UNIVERSIDADE DE SÃO PAULO
Faculdade de Ciências Farmacêuticas
Programa de Pós-Graduação em Farmácia (Fisiopatologia e Toxicologia)
Área de Toxicologia

Assessment of the single and mixture ecotoxicity of pharmaceuticals of environmental concern using aquatic test organisms

Avaliação da ecotoxicidade individual e das misturas de fármacos de preocupação ambiental usando organismos-teste aquáticos

Aline Andrade Godoy

Tese para obtenção do Título de Doutor
Orientador: Prof. Dr. Fábio Kummrow

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da
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Orientador/presidente

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São Paulo, 05 de julho de 2019

Dedico este trabalho aos meus pais Domingos
e Maria Conceição (in memoriam),
aos meus irmãos Henrique e Gustavo
e ao meu marido Luciano,
pelo amor e pela torcida incondicionais por mim.

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A Deus, pela vida que Ele me concedeu e pela força com que me sustenta para vencer todas as batalhas diárias.

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A todos, meu eterno MUITO OBRIGADA!

Man is a part of nature and his war against nature is inevitably a war against himself
(Rachel Carson, 1963)

RESUMO

GODOY, A.A. **Avaliação da ecotoxicidade individual e das misturas de fármacos de preocupação ambiental usando organismos-teste aquáticos.** 2019. 386 f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2019.

A contaminação ambiental por fármacos tem sido alvo de crescente preocupação pela comunidade científica. Fármacos de elevado consumo, incompleto metabolismo e remoção incompleta em estações de tratamento de esgoto, como é o caso da metformina (MET), bisoprolol (BIS), sotalol (SOT) e ranitidina (RAN), têm sido frequentemente detectados em matrizes aquáticas do mundo todo. Apesar disso, dados ecotoxicológicos consistentes para esses contaminantes são escassos, principalmente com relação a efeitos comportamentais e oriundos de estudos crônicos. Além disso, o entendimento dos efeitos de suas ações combinadas em organismos não-alvo é ainda incipiente, o que gera incertezas na avaliação dos seus riscos ambientais. Esta pesquisa teve por objetivo preencher essas lacunas de conhecimentos para esses quatro fármacos, por meio da realização de testes com cinco diferentes organismos-teste de três diferentes níveis tróficos. Foram analisados diferentes parâmetros avaliativos em testes com os organismos aquáticos *Raphidocelis subcapitata* (alga), *Lemna minor* (macrófita), *Daphnia similis* (crustáceo), *Hydra attenuata* (cnidário) e *Danio rerio* (peixe). As toxicidades agudas das misturas binárias e quaternárias desses quatro fármacos também foram avaliadas em testes com *D. similis* e embriões de *D. rerio*, respectivamente. Este trabalho também teve por objetivo avaliar a acurácia preditiva dos modelos de adição de concentração (CA) e ação independente (IA) e analisar a natureza das possíveis interações toxicológicas entre os fármacos, em misturas binárias, usando o modelo do Índice de Combinação (CI). A modelagem das relações concentração-resposta e as análises estatísticas associadas foram realizadas empregando-se a planilha automatizada ToxCalcMix versão 1.0 e o *software* OriginPro 2015. O *software* CompuSyn foi utilizado para as análises envolvendo o CI. O planejamento experimental dos testes de misturas binárias foi realizado por meio do *design* fatorial fracionado, a fim de cobrir diversas possíveis interações em várias proporções e níveis de efeitos, com a redução do número de organismos-teste. Os resultados desta pesquisa estão apresentados em quatro artigos. No artigo 1, realizou-se uma revisão crítica com relação às lacunas de conhecimentos e deficiências identificadas a partir da análise da literatura sobre a ecotoxicologia de misturas de fármacos e de produtos de higiene pessoal. Nos artigos seguintes, foram apresentados e discutidos os resultados oriundos dos testes com os quatro fármacos avaliados neste estudo. Os fármacos MET (artigo 2) e BIS (artigo 3) foram classificados como perigosos para o ambiente aquático, na categoria de toxicidade aguda. Contudo, um risco ecológico não é esperado para as espécies pelágicas de água doce expostas a esses dois fármacos, com base nos dados de toxicidade crônica obtidos. Os resultados dos testes de misturas (artigo 4) permitiram concluir que a maior parte dos efeitos observados das misturas binárias estiveram na zona entre os efeitos preditos pelos modelos clássicos de CA e IA. O modelo do CI mostrou-se uma ferramenta útil para descrever a natureza das possíveis interações toxicológicas que ocorrem entre os fármacos em ações combinadas. Mesmo concentrações de nenhum efeito estatisticamente significativo dos fármacos causaram efeitos adversos significativos quando em misturas (*something from nothing*). Concluiu-se que avaliações de risco ecológicas baseadas em efeitos tóxicos individuais de contaminantes ambientais podem subestimar o real impacto desses compostos em ecossistemas aquáticos.

Palavras-chave: Modelagem de misturas; sinergismo; comportamento locomotor; toxicidade crônica; avaliação de risco.

ABSTRACT

GODOY, A.A. **Assessment of the single and mixture ecotoxicity of pharmaceuticals of environmental concern using aquatic test organisms**. 2019. 386 f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2019.

Pharmaceuticals are contaminants of emerging concern which have been a target of increasing attention by the scientific community. Pharmaceuticals presenting high consumption, incomplete metabolism and incomplete removal at wastewater treatment plants have been frequently detected in aquatic ecosystems worldwide. This is the case of the pharmaceuticals metformin (MET), bisoprolol (BIS), sotalol (SOT) and ranitidine (RAN). However, ecotoxicity data for these contaminants are scarce, especially regarding behavior effects and chronic toxicity. In addition, the knowledge regarding the joint toxicity of these pharmaceuticals on non-target organisms is still incipient, which makes their environment risk assessment uncertain. This study aimed to fill these knowledge gaps for these four pharmaceuticals, by carrying out toxicity tests using five test organisms from three trophic levels. Different endpoints were assessed in tests with *Raphidocelis subcapitata* (algae), *Lemna minor* (macrophyte), *Daphnia similis* (crustacean), *Hydra attenuate* (cnidarian) and *Danio rerio* (fish). The binary and quaternary mixture acute toxicity for these pharmaceuticals were assessed on *D. similis* and *D. rerio* embryo tests, respectively. This study also aimed to evaluate the predictive accuracy of the Concentration addition (CA) and the Independent action (IA) classic models. In addition, the nature of the possible toxicological interactions between the pharmaceuticals in binary mixtures were also evaluated, using the Combination Index-isobologram (CI) method. The modelling of the concentration-response curves and the associated statistical analyses were performed using the automated spreadsheet ToxCalcMix v.1.0 and the software OriginPro 2015. The software CompuSyn was used for performing the mixture analyses with the CI method. The experimental planning of the binary mixture tests was performed using the fractioned factorial design, in order to cover several possible ratio and level-dependent effects with a reduced number of test organisms. The results obtained in this study are shown in four articles. In article 1, we provided a critical review and discussed the misunderstandings, deficiencies and data gaps on the ecotoxicity data of pharmaceuticals and personal care products mixtures published in the literature. In the following articles, the results obtained from the single and mixture toxicity tests performed in this study were presented and discussed. The pharmaceuticals MET (article 2) and BIS (article 3) were classified as hazardous to the aquatic environment, in the acute toxicity category. However, an ecological risk is not expected for the pelagic freshwater species exposed to these two pharmaceuticals, based on the chronic data obtained. The results obtained from the mixture toxicity tests (article 4) showed that most of the observed toxicity effects from the binary mixtures were in the zone between the predicted effects by the CA and IA models. The CI model showed to be an useful tool to describe the possible toxicological interactions occurring between the pharmaceuticals in joint action. Even statistically significant non-effect concentrations of the pharmaceuticals added up to induce significant adverse effects in mixtures (something from nothing). It was concluded that ecological risk assessment based on single toxic effects can underestimate the real impact of environmental contaminants on aquatic ecosystems.

Keywords: Mixture modelling; synergism; behavior locomotor; chronic toxicity; risk assessment.

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CONTENTS

1 INTRODUCTION

The scientific interest and public awareness on the problem represented by the presence of pharmaceuticals in the environment was highlighted initially in the 1970s, in a study on the biodegradability of steroid hormones (KÜMMERER, 2001). Yet, it was only in the middle of the 1990s that the concerning about the widespread environmental contamination caused by pharmaceutical substances grew, as a result of improvements in analytical science occurring in the end of the 20th century (SANTOS et al., 2010; TAYLOR and SENAC, 2014). Since then, advanced chromatographic techniques with detection limits within the ng L^{-1} to µg L^{-1} range have allowed researchers to quantify a large number of pharmaceuticals in several environmental matrices (SANTOS et al., 2010). As a result, the environmental pollution caused by pharmaceutical residues is an area of increasing concern (VASQUEZ et al., 2014).

This growing concern is justified since pharmaceuticals are designed to be biologically active by altering specific physiological functions and to resist to inactivation before reaching the therapeutic target (SANTOS et al., 2010; VASQUEZ et al., 2014). Therefore, there is a high probability that pharmaceuticals are biologically active towards non-target organisms as well, inducing toxic effects (VASQUEZ et al., 2014). In fact, a variety of organisms including amphibians, fishes, daphnids and algae have been predicted to possess evolutionary well-conserved drug targets with considerable degrees of similarity compared to humans (GUNNARSSON et al., 2008).

The widespread presence of pharmaceuticals in environmental matrices is a consequence of (i) the growing consumption of medicinal products, as a result of aging human demographic and medicinal development progress; (ii) their incomplete metabolism; (iii) their incomplete removal at wastewater treatment plants (WWTP) employing conventional activated sludge technique, (iv) the high polarity and low volatility of many of these compounds, which increases the probability that they can be transported to surface waters and (v) their consequent continuous release into aquatic environments, thus maintaining existing levels even of pharmaceuticals presenting relative short half-lives (BRAUSCH et al., 2012; HUGHES et al., 2013; STANKIEWICZ et al., 2015). The most significant entry route for pharmaceuticals into the aquatic environment is the release of final effluents from WWTP, since considerable proportions of these compounds can be excreted unaltered in the urine and feces to the sewage (BOUND and VOULVOULIS, 2004). In addition, another part of these pharmaceuticals is often excreted conjugated to polar molecules, which can easily be cleaved during sewage

treatment, thus releasing the original pharmaceuticals into the aquatic environments (HEBERER, 2002). Other possible sources for the occurrence of pharmaceuticals in the environment are the disposal of unused pharmaceuticals via the toilet (HEBERER, 2002), the hospital effluents (FRÉDÉRIC and YVES, 2014), industrial effluents (LARSSON et al., 2007), landfill leachate (BUSZKA et al., 2009), runoff following application of biosolids to the agriculture (SABOURIN et al., 2009), septic tanks, managed aquifer recharge (LAPWORTH et al., 2012) and livestock and aquaculture activities (KWON, 2016). As a result, pharmaceuticals have been detected, in the ng to µg L^{-1} range, in several environmental compartments worldwide, including fresh and estuarine/marine surface waters, groundwaters and even in drinking waters (UBA, 2019).

Despite this widespread contamination, ecotoxicological data for many pharmaceuticals are still scarce, mainly in relation to chronic and mixture effects (SANTOS et al., 2010; BRAUSCH et al., 2012). In addition, to date, very few studies have been carried out employing non-standardized endpoints such as behavior of non-target organisms exposed to environmental concentrations of pharmaceutical residues (HENRIQUES et al., 2016). Nonetheless, recent studies have pointed out that behavior is a crucial endpoint to be considered in future toxicity assays, since it has been showed to be more sensitive than developmental and biochemical endpoints to the effects induced by environmental contaminants (ANDRADE et al., 2016; HENRIQUES et al., 2016; SANCHES et al., 2018). Besides, behavior presents a high ecological relevance, since it is related to the survival of populations (ANDRADE et al., 2016). As a consequence of this lack of ecotoxicity data, robust ecological risk assessment for many pharmaceuticals is difficult to perform. In the absence of experimental data, this lack of information is often filled with data from quantitative structure-activity relationship (QSAR) predictions. However, this methodology is not sufficiently precise for accurate hazard and risk assessments of pharmaceuticals (FENT et al., 2016).

Another critical point regarding the presence of pharmaceuticals in the environment are the mixture toxicity effects. Pharmaceuticals typically do not occur isolated in the environment, but as mixtures. Therefore, aquatic organisms are usually exposed to complex mixtures of these environmental contaminants (BACKHAUS, 2014). Studying the joint toxicity effect of pharmaceutical mixtures is an important issue for matters of hazard and risk assessment because the ecotoxicity of a pharmaceutical mixture is normally higher than the single effects of each individual component. Thus, these mixtures can exert considerable toxic effects, even though the individual components are present at concentrations lower than the no observable effect concentration (NOEC) (BEYER et al., 2014; BACKHAUS, 2014). Therefore, compliance with

individual quality standards does not necessarily guarantee security against mixture toxic effects (BACKHAUS, 2016). Nonetheless, most research on the ecotoxic effects of pharmaceuticals is performed using only one compound a time (VASQUEZ et al., 2014), which may neglect their potential combined toxicity effects (BEYER et al., 2014).

To date, environmental risk assessment (ERA) for pharmaceuticals is performed based on toxicity effects of single compounds, according to protocols of the European Medicines Evaluation Agency (EMEA, 2006) or of the European Commission (2003) (VASQUEZ et al., 2014). A specific and uniform protocol for performing ERA for pharmaceutical mixtures is still not available. This is because data availability and acceptable methods often limit such task (BACKHAUS, 2016). Therefore, understanding the mixture toxicity effects of pharmaceuticals of environmental concern using robust predictive approaches that allows for possible interactive effects is indispensable for improving ERA and the regulatory toxicology for these compounds in combination (BEYER et al., 2014). Thus, generating robust data that might aid in the prediction of the effects of pharmaceutical mixtures on aquatic organisms is essential. For this purpose, studies with binary mixtures can be of special relevance, since they can elicit the toxicity effect of one specific chemical on the biological action of another (CEDERGREEN et al., 2007).

This study aimed to fill some of these pointed knowledge gaps, by carrying out ecotoxicity tests with 4 pharmaceuticals of environmental concerning, from different therapeutic classes. Single and mixture ecotoxicity tests were performed using different test organisms from 3 different trophic levels. Different endpoints from acute and chronic toxicity tests were assessed, in order to enhance the ecotoxicity knowledge for these compounds and provide a scientific basis to contribute for improving ERA for pharmaceutical mixtures. The reasons for the choice of these 4 pharmaceuticals used in this study will be shown and discussed below.

2 JUSTIFICATION

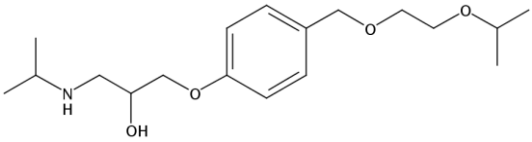
Over 4000 pharmaceuticals are used globally (ARNOLD et al., 2014). From these, more than 600 pharmaceutical substances have been shown to be present in the environment worldwide (KÜSTER and ADLER, 2014). In view of this large number of pharmaceutical compounds currently in use, it is essential to identify the priority compounds to be addressed in order to increase the effective deployment of resources in research and environmental regulation of pharmaceuticals. For this purpose, several prioritization schemes have been proposed, mostly in developed but also in developing countries (MANSOUR et al., 2016). Criteria used for the prioritization of pharmaceuticals for ERA or for monitoring programs usually include the number of sales/consumption/emissions, measured or predicted environmental occurrence, toxicity data, metabolism and excretion factors, physical-chemical properties, sewage treatment plant removal rates, environmental persistence and bioaccumulation (MANSOUR et al., 2016).

From these prioritization lists and considering the criteria before showed, pharmaceuticals occurring simultaneously in aquatic ecosystems, especially in fresh surface waters and for which ecotoxicity data were too limited were selected for this study. Following these premises and in order to assess the accuracy of the models used in the prediction and identification of toxicological interactions in the mixture toxicity studies, 4 pharmaceuticals belonging to 3 different therapeutic classes were chosen. The physical-chemical, pharmacological and environmental characteristics of these 4 chosen pharmaceuticals are described below.

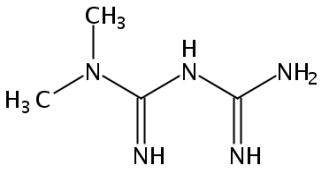
2.1 Physical-chemical and pharmacological properties of the selected pharmaceuticals

The pharmaceuticals prioritized in this study were the bisoprolol, sotalol, ranitidine and metformin. Their chemical structures are shown in Figure 1.

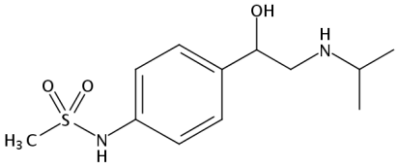
Figure 1 – Chemical structures of the pharmaceuticals bisoprolol, metformin, ranitidine and sotalol



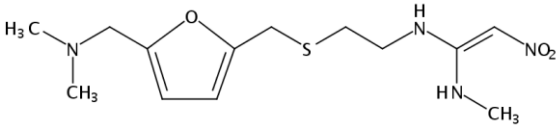
BISOPROLOL



METFORMIN



SOTALOL



RANITIDINE

Bisoprolol and sotalol are pharmaceuticals belonging to the therapeutic class known as β-adrenergic receptor antagonists or beta-blockers (WESTFALL and WESTFALL, 2012). Bisoprolol and sotalol are commonly used in the cardiovascular therapy for treating hypertension, ischemic heart disease, congestive heart failure and arrhythmias (WESTFALL and WESTFALL, 2012). About 50 - 60 % of bisoprolol is excreted unaltered in the urine (BÜHRING et al., 1986). This percent rate is of 80 - 90 % for sotalol (STANKIEWICZ et al., 2015).

Metformin is a pharmaceutical belonging to the biguanide class (POWERS and D´ALESSIO, 2012). It is the first-line oral therapy agent widely prescribed for treating type 2 diabetes (RENA et al., 2013; FORETZ et al., 2014). Metformin is excreted unaltered in the urine (BAILEY et al., 1996).

Ranitidine is a histamine H2-receptor antagonist, widely prescribed for treating gastric disturbances induced by the excessive stomach acid production (WALLACE and SHARKEY, 2012). The percentage of excretion of ranitidine in the unaltered form vary between 30 - 70 % after oral administration and between 70 – 80 % after intravenous usage (VEDIAPPAN and LEE, 2011).

The physical-chemical characteristics of toxicological importance of these pharmaceuticals are shown in Table 1.

Table 1 – Chemical Abstract Service (CAS) number, molecular formula and weight, octanol/water partition coefficient (log Kow), soil adsorption coefficient (log Koc), acid dissociation constant at logarithmic scale (pKa) and the water solubility of the pharmaceuticals bisoprolol, metformin, ranitidine and sotalol

Pharmaceutical	CAS number	Molecular formula (base)	Molecular weight (base)	Log Kow*	Log Koc*	pKa*	Water solubility* (g L^{-1})
Bisoprolol (fumarate)	104344-23-2	$C_{18}H_{31}NO_4$	650.88	1.87[a]	1.0	13.86	33[e]
Sotalol (hydrochloride)	959-24-0	$C_{12}H_{20}N_2O_3S$	272.36	0.24[b]	1.0	8.28	100-1000[f]
Metformin (hydrochloride)	66357-59-3	$C_4H_{11}N_5$	129.16	-4.3[c]	1.0	10.27 12.33	1000[c]
Ranitidine (hydrochloride)	1115-70-4	$C_{13}H_{22}N_4O_3S$	314.40	1.3[d]	1.0	8.35	660[g]

*Values predicted at 25 °C and at pH 7. Reference: SciFinder (https://sso.cas.org/).
[a]Lahti and Okari (2011); [b]Vieno et al. (2006); [c]ter Laak and Baken (2014); [d]Ferrari et al. (2011); [e]Charoo et al. (2014); [f]European Pharmacopoeia (2004); [g]Soleymani et al. (2013).

All the 4 pharmaceuticals selected for this study present certain polarity and are highly water-soluble compounds, which means that they tend to migrate in the aquatic environment and to move with surface water and groundwater (ATSDR-USA, 2005). Besides, their predicted log Koc indicate that their tendency to bond to organic matter is relatively low and a higher proportion of the pharmaceuticals is available to move into groundwater or surface water (ATSDR-USA, 2005). Regarding their Kow values, the 4 pharmaceuticals, especially metformin, are not likely to bioaccumulate in aquatic organisms. However, unexpected high concentrations of metformin (27.8 ng g^{-1}) were quantified in sculpin fishes from a large temperate estuary in USA (MEADOR et al., 2016). Bisoprolol and ranitidine have been recently reported to be present in the order of ng g^{-1} dry weight in aquatic invertebrate biota from streams receiving effluent from a WWTP employing tertiary treatment in Australia (RICHMOND et al., 2018). Therefore, these results indicate that the pelagic aquatic biota have been frequently exposed to these contaminants. However, the consequences of this chronic exposure are largely unknown.

