data to include more PPCPs in studies of life cycle impact assessment, characterization factors (human and ecotoxicological ones) were calculated using USEtox^{TM} methodology. These characterization factors were used to develop a classification with impact scores based on the occurrence of PPCPs in aquatic environments, air and soil in Spain (Chapter 6).

The characterization factors for ecotoxicity in freshwater were higher than those of human toxicity (with a difference on the order of 10^3 to 10^{12}), indicating that the effects of these compounds on aquatic life are much more relevant than their effects on human health. Hormones, antidepressants, fragrances, antibiotics, H_2 blockers, angiotensin receptor blockers and blood lipid regulators had the highest levels of impact on this categorization.

Despite the limitations and inherent differences of each methodology, a group of PPCPs are highlighted in the priority lists of this thesis: hormones, antibiotics, H_2 blockers and personal care products. These PPCPs can be considered priority compounds that should be subjected to more detailed studies of environmental impact with more stringent controls and regulations.

List of publications

The following publications are presented as part of the present thesis. Three of them are published in international journals indexed in ISI web of Knowledge (Papers I to III). Paper IV has been submitted for publication.

Paper I. Ortiz de García S, Pinto G, García-Encina P, Irusta-Mata R (2013) *Consumption and Occurrence of Pharmaceutical and Personal Care Products in the Aquatic Environment in Spain*. Sci Tot Environ 444:451-465. doi:10.1016/j.scitotenv.2012.11.057

Paper II. Ortiz de García S, Pinto G, García-Encina PA, Irusta RI (2013) *Ranking of concern, based on environmental indexes, for pharmaceutical and personal care products: an application to the Spanish case*. J Environ Manag 129:384–397. doi:10.1016/j.jenvman.2013.06.035

Paper III. Ortiz de García S, Pinto Pinto G, García-Encina P, Irusta-Mata R (2014) *Ecotoxicity and Environmental Risk Assessment of Pharmaceuticals and Personal Care Products in aquatic environments and wastewater treatment plants*. Ecotoxicol. 23(8):1517-33. doi: 10.1007/s10646-014-1293-8

Paper IV. Ortiz de García S, García-Encina P, Irusta-Mata R (2015) *Dose-response behavior of the bacterium Vibrio fischeri exposed to pharmaceuticals and personal care products*. Submitted for publication in Ecotoxicology.

Paper V. Ortiz de García S, García-Encina P, Irusta-Mata R (2015) *Human and ecotoxicological potential impact of pharmaceutical and personal care products from USEtox^{TM} life cycle impact assessment characterization factors.* Unpublished manuscript.

Chapter 1

Introduction

1.1. Pharmaceutical and personal care products (PPCPs) in the environment: Occurrence and fate

In recent years, concerns about the environmental fate and behavior of synthetic organic chemicals that have been detected in different ecological compartments have increased. Several of these compounds are used intensively, are persistent and bioactive, and exhibit bioaccumulation and endocrine-disrupting activity (Caliman and Gavrilescu, 2009). Some of these synthetic organic chemicals are PPCPs, which comprise an important group of environmental micro-pollutants. According to Silva et al. (2015), the environmental presence of PPCPs is a growing problem that must be addressed to meet Directive 2013/39/EU, minimize the resulting aquatic environmental contamination, and support future prioritization measures.

In some investigations that were carried out in Austria, Brazil, Canada, Croatia, England, Germany, Greece, Italy, Spain, Switzerland, The Netherlands, and the U.S., more than 80 pharmaceutical and drug metabolite compounds have been detected in the aquatic environment (Hereber, 2002), treated sewage, rivers and creeks, seawater, groundwater and even drinking water (Fent et al., 2006) with concentrations varying from nanograms per liter to micrograms per liter, and their occurrence in water varies greatly across regions and seasons (Zhu et al., 2013).

Generally, drugs are absorbed by an organism after intake and are subjected to metabolic reactions, such as hydroxylation, cleavage and glucuronation. However, a significant amount of the original substance will leave the organism unmetabolized via urine or feces and will therefore enter raw sewage (Hirsch et al. 1999).

The source of pharmaceutical active compounds (PhACs) can be divided into two: point source pollution and diffuse pollution. For instance, industrial effluent, hospital effluent and sewage treatment plants, as well as septic tanks, are the major point source to the soil zone and water resources. In contrast, for diffuse pollution, it is difficult to identify the emission location because it occurs over broad geographical scales (e.g., agricultural runoff from animal waste and manure, urban

runoff from domestic waste and leakage from waste treatment systems and plants) (Li, 2014).

PhACs also enter the environment from the disposal of unwanted medications directly into sewers and trash. The relative significance of this route compared to excretion and bathing is poorly understood and has been subjected to much speculation (Ruhoy and Daughton, 2008).

Previous studies argue that wastewater is the main sources of PhACs in the aquatic environment (Celle-Jeaton et al., 2014). The mass balances of the influents and effluents of drug residues as detected in wastewater treatment plants reveal that many pharmaceuticals are not completely eliminated by traditional treatment processes (Han et al., 2006).

Unlike PhACs, which are intended for internal use, personal care products (PCPs) are products that are intended for external use on the human body and thus are not subjected to metabolic alterations; therefore, large quantities of PCPs enter the environment unaltered through regular use (Brausch and Rand, 2011).

The interest in the occurrence of pharmaceuticals in the environment is ever increasing, and the number of reports on measurable concentrations of pharmaceuticals in environmental samples or reviews on pharmaceuticals found in the environment is growing (Carlsson et al., 2006). The concentration and fate of these products in the aqueous environment vary and depend on several parameters, such as (i) the geographical location, (ii) the fraction that leaves the user unchanged or as a conjugate and ends up in sewage, (iii) the effectiveness of wastewater treatment and proximity to wastewater plants, (iv) the volume of the water body, (v) the sorption and degradation processes in the environment, and (vi) the meteorological conditions (Kasprzyk-Hordern et al., 2008; ter Laak et al., 2010).

Another important aspect of the presence of PPCPs in the environment is the ability to detect them at their environmental concentrations. As state-of-the-art analytical techniques become more sensitive and more widely deployed, an increasing number of human and veterinary drugs are being detected in

environmental samples (Ankley et al., 2007). Recent trends have focused on the development and application of generic methods that permit the simultaneous analysis of multiclass compounds, including acidic, neutral, and basic pharmaceuticals (Gros et al., 2009). Many authors have published related results (Grabic et al., 2012; Gilart et al., 2013; Gros et al., 2009; Lajeunesse et al., 2008; López-Serna et al., 2010; Ternes et al., 2001; Valcárcel et al., 2011; Villaverde-de-Sáa et al., 2010; Weigel et al., 2004).

In addition to analytical procedures that require sophisticated equipment, a relatively long time, and high costs, estimation methodologies have been developed to predict the occurrence, concentrations, fate and effects of these compounds in nature. Reliably predicted or measured environmental concentrations (PECs or MECs) of chemicals are essential for exposure assessment, which is one of the two main pillars of ERA (Liebig et al., 2006). Various hydrological models have been developed for the calculation of PECs, and the resultant values are usually the maximum concentrations that are likely to occur (Ankley et al., 2007).

In this thesis, a wide variety of PPCPs and their metabolites have been studied. Tens of PPCPS have been analyzed in predictive assessments and in experimental assays. PPCPs and some metabolites were selected based on previous risk impact assessment studies, recent data for human consumption, and occurrence in aquatic environments in Spain. Many of these PPCPs coincide with the most commercialized compounds for human use worldwide and a few of their metabolites. The groups of PhACs under study include angiotensin-converting enzyme inhibitors, analgesics/antipyretics, angiotensin receptor blockers, antibiotics, antidepressants, antiepileptics, anxiolytics, blood lipid regulators, cytostatics/cancer therapeutics, H_2 blockers, hormones, platelet inhibitors, non-steroidal anti-inflammatory drugs/antirheumatics, and X-ray contrast media. PCPs include disinfectants, fragrances, preservatives and surfactants.

In this context, **Chapter 2** presents information on 88 PPCPs (See **Chapter 9**, appendix A, for more information of the compounds under study) with the following purposes:

- To propose different and novel methodologies to calculate the yearly amounts of sixty PhACs, twenty metabolites and eight PCPs in aquatic environments in Spain.
- To calculate their PECs.
- To compare PECs with MECs to verify the validity of the selected methods.

The occurrence in the aquatic environment was calculated through a mass balance approach considering the following: the number of pharmaceutical prescriptions issued; the amount of pharmaceutical discharged without consumption, consumption, self-medication, pharmacokinetics, treatment in WWTPs; and the amount discharged to the aquatic environment.

The estimation of the consumption of active compounds of pharmaceuticals was conducted using at least one of the following three methodologies: number of commercial packages sold, data for the number of defined daily doses per 1000 inhabitants per day (DHD), and pattern of treatment.

Pharmacokinetics consider the absorption or non-absorption of parent compounds and the excretion of unmetabolized or metabolized parent compounds. Data concerning the fate of PPCPs after excretion consider PPCPs or metabolites that are discharged directly into the environment or to treatment in WWTPs (three different options were considered according to the most common types of treatment facilities in Spain: (i) WWTPs with primary treatment, (ii) WWTPs with primary and secondary treatment and (iii) WWTPs with primary, secondary and tertiary treatment).

PEC values were calculated with the model that was proposed by the EMEA guidelines (EMEA, 2006). The environmental concentrations of PPCPs and metabolites were estimated and then compared with the environmental concentrations that were measured by several researchers and reported in recent Spanish and European literature.

The main results indicate that the compounds with the highest pharmaceutical occurrences in the aquatic environment were, in order, acetaminophen glucuronide, Galaxolide®, Iso-E-super®, acetaminophen, valsartan, amoxicillin, 2-

hydroxy-ibuprofen, iopromide, omeprazole, carbamazepine 10, 11-epoxide, iopamidol, salicylic β-D-O-glucuronide acid, Tonalide®, acetylsalicylic acid (ASA), clarithromycin and iohexol, with releases between 5 and 600 t y^{-1}. For almost 50% of the studied compounds, there were no MEC data or these data were not detected in aquatic environments. Metabolites also had high PECs, but there is little information on MECs. For approximately 60% of the compounds for which the PEC/MEC ratios were calculated, the models fit well, and the PECs were very close to the corresponding MECs with reasonable allowances for excess or deficit.

These results include relevant information about PPCPs and some of their metabolites, some of which have been poorly studied until now, at least in Spain and in many European countries, as well as updated data about consumption patterns, sampling campaigns and resource management.

1.2. Effects of pharmaceutical and personal care products in the environment

Pharmaceuticals are designed to stimulate a response in humans and animals at low doses with a very specific target; thus, the implications for human health and the environment need to be assessed (Calamari et al., 2003). The scientific community is in broad agreement with the possibility that adverse effects, not only for human health but also for aquatic organisms, may arise from the presence of pharmaceuticals (Santos et al., 2010).

Several almost negligible effects have been shown to occur from continuous exposure during the life cycle of aquatic vertebrates and invertebrates to sub-therapeutic drug concentrations. These effects slowly accumulate to manifest themselves into a final irreversible condition that is frequently only noticed several generations later, affecting the sustainability of aquatic organism populations (Santos et al., 2010). According to Kümmerer (2009), the amount of information that is available on the effects of active substances on organisms in the aquatic and terrestrial environment is increasing but still scarce. The high concentrations of some compounds, i.e., in the gram per liter range, produce acute effects on environmental organisms.

Recent studies have demonstrated that some metabolites are more lipophilic and more persistent than the original drugs from which they were derived (Han et al., 2006), increasing the complexity of the problem. Drug residues that are found in the aquatic environment usually occur as mixtures and not as single contaminants. Thus, a scientific assessment of risk to aquatic life should consider this complex exposure situation (Cleuvers, 2003). According to Fent et al. (2006), few studies consider the effects of mixtures of pharmaceuticals. These mixture have been found to be toxic at concentrations for which single compounds showed little or no effect. From a general risk assessment point of view, it would be interesting to see whether a mixture of substances may have adverse effects when test organisms are exposed to concentrations at or below their individual Non-Observed Effects Concentrations (NOECs) (Breitholtz et al. 2008).

Despite the varied studies existing to date, the adverse effects of many PPCPs and their metabolites remain unknown. Some authors have studied their harmful properties in detail, considering endocrine disruption, persistence (P), bioaccumulation (B), and toxicity (T) potential, among others. PBT substances are carbon-based chemicals that resist degradation in the environment and accumulate in the tissues of living organisms, where they can produce undesirable effects on human health or the environment at certain exposure levels (Pavan and Worth, 2006).

Pharmaceuticals are designed and manufactured to be resistant to biodegradation because metabolic stability usually improves their desired pharmacological action (causing a biological effect). Therefore, pharmaceuticals often have similar types of physico-chemical behavior that are characteristic of harmful xenobiotics (e.g., they are able to cross membranes). Their stability, however, contributes to their environmental persistence because the compounds are designed to avoid being inactivated before providing their therapeutic effect (Fatta-Kassinos et al., 2011; Sanderson et al., 2004a).

The persistence of a substance is the length of time that a substance remains in a particular environment before it is physically transported to another compartment or chemically or biologically transformed (Pavan and Worth, 2006). Persistence by

itself is not a problem if the compounds do not cause negative changes in the environment over time. The risk increases when a substance can cause ecotoxicity, bioaccumulation, and endocrine disruption, among other effects, in the time required for its (bio) degradation to safe concentrations.

Another important factor to consider is bioaccumulation. The term bioaccumulation is defined in many different ways. Bioaccumulation can be defined as the simple uptake of substances from the environment, their accumulation over time, or their retention. Bioaccumulation factors (BAFs) are ordinarily calculated as the ratio of the concentration of the compound of interest in the biota sample (plant, sand animals) to that in the surrounding media (e.g., soil or water) (Zenker et al. 2014). BAFs are commonly used metrics in risk assessments to predict the bioaccumulative potential and resultant potential toxicity of chemical contaminants in aquatic organisms. The impacts of BAF values are species-specific and depend on a range of factors, such as the habitat, reproductive status and life-stage of fish and the environmental behavior of pharmaceuticals (Liu et al., 2015).

An ecotoxic substance has the potential to generate adverse human health or environmental effects at specific exposure levels. The intrinsic toxicity of a substance can be identified by standard laboratory tests. For the environment, these properties include short-term (acute) or long-term (chronic) effects. For human health, these properties include toxicity through breathing or swallowing the substance and effects such as cancer, mutagenicity, reproductive toxicity and neurological effects (Pavan and Worth, 2006).

To be effective medicines, most pharmaceuticals are designed to cause minimal toxicity. As a consequence, most pharmaceuticals, irrespective of their primary mode of action (MOA), are toxic in short-term lethality assays only at concentrations that far exceed those in the environment. Many drugs, however, are designed to affect specific biological pathways in target organisms at relatively low doses and exposure concentrations. Some of these pathways are critical to the long-term homeostatic control of physiological function and can be highly conserved across phyla. As a consequence, long-term, sub lethal effects of

pharmaceuticals could be of much greater potential concern than acute effects in non-target animals (Ankley et al., 2007).

A comprehensive evaluation of ecotoxicity effects on non-target organisms must include the development of specific tests that evaluate either acute effects (where mortality rates are often registered) or chronic effects (by means of exposure to different concentrations of a chemical compound over a prolonged period of time) (Santos et al., 2010).

Ecotoxicological data are available in the open peer-reviewed literature and ecotoxicological databases (ECETOX (EU) and ECOTOX (US)) for less than 1% of pharmaceuticals, and only a small number of new pharmaceuticals have been subjected to a complete risk assessment, including a battery of appropriate ecotoxicological tests in the EU (Sanderson et al., 2004a). Therefore, additional effort is needed to obtain new ecotoxicological data of PPCPs (acute or chronic, experimental or predictive) from laboratory assays or predictive models, which allow estimating the potential negative effects of these compounds in different target organisms and environmental conditions.

With this background, this thesis presents three different studies that were designed to analyze the ecotoxicological effects of PPCPs on the environment.

1.2.1. Prediction of adverse effects of pharmaceutical and personal care products through quantitative structure-activity relationships

Future European Union legislations will enforce the fast hazard and risk assessment of thousands of existing chemicals. If conducted using the present data requirements, this assessment will use a huge number of test animals and will be neither cost- nor time-effective (Freidig et al., 2007). The experimental determination of the many adverse effects of PPCPs (as PBT potential) is generally expensive and demanding. Thus, measuring the potential PBT profiles of chemicals that are of potential regulatory interest experimentally is considered infeasible (Pavan and Worth, 2006). An attractive alternative to the use of animal testing has been the development of methodologies that enable predictions of

effects to be made directly from chemical structure. Predictions of effects from chemical structure encompass a broad range of techniques and methodologies, generally referred to as (quantitative) structure–activity relationships ((Q)SARs) (Cronin et al., 2002). (Q)SARs are models that enable the prediction of physical, chemical, and biological properties of non-assessed compounds by comparing structurally and/or quantitatively similar assessed compounds based on the structure and composition of the molecule (Sanderson et al., 2004b).

The use of (Q)SARs for classification and labeling and for hazard assessment and priority setting of chemicals is currently a hot topic within the EU due to the introduction of the Registration, Evaluation and Authorization of Chemicals (REACH) legislation (Freidig et al., 2007). Under REACH, the estimated data generated by (Q)SARs may be used both as a substitute for experimental data and as a supplement to experimental data in weight-of-evidence approaches (Pavan and Worth, 2006). Therefore, and according to Cronin et al. (2002), the future will almost certainly bring about the increased use of (Q)SARs by regulators to estimate the ecologic effects and environmental fate of chemical substances. Such activities may include the prioritization of existing chemical databases.

Several tools have been proposed for estimating the parameters and effects of chemicals on the environment from (Q)SAR methodology, including PBT potentials. One of these tools is the Estimation Programs Interface EPI Suite^{TM} that was developed by the Office of Pollution Prevention and Toxics of the US EPA and Syracuse Research Corporation. EPI Suite^{TM} software estimates physico-chemical properties, environmental fate and effects of molecules using models that are either fragment or K_{ow}-based (Q)SARs, expert systems, or some combination of the three (Pavan and Worth, 2006). This software or some of its modules have been widely used for estimating the effects of PPCPs in nature, confirming its versatility and acceptable predictions until there is experimental data.

According to Sanderson et al. (2004a), (Q)SARs and pharmacodynamic information should be used to prioritize and steer experimental risk assessments

of pharmaceuticals and could potentially be used in new drug discovery, optimizing the efficacy and minimizing the environmental hazards of new products.

Thus, in **Chapter 3** of this thesis, a (Q)SAR study was performed to assess the possible adverse effects of PPCPs and some of their metabolites (See **Chapter 9**, appendix B, for more information concerning the compounds under study). The main aspects of the methodology and results of this work will be explained in detail in the next section.

1.2.2. Persistence, bioaccumulation and ecotoxicity potential of pharmaceutical and personal care products

The experimental determination of P, B and T indexes is generally expensive and demanding to perform; therefore, **Chapter 3** presents the estimation of the PBT potentials (as extensive parameters) of relevant PPCPs from experimental results already published or from (Q)SAR estimation models to perform an environmental hazard classification of these compounds using novel tools (ranking techniques) to perform the decision analysis. Generally, the studies that report hazard/risk classifications use diverse adverse effects as environmental parameters; therefore, **Chapter 3** provides a new contribution, including the Occurrence (O) of PPCPs in the Spanish aquatic environments as another important extensive parameter to be considered in the different rankings of generated concern. Hence, the specific objectives of this study were as follows:

- To estimate the PBT potentials by (Q)SAR updated models and databases.
- To consider the Occurrence of PPCPs in aquatic Spanish environments as estimated using a mass balance approach (presented in **Chapter 2**) and incorporating it as an extensive environmental index to the PBT indexes.
- To generate rankings of concern of PBT and OPBT using the Decision Analysis by Ranking Techniques (DART) tool and to perform a sensitivity analysis considering several index weights.

In this research, 96 PPCPs and metabolites were considered to assess their possible environmental adverse effects. The PBT potential was calculated from the BIOWIN^{TM} biodegradability estimation program, BCFBAF v.3.00 routine and

the ECOSAR™ class program. These programs are part of the Estimation Programs Interface EPI Suite™ of the USEPA.

The physicochemical parameters of PPCPs and their metabolites were consulted in recognized databases or estimated with the EPI Suite™ interface. The DART tool, which was recently recommended by the European Commission, was used to rank the compounds according to their environmental and toxicological concern based on the most recent ranking theories. Partial and total rankings (through desirability and utility functions) were analyzed. These parameters were classified and grouped into four levels of concern. These levels were sufficiently broad to consider the uncertainties of each toxicological value. However, a sensitivity analysis for the index weights (eight different combinations) was conducted to verify their influence and the changes in the compound ranking list.

The persistence of a large number of the compounds under study (88.5%) merited the highest concern score. Only three compounds were in the highest level of the bioaccumulation index (tamoxifen, Galaxolide® and desogestrel), and a large percentage (96.8%) were located in levels 1 or 2, corresponding to low levels of concern. The distribution of toxicity results was more homogeneous across the different levels: 18.8% of PPCPs were in the higher concern score; 19.8% and 22.9% were in the second and third levels, respectively (middle concern score); and the remaining 38.5% were in the lowest score.

The principal PhACs that were placed in the highest level of risk (considering combined P, B and T indexes) were hormones, antidepressants and blood lipid regulators. The most relevant PCPs were triclosan (antimicrobial disinfectant), 4-nonylphenol (surfactant), and all of the considered fragrances. Some metabolites had a toxicity risk level equal to or greater than their parent compounds, such as N-desmethyl sertraline.

In general (including all PPCPs), the total hazard ranking score by desirability and utility functions and the partial hazard ranking score showed that fragrances, hormones, antidepressants, anxiolytics, blood lipid regulators and some of the metabolites that were considered in this study had the highest levels of risk. The

inclusion of occurrence in the ranking changed the top 25 compounds significantly, mainly by incorporating X-ray contrast media and antibiotics.

These rankings can be used to prioritize the PPCPs that require immediate attention to more deeply evaluate their effects on the environment (e.g., at the experimental level); to obtain preliminary results; to facilitate the decision-making processes in an ERA; and to perform preventive, corrective and regulatory actions.

Although the use of estimation models to predict the adverse effects of PPCPs is important and useful, experimental assays serve to improve these predictions and to determine the specific effects on target organisms.

1.2.3. Ecotoxicity of pharmaceutical and personal care products

Aquatic organisms are particularly important targets, as they are exposed via wastewater residues throughout their whole life (Fent et al. 2006). Acute and chronic ecotoxicity assessments have been implemented to evaluate the effects of these compounds on different species. The standard organisms that are used are fish, crustaceans and algae, which represent the principal three trophic levels. Although bacteria are less frequently used, many authors confirm the importance of considering them relevant ecotoxicological subjects (medium) (Backhaus and Grimme 1999; Choi and Meier 2001; Christofi et al. 2002; Ortiz de García et al. 2014; Parvez et al. 2006; van der Grinten et al. 2010; Vighi et al. 2009; Villa et al. 2012).

In the majority of aquatic ecosystems, the most important trophic level in terms of energy flow and nutrient cycles is bacteria. Hence, it is important to include representatives from this trophic level in a series of tests that are designed to protect aquatic ecosystems (Choi and Meier 2001). Vighi et al. (2001) assert that in view of the ecological importance of bacteria in all ecosystems, their exclusion from ecotoxicological risk assessments could, in some cases, result in the implementation of inadequate protective measures for the aquatic environment.

In this context, **Chapter 4** discusses the ecotoxicological effects (acute toxicity) of 26 PPCPs on *Vibrio fischeri* bioluminescence bacteria as a measure of the effect

on the aquatic environments using the Microtox® method, in addition to respirometry tests to determine the effects of these compounds on WWTP biota (See **Chapter 9**, appendix C, for the PPCPs list). The specific objectives were as follows:

- To determine the relevant ecotoxicological endpoints (Half-maximal effective concentration, EC_{50}) for *Vibrio fischeri* bacteria and the activated sludge of a WWTP and to compare these values with the ecotoxicity over other standard species.
- To classify the ecotoxicity values according to the Globally Harmonized System of Classification and Labeling of Chemicals (GHS).
- To relate experimental results to a representative physico-chemical property (K_{ow}) of a substance and to other ecotoxicity data that were obtained using a predictive ((Q)SAR) method in other species.
- To perform ERAs according to the EMEA guidelines.

The investigated PPCPs are some of the most important classes of drugs (non-steroidal anti-inflammatories, analgesics, antibiotics, H_2 blockers and blood lipid regulators) and personal care products (disinfectants and preservatives) worldwide. Their consumption and occurrence in aquatic environments and in WWTPs are relevant, at least in Spain, and have been previously reported (**Chapter 2**).

The determination of acute effects on the bioluminescence of *Vibrio fischeri* bacteria was performed using Microtox® equipment and the associated method. During these tests, the inhibition of light emission was measured in relative units of luminescence. The acute ecotoxicity endpoint was determined as the EC_{50} at 5 and 15 minutes for a 95% confidence interval using a linear regression model.

The activated sludge respirometry test is a more direct method for measuring sludge activity and thus the ecotoxicity of the sludge (Ren, 2004). A Strathtox Unit SI500 from Strathkelvin Instruments was used to carry out these assays according to a standardized method. The activated sludge that was used was obtained from the secondary treatment tank of Valladolid's WWTP.

The experimental ecotoxicity results in bacteria and activated sludge and the estimates obtained with ECOSAR^{TM} for algae, crustaceans and fish were classified as established by the GHS and were compared with each other.

The overall order of susceptibility as a function of the ecotoxicity results was as follows: *Vibrio fischeri* (5 min, MICROTOX®) > *Vibrio fischeri* (15 min, MICROTOX®) > Algae ((Q)SAR) > Crustacean ((Q)SAR) > Fish ((Q)SAR > Activated sludge of WWTP (respirometry assay).

The correlation between acute ecotoxicity in *Vibrio fischeri* and the compound's K_{ow} (physico-chemical property, descriptor of their hydrophobic/lipophilic activity) was extremely poor, suggesting that K_{ow} cannot be used to generate prediction models for *Vibrio fischeri* as has been done for other species in other studies. The relationship between more sensitive species in the experimental assays (bioluminescence acute ecotoxicity of *Vibrio fischeri* at 5 min) and in the predictive model (growth inhibition of green algae in 96 h) shows a better correlation (r^2= 0.9365), which may help to reduce the experimental test time (from 96 h with algae to 5 min with *Vibrio fischeri*) or to correlate existing models for algae with this bacteria or vice versa.

According to the GHS classification 1,4-Benzoquinone (transformation product of acetaminophen and clofibric acid) and triclosan were the most toxic compounds. In total, 65.4% of the PPCPs under study were classified (by GHS) between ''highly toxic'' and ''harmful to aquatic organisms'' according to at least two ecotoxicity values, which provides preliminary evidence concerning the negative effects of these compounds on the environment.

The ecotoxicity results are independent of the geographic area under study, as well as the consumption, occurrence and treatment of PPCPs, but they are strongly dependent on the laboratory conditions, testing species, methodologies and software that are used. Therefore, the GHS classification system is a useful tool to establish a reasonable range to classify ecotoxicity values and to compare results from different species and sources.

1.2.4. Dose-response behavior of individual and mixed PPCPs in *Vibrio fischeri* bacteria

A dose–response model is, in general, an equation describing the variation of a representative magnitude in an object population, with variation in the magnitude of an effector agent. A typical case is the inhibition of the growth of a microbial population by a chemical agent, but the same resource can often be applied, with minor changes, to stimulatory effects, mortality or survival, quantitative changes in cell components, the characters of macro-organisms, and different physical and chemical agents (Murado et al., 2002). Dose-response models are a common and statistically valid form with which to consider pharmaceutical data in medicine or other sciences. A set of points can be fitted to a function to determine what doses are considered effective and what doses might be considered toxic.

Dose-response curves are widely used to determine the behavior of substances in different conditions and concentrations, decreasing the number of experimental assays, costs and time. Through these curves, relevant ecotoxicological points (such as EC_{50}) can be obtained and used as the first step of an ERA. There are various types of models that can be used to fit the data; therefore, each compound and species under study must be evaluated to find a better adjustment.

In recent years, dose-response behavior has been studied not only in concentrations around the EC_{50} but also below the EC_0 (called the “Zero equivalent point (ZEP)), i.e., the dose at which the response crosses the control value. Calabrese and Baldwin (2002) demonstrated that there are numerous responses to chemical/physical agent exposures that occur below the traditional no-observed adverse effect levels (NOAEL). These authors affirm that these findings may also have profound effects on the health of individuals and present challenges to experimental design, the integration of data, and the application of biostatistical extrapolation models, as well as the definition of toxicology itself.

At low concentrations (below the ZEP), some species in the presence of certain compounds demonstrate a clear stimulatory effect. This phenomenon of low-dose stimulation and high-dose inhibition has been called hormesis. The phenomenon of hormesis has gained increased recognition during the past decade. Hormetic

responses can be found throughout the sciences, especially in the dose–response relationships of pharmacology, toxicology, agriculture, and nutrition (Qin et al. 2010). Hormesis has been hypothesized to be an overcompensation to an alteration in homeostasis (Stebbing et al., 1998).

At present, there is a lack of data of dose-response models of many PPCPs using *Vibrio fischeri* bacteria. The inhibitory effect is the most studied, but many PPCPs have not been evaluated using these bacteria, and dose-response models have established for the behavior of even fewer. The same affirmation applies to mixtures of PPCPs. Hashmi et al. (2013) report numerous references for the hormetic response of luminescent bacteria to different chemical compounds but not PPCPs.

Bacteria are indispensable microorganisms in the food chain. Therefore, any changes in bacteria might change the normal development of many species (including humans) and environmental physicochemical processes. Thus, ecotoxicological studies on bacteria are essential for deeply understanding the adverse, beneficial, or neutral effects of a wide variety of chemical compounds that can reach the environment, including PPCPs. These ecotoxicological results are also necessary for environmental risk/hazard assessments to prevent contamination affecting the ecosystem.

Therefore, in **Chapter 5**, the effects of PPCPs (single and mixture) on *Vibrio fischeri* bacteria at two different ranges of concentrations (environmental concentrations and those around the EC_{50}) have been studied to achieve the following goals for each PPCP (See **Chapter 9**, appendix D, for the PPCP list):

- To adjust dose-response data around the EC_{50} to a statistically validated model.
- To verify the dose-response behavior of PPCPs on bioluminescent bacteria at environmental concentrations (WWTPs and aquatic environments).
- To calculate relevant ecotoxicological data ($EC_{50,}$ EC_5, and EC_0).
- To evaluate the performance of a mixture of PPCPs at environmental concentrations.

- To discuss the relevance of these results in ecotoxicology and risk assessments studies.

Twenty PPCPs were selected in accordance with their high worldwide consumption and the evidence of their potential ecotoxicity in aquatic environments, as highlighted by Ortiz et al. (2014).

Two ecotoxicity tests were performed using Microtox® assays consisting of a basic test and a whole effluent toxicity (WET) test for the single PPCPs and their mixtures. Basic tests were performed at least twice each for four dilutions (5.6, 11.2, 22.6 and 45 % of the initial concentration) at 5 and 15 minutes to evaluate the different dose-response models above and below the ZEP value. The standard basic test procedure has been previously reported (Ortiz et al. 2014) and was performed in agreement with the manufacturer`s instructions and the ISO 11348-3:2007 protocol. The basic test is widely used to calculate the most relevant ecotoxicological point (EC_{50}) for a toxicant on *Vibrio fischeri* bacteria.

The principles of the WET test are similar to those of the basic procedure, but this test is carried out with three replicated samples, three control replicates and five dilutions (at 6, 12, 25, 50 and 100% of the initial concentration). Generally, the WET test is applied to samples of unknown behavior to determine the response, including the initial sample without dilution. Therefore, the WET test was applied to the PPCP mixture.

The dose-response data of the single PPCP solutions were fitted with three non-linear functions: a sigmoidal dose-response or three-parameter logistic model, a sigmoidal dose-response variable slope or four-parameter logistic model, and an asymmetrical or five-parameter logistic model. The goodness of fit was described by the correlation coefficient (R^2) and the sum of squares (SS). The 95% confidence intervals were calculated and plotted with the best non-linear function fit for each compound. The least-squares nonlinear regression assumes that the distribution of residuals follows a Gaussian distribution. This assumption was tested by running normality tests on the residuals (the D'Agostino-Pearson, Kolmogorov-Smirnov distance and Shapiro-Wilk). The mean results at 5 and 15 minutes and for the different range of concentrations considered were compared

with a two-way analysis of variance (Two-way ANOVA, α=0.05) to test for differences between times and among concentrations. All of the statistics were performed using the GraphPad Prism 6® software.

The results of the assay that was performed with the mixture of PPCPs were plotted as the dose-response behaviors and their standard deviations for different exposure time periods (six readings were taken from 0 to 445 minutes). The phenomenon of stimulation at low doses and the behavior of ZEP over time were analyzed.

Dose-response data around EC_{50} showed that the four-parameter regression model provided the best fit for most of the compounds (approximately 60%) (at 5 and 15 minutes), asymmetrical was the best fit model for approximately 30% of the compounds, and the three-parameter model was the best fit for approximately 10% of the PPCPs. Half of the compounds showed a very good adjustment ($R^2 \geq 0.99$) and, consequently, a low SS of residuals. These compounds also passed the normality tests for residuals.

The estimated EC_{50} of each PPCP with the corresponding model was compared with the corresponding value presented in a recent study (Ortiz et al. 2014), in which the acute ecotoxicity endpoint was determined using a linear regression model, as indicated in the Microtox® user's manual. After the confidence levels were considered, most of the estimated values were on the same order as those that were obtained in the aforementioned study. The EC_{50} values of acetaminophen, cefaclor, clofibrate, ethylparaben, ibuprofen sodium salt and propylparaben were outside the confidence limits of the previous cited study, possibly due to the inclusion of new data or deviations of the new models under study, but the values were located in the same level of ecotoxicity according to the classification used in Ortiz et al. (2014), with the exception of clofibrate and clofibric acid. This finding highlights the importance of adjusting the dose-response data for reliable results and the possible variations that can be observed using different models.

The dose-response results at environmental concentrations showed that 55% of the tested compounds (acetaminophen, ASA, ciprofloxacin HCl, clofibric acid,

diclofenac sodium salt, ibuprofen sodium salt, methylparaben, naproxen, norfloxacin, salicylic acid and sulfamethoxazole) exhibited at least two points (concentration mean) with a clear stimulatory effect, considering the standard deviation of data to ensure that this affirmation is statistically representative. The other 45% of the compounds showed an effect around zero, ranging between stimulatory or inhibitory when the standard deviation of each point was considered. Therefore, there was no clear trend in the behavior of these compounds over a range of concentrations, and the weak or null stimulatory effects could be considered noise within the system.

In the range of concentrations that were studied in this research, the PPCPs that presented the highest values of stimulatory effects were the analgesic/antipyretic compounds, the NSAIDs and the platelet aggregation inhibitors.

All of the data (results of the two ranges of concentrations) were adjusted with the three dose-response model. Fourteen compounds (70%) had the best fit with a four-parameter regression model, four (20%) fit best with an asymmetrical model, and two compounds (10%) fit best with a three-parameter model. With these new adjustments, ecotoxicological points (EC_{50}, EC_5 and EC_0) were calculated and compared with the results when only data around EC_{50} were considered. The dose-response fitting, including all data (which presented stimulation in some cases), generated slight changes in the statistical parameters compared with the fitted models for data around the EC_{50}. In those compounds where a stimulatory effect was presented, a β curve (an inverted U-shaped dose-response curve) was evident. If stimulation effects would have been higher than those obtained in this research, a specific model that includes U-shaped or J-shaped curves at low doses should have been used.

The ZEP values (EC_0) at exposure times of 5 and 15 minutes for 16 compounds were estimated from the best fit model of each PPCP, and these values can be used to determine the safe PPCP concentrations for bacteria.

The behavior of the mixture of these twenty PPCPs was quite different than the behavior of each singly tested compound. The stimulatory effect of the mixture was higher than the highest stimulatory effect of each single compound (single

bioassay), at least for the 15 minutes of response time. The mixture caused the greatest hormetic effect at a dilution of 25% of the initial concentration and with short exposure time periods (15 and 60 minutes). When the exposure time and concentration increased, the effects on *Vibrio fischeri* changed from stimulatory to inhibitory. As the time was further increased, the ZEP was reached at lower concentrations. At these doses, the compound began to be at least slightly ecotoxic.

Most of the studied PPCPs are found in the environment or in WWTPs at very low concentrations. In most cases, these concentrations are below the ZEP values, indicating that ecotoxicological studies must be performed for these concentrations not only to evaluate the potential hormetic effect but also to analyze other factors, such as chronic effects or the intra- and interspecies influences of these PPCPs on future generations.

1.3. Environmental risk assessment of pharmaceuticals and personal care products

Currently, risk assessment and risk management issues are gaining momentum (Kümmerer, 2009). Risk assessment studies identify potential hazardous consequences and determine both their likelihood to occur in a specific environment (i.e., exposure assessment) and their severity (i.e., toxicity) (Jjemba, 2006). It is desirable to be able to predict a compound's potential to cause adverse effects in the environment before these effects are observed. The probability of a compound causing undesired environmental effects can be estimated in an ERA (Carlsson et al., 2006). According to Muñoz et al. (2008), quantitative risk assessment approaches, such as those included in the EU Technical Guidance Document (TGD) and in the new EU chemicals regulation REACH, are considered appropriate tools to determine the health and environmental risks that are associated with chemicals.

Procedures for conducting ERA on pharmaceuticals are widely used in both Europe and the United States. The EMEA guidelines describe how to evaluate the potential risks of the medicinal product to the environment. This guideline focuses only on the environmental risks that are associated with the use of medicinal

products not arising from storage, disposal, synthesis or manufacture of medicinal products (Grung et al., 2008).

According to the EMEA guidelines for the ERA of pharmaceuticals, data that are used for effect analysis in the lower tier should preferably follow standard testing protocols. Standard tests are generally more accepted across jurisdictions, and comparisons across substances are easier. Standard tests also promote the reproducibility of data due to the detailed test procedures and reporting requirements. A disadvantage of standard tests is that they do not always use the most sensitive species or represent the most relevant testing approach considering the type of endpoint under investigation. There are cases where nonstandard tests can be more sensitive and thereby contribute additional and significant information to risk assessment (Ågerstrand et al., 2011), which could be true for *Vibrio fischeri* bacteria or the activated sludge from the secondary treatment stage of WWTPs. Therefore, in **Chapter 4**, bacteria (in aquatic environments) and the biomass of WWTPs were included as other important species (in addition to predictive ecotoxicity values of algae, crustacean and fish) to perform ERAs of PPCPs.

The basic principle of ERAs is the comparison of a PEC or MEC of a substance with a predicted no-effect concentration (PNEC) and the concentration at which no effects on environmental organisms are expected to occur (Liebig et al., 2006). The assessment of the potential risks to the environment of this type of compound is a stepwise process that consists of two phases.

In Phase I (pre-screening), the estimation is only based on the substance's structural characteristics, irrespective of its route of administration, pharmaceutical form, metabolism and excretion. If the PEC value is below 0.01 μg L^{-1} and no other environmental concerns are apparent, it is assumed that the medicinal product is unlikely to represent a risk to the environment following its prescribed usage in patients. If the PEC value is equal to or greater than 0.01 μg L^{-1}, then a Phase II environmental fate and effect analysis should be performed.

Phase II has two tiers (A and B). Tier A is a risk screening in which a simple PEC is calculated (metabolization in humans and removal in WWTPs are excluded from

calculations). Tier B is an extended screening in which a refined PEC is calculated (metabolization in humans and removal in WWTPs are considered in calculations). PEC values in aquatic environments and in WWTPs were obtained from the **Chapter 2** results for both tiers of the second phase; PNECs for aquatic environments were obtained from the lower value of ecotoxicity (EC_{50} or LC_{50}) (the worst case, among the estimated acute ecotoxicity values by (Q)SAR in fish, crustaceans, and algae, as well as the experimental acute ecotoxicity values of the Microtox® assay); and PNECs for WWTPs were calculated from respirometry test results. PNECs calculation also considers a standard dilution assessment factor as recommended by the EMEA for each case. In Phase II, the risk quotients (RQs) (the PEC:PNEC or MEC:PNEC ratio that indicates the greatest toxicity) were calculated to predict the PPCP risk.

According to the RQ classification of the European Medicines Agency (2006), if the RQ is below 1, further testing in the aquatic compartment will not be necessary, and it can be concluded that the drug substance and/or its metabolites are unlikely to represent risks to the aquatic environment. If the RQ is above 1, further evaluation, preferably on the fate of the drug substance and/or its metabolites in the aquatic environment, is required in Tier B. The results also can be classified according to more restrictive ERA criteria (MRERA): high toxicity (RQ>1), medium toxicity (0.1<RQ<1) and low toxicity (0.01<RQ<0.1).

Through these principles and guidelines, the ERAs of 26 PPCPs in aquatic environments and WWTP are presented and discussed in **Chapter 4** to accomplish the following specific objectives:

- To predict whether the PPCP/metabolite requires more attention.
- To predict whether other tests must be performed to demonstrate its adverse effects on the environment or otherwise.
- To predict whether the PPCP/metabolite is not harmful.

Phase I of the ERA in aquatic environments showed that when a simple PEC value was used, all of the compounds continued to phase B; however, with refined PEC values, cefaclor, clofibric acid and clofibrate were classified as risk-free. When phase II Tier A were applied, acetaminophen, ibuprofen and omeprazole

exhibited a RQ>1, therefore, further evaluation was required to go to Tier B. When Tier B was applied only 1,4 benzoquinone (a transformation product of acetaminophen and clofibric acid) proved that it should be referred to the committee for proprietary medicinal products for safety measures. When MRERA criteria were used with the refined data, in addition to 1,4-benzoquinone (high risk), omeprazole and triclosan had medium risk, and clarithromycin, ethylparaben and methylparaben had low risk. The application of MRERA to simple PEC data substantially increases the number of PPCPs that generated some type of risk (82.4% of the total compounds under study).

The ERA of PPCPs in WWTPs highlights that any compound represents a risk in aquatic environments. Following the MRERA classification and excluding metabolization in humans, ibuprofen, ciprofloxacin, naproxen and acetaminophen showed some type of risk in these facilities, and when the metabolization was considered, only ibuprofen and ciprofloxacin were highlighted with a low risk.

EMEA methodology has proved to be a useful and powerful tool to make ERAs for those compounds of recent concern, especially for those for which there is still a lack of experimental data concerning their occurrence, fate and effects in nature as PPCPs. Despite this utility, it is necessary to take into account all of the limitations and assumptions made for comparison with other methodologies and results.

ERA is a geographic-dependent tool due to the different data concerning the consumption, occurrence and treatment for the area under study. The RQ values can substantially vary if these values use a simple or a refined PEC or MEC approximation. Therefore, a further improvement of these parameters and of the ecotoxicity data (acute and chronic) of these compounds, particularly their metabolites, transformation products and mixtures that have been less investigated, is required.

1.4. Pharmaceutical and personal care products in life cycle impact assessment

Life Cycle Assessment (LCA) is a methodology developed for the environmental assessment of products/functions during their life cycles. LCA is thus an important tool in the product-oriented environmental efforts. Aspects of application and practical use have been coordinated by preparation of standards in International Standardization Organization (ISO) (Olsen et al., 2001). LCA is a ‘‘cradle-to-grave’’ approach that evaluates several potential environmental impacts, such as those listed above, of a product, process or service. To ensure a credible evaluation and comparison, methodological rules have to be followed that are developed within the framework of the ISO 14040 standards (Renou et al., 2008).

In LCAs, characterization factors (CFs) are used to determine the relative importance of a substance to toxicity related impact categories, such as human toxicity and freshwater ecotoxicity (Huijbregts et al., 2005a).

CFs can be converted in toxicity potentials that, according to Huijbregts et al. (2000), are substance-specific, quantitative representations of potential impacts per unit emission of a toxic substance. In LCAs of products, these potentials are used as weighting factors to determine the relative contribution of a substance to toxicity related impact category.

Recently, the incorporation of PPCPs in LCAs studies has been increasing significantly, which necessitates determining the CFs of these compounds (and their corresponding toxicity potentials) to verify their contributions in this type of assessment. LCA studies in pharmaceutical industries or for comparing different treatment processes or for evaluating the environmental impacts of WWTPs are some types of analysis where PPCPs should be included. There is an extensive CF database available from different estimation methods, but the number of CFs for PPCPs included in this database is relatively small considering the large amount of such existing compounds. Therefore, it is necessary to complement this information to include these compounds in new and future investigations in this field of knowledge.

In view of this requirement, in **Chapter 6** has been developed a research with the following specific objectives:

- To calculate the human and ecotoxicological CFs of 28 PPCPs from USEtox^{TM} method.
- To estimate the potential impact of 49 PPCPs with CFs already published and the new ones.
- To establish rankings of concern, from human and ecotoxicological CFs, using the occurrence of these compounds in the Spanish aquatic environments.
- To compare the ecotoxicological ranking of concern obtained from CFs with other methodologies.

The PPCPs considered in this study (See **Chapter 9**, appendix E, for the PPCPs list) have been classified into three categories: (i) PPCPs for which CF values are unknown, (ii) PPCPs for which CF values are available in the USEtox^{TM} database, (iii) PPCPs for which CF values are available in other bibliographic sources.

The input parameters that must be supplied by the user to the USEtox^{TM} program are: molecular weight, partition coefficient between organic carbon and water (K_{ow}), Henry law coefficient, vapor pressure, solubility, degradation rate in air, degradation rate in water, BAF, water ecotoxicity (chronic and acute) and human carcinogenic and non-carcinogenic effects.

The methodology and models used to calculate the unknown CFs are widely explained in Huijbregts et al. (2005b; 2010a; 2010b) and Rosenbaum et al. (2008). CFs of chemicals include a fate factor (FF), an exposure factor (XF) and an effect factor (EF). CFs for ecotoxicity were calculated for freshwater aquatic ecotoxicological effects and include impacts for emissions to urban air, rural air, freshwater and/or agricultural soil in different scales (urban, continental and global).

CFs for human toxicity were estimated for carcinogenic and non-carcinogenic effects, and they also take into account emissions on different scales. The FF and

EF are combined to reflect the intake fraction (iF) of a chemical, representing the fraction of the emitted mass that enters the human population.

The FF is equal to the compartment-specific residence time (in days) of a chemical. The longer the residence time, the longer a chemical remains in the environment. Within the consensus model, the residence time of a chemical depends on (i) the properties of the chemical, (ii) the selected emission compartment, and (iii) the selected receiving compartment. XF for ecotoxicity depends on the partition coefficient between water and suspended solids, the suspended matter concentration in freshwater, the partitioning coefficient between dissolved organic carbon and water, the dissolved organic carbon concentration in freshwater, the bioconcentration factor in fish and the concentration of biota in water. The XF for humans depends on the rate at which a pollutant is able to transfer from a receiving compartment into the human population through a series of exposure pathways (air, drinking water, food, and inhalation). Ecotoxicological EF is estimated by acute and chronic data from recognized database and published literature. Human-toxicological EF reflects the change in life time disease probability due to change in life time intake of a pollutant. In this research, carcinogenic and non-carcinogenic effects and ingestion as route of exposure are considered (due to the lack of data for inhalation as route of exposure).

The potential impact is measured with the impact score (IS). The IS for ecotoxicity is calculated with the CF and the emission of each PPCP released to each compartment. ISs were estimated for ecotoxicity and human toxicity with their respective CFs. The emissions of PPCPs were taken from **Chapter 2**.

Emissions from continental urban air, continental rural air, continental freshwater, continental natural soil and continental agricultural soil generate freshwater aquatic ecotoxicological CFs in the order of 10^{-1} to 10^{4}, where the highest CFs are those from continental freshwater due to the direct contact between the source of emission and the compartment affected.

Freshwater aquatic ecotoxicological CFs are much higher than human health CFs due to the low tolerance of aquatic organisms to this compounds and their persistence in this medium.

The compounds with the highest impact scores are also compounds of concern in other rankings performed by different methodologies reported in previous studies. In comparison with the ranking of concern developed in **Chapter 3,** it is observed that hormones, antidepressants, blood lipid regulators and personal care products are at the highest levels of risk, similar to this new ranking.

1.5. References

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Chapter 2

Paper I

Consumption and Occurrence of Pharmaceutical and Personal Care Products in the Aquatic Environment in Spain.

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Consumption and occurrence of pharmaceutical and personal care products in the aquatic environment in Spain

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HIGHLIGHTS

- Consumption of pharmaceutically active compounds was estimated.
- The occurrence in the environment of PPCPs and metabolites was estimated.
- Predicted and measured environmental concentrations were compared.
- PECs were close to MECs for 64.7% of compounds.
- PECs are a useful tool for prioritizing the study of PPCPs in the environment.

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Occurrence in aquatic environment

ABSTRACT

The occurrence of sixty pharmaceutically active compounds (PhACs), twenty metabolites and eight personal care products (PCPs) in the aquatic environment in Spain and their predicted environmental concentrations (PECs) were calculated and compared with measured environmental concentrations (MECs) obtained from relevant published research. The occurrence in the aquatic environment was calculated through a mass balance approach considering the following: the number of pharmaceutical prescriptions issued, the amount of pharmaceutical discharged without consumption, consumption, self-medication, pharmacokinetics, treatment in wastewater facilities and discharged to aquatic environment. The estimation of consumption of active compounds of pharmaceuticals was conducted by at least one of the following methodologies: number of commercial packages sold, data for the number of defined daily dose per 1000 inhabitants per day (DHD), and pattern of treatment. Comparison of these methodologies for some compounds showed similar estimated consumption ranges. The highest pharmaceutical occurrence in the aquatic environment was for acetaminophen glucuronide, Galaxolide®, Iso-E-super®, acetaminophen, valsartan, amoxicillin, 2-hydroxy-ibuprofen, iopromide, omeprazole, carbamazepine 10, 11-epoxide, iopamidol, salicylic β-D-O-glucuronide acid, Tonalide®, acetylsalicylic acid (ASA), clarithromycin and iohexol, with releases between 5 and 600 t y^{-1}. The relation of PEC/MEC was calculated for 58% of the compounds under study, and 64.7% of them had PEC/MEC ratios between 0.5 and 2. PEC values were mostly overestimated (57.4%). The predicted concentrations for pharmaceutical and personal care products (PPCPs) that had a high occurrence in the aquatic environment were very close to the measured concentrations. This research provides information that had not been calculated and analyzed previously, at least for Spain. Estimation of the PECs for pharmaceutical,

Abbreviations: AD, Average dose in milligrams per body surface area; ADDD, Amount of one defined daily dose for each pharmaceutically active compound; ATC, Anatomical Therapeutic Chemical Classification System; ASA, Acetylsalicylic acid; BSA, Body surface area; DDD, Defined daily dose; DHD, Number of defined daily dose per 1000 inhabitants per day; EAC, Estimation of pharmaceutical active compound consumption; ENH, Estimated excretion of natural hormones; EPA EPI Suite™, Estimation Programs Interface Suite™ developed by the EPA's Office of Pollution Prevention Toxics and Syracuse Research Corporation; IA, Estimate of total number of cases with probability to receive treatment; LOQ, Limit of quantitation; MAD, Mass of active ingredient per dose; ME, Amount of metabolite excretion; MECs, Measured environmental concentrations; NDC, Number of doses per cycle; NP, Number of packages; NPA, Number of people who used different types of antidepressants; NPP, Number of pills in the most commercialized package; NSAIDs, Non-steroidal anti-inflammatory drugs; OAE, Occurrence in aquatic environment; PA, Percentage of absorption; PCAU, Amount of parent compound absorbed but unmetabolized; PCPs, Personal care products; PECs, Predicted environmental concentrations; PFM, Percentage of formation of each metabolite; PhAC, Pharmaceutical active compound; PPCPs, Pharmaceutical and personal care products; PPEU, Percentage of pharmaceutical excreted unchanged; R, Ratio of the number of cases treated with a particular drug and the number of total cases receiving chemotherapy; SM, Self-medication; STPWIN™, Sewage treatment plant program in EPA EPI Suite™; TC, Treatment cycles in one year; UPC, Unabsorbed parent compound; WWTP, Wastewater treatment plant.

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personal care products and metabolites is a useful tool for identifying compounds that should be considered for environmental concern, and such estimations could be used to improve environmental risk assessment studies.

1. Introduction

Concerns about the presence and possible harmful effects of active pharmaceuticals and personal care products (PPCPs) in the environment, have arisen in recent years. Several studies have demonstrated adverse effects from longstanding, low-dose exposures in both aquatic and terrestrial wildlife, although human toxicity related to trace levels of pharmaceuticals in the water supply remains unknown (Strauch, 2011). It is now well-established that these compounds are introduced into the environment, mainly through wastewater effluent from municipal treatment plants, hospital effluents and livestock activities (Halling-Sorensen et al. 1998; Ternes, 1998; Hereber, 2002; Fent et al. 2006; Besse et al. 2008, 2012; Besse and Garric, 2009; Kümmerer and Al-Ahmad, 2010; Vulliet and Cren-Olivé, 2011; Brausch and Rand, 2011). Water effluents are then discharged into rivers, and sludge is spread on the soil as fertilizer, which means these compounds can reach all environmental compartments.

Physicochemical analyses have confirmed the presence of drug residues and their metabolites in all the different compartments of the aquatic environment: wastewater, groundwater, surface water, and drinking water (Houeto et al. 2012). These analyses require highly specialized equipment, and the time and costs associated are also relatively high.

The level of these compounds in the natural environment depends on many factors: their consumption pattern and use, the percentage of wastewater that is collected, the characteristics of the processes used for wastewater treatment and legislation. These features are characteristic of each population, although the trend in the use/consumption of major PPCPs worldwide tends to be similar due to the globalization of the chemical and pharmaceutical industries. Barral and Cohen (1998) claimed that a study of the pharmaceutical industry has shown an ever increasing globalization, particularly for the most innovative drugs. This global reorganization of the pharmaceutical industry is ongoing.

Besse et al. (2008) estimated the consumption of pharmaceutically active compounds (PhACs) and the excretion of some metabolites; they also calculated ratios of predicted environmental concentrations (PECs) with respect to measured environmental concentrations (MECs) in France. In other research, they provided an overview of the occurrence of anticancer drugs in the aquatic environment by calculating PECs based on French consumption data (Besse et al. 2012). Carballa et al. (2008) calculated consumption and excretion rates of some PhACs in Spain in 2003, Kasprzyk-Hordern et al. (2009) estimated the use of drugs in local communities, ter Laak et al. (2010) related environmental concentrations of pharmaceuticals to consumption for the river Rhine and Baran et al. (2011) reported the use and occurrence of sulfonamides in different countries. These studies yielded interesting results and showed that, due to the large amount of PPCP use, more research (experimental and theoretical) is needed.

Generally, the data for PPCP consumption are dispersed and this information is essential for calculating relevant PECs. Therefore, an objective of this work was the use of three different methodologies to estimate the consumption of PhACs: (1) use of the number of packages of PhACs sold based on information from the Ministry of Health and Social Policy (2010), (2) use of parameter "number of defined daily dose per 1000 inhabitants per day" (DHD), and (3) treatment patterns. Consumption of personal care products (PCPs) was estimated by extrapolating data for other countries and years. The first two methodologies have been used in other studies. Stuer-Lauridsen et al. (2000) calculated the annual consumption of pharmaceuticals in Denmark using the numbers of defined daily doses (DDD), Carlsson et al. (2006) used DDD to obtain the total weight of the 100 most sold active pharmaceutical ingredients for human use in Sweden in 2002, Carballa et al. (2008) performed a review of consumption of 17 pharmaceuticals, two musk fragrances and two hormones by the Spanish population in 2003 and ter Laak et al. (2010) calculated and predicted loads of pharmaceuticals in the river Rhine from pharmaceuticals sales. The third methodology is novelty.

The pharmacokinetics in humans, removal in different wastewater facilities and discharge to the aquatic environment for each PPCP were used to estimate their environmental occurrence and to calculate the PECs. These PECs were compared with MECs published in other studies to verify the estimated predictions from the different models.

Consequently, the goals of this work were (1) to use different methodologies to calculate yearly amounts of PPCPs and metabolites in aquatic environments in Spain, (2) calculation of PECs and (3) comparison of PECs with MECs to verify the validity of the selected methods.

This work includes relevant information about PPCPs, some of which have been poorly studied until now, at least in Spain and in many European countries, as well as updated data about consumption patterns, sampling campaigns and resource management.

2. Materials and methods

A diagram showing the methodological procedure used to estimate PPCPs and metabolites in the aquatic environment from their prescription, sale or excretion is shown in Fig. 1. PPCPs selected for this study were based on their human consumption in Spain and their occurrence in the aquatic European environment as shown in the literature. The compounds selected are presented in Tables 1–5.

2.1. Estimation of consumption of pharmaceutically active compounds (EACs)

2.1.1. Number of packages sold (EAC_{NP}) method

To estimate PhAC consumption, the following information was collected: the number of packages (NP) of each type of PhAC sold or dispensed in Spain in 2009 as charged to the National Health System (Ministry of Health and Social Policy, 2010), the number of pills in the most-often sold package (NPP) and the mass of active ingredient per dose (MAD), usually expressed as milligrams per pill. With these data, the estimated amount of PhACs prescribed (EAC_{NP}) was calculated, in kilograms per year, according to Eq. (1) for PhACs shown in Table 1.

$$EAC_{NP}\left(\text{kg y}^{-1}\right) = NP\left(\text{No.packages y}^{-1}\right) * NPP\left(\text{No.pills package}^{-1}\right) * MAD\left(\text{mg pill}^{-1}\right) * 10^{-6}\left(\text{kg mg}^{-1}\right). \quad (1)$$

In this study, the oral administration of PhACs in humans was considered for most compounds. The exception was for X-ray contrast media and cytostatics/cancer therapeutics (can use other routes of administration, e.g., parenteral).

The EAC_{NP} calculation varied for some PhACs that had more than one presentation, such as differing NPPs or MADs; in these cases, the EAC was calculated considering a range of values. EACs were calculated with Eq. (1) for the PhACs shown in Table 1.

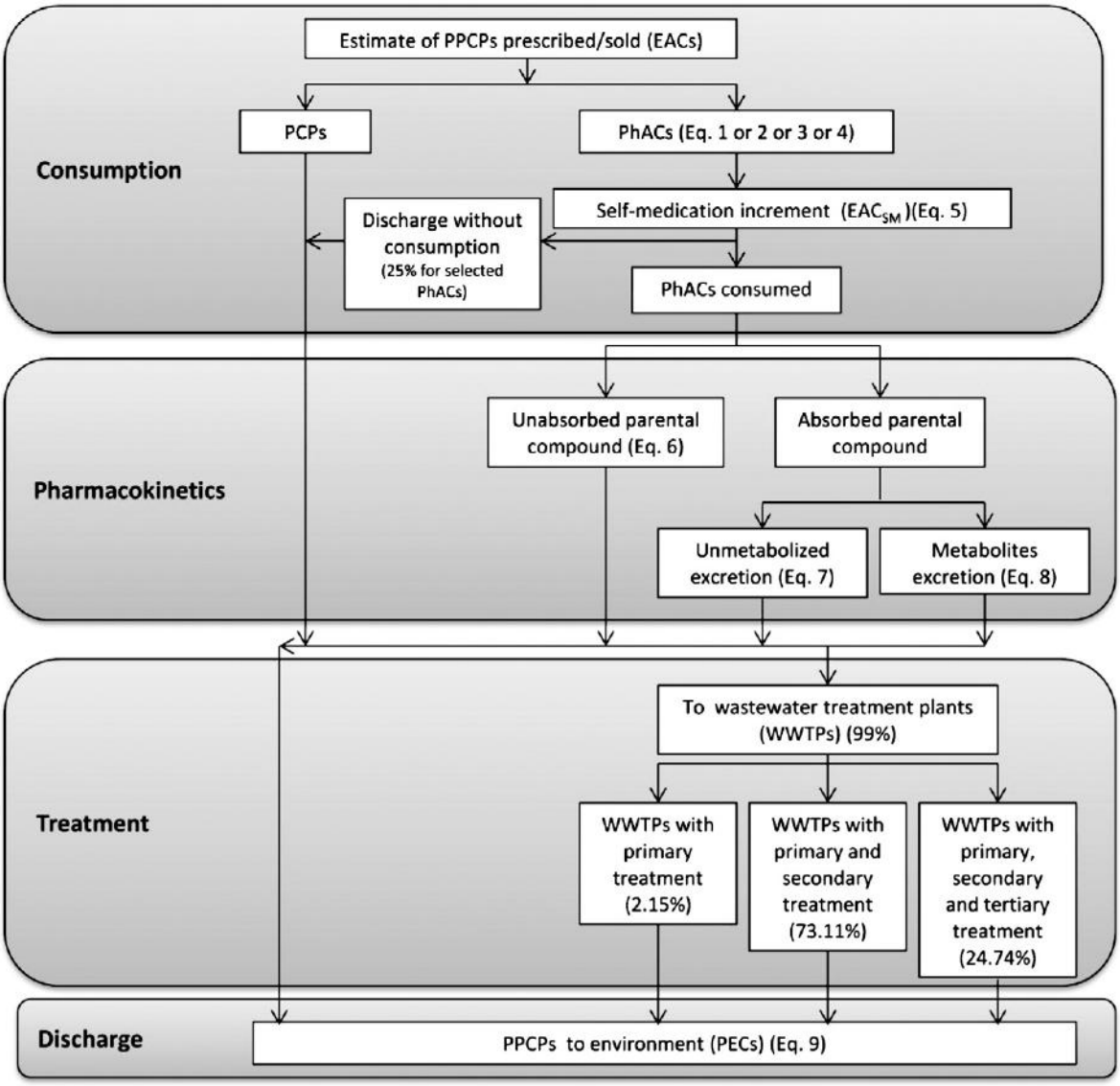

Fig. 1. Diagram of the methodological procedure used to estimate PPCPs and metabolites in the aquatic environment.

X-ray contrast media (iopromide, iohexol and iopamidol) EACs were estimated based on consumption data from a regional government in Spain (Technical Specification 10 DG/06 of the Aragon Government). The consumption per capita was calculated, and then the EACs were extrapolated for the Spanish population as of January 2010.

2.1.2. Number of defined daily dose per 1000 inhabitants per day (EAC_{DHD}) method

The Spanish Agency of Medicines and Health Products uses a "number of defined daily dose per 1000 inhabitants per day (DHD)", for measuring the PhACs consumption. According to the World Health Organization (WHO), sales or prescription data presented in DHDs may provide a rough estimate of the proportion of the studied population treated daily with a particular drug or group of drugs. The DDD is the assumed average maintenance dose per day for a drug used for its main indication in adults. DDD values were consulted on the website of the WHO Collaborating Center for Drug Statistics Methodology (2011) with ATC/DDD index 2012.

The Spanish Agency of Medicines and Health Products reports DHD for different drugs and different years; with this information and the amount of one DDD for each PhAC (ADDD), twenty-one $EACs_{DHD}$ for PhACs were calculated using Eq. (2).

$$EAC_{DHD}\left(\text{kg y}^{-1}\right) = DHD\left(\text{DDD (1000 inh)}^{-1}\text{ d}^{-1}\right) * 365\left(\text{d y}^{-1}\right) * \text{Population(1000 inh)} * ADDD\left(\text{mg DDD}^{-1}\right) * 10^{-6}\left(\text{kg mg}^{-1}\right). \quad (2)$$

The official Spanish population in January 2010 (47,021,031 inhabitants) was obtained from the Spanish National Institute of Statistics (2012). The DHD for the antibiotics sulfamethoxazole, trimethoprim, cefaclor, azithromycin, clarithromycin, erythromycin, roxithromycin, ciprofloxacin, levofloxacin, norfloxacin and moxifloxacin were collected from the Spanish Agency of Medicines and Health Products (2007a, 2009). The DHD for the antiepileptics gabapentin, valproic acid, carbamazepine, topiramate and pregabalin were obtained from the Spanish Agency of Medicines and Health Products (2007b). The DHDs for the

Table 1
Estimation of consumption of pharmaceutically active compounds sold taking into account the number of packages commercialized.

PhACs	NP[a] (No. packages y^{-1})	NPP[b] (No. pills $package^{-1}$)	MAD[c] (mg)	EAC_{NP}[d] (kg y^{-1})
Analgesic/antipyretic				
Acetaminophen	35,527,800	20 or 40	1000	710,560–1,421,110
Antibiotic				
Amoxicillin	10,431,300	12 or 24	1000	125,170–250,350
NSAID				
Ibuprofen	25,222,100	10 or 20	400	100,890–201,780
Platelet aggregation inhibitor				
Acetylsalicylic Acid	25,000,000	30	100 or 300	75,000–225,000
Angiotensin receptor blockers				
Valsartan	4,418,550	28	80–160	9900–19,800
Valsartan with diuretics	4,557,080	28	80–160	10,210–20,420
Irbesartan	3,203,540	28	75–300	6720–26,910
Irbesartan with diuretics	2,751,550	28	75–300	5780–23,110
H_2 blockers				
Omeprazole	49,483,960	14 or 28	20	13,850–27,710
Pantoprazole	6,091,280	14 or 28	40	3410–6820
Lanzoprazole	4,809,220	28	15	2020
Esomeprazole	2,880,150	14 or 28	20 or 40	806–3226
Blood lipid regulators				
Simvastatin	19,056,990	28	10, 20 or 40	5336–21,344
Fluvastatin	3,019,410	28	20, 40 or 80	1691–6763
Atorvastatin	15,281,220	28	10–20	4279–8557
Angiotensin-converting enzyme inhibitor				
Enalapril	13,062,000	30	10	3918.6
Antidepressants and anxiolytics				
Paroxetine	3,620,370	28	10–50	1010–5070
Lorazepam	14,015,690	20 or 50	1 or 5	280.3–3503.9
Bromazepam	12,062,940	20 or 30	1.5, 3 or 6	14–5428
Alprazolam	12,937,060	30 or 50	0.25–1	97–646.85
Escitalopram	4,862,430	28	5–20	680–2720
Hormones				
Levonorgestrel (only for emergency pill)[e]	489,829	1	1.5	0.7347

[a] Number of packages of pharmaceuticals sold or dispensed in Spain in 2009. Ministry of Health and Social Policy (2010). Except for acetylsalicylic acid, enalapril, lorazepam, bromazepam and alprazolam, which were estimated from information reported by General Council of Official Pharmacists Associations (2009).
[b] Number of pills in the most commercialized packages.
[c] Mass of active ingredient per dose.
[d] Estimation of consumption of PhACs by number of packages sold. For occurrence calculations, the average of the range reported was used.
[e] Data estimated in January 1, 2010 from the information of Toquero de la Torre and Zarco Rodríguez (2005).

blood lipid regulators bezafibrate and clofibrate were obtained from the Spanish Agency of Medicines and Health Products (2007c) and Siles Gutiérrez et al. (2001). The DHDs for the non-steroidal anti-inflammatories (NSAIDs) naproxen, diclofenac and ketorolac, were obtained from the Spanish Agency of Medicines and Health Products (2007d), and the DHDs for the anxiolytics alprazolam, bromazepam and lorazepam, were obtained from the from Spanish Agency of Medicines and Health Products (2007e). The DHDs from different years were extrapolated to the population in January 2010.

The Spanish Ministry of Health, Social Services and Equality supplied DHDs for sixteen groups based on the Anatomical Therapeutic Chemical Classification System (ATC), including the hormones 17-α-ethynylestradiol, 17-β-estradiol, levonorgestrel, desogestrel, testosterone, progesterone, norethisterone and megestrol (personal communication, 2012).

2.1.3. Treatment patterns method

Alloza (2009) showed trends in antidepressant use in Spain (approximate number of people who use different types of antidepressants, NPA). It was assumed that, on average, a person follows this type of treatment for eight months per year, based on an analysis of clinical practice guidelines by Qaseem et al. (2008). The $EAC_{Antidepressant}$ was calculated using Eq. (3).

$$EAC_{Antidepressant}\left(\text{kg y}^{-1}\right) = \text{NPA(inh)} * \text{DDD}\left(\text{g inh}^{-1}\ \text{d}^{-1}\right) * 30\left(\text{d month}^{-1}\right) * 8\left(\text{month y}^{-1}\right) * 10^{-3}\left(\text{kg g}^{-1}\right). \quad (3)$$

Cytostatics/cancer therapeutics EACs were estimated using the following information:

- Incidence of different types of cancer in Spain per 100,000 inhabitants (Cabanes et al. 2009). With these data, and the Spanish population in January 2010, the total number of cases with probability to receive treatment was estimated (IA).
- Ratio of the number of cases treated with a particular drug and the quantity of total cases receiving chemotherapy (R). This ratio was calculated using information from Piedra Sánchez (2004).
- Information about cancer types and treatment. Average doses (ADs) were expressed as milligrams per body surface area (BSA). The male BSA (for prostate cancer) was 1.8 m^2, the female BSA (for cervical and breast cancer) was 1.6 m^2 and the average BSA (all other types of cancer) was 1.7 m^2. Other information included the number of treatment cycles in one year (TC) and the number of doses per cycle (NDC).

The cytostatics/cancer therapeutics $EACs_{Cancer}$ were calculated using Eq. (4).

$$EAC_{Cancer}\left(\text{kg y}^{-1}\right) = \text{IA}\left(\text{inh y}^{-1}\right) * \text{R}\left(\text{inh inh}^{-1}\right) * \text{AD}\left(\text{mg No. dose}^{-1}\ \text{m}^{-2}\right) * \text{BSA}\left(\text{m}^2\right) * \text{TC(cycle)} * \text{NDC}\left(\text{No. dose cycle}^{-1}\ \text{inh}^{-1}\right) * 10^{-6}\left(\text{kg mg}^{-1}\right). \quad (4)$$

Table 2
Estimation of consumption of pharmaceutically active compounds sold taking into account the DHD (EAC_{DHD}).