2.2 Pharmaceuticals of environmental concern

Bisoprolol, sotalol, ranitidine and metformin are pharmaceuticals of environmental concern because of their widespread occurrence in aquatic environments (BERGHEIM et al., 2012; OOSTERHUIS et al., 2013; GODOY et al., 2015; UBA, 2019). Some factors that

contribute to this occurrence are (i) their high consumption and incomplete metabolism, leading to expressive WWTP influent loads, (ii) their incomplete removal at municipal WWTP and (iii) their resistance to abiotic and biotic degradation.

Pharmaceutically active substances for treating gastric, metabolic and cardiovascular diseases were among the four most consumed therapeutic classes in Brazil in 2016 and 2017 (ANVISA, 2017; 2018). Metformin, for example, was the 4th pharmaceutically active substance most sold in Brazil in 2017 (ANVISA, 2018). Not only in Brazil, metformin presents one of the highest consumption rates of all pharmaceuticals worldwide, with over 100 million patients consuming this pharmaceutical worldwide annually (SCHEURER et al., 2012; RENA et al., 2013). Beta-blockers, including sotalol and bisoprolol, are also among widely consumed pharmaceuticals worldwide (OOSTERHUIS et al., 2013; MASZKOWSKA et al., 2014a). Ranitidine is one of the most popular pharmaceuticals on the planet (BOJIĆ et al., 2015). Besides their high consumption, these pharmaceuticals are released to domestic sewage in the unaltered form at expressive percentage rates from urine and feces of patients, as cited before. Once at the municipal WWTP, ranitidine, metformin, bisoprolol and sotalol are not completely removed. Radjenović et al. (2009), Scheurer et al. (2010) and Lara-Martín et al. (2014) have reported removal rates for sotalol varying from 0 to a maximum of 59 % in WWTP employing activated sludge, advanced membrane bioreactor and oxidation ditch treatments. Removal rates for bisoprolol using activated sludge treatment was reported to be of only 36 % in a German WWTP (SCHEURER et al., 2010). Using tertiary treatments (such as reverse osmosis, ozonation, activated carbon), these removal rates varied from 40 to 70 % for bisoprolol (GABET-GIRAUD et al., 2010). Removal of ranitidine in a WWTP in Spain employing conventional activated sludge was considered poor, reaching only 25 % (RADJENOVIĆ et al., 2009). Using pilot-scale membrane bioreactors treatments operating in parallel with the conventional activated sludge, these removal rates for ranitidine varied from around 29.5 to a maximum of 44 % (RADJENOVIĆ et al., 2009). Finally, metformin has been reported to present high removal rates in WWTP (93 – 97 %) due to its microbiological transformation into guanylurea (TRAUTWEIN et al., 2014). However, due to its high influent loads, metformin has been detected at expressive concentrations in surface waters (OOSTERHUIS et al., 2013).

As a result of their high consumption, incomplete metabolism and incomplete removal at WWTP, the mass output loads and concentrations of these pharmaceuticals quantified in WWTP effluents worldwide are relevant. For ranitidine, this average daily output effluent load was quantified at the range of 0.55 to 5.30 g day^{-1} for a WWTP in Barcelona, Spain (RADJENOVIĆ et al., 2007). For sotalol, this load effluent was quantified at the range of 1.9

to 24.9 g day^{-1} in another WWTP in Spain (RADJENOVIĆ et al., 2009). Bisoprolol has been detected at concentrations of over 24 µg L^{-1} in WWTP municipal effluents in Portugal (SOUSA et al., 2013). For metformin, concentrations in WWTP effluents have reached up to 82.7 µg L^{-1} in USA cities (MEADOR et al., 2016).

In addition to these factors here presented, the resistance to biotic and or abiotic degradation contribute to the widespread and frequent presence of these pharmaceuticals in aquatic ecosystems. Metformin lacks functional groups that hydrolyze under environmental conditions (TER LAAK and BAKEN, 2014). Therefore, hydrolysis is not probable to occur to metformin in the environment. The beta-blockers bisoprolol and sotalol only absorb radiation in the UV-C range. Therefore, photodegradation by direct photolysis is not probable to occur with these pharmaceuticals in environmental waters (PIRAM et al., 2008). Besides, in a study using inoculum taken from activated and digested sludge processes, the biotransformation rate of bisoprolol showed to be slow and incomplete, with 37 % removal during 75 days in aerobic low-carbon conditions and only 14 % removal in anaerobic biotransformation during 161 days (LAHTI and OKARI, 2011). Sotalol is equally only slightly biodegraded and hydrolyzed (FEINER et al., 2014). Ranitidine presents prolonged stability in water, being stable during 160 h in pH 6.18 and at 65°C (FERRARI et al., 2011).

As a result of these resistance to chemical and biological degradation, the pharmaceuticals ranitidine, bisoprolol, sotalol and metformin can be found in several aquatic matrices, many times simultaneously, including fresh surface waters (e.g. GINEBREDA et al., 2010; FICK et al., 2011; VALCÁRCEL et al., 2011a; 2011b; GONÇALVES et al., 2013; RUFF et al., 2015), WWTP effluents (RADJENOVIĆ et al. 2009; de LA CRUZ et al., 2012; OOSTERHUIS et al., 2013), hospital effluents (VERLICCHI et al., 2012; SANTOS et al., 2013; OLIVEIRA et al., 2015), groundwater (LÓPEZ-SERNA et al., 2013) and even drinking water (KOT-WASIK et al., 2016). Therefore, since these pharmaceuticals can co-occur in aquatic ecosystems, their mixture ecotoxicity effects must be also investigated.

2.3 Lack of ecotoxicity data

Despite their widespread occurrence in aquatic environments globally, ecotoxicological investigations into the biological effects of the pharmaceuticals bisoprolol, sotalol, ranitidine and metformin are still scarce, mainly considering sub-lethal endpoints. Databases such as USEPA Ecotox Knowledgebase (https://cfpub.epa.gov/ecotox/) and Wikipharma

(http://www.wikipharma.org/api_data.asp) do not present ecotoxicity data for bisoprolol. This lack of data is commented by Lahti and Oikari (2011), who recognized the difficult to assessing the realism of the risk posed by bisoprolol to aquatic organisms in view of the knowledge gaps regarding the ecotoxicity effects induced by this beta-blocker.

Sotalol is another beta-blocker lacking additional information on its toxicity to non-target organisms, especially concerning their possible sublethal effects (FEINER et al., 2014; GODOY et al., 2015). Since a study performed with the New Zealand *Potamopyrgus antipodarum* showed that sotalol can alter the reproduction of this aquatic organism, other species could also be adversely affected by this beta-blocker.

Besse and Garric (2008), who included ranitidine in their list of priority pharmaceuticals for environmental monitoring and ERA, highlighted the need to build ecotoxicological data for this pharmaceutical. This task began with the tests performed with the rotifer *Brachionus calyciflorus* and with the crustacean *Ceriodaphnia dubia* by Isidori et al. (2009). However, additional experiments using test-organisms from different trophic levels are still needed in order to conclude about the risk posed by ranitidine in aquatic environments.

Likewise, the lack of knowledge on the ecotoxicity effects, especially the sub-lethal ones, induced by metformin on aquatic organisms still hampers interpretation of its risk (TER LAAK and BAKEN, 2014). Thus, these knowledge gaps need to be filled so that proper interpretation of environmental monitoring results and robust ERA for these pharmaceuticals can be achieved.

Moreover, evolutionarily well-conserved drug targets were predicted in aquatic species including fish, daphnid and algae, with considerable similarity compared to humans (GUNNARSSON et al., 2008). This prediction included targets for the pharmaceuticals metformin (5'-AMP-activated protein kinase), bisoprolol (β1 and β2-adrenergic receptors), sotalol (Potassium voltage-gated channel) and ranitidine (histamine H2-receptor). For the fish *Danio rerio*, for example, the similarity of the respective drug targets compared to humans was of around 43 % for ranitidine, 53 % - 57 % for the beta-blockers and more than 78 % for metformin target. This means that these aquatic organisms can be adversely affected by these pharmaceuticals acting on these predicted targets.

Finally, it is worth remembering that the pharmaceuticals chosen for this study do not occur isolated in aquatic environments, as it was commented before. Therefore, it is important to identify possible changes on the ecotoxicological behavior of these pharmaceuticals in combination with each other. Current understanding of the ecotoxicology of pharmaceutical mixtures is still in its infancy (BACKHAUS, 2014). Sound experimental data addressing

mixture toxicity of pharmaceuticals are often missing (BACKAHUS, 2016). Besides, there is not a consensus about the predictive accuracy of the classical mathematical models used to describe the mixture toxicity effects (RODEA-PALOMARES et al., 2010). Consequently, no uniform regulation for ERA of pharmaceutical mixtures is currently available, in spite of some efforts at the European Commission level (VASQUEZ et al., 2014). Thus, it is necessary to carry out additional studies that add up to increase the still incipient knowledge on the mixture toxicity of pharmaceuticals on non-target organisms, in order to contribute for the development of relevant guidelines for assessing mixture ecotoxicity of these compounds.

3 OBJECTIVES

This study aimed to assess and model the single and mixture lethal and sub-lethal effects induced by the pharmaceuticals metformin, bisoprolol, sotalol and ranitidine on aquatic organisms from 3 different trophic levels, in order to contribute for the implementation of robust ERA for these pharmaceuticals and subsidize possible regulatory actions.

The specific objectives were:

- To provide a critical review on the updated state of the knowledge on the ecotoxicity of pharmaceuticals and personal care product mixtures;
- To assess the toxicity of the pharmaceuticals bisoprolol, sotalol, metformin and ranitidine on the growth of the algae *Raphidocelis subcapitata* and the macrophyte *Lemna minor*, on the morphology of the cnidarian *Hydra attenuate*, on the immobilization of the crustacean *Daphnia similis* and on the development and locomotor behavior of the *Danio rerio* fish embryo;
- To assess the chronic toxicity of the pharmaceuticals bisoprolol and metformin in additional tests of reproduction with *D. similis* and *H. attenuate*;
- To assess the ecological risk posed by the pharmaceuticals metformin and bisoprolol to pelagic aquatic biota, considering a worst-case scenario;
- To derive updated environmental quality standards (EQS) for protecting freshwater pelagic community from adverse effects of metformin;
- To assess the binary mixture toxicities of the 4 pharmaceuticals in acute tests with *D. similis* and to compare the observed effects with those predicted by the Concentration addition (CA) and Independent action (IA) models and respective synergistic, antagonistic, dose ratio and dose level-dependent deviations;
- To identify and describe the toxicological interactions occurring between the pharmaceuticals in binary combinations using the Combination Index-isobologram (CI) method;
- To assess the locomotor behavior of *D. rerio* larvae exposed to environmental concentrations of the pharmaceuticals in quaternary mixture exposure.

4 PUBLISHED AND SUBMITTED ARTICLES

The results obtained in this study were described in 4 articles. The first article is a critical review entitled *What do we know about the ecotoxicology of pharmaceutical and personal care product mixtures? A critical review*. It presents an updated state of knowledge on the ecotoxicity of pharmaceuticals and personal care product mixtures. The misunderstandings, deficiencies and data gaps identified from the studies published in the literature during 2000 - 2017 were critically discussed. This article was published in the journal *Critical Reviews in Environmental Science and Technology* (2017), v. 47, n. 16, p. 1453 - 1496. DOI: 10.1080/10643389.2017.1370991.

The second article is entitled *Ecotoxicological effects, water quality standards and risk assessment for the anti-diabetic metformin*. It presents the results and the analyses of the ecotoxicity effects performed with metformin, the environmental quality standards for protection of pelagic freshwater biota against the adverse effects induced by this pharmaceutical and an environmental risk assessment performed considering a worst-case scenario for the presence of metformin in fresh surface waters. This article was published in the journal *Environmental Pollution* (2018), v. 243, p. 534 - 542. DOI: 10.1016/j.envpol.2018.09.03.

The third article is entitled *Assessment of the ecotoxicity of the pharmaceuticals bisoprolol, sotalol and ranitidine using standard and behavioral endpoints*. It presents the results of the ecotoxicity tests performed with the single pharmaceuticals bisoprolol, sotalol and ranitidine, using 5 different aquatic organisms from 3 trophic levels. The result of a preliminary ecological risk assessment performed for bisoprolol based on chronic data generated in this study was also shown in this article. This article was submitted to the journal *Environmental Science and Pollution Research.*

The fourth article is entitled *Single and mixture toxicity of four pharmaceuticals of environmental concern to aquatic organisms, including a behavioral assessment*. It presents the results of the binary and quaternary mixture toxicity tests performed with *D. similis* and *D. rerio* embryos and the assessment of predictive the accuracy of the classical mathematical models CA and IA and of their respective deviations. It also presents the nature of the toxicological interactions between the pharmaceuticals in binary mixtures using the CI model. This article was published in the journal *Chemosphere* (2019), v. 235, p. 373-382. DOI: 10.1016/j.chemosphere.2019.06.200

4.1 Article I

What do we know about the ecotoxicology of pharmaceutical and personal care product mixtures? A critical review

Aline A. Godoy[a,b], Fábio Kummrow[c]

[a]Department of Clinical and Toxicological Analysis, Faculty of Pharmaceutical Sciences, University of São Paulo (USP), Avenida Professor Lineu Prestes, 580, São Paulo, SP, 05508-000, Brazil.

[b]Science and Technology Institute, Federal University of Alfenas (Unifal-MG – *campus* Poços de Caldas), Rodovia José Aurélio Vilela, 11,999, Poços de Caldas, MG, 37715-400, Brazil.

[c]Institute of Environmental, Chemical and Pharmaceutical Sciences, Federal University of São Paulo (Unifesp – *campus* Diadema), Rua São Nicolau, 210, 09972-270, Diadema, SP, Brazil.

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ABSTRACT

No uniform regulation for risk assessment of pharmaceutical and personal care products (PPCP) mixtures is currently available. Hence, a large diversity of strategies can be used for studying cocktail effects of PPCP, which makes difficult the task of incorporating this challenging issue into regulatory frameworks. This review provides an updated state of knowledge on the ecotoxicity of PPCP mixtures, including 194 assessments of the toxicity of mixtures from 65 articles published during 2000-2017. The misunderstandings, deficiencies and data gaps identified from those studies were critically discussed based on the models/tools used to predict/assess the joint effects and the interpretation and presentation of the effect profiles, the experimental designs used, the qualitative and quantitative composition of the PPCP mixtures and the type of bioassays performed regarding test duration, endpoints and levels of biological organization. Possible approaches pointed out in the literature to deal with the identified critical issues were also discussed. Overall, we have identified that further advances in this field of research still lack robust and consistent studies regarding the experimental design and the approaches and terminologies used to calculate, interpret and report the joint effects.

Keywords: Experimental designs; Non-additive interaction; Synergism

1. INTRODUCTION

Pharmaceuticals and personal care products (PPCP) are among the environmental contaminants of emerging concern which have been a target of increasing attention by the scientific community. Among the reasons that have been pointed out for justifying this attention are the growing consumption of various medicinal products, in addition to the frequent incomplete metabolism of several pharmaceuticals and their also frequent incomplete removal at the wastewater treatment plants (Halling-Sørensen et al., 1998; Fent et al., 2006a; Godoy et al., 2015a). In addition, PPCP are designed to alter particular physiological functions which probably makes them biologically active also for non-target species (Santos et al., 2010) and to possess some resistance to biotransformation processes in order that they can exert their therapeutic effects. However, this resistance to degradation processes also contributes to their environmental persistence (Fatta-Kassinos et al., 2011). Even those pharmaceuticals that have an environmentally relative short half-life may be continuously found in different compartments, which makes them to be considered pseudo-persistent compounds. This is due to their constant release, which leads to a continual replacement of the degraded molecules, hence maintaining existing levels of the parent compounds, especially in aquatic environments (Oosterhuis et al., 2013; Daughton, 2016).

Of special relevance is the fact that PPCP residues are usually found as mixtures and not isolated in the environment (Kümmerer et al., 2009; López-Serna et al., 2012). Hence, although it is a common practice to evaluate the possible environmental risk of these compounds individually, according to specific guidelines (EMEA, 2006;2016), this approach may over or underestimate the real environmental impact of PPCP mixtures (Godoy et al., 2015a). This is because this practice ignores the fact that the ecotoxicity of a PPCP mixture is usually higher than the effects of each single component and that considerable adverse effects can occur even if all the components of such a mixture are present below their individual non-observed effect concentration (NOEC) (Backhaus, 2014). In addition, mixture toxicity effects may occur on non-target organisms that cannot be predicted from the individual effects posed by their components (González-Pleiter et al., 2013; Brezovšek et al., 2014; Godoy et al., 2015b).

In this context, the adverse effects posed by PPCP mixtures towards non-target organisms is an area of increasing concerning (Rodea-Palomares et al., 2010). Currently, some efforts have been made to establish regulations for the risk assessment of chemical mixtures released into the environment (Vasquez et al., 2014), such as the State-of-the-Art Report on Mixture Toxicity (Kortenkamp et al., 2009) and the Communication from the Commission on

Combination effects of chemicals (European Commission, 2012). However, no uniform regulation is currently available for risk assessment of joint effects from PPCP substances (Vasquez et al., 2014; Hoyett et al., 2016). This implies that a variety of strategies can be used for studying mixture toxicity of these compounds, which makes even more difficult the task of incorporating this challenging issue into regulatory frameworks.

The aim of this review is to present an updated state of knowledge on the ecotoxicity of PPCP mixtures, based on studies from the last 17 years, in order to critically discuss the misunderstandings, deficiencies and data gaps and point out the need for future research in this field in order that optimized and robust criteria for designing, predicting and understanding the effects of PPCP mixtures can be achieved, especially for aquatic environments. In this sense, we will address the specific following topics: (1) the models or approaches used to predict and assess the PPCP mixture effects; (2) the experimental designs generally employed in PPCP mixture studies; (3) the interpretation and presentation of the observed effect profiles for the PPCP mixtures regarding the model/approach used and deviations; (4) the pharmacological classes usually covered in ecotoxicological mixture studies, comprising both human and veterinary drugs; (5) the number of components in the analyzed PPCP mixtures and (6) the bioassays commonly used to evaluate the mixture toxicity of PPCP.

2. RETRIEVED DATA PUBLISHED IN THE INTERNATIONAL LITERATURE ON MIXTURE TOXICITY OF PPCP

We collected data on ecotoxicological studies of PPCP mixtures reported in the literature from 65 international articles, retrieved from several databases (among them ScienceDirect, Scopus, SciELO, SpringerLink, Web of Science and Wiley Online Library), covering a period from 2000 to 2017. PPCP mixtures included in our review were composed exclusively of personal care products, human and veterinary pharmaceuticals, as well as their metabolites and transformation products. Mixtures comprising PPCP and chemicals from other classes such as pesticides, metals, hydrocarbon compounds etc. were not included. The data from these 65 scientific publications are reported in Table A.1 (Appendix A of Supplementary Material). For this survey, the following key-words were looked for in the databases: "Pharmaceutical mixtures"; "Mixture toxicity"; "Emerging contaminants"; "Pharmaceutical cocktails"; "Combination effects"; "Additive effects"; "Interactive effects"; "Joint effects".

A total of 194 assessments of the toxicity of PPCP mixtures were retrieved from these 65 papers. From these data, we first identified the different mathematical models and/or

approaches used to predict and/or assess the ecotoxicological effects of the PPCP mixtures. We also identified, quantified and analyzed the types of experimental designs used for assessing the PPCP mixtures. In addition, we identified the effect profiles of the PPCP mixtures regarding the model or approach used and quantified the deviation between observed and predicted mixture toxicity from reference models for the fraction of papers reporting such data. Afterwards, we identified the therapeutic class of each component of the PPCP mixtures and classified them according to their pharmacology, following the Anatomical Therapeutic Chemical (ATC) classification system (WHO, 2013). Then, we calculated the percentage frequency of presence of each therapeutic class in the retrieved mixture studies. We also quantified the total of each type of PPCP mixture study according to the number of their components and according to the type of effects posed to the biological component (*in vivo* acute or chronic effects and *in vitro* assays).We referred as "*in vivo*" to those studies that were performed on a living organism, including bacteria, protozoa and unicellular algae, while we classified as "*in vitro*" those ones that were performed in an isolated organ, tissue (e.g., hemolymph collected from mussels), cell from multicellular organisms (e.g., fish liver cells) or biochemical systems (e.g., recombinant yeast system expressing human progesterone/estrogen/androgen receptors), according to the International Union of Pure and Applied Chemistry (IUPAC) definition (Duffus et al., 2007).The classification of the *in vivo* assays into acute or chronic was based on OECD (Organisation for Economic Co-operation and Development) protocols as well as on the European technical guidance document for deriving environmental quality standards under the Water Framework Directive (European Commission, 2011a) and on other protocols published in the international literature, used for performing the bioassays. According to these protocols, the EC_{50} from the 72-h algae test or from the 7 d Lemna sp. test is considered as acute, while the NOEC or EC_{10} from the same tests is regarded as a chronic value. Therefore, in this review, the algae and macrophyte test were classified as acute and/or chronic according to the toxicological values (EC_{50}, EC_{10} or NOEC) used in the respective experimental designs of each mixture data. The designation as "unclassified" was attributed to those tests for which a classification was not possible based on protocols described in the international literature.

3.RESULTS AND DISCUSSION

3.1 The approaches currently used to predict/assess the PPCP mixture effects

Two classical models have been commonly used in the prediction/assessment of PPCP mixture effects and are the current standard supporting mixture risk assessment in ecotoxicology. They are named Concentration Addition (CA), also known as Loewe additivity or dose addition, and Independent Action (IA), synonymous to Bliss independence and to response addition. The CA model was originally reported in the early works of the pharmacologists Loewe and Muischnek (1926). The CA model can be mathematically expressed as the equation (1) (Berenbaum, 1985):

$$\sum_{i=1}^{n} \frac{c_i}{EC_{xi}} = 1 \qquad (1)$$

where c_i represents the dose/concentration of the *i* component in a *n*-compound mixture with a total effect of *x* % and EC_{xi} denotes those concentrations of the single substances that would alone induce the same effect *x* as observed for the mixture. Since the fraction c/EC_x is termed a "toxic unit", the CA model is also known as "toxic unit summation" (Backhaus, 2014).This approach assumes that all components in a mixture have the same molecular site of action and, therefore, they behave as if they are simple dilutions of one another (Cedergreen et al., 2007; Rodea-Palomares et al., 2015). Although the CA model assumes similar molecular target sites in a strict sense, it has been also proposed to apply to mixture components which can cause the same toxicological response (Cleuvers, 2003).

The alternative concept of IA was originally proposed by Bliss (1939) and can be mathematically represented by the equation (2):

$$E(C_{mix}) = 1 - \prod_{i=1}\left(1 - E(c_i)\right) \qquad (2)$$

where E(c_{mix}) is the total effect of the mixture and $E(c_i)$ corresponds to the effects that the individual components would induce if applied singly at the concentration at which they are present in the mixture. The IA approach assumes that the compounds in a mixture cause a common effect through different molecular target sites and modes of action (Cleuvers, 2003).

Both CA and IA concepts are only applicable to mixtures of known composition and presume that each individual component of a mixture is toxic if applied singly, i.e., inert

compounds do not contribute to the toxicity of a mixture (Backhaus, 2014; 2016). However, Backhaus et al. (2016) draws attention to an important point of difference between both models, regarding the contribution of low, non-toxic effect concentrations, i.e., at concentrations that did not result in a statistically significant effect in a particular experiment, under defined conditions of exposure (OECD, 2003). The IA model, which is effect-based, presumes that such non-toxic effect concentrations do not contribute to the joint toxicity of a mixture, i.e., if $E(ci) = 0$ (equation 2), while the CA model assumes that every component, even when applied at levels below its toxicity threshold, can nevertheless contribute to the overall toxicity of a mixture, in strict direct proportion to its toxic unit (TU) (equation 1) (Backhaus, 2014; 2016). The apparent absence of effect may be actually a mere consequence of the limited statistical power of a bioassay in demonstrating a toxic effect (Backhaus, 2016). In other words, statistical insignificance must not be considered as a proof for the absence of any effect (Faust et al., 2003).

Besides these two prominent concepts, other approaches have been also used for the analysis, prediction and/or interpretation of the joint action of PPCP in ecotoxicology, including other models, graphical approaches, and the use of indices (Table 1). Many of these approaches are equivalent to or are an extension of the CA model and its graphical representation, the isobologram. An isobologram is defined as a graph in Cartesian coordinates consisting of a line or a curve that represents dose/concentration pairs, giving a specified effect level for compounds acting independently (Tallarida, 2012).

Table 1 Models/approaches used to assess/predict the PPCP mixture toxicity effects retrieved from 65 international articles covering a period from 2000 - 2017

Model/Approach used	References
Concentration addition (CA) model, including its graphical representation (isobologram)	Thorpe et al. (2003); DeLiguoro et al. (2009; 2010); Runnalls et al. (2015); Zhao et al. (2015); Hinfray et al. (2016)
Independent action (IA) model	Parrella et al. (2014)
Both CA and IA models	Various[c]
CA and Toxic Unit (TU) approach	Rossier et al. (2016); Siegenthaler et al. (2017)
Combination-Index Isobologram (CI) model only	Rodea-Palomares et al. (2010)
All the three CA, IA and CI models	González-Pleiter et al. (2013); Di Nica et al. (2017); Geiger et al. (2016)
Principal Component Analysis/ Cluster Analysis	Pomati et al. (2008); Franzellitti et al. (2013); Gonzalez-Rey et al. (2014); Zucchi et al. (2014); Ding et al. (2016)
Specific equations based on Toxic Units (TU) of the mixture	Zou et al. (2012)
Additive Index and the Modified TU approach	DeLorenzo and Fleming (2008)
Comparison between observed and predicted additivity from the effects caused by 1 TU for each individual component	Bisesi Jr et al. (2016)
Statistical comparison between individual and mixture effects, using statistical methods such as e.g. Student´s T test, Analysis of variance (followed by post-hoc test) or the Fisher method	Various[d]
Overlap analysis of the 95 % confidence intervals of the individual and the mixture effects	Luna et al. (2013; 2015);
Comparison of the toxicity threshold values (calculated from the square root of the product between the NOEC[a] and LOEC[b]) between the mixture and the individual effects of each component	Quinn et al. (2009)
Comparison between mixture and single effects of each component by means of simple percent calculation	Parolini and Binelli (2012)
Empiric comparison between mixture and single effects of each component without using a mathematical approach or a direct statistical comparison	Ericson et al. (2010); Galus et al. (2013); Li and Lin (2015); Chiffre et al.(2016)
The mixture toxicity was statistically compared to the individual toxicity of just one of the mixture compounds (the parental compound)	Almeida et al. (2017)
The whole-mixture approach was used. The mixture toxicity was not compared to the individual effects of the components	Brain et al. (2005); Borgmann et al. (2007); Pomati et al. (2007); Gust et al. (2013); Melvin (2016)

[a]NOEC - Non-observed effect concentration [b]LOEC - Lowest observed effect concentration [c]Various - Backhaus et al. (2000; 2011); Cleuvers (2003; 2004; 2005); Christensen et al. (2006; 2007); Fent et al. (2006b);Henry and Black (2007); Schnell et al. (2009); Brezovšec et al. (2014); Villa et al. (2014); Godoy et al. (2015b); Guo et al. (2016); Nieto et al. (2016); Watanabe et al. (2016); Bialk-Bielińska et al. (2017)

[d]Various- Brain et al. (2004); Eguchi et al. (2004); Flaherty and Dodson (2005); Dietrich et al. (2010); Gust et al. (2012); Láng and Kőhidai (2012); Melvin et al. (2014); Säfholm et al. (2015); Wolfe et al. (2015); González-Ortegón et al. (2016); Hua et al. (2016); Örn et al. (2016); Rossier et al. (2016); Liang et al. (2017); Siegenthaler et al. (2017)

Source: Godoy and Kummrow (2017)

As an extension of the CA model, the Isobologram-Combination Index (CI), first introduced by Chou and Talalay (1983; 1984), is based on median-effect principle (mass-action law) and allows quantitative determination of drug-interactions. The mathematical formulation for the CI model, for *n*-compounds combination at *x* % inhibition is described as:

$$^{n}(CI)_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j} = \sum_{j=1}^{n} \frac{(D_x)_{1-n\{[D]_j/\sum_1^n[D]\}}}{(D_m)_j\{(f_{ax})_j/[1-(f_{ax})_j]\}^{1/m_j}} \quad (3)$$

where $^{n}(CI)_x$ is the combination index for a *n* compounds at *x* % inhibition; $(D_x)_{1\text{-}n}$ is the sum of the dose/concentration of *n* drugs that elicits *x* % inhibition in combination; $\{[D]_j/\sum_1^n[D]\}$is the proportionality of the dose/concentration of each of *n* compounds that elicits *n* % inhibition in combination and $(D_m)_j\{(f_{ax})_j/[1\text{-}(f_{ax})_j]\}^{1/mj}$ is the dose/concentration of each compound alone that elicits *x* % inhibition, where D_m is the median-effect dose/concentration, f_{ax} is the fractional inhibition at *x* % inhibition and *m* is the slope of the median-effect plot (Chou, 2006). From equation 3, CI <1, =1, and >1 indicate synergism, additive effect, and antagonism, respectively (Chou, 2006).Therefore, this method considers both the potency (D_m) and the shape (*m*) of the dose/concentration-effect curve of each compound, where *m* =1, >1 and <1 indicate hyperbolic, sigmoidal and flat sigmoidal curve, respectively (Chou, 2006).Usually employed in the pharmacology field, this method has been recently also used to assess ecotoxicological interactions of pharmaceuticals (e.g., Rodea-Palomares et al., 2010; González-Pleiter et al., 2013).

The indices of mixture toxicity usually used in ecotoxicity studies with PPCP, such as the Sum of Toxic Units (STU), the Additivity Index (AI), and the Modified Toxic Unit approach, relate expected and observed responses in quantitative terms (Altenburger et al., 2003). The equation (4) represents the mathematical definition of STU:

$$S = \sum TU_i = \sum \frac{c_i}{EC50_i} \quad (4)$$

where c_i is the concentration of component *i* and $EC50_i$ is the 50 % effect concentration of component *i*. The AI is calculated based on the S values from equation (4). If S ≤1, then AI = (1/S) - 1; if S ≥ 1, then AI = S (-1) +1 (Marking, 1977). The Modified Toxic Unit approach builds on comparisons between observed and predicted response of the mixture based on toxic units, being the percent effect of each mixture treatment calculated and graphed as a

dose/concentration-response curve (DeLorenzo and Fleming, 2008). According to Altenburger et al. (2003), most of these indices are algebraic equivalents of the isobologram method, differing only in scaling of the quantitative deviations from CA.

As fundamentally different approaches from the previous ones, multivariate data analysis tools, such as the cluster analysis and the Principal Component Analysis (PCA), have been employed in a few studies aiming to assess the effects of pharmaceutical mixtures on non-target organisms (Pomati et al., 2008; Franzellitti et al., 2013; Gonzalez-Rey et al., 2014; Ding et al.,2016). Briefly, cluster analysis is a multivariate statistical technique used to identify or structure groups or clusters based on similarities (Sparks et al., 1999). PCA is an ordination technique in which linear combinations of the variables of a large multivariate dataset are created that explain the major factors responsible for the variance in the original data (Sparks et al., 1999). In general, these approaches have been used in PPCP mixture toxicity studies in order to test the adverse effects associated with single pharmaceuticals present in a mixture (Pomati et al., 2008); to determine if significantly different effects of the mixture with respect to single components exposure occurred (Franzellitti et al., 2013); to compare biomarker/transcriptional responses to single pharmaceuticals exposure with their mixtures (Gonzalez-Rey et al., 2014; Zucchi et al., 2014); and to evaluate the variability associated with each biomarker factorial weight in each isolated pharmaceutical and binary mixture treatment (Ding et al., 2016).

Finally, there are also a few mixture ecotoxicity studies with PPCP in which no mathematical approach or a direct statistical comparison is used to link the toxicity of the individual components and the effects of the mixture (Table 1). In such cases, a sound discussion about possible interactions among the mixture constituents is not possible.

Toxicological interactions are defined as responses that deviate from those expected under a specified definition of additivity, based on the dose/concentration-response relationships of the individual components (ATSDR, 2004). Additivity, in turns, occurs when the effect of a mixture can be estimated from the sum of the exposure levels or the effects of the individual components (ATSDR, 2004). Thus, interaction can be greater-than-additive (synergistic) or less-than-additive (antagonistic) (Ragas et al., 2011). Herein, we refer to interaction as the deviations from the expected additivity of effects based on using a certain reference model, e.g., CA, IA or CI.

It is worth highlighting that in addition to these component-based approaches, some mixture ecotoxicity studies with PPCP have employed the whole-mixture based approach, i.e., they are based on the direct ecotoxicological assessment of a given PPCP mixture (Table 1).

Whole-mixture approaches are frequently used, for instance, in studies with complex test systems or in studies employing semi-quantitative endpoints, such as histopathological data (Backhaus, 2014). Although this approach may account for any possible interactions between the component chemicals that might have been missed if a component-based approach was used, it does not allow the identification of the toxicant responsible for a certain outcome (Heys et al., 2016). In addition, results using this approach are usually applicable to the specifically tested mixtures, thus lacking generalizability (Backhaus, 2014).

Advantages and limitations have been pointed out in the literature also for the tools used in the component-based approaches. For instance, indices of mixture toxicity allow numerical quantification of the degree of deviation from a certain reference model. On the other hand, they present the limitation of providing only point-wise assessments when only the EC_{50} level is considered, thus leading to loss of information that could be derived from a concentration-response relationship (Altenburger et al., 2003). Regarding the CI method, it presents the advantage of considering the potency and the shape of the dose-effect curve for each mixture component, which is an important prerequisite for synergism/antagonism determination (Chou, 2006). Furthermore, based on this same theorem, Chou and Martin (2005) developed the CompuSyn software, which allows construction of polygonograms, depicting interactions for multi-compound combinations (Chou, 2006). However, according to Backhaus (2014), this method based on the law of mass action is a quite rigid approach since the shape of the individual concentration-response curve is usually captured within only one parameter.

It deserves special discussion the advantages and criticisms that have been systematically point out in the literature regarding the classical IA and mainly the CA model. Altenburger et al. (2013) have recently reported that the observed combined effects of the multi-component mixtures were almost perfectly depicted in several investigations by the predictions based on the CA model for the mixtures of similar modes of action and IA for those of dissimilar modes of action, regardless of the actual mixture ratios, effect level, chemical composition, biological endpoint, and test organism. Those authors defending the evidence on the applicability of the IA and mainly the CA model for estimating the ecotoxicity of pharmaceutical mixtures have cited studies published by, for example, Backhaus et al. (2000) and Cleuvers (2004; 2005). On the other hand, these two classical additive mixture models are based on the notion of non-interaction, i.e., they both assume that the compounds in a mixture do not interact, i.e., that mixture components do not interfere with each other, neither in the uptake nor in the toxicokinetic and/or toxicodynamic phases (Backhaus, 2016).Consequently, deviations from the predictions derived from CA and/or IA have been reported (e.g., Cleuvers,

2003; Christensen et al., 2006; Fent et al., 2006b; Gonzalez-Pleiter et al., 2013; Brezovšek et al., 2014; Godoy et al., 2015b; Nieto et al., 2016; Rodea-Palomares et al., 2016), which might indicate that interactions occurred.

It must also be highlighted that, although it has been argued that interactions such as antagonism, potentiation and synergism are either unlikely to occur or are toxicologically insignificant at low exposure levels (European Commission, 2011b), this supposed low incidence of interaction might be simply a consequence of the lack of systematic experimental evaluations and/or of the large uncertainties in existing experimental methods. Particularly for pharmaceutical mixtures, currently it is unknown how frequent is the occurrence of synergistic and antagonistic interactions in non-target organisms (Backhaus, 2016). In addition, it is also worth mentioning that the scientific priority question of dealing with the effects of long-term exposure to low concentrations of PPCP mixtures on non-target organisms may not be adequately addressed by using the additive approach. This is because the CA model does not allow for low-dose nonlinear/nonadditive sub-lethal effects, which has limited the study of the sub-lethal region of the concentration-response curves, termed the "gray zone" (Kortenkampet al., 2009; Fagin, 2012; Rodea-Palomares et al., 2016).

In this sense, Rodea-Palomares et al. (2016) have recently proposed a new tool consisting of global sensitivity analysis coupled with quantitative high-throughput screening (GSA-QHTS) in order to deal with the discussed limitations in the study of sub-lethal effects of low-dose PPCP mixtures. The GSA-QHTS consists of a tool that couples computational global sensitivity analysis (GSA) techniques for generating experimental design templates (e.g., low-dose PPCP mixture experimental design) with quantitative high-throughput screening (QHTS) experiments to identify the main effects and interactions of combinations of chemical compounds, biotic or abiotic factors etc., called input factors (Rodea-Palomares et al., 2016). GSA computational methods allow assessing how the variation of the input factors influence the model outputs and help modellers in distinguishing factors of major influence from those considered as non-influential ones, as well as help identifying interactions among these factors (Vanrolleghem et al., 2015). QHTS assays are multiple-concentration experiments that enable the simultaneous assessment of a large number of compounds (Shockley, 2012). Rodea-Palomares et al. (2016) applied this screening method to study a set of realistic low-dose mixtures of 16 commonly found PPCP in Spanish freshwaters, by using responses from the high-throughput configuration of a bioluminescent whole-cell biosensor which detects metabolic toxicity based on the freshwater cyanobacterium *Anabaena* CPB4337. Overall, by using this method, Rodea-Palomares et al. (2016) were able to identify the main pharmaceutical

pollutants and their interactions driving biological effects on the microbial population. They also found nonlinear/nonadditive effects resulting from low-dose mixtures of PPCP and suggested that linear/additive chemical risk assessment approaches may neglect a considerable number of ecologically dangerous chemical pollutants that may be important under real low-dose environmental conditions. Therefore, methods such as the GSA-QHTS appear to be promising tools of interest to researchers dealing with the effects of combined chemical stressors. However, additional studies using other biological systems as well as an implementation of derived methods that allow quantitative ranking of the drivers of low-dose pharmaceutical pollutant mixtures are still needed (Rodea-Palomares et al., 2016).

3.2 The experimental designs generally used in PPCP mixture studies

An important issue generally neglected but that deserves attention regarding ecotoxicological studies with mixtures is the experimental design employed to quantify the combined effects. As an essential prerequisite, optimal results in mixture experiments depend on a rational experimental design and this, in turns, depends on underlying hypothesis and reference models adopted in the study (Altenburger et al., 2003). Several types of experimental designs have been used in PPCP mixture studies, as is shown in Table 2.

Table 2 Types and percentage frequency of experimental designs employed in the 194 assessments of the toxicity of pharmaceutical and personal care (PPCP) mixtures retrieved from the international literature

Types of experimental design	Number of experimental data	Percentage frequency (%)	References
Fixed ratio design based on the NOEC[a]/EC_{01}[b] values of each compound	2	1.0	Backhaus et al. (2000b; 2011)
Fixed ratio design based on the LOEC[c] values of each compound	1	0.5	Bisesi Jr et al. (2016)
Fixed ratio design based on the EC_{10}[d] values of each compound	10	5.2	Di Nica et al. (2017)
Fixed ratio design based on the EC_{50}[e] values of each compound (including the isobologram method)	77	39.7	Various[g]
Fixed ratio design based on different EC_x[f] values besides the EC_{50}[c] of each compound (including, e.g., EC_5, EC_{10}, EC_{20}, EC_{80} and EC_{90})	22	11.3	Cleuvers (2003; 2004; 2005); Brezovšec et al. 2014; Godoy et al.(2015b); Nieto et al. (2016); Rossier et al. (2016); Siegenthaler et al. (2017)
Fixed ratio design based on the individual predicted no-effect concentration (PNEC) values	1	0.5	Di Nica et al. (2017)
Fixed ratio design based on the maximum aquatic environmental concentration of the compounds reported in the literature	3	1.5	Watanabe et al. (2016)
Fixed ratio design based on a specific exposure modeling	3	1.5	Zucchi et al. (2014); Runnalls et al. (2015); Guo et al. (2016)
Two-factor fractional-factorial design	2	1.0	Pomati et al. (2008)
Ray design consisting of multiple ratios based on the effective concentrations of the single compounds	13	6.7	Christensen et al. (2006; 2007); Hinfray et al. (2016)
Multiple combination ratios (based on the EC_{50}[d] of the single compounds) equidistantly distributed on the additivity line of the isobologram	8	4.1	De Liguoro et al. (2009; 2010)
Multiple ratios based on the 0.05, 1, 10, 20, 25 and/or 50 % value of the maximum effect concentration of the standard compound established (reference)	12	6.2	Fent et al. (2006b)
The concentration of one of the components was fixed at their NOEC[a] value while the concentration of the other compound was altered	3	1.5	Eguchi et al. (2004)
The concentrations of the components were based on available data for aquatic environments and/or on those able to elicit measurable toxic responses	29	14.9	Various[h]
The concentrations of the components were based on choices whose reasons were not specified in the corresponding paper	8	4.1	Ericson et al. (2010); Melvin et al. (2014; 2016); Li and Lin (2015); Ding et al. (2016); Liang et al. (2017)

[a] NOEC - Non-observed effect concentration [b] EC_{01} - Effect concentration at 1 % [c] LOEC - Lowest observed effect concentration

[d] EC_{10} - Effect concentration at 10 %

[e] EC_{50} - Effect concentration at 50 %

[f] ECx - Effect concentration at x %

[g]Various - Backhaus et al. (2000b); Thorpe et al. (2003); Henry and Black (2007); DeLorenzo and Fleming (2008); Schnell et al. (2009); Rodea-Palomares et al. (2010);Láng and Kőhidai (2012); Zou et al. (2012); González-Pleiter et al. (2013); Parrella et al. (2014); Villa et al. (2014); Geiger et al. (2016); Bialk-Bielińska et al. (2017)
[h] Various - Brain et al. (2004; 2005); Flaherty and Dodson (2005); Borgmann et al. (2007); Pomati et al. (2007); Quinn et al. (2009); Dietrich et al. (2010); Parolini and Binelli (2012); Franzelliti et al. (2013); Galus et al. (2013); Gust et al. (2012; 2013); Luna et al.(2013; 2015); Gonzalez-Rey et al. (2014); Säfholm et al. (2015); Wolfe et al. (2015); Zhao et al. (2015); Chiffre et al. (2016); González-Ortegónet al. (2016); Hua et al. (2016); Örn et al. (2016); Rossier et al. (2016); Almeida et al. (2017); Siegenthaler et al. (2017)

Source: Godoy and Kummrow (2017)

From the Table 2 data, we can conclude that most of the experimental designs (over 60 %) used in the retrieved PPCP mixture studies are based on constant mixture ratios. This type of design is usually employed for comparing observed responses with predicted ones from reference models (Altenburger et al., 2003). On the other hand, although the fixed ratio design allows the assessment of toxic interactions across mixture effect levels, it can underestimate toxic interactions across different mixture ratios (Barata et al., 2006).