PhACs	DHD[a]	ADDD[b]	EAC_{DHD}[c]
	(DDD(1000 inh)$^{-1}$ d^{-1})	(g DDD^{-1})	(kg y^{-1})
Antibiotics			
Azithromycin	0.9	0.3	4633.92
Cefaclor	0.01	1	171.63
Ciprofloxacin	1.1	1	18,878.9
Clarithromycin	0.6	1	10,297.6
Erythromycin	0.1	1	1716.3
Levofloxacin	0.6	0.5	5148.8
Moxifloxacin	0.3	0.4	2059.5
Norfloxacin	0.3	0.8	4119.0
Roxythromycin	0.01	0.3	51.5
Sulfametoxazole	0.3	2	10,297.6
Trimethoprim	0.01	0.4	68.65
Antiepileptics			
Carbamazepine	1.2	1	20,595.2
Gabapentin	1.6	1.8	49,428.5
Pregabalin	1	0.3	5148.8
Topiramate	1.1	0.3	5663.68
Valproic acid	1.8	1.5	46,339.2
Anxiolytics			
Alprazolam	17.64	0.001	302.75
Bromazepam	2.2	0.01	377.58
Lorazepam	19.67	0.0025	843.97
Blood lipid regulators			
Bezafibrate	0.6	0.6	6178.56
Clofibrate	0.01	2	343.25
NSAIDs			
Diclofenac	7.9	0.1	13,558.5
Ketorolac	0.54	0.03	278.04
Naproxen	5.15	0.5	44,193.9

[a] Number of defined daily dose per 1000 inhabitants per day.
[b] Amount of one defined daily dose to each PhAC, in grams.
[c] Estimation of consumption of PhACs by DHD method.

Table 4
Estimation of consumption of antidepressant taking into account the treatment pattern ($EAC_{Antidepressant}$).

Antidepressant	People using antidepressants (NPA) (inh)	DDD[a] (g inh^{-1} d^{-1})	Average time of treatment (month)	$EAC_{Antidepressant}$[b] (kg y^{-1})
Fluoxetine	600,000	0.02	8	2880
Paroxetine	680,000	0.02	8	3264
Fluvoxamine	80,000	0.10	8	1920
Escitalopram	760,000	0.01	8	1824
Sertraline	400,000	0.05	8	4800

[a] Defined daily dose.
[b] Estimation of consumption of PhACs by treatment pattern method.

2.2. Consumption of PCPs

Six fragrances and two bactericides/preservatives were considered in this study. Eriksson et al. (2008) reported that methylparaben and propylparaben consumption was 0.3 and 0.1 t y^{-1}, respectively, in Denmark in 2004. The fragrances studied were 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta(g)-2-benzopyran (HHCB or Galaxolide®), 7-acetyl-1,1,3,4,4,6-hexamethyltetrahydronaphthalene (AHTN or Tonalide®), [1,2,3,4,5,6,7,8-octahydro-2,3,8,8-tetramethyl naphthalen-2yl] ethan-1-one (OTNE or Iso-E-super®), acetyl-1,1,2,3,5-hexamethyldihydroindene (AHMI or Phantolide®), musk xylene and musk ketone, which are widely used in Europe. Bester (2009) and Alder et al. (2007) indicate that the consumptions of OTNE, HHCB, AHTN, musk xylene and musk ketone in Europe in 1998 were 3000, 1473, 385, 86 and 40 t annually, respectively. For the same year and geographic area, the AHMI consumption was 19 t y^{-1} (Alder et al. 2007).

The methodology described by Carballa et al. (2008) was used to determine PCP consumption in Spain in 2010. The estimation of

Table 3
Estimation of consumption of hormones sold taking into account the DHD (EAC_{DHD}).

ATC group[a]	Base formulation	DHD[b]	ADDD[c]	EAC_{DHD}[d]
		(DDD(1000 inh)$^{-1}$ d^{-1})	(mg DDD^{-1})	(kg y^{-1})
	One compound			
G03AA91[e]	17-β estradiol	0.000008	10	0.0013
G03AC09	Desogestrel	0.83	0.075	1.0684
G03BA03	Testosterone	0.30	0.3	1.5446
G03CA03	17-β estradiol	0.38	0.050	0.3261
G03DA04	Progesterone	0.3547	300	1826.3
G03DC02	Norethisterone	0.26	5	22.310
G03FA15[e]	17-β estradiol	0.04	2	1.5446
G03FB06[e]	17-β estradiol	0.024	2	0.8238
G03HB01[e]	17-β estradiol	0.069	2	2.3684
G03HB01[e]	17-α ethinylestradiol	2.44	0.026	1.0888
L02AB01	Megestrol	0.4955	160	1360.7
	Two compounds			
G03AA07	17-α ethinylestradiol	0.21	0.030	0.1081
	Levonorgestrel	0.21	0.100	0.3604
G03FA01	17-β estradiol	0.16	1	2.7460
	Norethisterone	0.16	0.5	1.3730
G03FA01	17-β estradiol	0.007	1.5	0.1802
	Norethisterone	0.007	0.7	0.0841
G03FA11	17-β estradiol	0.118	2	4.0504
	Levonorgestrel	0.118	0.075	0.1519
G03FB05	Norethisterone	0.096	0.36	0.5931
	17-β estradiol	0.096	1.8	2.9657

[a] The Anatomical Therapeutic Chemical (ATC) Classification System.
[b] Number of defined daily dose per 1000 inhabitants per day from Spanish Ministry of Health, Social Services and Equality (Personal communication, February 13, 2012).
[c] Amount of one defined daily dose to each PhAC, in milligrams. The DDDs of many of the hormone preparations may vary considerably with the route of administration due to substantial differences in bioavailability. These values are the average of the most commercialized presentations.
[d] Estimation of consumption of PhACs by DHD method calculated for the Spanish population on January 1, 2010.
[e] These products contain another compound not considered in this study.

Table 5
Estimation of consumption of cytostatics/cancer therapeutics taking into account the treatment pattern (EAC_{Cancer}).

Type of cancer	Total incidence	IA[a]	Pharmaceutically active compounds																	
			Mitomycin					Gemcitabine				Cyclophosphamide				Ifosfamide				
			R[b]	AD[c]	TC[d]	NDC[e]	EAC[f]	R	AD	TC	NDC	EAC	R	AD	TC	NDC	EAC	R	AD	TC	NDC	EAC
Stomach	24.3	11,426	0.23045	10	3	1	0.13	0.00412	1200	6	2	1.15	0		0	0	0	0		0	0	0
Colon and rectal	79.8	37,523	0.00303	20	6	1	0.02	0	0	0	0	0	0		0	0	0	0		0	0	0
Lung	82.1	38,604	0	0	0	0	0	0.45584	1250	6	2	448.73	0	1000	4	1	0	0.00997	1600	6	2	12.56
Cervical	24.5	11,520	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05263	1000	6	2	11.64
Breast	93.6	44,012	0.02539	10	6	1	0.11	0.03440	2500	6	1	36.33	0.34808	600	6	1	30.71	0.00737	1000	15	3	23.36
Prostate	77.2	36,300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Others	680.3	319,884	0.04708	30	17	1	13.06	0.05018	1200	6	2	392.96	0.25504	1500	6	1	1248.24	0.02069	1500	6	3	303.83
Total (kg y^{-1})							13.32					879.17					1278.95					351.39

[a] IA: Estimate of total number of cases with probability to receive treatment (inh y^{-1}).
[b] R: Ratio of the number of cases treated with a particular drug and the number of total cases receiving chemotherapy.
[c] AD: Average dose (mg no. dose^{-1} m^{-2}).
[d] TC: Treatment cycles (cycle).
[e] NDC: Number of doses per cycle per inhabitant (no. doses cycle^{-1} inh^{-1}).
[f] EAC_{Cancer}: Estimation of consumption of cancer PhACs by treatment pattern (kg y^{-1}).

local consumption was based on the per-capita use in the EU and Denmark and extrapolated to the number of inhabitants in Spain in January 2010.

2.3. Self-medication and discarding without consumption

Self-medication was estimated from information from the Spanish National Health System as reported in the National Health Survey 2006 (Spanish National Institute of Statistics, 2012). This survey reported the percentage of self-medication (SM) of some non-prescribed and prescribed medicines. These percentages were considered for calculations to estimate PhAC total consumption including self-medication (EAC_{SM}) using Eq. (5).

$$EAC_{SM}\left(\text{kg } y^{-1}\right) = EAC\left(\text{kg } y^{-1}\right) * \{1 + [SM(\%)/100]\}. \quad (5)$$

There were few data available to estimate the relative contribution of improper disposal of PhACs (intentional releases) with regard to the total release into the environment (NRDC, 2009). Barceló and López de Alda (2008) reported as a first estimate that 25–30% of the Spanish population discarded expired PhACs in sewers and therefore to the local wastewater treatment plant. For this study, it was assumed that 25% of over-the-counter pharmaceuticals that do not require prescriptions (analgesics, antipyretics and NSAIDs), would be disposed of as expired pharmaceuticals into the sewer.

2.4. Pharmacokinetics

The quantity of PhACs and metabolites that are discharged into the environment depends on their pharmacokinetics in humans and animals. Parent compounds can be excreted directly or can be metabolized to a few or many metabolites after their absorption. Both processes usually occur. Percentages of PhACs absorption, excretion and metabolite formation were obtained from different authors and the database. The following relations were considered:

$$UPC\left(\text{kg } y^{-1}\right) = EAC\left(\text{kg } y^{-1}\right) * [1-(PA(\%)/100)] \quad (6)$$

$$PCAU\left(\text{kg } y^{-1}\right) = EAC\left(\text{kg } y^{-1}\right) * [PA(\%)/100] * [PPEU(\%)/100] \quad (7)$$

$$ME\left(\text{kg } y^{-1}\right) = EAC\left(\text{kg } y^{-1}\right) * [PA(\%)/100] * [PFM(\%)/100] \quad (8)$$

where UPC is the unabsorbed parent compound, in kg y^{-1}; PA is the percentage of absorption of the PhAC; PCAU is the amount of the parent compound absorbed but unmetabolized, in kg y^{-1}; PPEU is the percentage of PhACs excreted unchanged; ME is the amount of metabolites excretion, in kg y^{-1}, and PFM is the percentage of formation of each metabolite.

To estimate the excretion of natural hormones (ENH), the methodology described in Carballa et al. (2008) was used. These authors reported the excretion rates of estrone and 17-β estradiol by five population groups: men, menstruating women, pregnant women, menopausal women and postmenopausal women, based on hormone replacement therapies that were studied in detail in Johnson and Williams (2004). The total amounts excreted by the Spanish population were calculated by multiplying the reported excretion rates by the number of persons belonging to each group (Carballa et al. 2008). Natural excretion of 17-β estradiol was added to the excretion estimate based on consumption of medicines that contain this compound.

Pharmacokinetic parameters were not considered for PCPs. It was considered that PCPs consumed are discharged to the wastewater treatment plant or environment without changes.

Metabolites studied in this research were selected based on one or more of the following reasons: (1) high consumption of the parent compound, (2) high percentage of formation, (3) high occurrence in aquatic environments, (4) high removal difficulty in a wastewater treatment plant (WWTP), and (5) negative environmental impact.

Metabolites that are conjugates with a functional group (such as glucuronide, sulfate, or acetate) were excluded except for acetaminophen glucuronide due to the high occurrence of its parent compound. It is known that cleavage of conjugates can occur in the sewer system or during primary treatment (Suárez et al. 2008).

2.5. Treatment

The amount of each PPCP that is finally discharged to aquatic environments (with or without treatment) can be estimated with the following data: (1) percentage of wastewater treated, (2) types of wastewater treatment plants and (3) total number of each type of wastewater treatment plant installed in the geographic area and in the period of time under study. In Spain, approximately 99% of the produced wastewater is discharged to treatment plants (DG environment, 2011); therefore, 1% is discharged directly to the environment without treatment. According to the Observatory of Sustainability in Spain (2009), for a total of 1714 urban WWTPs, 37 (2.16%) have only primary (1°) treatment, 1253 (73.10%) have 1° plus secondary (2°) treatment and 424 (24.74%) have 1°, 2° and tertiary (3°) treatment.

The removal ratios of each compound and process were collected from the literature. In the absence of available data, STPWIN™ of the U.S. Environmental Protection Agency (US EPA, 2009) was used for estimating these removal rates.

According to information from the US EPA EPI Suite™ (2009), the STP Model is based on fugacity principles and, similar to other such models, attempts to predict the fate of an organic chemical in a particular environment. In this case, the environment is a conventional WWTP that uses activated sludge as a secondary treatment, as most do in Spain. The model estimates the fate of a chemical present in the influent as it becomes subject to removal by evaporation, biodegradation or other degradation processes, sorption to sludge, and loss in the final effluent. The most critical and uncertain variable is the biodegradation rate and its dependence on biomass concentration.

2.6. Occurrence in aquatic environment (OAE), PECs and MECs

The OAE was calculated for each compound according to the methodology described in Fig. 1 and by using EAC_{SM}, pharmacokinetics and treatment data.

The PEC for each PPCP and metabolite was calculated by dividing the OAE by an estimate of the annual (2009) Spanish wastewater production (4.67×10^{12} L y^{-1}) (Spanish National Institute of Statistics, 2012) (see Eq. (9)).

$$\mathrm{PEC}\left(\mathrm{ng\ L^{-1}}\right) = \left[\mathrm{OAE}\left(\mathrm{kg\ y^{-1}}\right) * 10^{12}\left(\mathrm{ng\ kg^{-1}}\right)\right] / \left[4.67\times10^{12}\left(\mathrm{L\ y^{-1}}\right) * \mathrm{Dilution\,factor}\right]. \quad (9)$$

This calculation was adapted from Kostich et al. (2010), and it agrees with the model proposed by the European Medicine Agency guideline (EMEA, 2006) that proposes a dilution factor from WWTP effluent to surface waters (default value set to 10). The PECs of PPCPs and metabolites were compared with MECs. MECs were reviewed in twenty-five published papers; at least 90% of them correspond to data supplied to Spain and Europe, and they present the analytical methodology that was conducted for obtaining their results; 96% of them were published between 2004 and 2012. Only data for surface water was used, except for (1) two compounds (amoxicillin, and simvastatin) for which surface water data were not available and WWTP concentrations of effluents were used, and (2) compounds that did not have MECs available (flutamide, gemcitabine) so PECs calculated by other authors were used.

The ratio PEC/MEC was calculated to obtain the proportion between these two parameters and to verify the mass-balance approach and the viability of models.

3. Results and discussion

3.1. Estimation of PPCP consumption or use

Tables 1–5 show the results for EACs for a wide variety of pharmaceutically active compounds evaluated using the various methodologies previously described. The three different methods were used because the data were dispersed, in different units, and differences between the compounds in their use commercial presentation made it impossible to use all three methodologies for all PPCPs.

The EAC_{NP} is a quick and straightforward methodology, but there are factors such as a large range of different commercial presentations of some PhACs or the dispersion of economic data that limits its use to the most commonly used drugs. Table 1 presents twenty EACs calculated through the EAC_{NP} methodology. The EAC_{NP} values have upper and lower limits based on differing NPP and MAD. An average of this range was used for occurrence calculations.

The DHD parameter is an indirect measure of the number of packages sold for a defined population group and period of time (e.g., a year). Generally, DHD data were found for most commercialized PhACs or for those where consumption control is necessary because of possible adverse effects (e.g., microbial resistance caused by antibiotics). EAC_{DHD} for twenty-four PhACs are presented in Table 2. DHD information for more specific PhACs could be obtained from the pharmaceutical industry or government offices, as shown in Table 3 for hormones.

If the DHD or the number of packages prescribed or sold is unknown, another way to estimate the amount of PhACs consumed is through an analysis of studies of the incidence of diseases that are treated with these PhACs and their pattern of treatment. These two parameters could vary for different populations. To verify the possibility of using this methodology, EACs were calculated for antidepressants (Table 4) and cytostatic/cancer therapeutic pharmaceuticals (Table 5).

For some compounds, it was possible to estimate their consumption using more than one methodology so that results could be compared. Anxiolytic consumption (lorazepam, alprazolam and bromazepam) was estimated using both EAC_{NP} and EAC_{DHD} methodologies (Tables 1 and 2). For these three compounds, the EAC_{DHD} values were within the ranges estimated with the EAC_{NP} methodology. The alprazolam EAC_{DHD} estimate was close to the mean value of the EAC_{NP}, in contrast to lorazepam and bromazepam, whose EAC_{DHD} values were closer to the lower limits of the EAC_{NP}. Obviously, the wide range of values of the EAC_{NP} methodology makes it less precise.

Antidepressant (paroxetine and escitalopram) consumption was estimated with EAC_{NP} (Table 1) and the pattern of treatment (Table 4) methodologies. The pattern of treatment consumption results for both compounds were in the ranges of the EAC_{NP} estimations and very close to the mean values. Other comparisons could not be made due to the unavailability of data for the remaining compounds.

EACs for acetaminophen, ibuprofen, amoxicillin, acetylsalicylic acid (ASA), Iso-E-super® and Galaxolide® were in the range of 100 to 1500 t y^{-1} and had the highest values for the studied compounds. The EACs for naproxen, gabapentin, Tonalide®, valproic acid, irbesartan, valsartan, iopromide, omeprazole, carbamazepine, ciprofloxacin, diclofenac, iopamidol, simvastatin, clarithromycin, sulfamethoxazole, musk xylene, iohexol, atorvastatin, bezafibrate, topiramate, pantoprazole, levofloxacin, pregabalin and musk ketone were between 5 and 60 t y^{-1}. Azithromycin, sertraline, norfloxacin, fluvastatin,

enalapril, paroxetine, fluoxetine, methylparaben, Phantolide®, moxifloxacin, lanzoprazole, esomeprazole, flutamide, lorazepam, fluvoxamine, progesterone, erythromycin, escitalopram, megestrol, cyclophosphamide and bromazepam EACs were between 1 and 5 t y^{-1}. The EACs of the other compounds considered are less than 1 t y^{-1}.

The EACs of some PhACs estimated for Spain were compared with EACs for other European countries (Table 6). In general, the estimated consumption in countries such as Germany, France, Sweden and Switzerland were higher than the estimated consumption in Spain, taking into account population densities and the year for which these values were reported. Consumption of diclofenac in Germany and France, clarithromycin in Switzerland and France and sulfamethoxazole and tamoxifen in France were similar to consumption in Spain. Other compounds such as ibuprofen showed less consumption in Germany, Switzerland and France than in Spain.

Besse et al. (2012) and Besse et al. (2008) calculated consumption and PECs of PhACs in 2004 and 2008 in France. For gemcitabine, ifosfamide, flutamide, and mitomycin, consumption in Spain was about three, four, five and six times higher than in France, respectively. The consumptions of atorvastatin, clarithromycin, diclofenac, fluoxetine, sertraline and tamoxifen in these two countries were of the same order of magnitude, as shown in Table 6.

Twelve compounds included in this research were studied before for Spain in a similar analysis conducted by Carballa et al. (2008). In that earlier study, the authors calculated EACs by the annual prescribed items of selected PhACs and total quantities consumed in Spain. For five of these compounds (bezafibrate, carbamazepine, ibuprofen, sulfamethoxazole and iopromide), the EAC values in both studies were of the same order taking into account that the studies were performed in different years.

The differences observed within consumption data between European countries or in the same country but in different years could be due to changes in prescribing patterns (e.g., sale of packages with the exact number of pills required based on the disease and not a standard number of pills), decreased doses in new formulations (e.g., drugs containing hormones may have improved formulations to decrease secondary effects, as in progestins), or differences in the information sources.

Table 6
PhACs consumption for European countries.

Compound	EACs[a]									
	Germany		Switzerland		France		Sweden	Spain		
	(kg y^{-1})	(mg y^{-1} inh^{-1})	(kg y^{-1})	(mg y^{-1} inh^{-1})	(kg y^{-1})	(mg y^{-1} inh^{-1})	(mg y^{-1} inh^{-1})[b]	(mg y^{-1} inh^{-1})[b]	(kg y^{-1})[c]	(mg y^{-1} inh^{-1})[c]
Acetaminophen	n.a.	n.a.	n.a.	n.a.	3,303,077[d]	54,389.5	n.a.	n.a.	1,065,835	22,667.7
Acetylsalicylic acid	n.a.	n.a.	n.a.	n.a.	396,212[d]	6524.2	n.a.	n.a.	150,000.0	3190.1
Alprazolam	n.a.	n.a.	n.a.	n.a.	178[d]	2.9	n.a.	n.a.	302.8	6.4
Amoxicillin	n.a.	n.a.	n.a.	n.a.	333,223[d]	5487.0	n.a.	n.a.	187,760	3993.2
Atorvastatin	n.a.	n.a.	n.a.	n.a.	7924[d]	130.5	n.a.	n.a.	6418.0	136.5
Azithromycin	n.a.	n.a.	n.a.	n.a.	4073[d]	67.1	n.a.	n.a.	4634.0	98.6
Bezafibrate	39,158[e]	475.2	1574[e]	215.6	20,852[d]	343.4	66.7	92.6	6254.6	133.0
Bromazepam	n.a.	n.a.	n.a.	n.a.	2604[d]	42.9	n.a.	n.a.	377.6	8.03
Carbamazepine	83,299[e]	1010.9	6260[e]	857.5	33,364[e]	554.3	820.2	463.0	20,595.2	438.0
Clarithromycin	12,360[e]	150.0	1700[e]	232.9	16,889[e]	276.1	n.a.	n.a.	10,864.0	231.0
Ciprofloxacin	n.a.	n.a.	n.a.	n.a.	12,186[d]	200.7	n.a.	n.a.	18,879.0	401.5
Cyclophosphamide	n.a.	n.a.	n.a.	n.a.	305.7[f]	4.9	n.a.	n.a.	1279.0	27.2
Diclofenac	78,579[e]	953.6	6819[e]	934.1	22,640[e]	370.1	375.9	747.7	17,395.6	369.9
Escitalopram	n.a.	n.a.	n.a.	n.a.	4.6[d]	0.08	n.a.	n.a.	1824.0	38.8
Fluoxetine	n.a.	n.a.	n.a.	n.a.	3740[d]	61.6	n.a.	97.2	2914.6	62.0
Flutamide	n.a.	n.a.	n.a.	n.a.	521[f]	8.3	n.a.	n.a.	1987.4	42.3
Fluvoxamine	n.a.	n.a.	n.a.	n.a.	1121[d]	18.5	n.a.	n.a.	1920.0	40.8
Gemcitabine	n.a.	n.a.	n.a.	n.a.	379.3[f]	6.1	n.a.	n.a.	879.2	18.6
Ibuprofen	250,792[e]	3043.6	22,471[e]	3078.2	58,353[e]	953.8	7864.3	6391.2	218,527	4647.5
Ifosfamide	n.a.	n.a.	n.a.	n.a.	121.4[f]	1.9	n.a.	n.a.	351.4	7.5
Iohexol	8053[e]	97.7	4614[e]	632.1	46,774[e]	764.5	n.a.	n.a.	10,761.0	228.9
Iopamidol	38,165[e]	463.2	2739[e]	375.2	34,540[e]	564.6	n.a.	n.a.	14,866.7	316.2
Iopromide	97,817[e]	1187.1	8965[e]	1228.1	12,810[e]	209.4	n.a.	463.0	23,710.5	504.3
Levonorgestrel	n.a.	n.a.	n.a.	n.a.	90[g]	1.38	n.a.	n.a.	1.2	0.03
Lorazepam	n.a.	n.a.	n.a.	n.a.	585[d]	9.6	n.a.	n.a.	844.0	17.9
Mitomycin	n.a.	n.a.	n.a.	n.a.	3.01[f]	0.05	n.a.	n.a.	13.3	0.3
Naproxen	n.a.	n.a.	n.a.	n.a.	37,332[d]	614.7	n.a.	986.1	56,700.8	1205.9
Omeprazole	n.a.	n.a.	n.a.	n.a.	8045[d]	132.5	n.a.	n.a.	20,780	441.9
Pantoprazole	n.a.	n.a.	n.a.	n.a.	5287[d]	87.1	n.a.	n.a.	5115.0	108.8
Paroxetine	n.a.	n.a.	n.a.	n.a.	5515[d]	90.8	n.a.	n.a.	3264.0	69.4
Progesterone	n.a.	n.a.	n.a.	n.a.	10,000[g]	153.7	n.a.	n.a.	1826.3	38.8
Roxythromycin	7359[e]	89.3	149[e]	20.4	4182[e]	68.4	n.a.	9.3	54.3	1.2
Sertraline	n.a.	n.a.	n.a.	n.a.	6224[d]	102.5	n.a.	n.a.	4800.0	102.1
Simvastatin	n.a.	n.a.	n.a.	n.a.	6943[d]	114.3	n.a.	n.a.	13,340	283.7
Sulfamethoxazole	53,600[e]	650.5	2300[e]	315.1	17,519[e]	286.4	160.4	294.0	10,864	231.0
Tamoxifen	n.a.	n.a.	n.a.	n.a.	377[f]	6.0	n.a.	n.a.	257.0	5.5
Trimethoprim	12,183[e]	147.8	520[e]	71.2	20,603[e]	336.8	n.a.	n.a.	72.5	1.5
17-α thynylestradiol	48.2[b]	0.58	3.96[b]	0.54	n.a.	n.a.	0.11	0.3	1.2	0.03

n.a.: Data not available.
[a] EACs: Estimation of consumption of PhACs.
[b] Data from Carballa et al. (2008) for 2005 in Sweden for 2001 in Germany, for 2000 in Switzerland and for 2003 in Spain.
[c] Data calculated in this study for Spanish population in January 2010: 47.02×10^6 inhabitants.
[d] Data from Besse et al. (2008) for 2004 in France.
[e] Data from ter Laak et al. (2010) for Germany, Switzerland and France.
[f] Data from Besse et al. (2012) for 2008 in France.
[g] Data from Vulliet and Cren-Olivé (2011) for 2008 in France.

The consumption of most of these PhACs has increased in recent years. Spanish statistics reported that consumption of NSAIDs, from 1999 to 2003, increased by 93.6% (García del Pozo and De Abajo, 2005). The use of H_2 blockers grew by 200.8% from 2000 to 2008 (García del Pozo, 2009). The total consumption of antidepressants increased by 107% between 1997 and 2002 (Martín, 2005). Between 1997 and 2006, the use of anxiolytics and hypnotics increased by 113.6% (García del Pozo et al. 2006). It is expected that the use of ASA will increase due to population aging and prescription versatility.

Antibiotic consumption is another aspect to highlight. Spain, as is the case for other countries in southern Europe, has been characterized by a high use of antibiotics and, at the same time, a high rate of resistance (Lázaro and Montero, 2010).

Therefore, generation of waste of PhACs is an issue that will continue to increase significantly.

3.2. Self-medication

Self-medication is a widespread practice in today's society. For instance, in Spain, pain reliever consumption by self-medication is almost equivalent to the consumption by prescription (Hours Pérez et al. 2007). Self-medication with pain relief drugs falls into the following percentages: 40.4% for ibuprofen, 37% for acetaminophen and 22.5% for acetylsalicylic acid (Hours Pérez et al. 2007).

Similarly, the self-medication (SM) percentages for other non-prescription medicines are 6.6% for antibiotics; 3.7% for tranquilizers, relaxing and sleeping pills; 0.76% for blood pressure control; 7.3% for stomach medicines or digestive disorder medications; 1.2% for antidepressants and stimulants; 5.3% for contraceptives; and 1.2% for blood lipid regulators (Spanish National Institute of Statistics, 2012). These differences in SM made more inaccurate the estimation of PPCPs in the environment and could be the difference between to have adverse effects and not.

Self-medication percentages were considered for consumption calculations of PhACs (EAC_{SM}). Self-medication was not considered for antiepileptics, hormones (except 17-α-ethynylestradiol), cytostatics/anti-cancer pharmaceuticals and X-ray contrast media because these drugs are for hospital use or have controlled prescription and sale.

Patient non-compliance with treatment directions is an additional aspect of self-medication and another variable that could have been considered. However, it was excluded in this study because there are little data available in Spain on this topic.

3.3. Pharmacokinetics

Pharmacokinetics is an important and decisive factor in the occurrence of PhACs and their metabolites in the environment.

Pharmacokinetic processes are quite complex. For this study, three pharmacokinetic parameters (absorption, metabolism and elimination) are considered to estimate PhACs and metabolite occurrence. The pharmacokinetics are analyzed based on the following steps: (1) each PhAC has a particular absorption rate and any compound not absorbed is excreted in its parent form, (2) a fraction of the absorbed compound is excreted as a metabolite or parent compound, and (3) the average rates of absorption, excretion and formation of metabolites are considered when data are available from multiple sources. These calculations and estimations are shown in Table 7 for natural hormones, Table 8 for PhACs and in Table 9 for metabolites.

The absorption rates of thirty-nine PhACs are greater than 70%. After absorption, only 23% of PhACs are going to be excreted as parent compounds at a high proportion (formation of metabolites below 30%). This finding indicates that metabolite formation is high for many compounds. Therefore, if absorption and metabolite formation are excluded from the estimates of occurrence, their exclusion can cause significant deviations from reality. Despite this fact, some recent studies have considered pharmacokinetic parameters in their estimates (Besse et al. 2008; Carballa et al. 2008) while another has not (Kostich et al. 2010).

The rates of excretion of nineteen metabolites were estimated using the pharmacokinetic parameters and EACs of acetaminophen, ibuprofen, diclofenac, ASA, carbamazepine, clofibrate, sulfamethoxazole, escitalopram, paroxetine, sertraline and fluoxetine.

Estrone and 17-β estradiol were included as natural hormones in human excretion, and estimates of their excretion, based on the particular characteristics of the Spanish population, are shown in Table 7. The results show a similar behavior as reported by Carballa et al. (2008) for the year 2003, with the only difference being that the percentage of menstruating females was 6.2% less in 2009 than in 2003. Hence, the estimation of the occurrence of these two compounds is not directly proportional to the growth in the total population but depends mainly on the menstruating, pregnant or postmenopausal female populations because of the high excretion rate of these population groups.

Acetaminophen glucuronide was the metabolite that had the highest amount excreted, approximately 600 t y^{-1}. Amounts excreted of ibuprofen carboxylic acid, 2-hydroxy-ibuprofen, carbamazepine 10, 11-epoxide, salicylic β-D-O glucuronide acid, salicylic acid, 4-hydroxy-diclofenac, sertraline carbamoyl glucuronide, a metabolite from paroxetine (4-[[(3S,4R)-4-(4-fluorophenyl)piperidin-3-yl] methoxy]-2-methoxyphenol) and N-desmethyl sertraline were between 1 and 51 t y^{-1}. The other ten metabolites had occurrence of less than 1 t y^{-1} (Table 9).

There are few evidences of similar previous studies that have calculated the occurrence in aquatic environment of most metabolites considered in this research. Only four metabolites included in this study were analyzed before in France (Besse et al. 2008). Excretion rates were on the same range in both studies.

Table 7
Estimation of natural excretion of estrone and 17-β estradiol.

Population group	Percentage of total population (%)[a]	Population in each category (inh)[a]	Estrone		17-β estradiol	
			Excretion (μg inh^{-1} d^{-1})	ENH[b] (kg y^{-1})	Excretion (μg inh^{-1} d^{-1})	ENH[b] (kg y^{-1})
Males	49.4	23,228,389	2.6	22.04	1.8	15.26
Females	50.6	23,792,642				
Menstrual[c]	25.0	11,747,631	11.7	50.17	3.2	13.72
Pregnant	1	470,210	550	94.39	393	67.45
Menopausal	17.5	8,229,713	1.8	5.41	1	3.00
Postmenopausal[d]	2.8	1,316,589	28.4	13.65	56.1	26.96
Total				185.66		126.39

[a] Estimated according to Spanish National Institute of Statistics (2012) data and Carballa et al. (2008) methodology.
[b] ENH: Excretion of natural hormones.
[c] Females between 15 and 50 years old.
[d] Hormone replacement therapy.

3.4. Treatment

For primary and secondary conventional treatments, the removal rates for each compound were considered based on the published research in Spanish treatment plants or, if there were not enough data available, reference values were taken from other countries or estimated from the US EPA EPI Suite™ (STPWIN™). These values are shown in Table 8 for PhACs and in Table 9 for metabolites and PCPs.

Although real data for PPCPs removal in treatment facilities are the most appropriate values to estimate their occurrence in aquatic environments, to date, these figures have not been sufficiently investigated for many PPCPs, so models are needed to estimate their behavior, at least in conventional WWTPs. While STPWIN™ presents some deficiencies (e.g., low and similar percentage of primary removal for most PPCPs, which can be questioned), it has been successfully used in other studies (Jones et al. 2002) and by the US Environmental Protection Agency.

The removal rates for some compounds (approximately 50% of the PhACs, primarily the most known and most consumed, e.g., analgesics, antipyretics and antibiotics) have been obtained from different

Table 8
PECs of PhACs under study, based on consumption, pharmacokinetics and elimination in wastewater facilities in Spain in 2009, and MECs from published data.

PhACs	Pharmacokinetics (%)		Elimination in urban WWTP* (%)		EAC_{SM}^{*} (kg y^{-1})	OAE*** (kg y^{-1})	PECs† (ng L^{-1})	MECs†† (ng L^{-1})
	Absorption	Excretion[c]	Primary (1°)	1° plus secondary++				
Acetaminophen	95[a]	6	0.26+	97[s,t,u]	1,460,193.95	23,267.40	498.23	307.0[ah]
Acetylsalicylic acid	65[a,b,c]	0	0.29+	90[u]	183,750.00	10,563.69	226.20	160[am]
Alprazolam	90[c,§]	10	0.56+	2.37+	385.81	60.22	1.29	n.d.[aj]
Amoxicillin	100[a,d]	60	0.27+	88[s]	198,086.80	15,256.93	326.70	40[j,•]
Atorvastatin	70[e,] •••	1[ay]	56.25+	58[v]	6496.94	715.38	15.31	2.99[ah]
Azithromycin	38[a,d,e]	12	17.87+	30+	4888.79	1933.32	41.40	14.73[ah]
Bezafibrate	100[e]	50	25.06+	90[s,t,w]	6254.56	234.02	5.01	3.4[aj]
Bromazepam	90[c,e]	2	0.52+	2.29+	1032.11	100.13	2.144	13[as]
Carbamazepine	90[c,g,h]	12	0.92+	29[t,x,y]	20,595.21	2595.31	55.57	31.28[ah]
Cefaclor	100[a,c]	80	0.25+	1.86+	181.07	119.60	2.56	n.a.
Ciprofloxacin	70[a,c]	70	0.25+	85[s,z]	19,917.29	2402.03	51.44	28.02[ah]
Clarithromycin	50[e]	58	3.51+	20[s,aa]	10,863.97	5820.23	124.63	88.83[ah]
Clofibrate	100[a,i]	1	8.78+	16.47+	347.48	2.45	0.052	n.a.
Cyclophosphamide	95[a,i]	15	0.26+	1.86+	1278.96	203.28	4.35	<0.05 to 10.1[ap]
Desogestrel	100[a,i,j,k]	2	56.74+	89.91+	1.06	0.00215	0.00005	n.a.
Diclofenac	55[c,d,h]	15	20	60[t,u,w,ab]	17,395.57	3963.10	84.86	89.53[ah]
Enalapril	60[e]	20	0.92+	60[s]	3929.64	725.20	15.53	n.d[ah]
Erythromycin	45[c,d]	25	2.87+	10[ac,ad]	1810.66	910.75	19.50	50.38[ah]
Escitalopram	80[a,e]	8	11+	19.74+	1720.40	308.24	6.60	n.a.
Esomeprazole	70[e]	20	0.65+	2.52+	2162.97	780.65	16.72	n.a.
Fluoxetine	85[c,f,l]	3	18.74+	25[ac]	2914.56	324.51	6.95	n.d.[ah];1.3[af]
Flutamide	100[c,i]	2	5.16+	10.04+	1987.44	30.15	0.646	<1.2[at,••]
Fluvastatin	70[a,c,] •••	2[ay]	44.35+	72.39+	4278.99	329.66	7.06	n.a.
Fluvoxamine	53[c,f]	4	3.05+	63[s]	1943.04	315.11	6.75	12[au]
Gabapentin	60[a,m]	100	0.25+	99[s]	49,428.51	1943.87	41.62	n.a.
Gemcitabine	98[a,e]	10	0.25+	1.85+	879.17	85.67	1.83	0.52[at,••]
Ibuprofen	85[a,c,d]	10	16.48+	97[t,u,w]	218,527.74	4849.50	103.84	134.75[ah]
Ifosfamide	98[a,§]	61	0.27+	3[s]	351.40	177.25	3.80	<0.05 to 10.1[ap]
Iohexol	100[e]	100	0.25+	45[s]	10,760.97	5127.22	109.79	100[an]
Iopamidol	100[e]	100	0.25+	9[s,ad]	14,866.67	11,415.95	244.45	157.25[ai]
Iopromide	100[e]	100	0.25+	27[s,ad]	23,710.49	14,752.11	315.89	574.5[ai]
Irbesartan	70[n]	82	53.4+	85.36+	31,497.58	3810.87	81.60	260 to 685[ak]
Ketorolac	100[a,c]	66	0.74+	2.68+	356.72	217.64	4.66	n.a.
Lanzoprazole	80[c]	70	9.85+	43[ac]	2167.26	808.09	17.30	n.d.[aw]
Levofloxacin	99[a,c]	90	0.25+	1.85+	5431.99	404.15	8.65	11.9[av]
Levonorgestrel	100[i,j]	20	6.69+	12.58+	1.24	0.17	0.0036	0.4 to 11.1[aj]
Lorazepam	90[c,e]	10	0.83+	2.82+	1962.86	304.99	6.53	11[ax]
Megestrol	97[a,o]	92	17.31+	30.06+	1360.7	745.29	15.96	n.a.
Mitomycin	98[e,§]	10	0.25+	1.85+	13.32	1.30	0.028	n.a.
Moxifloxacin	90[a,c]	45	0.27+	1.88+	2172.79	905.81	19.40	n.a.
Naproxen	95[a,c]	3	10	71[u,w,ab]	56,700.76	4196.75	89.87	81.05[ah]
Norethisterone	64[i,j]	10	2.4+	5.44+	24.36	8.19	0.175	n.a.
Norfloxacin	40[a,c]	60	0.25+	66[y]	4345.59	1118.69	23.95	15.83[ah]
Omeprazole	35[c]	30	0.65+	8.5[ae]	22,294.86	12,992.28	278.21	n.d.[ar]
Pantoprazole	77[c]	20	0.64+	2.51+	5487.88	1728.75	37.02	13[aw]
Paroxetine	100[a,c,p]	3	15.94+	25[ac]	3076.48	58.61	1.26	n.d.[ah];1.3[af]
Pregabalin	90[a,e]	98	0.25+	1.85+	5148.80	4175.19	89.40	n.a.
Progesterone	99[a,j]	10	13.91+	24.5+	1826.3	127.3	27.3	11.1[aj]
Roxythromycin	50[q]	66	1.56+	10[t,ac]	54.32	34.24	0.733	n.d.[ah]
Sertraline	100[a,l,r]	14	1.86+	15[af]	4857.60	488.95	10.47	2.4[af]
Simvastatin	70[c,e,]•••	1[ay]	39.45+	65.05+	13,504.08	1267.82	27.13	n.d.[j,•]
Sulfamethoxazole	80[a]	30	0.27+	50[s,t,z,ac]	10,863.97	2084.07	44.63	39.7[ah]
Tamoxifen	98[c,§]	50	59.16+	93.09+	257.03	9.78	0.209	n.d[ah]; <10[aq]
Testosterone	98[a,j]	10	4.86+	9.54+	1.54	0.14	0.03	0.3[aj]
Topiramate	80[a,c,e]	75	0.25+	1.85+	5663.68	3741.51	80.12	n.a.
Trimethoprim	95[a,c]	80	0.27+	10[t,z,ac]	72.46	44.57	0.954	0.9[aj]
Valproic acid	90[a,c]	3	1.56+	99[s]	46,339.23	229.80	4.92	n.a.
Valsartan	23[a,e]	80	9.3+	16.92+	30,394.25	20,350.90	435.78	260 to 685[ak]
17-α ethynylestradiol	45[e,q]	40	9.66+	68[s,t]	1.1969	0.29	0.006	0.5–2.6[aj]; n.d.[ao]
17-β estradiol	43[i]	30	17.59+	41[t,ag]	136.83+++	69.12	1.48	1.3[aj]; n.d.

sources. For these compounds, the data were highly variable, so averages of values available in the different cited studies are considered. Most removal percentages for primary treatment of PhACs and primary and secondary treatment of metabolites have been estimated with the STPWIN™, and they are particularly low; in these cases, it is advisable to verify these estimates when there are real data available.

Primary treatment has a low removal efficiency for PPCPs, with 83% of the PPCPs having removal rates below 20%. Secondary treatment increases removal rates up to 99%. Removal rates for 17% of the PPCPs in this study are higher than 80%, another 17% of the compounds have removal rates between 50 and 80%, another 19.3% have removal rates between 20 and 50%, and the remaining 46.7% have removal rates below 20%. Tertiary or advanced treatments (e.g., ozonation, chlorination, ultraviolet light, photocatalysis, membrane filtration and activated carbon) increase the removal of these compounds but are typically not yet widely used in Spain. For each compound, the removal rate varies with the type of advanced treatment; some of them are more efficient than others, and for some compounds, the removal rate is still unknown. Therefore, in this study, a conservative value of removal rate of 65%, as an average for different removal rates in the bibliography, was assumed for tertiary treatment.

Despite these high removal rates, it is important to consider that most of these compounds have low rates of biodegradation and high soil/water partition coefficients. Therefore, a high proportion of them are adsorbed, mainly in secondary sludge in WWTPs.

3.5. OAE, PECs and MECs

The OAE and PECs were calculated and are shown in Tables 8 and 9. The maximum OAE was for acetaminophen glucuronide (up to 600 t y^{-1}), followed by Galaxolide®, Iso-E-super®, acetaminophen, valsartan, amoxicillin, 2-hydroxy-ibuprofen, iopromide, omeprazole, carbamazepine 10, 11-epoxide, iopamidol, salicylic β-D-O-glucuronide acid, Tonalide®, ASA, clarithromycin, iohexol and hydroxylamine sulfamethoxazole between 5 and 70 t y^{-1}. High formations of metabolites, solubility in water, low soil/water partition coefficients and PPCP removal efficiencies for different treatments (including biodegradation of some compounds) are the main reasons for differences between the EACs and OAEs. Not all compounds exhibit this behavior, cefaclor, ketorolac, pregabalin and roxythromycin have shown EACs similar to OAEs.

The PECs can be higher or lower than the MECs, as others authors have found (ter Laak et al. 2010; Carballa et al. 2008; Liebig et al. 2006). PEC/MEC ratios were calculated with the MECs reported in Tables 8 and 9; if the literature reported a range of values for the MEC, a mean of the range was used. There were no MEC data available for 26.1% of the compounds studied, and for 12.5% of them, different authors have shown concentrations below the limit of quantitation. So, PEC/MEC ratios were calculated for 61.4% of the compounds.

Fig. 2 shows PEC/MEC ratios for 54 compounds. Thirty-three of these compounds (61.1%) had PEC/MEC ratios between 0.5 and 2. This group includes X-ray contrast media (iopromide, iopamidol, iohexol) and anti-cancer (ifosfamide, cyclophosphamide, flutamide) PhACs. Calculations made using data from a Spanish regional government and treatment pattern data showed that the relationship between PECs and MECs for the media and the anti-cancer compounds was close. The PEC/MEC ratios for ibuprofen and trimethoprim were also approximately one; these differ from the results of ter Laak et al. (2010), who obtained ratios of 7 and 5, respectively. PPCPs that had a high OAE (acetaminophen, Galaxolide®, ibuprofen, valsartan and ASA) also had PEC/MEC ratios close to one; nevertheless, amoxicillin had a high occurrence as well, but the PEC/MEC ratio was 8. This high ratio could be due to photodegradation, adsorption or biodegradation processes after discharge to the environment. Besse et al. (2008) found a similar behavior for amoxicillin in their study.

The PEC/MEC ratios for hormones were highly variable. 17-β Estradiol and the metabolite estrone had ratios close to 1, while testosterone and progesterone had ratios of 0.1 and 2.5, respectively. In a recent screening study of pharmaceuticals and hormones in France (Vulliet and Cren-Olivé, 2011), 17-α-ethynylestradiol, 17α- and 17β-estradiol were quantified in only a few samples, and progestagens, such as progesterone and levonorgestrel, were rarely monitored in surface waters. That study found highly variable concentrations for these two compounds (in most cases between ≤1 ng L^{-1} and 10 ng L^{-1}). The median concentration of 17-α-ethynylestradiol in sewage effluents in Germany, England, the Netherlands and the U.S. is approximately 1 to 3 ng L^{-1} or even lower (below the analytical detection limit) (Hereber, 2002). This low frequency of detection and variable concentration of hormones in aquatic environments produces a large uncertainty for the PEC/MEC ratios. For example, for 17-α-ethynylestradiol and levonorgestrel, the PEC/MEC ratio values are underestimated if a mean value for the MEC range is used. Therefore, the MECs of hormones should be monitored to allow better comparison with PEC values. For natural estrogens, Carballa et al. (2008) found a similar variation with

Notes to Table 8

*Wastewater treatment plant.

**Estimation of consumption of PhACs including self-medication (EAC_{SM}). Self-medication was considered for all PhACs except the following: antiepileptics, cytostatics/anti-cancer, X-ray contrast media and all hormones except 17-α ethynylestradiol.

***Occurrence in aquatic environment.

†Predicted environmental concentrations.

††Measured environmental concentrations.

ᶜThe references for these data are the same as for absorption.

§Pharmacokinetic parameters estimated according to the values reported for other compounds of the same pharmaceutical group.

⁺Estimated with STPWIN™ (US EPA, 2009).

⁺⁺References for these data include tertiary treatment information.

⁺⁺⁺This value includes 17-β estradiol of human natural excretion (see Table 6) and pharmacokinetic effects of drug consumption referred to Table 3.

n.a.: Data not available.

n.d.: Not detected.

•Values in effluents of WWTP.

••PECs calculated by other authors.

•••Approximate value due to scarce data and diverse information available.

[a]WebMD (2011), [b]Benedek et al. (1995), [c]MERCK (2011), [d]World Health Organization (2011), [e]Spanish Agency of Medicines and Health Products (2011), [f]Otero et al. (1996), [g]Pelkonen et al. (2001), [h]Hereber and Feldmann (2005), [i]Wishart et al. (2008), [j]Besse and Garric (2009), [k]Verhoeven et al. (2001), [l]International Program on Chemical Safety (2011), [m]Medicines and Healthcare products Regulatory Agency (2007), [n]European Medicines Agency (2011), [o]Abrams et al. (1999), [p]Cunningham et al. (2004), [q]Carballa et al. (2008), [r]Hiemke and Härtter (2000), [s]Onesios et al. (2009), [t]Esplugas et al. (2007), [u]Ziylan and Ince (2011), [v]Gros et al. (2010), [w]Quintana et al. (2005), [x]Miao et al. (2005), [y]Kosjek et al. (2009), [z]Batt et al. (2007), [aa]Lange et al. (2006), [ab]Radjenović et al. (2008), [ac]Gros et al. (2007), [ad]Ternes et al. (2003), [ae]Rosal et al. (2010), [af]Lajeunesse et al. (2008), [ag]Mouatassim-Souali et al. (2003), [ah]López-Serna et al. (2010), [ai]Teijon et al. (2010), [aj]Vulliet and Cren-Olivé (2011), [ak]Huerta-Fontela et al. (2011), [al]Valcárcel et al. (2011), [am]Ternes (1998), [an]ter Laak et al. (2010), [ao]Liebig et al. (2006), [ap]Kümmerer and Al-Ahmad (2010), [aq]Ashton et al. (2004), [ar]Ternes et al. (2001), [as]Kosjek et al. (2012), [at]Besse et al. (2012), [au]Kosjek and Heath (2010), [av]Conley et al. (2008), [aw]Barreiro et al. (2011), [ax]Gros et al. (2009), and [ay]Besse et al. (2008).

Table 9
PECs for metabolites and PCPs under study, based on consumption, pharmacokinetics and elimination in urban WWTP in Spain in 2009, and MECs from published data.

Compound	Excretion (%)*	Elimination in urban WWTP (%)		Metabolites excreted or EAC for PCPs (kg y^{-1})	OAE** (kg y^{-1})	PECs† (ng L^{-1})	MECs†† (ng L^{-1})
		Primary (1°)§	1° plus secondary				
Metabolites							
2-Hydroxy carbamazepine	1[a]	0.31+	1.96+	185.36	152.89	3.27	n.d.[x]
2-Hydroxy ibuprofen	25[b,c,d]	0.71+	50[n,o]	34,827.86	15,181.13	325.08	18 to 101[ac]
3-Hydroxy carbamazepine	1[a]	0.31+	1.96+	185.36	152.89	3.27	n.d.[x]
4-Hydroxy diclofenac	27[c,d,e]	10.22+	18.44+	6315.05	4353.27	93.22	<LOQ††† to 71[af,•]
5-Hydroxy diclofenac	1[c,d,e]	5.7+	10.95+	71.76	53.89	1.15	<LOQ to 86[af,•]
Acetaminophen glucuronide	70[b]	0.25+	1.85+	728,271.73	601,384.41	12,877.6	n.a.
Carbamazepine 10, 11-epoxide	80[a]	0.27+	1.89+	14,828.55	12,240.09	262.10	n.d.[x]
Carbamazepine 10,11-dihydrodiol	2[a]	0.25+	1.85+	370.71	306.12	6.56	2.2[x]
Clofibric acid from clofibrate	99[f]	1.12+	51[n,p]	156.52	66.95	1.43	3[ag]
Clofibric acyl-β-D-glucuronide acid	54.5[g]	0.25+	1.85+	187.48	154.82	3.32	n.a.
Estrone	++	3.31+	85[p,s,t]	185.66	28.22	0.604	0.1 to 1[v]
Hydroxylamine sulfamethoxazole	2.4[ab]	0.26+	1.87+	208.59	172.21	36.86	n.a.
Ibuprofen carboxylic acid	37[b,c,d]	0.47+	100[n]	51,545.23	1607.44	34.42	n.a.
MET from paroxetine+++	90[h]	18.16+	31.45+	2768.83	1612.40	34.53	n.a.
N-desmethyl escitalopram	30[b,i]	7.38+	13.74+	412.90	300.59	6.44	n.a.
N-desmethyl sertraline	22[j,k]	1.86+	28[q]	1068.67	655.89	14.04	7[ad]
Norfluoxetine	10[c,l,m]	22.76+	57[q]	247.74	92.79	1.99	1.3[ae]
Salicylic acid	10[c]	0.68+	92[r]	8957.81	859.07	18.40	6.7[v]
Salicylic β-D-O-Glucuronide acid	15[m]	0.25+	1.86+	13,436.72	11,094.54	237.57	n.a.
Sertraline carbamoyl glucuronide	64[k]	13.66+	24.1+	3108.86	1998.37	42.79	n.a.
PCPs							
Iso-E-super®	NA	51.39+	87[r]	375,838.11	47,199.01	1010.69	29 to 810[ae]
Galaxolide®	NA	50+	56[r,s,u]	184,536.51	69,221.20	1482.25	320 to 3150[aa]
Musk ketone	NA	27[u]	21[u]	5011.17	2480.81	52.01	4.8 to 390[z]
Methylparaben	NA	0.47+	2.22+	2611.64	2148.67	46.01	n.d./54[w]
Musk xilene	NA	29[u]	34[u]	10,774.03	4369.77	93.57	1.1 to 180[z]
Phantolide®	NA	28[r]	40[r]	2380.31	1201.29	25.72	30 to 170[aa]
Propylparaben	NA	2.76+	6.05+	870.55	688.81	14.75	n.d./105[w]
Tonalide®	NA	57.05+	74[r,s,u]	48,232.56	11,074.93	237.15	160[y]

*Percentage of metabolite excreted from parent compound.
**Occurrence in aquatic environment.
†Predicted environmental concentrations.
††Measured environmental concentrations.
†††Limit of quantitation.
§According to Anca Caliman and Gavrilescu (2009) the most of EDCs, PhACs and PCPs have poor/low removal efficiency in primary treatments.
+Estimated with STPWIN™ (US EPA, 2009).
++Only natural excretion was considered (see Table 6).
+++4-[[(3S,4R)-4-(4-fluorophenyl)piperidin-3-yl]methoxy]-2-methoxyphenol.
•Values in effluents of WWTP adjusted with a dilution factor (10).
NA: Does not apply.
[a]Pelkonen et al. (2001), [b]WebMD (2011), [c]MERCK (2011), [d]World Health Organization (2011), [e]Hereber and Feldmann (2005), [f]Wishart et al. (2008), [g]Faed and Mc Queen (1978), [h]Cunningham et al. (2004), [i]Spanish Agency of Medicines and Health Products (2011), [j]Hiemke and Härtter (2000), [k]Obach et al. (2005), [l]Otero et al. (1996), [m]International Program on Chemical Safety (2011), [n]Radjenović et al. (2008), [o]Hereber (2002), [p]Esplugas et al. (2007), [q]Lajeunesse et al. (2008), [r]Onesios et al. (2009), [s]Ternes et al. (2003), [t]Barceló and Petrović (2007), [u]Yang and Metcalfe (2006), [v]Vulliet and Cren-Olivé (2011), [w]Villaverde-de-Sáa et al. (2010), [x]Miao and Metcalfe (2003), [y]Ternes (2004), [z]Brausch and Rand (2011), [aa]Fromme et al. (2001), [ab]Van der Ven et al. (1994), [ac]Weigel et al. (2004), [ad]Kosjek and Heath (2010), [ae]Bester et al. (2008), [af]Stülten et al. (2008), and [ag]Gros et al. (2009).

MECs and PECs to that observed in the present study, but their PECs were higher than the MECs. For desogestrel and megestrol, there were no MECs available.

Sertraline, atorvastatin and fluoxetine PEC/MEC ratios were highly overestimated (4.4, 5 and 5.35, respectively). These may be due to low solubility in water and high values of the octanol/water and soil/water partition coefficients for these compounds; therefore, they could be bioaccumulated in fauna and flora or adsorbed in soils or sediments, thereby reducing their concentrations in water.

Johnson et al. (2005) studied the exposure and fate of selective serotonin reuptake inhibitors in microcosm model ecosystems. Their results confirm that sertraline concentration in the aqueous compartment at all treatments tended toward zero, while fluoxetine and fluvoxamine did not. They suggest that while sertraline could be completely degraded or completely sequestered, flouxetine was sequestered, possibly via adsorption to biomass and sediments. Kwon and Armbrust (2006) indicated that flouxetine is relatively recalcitrant to hydrolysis, photolysis, and microbial degradation and that it is rapidly removed from surface waters by adsorption to sediment, where it appears to be persistent. For atorvastatin, the photodegradation process could be the reason for an overestimated PEC. Some studies have been reported direct and indirect photolysis of this compound (Lam et al. 2004; Lam and Mabury, 2005; Cermola et al. 2006; Razavi et al. 2011).

Because of the few data available for the MECs of metabolites, the verification of calculations of PECs was more difficult.

There were not found MECs of acetaminophen glucuronide in the literature (where the OAE was more than 600 t y^{-1}) and therefore, PEC value for this compound could not be verified. Processes such as hydrolysis in the environment or cleavage in the sewer system can occur with this glucuronide, and these variables were not taken into account in the STPWIN™ estimate. Besse et al. (2008) considered reasonable to assume that glucuronide conjugates of pharmaceutical compounds show similar behavior to those for estrogen glucuronide metabolites. The latter could be cleaved in the environment and thus regenerate the parent compound. Nevertheless, in this study, it was considered that acetaminophen glucuronide remains in the metabolite form, at least until it enters to the aquatic environment. This assumption was based on the PEC/MEC ratio for acetaminophen

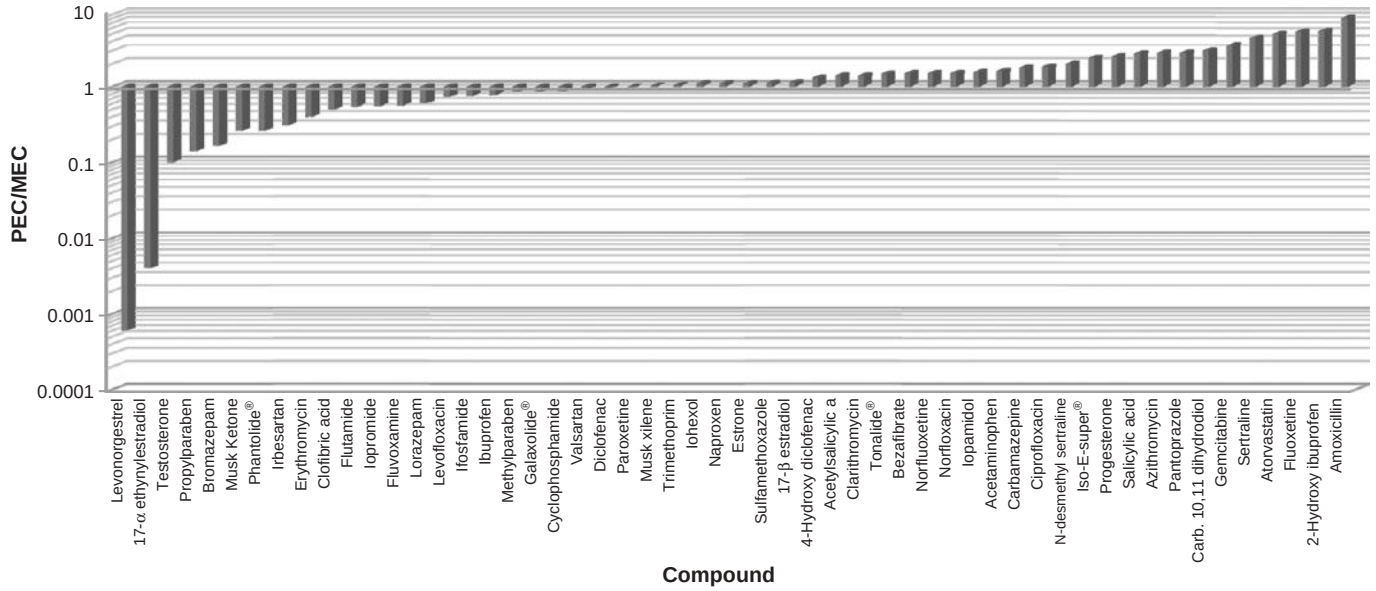

Fig. 2. Comparison of PEC/MEC ratios.

being approximately 1, which confirms that the estimation of the parent compound is close to its actual occurrence, without considering the potential transformation of glucuronide. Detailed studies on possible transformations of acetaminophen and its metabolites in the wastewater facilities and in the environment should be perform to properly adjust these estimations.

For the other metabolites, the OAE values ranged from a few kilograms to 15 t y^{-1}. Although the metabolites tend to be more easily degraded than the parent compounds, the large amount excreted should be reason enough to analyze their presence and effects in the environment.

In this study, the PEC values tend to be overestimated (57.4%) compared with MEC values. There are many reasons for overestimating or underestimating these values. In this study, the MECs were taken from other research and were obtained with different methodologies and degrees of uncertainty. The percentages of pharmacokinetics and treatment parameters are very specific for the type of population and processes used, and average values were used or estimated with EPA STPWIN™. There is little information on the percentages of self-medication and expired drugs released into the environment. PPCPs for veterinary uses were also not considered, and the consumption data for PCPs was not updated. All of these uncertainties affect the calculation of the occurrence of PPCPs and hence the PEC values. Nevertheless, the large number and variety of PPCPs that exist at present makes it necessary to implement predictive models for their occurrence. These methodologies can simplify the identification and ranking of priority and sensitive substances that affect the environment so that they can then be analyzed thoroughly.

4. Conclusions

In this study, a mass balance approach was carried out to assess the occurrence of eighty-eight pharmaceutical and personal care product compounds and some of their metabolites, considering updated data of pharmacokinetics, removal in wastewater facilities and measured concentrations in the aquatic environment in Spain for each compound.

Three methodologies were used to estimate pharmaceutically active compounds consumption, with data allowing for method comparison in some cases. The values calculated with DHD and treatment pattern methodologies were on the same order as the range of values obtained using the number of packages sold methodology. This allowed estimation of the PPCPs consumption using different methods and references based on the available data with the confidence that results could be reasonably used and compared.

Environmental concentrations of PPCPs and metabolites were estimated and then compared with the environmental concentrations measured by several researchers and reported in recent Spanish and European literature. This comparison allowed verification of the predictions made according to the methodologies used in this work.

Analgesic/antipyretics (acetaminophen, ibuprofen, ASA), antibiotics (amoxicillin), and personal care products (Galaxolide®, Iso-E-super®, Tonalide®) had the highest occurrence. Metabolites also had high PECs, but there was less information for the MECs; therefore, it will be necessary to obtain more data of their occurrence before concluding if the models are adjusted to them.

For almost 50% of the studied compounds, there were no MEC data or they were not detected in aquatic environments; therefore, efforts should be increased to determine these MECs. For approximately 60% of compounds for which PEC/MEC ratios were calculated, it was shown that the models fit well and that the PECs were very close to the corresponding MECs with reasonable allowances for excess or deficit.

Many factors cause underestimated or overestimated PECs, but each new study can be used for understanding the complex behavior of these compounds in the environment. With additional studies, estimations could be improved and provide valuable information (PECs closer to MECs) for the prioritization of future investigations, such as risk assessment for pharmaceuticals, personal care products and metabolites. Therefore, currently, when there are high discrepancies between MECs and PECs, environmental chemical analyses should be implemented; whereas, for compounds with similar MECs and PECs, mass balance approaches may be adequate, allowing avoiding systematic chemical analyses.

The information and the analysis provided in this work was unknown at least in the Spanish region and could be used as a model for other geographic areas and to further improve predictive models of these variables.

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Chapter 3

Paper II

Ranking of concern, based on environmental indexes, for pharmaceutical and personal care products: an application to the Spanish case.

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Ranking of concern, based on environmental indexes, for pharmaceutical and personal care products: An application to the Spanish case

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ABSTRACT

A wide range of Pharmaceuticals and Personal Care Products (PPCPs) are present in the environment, and many of their adverse effects are unknown. The emergence of new compounds or changes in regulations have led to dynamical studies of occurrence, impact and treatment, which consider geographical areas and trends in consumption and innovation in the pharmaceutical industry. A Quantitative study of Structure–Activity Relationship ((Q)SAR) was performed to assess the possible adverse effects of ninety six PPCPs and metabolites with negligible experimental data and establish a ranking of concern, which was supported by the EPA EPI Suite™ interface. The environmental and toxicological indexes, the persistence (P), the bioaccumulation (B), the toxicity (T) (extensive) and the occurrence in Spanish aquatic environments (O) (intensive) were evaluated. The most hazardous characteristics in the largest number of compounds were generated by the P index, followed by the T and B indexes. A high number of metabolites has a concern score equal to or greater than their parent compounds. Three PBT and OPBT rankings of concern were proposed using the total and partial ranking method (supported by a Hasse diagram) by the Decision Analysis by Ranking Techniques (DART) tool, which was recently recommended by the European Commission. An analysis of the sensibility of the relative weights of these indexes has been conducted. Hormones, antidepressants (and their metabolites), blood lipid regulators and all of the personal care products considered in this study were at the highest levels of risk according to the PBT and OPBT total rankings. Furthermore, when the OPBT partial ranking was performed, X-ray contrast media, H_2 blockers and some antibiotics were included at the highest level of concern. It is important to improve and incorporate useful indexes for the predicted environmental impact of PPCPs and metabolites and thus focus experimental analysis on the compounds that require urgent attention.

Abbreviations: ATC, Therapeutic Chemical Classification System; B, bioaccumulation; BCF, bioconcentration factor; BIOWIN™, software by US EPA for estimates probability of rapid aerobic and anaerobic biodegradation; CAS, chemical abstracts; ChV, chronic toxicity; DART, Decision Analysis by Ranking Techniques; ECOSAR, US EPA ecological structure–activity relationship; EMEA, European Medicines Agency; EPA EPI Suite™, Estimation Programs Interface Suite™ developed by the EPA's Office of Pollution Prevention Toxics and Syracuse Research Corporation; ERA, Environmental Risk Assessment; LC, level of concern; NOEC, no-observed effect concentration; NSAIDs, non-steroidal anti-inflammatory drugs; O, occurrence; OECD, Organization for Economic Co-operation and Development; P, persistence; PCPs, Personal Care Products; PECs, predicted environmental concentrations; PERs, physical–chemical property estimation routines; PhACs, Pharmaceutically Active Compounds; PNEC, predicted no effect concentration; POR, partial order ranking; PPCPs, Pharmaceutical and Personal Care products; (Q)SARs, Quantitative Structure–Activity Relationships; REACH, European Regulation for Registration Evaluation Authorization and Restriction of Chemicals; SARs, Structure–Activity Relationships; SMILES, Simplified Molecular Input Line Entry System; SRC, Syracuse Research Corporation; T, Toxicity; US EPA, United States Environmental Protection Agency; WWTP, Wastewater Treatment Plant.

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1. Introduction

Intensive research on pharmaceuticals in the environment started approximately 15 years ago. Since then, a vast amount of literature has been published (Kümmerer, 2009). Pharmaceuticals and personal care products (PPCPs) are being used extensively and increasingly in human and veterinary medicine (Fent et al., 2006) and have been identified as an emerging class of potential pollutants for the aquatic environment (Gagné et al., 2005).

These compounds and their bioactive metabolites can be continually introduced to the aquatic environment as complex mixtures via a number of routes but primarily by both untreated and treated sewage (Daughton and Ternes, 1999). Hundreds of PPCPs and metabolites have been detected in the aquatic environment in various studies performed worldwide (Ternes et al., 2001; Hereber, 2002; Kümmerer, 2009; Gros et al., 2010; Brausch and Rand, 2011).

This concern has led to the development of an extensive area of research, including chemical identification and quantification of these compounds, the elucidation of transformation pathways when these compounds are present in wastewater treatment plants (WWTPs) or in environmental matrices, the assessment of their potential biological effects, and the development and application of advanced treatment processes for their removal and mineralization (Fatta-Kassinos et al., 2011).

Despite these efforts, the adverse effects of many PPCPs and their metabolites are still unknown. Some authors have studied their harmful properties in detail: endocrine disruptions, persistence (P), toxicity (T) and bioaccumulation potential (B) (Gagné et al., 2005; Fent et al., 2006; Bruce et al., 2010; Kümmerer and Al-Ahmad, 2010).

Increasingly restrictive regulations in Europe, such as the European Regulation for Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) are considering the problem of releasing chemicals, especially organic chemicals, into the environment (Dévier et al., 2011). However, to date, approximately 16 million compounds are known and registered in the Chemical Abstracts Service (CAS), which indicates a tremendous discrepancy between the number of compounds potentially present in the environment and the number of priority pollutants that have been regularly monitored (Brack et al., 2005). Voigt and Brueggemann (2008) concluded in their research that the issue of pharmaceuticals in the environment and the unavailability of data necessitate much closer communication between scientists, medical healthcare professionals and politicians in the future.

Members countries of the Organization for Economic Cooperation and Development (OECD) agreed on the principles for validating models of Quantitative Structure–Activity Relationships ((Q)SARs) to be used in the regulatory assessments of chemical safety (Freidig et al., 2007). Thus, the REACH regulation (European Parliament and Council Regulation, 2006) allows researchers to utilize data generated by (Q)SAR as a substitute for experimental data and as a supplement to experimental data in the weight-of-evidence approach (Pavan and Worth, 2006).

The experimental determination of the PBT is generally expensive and demanding to perform. For this reason, measuring the potential PBT profiles of chemicals that are of potential regulatory interest experimentally is considered infeasible (Pavan and Worth, 2006). The Stockholm County Council (2012) has performed an environmental hazard classification of pharmaceuticals by PBT index, but it uses mainly experimental data, which leads to a lack of a PBT index for many of these compounds due to the lack of data. Therefore, (Q)SAR models are a powerful tool that could be used to prioritize chemicals for further testing, to identify certain types of toxic hazards (possibly to derogate from further testing) or to provide estimates of toxic potency for use in risk assessments (Cronin et al., 2003), bearing in mind its advantages and limitations.

It is widely agreed that (Q)SARs in the field of aquatic toxicity are valid for prediction within their restricted applicability domain. Although this restricted applicability might seem a disadvantage at first, it can be advantageous because the predictions of (Q)SARs can be more stringently tested and the uncertainties (and specificity) are much easier to quantify and explain (Freidig et al., 2007).

If the study of PPCPs in the environment is a relatively recent issue, the detection and evaluation of the risk of their metabolites is even more so. Escher and Fenner (2011) demonstrated how effect data for parent compounds can be used in combination with the analysis of toxicophore structures and the bioconcentration potential to facilitate the assessment of the effect of the transformation product. On the basis of the large number of transformation products that originate from PPCPs, it is appropriate and necessary to include them in all research to establish better and more realistic environmental impact assessments.

Other studies have been performed with similar objectives. Cooper et al. (2008) created a preliminary risk assessment database for common pharmaceuticals and put it into a web-accessible database. The pharmaceuticals were ranked using five different combinations of physical–chemical and toxicological data, which emphasized different risks. Kumar and Xagoraraki (2010) developed a comprehensive ranking system for prioritizing PPCPs and endocrine-disrupting chemicals in stream water/source water and finished drinking water using four criteria (occurrence, treatment, ecological effects and human health effects) and used it to rank emerging organic pollutants in U.S. stream water/source water and finished drinking water. Sanderson et al. (2004a) estimated the toxicity of many pharmaceuticals with the U.S. Environmental Protection Agency (US EPA) ecological structure–activity relationship (ECOSAR) and then classified and evaluated them. In another study (Sanderson et al., 2004b), they obtained a predicted risk ranking by means of QSAR modeling using the US EPA EPIWIN package (now EPI Suite™) especially the ECOSAR program to assess the toxicity to the aquatic environment.

An environmental risk assessment (ERA) by the European Medicines Agency (EMEA) guidelines was conducted by Carlsson et al. (2006) for 27 active pharmaceutical ingredients by considering half-lives/biodegradability, the environmental occurrence, and Swedish sales statistics. They used EPI Suite™ when experimental data were lacking.

More recently, Jean et al. (2012) presented a method to select the pharmaceuticals discharged in hospital effluents that have a higher impact on the aquatic ecosystem, primarily based on their bioaccumulation potential.

None of these publications created a risk categorization in the framework of the recent REACH guidelines or used a Decision Analysis by Ranking Techniques (DART) tool, which was recently recommended by the European Commission (Pavan and Worth, 2008). Very few of such publications have included metabolites. Therefore, this study considers some PPCPs widely used worldwide, the most widely consumed PPCPs in Spain, others that were recently synthesized or whose prescription rates have increased in recent years, and some of their metabolites, which have not been considered previously, at least in this geographic area. Updated data of occurrence were used in conjunction with the PBT values estimated by the (Q)SAR methodology according to the recent recommendations of the European regulations.

In this way, the aims of this research on the PPCPs and metabolites under study were: (1) estimating the PBT potentials by (Q)SAR updated models and databases; (2) considering the occurrence (O) of PPCPs in aquatic Spanish environments, as estimated by using a mass balance approach presented in a recent study (Ortiz

et al., 2013), and incorporating it as an extensive environmental index to the PBT indexes; (3) generating rankings of concern of PBT and OPBT by using the DART tool, which was recently recommended by the European Commission (Pavan and Worth, 2008) to perform a sensitivity analysis that considered several index weights.

These rankings can be used to prioritize substances that require immediate attention to further evaluate their effects on the environment (at the experimental level), to obtain preliminary results to facilitate the decision making processes in an environmental risk assessment and to perform preventive, corrective and regulatory actions.

2. Materials and methods

2.1. Selection of PPCPs and metabolites

PPCPs and metabolites were selected on the basis of previous studies of risk impact assessment and recent data of human consumption and occurrence in aquatic environments in Spain. Many of these PPCPs coincide with the most commercialized compounds for human use worldwide and a few of their metabolites that have been reported by some authors (Celiz et al., 2009; Quintana et al., 2005; Radke et al., 2009). The list of the selected compounds is shown in Table 1.

2.2. Occurrence of PPCPs and metabolites in the aquatic environment

The methodology and the data of occurrence of the selected PPCPs and metabolites in aquatic environments in Spain are explained in detail in Ortiz et al. (2013). They proposed the estimation of the consumption of active compounds of pharmaceuticals by at least one of the following methodologies: the number of commercial packages sold, the data for the number of defined daily doses per 1000 inhabitants per day, or the pattern of treatment. The occurrence in the aquatic environment was calculated through a mass balance approach by considering the following factors: the number of pharmaceutical prescriptions issued, the amount of pharmaceuticals discharged without consumption, consumption, self-medication, pharmacokinetics, treatment in wastewater facilities and the introduction of treated wastewater to the aquatic environment.

The occurrence was estimated only for PPCPs used by humans and their main excreted metabolites.

2.3. Physicochemical properties

The physicochemical parameters of PPCPs and their metabolites were consulted in a recognized data-base, Chem ID Plus Lite (United States National Library of Medicine, 2010), PubChem (National Center for Biotechnology Information, 2011), PhysProp Database (SRC, 2010), or estimated with the EPI Suite™ interface (US EPA, 2009), which gave those properties when the experimental data were lacking. The general information collected was the CAS number, the chemical formula, the molecular mass, the solubility in water, the dissociation constant (as pK_a), and the logarithm of octanol/water and soil/water partition coefficients (log K_{ow} and log K_{oc}, respectively).

2.4. Estimation of PBT indexes by using (Q)SAR models and categorization in levels of concern

(Q)SAR analyses were conducted with the support of the Estimation Programs Interface EPI Suite™ that was developed by the Office of Pollution Prevention and Toxics of the US EPA and

Table 1
Pharmaceutical and personal care products selected.

PPCP group	Compound name
PhAC	
ACE[a] inhibitors	Enalapril
Analgesic/antipyretic	Acetaminophen, acetaminophen glucuronide[c], 1, 4 benzoquinone[d]
Angiotensin receptor blockers	Irbesartan, valsartan
Antibiotics	Amoxicillin, azithromycin, cefaclor, ciprofloxacin, clarithromycin, erythromycin, levofloxacin, moxifloxacin, norfloxacin, roxythromycin, sulfamethoxazole, sulfamethoxazole hydroxylamine[c], trimethoprim
Antidepressants	Escitalopram, fluoxetine, fluvoxamine, metabolite paroxetine[c,e], N-desmethyl escitalopram[c], N-desmethyl sertraline[c], norfluoxetine[c], paroxetine, sertraline, sertraline carbamoyl glucuronide[c]
Antiepileptics	Carbamazepine, carbamazepine 10, 11 dihydrodiol[c], carbamazepine 10, 11 epoxide[c], 2-hydroxy carbamazepine[c], 3-hydroxy carbamazepine[c], gabapentin, pregabalin, topiramate, valproic acid
Anxiolytics	Alprazolam, bromazepam, lorazepam
Blood lipid regulators	Atorvastatin, bezafibrate, clofibrate, clofibric acid[c], clofibric acyl-β-D-glucuronide acid[c], 4-chlorobenzoic acid[c], fluvastatin, simvastatin
Cytostatics/cancer therapeutics	Cyclophosphamide, flutamide, gemcitabine, ifosfamide, mitomycin, tamoxifen
H_2 blockers	Esomeprazole, lanzoprazole, omeprazole, pantoprazole
Hormones	Desogestrel, diethylstilbestrol, estrone[c], gestodene, levonorgestrel, megestrol, norethisterone, progesterone, testosterone, 17-α ethynylestradiol, 17-β estradiol
Platelet inhibitor	Acetylsalicylic acid, salicylic acid[c], salicylic β-D-O-glucuronide acid[c]
NSAIDs[b]/antirreumatics	Diclofenac, 4-hydroxy diclofenac[c], 5-hydroxy diclofenac[c], ibuprofen, Ibuprofen carboxylic acid[c], 2-hydroxy ibuprofen[c], ketorolac, naproxen
X-ray contrast media	Iohexol, iopamidol, iopromide
PCP	
Disinfectant	Triclosan
Fragrances	Galaxolide®, Iso-E-super®, musk ketone, musk xilene, Phantolide®, Tonalide®
Preservatives	Ethylparaben, methylparaben, p-hydroxybenzoic acid[c,d], propylparaben
Surfactant	4- nonylphenol[d]

[a] Angiotensin converting enzyme.
[b] Non-steroidal anti-inflammatory drugs.
[c] Metabolite.
[d] Product of transformation.
[e] Metabolite paroxetine: 4-[[(3S,4R)-4-(4-fluorophenyl)piperidin-3-yl]methoxy]-2-methoxyphenol.

Syracuse Research Corporation (SRC). This software was downloaded from the US EPA website: http://www.epa.gov/oppt/exposure/pubs/episuite.htm.

The software consists of physical–chemical property estimation routines (PERs) and mass balances based on environmental fate models. EPI Suite™ is utilized by various agency program offices, US federal agencies, state regulatory agencies, foreign countries and the private sector (Granger and McFarland, 2007) to support the assessment of new and existing industrial chemicals. It requires the CAS number, the name of the chemical compound or the Simplified Molecular Input Line Entry System (SMILES) notation as input data.

According to Granger and McFarland (2007), the scientific community that understands the role and accuracy limitations of screening models used in regulatory decision-making generally accepts the EPI Suite™ module results for many classes of organic chemicals. The EPI Suite™ modules are also generally accepted among regulators and by OECD and are being tested for implementation in relation to high production volume chemicals and the Globally Harmonized System for the classification and labeling of chemicals by OECD.

Nevertheless, EPI Suite™ (Q)SARs do not provide adequate coverage of nanoparticles, inorganic compounds, organo-metallic and certain other classes of chemicals as polymers. Under most circumstances, the PERs predict the measured property value within an order of magnitude, a standard of accuracy that is generally acceptable for screening level decision-making. However, it would be inappropriate to use PERs to predict physical–chemical properties of chemicals whose characteristics are significantly different than those found in the module training set because the difference between the predicted and measured values may be great (Granger and McFarland, 2007). In this study, all of the compounds are organic molecules formed by types of structures (fragments) that are included in the database of EPI Suite™.

2.4.1. Persistence

Pharmaceuticals are designed to be resistant to biodegradation because the metabolic stability usually improves their desired pharmacological action. This stability, however, contributes to their environmental persistence (Fatta-Kassinos et al., 2011). For persistence, Pavan and Worth (2006) recommended, (Q)SAR models through the BIOWIN™ program (specifically BIOWIN 2,3 and 6, see Table 2) in a preliminary assessment to estimate the potential for biodegradation in the environment for those substances with no available data or with information that is difficult to interpret.

The BIOWIN™ biodegradability estimation is based upon fragment constants that were developed using multiple linear or non-linear regression analyses that depend on the model. A discussion of the methodology used to derived the linear and non-linear fragment constants is presented in Howard et al. (1992), Boethling et al. (1994), Tunkel et al. (2000) and Meylan et al. (2007).

The estimation of biodegradation has inherent problems, one of which is the lack of reproducibility of measured biodegradation data. However, the BIOWIN model is reasonably well accepted and generally performs as well as or better than the available models (Granger and McFarland, 2007).

Moreover, the annex XXIII of REACH (European Parliament and Council Regulation, 2006) establishes the criteria for the identification of the persistence potential for substances. A compound fulfills the persistence criterion when (i) its half-life in marine water is higher than 60 days, (ii) its half-life in fresh or estuarine water is higher than 40 days, (iii) its half-life in marine sediment is higher than 180 days, (iv) its half-life in fresh or estuarine water sediment is higher than 120 days or (v) its half-life in soil is higher than 120 days.

In this study, biodegradation estimations according BIOWIN 2, 3 and 6 were obtained from EPI Suite™ for each compound, and then these values were classified between 1 and 4 according to the PBT concern score shown in Table 2.

Table 2
Conversion of PBT to levels of concern[a]

Persistence	Bioaccumulation	Toxicity	Level of concern[b]
BIOWIN 2 (non-linear model) <0.5 or BIOWIN 6 (MITI non-linear model) <0.5 and BIOWIN 3 (ultimate biodegradation) <2.2	BCF[c] > 2000	ChV[d] < 0.1	4
2.2 ≤ BIOWIN 3 < 3	1500 < BCF ≤ 2000	0.1 ≤ ChV < 1	3
3 ≤ BIOWIN 3 < 3.5	1000 < BCF ≤ 1500	1 ≤ ChV < 10	2
BIOWIN 3 ≥ 3.5	BCF ≤ 1000	ChV > 10	1

[a] Adapted from Pavan and Worth (2008).
[b] Level of risk of PBT: 4 is the maximum, and 1 is the minimum.
[c] Bioconcentration factor in L (kg wet-wt)$^{-1}$.
[d] Chronic toxicity in mg/L.

2.4.2. Bioaccumulation

The assessment of bioaccumulation shall be based on measured data on bioconcentration in aquatic species. Data from freshwater and marine water species can be used (European Parliament and Council Regulation, 2006).

The EPI Suite™ interface includes the BCFBAF v.3.00 routine. The BCFBAF Program is an update and an expansion of the previous BCFWIN Program that was part of the EPI Suite™ version 3.20. The BCFBAF program estimates the BCF of an organic compound using the log K_{ow} value of the compound. For the update, a more recent and better evaluated database of BCF values was used for both training and validation. The details of the methods to review the data-quality are described in Arnot and Gobas (2006). BCFBAF requires only a chemical structure, entered by SMILES, to estimate BCF.

The BCFBAF method classifies a compound as either ionic or non-ionic. Ionic compounds include carboxylic acids, sulfonic acids, salts of sulfonic acids and charged nitrogen compounds (nitrogen with a +5 valence such as quaternary ammonium compounds). All other compounds are classified as non-ionic. The training dataset included 466 non-ionic compounds and 61 ionic compounds. The methodology for the non-ionic compounds separates them into three groups in terms of their log K_{ow} values as follows: log $K_{ow} < 1.0$; log K_{ow} from 1.0 to 7.0; log $K_{ow} > 7.0$. For log $K_{ow} < 1.0$, an estimated log BCF of 0.50 is assigned, and the other two categories have their respective regression equations. For ionic compounds, if the log K_{ow} is lower than 5.0, an estimated log BCF of 0.50 is assigned (US EPA, 2009).

It is important to take into account that BCFBAF v.3.00 presents reasonable regression equations for non-ionic compounds but no acceptable regression for ionic ones. Therefore, for the ionic compounds under study, the bioconcentration factor in previous studies was verified if available.

According to annex XXIII of REACH (European Parliament and Council Regulation, 2006) a substance fulfills the bioaccumulation criterion when its bioconcentration factor (BCF) is higher than 2000. In this research, the BCF values were estimated with the EPI Suite™ BCFBAF routine and classified as shown in Table 2.

2.4.3. Toxicity

Usually, one or several toxicity profile(s) for aquatic organism is (are) used in risk assessment. Beyond laboratory investigations,

some mathematical models were developed to estimate or predict ecotoxicological effects. The most often applied (Q)SAR program is ECOSAR (Fent et al., 2006). The ECOSAR Class Program is a computerized version of the ecotoxicity analysis procedures as currently practiced by the Office of Pollution Prevention and Toxics (OPPT) of the EPA when there is a lack of data for regulatory endpoints. It has been developed within the regulatory constraints of the Toxic Substances Control Act (Mayo-Bean et al., 2009).

The structure–activity relationships (SARs) presented in this program was used to predict the aquatic toxicity of chemicals based on their similarity of structure to chemicals for which the aquatic toxicity has been previously measured. Most SAR calculations in the ECOSAR Class Program are based upon K_{ow} (US EPA, 2009). Sanderson et al. (2004a, 2004b) have used and reported many advantages of this program, and it has recently been used in similar studies (Cooper et al., 2008; Ginebreda et al., 2010).

The mode of toxic action for most neutral organic chemicals is narcosis, and many other types of chemicals are toxic to organisms in this way (i.e., ethers, alcohols, ketones). However, some organic chemicals have been identified as having a more specific mode of toxicity. These chemicals are typically organics that are reactive and/or ionizable, which exhibit excess toxicity in addition to narcosis (i.e., acrylates, epoxides, anilines) (Mayo-Bean et al., 2009).

According to annex XXIII of REACH (European Parliament and Council Regulation, 2006) a compound is toxic if (i) its long-term no-observed effect concentration (NOEC) for marine or freshwater organisms is less than 0.01 mg/l; (ii) it is classified as carcinogenic (category 1 or 2), mutagenic (category 1 or 2), or toxic for reproduction (category 1, 2, or 3); or (iii) there is other evidence of chronic toxicity, as identified by the classifications T, R48, or Xn, and R48 according to Directive 67/548/EEC.

In this work, a lower value of chronic toxicity (the worst case between narcosis and other types of toxicity) was assumed for algae, crustaceans (daphnids) and fish as an endpoint either from the baseline or from another type of fragment. The value of chronic toxicity for each species was partially classified according to Table 2. The global level of concern for each PPCP was the level that dominated in the partial classification.

According to P, B and T values and in order to classify PPCPs by (Q)SAR and REACH regulation, Pavan and Worth (2008) recommend a concern score by combining PBT indicators, as shown in Table 2. This concern score is used in the assessment presented in this research. Nevertheless, the occurrence, in kilograms per year, is an extensive variable and is another important indicator that should be evaluated; thus, another additional classification (OPBT) was included and analyzed to assess the influence of the occurrence on the ranking of PBT.

2.5. Rankings of PPCPs

In recent years, some researchers have proposed different ranking methods for prioritizing organic compounds. These rankings can be used to check the environmental effect of the PPCPs and their metabolites. DART is software designed to rank the chemicals according to their environmental and toxicological concern based on the most recent ranking theories. DART can be downloaded from the Joint Research Centre website (see details at http://ihcp.jrc.ec.europa.eu/our_labs/computational_toxicology/qsar_tools/DART).

In Appendix A (supplementary material), the general principles of DART are explained according to the information included in the DART manual (TALETE, 2007) and Pavan and Worth (2008).

The Desirability and Utility functions (Total rankings) and partial order ranking (supported by Hasse Diagram) were used in this study.

Pavan and Worth (2008) have shown the procedure to implement this tool to generate a chemical total ranking of concern according to the PBT behavior. As shown in Table 2, the "best" condition for each P, B and T property is related to the minimum score. Then, in this study each property was independently transformed into a desirable value (Eq. (A.2), Appendix A) or a useful one (Eq. (A.3), Appendix A) by means of an inverse linear transformation with equal weight:

$$d_{ir}(\text{or } u_{ir}) = (-1/3)y_{ir} + (4/3) \quad (1)$$

where d_{ir}, u_{ir} and y_{ir} are the desirability, utility and the actual (real) value for the i-th element for the r-th criterion, respectively.

Thus, the best condition, which corresponds to the predicted safest chemicals, has a desirability value equal to 1, whereas the worst condition, corresponding to the chemicals predicted to be the most hazardous, has a desirability value of 0. The three properties were initially equally weighted in the ranking procedure, and the PBT hazard score was calculated as $1-Di$ (or $1-Ui$) for each chemical, where Di is the overall desirability (Eq. (A.2), Appendix A) and Ui is the overall utility (Eq. (A.3), Appendix A) of the chemicals. Therefore, the PBT hazard score ranges from 0 for chemicals with the least PBT concern to a maximum of 1 for chemicals with the highest PBT or OPBT concern.

The ranking based on the desirability function is severe: it gives a PBT hazard score of 1 ($D_i = 0$) if any of the three properties (P, B and T) has a score of 4 and only gives a PBT hazard score of 0 if all of the three properties have a score of 1 ($D_i = 1$). The ranking based on the utility function is less severe than that based on the desirability function, giving a PBT hazard score of 1 if (and only if) all of the three properties (P, B and T) have a score of 4.

Partial order ranking (POR) methods are vectorial approaches that recognize that different criteria are not always in agreement but can be conflicting, which means that not every substances can be directly compared with others (Pavan and Worth, 2008). The Hasse Diagram is a way to represent the results of this method graphically. Incomparable alternatives are not connected by lines and are located at the same geometrical height and as high as possible in the diagram, resulting in a structure of levels. Alternatives belonging to the same level are incomparable (Pavan and Worth, 2008). The general principles of this method are found in Appendix A (supplementary material).

The occurrence is a fourth property included in this study in the three proposed ranking scores (ranking of hazard OPBT). The occurrence was not transformed to a defined level of concern as PBT indexes because there is not a defined scale that suggests ranges of amounts of PPCPs that should be released in the environment. Thus, the best condition matches the smallest amount of compound discharged to aquatic environments, and the worst matches the highest quantity. The values of occurrence of PPCPs and metabolites in the Spanish aquatic environment were taken from Ortiz et al. (2013).

Finally, PPCPs are ordered by each hazard score (total rankings) or level (partial ranking) and numbered consecutively. The compound that is located in the highest level of hazard will be number 1 and so on to the last PPCP or metabolite of the classification. Compounds that belong to the same level (in total rankings) will have the same number in the ranking score.

2.6. Uncertainties of models and rankings

All of the models used (EPIWIN™, ECOSAR and BCFBAF) have some degree of uncertainty and limitations that have been reported in detail for each methodology (US EPA, 2009) and summarized in the previous sections. However, in this work, all of these

parameters were classified and grouped in four levels of concern before developing the ranking. These levels are sufficiently wide to consider the uncertainties of each toxicological value, which is considered to be approximately 20% in a recent study of methodology to account for uncertainties and tradeoffs in pharmaceutical environmental hazards (Coutu et al., 2012), for which the final ranking will not change significantly.

However, the assignment of weights and its uncertainty is a complex issue that has generally been evaluated by expert judgments (panel of experts) (Coutu et al., 2012). For this reason, the same weight is generally assigned to each index to evaluate all of them with the same relevance (Kumar and Xagoraraki, 2010). In this study, a sensitivity analysis for the index weights was conducted to verify their influence and changes in the compounds ranking list. The DART utility function was used for eight different combinations of PBT weights, as shown in Fig. 1.

3. Results and discussion

3.1. PPCP selection, physicochemical properties and occurrence in Spanish aquatic environments

Antibiotics, hormones, analgesics, antipyretics, some antiepileptics (carbamazepine), angiotensin converting enzyme (enalapril) and blood lipid regulators (clofibrate, clofibric acid) are the most studied pharmaceuticals in accordance to their occurrence, treatment and potential negative environmental impacts. More recently, there has been an increase in interest in antidepressants, cytostatics, anxyolitics, angiotensin receptor blockers, H_2 blockers, and others types of blood lipid regulators (simvastatin, fluvastatin, atorvastatin) due to changes in prescription drug protocols, increased consumption, and the recent detection of them in the environment.

All compounds considered in this study are organic molecules and mostly contain cyclic structures: cycloalkanes, benzene rings and polynuclear hydrocarbons. Oxygen, nitrogen, halogens and sulfur are the main heteroatoms present. These structural characteristics directly influence their physical, chemical or biological behavior, combined with other factors, such as interaction with complex matrices, pH, the temperature, their physical condition and the environment where they are discharged. Appendix B (supplementary material) collects the physicochemical properties of pharmaceutically active compounds (PhACs) (Table B1, Appendix B), personal care products (PCPs) and metabolites (Table B2, Appendix B). These data are relevant to estimate and evaluate the potential of PBTs of PPCPs according the (Q)SAR methodology.

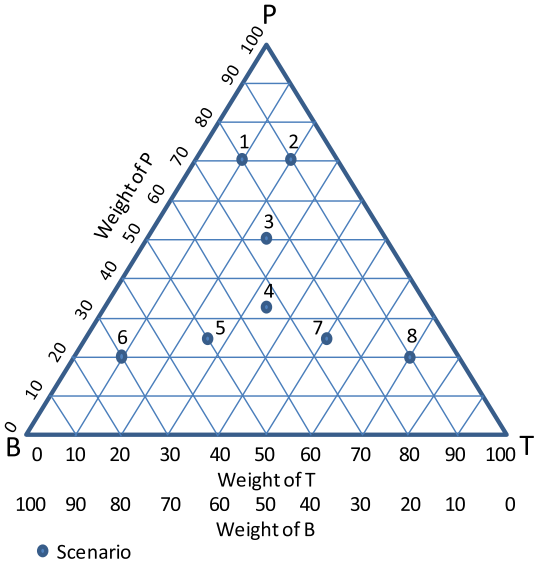

Fig. 1. Scenarios evaluated in the weight sensitivity analysis of PBT.

The PPCPs evaluated in this study have been classified into three general types: PhACs ($n = 62$; 64.6%), which are subclassified in fourteen types, according to the Anatomical, Therapeutic, Chemical Classification System (ATC); metabolites ($n = 22$; 22.9%) and PCPs ($n = 12$; 12.5%), which are subclassified in four types (Table 1).

The occurrence of PPCPs and metabolites that are being discharged into Spanish aquatic environments was evaluated according to the methodology of Ortiz et al. (2013). These values are listed in Table 3 for PhACs, PCPs and metabolites according to the data calculated for January 1, 2010. The compounds that had the highest occurrence (over 10 t y^{-1}) were the PhACs acetaminophen, valsartan, amoxicillin, iopromide, omeprazole, iopamidol and acetylsalicylic acid, the PCPs Galaxolide®, Iso-E-super® and Tonalide® and the metabolites acetaminophen glucuronide, salicylic β-D-O-glucuronide acid, 2-hydroxy ibuprofen and carbamazepine 10, 11-epoxide.

Twenty-five compounds had occurrences between 1 and 10 t y^{-1}, and fifty two were below 1 t y^{-1}. The occurrence of diethylstilbestrol from human consumption was considered zero even though its presence has been reported (Petrović et al., 2002) due perhaps to veterinarian consumption or fraudulent use. Insufficient data are available for gestodene, ethylparaben, p-hydroxybenzoic acid, triclosan and 4-nonylphenol; therefore, the occurrence of these compounds could not be determined.

3.2. PBT of PPCPs and metabolites by (Q)SAR models

(Q)SARs models were applied for the PPCPs and metabolites under study to estimate the P, B and T values. These results are shown in Table 3 with their corresponding levels of concern according to information shown in Table 2. Fig. 2 shows the number of PPCPs grouped by different levels of concern for each environmental index (PBT).

Fig. 2 shows the persistence of a large amount of compounds, 88.5% of them (85 of the 96 PPCPs studied) located in levels 3 and 4 of the classifications (highest concern scores). The persistence of the remaining 11.5% of PPCPs investigated resulted in lower concern (at the second level of concern) according the (Q)SAR prediction assessment performed. This group included the following compounds: gabapentin, ethylparaben, methylparaben, acetilsalicylic acid, salicylic acid, pregabalin, valproic acid, and four metabolites: acetaminophen glucuronic, ibuprofen carboxylic acid, salicylic β-D-O-glucuronide acid and p-hydroxybenzoic acid. The metabolites mentioned above are hydrophilic molecules that can react and decompose rapidly in an aquatic environment. The other metabolites considered were found to be as persistent as their parent compound.

Several authors have related the PPCP persistence in their studies, i.e., Cooper et al. (2008) and Homem et al. (2010) for antibiotics, Liebig et al. (2006) for 17-α ethinylestradiol and iopromide, Redshaw et al. (2008) for some antidepressants and their human metabolites. These PPCPs have the highest persistence index among the compounds included in this study.

The persistence itself does not represent environmental risk, but it indicates that PPCPs will be available in different media and therefore may be a negative factor for other impacts such as bioaccumulation or chronic toxicity. The long-term negative effects caused by many of these persistent compounds are still unknown. Kümmerer (2009) shows that the persistence of organic pollutants increases their long term contaminant potential, and, as

Table 3
Values of OPBT endpoints and levels of concern of the PPCPs under study.

Type	Compound name	Occurrence[g] (kg L^{-1})	Persistence						Bioaccumulation (L kg wet-wt^{-1})		Toxicity (ChV[f]) (mg L^{-1})	
			BIO2[a]	BIO3[b]	BIO6[c]	LC[d]	BCF[e]	LC	Fish	Daphnid	Algae	LC
PhACs												
Analgesic/antipyretic	Acetaminophen	23,267.40	0.99	2.9	0.510	3	3.162	1	335.3	108.2	88.92	1
Angiotensin converting enzyme (ACE) inhibitors	Enalapril	725.20	0.99	2.7900	0.080	3	3.162	1	11.44	7.410	15.06	1
Angiotensin receptor blockers (ARBs)	Valsartan	20,350.90	0.88	2.8400	0	3	3.162	1	1.235	1.175	3.940	2
	Irbesartan	3810.87	0.43	2.3000	0	3	1470	2	0.046	0.075	0.502	4
Antibiotics	Amoxicillin	15,256.93	0.98	2.2500	0.020	3	3.162	1	202.0	81.70	90.00	1
	Azithromycin	1933.32	0	0.9700	0	4	208.6	1	4.730	3.950	11.20	2
	Cefaclor	119.60	0.99	2.5100	0.005	3	3.162	1	695.2	230.3	195.7	1
	Ciprofloxacin	2402.03	0	1.9000	0	4	3.162	1	1249	369.9	271.8	1
	Clarithrormycin	5820.23	0	1.2000	0	4	56.49	1	5.370	4.400	12.12	2
	Erythromycin	910.75	0	1.2400	0	4	48.53	1	20.91	13.68	28.20	1
	Levofloxacin	404.15	0	1.5100	0	4	3.162	1	2017	560.3	378.9	1
	Moxifloxacin	905.81	0	1.7000	0	4	3.162	1	231.6	92.98	101.6	1
	Norfloxacin	1118.69	0	1.9400	0	4	3.162	1	2197	590.0	381.8	1
	Roxythromycin	34.24	0	1.0200	0	4	30.34	1	13.94	9.950	22.98	1
	Sulfamethoxazole	2084.07	0.13	2.4300	0	3	3.162	1	367.4	127.0	114.2	1
	Trimethoprim	44.57	0.92	2.0400	0.020	4	3.162	1	259.9	97.17	96.73	1
Antidepressants	Escitalopram	308.24	0	1.5000	0	4	136.8	1	0.770	0.750	2.620	3
	Fluoxetine	324.51	0.13	2.0000	0	4	218.4	1	0.122	0.160	0.816	3
	Fluvoxamine	315.11	0	2.0000	0	4	50.41	1	2.744	2.180	5.784	2
	Paroxetine	58.61	0	1.9000	0	4	188.6	1	0.513	0.538	2.050	3
	Sertraline	488.95	0	2.0600	0	4	1429	2	0.034	0.055	0.370	4
Antiepileptics	Carbamazepine	2595.31	0.41	2.6800	0.040	3	19.21	1	10.60	6.440	12.06	1
	Gabapentin	1943.87	0.65	3.0000	0.700	2	3.162	1	9589	1832	761.6	1
	Pregabalin	4175.19	0.92	3.2400	0.500	2	3.162	1	20,183	3378	1182	1
	Topiramate	3741.51	0	1.7700	0.002	4	3.162	1	2430	648.3	416.0	1
	Valproic acid	229.80	0.83	3.2500	0.760	2	3.162	1	17.66	13.94	33.40	1
Anxiolytics	Alprazolam	60.22	0.27	2.2600	0	3	11.64	1	0.570	0.580	2.130	3
	Bromazepam	100.13	0.11	2.1000	0.030	4	10.46	1	26.56	14.58	23.90	1
	Lorazepam	304.99	0.14	2.1800	0.010	4	17.54	1	10.51	6.720	13.46	1
Blood lipid regulators	Atorvastin	715.38	0	2.1600	0	4	56.23	1	0.008	0.017	0.179	4
	Bezafibrate	234.02	0.57	2.1580	0.043	4	3.160	1	0.312	0.360	6.450	3
	Clofibrate	2.45	0.90	2.3300	0.610	3	113.8	1	0.727	0.686	2.275	3
	Fluvastatin	329.66	0	2.5700	0	3	3.162	1	0.110	0.154	0.849	3
	Simvastatin	1267.82	0.99	2.5000	0.120	3	568.7	1	0.057	0.088	0.564	4
Cytostatics/cancer therapeutics	Cyclophosphamide	203.28	0.01	2.2800	0.020	3	3.162	1	144.3	58.35	64.31	1
	Flutamide	30.15	0	1.8500	0	4	75.39	1	1.036	0.942	2.980	2
	Gemcitabine	85.67	0.02	2.7200	0	3	3.162	1	52,522	8165	2595	1
	Ifosfamide	177.25	0.01	2.2800	0.021	3	3.162	1	144.3	58.35	64.31	1
	Mitomycin	1.30	0	1.9400	0	4	3.162	1	12,766	2596	1170	1
	Tamoxifen	9.78	0.80	2.1000	0	4	6689	4	0.006	0.013	0.127	4
H_2 blockers	Esomeprazole	780.65	0.89	1.9600	0.010	4	13.75	1	1.610	1.410	4.270	2
	Lanzoprazole	808.09	0	1.5200	0	4	123.7	1	0.990	0.950	3.235	3
	Omeprazole	12,992.28	0.89	2.0000	0.010	4	13.75	1	1.610	1.413	4.300	2
	Pantoprazole	1728.75	0.93	1.9600	0	4	13.64	1	18.01	10.86	20.15	1
Hormones	Desogestrel	0.00215	0	2.1000	0.002	4	2489	4	0.017	0.031	0.240	4
	Diethylstilbestrol	0	0.73	2.7200	0.080	3	1030	2	0.015	0.027	0.210	4
	Gestodene	n.a.	0	2.0700	0.030	4	66.24	1	1.880	1.580	4.520	2
	Levonorgestrel	0.17	0	2.0600	0.050	4	91.83	1	1.237	1.115	3.500	2
	Megestrol	745.29	0.01	1.8000	0.110	4	202.9	1	0.544	0.580	2.250	3
	Norethisterone	8.19	0	2.1000	0.050	4	42.33	1	3.110	2.400	6.110	2
	Progesterone	127.3	0	2.0400	0.070	4	166.1	1	0.862	0.825	2.800	3
	Testosterone	0.14	0.02	2.3000	0.150	3	72.03	1	1.730	1.460	4.170	2
	17-α ethynylestradiol	0.29	0.07	2.0300	0.040	4	122.6	1	0.335	0.370	1.510	3
17-β estradiol	69.12	0.65		2.4500	0.100	3	205.5	1	0.433	0.453	1.720	3
Inhibiting platelet aggregation	Acetylsalicylic acid	10,563.69	0.99	3.0300	0.940	2	3.162	1	73.35	31.17	36.64	1
Non-steroidal	Diclofenac	3963.10	0	2.2900	0.003	3	3.162	1	4.580	4.660	17.79	2
Antiinflamatories	Ibuprofen	4849.50	0.87	2.9600	0.150	3	3.162	1	4.940	4.760	16.50	2
(NSAIDs)/	Ketorolac	217.64	0.88	2.9000	0.210	3	3.162	1	9.920	6.170	11.92	2
Antirreumatics	Naproxen	4196.75	0.96	2.9000	0.340	3	3.162	1	21.31	17.40	44.40	1
X-ray contrast media	Iohexol	5127.22	0	2.0500	0	4	3.162	1	779,000	94,025	21,503	1
	Iopamidol	11,415.95	0	1.9800	0	4	3.162	1	44013	8394	3481	1
	Iopromide	14,752.11	0	1.7800	0	4	3.162	1	402,000	53,741	14,019	1
Metabolites	Acetaminophen glucuronide	601,384.41	0.9795	3.2000	0.3054	2	3.160	1	888.9	5.468	5.468	2
	Carbamazepine 10,11 dihydrodiol	306.12	0.8000	2.9200	0.1439	3	3.160	1	11.45	6.714	0.452	2
	Carbamazepine 10, 11 epoxide	12,240.09	0.1790	2.6330	0.0330	3	3.160	1	0.044	31.45	214.4	1
	Clofibric acid	66.95	0.29	2.6100	0.370	3	3.162	1	33.00	25.34	57.59	1
	Clofibric acyl-β-D-glucuronide acid	154.82	0.6493	2.8348	0.2901	3	3.160	1	764.0	14305	1045	1
	Estrone	28.22	0.28	2.3000	0.110	3	53.97	1	1.180	1.050	3.200	2
	Ibuprofen carboxylic acid	1607.44	0.8900	3.2566	0.1872	2	3.160	1	201.3	125.4	193.6	1

Table 3 (*continued*)

Type	Compound name	Occurrence[g] (kg L^{-1})	Persistence						Bioaccumulation (L kg wet-wt^{-1})		Toxicity (ChV[f]) (mg L^{-1})	
			BIO2[a]	BIO3[b]	BIO6[c]	LC[d]	BCF[e]	LC	Fish	Daphnid	Algae	LC
	Metabolite paroxetine[h]	1612.40	0.0058	1.9496	0.0024	4	211.0	1	0.224	0.233	2.129	3
	N-desmethyl escitalopram	300.59	0	1.8270	0.0003	4	99.20	1	0.128	0.026	0.168	3
	N-desmethyl sertraline	655.89	0.0298	2.0889	0.0080	4	704.0	1	0.023	0.018	0.037	4
	Norfluoxetine	92.79	0.1550	2.0200	0	4	267.0	1	0.052	0.021	0.077	4
	Salicylic acid	859.07	0.9870	3.0382	0.8757	2	3.160	1	10.87	9.982	110.4	1
	Salicylic β-D-O-glucuronide acid	11,094.54	0.9778	3.3700	0.7490	2	3.160	1	60,250	19,371	8798	1
	Sertraline carbamoyl glucuronide	1998.37	0.0007	2.2100	0.0005	3	3.162	1	0.962	0.978	3.576	3
	Sulfamethoxazole hydroxylamine	172.21	0.4400	2.5293	0.0129	3	3.160	1	3.503	2.570	0.450	2
	1,4 benzoquinone	2927.78[i]	0.6382	2.9153	0.7660	3	3.160	1	0.012	0.041	0.996	4
	4-chlorobenzoic acid	29.09[i]	0.7672	2.7344	0.6708	3	3.160	1	45.49	32.32	63.62	1
	2-hydroxy carbamazepine	152.89	0.5800	2.6980	0.0332	3	4.000	1	0.889	0.584	1.223	3
	3-hydroxy carbamazepine	152.89	0.5800	2.6980	0.0332	3	4.000	1	0.889	0.584	1.223	3
	4-hydroxy diclofenac	4353.27	0.0053	2.3070	0.0026	3	3.160	1	6.732	6.608	66.03	2
	5-hydroxy diclofenac	53.89	0.0053	2.4427	0.0176	3	3.160	1	0.270	1.843	3.804	2
	2-hydroxy ibuprofen	15,181.13	0.4816	2.7100	0.1569	3	3.160	1	101.0	67.93	120.9	1
PCPs												
Biocide	Triclosan	n.a.	0.0180	1.9400	0.0187	4	642.0	1	0.082	0.089	0.756	4
Fragrances	Galaxolide® (HHCB)	69,221.20	0.0009	2.1200	0.0255	4	3639	4	0.005	0.009	0.088	4
	Iso-E-super® (OTNE)	47,199.01	0.0145	2.2345	0.1886	3	1220	2	0.037	0.049	0.317	4
	Musk ketone	2480.81	0.0007	1.8253	0.0001	4	131.0	1	0.007	0.038	0.042	4
	Musk xilene	4369.77	0.0001	1.6700	0	4	401.0	1	0.006	0.033	0.039	4
	Phantolide® (AHDI)	1201.29	0.0220	2.1376	0.0860	4	880.0	1	0.010	0.016	0.140	4
	Tonalide® (AHTN)	11,074.93	0.0183	2.1066	0.0737	4	696.0	1	0.004	0.007	0.079	4
Preservatives	Ethylparaben	n.a.	0.9964	3.0285	0.8310	2	19.80	1	0.935	0.874	9.418	3
	Methylparaben	2148,67	0.9970	3.0600	0.8275	2	9.120	1	1.868	26.98	4.586	2
	P-hydroxybenzoic acid	n.a.	0.9870	3.0380	0.8760	2	3.160	1	33.33	15.42	20.24	1
	Propylparaben	688.81	0.9957	2.9970	0.8344	3	47.10	1	0.516	0.499	5.098	3
Surfactant	4-nonylphenol	n.a.	0.9612	2.9900	0.5100	3	124.0	1	0.006	0.012	0.111	4

n.a.: Data not available.
[a] Biowin 2.
[b] Biowin 3.
[c] Biowin 6.
[d] Level of concern.
[e] Bioconcentration factor.
[f] Chronic toxicity.
[g] Data of occurrence from Ortiz et al. (2013).
[h] 4-[[(3S,4R)-4-(4-fluorophenyl)piperidin-3-yl]methoxy]-2-methoxyphenol.
[i] Calculate in this study based on the methodology of Ortiz et al. (2013).

a consequence, longer exposure increases the potential for multiple contaminations of the ecosystem.

Bioaccumulation is the second environmental index evaluated. The results indicate that 96.8% of the PPCPs analyzed in this study are located in levels 1 or 2, corresponding to low levels of concern. Only three compounds have the highest level: tamoxifen, Galaxolide® and desogestrel. Brausch and Rand (2011) confirm in their (Q)SAR study that nitro and polycyclic musks (as Galaxolide®) have high log K_{ow} values, which indicates a great potential for their bioaccumulation in aquatic species. The occurrence of the antiestrogen tamoxifen was reported in U.K. wastewater and was not reduced in the sewage treatment plants (Fent et al., 2006).

Despite the classification obtained, specific research shows that compounds with concern score levels of 1 or 2 can also bioaccumulate. Thus, fluoxetine and sertraline and their metabolites norfluoxetine and N-desmethyl sertraline have been detected in wild fish sampled in the U.S. (Fent et al., 2006) and in wastewater treatment plant effluents (Celiz et al., 2009). Ethylparaben, methylparaben, and triclosan exhibit bioaccumulation potential (Anca and Gravilescu, 2009) as well as ciprofloxacin and erythromycin (Gao et al., 2012).

Information about the bioaccumulation of pharmaceuticals in biota or food webs is still limited (Nikolaou et al., 2007), and the data are dispersed and calculated for different species. In addition, the ranges of values of bioconcentration or bioaccumulation factors for many PPCPs are very wide, and thus a compound can be classified in different levels of concern according to the data source. Thus, the values calculated using the BCFBAF method could be verified with real data in a specific geographical area to achieve a specific ranking.

Most of the compounds exhibiting these divergent results for the bioconcentration factor were ionic compounds, confirming the poor correlation reported by the BCFBAF method. Therefore, it is necessary to improve the estimation of the bioconcentration factor for these types of substances to obtain an estimation and a ranking process more adjusted to reality. Daughton and Brooks (2010) have indicated that predictive models for bioconcentration in fish are not yet up to the task; therefore, empirical data are needed at least to validate computational approaches.

Toxicity is the third index under evaluation. Fig. 2 shows that 18.8% ($n = 18$) of PPCPs are in level 4 of the classification (higher concern score), 19.8% ($n = 19$) are in level 3, 22.9% ($n = 22$) are in the second level, and the remaining 38.5% ($n = 37$) are in the lowest score.

The principal PhACs placed in the highest level of risk are the hormones, antidepressants and blood lipid regulators. The most relevant PCPs are triclosan (antimicrobial disinfectant), 4-nonylphenol (surfactant) and all fragrances considered.

It is important to highlight that some metabolites have a toxicity level of risk equal to or higher than their parent compounds. N-desmethyl sertraline is located at the same level of concern as its parent compound (sertraline) (level 4) as along with paroxetine

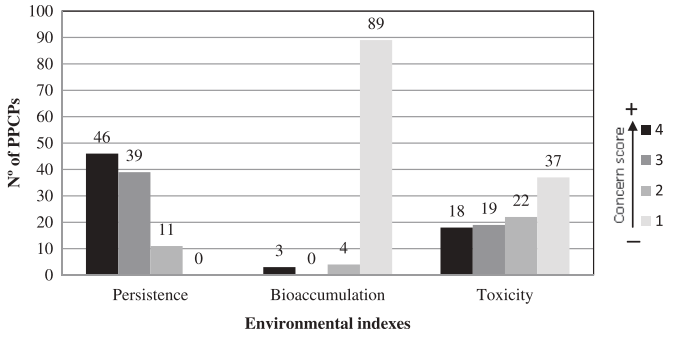

Fig. 2. Number of PPCPs grouped by each environmental index and concern score.

and escitalopram and their metabolites (level 3). Norfluoxetine is in level 4, and fluoxetine is in a lower level (3); 2 and 3 hydroxy carbamazepine are in level 3, and carbamazepine is in level 1. Sulfamethoxazole hydroxylamine is in level 2, and its parent compound is in level 1. The transform byproduct of acetaminophen, 1,4 benzoquinone, has a level of concern of 4 while its parent compound is in level 1. Celiz et al. (2009) confirm that 1, 4 benzoquinone and at least other metabolite can be formed during the chlorination of wastewater containing acetaminophen, and they have toxicities higher than their parent drug.

Desogestrel and mestranol (progestins) are in the fourth and third levels of concern of toxicity, respectively. Generally, most studies of the environmental impact of hormones have been conducted for estrogens, and there is less for progestins, although many hormonal medicine formulations have a higher content of progestins than estrogens. However, some studies have found that natural steroids and artificial hormones are removed by wastewater treatment processes with variable percentages of elimination of 38–83% (Bound and Voulvoulis, 2004). Besse and Garric (2009) recommend studies on occurrence, toxicity and degradation time for several of these compounds, and Streck (2009) confirms that research on progestagenic and androgenic compounds is only just beginning. Therefore, it is important to consider especially progestins in environmental impact assessments.

Antidepressant use and its appearance in different aquatic environments (including metabolites) has increased in recent years. Sertraline, its metabolite N-desmethyl sertaline and norfluoxetine (fluoxetine metabolite) are at a higher level of concern of toxicity in this study. Recently, Santos et al. (2010) made some comments on studies where sertraline exhibits highly toxic properties. Doerr-MacEwen and Haight (2006) found that the antidepressant fluoxetine induce mussel spawning in laboratory studies. However, Cunningham et al. (2004) found that paroxetine and its major residual metabolite will not persist in the aquatic environment after discharge from a WWTP.

Blood lipid regulators are included in cardiovascular pharmaceuticals, which are highly prescribed compounds; five of them are in the top ten products on the top 200 US Rx list (Sanderson et al., 2004a). Attention has been recently focused on the occurrence and treatment of these compounds, specifically clofibrate and its derivatives. However, these compounds have been progressively replaced by statins. This class of drugs was predicted as the most hazardous of all therapeutic pharmaceutical compounds in a recent comprehensive (Q)SAR screening (Sanderson et al., 2004b). According to our results, atorvastatin and simvastatin show a greater potential risk of environmental toxicity than clofibrate and its derivates, which have been studied extensively.

In the case of PCPs, musk xilene and ketone (nitro musk) show more toxicity than Tonalide®, Galaxolide®, Phantolide® and Iso-E-super®, and the less toxic compound was triclosan, but all of them were at the highest toxicity level. However, according to Brausch and Rand (2011), polycyclic musks are more acutely toxic than nitro musks, and they have relatively low or no propensity to cause acute toxicity to aquatic taxa, although they are potentially toxic to aquatic organisms over longer periods of time. In view of these results, it is necessary to review the fate of these compounds in the environment, verify acute and chronic toxicity for a wide range of organisms and study the possible reactions and transformation products formed.

3.3. PBT rankings by total and partial DART

According to the total ranking by desirability function evaluated with equal weights for all PBT indexes, approximately 50% of compounds are located at the highest level of hazard because these substances have at least one index with a level of concern of 4. Antibiotics, antidepressants (and its metabolites), anxiolytics (and its metabolites), hormones, blood lipid regulators, H_2 blockers and fragrances are the principal compounds that highlight in this level of concern.

The total ranking by utility function provides a more specific ranking that is organized in more levels of concern and is less severe than the desirability function.

The total ranking by utility function estimated with equal weights for all PBT indexes has shown that the twenty five most hazardous PPCPs and metabolites distributed in the first five levels of concern were desogestrel, Galaxolide®, tamoxifen, sertraline, atorvastatin, musk ketone, musk xilene, N-desmethyl sertraline, norfluoxetine, Phantolide®, Tonalide®, triclosan, diethylstilbestrol, irbesartan, Iso-E-super®, bezafibrate, escitalopram, fluoxetine, lanzoprazole, megestrol, metabolite of paroxetine, N-desmethyl escitalopram, paroxetine, progesterone and 17-α ethynylestradiol.

The top 25 compounds of the total ranking by utility function are in concordance with the results of the partial hazard ranking score (although the compounds are grouped in fewer levels) performed with the Hasse Diagram for the PBT indexes (See Fig. 3). According to this diagram, only 3.1% ($n = 3$) of the PPCPs studied are at the highest level of concern (level 1), desogestrel, Galaxolide® and tamoxifen, because they have the maximum score in all indexes, and 12.5% ($n = 12$) of PPCPs are at the next two levels of hazard (levels 2 and 3). In the lower levels (level 7 and 8) are 25% ($n = 24$) of the studied compounds studied, and the remaining 59.4% ($n = 57$) are in the middle.

Fig. 3 illustrates the ranking of the PPCPs evaluated by levels of concern and also interrelates them according to the common indexes. Thus, each case could be analyzed or discarded according to the particular assessment required. Only PPCPs belonging to level 1 show the highest P, B and T levels of concern. Bioaccumulation is less relevant in the other levels. On the left side of the Hasse diagram are those PPCPs and metabolites that exhibit a high persistence index while the toxicity index decreases from top to bottom. In contrast, the right column has those PPCPs that have a high toxicity index, and the persistence index decreases from top to bottom. This graph is an easy way to identify those compounds that share similar levels of concern or to know those that are highlighted as hazardous in terms of one, two or three criteria.

3.4. Sensitivity analysis of the PBT rankings

The weights of the indexes are an interesting feature to evaluate in ranking methodologies. Kumar and Xagoraraki (2010) have considered weights equally to avoid any judgment bias. More recently, Coutu et al. (2012) have considered the weights as a random variable, and their weight uncertainties are based on a hazard ranking from a panel of experts. In this study, eight different

weight combinations for PBT (See Fig. 1) were considered to evaluate the impact of these scenarios on the total hazard score by utility function. Appendix C (supplementary material) shows these results for seven of the eight scenarios. Scenario 2 is not shown because it is quite similar to scenario 1.

For all cases, the 25 most hazardous compounds according to the utility function remain at the highest levels of the classifications except for two groups of compounds: (i) diethylstilbestrol, irbesartan, and Iso-E-super® and (ii) simvastatin, 1,4 benzoquinone, and 4-nonylphenol, which have high mobility through the different scenarios when the PBT weights change (See Appendix C). In scenarios with high weights of persistence, these two groups of compounds are located in the middle of the ranking, and, for high weights of B and T, these compounds remain at the top of the rankings. A similar behavior occurs with ethylparaben, acetaminophen glucuronide and methylparaben in the lower half of the rankings. This higher or lower dispersion of some compounds in the ranking of hazardous levels has been found in another study (Coutu et al., 2012), although the group of compounds studied and the methodology were different.

Therefore, with the uncertainty analysis performed in this work, the top 25 most hazardous compounds of the ranking could vary by 24%, if the PBT weights change significantly (more than 50% in one index), as shown in Appendix C (supplementary material).

Other researchers have developed concern rankings through different methodologies and specific lists of compounds based on their interest and geographic area. Many of PPCPs that are in our top 25 according to the level of concern are highlighted in these studies. It must be pointed that metabolites have been less often compared because these compounds have not been widely considered in such research. In the priority ranking, the following 8 compounds in the top 25 of Kumar and Xagoraraki (2010) for ecological effects match those of this study: Tonalide®, Galaxolide®, 4-nonylphenol, fluoxetine, triclosan, 17-α ethynylestradiol, musk xilene, and musk ketone. The same research in the ranking of stream water found the aforementioned compounds in addition to bezafibrate, atorvastatin and norfluoxetine. In Coutu et al. (2012), there are fewer matches in their environmental ranking (17-α ethynylestradiol, simvastatin, irbesartan and fluoxetine), but it should be emphasized that this work did not consider PCPs and metabolites, which occupy important positions in our study.

Although antibiotics are ranked in the middle of the classification, those with the highest risk score have also been identified as priorities in other studies (Cooper et al., 2008; Coutu et al., 2012; Kumar and Xagoraraki, 2010), mainly the macrolides and quinolones.

According to Cooper et al. (2008), the most noteworthy dilemma with any risk ranking is the lack of data available for a large number of pharmaceuticals. Kumar and Xagoraraki (2010) assumed an intermediate neutral value between 0 and 1, i.e., 0.5, of an attribute in the utility function in the absence of any data. Coutu et al. (2012) established hazard rankings from a limited number of criteria and decision makers that were dictated by data availability on pharmaceutical properties, time, and the availability of decision makers. In this work, this limitations were avoided by using the (Q)SARs methodologies, the predicted values of P, B, T and the levels of concern recommended by European policies, but these tools have their own uncertainties. Therefore, all of these rankings are powerful tools to provide a hierarchical list of hazardous compounds in a short time and with a wide range of criteria, but it will be experimental evidence that allows us to conclude the extent and magnitude of the adverse effects of each compound.

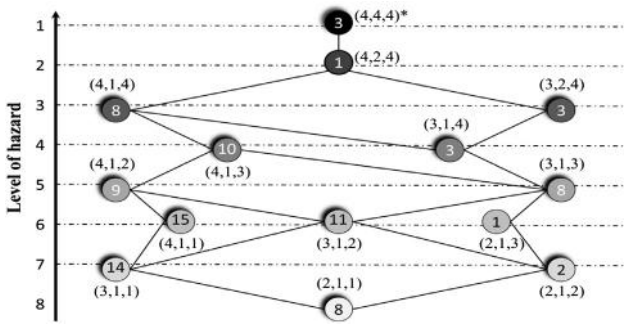

Fig. 3. Partial ranking of concern of PPCPs according to the Hasse diagram for the PBT indexes. *(P,B,T) concern score. The PPCP level of hazard increases from the bottom to the top. The numbers inside the circles are the amounts of PPCPs in each group. The union lines indicate that these PPCPs have two parameters in common.

3.5. OPBT rankings by total and partial DART

The occurrence was included in three rankings of concern as another environmental index with equal weights for O, P, B and T: two rankings with total DART methods by desirability and utility functions and another with the partial DART method.

The OPBT ranking by desirability function has fourteen levels. This ranking placed 50 compounds (55% of total evaluated) in the highest level of hazard (level 1), where antibiotics, hormones, anxiolytics (and some of their metabolites), antidepressants, H_2 blockers and PCPs predominate. The results of the OPBT ranking by desirability function are similar to those of the PBT ranking. The principal differences between the OPBT and PBT ranking by desirability function are (i) the inclusion of acetaminophen glucuronide in the highest level of hazard for its high occurrence and (ii) the higher number of levels of the OPBT ranking due to the incorporation of the occurrence as an index because these values were very particular for each compound. PPCPs with a high desirability indicate that such compounds have at least one index (O, P, B or T) at the highest level of hazard, but the method does not discriminate based upon which type of index it is.

The OPBT ranking by utility function has thirty eight levels, and the compounds located in the 25 first places of this classification are very similar to those obtained in PBT ranking, as evidenced by the comparison between scenario 4 (equal weights of indexes) in Appendix C (supplementary material) and in the OPBT ranking by the utility function shown in Fig. 4. In this ranking, fragrances, a few hormones, blood lipid regulators, antidepressants and anxiolytics (and some of their metabolites) predominate in the first positions.

Although utility is more specific than the desirability function and allows a more specific classification, a partial ranking is recommended for the disagreement that can be presented between the levels of concern of PBT indexes and the values of occurrence. The total and partial rankings have a different focus and principles, the Hasse diagram technique (partial ranking) does not require any transformation function, and the criteria are not weighted. Partial ranking can detect incomparabilities, contradictions or conflicts among the criteria (Pavan and Worth, 2007). Lerche et al. (2002) have indicated that the Hasse diagram technique needs the least external input, is the most transparent and is the least subjective. However, this technique has some weaknesses if there are criteria that exclude each other. Then, weighting is needed, and total ranking can handle such mutual exclusions because their formalisms to quantify preferences allow participation, e.g., weighting of criteria.

The OPBT ranking by partial DART has 21 levels, as shown in Fig. 4 and Appendix D (supplementary material), and the top 25 differ by 40% of the compounds in comparison with the OPBT ranking by utility function, which shows the great difference between these two ranking techniques and the need to apply several

ranking techniques to identify priority compounds according to the focus of the study. Some examples are taken from Fig. 4 and the data from Table 3 to explain the differences among these classifications. Desogestrel and tamoxifen are located at the second level of concern in the OPBT utility ranking score but at the 18th level in the partial ranking. Though these compounds have the highest levels of concern according to P, B, T criteria as Galaxolide® (placed at the first level in the utility ranking score), the occurrence between them differs substantially, as shown in Table 3 data. Therefore, desogestrel (ID: 71) and tamoxifen (ID: 78) are connected by a

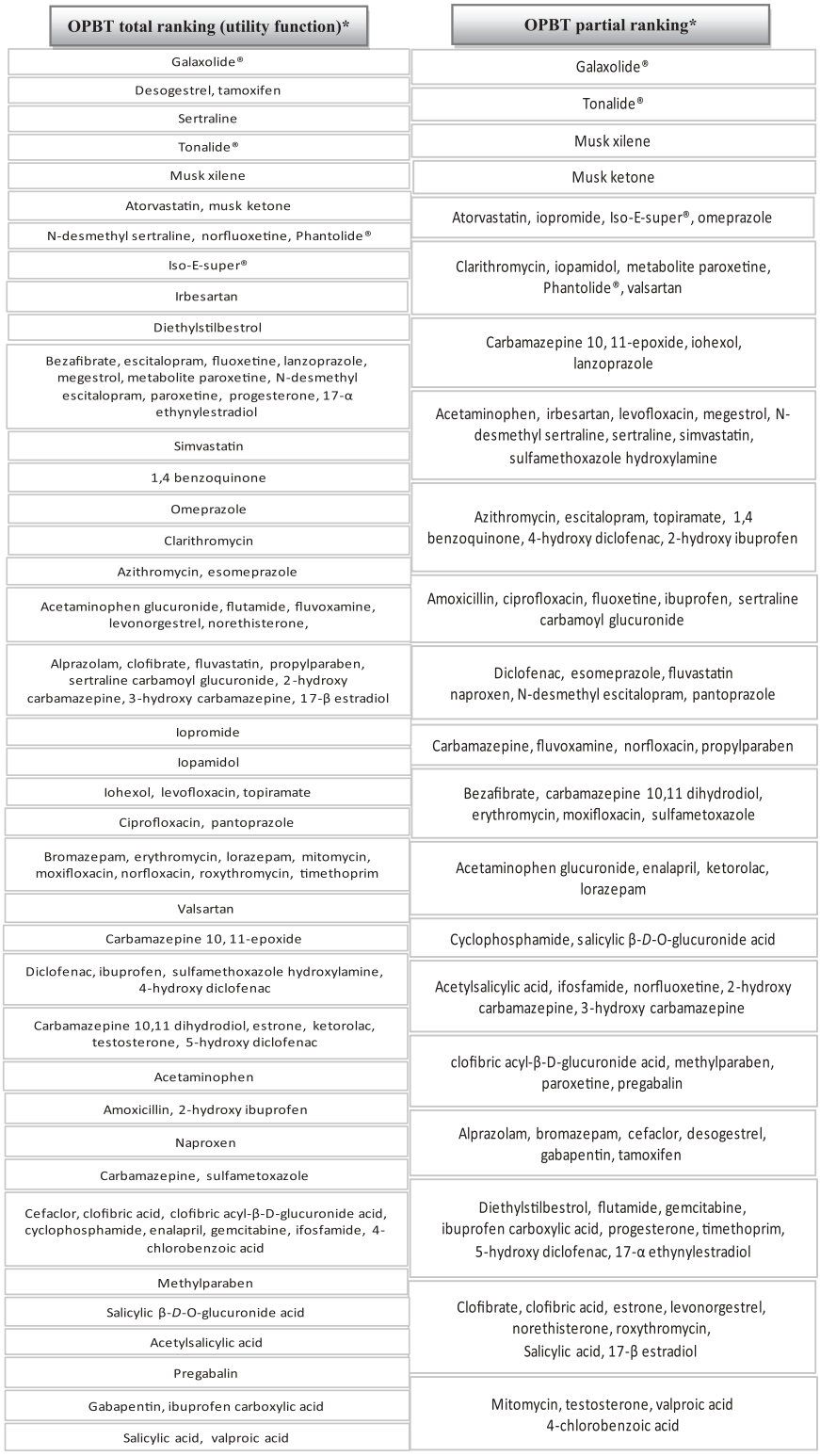

Fig. 4. OPBT partial and total rankings of hazard. *These rankings do not include gestodene, ethylparaben, p-hydroxybenzoic acid, 4-nonylphenol and triclosan due to the lack of data. PPCP Level of hazard increases from the bottom to the top.

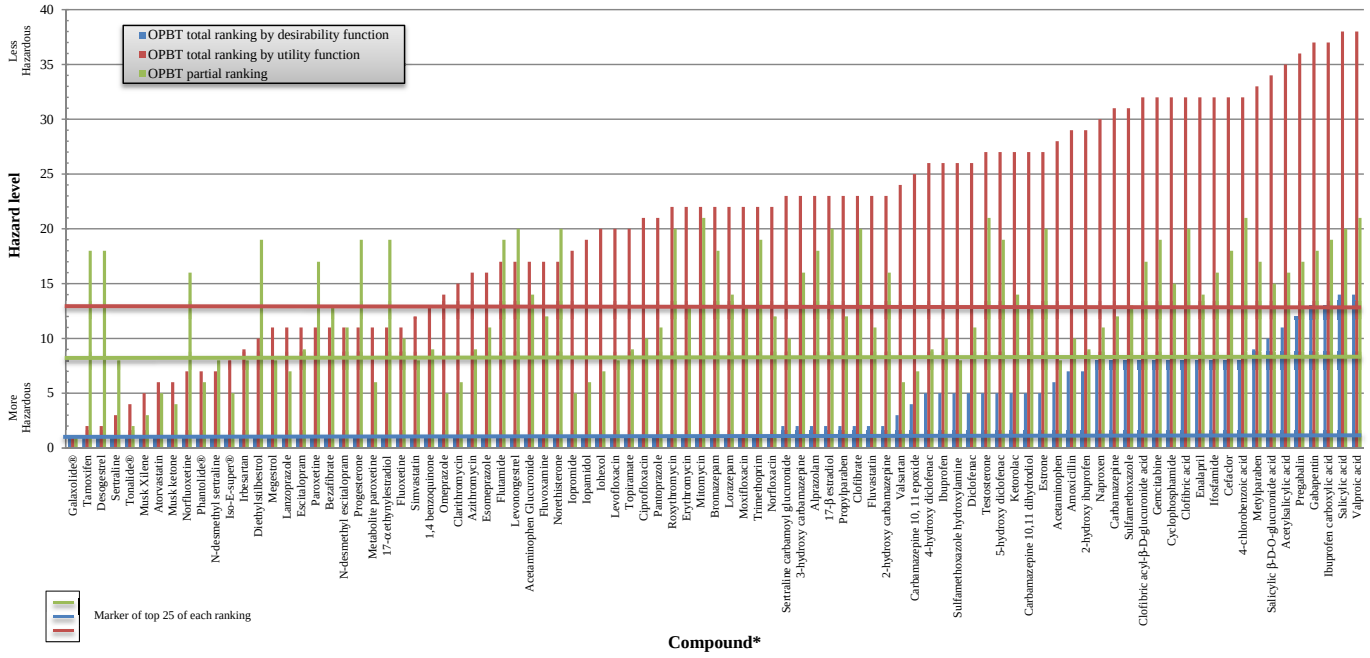

Fig. 5. OPBT partial and total rankings of PPCPs. *These rankings do not include gestodene, ethylparaben, p-hydroxybenzoic acid, 4-nonylphenol and triclosan due to the lack of data.

line with Galaxolide® (ID: 86) in the Hasse diagram (Appendix D), but it is located at a level with other compounds with similar occurrences. In partial ranking, compounds located at the same level present contradictions or conflicts and are therefore incomparable, i.e., atorvastatin (ID: 37) has the same P and B levels of concern as iopromide (ID: 81), but it has a lower occurrence and greater T that the latter. Accordingly, when these two compounds are compared, they are placed at the same level (5th level in Hasse diagram, Appendix D) but for different reasons.

Moreover, Fig. 3 and the Hasse diagram (Appendix D) highlight the great difference between PBT and OPBT partial rankings, when the occurrence has been included as other index for the hazard prioritization, the interactions are very complex and the location of the compounds change significantly.

In partial ranking, hormones, antidepressant and a few metabolites were displaced by X-ray contrast media (iopromide, iopamidol, iohexol), some antibiotics, the analgesic acetaminophen, the H_2 blocker omeprazol, and a few metabolites due to the high differences in the occurrence between them.

Fig. 5 shows the hazard score for the three DART rankings mentioned above for 91 compounds, and it also marked the top 25 for each classification. This figure shows the restrictiveness or not of each methodology and the location of each compound according to the function used. In all classifications, the first five compounds are the same, but the remaining compounds of the list may change considerably, making it clear that the technique of multi-criteria analysis used can also affect the level of risk of the substance evaluated.

Other studies have included the occurrence or the predicted environmental concentration (PEC) in their rankings or analysis of risk assessments (Calamari et al., 2003; Bound and Voulvoulis, 2004; Sanderson et al., 2004b; Carlsson et al., 2006; Liebig et al., 2006; Besse and Garric, 2009; Cooper et al., 2008; Kumar and Xagoraraki, 2010; Murray et al., 2010; Coutu et al., 2012). Some of these studies used the EMEA guidelines in the environmental risk assessment of medicinal products for human use, a methodology with two phases, where mainly the occurrence of the compound and its toxicological information are considered. Only for those compounds that have a log K_{ow}>4.5 is a screening for PBT recommended. Then, the PECs values have to be calculated along with the predicted no effect concentration (PNEC), which includes the toxicity value (chronic or acute, according to the information available) (European Medicines Agency, 2006).

The EMEA guidelines, as a methodology of steps, do not consider the evaluation of all possible indexes of hazard at the same time, which generates a methodological difference between the studies that used the occurrence as a parameter for the identification of risks according to this guideline and the multi-criteria approaches. Thus, it is more difficult to compare the results of the different studies.

Moreover, the occurrence of PPCPs in different geographic areas can vary significantly, which generates rankings that are highly specific due to the trends of consumption of these compounds in each population.

Despite these differences, antidepressants, anxiolytics, hormone and antibiotics are highlighted as possible hazardous compounds in aquatic environments, and we complemented this list of concern with fragrances, X-ray contrast media and some metabolites in this study. These compounds should be considered more carefully in all environmental impact studies and should be subject to more detailed experimental analysis.

4. Conclusions

The occurrence of PPCPS in the environment is an increasingly interesting area of study. New advances in detection, negative impacts and removal/degradation treatments have confirmed that it is an issue that requires urgent attention. In Spain, fragrances, analgesics, X-ray contrast media, antibiotics, H_2 blockers and some metabolites from analgesic/antipyretics and antiepileptics are the compounds with the highest occurrence in aquatic environments.

The development of methodologies to evaluated environmental indexes has increased in recent years, and these methodologies are

able to predict the negative environmental impacts of PPCPs and metabolites. In many cases, the (Q)SAR tool has been used to make these predictions in the absence of data or to perform preliminary lists of compounds for environmental impact assessment or the level of hazard. Although (Q)SAR is a useful tool, the uncertainties associated with these methods should be considered to analyze the results.

P, B and T are (intensive) indexes that were recommended recently by the REACH regulation to estimate the potential negative impact of compounds on the environment. In this study, the PBT values were estimated by applying (Q)SARs models with the EPA EPI Suite™ interface. Then, they were turned into indexes of environmental concern according to this regulation. The occurrence was incorporated and analyzed in conjunction with the PBT indexes as another extensive index. Persistence was the environmental index with the highest level of hazard in most of the PPCPs under study, followed by toxicity and bioaccumulation. Many metabolites have a concern score equal to or greater than that of their parent compounds. Therefore, due to the great capacity of transformation of many parental compounds of PPCPs to metabolites, it is always necessary to consider these compounds to perform a hazard or risk impact assessment. Twenty-two metabolites were analyzed in this work to generate information on their impact in aquatic environments.

A total hazard ranking score by desirability and utility functions, a partial hazard ranking score and a Hasse diagram were generated by DART tools. These rankings show that fragrances, hormones, antidepressants, anxiolytics, blood lipid regulators and some metabolites considered in this study have the highest levels of risk. The inclusion of the occurrence in the ranking changed the top 25 compounds significantly, mainly by incorporating X-ray contrast media and antibiotics.

The methodologies used in this work provide preliminary rankings of concern for the PPCPs most consumed in Spain, widely used in worldwide, and metabolites with high excretion. There are some uncertainties in the models used because different PBT weights and different ranking techniques were evaluated. As in other rankings proposed, the rankings obtained in this study cannot replace the experimental determination of P, B and T, but they are a powerful tool to identify those compounds that require immediate attention in aquatic environments among the hundreds of thousands of PPCPs that are currently on the market.

It is important to highlight that PBT indexes estimated by the (Q)SAR method and that the ranking obtained using the DART method, do not depend of the geographic area. When occurrence index is also evaluated (OPBT ranking) the results are exclusive to the country or region where the assessment was been performed. This may be a matter to be considered for next studies involving the O, P, B and T indexes, because the methodology presented in this paper can be used systematically in other scenarios to compare the results.

It is necessary to further improvements of the predictive models to estimate the PBT environmental impact indexes and further progress in classification techniques to focus the efforts of experimental work and field work on compounds of urgent attention. However, these experimental results must simultaneously complement the predictive models. These actions will allow the optimization of costs and time, minimize uncertainties and provide support to continue to enhance the current regulations.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.jenvman.2013.06.035.

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Chapter 4

Paper III

Ecotoxicity and Environmental Risk Assessment of Pharmaceuticals and Personal Care Products in aquatic environments and wastewater treatment plants.

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Ecotoxicity and environmental risk assessment of pharmaceuticals and personal care products in aquatic environments and wastewater treatment plants

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Abstract A wide range of pharmaceuticals and personal care products (PPCPs) are present in the environment, and many of their adverse effects are unknown. The environmental risk assessment of 26 PPCPs of relevant consumption and occurrence in the aquatic environment in Spain was accomplished in this research. Based on the ecotoxicity values obtained by bioluminescence and respirometry assays and by predictions using the US EPA ecological structure–activity relationship (ECOSAR^{TM}), the compounds were classified following the Globally Harmonized System of Classification and Labelling of Chemicals. According to the criteria of the European Medicines Agency, the real risk of impact of these compounds in wastewater treatment plants (WWTPs) and in the aquatic environment was predicted. In at least two ecotoxicity tests, 65.4 % of the PPCPs under study showed high toxicity or were harmful to aquatic organisms. The global order of the species' sensitivity to the PPCPs considered was as follows: *Vibrio fischeri* (5 min) > *Vibrio fischeri* (15 min) > algae > crustaceans > fish > biomass of WWTP. Acetaminophen, ciprofloxacin, clarithromycin, clofibrate, ibuprofen, omeprazole, triclosan, parabens and 1,4-benzoquinone showed some type of risk for the aquatic environments and/or for the activated sludge of WWTPs. Development of acute and chronic ecotoxicity data, the determination of predicted and measured environmental concentrations of PPCPs, the inclusion of metabolites and transformation products and the evaluation of mixtures of these compounds will allow further improvements of the results of the ERAs and, finally, to efficiently identify the compounds that could affect the environment.

Keywords Bioluminescence · Ecotoxicity · Environmental risk assessment · Pharmaceuticals and personal care products · QSAR · Respirometry

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Abbreviations

ASA	Acetylsalicylic acid
B	Bioaccumulation
EC50	Half maximal effective concentration
ECOSAR, US EPA	Ecological structure–activity relationship
EMEA	European medicines agency
EPA	United States environmental protection agency
EPA EPI SuiteTM	Estimation programs interface suite^{TM} developed by the EPA's Office of Pollution Prevention Toxics and Syracuse Research Corporation
ERA	Environmental risk assessment
EU TGD	European Union Technical Guidance Document

GHS	Globally harmonized system of classification and labeling of chemicals
LC50	Half maximal lethal concentration
MLSS	Mixed liquor suspended solids
MRERA	More restrictive ranking of environmental risk assessment
NOEC	No observed effect concentration
P	Persistence
PCPs	Personal care products
PEC	Predicted environmental concentration
PEC_{Simple}	Simple predicted environmental concentration
PEC_R	Refined predicted environmental concentration
PhAC	Pharmaceutical active compound
PNEC	Predicted no effect concentration
PPCPs	Pharmaceutical and personal care products
(Q)SARs	Quantitative structure–activity relationships
RQ	Risk quotient
RQS	Simple risk quotient
RQ_R	Refined risk quotient
RQ_{MEC}	Risk quotient calculated with MEC
RQ_{WM}	Risk quotient in WWTPs without metabolization in humans
RQ_{WWTPs}	Risk quotient in the influent of WWTPs considering metabolization in humans
SARs	Structure activity relationships
T	Toxicity
WWTP	Wastewater treatment plant

Introduction

The generation and consumption/use of a large amount of synthetic chemicals, specifically pharmaceuticals and personal care products (PPCPs), have led to the detection of these substances with greater frequency and persistence in natural water, wastewater and drinking water systems. In recent years, the occurrence and fate of pharmaceutically active compounds (PhACs) in the aquatic environment have been recognized as one of the emerging issues in environmental chemistry (Hereber 2002), and this issue has been published a wide variety of literature (Anca Caliman and Gavrilescu 2009; Besse and Garric 2009; Fent et al. 2006; Halling-Sorensen et al. 1998; Hereber 2002; Kümmerer 2009; McClellan and Halden 2010; Ortiz de García et al. 2013a; Ternes 1998).