Indeed, statistical interactions can be concentration level and toxicant ratio dependent (Jonker, 2003). For instance, evaluating the combined effect of the antimicrobials sulfaquinoxaline and sulfaguanidine on the algae *Raphidocelis subcapitata* by employing five exposure levels and three selected combination ratios, De Liguoro et al. (2010) found that the interaction was mixture-ratio dependent. A similar finding was observed for the complex interaction of the antimicrobials sulfamethazine and sulfaquinoxaline in the *D. magna* acute toxicity test, in which superadditivity, additivity or subadditivity were observed at the three different combination ratios tested (De Liguoro et al., 2009). In this sense, composite designs covering several possible interactions at various mixture ratios, such as those selected by factorial strategies, are probably most useful since they make it possible to cover complete response surfaces (Jonker, 2003). However, less than 20 % of the studies retrieved for this review employed design strategies aiming to evaluate multiple mixture ratios (Table 2).

It is also worth highlighting that about 4 % of the retrieved mixture studies used experimental designs in which the concentrations of the mixture components seemed to be randomly chosen, i.e., they were not explicitly based on environmentally relevant concentrations or on effect concentrations of the single compounds (Table 2 and Table A.1 of the Supplementary Material). Therefore, some experimental designs currently employed in mixture ecotoxicological studies may not be adequate to evaluate the joint effects of PPCP of environmental concern. Moreover, the great diversity of experimental designs employed makes it difficult to compare and conclude about results obtained in the PPCP mixture studies.

3.3 The interpretation and presentation of the effect profile for the PPCP mixtures regarding the model/approach used and deviations

Besides using appropriate experimental designs and approaches to predict/assess mixture toxicity, as discussed before, a satisfactory and comparable report of the outcome of this type of study also depends on a consistent and clear terminology to describe the toxicological effects, particularly regarding the terms antagonism and synergism. There are many different definitions of both terms, which has generated some confusion in the

presentation of the results. Synergism is often used as a synonym of potentiation, augmentation, sensitization, suppraaditiveness, superadditivity and potentiated summation, while antagonism is frequently referred to as depotentiation, desensibilization, infraadditiveness, subadditivity, negative synergy, among others (Rodea-Palomares et al., 2015).

However, the ATSDR (2004) defined that synergism and antagonism can be considered when the effect of the mixture is respectively greater and less than that estimated for additivity based on the toxicities of the components. Still according to the ATSDR (2004), potentiation is not synonymous with synergism, but it is the situation when a component without a toxic effect if applied singly on a system increases the effect of a second chemical. On that basis, synergism and antagonism may be considered as departures from additivity and are often defined in relation to the basic concepts of CA and IA (Cedergreen, 2014). Therefore, Backhaus (2014) draws attention to the fact that it is critically important to specify the frame/model of reference against which a mixture is evaluated. In practice, however, these terms have been sometimes used without explicitly referring to any reference model (Table A.1 of Supplementary Material).

It also deserves attention the recent criticisms that have been pointed out regarding the usual practice of considering synergism/antagonism in relation to the CA and/or IA models. Berthoud (2013) argues that these approaches are overly simplistic since they may fail to capture the biological complexity of a certain system. Indeed, Backhaus (2014) states that neither CA nor IA make any conjecture on the target biological system and therefore it is likely that they may describe the reality only in biologically extremely simple systems. In addition, additive predictions depend on the types or shapes of the dose/concentration-response of the individual compounds being tested (Berthoud, 2013). For nonlinear dose/concentration-response relationships, response additivity gives incorrect results and unfortunately this is the case of almost all dose-effect curves if considering the region of threshold doses/concentrations (Berthoud, 2013; Geary, 2013). Berthoud (2013) and Geary (2013) also highlight that the practice of considering interaction based on dose additivity is equally problematic because it is generally assumed that isobolograms are linear and do not dependent on the form of the dose-effect curves of the two compounds tested. However, Tallarida (2012) draws attention to the fact that linear isoboles occur only when the potency ratio of the individual compounds is a constant. When individual components do not have a constant relative potency, additive isoboles are not straight lines but are curves (Grabovsky and Tallarida, 2004). Nonlinear (curvilinear) isobole is the case that would apply when the individual log dose/concentration-response curves of the tested compounds are not parallel or when the individual chemical maximum effects differ (Grabovsky and Tallarida, 2004; Tallarida, 2012). This is a critical

point to be considered, because assuming that the additive isobole is always linear may lead to misinterpreted indications of synergism when the combination is actually additive (Grabovsky and Tallarida, 2004; Tallarida, 2012).

Hence, the probability that both response and dose additivity fail to offer a generally valid approach to interactions also in ecotoxicology should be considered. Anyway, considering the intuitive appeal of either simple IA and CA-isobologram models and considering the actual common practice of reporting synergism in relation to an additive model (or in relation to variants of these, such as the CI model), the percentage of synergisms at all effect levels tested, including the lowest ones, reported in the literature for mixture ecotoxicity of PPCP seems not to be irrelevant (Table S1 - Supplementary material). A possible synergism regarding one or more models/approaches was reported at all or in most of the effect levels tested in around 9.4 % of the 194 assessments of the toxicity of mixtures retrieved from 65 articles (Christensen et al. 2006; Zou et al., 2012; González-Pleiter et al., 2013; Brezovšec et al., 2014; Parrella et al., 2014; Geiger et al., 2016).Furthermore, if we consider other definitions of synergism such as that defended by Geary (2013), in which synergism is defined simply as a combination of drugs that lead to statistically significant increases over the effect of either agent alone, as it is usual in drug development, this percentage increases to around 11.0 % (Eguchi et al., 2004; Ericson et al., 2010; Luna et al., 2015; Liang et al., 2017). It must be highlighted that these percentages are without considering the synergism effect level-dependent and the overestimations of the toxicity by a specific model, as strictly reported by the authors. In spite of the limitations discussed in this review regarding the design and approaches usually adopted in mixture toxicity studies, such results deserve more attention.

Unfortunately, it was not possible to calculate the degree of deviation between observed and predicted mixture toxicity regarding one or more reference models for the whole of the retrieved studies because of the large diversity of experimental designs, approaches used in the retrieved studies and the form of presentation of results. However, such calculations of magnitude of deviation were possible for 13 papers, comprising 64 assessments of the toxicity of PPCP mixtures tested on 10 aquatic species, 1 marine periphyton and 1 recombinant yeast system (Table 3). The magnitude of deviations from reference models (CA, IA and/or CI) described in Table 3 were calculated or retrieved from data reported in the respective papers and grouped according to two methods of characterizing such deviations, according to Boobis et al. (2011). Method A depicts the magnitude of deviation as the ratio of predicted to observed concentration associated with a specific mixture response, while method B expresses this

magnitude of deviation as the ratio of observed to predicted response at a specific mixture concentration.

Table 3 Summary of pharmaceuticals and personal care products (PPCP) mixture toxicity studies reporting magnitude of deviation from reference models (CA = concentration addition; IA = independent action; CI = combination index - isobologram)

PPCP mixture	Endpoint/Species/Time duration	Effect levels/concentrations evaluated	Reference model	Magnitude of deviation*	Reference
Studies that report magnitude of deviation using Method A (ratio of predicted to observed concentration associated with a fixed mixture response)					
Sulpiride+ Clarithromycin+ Diphenhydramine+ Benzafibrate+ Acetaminophen+ Ketoprofen+ Phenytoin+ Etodolac+ Crotamiton+ Epinastine	Growth inhibition of algae *Raphidocelis subcapitata*/72 h	The pharmaceuticals were mixed in a ratio that was based on their maximum detected concentration in the Tama River (Tokyo, Japan) and effluent samples. Observed and predict mixture toxicity were compared at the mixture inhibition concentration at 5 % (IC_5) and 50 % (IC_{50}) levels	CA IA	1.0 1.1 - 1.3	Watanabe et al. (2016)
Sulpiride+ Clarithromycin+ Diphenhydramine+ Benzafibrate+ Acetaminophen+ Ketoprofen+ Phenytoin+ Etodolac+ Crotamiton+ Epinastine	Inhibition of reproduction of the crustacean *Ceriodaphnia dubia*/6 - 8 d	The pharmaceuticals were mixed in a ratio that was based on their maximum detected concentration in the Tama River (Tokyo, Japan) and effluent samples. Observed and predict mixture toxicity were compared at the mixture inhibition concentration at 25 % (IC_{25}) and 50 % (IC_{50}) levels	CA IA	1.5 - 2.3 2.2 - 3.4	
Sulpiride+ Clarithromycin+ Diphenhydramine+ Benzafibrate+ Acetaminophen+ Ketoprofen+ Phenytoin+ Etodolac+ Crotamiton+ Epinastine	Survival of the larvae of *Danio rerio*/9 d	The pharmaceuticals were mixed in a ratio that was based on their maximum detected concentration in the Tama River (Tokyo, Japan) and effluent samples. Observed and predict mixture toxicity were compared at the mixture inhibition concentration at 10 % (IC_{10}) and 50 % (IC_{50}) levels	CA IA	0.5 - 0.7 1.0 - 1.4	

(to be continued)

PPCP mixture	Endpoint/Species/Time duration	Effect levels/concentrations evaluated	Reference model	Magnitude of deviation*	Reference
Studies that report magnitude of deviation using Method A (ratio of predicted to observed concentration associated with a fixed mixture response)					
Ciprofloxacin+ Ibuprofen	Growth rate inhibition of the algae *Chlorella vulgaris*/96 h	Equal proportions of the respective IC_{50} (inhibitory concentration at 50%) of each component were used, comprising the sum of 0.25, 0.5, 1.0, 2.0 and 4.0 toxic units. Deviations were calculated for the range between 1 and 95 % of growth inhibition	CA IA	0.5 - 1.3 0.1 - 0.9	Geiger et al. (2016)
Chlortetracycline+ Diclofenac	Bioluminescence inhibition of the marine bacteria *Aliivibrio fischeri*/15 min	The pharmaceuticals were mixed at an equitoxic ratio corresponding to their individual inhibitory concentration at 10 % (IC_{10}). Predicted and observed values were compared at the mixture inhibition concentrations at 10 % (IC_{10}) and 50 % (IC_{50}) levels	CA IA	1.5 - 6.1 1.9 - 10	Di Nica et al. (2017)
Diclofenac+ Sulfamethizole			CA IA	0.9 - 1.8 0.9 - 2.4	
Acetylsalycilic acid+ Chlortetracycline			CA IA	0.4 0.4	
Acetylsalycilic acid+ Sulfamethizole			CA IA	0.4 - 1.0 0.5 - 1.0	
Chlortetracycline+ Amoxicillin			CA IA	0.5 - 11.2 0.6 - 12.6	
Acetylsalycilic acid+ Diclofenac			CA IA	0.9 - 1.5 1.0 - 2.0	
Chlortetracycline+ Sulfamethizole			CA IA	0.8 - 0.9 0.8 - 0.9	
Diclofenac+ Amoxicillin			CA IA	1.4 - 4.6 1.7 - 6.6	
Sulfamethizole+ Amoxicillin			CA IA	0.7 - 2.2 0.8 - 2.5	

(To be continued)

PPCP mixture	Endpoint/Species/Time duration	Effect levels/concentrations evaluated	Reference model	Magnitude of deviation*	Reference
Acetylsalycilic acid+ Amoxicillin			CA IA	0.5 - 1.3 0.6 - 1.4	
Chlortetracycline+ Diclofenac+ Acetylsalycilic acid+ Sulfamethizole+ Amoxicillin		The pharmaceuticals were mixed at an equitoxic ratio corresponding to their individual predicted non-effect concentration (PNEC) values	CA IA	0.7 - 1.3 0.8 - 1.4	
Cinoxacin+ Enoxacin+ Flumequine+ Lomefloxacin+ Nalidixic acid+ Norfloxacin+ Ofloxacin+ Oxolinic acid+ Pipemidic acid+ Piromidic acid	Bioluminescence inhibition of the marine bacteria *A. fischeri*/24 h	Three different mixture ratios based on the toxicity of each pharmaceutical compound were applied in mixture: effect concentrations at 1 % and 50 % (EC_{01} and EC_{50}) and non-observed effect concentration (NOEC). Predicted and observed mixture EC_{50} values for the three mixture ratios were reported by the authors	CA IA	0.8 - 0.9 2.4 - 2.7	Backhaus et al. (2000)
Clotrimazole+ Triclosan+ Zinc-pyrithione+ Fluoxetine+ Propranolol	Total pigment content and specific pigments (Chlorophyll a, Diadinoxanthin, Diatoxanthin, Fucoxanthin, Prasinoxanthin, Zeaxanthin, and β-carotene)/96 h of microalgae from marine periphyton	A fixed-ratio design was used based on the non-observed effect concentration (NOEC) values of the compounds. Predicted and observed concentrations were reported for the mixture effect level at 50 % (EC_{50})	CA IA	0.6 0.9	Backhaus et al. (2011)
Triclocarban+ Triclosan+ Methyltriclosan (metabolite)	Bioluminescence inhibition of *A. fischeri*/15 min	Equitoxic concentration ratio was used corresponding to the individual inhibitory concentration at 50 % (IC_{50}) of each compound. Predicted and observed concentrations were reported for the 10 % and 50 % mixture effect levels (IC_{10} and IC_{50})	CA IA	1.3 - 1.4 0.9 - 1.1	Villa et al. (2014)

(To be continued)

PPCP mixture	Endpoint/Species/Time duration	Effect levels/concentrations evaluated	Reference model	Magnitude of deviation*	Reference
Studies that report magnitude of deviation using Method A (ratio of predicted to observed concentration associated with a fixed mixture response)					
Sertraline+ Fluoxetine	Mortality of the crustacean *C. dubia*/48 h	Equitoxic concentration ratio was used corresponding to the individual lethal concentration at 50 % (LC_{50}) of each pharmaceutical compound. Predicted and observed concentrations were reported for the 50 % mixture lethality level (LC_{50})	CA IA	1.2 1.4	Henry and Black (2007)
Sertraline+ Paroxetine			CA IA	1.2 1.3	
Sertraline+ Citalopram			CA IA	1.2 1.4	
Fluoxetine+ Paroxetine			CA IA	1.1 1.3	
Fluoxetine+ Citalopram			CA IA	1.2 1.5	
Paroxetine+ Citalopram			CA IA	0.8 1.2	
Sertraline+ Fluoxetine+ Paroxetine+ Citalopram			CA IA	1.2 - 2.5 1.2 - 2.8	
Tylosin+ Lincomycin+ Trimethoprim	Growth inhibition of the cyanobacteria *Anabaena flos-aquae*/96 h	Compounds were applied at the mixture ratio comprising 1 part of tylosin: 4.31 parts trimethoprim: 6.65 parts lincomyin, based on exposure models. Predicted and observed values were reported for the 5 % and 50 % mixture effective concentration (EC_5 and EC_{50}) levels	CA IA	0.8 - 1.2 1.4 - 2.4	Guo et al. (2016)

(To be continued)

PPCP mixture	Endpoint/Species/Time duration	Effect levels/concentrations evaluated	Reference model	Magnitude of deviation*	Reference
Studies that report magnitude of deviation using Method B (ratio of observed to predicted response at a specific mixture concentration)					
Furosemide+ 17 β estradiol	Estrogenic activity assessed in recombinant yeast (*Saccharomyces cerevisiae*) cells containing the human estrogen receptor/72 h	Equipotent mixtures in concentrations of each compound varying from 20 to 80 % of the maximal induction by 17 β estradiol	CA IA	0.9 - 1.1 1.0 - 1.5	Fent et al. (2006b)
Furosemide+ Phenazone		Equipotent mixtures in concentrations of each compound corresponding to 20 % and 25 % of the maximal induction by the standard 17 β estradiol	CA IA	1.0 - 1.1 1.1 - 1.2	
Furosemide+ Cimetidine		Equipotent mixtures in concentrations of each compound corresponding to 1 % and 10 % of the maximal induction by the standard 17 β estradiol	CA IA	1.0 1.2 - 1.7	
Furosemide+ Fenofibrate		Equipotent mixtures in concentrations of each compound corresponding to 1 % and 10 % of the maximal induction by the standard 17 β estradiol	CA IA	0.7 - 1.3 1.0 - 1.6	
Furosemide+ Paracetamol		Equipotent mixtures in concentrations of each compound corresponding to 1 % and 10 % of the maximal induction by the standard 17 β estradiol	CA IA	1.1 - 1.2 2.7 - 3.1	
Cimetidine+ Fenofibrate		Equipotent mixtures in concentrations of each compound corresponding to 1 % and 10 % of the maximal induction by the standard 17 β estradiol	CA IA	1.1 - 1.8 0.9 - 2.9	
Furosemide+ Cimetidine+ Fenofibrate		Equipotent mixtures in concentrations of each compound corresponding to 10 % of the maximal induction by the standard 17 β estradiol	CA IA	2.7 1.7	

(To be continued)

PPCP mixture	Endpoint/Species/Time duration	Effect levels/concentrations evaluated	Reference model	Magnitude of deviation*	Reference
Furosemide+ Phenazone+ Fenofibrate		Equipotent mixtures in concentrations of each compound corresponding to 10 % of the maximal induction by the standard 17 β estradiol	CA IA	4.0 1.4	
Phenazone+ Cimetidine+ Fenofibrate		Equipotent mixtures in concentrations of each compound corresponding to 10 % of the maximal induction by the standard 17 β estradiol	CA IA	2.2 1.4	
Phenazone+ Cimetidine+ Furosemide		Equipotent mixtures in concentrations of each compound corresponding to 10 % of the maximal induction by the standard 17 β estradiol	CA IA	1.7 1.9	
Furosemide+ Phenazone+ Cimetidine+ Fenofibrate		Equipotent mixtures in concentrations of each compound corresponding to 0.05 % (non-effect concentration) and 1 % of the maximal induction by the standard 17 β estradiol	CA IA	1.1 - 2.7 2.8 - 4.7	
Furosemide+ Phenazone+ Cimetidine+ Fenofibrate+ Paracetamol		Equipotent mixtures in concentrations of each compound corresponding to 1 % and 10 % of the maximal induction by the standard 17 β estradiol	CA IA	2.0 - 2.7 1.8 - 5.1	
Diclofenac+ Ibuprofen	Lethality of the shrimp *Atyaephyra desmarestii* at 20 and 25 ºC/96 h	Equipotent mixtures corresponding to half of the lethal concentrations ($LC_x/2$) at 5 %, 10 %, 20 %, 50 % and 80 %, obtained from the concentration-response curves for the individual pharmaceuticals	CA IA	0.3 - 5.6 1.7 - 19.9	Nieto et al. (2016)

(To be continued)