Many effects and negative impacts of PPCPs on the environment remain unknown. Depending on their physicochemical properties, most of these substances become part of the municipal wastewater once these substances have been consumed, metabolized and excreted by living organisms. The balances of the influents and effluents of drug residues detected in wastewater treatment plants (WWTPs) reveal that many pharmaceuticals are not completely eliminated by traditional treatment processes (Han et al. 2006). In many cases, these compounds are adsorbed by the primary and secondary sludge; however, these compounds can also remain in the treated wastewater and can be distributed in surface waters, groundwaters, sediments and tissues of exposed wildlife as has been described in previous studies (Fent et al. 2006; Gros et al. 2010; Hereber 2002; Santos et al. 2010).

The risks associated with the discharge of pharmaceuticals into the environment are due to not only their acute ecotoxicity but also their genotoxicity, development of pathogen resistance, and endocrine disruption (Rosal et al. 2010a).

Standard ecotoxicity assays are a way to determine some PPCP effects, such as acute or chronic ecotoxicity, on organisms of different trophic levels. Different species of fish, crustaceans and algae are often used for this purpose; however, other microorganisms, such as bacteria, have also been used in these studies. For example, the luminescence inhibition bioassay with marine *Vibrio fischeri* photobacteria has proven to be a useful way of estimating the acute ecotoxicity of many chemicals (Rosal et al. 2010b). When these ecotoxicity values are unknown, the methodology of "quantitative structure–activity relationship" ((Q)SAR) is an alternative approach to estimate the fate and the negative effects of these substances in the environment. The (Q)SAR methodology considers the physicochemical properties and molecular structures of the compounds to evaluate their biodegradability, biological ecotoxicity, mutagenicity and carcinogenicity, among other adverse effects. The combination of this predictive model with experimental data of ecotoxicity, generally supplied with tests of low complexity and cost, provide a preliminary scientific approach to rapidly identify those compounds that require immediate attention.

It is desirable to be able to predict a compound's potential to cause adverse effects in the environment before effects are observed. The probability of a compound to cause undesired environmental effects can be estimated by an environmental risk assessment (ERA) (Carlsson et al. 2006). The development of specific ERA for pharmaceuticals began in Europe in the early 1990s (Bound and Voulvoulis 2004). More recently, the Committee for Medicinal Products for Human Use of the European Medicines Agency (EMEA) has created a guideline for the ERAs of medicinal products for human use, which must be performed for all new marketing authorization applications for a medicinal product or if there is an increase in the environmental exposure (European Medicines Agency 2006).

The need for information on the negative effects of PPCPs on the environment and to improve the available ERA methodologies are the basis of this work. This study focuses on the ERAs of 26 PPCPs of relevant consumption and occurrence in Spain, using EMEA guidelines and considering the (Q)SAR methodology and the experimental data regarding their acute ecotoxicity on *Vibrio fischeri* photobacteria for aquatic environments and respirometry assays with biomass from the secondary treatment of WWTPs. However, because the considered PPCPs are being widely consumed around the world, the methodology used can be applied in other geographic areas, and the results can be compared with existing data.

Materials and methods

Selection of PPCPs

The investigated PPCPs are some of the most important classes of drugs (non-steroidal anti-inflammatories, analgesics, antibiotics, H_2 blockers and blood lipid regulators) and personal care products (disinfectants and preservatives) worldwide. Their consumption and occurrence in aquatic environments and in WWTPs are relevant, at least in Spain, and have been previously reported (Ortiz de García et al. 2013a). Additionally, their potential toxicity has been analyzed in previous studies (Cleuvers 2004; Fent et al. 2006; Ortiz de García et al. 2013b; Sanderson et al. 2004a; Santos et al. 2013), showing different behaviors for the different species under study. Moreover, it is important to assess the impact of some of their metabolites, transformation products, and drug molecules in their neutral and ionic forms. These reasons justify their selection.

The 26 PPCPs examined in this study were as follows: acetaminophen, 1,4-benzoquinone (as acetaminopheńs transformation product), ibuprofen, ibuprofen sodium salt, diclofenac sodium salt, naproxen, naproxen sodium salt, acetylsalicylic acid (ASA), salicylic acid, amoxicillin, sulfamethoxazole, cefaclor, ciprofloxacin, ciprofloxacin hydrochloride monohydrate, clarithromycin, erythromycin, levofloxacin, norfloxacin, omeprazole, clofibrate, clofibric acid, methylparaben, ethylparaben, propylparaben, *p*-hydroxybenzoic acid (parabens metabolite), and triclosan.

Chemicals, test organisms and media

Analytical or technical grade PPCPs with purity ≥95 %, which were obtained from Sigma-Aldrich and Fluka Chemicals, were used to perform the ecotoxicity tests.

Microtox® acute ecotoxicity tests were performed using the marine bioluminescent bacteria *Vibrio fischeri* (strain NRRL B-11177) as the test organism. The bacteria were supplied in a freeze-dried form by Instrumentación Analítica S.A. and were stored at −20 to −25 °C to preserve their microbial activity. All of the PPCP solutions were prepared with Milli-Q® water. Two solutions of NaCl (2 and 22 % w/v) were used as a saline medium and for osmotic adjustment, respectively.

The respirometry tests were performed with: (i) aerobic sludge obtained from the secondary treatment tank of Valladolid's WWTP, (ii) synthetic wastewater according to the EPA 712-C-014 OCSPP 850.3300 procedure (EPA 2012) and (iii) distilled water.

Ecotoxicity tests and estimations

Two ecotoxicity tests were selected to evaluate the ERA of the PPCPs under study. For measuring the impact of PPCP's on aquatic environments, the Microtox® acute ecotoxicity test was performed, and for determining their impact on WWTPs respirometry assays were carried out.

The standard Microtox® bioassay is claimed to be reliable, rapid, and sensitive (Fulladosa et al. 2005). Parvez et al. (2006) concluded in their research that out of the various available bioassays, *Vibrio fischeri* based luminescent inhibition test is sensitive, rapid, cost effective, reproducible and without ethical problems ensuing from the use of higher organisms such as fish and rat. Moreover, microbiotests (as Microtox®) require smaller volumes for testing, which is useful for ecotoxicity screening and environmental biomonitoring (Radix et al. 2000). In addition, the genus *Vibrio* (used for this test) play an important role in nutrient regeneration in the aquatic milieu by taking up dissolved organic matter, producing essential polyunsaturated fatty acids needed in the aquatic food web, and degrading chitin (Milton 2006).

In contrast to bioluminescence, the activated sludge respirometry test is a more direct method for measuring sludge activity and, thus, toxicity to sludge (Ren 2004). The use of respirometry, based on measuring the consumed amount of oxygen by a sample of activated sludge for the metabolism of a given amount of substrate, seems to be able to contribute to the improvement of WWTP management. In fact, it enables the estimation of certain characteristic variables for a good process or the detection of the influence of the physico-chemical conditions such as pH, salinity, metal toxicity (Zerdazi et al. 2012) or other contaminants toxicity as the PPCP's. Other advantage of respirometry is related to the use of bacteria present in WWTPs, without need of using pure bacterial strains different from activated sludge (Andreottola et al. 2008). As Microtox® assay, the respirometry is sensitive, rapid and cost effective. For these reasons, these two assays were selected.

The determination of acute effects on the bioluminescence of *Vibro fischeri* bacteria was performed at 15 °C

using a Microtox® Model 500 ecotoxicity analyzer according to the manufacturer's instructions (Azur Environmental, Newark, Delaware, USA) and ISO 11348-3:2007 protocol (ISO 2007). The bacteria were reconstituted and incubated at 5 °C in the ecotoxicity analyzer.

During the Microtox® test, the inhibition of light emission was measured in relative units of luminescence. The data were used to calculate the half-maximal effective concentration (EC_{50}), which is the mean sample concentration that causes a 50 % reduction in bacteria bioluminescence. Tests were performed in duplicate at five different concentrations, which were obtained by serial dilution from the prepared stock solution (basic test). A reference toxicant, zinc sulfate ($ZnSO_4 \cdot 7H_2O$), was used as the positive control. The positive control was concurrently performed with the sample as a quality control test. For those compounds with low solubility or low ecotoxicity at high concentrations, the highest percentage of inhibition was calculated (inhibition test). Temperature, pH, solubility, turbidity and color were adjusted or measured when necessary. The dose–response curves for PPCPs were obtained at 5 and 15 min. The acute ecotoxicity endpoint was determined as the EC_{50} at both times for a 95 % confidence interval using a linear regression model, as indicated in the user's manual for the Microtox® Model 500 analyzer.

The respiration inhibition test (immediate) was used in this study to measure the ecotoxicity of PPCPs on the activated sludge obtained from the secondary treatment tank of Valladolid's WWTP and was accomplished using a Strathtox Unit SI500 from Strathkelvin Instruments (Lanarkshire, Scotland) according to its procedure manual and the EPA 712-C-014 OCSPP 850.3300 method (EPA 2012). The respiration inhibition test calculates EC_{50}, EC_{20} and EC_{10} values, i.e., the PPCP concentration in wastewater that causes 50, 20 and 10 % inhibition of the respiration rate, respectively. The activated sludge was kept fully aerated during the test, and the mixed liquor suspended solids concentration (MLSS) was kept between 2 and 4 g L^{-1}. The PPCP solutions were directly added to the respirometer tubes and were mixed with distilled water to obtain five different dilutions, with a total volume of 10 mL. Then, the synthetic doped wastewater and the activated sludge were mixed to measure the respiration rate of the microorganisms (based on the oxygen concentration decrease over time). The respiration inhibition tests were evaluated in triplicate for each PPCP solution at each concentration. The EC_{50} was calculated at a 95 % confidence level using a linear regression model, similar to the Microtox® test.

Beyond laboratory assays, some mathematical models were developed to estimate or predict ecotoxicological effects (Ortiz de García et al. 2013b). The most often applied (Q)SAR program is the US EPA ecological structure–activity relationship (ECOSAR™) (Fent et al. 2006). Acute ecotoxicity values of the PPCPs under study for fish (half-maximal lethal concentration, LC_{50}), crustaceans (EC_{50}) and algae (EC_{50}) were calculated using the ECOSAR™ program according to the methodology described in Ortiz de García et al. (2013b). These theoretical values have been compared in this research with the ecotoxicity results for *Vibrio fischeri* bacteria and for activated sludge of the WWTP.

Ecotoxicity levels

Based on ecotoxicity values obtained by bioluminescence and respirometry assays, the compounds were classified as established by the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (United Nations 2011):

(i) highly toxic: $EC_{50} \leq 1$ mg L^{-1};
(ii) toxic: 1 mg $L^{-1} < EC_{50} \leq 10$ mg L^{-1};
(iii) harmful to aquatic organisms: 10 mg $L^{-1} < EC_{50} \leq 100$ mg L^{-1}.

Some regulatory systems include a fourth category (non-toxic) for those compounds having an $EC_{50} > 100$ mg L^{-1}.

These ecotoxicity levels have been used in previous studies (Cleuvers 2004; Han et al. 2006; Hernando et al. 2006, 2007; Rosal et al. 2010b; Sanderson et al. 2004a).

ERAs of PPCPs performed considering the framework of the EMEA for the Evaluation of Medicinal Products

Figure 1 summarizes the schematic procedure to perform an ERA of medicinal products for human use following the EMEA guidelines (European Chemicals Bureau 2003; European Medicines Agency 2006). The assessment of the potential risks to the environment of this type of compound is a step-wise process that consists of two phases.

In Phase I, the estimation was only based on the substance's structural characteristics, irrespective of its route of administration, pharmaceutical form, metabolism and excretion. If the predicted environmental concentration (PEC) value is below 0.01 μg L^{-1}, and no other environmental concerns are apparent, it is assumed that the medicinal product is unlikely to represent a risk for the environment following its prescribed usage in patients. If the PEC value is equal to or above 0.01 μg L^{-1}, then a Phase II environmental fate and effect analysis should be performed. In some cases, the action limit may not be applicable. Some drug substances may affect the reproduction of vertebrates or lower animals at concentrations lower than 0.01 μg L^{-1}. These substances should enter

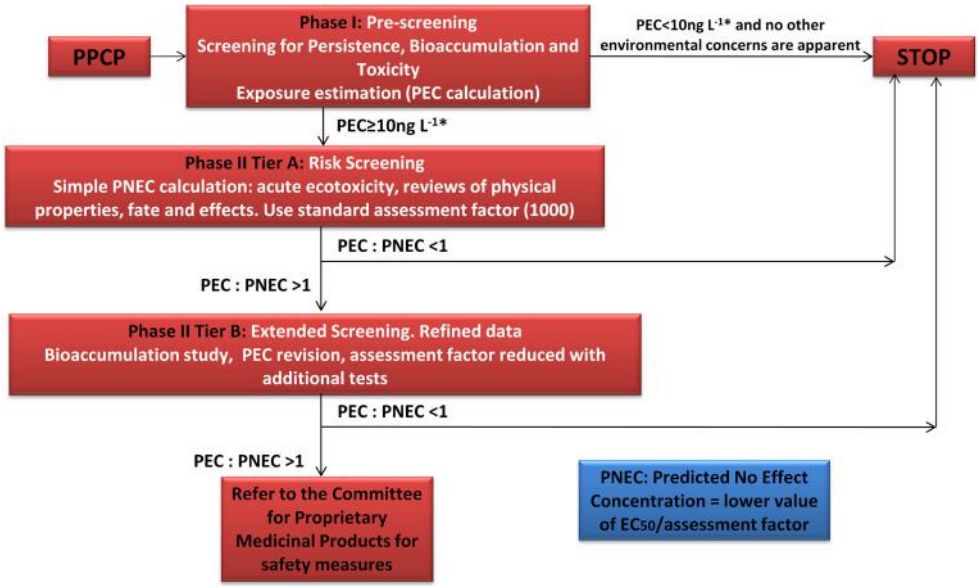

Fig. 1 Diagram of EMEA guidelines for ERAs of PPCPs. *In some cases, the action limit may not be applicable. Some drug substances may affect the reproduction of vertebrates or lower animals at concentrations lower than 0.01 μg L^{-1}. These substances should enter into Phase II, and a tailored risk assessment strategy should be followed to address its specific mechanism of action. In these cases, the applicant should justify all actions taken (European Medicines Agency 2006). Moreover, following the guidelines of the EMEA, an evaluation of persistence (P), bioaccumulation (B) and toxicity (T) according to European Union Technical Guidance Document (EU TGD) (European Chemicals Bureau 2003) was performed for those PPCPs with a logarithm octanol–water partition coefficient (log K_{ow}) greater than 4.5. The PBT indexes were evaluated using the (Q)SAR models implemented in the EPI Suite™ interface (EPA 2009). In the second stage (Phase II), information concerning measured environmental concentrations (MECs), fate and effects in the environment were obtained and assessed. Phase II is divided into two parts, Tiers A and B (European Medicines Agency 2006)

Phase II, and a tailored risk assessment strategy should be followed that addresses its specific mechanism of action. In these cases, the applicant should justify all actions taken (European Medicines Agency 2006).

In this study, PEC values in aquatic environments and in WWTPs were obtained from a recent study (Ortiz de García et al. 2013a) for both tiers of the second phase, II A (simple PEC: metabolization in humans and removal in WWTPs were excluded from calculations) and II B (refined PEC: metabolization in humans and removal in WWTPs were considered in calculations). Predicted no effect concentrations (PNECs) for aquatic environments were obtained as the ratio of the lower value of ecotoxicity (EC_{50} or LC_{50}) (the worst case, among the estimated acute ecotoxicity values by (Q)SAR in fish, crustaceans, and algae, as well as the experimental acute ecotoxicity values of the Microtox® assay) and the standard dilution assessment factor recommended by the EMEA (1000). PNECs for WWTPs were calculated from respirometry test results and from the standard assessment factor recommended by the EMEA (100).

In Phase II A and II B, the risk quotients (RQs) (the PEC:PNEC or MEC:PNEC ratio that indicates the greatest toxicity) were calculated to predict: (i) whether the compound requires more attention, (ii) whether other tests must be performed to demonstrate its adverse effects on the environment or otherwise (iii) whether the compound is not harmful. If the ratio PEC:PNEC (or MEC:PNEC) for the drug substance is below 1, then further testing in the aquatic compartment will not be necessary, and it can be concluded that the drug substance and/or its metabolites are unlikely to represent risks to the aquatic environment. If the ratio PEC:PNEC (or MEC:PNEC) for the drug substance is above 1, then further evaluation, preferably on the fate of the drug substance and/or its metabolites in the aquatic environment, are required in Tier B (European Medicines Agency 2006).

The more restrictive ranking of environmental risk assessment (MRERA) (EC 1996; Hernando et al. 2006) establishes the following classification of RQs: (i) High toxicity: $RQ > 1$, (ii) medium toxicity: $0.1 < RQ < 1$ and (iii) low toxicity: $0.01 < RQ < 0.1$.

These two classifications were used in this work to rank the risk of the PPCPs under study.

Results and discussion

Ecotoxicity tests and (Q)SAR predictions

The results of the acute ecotoxicity estimated by (Q)SAR for fish (LC_{50}), crustaceans (*Daphnia magna*) and algae (EC_{50}) and of the acute ecotoxicity obtained by

bioluminescence and respirometry laboratory assays (EC_{50}) have been plotted in Fig. 2 and tabulated with their confidence intervals in Table 1. These values have been grouped by levels according to the GHS classification. In Table 1, the experimental EC_{50} values of *Vibrio fischeri* were provided for 22 compounds. The EC_{50} values were not determined for the other four compounds (amoxicillin, ciprofloxacin, erythromycin, *p*-hydroxybenzoic acid) due to their low water solubility or low ecotoxicity at high concentrations. However, the greatest effects for these four compounds are shown in Table 1 and in Fig. 2, and these compounds have been classified as "non-toxic" according to the GHS classification. Experimental and predicted values of bioluminescence and respirometry, with their confidence intervals, are shown in Table 1.

Predicted values for fish, crustaceans and algae were determined only for neutral compounds because ECOSAR™ has been primarily developed for the evaluation of these types of organic molecules; therefore, sodium salts of diclofenac, ibuprofen and naproxen and ciprofloxacin hydrochloride monohydrate were excluded.

Experimental ecotoxicity values show that *Vibrio fischeri* is more sensitive than biomass microorganisms from the secondary treatment of WWTPs because this mixture of microorganisms is adapted and acclimated to more toxic compounds from urban and industrial wastewaters.

The order of susceptibility for the predicted ECOSAR values for most compounds (68.2 %, 15 of 22 compounds analyzed) was as follows: algae > crustaceans > fish, which is in agreement with Sanderson et al. (2003); however, this result is inconsistent with Sanderson et al. (2004a, b). Although the same tool and methodology for estimating the ecotoxicity values of algae, crustaceans and fish (ECOSAR™ software) were used in these three publications (Sanderson et al. 2003, 2004a, b), the number of compounds under study were different (<100, 671 and 2,986, respectively), which is presumed to have caused the different orders of susceptibility because PPCPs represent a heterogeneous group of chemical compounds. Consequently, according to the PPCP class under study in this work, some species may be more affected than others, which generates particular orders of susceptibility in each study.

The results show the following behavior, according to the GHS classification (22 compounds were considered for algae, crustaceans and fish, and 26 compounds were considered for *Vibrio fischeri* and active WWTP biomass):

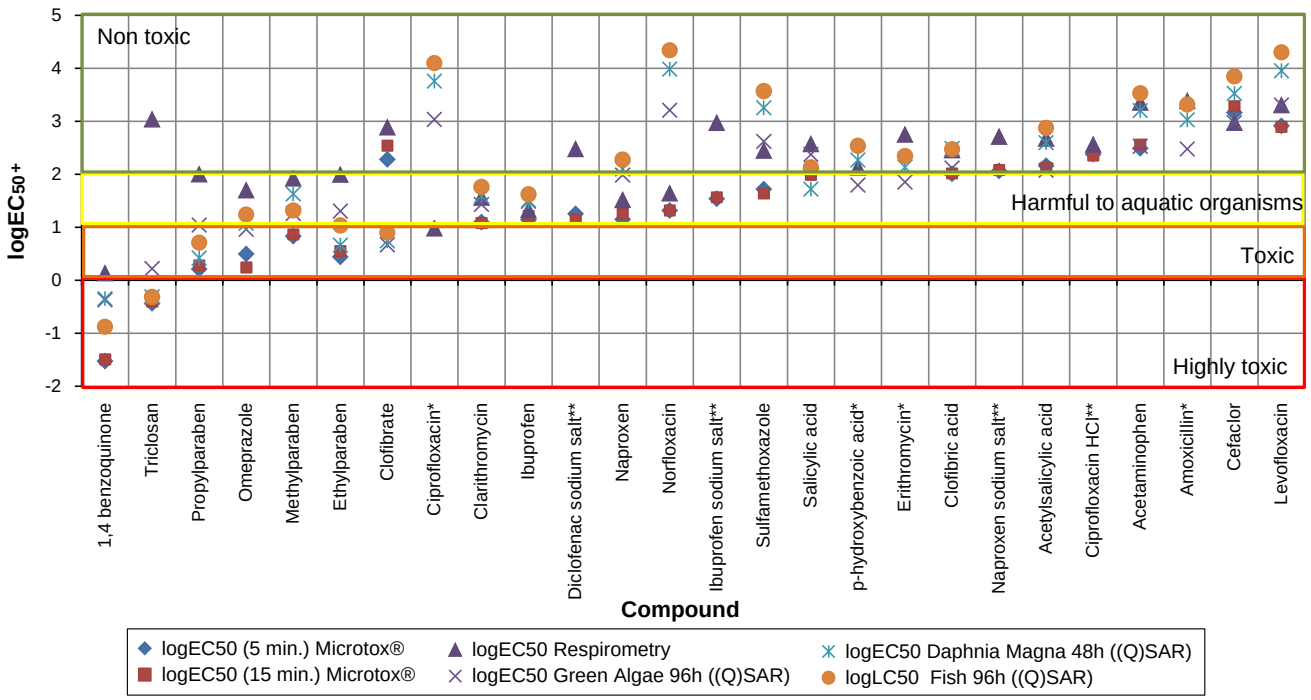

Fig. 2 Ecotoxicity results from (Q)SAR (fish, crustaceans and algae), bioluminescence (*Vibrio fischeri*) and respirometry (activated sludge) assays and from the GHS classification $^{+}EC_{50}$ and LC_{50} units: mg L^{-1}. *For these compounds, the EC_{50} values of bioluminescence were not determined; however, the highest effect was provided (at a 95 % Confidence interval): amoxicillin $EC_{33\pm2}$ (2,019 mg L^{-1}) at 5 min and $EC_{33\pm4}$ (2,019 mg L^{-1}) at 15 min, ciprofloxacin $EC_{13\pm4}$ (63 mg L^{-1}) at 5 and 15 min; erythromycin $EC_{31\pm4}$ (900 mg L^{-1}) at 5 min and $EC_{45\pm7}$ (900 mg L^{-1}) at 15 min, *p*-hydroxybenzoic acid $EC_{27\pm2}$ (391.18 mg L^{-1}) at 5 min and $EC_{29\pm8}$ (391.18 mg L^{-1}) at 15 min. **For these compounds, the predicted ecotoxicity values were not estimated because ECOSAR™ has been primarily developed for the evaluation of neutral organic molecules

Table 1 Ecotoxicity values obtained by luminescence and respirometry, as well as predicted values using ECOSAR™, for the compounds under study

Compound	Bioluminescence						Respirometry			Predicted by ECOSAR		
	5 min			15 min			Immediate test					
	EC_{50}^{a} (mg L^{-1})	Confidence levels		EC_{50} (mg L^{-1})	Confidence levels		EC_{50} (mg L^{-1})	Confidence levels		LC_{50}^{b} Fish (96 h) (mg L^{-1})	EC_{50} Crustacean *Daphnia magna* (48 h) (mg L^{-1})	EC_{50}Algae Green algae (96 h) (mg L^{-1})
		Min	Max		Min	Max		Min	Max			
Acetaminophen	310.70	289.64	333.35	363.30	342.10	385.80	2,255.00	2,253.00	2,258.00	3,398.99	1,590.91	337.05
Acetylsalicylic acid	146.13	113.57	188.02	133.59	101.20	176.34	471.68	467.59	475.77	758.00	393.70	118.70
Amoxicillin[c]	n.a	n.a.	n.a.	n.a.	n.a.	n.a.	2,423.29	2,403.55	2,443.04	2,079.77	1,060.10	299.90
Cefaclor	1,469.40	1,149.20	1,878.90	1,930.20	1,451.10	2,567.40	940.27	937.96	942.58	7,061.40	3,337.70	731.00
Ciprofloxacin[c]	n.a	n.a.	n.a.	n.a.	n.a.	n.a.	9.55	7.21	11.89	1,2591.00	5,704.00	1,082.13
Ciprofloxacin HCl[d]	241.37	205.26	283.85	226.67	193.73	265.20	363.55	362.01	365.10	n.a.	n.a.	n.a.
Clarithromycin	12.65	6.24	25.66	12.08	5.49	26.57	36.40	25.72	47.08	58.09	38.65	27.01
Clofibric acid	102.70	84.00	125.00	104.11	80.00	135.54	285.40	281.67	289.13	299.34	306.80	130.40
Clofibrate	192.27	167.99	220.05	348.87	289.44	420.50	769.28	765.93	772.64	7.94	5.58	4.67
Diclofenac Na[d]	18.08	14.80	22.09	14.31	11.71	17.49	299.61	298.13	301.08	n.a.	n.a.	n.a.
Erythromycin[c]	n.a	n.a.	n.a.	n.a.	n.a.	n.a.	561.91	549.63	574.20	222.65	136.13	71.41
Ethylparaben	2.78	2.50	3.09	3.59	2.90	4.44	98.64	96.82	100.46	10.88	4.60	20.17
Ibuprofen	13.87	10.88	17.70	16.49	11.26	24.16	21.00	n.a.	n.a.	42.04	30.90	31.95
Ibuprofen Na[d]	34.81	25.87	48.34	37.03	27.73	49.45	937.15	936.04	938.25	n.a.	n.a.	n.a.
Levofloxacin	825.52	674.91	1,009.75	788.30	598.04	1,039.09	2,021.41	2,018.89	2,023.93	2,0236.00	8,950.00	1,564.70
Methylparaben	6.85	4.80	9.77	7.36	3.31	16.34	83.72	81.91	85.53	20.78	42.72	18.21
Naproxen	14.20	6.00	33.60	17.92	9.33	34.41	32.82	30.23	35.41	190.00	126.10	96.63
Naproxen Na[d]	116.73	106.31	128.17	120.58	109.79	128.17	512.81	511.18	514.44	n.a.	n.a.	n.a.
Norfloxacin	20.87	2.52	172.99	20.81	4.59	94.24	43.81	37.78	49.84	2,1993.00	9,603.40	1,607.30
Omeprazole	3.16	2.97	3.35	1.76	1.65	1.88	50.05	44.49	55.60	17.48	11.95	9.14
P-hyd.benzoic acid[c,e]	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	134.68	131.71	137.65	346.41	185.77	62.46
Propylparaben	1.64	1.53	1.77	1.92	1.76	2.10	100.16	98.64	101.69	5.16	2.63	10.99
Salicylic acid	126.60	99.70	160.80	98.10	66.70	144.30	372.76	369.55	375.98	136.3	52.49	235.76
Sulfamethoxazole	52.92	48.27	57.68	43.56	35.15	53.98	279.81	270.93	288.69	3,742.30	1,797.80	416.10
Triclosan	0.37	0.32	0.43	0.40	0.31	0.43	1,089.07	1,071.73	1,106.41	0.48	0.48	1.66
1,4-Benzoquinone	0.03	0.02	0.04	0.03	0.01	0.08	1.36	0.00	8.28	0.13	0.45	0.43

n.a.: Data not available

[a] Half maximal effective concentration

[b] Half maximal lethal concentration

[c] For these compounds, the EC_{50} values of bioluminescence were not determined; however, the highest effect is provided (at a 95 % Confidence interval): amoxicillin $EC_{33\pm2}$ (2,019 mg L^{-1}) at 5 min and $EC_{33\pm4}$ (2,019 mg L^{-1}) at 15 min, ciprofloxacin $EC_{13\pm4}$ (63 mg L^{-1}) at 5 and 15 min; erythromycin $EC_{31\pm4}$ (900 mg L^{-1}) at 5 min and $EC_{45\pm7}$ (900 mg L^{-1}) at 15 min, *p*-hydroxybenzoic acid $EC_{27\pm2}$ (391.18 mg L^{-1}) at 5 min and $EC_{29\pm8}$ (391.18 mg L^{-1}) at 15 min

[d] ECOSAR™ has been primarily developed for the evaluation of neutral compounds; therefore, these molecules were not screened

[e] *P*-hydroxybenzoic acid

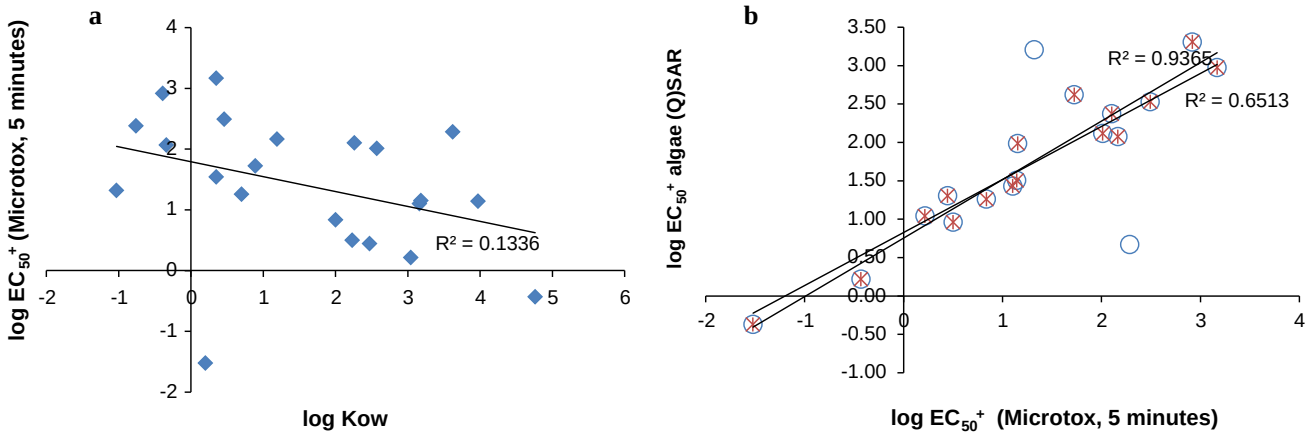

Fig. 3 Relation of acute ecotoxicity on *Vibrio fischeri* at 5 min with the compounds' hydrophobicity (log K_{ow}) (**a**) and with predictive acute ecotoxicity on green algae at 96 h (**b**) $^{+}EC_{50}$ units: mg L^{-1}. *Data marked with *circles* (*blue*) represent 18 compounds, excluding four compounds with low ecotoxicities (*Vibrio fischeri* EC_{50} were not determined) and four compounds whose predictive values were not estimated (ionic compounds). Data marked with blades (*red*) represent 16 compounds, excluding identical compounds in circles and also norfloxacin and clofibrate, which have shown high deviations between predictive results and all of the experimental data consulted

- For algae: 4.55 % of PPCPs are highly toxic; 13.64 % toxic; 36.36 % harmful to aquatic organisms and 45.45 % nontoxic.
- For crustaceans: 9.09 % of PPCPs are highly toxic; 13.64 % toxic; 27.27 % harmful to aquatic organisms and 50 % nontoxic.
- For fish: 9.09 % of PPCPs are highly toxic; 9.09 % toxic; 22.73 % harmful to aquatic organisms and 59.09 % nontoxic.
- For *Vibrio fischeri* (5 and 15 min): 7.7 % of PPCPs are highly toxic; 15.38 % toxic; 26.92 % harmful to aquatic organisms and 50 % nontoxic.
- For active WWTP biomass: 7.7 % of PPCPs are toxic; 30.8 % harmful to aquatic organisms and 61.5 % nontoxic.

Therefore, the overall order of susceptibility was as follows: *Vibrio fischeri* (5 min, MICROTOX®) > *Vibrio fischeri* (15 min, MICROTOX®) > Algae ((Q)SAR) > crustacean ((Q)SAR) > fish ((Q)SAR) > activated sludge of WWTP (respirometry assay).

The research results indicate that for 85 % of the compounds under study, at least four of the 6 values of ecotoxicity, which were classified according to the GHS, matched in the same category, and for 100 % of compounds, at least three of the six values of ecotoxicity coincided in the same category, which suggest that this classification can be used for a wide range of species without substantial changes in the final results and can be used to compare different studies with similar goals.

In total, 65.4 % of PPCPs under study were classified between "highly toxic" and "harmful to aquatic organisms" in at least two ecotoxicity values, which provides preliminary evidence concerning the negative effects of these compounds on the environment.

A significant physico-chemical property of a chemical in relation to exposure and to baseline ecotoxicity assessment is the solubility of the compound. The log K_{ow} value is a commonly used descriptor of the hydrophobic/lipophilic property of the compound. Generally, the (Q)SAR methodology will begin by identifying a possible linear relation between K_{ow} and the observed ecotoxicity (baseline narcosis), when the ecotoxicity of an active compound can be explained by its lipophilicity (narcosis effect) or when the chemical mode of action is unknown (Sanderson et al. 2004b). In this study, the relation between acute ecotoxicity in *Vibrio fischeri* and the compound's hydrophobicity can be seen in Fig. 3a. The correlation is extremely poor ($r^2 = 0.1336$), as has been found in previous research with parabens (Terasaki et al. 2009). A likely reason is that the test system with *Vibrio fischeri* works with a unicellular organism with low lipid content, and hence, both hydrophobic and hydrophilic parabens (and other similar chemical compounds) can easily cross the cell membrane. Therefore, in this case, parabens accumulation does not greatly increase with increasing hydrophobicity (Terasaki et al. 2009). This phenomenon could occur with other PPCPs with similar chemical structures. The relation between more sensitive species in the experimental assays (bioluminescence acute ecotoxicity of *Vibrio fischeri* in 5 min) and in the predictive model (growth inhibition of green algae in 96 h) shows a better correlation (Fig. 3b), which may help to reduce the experimental test time (96 h with algae to 5 min with *Vibrio fischeri*) or to correlate predictive models with bacteria ecotoxicity. Figure 3b shows two correlations. The data marked with circles

represent 18 compounds, excluding four compounds with low ecotoxicities (*Vibrio fischeri* EC_{50} were not determined) and four compounds whose predictive values were not estimated (ionic compounds), with $r^2 = 0.6513$. Additionally, the data marked with circles and blades represent 16 compounds, excluding identical compounds that are in *circles*, as well as norfloxacin and clofibrate, which show a high deviation between predictive results. All of these experimental data had $r^2 = 0.9365$. The difference between r^2 values for these two correlations shows the importance of considering the comparisons among predictive models and experimental results. Parvez et al. (2006) commented that a good correspondence for EC_{50} exists based on *Vibrio fischeri*, with LC_{50} based on other aquatic species, i.e., fathead minnow, bluefill, catfish, goldfish, goldorfe, guppy, killifish, rainbow trout, sheepshead minnow, zebrafish, the water flea *Daphnia* species, the ciliate *Tetrahymena pyriformis* and algae. Similarly, Radix et al. (2000) found high correlation between the chronic ecotoxicity of different species (bacteria, algae, crustaceans and rotifers) for 16 chemicals.

According to the ecotoxicity values (predicted and experimental), the most toxic chemical (lower EC_{50}) was 1,4-benzoquinone, which was reported as a transformation product of acetaminophen during water chlorination treatment (Bedner and MacCrehan 2006; Xagoraraki et al. 2008) or as an intermediate compound in the degradation of clofibric acid by photolysis (Nikolaou et al. 2007). 1,4-benzoquinone was found to be "highly toxic" using predicted and bioluminescence values and was "toxic" for respirometry assays. This compound has been poorly studied in ERAs of PPCPs; hence, the information found in this research can be used to increase the knowledge regarding the adverse effects of acetaminophen and its transformation product (1,4-benzoquinone) because acetaminophen is one of the most consumed analgesics worldwide.

Omeprazole, which is a poorly studied compound that is daily consumed in high quantities, has also shown high ecotoxicity. Woldegiorgis et al. (2009) confirmed that the ecotoxicity tests and the corresponding "no observed effect concentration" (NOEC), EC_{50} or even LC_{50} values, reflected acute ecotoxicity and that this compound may, in fact, also trigger receptors in aquatic organisms that regulate the proton flux in the lumen.

The results of acute ecotoxicity obtained in this research can be compared with other studies with similar goals. Cleuvers (2004) evaluated non-steroidal anti-inflammatory drugs and ASA with algae and with *Daphnia magna*. The author's EC_{50} values using *Daphnia magna* were in the range from 68 to 166 mg L^{-1} and from 72 to 626 mg L^{-1} in the algal test. Diclofenac, ibuprofen and naproxen were more toxic for *Vibrio fischeri* (below 20 mg L^{-1} for 5 and 15 min tests in this study) than for algae and for *Daphnia magna*. ASA showed the opposite behavior: 106.7 mg L^{-1} in the algae test and 88.1 m L^{-1} in the *Daphnia* test (Cleuvers 2004) versus 146.13 and 133.59 mg L^{-1} in the 5 and 15 min MICROTOX® tests, respectively (this work). Claessens et al. (2013) analyzed salicylic acid and acetaminophen acute ecotoxicity with the marine diatom *P. tricornutum*. For salicylic acid, the marine diatom was less sensitive than *Vibrio fischeri*, and for acetaminophen, both species yielded similar results. Santos et al. (2010) presented a large variety of data for the ecotoxicity of naproxen, ibuprofen and acetaminophen. The obtained values were in a wide range depending on the species tested and on the toxicological endpoint. The EC_{50} of the acetaminophen for *Vibrio fischeri* in the present study (310.70 and 363.30 mg L^{-1} in 5 and 15 min MICROTOX® tests) was minor compared with that reported by Santos et al. (2010) (567.5 and 650 mg L^{-1} for 15 and 30 min bioluminescence tests); However, this compound was considered nontoxic in both studies according to the GHS classification. Values of ASA and salicylic acid ecotoxicities were in the same order and were classified between "harmful to aquatic organism" and "nontoxic". Pounds et al. (2008) reported an acute ecotoxicity of ibuprofen on the mollusk *Planorbis carinatus*, finding that the 48 and 72 h LC_{50} values were both 17.1 mg L^{-1}, which is similar to the value obtained for *Vibrio fischeri* in this study.

Antibiotics belong to a therapeutic class where human health preservation and environmental disturbance are closely related. The major concern is associated with the development of resistance mechanisms by bacteria, which can subsequently compromise public health by limiting the effectiveness of the treatment (Santos et al. 2010). Thus, in this study, nine antibiotics widely used worldwide have been studied. The acute ecotoxicities of levofloxacin, which were determined by Kim et al. (2009) in crustaceans and in fishes (EC_{50}, $LC_{50} > 100$ mg L^{-1}), were in the same GHS classification as in this study ($EC_{50} =$ 825.52 mg L^{-1} and $EC_{50} = 788.30$ mg L^{-1} for 5 and 15 min of MICROTOX® tests, respectively). However, the EC_{50} for algae that was reported by Yamashita et al. (2006) (1.2 mg L^{-1}) was much lower than the value in this study for *Vibrio fischeri*. The EC_{50} of norfloxacin for rotifers and algae (Santos et al. 2010) were in the same GHS range (between 10 and 100 mg L^{-1}) as in this study for *Vibrio fischeri*. Ciprofloxacin showed more sensitivity for the cyanobacterium *Anabaena flos-aquae* ($EC_{50} = 10.2$ μg L^{-1}) and for the monocotyledonous macrophyte *Lemna minor* ($EC_{50} = 62.5$ μg L^{-1}) (Ebert et al. 2011) than in this study for *Vibrio fischeri* ($EC_{13} = 63$ mg L^{-1}). It is important to emphasize the high values of ECOSAR™-predicted ecotoxicities of ciprofloxacin, levofloxacin and

norfloxacin (fluoroquinolone antibiotics), which do not agree with the experimental results for different species, including the *Vibrio fischeri* bacteria. Therefore, these predicted values should be revised considering the experimental data available.

For sulfamethoxazole, some authors reported values of EC_{50} in different species. Kim et al. (2007) found that the acute ecotoxicity of *Vibrio fischeri* (78.1 mg L^{-1} in 15 min.) was 1.8 times higher than in this study; however, both values match in the same GHS category (Harmful to aquatic organism). In the same study, the EC_{50} of crustaceans and the LC_{50} of fish were over 100 mg L^{-1}. Isidori et al. (2005) found lower EC_{50} values than Kim et al. (2007), with values between 9 and 35 mg L^{-1} for rotifers and crustaceans and as low as 0.52 mg L^{-1} in 72 h growth inhibition for the algae *P. Subcapitata*. In another study (Park and Choi 2008), the EC_{50} for *Daphnia magna* in 48 h was higher than 100 mg L^{-1}, and for *M. Macrocopa*, the EC_{50} was approximately 80 mg L^{-1}. These experimental results for sulphametoxazole substantially deviate from the predicted values achieved with ECOSAR™, as in the case of fluoroquinolone antibiotics.

The ecotoxicity values for the macrolide clarithromycin in different species (rotifers and crustaceans from Isidori et al. 2005 and Kim et al. 2009) and those values found in this work coincide in the same GHS category "harmful to aquatic organism". For other species, this compound was found in other categories of classification. For fish mortality, Kim et al. (2009) found $LC_{50} > 100$ mg L^{-1} (nontoxic), and for algae, Isidori et al. (2005) reported 2 μg L^{-1} in 72 h growth inhibition for *P. Subcapitata* (highly toxic). The other macrolide under study (erythromycin) also showed significantly different experimental results among species and among the different acute ecotoxicity endpoints (e.g., mortality in 24 or 96 h, population growth inhibition in 48 or 72 h, immobilization in 24 h). Ecotoxicity values for erythromycin ranged from "nontoxic" (for crustaceans and fish, according to Kim et al. 2009) to "highly toxic" (for algae, according to Isidori et al. 2005) to "harmful to aquatic organisms" (in rotifers and crustaceans, according to Isidori et al. 2005). In this study, erythromycin was classified as "nontoxic" based on *Vibrio fischeri* results (see Table 1; Fig. 2). Therefore, according to the GHS classification, no definitive conclusion as to the grade of these compounds (macrolides) can be made. Despite this limitation, and in contrast to fluoroquinolones, the ECOSAR™-predicted ecotoxicity values for macrolides are more consistent with the experimental results.

There are fewer data in the literature regarding the acute ecotoxicity of amoxicillin and cefaclor; amoxicillin seems to be nontoxic when considering the predicted ecotoxicity values and studies for *Vibrio fischeri* (this research and Park and Choi 2008) but is highly toxic for some algae (Andreozzi et al. 2004). Cefaclor was found to be nontoxic in the predictive model results and in the bioluminescence assays.

The contradictory behavior of amoxicillin compared with other antibiotics may be explained because there is no "typical" antibiotic. (Q)SAR models have been developed considering low hydrophobicity is required for antibacterial activity; however, the relation between the decreasing log K_{ow} value and the increasing antibiotic activity has not been elucidated (Sanderson et al. 2004a).

The ecotoxicity of clofibric acid was evaluated by Rosal et al. (2010b) using three different aquatic microorganisms (one of the aquatic microorganisms was *Vibrio fischeri* bacteria), and the EC_{50} was found to be 2.8 times lower than ours. However, the risk classification of this PPCP is similar in both studies: "nontoxic". The clofibric acid EC_{50} values obtained by Ferrari et al. (2003) and by Santos et al. (2010) were in the same order as that of the present research. The predicted ecotoxicity values of clofibric acid are similar to the experimental results, and therefore, the GHS classification agrees. For clofibrate, there is less ecotoxicity information available; clofibrate has been reported as toxic for fish (Raldúa et al. 2008) (similar to the predicted values from (Q)SAR). However, clofibrate has been found to be "nontoxic" for *Vibrio fischeri* and for biomass microorganisms (respirometry assay) in the present work.

Regarding personal care products (PCPs), triclosan was reported as "very toxic" according to acute ecotoxicity in different species and exposure times (Brausch and Rand 2011) are in line with the results for *Vibrio fischeri* in this study ("toxic" in the GHS classification). Parabens could cause adverse effects on environment (Terasaki et al. 2009), and the ecotoxicity values reported by Brausch and Rand (2011) confirm the results for *Vibrio fischeri* in this study ("toxic" in the GHS classification). Yamamoto et al. (2007) found higher acute ecotoxicity values for algae (72 h-EC_{50}), *Daphnia* (48 h-EC_{50}) and fish (96 h-LC_{50}) because these species are less sensitive than the bacteria *Vibrio fischeri*. However, when respirometry assays were performed in this study, the parabens were classified as "harmful to aquatic organism", and triclosan was classified as "nontoxic".

Overall, discrepant experimental values of EC_{50} can be attributed to the complexity of the biological tests, to changes in organism sensibilities and to inter-laboratory differences. Additionally, intra- and inter-laboratory variability of standard single species toxicity tests must be considered when assessing the sensitivity and quality of structure activity relationship (SAR) estimates versus experimental values (Sanderson et al. 2003). Despite the discrepancies between predicted and experimental ecotoxicity values for a variety of species, the GHS

classification system is a useful tool to establish a reasonable range to classify the ecotoxicity values and their adverse effects, as well as to compare results from different studies. Therefore, the GHS classification system could be used to make decisions concerning the potential impact of these compounds on the environment.

ERA of PPCPs in aquatic environments using the EMEA framework

Table 2 shows the ERA parameters used for the PPCPs under study following the EMEA guidelines in aquatic environments. According to Phase I (see Fig. 1), the PEC values were verified for the compounds investigated in this study. When a simple PEC value (PEC_S) was used, all compounds continued to the next phase; however, with refined PEC values (PEC_R), cefaclor, clofibric acid and clofibrate had a $PEC_R < 0.01$ μg L^{-1}, and therefore, these compounds were classified as risk-free.

For the PBT screening, the octanol–water partition coefficient was verified using a recent study (Ortiz de García et al. 2013b), and only triclosan had a log $K_{ow} \geq 4.5$. Therefore, a PBT analysis was performed for this compound using the EPI Suite™ interface (EPA 2009). The results indicated that triclosan is persistent and toxic but not bioaccumulative. Despite this finding, a recent study (Brausch and Rand 2011) noted that there are contradictory studies concerning the bioaccumulation potential of triclosan. As a result, bioaccumulation tests of triclosan should be performed for specific species and geographic areas under study. Kosma et al. (2014), in an ERA performed in Greece, found that triclosan was the most critical compound in terms of its contribution and environmental risk, concluding that triclosan should be seriously considered a candidate for regulatory monitoring and prioritization on a European scale from realistic PNECs. In this study, the triclosan RQ value was calculated from MEC, and the result was less than one. In the MRERA classification, triclosan has been found with "medium toxicity".

When the phase II EMEA guidelines were applied to the 26 PPCPs under study and their RQ ratios were calculated, excluding pharmacokinetics in humans and their removal in WWTPs (RQ_S), then only acetaminophen, ibuprofen and omeprazole have a $RQ > 1$. Therefore, these compounds should be evaluated according to Tier B with a refined PEC. Nevertheless, when a MRERA classification (EC 1996) is applied for RQ_S, RQ_R and RQ_{MEC}, then 82.4, 20 and 28.57 % of compounds investigated, respectively, have some type of risk. A RQ calculated with refined data from PEC is congruent with the RQ calculated with MECs data for 65 % of the compounds for which both values were known.

In Tier B (with refined PEC_R data) -Phase II- of the EMEA methodology, only 1,4-benzoquinone was found as a compound that must be referred to the committee for proprietary medicinal products for safety measures, although 1,4-benzoquinone is a product of the transformation of acetaminophen and clofibric acid.

When MRERA criteria were used with refined data, in addition to 1,4-benzoquinone (high risk), omeprazole and triclosan had medium risk, and clarithromycin, ethylparaben and methylparaben had a low risk.

All of these results are characteristic of a specific geographic area because the PEC or MEC values are dependent on the particular pattern of treatment, consumption and wastewater management. Thus, the results for some compounds could be similar in different countries, but the results for other compounds could substantially differ.

Other studies concerning aquatic environments have reported the RQ ratio according to the EMEA guidelines for different PPCPs and metabolites. In Switzerland (Tauxe-Wuersch et al. 2005), the environmental risk of five pharmaceutical compounds was analyzed in three WWTPs. The authors predicted the PECs values (refined or not) and measured the concentrations of the substances in the effluent of the WWTPs, and the PNEC values were calculated from acute or chronic values of standard species or with ECOSAR™ predictions. Their main results show that the concentrations of ibuprofen, mefenamic acid and diclofenac were relatively high in the effluents (150–2,000 ng L^{-1}), showing a potential contamination of surface water. Mefenamic acid seemed to present a risk for the aquatic environment, with a ratio PEC/PNEC higher than one.

RQ ratios were estimated for effluents of WWTPs in the principal cities of the Ebro River Basin (Spain) (Gros et al. 2010). The RQ values were calculated with the detected MECs and with the estimated PNECs for fish, *Daphnia* and algae acute ecotoxicity values.

Another study (Hernando et al. 2006), presents an overview of the environmental occurrence and ecological risk assessment of pharmaceutical residues from the literature and discusses the potential environmental impact of WWTP effluents, surface water and sediments, using acute ecotoxicity values for fish, *Daphnia* and algae. The authors found that high risk is suspected in surface waters for anti-inflammatory drugs (ibuprofen, naproxen, diclofenac, and ketoprofen) and for antiepileptics (carbamazepine). Additionally, medium risk was suspected in sediments for antibiotics (oxytetracycline and flumequine) and for β-blockers (propanolol) in surface waters. In WWTP effluents, high risk is suspected to be induced for the following drugs: antibiotics (erythromycin), anti-inflammatories (ibuprofen, naproxen, diclofenac, and ketoprofen), lipid regulators agents (gemfibrocil and clofibric acid), β-blockers (propanolol and metoprolol) and antiepileptics (carbamazepine).

Table 2 Parameters used in the ERA studies according to the EMEA guidelines in aquatic environments and WWTPs

Compound[a]	Aquatic environments								WWTPs				
	PEC_S^b (ng L^{-1})	PEC_R^c (ng L^{-1})	MEC^d (ng L^{-1})	Lowest EC_{50} (mg L^{-1})	$PNEC^e$ (ng L^{-1})	RQ_S^f	RQ_R^g	RQ_{MEC}^h	EC_{50} respirometry (mg L^{-1})	PEC_{WWTPs} (ng L^{-1})[i]	$PNEC_{WWTPs}$ (ng L^{-1})[j]	RQ_{WM}^k	RQ_{WWTPs}^l
Acetaminophen	312,675.36	498.23	307.00	310.70[o]	3,10700	**1.0064**	0.0016	0.0010	2,255.00	102,228	22,550,000	0.0139	0.0045
Acetylsalicylic acid	39,346.90	226.20	160.00	118.70[n]	118,700	0.3315	0.0019	0.0013	471.68	19,964	4,716,794	0.0083	0.0042
Amoxicillin	42,416.87	326.50	40.00	299.90[n]	299,900	0.1414	0.0011	0.0001	2,423.29	25,196	24,232,900	0.0018	0.0010
Cefaclor	38.77	2.56	200.00	731.00[n]	731,000	0.00005	0.000004	0.0003	940.27	30.71	9,402,700	0.000004	0.000003
Ciprofloxacin (HCl)	4264.94	51.44	28.02	226.67[o]	135,050	0.0307	0.0007	0.0002	9.55	3336	95,500	0.0447	0.0175
Clarithromycin	2326.33	124.63	88.83	12.08[o]	12,080	0.1926	0.0100	0.0074	36.40	1819	364,003	0.0064	0.0050
Clofibric acid	n.a	1.43	3.00	102.70[p]	102,699	n.a.	0.00001	0.00003	285.4	72.93	2854000	n.a.	0.00003
Clofibrate	74.41	0.0520	80.00	4.67[n]	4674	0.0159	0.00001	0.0171	769.28	0.74	7692,800	0.00001	0.0000001
Diclofenac Na	3724.96	84.86	89.53	14.31[o]	14,310	0.2603	0.0059	0.0063	299.61	2395	2996,100	0.0012	0.0008
Erythromycin	387.72	19.50	50.38	71.41[n]	71,400	0.0054	0.0003	0.0007	561.91	254	5619,100	0.0001	0.00005
Ethylparaben	n.a.	n.a.	30.00	2.78[p]	2780	n.a.	n.a.	0.0108	98.64	1943[d]	986,400	n.a.	0.0020
Ibuprofen	46,793.95	103.84	134.75	13.87[p]	13,875	**3.3726**	0.0075	0.0097	21.00	19746	210,000	0.2228	0.0940
Levofloxacin	1163.67	86.50	11.90	788.30[o]	788,300	0.0015	0.0001	0.00002	2021.41	1038	20,214,073	0.0001	0.00005
Methylparaben	559.24	46.01	54.00	6.85[o]	6846	0.0817	0.0067	0.0079	83.72	554	837,200	0.0007	0.0007
Naproxen	12,141.49	89.87	81.05	14.20[p]	14,199	0.8551	0.0063	0.0057	32.82	1856	32,8206	0.0370	0.0056
Norfloxacin	930.53	23.95	15.83	20.81[o]	20,810	0.0447	0.0012	0.0008	43.81	774	438,114	0.0021	0.0018
Omeprazole	4774.06	278.21	33.00	1.76[o]	1760	**2.7125**	0.1581	0.0188	50.05	3568	50,0473	0.0095	0.0071
P-hyd.benzoic acid[m]	n.a	n.a.	n.a.	62.50[n]	62,500	n.a.	n.a.	n.a.	134.68	n.a.	134,680	n.a.	n.a.
Propylparaben	186.41	14.75	105.00	1.64[p]	1642	0.1135	0.0090	0.0640	100.16	185	1001,600	0.0002	0.0002
Salicylic acid	n.a.	18.40	6.70	52.49[q]	118,700	n.a.	0.0002	0.00006	372.76	1899	3727,600	n.a.	0.0005
Sulfamethoxazole	2326.33	44.63	39.70	43.56[o]	43,560	0.0534	0.0010	0.0009	279.81	1013	2798100	0.0008	0.0003
Triclosan	n.a.	n.a.	138.00	0.37[p]	372	n.a.	n.a.	0.3708	1089.07	1142[d]	10890,700	n.a.	0.0001

Table 2 continued

Compound[a]	Aquatic environments								WWTPs				
	PEC_S^b (ng L^{-1})	PEC_R^c (ng L^{-1})	MEC^d (ng L^{-1})	Lowest EC_{50} (mg L^{-1})	$PNEC^e$ (ng L^{-1})	RQ_S^f	RQ_R^g	RQ_{MEC}^h	EC_{50} respirometry (mg L^{-1})	PEC_{WWTPs} (ng L^{-1})[i]	$PNEC_{WWTPs}$ (ng L^{-1})[j]	RQ_{WM}^k	RQ_{WWTPs}^l
1,4-Benzoquinone	n.a.	62.66	n.a.	0.03[p]	27.86	n.a.	2.2489	n.a.	1.36	[r]	13,600	n.a.	n.a.

n.a.: Data not available

[a] For those compounds that were analyzed as neutral (ciprofloxacin, ibuprofen and naproxen) and as ionized molecules (ciprofloxacin HCl, ibuprofen sodium salt and naproxen sodium salt), the ERA was performed only for one compound (the neutral or the ionized), i.e., the compound that showed the highest ecotoxicity value (the lowest EC_{50})

[b] Simple predicted environmental concentration: metabolization in humans and removal in WWTPs were excluded for PEC calculations. Based on data reported by Ortiz de García et al. 2013a

[c] Refined predicted environmental concentration: metabolization in humans and removal in WWTPs were considered in PEC calculations. Based on data reported by Ortiz de García et al. 2013a

[d] Measured Environmental Concentration. The highest MECs in aquatic environments and in the influents of WWTPs were taken as the worst condition for the environment from the following publications: Brausch and Rand (2011), Fatta-Kassinos et al. (2011), García-Galán et al. (2011), Ginebreda et al. (2010), González-Mariño et al. (2011), Gros et al. (2010), Ortiz de García et al. (2013a), Pothitou and Voutsa (2008), Raldúa et al. (2008), Rosal et al. (2010b), Teijon et al. (2010), Valcárcel et al. (2011), and Villaverde-de-Sáa et al. (2010)

[e] Predicted no effect concentration

[f] Risk quotient with simple PEC (PECS). Values higher than 1 in bold (see Fig. 1)

[g] Risk quotient with refined PEC (PECR)

[h] Risk quotient with MEC

[i] Predicted environmental concentration in the influents of WWTPs: metabolization in humans was considered in this calculation

[j] Predicted no effect concentration on biomass of WWTPs

[k] Risk Quotient in WWTPs with simple PEC (PECs): without human metabolization, without WWTPs removal (Influent and effluent PPCP concentrations are identical)

[l] Risk Quotient with PEC_{WWTPs}

[m] *P*-Hydroxybenzoic acid

[n] Algae, predicted value, after 96 h

[o] *Vibrio fischeri*, experimental value, after 5 min

[p] *Vibrio fischeri*, experimental value, after 15 min

[q] Crustaceans, predicted value, 48 h

[r] According to the literature (Bedner and MacCrehan 2006; Xagoraraki et al. 2008) 1,4-benzoquinone is formed from the chlorination of acetaminophen or after the irradiation of clofibric acid (Nikolaou et al. 2007); therefore, this compound was not considered present in the influent of a WWTP, at least as a transformation product of acetaminophen and clofibric acid

In Norway, an ERA of eleven pharmaceuticals was performed (Grung et al. 2008) according to the EMEA guidelines. Risk quotients greater than 1 were obtained for ciprofloxacin, diclofenac, ethinylestradiol, sulfamethoxazole and tetracycline according to the EMEA guidelines. MEC values confirmed that the release of ciprofloxacin from WWTPs might potentially be of environmental concern in Norway.

Comparing these aforementioned studies, the results show a wide divergence among their RQs primarily due to the assumptions made, the different methods to calculate PECs, the different values of ecotoxicity available or that were determined in laboratory assays and the available data. Despite this divergence, non-steroidal anti-inflammatories, analgesics, antibiotics and PCPs coincide with this study as some of the main compounds that require attention in future studies because of their potential negative impacts.

Notably, the veterinary consumption of PPCPs was not considered in this study, which could increase the PECs values (particularly for anti-inflammatories, analgesics and antibiotics) and modify the RQ values. The ecotoxicity effect of mixtures is another aspect that was not considered in this work.

ERA of PPCPs in WWTPs using the EMEA framework

The ERA of PPCPs in WWTPs has been performed based on the occurrence of the compounds under study in the influents of these facilities in Spain, with or without considering their metabolization in humans. Respirometry is the most realistic ecotoxicity test to calculate the RQ in a WWTP because the results indicate the effect of these compounds on the active biomass of the biological treatment (In Spain, the most used secondary treatment in WWTPs includes aerobic processes). Thus, these ERAs are strongly dependent on consumption data, management of wastewater and particular characteristics of the WWTP microorganisms.

According to the EMEA guidelines, any compound having a RQ $>$ 1 (in Phase I and Phase II) will not act on this environment (see Table 2). Despite this classification, some compounds showed high ecotoxicity in the respirometry tests and presented some type of risk in the GHS classification: 1,4-benzoquinone (toxic), ciprofloxacin (toxic) and clarithromycin, ibuprofen, naproxen, norfloxacin, omeprazole (harmful to aquatic organism). The result highlights triclosan, which had high ecotoxicity over the aquatic species but not over the activated sludge, which showed a high tolerance to this compound.

Following the MRERA classification, excluding the metabolization in humans, ibuprofen, ciprofloxacin, naproxen and acetaminophen showed some type of risk in these facilities, and when the metabolization was considered, then ibuprofen and ciprofloxacin were highlighted with a low risk.

Respirometry ecotoxicity data (using real activated sludge and synthetic wastewater contaminated with PPCPs, metabolites and transformation products) in ERA studies have been poorly investigated, at least in Spain. Hernando et al. (2006) performed a risk characterization of WWTP effluents and found that most of the drug residues, including antibiotics, anti-inflammatory, lipid regulators agents, β-blockers and antiepileptics, are suspected to produce high ecological risks to representative species of the food chain.

The inhibition of the biomass respiration assays has a high dependence on laboratory conditions: temperature, equipment calibration, agitation, and, in particular, the biomass, which is specific for each WWTP. However, the respirometry results obtained using a standard methodology serve as a preliminary assessment that allows: (i) the prediction of the behavior of these compounds in the WWTPs (ii) the implementation of actions to improve their performance (iii) the prevention of the negative effect of PPCPs on the biological treatment and (iv) the avoidance of these compounds reaching the aquatic environment. Moreover, the obtained classification could be used as a starting point for a more detailed analysis, including the long-term occurrence, fate and effects of these compounds and their mixtures in WWTPs, with a particular emphasis on the secondary treatment of these facilities.

Conclusions

The ecotoxicities of 26 PPCPs, metabolites and transformation products of interest in Spain were determined using bioluminescence and respirometry assays and were theoretically predicted using the ECOSAR™ software. The experimental ecotoxicity results showed that 65.4 % of PPCPs under study were at least harmful to aquatic organisms according to the GHS classification based on two different ecotoxicity tests, which provides preliminary evidence concerning the negative effects of these compounds on the environment. 1,4-Benzoquinone (transformation product of acetaminophen and clofibric acid) and triclosan were found as the most toxic compounds according to the GHS classification. The overall order of the species susceptibility was as follows: *Vibrio fischeri* (5 min) $>$ *Vibrio fischeri* (15 min) $>$ algae $>$ crustaceans $>$ fish $>$ activated sludge of WWTP (respirometry assay). The ecotoxicity results and the GHS classification are independent of the geographic area under study, as well as the consumption, occurrence and treatment of PPCPs,

but are strongly dependent on the laboratory conditions, testing species, methodologies and software used.

ERAs of PPCPs in aquatic environments and WWTPs were performed following the EMEA guidelines using the previously obtained ecotoxicity values. Acetaminophen, ciprofloxacin, clarithromycin, clofibrate, ibuprofen, omeprazole, triclosan, parabens and 1,4-benzoquinone showed some type of risk for aquatic environments and for activated sludge from the secondary treatment of WWTPs. Triclosan was found to be persistent and toxic but not bioaccumulative, although there is contradictory information concerning the bioaccumulation of this compound. The respirometry assays to determine the RQs of PPCPs in WWTPs have demonstrated to be useful tools for studying the effect of PPCPs in these facilities and complement the available ecotoxicity information for such compounds. ERA is a geographic-dependent tool due to the different data concerning the consumption, occurrence and treatment for the area under study. The RQ values can substantially vary if these values use a simple or a refined PEC or MEC approximation. Therefore, a further improvement of these parameters and of the ecotoxicity data (acute and chronic) of these compounds, particularly their metabolites, transformation products and mixtures, which have been less investigated, is required.

Despite the limitations of the EMEA guidelines, these guidelines are a useful method for ERA studies, which is the reason these guidelines are increasingly used.

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Chapter 5

Paper IV

Dose-response behavior on bacterium *Vibrio fischeri* exposed at single and mixtures of Pharmaceuticals and Personal Care Products.

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Dose-response behavior of the bacterium *Vibrio fischeri* exposed to pharmaceuticals and personal care products

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Abstract

The presence of pharmaceuticals and personal care products (PPCPs) in the environment has become a real and broad concern in recent years. Therefore, the main goal of this study was to investigate the behavior of 20 common and highly consumed PPCPs both individually and their mixture to assess their effect on important specie in trophic level: bacteria. The ecotoxicological data of PPCPs were determined in *Vibrio fischeri* bacteria by Microtox® and were statistically analyzed using three models in the GraphPad Prism 6 program for Windows, v.6.03 in two different ranges of concentrations. Four parameter model was the best fit model for the majority of compounds. Half maximal effective concentration (EC_{50}) of each PPCP has been estimated by the best fit model and has been compared with a recent study. Most compounds showed the same level of toxicity when the comparative analysis has been made. Moreover, the stimulation effects of PPCPs at environmental concentrations (low-doses) were assessed. The results showed that some compounds have traditional inverted U- or J-shaped dose-response curves, and 55% of them presented a stimulatory effect under zero effect-concentration point. The effective concentrations at 0 (EC_0), 5 (EC_5) and 50% (EC_{50}) were calculated for each PPCP as ecotoxicological points. All compounds that presented narcosis as a mode of toxic action at high doses also showed some stimulation at low concentrations. The maximum stimulatory effect of a mixture was higher than the highest stimulatory effect of each individually tested compound. Moreover, when the exposure time was increased, the hormetic effect decreased. Hormesis is being increasingly included in dose-response studies because this effect could be harmful, beneficial or indifferent effect in the environmental. Despite the results obtained in this research still there is plenty to investigated in order to fully understand the behavior of PPCPs in the aquatic environments.

Keywords: Bioluminescence, Ecotoxicity, Hormesis, Pharmaceuticals and Personal Care Products.

Highlights:

- Relevant ecotoxicological information has been calculated for 20 PPCPs.
- All of the PPCPs investigated were correlated with models of the nonlinear functions analyzed.
- A stimulatory effect at low concentrations was detected in 55% of the PPCPs studied.
- All PPCPs that presented narcosis as a toxic mode of action at high doses also showed stimulation at low concentrations.
- The PPCP mixture investigated showed a hormetic effect that was higher than the maximum effects of the single compounds of the mixture.

1. Introduction

Pharmaceuticals and personal care products (PPCPs) are an important, diverse and large group of chemical compounds that are present as micro-pollutants in different aquatic environments. The input and presence of pharmaceutical active compounds (PhACs) and their fate in the environment were and are still of high interest (Kümmerer 2009). Several investigations have shown some evidence that substances of pharmaceutical origin are often not eliminated during wastewater treatment and are not biodegraded in the environment (Hereber 2002).

Generally, PPCPs have been found in aquatic environments in a very low and wide range of concentrations (mostly in ng L^{-1} and μg L^{-1}). As a result, a variety of literature show advanced detection methods that have been refined to demonstrate their presence at these levels of concentrations in the environment (Batt et al. 2008; Dévier et al. 2011; González-Mariño et al. 2011; Kot-Wasik et al. 2006, 2007; Ternes et al. 2001). In contrast, little is known about the ecotoxicological effects of pharmaceuticals on aquatic and terrestrial organisms and wildlife, and a comprehensive review of the ecotoxicological effects is lacking (Fent et al. 2006). In particular, it is necessary to elucidate the significance of low-level effects in the range where so-called ‘‘paradoxical’’ dose-responses are prevalent [e.g., at levels of nM–pM (mg–ng L^{-1}) and below, where the U- or J-shaped nature of the dose-response curves becomes evident]. An example is hormesis, a dose-response phenomenon in which non-inhibitory effects occur below previously established levels of ‘‘no-observed effects’’ (Daughton 2004). The fundamental nature of the dose-response is neither linear nor threshold, but, rather, it is U-shaped.

When studies are properly designed to evaluate biological activity below the traditional ecotoxicological threshold, low-dose stimulatory responses are observed with high frequency and display specific quantitative features (Calabrese and Baldwin 2001)**.** The ubiquity of hormesis is well established and there are many examples cited in the literature (Stebbing 2000).

Aquatic organisms are particularly important targets, as they are exposed via wastewater residues over their whole life (Fent et al. 2006). Acute and chronic ecotoxicity assessments have been implemented to evaluate the effects of these compounds on different species. The standard organisms used are fish, crustaceans and algae, which represent the principal three trophic levels. It is clear that aquatic life can be exquisitely sensitive to at least some PPCPs. Between-species, between-sex, and between-drug effects can also vary widely (Daughton and Ternes 1999).

Although bacteria are less used, many authors confirm the importance of take them into account as a relevant ecotoxicological subject (medium) (Backhaus and Grimme 1999; Bouki et al. 2013; Choi and Meier 2001; Christofi et al. 2002; Deng et al. 2012; Liu et al. 2009, 2013; Ortiz de García et al. 2014; Parvez et al. 2006; Shen et al. 2009; van der Grinten et al. 2010; Vighi et al. 2009; Villa et al. 2012; Zhang et al. 2011; Zou et al. 2013). In the majority of aquatic ecosystems, the most important trophic level in terms of energy flow and nutrient cycles is the bacteria. Hence, it is important to include representatives from this trophic level in a series of tests designed for protecting the aquatic ecosystems (Choi and Meier 2001). Vighi et al. (2001) assert that in view of the ecological importance of bacteria in all ecosystems, their exclusion from ecotoxicological risk assessments could, in some cases, result in the implementation of

inadequate protective measures for the aquatic environment. Therefore, it is important to study and know the effects that PPCPs have on these microorganisms at the real environmental concentration levels.

Bacteria in general (and more specifically, *Vibrios*) have important functions in aquatic environments. *Vibrios* play a role in nutrient regeneration in the aquatic milieu by taking up dissolved organic matter, producing essential polyunsaturated fatty acids needed in the aquatic food web, and degrading chitin. Some *Vibrios* bacteria have a role in the biodegradation of polycyclic aromatic hydrocarbons in polluted marine sediments. Among the marine bacteria, they are prolific producers of antimicrobials as well as the most resistant (Milton 2006). *Vibrio fischeri* and other luminous bacteria also form a variety of pathogenic and cooperative associations with marine animals: they are increasingly recognized as causes of invertebrate diseases; they are a common constituent of the microbial consortia of the enteric tract; and they form stable, cooperative associations in specialized symbiotic organs of marine squids and fishes (Ruby and Lee 1998).
Milton (2006) reviewed that the genus *Vibrio* contains more than 50 free-living species that are found in aquatic habitats, such as marine coastal waters, estuaries, sediments, and aquaculture settings, or in association with marine organisms such as coral, fish, mollusk, seagrass, zooplankton, and shrimp. These species associate with marine animal tissues as commensal microflora on fish mucosal surfaces, as symbionts in the light organs of fish and squid, and as pathogens causing disease in fish, coral, and crustaceans. They may also be bound as a biofilm on inanimate surfaces such as the exoskeletons of crustaceans, aiding survival during starvation and environmental stress. *Vibrio fischeri* is not pathogenic, which facilitates its use in laboratory ecotoxicological tests. However, many species of the genus *Vibrio* (*harveyi*, *cholera*, *anguillarum*, *vulnificus*) that could be disturbed by PPCPs in aquatic environments can cause notable adverse effects in different organisms, including humans.

In this context, any disturbance due to external agents suffered by populations of these bacteria can positively or negatively affect the ecosystem where they are present. According to Kefford et al. (2008), the goal of ecotoxicology is usually not to protect any one species but to protect communities of many species. Moreover, not all species have equal sensitivity to a toxicant. Stimulatory effects that have real consequences on individual organisms do not necessarily mean that there will be effects at the population and community levels. Nonetheless, these effects could still occur, affecting these individual organisms across a very wide range of taxa, toxicants and endpoints.

One of the most widely used biotests to determine the aquatic toxicity of chemicals towards bacteria is the acute bioluminescence inhibition assay with *Vibrio fischeri*, formerly *Photobacterium phosphoreum* (Backhaus and Grimme 1999), which involves measuring the reduction in light output when the organisms are exposed to a toxic sample (Christofi et al. 2002). The Microtox® assay has been widely applied as a rapid, economical monitoring tool for toxicity of environmental contaminants (organic and inorganic) in different compartments (surface water, groundwater, wastewater facilities, sediments). It has a considerably lower coefficient of variance than other bioassays because of the highly formalized and standardized reagents that are less susceptible to variation (Choi and Meier 2001). Furthermore, researchers have reported the *Vibrio fischeri* bioluminescence assay as the most sensitive across a wide range of chemicals compared to other

bacterial assays such as nitrification inhibition, respirometry, ATP luminescence and enzyme inhibition (Parvez et al. 2006).

In a recent study, the advantages of using this assay were explained (Ortiz et al. 2014). In addition, bacteria are important microorganisms in many ecosystems and are essential in the food chain; therefore, it is crucial to study the effects that some PPCPs used worldwide have on these microorganisms at the real environmental concentration levels, which are much lower than their EC_{50}.

The most often ecotoxicological studies of PPCPs have determined some relevant environmental parameters as the half maximal lethal concentration (LC_{50}), the half maximal effective concentration (EC_{50}), the non-observed effect concentration (NOEC), the non-observed (adverse) effect level (NO(A)EL), the lowest observable effect concentration (LOEC) as seen in the work of Santos et al. (2010) who conducted an extensive review of existing data of acute and chronic effects on non-target organisms for many therapeutic classes. More recently, the zero equivalent point (ZEP or EC_0) is an important parameter that has been estimated instead of the NOEC. Despite this, there is a scarce evidence for the effects of a single compound or a complex mixture of PPCPs below the NO(A)EL or the LOEC values.

During the last 10 years, mixture ecotoxicology has undergone a remarkable and productive development. While earlier experimental studies have focused mainly on combinations of only two chemicals, a significant number of well-designed and decisive studies have been carried out that involve multi-component mixtures. There is strong evidence that chemicals with common specific modes of action work together to produce combination effects that are larger than the effects of each mixture component applied singly (Kortenkamp et al. 2009). However, a significant lack of knowledge persists particularly concerning ecotoxicological data from synergistic pharmaceuticals interactions (Santos et al. 2010). Because pharmaceuticals in the aquatic environment occur usually as mixtures, an accurate prediction of the mixture ecotoxicity is indispensable to perform environmental risk assessment (Cleuvers 2004). Backhaus et al. (2011) investigated the single-substance and mixture ecotoxicity of five PPCPs over marine microalgal communities (periphyton). They found that all compounds proved to be ecotoxic. Moreover, the mixture provoked stimulating effects in the lower effect range.

According to Kortenkamp et al. (2009) scientific research has repeatedly demonstrated that the effects of mixtures are considerably more pronounced than the effect of each of its individual components and that environmental pollution is from chemical mixtures and not from individual substances. Parvez et al. (2008, 2009) found synergistic, antagonistic and additive effects of different chemicals mixture on *Vibrio fischeri* bacteria. In the research of Villa et al. (2012) the responses to complex mixtures of a high number of individual components with different chemical and toxicological characteristics, were tested on the bacterium *Vibrio fisheri*. They found that even extremely low concentrations (far below NOEC) of individual chemicals contributed to the effect of the mixtures; additionally, synergistic effects were not observed in any of the tested mixtures. Moreover, Breitholtz et al. (2008) concluded in their study on mixture ecotoxicity of brominated flame-retardants in the copepod *Nitocra spinipes* that low concentrations of individual substances can cause ecotoxicity if exposed in mixtures, which highlights the need to consider mixture ecotoxicity to a greater extent in regulatory works. The evaluation by

Cleuvers (2003) of the ecotoxicological potential of 10 prescription drugs against aquatic organisms from different taxonomical classes has shown that the measured acute effects of single pharmaceuticals in the aquatic environment are very unlikely. Nevertheless, it should keep in mind that considerable combination effects can occur.

Scarce information exists concerning the behavior of individual PPCPs and their mixtures on bacteria, therefore, in this framework, the objective of the present study was to investigate the dose-response relationships (by different models) of 20 common and highly consumed PPCPs (individually and its mixture) at concentrations around those found in aquatic environments or in wastewater treatment plants (WWTPs) (real environmental concentrations) and concentrations to estimate relevant ecotoxicological endpoints (as EC_{50}). Mixture behavior of these PPCPs was evaluated at different times and environmental concentrations levels to know the real effect on these bacteria. First, ecotoxicological data for single and mixtures of the PPCPs under study were determined on *Vibrio fischeri* bacteria by Microtox® at different times. The experimental data were statistically analyzed by three models (sigmoidal dose-response or three parameter logistic model, sigmoidal dose-response variable slope or four parameter logistic model and asymmetrical or five parameter logistic model) using GraphPad Prism 6 for Windows, v.6.03. Different statistical parameters have been calculated to support the results obtained. In addition, the bacteria response (stimulatory/inhibitory) of single compounds was assessed and was correlated with the chemical classes of the PPCPs using the program "Ecological Structure Activity Relationship" (ECOSAR™). Ecotoxicological endpoints (EC_5, EC_{50}, maximum stimulatory effect (MSE), maximum stimulatory effect concentration (MSEC) and ZEP) were estimated considering the data at different concentrations. Finally, the behavior of PPCPs mixture at environmental concentrations was analyzed.

2. Materials and methods

2.1. Chemicals

Twenty PPCPs were selected in accordance with their high worldwide consumption and the evidence of their potential ecotoxicity in aquatic environments, which has been highlighted by Ortiz et al. (2014). Of the PPCPs analyzed, seven were antibiotics (amoxicillin trihydrate, cefaclor, ciprofloxacin hydrochloride monohydrate, clarithromycin, erythromycin, norfloxacin, sulphametoxazole), six were non-steroidal anti-inflammatory drugs (NSAIDs)/analgesics (acetaminophen, acetylsalicylic acid (ASA), diclofenac sodium salt, ibuprofen sodium salt, naproxen and salicylic acid), two were blood lipid regulators (clofibrate and clofibric acid), one was an H_2 blocker (omeprazole), three were preservatives (methylparaben, ethylparaben and propylparaben) and one was an antimicrobial/disinfectant (triclosan). All of these compounds were purchased from Sigma-Aldrich® and Fluka in either analytical or technical grade purity (≥95%).

2.2. Test organism, culture and solutions

The test organism, culture and standard solutions used for the Microtox® ecotoxicity tests were reported in a previous publication (Ortiz et al. 2014). Stock solutions of single and mixtures of PPCPs were prepared in Milli-Q® water such that all compounds (except clofibrate and clarithromycin) were tested below their solubility values. Clofibrate and clarithormycin were dissolved in ethanol and then Milli-Q® water was used to prepare the stock solution.

Stock solutions were prepared at concentrations ranging from between 0.05 and 2 g L^{-1}. Preliminary assays (did not show in this study) have been made for establish the range of concentrations around the EC_{50} of each PPCP. Range of concentrations at environmental levels (aquatic environments and WWTPs) were consulted in published researches (Bouki et al.2013; Fent et al. 2006; González Mariño et al. 2011; Hereber 2002; Kot-Wasik et al. 2006, 2007; Kümmerer 2009; Ortiz et al. 2014; Ternes et al. 2001; Santos et al. 2010). According to the concentration range of each assay, the initial concentration of each PPCP solution was directly taken from its stock (in the case where the initial concentration coincide with the stock concentration) or was prepared by dilution (in the case of initial concentration had to be less than stock solution). Then, serial dilutions were made to generate four different concentrations as Microtox® basic test procedure indicates. The 20 PPCPs in the mixture were evaluated at a range of concentrations that included those values reported in literature for WWTPs and for some aquatic environments, where these compounds were found.

Product identification of the PPCPs and their principal physicochemical properties are presented in **Table 1**. All the concentrations (in the two ranges analyzed) used in the single PPCP assays and the initial concentration of each PPCP for the mixture ecotoxicity test are shown in **Table 2**.

2.3. Ecotoxicity tests

Two ecotoxicity tests using the Microtox® assays were performed, consisting of a 45% basic test for single assays and a whole effluent toxicity test (WET) for the mixture of PPCPs.

The standard basic test procedure has been previously reported (Ortiz et al. 2014) and it was performed here in agreement with the manufacturer`s instructions (Azur Environmental 1999) and the ISO 11348-3:2007 protocol (ISO 2007). In the basic test using the single PPCPs, bacteria were exposed for periods of 5 and 15 minutes. 45% basic test was performed two times in duplicate for two control samples and four dilutions of the each PPCP initial concentration (5.6, 11.2, 22.4 and 44.8%).

The results of the single 45% basic test assays were plotted as the dose-response behavior with their standard deviations separately, for the two exposure times and the two concentration ranges considered (See **Table 2**).

Additionally, the behavior of PPCPs environmental concentrations were correlated with the toxic modes of action at high concentrations using the ECOSAR™ program, which has been used in previous investigations to estimate the ecotoxicity of the PPCPs (Ortiz et al. 2013; 2014).

The ecotoxicity of the mixture of PPCPs was assessed following the WET test. According to the Environmental Protection Agency (EPA), WET is the aggregate toxic effect of an aqueous sample (e.g., a reference toxicant, an effluent, or a receiving water) measured directly by an aquatic toxicity test. The Convention for the Protection of the Marine Environment of the North-East Atlantic (the "OSPAR Convention"), in its Practical Guidance Document on Whole Effluent Assessment (OSPAR, 2007) (equivalent to the EPA WET guidance), considers the acute toxicity test using bacteria (*Vibrio fischeri*) as one of the key ecotoxicity procedures for use in identifying compounds of possible environmental concern. In this sense, Microtox® has developed its own WET testing protocols. In general, WET test is similar than Microtox® basic test but exist few differences in the experimental

procedure. WET implies three replicated samples, three control replicates and five dilutions (at 6, 12, 25, 50 and 100% of the mixture initial concentration. Initial concentration of each PPCP in the mixture is shown in **Table 2**) and generally is used to study the behavior of a compound (or mixture of them or samples of unknown composition) when it is important know the effects on bacteria. Basic test is generally used to estimate the effective concentration of PPCP that gives a bioluminescence inhibition of F percent (EC_f) and correlated the data to mathematical models.

The analysis of the behavior of the PPCPs mixture in preliminary assays with Microtox® (not shown) and those previously published in the literature served as the basis for designing WET a short-chronic bioassay used to study the behavior of this mixture.

Initial concentrations of each PPCP in the mixture assay were close to initial concentration in the individual assays at real environmental concentrations to make comparisons between single and mixture assays.

Table 1. Identification and physicochemical properties of PPCPs

PPCP	CAS number[+]	Chemical formula[+]	Mol. weight[+]	$S^{++,+++}$ (mg L^{-1})	$logK_{ow}$ †,†††	pK_a ††,†††	$logK_{oc}$ †††,†††
PhACs							
Analgesic/antipyretic							
Acetaminophen	103-90-2	$C_8H_9NO_2$	151.16	14000	0.46	9.38	1.320
Antibiotics							
Amoxicillin Trihydrate	61336-70-7	$C_{16}H_{19}N_3O_5S{\cdot}3H_2O$	419.45	4000	0.87	--	0.709
Cefaclor	53994-73-3	$C_{15}H_{14}ClN_3O_4S$	367.81	10000	0.35	2.4/7.2	0.255
Ciprofloxacin HCl	86393-32-0	$C_{17}H_{18}FN_3O_3{\cdot}HCl{\cdot}H_2O$	385.82	30000	0.28	6.09	-0.004
Clarithromycin	81103-11-9	$C_{38}H_{69}NO_{13}$	747.95	0.342[a]	3.16	8.99	2.174
Erythromycin	114-07-8	$C_{37}H_{67}NO_{13}$	733.93	2000	3.06	8.88	2.754
Norfloxacin	70458-96-7	$C_{16}H_{18}FN_3O_3$	319.33	177900	-1.03	--	-0.392
Sulfamethoxazole	723-46-6	$C_{10}H_{11}N_3O_3S$	253.28	610.0	0.89	--	1.536
Blood lipid regulators							
Clofibrate	637-07-0	$C_{12}H_{15}ClO_3$	242.70	69.12[a]	3.62	--	2.918
Clofibric acid	882-09-7	$C_{10}H_{11}ClO_3$	214.65	582.5	2.57	--	1.633
H_2 Blocker							
Omeprazole	73590-58-6	$C_{17}H_{19}N_3O_3S$	345.42	82.28	2.23	--	2.940
Platelet aggregation inhibitors							
Acetylsalicylic acid	50-78-2	$C_9H_8O_4$	180.16	4600	1.19	3.49	0.784
Salicylic acid	69-72-7	$C_7H_6O_3$	138.12	2240	2.26	2.97	1.573
Non-steroidal Anti-inflammatory drugs							
Diclofenac sodium salt	15307-79-6	$C_{14}H_{10}Cl_2NNaO_2$	318.13	2425	4.51	4.15	2.607
Ibuprofen sodium salt	31121-93-4	$C_{13}H_{17}O_2Na$	228.26	100000	3.97	4.91	2.352
Naproxen	22204-53-1	$C_{14}H_{14}O_3$	230.26	15.90	3.18	4.15	1.971
PCPs							
Biocide							
Triclosan	3380-34-5	$C_{12}H_7Cl_3O_2$	289.54	10.00	4.76	--	4.760
Preservatives							
Ethylparaben	120-47-8	$C_9H_{10}O_3$	166.17	885.0	2.47	8.34	2.393
Methylparaben	99-76-3	$C_8H_8O_3$	152.15	2500	2.00	--	2.111
Propylparaben	94-13-3	$C_{10}H_{12}O_3$	180.20	500.0	3.04	7.91	2.708

[+] The safety data sheet information was obtained from Sigma-Aldrich.
[++]Solubility in water at 25ºC except for triclosan (20ºC) and sulfamethoxazole (37ºC).
[+++]Data obtained from the SRC PhysProp Database (2014) or from the Sigma-Aldrich product information, or estimated using the US EPA Estimation Programs Interface Suite™ (2009).
[†]Logarithm of the octanol/water partition coefficient.
[††]The negative logarithm of the acid dissociation constant (K_a).
[†††]Logarithm of the soil/water partition coefficient.
-- Not Available.
[a] Solubility in water. For initial high concentrations, these compounds were dissolved in ethanol and subsequently diluted.

Table 2. PPCPs concentrations tested in ecotoxicity tests

PPCP	Concentration for Individual tests		Initial concentration for each PPCP in the mixture* assay (mg L^{-1}) x 10^{-3}
	Around environmental concentrations (mg L^{-1}) x 10^{-3}	Above EC_0/Around EC_{50} (mg L^{-1})	
PhACs			
Analgesic/antipyretic			
Acetaminophen	25.0±0.3; 50.0±0.5; 100±1; 200±2	234±1; 468±2; 936±4; 1872±7	200±1
Antibiotics			
Amoxicillin trihydrate	0.53±0.03; 1.07±0.06; 2.1±0.1;4.3±0.2	126±1; 252±1; 505±2; 1010±4	4.8±0.3
Cefaclor	0.45±0.02; 0.90±0.05; 1.8±0.1; 3.6±0.2	117±1; 233±1; 467±2; 934±3	4.0±0.2
Ciprofloxacin HCl	2.3±0.1; 4.5±0.2; 9.0±0.4; 18.1±0.8	94.7±0.5; 189±1; 379±2; 758±3	17±1
Clarithromycin	0.56±0.03; 1.13±0.06; 2.3±0.1; 4.5±0.2	2.5±0.1; 5.0±0.2; 9.9±0.5; 19.8±0.2	5.0±0.3
Erythromycin	1.05±0.04; 2.11±0.09; 4.2±0.2; 8.4±0.3	225±1; 450±2; 900±3; 1800±4	8.8±0.4
Norfloxacin	0.45±0.03; 0.90±0.06; 1.8±0.1; 3.6±0.2	3.9±0.1; 7.9±0.2; 15.8±0.5; 32±1	3.2±0.2
Sulfamethoxazole	0.84±0.03; 1.69±0.06; 3.4±0.1; 6.8±0.2	17.8±0.1; 35.7±0.2; 71.3±0.4; 143±1	7.5±0.3
Blood lipid regulators			
Clofibrate	2.7±0.1; 5.3±0.2; 10.6±0.4; 21.3±0.8	108±1; 216±1; 432±2; 864±3	20±1
Clofibric acid	0.56±0.03; 1.13±0.06; 2.3±0.1; 4.5±0.2	13.5±0.1; 27.0±0.3; 54.0±0.5; 108±1	5.0±0.3
H_2 Blocker			
Omeprazole	0.57±0.02; 1.15±0.05; 2.3±0.1; 4.6±0.2	0.86±0.01; 1.72±0.01; 3.44±0.03; 6.9±0.1	4.8±0.2
Non-steroidal Anti-Inflammatory drugs			
Diclofenac sodium salt	36.4±0.5; 73±1; 146±2; 291±4	5.19±0.04; 10.4±0.1; 20.8±0.2; 41.5±0.3	285±3
Ibuprofen sodium salt	126±1; 251±3; 502±5; 1003±10	30.4±0.2; 60.8±0.3; 122±1; 243±1	984±10
Naproxen	1.02±0.01; 2.05±0.02; 4.13±0.05; 8.2±0.1	0.90±0.01; 1.80±0.01; 3.60±0.03; 7.20±0.05	10.1±0.1
Platelet aggregation inhibitors			
Acetylsalicylic acid	1.08±0.01; 2.16±0.03; 4.3±0.1; 8.6±0.1	16.9±0.1; 33.8±0.2; 67.5±0.3; 135.0±0.5	8.6±0.1
Salicylic acid	56.2±0.4; 113±0.7; 225±1; 450±2	35.6±0.2; 71.3±0.3; 142±1; 285±1	455±3
PCPs			
Biocide			
Triclosan	0.39±0.01; 0.79±0.02; 1.58±0.05; 3.2±0.1	0.28±0.01; 0.56±0.01; 1.13±0.02; 2.25±0.05	3.5±0.1
Preservatives			
Ethylparaben	0.65±0.02; 1.31±0.05; 2.6±0.1; 5.2±0.2	1.86±0.06; 3.7±0.1; 7.4±0.2; 14.9±0.5	5.8±0.2
Methylparaben	2.8±0.1; 5.6±0.2; 11.3±0.5; 23.5±0.9	1.33±0.01; 2.66±0.02; 5.31±0.03; 10.62±0.06	25±1
Propylparaben	0.56±0.02; 1.13±0.05; 2.3±0.1; 4.5±0.2	0.61±0.01; 1.23±0.01; 2.45±0.02; 4.91±0.05	5.0±0.2

* These are the initial concentrations of each PPCP in a one liter of mixture solution. According to the assay procedure the dilutions are 100, 50, 25, 12 and 6% of these initial concentrations.

The time-dependent dose-response was analyzed by taking continuously readings of the light intensity of the traditional Microtox® assay over an extended period of time: six readings were taken from 0 to 445 minutes, taking into account that these readings needed to achieve the minimum light level established in the methodology. The software provided by the manufacturer of the luminometer (MicrotoxOmni^{TM}) (Azur environmental 1999) calculated the effect of each light level, estimated the EC_{50} or provided the highest effect depending on the range of concentrations analyzed, and performed the statistical calculation. Moreover, this software highlighted the presence of stimulatory (hormetic) effects. All of the bioluminescence data were recorded to obtain a thorough statistical analysis.

2.4. Statistical data analysis for the single PPCPs tests

The inhibition or stimulation of bioluminescence was calculated based on the measure of the bioluminescence at different concentrations in function of the corresponding controls (as Microtox® procedure indicates) and was reported as a

mean percentage effect with their standard deviations (SD) (see section **A1** and **A2** in **Appendix A** for statistics equations) and plotted against the nominal concentrations of PPCP.

The dose-response data of the single PPCP solutions were fitted with three non-linear functions for two conditions: (i) using the results at concentrations around EC_{50}, to calculate the EC_{50} and to determine the best function for these data, and (ii) using all data (results around EC_{50} plus results for environmental concentrations) to calculate the ZEP, $EC_{5,}$ EC_{50}, and to determine the best function in this case and compare with (i).
The three non-linear functions considered were: a sigmoidal dose-response or three parameter logistic model (**Eq. 1**); a sigmoidal dose-response variable slope or four parameter logistic model (**Eq. 2**); and an asymmetrical or five parameter logistic model (**Eq. 3** and **Eq. 4**).

$$Y = Y_B + \frac{Y_T - Y_B}{\left[1+10^{(log\ EC_{50}-X)}\right]} \qquad \textbf{Eq. 1}$$

$$Y = Y_B + \frac{Y_T - Y_B}{\left[1+10^{(log\ EC_{50}-X)\cdot m}\right]} \qquad \textbf{Eq. 2}$$

$$Y = Y_B + \frac{Y_T - Y_B}{\left[1+10^{(log\ X_b-X)\cdot m}\right]^S} \qquad \textbf{Eq. 3}$$

Here, log X_b is given by Eq. 4:

$$log\ X_b = log\ EC_{50} + \frac{1}{m}\cdot log\ \left(2^{\frac{1}{S}} - 1\right) \qquad \textbf{Eq. 4}$$

In these equations, **Y** is the effect on *Vibrio fischeri* (%), **X** is the logarithm of the molar concentration of the PPCPs that induces the Y effect, $\mathbf{Y_B}$ and $\mathbf{Y_T}$ are the plateaus at the left and right ends of the dose-response curve in the same units as Y, and **m** describes the slope of the curve: if m is positive, the curve increases as X increases, and if m is negative, the curve decreases as X increases. A standard sigmoidal dose-response curve (**Eq. 1**) has a slope of m=1. When the slope m<1.0, the curve is shallower. When the slope is >1.0, the curve is steeper. Slope has no units. Finally, **S** is the unitless symmetry parameter.

The goodness of fit was described by the sum of squares (SS) (**Eq. A2**, **Appendix A**) and the correlation coefficient (R) (**Eq. A3**, **Appendix A**). Higher values of R and lower values of SS indicate a better fit of the data. The 95% confidence intervals were calculated and plotted with the best non-linear function fit for each compound. The least-squares nonlinear regression assumes that the distribution of residuals follows a Gaussian distribution. This assumption was tested by running a normality test on the residuals (NTR). The D'Agostino-Pearson (omnibus K2), Kolmogorov-Smirnov distance and Shapiro-Wilk normality tests were all applied (more information for normality test procedure is shown in section **A4**, **Appendix A**).

Mean results at 5 and 15 minutes and for the different range of concentrations considered were compared with a two-way analysis of variance (Two-way ANOVA, α=0.05) to test for differences between times and among concentrations. This analysis was done to those data that passed the normality test. Means were considered significantly different if P<0.05, sum of square (SS), degrees of freedom (DF), mean of square (MS), F-ratio and P-value were reported (more information for two-way ANOVA procedure is shown in section **A5**, **Appendix A**). All statistics were performed using the GraphPad Prism 6 software (GraphPad Software Inc. San Diego, California, USA).

2.5. Mixture of PPCPs analysis

The results of the assay performed with the mixture of PPCPs were plotted as the dose-response behaviors (mean effect at each concentration with their SD, see section **A1** and **A2** in **Appendix A** for statistics

equations) for the different exposure time periods. The results observed at low doses and the behavior of ZEP over time were analyzed.

3. Results and discussion

3.1. Dose-response behavior of single PPCPs around EC_{50}

The dose-response behavior of *Vibrio fischeri* bacteria exposed to 20 PPCPs were analyzed at concentrations around the EC_{50} using bioluminescence assays (Microtox®).

Dose-response curves for the first interaction of a chemical with a biochemical target molecule are usually monotonic, i.e., they increase or decrease over the entire range of doses (Conolly and Lutz 2004). In this case, in the range of concentrations under study (around EC_{50}) the inhibition of luminescence increases with the concentration increase, at both times 5 and 15 minutes. These results were plotted in **Figure B1**, **Appendix B**. All compounds present the trend of α curve that is described for Christofi et al. (2002) as the general dose-response observed in the presence of a toxic substance, indicating inhibition above a threshold concentration. According to Microtox® acute toxicity test user guide (Azur environmental, 1999) different chemicals affect living organisms at different rates, reflecting differences in mechanism of action. For some classes of chemicals, the effect on light output is complete in 5 minutes. For other classes of chemicals, the light output is still decreasing rapidly at 5 minutes. In these cases, 15 minutes data may be more reliable. Therefore, 5 and 15 minutes have been considered to establish the effect on *Vibrio fischeri*'s light output in these two times.
Despite that all PPCPs under study are organic molecules, they have different chemical structures, that could generate different mechanism of action on bacteria in function of range of concentration under study or time.

The concentration range around the EC_{50} is very different among the compounds, therefore, preliminary dose-response assays were made to find the range of concentrations where the EC_{50} were located. Range of concentration tested for each PPCP was very different among them in function of the toxicity of each compound. Acetaminophen, amoxicillin, cefaclor, ciprofloxacin HCl and clofibrate were tested between 90 and 2000 mg L^{-1}, the assays of sulphametoxazole, clofibric acid ibuprofen sodium salt, ASA and salicylic acid were among 10 and 150 mg L^{-1} and clarithromycin, norfloxacin, omeprazole, diclofenac, naproxen, triclosan, ethylparaben, methylparaben and propylparaben between 0 and 50 mg L^{-1} (See **Table 2**). These concentration ranges give a preliminary idea of the level of toxicity of each compound when they are compared among them.

3.1.1. Non-linear fit of the dose-response data around EC_{50}

Results of *Vibrio fischeri* dose-response around the EC_{50} allowed evaluate models to describe mathematically the behavior of each PPCP and can describe the effect at different concentrations without experimental procedure. Three models were fitted and the best fit model was chosen for each PPCP. **Table 3** shows the statistical parameters (R, SS, NTR) for the best fit model for each PPCP and the estimated EC_{50}. **Figure B1** (**Appendix B**) present the plots for the Best-fit dose-response curves of the Microtox® ecotoxicity tests of each PPCPs with their 95% confidence intervals at 5 and 15 minutes.

The R square and the SS were used to verify the goodness of fit. The best fit model was chosen for each time according to highest R

square the lowest NTR. Only for two compounds the best fit model did not coincide among 5 and 15 minutes (omeprazole and ASA). Four parameter is the model that best fits in most cases for both times 5 and 15 minutes (55% of compounds to 5 minutes and 65% to 15 minutes), then, asymmetric model is the best fits of 35% of PPCPs at 5 minutes and 30% at 15 minutes. Three parameter only fits well in two compounds at 5 minutes (10%) and one compound at 15 minutes (5%). R square was higher or equal than 0.99 of 50% of compounds at 5 minutes and for 40% of PPCPs at 15 minutes. R square was between 0.94 and 0.99 for 45% of PPCPs at 5 and 15 minutes data, and it was below 0.94 to one PPCP at 5 minutes (5%) and for three compounds at 15 minutes (15%).

All data passed the normality test (See **Table 3**). Therefore, a two-way ANOVA was done in order to establish the significance of two important factors: concentration and time.

Table B.1 in **Appendix B** shows the main results of this statistical analysis (SS, DF, F-ratio and P-value). Concentration factor resulted to be significant for all compounds in the range of concentration tested, therefore, an increase or decrease in concentration generated a significant change in the effect on *Vibrio fischeri*. Time factor was significant to nine compounds (cefaclor, clarithromycin, clofibrate, omeprazole, ASA, salicylic acid, diclofenac sodium salt, ethylparaben and propylparaben) for the other eleven compounds there was not significance of difference between 5 and 15 minutes.

EC_{50}'s were estimated with each best fit model equation at 5 and 15 minutes for eighteen compounds, amoxicillin and naproxen highest effect was lower than 50% in the range of concentration tested, thus, their EC_{50} were not estimated.

The estimated EC_{50} of each PPCP was compared with its corresponding value presented in a recent study (Ortiz et al. 2014) in which the acute ecotoxicity endpoint was determined using a linear regression model, as indicated in the user's manual for the Microtox® Model 500 analyzer. After the confidence levels were taken into account, the EC_{50} of most compounds were on the same order as those obtained in the aforementioned previous studies.

EC_{50} of acetaminophen, cefaclor, clofibrate, ethylparaben, ibuprofen sodium salt and propylparaben were found outside the confidence limits of the previous cited study maybe due to the inclusion of new data or deviations of the new models under study but they were located in the same level of ecotoxicity by the classification used in Ortiz et al. (2014) with the exception of clofibrate and clofibric acid. This fact highlights the importance of adjusting the dose-response data for find reliable results and the possible variations that can be observed using different models. The level of toxicity of each PPCP was: acetaminophen, ASA, cefaclor, ciprofloxacin HCl and salicylic acid are "non toxic", clarithromycin, clofibric acid, clofibrate, diclofenac, ibuprofen sodium salt, norfloxacin and sulphametoxazole are "harmful to aquatic organisms", ethylparaben, methylparaben, omeprazole and propylparaben are "toxic" and triclosan were found "highly toxic" for aquatic organisms.

3.2. Dose-response behavior of single PPCPs at environmental concentrations

Low-dose response on bacteria was studied at environmental concentrations. The mean of the acute effects and their standard deviations are shown in **Figure B2** (**Appendix B**) for 5 and 15 minutes of testing time.

Table 3. Parameters of the best non-linear fit dose-response curves from concentration range around the EC_{50}

Compound*	Model	R^2		SS**		NTR***		EC_{50}^{+} (mg L^{-1})	
		5 min	15 min	5 min	15 min	5 min	15 min	5 min	15 min
Antibiotics									
Amoxicillin	Four parameter	0.8557	0.8703	53.42	84.83	b,c	c	na	na
Cefaclor	Four parameter	0.9461	0.9220	132.9	153.7	a,b,c	a,b,c	344.93	323.64
Ciprofloxacin HCl	Asymmetric	0.9818	0.9906	136.5	82.41	a,b,c	a,b,c	257.84	234.96
Clarithromycin	Four parameter	0.9941	0.9864	127.6	272.4	a,b,c	a,b,c	12.76	12.03
Erythromycin	Four parameter	0.9478	0.9593	379.7	329.3	a,b,c	a,b,c	1683.9	1185
Norfloxacin	Four parameter	0.9808	0.9760	408.4	502.5	a,b,c	a,b,c	23.68	23.55
Sulphametoxazole	Asymmetric	0.9976	0.9728	1337	212.2	a,b,c	a,b,c	45.21	49.49
Blood lipid regulators									
Clofibrate	Four parameter	0.9768	0.9631	100.1	140.0	a,b,c	a,b,c	73.59	128.44
Clofibric acid	Four parameter	0.9866	0.9910	167.9	117.9	a,b,c	a,b,c	87.51	91.01
H_2Blocker									
Omeprazole	Three/Four parameter	0.9974	0.9956	17.3	35.06	a,b,c	a	2.90	3.71
NSAIDs/ Analgesics									
Acetaminophen	Asymmetric	0.9972	0.9969	24.45	29.00	a,b,c	a,b,c	248.50	301.41
ASA	Asymmetric/Four parameter	0.9950	0.9850	24.65	71.17	a,b,c	a,b,c	134.08	138.11
Diclofenac Na	Four parameter	0.9961	0.9954	54.35	63.35	a,b,c	a,b,c	21.29	25.36
Ibuprofen Na	Four parameter	0.9977	0.9959	22.05	39.69	a,b,c	a,b,c	62.20	50.79
Naproxen	Three parameter	0.9555	0.9189	43.94	69.34	a,b,c	a,b,c	na	na
Salicylic Acid	Four parameter	0.9510	0.9412	527.3	625.1	c	a,c	138.81	125.47
PCPs									
Ethylparaben	Asymmetric	0.9931	0.9754	46.81	181.9	a,b,c	a,c	3.37	4.13
Methylparaben	Four parameter	0.9800	0.9767	129.2	190.5	a,b,c	a,b,c	9.25	5.76
Propylparaben	Asymmetric	0.9968	0.9930	22.53	45.34	a,b,c	a,b,c	1.28	1.36
Triclosan	Asymmetric	0.9987	0.9987	17.85	22.77	a,b,c	b,c	0.38	0.42

*Number of data for all compounds (n) = 17.
**Sum of Squares.
***Normality test of residuals passed.
[a]D'Agostino & Pearson omnibus K2.
[b]Shapiro-Wilk.
[c]Kolmogorov-Smirnov distance.
[+] Half maximal effective concentration.
na: Data not available.

As it was done at concentrations around EC_{50}'s a two-way ANOVA was evaluated for environmental concentrations. In this case, five compounds did not show significant difference among concentrations and thirteen PPCPs had not significant differences between 5 and 15 minutes. Therefore, for the majority of compounds a change in the concentration generates a significant change in the bioluminescence. Time is less significant than concentration probably due to the short difference between 5 and 15 minutes. Calabrese and Baldwin (2001) and Stebbing (2000) reported and explained the influence of these two factors at low-dose response when stimulation has been presented.

As can be seen in **Figure B2** (**Appendix B**) eleven of the 20 (55%) compounds tested (acetaminophen, ASA, ciprofloxacin HCl, clofibric acid, diclofenac sodium salt, ibuprofen sodium salt, methylparaben, naproxen, norfloxacin, salicylic acid and sulphametoxazole) showed at least two points (concentration mean) with a clear stimulatory effect (bacteria showed more

luminescence when they are exposed at certain concentrations of PPCP than control) taking into account the standard deviation of data to ensure that this affirmation are statistically representative. The other 45% of the compounds showed an effect around zero, ranging between stimulatory or inhibitory when the standard deviation of each point was taken into account. Therefore, there was no clear trend in the behavior of these 9 compounds over a range of concentrations, and the weak or null stimulatory effects could be considered to be noise of the system (Calabrese 2005).

In the range of concentrations studied in this research, the PPCPs that present the highest values of stimulatory effects were the analgesic/antipyretic compounds, the NSAIDs and the platelet aggregation inhibitors. The compound with the maximum effect in this regard was the diclofenac sodium salt. **Table 4** contains the maximum stimulatory effects (MSE) and the maximum stimulatory effect concentrations (MSEC) for each PPCP at 5 and 15 minutes of exposure. These values could not be compared with other studies because the compounds have not been previously evaluated using this methodology, in this biological target, or in these ranges of concentrations. Nonetheless, these findings may serve as the basis for further investigations.

3.3. Analysis of the stimulation/inhibition in dose-response curves of PPCPs on bacteria

A dose-response curve that is characterized by stimulation at a low dose/concentration (environmental concentrations in this study) and inhibition at a high dose/concentration (concentrations around EC_{50}) is known as hormesis. In the literature, four types of concentration-response curves have been identified. The α curve is the general dose-response observed in the presence of a toxic substance, indicating inhibition above a threshold concentration. The β, γ, and δ, curves have been described previously by other authors and indicate some type of hormesis, but the most frequently observed is the β curve (an inverted U-shaped dose-response curve) (Christofi et al. 2002). According to Calabrese and Baldwin (2001), hormesis is characterized by a U-shaped or J-shaped dose response curve if the stimulation is calculated as a negative percentage of the effect, as it was calculated in this research. In the present study, the eleven compound that show stimulatory effects at low-dose in some concentrations (between 0 and -20%) presented a trend similar than a U-shaped or J-shaped curves or at least a section of them.

Other authors have found a similar behavior in bacteria using different compounds (Deng et al. 2012; Ma et al. 2012; Zhang et al. 2013a; 2013b), though these results were generally obtained over more extended periods of time, where this effect is much more obvious than over the short term periods (acute toxicity) evaluated in our study.

Though different authors have widely studied the hormesis phenomenon (Belz et al. 2008; Calabrese 1999, 2005, 2008a; Calabrese and Baldwin 2000, 2001, 2003; Calabrese and Blain 2005; Kefford et al. 2008; Mattson 2008; Stebbing 1998, 2000; Zou et al. 2013), acceptance that hormesis like U-shaped dose-response is widespread and real has been difficult to achieve (Calabrese and Baldwin 2003).

Table 4. Parameters of the best non-linear fit dose-response curves and the relevant environmental ecotoxicological points considering effects at environmental concentrations and around the EC_{50}*

Compound	Model	R^2		SS**		NTR***		EC_{50}^{+} (mg L^{-1})		EC_{5}^{++} (mg L^{-1})		ZEP^{+++}(mg L^{-1})		$MSE^{\dagger}$ (%)/MSEC (µg L^{-1})	
		5 min	15 min	5 min	15 min	5 min	15 min	5 min	15 min	5 min	15 min	5 min	15 min	5 min	15 min
Antibiotics															
Amoxicillin	Four parameter	0.8475	0.8743	104.9	148.2	a,b,c	c	na	na	361.14	277.12	23.59	29.02	-2.80/1.07	-2.44/1.07
Cefaclor	Four parameter	0.9735	0.9623	216.5	222.9	a,b,c	a,b,c	349.24	323.53	45.25	46.30	8.62	4.37	-5.10/0.45	-4.76/0.90
Ciprofloxacin HCl	Asymmetric	0.9914	0.9942	248.24	192.0	a,b,c	a,b,c	233.04	209.54	3.97	9.04	0.24	1.93	-6.32/2.26	-7.82/4.52
Clarithromycin	Four parameter	0.9842	0.9611	476.5	1242	a	a	12.39	11.56	1.18	2.59	na	na	-2.56/2.25	-4.58/4.5
Erythromycin	Four parameter	0.9640	0.9704	385	509.6	b,c	a,b,c	1681.4	1199.2	153.74	133.55	16.81	33.55	-4.92/4.28	-8.29/4.28
Norfloxacin	Four parameter	0.9607	0.9503	1254	1625	a,b,c	b	21.52	21.33	4.95	4.41	na	na	-8.01/0.45	-8.10/3.60
Sulphametoxazole	Asymmetric	0.9955	0.9855	133.7	402.3	c	a,b,c	57.92	44.17	9.32	7.30	5.90	3.46	-8.79/1.69	-3.58/0.84
Blood lipid regulators															
Clofibrate	Four parameter	0.9944	0.9905	160.2	216.6	a,b	a,b,c	72.06	107.13	9.66	7.00	1.53	1.08	-5.45/10.6	-4.42/21.2
Clofibric acid	Four parameter	0.9639	0.9592	675.6	772.7	a,b,c	a,b,c	82.23	85.45	13.24	18.70	na	na	-8.45/1.13	-11.30/1.13
H_2Blocker															
Omeprazole	Four parameter	0.9970	0.9968	64.26	98.51	a,b,c	a,b,c	3.07	1.95	0.17	0.10	0.01	na	-2.65/4.50	-3.84/4.50
NSAIDs/ Analgesics															
Acetaminophen	Four parameter	0.993	0.9958	370.6	187.3	a,b,c	a,b,c	272.84	349.94	34.19	27.39	20.07	17.31	-16.67/200	-10.65/200
ASA	Four parameter	0.9702	0.9601	382.8	625.2	a,b,c	a,b,c	125.82	114.28	19.30	14.99	11.37	9.03	-14.11/4.30	-17.41/4.30
Diclofenac Na	Four parameter	0.9869	0.9874	471.3	579.4	a,b,c	c	22.11	22.80	4.25	3.26	2.97	2.34	-17.68/291.5	-20.22/291.5
Ibuprofen Na	Four parameter	0.9957	0.9951	235.7	269.6	c	c	41.61	43.53	2.51	2.74	1.38	1.58	-12.93/0.13	-14.34/0.13
Naproxen	Four parameter	0.8693	0.8507	286.7	344.6	a,c	a,b,c	na	na	1.52	1.39	0.58	0.58	-8.12/1.02	-9.80/1.02
Salicylic Acid	Three parameter	0.9645	0.9676	1231	1309	a,b,c	a,b,c	170.58	105.5	10.36	9.56	3.80	4.79	-11.21/450.0	-15.40/450.0
PCPs															
Ethylparaben	Asymmetric	0.9933	0.9897	219.4	341.3	a,b,c	b,c	3.08	4.05	0.22	0.35	na	0.32	-6.03/2.61	-6.40/2.61
Methylparaben	Four parameter	0.9871	0.9862	229.3	242.0	a,b,c	b,c	9.98	5.86	0.50	1.02	0.15	0.44	-5.97/11.25	-4.95/22.50
Propylparaben	Asymmetric	0.9930	0.9906	201.1	240.0	a,b,c	a,b,c	1.24	1.28	0.22	0.22	0.13	0.13	-8.41/0.56	-8.42/1.13
Triclosan	Three parameter	0.9976	0.9940	124.1	223.2	a,b,c	a,b	0.38	0.42	0.04	0.04	0.0014	0.0055	-4.86/3.15	-9.31/3.15

*Number of data for all compounds: 33.
**Sum of Squares.
***Normality test of residuals passed.
[a]Kolmogorov-Smirnov distance.
[b]D'Agostino & Pearson omnibus K2.
[c]Shapiro-Wilk.
[+] Half maximal effective concentration.
[++]Effective Concentration of PPCP that gives a bioluminescence inhibition of 5%.
[+++]Zero effect concentration point.
[†]MSE: Maximum stimulatory effect. MSEC: Maximum stimulatory effect concentration.
na: Data not available.

At low levels of disruption or toxicity, many biological systems display an overcompensated response (other common terms in the literature are "overshoot" and "rebound"), which results in the apparent low-dose stimulation component of the response curve. At higher doses with greater initial toxicity, the system often displays a more limited capacity for a compensatory response, which is usually insufficient to return to the control values (Calabrese and Baldwin 2001). Additionally, Calabrese (2005) indicated that such compensatory (i.e., overcompensatory) responses are not usually achieved at the higher doses, leading to the hormetic biphasic dose response. The nature of the overcompensation response appears to result from biological compensatory processes that allocate resources slightly in excess of those that ensure a return to homeostasis. This "extra" allocation of resources (i.e., an adaptive response) leads to the hormetic stimulation. Calabrese (1999) did experimental assays that supported and corroborated the hypothesis that growth hormesis represents an overcompensation to a disruption in homeostasis.

According to Calabrese (2005), the hormetic effects start to occur immediately below the NO(A)EL. In the design of the present study, the low range of concentrations for each PPCP evaluated did not necessarily coincide with the concentrations immediately below the NO(A)EL because the principal goal was to study the effect of toxicants on *Vibrio fischeri* in the range of concentrations present in WWTPs and in some aquatic environments. Therefore, it is possible that the stimulatory effect was more evident for some compounds than other in function of the proximity of the NO(A)EL with the range of concentration tested. Thus, it could not be ruled out that the compounds that showed some degree of stimulation at concentrations far below the NO(A)EL could present major stimulatory effects at concentrations near this point. For example, Zou et al. (2013) reported that sulfametoxazole, which was also studied in the present research, inhibited *Vibrio fischeri* by -112.804 % at a concentration of $9.094x10^{-6}$ M. However, we did not test this concentration in the present work for the reasons given above.

In any case, there are scarce data for the hormesis effect of PPCPs specifically obtained from the *Vibrio fischeri* bioassay. According to Christofi et al. (2002), a reason why hormesis is not often reported in the *Vibrio fischeri* assay and other microbial-based bioassays is probably due to the following: (i) lethal and inhibitory concentrations of toxicants are more frequently studied; (ii) a hormesis result may be dismissed as experimental error or a distortions of the results, and data that show an elevated response are thus ignored in toxicity calculations; and (iii) the experimental design affects the observation of hormesis when the range of concentrations of the toxins tested is too wide, resulting in no observation of the hormetic response.

Antibiotics have been the most studied PhACs. Zou et al. (2013) determined the hormetic effects of six antibiotics and their mixtures, while Deng et al. (2012) proposed a novel parametric model with a mechanistic basis and two model-based parameters for hormesis, successfully applying these models to the hormetic dose-response observed in the chronic ecotoxicity of sulfonamides on *Photobacterium phosphoreum*.

Calabrese and Baldwin (2001) commented that biphasic responses are observed frequently with antibiotics, antivirals, NSAIDs and numerous other agents. These background data and the results of the present study lead us to affirm that many PPCPs can generate the hormetic response. Despite this, the effect is not observed in some cases; therefore, the presence or

absence of the hormetic effect depends on multiple factors simultaneously, such as the chemical structure of the toxicant, the experimental design of each assay, and the biological system used. Calabrese and Baldwin (2003) argue that a continued search and demand for a single molecular explanation to account for hormesis is a belief in an incorrect paradigm, while Deng et al. (2012) consider the mechanistic understanding of hormesis to remain extremely limited. Many believe that a single mechanism accounting for the hormetic dose-response is not required.

3.3.1. Non-linear fit including the dose-response data around EC_{50} and at environmental concentrations

Three non-linear regression models were used to fit the observed concentration-effect response including data around EC_{50} and at environmental concentrations for each PPCP. **Table 4** shows the main statistical parameters used in the best-fit dose-response model (R^2, SS of residuals and NTR passed) and some relevant environmental ecotoxicological points such as the EC_{50}, EC_5 and ZEP, which were calculated from the equation of the best fit regression model of each PPCP. ZEP (EC_0) (i.e., the dose where the response crosses the control value used by Calabrese 2005; Deng et al. 2012 and Zou et al. 2013), was calculated in place of the NOEC data and EC_5 was calculated in place of the LOEC data, thus, according to Warne and van Dam (2008), the use of the NOEC and LOEC data in ecotoxicology and in particular, the regulatory aspects of ecotoxicology, has been severely criticized through a series of articles in the 1990s. There are now several alternatives to the NOEC and LOEC data, including low percent effect point estimates.

Fourteen compounds (70%) had the best fit with a four parameter regression model, four (20%) fit best with an asymmetrical model and two compounds (10%) fit best with a three parameter model. The four parameter regression model was the best fit model in most cases, and van der Grinten et al. (2010) used this model in their research of antibiotics, finding similar results. Although in some cases, the asymmetrical and the three parameter models showed a better statistical fit than the four parameter model, the differences between the models with better fit and the four parameter regression model were small. Therefore, the four parameter model could be used as a standard model in these types of compounds.

Nine of the 20 tested compounds (45%) that were fitted to models showed a very good adjustment (R square $\geq$ 0.99) at 5 minutes and 35% of them at 15 minutes and they showed a low SS of residuals. These compounds also passed the normality tests for residuals. Therefore, these compounds had an excellent fit according to statistical parameters. Nine compounds (45%) at 5 minutes showed a worse fit (0.94 $\leq$ R square $<$ 0.99, and therefore a higher SS of residuals) and 55% of them at 15 minutes. Finally, naproxen and amoxicillin had the worst fit (R square $\leq$0.9) because these two compounds presented the maximum effect below 30% and the models require the top of the curve (Y_T) near of 100% of effect.

The dose-response fitted including the low-dose behavior (which presented stimulation in some cases) generated slight changes in the statistical parameters when they were compare with the fitted models and the statistical parameters of only data around the EC_{50}. If stimulation effects would have been higher than those obtained in this research a specific model that includes U-shaped or J-shaped curves at low-dose should have been used.

For the hormetic effect, some authors have proposed different models that take into

account both the hormesis effect at low concentrations and the usual dose-response behavior when the response is higher than zero. According to Stebbing (1998), other statistical non-linear regression models have been developed based on the logistic equation used to describe data exhibiting hormesis. Deng et al. (2012) have proposed a novel bilogistic model, which was obtained via the algebraic summation of two logistic functions representing the stimulation and the inhibition. This model was successfully applied to the hormetic dose-response behaviors observed in the chronic ecotoxicity of sulfonamide exposure in *Photobacterium phosphoreum*. Zou et al. (2013) also applied a logistic-model to data obtained for antibiotics that indicated no hormetic effects, whereas the data indicating a relationship between the concentration and hormetic effects were fitted with Brain-Cousens Model. Cedergreen et al. (2005) developed a new empirical model that can describe the hormetic responses in all types of dose-response data irrespective of their slopes. Moreover, Beckon et al. (2008) suggested the use of a simple, general, and biologically reasonable modeling approach. This resulted in a family of mathematical models that combine log-logistic functions to give at least one model for the up slope and one model for the down slope, which could then be used to model the biphasic relationships more closely over wider ranges of exposures.

Despite the existence of complex models that account for the hormetic effect, the models used in this study showed good functional correlation with the experimental data as it has been described (see **Table 4**). Based on one of the three simple models described in the methodology, the ecotoxicological parameters (EC_{50}, EC_5 and ZEP) were calculated (see **Table 4**). The estimated EC_{50} of each PPCP was compared with its corresponding value presented in a recent study (Ortiz et al. 2014) in which the acute ecotoxicity endpoint was determined using a linear regression model, as indicated in the user's manual for the Microtox® Model 500 analyzer. After the confidence levels were taken into account, these estimated values were on the same order as those obtained in the aforementioned previous studies, which suggests that the incorporation of new data at low concentrations in the models under study did not significantly influence the estimation of the EC_{50} values.

Increasingly, other ecotoxicological points are being estimated or calculated instead of the LOEC and NOEC. According to Warne and van Dam (2008), there are several alternatives to the NOEC and LOEC data, including low X-percent effect point estimates (e.g., 5, 10 and even 20% levels) that are termed EC_x; these data were used in the present study. Warne and van Dam (2008) have explained that the main problems with the NOEC and LOEC data fall into the next three aspects: (i) the misleading nature of their names; (ii) the inappropriateness of the method by which they were calculated; and (iii) the validity of the statistical methods used. In fact, the EC_x approach is generally more sensitive to detecting low effects because the NOEC data were dependent on actual selected test concentrations and were computed by separately comparing each treatment to the control (Cleuvers 2004). According to Backhaus et al. (2011), NOECs are the result of a statistical comparison of the data from treated samples with those from untreated controls. The highest tested concentration at which this comparison does not result in a statistically significant difference is denoted the NOEC. Such a failure to detect a statistically significant effect does not prove that there is no effect in reality.

Therefore, instead of the LOEC and NOEC, the EC_5 and ZEP (EC_0) were calculated in this study as the ecotoxicological points for

Vibrio fischeri. The EC_x approach has been included in other research studies (Calabrese and Blain 2009; Cedergreen et al. 2005; Cleuvers 2004; Ge et al. 2011; Silva et al. 2002; Qin et al. 2010), and with the acceptance of the hormetic effect in the dose-response curves of many compounds. A hormetic dose-response relationship has well-defined quantitative features, including the magnitude and the width of the stimulatory zone and, in certain features, its equivalent called the ZEP (Deng et al. 2012). ZEP is a parameter that can be used to well fit the hormesis dose-response curve.

Moreover, ZEP can be used as a safety level of concentration of a compound in aquatic environments, at that point there is no effects of stimulation and inhibition. In this research ZEP could be calculated in all cases where the curves of the model cross the line of zero effect due to the stimulation effect. Hence, the importance of including the stimulatory effect (if they exist) in the dose-response studies.
The ZEP values at exposure times of 5 and 15 minutes for 16 compounds were estimated from the models studied (**Table 4**). When these values were compared, it was observed that seven PPCPs presented ZEP values that were higher at 15 minutes than at 5 minutes, six compounds presented ZEP at 5 minutes higher than ZEP at 15 minutes, ZEP of two compounds were equal and five of them could not be compared because some ZEP could not be calculated. Therefore, for short time intervals in acute ecotoxicity studies, a trend about the evolution of ZEP over the time of exposure cannot be established.

The parameters obtained from the models studied in this research are not only important and valuable information for modeling the effects of PPCPs on *Vibrio fischeri*, but they also complement the existing ecotoxicological data. This allows for a deeper understanding of how these compounds could be affect bacteria in the aquatic environments at different concentrations.

There is a need for a widely accessible interpretation of the dose–response (concentration–effect) curve that includes stimulatory and inhibitory effects as part of a continuum of exposure to a full range of concentrations, and that such an explanation should be readily communicable, allowing stimulation and inhibition to co-exist in the mind of the layperson, within the same paradigm (Stebbing, 2000).

3.3.2. Correlation of PPCPs stimulation/inhibition dose-responses with their chemical classes using ECOSAR™.

In the specific case of *Vibrio fischeri* bacteria and on the basis of the methods of molecular docking and quantitative structure-activity relationships (QSARs), Deng et al. (2012) proposed a mechanistic hypothesis for hormesis that introduces the concept of quorum sensing to ecotoxicological studies for the first time in order to explain the mechanism at the receptor level. Quorum sensing bacteria produce and release chemical signal molecules called autoinducers (AI) that increase in concentration as a function of cell density. The detection of a minimal-threshold stimulatory concentration of an AI leads to an alteration in the gene expression of luminescent proteins. Zou et al. (2013) have affirmed that according to this mechanistic hypothesis, the transcriptional level could be excited by specific target binding at the low dose exposure of the antibiotics they studied, which would further enhance the luciferase activity. Similarly, this hypothesis may explain the behavior of the PPCPs involved in the present investigation.

There is another possible explanation that the induction in bioluminescence might be a

direct reflection of the hormetic/stimulating effect of an external factor on the metabolism of the organism. This explanation matches better with the finding that bioluminescence was stimulated in both natural and recombinant bioluminescent organisms by rather different substances (heavy metals and organic chemicals) (Fernández-Piñas et al. 2014).

The influence of the chemical structures of PPCPs can be analyzed by the mode of toxic action. Escher et al. (2005) indicated that there are three types of interactions between a pollutant and a biological target: these are nonspecific and specific interactions as well as chemical reactions. The most important nonspecific mode of action is the baseline toxicity, or narcosis. On the basis of these interactions, ECOSAR™ derives the ecotoxicity values for three general types of chemicals: neutral organics, which are nonionizable and nonreactive and act via simple nonpolar narcosis (this general narcosis is often referred to as baseline toxicity); organic chemicals with excess toxicity, including molecules that present more specific modes of toxicity based on the presence of reactive functional groups (which can be more toxic to one or more aquatic organisms than predicted by the baseline toxicity equations); and surfactant organic chemicals, which were not considered in this study (EPA 2009).
It is necessary to highlight that ECOSAR™ used the acute and chronic ecotoxicity data from standard tests performed on fish, daphnia and algae, to predict ecotoxicity in a general aquatic community.

Table 5 has been compiled based on the information in the ECOSAR™ manual and the dose-response behavioral results at environmental concentrations obtained in the present study. As shown in **Table 5**, all compounds that present narcosis as mode of toxic action (in the inhibition dose-response range of concentrations) showed stimulation at environmental concentrations. This allowed for the inference that there might be a relationship between the toxic mode of action of certain compounds at high concentrations and the stimulation phenomenon at low doses. Most compounds that present excess toxicity as a toxic mode of action did not show stimulation at low doses, though acetaminophen, ciprofloxacin HCl, norfloxacin, sulphametoxazole and methylparaben.

On the other hand, most compounds that did not show stimulation at environmental concentrations (in the range evaluated in this research) were the most toxic, they presented the lowest EC_{50} (clarithromycin, ethylparaben, naproxen, propylparaben, triclosan, omeprazole).

A larger amount of PPCPs and different ranges of concentrations should be studied to see if these correlations are maintained, and based on them QSAR's methodologies could be complemented.

3.4. An initial approach on dose-response behavior of PPCPs mixture at environmental concentrations.

Aquatic environmental pollution is a very complex issue. Hundreds of thousands of contaminants are present in varied forms and concentrations, and their interactions with the environment are determined by their physicochemical properties. In this sense, it is interesting to study the behavior of a complex mixture of some of these compounds that are present in aquatic environments at their actual environmental concentration levels. This is especially true when each compound has a different behavior, such as the hormetic effect, at low concentrations.

Table 5. QSAR data from the ECOSAR™ models used to correlate ecotoxicity at high PPCPs concentrations with stimulation at low PPCPs concentrations

Type of PPCP	Common name	Chemical class*	Type of toxicity*	Stimulation**
PhACs				
Analgesic/ antipyretic	Acetaminophen	Phenols, Amides, Phenol Amines, Neutral organic	Organic chemicals with excess toxicity	Yes
Antibiotics	Amoxicillin Trihydrate	Aliphatic Amines-acid, Phenols-acid, Benzyl Amines-acid, Amides –acid, Phenol Amines –acid	Organic chemicals with excess toxicity	No
	Cefaclor	Aliphatic Amines-acid, Vinyl/Allyl Halides-acid, Benzyl Amines-acid, Amides -acid	Organic chemicals with excess toxicity	No
	Ciprofloxacin HCl	Aliphatic Amines-acid, Vinyl/Allyl Ketones-acid, Vinyl/Allyl Amines-acid, Neutral organic	Organic chemicals with excess toxicity	Yes
	Clarithromycin	Aliphatic Amines, Esters, a-,b-Ketone alcohol	Organic chemicals with excess toxicity	No
	Erythromycin	Aliphatic Amines, Esters, a-,b-Ketone alcohol, Neutral organic	Organic chemicals with excess toxicity	No
	Norfloxacin	Aliphatic Amines-acid, Vinyl/Allyl Ketones-acid, Vinyl/Allyl Amines-acid , Neutral organic	Organic chemicals with excess toxicity	Yes
	Sulfamethoxazole	Anilines (Aromatic Amines), Amides, Neutral organic	Organic chemicals with excess toxicity	Yes
Blood lipid regulators	Clofibrate	Esters, Neutral organic	Organic chemicals with excess toxicity	No
	Clofibric acid	Neutral organics-acid	Narcosis	Yes
H_2 Blocker	Omeprazole	Imidazoles, Neutral organic	Organic chemicals with excess toxicity	No
Platelet aggregation inhibitors	Acetylsalicylic acid	Esters acid, Neutral organic	Narcosis	Yes
	Salicylic acid	Phenols acid, Neutral organic	Narcosis	Yes
Non-steroidal anti-inflammatory drugs	Diclofenac	Neutral organics, Acids	Narcosis	Yes
	Ibuprofen	Neutral organics	Narcosis	Yes
	Naproxen	Neutral organics	Narcosis	Yes
PCPs				
Biocide	Triclosan	Phenol, Neutral organic	Organic chemicals with excess toxicity	No
Preservatives	Ethylparaben	Esters, Phenol, Neutral organic	Organic chemicals with excess toxicity	No
	Methylparaben	Esters, Phenol, Neutral organic	Organic chemicals with excess toxicity	Yes
	Propylparaben	Esters, Phenol, Neutral organic	Organic chemicals with excess toxicity	No

*According to the ECOSAR™ software.
**In at least two points tested at environmental concentrations.

Escher et al. (2005) asserted that pharmaceuticals never occur alone in the environment but always in combination with other compounds, including not only other pharmaceuticals but also their own metabolites or other environmental pollutants such as industrial chemicals, pesticides, or personal care products. Possible mixture effects are therefore relevant.

The principles of hazard assessment of chemical mixtures have recently been reviewed. Predicated on the knowledge that complete testing is not feasible, the strategies are designed to characterize the interactions between constituents as non-interactive, synergistic, and/or antagonistic so that these types of interactions can be applied generally to mixtures of similar composition (Teeguarden et al. 2000). According to Zou et al. (2013), only a small number of studies have investigated the hormetic features of mixtures, although the hormetic effects have been reported in some mixtures that include heavy metals, polychlorinated biphenyls (PCBs) and persistent organic pollutants.

Studying time-dependency is essential for understanding the biological process of hormetic effects (Zhang et al. 2013). This is especially true for mixtures of compounds that have the potential to affect the environment, even though these cases are less studied. The study of the behavior of micropollutants mixture in nature is very important, due it is the real situation that is presented in the different compartments of the environment. In the research presented in this paper, an assay was designed in order to study the dose-response behavior of a mixture of the 20 PPCPs. **Figure 1** shows the results for the dose-response behavior of the PPCPs mixture over time obtained from the short-term chronic ecotoxicity bioassays described in the methodology.

As shown in **Figure 1**, the mixture causes the highest hormetic effect at low concentrations and short exposure time periods (15 and 60 minutes). When the exposure time and the concentration were increased, the effects on *Vibrio fischeri* changed from stimulatory to inhibitory. As the time was further increased, the ZEP was reached at lower concentrations. At these doses, the compound began to be at least slightly ecotoxic. Calabrese (2008b) indicated that an agent could induce hormesis in the first part of an experiment but ecotoxicity results from a longer-term exposure if the agent accumulates and transitions to a toxic concentration in the target organism. Thus, the occurrence of hormesis is highly dependent on the pharmacokinetics of the agent in the biological model. The impact of pharmacokinetics has even been reported during the course of a single administration. According to the present results, these phenomena occurred for the PPCPs under study in *Vibrio fischeri* bacteria, and they were more evident for the mixture of PPCPs studied in the short-chronic test.

The evident phenomenon observed in Figure 3 has also been described by Calabrese (2005) as the compensatory response that occurs with low doses over time, eventually leading to the low dose stimulation characteristics of hormesis. Such compensatory and overcompensatory responses are not usually achieved at higher doses, leading to the hormetic biphasic dose-response.

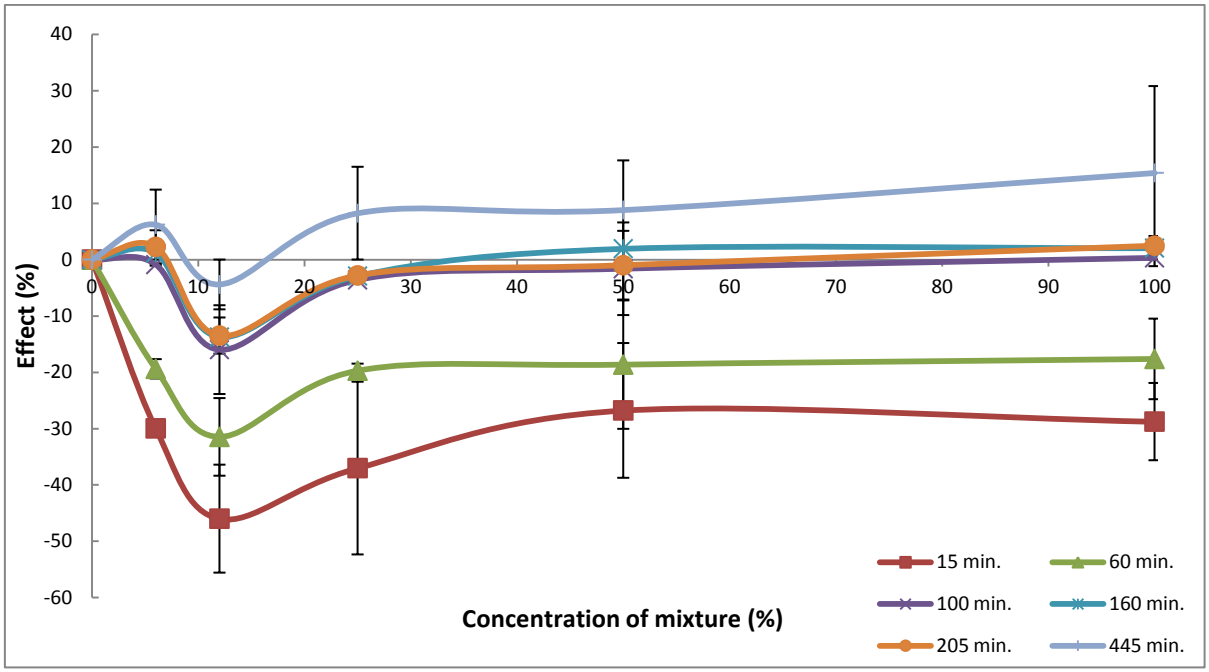

Figure 1. The dose-response behavior of the mixture of PPCPs over time. The results were obtained from short chronic ecotoxicity bioassays.

The behavior of the mixture of PPCPs was quite different than the behavior of each singly tested compound. The stimulatory effect of the mixture was higher than the highest stimulatory effect of each single compound (single bioassay) at least for the 15 minutes of response time. Some authors (Belz et al. 2008; Calabrese 2008a; Liu et al. 2009; Silva et al. 2002; Villa et al. 2012; Zhang et al. 2011) have suggested that antagonistic and synergistic phenomena could be present. Synergism or antagonism can then be defined, respectively, as upward or downward deviations from the additivity.

Dose (concentration) addition is thought to be applicable to mixtures composed of chemicals with similar modes of action. Conversely, independent action is applied to chemicals with diverse modes of action (Kortencamp et al. 2009). A more thorough study of PPCPs mixtures can confirm the real behavior and the best predictive model for these phenomena.

The results shown in **Figure 1** are the consequence of the mixture's complexity and the various modes of action of the compounds. Even after accounting for each dilution percentage in the mixture, not all compounds behaved in the same manner. The concentrations of some PPCPs could be stimulatory whereas other could be inhibitory, as shown by a comparison of **Figures 1** and **B2** (in **Appendix B**).

Other factors for the different behavior between the single PPCPs and the mixture of PPCPs in the bioassays could be the formation of transformation products due to the natural interaction of the different compounds or due to the change of some physicochemical properties or environmental conditions as pH, pKa and temperature.

In the present study, according to the ECOSAR™ methodology, 6 PPCPs presented narcosis as the toxic mode of action while the remaining 14 PPCPs were

organic chemicals with excess toxicity. Accordingly, these differences in the PPCPs' toxic modes of action became more complex when mixed, thus making it difficult to predict the behavior of the mixture.

Therefore, the biological, chemical and physical aspects of each individual mixture must be carefully studied in order to establish the endpoint for each different assay as well as the range of concentrations, types of compounds, types of mode of toxic action, etc.

3.5. Theoretical implications of the hormesis of PPCPs and their mixture in *Vibrio fischeri* bacteria

While many works in the scientific and popular literature have defined hormesis as a beneficial effect at low doses, a decision on whether a hormetic dose-response exists or not should precede any subsequent, independent decision as to whether the response is potentially beneficial, harmful or neutral to human health or different organisms in the environment (Calabrese 2005). Therefore, it is important to use the studies conducted to date and future toxicological assessments to determine the effects of hormesis on different organisms in the food chain (especially bacteria, which are indispensable microorganisms within ecosystems). Kefford et al. (2008) noted that short-term stimulatory effects may not necessarily affect populations in the long term. It is possible that hormesis led to increases in reproduction in one generation, but do not necessarily improve the evolutionary fitness over many generations. Thus, thus hormesis may not operate at the population level even with extended exposure. Indeed, there may be negative consequences to positive changes in some life history traits. Therefore, there may be no net gain to the organism's population over the long term.

Calabrese and Baldwin (2003) analyzed whether hormetic effects have evolutionary, biological or toxicological implications, implying that hazard assessment strategies should include protocols to assess the possible occurrence of hormesis. This has practical importance because hormetic effects may affect both the concept and derivation of the NO(A)EL data. The derivation of the NO(A)EL data could change if the low-dose stimulation was determined to have an adverse effect.

An additional aspect to be considered is the very low stimulatory, inhibitory or neutral effect that certain antibiotic compounds had on bacteria at low concentrations. In the present study, Figure B2 (Appendix B) shows this result for the antibiotics evaluated in *Vibrio fischeri*. Only norfloxacin and sulphametoxazole showed a slight stimulation at some low concentration tested (**Figure B2**). Currently, there are a number of bacterial species that have exhibited acquired resistance to one or more classes of antibiotics. The emergence and rapid spread of antibiotic-resistant bacteria (ARB) has led to increasing concern about the potential environmental and public health risks. Due to bacterial resistance, treatment often fails, which can induce consequences within the community. Furthermore, the spread of resistant bacteria poses obvious additional problems for controlling infections. ARB and antibiotic resistant genes have been detected extensively in wastewater samples (Bouki et al. 2013).

Thus, the inclusion of the hormetic effects in ecotoxicological risk or hazard assessments for different trophic levels and pollutants should be evaluated if homeostasis disruption is suspected. Chapman (2002) proposed a detailed level ecological risk assessment (DLERA), which involves several key aspects: the establishment of the hormetic dose/concentration-response curves; the

determination of the extent and range of hormesis in the selected organisms; and the determination of whether a given low dose-response has a positive, neutral or negative effect on the overall health of both individual organisms and populations/communities.

The hormetic concept has numerous applications in multiple areas of the biological sciences and also provides a basis for theoretical foundations within the broader evolutionary biological-toxicological-medical continuum. Several examples in fields such as experimental psychology, plant biology, and chemotherapy illustrate the rich generalizability of the hormesis concept (Calabrese and Baldwin 2003). Therefore, Calabrese and Baldwin (2001) also affirmed that future toxicology and pharmacology studies will have to come to terms with the emerging reality that the toxicological dose-response relationships are more complex than previously recognized. Moreover, the traditional evaluative extrapolation procedures are often no longer viewed as providing accurate estimates of responses at low doses.

Conclusion

Ecotoxicological assessments of 20 individual PPCPs and a mixture of these compounds were performed for the bioluminescent *Vibrio fischeri* bacteria. All compounds could be fitted by different dose-response models with the data around EC_{50}. Results in this range of concentrations showed a decrease in the bioluminescence with an increase in the concentrations of the compounds (inhibition). In this sense, four parameter model was the best fit for most compounds. The EC_{50} for each PPCP was estimated by the best fit model. The level of toxicity on bacteria by each PPCP was in the same order than in other studies performed by other methodologies.

Environmental concentrations were tested to evaluate the behavior of PPCP at these low concentrations. A 55% of the single compounds under study showed a stimulating effect in these low-doses. This effect has not often been overlooked in other ecotoxicological studies and is considered to be the widely recognized phenomenon called the hormetic effect.

All data (effects at environmental concentrations and around the EC_{50}) were fitted by the three models considered. Even though stimulatory effects were observed for many of the studied PPCPs, the dose-response curves of 90% of the tested compounds could be well adjusted with some of the non-linear functions proposed in this research. In this case, four parameter model was the best fit for most compounds. The best fitted curve of each PPCP allowed the estimation of several EC_x values (EC_0 or ZEP, EC_5 and EC_{50}) as relevant ecotoxicological points to determine the behavior of the studied compounds in *Vibrio fischeri* bacteria.

Despite the scarcity of data regarding the hormetic effect that PPCPs have on some organisms (such as bacteria) at actual environmental concentrations, the results could be widely compared and analyzed. Antibiotics are the most studied pharmaceutical compounds in this sense because these types of drugs are well known to cause bacterial resistance. For the remaining PPCPs, it is important to continue studying these topics in order to better understanding these complex phenomena.