PPCP mixture	Endpoint/Species/Time duration	Effect levels/concentrations evaluated	Reference model	Magnitude of deviation*	Reference
Diclofenac+ Carbamazepine		Equipotent mixtures corresponding to half of the lethal concentrations ($LC_x/2$) at 5 %, 10 %, 20 %, 50 % and 80 %, obtained from the concentration-response curves for the individual pharmaceuticals	CA IA	0.4 - 1.2 1.4 - 11.8	
Diclofenac+ Ibuprofen+ Carbamazepine		Equipotent mixtures corresponding to a third of the lethal concentrations ($LC_x/3$) at 5 %, 10 %, 20 %, 50 % and 80 %, obtained from the concentration-response curves for each of the individual pharmaceuticals	CA IA	0.0 - 4.5 0.0 - 16.6	
Propranolol+ Losartan	Growth inhibition of the macrophyte *Lemna minor* based on frond number/7 d	The compounds were combined in five effect concentration levels, using half of the effect concentrations ($EC_x/2$) at 10, 20, 50, 70 and 80 % of each pharmaceutical, based on the individual concentration-response curves	CA IA	0.3 - 0.6 0.3 - 0.7	Godoy et al. (2015b)
Erythromycin+ Levofloxacin	Inhibition of luminescence of the cyanobacterium *Anabaena* CPB4337/72 h	The pharmaceuticals were mixed at a fixed constant ratio (1:1) based on the individual EC_{50} values (mg L^{-1})	CA IA CI	0.8 - 1.1 1.0 - 1.1 0.9 - 1.0	González-Pleiter et al. (2013)
Erythromycin+ Norfloxacin			CA IA CI	0.9 - 1.0 0.9 - 1.1 1.0 - 1.1	
Erythromycin+ Tetracycline			CA IA CI	0.9 - 28.3 0.9 - 28.0 1.0 - 2.1	
Levofloxacin+ Norfloxacin			CA IA CI	0.8 - 1.6 1.0 - 2.0 0.7 - 1.4	
Levofloxacin+ Tetracycline			CA IA	2.9 - 23.0 4.4 - 30.5	(To be continued)

PPCP mixture	Endpoint/Species/Time duration	Effect levels/concentrations evaluated	Reference model	Magnitude of deviation*	Reference
			CI	1.2 - 2.0	
Norfloxacin+ Tetracyclin			CA	1.4 - 1.9	
			IA	1.7 - 2.7	
			CI	1.3 - 2.1	
Amoxicillin+ Erythromycin			CA	0.9 - 1.1	
			IA	0.9 - 1.1	
			CI	0.9 - 1.2	
Amoxicillin+ Norfloxacin			CA	0.8 - 1.2	
			IA	0.8 - 1.1	
			CI	1.0 - 1.1	
Amoxicillin+ Levofloxacin			CA	1.2 - 1.8	
			IA	1.3 - 1.9	
			CI	0.9 - 1.2	
Amoxicillin+ Tetracyclin			CA	1.2 - 4.5	
			IA	1.2 - 5.9	
			CI	0.9 - 1.1	
Erythromycin+ Levofloxacin+ Norfloxacin+ Tetracyclin+ Amoxicillin			CA	1.0 - 1.6	
			IA	1.2 - 2.3	
			CI	0.9 - 1.1	
Erythromycin+ Levofloxacin	Growth rate inhibition of *R. subcapitata*/72h		CA	0.2 - 1.2	
			IA	0.2 - 1.1	
			CI	0.6 - 1.5	
Erythromycin+ Norfloxacin			CA	1.2 - 1.8	
			IA	1.2 - 1.8	
			CI	0.9 - 1.2	(To be continued)
Erythromycin+			CA	1.3 - 2.6	

PPCP mixture	Endpoint/Species/Time duration	Effect levels/concentrations evaluated	Reference model	Magnitude of deviation*	Reference
Tetracycline			IA CI	1.1 - 2.3 0.7 - 1.5	
Levofloxacin+ Norfloxacin			CA IA CI	1.6 - 2.4 1.6 - 2.4 0.9 - 1.1	
Levofloxacin+ Tetracycline			CA IA CI	0.9 - 1.6 0.6 - 1.3 0.7 - 1.1	
Norfloxacin+ Tetracyclin			CA IA CI	0.2 - 2.1 0.2 - 2.1 0.6 - 0.9	
Erythromycin+ Levofloxacin+ Norfloxacin+ Tetracyclin			CA IA CI	1.0 - 1.4 0.8 - 1.1 0.9 - 1.2	
Sulfathiazole+ Sulfamerazine+ Sulfadimidine+ Sulfamethoxazol+ Sulfadimethoxine+ Sulfapyridine	Growth inhibition of the algae *Scenedesmus vacuolatus*/24h (corresponding to the 96h standard test)	A fixed ratio design was used, in which each of the pharmaceutical compounds was mixed in a concentration equal to 1/6 of its EC_{50} value	CA IA	0.7 0.2	Białk-Bielińska et al. (2017)
Sulfathiazole+ Sulfamerazine+ Sulfadimidine+ Sulfamethoxazol+ Sulfadimethoxine+ Sulfapyridine+ Sulfanilamid	Growth inhibition of *S. vacuolatus*/24h (corresponding to the 96h standard test)	A fixed ratio design was used, in which each of the compounds was mixed in a concentration equal to 1/7 of its EC_{50} value	CA	0.5	

(To be continued)

PPCP mixture	Endpoint/Species/Time duration	Effect levels/concentrations evaluated	Reference model	Magnitude of deviation*	Reference
Sulfathiazole+ Sulfamerazine+ Sulfadimidine+ Sulfamethoxazol+ Sulfadimethoxine+ Sulfapyridine+ Sulfanilamide (photodegradation product)	Growth inhibition of the macrophyte *L. minor*/7d		CA IA	0.6 4.6	

* Where observed/predicted values were reported with an associated confidence interval, the mean reported values were used for calculating the respective deviation.

Source: Godoy and Kummrow (2017)

It is worth mentioning that there is not a defined size from which the synergistic interaction should be considered of biological quantitative importance. However, some authors have proposed a more than two-fold deviation from CA as a reference value for pesticides, metals, and antifouling mixtures (e.g., Belden et al. 2007; Cedergreen, 2014). Therefore, considering this same scheme for comparison, from Table 3 we can conclude that 32.8 % (21 out of the 64) of all assessments of the toxicity of PPCP mixtures had a magnitude of deviation value greater than a factor of 2 regarding the CA model (out of the 0.5 - 2.0 range), for at least one effect level tested. This percentage reached 45.3 % (29 out of the 64 mixtures) regarding the IA model. It is particularly notable the magnitude of deviation, greater than 20.0, calculated for the observed/predicted effects of the binary antimicrobial mixtures of erythromycin - tetracycline and levofloxacin - tetracycline on the cyanobacterium *Anabaena* (González-Pleiter et al., 2013). In fact, González-Pleiter et al. (2013) reported that a strong synergism (confirmed by applying the CI model) was observed for both antimicrobial binary mixtures at very low effect levels, comprising environmentally relevant concentrations. It must be highlighted, however, that most of the studies with PPCP mixtures reporting comparison values between predicted/observed toxicity assessed the joint effect of only 2 to 3 compounds and most of them employed a fixed-ratio experimental design (Tables 2 and 3). However, real environmental PPCP mixtures are often composed of multiple compounds (Kümmerer, 2009). Furthermore, mixture ratios in environmental samples are unlikely to occur at equitoxic ratios (Kortenkamp and Altenburger, 2011).

Based on what was discussed, further sound conclusions about how frequently antagonistic and synergistic interactions occur in non-target organisms exposed to relevant PPCP mixtures, and what their quantitative consequences are, should be underpinned on further PPCP mixture studies employing optimized experimental designs (e.g., factorial strategies) and on defined and clear criteria for interpreting/reporting the results of such studies, particularly regarding the terminology used to describe the toxicological effects. In addition, risk assessment frameworks should, otherwise, endorse integration of existing information on chemical non-additive interactions, similar to what happens in the Binary Weight of Evidence (WOE) approach, from the US Department of Human Health and Services (Mumtaz and Durkin, 1992; Rodea-Palomares et al., 2015). This approach incorporates information on binary mixtures in order to allow a prediction of mixture effects different from linear additivity, by including an interaction-based hazard index. Another approach that allows someone to integrate interactions in the prediction of mixture toxicity and risk is the physiologically-based pharmacokinetic/dynamic (PBPK/PD) model. This approach allows extrapolation of

interactions from binary to more complex mixtures, making it possible to predict the magnitude of chemical interactions and assisting in determining mechanisms for the interactions (Moser and Krishnan, 2007). However, it must be highlighted that this approach demands detailed knowledge on the physiology of organisms and requires a considerable number of input parameters such as partitioning coefficients and metabolic rate constants (Backhaus et al., 2008). Perhaps because of this, PBPK/PD models have not yet been used for the modeling of PPCP mixtures in an environmental context.

It is important to mention that although these interaction-based risk prediction approaches can be promising and important methods to be considered in ERA of chemical mixtures, they require a lot of data and specialist professionals in modeling and interpreting the outcomes, hampering their current use as a standard protocol (Heys et al., 2016). In view of this, approaches for ERA of pharmaceutical mixture have still been proposed assuming a concentration-additive behavior, such as the mixture-specific assessment factor reported by Backhaus (2016).

3.4 Therapeutic classes usually covered in ecotoxicological studies with PPCP mixtures

The potential harmful effects of PPCP to non-target organisms remain largely unknown. However, it is estimated that 10-15% of the pharmaceuticals found in surface waters are acutely or chronically toxic for specific endpoints, when they are evaluated in standardized ecotoxicological biosystems (Brausch et al., 2012; Wilkinson et al., 2016). Moreover, studies on ecotoxicological effects available both for the individual PPCP (Santos et al., 2010) and for their mixtures are quite concentrated in certain pharmacological classes such as antibiotics, antiinflammatory and blood lipid regulator agents. Figure 1 illustrates the clear predominance of antimicrobial agents in ecotoxicological studies with PPCP mixtures.

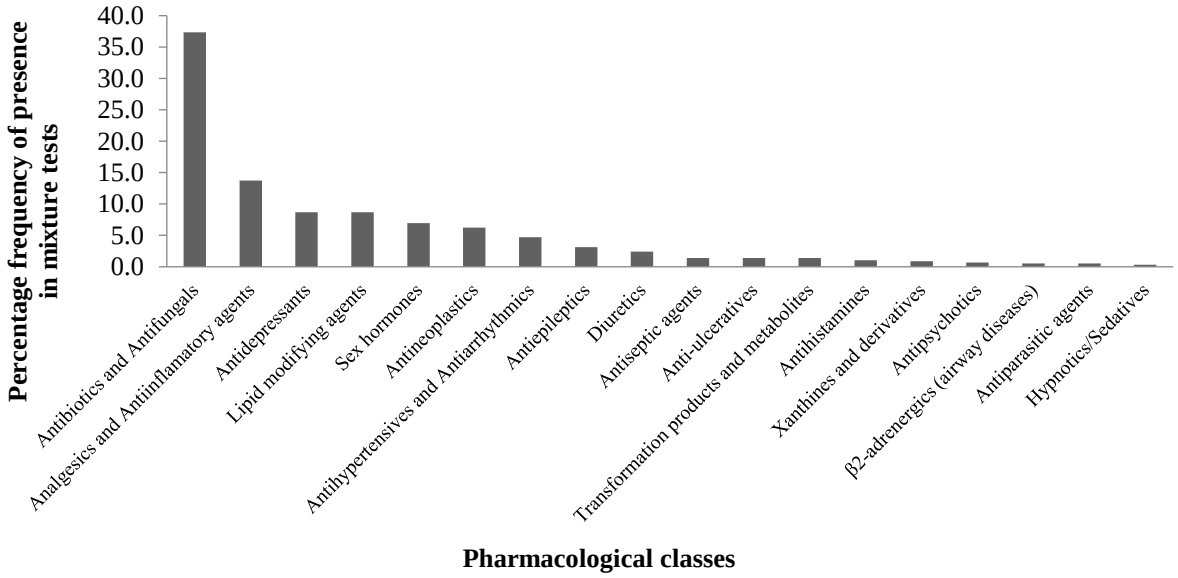

Fig. 1 Percentage frequency of the therapeutic classes of the pharmaceuticals and personal care products (PPCP) addressed in the retrieved mixture toxicity studies (data collected from 65 articles published between 2000-2017)

Source: Godoy and Kummrow (2017)

PPCP mixtures of relevance for the environment can be considered those ones whose compounds co-occur in a specific medicine or personal care product or those mixtures that are emitted from a common process or emission source, besides those PPCP mixtures that happen to co-occur in a certain environmental compartment (Kortenkamp et al., 2009; Backhaus, 2016). In this sense, one possible reason for the large predominance of antimicrobial PPCP in ecotoxicological studies on mixture effects is that some antibiotics are used in association to increase their efficacy, which in turn increases the likelihood that potential interactions between these chemicals can occur also in non-target organisms when they co-occur in the environment (Heys et al., 2016). This is the case with the combination effects between sulfonamides and the diaminopyrimidine antibiotic trimethoprim. Sulfonamides and trimethoprim both act in the folate biosynthetic pathway, thus impairing the synthesis of nucleic acids, amino acids and pantothenate in bacteria. Therefore, it is common the association of sulfonamides and trimethoprim in order to potentiate the antimicrobial activity, both in human and in the veterinary medicine. In addition, these antimicrobials have been frequently detected simultaneously, especially in aquatic environments (Lindberg, 2006; terLaak et al., 2010; Giang et al., 2015; Tlili et al., 2016). Thus, it is not surprising that the sulfonamides and trimethoprim are the antimicrobials most frequently tested in mixture studies, being responsible for 46 % of the compounds evaluated within this therapeutic class (Brain et al., 2004; Eguchi et al., 2004;

Flaherty and Dodson, 2005; Pomati et al., 2007; De Liguoro et al., 2009; Quinn et al., 2009; De Liguoro et al., 2010; Gust et al., 2012; Zou et al., 2012;Gust et al., 2013; Melvin et al., 2014;Li and Lin, 2015; Wolfe et al., 2015; Di Nica et al., 2017; Guo et al., 2016; Bialk-Bielińska et al., 2017).

Another important reason that makes the PPCP mixtures involving antimicrobials a key environmental issue is that these compounds may also increase the persistence of other PPCP, which can affect the overall environmental risk. This issue was exemplified in a study carried out by Monteiro and Boxall (2009), in which the authors used soil and soil-biosolid mixtures spiked with carbamazepine, fluoxetine, and naproxen as well as with the antibacterial compound sulfamethazine in order to explore the effects of pharmaceutical mixtures on the persistence of these compounds. The authors observed that the degradation of the anti-inflammatory naproxen was significant slower than in the single-compound studies. Thus, Monteiro and Boxall (2009) attributed their findings to the presence of the antimicrobial sulfamethazine, which has been shown to decrease soil bacterial populations.

On the other hand, not only antimicrobial agents are used in combination or are emitted and occur together in the same environmental compartment. Pharmaceuticals belonging to other therapeutic classes of environmental concern are still much less addressed in the ecotoxicological studies with PPCP mixtures, such as the sex hormones (Fig. 1). Natural and synthetic sex hormones, especially estrogens and progestogens, are frequently used combined with each other in oral contraceptives and in hormone replacement therapy, among other therapeutic uses (Runnalls et al., 2010). Some important physical-chemical properties make this class of pharmaceuticals of special environmental concern. Runnalls et al. (2010) state that the relative high degree of hydrophobicity, small molecular weight and their affinity for sex steroid binding protein are factors that suggest that most of the sex steroids should readily pass across the gills of fishes and bioconcentrate in these aquatic organisms. Besides, steroid hormones are generally extremely potent even at very low concentrations (in the µg or ng L^{-1} ranges), as in the case of the synthetic estrogen ethinylestradiol, which may induce adverse effects on reproduction of fish at concentrations less than 1 ng L^{-1} (Sumpter and Johnson, 2005; Runnalls et al., 2010). When it comes to their combined effects, these pronounced effects become even more worrying. Mixtures of compounds belonging to this class of pharmaceuticals (including estrogens, progestogens and androgens) have been shown to additively affect reproduction and lead to histological and transcriptional alterations in fishes and amphibians at environmentally relevant concentrations (Zucchi et al., 2014; Runnalls et al., 2015; Säfholm et al., 2015; Zhao et al., 2015; Hua et al., 2016; Örn et al., 2016). In this sense, the statement that steroid hormones

should be a high priority for research (Runnalls et al., 2010) could be further strengthened when it comes to their combined effects.

Also, metabolites and transformation products co-occurring in the environment are still scarcely evaluated in mixture studies. In a study carried out in the Ebro River basin (Spain), López-Serna et al. (2012) found that in all sampling sites the metabolites and transformation products detected were present at the same concentration level as the parent compounds. Therefore, these authors reported that the metabolites and transformation products represented an average 30-50% of the total pharmaceutical load (this total included the parent compounds). However, from the published articles retrieved for this review, only one included a metabolite in the evaluated mixture, namely methyl-triclosan, which is a methylated derivative from triclosan (Villa et al., 2014). Villa et al. (2014) found that methyl-triclosan acted additively (according to the CA model) in an equitoxic mixture with the PPCP triclosan and triclocarban towards the bacteria *Aliivibrio fischeri*, thus contributing to the overall acute toxicity of the evaluated mixture. Likewise, transformation products have been evaluated in mixtures in only two included articles, which were published recently (Almeida et al., 2017; Białk-Bielińska et al., 2017). Białk-Bielińska et al. (2017) found evidence that sulfanilamide, a degradation product from antimicrobial sulfonamides, also acted additively (according to the CA model) in a mixture with six sulfonamides (sulfathiazole, sulfamerazine, sulfadimidine, sulfamethoxazole, sulfadimethoxine and sulfapyridine), in the growth inhibition test with the macrophyte *Lemna minor* and the green algae *Scenedesmus vacuolatus*. Therefore, this transformation product contributed to the toxic potential of the PPCP mixture. Those examples demonstrate the importance of increasingly considering metabolites and transformation products in ecotoxicological assays and environmental risk assessment (ERA) of PPCP mixtures, especially because there is evidence that the ecotoxicity of some degradation products can be greater than of their respective parent compounds (Isidori et al., 2005; 2009). In addition, as it was well reminded by López-Serna et al. (2012), the combined concentrations from these compounds sharing a common mode of action or even acting through additional unknown modes of toxic action on non-target organisms could be considerable.

In addition to those substantial identified gaps of knowledge regarding ecotoxicological studies with PPCP mixtures, it must be highlighted that only a small number of different molecules have been covered in such investigations. From the approximately 3000 - 5000 pharmacologically active substances currently on the European Union market, among which are more than 600 that have already shown to be present in the environment worldwide (Küster and Adler, 2014; Donnachie et al., 2016), only 110 molecules (parental compounds) have been

ecotoxicologically evaluated in mixtures in the papers included in our review. This lack of data makes it difficult to evaluate the real frequency of interactions and their quantitative consequences in non-target organisms exposed to environmental pharmaceutical mixtures. Moreover, this also implies that any statement regarding the magnitude and importance of the possible interactions that occur among pharmaceuticals in the environment represents only a still partial and inconclusive picture of this challenging issue. On the other hand, it is unfeasible to test all possible PPCP mixtures that occur in environmental compartments. In addition, although monitoring surveys have detected pharmaceutical mixtures with a widely variable number of compounds, frequently the overall toxicity of chemical mixtures is dominated by only a few components (Backhaus, 2016). In this sense, the need emerges to establish criteria of prioritizations for PPCP mixtures of environmental concern to be evaluated in ecotoxicological studies, focusing mainly on their likelihood for inducing additive or synergistic adverse effects on non-target organisms, for matters of ERA.

An important approach that could support the prioritization of PPCP mixtures for such studies is based on the knowledge of evolutionary conserved molecular drug targets. Pharmaceuticals are designed to interact with specific molecular targets, which are often evolutionary conserved in many non-target organisms (Gunnarsson et al., 2008; Furuhagen et al., 2014). Thus, two or more PPCP compounds that would bind to the conserved active site of a same protein or enzyme could potentially induce an additive effect that should be investigated in ERA (Walker and McEldowney, 2013). For instance, because of the bacterial ancestry of plastid organelles and conservation of several metabolic pathways in plant cells, several classes of antimicrobials are believed to target these pathways or processes, such as sulfonamides, fluoroquinolones, macrolides, tetracyclines and the antimicrobials trimethoprim and triclosan, among other ones (Brain et al., 2008). Thus, the possibility emerges that pharmacodynamic interactions in plants can occur among the several antimicrobials that co-occur in each environmental compartment, both by the action of the antimicrobials on the same or in different metabolic pathways within a plant cell. In fact, the six sulfonamides evaluated using *L. minor* growth inhibition test by Białk-Bielińska et al. (2017) acted additively in an equitoxic mixture, i.e., their combined toxicity could be reasonably estimated by the CA model. These outcomes are not surprising considering that sulfonamides are known to inhibit the enzyme dihydropteroate synthase in the folate biosynthetic pathway, which in turns has an essential similarity in plants and bacteria (Brain et al., 2008). Thus, it is probable that the sulfonamides evaluated in mixture by Białk-Bielińska et al. (2017) acted in the same enzymatic pathway, which is evolutionary conserved between bacteria and plants, causing *L. minor* growth

inhibition effects even stronger than those previously observed for the individual pharmaceuticals (Białk-Bielińska et al., 2011) at the concentrations at which they were presented in the mixture.

Also in invertebrates, evolutionary conserved molecular drug targets have been found to be present. For example, serotonin transporters, which are the pharmacological target of the serotonin reuptake inhibitors (SSRI), such as fluoxetine, are highly conserved between vertebrates and invertebrates (Campos et al., 2012). Consequently, pharmaceuticals belonging to this therapeutic class also exert different physiological effects in invertebrates, such as the increased production of smaller offspring in *Daphnia magna* (Campos et al., 2012). Based on these findings, Campos et al. (2012) hypothesized that the SSRI act in *D. magna* following a similar mode of action as observed in humans, i.e., by blocking serotonin reuptake and increasing serotonin postsynaptic activity. Under these assumptions, it is possible that the simultaneous presence of SSRI compounds in environmental compartments can result in additive joint effects on invertebrates having serotonin transporters. In fact, the additive acute toxicity (well predicted by the CA model) of mixtures of SSRI pharmaceuticals such as fluoxetine, paroxetine, sertraline, and citalopram towards crustaceans such as *D. magna* and *Ceriodaphnia dubia* have been demonstrated by Christensen et al. (2007) and Henry and Black (2007).

It is also worth mentioning that fishes and amphibians may be also important target of PPCP mixtures since they have been predicted to have the greatest number of human drug target conserved proteins (called orthologs), with the highest degree of similarity (Gunnarsson et al., 2008). Therefore, it is more likely that low levels of pharmaceuticals and their mixtures would act specifically in conserved drug targets of aquatic vertebrates than in other species commonly used for aquatic ERA (Gunnarsson et al., 2008). However, fishes and principally amphibians are still much less addressed in PPCP mixture toxicity studies compared to primary producers, invertebrates, fungi and bacteria (Figure 2).

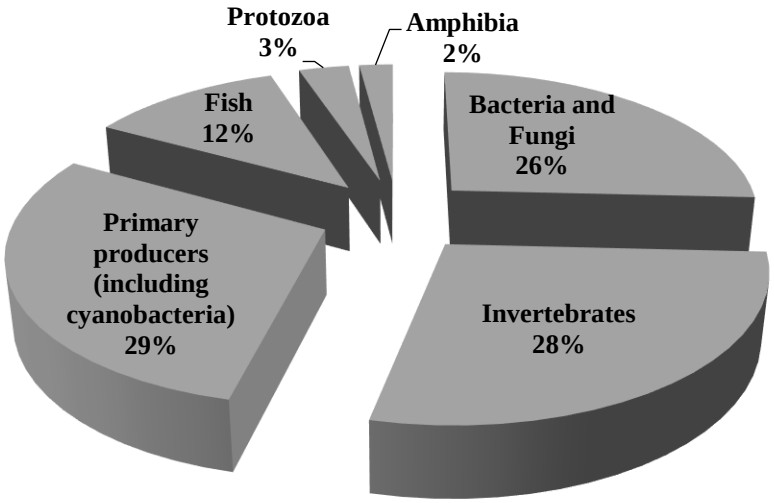

Fig. 2 Principal types of test-organisms used in pharmaceuticals and personal care products (PPCP) mixture studies, expressed in relative percentage (data collected from 65 articles published between 2000-2017)

Source: Godoy and Kummrow (2017)

Finally, it must be addressed that there has been recently a recognition that grouping chemicals for environmental mixture risk assessment would better focuses on common adverse outcome pathways (AOP) rather than on chemical similarity or similar mechanisms of action (Kortenkamp et al., 2009). This is because grouping PPCP compounds for mixture risk assessment based solely on chemical similarity or similar mechanisms of action may neglect compounds that also might contribute to joint effects, since compounds sharing a similar biological effect may act by toxicological mechanisms profoundly different in many aspects (Kortenkamp et al., 2009). Thus, the AOP concept appears as a systematic approach describing key event relationships linking a direct molecular initiating event (MIE) to an adverse outcome considered relevant to risk assessment (Ankley et al., 2010; Conolly et al., 2017). Connoly et al. (2017) describe that this linkage can be quantitatively made by using one or more biologically based, computational models, called quantitative AOP (qAOP). Since a qAOP is not chemical or stressor-specific (Connoly et al., 2017), it could be used in the predictive toxicology of chemical mixtures regardless of the specific identity of the components that disrupt the key events leading to a common adverse outcome. In the particular case of mixtures, however, it is important to remember that adverse outcomes may be triggered by different MIEs and thus, key events are considered those observations aggregating several potential MIEs (Altenburger et al., 2015; Escher et al., 2017). Escher et al. (2017) highlight that there are no deviations between requisites for AOPs to be used for mixtures and for single compounds and

emphasize that experimental methods should focus on key events affected by different compounds binding to diverse target sites, but that these can converge into the same adverse outcome.

To date, most of the applications of the AOP concept in risk assessment have been limited to individual chemical compounds, lacking considerations for combined effects of chemicals in mixtures (Escher et al., 2017). However, a potential application of a qAOP in the PPCP mixture field have been recently pointed out by Conolly et al. (2017). These authors generated quantitative response-response functions (such as for plasma 17β-estradiol in *Pimephales promelas* as a function of aromatase inhibition, fecundity as a function of plasma vitellogenin, etc.), developed from aromatase inhibition qAOP models, and proposed that those functions are applicable to the predictive assessment of multiple aromatase inhibitors occurring as a mixture in the environment, by summing the derived toxicity equivalent factor values in order to achieve an estimated "total" aromatase inhibition.

It is also worth mentioning the importance of considering the narcosis AOP for ecotoxicological predictions and for prioritization of PPCP mixtures for matters of ERA. Narcosis or baseline toxicity is a non-specific toxicity that occurs with chemicals that do not interact with specific receptors in the organism (Cleuvers, 2003; Ankley et al., 2010). This AOP involves weak and reversible hydrophobic interactions between chemicals and cellular membranes and is observed for a diverse set of chemical structures, making it unlikely that a specific receptor linkage is involved on the event (Ankley et al., 2010). In the PPCP mixture context, the narcosis AOP is relevant because, as many of other types of industrial organic chemicals, PPCP and their mixtures, including their metabolites and transformation products, may also act via narcosis (Escher et al., 2006; Furuhagen et al., 2014; Villa et al., 2014; Neale et al., 2017) and may contribute to the overall toxicity of PPCP environmental mixtures. For instance, baseline toxicity was observed in a mixture study carried out by Cleuvers (2003) with the pharmaceuticals carbamazepine and clofibrinic acid in the *D. magna* acute test. The author was able to show that both pharmaceuticals acted in combination by narcosis. Also, Neale et al. (2017) showed that an equipotent mixture of five pharmaceuticals (carbamazepine, diclofenac, fluoxetine, gemfibrozil and naproxen), acting as baseline toxicants on the bioluminescent bacteria *Photobacterium leiognathi*, acted in combination showing additive (according to the CA model) acute and chronic effects. Similarly, these authors also found that an equipotent mixture containing these 5 baseline toxicants plus 5 specifically acting antibiotics (doxycycline, monensin, sulfamethizole, sulfamethoxazol and tetracycline) also acted additively in the tests performed with the same bacteria, thus showing that the baseline toxicants contributed to the

overall toxicity of the mixture of pharmaceuticals. Finally, Ankley et al. (2010) highlighted that understanding of narcosis can be useful as a reference point for identifying specific toxicity. This may also apply to PPCP mixtures. For instance, Escher et al. (2006) evaluated the effects of the three beta-blockers propranolol, atenolol, and metoprolol (all of them previously identified as baseline toxicants in a screening test battery), in the inhibition of the photosynthesis efficiency in green algae *Desmodesmus subspicatus* and found that all of them were 10-fold more toxic than their modeled baseline toxicity. Moreover, these authors also found that the mixture toxicity of the three beta-blockers followed the CA model, indicating that a mutual specific nontarget effect, unrelated to their therapeutic effects, was induced on algae. Therefore, the relevance of narcosis AOP and the understanding of baseline toxicity for PPCP mixture cannot be neglected.

3.5 Number of components in the analyzed PPCP mixtures

From the 194 assessments of the toxicity of PPCP mixtures included in this manuscript, a total of 143 were binary mixtures, corresponding to nearly 74 %. Mixture studies involving ten or more components corresponded to only around 5 % of the data (Figure 3). This implies that most of the current knowledge concerning the effects of PPCP mixtures on non-target organisms originated from mixtures with only two compounds.

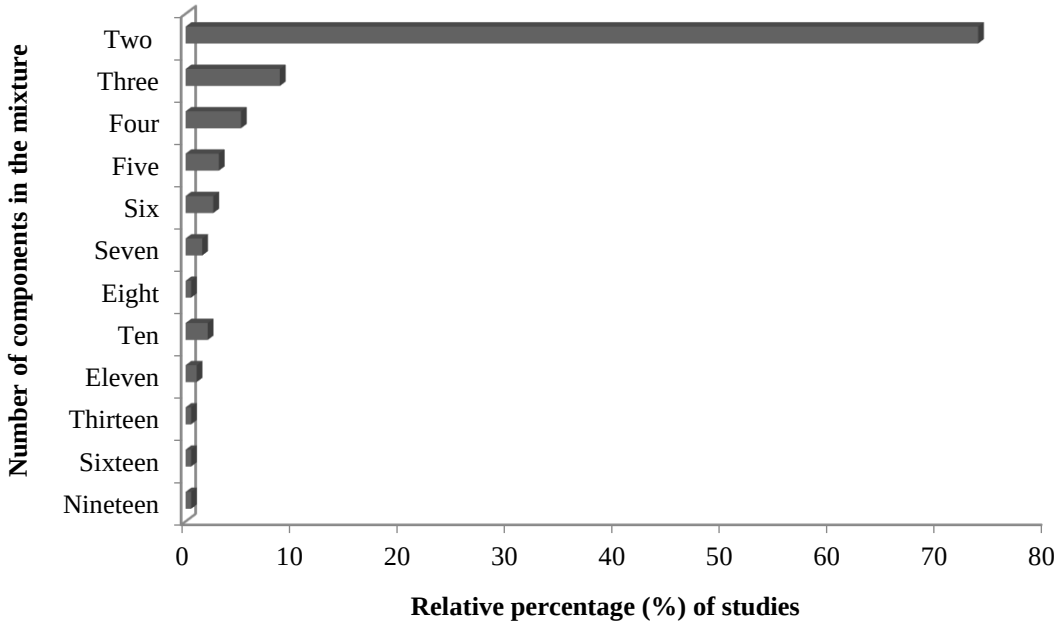

Fig. 3 Number of components in the 194 assessments of the toxicity of pharmaceuticals and personal care products (PPCP) mixtures retrieved from 65 articles published between 2000-2017, expressed in relative percentage

Source: Godoy and Kummrow (2017)

According to Cedergreen et al. (2007), mixture toxicity studies can be divided into two categories: those investigating the effect of multiple components at predefined mixture ratios and those which aims to investigate binary mixtures at various mixture ratios. Binary mixture studies offer the advantage of allowing the elucidation of the effect of one specific chemical on the biological action of another, while little can be concluded from mixtures with several components (Cedergreen et al., 2007). On the other hand, environmental mixtures are often highly complex, composed by tens to almost a hundred compounds (Backhaus, 2014). Therefore, in an environmental context, the potential interactions between the numerous components are more likely to occur (Heys et al., 2016).

Indeed, some ecotoxicological studies on the interaction of pharmaceutical compounds in mixtures have shown an increase of the degree of the departures from additivity with the number of mixture components. For instance, Rodea-Palomares et al. (2010) found that ternary mixtures of pharmaceuticals of the fibrate family, as well as mixtures of fibrates and wastewater (complex mixture) were more synergistic than the corresponding binary mixtures in studies carried out with the cyanobacteria *Anabaena*. Studies such as this one have provided the basis for some reformulations of the "funnel hypothesis", which predicts that the range of deviation from toxic additivity decreases as the number of components in a mixture of narcotic toxicants increases (Warne and Hawker, 1995). For instance, Cedergreen et al. (2012) proposed to shift the focus from number of components to number of possible interactions. These authors postulated that the chance of large joint deviation from the toxic additivity decreases as the number of possible interactions in a mixture increases. Rodea-Palomares et al. (2015) also proposed a revised funnel hypothesis, but unlike the proposal reported by Cedergreen et al. (2012), their premise is not centered on additivity. Instead, they postulated that the interaction of compounds in a multicomponent mixture may not necessarily converge to additivity values, depending on the ratio and the intensity of synergistic or antagonistic component-component interactions present in the mixture. Accordingly, although in the European legislation it is usually assumed that the incidence of departures from additivity is irrelevant for matters of ecological risk assessment, predicting the resulting type of interaction in multicomponent mixtures emerges as a challenge that should be considered in ecotoxicology (Rodea-Palomares et al., 2015). In this context, some approaches that consider binary mixture interactions to predict interactions in multicomponent mixtures, such as the Hazard Index (HI) or the WOE HI modification and the PBPK/PD modeling, emerge as possible approaches aiming to predict interaction-based ecological risk, despite their previously discussed limitations.

3.6 Bioassays commonly used to evaluate the mixture toxicity of PPCPs

Regarding the type of assays usually employed to evaluate PPCP mixture effects, both *in vivo* and *in vitro* assays have been used, with the first one largely predominating (Table A.1 of Supplementary Material). *In vitro* assays can be useful for a first screening of mixture toxicity of PPCP since this type of assay generally can allow the cost-effective testing of a wide range of chemicals and concentrations (Schnell et al., 2009), consequently allowing the assessment of toxic joint effects across a wider range of mixture effect levels and ratios. However, *in vivo* and *in vitro* assays with PPCP mixtures may produce different results. For instance, Canesi et al. (2007) evaluated *in vitro* and *in vivo* immunomodulation of *Mytilus galloprovincialis* hemocytes after exposure to a mixture of estrogenic compounds (including the steroids 17β-estradiol and 17α-ethinylestradiol) and found that the additive effects observed on hemocyte parameters after *in vivo* long-term exposure could not be observed *in vitro* after short-term exposure. This is because *in vitro* assays may not account for metabolism and indirect additional effects occurring at the organism level (Fent et al., 2006b; Canesi et al., 2007). In this sense, also for PPCP mixtures a greater use of alternative tools including the *in vitro* assays should be made (Ankley et al., 2010), although the *in vitro* assays will need to be extensively validated before use for matters of ERA (Boxall et al., 2012).

Regarding the *in vivo* bioassays, the results showed that the acute mixture bioassays largely predominate in relation to the chronic ones (Figure 4a).

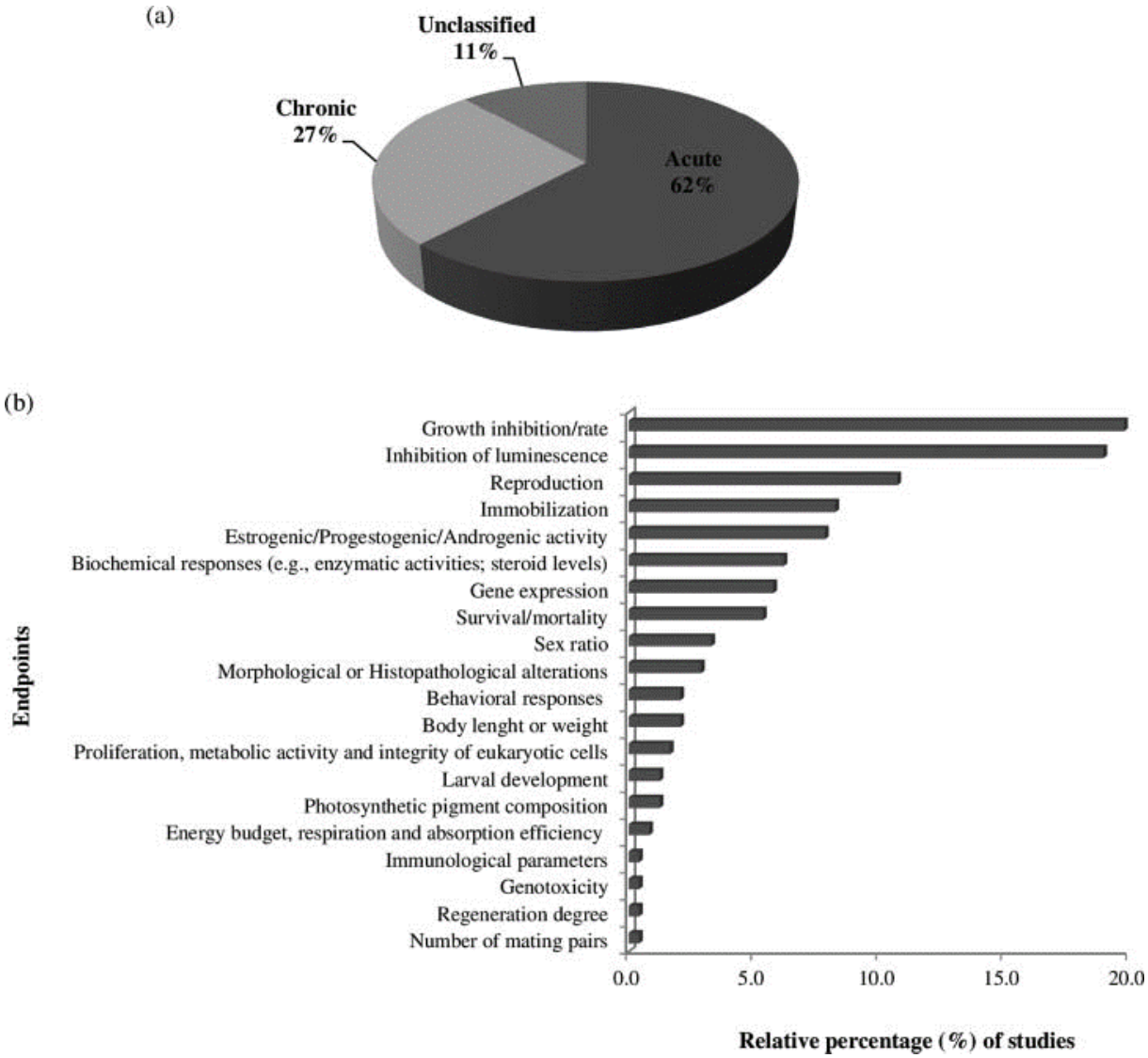

Fig. 4 (a) Percentage of acute x chronic studies obtained from the 194 assessments of the toxicity of pharmaceuticals and personal care products (PPCP) mixtures retrieved from 65 articles published between 2000-2017. Those studies not possible to be classified into these two categories according to international protocols were referred to as unclassified. (b) Principal endpoints used in the retrieved studies, expressed in relative percentage

Source: Godoy and Kummrow (2017)

According to Figure 4, acute ecotoxicological studies with PPCP mixtures represented 62 % of the evaluated *in vivo* bioassays data. Similarly to what occurs with single pharmaceutical ecotoxicological studies, chronic toxicity tests with PPCP mixtures are much less addressed probably due to the higher costs, complexity and time involved in this type of experiment. However, non-target organisms are typically exposed to PPCP mixtures over long periods of time or even during all their life cycle due to the continuous entry of these contaminants into aquatic environments (Dietrich et al., 2010). Moreover, PPCP usually cause sub-lethal effects even at low concentrations, while lethality is not usually evident even at

unrealistically high concentrations (Rodea-Palomares et al., 2016). Therefore, for risk assessment purposes it would be better to perform chronic mixture studies, including those aiming to evaluate multigenerational long-term toxicity, instead of the acute ones. Moreover, differences between acute and chronic mixture toxicity mechanisms can lead to different results. For instance, Zou et al. (2012) showed that the joint effects of sulfonamide antimicrobials and their potentiator trimethoprim on *Photobacterium phosphoreum* varied markedly with changes in exposure time. The authors found that the antagonistic interaction observed in acute mixture toxicity became synergistic in chronic mixture toxicity due to dissimilarities between the mechanisms involved in the two types of test. In this sense, the results obtained from acute mixture toxicity tests, which represent most of the studies reported in the literature, may not necessarily be representative for an environmentally relevant scenario.

The PPCP mixture studies employing endpoints at the individual-level largely predominate compared to the sub-individual level ones (Figure 4 b). Considering data from these different levels of biological organization for matters of ERA has interesting positive, negative and unknown implications. In a critical review recently published, Rohr et al. (2016) stated that biomarkers and other sub-organismal level responses such as mRNA transcripts, proteins and metabolites are important tools to assist in the understanding of potential mechanisms of toxicity or in the knowledge whether exposure to a set of contaminants has occurred. On the other hand, the authors remember that the relevance for ERA of this type of response is still largely uncertain. This is because biomarkers and molecular initiating events might miss important physiological positive and negative feedbacks that often occur and can counter adverse effects at the level of the whole organism. In addition, Rohr et al. (2016) report that although the most common tests at the individual-level can reflect physiological feedbacks within the whole organism, they lack important occurrences at the population or community levels.

Hence, an effective ERA for mixture of PPCP needs to relate the knowledge from biochemical, physiological, and individual-level endpoints to higher levels of biological organization, which are of greater ecological relevance. In addition to the need of more studies employing biomarkers and other sub-organismal responses, more microcosm and mesocosm mixture toxicity studies are also an urgent need for a satisfactory understanding of adverse effects of the joint action of PPCP.

4. CONCLUSION AND FUTURE OUTLOOK

Mixture toxicity is still a very complex challenge to be integrated into regulatory frameworks. It is a consensus that this issue is still incipient, particularly for PPCP. Thus, understanding the mechanisms and interactions involved in the joint action of these compounds of environmental concern is an urgent need.