Dose-response behavior of mixture of PPCPs at environmental concentrations was evaluated preliminarily. In this assay it was noted that the stimulating effect of the mixture was higher than the maximum effect of the single compounds, while the hormetic effect and the ZEP decreased when the

exposure time increased to the point that light emission was inhibited.

Most of the PPCPs studied are found in the environment or in the WWTPs at very low concentrations. In most cases, these concentrations are under the NO(A)ELs values, indicating that ecotoxicological studies must be performed for these concentrations not only to evaluate the potential hormetic effect but also to analyze other factors such as chronic effects or the intra- and interspecies influences of these PPCPs on upcoming generations.

Bacteria are indispensable microorganisms in the food chain. Therefore, any changes in them might change the normal development of many species (including humans) and environmental physicochemical processes. Thus, ecotoxicological studies on bacteria are essential for knowing deeply about the adverse, beneficial or neutral effects of a wide variety of chemical compounds that can reach the environment, including PPCPs. These ecotoxicological results are also necessary for environmental risk/hazard assessments to prevent contamination that affects the ecosystem.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Chapter 6

Paper V

Human and ecotoxicological potential impact of pharmaceutical and personal care products from USEtox™ life cycle impact assessment characterization factors

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Unpublished manuscript.

Human and ecotoxicological potential impact of pharmaceutical and personal care products from USEtox™ life cycle impact assessment characterization factors

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Abstract

Pharmaceutical and personal care products (PPCPs) are being increasingly included in Life Cycle Assessments (LCAs) since they have evidenced ecological and human adverse effects and due to their presence in different environmental compartments, wastewater facilities and industry. Therefore, the main goal of this research was to estimate characterization factors (CFs) of 27 PPCPs widely used worldwide for incorporating these values in LCIA studies or to generate impact score rankings. Physicochemical properties, degradation rates, bioaccumulation, ecotoxicity and human health effects were collected from experimental data, recognized databases or estimated by EPI Suite™. USEtox™ software was used for estimating CFs. An impact score ranking was done for 49 PPCPs using the new CFs calculated and the CFs already available and besides the data of PPCPs occurrence in the environment in Spain. Emission to continental freshwater compartment showed the highest CFs for human effects (ranging on 10^{-9} to 10^{-3}), following by air (10^{-9} to 10^{-5}), soil (10^{-11} to 10^{-5}) and sea water (10^{-12} to 10^{-4}). CFs of the affectation of freshwater aquatic environments were the highest from emission to continental freshwater (between 1 to 10^{4}) due to the direct contact between the source of emission and the compartment affected, followed by soil (among 10^{-1} to 10^{4}), air (among 10^{-2} to 10^{4}) and the lowest were continental sea water CFs (among 10^{-28} to 10^{-3}). Freshwater aquatic ecotoxicological CFs are much higher than human toxicity CFs which involves that ecological impact of PPCPs in aquatic environments is a matter of urgent attention. PPCPs with the highest impact are hormones,

α, log of HC50; AF_a, Extrapolation factor for interspecies differences; AF_N, Extrapolation factor for NOAEL to ED_{50}; AF_q, Extrapolation factor for 1/q* to ED_{50}; AF_t, Extrapolation factor for differences in time of exposure; EMEA, European Agency for the Evaluation of Medicinal Products; BAF, Bioaccumulation factor of the chemical; BCF_{fish}, Bioconcentration Factor in fish; BIOmass, Concentration of biota in water; BW, Average body weight of humans; CF, Characterization Factor; CRA, Comparative Risk Assessment; CTU, Comparative Toxic Units, DOC, Dissolved Organic Carbon concentration in freshwater; EC_{50}, Water concentration at which 50% of a population displays an effect; ED_{50}, Daily dose that causes a disease probability of 50% of population; EF, Effect Factor; FDA, Food and Drugs Administration; FF, Fate Factor; FRw.w, Fraction of a chemical dissolved in freshwater; GEM, Genetically-Engineered Mouse; HC50, Hazardous concentration of chemical at which 50% of the species are exposed above their EC50; IARC, International Agency for Research on Cancer; iF, Intake Fraction; INH, Average inhalation rate of a person; IS, Impact Score; KdegA, Degradation rate in air; KdegSI, Degradation rate in soil; KdegSd, Degradation rate in sediment, KdegW, Degradation rate in water; KH, Henry law coefficient; K_{doc}, Partitioning coefficient between dissolved organic carbon and water; K_{oc}, Partition coefficient between organic carbon and water; K_{OH}, Hydroxyl radical rate constant; K_{ow}, Partition coefficient between octanol and water; K_p, Partition coefficient between water and suspended solids; LCA, Life Cycle Assessment; LCI, Life Cycle Inventory; LCIA, Life Cycle Impact Assessment; LOAEL, Lowest Observed Adverse Effect Level; LT, Average lifetime of humans; M, Mass emission; MASS, Mass of compartment; MTD, Maximum Tolerated Dose; MW, Molecular Weight; N, Number of days per year; n_s, Number of species; NOAEL, No-observed Adverse Effect Level; NSAIDs, non-steroidal anti-inflammatory drugs; [OH], Hydroxyl radical concentration; PAF, Potentially Affected Fraction; PCPs, Personal Care Products; PhACs, Pharmaceutical active compounds; POP, Population number; PPCPs, Pharmaceutical and Personal Care Products; PROD, Production per person; P_{vap}, Vapor pressure; q^*, carcinogenic low dose slope factor; QSAR, quantitative structure–activity relationship; Sol, Solubility; SUSP, Suspended matter concentration in freshwater; US FDA, United State Food and Drug Administration; VOLUMEair, Volume of the air compartment; WWTPs, Wastewater Treatment Plants; XF, Exposure Factor.

antidepressants, fragrances, antibiotics, angiotensin receptor blockers and blood lipid regulators, which have been already found in other ranking scores. These results, not available until now, are useful to do better LCIAs incorporating these pollutants in these studies or for assessing single hazard/risk environmental impact assessments.

Keywords: Characterization Factor, Ecotoxicity, Human toxicity, Life Cycle Impact Assessment, Pharmaceuticals and Personal Care Products.

1. Introduction

In the last years pharmaceutical and personal care products (PPCPs) have been found at different level of concentrations in all environmental compartment (air, water and soil), and many of their impacts are still unknown or they are under analysis. The primary routes for pharmaceuticals into the environment are through human excretion, disposal of unused products and through agricultural usage. A wide range of pharmaceutical products have been detected in surface and groundwater, associated with wastewater disposal (Stuart et al., 2012) but can also be found in soil, sediments and to a lesser extent in the air. Due to the large amount of these type of compounds, the possibility of known the potential impact that would generate all the PPCPs in nature is almost impossible without spend large amounts of money, resources and time.

One tool used for estimated the potential impact of PPCPs in the environment is the life cycle assessment (LCA). LCA is increasingly gaining acceptance as a holistic environmental evaluation of chemicals and chemical processes. LCA does not substitute other methodologies, since the different tools fulfill different purposes and they can rather play complementary roles and benefit from each other (Muñoz et al., 2008).

The guidelines of LCA studies are establish in the collection of ISO 14000 specifically in the ISO 14040:2006 to 14044:2006 standard procedure, which specifies the phases of LCA: (i) requirements of definition of the goal and scope of the LCA, (ii) the life cycle inventory analysis (LCI) phase, (iii) the life cycle impact assessment (LCIA) phase, (iv) the life cycle interpretation phase, (v) reporting and critical review of the LCA, (vi) limitations of the LCA, relationship between the LCA phases, and (vii) conditions for use of value choices and optional elements (ISO, 2006).

The production and use of PPCPs are widespread worldwide so the use of this LCA methodology in this industry and for these compounds can be a powerful tool: (i) to identify the type of (negative) impact of these compounds in different environmental scenarios; (ii) to compare these impacts with those from other compounds; (iii) to implement preventive or corrective actions to minimize the potential or real adverse effect caused by them.

Some authors have successfully used LCAs in systems involving wastewater treatments and PPCPs. For treatment facilities, Corominas et al. (2013) presented a comprehensive review of 45 papers dealing with wastewater treatment plants (WWTPs) and LCA, Niero et al. (2014) did a comparative LCA of wastewater treatment in Denmark including sensitivity and uncertainty analysis and Loubet et al. (2014) did a comparative analysis of selected peer-reviewed literature of LCA of urban water systems.

More specifically, Igos et al. (2012) did a LCA comparison between the removal of pharmaceuticals in decentralized or conventional WWTPs, Muñoz et al. (2008) used in their work the LCIA, a feature of the LCA methodology (outside the LCA framework) as a means to quantify the

potential environmental impacts on ecotoxicity and human toxicity of wastewater containing priority and emerging pollutants. LCA methodology was also used to compare three disposal options for unused pharmaceuticals in Cook et al. (2012).

In biopharmaceutical industry Ramasamy et al. (2014) used the LCA as a tool to support decision making and Jiménez-González et al. (2004) analyzed the LCI of pharmaceutical compounds.

A methodological comparison has been made by Olsen et al. (2001) between LCIA and risk assessment of chemicals, they concluded that the conceptual background and the purpose of the tools are different, but there are overlaps and they may benefit from each other and complement each other in an overall environmental effort.

In the LCIA phase, the emissions inventory data are multiplied by characterization factors (CFs) to provide indicators in the context of various impact categories (such as global warming, stratospheric ozone depletion, tropospheric ozone creation, eutrophication/nitrification, acidification, toxicological impacts to humans, and toxicological impacts to ecosystems) (Pennington et al., 2004).

The calculation or estimation of CFs is essential to obtain the human and ecological potential impact of substances on different environments (air, freshwater, seawater, natural soil, agricultural soil, etc.) and it is indispensable include them in LCIA investigations.

In environmental LCAs, CFs (alternatively referred to as equivalency factors) are used to determine the relative importance of a substance to toxicity related impact categories, such as human toxicity and freshwater ecotoxicity. The CF accounts for the environmental persistence (fate) and accumulation in the human food chain (exposure), and toxicity (effect) of a chemical. Fate and exposure factors can be calculated by means of "evaluative" multimedia fate and exposure models, while effect factors can be derived from ecotoxicity data on human beings and laboratory animals (Huijbregts et al. 2005a).

In this sense, the USEtox™ model is a powerful tool to calculate CFs. It is an environmental model for characterization of human and ecotoxicological impacts in LCIA and comparative risk assessment (CRA). It has been developed by a team of researchers from the Task Force on Toxic Impacts under the UNEP-SETAC Life Cycle Initiative. USEtox™ is designed to describe the fate, exposure and effects of chemicals. The UNEP-SETAC initiative supports the development, evaluation, application, and dissemination of USEtox™ to improve understanding and management of chemicals in the global environment (Huijbregts et al., 2010a).

USEtox™ provides a parsimonious and transparent tool for human health and ecosystem CF estimation. Based on a referenced database, it has now been used to calculate CFs for several thousand of substances and forms the basis of the recommendations from UNEP-SETAC's Life Cycle Initiative regarding characterization of toxic impacts in LCA (Rosenbaum et al., 2008).

Despite the large number of substances which have been considered in the USEtox™ database (more than 3000 in the USEtox™ organic database 1.01) a small amount of PPCPs have been considered (approximately less than 2% of the organic database corresponds to this group of compounds). Therefore, the CFs of many PPCPs have not been calculated.

LCIA conducted in systems containing PPCPs may be incomplete or unrealistic if these compounds are not considered. Therefore, CFs estimation is a very important issue and a novel contribution in this research field.

Recently, Alfonsín et al. (2014) provide CFs for the toxicity related impact categories in LCA for 23 PPCPs. The CFs already available in databases were updated whereas others were implemented for the first time by means of USEtox™ and USES-LCA 2.0 methodologies. They cited only five previous researches that calculated a limited number of PPCPs' CFs by different methodologies. This indicates that there is still a lack of data in this topic.

Therefore, in this research CFs for 27 PPCPs have been calculated by USEtox™ methodology to complement its database and thus be able to incorporate these compounds into LCIA studies. Five compartments were considered to which emission can take place (continental urban air, continental rural air, continental freshwater, continental sea water, continental natural soil and continental agricultural soil) and toxicity potentials were estimated for two different impact categories: human health and freshwater aquatic environments.

Additionally, using the new CFs calculated in this research, those CFs existing in the software database of USEtox™, the newly CFs calculated by Alfonsín et al. (2014) and the data of occurrence of PPCPs in the aquatic environments in Spain (Ortiz et al., 2013a) have been used to estimate the human toxicity and ecotoxicity impact scores (IS) for 49 PPCPs. These ISs have been used to develop and analyzed ranking scores from CFs and then, compare the relative toxicity between these compounds and compare with other ranking of concern such as the established in Ortiz et al. (2013b).

2. Materials and methods

The main steps carried out for the realization of this research is summarized below. The procedure to collect the input parameters, run the software and analyze the results of USEtox™ model were consulted in the published literature for this purpose (Huijbretgs et al., 2005a; 2005b; 2010a; 2010b; Rosenbaum et al., 2008).

2.1. Selection of PPCPs

Similar to previous researches (Ortiz et al. 2013a, 2013b; 2014) the PPCPs selected are some of the worldwide more important pharmaceutical active compounds (PhACs) and personal care products (PCPs), their consumption and occurrence in aquatic environments and in WWTPs are relevant and there are evidences of their potential ecotoxicity in the different compartments of environment.

Table 1 shows the PPCPs used to estimate the human toxicity and ecotoxicity ISs in this study. For some PPCPs the CFs were calculated, but other values were obtained from bibliography (Alfonsin et al 2014 and the database of USEtox™). A total of 49 compounds from 14 different therapeutic classes have been considered: analgesic/antipyretic (1), Angiotensin converting enzyme inhibitor (1), angiotensin receptor blockers (2), antibiotics (11), antidepressants (3), antiepileptics (4), anxiolytics (3), blood lipid regulators (3), cytostatics/cancer therapeutic (2), H_2 blocker (1), hormones (4), Platelet inhibitor (1), non-steroidal anti-inflammatory drugs (NSAIDs)/antirreumatics (4), X-ray contrast media (3) and PCPs (6) were considered in this study.

Table 1. Pharmaceutical and personal care products under study

Compounds	Estimation in this study	Reference
Acetaminophen, alprazolam, amoxicillin, atorvastatin, azithromycin, bromazepam, cefaclor, ciprofloxacin, clarithromycin, enalapril, ethylparaben, gabapentine, iohexol, iopamidol, irbesartan, ketorolac, levofloxacin, lorazepam, methylparaben, norfloxacin, omeprazol, paroxetine, pregabalin, propylparaben, sertraline, simvastatin and valsartan.	New CFs*, human toxicity and ecotoxicity IS	This study
17α-ethinylestradiol, 17β-estradiol, carbamazepine, diclofenac, erythromycin, estrone, galaxolide, ibuprofen, iopromide, naproxen, roxythromycin, sulphametoxazole, tonalide, triclosan, trimethoprim.	Human toxicity and ecotoxicity IS**	CFs from Alfonsin et al. (2014)
Clofibrate, cyclophosphamide, fluoxetine, salicylic acid, tamoxifen, testosterone, valproic acid.	Human toxicity and ecotoxicity IS	CFs from USEtox™ database

*CFs: Characterization factors.
**IS: Impact Score.

2.2. Impact score

The IS is a weighted summation of the releases of pollutants of a product system with help of CFs (Huijbregts et al. 2010a). IS allows be grouped into a single index the impact (ecotoxicity or human toxicity) of a compound released to the different compartments of nature. The equation and procedure to calculate IS for ecotoxicity (Eq. 1) and for human toxicity (Eq. 6) are shown in Table 2. IS is reported as comparative toxic units (CTU) that can be compared with IS from other methodologies.

In a recent study (Ortiz et al., 2013a) it has been estimated the occurrence (mass year^{-1}) of 88 PPCPs and metabolites in the aquatic environment in Spain. These results of occurrence in aquatic environments were used to estimate ISs (in CTU year^{-1}) for ecotoxicological and human toxicity for the PPCPs considered in this study. The mass balance approach used for estimating the occurrence in aquatic environments used in Ortiz et al. (2013a) and the data for removal in WWTPs estimated by EPI Suite™ allowed the calculation of the mass of PPCPs adsorbed into sludge and the volatilization to air. In this way, it is possible to obtain a total ecotoxicological IS that included three compartments: water, soil and air. For this case study, the emission was considered to the following environmental compartments: continental freshwater (water), continental natural soil (soil) and continental urban air (air).

2.3. Characterization factor

The potential to increase the ecological toxicity and the human toxicity are estimated through CFs of chemicals that includes a fate factor (FF), an exposure factor (XF) and an effect factor (EF). Ecotoxicological and human toxicity CFs are shown in Table 2 (Eq. 2 and Eq. 7, respectively). FF, XF and EF are related as shown in Figure 1.

CFs for ecotoxicity are reported for freshwater aquatic ecotoxicological effects and include impacts for emissions to urban air, rural air, freshwater and/or agricultural soil in different scales (Figure 2).

Table 2. Main equations of the concept and model of USEtox™ for human toxicity and ecotoxicity

Life cycle impact assessment for ecotoxicity		
Parameter (Acronym)	**Equation (Nº)**	**Explanation**[+]
Impact score (IS_{eco})	$IS_{eco} = \sum_i \sum_x CF_{x,i} \cdot M_{x,i}$ (1)	IS_{eco}: Impact score for ecotoxicity (PAF[++] m^3 day or comparative toxic units, CTU_e). $CF_{x,i}$: Ecotoxicity characterization factor of substance *x* released to compartment *i* (for freshwater aquatic ecotoxicity: PAF m^3 day $kg_{emission}^{-1}$ or CTU_e kg^{-1}). $M_{x,i}$: Emission of *x* to compartment *i* (kg). The summation holds for substances and emission compartments.
Characterization factor (CF_{eco})	$CF_{eco} = FF_{eco} \cdot XF_{eco} \cdot EF_{eco}$ (2)	FF_{eco}: Ecotoxicity fate factor (day). XF_{eco}: Ecotoxicity exposure factor (unitless). EF_{eco}: Ecotoxicity effect factor (PAF m^3 kg^{-1}).
Fraction of a chemical dissolved in freshwater ($FR_{w.w}$)	$FR_{w.w} = \frac{1}{1+(K_p \cdot SUSP + K_{doc} \cdot DOC + BCF_{fish} \cdot BIOmass)/1 \cdot 10^6}$ (3)	The XF_{eco} for freshwater ecotoxicity is the $FR_{w.w}$. Kp: Partition coefficient between water and suspended solids (L kg^{-1}). SUSP: Suspended matter concentration in freshwater (= 15 mg L^{-1} in USEtox™). K_{doc}: Partitioning coefficient between dissolved organic carbon and water. DOC: Dissolved organic carbon concentration in freshwater (= 5 mg L^{-1} in USEtox™). BCF_{fish}: Bioconcentration factor in fish (L kg^{-1}). BIOmass: Concentration of biota in water (= 1 mg L^{-1} in USEtox™).
Ecotoxicity effect factor (EF_{eco})	$EF_{eco} = \frac{0.5}{HC50}$ (4)	The EF_{eco} reflects the change in the PAF of species due to change in concentration. In USEtox™, the EF_{eco} is calculated by determining the linear slope along the concentration–response relationship up to the point where the fraction of effected species is 0.5. HC50: Hazardous concentration of chemical at which 50% of the species are exposed above their EC_{50}, based on species-specific EC_{50} data. EC_{50}: Water concentration at which 50% of a population displays an effect (e.g. mortality). Aquatic EF_{eco} is calculated based on geometric means of single species EC_{50} tests data. Chronic values have priority as long as they represent measured EC_{50} values but chronic EC_{50} values are seldom reported. Second-order priority is given to acute data, applying an acute-to-chronic extrapolation factor that is set to a default factor of 2.
Log of HC50 (α)	$\propto = \frac{1}{n_s} \cdot \sum_s logEC_{50_s}$ (5)	n_s: Number of species.

[+]Huijbregts et al. (2005b); Huijbregts et al. (2010a); Huijbregts et al. (2010b); Rosenbaum et al. (2008).
[++]Potentially affected fraction.

Table 2. Cont. Main equations of the concept and model of USEtox™ for human toxicity and ecotoxicity

Life cycle impact assessment for human toxicity

Parameter (Acronym) (Units)	Equation (Nº)	Explanation[+]
Impact score (IS_{hum}) (CTU_h)	$IS_{hum} = \sum_i \sum_x CF_{x,i,hum} \cdot M_{x,i}$ (6)	IS_{eco}: Impact score for human toxicity (cases or CTU_h). $CF_{x,i,hum}$: Human toxicity characterization factor of substance *x* released to compartment *i* (cases kg^{-1} or CTU_h kg^{-1}). $M_{x,i}$: Emission of *x* to compartment *i* (kg). The summation holds for substances and emission compartments.
Characterization factor (CF_{hum})	$CF_{hum} = FF_{hum} \cdot XF_{hum} \cdot EF_{hum}$ (7)	In USEtox™, chemicals that have a potential to increase human disease have a CF_{hum} that includes a fate factor (FF_{hum}), an exposure factor (XF_{hum}) and an effect factor (EF_{hum}) similar than ecotoxicity. Both the human and ecotoxicity CFs are calculated using standard matrix algebra.
Intake factor (iF)	$iF = FF_{hum} \cdot XF_{hum}$ (8)	iF: Intake fraction, fraction of the emitted mass that enters the human population (kg_{intake} $kg_{emitted}^{-1}$) . Intake through inhalation and ingestion is commonly considered in iF calculations.
Exposure factor via inhalation of air ($XF_{hum,air}$)	$XF_{hum,air} = \frac{INH \cdot POP}{Volume_{air}}$ (9)	INH: Average inhalation rate of a person (=13 m^3day^{-1} in USEtox™). POP: Population number (e.g. 900 million on the continental scale). $VOLUME_{air}$: Volume of the air compartment (e.g. $5.76 \cdot 10^{10}$ m^3 at the urban scale).
Exposure factor for a specific food or drinking water at a specific scale (e.g. continent) ($XF_{hum,i,r}$)	$XF_{hum,i,r} = \frac{BAF_{i,r} \cdot PROD_i \cdot POP}{MASS_r}$ (10)	$BAF_{i,r}$: Bioaccumulation factor of the chemical of exposure pathway *i* (e.g. fish) via compartment r (e.g. freshwater) (kg kg^{-1}). $PROD_i$: Production per person of item i in the exposure pathway (e.g. 0.04 kg day^{-1} $person^{-1}$ for freshwater fish). $MASS_r$: Mass of compartment r (e.g. $6.8 \cdot 10^{14}$ kg for continental freshwater).
Human-toxicological effect factor of a chemical (EF_{hum})	$EF_{hum} = \frac{0.5}{ED50}$ (11)	EF_{hum}: Reflects the change in life time disease probability due to change in life time intake of a pollutant (cases kg_{intake}^{-1}). In USEtox™, separate EFs are derived for non-carcinogenic effects and carcinogenic effects. Furthermore, for each effect type (non-carcinogenic and carcinogenic) the two exposure routes, i.e. inhalation and ingestion are addressed separately. The EF_{hum} is calculated under the assumption of linearity in concentration–response up to the point at which the life time disease probability is 0.5.
Daily dose for a chemical per person (human) in its lifetime for carcinogenic and non-carcinogenic effects related to inhalation or oral exposure ($ED_{50h,j}$) (kg $person^{-1}$ $lifetime^{-1}$)	$ED_{50_{h,j}} = \frac{ED_{50_{a,t,j}} \cdot BW \cdot LT \cdot N}{AF_a \cdot AF_t \cdot 10^6}$ (12)	$ED_{50a,t,j}$: Daily dose for animal *a* (e.g. rat) and time duration *t* (e.g. subchronic) per kg body weight that causes a disease probability of 50% for exposure route *j* (mg $kg^{-1}day^{-1}$). AF_a: Extrapolation factor for interspecies differences[+++]. AF_t: Extrapolation factor for differences in time of exposure (i.e. a factor of 2 for subchronic to chronic exposure and a factor of 5 for subacute to chronic exposure). BW: Average body weight of humans (70 kg). LT: Average lifetime of humans (70 years). N: Number of days per year (365 days $year^{-1}$).

[+]Huijbregts et al. (2005b); Huijbregts et al. (2010a); Huijbregts et al. (2010b); Rosenbaum et al. (2008).

[+++]Human=1.0. Dog=1.5. Rabbit=2.4. Rat=4.1. Mouse=7.3.

Table 2. Cont. Main equations of the concept and model of USEtox™ for human toxicity and ecotoxicity

Life cycle impact assessment for human toxicity		
Parameter (Acronym) (Units)	**Equation (Nº)**	**Explanation**[+]
Daily dose for a chemical per person in its lifetime for carcinogenic and non-carcinogenic effects related to inhalation exposure ($ED_{50h,inh}$) (kg person^{-1} lifetime^{-1})	$ED_{50\,h,inh} = \frac{ED_{50\,a,t,inh} \cdot INH \cdot LT \cdot N}{AF_a \cdot AF_t \cdot 10^6}$ (13)	$ED_{50\,a,t,inh}$: Concentration in air (mg m^{-3}) which has been exposed to an animal *a* and time duration *t*. INH: Average human inhalation rate (13 m^3 day^{-1}). AF_a: The extrapolation factor for interspecies differences is by default 1 if the ED_{50} is given as concentration in the air. Metabolic activity and inhalation rate are assumed to have the same ratio for all species.
Daily dose for animal *a* and time duration *t* per kg body weight that causes carcinogenic effects of 50% for exposure route *j* ($ED_{50a,t,j(cancer)}$) (mg kg^{-1}day^{-1})	$ED_{50\,a,t,j(cancer)} = \frac{1}{q^*_{a,t,j}} \cdot AF_q$ (14)	For carcinogenic effects, the ED_{50} can also be estimated from the carcinogenic, low-dose, slope factor q* by the 1/q*-to-ED_{50} extrapolation factor. $q^*_{a,t,j}$: Carcinogenic, low-dose, slope factor for animal *a* (e.g. rat) and time duration *t* (e.g. chronic) for exposure route *j* (kg day mg^{-1} or m^3 mg^{-1}). AF_q: Extrapolation factor for 1/q* to ED_{50}, which is a factor of 0.8.
Daily dose for animal *a* and time duration *t* per kg body weight that causes non-carcinogenic effects of 50% for exposure route *j* ($ED_{50a,t,j(non-cancer)}$) (mg kg^{-1}day^{-1})	$ED_{50\,a,t,j\,(non-cancer)} = NOAEL_{a,t,j} \cdot AF_N$ (15)	For non-carcinogenic effects, the ED_{50} can also be estimated from the No-Observed Adverse Effect Level (NOAEL) by a NOAEL-to-ED_{50} extrapolation factor. $NOAEL_{a,t,j}$: Daily dose per kg body weight or concentration for animal *a* (e.g. rat) and time duration *t* (e.g. chronic) that causes No Observed Effects for exposure route *j* (mg kg^{-1} day^{-1} or mg m^{-3}). AF_N: Extrapolation factor for NOAEL to ED_{50}, which is a factor of 9.
LOAEL-to-NOAEL extrapolation (mg kg^{-1}day^{-1})	$NOAEL_{a,t} = \frac{LOAEL_{a,t}}{AF_L}$ (16)	For some chemicals only the Lowest Observed Adverse Effect Level (LOAEL) is available. $LOAEL_{a,t}$: Daily dose per kg body weight or concentration for animal *a* and time duration *t* that causes Lowest Observed Adverse Effect level. In these cases, the NOAEL can be derived by a LOAEL-to-NOAEL extrapolation factor. AF_L: Extrapolation factor from LOAEL to NOAEL, which is a factor of 4.

[+]Huijbregts et al. (2005b); Huijbregts et al. (2010a); Huijbregts et al. (2010b); Rosenbaum et al. (2008).

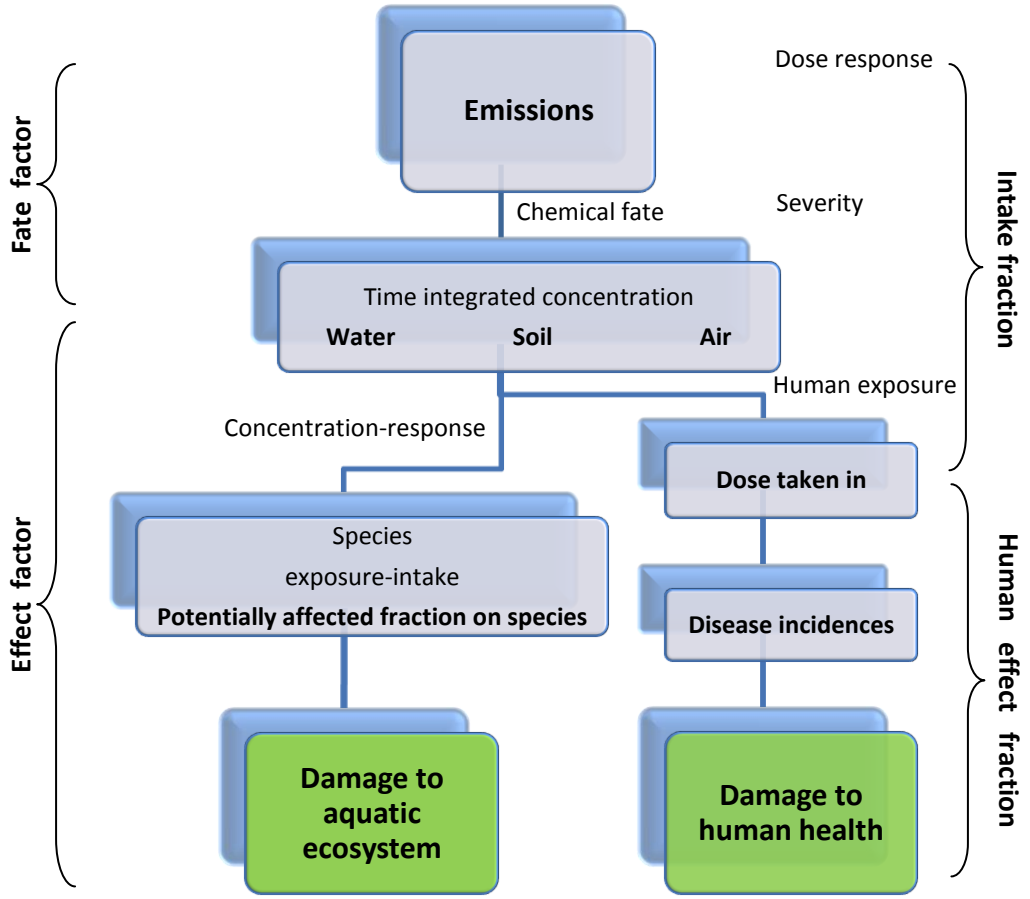

Figure 1. Main Steps of the USEtox™ assessment
(Adapted from Huijbregts et al., 2010a)

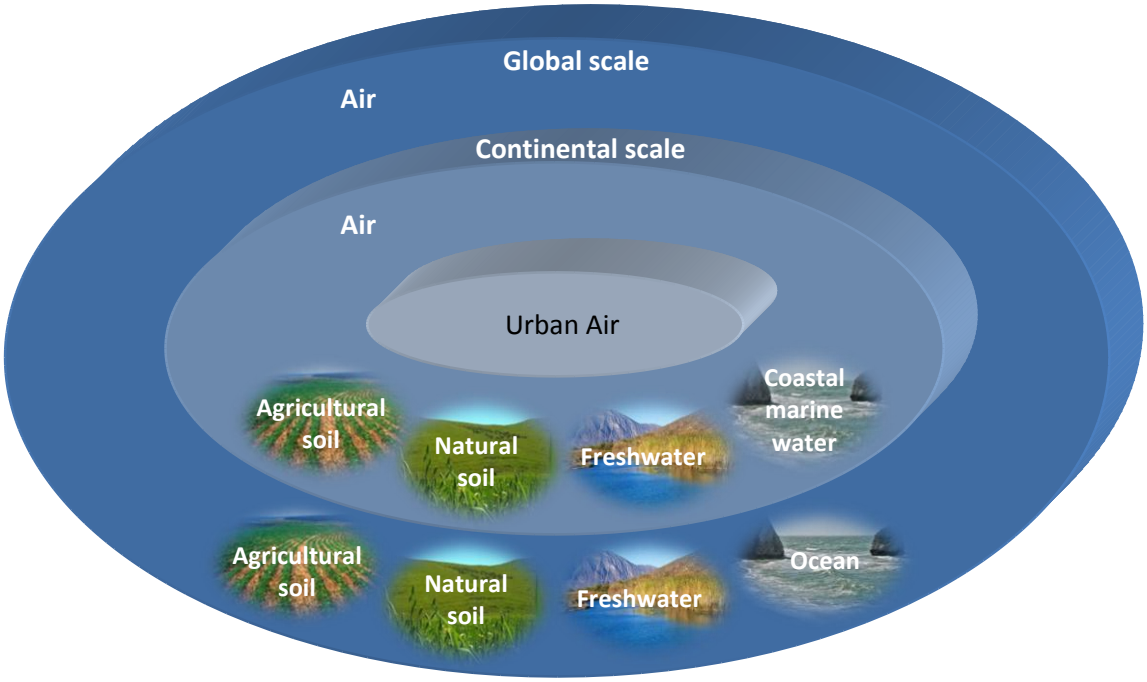

Figure 2. Nested structure of the USEtox™
(Adapted from Rosenbaum et al., 2008)

CFs for human toxicity are estimated for carcinogenic and non-carcinogenic effects and take into account emissions in different scales (Figure 2). The main CFs are reported for emissions to urban air, continental rural air, continental freshwater, continental sea water, continental natural soil and continental agricultural soil. The FF and EF are combined to reflect the intake fraction (iF) of a chemical, representing the fraction of the emitted mass that enters the human population (Eq. 8, Table 2).

2.4. Fate factor

The fate factor is the same for ecotoxicity and human toxicity. Two geographical scales are specified: (i) the continental scale with the following compartments: urban air, rural air, freshwater, sea, natural soil and agricultural soil and; (ii) the global scale with the following compartments, air, freshwater, ocean, natural soil and agricultural soil. The continental scale is nested in the global scale (see Figure 2). "Nested" means that chemicals can be transported from one scale to a higher scale and vice versa (Huijbregts et al., 2010a).

The fate factor is equal to the compartment-specific residence time (in days) of a chemical. The longer the residence time, the longer a chemical remains in the environment. Within the consensus model, the residence time of a chemical depends on (i) the properties of the chemical, (ii) the selected emission compartment, and (iii) the selected receiving compartment.

The fate model part of USEtox™ calculates the residence time of a chemical, by solving the mass balance under steady state conditions with the help of linear algebra calculation rules and based on the quantification of environmental processes such as: (bio) degradation by micro-organisms, transport of the chemical to the sediment, leaching to the groundwater and escape to the stratosphere (removal processes) and intermedia transport processes (advective and diffusive transport).

2.5. Exposure factor

The exposure factor for ecotoxicity CF is calculated by the Eq. 3 (Table 2). According to Huijbregts et al. (2010a) CFs for human toxicity reflect the rate at which a pollutant is able to transfer from a receiving compartment into the human population through a series of exposure pathways: air (inhalation), drinking water, above-ground leaf crops (including fruit and cereals), crops below ground (root crops), meat, dairy products, fish. For exposure via inhalation of air, the exposure factor is calculated by Eq. 8 (Table 2) and the exposure factor for a specific food item or drinking water at a specific scale (e.g. continent) is calculated by Eq. 9 (Table 2).

2.6. Effect factor

The ecotoxicological EF is estimated by Eq. 4 and Eq. 5 (Table 2). In this study acute and chronic data were used to estimate this parameter. The US EPA ECOTOX database (US EPA, 2015) was consulted as first option, if no data exists in ECOTOX other researches were consulted (Dobbins et al., 2009; Iannacone and Alvariño, 2009; Ortiz et al., 2014; Santos et al., 2010; Terasaki et al., 2009) or the ecotoxicity of chemicals was estimated by the EPI Suite™ software (US EPA, 2012). According to Huijbregts et al. (2010b) aquatic ecotoxicological CFs are specified as "interim", if EFs are based on species toxicity data covering less than three different trophic levels. This is to ensure a minimum variability of biological responses. In this study, at least three thropic levels have been considered to calculate ecotoxicological EF, therefore, the

ecotoxicological CFs calculated may be considered as "recommended".

The human-toxicological EF reflects the change in life time disease probability due to change in life time intake of a pollutant. In USEtox™, separate effect factors are derived for non-carcinogenic and carcinogenic effects. Furthermore, for each effect type (non-carcinogenic and carcinogenic) the two exposure routes, i.e. inhalation and ingestion are addressed separately (Huijbregts et al. 2010a). In this research carcinogenic and non-carcinogenic effects and ingestion as route of exposure are considered (due to the lack of data for inhalation as route of exposure) thus, the CFs of human toxicity calculated in this research should be taken as "interim". Equations 11 to 16 (Table 2) are used to calculate the human-toxicological EFs.

2.7. Input data

The input parameters that must be supplied by the user for the USEtox™ program are: molecular weight (MW), partition coefficient between octanol and water (K_{ow}), partition coefficient between organic carbon and water (K_{oc}), Henry law coefficient at 25ºC (K_H), vapor pressure at 25ºC (P_{vap}), solubility at 25ºC (Sol), degradation rate in air (K_{degA}), degradation rate in water (K_{degW}), bioaccumulation factor of the chemical (BAF), water ecotoxicity (chronic and acute) and human carcinogenic and non-carcinogenic effects.

In this study, the experimental value (if it is available) or the estimation values for the physicochemical properties (MW, K_{ow}, K_{oc}, K_H, P_{vap} and Sol) and BAF have been taken for EPI Suite™ as it is recommended by Huibregts et al. (2010a, 2010b).

For air degradation rates, experimental values for the hydroxyl radical rate constant (K_{OH}) are available for some chemicals in EPI Suite™. To derive the K_{degA}, the K_{OH} was multiplied by the hydroxyl radical concentration [OH]. The default [OH] was set at $1.5 \cdot 10^6$ molecules (radicals) cm^{-3} per 12h of daylight. K_{degW}, soil (K_{degSl}) and sediment (K_{degSd}) were estimated by biodegradation half life with EPI Suite™, the Biowin3 model is used for USEtox™ input to convert the ultimate biodegradation probability in half-lives for all chemicals in the database. In addition, division factors of 1:2:9 are used to extrapolate biodegradation rates for water, soil and sediment compartments respectively, as suggested in EPI Suite™ (Huijbregts et al., 2010b).

Water ecotoxicity for different species and trophic levels (bacteria, algae, crustacean, rotifer, mollusk and fish) have been collected as it was explained in the effect factor section. The calculation steps of the logHC50 (α) according to Huijbregts et al. (2010a) are: (i) gather experimental or estimated EC_{50} data for the chemical of interest; (ii) specify for every EC_{50}-value whether it is chronic or acute exposure; (iii) calculate the geometric mean chronic or acute EC_{50} for every individual species (in case of acute EC_{50}-data, derive the chronic-equivalent EC_{50} per species by dividing by a factor of 2, acute-to-chronic extrapolation factor) and (iv) take the log of the geometric mean EC_{50}s and calculate the average of the log-values (this average equals the logHC50).

Non-carcinogenic data for rat, mouse, rabbit, dog and monkey were collected from different sources (World Health Organization database, European Agency for the Evaluation of Medicinal Products (EMEA) reports, toxicology studies of the US Food and Drugs Administration (FDA), reports of Scientific Committee on Consumer Safety of European Commission, United States Pharmacopeia monographs, material safety data sheet of Merck, Pifzer, Medsafe and La Roche; Rashmi et al., 2012). From these sources the daily dose that causes a disease probability of 50% of

population (ED_{50}) was estimated from no-observed adverse effect level (NOAEL) or the lowest observed adverse effect level (LOAEL) (chronic toxicity data). Carcinogenic data (as ED_{50}) was obtained from Brambilla et al. (2012).

In this study, the steps for the ED_{50} estimation according to Huijbregts et al. (2010a) guidelines are: (i) gather experimental non-carcinogenic oral ED_{50} data, (ii) specify for every ED_{50}-value whether it is chronic, subchronic or subacute exposure; (iii) as the chronic value is needed, subchronic and subacute data have to be extrapolated to chronic ED_{50}. According to the type of ED_{50}-data found in the literature or database (non-human ED_{50}-data, NOAEL or LOAEL) the chronic-equivalent ED_{50} has to be derived using equations 12 to 16 (Table 2).

The USEtox™ software calculates the remainder of the necessary data, from the input parameters supplied of users above referred. More information about procedure, equations, considerations, estimations and the methodology can be consulted in the main literature that supports the software ((Huijbretgs et al., 2005a; 2005b; 2010a; 2010b; Rosenbaum et al., 2008).

3. Results and discussion

In recent years the occurrence, fate and adverse impact of PPCPs in the environmental is an important topic that generated increasing interest.

In the last years some studies have generated and updated the CFs from different methodologies (Alfonsín et al. 2014; Hospido et al. 2010; Igos et al. 2012; Larsen et al. 2009; Morais et al. 2013; Muñoz et al. 2008) but still there is lack of data of some PPCPs widely used worldwide, which does not allow include these compounds in studies of LCA or LCIA to verify and compare their negative impact with other compounds or in different processes.

In this sense, Table 3 shows the input data collected for estimating CFs of 27 PPCPs through the models of the methodology of USEtox™. The required parameters are physicochemical properties, biodegradation rates, bioaccumulation factor, ecotoxicity and human toxicity (carcinogenic and non-carcinogenic).
In general, the PPCPs under study presented a high variable solubility, K_{oc} and K_{ow}. These parameters provide an estimation of the mobility of the PPCPs in water environments, soils and sediments. Atorvastatin, azithromycin, clarithromycin, irbesartan, paroxetine, simvastatin, valsartan and sertraline showed the highest values of K_{ow} and K_{oc} and low solubility, therefore, these compounds will be probably located in soils, sediments or bioaccumulated.

Degradation rates in water, soils and sediments were in the same order although slightly higher in water than in soil and sediments in most cases. Degradation rates in air are the highest among all the compartments, possibly by the high dispersion in this medium and the different photochemical effects and reactions. Despite this, all compounds presented low P_{vap} and K_H which indicates that they will not be found in large quantities in the air.

Alprazolam, atorvastatin, azithromycin, clarithromycin, irbesartan, omeprazole, paroxetine, sertraline and simvastatin were the compounds most ecotoxic and human toxic according to the parameters collected following the USEtox™ methodology.

Table 3. Input parameters required for the ecotoxicological and human toxicity characterization factors calculation in USEtox™ for the pharmaceuticals and personal care products under study

Compound	CAS	Physicochemical parameters						Degradation rates				Ecotoxicity	Human Toxicity•		Bioaccumulation
		MW* g/mol	Kow**	Koc*** L kg^{-1}	K_H $^{+}$ Pa m^3 mol^{-1}	P_{vap} $^{++}$ Pa	Sol.$^{+++}$ mg L^{-1}	K_{degA} † s^{-1}	K_{degW} †† s^{-1}	K_{degSd} ††† s^{-1}	K_{degSl} †††† s^{-1}	$\alpha^{\ddagger}$ log mg L^{-1}	$ED_{50ing, NonCancer}$•• kg/lifetime/person	$ED_{50ing, Cancer}$•• kg/lifetime/person	BAF••• L kg^{-1}
Acetaminophen	103-90-2	151.17	2.88	45.09	$6.51\cdot10^{-8}$	$2.59\cdot10^{-4}$	14000	$2.65\cdot10^{-5}$	$5.30\cdot10^{-7}$	$5.89\cdot10^{-8}$	$2.65\cdot10^{-7}$	1.8800	673	--	0.98
Alprazolam	28981-97-7	308.77	131.82	98320	$5.19\cdot10^{-5}$	$2.21\cdot10^{-6}$	13.10	$1.14\cdot10^{-5}$	$2.10\cdot10^{-7}$	$2.33\cdot10^{-8}$	$1.05\cdot10^{-7}$	-0.4360	8.11	--	14.10
Amoxicillin	26787-78-0	365.41	7.41	108.40	$2.52\cdot10^{-16}$	$6.26\cdot10^{-15}$	4000.00	$2.08\cdot10^{-4}$	$2.10\cdot10^{-7}$	$2.33\cdot10^{-8}$	$1.05\cdot10^{-7}$	1.7800	1960	--	1.10
Atorvastatin	134523-00-5	558.66	2290867	28570	$4.64\cdot10^{-17}$	$9.26\cdot10^{-23}$	0.0011	$3.42\cdot10^{-4}$	$1.30\cdot10^{-7}$	$1.44\cdot10^{-8}$	$6.50\cdot10^{-8}$	-0.6160	30.70	1.23	104.00
Azithromycin	83905-01-5	749.00	10471	3135.00	$4.27\cdot10^{-18}$	$3.53\cdot10^{-22}$	0.0620	$6.35\cdot10^{-4}$	$4.50\cdot10^{-8}$	$5.00\cdot10^{-9}$	$2.25\cdot10^{-8}$	0.0669	16.20	--	12.50
Bromazepam	1812-30-2	316.16	112.20	3605.00	$4.57\cdot10^{-7}$	$2.53\cdot10^{-7}$	175.20	$8.68\cdot10^{-6}$	$1.30\cdot10^{-7}$	$1.44\cdot10^{-8}$	$6.50\cdot10^{-8}$	0.7080	103.00	--	9.94
Cefaclor	53994-73-3	367.81	2.24	104.30	$9.15\cdot10^{-13}$	$2.96\cdot10^{-13}$	10000	$2.02\cdot10^{-4}$	$2.10\cdot10^{-7}$	$2.33\cdot10^{-8}$	$1.05\cdot10^{-7}$	3.0600	2680.00	--	0.98
Ciprofloxacin	85721-33-1	331.35	1.91	10.00	$1.10\cdot10^{-12}$	$3.80\cdot10^{-11}$	30000	$4.70\cdot10^{-4}$	$1.30\cdot10^{-7}$	$1.44\cdot10^{-8}$	$6.50\cdot10^{-8}$	0.4450	506.00	--	0.98
Clarithormycin	81103-11-9	747.97	1445.44	149.40	$6.77\cdot10^{-20}$	$3.10\cdot10^{-23}$	0.3420	$5.97\cdot10^{-4}$	$4.50\cdot10^{-8}$	$5.00\cdot10^{-9}$	$2.25\cdot10^{-8}$	-0.1480	344.00	--	15.30
Enalapril	75847-73-3	376.46	1.17	348.50	$2.92\cdot10^{-10}$	$1.41\cdot10^{-10}$	16400	$1.77\cdot10^{-4}$	$5.30\cdot10^{-7}$	$5.89\cdot10^{-8}$	$2.65\cdot10^{-7}$	2.0000	353.00	--	0.916
Ethylparaben	120-47-8	166.18	295.12	162.18	$4.86\cdot10^{-4}$	$1.24\cdot10^{-2}$	885.00	$1.89\cdot10^{-5}$	$5.30\cdot10^{-7}$	$5.89\cdot10^{-8}$	$2.65\cdot10^{-7}$	0.8930	3930.00	--	8.15
Gabapentin	60142-96-3	171.24	0.0794	53.14	$9.42\cdot10^{-7}$	$3.91\cdot10^{-8}$	4491.00	$6.02\cdot10^{-5}$	$5.30\cdot10^{-7}$	$5.89\cdot10^{-8}$	$2.65\cdot10^{-7}$	3.5100	981.00	436.22	0.90
Iohexol	66108-95-0	821.15	0.0009	10.00	$4.17\cdot10^{-26}$	$5.41\cdot10^{-27}$	106.50	$1.04\cdot10^{-4}$	$1.30\cdot10^{-7}$	$1.44\cdot10^{-8}$	$6.50\cdot10^{-8}$	4.1000	29.70	--	0.89
Iopamidol	60166-93-0	777.09	0.0038	10.00	$2.26\cdot10^{-27}$	$1.78\cdot10^{-28}$	140000	$8.66\cdot10^{-5}$	$1.30\cdot10^{-7}$	$1.44\cdot10^{-8}$	$6.50\cdot10^{-8}$	3.1800	68.90	--	0.89
Irbesartan	138402-11-6	428.54	204173	1337000	$1.17\cdot10^{-9}$	$1.64\cdot10^{-13}$	0.0599	$5.58\cdot10^{-5}$	$2.10\cdot10^{-7}$	$2.33\cdot10^{-8}$	$1.05\cdot10^{-7}$	-1.3100	43.30	--	2480
Ketorolac	74103-06-3	255.28	208.93	428.80	$8.74\cdot10^{-6}$	$1.96\cdot10^{-5}$	572.30	$3.05\cdot10^{-4}$	$5.30\cdot10^{-7}$	$5.89\cdot10^{-8}$	$2.65\cdot10^{-7}$	0.6960	12.50	--	22.00
Levofloxacin	100986-85-4	361.38	0.4074	0.99	$1.68\cdot10^{-12}$	$1.31\cdot10^{-10}$	28260	$2.95\cdot10^{-4}$	$4.50\cdot10^{-8}$	$5.00\cdot10^{-9}$	$2.25\cdot10^{-8}$	0.9650	38.20	--	0.90
Lorazepam	846-49-1	321.16	245.47	944.40	$1.58\cdot10^{-9}$	$4.11\cdot10^{-10}$	80.00	$1.68\cdot10^{-5}$	$1.30\cdot10^{-7}$	$1.44\cdot10^{-8}$	$6.50\cdot10^{-8}$	0.8950	67.10	--	25.00
Methylparaben	99-76-3	152.15	91.20	86.29	$2.90\cdot10^{-3}$	$1.14\cdot10^{-1}$	2500.00	$1.66\cdot10^{-5}$	$5.30\cdot10^{-7}$	$5.89\cdot10^{-8}$	$2.65\cdot10^{-7}$	1.0600	4480.00	--	3.88
Norfloxacin	70458-96-7	319.34	0.0933	18.68	$1.99\cdot10^{-12}$	$1.11\cdot10^{-9}$	177900	$4.82\cdot10^{-4}$	$1.30\cdot10^{-7}$	$1.44\cdot10^{-8}$	$6.50\cdot10^{-8}$	1.0200	188.00	--	0.890
Omeprazole	073590-58-6	345.42	169.82	1455.00	$3.08\cdot10^{-14}$	$1.55\cdot10^{-9}$	82.28	$1.43\cdot10^{-4}$	$1.37\cdot10^{-8}$	$1.52\cdot10^{-9}$	$6.85\cdot10^{-9}$	-0.1690	21.70	3.49	3.46
Paroxetine	61869-08-7	329.37	8912.50	12360	$5.96\cdot10^{-5}$	$6.39\cdot10^{-6}$	35.27	$2.45\cdot10^{-4}$	$1.30\cdot10^{-7}$	$1.44\cdot10^{-8}$	$6.50\cdot10^{-8}$	-0.4580	3.93	4.36	624.00
Pregabalin	148553-50-8	159.23	0.0166	25.05	$2.19\cdot10^{-9}$	$2.69\cdot10^{-7}$	19630	$6.12\cdot10^{-5}$	$5.30\cdot10^{-7}$	$5.89\cdot10^{-8}$	$2.65\cdot10^{-7}$	3.7500	139.00	--	0.89
Propylparaben	94-13-3	180.21	1096.48	286.60	$1.39\cdot10^{-2}$	$4.09\cdot10^{-2}$	500.00	$2.11\cdot10^{-5}$	$5.30\cdot10^{-7}$	$5.89\cdot10^{-8}$	$2.65\cdot10^{-7}$	0.4330	39.30	--	15.60
Sertraline	79617-96-2	306.24	194984	170800	$1.36\cdot10^{-2}$	$1.56\cdot10^{-4}$	3.52	$1.47\cdot10^{-4}$	$1.30\cdot10^{-7}$	$1.44\cdot10^{-8}$	$6.50\cdot10^{-8}$	-0.4870	34.20	1.23	50800
Simvastatin	79902-63-9	418.58	47863	10940	$3.09\cdot10^{-7}$	$5.65\cdot10^{-10}$	0.0300	$3.44\cdot10^{-4}$	$2.10\cdot10^{-7}$	$2.33\cdot10^{-8}$	$1.05\cdot10^{-7}$	-0.3970	109.00	5.45	151.00
Valsartan	137862-53-4	435.53	4466.84	22630	$3.38\cdot10^{-11}$	$1.09\cdot10^{-13}$	1.41	$6.42\cdot10^{-5}$	$5.30\cdot10^{-7}$	$5.89\cdot10^{-8}$	$2.65\cdot10^{-7}$	0.2420	3.93	--	215.00

*Molecular weight.
**Partitioning coefficient between octanol and water.
***Partitioning coefficient between organic carbon and water.
$^{+}$ Henry law coefficient at 25ºC.
$^{++}$ Vapor pressure at 25ºC.
$^{+++}$ Solubility in water at 25ºC.
† Degradation rate in air.
†† Degradation rate in water. Results of BIOWIN3 → Biodegradation rates in USEtox™: Hours →$4.7\cdot10^{-5}$; hours to days → $6.4\cdot10^{-6}$; days →$3.4\cdot10^{-6}$; days to weeks → $9.3\cdot10^{-7}$; weeks→ $5.3\cdot10^{-7}$; weeks to months →$2.1\cdot10^{-7}$; months →$1.3\cdot10^{-7}$; recalcitrant →$4.5\cdot10^{-8}$.
††† Degradation rate in sediment.
†††† Degradation rate in soil.
‡ log of HC50 (HC50: Hazardous concentration of chemical at which 50% of the species are exposed above their EC_{50}. The EC_{50} is the water PPCP concentration at which 50% of a population displays an effect).
• According to the USEtox™ methodology the human toxicity is calculated for carcinogenic and non-carcinogenic effects and for inhalation and ingestion (exposure routes). This table only shows human toxicity for non-carcinogenic effect by ingestion, due to the lack of data of cancer effect and inhalation route. For more information see methodology and discussion section.
•• Daily dose (by ingestion) of PPCP that causes a disease (carcinogenic or non-carcinogenic) probability of 50% in a person in its lifetime.
••• Bioaccumulation factor of the chemical in fish.
-- : Negative for cancer or not available.

Data for human cancer have been found in lower quantities than for other effects, only for atorvastatin, gabapentin, omeprazole, paroxetine, sertraline, and simvastatin could be calculated the $ED_{50ing, Cancer}$.

According to USEtox™ methodology and software, both the human and ecotoxicity CFs were calculated with all these input data using standard matrix algebra. This means that each of the mentioned factors in the methodology (e.g. fate, exposure, and effect factors) is represented as matrices which are multiplied to obtain a CF matrix, as the final result. This optimizes calculation efficiency (i.e. only one model run for all emission scenarios), transparency, and interpretability of results (Huijbregts et al., 2010a)

3.1. Human Health effects

It is known that chemicals may pose hazards to organisms including humans, as indicated by observable effects (e.g. in vivo and in vitro bioassays). The application of bioassays indicating effects on cellular, organism or population level in laboratory test systems and linking measurable effects of complex environmental samples to different toxicants are required to bridge the gap between chemical contamination and ecological status (González et al. 2012). Different available databases include human health effects that generally are an approximation of bioassays in some typical species used for this purpose.

In addition, currently, there is a wide range of endpoints available from predictive quantitative structure–activity relationship (QSAR) models driven by many different computational software programs and data sources grouped under the term "in silico toxicology" (Valerio, 2009). These tools also are used for PPCPs that are already on the market and for estimated human effects.

In USEtox™, and therefore in this research, human toxicity includes carcinogenic and non-carcinogenic effects.

3.1.1. Carcinogenic effects

In this study, six compounds present evidence of carcinogenic effects (atorvastatin, gabapentin, omeprazole, paroxetine, sertraline and simvastatin) in rats or mice in a long term studies (chronic), fourteen have not evidence of carcinogenic effects and for eight compounds there were not data available (bromazepam, ciprofloxacin, clarithromycin, fluoxetine, iohexol, iopamidol, norfloxacin and valsartan) according to the information (ED_{50}) reported in Brambilla et al. (2012) and the database consulted and recommended by the USEtox™ users' manual.

The assessment of the carcinogenic potential of pharmaceuticals and evaluation of potential risk to humans is a major challenge for the scientific community, industry and regulatory agencies. The importance of reaching appropriate conclusions and balancing those conclusions with benefit, and the potential impact that those decisions may have on public health cannot be overstated (DeGeorge, 1998). Abraham and Ballinger (2012) affirm that human exposure to pharmaceuticals can cause cancer, so modern societies have assessed the carcinogenicity of new drugs since the 1960s.

Recent studies provide evidence of the carcinogenic effect of some PPCPs. In October, 2008, 21 scientists from nine countries met at the International Agency for Research on Cancer (IARC) and reaffirmed the Group 1 classification "carcinogenic to humans" of 20 pharmaceutical agents (this group included estrogens, analgesic mixtures with phenacetin and antineoplastic drugs) (Grosse et a l. 2009). Brambilla and Martelli (2009) did a compendium of genotoxicity and carcinogenicity information of 838

marketed drugs, whose expected clinical use is continuous for at least 6 months or intermittent over an extended period of time. Of these 838 drugs they founded that 366 (43.7%) do not have retrievable genotoxicity or carcinogenicity data. The remaining 472 (56.3%) have at least one genotoxicity or carcinogenicity test result, a fairly high percentage for this type of chemical compounds. This information supports the evidence of cancer in some of the compounds under study.

The traditional approach to carcinogenicity test for pharmaceuticals is relatively standardized. It relied on testing at the maximum tolerated dose (MTD) and, usually, in two rodent species for 2 years. The results of these studies were viewed as either positive or negative, with only minimal attempts to evaluate the relevancy of the findings for humans (DeGeorge, 1998). In this sense, Abraham and Ballinger (2012) worked on the validation and application of new techno-regulatory testing standards, specifically use of genetically-engineered mouse (GEM) models in pharmaceutical carcinogenic risk assessment, this methodology focuses on the replacement of long-term carcinogenicity tests in rodents (especially mice) with shorter-term tests involving genetically-engineered mice. This methodology or other more traditional, experimental or not, may be used to know or predict the possible carcinogenic potential of PPCPs and for including carcinogenic data in studies of environmental risk/hazard assessment as LCIA.

Despite of this, there are not enough studies that evidence if many PPCP are or are not carcinogenic and the minimum doses which cause this adverse effect. In this research, there was lack of data of carcinogenic effects for 30% of PPCPs under study.

3.1.2. Non-carcinogenic effects

PPCPs must undergo through strict controls to approve their use in animals and humans, non-carcinogenic effects on human or non human are some of these important data that must be reported for the safe use of these compounds. In general the animal data used for making judgments with respect to the registration of drugs is frequently augmented with documentation of effects that have been observed in clinical practice. This information may be gathered in clinical trials or through the US Food and Drug Administration's (US FDA's) adverse drug reaction reporting system (Bull et al. 2011).

In this study, for all PPCPs, non-carcinogenic data were available as NOAEL or LOAEL and were reported for different species (mouse, rabbit, rat, dog and monkey) and for different times of exposure (sub-acute, sub-chronic and chronic). These data were converted to the chronic-equivalent ED50 by using Eq. 15 and 16 (Table 2). Ingestion route was only considered due to the lack of data for other routes of exposition as inhalation.

Affectation on liver, kidney, testicle, lung, eyes and central nervous system and symptoms as: sedation, ataxia, convulsive seizures, abnormal secretion of sex hormones, decreasing in blood pressure, hyperplasia of the juxtaglomerular apparatus, pallor, hematologic and pathologic alterations, benign tumors, cardiovascular malformations, embryotoxicity and teratogenicity were the main adverse effects reported in the literature consulted and cited in the material and methods section for this parameter.

3.1.3. Human health CF for PPCPs under study

Table 3 show carcinogenic and non-carcinogenic ED_{50} used to calculate human health CFs. These CFs estimated by

USEtox™ have been calculated for different media of release: continental urban air, continental rural air, continental freshwater, continental sea water, continental natural soil and continental agricultural soil. Carcinogenic and non-carcinogenic CFs are summed as well, assuming equal weighting between cancer and non-cancer effects in those compounds where both effects were present. This results in a single characterization factor per emission compartment (Huijbregts et al., 2010a) as cases $kg_{emitted}^{-1}$ shown in Table 4 for each PPCP.

Emission to continental freshwater compartment showed the highest CFs of human health for most compounds ranging on 10^{-9} to 10^{-3} followed by air compartment (urban and rural that had values in the same order: 10^{-9} to 10^{-5}), soil compartment (agricultural and natural) in the order of 10^{-11} to 10^{-5} and finally, continental sea water in the order of 10^{-12} to 10^{-4}. These results indicate the relative order of importance of different PPCP emissions and to what grade (magnitude) can affect the human health, being the drug emission to continental freshwater the most important compartment.

Table 4. Human health characterization factor for PPCPs under study

Compound	Human health characterization factor (cases $kg_{emitted}^{-1}$)[a]					
	ECUair*	ECRair**	ECFW***	ECSW+	ECNS++	ECAS+++
Acetaminophen	$5.6 \cdot 10^{-9}$	$6.00 \cdot 10^{-9}$	$2.90 \cdot 10^{-8}$	$5.40 \cdot 10^{-12}$	$3.40 \cdot 10^{-9}$	$6.10 \cdot 10^{-9}$
Alprazolam	$1.39 \cdot 10^{-6}$	$1.50 \cdot 10^{-6}$	$2.12 \cdot 10^{-6}$	$1.41 \cdot 10^{-8}$	$6.31 \cdot 10^{-10}$	$1.80 \cdot 10^{-9}$
Amoxicillin	$1.75 \cdot 10^{-8}$	$1.80 \cdot 10^{-8}$	$2.11 \cdot 10^{-8}$	$4.84 \cdot 10^{-12}$	$2.74 \cdot 10^{-9}$	$4.50 \cdot 10^{-9}$
Atorvastatin	$5.10 \cdot 10^{-5}$	$5.28 \cdot 10^{-5}$	$4.72 \cdot 10^{-5}$	$9.47 \cdot 10^{-7}$	$6.10 \cdot 10^{-8}$	$2.82 \cdot 10^{-6}$
Azithromycin	$4.60 \cdot 10^{-6}$	$4.72 \cdot 10^{-6}$	$6.11 \cdot 10^{-6}$	$2.10 \cdot 10^{-8}$	$1.76 \cdot 10^{-7}$	$9.44 \cdot 10^{-7}$
Bromazepam	$1.99 \cdot 10^{-7}$	$2.06 \cdot 10^{-7}$	$5.60 \cdot 10^{-7}$	$1.25 \cdot 10^{-9}$	$5.08 \cdot 10^{-9}$	$9.60 \cdot 10^{-9}$
Cefaclor	$1.24 \cdot 10^{-8}$	$1.28 \cdot 10^{-8}$	$1.54 \cdot 10^{-8}$	$3.17 \cdot 10^{-12}$	$2.06 \cdot 10^{-9}$	$3.02 \cdot 10^{-9}$
Ciprofloxacin	$1.11 \cdot 10^{-7}$	$1.15 \cdot 10^{-7}$	$1.13 \cdot 10^{-7}$	$2.51 \cdot 10^{-11}$	$4.46 \cdot 10^{-8}$	$6.17 \cdot 10^{-8}$
Clarithormycin	$2.92 \cdot 10^{-7}$	$3.01 \cdot 10^{-7}$	$3.14 \cdot 10^{-7}$	$1.21 \cdot 10^{-9}$	$8.60 \cdot 10^{-8}$	$1.95 \cdot 10^{-7}$
Enalapril	$6.58 \cdot 10^{-9}$	$6.92 \cdot 10^{-9}$	$5.55 \cdot 10^{-8}$	$9.64 \cdot 10^{-12}$	$1.20 \cdot 10^{-9}$	$1.98 \cdot 10^{-9}$
Ethylparaben	$1.14 \cdot 10^{-9}$	$1.21 \cdot 10^{-9}$	$5.33 \cdot 10^{-9}$	$7.73 \cdot 10^{-12}$	$2.32 \cdot 10^{-10}$	$7.27 \cdot 10^{-10}$
Gabapentin	$5.86 \cdot 10^{-9}$	$6.57 \cdot 10^{-9}$	$6.53 \cdot 10^{-8}$	$1.11 \cdot 10^{-11}$	$6.83 \cdot 10^{-9}$	$1.01 \cdot 10^{-8}$
Iohexol	$1.93 \cdot 10^{-6}$	$2.00 \cdot 10^{-6}$	$1.93 \cdot 10^{-6}$	$3.89 \cdot 10^{-10}$	$7.59 \cdot 10^{-7}$	$8.81 \cdot 10^{-7}$
Iopamidol	$8.32 \cdot 10^{-7}$	$8.59 \cdot 10^{-7}$	$8.30 \cdot 10^{-7}$	$1.68 \cdot 10^{-10}$	$3.27 \cdot 10^{-7}$	$3.79 \cdot 10^{-7}$
Irbesartan	$7.28 \cdot 10^{-7}$	$7.49 \cdot 10^{-7}$	$9.36 \cdot 10^{-7}$	$2.57 \cdot 10^{-7}$	$9.90 \cdot 10^{-11}$	$1.77 \cdot 10^{-9}$
Ketorolac	$4.62 \cdot 10^{-8}$	$3.61 \cdot 10^{-8}$	$1.87 \cdot 10^{-6}$	$6.56 \cdot 10^{-9}$	$3.33 \cdot 10^{-8}$	$8.94 \cdot 10^{-8}$
Levofloxacin	$2.17 \cdot 10^{-6}$	$2.26 \cdot 10^{-6}$	$2.51 \cdot 10^{-6}$	$6.45 \cdot 10^{-10}$	$1.20 \cdot 10^{-6}$	$1.41 \cdot 10^{-6}$
Lorazepam	$5.42 \cdot 10^{-7}$	$5.52 \cdot 10^{-7}$	$1.02 \cdot 10^{-6}$	$4.81 \cdot 10^{-9}$	$3.30 \cdot 10^{-8}$	$6.14 \cdot 10^{-8}$
Methylparaben	$1.04 \cdot 10^{-9}$	$1.09 \cdot 10^{-9}$	$4.51 \cdot 10^{-9}$	$3.27 \cdot 10^{-12}$	$3.36 \cdot 10^{-10}$	$8.13 \cdot 10^{-10}$
Norfloxacin	$1.20 \cdot 10^{-7}$	$1.25 \cdot 10^{-7}$	$3.05 \cdot 10^{-7}$	$6.17 \cdot 10^{-11}$	$1.09 \cdot 10^{-7}$	$1.28 \cdot 10^{-7}$
Omeprazole	$3.61 \cdot 10^{-5}$	$3.71 \cdot 10^{-5}$	$4.25 \cdot 10^{-5}$	$5.39 \cdot 10^{-8}$	$6.38 \cdot 10^{-6}$	$1.09 \cdot 10^{-5}$
Paroxetine	$2.92 \cdot 10^{-6}$	$1.09 \cdot 10^{-6}$	$1.46 \cdot 10^{-4}$	$3.83 \cdot 10^{-6}$	$4.06 \cdot 10^{-7}$	$9.01 \cdot 10^{-7}$
Pregabalin	$1.35 \cdot 10^{-8}$	$1.54 \cdot 10^{-8}$	$1.42 \cdot 10^{-7}$	$2.40 \cdot 10^{-11}$	$2.34 \cdot 10^{-8}$	$3.44 \cdot 10^{-8}$
Propylparaben	$1.03 \cdot 10^{-7}$	$1.09 \cdot 10^{-7}$	$5.62 \cdot 10^{-7}$	$1.50 \cdot 10^{-9}$	$1.58 \cdot 10^{-8}$	$7.36 \cdot 10^{-8}$
Sertraline	$6.70 \cdot 10^{-5}$	$1.18 \cdot 10^{-5}$	$4.77 \cdot 10^{-3}$	$4.85 \cdot 10^{-4}$	$1.62 \cdot 10^{-6}$	$2.03 \cdot 10^{-6}$
Simvastatin	$4.32 \cdot 10^{-6}$	$4.31 \cdot 10^{-6}$	$1.55 \cdot 10^{-5}$	$2.48 \cdot 10^{-7}$	$3.01 \cdot 10^{-8}$	$5.20 \cdot 10^{-7}$
Valsartan	$3.86 \cdot 10^{-6}$	$3.85 \cdot 10^{-6}$	$1.18 \cdot 10^{-5}$	$2.01 \cdot 10^{-10}$	$4.62 \cdot 10^{-9}$	$2.56 \cdot 10^{-8}$

[a] Total for cancer and non-cancer effect for those compounds that exhibit both effects (See Table 3).
*Emission to continental urban air.
**Emission to continental rural air.
***Emission to continental freshwater.
+Emission to continental sea water.
++Emission to continental natural soil.
+++Emission to continental agricultural soil.

PPCPs listed in Table 4 can be compared for each emission compartment if it is considered that all compounds are emitted in the same quantity. In this case CF can be used as a final result for prioritizing substance under the same condition of emission. Omeprazole, atorvastatin, sertraline, azithromycin and simvastatin are the compounds that highlight with the highest CFs under this assumption. Nevertheless in nature or in industrial processes, the emission in each compartment and for each compound is different and variable which imply that this amount emitted must be known for generating a human hazard ranking score under real conditions.

3.2. Ecotoxicological effects

Ecotoxicological effects of PPCPs on environment are an aspect that has had a lot of interest in recent years due to the detection of these compounds in nature. Some authors (Brausch and Rand, 2011; Cleuvers, 2003, 2004; Daughton and Brooks, 2010; Fent et al., 2006; Sanderson et al., 2004a; Santos et al., 2010; Vasquez and Fatta-Kassinos, 2013) have reported ecotoxicogical data from different types of assays (for single PPCP or their mixtures) including different species (trophic levels), times of exposure (chronic, subchronic or acute) and endpoints (EC_{50}, LC_{50}, NOAEL, LOAEL). QSARs methodologies also have been used to estimate these environmental impacts as was used in Carlsson et al. (2006), Cronin et al., (2003), Freidig et al. (2007), Ortiz et al. (2013b), Sanderson et al. (2004a, 2004b), Ursem et al. (2009), among other researches.