In this sense, so that further advances can be achieved in this field, future studies need to establish consistent criteria for prioritizing mixture components or samples of environmental concern, planning the most adequate type of test regarding time duration, endpoint and level of biological organization, choosing the optimal experimental design and the adequate tools to predict/assess the data. More specifically, future studies should focus on prioritizing the following aspects:

- To perform further PPCP mixture studies using biological systems and approaches (e.g., GSA-QHTS) that allow studying the sub-lethal region of the concentration-response curves, in order to adequately predict/evaluate the low-dose nonlinear/nonadditive sub-lethal effects of PPCP mixtures of environmental relevance;
- To employ experimental designs (e.g., factorial design strategies) covering possible interactions at various mixture ratios, in order to evaluate complete concentration-response surfaces, i.e., at different effect levels and different mixture ratios;
- To evaluate species and endpoints considering the probable adverse effects induced by PPCP mixtures, based on evolutionary conserved drug targets;
- To focus on developing more qAOPs that can be applied to predict combined effects of PPCP mixtures;
- To apply adequate approaches (e.g., HI and WOE HI) in order to enlarge the knowledge regarding interactions in multicomponent mixtures;
- To focus on chronic PPCP mixture effects rather than the acute ones, especially those aiming to evaluate multigenerational long-term toxicity;
- To further evaluate PPCP mixture adverse effects at the sub-organismal level (e.g., by using biomarkers) as well as at higher levels of biological organization (e.g., micro and mesocosms), in order to link knowledge from several levels of biological organization for an effective ERA for mixture of PPCP.

Moreover, so that trustworthy conclusions can be achieved, it is essential to attempt to use harmonized terminology for describing PPCP mixture toxicity effects, particularly regarding the term synergism. In addition, setting quantitative criteria from which a non-

additive interaction can be considered relevant is an open critical point to be solved, so that an uniform and robust regulation can be achieved for risk assessment of combined effects from PPCP compounds. While, however, this is not satisfactorily achieved, it is still premature to consider possible synergistic/antagonistic interactions at low effect levels negligible, at least for PPCP.

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Supplementary material:

What do we know about the ecotoxicology of pharmaceutical and personal care product mixtures? A critical review

Aline A. Godoy • Fábio Kummrow

Table A.1 - Raw data on the assessments of the toxicity of pharmaceutical and personal care products (PPCP) mixtures retrieved from 65 international articles covering a period from 2000 - 2017 (Definitions for the abbreviations are described in table notes below the table body).

***In vivo* tests**								
Pharmaceutical mixture	**Species**	**Taxonomic group**	**Endpoint/ Duration of the test**	**Type of test**	**Experimental design of the mixture test**	**Model/ software used to assess combination effects**	**Model/ Deviation that better explained the mixture effect**	**Reference**
Cinoxacin+ Enoxacin+ Flumequine+ Lomefloxacin+ Nalidixic acid+ Norfloxacin+ Ofloxacin+ Oxolinic acid+ Pipemidic acid+ Piromidic acid	*Aliivibrio fischeri*	Bacteria	Reduction in luminescence / 24 h	Chronic	Three different mixture fixed ratios (based on the relative EC_{50}, $EC_{01}$and NOEC values of the components)	Regression models (Weibull; Generalized Logit; Box-Cox Weibull). Statistical uncertainty was estimated using the bootstrap approach. The CA and IAmodels were used to predict the toxicity of the mixture	Better predicted by the CA model. The IA model underestimated the toxicity of the mixture	Backhaus et al. (2000)
Gemfibrozil+ Benzafibrate	*A. fischeri*	Bacteria	Reduction in luminescence / 30 min	Acute	A fixed constant ratio (1:1), based on the individual EC_{50} values of each compound, comprising the sum of 0.25, 0.5, 1, 2 and 4 TU of each single pharmaceuticalwere applied in the mixture	CI-isobologram equation	Additive effect was observed at the EC_{10} and EC_{50} levels. Synergism was observed at EC_{90} level regarding the CI model	Rodea-Palomares et al. (2010)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Fenofibric acid+ Benzafibrate	*A. fischeri*	Bacteria	Reduction in luminescence / 30 min	Acute	A fixed constant ratio (1:1), based on the individual EC_{50} values of each compound, comprising the sum of 0.25, 0.5, 1, 2 and 4 TUof each single pharmaceuticalwere applied in the mixture	CI-isobologram equation	Antagonism was observed at the EC_{10}, EC_{50} and EC_{90} levels regarding the CI model	Rodea-Palomares et al. (2010)
Fenofibric acid+ Gemfibrozil	*A. fischeri*	Bacteria	Reduction in luminescence / 30 min	Acute	A fixed constant ratio (1:1), based on the individual EC_{50} values of each compound, comprising the sum of 0.25, 0.5, 1, 2 and 4 TU of each single pharmaceutical were applied in the mixture	CI-isobologram equation	Additive effect was observed at the EC_{10} level. Synergism was observed at the EC_{50} and EC_{90} levels regarding the CI model	Rodea-Palomares et al. (2010)
Fenofibric acid+ Gemfibrozil+ Benzafibrate	*A.fischeri*	Bacteria	Reduction in luminescence / 30 min	Acute	A fixed constant ratio (1:1), based on the individual EC_{50} values of each compound, comprising the sum of 0.25, 0.5, 1, 2 and 4 TU of each single pharmaceutical were applied in the mixture	CI-isobologram equation	Antagonism was observed at the EC_{10} level. Additive effects were observed at the EC_{50} and EC_{90} levels regarding the CI model	Rodea-Palomares et al. (2010)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Triclocarban+ Triclosan+ Methyltriclosan (metabolite)	*A. fischeri*	Bacteria	Reduction in luminescence / 15 min	Acute	The three compounds were mixed in an equitoxic ratio based on the EC_{50} of the individual components	The non-linear Weibull function was fitted to the data to describe the concentration response curves and the observed data were compared to the predicted ones using the CA and the IA equations	Equally well predicted by both the CA and the IA models	Villa et al. (2014)
Chlortetracycline+ Diclofenac	*A. fischeri*	Bacteria	Reduction in luminescence / 15 min	Acute	The two compounds were mixed in an equitoxic ratio based on the EC_{10} of the individual components	The non-linear Weibull function was fitted to the data. The observed data were compared to the predicted ones using the CA and the IA equations. The CI model was used to describe the nature of the possible interactions	Both CA and IA underestimated the mixture effects, especially at lower concentrations. Clear synergistic effects were observed up to the effect level of IC_{50} and a nearly additive effect was observed at higher effect levels	Di Nica et al. (2017)
Acetylsalycilic acid+ Diclofenac	*A. fischeri*	Bacteria	Reduction in luminescence / 15 min	Acute	The two compounds were mixed in an equitoxic ratio based on the EC_{10} of the individual components	The non-linear Weibull function was fitted to the data. The observed data were compared to the predicted ones using the CA and the IA equations. The CI model was used to describe the nature of the possible interactions	The observed effects were equally well predicted by both the CA and the IA models. The mixture effects were fairly additive at all the effect levels considered regarding the CI model	Di Nica et al. (2017)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Diclofenac+ Sulfamethizole	*A. fischeri*	Bacteria	Reduction in luminescence / 15 min	Acute	The two compounds were mixed in an equitoxic ratio based on the EC_{10} of the individual components	The non-linear Weibull function was fitted to the data. The observed data were compared to the predicted ones using the CA and the IA equations. The CI model was used to describe the nature of the possible interactions	The observed effects were equally well predicted by both the CA and the IA models. The mixture effects were fairly additive at all the effect levels considered regarding the CI model	Di Nica et al. (2017)
Chlortetracycline+ Sulfamethizole	*A. fischeri*	Bacteria	Reduction in luminescence / 15 min	Acute	The two compounds were mixed in an equitoxic ratio based on the EC_{10} of the individual components	The non-linear Weibull function was fitted to the data. The observed data were compared to the predicted ones using the CA and the IA equations. The CI model was used to describe the nature of the possible interactions	The observed effects were equally well predicted by both the CA and the IA models. The mixture effects were fairly additive at all the effect levels considered regarding the CI model	Di Nica et al. (2017)
Acetylsalycilic acid+ Chlortetracycline	*A. fischeri*	Bacteria	Reduction in luminescence / 15 min	Acute	The two compounds were mixed in an equitoxic ratio based on the EC_{10} of the individual components	The non-linear Weibull function was fitted to the data. The observed data were compared to the predicted ones using the CA and the IA equations. The CI model was used to describe the nature of the possible interactions	Both the CA and the IA models clearly overestimated the mixture effects at all concentrations tested. Antagonism was observed over the whole range of effect levels regarding the CI model	Di Nica et al. (2017)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Diclofenac+ Amoxicillin	*A. fischeri*	Bacteria	Reduction in luminescence / 15 min	Acute	The two compounds were mixed in an equitoxic ratio based on the EC_{10} of the individual components	The non-linear Weibull function was fitted to the data. The observed data were compared to the predicted ones using the CA and the IA equations. The CI model was used to describe the nature of the possible interactions	Both the CA and the IA models underestimated the mixture effects, especially at lower concentrations. Clear synergistic effects were observed at combinations up to the IC_{50} and a nearly additivity was observed at higher effect levels	Di Nica et al. (2017)
Acetylsalycilic acid+ Sulfamethizole	*A. fischeri*	Bacteria	Reduction in luminescence / 15 min	Acute	The two compounds were mixed in an equitoxic ratio based on the EC_{10} of the individual components	The non-linear Weibull function was fitted to the data. The observed data were compared to the predicted ones using the CA and the IA equations. The CI model was used to describe the nature of the possible interactions	Both the CA and the IA models overestimated the mixture effects, especially at higher concentrations. However, a complete concentration-response curve was not observed for the mixture effects. Synergism was observed at low effect levels (0.1 to 0.3), turning into antagonism within a narrow range of concentrations regarding the CI model	Di Nica et al. (2017)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Sulfamethizole+ Amoxicillin	*A. fischeri*	Bacteria	Reduction in luminescence / 15 min	Acute	The two compounds were mixed in an equitoxic ratio based on the EC_{10} of the individual components	The non-linear Weibull function was fitted to the data. The observed data were compared to the predicted ones using the CA and the IA equations. The CI model was used to describe the nature of the possible interactions	Both the CA and the IA models underestimated the mixture effects at lower tested concentrations. Synergism was observed at low effect levels (0.1 to 0.3), turning into antagonism within a narrow range of concentrations regarding the CI model	Di Nica et al. (2017)
Chlortetracycline+ Amoxicillin	*A. fischeri*	Bacteria	Reduction in luminescence / 15 min	Acute	The two compounds were mixed in an equitoxic ratio based on the EC_{10} of the individual components	The non-linear Weibull function was fitted to the data. The observed data were compared to the predicted ones using the CA and the IA equations. The CI model was used to describe the nature of the possible interactions	Both the CA and the IA models underestimated the mixture effects at lower tested concentrations. Synergism was observed at low effect levels (0.1 to 0.3), turning into antagonism within a narrow range of concentrations regarding the CI model	Di Nica et al. (2017)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Acetylsalycilic acid+ Amoxicillin	*A. fischeri*	Bacteria	Reduction in luminescence / 15 min	Acute	The two compounds were mixed in an equitoxic ratio based on the EC_{10} of the individual components	The non-linear Weibull function was fitted to the data. The observed data were compared to the predicted ones using the CA and the IA equations. The CI model was used to describe the nature of the possible interactions	Both the CA and the IA models overestimated the mixture effects, especially at higher concentrations, but a complete concentration-response curve was not observed for the mixture effects. Synergism was observed at low effect levels (0.1 to 0.3), turning into antagonism within a narrow range of concentrations regarding the CI model	Di Nica et al. (2017)
Amoxicillin+ Acetylsalycilic acid+ Chlortetracycline+ Diclofenac+ Sulfamethizole	*A. fischeri*	Bacteria	Reduction in luminescence / 15 min	Acute	The mixture was prepared at a ratio corresponding to the individual predicted no-effect concentration (PNEC)	The non-linear Weibull function was fitted to the data. The observed data were compared to the predicted ones using the CA and the IA equations. The CI model was used to describe the nature of the possible interactions	Both the CA and the IA model predicted equally well the mixture effects. No strong synergism or antagonism was observed regarding the CI model.	Di Nica et al. (2017)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Erythromycin+ Levofloxacin	*Anabaena* sp. PCC 7120 strain CPB4337	Bacteria	Inhibition of luminescence / 72 h	Acute	A fixed constant ratio (1:1) was used based on the individual EC_{50} values of each component	CI-isobologram equations and the CA and IA models	The three methods tested, i.e., CA, IA and CI predicted very similarly the mixture toxicity. Antagonism was observed at very low to low effect levels and a near additivity was observed at effect levels above 0.4	González-Pleiter et al. (2013)
Erythromycin+ Norfloxacin	*Anabaena* sp. PCC 7120 strain CPB4337	Bacteria	Inhibition of luminescence / 72 h	Acute	A fixed constant ratio (1:1) was used based on the individual EC_{50}values of each component	CI-isobologram equations, and CA and IA models	The three methods tested, i.e., CA, IA and CI predicted very similarly the mixture toxicity. A near additivity was observed in the whole range of the effect levels	González-Pleiter et al. (2013)
Erythromycin+ Tetracyclin	*Anabaena* sp. PCC 7120 strain CPB4337	Bacteria	Inhibition of luminescence / 72 h	Acute	A fixed constant ratio (1:1) was used based on the individual EC_{50} values of each component	CI-isobologram equations and CA and IA models	Both the CA and the IA models underestimated the mixture toxicity at effect levels < 0.5 and overestimated it at effect levels > 0.6. A strong synergism was observed at effect levels < 0.6, which turned into antagonism at higher effect levels according to CI	González-Pleiter et al. (2013)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Levofloxacin+ Norfloxacin	*Anabaena* sp. PCC 7120 strain CPB4337	Bacteria	Inhibition of luminescence / 72 h	Acute	A fixed constant ratio (1:1) was used based on the individual EC_{50} values of each component	CI-isobologram equations and CA and IA models	The three methods tested, i.e., CI, CA and IA underestimated the actual synergism of the mixture, except at very low effect levels, where it approached the additivity	González-Pleiter et al. (2013)
Levofloxacin+ Tetracyclin	*Anabaena* sp. PCC 7120 strain CPB4337	Bacteria	Inhibition of luminescence / 72 h	Acute	A fixed constant ratio (1:1) was used based on the individual EC_{50} values of each component	CI-isobologram equations and CA and IA models	Both CA and IAmodels underestimated the mixture toxicity. A strong synergism was observed in the whole range of effect levels, according to the CI model	González-Pleiter et al. (2013)
Norfloxacin+ Tetracyclin	*Anabaena* sp. PCC 7120 strain CPB4337	Bacteria	Inhibition of luminescence / 72 h	Acute	A fixed constant ratio (1:1) was used based on the individual EC_{50}values of each component	CI-isobologram equations and the CA and IA models	The three models tested, i.e., CI, CA and IA underestimated the mixture toxicity. A synergism was observed, based on the CI calculation	González-Pleiter et al. (2013)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Amoxicillin+ Erythromycin	*Anabaena* sp. PCC 7120 strain CPB4337	Bacteria	Inhibition of luminescence / 72 h	Acute	A fixed constant ratio (1:1) was used based on the individual EC_{50} values of each component	CI-isobologram equations and CA and IA models	A strong antagonism was observed at effect levels < 0.6, which turned into a slight synergism at higher effect levels, according to the CI model. All the three models tested, i.e., CI, CA and IA predicted very similar values for the mixture toxicity.	González-Pleiter et al. (2013)
Amoxicillin+ Norfloxacin	*Anabaena* sp. PCC 7120 strain CPB4337	Bacteria	Inhibition of luminescence / 72 h	Acute	A fixed constant ratio (1:1) was used based on the individual EC_{50} values of each component	CI-isobologram equations, and CA and IA models	A strong antagonism was observed at effect levels < 0.6, which turned into a slight synergism at higher effect levels, according to the CI model	González-Pleiter et al. (2013)
Amoxicillin+ Levofloxacin	*Anabaena* sp. PCC 7120 strain CPB4337	Bacteria	Inhibition of luminescence / 72 h	Acute	A fixed constant ratio (1:1) was used based on the individual EC_{50} values of each component	CI-isobologram equations and CA and IA models	A strong antagonism was observed at low effect levels < 0.2, which turned into a slight synergism at higher effect levels, according to the CI model. Both CA and IA models underestimated the mixture toxicity at all the effect levels tested	González-Pleiter et al. (2013)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Amoxicillin+ Tetracyclin	*Anabaena* sp. PCC 7120 strain CPB4337	Bacteria	Inhibition of luminescence / 72 h	Acute	A fixed constant ratio (1:1) was used based on the individual EC_{50} values of each component	CI-isobologram equations and CA and IA models	A strong synergism was observed in the whole range of effect levels, according to the CI model. The mixture toxicity was better predicted by the CI model, while both the CA and IA underestimated the mixture toxicity at all the effect levels tested	González-Pleiter et al. (2013)
Erythromycin+ Levofloxacin+ Norfloxacin+ Tetracyclin+ Amoxicillin	*Anabaena* sp. PCC 7120 strain CPB4337	Bacteria	Inhibition of luminescence / 72 h	Acute	A fixed constant ratio (1:1) was used based on the individual EC_{50} values of each component	CI-isobologram equations and CA and IA models	Antagonism was observed at low effect levels which turned into synergism at effect levels values above 0.25, according to the CI model. The mixture toxicity was accurately predicted by CI, while both CA and IA underestimated it	González-Pleiter et al. (2013)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Tylosin+ Lincomycin+ Trimethoprim	*Anabaena flos-aquae*	Bacteria	Growth inhibition/ 96 h	Acute/ Chronic*	The molar ratio applied in mixture was tylosin: trimethoprim: lincomycin = 1:4.31:6.65, chosen based on exposure models	Concentration-response curves were generated by a sigmoidal regression (Three-parameter Hill). The observed concentration-response curves were compared to the ones based on predictions by the CA and the IA models, by means of comparing the slope (EC_{50}/EC_{05} ratios) of the predicted and experimental data	Better predicted by the CA model	Guo et al. (2016)
Gemfibrozil+ Benzafibrate	*Anabaena* strain CPB4337	Bacteria	Inhibition of luminescence/ 1 h	Acute	A fixed constant ratio (1:1), based on the individual EC_{50} values of each compound, comprising the sum of 0.25, 0.5, 1, 2 and 4 TU of each single pharmaceutical were applied in the mixture	CI-isobologram equation	Additive effects were observed at the EC_{10} level. Antagonism was observed at the EC_{50} and EC_{90} levels.	Rodea-Palomares et al. (2010)
Fenofibric acid+ Benzafibrate	*Anabaena* strain CPB4337	Bacteria	Inhibition of luminescence/ 1 h	Acute	Fixed constant ratio (1:1), based on the individual EC_{50} values of each compound, summing 0.25, 0.5, 1, 2 and 4 TU of each single pharmaceutical	CI-isobologram equation	Synergism was observed at the EC_{10} level. Antagonistic effects were observed at the EC_{50} and EC_{90}levels.	Rodea-Palomares et al. (2010)