At the moment, ecotoxicological effects (from bioassays or estimated by software) are always taking into account to evaluated the effects of PPCPs in the environmental. Specifically for LCIA many different categories exist to measure the impact of contaminant substances in the nature. Carvalho et al. (2014) verified a total of 167 impact categories presented in 25 methods that they have been studied and which they have been classified into 3 main classes (ecological, human health and resources). They verified that the majority of the methods gave more importance to the ecological class. This is the case of USEtox™ model which calculate the freshwater aquatic ecotoxicological CFs when the compound under study has been released to different environmental compartments (air, water and soil). Table 5 shows the results of these PPCPs' CFs

Emissions to continental urban and rural air presented CFs among 10^{-2} to 10^{4}, continental freshwater between 1 to 10^{4}, continental sea water among 10^{-28} to 10^{-3} and continental natural soil and continental agricultural soil in the same order between 10^{-1} to 10^{4}, being the highest CFs those from continental freshwater due to the direct contact between the source of emission and the compartment affected. CFs of continental sea water as emission source are the lowest, probably by the difficulty in the inter-compartment transfer (continental sea water to continental fresh water) and therefore, the low bioavailability of the compounds in fresh water from sea water. These phenomena were considered in calculations in the fate factor and the exposure factor.

Freshwater aquatic ecotoxicological CFs are much higher than human health CFs due to the low tolerance of aquatic organisms to these compounds and the persistence of them in this media.

The results of Table 4 and 5 are in the same order as those calculated in other research (Alfonsín et al. 2014) and in USEtox™ database for other PPCPs. There are few studies that calculate CFs for PPCPs to include them in LCIA studies. LCIA in WWTPs are researches more generalized but these studies are not complete if PPCPs are excluded.

Table 5. Freshwater aquatic ecotoxicological characterization factor for pharmaceuticals and personal care products

Compound	Ecotoxicological characterization factor (CTU[a] = PAF m^3 day kg^{-1})					
	ECUair*	ECRair**	ECFW***	ECSW^{+}	ECNS^{++}	ECAS^{+++}
Acetaminophen	5.07	3.84	$1.25\cdot10^{2}$	$4.09\cdot10^{-9}$	$1.47\cdot10^{1}$	$1.47\cdot10^{1}$
Alprazolam	$5.39\cdot10^{2}$	$2.67\cdot10^{2}$	$2.01\cdot10^{4}$	$3.94\cdot10^{-4}$	5.99	5.99
Amoxicillin	$4.37\cdot10^{1}$	$4.04\cdot10^{1}$	$3.33\cdot10^{2}$	$2.94\cdot10^{-16}$	$4.32\cdot10^{1}$	$4.32\cdot10^{1}$
Atorvastatin	$1.76\cdot10^{3}$	$1.10\cdot10^{3}$	$4.53\cdot10^{4}$	$1.62\cdot10^{-15}$	$5.85\cdot10^{1}$	$5.85\cdot10^{1}$
Azithromycin	$2.19\cdot10^{3}$	$1.66\cdot10^{3}$	$3.68\cdot10^{4}$	$5.59\cdot10^{-16}$	$1.06\cdot10^{3}$	$1.06\cdot10^{3}$
Bromazepam	$1.70\cdot10^{2}$	$1.02\cdot10^{2}$	$5.00\cdot10^{3}$	$2.12\cdot10^{-6}$	$4.53\cdot10^{1}$	$4.53\cdot10^{1}$
Cefaclor	2.27	2.10	$1.71\cdot10^{1}$	$5.53\cdot10^{-14}$	2.28	2.28
Ciprofloxacin	$3.05\cdot10^{3}$	$3.04\cdot10^{3}$	$9.84\cdot10^{3}$	$1.52\cdot10^{-10}$	$3.88\cdot10^{3}$	$3.88\cdot10^{3}$
Clarithormycin	$1.52\cdot10^{4}$	$1.49\cdot10^{4}$	$6.46\cdot10^{4}$	$7.95\cdot10^{-17}$	$1.77\cdot10^{4}$	$1.77\cdot10^{4}$
Enalapril	2.01	$8.31\cdot10^{-1}$	$9.40\cdot10^{1}$	$2.92\cdot10^{-12}$	2.04	2.04
Ethylparaben	$3.85\cdot10^{1}$	$2.42\cdot10^{1}$	$1.21\cdot10^{3}$	$1.87\cdot10^{-4}$	$5.23\cdot10^{1}$	$5.23\cdot10^{1}$
Gabapentin	$7.94\cdot10^{-2}$	$4.87\cdot10^{-2}$	2.94	$7.22\cdot10^{-10}$	$3.08\cdot10^{-1}$	$3.08\cdot10^{-1}$
Iohexol	$6.97\cdot10^{-1}$	$6.96\cdot10^{-1}$	2.17	$9.73\cdot10^{-28}$	$8.54\cdot10^{-1}$	$8.54\cdot10^{-1}$
Iopamidol	5.85	5.84	$1.82\cdot10^{1}$	$4.50\cdot10^{-28}$	7.17	7.17
Irbesartan	$6.39\cdot10^{2}$	$3.94\cdot10^{2}$	$1.68\cdot10^{4}$	$6.56\cdot10^{-9}$	1.78	1.78
Ketorolac	$3.48\cdot10^{1}$	6.24	$1.89\cdot10^{3}$	$7.50\cdot10^{-7}$	$3.37\cdot10^{1}$	$3.37\cdot10^{1}$
Levofloxacin	$1.67\cdot10^{3}$	$1.68\cdot10^{3}$	$4.99\cdot10^{3}$	$2.83\cdot10^{-10}$	$2.38\cdot10^{3}$	$2.38\cdot10^{3}$
Lorazepam	$2.12\cdot10^{2}$	$1.66\cdot10^{2}$	$3.42\cdot10^{3}$	$1.20\cdot10^{-8}$	$1.11\cdot10^{2}$	$1.11\cdot10^{2}$
Methylparaben	$3.24\cdot10^{1}$	$2.34\cdot10^{1}$	$8.27\cdot10^{2}$	$1.11\cdot10^{-3}$	$6.02\cdot10^{1}$	$6.02\cdot10^{1}$
Norfloxacin	$3.68\cdot10^{2}$	$3.59\cdot10^{2}$	$2.64\cdot10^{3}$	$3.27\cdot10^{-11}$	$9.42\cdot10^{2}$	$9.42\cdot10^{2}$
Omeprazole	$1.29\cdot10^{4}$	$1.21\cdot10^{4}$	$8.84\cdot10^{4}$	$8.25\cdot10^{-11}$	$1.33\cdot10^{4}$	$1.33\cdot10^{4}$
Paroxetine	$1.10\cdot10^{3}$	$1.59\cdot10^{2}$	$6.25\cdot10^{4}$	$4.24\cdot10^{-4}$	$1.74\cdot10^{2}$	$1.74\cdot10^{2}$
Pregabalin	$5.36\cdot10^{-2}$	$3.90\cdot10^{-2}$	1.67	$1.37\cdot10^{-12}$	$2.76\cdot10^{-1}$	$2.76\cdot10^{-1}$
Propylparaben	$8.11\cdot10^{1}$	$4.22\cdot10^{1}$	$3.44\cdot10^{3}$	$9.06\cdot10^{-3}$	$8.88\cdot10^{1}$	$8.88\cdot10^{1}$
Sertraline	$2.43\cdot10^{2}$	$2.75\cdot10^{1}$	$1.80\cdot10^{4}$	$1.50\cdot10^{-2}$	6.11	6.11
Simvastatin	$1.39\cdot10^{3}$	$8.08\cdot10^{2}$	$4.08\cdot10^{4}$	$6.79\cdot10^{-6}$	$7.92\cdot10^{1}$	$7.92\cdot10^{1}$
Valsartan	$1.67\cdot10^{2}$	$1.03\cdot10^{2}$	$4.37\cdot10^{3}$	$3.96\cdot10^{-11}$	1.71	1.71

[a]Comparative toxic units
*Emission to continental urbar air.
**Emission to continental rural air.
***Emission to continental freshwater.
$^{+}$Emission to continental sea water.
$^{++}$Emission to continental natural soil.
$^{+++}$Emission to continental agricultural soil.

In the last years, some of these micro pollutants have been incorporated in LCIA studies of wastewater systems, pharmaceutical industry or as contaminants in the different compartments of environmental (Alfonsín et al., 2014; Igos et al., 2012; Jiménez-González et al., 2004; Loubet et al., 2014; Muñoz et al., 2008; Niero et al., 2014; Ramasamy et al., 2014) but still there is too much to investigate. More compounds have to be incorporated in the different database of LCA methods as well as other relevant methods for environmental risk/hazard assessments. New experimental data of impact of PPCPs in nature and QSAR applied in this field must be improved continuously.

Ecotoxicological and human effects of mixtures (synergistic or antagonistic) is another important aspect to be considered and must be annexed in risk/hazard environmental assessments since it has been found that mixture behavior and the possible negative effects are different (in many cases more pronounced) that the effects of single compounds; e.g., Mater et al. (2014) did in vitro tests aiding ecological risk assessment of ciprofloxacin, tamoxifen and cyclophosphamide and they found that an

individual drug does not induce any DNA breaks on hepatic cells, whereas a mixture leads to a dose dependent increase of DNA breaks, Cleuvers (2003) found that tests with combinations of various pharmaceuticals generated stronger effects than those expected from single compounds.

According to Vasquez et al. (2014) the assessment of the toxicity of pharmaceutical mixtures is both an urgent need and a great challenge to achieve more progressive and proactive risk assessment. Kortenkamp et al. (2009) indicated that there is a consensus in the field of mixture toxicology that the customary chemical-by-chemical approach to risk assessment might be too simplistic. The risk of chemicals to human health and to the environment can be underestimated. Therefore, these new findings will have to be included in all environmental impact assessment methodologies as LCIA.

3.3. Impact score of PPCPs

The growing public awareness of the importance of protecting both ecosystems and human health from the risks associated to chemical exposure has given rise to the development of an increasingly important body of regulations in the last several years, especially in developed countries. In this context, risk assessment (and hence the elaboration of priority lists of chemical substances) provides the necessary scientific basis for more regulations (Guillen et al. 2012). Therefore, in this study an impact score for common and extensively PPCPs used worldwide has been done from the CFs available in the database of USEtox™, the USEtox™ CFs available in the literature (Alfonsín et al., 2014), and the CFs newly calculated in this research. The mass emitted into the air, freshwater and soil, in Spain, in a year (estimated in a previous study: Ortiz et al., 2013a) was used to obtained the impact score (human and ecotoxicological) in CTU $year^{-1}$.

The results are shown in Table 6 and in Figures 1 and 2. The compounds with highest impact score are also concern compounds in other rankings done by different methodologies. Ortiz et al. (2013b) did a ranking of concern considered occurrence, persistence, bioaccumulation and toxicity as environmental and toxicological indexes, they found that hormones, antidepressants, blood lipid regulators and personal care products were at the highest levels of risk (similar that in this new ranking). In the first 20 priority compounds ranked by Kumar and Xagoraraki (2010) appear some coincident compounds with this study: 17β-Estradiol, estrone, carbamazepine, azithromycin and flouxetine.

The present results also match with the ranking of Sanderson et al. (2004b) in gastrointestinal drugs (e.g omeprazole) and hormones. Muñoz et al. (2008) did a ranking potential impacts of priority and emerging pollutants in urban wastewater through LCIA, they used two characterization models (EDIP97 and USES-LCA) and found that PPCPs are very important contributors to toxicity in WWTPs being ciprofloxacin, fluoxetine and nicotine (not considered in this study) the main PPCPs of concern. In the present study these two compounds also appear in the top 20 of ecotoxicological potential impact. Cooper et al. (2008) indicate that anti-infective (antibiotics) may pose the greatest overall risk based upon their results using a combination of factors that measure environmental transport, fate, and aquatic toxicity.

In this study most antibiotics are located in the top 20 of the ecotoxicity impact score and for human toxicity impact score azithromycin and levofloxacin are in the top 10.

Although it is not surprising that some of the compounds studied in this research occupy the top ranking (by previous researches) even their CFs was not known. The estimation of new CFs should be continued, either for compounds that are already marketed as for the new ones.

Table 6. Impact score of PPCPs from human health and ecotoxicity characterization factors and

Compound	Human health characterization factor (CTU^{+}_{h} kg^{-1})* and mass emitted in the different compartments (kg $year^{-1}$)**						Human Toxicity impact score (CTU^{+}_{h} $year^{-1}$)	Ecotoxicity characterization factor (CTU^{+}_{e} kg^{-1})* and mass emitted in the different compartments (kg $year^{-1}$)**						Ecotoxicity impact score (CTU^{+}_{e} $year^{-1}$)
	ECUair*,a	M_{Air}**	ECFW*,b	M_{water}**	ECNS*,c	M_{Soil}**		ECUair *,a	M_{Air}**	ECFW*,b	M_{water}**	ECNS*,c	M_{Soil}**	
17α-ethinylestradiol	$6.79\cdot10^{-2}$	na	$2.45\cdot10^{-2}$	0.29	$3.02\cdot10^{-6}$	0.66	$7.11\cdot10^{-3}$	$3.02\cdot10^{4}$	na	$1.69\cdot10^{6}$	0.29	$2.56\cdot10^{5}$	0.66	$6.59\cdot10^{5}$
17β-estradiol	$7.76\cdot10^{-4}$	na	$2.18\cdot10^{-3}$	69.12	$3.02\cdot10^{-6}$	54.86	$1.51\cdot10^{-1}$	$3.30\cdot10^{6}$	na	$1.84\cdot10^{8}$	69.12	2.56	54.86	$1.27\cdot10^{10}$
Acetaminophen	$5.6\cdot10^{-9}$	na	2.90·10-8	23267	$3.40\cdot10^{-9}$	453155	$2.22\cdot10^{-3}$	5.07	na	$1.25\cdot10^{2}$	23267	$1.47\cdot10^{1}$	453155	$9.57\cdot10^{6}$
Alprazolam	$1.39\cdot10^{-6}$	na	$2.12\cdot10^{-6}$	60.21	$6.31\cdot10^{-10}$	1.69	$1.28\cdot10^{-4}$	$5.39\cdot10^{2}$	na	$2.01\cdot10^{4}$	60.21	5.99	1.69	$1.21\cdot10^{6}$
Amoxicillin	$1.75\cdot10^{-8}$	na	$2.11\cdot10^{-8}$	15257	$2.74\cdot10^{-9}$	101325	$5.99\cdot10^{-4}$	$4.37\cdot10^{1}$	na	$3.33\cdot10^{2}$	15257	$4.32\cdot10^{1}$	101325	$9.45\cdot10^{6}$
Atorvastatin	$5.10\cdot10^{-5}$	na	$4.72\cdot10^{-5}$	715.39	$6.10\cdot10^{-8}$	1145.81	$3.38\cdot10^{-2}$	$1.76\cdot10^{3}$	na	$4.53\cdot10^{4}$	715.39	$5.85\cdot10^{1}$	1145.81	$3.24\cdot10^{7}$
Azithromycin	$4.60\cdot10^{-6}$	na	$6.11\cdot10^{-6}$	1933.32	$1.76\cdot10^{-7}$	958.03	$1.20\cdot10^{-2}$	$2.16\cdot10^{3}$	na	$3.68\cdot10^{4}$	1933.32	$1.06\cdot10^{3}$	958.03	$7.22\cdot10^{7}$
Bromazepam	$1.99\cdot10^{-7}$	na	$5.60\cdot10^{-7}$	100.13	$5.08\cdot10^{-9}$	2.72	$5.61\cdot10^{-5}$	$1.70\cdot10^{2}$	na	$5.00\cdot10^{3}$	100.13	$4.53\cdot10^{1}$	2.72	$5.01\cdot10^{5}$
Carbamazepine	$2.08\cdot10^{-6}$	na	$7.68\cdot10^{-6}$	2595.31	$1.12\cdot10^{-7}$	1204.28	$2.01\cdot10^{-2}$	$1.65\cdot10^{1}$	na	$8.54\cdot10^{2}$	2595.31	$1.25\cdot10^{1}$	1204.28	$2.23\cdot10^{6}$
Cefaclor	$1.24\cdot10^{-8}$	na	$1.54\cdot10^{-8}$	119.60	$2.06\cdot10^{-9}$	2.62	$1.85\cdot10^{-6}$	2.27	na	$1.71\cdot10^{1}$	119.60	2.28	2.62	$2.05\cdot10^{3}$
Ciprofloxacin	$1.11\cdot10^{-7}$	na	$1.13\cdot10^{-7}$	2402.03	$4.46\cdot10^{-8}$	12957	$8.50\cdot10^{-4}$	$3.05\cdot10^{3}$	na	$9.84\cdot10^{3}$	2402.03	$3.88\cdot10^{3}$	12957	$7.39\cdot10^{7}$
Clarithormycin	$2.92\cdot10^{-7}$	na	$3.14\cdot10^{-7}$	5820.23	$8.60\cdot10^{-8}$	1669.22	$1.97\cdot10^{-3}$	$1.52\cdot10^{4}$	na	$6.46\cdot10^{4}$	5820.23	$1.77\cdot10^{4}$	1669.22	$4.05\cdot10^{8}$
Clofibrate	$2.07\cdot10^{-7}$	$1.11\cdot10^{-2}$	$3.67\cdot10^{-7}$	2.45	$2.58\cdot10^{-8}$	0.56	$9.16\cdot10^{-7}$	na	$1.11\cdot10^{-2}$	na	2.45	na	0.56	na
Cyclophosphamide	$2.45\cdot10^{-6}$	na	$7.33\cdot10^{-6}$	9.78	$1.03\cdot10^{-6}$	119.86	$1.95\cdot10^{-4}$	na	na	na	9.78	na	119.86	na
Diclofenac	$3.08\cdot10^{-7}$	na	$1.22\cdot10^{-6}$	3963.10	$4.84\cdot10^{-8}$	6613.79	$5.16\cdot10^{-3}$	$5.03\cdot10^{1}$	na	$2.67\cdot10^{3}$	3963.10	$1.05\cdot10^{2}$	6613.79	$1.13\cdot10^{7}$
Enalapril	$6.58\cdot10^{-9}$	na	$5.55\cdot10^{-8}$	725.20	$1.20\cdot10^{-9}$	1188.09	$4.17\cdot10^{-5}$	2.01	na	$9.40\cdot10^{1}$	725.20	2.04	1188.09	$7.06\cdot10^{4}$
Erythromycin	na	na	na	910.75	na	116.94	na	$3.22\cdot10^{3}$	na	$2.49\cdot10^{4}$	910.75	$3.15\cdot10^{3}$	116.94	$2.30\cdot10^{7}$
Estrone	$2.64\cdot10^{-4}$	na	$3.17\cdot10^{-4}$	28.22	$5.37\cdot10^{-7}$	153.00	$9.03\cdot10^{-3}$	$4.39\cdot10^{1}$	na	$2.14\cdot10^{4}$	28.22	$1.93\cdot10^{1}$	153.00	$6.07\cdot10^{5}$
Ethylparaben	$1.14\cdot10^{-9}$	na	$5.33\cdot10^{-9}$	na	$2.32\cdot10^{-10}$	na	na	$3.85\cdot10^{1}$	na	$1.21\cdot10^{3}$	na	$5.23\cdot10^{1}$	na	na
Fluoxetine	$2.49\cdot10^{-5}$	na	$2.6\cdot10^{-5}$	324.51	na	125.92	$8.44\cdot10^{-3}$	$3.82\cdot10^{2}$	na	$4.64\cdot10^{4}$	324.51	$7.32\cdot10^{1}$	125.92	$1.51\cdot10^{7}$
Gabapentin	$5.86\cdot10^{-9}$	na	$6.53\cdot10^{-8}$	1943.87	$6.83\cdot10^{-9}$	47406	$4.51\cdot10^{-4}$	$7.94\cdot10^{-2}$	na	2.94	1943.87	$3.08\cdot10^{-1}$	47406	$2.03\cdot10^{4}$
Galaxolide	$6.95\cdot10^{-7}$	na	$5.00\cdot10^{-7}$	69221	$4.69\cdot10^{-9}$	102389	$3.51\cdot10^{-2}$	$2.19\cdot10^{1}$	na	$1.01\cdot10^{4}$	69221	$1.72\cdot10^{1}$	102389	$7.01\cdot10^{8}$
Ibuprofen	$4.16\cdot10^{-7}$	6.74	$3.71\cdot10^{-7}$	4849.50	$1.74\cdot10^{-8}$	87853	$3.33\cdot10^{-3}$	3.25	6.74	$2.09\cdot10^{2}$	4849.50	3.65	87853	$1.33\cdot10^{6}$
Iohexol	$1.93\cdot10^{-6}$	na	$1.93\cdot10^{-6}$	5127.22	$7.59\cdot10^{-7}$	4691.52	$1.34\cdot10^{-2}$	$7.00\cdot10^{-1}$	na	2.17	5127.22	$8.54\cdot10^{-1}$	4691.52	$1.51\cdot10^{4}$
Iopamidol	$8.32\cdot10^{-7}$	na	$8.30\cdot10^{-7}$	11416	$3.27\cdot10^{-7}$	1296.93	$9.90\cdot10^{-3}$	5.90	na	$1.82\cdot10^{1}$	11416	7.17	1296.93	$2.17\cdot10^{5}$
Iopromide	$2.29\cdot10^{-7}$	na	$1.86\cdot10^{-7}$	14752	$7.29\cdot10^{-8}$	6202.81	$3.20\cdot10^{-3}$	5.57	na	$1.74\cdot10^{1}$	14752	6.82	6202.81	$2.99\cdot10^{5}$
Irbesartan	$7.28\cdot10^{-7}$	na	$9.36\cdot10^{-7}$	3810.87	$9.90\cdot10^{-11}$	23076	$3.57\cdot10^{-3}$	$6.39\cdot10^{2}$	na	$1.68\cdot10^{4}$	3810.87	1.78	23076	$6.42\cdot10^{7}$
Ketorolac	$4.62\cdot10^{-8}$	na	$1.87\cdot10^{-6}$	217.64	$3.33\cdot10^{-8}$	6.94	$4.06\cdot10^{-4}$	$3.48\cdot10^{1}$	na	$1.89\cdot10^{3}$	217.64	$3.37\cdot10^{1}$	6.94	$4.12\cdot10^{5}$
Levofloxacin	$2.17\cdot10^{-6}$	na	$2.51\cdot10^{-6}$	4041.49	$1.20\cdot10^{-6}$	87.97	$1.03\cdot10^{-2}$	$1.67\cdot10^{3}$	na	$4.99\cdot10^{3}$	4041.49	$2.38\cdot10^{3}$	87.97	$2.04\cdot10^{7}$
Lorazepam	$5.42\cdot10^{-7}$	na	$1.02\cdot10^{-6}$	304.99	$3.30\cdot10^{-8}$	10.25	$3.11\cdot10^{-4}$	$2.12\cdot10^{2}$	na	$3.42\cdot10^{3}$	304.99	$1.11\cdot10^{2}$	10.25	$1.05\cdot10^{6}$
Methylparaben	$1.04\cdot10^{-9}$	na	$4.51\cdot10^{-9}$	2148.67	$3.36\cdot10^{-10}$	56.43	$9.70\cdot10^{-6}$	$3.24\cdot10^{1}$	na	$8.27\cdot10^{2}$	2148.67	$6.02\cdot10^{1}$	56.43	$1.78\cdot10^{6}$
Naproxen	$1.42\cdot10^{-7}$	na	$2.95\cdot10^{-7}$	4196.75	$6.61\cdot10^{-9}$	12592	$1.32\cdot10^{-3}$	3.94	na	$2.18\cdot10^{2}$	4196.75	4.86	12592	$9.76\cdot10^{5}$
Norfloxacin	$1.20\cdot10^{-7}$	na	$3.05\cdot10^{-7}$	1118.69	$1.09\cdot10^{-7}$	2334.02	$5.94\cdot10^{-4}$	$3.68\cdot10^{2}$	na	$2.64\cdot10^{3}$	1118.69	$9.42\cdot10^{2}$	2334.02	$5.15\cdot10^{6}$
Omeprazole	$3.61\cdot10^{-5}$	na	$4.25\cdot10^{-5}$	12992	$6.38\cdot10^{-6}$	1388.34	$5.61\cdot10^{-1}$	$1.29\cdot10^{4}$	na	$1.63\cdot10^{1}$	12992	$3.92\cdot10^{2}$	1388.34	$7.55\cdot10^{5}$
Paroxetine	$2.92\cdot10^{-6}$	na	$1.46\cdot10^{-4}$	58.60	$4.06\cdot10^{-7}$	22.66	$8.54\cdot10^{-3}$	$1.10\cdot10^{3}$	na	$6.25\cdot10^{4}$	58.60	$1.74\cdot10^{2}$	22.66	$3.66\cdot10^{6}$
Pregabalin	$1.35\cdot10^{-8}$	na	$1.42\cdot10^{-7}$	4175.19	$2.34\cdot10^{-8}$	90.88	$5.95\cdot10^{-4}$	$5.36\cdot10^{-2}$	na	1.67	4175.19	$2.76\cdot10^{-1}$	90.88	$7.00\cdot10^{3}$
Propylparaben	$1.03\cdot10^{-7}$	na	$5.62\cdot10^{-7}$	688.81	$1.58\cdot10^{-8}$	51.53	$3.88\cdot10^{-4}$	$8.11\cdot10^{1}$	na	$3.44\cdot10^{3}$	688.81	$8.88\cdot10^{1}$	51.53	$2.37\cdot10^{6}$
Roxythromycin	na	na	na	34.24	na	4.38	na	$9.84\cdot10^{1}$	na	$2.18\cdot10^{3}$	34.24	$2.21\cdot10^{1}$	4.38	$7.47\cdot10^{4}$
salicylic acid	na	na	na	859.07	na	7984.66	na	$1.35\cdot10^{1}$	na	$1.61\cdot10^{2}$	859.07	$2.82\cdot10^{1}$	7984.66	$3.63\cdot10^{5}$
Sertraline	$6.70\cdot10^{-5}$	na	$4.77\cdot10^{-3}$	488.95	$1.62\cdot10^{-6}$	99.09	2.33	$2.43\cdot10^{2}$	na	$1.80\cdot10^{4}$	488.95	6.11	99.09	$8.82\cdot10^{6}$
Simvastatin	$4.32\cdot10^{-6}$	na	$7.55\cdot10^{-5}$	1267.82	$3.01\cdot10^{-8}$	2647.25	$1.97\cdot10^{-2}$	$1.39\cdot10^{3}$	na	$4.08\cdot10^{4}$	1267.82	$7.92\cdot10^{1}$	2647.25	$5.20\cdot10^{7}$
Sulphametoxazole	$3.24\cdot10^{-8}$	na	$1.58\cdot10^{-7}$	2084.07	$1.03\cdot10^{-8}$	2315.58	$3.53\cdot10^{-4}$	$6.07\cdot10^{1}$	na	$2.99\cdot10^{3}$	2084.07	$1.95\cdot10^{2}$	2315.58	$6.68\cdot10^{6}$
Tamoxifen	na	na	na	9.78	na	119.86	na	$2.82\cdot10^{2}$	na	$1.99\cdot10^{4}$	9.78	3.08	119.86	$1.95\cdot10^{5}$
Testosterone	na	na	na	0.14	na	0.02	na	$2.37\cdot10^{2}$	na	$1.30\cdot10^{4}$	0.14	$1.17\cdot10^{2}$	0.02	$1.82\cdot10^{3}$
Tonalide	$1.04\cdot10^{-6}$	$9.43\cdot10^{1}$	$2.77\cdot10^{-5}$	11075	$1.82\cdot10^{-7}$	35161	$3.13\cdot10^{-1}$	$3.00\cdot10^{1}$	$9.43\cdot10^{1}$	$1.20\cdot10^{4}$	11075	$4.26\cdot10^{1}$	35161	$1.34\cdot10^{8}$
Triclosan	$1.11\cdot10^{-7}$	na	$2.21\cdot10^{-7}$	na	$5.01\cdot10^{-10}$	na	na	$2.58\cdot10^{3}$	na	$1.06\cdot10^{5}$	na	$1.61\cdot10^{1}$	na	na
Trimethoprim	$9.16\cdot10^{-8}$	na	$5.66\cdot10^{-7}$	44.57	$2.29\cdot10^{-8}$	5.69	$2.54\cdot10^{-5}$	9.11	na	$4.74\cdot10^{2}$	44.57	$1.92\cdot10^{1}$	5.69	$2.12\cdot10^{4}$
Valproic acid	na	8.52	na	229.80	na	5645.91	na	2.14	8.52	$1.21\cdot10^{2}$	229.80	$1.53\cdot10^{1}$	5645.91	$1.14\cdot10^{5}$
Valsartan	$3.86\cdot10^{-6}$	na	$1.18\cdot10^{-5}$	20351	$4.62\cdot10^{-9}$	4810.05	$2.40\cdot10^{-1}$	$1.67\cdot10^{2}$	na	$4.37\cdot10^{3}$	20351	1.71	4810.05	$8.89\cdot10^{7}$

$^{+}$Comparative toxic units. [a] Emission to continental urbar air. [b] Emission to continental freshwater. [c] Emission to continental natural soil.

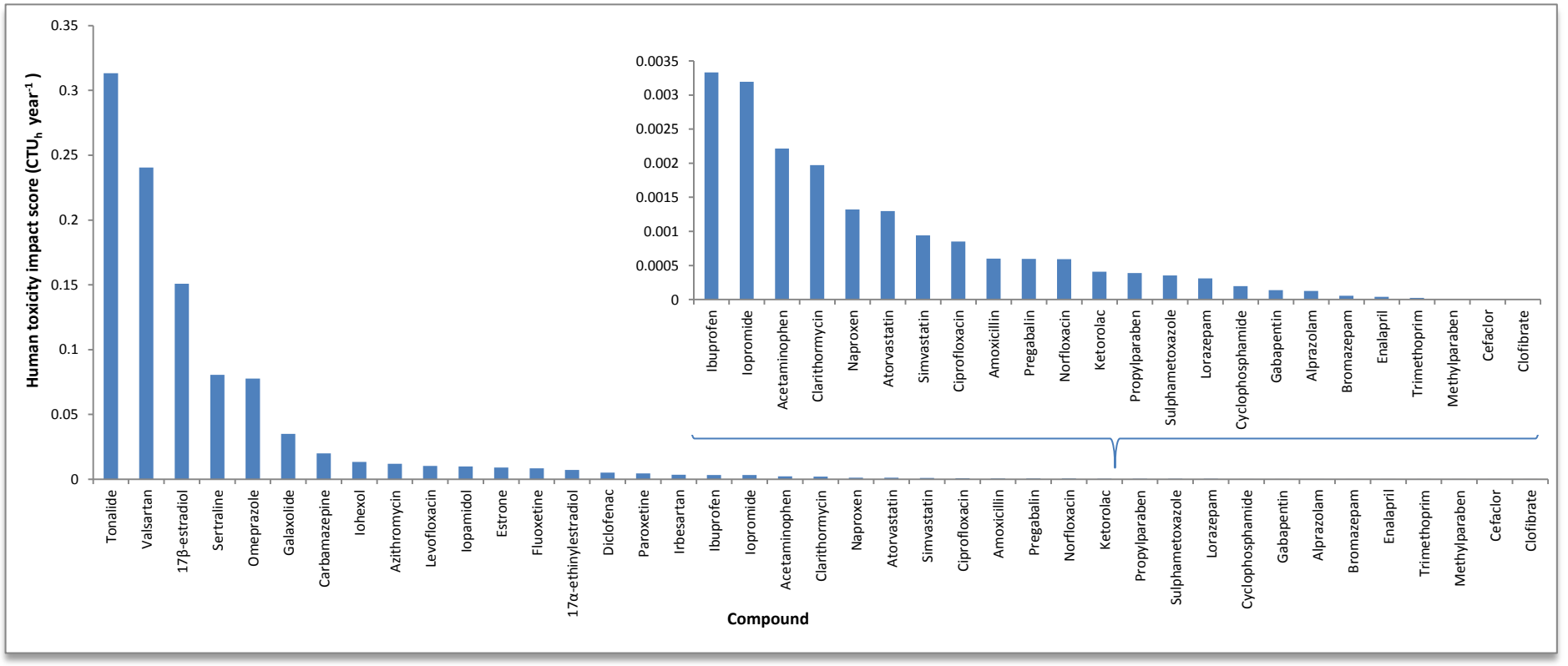

Figure 1. Human toxicity impact score for the selected PPCPs

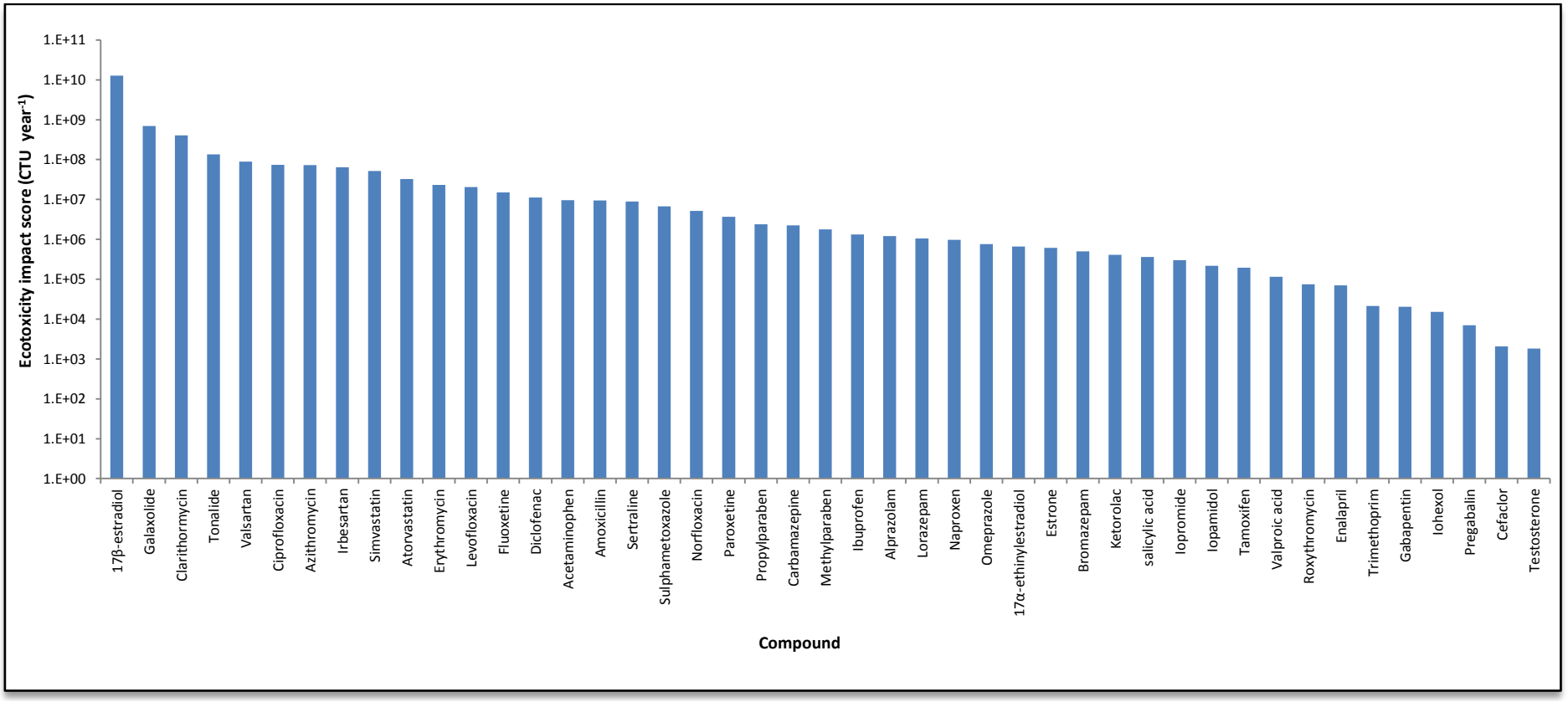

Figure 2. Ecotoxicity impact score for the selected PPCPs

Conclusion

PPCPs are a large group of compounds which are present in all compartments of nature. It is impossible analyzed all the interactions and effects of these compounds in the environment, therefore, the use of LCIA studies and risk/hazard assessment are very useful tools to predict their ecotoxicological and human effects. To implement these methodologies, it is necessary estimate CFs, therefore, USEtox^{TM} CFs have been calculated for 27 PPCPs widely used at present. A ranking impact score was done for ecotoxicological and human toxicity for 49 PPCPs as a case study, using the new CFs and the existing ones in the literature and data of occurrence of these compounds in the Spanish environmental.

There is still lack of experimental data of carcinogenic and non-carcinogenic effects of PPCPs to predict these effects on humans. In ecotoxicology more efforts should be made to know chronic effects and PPCPs mixture effects on different organisms and trophic levels. With these values, the CFs calculation will be more adjusted to reality.

In this study, emissions to continental freshwater originated the highest CFs, for both impacts human and ecological ones. Ecotoxicological CFs were much higher than human toxicity CFs, since the human tolerance of PPCPs is higher than environmental biota.

The CFs estimated in this research offers the possibility to incorporate these PPCPs in new LCIA studies or to do ranking impact score using the CFs individually.

In the case of study, the score done with the USEtox^{TM} CFs places the fragrances, hormones, antibiotics, antidepressants, angiotensin receptor blockers and blood lipid regulators in the top of the ranking, similar to other rankings generated with other methodologies.

The presence and the possible negative effects of PPCPs in the environmental is an important issue that currently must be in continuous discussion, the ability to include these compounds in LCIA or risk/hazard assessment is absolutely necessary and this is accomplished with experimental assays and improving models to estimate the occurrence, fate and effects of these compounds in the environmental.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Chapter 7

Conclusions and future work

The results obtained in this thesis confirm by experimental and predictive tools the relevance of the effects of PPCPs on different species and in the different compartments of the environment, as well as the advantages and disadvantages of the use of different models, software, methodologies and tools, to predict these effects and to generate impact scores, rankings of concern, and environmental risk/hazard assessments.

The prediction of PPCP occurrence in the environment is an aspect of great interest that could save time and money by minimizing sample collections and experimental assays. The occurrence of PPCPs in aquatic environments begins mainly with their consumption and their improper disposal to the environment. In this sense, three different methodologies were used to estimate the consumption of PPCPs; then, the occurrence of these compounds in Spanish aquatic environments were estimated. Analgesic/antipyretics, antibiotics, and PCPs had the highest occurrence. It is important to highlight that metabolites are less considered in studies of occurrence and hazard/risk assessments and in this thesis, it has been found that these compounds have high occurrence in aquatic environments. The predicted models showed a good fit for most compounds when they were compared with real values. Although the data used were from Spain, these models could be used for other geographic areas. It has been demonstrated that the estimation of the occurrence of PPCPs and their metabolites is a useful tool for identifying compounds that should be considered for environmental concern, and such estimations could be used to improve environmental risk assessment studies (**Chapter 2**).

The occurrence of PPCPs in aquatic environments does not mean that these compounds may cause adverse effects in this compartment of nature. Therefore, a (Q)SAR study was performed in **Chapter 3** to assess the possible adverse effects of PPCPs and some of their metabolites, through ecotoxicological indexes: persistence, bioaccumulation and toxicity, recently recommended by the REACH regulation, and the inclusion of occurrence as a novel environmental index. The most hazardous toxicological characteristic in the largest number of compounds was the persistence, followed by the toxicity and bioaccumulation. A high number of metabolites have a concern score equal to or greater than their parent

compounds. Different rankings of concern were proposed, and it was found that hormones, antidepressants (and their metabolites), blood lipid regulators and all of the PCPs considered in this study were at the highest levels of risk. Furthermore, when the occurrence was included, X-ray contrast media, H_2 blockers and some antibiotics were included at the highest level of concern. The methodologies used in this work provide preliminary rankings of concern for the PPCPs most consumed in Spain and widely used worldwide, was well as metabolites with high excretion. The rankings proposed cannot replace the experimental determination of PPCPs' adverse effects, but they are a powerful tool to identify those compounds that require immediate attention in aquatic environments, among the hundreds of thousands of PPCPs that are currently on the market.

Predicted values of occurrence, ecotoxicity, and some physicochemical characteristics were the basis to perform an ecotoxicity classification and an ERA of PPCPs in aquatic environments and in WWTPs using bioluminescence and respirometry assays and the US EPA ecological structure–activity relationship predictions. The experimental ecotoxicity results showed that 65.4 % of PPCPs under study were at least harmful to aquatic organisms, which provides evidence concerning the negative effects of these compounds on the environment. *Vibrio fischeri* bacteria proved to be the most sensitive species; therefore, it could be interesting to include this microorganism in further environmental studies, such as ERAs. In the ERAs of PPCPs in aquatic environments and WWTPs, analgesics/antipyretics, some antibiotics, H_2 blockers, PCPs and one transformation product of PPCPs showed some type of risk. The respirometry assays used to perform the WWTP ERA proved to be useful tools for studying the effect of PPCPs in these facilities and to complement the available ecotoxicity information for such compounds (**Chapter 4**).

In view of the ecotoxicological results obtained with *Vibrio fischeri* bacteria, a comprehensive study was performed to provide deeper knowledge regarding the dose-response effect of PPCPs (individually and in a mixture) on this microorganism (**Chapter 5**). Four parameters provided the best-fit model for the majority of compounds. The EC_{50} of each PPCP was estimated by the best fit model and compared with the results provided in **Chapter 4**, showing that the

majority of the compounds were located in the same range of risk classification. When dose-response was analyzed at PPCP environmental concentrations, 55% of the studied PPCPs presented a stimulatory effect. This behavior, a dose-response inhibition at high concentrations and dose-response stimulation at low doses, is called hormesis; this phenomenon has been less studied in ecotoxicology, especially for PPCPs. All compounds that presented narcosis as a mode of toxic action at high doses also showed some stimulation at lower concentrations; therefore, it a relationship between these two behaviors may exist, but more assays must be performed. An assay of PPCPs mixed at environmental concentrations showed a stimulatory effect higher than the highest stimulatory effect of each individually tested compound. Moreover, when the exposure time was increased, the hormetic effect decreased. The effects of PPCPs at environmental concentrations, individually or their mixtures, give a more realistic result concerning the affectation of these compounds in nature.

Finally, **Chapter 6** contributes to improving the LCAs through the estimation of the CFs of PPCPs to include these compounds in the aforementioned methodology. These factors can also be used to generate impact score rankings. Emission to the continental freshwater compartment showed the highest CFs for human effects, following by air, soil, and seawater. The CFs for effects on freshwater aquatic environments were the highest from emission to continental freshwater due to the direct contact between the source of emission and the compartment affected, followed by soil and air, and the lowest values were for continental sea water CFs. Freshwater aquatic ecotoxicological CFs are much higher than human toxicity CFs, suggesting that the ecological impact of PPCPs in aquatic environments is a matter of urgent attention. PPCPs with the highest impact are hormones, antidepressants, fragrances, antibiotics, angiotensin receptor blockers and blood lipid regulators, which were already found in other ranking scores provided in **Chapters 2, 3** and **4**.

Despite the estimates, findings and the different information obtained in this thesis, some topics must be complemented or more deeply investigated. In this regard the following aspects may be considered for future investigations:

- On the basis of advances in analytical methods, the real concentrations of PPCPs (their metabolites and transformation products) that significantly deviated from the models studied in this thesis must be determined to improve these estimates or those provided in other studies.

- Acute and chronic ecotoxicity data must be obtained for those PPCPs less studied, and these studies must include their metabolites and transformation products.

- Hormesis caused by PPCps should be further studied and included in ecotoxicological studies to establish how this effect could impact bacteria and other species, especially in the long term (chronic effects).

- The behavior of PPCP mixtures and their effects in the different compartments of environment (soil, sediment, fresh water and sea water) and their biota should be further studied, with the purpose of better understanding the real effects and interactions of these compounds in nature.

- All limitations and uncertainties highlighted in each chapter can be minimized with new studies focusing on this consideration.

- New characterization factors can be calculated by USEToxTM (or other methodologies) to increase their database and improve the LCA studies.

Chapter 8

About the author

Bio

Sheyla Andrea Ortiz de García (Valencia, Venezuela, 1974) studied Chemical Engineering in the Faculty of Engineering of Carabobo University (Venezuela). She was student assistant in Analytical Chemistry Laboratory during her engineering studies. She was placed in the third position of her Engineer promotion and she was received honorific mention of her thesis.

She has worked in Carabobo University (Venezuela) for the last 14 years being at present an Associate professor of the Faculty of Science and Technology.

Sheyla has two master degree: Environmental Engineering (Venezuela, 2009) and Engineering Research of Processes and Systems (Valladolid, 2011). Her Phd started in 2011 in the University of Valladolid and it is focused on pharmaceuticals and personal care products in aquatic environments and wastewater treatment plants, under the supervision of prof. Rubén Irusta Mata and Pedro García Encina.

Other relevant information:

Key qualifications/skills

Environmental engineering: Environmental management, environment and society, environmental legislation, formulation, preparation and evaluation of environmental projects, treatment of sewage, industrial and wastewater, management of urban and industrial solid waste, saving and efficient use of energy, design and analysis of ecotoxicity assays in aquatic environments, life cycle analysis, risk assessments and environmental impact.

Chemical engineering: Mass, moment and heat transfer balances, management and evaluation of mass and energy transfer equipments, analysis and design of chemical reactors, topics of industrial chemistry.

Other skills: Personal Growth and community management, effective communication, developing management skills, conflict resolution, teamwork, diagnosis of community participatory projects, formulation, preparation and evaluation of environmental projects, development of plans, programs and projects of environment-society, effective Leadership, social responsibility.

Publications

Consumption and occurrence of pharmaceutical and personal care products in the aquatic environment in Spain. 2012. Sheyla Ortiz de García, Gilberto Pinto Pinto, Pedro A. García-Encina, Rubén Irusta Mata. Science of the Total Environment, 444:451-465.

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Referee/reviewer in congress/journals

Environmental Science: Processes & Impacts (Reviewer)
Ecotoxicology (Reviewer)
VI Congress of Research at the University of Carabobo (Referee)

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Conference organization

International Conference IWA-WATER & INDUSTRY 2011. Member of the organization committee. 2-4.05.2011.

Participation in I+Di Projects

Waste recovery of Agro-food and bioenergy generation and bioproducts in microalgae processes. Junta de Castilla y Leon. Spain. 2014-2017. 33000$

Advanced biological processes for the removal of greenhouse gases CH_4 and N_2O: exploring the direct transport gas-cell and microbiology of the process. Ministry of Economy and Finance. National Plan I+D+I 2008-2011. Spain. 2013-2015. 300000$.

Eco-toxicological evaluation of Pharmaceutical and Personal Care Products (PPCPs) as a measure for the prevention of pollution in aquatic environments. Spain. MAPFRE fundation. 01.01.2012-10.12.2012. 17000$.

Assessment of municipal solid waste to improve the quality of life of the inhabitants of the community of Las Brisas, Miguel Peña, Valencia, Carabobo state. Ministry of Science and Technology of the Bolivarian Republic of Venezuela. 2008-2010. 8000$.

Creation of the Laboratory of Chemical Technology III of the Department of Chemistry FACYT. University of Carabobo. Shell de Venezuela. 01.01.2007- 31.12.2010. 65000$.

Thesis and tutorials of industry practices

Tutor of more than 15 theses and 10 industry practices in the environmental area in Venezuela and Spain. Jury of more than 30 undergraduate theses in Venezuela.

Schoolarship

Scholarship-salary from University of Carabobo for PhD studies in University of Valladolid (01.10.2010-30.09.2014).

Stays abroad

Study tours to Germany for foreign students ("Studienreisen von ausländischen Studierenden nach Deutschland") for 15 days (October 05 to 21, 2015) through a grant from the DAAD (Deutscher Akademischer Austauschdienst, German Academic Exchange Service).

Chapter 9

Supplementary material

9.1. Appendix A. Supplementary material Chapter 2

Consumption and occurrence of pharmaceutical and personal care products in the aquatic environment in Spain

Pharmaceutical active compounds under study in Chapter 2

Acetaminophen, acetylsalicylic acid, alprazolam, amoxicillin, atorvastatin, azithromycin, bezafibrate, bromazepam, carbamazepine, cefaclor, ciprofloxacin, clarithromycin, clofibrate, cyclophosphamide, desogestrel, diclofenac, enalapril, erythromycin, escitalopram, esomeprazole, fluoxetine, flutamide, fluvastatin, fluvoxamine, gabapentin, gemcitabine, ibuprofen, ifosfamide, iohexol, iopamidol, iopromide, irbesartan, ketorolac, lanzoprazole, levofloxacin, levonorgestrel, lorazepam, megestrol, mitomycin, moxifloxacin, naproxen, norethisterone, norfloxacin, omeprazole, pantoprazole, paroxetine, pregabalin, progesterone, roxythromycin, sertraline, simvastatin, sulfamethoxazole, tamoxifen, testosterone, topiramate, trimethoprim, valproic acid, valsartan,17-α ethynylestradiol, 17-β estradiol.

Metabolites under study in Chapter 2

2-Hydroxy carbamazepine, 2-Hydroxy ibuprofen, 3-Hydroxy carbamazepine, 4-Hydroxy diclofenac, 5-Hydroxy diclofenac, acetaminophen Glucuronide, carbamazepine 10, 11-epoxide, carbamazepine 10,11-dihydrodiol, clofibric acid from clofibrate, clofibric acyl-β-D-glucuronide acid, estrone, hydroxylamine sulfamethoxazole, ibuprofen carboxylic acid, 4-[[(3S,4R)-4-(4-fluorophenyl)piperidin-3-yl]methoxy]-2-methoxyphenol,N-desmethyl escitalopram, N-desmethyl sertraline, norfluoxetine, salicylic acid, salicylic β-D-O-Glucuronide acid, sertraline Carbamoyl glucuronide.

Personal care products under study in Chapter 2

Iso-E-super®, galaxolide®, musk ketone, methylparaben, musk xilene, phantolide®, propylparaben, tonalide®.

9.2. Appendix B. Supplementary material Chapter 3

Ranking of concern, based on environmental indexes, for pharmaceutical and personal care products. An application to the Spanish case

Appendix A

General principles of DART

DART

The general principles and the interpretation of results of DART are explained according to the information in the DART manual (TALETE, 2007) and Pavan and Worth (2008).
DART introduces partial and total ordering techniques. The total order ranking methods, which are scalar methods, combine the different criteria values into an index, the ranking index Γ, and the element comparison and ordering are performed according to the numerical value of Γ. In this way, the elements are always ranked in a total or linear ordered sequence, but the information on conflict among the criteria is inevitably lost.
Desirability functions are a well-known method of multicriteria-decision making. The approach is based on the definition of a desirability function for each criterion to transform values of the criteria to the same scale. Each criterion (*r*) is independently transformed into a desirability value d_{ir} by an arbitrary function that transforms the actual value of each element (*i*) into a value between 0 and 1:

$$d_{ir} = f_r(y_{ir}) \qquad 0 \le d_{ir} \le 1;\ r = 1,2,\ldots,R. \qquad \text{Eq. A.1}$$

where *r* is the selected criterion, *f* is the function chosen and y_{ir} is the actual value of the *i*-th element for the *r*-th criterion. Once the type of function and its trend for each criterion are defined, the global desirability *D* of each *i*-th element and of each weight *w* can be evaluated as follows:

$$D_i = d_{1i}^{w1} * d_{2i}^{w2} * \ldots * d_{R1}^{wR} \qquad 0 \le D_i \le 1 \qquad \text{with} \quad \sum_{r=1}^{R} w_r = 1 \qquad \text{Eq. A.2}$$

It must be highlighted that the desirability product is very strict: if an element is poor with respect to one criterion, its overall desirability will be poor. If any desirability d_i is equal to 0, the overall desirability D_i will be zero, whereas the D_i will be equal to one only if all of the desirabilities have the maximum value of one.

For the utility functions, the approach is very similar to that of the desirability functions; each criterion (r) with its respectively weight (w_r) is independently transformed into a utility value u_{ir} by a function that transforms the actual value of each element (i) into a value between 0 and 1. Once the type of function and its trend for each criterion has been defined, the overall utility U of each i-th element is defined as

$$U_i = \sum_{r=1}^{R} w_r * u_{ir} \qquad 0 \le U_i \le 1 \qquad \text{with} \qquad \sum_{r=1}^{R} w_r = 1 \qquad \text{Eq. A.3}$$

Moreover, partial order ranking is a vectorial approach that recognizes that not all elements can be directly compared with all other elements because contradictions in the ranking can be present when many criteria are used. Compounds belonging to the same level do not necessarily have the same level of concern for each environmental index.

The Hasse diagram is a technique of ranking of partial order introduced in environmental sciences by Halfon and Reggiani (1986) and refined by Bruggemann and Bartel (1999). It is based on a specific order relation, called the product order, and it provides a diagram that visualizes the results of the sorting.

In this approach, the basis for the ranking is the information collected in the full set of criteria, called even “attributes”, *E*, which is called the "information basis" of the comparative evaluation of elements.

The processed data matrix *Q (N x R)* contains ***N*** elements (rows) and *R* attributes (columns). The entry $y_{i,r}$ of *Q* is the numerical value of the *r*-th attribute of the *i*-th element. According to the product order relation, on which the Hasse diagram technique is based, *IB* is the information basis of evaluation, and *E* is the set of *N* elements. The two elements s and t are comparable if for all $y_r \in IB$ either $y_r(s) \le y_r(t)$ *or* $y_r(t) \ge y_r(s)$. If $y_r(s) \le y_r(t)$ for all $y_r \in IB$, then $s \le t$. The request "for all" is very important and is called the generality principle:

$s, t \in E;\ s \le t \leftrightarrow y(s) \le y(t)$

$y(s) \le y(t) \leftrightarrow y_r(s) \le y_r(t)$ for all $y_r \in IB$

If there are some y_r for which $y_r(s) < y_r(t)$ and some others for which $y_r(s) > y_r(t)$, then *s* and *t* are not comparable, and the common notation is “*st*”. If only one attribute is used or all of the attributes are perfectly correlated, then the total order is obtained, and all of the elements are comparable.

In this study, the PPCPs are the elements (*N*), and the attributes are O, P, B and T.

Appendix B

Table B.1. Physicochemical properties of pharmaceutically active compounds

Type	Compound common name	CAS number[+]	Molecular formula[++]	Molecular weight[++] (g gmol^{-1})	S[+++,*,**] (mg L^{-1})	logK_{ow}[†,*,**]	pKa[††,*]	logK_{oc}[†††,**]
Analgesic/antipyretic	Acetaminophen	103-90-2	$C_8H_9NO_2$	151.16	14,000	0.46	9.38	1.320
Angiotensin converting enzyme (ACE) inhibitors	Enalapril	75847-73-3	$C_{20}H_{28}N_2O_5$	376.45	16,400	0.07	--	1.354
Angiotensin receptor blockers (ARBs)	Valsartan	137862-53-4	$C_{24}H_{29}N_5O_3$	435.52	1.40	3.65	--	2.200
	Irbesartan	138402-11-6	$C_{25}H_{28}N_6O$	428.53	0.06	5.30	--	3.888
Antibiotics	Amoxicillin	26787-78-0	$C_{16}H_{19}N_3O_5S$	365.40	3430	0.87	--	0.709
	Azithromycin	83905-01-5	$C_{38}H_{72}N_2O_{12}$	748.98	7.09	4.02	8.74	1.676
	Cefaclor	53994-73-3	$C_{15}H_{14}ClN_3O_4S$	367.81	10,000	0.35	2.43/7.16	0.255
	Ciprofloxacin	85721-33-1	$C_{17}H_{18}FN_3O_3$	331.34	30,000	0.28	6.09	-0.004
	Clarithrormycin	81103-11-9	$C_{38}H_{69}NO_{13}$	747.95	0.342	3.16	8.99	1.371
	Erythromycin	114-07-8	$C_{37}H_{67}NO_{13}$	733.92	1.44	3.06	8.88	1.406
	Levofloxacin	100986-85-4	$C_{18}H_{20}FN_3O_4$	361.37	28,300	-0.39	--	-0.004
	Moxifloxacin	151096-09-2	$C_{21}H_{24}FN_3O_4$	401.43	453.7	0.95	--	0.423
	Norfloxacin	70458-96-7	$C_{16}H_{18}FN_3O_3$	319.33	178,000	-1.03	--	-0.392
	Roxythromycin	80214-83-1	$C_{41}H_{76}N_2O_{15}$	837.04	0.0189	2.75	--	0.858
	Sulfamethoxazole	723-46-6	$C_{10}H_{11}N_3O_3S$	253.27	610.0	0.89	--	1.536
	Trimethoprim	738-70-5	$C_{14}H_{18}N_4O_3$	290.32	400.0	0.91	7.20	1.896
Antidepressants	Escitalopram	128196-01-0	$C_{20}H_{21}FN_2O$	324.39	31.09	3.74	--	3.230
	Fluoxetine	54910-89-3	$C_{17}H_{18}F_3NO$	309.33	60.30	4.05	--	3.178
	Fluvoxamine	54739-18-3	$C_{15}H_{21}F_3N_2O_2$	318.33	22.22	3.09	--	2.522
	Paroxetine	61869-08-7	$C_{19}H_{20}FNO_3$	329.37	35.30	3.95	9.00	3.088
	Sertraline	79617-96-2	$C_{17}H_{17}Cl_2N$	306.23	3.52	5.29	--	3.808
Antiepileptics	Carbamazepine	298-46-4	$C_{15}H_{12}N_2O$	236.27	112.0	2.45	--	2.227
	Gabapentin	60142-96-3	$C_9H_{17}NO_2$	171.24	4490	-1.10	3.68	-0.475
	Pregabalin	148553-50-8	$C_8H_{17}NO_2$	159.23	19,600	-1.78	--	-0.850
	Topiramate	97240-79-4	$C_{12}H_{21}NO_8S$	339.36	13,600	-0.33	--	0.451
	Valproic acid	99-66-1	$C_8H_{16}O_2$	144.21	894.6	2.75	4.60	1.677
Anxiolytics	Alprazolam	28981-97-7	$C_{17}H_{13}ClN_4$	308.76	13.10	2.12	--	2.171
	Bromazepam	1812-30-2	$C_{14}H_{10}BrN_3O$	316.15	175.0	2.05	--	2.210
	Lorazepam	846-49-1	$C_{15}H_{10}Cl_2N_2O_2$	321.17	80.00	2.39	13.00	1.810
Blood lipid regulators	Atorvastin	134523-00-5	$C_{33}H_{35}FN_2O_5$	558.64	0.001	6.36	--	2.600

	Bezafibrate	41859-67-0	$C_{19}H_{20}ClNO_4$	361.82	0.355	4.25	--	2.311
	Clofibrate	637-07-0	$C_{12}H_{15}ClO_3$	242.70	69.10	3.62	--	2.918
	Clofibric acid	882-09-7	$C_{10}H_{11}ClO_3$	214.65	583.0	2.57	--	1.633
	Fluvastatin	93957-54-1	$C_{24}H_{26}FNO_4$	411.47	0.468	4.85	--	2.016
	Simvastatin	79902-63-9	$C_{25}H_{38}O_5$	418.57	0.030	4.68	--	2.971
Cytostatics/	Cyclophosphamide	50-18-0	$C_7H_{15}Cl_2N_2O_2P$	261.09	40,000	0.63	--	1.312
cancer therapeutics	Flutamide	13311-84-7	$C_{11}H_{11}F_3N_2O_3$	276.21	9.45	3.35	--	2.972
	Gemcitabine	95058-81-4	$C_9H_{11}F_2N_3O_4$	263.20	51,000	-2.01	--	-1.166
	Ifosfamide	3778-73-2	$C_7H_{15}Cl_2N_2O_2P$	261.08	3780	0.86	--	1.439
	Mitomycin	1404-00-8	$C_{15}H_{18}N_4O_5$	334.33	8430	-0.40	--	1.300
	Tamoxifen	10540-29-1	$C_{26}H_{29}NO$	371.51	0.19	6.30	8.87	4.400
H_2 Blockers	Esomeprazole	119141-88-7	$C_{17}H_{19}N_3O_3S$	345.42	82.3	2.23	--	2.940
	Lanzoprazole	103577-45-3	$C_{16}H_{14}F_3N_3O_2S$	369.36	3.43	3.68	--	3.686
	Omeprazole	73590-58-6	$C_{17}H_{19}N_3O_3S$	345.42	82.3	2.23	--	2.940
	Pantoprazole	102625-70-7	$C_{16}H_{15}F_2N_3O_4S$	383.37	48.8	2.22	--	2.935
Hormones	Desogestrel	54024-22-5	$C_{22}H_{30}O$	310.47	0.27	5.65	--	6.639
	Diethylstilbestrol	56-53-1	$C_{18}H_{20}O_2$	268.35	12.0	5.07	--	4.063
	Estrone	53-16-7	$C_{18}H_{22}O_2$	270.37	30.0	3.13	--	3.019
	Gestodene	60282-87-3	$C_{21}H_{26}O_2$	310.43	8.12	3.26	--	2.512
	Levonorgestrel	797-63-7	$C_{21}H_{28}O_2$	312.44	2.05	3.48	--	2.634
	Megestrol	3562-63-8	$C_{22}H_{30}O_3$	342.47	0.27	5.65	--	3.463
	Norethisterone	68-22-4	$C_{20}H_{26}O_2$	298.42	7.04	2.97	--	2.352
	Progesterone	57-83-0	$C_{21}H_{30}O_2$	314.46	8.81	3.87	--	3.457
	Testosterone	58-22-0	$C_{19}H_{28}O_2$	288.42	23.4	3.32	--	2.546
	17-α ethynylestradiol	57-63-6	$C_{20}H_{24}O_2$	296.40	11.3	3.67	--	2.710
	17-β estradiol	50-28-2	$C_{18}H_{24}O_2$	272.38	3.60	4.01	--	2.899
Inhibiting platelet aggregation	Acetylsalicylic acid	50-78-2	$C_9H_8O_4$	180.16	4600	1.19	3.49	0.784
Non steroidal	Diclofenac	15307-86-5	$C_{14}H_{11}Cl_2NO_2$	296.15	2.37	4.51	4.15	2.607
Antiinflamatories (NSAIDs)/	Ibuprofen	15687-27-1	$C_{13}H_{18}O_2$	206.28	21.00	3.97	4.91	2.352
Antirreumatics	Ketorolac	74103-06-3	$C_{15}H_{13}NO_3$	255.27	298.0	2.32	3.49	1.635
	Naproxen	22204-53-1	$C_{14}H_{14}O_3$	230.26	15.90	3.18	4.15	1.971
X ray contrast media	Iohexol	66108-95-0	$C_{19}H_{26}I_3N_3O_9$	821.14	107.0	-3.05	--	-2.134
	Iopamidol	60166-93-0	$C_{17}H_{22}I_3N_3O_8$	777.08	140,000	-2.42	--	-1.764
	Iopromide	73334-07-3	$C_{18}H_{24}I_3N_3O_8$	791.11	23.80	-2.05	--	-1.672

[+] United States National Library of Medicine. ChemID Plus Lite (2010). [++] National Center for Biotechnology Information. PubChem Compound Database. (2011).

[+++]Solubility at 25ºC. For 17-α ethynylestradiol and 17-β estradiol at 27ºC. [†]Logarithm of octanol/water partition coefficient.

[††]The negative logarithm of the acid dissociation constant (K_a). [†††]Logarithm of soil/water partition coefficient.

* SRC PhysProp Database (2010). **Estimated with US EPA Estimation Programs Interface Suite™ (2009).

-- Not Available.

Table B.2. Physicochemical properties of metabolites and personal care products

Type	Compound common name	CAS number[+]	Molecular formula[++]	Molecular weight[++] (g gmol^{-1})	S[+++,*,**] (mg L^{-1})	logK_{ow}[†,*]	pKa[††,*]	logK_{oc}[†††,**]
Metabolites	Acetaminophen glucuronide	120595-80-4	$C_{14}H_{16}NO_8 \cdot Na$	349.27	94680	-1.23	--	-1.408
	Carbamazepine 10,11 dihydrodiol	35079-97-1	$C_{15}H_{14}N_2O_3$	270.28	103.9	-0.21	--	-0.067
	Carbamazepine 10, 11-epoxide	36507-30-9	$C_{15}H_{12}N_2O_2$	252.27	276.8	0.95	--	1.307
	Clofíbric acyl-β-D-glucuronide acid	72072-47-0	$C_{16}H_{19}ClO_9$	390.77	5640	0.22	--	-0.646
	Ibuprofen carboxylic acid	15935-54-3	$C_{13}H_{16}O_4$	236.26	1453	1.97	--	3.100
	Metabolite paroxetine***	112058-90-9	$C_{19}H_{22}FNO_3$	331.38	29.72	4.02	--	3.389
	N-desmethyl escitalopram	144025-14-9	$C_{19}H_{19}FN_2O$	310.36	57.01	3.53	--	3.136
	N-desmethyl sertraline	91797-57-8	$C_{16}H_{16}Cl_3N$	328.66	10.61	4.82	--	3.569
	Norfluoxetine	126924-38-7	$C_{16}H_{16}F_3NO$	295.30	35.70	4.20	--	3.271
	Salicylic acid	69-72-7	$C_7H_6O_3$	138.12	2240	2.26	2.97	1.573
	Salicylic β-D-O-glucuronide acid	7695-70-7	$C_{13}H_{14}O_9$	314.24	120,100	-0.79	--	-1.144
	Sertraline carbamoyl glucuronide	119733-44-7	$C_{24}H_{25}Cl_2NO_8$	526.36	0.776	3.86	--	1.251
	Sulfamethoxazole hydroxylamine	114438-33-4	$C_{10}H_{11}N_3O_4S$	269.28	7745	0.44	--	1.287
	1,4 benzoquinone	106-51-4	$C_6H_4O_2$	108.09	11,000	0.20	--	1.935
	4-chlorobenzoic acid	74-11-3	$C_7H_5ClO_2$	156.56	72.00	2.65	3.98	1.622
	2-hydroxy carbamazepine	68011-66-5	$C_{15}H_{12}N_2O_2$	252.27	109.9	1.42	--	1.824
	3-hydroxy carbamazepine	68011-67-6	$C_{15}H_{12}N_2O_2$	252.27	109.9	1.42	--	1.824
	4-hydroxy diclofenac	64118-84-9	$C_{14}H_{11}Cl_2NO_3$	312.1	17.90	3.70	--	2.326
	5-hydroxy diclofenac	--	$C_{14}H_9Cl_2NO_3$	310.13	32.99	3.40	--	2.232
	2-hydroxy ibuprofen	51146-55-5	$C_{13}H_{18}O_3$	222.28	2974	2.30	--	1.011
PCPs								
Biocide	Triclosan	3380-34-5	$C_{12}H_7Cl_3O_2$	289.54	10.00	4.76	--	4.760
Fragrances	Galaxolide® (HHCB)	1222-05-5	$C_{18}H_{26}O$	258.40	1.750	5.90	--	4.098
	Iso-E Super® (OTNE)	54464-57-2	$C_{16}H_{26}O$	234.37	1.077	5.18	--	3.986
	Musk ketone	81-14-1	$C_{14}H_{18}N_2O_5$	294.30	0.460	4.30	--	4.103
	Musk xilene	81-15-2	$C_{12}H_{15}N_3O_6$	297.26	0.472	4.45	--	3.825
	Phantolide® (AHDI)	15323-35-0	$C_{17}H_{24}O$	244.37	0.255	5.85	--	4.357
	Tonalide® (AHTN)	21145-77-7	$C_{18}H_{26}O$	258.39	1.250	5.70	--	4.274
Preservatives	Ethylparaben	120-47-8	$C_9H_{10}O_3$	166.18	885.0	2.47	8.34	2.393
	Methylparaben	99-76-3	$C_8H_8O_3$	152.14	5981	2.00	--	2.111
	P-hydroxybenzoic acid	99-96-7	$C_7H_6O_3$	138.12	5000	1.58	4.54	1.430
	Propylparaben	94-13-3	$C_{10}H_{12}O_3$	180.20	500.0	3.04	7.91	2.708
Surfactant	4- nonylphenol	104-40-5	$C_{15}H_{24}O$	220.36	7.000	5.92	11.10	5.760

[+] United States National Library of Medicine. ChemID Plus Lite (2010).
[++] National Center for Biotechnology Information. PubChem Compound Database. (2011).
[+++]Solubility at 25ºC.
[†]Logarithm of octanol/water partition coefficient.
[††]The negative logarithm of the acid dissociation constant (K_a) at 25ºC.
[†††]Logarithm of soil/water partition coefficient.
* SRC PhysProp Database (2010).
**Estimated with US EPA Estimation Programs Interface Suite™ (2009).
***4-[[(3S,4R)-4-(4-fluorophenyl)piperidin-3-yl]methoxy]-2-methoxyphenol.
-- Not Available.

Appendix C

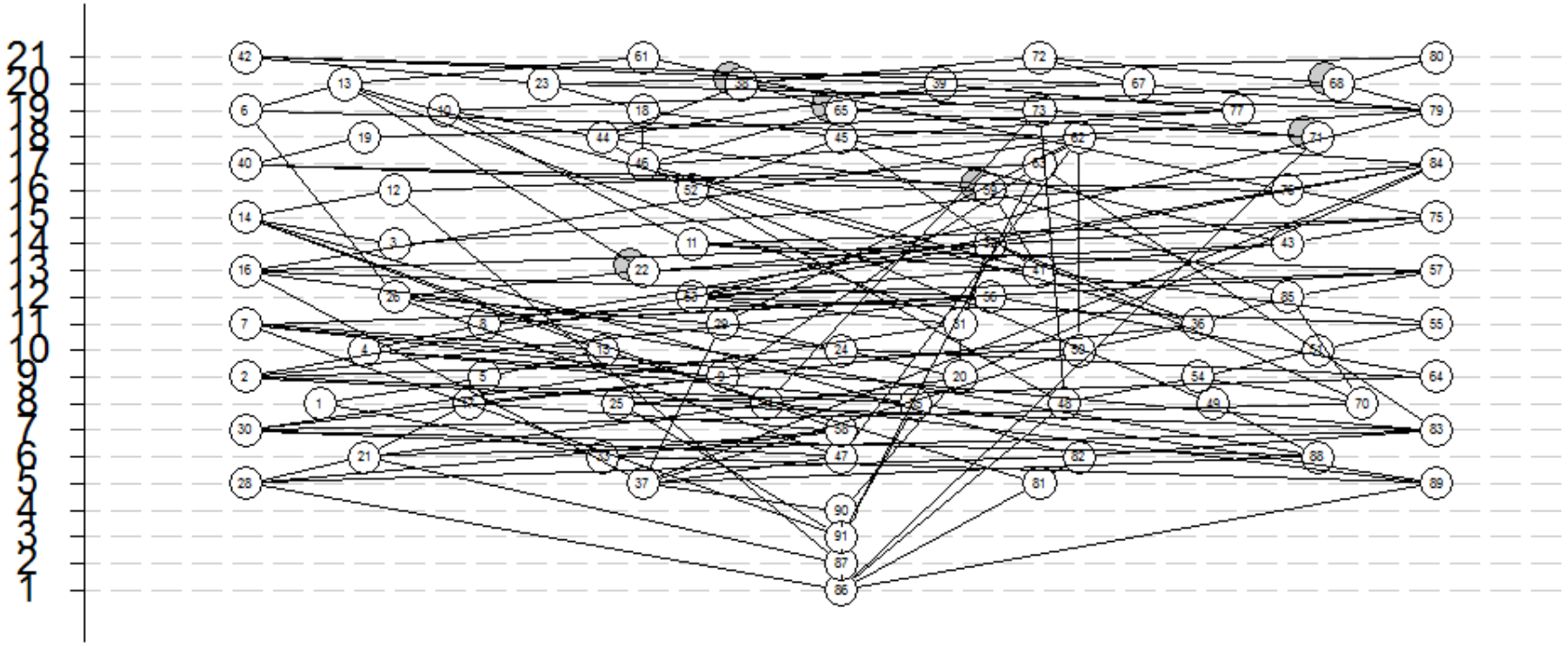
Rankings of concern by the DART utility function when different weights of PBT are used

Scenario 1 (70.20.10)*	Scenario 3 (50.25.25)	Scenario 4 (33.33.33)	Scenario 5 (25.50.25)	Scenario 6 (20.70.10)	Scenario 7 (25.25.50)	Scenario 8 (20.10.70)
Desogestrel, Galaxolide®, tamoxifen	Desogestrel, Galaxolide®, tamoxifen	Desogestrel, Galaxolide®, tamoxifen	Desogestrel, Galaxolide®, tamoxifen	Desogestrel, Galaxolide®, tamoxifen	Desogestrel, Galaxolide®, tamoxifen	Desogestrel, Galaxolide®, tamoxifen
Sertraline	Sertraline	Sertraline	Sertraline	Sertraline	Sertraline	Sertraline
Atorvastatin, musk ketone, musk xilene, N-desmethyl sertraline, norfluoxetine, Phantolide®, Tonalide®, triclosan	Atorvastatin, musk ketone, musk xilene, N-desmethyl sertraline, norfluoxetine, Phantolide®, Tonalide®, triclosan	Atorvastatin, musk ketone, musk xilene, N-desmethyl sertraline, norfluoxetine, Phantolide®, Tonalide®, triclosan	Diethylstilbestrol, irbesartan, Iso-E-super®	Diethylstilbestrol, irbesartan, Iso-E-super®	Atorvastatin, musk ketone, musk xilene, N-desmethyl sertraline, norfluoxetine, Phantolide®, Tonalide®, triclosan	Atorvastatin, musk ketone, musk xilene, N-desmethyl sertraline, norfluoxetine, Phantolide®, Tonalide®, triclosan
Bezafibrate, escitalopram, fluoxetine, lanzoprazole, megestrol, metabolite paroxetine, N-desmethyl escitalopram, paroxetine, progesterone, 17-α ethynylestradiol	Bezafibrate, escitalopram, fluoxetine, lanzoprazole, megestrol, metabolite paroxetine, N-desmethyl escitalopram, paroxetine, progesterone, 17-α ethynylestradiol	Diethylstilbestrol, irbesartan, Iso-E-super®	Atorvastatin, musk ketone, musk xilene, N-desmethyl sertraline, norfluoxetine, Phantolide®, Tonalide®, triclosan	Atorvastatin, musk ketone, musk xilene, N-desmethyl sertraline, norfluoxetine, Phantolide®, Tonalide®, triclosan	Diethylstilbestrol, irbesartan, Iso-E-super®	Diethylstilbestrol, irbesartan, Iso-E-super®
Azithromycin, clarithromycin, esomeprazole, gestodenol, flutamide, fluvoxamine, levonorgestrel, norethisterone, omeprazole	Azithromycin, clarithromycin, diethylstilbestrol, esomeprazole, gestodenol, flutamide, fluvoxamine, irbesartan, Iso-E-super®, levonorgestrel, norethisterone, omeprazole	Bezafibrate, escitalopram, fluoxetine, lanzoprazole, megestrol, metabolite paroxetine, N-desmethyl escitalopram, paroxetine, progesterone, 17-α ethynylestradiol	Bezafibrate, escitalopram, fluoxetine, lanzoprazole, megestrol, metabolite paroxetine, N-desmethyl escitalopram, paroxetine, progesterone, 17-α ethynylestradiol	Bezafibrate, escitalopram, fluoxetine, lanzoprazole, megestrol, metabolite paroxetine, N-desmethyl escitalopram, paroxetine, progesterone, 17-α ethynylestradiol	Simvastatin, 1,4 benzoquinone, 4-nonylphenol	Simvastatin, 1,4 benzoquinone, 4-nonylphenol
Bromazepam, ciprofloxacin, erythromycin, iohexol, iopamidol, iopromide, levofloxacin,lorazepam, mitomycin, moxifloxacin, norfloxacin, pantoprazole, roxythromycin, timethoprim, topiramate	Bromazepam, ciprofloxacin, erythromycin, iohexol, iopamidol, iopromide, levofloxacin, lorazepam, mitomycin, moxifloxacin, norfloxacin, pantoprazole, roxythromycin, simvastatin, timethoprim, topiramate, 1,4 benzoquinone, 4-nonylphenol	Simvastatin, 1,4 benzoquinone, 4-nonylphenol	Simvastatin, 1,4 benzoquinone, 4-nonylphenol	Azithromycin, clarithromycin, esomeprazole, gestodenol, flutamide, fluvoxamine, levonorgestrel, norethisterone, omeprazole	Bezafibrate, escitalopram, fluoxetine, lanzoprazole, megestrol, metabolite paroxetine, N-desmethyl escitalopram, paroxetine, progesterone, 17-α ethynylestradiol	Bezafibrate, escitalopram, fluoxetine, lanzoprazole, megestrol, metabolite paroxetine, N-desmethyl escitalopram, paroxetine, progesterone, 17-α ethynylestradiol
Diethylstilbestrol, irbesartan, Iso-E-super®	Alprazolam, clofibrate, fluvastatin, propylparaben, sertraline carbamoyl glucuronide, 2-hydroxy carbamazepine, 3-hydroxy carbamazepine, 17-β estradiol	Azithromycin, clarithromycin, esomeprazole, gestodenol, flutamide, fluvoxamine, levonorgestrel, norethisterone, omeprazole	Azithromycin, clarithromycin, esomeprazole, gestodenol, flutamide, fluvoxamine, levonorgestrel, norethisterone, omeprazole	Bromazepam, ciprofloxacin, erythromycin, iohexol, iopamidol, iopromide, levofloxacin,lorazepam, mitomycin, moxifloxacin, norfloxacin, pantoprazole, roxythromycin, simvastatin, timethoprim, topiramate, 1,4 benzoquinone, 4-nonylphenol	Alprazolam, clofibrate, fluvastatin, propylparaben, sertraline carbamoyl glucuronide, 2-hydroxy carbamazepine, 3-hydroxy carbamazepine, 17-β estradiol	Alprazolam, clofibrate, fluvastatin, propylparaben, sertraline carbamoyl glucuronide, 2-hydroxy carbamazepine, 3-hydroxy carbamazepine, 17-β estradiol
Simvastatin, 1,4 benzoquinone, 4-nonylphenol	Carbamazepine 10,11 dihydrodiol, carbamazepine 10, 11-epoxide, diclofenac, estrone, ibuprofen, ketorolac, sulfamethoxazole hydroxylamine, testosterone, valsartan, 4-hydroxy diclofenac, 5-hydroxy diclofenac	Alprazolam, clofibrate, fluvastatin, propylparaben, sertraline carbamoyl glucuronide, 2-hydroxy carbamazepine, 3-hydroxy carbamazepine, 17-β estradiol	Alprazolam, clofibrate, fluvastatin, propylparaben, sertraline carbamoyl glucuronide, 2-hydroxy carbamazepine, 3-hydroxy carbamazepine, 17-β estradiol	Alprazolam, clofibrate, fluvastatin, propylparaben, sertraline carbamoyl glucuronide, 2-hydroxy carbamazepine, 3-hydroxy carbamazepine, 17-β estradiol	Azithromycin, clarithromycin, esomeprazole, gestodenol, flutamide, fluvoxamine, levonorgestrel, norethisterone, omeprazole	Ethylparaben
Alprazolam, clofibrate, fluvastatin, propylparaben, sertraline carbamoyl glucuronide, 2-hydroxy carbamazepine, 3-hydroxy carbamazepine, 17-β estradiol	Acetaminophen, amoxicillin, carbamazepine, cefaclor, clofibric acid, clofibric acyl-β-D-glucuronide acid, cyclophosphamide, enalapril, gemcitabine, ifosfamide, naproxen, sulfametoxazole, 4-chlorobenzoic acid, 2-hydroxy ibuprofen	Bromazepam, ciprofloxacin, erythromycin, iohexol, iopamidol, iopromide, levofloxacin, lorazepam, mitomycin, moxifloxacin, norfloxacin, pantoprazole, roxythromycin, timethoprim, topiramate	Bromazepam, ciprofloxacin, erythromycin, iohexol, iopamidol, iopromide, levofloxacin,lorazepam, mitomycin, moxifloxacin, norfloxacin, pantoprazole, roxythromycin, timethoprim, topiramate	Carbamazepine 10,11 dihydrodiol, carbamazepine 10, 11-epoxide, diclofenac, estrone, ibuprofen, ketorolac, sulfamethoxazole hydroxylamine, testosterone, valsartan, 4-hydroxy diclofenac, 5-hydroxy diclofenac	Ethylparaben	Azithromycin, clarithromycin, esomeprazole, gestodenol, flutamide, fluvoxamine, levonorgestrel, norethisterone, omeprazole
Carbamazepine 10,11 dihydrodiol, carbamazepine 10, 11-epoxide, diclofenac, estrone, ibuprofen, ketorolac, sulfamethoxazole hydroxylamine, testosterone, valsartan, 4-hydroxy diclofenac, 5-hydroxy diclofenac	Ethylparaben	Carbamazepine 10,11 dihydrodiol, carbamazepine 10, 11-epoxide, diclofenac, estrone, ibuprofen, ketorolac, sulfamethoxazole hydroxylamine, testosterone, valsartan, 4-hydroxy diclofenac, 5-hydroxy diclofenac	Carbamazepine 10,11 dihydrodiol, carbamazepine 10, 11-epoxide, diclofenac, estrone, ibuprofen, ketorolac, sulfamethoxazole hydroxylamine, testosterone, valsartan, 4-hydroxy diclofenac, 5-hydroxy diclofenac	Acetaminophen, amoxicillin, carbamazepine, cefaclor, clofibric acid, clofibric acyl-β-D-glucuronide acid, cyclophosphamide, enalapril, gemcitabine, ifosfamide, naproxen, sulfametoxazole, 4-chlorobenzoic acid, 2-hydroxy ibuprofen	Carbamazepine 10,11 dihydrodiol, carbamazepine 10, 11-epoxide, diclofenac, estrone, ibuprofen, ketorolac, sulfamethoxazole hydroxylamine, testosterone, valsartan, 4-hydroxy diclofenac, 5-hydroxy diclofenac	Carbamazepine 10,11 dihydrodiol, carbamazepine 10, 11-epoxide, diclofenac, estrone, ibuprofen, ketorolac, sulfamethoxazole hydroxylamine, testosterone, valsartan, 4-hydroxy diclofenac, 5-hydroxy diclofenac
Acetaminophen, amoxicillin, carbamazepine, cefaclor, clofibric acid, clofibric acyl-β-D-glucuronide acid, cyclophosphamide, enalapril, gemcitabine, ifosfamide, naproxen, sulfametoxazole, 4-chlorobenzoic acid, 2-hydroxy ibuprofen	Acetaminophen glucuronide, methylparaben	Ethylparaben	Ethylparaben	Ethylparaben	Bromazepam, ciprofloxacin, erythromycin, iohexol, iopamidol, iopromide, levofloxacin,lorazepam, mitomycin, moxifloxacin, norfloxacin, pantoprazole, roxythromycin, timethoprim, topiramate	Acetaminophen glucuronide, methylparaben
Ethylparaben	Acetylsalicylic acid, gabapentin, ibuprofen carboxylic acid, p-hydroxybenzoic acid, pregabalin, salicylic acid, salicylic β-*D*-O-glucuronide acid, valproic acid	Acetaminophen, amoxicillin, carbamazepine, cefaclor, clofibric acid, clofibric acyl-β-D-glucuronide acid, cyclophosphamide, enalapril, gemcitabine, ifosfamide, naproxen, sulfametoxazole, 4-chlorobenzoic acid, 2-hydroxy ibuprofen	Acetaminophen, amoxicillin, carbamazepine, cefaclor, clofibric acid, clofibric acyl-β-D-glucuronide acid, cyclophosphamide, enalapril, gemcitabine, ifosfamide, naproxen, sulfametoxazole, 4-chlorobenzoic acid, 2-hydroxy ibuprofen	Acetaminophen glucuronide, methylparaben	Acetaminophen glucuronide, methylparaben	Bromazepam, ciprofloxacin, erythromycin, iohexol, iopamidol, iopromide, levofloxacin,lorazepam, mitomycin, moxifloxacin, norfloxacin, pantoprazole, roxythromycin, timethoprim, topiramate
Acetaminophen glucuronide, methylparaben		Acetaminophen glucuronide, methylparaben	Acetaminophen glucuronide, methylparaben	Acetylsalicylic acid, gabapentin, ibuprofen carboxylic acid, p-hydroxybenzoic acid, pregabalin, salicylic acid, salicylic β-*D*-O-glucuronide acid, valproic acid	Acetaminophen, amoxicillin, carbamazepine, cefaclor, clofibric acid, clofibric acyl-β-D-glucuronide acid, cyclophosphamide, enalapril, gemcitabine, ifosfamide, naproxen, sulfametoxazole, 4-chlorobenzoic acid, 2-hydroxy ibuprofen	Acetaminophen, amoxicillin, carbamazepine, cefaclor, clofibric acid, clofibric acyl-β-D-glucuronide acid, cyclophosphamide, enalapril, gemcitabine, ifosfamide, naproxen, sulfametoxazole, 4-chlorobenzoic acid, 2-hydroxy ibuprofen
Acetylsalicylic acid, gabapentin, ibuprofen carboxylic acid, p-hydroxybenzoic acid, pregabalin, salicylic acid, salicylic β-*D*-O-glucuronide acid, valproic acid		Acetylsalicylic acid, gabapentin, ibuprofen carboxylic acid, p-hydroxybenzoic acid, pregabalin, salicylic acid, salicylic β-*D*-O-glucuronide acid, valproic acid	Acetylsalicylic acid, gabapentin, ibuprofen carboxylic acid, p-hydroxybenzoic acid, pregabalin, salicylic acid, salicylic β-*D*-O-glucuronide acid, valproic acid		Acetylsalicylic acid, gabapentin, ibuprofen carboxylic acid, p-hydroxybenzoic acid, pregabalin, salicylic acid, salicylic β-*D*-O-glucuronide acid, valproic acid	Acetylsalicylic acid, gabapentin, ibuprofen carboxylic acid, p-hydroxybenzoic acid, pregabalin, salicylic acid, salicylic β-*D*-O-glucuronide acid, valproic acid

*In brackets, the weight (in percentage) of P, B and T used in the sensitivity analysis. PPCP Level of hazard increases from the bottom to the top.

Appendix D

Partial ranking of concern of PPCPs according to the Hasse diagram for the OPBT indexes



The PPCP level of hazard increases from the top to the bottom.

Compounds legend: (1)Acetaminophen (2)1,4 benzoquinone (3)Acetaminophen Glucuronide (4)Ibuprofen (5)2-Hydroxy ibuprofen (6)Ibuprofen carboxylic acid (7)Naproxen (8)Diclofenac (9)4-Hydroxy diclofenac (10)5-hydroxy diclofenac (11)Ketorolac (12)Acetylsalicylic acid (13)Salicylic acid (14)Salicylic ß-D-O-Glucuronide acid (15)Amoxicillin (16)Sulfamethoxazole (17)Hydroxylamine sulfamethoxazole (18)Trimethoprim (19)Cefaclor (20)Azithromycin (21)Clarithromycin (22)Erythromycin (23)Roxythromycin (24)Ciprofloxacin (25)Levofloxacin (26)Norfloxacin (27)Moxifloxacin (28)Omeprazole (29)Pantoprazole (30)Lanzoprazole (31)Esomeprazole (32)Enalapril (33)Valsartan (34)Irbesartan (35)Simvastatin (36)Fluvastatin (37)Atorvastatin (38)Clofibrate (39)Clofibric acid (40)Clofibric acyl-ß-D-glucuronide acid (41)Bezafibrate (42)4-chlorobenzoic acid (43)Lorazepam (44)Alprazolam (45)Bromazepam (46)Paroxetine (47)4-[[(3S,4R)-4-(4-fluorophenyl)piperidin-3-yl]methoxy]-2-methoxyphenol (48)Sertraline (49)N-desmethyl sertraline (50)sertraline Carbamoyl glucuronide (51)Fluoxetine (52)Norfluoxetine (53)Fluvoxamine (54)Escitalopram (55)N-Desmethyl Escitalopram (56)Carbamazepine (57)Carbamazepine 10,11 dihydrodiol (58)Carbamazepine 10, 11-epoxide (59) 2-Hydroxy carbamazepine (60) 3-hydroxy carbamazepine (61)Valproic acid (62)Gabapentin (63)Pregabalin (64)Topiramate (65) 17-α etinylestradiol (66)17-ß estradiol (67)Estrone (68)Levonorgestrel (69)Norethisterone (70)Megestrol (71)Desogestrel (72)Testosterone (73)Diethylstilbestrol (74)Progesterone (75)Cyclophosphamide (76)Ifosfamide (77)Gemcitabine (78)Tamoxifen (79)Flutamide (80)Mitomycin (81)Iopromide (82)Iopamidol (83)Iohexol (84)Metylparaben (85)Propylparaben (86)Galaxolide® (87)Tonalide® (88)Phantolide® (89)Iso-E super® (90)Musk ketone (91)Musk Xilene.

9.3. Appendix C. Supplementary material Chapter 4

Ecotoxicity and environmental risk assessment of pharmaceuticals and personal care products in aquatic environments and wastewater treatment plants

PPCPs considered in Chapter 4

Acetaminophen, 1,4-benzoquinone (as acetaminophen´s transformation product), ibuprofen, ibuprofen sodium salt, diclofenac sodium salt, naproxen, naproxen sodium salt, acetylsalicylic acid (ASA), salicylic acid, amoxicillin, sulfamethoxazole, cefaclor, ciprofloxacin, ciprofloxacin hydrochloride monohydrate, clarithromycin, erythromycin, levofloxacin, norfloxacin, omeprazole, clofibrate, clofibric acid, methylparaben, ethylparaben, propylparaben, p-hydroxybenzoic acid (parabens metabolite), and triclosan.

9.4. Appendix D. Supplementary material Chapter 5

Dose-response behavior of the bacterium *Vibrio fischeri* exposed to pharmaceuticals and personal care products

APPENDIX A

STATISTICAL EQUATIONS

A1. Mean effect

$$\bar{X} = \frac{X_1+X_2+X_3+\cdots+X_N}{N} \qquad \textbf{Eq. A1}$$

$\bar{X}$ is the mean effect on bacteria to a specific concentration.

X_1, X_2, X_3….X_N are the different effects on bacteria from replicate assays or independent assays at the same concentration.

N is the number of results (effect) for the same concentration.

A2. Standard Deviation (SD) [1]

The standard deviation (SD) quantifies variability or scatter, and it is expressed in the same units as the data analyzed.

SD was calculated following the next steps:

- It is calculated the square of the difference between each value and the sample mean.
- It is added those values up.
- It is divided the sum by N-1. This is the variance.
- It is taked the square of the root to obtain the SD

A3. Goodness of fit

Sum of Square (SS):

$$SS = \sum_{i=1}^{n}(y_i - \bar{y})^2 \qquad \textbf{Eq. A2}$$

Where y_i is each value of the effect on Vibrio fischeri (%) and $\bar{y}$ is the mean value of *y*.

Correlation coefficient (R):

$$R = \sqrt{1 - \frac{SSE}{SS}} \qquad \textbf{Eq. A3}$$

Where SSE is the sum of squared errors calculated with equation A3.

Sum of square errors (SSE):

$$SSE = \sum_{i=1}^{n} (y_i - yf_i)^2 \qquad \textbf{Eq. A4}$$

Where yf_i are the fitted values of *y*.

A4. Normality tests [1,2]

Test of normality are statistical inference procedures designed to test that the underlying distribution of a random variable is normally distributed. GraphPad Prism 6 offers three normality tests.

D'Agostino-Pearson normality test. It first computes the skewness and kurtosis to quantify how far the distribution is from Gaussian in terms of asymmetry and shape. It then calculates how far each of these values differs from the value expected with a Gaussian distribution, and computes a single P value from the sum of these discrepancies. It is a versatile and powerful normality test, and is recommended. D'Agostino developed several normality tests. The one used by Prism is the "omnibus K2" test.

An alternative is the Shapiro-Wilk normality test that works very well if every value is unique, it does not work as well when several values are identical. The basis of this test is hard to understand. Finally, the Kolmogorov-Smirnov test is the third option. It computes a P value from a single value: the largest discrepancy between the cumulative distribution of the data and a cumulative Gaussian distribution.

The Kolmogorov-Smirnov method as originally published assumes that the mean and SD of the overall population (perhaps from prior work) are known. When analyzing data, rarely it is known the overall population mean and SD. It is only known the mean and SD of the sample. To compute the P value, therefore, Prism uses the Dallal and Wilkinson approximation to Lilliefors' method. Since that method is only accurate with small P values, Prism simply reports “P>0.10” for large P values.

A5. Two way analysis of variance (Two-way ANOVA)[1,3]

Two-way ANOVA determines how a response is affected by two factors.
In this research, time (5 and 15 minutes) and concentrations (four different concentrations for each PPCP at environmental level and four different concentrations around EC_{50} level) are the factors. The response is the variation of bioluminescence expressed in percentage. The variability among times and among concentrations for the two range of concentrations studied were analyzed by this method. SS, degrees of freedom, mean square, F-ratio and P-value were calculated and reported.

To determine whether the differences between some of the means are statistically significant it has been compare the p-value to the significance level to assess the null hypothesis. The null hypothesis is that the group means are all equal. In this research, the significance level (denoted as α or alpha) of 0.05 was chosen. A significance level of 0.05 indicates a 5% risk of concluding that a difference exists when there is no actual difference.

P-value ≤ α: The differences between some of the means are statistically significant.

P-value > α: The differences between the means are not statistically significant.

References

[1] *GraphPad Prism® statistics guide.
[2] D'Agostino RB et al. (1990) A suggestion for Using Powerful and Informative Test of Normality. The American Statistician 44(4):316-321.
[3] Minitab Express Support. Available at: http://support.minitab.com/en-us/minitab-express/1/help-and-how-to/modeling-statistics/anova/how-to/one-way-anova/interpret-the-results/key-results/. Accessed on May 26, 2015.

APPENDIX B

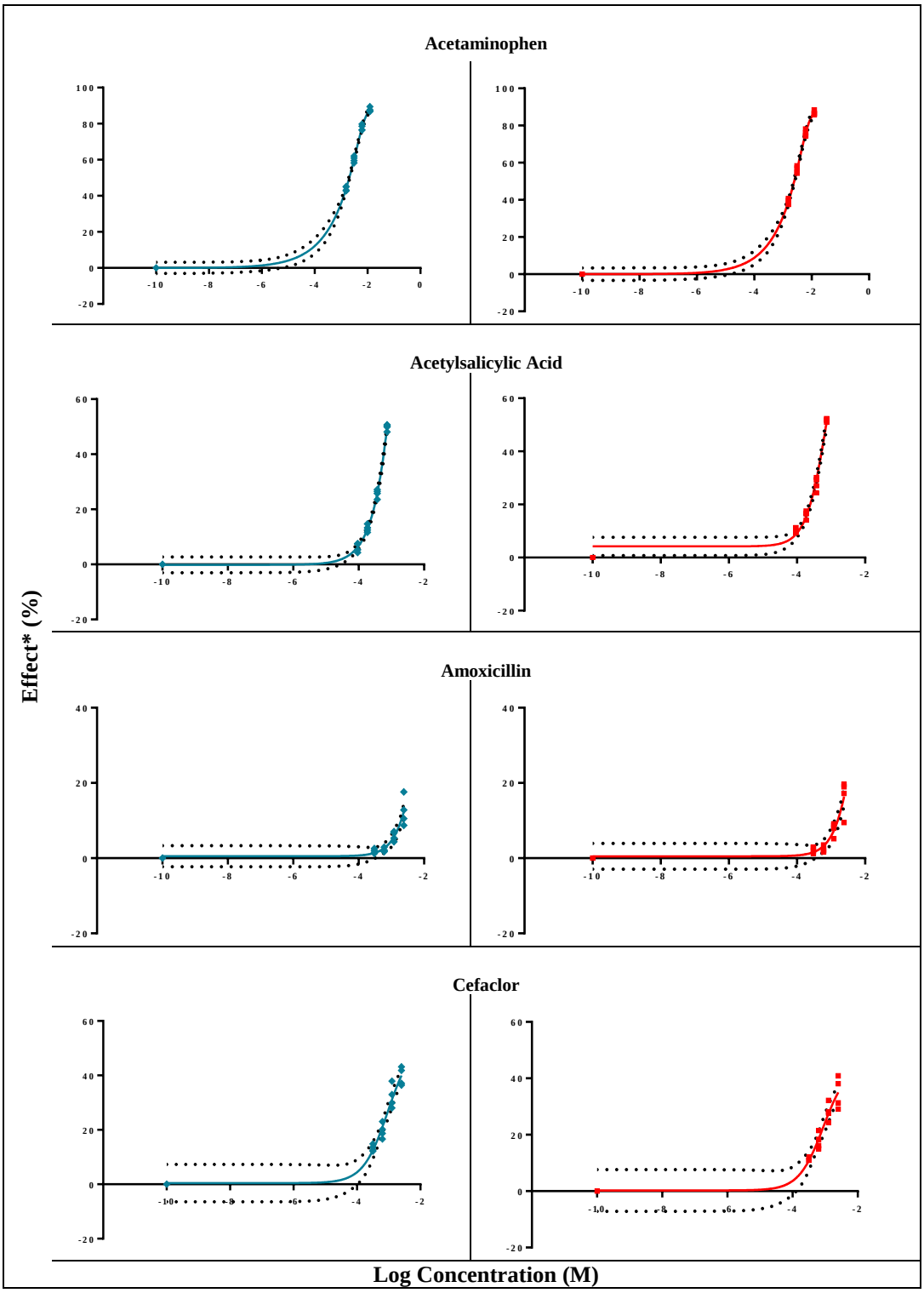


Figure B.1. Best-fit dose-response curves of the Microtox® ecotoxicity test results for PPCPs with their 95% confidence intervals.
Blue line: 5 minutes data. Red line: 15 minutes data.
* Positive effect means bioluminescence inhibition. Negative effect means bioluminescence stimulation.

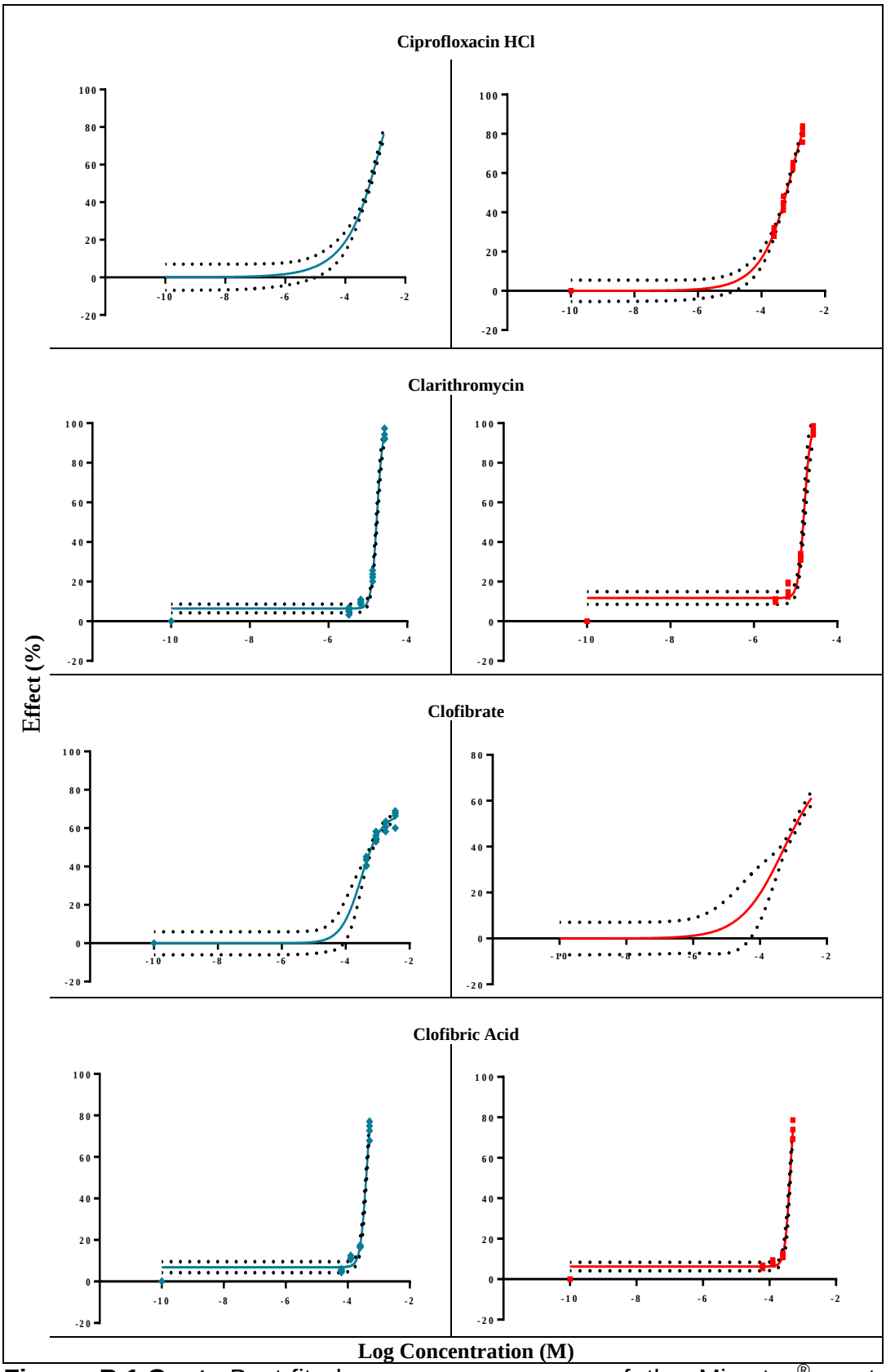


Figure B.1.Cont. Best-fit dose-response curves of the Microtox® ecotoxicity test results for PPCPs with their 95% confidence intervals. Blue line: 5 minutes data. Red line: 15 minutes data.

Blue line: 5 minutes data. Red line: 15 minutes data.

* Positive effect means bioluminescence inhibition. Negative effect means bioluminescence stimulation.

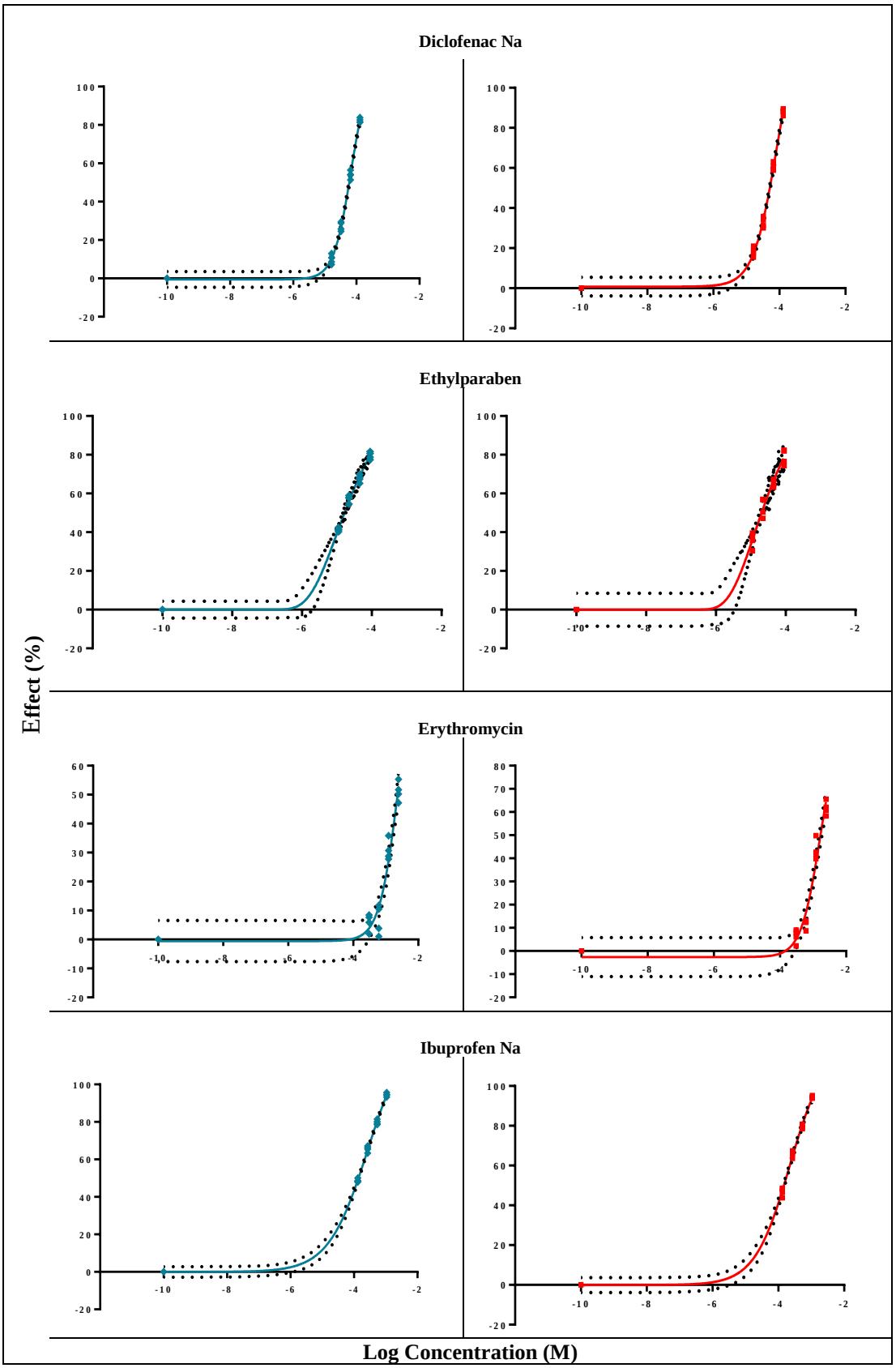


Figure B.1.Cont. Best-fit dose-response curves of the Microtox® ecotoxicity test results for PPCPs with their 95% confidence intervals. Blue line: 5 minutes data. Red line: 15 minutes data.

Blue line: 5 minutes data. Red line: 15 minutes data.

* Positive effect means bioluminescence inhibition. Negative effect means bioluminescence stimulation.

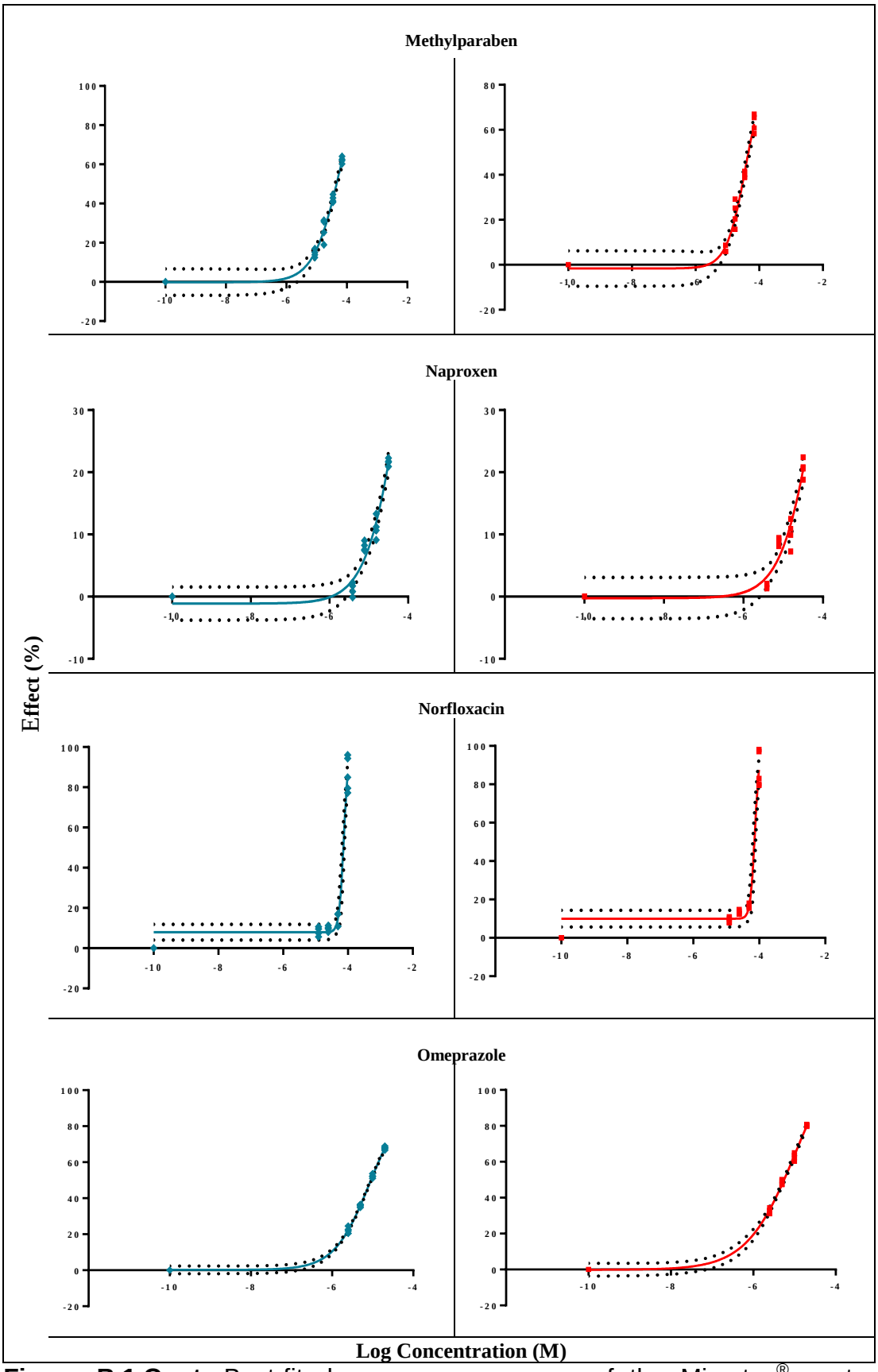


Figure B.1.Cont. Best-fit dose-response curves of the Microtox® ecotoxicity test results for PPCPs with their 95% confidence intervals. Blue line: 5 minutes data. Red line: 15 minutes data.

Blue line: 5 minutes data. Red line: 15 minutes data.

* Positive effect means bioluminescence inhibition. Negative effect means bioluminescence stimulation.

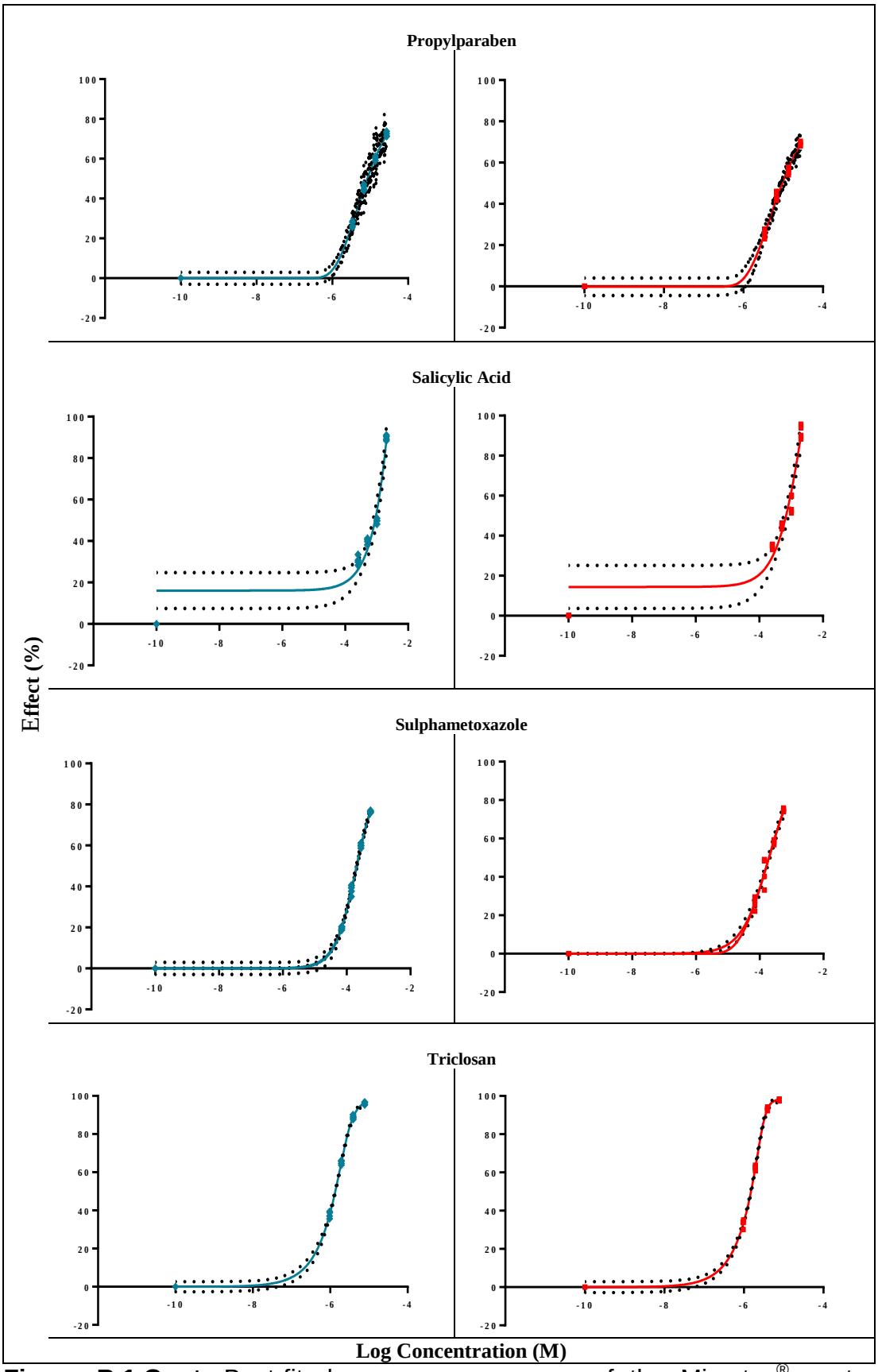


Figure B.1.Cont. Best-fit dose-response curves of the Microtox® ecotoxicity test results for PPCPs with their 95% confidence intervals. Blue line: 5 minutes data. Red line: 15 minutes data.

Blue line: 5 minutes data. Red line: 15 minutes data.

* Positive effect means bioluminescence inhibition. Negative effect means bioluminescence stimulation.

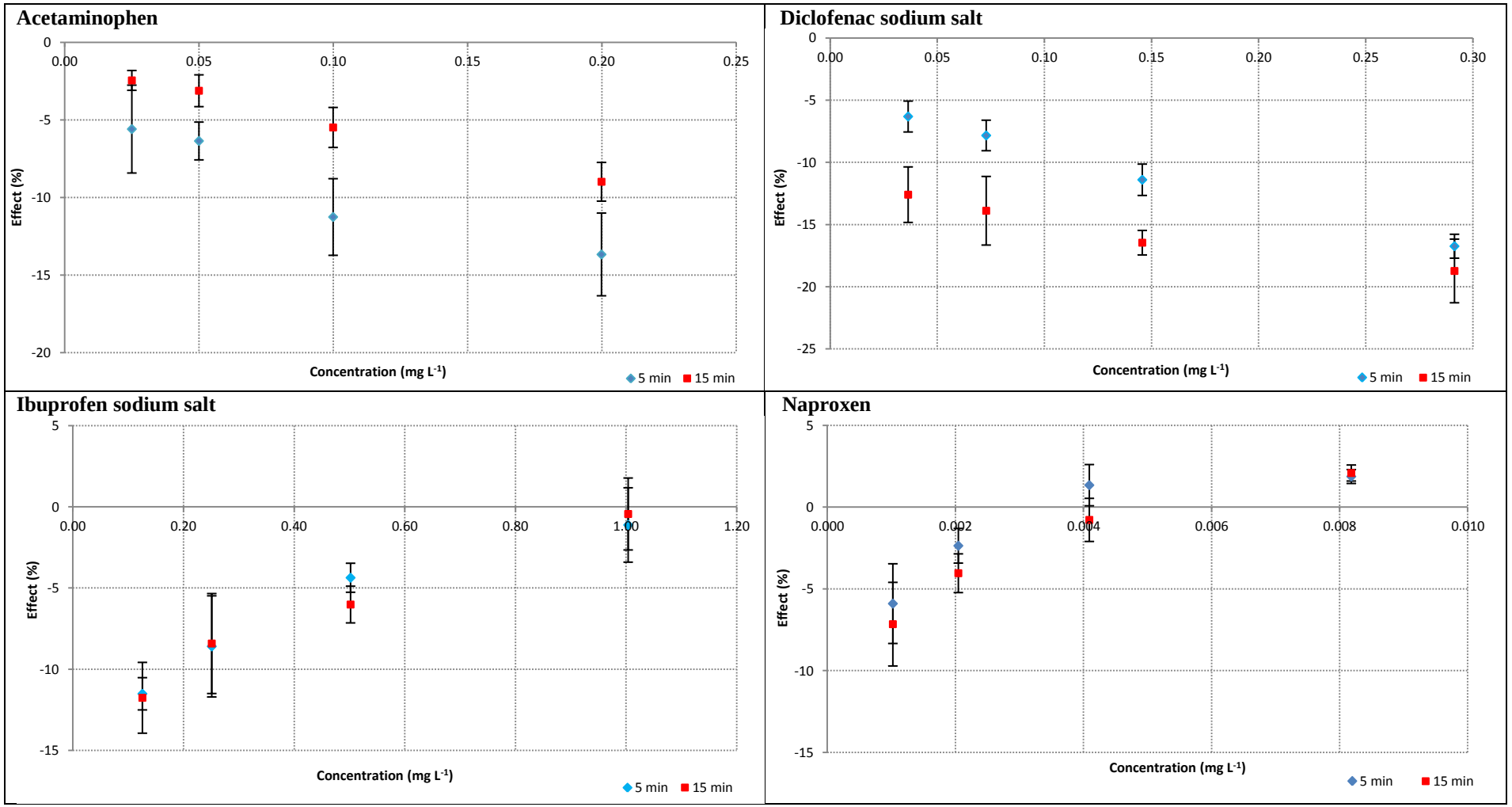


Figure B2. Dose-response from Microtox® ecotoxicity tests at environmental concentrations.
Data are given as mean effects with their standard deviation. Positive effect means bioluminescence inhibition. Negative effect means bioluminescence stimulation.

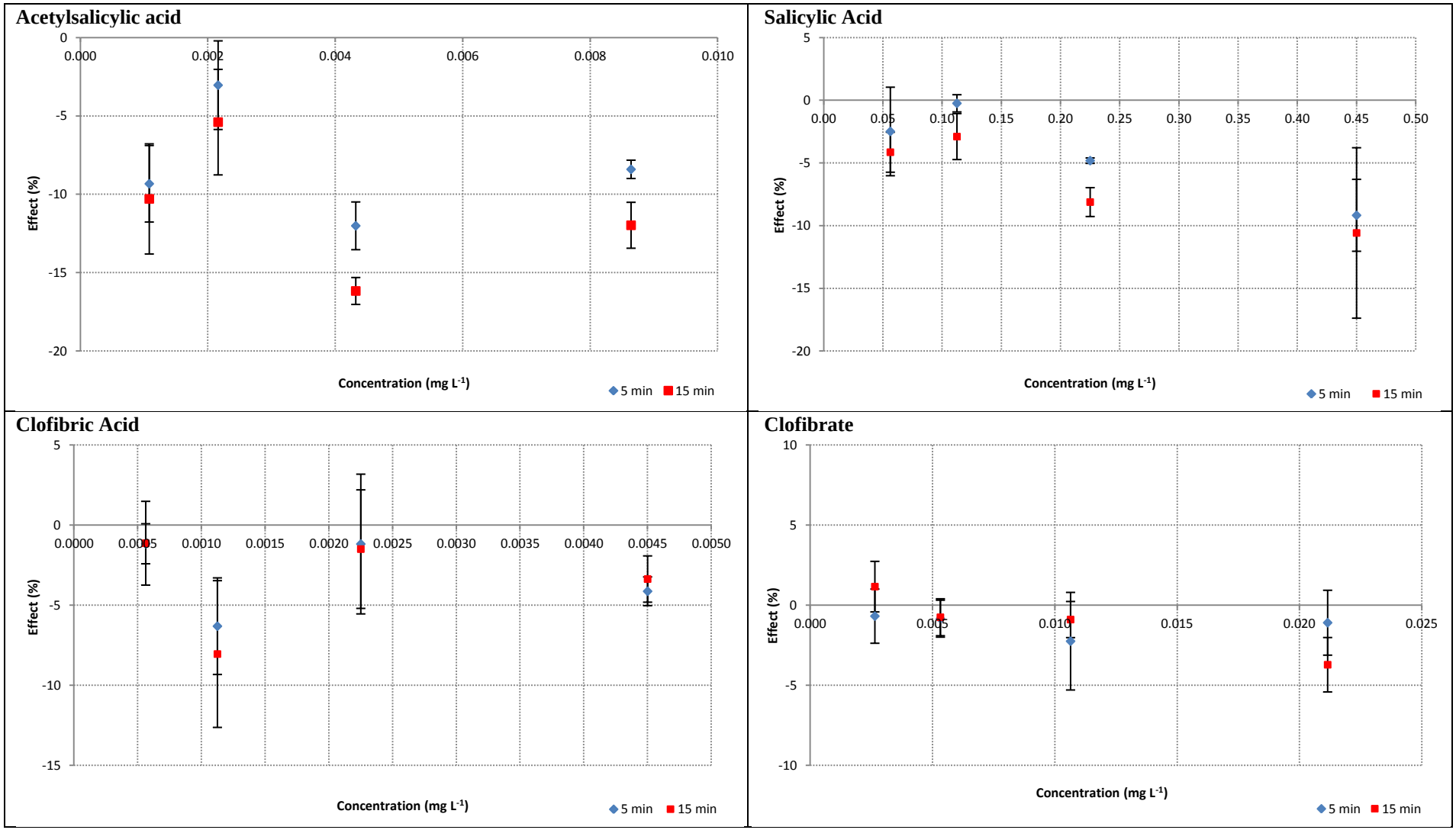


Figure B2. Cont. Dose-response from Microtox® ecotoxicity tests at environmental concentrations.
Data are given as mean effects with their standard deviation. Positive effect means bioluminescence inhibition. Negative effect means bioluminescence stimulation.

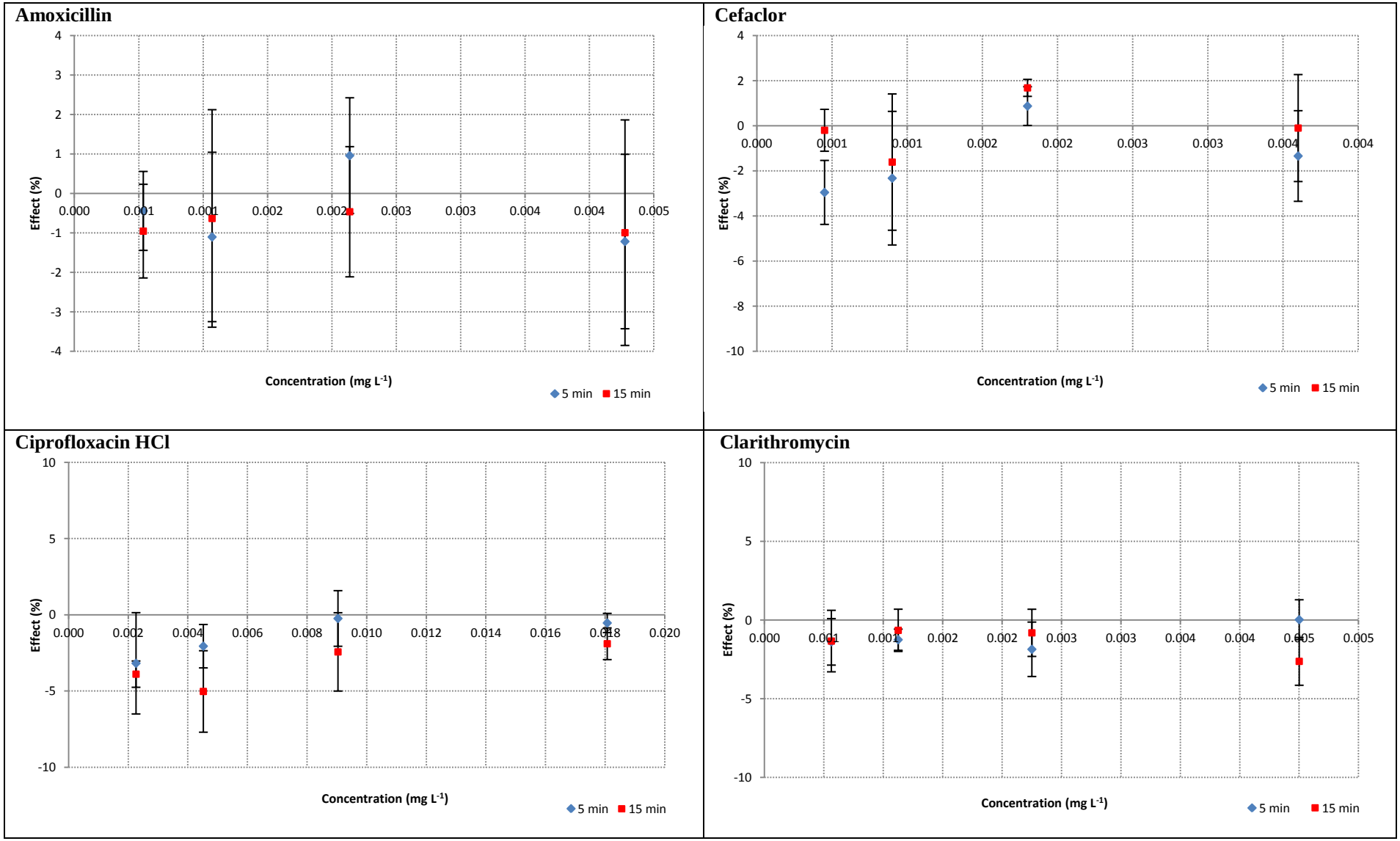


Figure B2. Cont. Dose-response from Microtox® ecotoxicity tests at environmental concentrations.
Data are given as mean effects with their standard deviation. Positive effect means bioluminescence inhibition. Negative effect means bioluminescence stimulation.

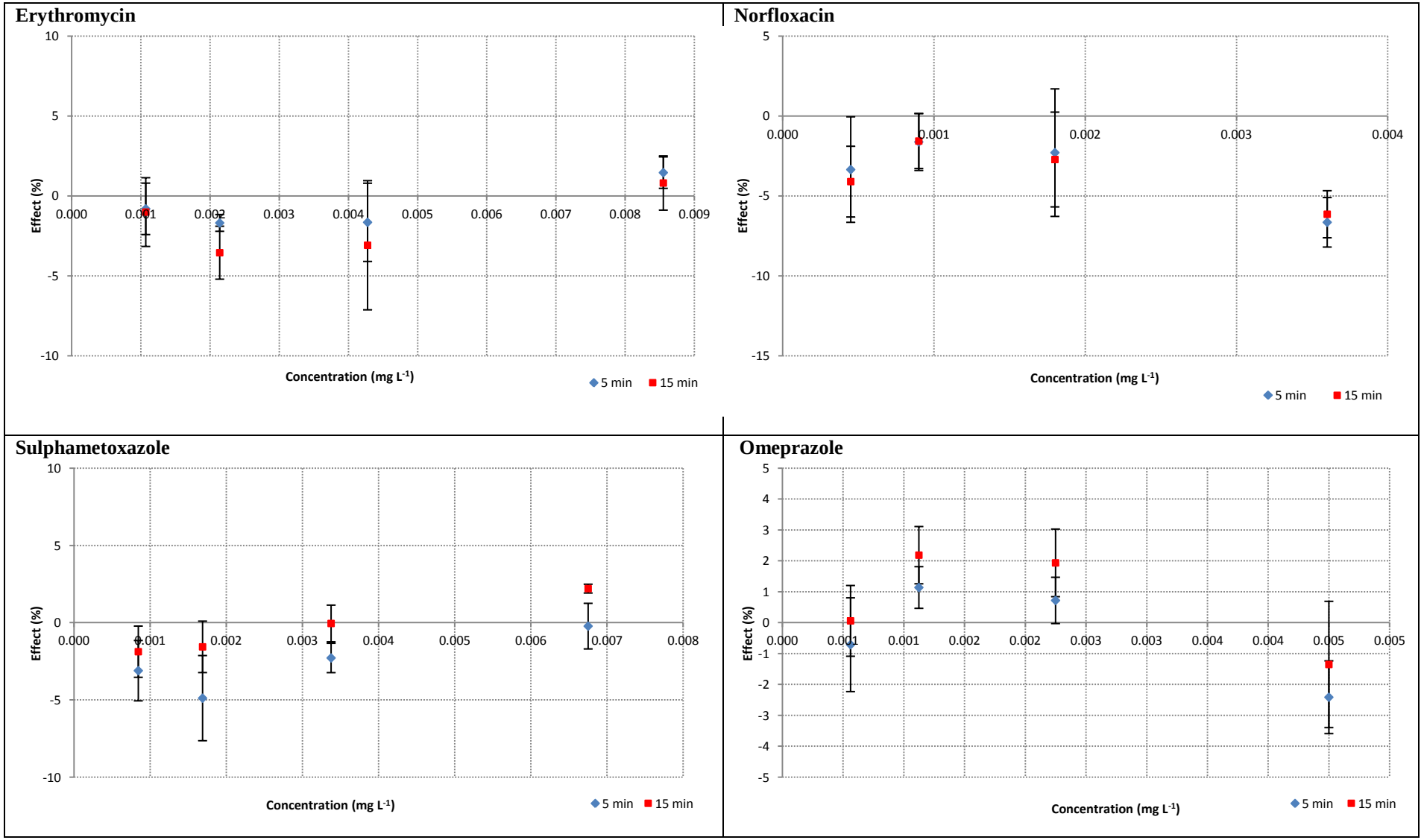


Figure B1. Cont. Dose-response from Microtox® ecotoxicity tests at environmental concentrations.
Data are given as mean effects with their standard deviation. Positive effect means bioluminescence inhibition. Negative effect means bioluminescence stimulation.

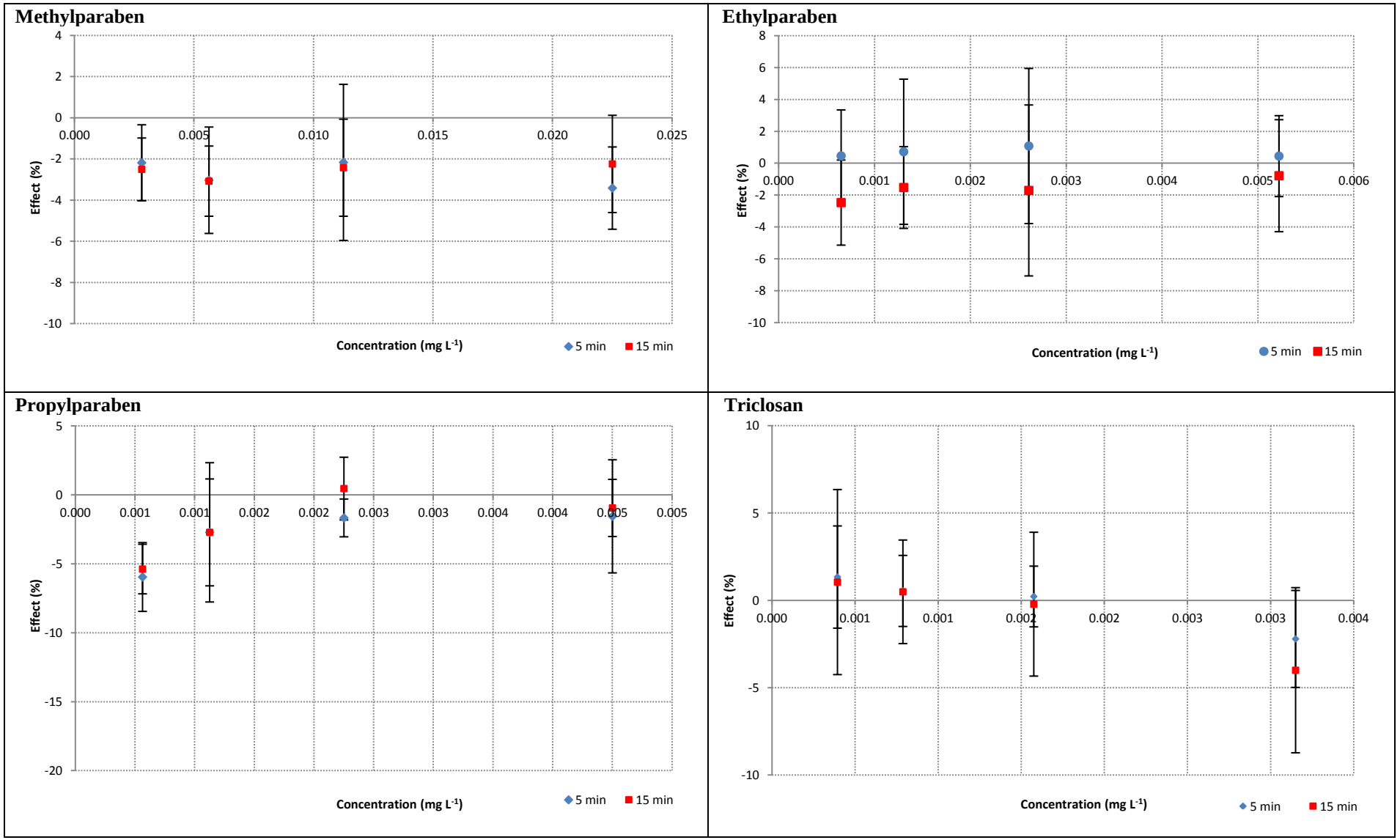


Figure B1. Cont. Dose-response from Microtox® ecotoxicity tests at environmental concentrations.
Data are given as mean effects with their standard deviation. Positive effect means bioluminescence inhibition. Negative effect means bioluminescence stimulation.

Table B1. Two-way Analysis of variance (Two-way ANOVA) for the dose-response data around EC_{50}

Compound \ Factor	Concentration						Time					
	SS*	DF**	MS***	F+	P value++	Significant	SS	DF	MS	F	P value	Significant
Analgesic/antipyretic												
Acetaminophen	2463	3	821	478	0.0002	Yes	17.23	1	17.23	10.03	0.0506	No
Antibiotics												
Amoxicillin Trihydrate	199.8	3	66.6	45.00	0.0054	Yes	5.036	1	5.0360	3.4030	0.1623	No
Cefaclor	753.8	3	251.3	229.9	0.0005	Yes	20.47	1	20.47	18.73	0.0227	Yes
Ciprofloxacin	2667	3	889.1	314.7	0.0003	Yes	7.452	1	7.4520	2.6380	0.2028	No
Clarithromycin	9750	3	3250	705.9	0.0001	Yes	71.02	1	71.02	15.43	0.0294	Yes
Erythromycin	2802	3	934.1	-	<0.0001	Yes	0	1	0	-	>0.9999	No
Norfloxacin	8402	3	2801	2505	<0.0001	Yes	0.0496	1	0.0496	0.3287	0.6066	No
Sulphametoxazole	3123	3	1041	117.1	0.0013	Yes	6.7650	1	6.7650	0.7609	0.4472	No
Blood lipid regulators												
Clofibrate	636.1	3	212	97.32	0.0017	Yes	84.74	1	84.74	38.89	0.0083	Yes
Clofibric acid	6032	3	2011	529.7	0.0001	Yes	7.1640	1	7.1640	1.8870	0.2632	No
H_2 blocker												
Omeprazole	2395	3	798.3	1058	<0.0001	Yes	265	1	265	351	0.0003	Yes
Platelet aggregation inhibitors												
Acetylsalicylic acid	2117	3	705.6	1464	<0.0001	Yes	15.33	1	13.33	31.81	0.0110	Yes
Salicylic acid	3900	3	1300	862.0	<0.0001	Yes	40.30	1	40.30	26.72	0.0140	Yes
Non-steroidal Anti-inflammatory drugs												
Diclofenac sodium salt	5880	3	1960	2728	<0.0001	Yes	85.94	1	85.94	119.6	0.0016	Yes
Ibuprofen sodium salt	2373	3	791.0	1628	<0.0001	Yes	0.5440	1	0.5440	1.1200	0.3676	No
Naproxen	399	3	133.0	277.1	0.0004	Yes	0.0334	1	0.0334	0.0696	0.8091	No
PCPs												
Biocide												
Triclosan	4851	3	1617	193.5	0.0006	Yes	0.1871	1	0.1871	0.0224	0.8905	No
Preservatives												
Ethylparaben	2196	3	731.9	2281	<0.0001	Yes	14.93	1	14.93	46.53	0.0064	Yes
Methylparaben	2999	3	999.8	154.3	0.0009	Yes	22.55	1	22.55	3.4810	0.1589	No
Propylparaben	2196	3	731.9	2281	<0.0001	Yes	14.93	1	14.93	46.53	0.0064	Yes

*Sum of Square. **Degree of Freedom. ***Mean of Square. +F-ratio. ++P-value.

Table B2. Two-way Analysis of variance (Two-way ANOVA) for the dose-response data at environmental concentrations

Compound \ Factor	Concentration						Time					
	SS*	DF**	MS***	F+	P value++	Significant	SS	DF	MS	F	P value	Significant
Analgesic/antipyretic												
Acetaminophen	69.06	3	23.02	28.96	0.102	Yes	35.41	1	35.41	44.54	0.0069	Yes
Antibiotics												
Amoxicillin Trihydrate	2.112	3	0.7041	1.946	0.2992	No	0.1928	1	0.1928	0.532	0.5183	No
Cefaclor	12.55	3	4.184	9.357	0.0494	Yes	3.8040	1	3.804	8.507	0.0617	No
Ciprofloxacin	10.26	3	3.42	7.07	0.0712	No	6.5510	1	6.551	13.54	0.0348	Yes
Clarithromycin	0.2211	3	0.0737	0.0536	0.9808	No	0.1140	1	0.114	0.0829	0.7921	No
Erythromycin	17.84	3	5.946	21.15	0.0161	Yes	2.1550	1	2.155	7.665	0.0696	No
Norfloxacin	26.13	3	8.711	57.72	0.0038	Yes	0.0496	1	0.0496	0.3287	0.6066	No
Sulphametoxazole	20.54	3	6.8480	18.59	0.0193	Yes	10.60	1	10.6	28.76	0.0127	Yes
Blood lipid regulators												
Clofibrate	7.589	3	2.53	1.267	0.4251	No	0.0548	1	0.0548	0.0274	0.8790	No
Clofibric acid	47.46	3	15.82	28.64	0.0104	Yes	0.1980	1	0.1980	0.3585	0.5915	No
H_2 blocker												
Omeprazole	16.05	3	5.35	306.0	0.0003	Yes	2.1010	1	2.1010	120.2	0.0016	Yes
Platelet aggregation inhibitors												
Acetylsalicylic acid	99.07	3	33.02	33.09	0.0085	Yes	15.27	1	15.27	15.30	0.0297	Yes
Salicylic acid	80.36	3	26.79	68.65	0.0029	Yes	10.17	1	10.17	26.07	0.0145	Yes
Non-steroidal Anti-inflammatory drugs												
Diclofenac sodium salt	80.94	3	26.98	13.83	0.0291	Yes	46.95	1	46.95	24.06	0.0162	Yes
Ibuprofen sodium salt	129.6	3	43.19	86.29	0.0021	Yes	0.1348	1	0.1348	0.2692	0.6397	No
Naproxen	85.86	3	28.62	55.46	0.0040	Yes	2.9280	1	2.9280	5.6740	0.0974	No
PCPs												
Biocide												
Triclosan	21.65	3	7.2160	23.54	0.0138	Yes	0.8240	1	0.8240	2.6880	0.1996	No
Preservatives												
Ethylparaben	30.43	3	10.14	24.53	0.0130	Yes	1.3780	1	1.3780	3.3320	0.1654	No
Methylparaben	0.8281	3	0.2760	1.1310	0.4610	No	0.0378	1	0.0378	0.1549	0.7202	No
Propylparaben	30.43	3	10.14	24.53	0.0130	Yes	1.3780	1	1.378	3.3320	0.1654	No

*Sum of Square. **Degree of Freedom. ***Mean of Square. +F-ratio. ++P-value.

9.5. Appendix E. Supplementary material Chapter 6

Human and ecotoxicological potential impact of pharmaceutical and personal care products from USEtox™ life cycle impact assessment characterization factors

PPCPs considered in Chapter 6

Acetaminophen, alprazolam, amoxicillin, atorvastatin, azithromycin, bromazepam, carbamazepine, cefaclor, ciprofloxacin, clarithromycin, clofibrate, cyclophosphamide, diclofenac, enalapril, erythromycin, estrone, ethylparaben, fluoxetine, gabapentine, galaxolide®, ibuprofen, iohexol, iopamidol, iopromide, irbesartan, ketorolac, levofloxacin, lorazepam, methylparaben, naproxen, norfloxacin, omeprazol, paroxetine, pregabalin, propylparaben, roxythromycin, salicylic acid, sertraline, simvastatin, sulphametoxazole, tamoxifen, testosterone, tonalide®, triclosan, trimethoprim, valproic acid, valsartan, 17α-ethinylestradiol, 17β-estradiol.

Occurrence and effects of pharmaceuticals and personal care products: new contributions in predictive models, potential risks assessments and rankings of hazard

Author: Sheyla Ortiz de García
Directors: Dr. Rubén Irusta Mata
Dr. Pedro García Encina

Valladolid, October 2015