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Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Fenofibric acid+ Gemfibrozil	*Anabaena* strain CPB4337	Bacteria	Inhibition of luminescence/ 1 h	Acute	A fixed constant ratio (1:1), based on the individual EC_{50} values of each compound, comprising the sum of 0.25, 0.5, 1, 2 and 4 TU of each single pharmaceutical were applied in the mixture	CI-isobologram equation	Synergistic effects were observed at the EC_{10}level. Antagonism was observed at the EC_{50} and EC_{90} levels.	Rodea-Palomares et al. (2010)
Fenofibric acid+ Gemfibrozil+ Benzafibrate	*Anabaena* strain CPB4337	Bacteria	Inhibition of luminescence/ 1 h	Acute	A fixed constant ratio (1:1), based on the individual EC_{50} values of each compound, comprising the sum of 0.25, 0.5, 1, 2 and 4 TU of each single pharmaceutical were applied in the mixture	CI-isobologram equation	Synergistic effects were observed at the EC_{10} and EC_{50} levels. Antagonistic effects were observed at the EC_{90} level.	Rodea-Palomares et al. (2010)
Atenolol+ Lincomycin	*Escherichia coli*	Bacteria	Proliferation (reproduction) / 3 h	Unclassified	A two-factor fractional-factorial design was used to detect interactive effects. Experimental blocks were used to allow for all sources of variance.	Polynomial function of the general linear model was used. Data were considered statistically significant with a confidence limit of $p \leq 0.1$. A component interaction analysis was performed to identify the components able to interact in mixture	N/A. An antagonistic interaction was reported considering the experimental design and significance level used.	Pomati et al. (2008)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Cyclophosphamide+ Salbutamol	*E. coli*	Bacteria	Proliferation (reproduction) / 3 h	Acute	A two-factor fractional-factorial design was used to detect interactive effects. Experimental blocks were used to allow for all sources of variance.	Polynomial function of the general linear model was used. Data were considered statistically significant with a confidence limit of $p \leq 0.1$. A component interaction analysis was performed to identify the components able to interact in mixture	N/A. An antagonistic interaction was reported considering the experimental design and significance level used.	Pomati et al. (2008)
Trimethoprim+ Sulfamethazine	*Photobacterium phosphoreum*	Bacteria	Inhibition of the bioluminescence / 15 min	Acute	An equitoxic ratio was used (1:1) based on the EC_{50} results of the single experiments	The mixture toxicity was described using toxicity units and media effective concentrations of each compound, according to specific equations. Simple addition was considered when toxicity unit was between 0.8 and 1.2; toxicity unit > 1.2 was considered antagonism and <0.8 was described as synergism	N/A. Antagonism was observed according to the toxicity units approach adopted by the authors	Zou et al. (2012)
Trimethoprim+ Sulfamethazine	*P. phosphoreum*	Bacteria	Inhibition of the bioluminescence / 24 h	Chronic	An equitoxic ratio was used (1:1) based on the EC_{50} results of the single experiments	The mixture toxicity was described using toxicity units and media effective concentrations of each compound, according to specific equations.	N/A. Synergism was observed according to the toxicity units' approach adopted by the authors	Zou et al. (2012)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Trimethoprim+ Sulfapyridine	*P. phosphoreum*	Bacteria	Inhibition of the bioluminescence/ 15 min	Acute	An equitoxic ratio was used (1:1) based on the EC_{50} results of the single experiments	The mixture toxicity was described using toxicity units and media effective concentrations of each compound, according to specific equations. Simple addition was considered when toxicity unit was between 0.8 and 1.2; toxicity unit > 1.2 was considered antagonism and <0.8 was described as synergism	N/A. Antagonism was observed according to the toxicity units' approach adopted by the authors	Zou et al. (2012)
Trimethoprim+ Sulfapyridine	*P. phosphoreum*	Bacteria	Inhibition of the bioluminescence / 24 h	Chronic	An equitoxic ratio was used (1:1) based on the EC_{50} results of the single experiments	The mixture toxicity was described using toxicity units and media effective concentrations of each compound, according to specific equations.	N/A. Synergism was observed according to the toxicity units' approach adopted by the authors	Zou et al. (2012)
Trimethoprim+ Sulfamethoxazole	*P. phosphoreum*	Bacteria	Inhibition of the bioluminescence / 15 min	Acute	An equitoxic ratio was used (1:1) based on the EC_{50}results of the single experiments	The mixture toxicity was described using toxicity units and media effective concentrations of each compound, according to specific equations. Simple addition was considered when toxicity unit was between 0.8 and 1.2; toxicity unit > 1.2 was considered antagonism and <0.8 was described as synergism	N/A. Antagonism was observed according to the toxicity units' approach adopted by the authors	Zou et al. (2012)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Trimethoprim+ Sulfamethoxazole	*P. phosphoreum*	Bacteria	Inhibition of the bioluminescence / 24 h	Chronic	An equitoxic ratio was used (1:1) based on the EC_{50}results of the single experiments	The mixture toxicity was described using toxicity units and media effective concentrations of each compound, according to specific equations.	N/A. Synergism was observed according to the toxicity units' approach adopted by the authors	Zou et al. (2012)
Trimethoprim+ Sulfadiazine	*P. phosphoreum*	Bacteria	Inhibition of the bioluminescence / 15 min	Acute	An equitoxic ratio was used (1:1) based on the EC_{50} results of the single experiments	The mixture toxicity was described using toxicity units and media effective concentrations of each compound, according to specific equations. Simple addition was considered when toxicity unit was between 0.8 and 1.2; toxicity unit > 1.2 was considered antagonism and <0.8 was described as synergism	N/A. Antagonism was observed according to the toxicity units' approach adopted by the authors	Zou et al. (2012)
Trimethoprim+ Sulfadiazine	*P. phosphoreum*	Bacteria	Inhibition of the bioluminescence / 24 h	Chronic	An equitoxic ratio was used (1:1) based on the EC_{50} results of the single experiments	The mixture toxicity was described using toxicity units and media effective concentrations of each compound, according to specific equations.	N/A. Synergism was observed according to the toxicity units' approach adopted by the authors	Zou et al. (2012)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Trimethoprim+ Sulfisoxazole	*P. phosphoreum*	Bacteria	Inhibition of the bioluminescence / 15 min	Acute	An equitoxic ratio was used (1:1) based on the EC_{50} results of the single experiments	The mixture toxicity was described using toxicity units and media effective concentrations of each compound, according to specific equations. Simple addition was considered when toxicity unit was between 0.8 and 1.2; toxicity unit > 1.2 was considered antagonism and <0.8 was described as synergism	N/A. Antagonism was observed according to the toxicity units' approach adopted by the authors	Zou et al. (2012)
Trimethoprim+ Sulfisoxazole	*P. phosphoreum*	Bacteria	Inhibition of the bioluminescence / 24 h	Chronic	An equitoxic ratio was used (1:1) based on the EC_{50} results of the single experiments	The mixture toxicity was described using toxicity units and media effective concentrations of each compound, according to specific equations.	N/A. Synergism was observed according to the toxicity units' approach adopted by the authors	Zou et al. (2012)
Trimethoprim+ Sulfamonomethoxine	*P. phosphoreum*	Bacteria	Inhibition of the bioluminescence / 15 min	Acute	An equitoxic ratio was used (1:1) based on the EC_{50} results of the single experiments	The mixture toxicity was described using toxicity units and media effective concentrations of each compound, according to specific equations. Simple addition was considered when toxicity unit was between 0.8 and 1.2; toxicity unit > 1.2 was considered antagonism and <0.8 was described as synergism	N/A. Antagonism was observed according to the toxicity units' approach adopted by the authors	Zou et al. (2012)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Trimethoprim+ Sulfamonomethoxine	*P. phosphoreum*	Bacteria	Inhibition of the bioluminescence / 24 h	Chronic	An equitoxic ratio was used (1:1) based on the EC_{50} results of the single experiments	The mixture toxicity was described using toxicity units and media effective concentrations of each compound, according to specific equations.	N/A. Synergism was observed according to the toxicity units' approach adopted by the authors	Zou et al. (2012)
Trimethoprim+ Sulfachloropyridazine	*P. phosphoreum*	Bacteria	Inhibition of the bioluminescence / 15 min	Acute	An equitoxic ratio was used (1:1) based on the EC_{50} results of the single experiments	The mixture toxicity was described using toxicity units and media effective concentrations of each compound, according to specific equations. Simple addition was considered when toxicity unit was between 0.8 and 1.2; toxicity unit > 1.2 was considered antagonism and <0.8 was described as synergism	N/A. Antagonism was observed according to the toxicity units' approach adopted by the authors	Zou et al. (2012)
Trimethoprim+ Sulfachloropyridazine	*P. phosphoreum*	Bacteria	Inhibition of the bioluminescence / 24 h	Chronic	An equitoxic ratio was used (1:1) based on the EC_{50} results of the single experiments	The mixture toxicity was described using toxicity units and media effective concentrations of each compound, according to specific equations.	N/A. Synergism was observed according to the toxicity units' approach adopted by the authors	Zou et al. (2012)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
5-Fluorouracil+ Cisplatin	*Synechococcus leopoliensis*	Bacteria	Growth rate inhibition / 72 h	Acute/ Chronic*	Half of the effective concentrations of each component ($EC_5/2$; $EC_{10}/2$; $EC_{20}/2$; $EC_{50}/2$; $EC_{90}/2$) calculated from the dose response curves of the single compounds	Nonlinear regression model was fitted to the data and compared to the CA and IA models	Better predicted by the IA model at the lower effective concentrations (EC_5 - EC_{20}). At effective concentrations ≥ EC_{50}both CA and IA underestimated the mixture toxicity. Synergistic interaction was suggested at these levels regarding these two models	Brezovšec et al. (2014)
5-Fluorouracil+ Imatinib	*S. leopoliensis*	Bacteria	Growth rate inhibition / 72 h	Acute/ Chronic*	Half of the effective concentrations of each component ($EC_5/2$; $EC_{10}/2$; $EC_{20}/2$; $EC_{50}/2$; $EC_{90}/2$) calculated from the dose response curves of the single compounds	Nonlinear regression model was fitted to the data and compared to the CA and IA models	Both the CA and the IA overestimated the mixture toxicity. Antagonistic interaction was suggested regarding these two models	Brezovšec et al. (2014)
Ibuprofen+ Ciprofloxacin	*Chlorella vulgaris*	Algae	Growth rate inhibition / 96 h	Acute*	Equal proportions of the respective IC_{50} (inhibitory concentration at 50%) of each component were used, comprising the sum of 0.25, 0.5, 1.0, 2.0 and 4.0 TU[f]	CI-isobologram equation. The CA and IA equations were also used to predict the mixture toxicity	Better predicted by CI. Strong synergism observed for inhibitory concentrations from 5 to 75%, according to CI. Both CA and IA failed to predict the synergism, especially at low effect levels (<10 % inhibition)	Geiger et al. (2016)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Clofibrinic acid+ Carbamazepine	*Desmodesmus subspicatus*	Algae	Growth rate inhibition / 72 h	Acute/ Chronic*	Half of the calculated effect concentrations from the single components (EC_5, EC_{10}, EC_{20}, EC_{50}, EC_{80}) were used in the mixture test	Nonlinear curve fitting (four-parameter logistic function) was fitted to the data and compared to the CA and IA models	Better predicted by the IA model	Cleuvers (2003)
Ibuprofen+ Diclofenac	*D. subspicatus*	Algae	Growth rate inhibition/ 72 h	Acute/ Chronic*	Half of the calculated effect concentrations from the single components (EC_5, EC_{10}, EC_{20}, EC_{50}, EC_{80}) were used in the mixture test	Nonlinear curve fitting (four-parameter logistic function) was fitted to the data and compared to the CA and IA models	Better predicted by the CA model	Cleuvers (2003)
Diclofenac+ Ibuprofen+ Naproxen+ Acetylsalicylicacid	*D. subspicatus*	Algae	Growth rate inhibition/ 72 h	Acute/ Chronic*	A quarter of the calculated effect concentrations ($EC_5/4$, $EC_{10}/4$, $EC_{20}/4$, $EC_{50}/4$, $EC_{80}/4$) of each component was used in the mixture tests.	Probit analysis (normal sigmoid, maximum-likelihood regression) was used to describe the concentration-response curves. Data were compared to the CA and IA models	Well predicted by the CA model	Cleuvers (2004)
Propranolol+ Metoprolol+ Atenolol	*D. subspicatus*	Algae	Growth rate inhibition/ 72 h	Acute/ Chronic*	A third of the calculated effect concentrations from the single components ($EC_5/3$, $EC_{10}/3$, $EC_{20}/3$, $EC_{50}/3$, $EC_{80}/3$) was used in the mixture test	Nonlinear curve fitting (four-parameter logistic function) was used to describe the concentration-response curves. Data were compared to the CA and IA models	Better predicted by the CA at the EC_{50} and EC_{80} levels, but at lower levels both CA and IA underestimated the mixture toxicity	Cleuvers (2005)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Triclosan+ Fluoxetin	*Dunaliella tertiolecta*	Algae	Cell density reduction/ 96 h	Acute*	Proportions of the respective EC_{50}, summing up the TU (0.25 TU, 0.5 TU, 1.0 TU, 2.0 TU and 4.0 TU) of the single components	Additive Index and the Modified toxic unit approach	N/A. An additive effect was observed	DeLorenzo and Fleming (2008)
Simvastatin+ Clofibrinic acid	*D. tertiolecta*	Algae	Cell density reduction / 96 h	Acute*	Proportions of the respective EC_{50}, summing up the TU (0.25 TU, 0.5 TU, 1.0 TU, 2.0 TU and 4.0 TU) of the single components	Additive Index and the Modified toxic unit approach	N/A. An additive effect was observed	DeLorenzo and Fleming (2008)
5-Fluorouracil+ Cisplatin	*Raphidocelis subcapitata*	Algae	Growth rate inhibition / 72 h	Acute/ Chronic*	Half of the effective concentrations of each component ($EC_5/2$; $EC_{10}/2$; $EC_{20}/2$; $EC_{50}/2$; $EC_{90}/2$) calculated from the dose response curves of the single compounds	Nonlinear regression model was fitted to the data and compared to the CA and IA models	Both the CA and IA underestimated the observed mixture toxicity. A synergistic interaction was suggested	Brezovšec et al. 2014
5-Fluorouracil+ Imatinib	*R. subcapitata*	Algae	Growth rate inhibition / 72 h	Acute/ Chronic*	Half of the effective concentrations of each component ($EC_5/2$; $EC_{10}/2$; $EC_{20}/2$; $EC_{50}/2$; $EC_{90}/2$) calculated from the dose response curves of the single compounds	Nonlinear regression model was fitted to the data and compared to the CA and IA models	Both the CA and IA underestimated the observed mixture toxicity. A synergistic effect was suggested	Brezovšec et al. 2014

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Cisplatin+ Etoposide	*R. subcapitata*	Algae	Growth rate inhibition / 72 h	Acute/ Chronic*	Half of the effective concentrations of each component ($EC_5/2$; $EC_{10}/2$; $EC_{20}/2$; $EC_{50}/2$; $EC_{90}/2$) calculated from the dose response curves of the single compounds	Nonlinear regression model was fitted to the data and compared to the CA and IA models	Both the CA and IA models overestimated the observed toxicity. An antagonistic interaction was suggested.	Brezovšec et al. 2014
Florfenicol+ Oxytetracycline	*R. subcapitata*	Algae	Growth rate inhibition/ 48 h	Acute*	Ray design consisting of seven different mixture ratios based on the effect concentrations of the two single components (100:0; 83:17; 67:33; 50:50; 33:67; 17:83; 0:100)	Isobologram built from calculated $EC_{50}$from concentration-response curves. The results were compared to the CA and IA predictions	Better predicted by the CA model. An additive effect was observed	Christensen et al. (2006)
Oxytetracycline+ Flumequine	*R. subcapitata*	Algae	Growth rate inhibition/ 48 h	Acute*	Ray design consisting of seven different mixture ratios based on effect concentrations of the two single components (100:0; 83:17; 67:33; 50:50; 33:67; 17:83; 0:100)	Isobologram built from calculated EC_{50} from concentration-response curves. The results were compared to the CA and IA predictions	Both the CA and IA overestimated the mixture effect. An antagonistic effect was observed regarding both models.	Christensen et al. (2006)
Erythromycin+ Florfenicol	*R. subcapitata*	Algae	Growth rate inhibition/ 48 h	Acute*	Ray design consisting of seven different mixture ratios based on effect concentrations of the two single components	Isobologram built from calculated $EC_{50}$from concentration-response curves. The results were compared to the CA and IA predictions	Both the IA and CA predicted well the mixture toxicity. An additive effect was observed	Christensen et al. (2006)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Erythromycin+ Oxolinic acid	*R. subcapitata*	Algae	Growth rate inhibition/ 48 h	Acute*	Ray design consisting of seven different mixture ratios based on effect concentrations of the two single components (100:0; 83:17; 67:33; 50:50; 33:67; 17:83; 0:100)	Isobologram built from calculated EC_{50} from concentration-response curves. The results were compared to the CA and IA predictions	Both the IA and CA predicted well the mixture toxicity. An additive effect was observed	Christensen et al. (2006)
Erythromycin+ Flumequine	*R. subcapitata*	Algae	Growth rate inhibition/ 48 h	Acute*	Ray design consisting of seven different mixture ratios based on effect concentrations of the two single components (100:0; 83:17; 67:33; 50:50; 33:67; 17:83; 0:100)	Isobologram built from calculated EC_{50} from concentration-response curves. The results were compared to the CA and IA predictions	Both the CA and IA overestimated the mixture toxicity. An antagonistic effect was observed	Christensen et al. (2006)
Erythromycin+ Oxytetracycline	*R. subcapitata*	Algae	Growth rate inhibition/ 48h	Acute*	Ray design consisting of seven different mixture ratios based on effect concentrations of the two single components (100:0; 83:17; 67:33; 50:50; 33:67; 17:83; 0:100)	Isobologram built from calculated EC_{50} from concentration-response curves. The results were compared to the CA and IA predictions	Both the CA and IA underestimated the mixture toxicity. A synergistic effect was observed	Christensen et al. (2006)
Sertraline+ Citalopram	*R. subcapitata*	Algae	Growth rate inhibition/ 48h	Acute*	Ray design consisting of five different mixture ratios based on the single-compound effect concentration	Isobologram built from calculated EC_{50} from concentration-response curves. The results were compared to the CA and IA predictions	Better predicted by the CA model. An additive effect was observed.	Christensen et al. (2007)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Sertraline+ Fluoxetine	*R. subcapitata*	Algae	Growth rate inhibition/ 48h	Acute*	Ray design consisting of five different mixture ratios based on the single-compound effect concentration (100:0; 67:33; 50:50; 33:67; 0:100)	Isobologram built from calculated EC_{50} from concentration-response curves. The results were compared to the CA and IA predictions	Better predicted by the CA model. An additive effect was observed.	Christensen et al. (2007)
Fluoxetine+ Citalopram	*R. subcapitata*	Algae	Growth rate inhibition/ 48h	Acute*	Ray design consisting of five different mixture based on the single-compound effect concentration (100:0; 67:33; 50:50; 33:67; 0:100)	Isobologram built from calculated EC_{50} from concentration-response curves. The results were compared to the CA and IA predictions	Better predicted by the CA model. An additive effect was observed	Christensen et al. (2007)
Sulfamethoxazole+ Trimethoprim	*R. subcapitata*	Algae	Growth inhibition / 72 h	Chronic*	The concentration of trimethoprim was fixed at their NOEC (25.5 mg L^{-1}) calculated in a test of single effect and the concentration of the sulfa was altered (not well specified)	The logit method was used to calculate de growth inhibition and the significant differences between groups were determined using the Student´s T- test. Individual effects were statistically compared to the mixture ones	N/A. The inhibitory activity caused by the mixture was significantly increased in comparison with the individual activities caused by each component. The authors considered that there was a synergistic effect	Eguchi et al. (2004)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Sulfadiazine+ Trimethoprim	*R. subcapitata*	Algae	Growth inhibition / 72 h	Chronic*	The concentration of trimethoprim was fixed at their NOEC (25.5 mg L^{-1}) calculated in a test of single effect and the concentration of the sulfa was altered (not well specified)	The logit method was used to calculate de growth inhibition and the significant differences between groups were determined using the Student´s T- test. Individual effects were statistically compared to the mixture ones	N/A. The inhibitory activity caused by the mixture was significantly increased in comparison with the individual activities caused by each component. The authors considered that there was a synergistic effect	Eguchi et al. (2004)
Sulfadimethoxine+ Pyrimethamine	*R. subcapitata*	Algae	Growth inhibition / 72 h	Chronic*	The concentration of pyrimethamine was fixed at their NOEC (0.752 mg L^{-1}) calculated in a test of single effect and the concentration of the sulfa was altered (not well specified)	The logit method was used to calculate de growth inhibition and the significant differences between groups were determined using the Student´s T- test. Individual effects were statistically compared to the mixture ones	N/A. It was not observed a statistically significant difference between individual and mixture effects posed by the pharmaceuticals	Eguchi et al. (2004)
Erythromycin+ Levofloxacin	*R. subcapitata*	Algae	Growth rate inhibition / 72 h	Acute*	A fixed constant ratio (1:1) was used based on the individual EC_{50} values of the components	CI-isobologram equations and the CA and IA models	A clear antagonism was observed at low effect levels, becoming synergistic at effect level values above 0.3 (regarding CI). CI model better predicted the mixture toxicity at effect levels < 0.2 and IA better predicted at effect levels > 0.2	González-Pleiter et al. (2013)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Erythromycin+ Norfloxacin	*R. subcapitata*	Algae	Growth rate inhibition / 72 h	Acute*	A fixed constant ratio (1:1) was used based on the individual EC_{50} values of the components	CI-isobologram equations and the CA and IA models	The mixture was nearly additive in the whole range of effect levels, according to the CI model. Both CA and IA underestimated the mixture effects, except at the lowest effect level	González-Pleiter et al. (2013)
Erythromycin+ Tetracycline	*R. subcapitata*	Algae	Growth rate inhibition / 72 h	Acute*	A fixed constant ratio (1:1) was used based on the individual EC_{50} values of the components	CI-isobologram equations and the CA and IA models	The mixture showed a clearly synergistic interaction, especially at very low effect levels, according to the CI model. Both the CA and IA underestimated the mixture toxicity	González-Pleiter et al. (2013)
Levofloxacin+ Norfloxacin	*R. subcapitata*	Algae	Growth rate inhibition / 72 h	Acute*	A fixed constant ratio (1:1) was used based on the individual EC_{50} values of the components	CI-isobologram equations and the CA and IA models	The mixture was clearly synergistic in the whole range of effect levels, according to the CI model. Both CA and IA underestimated the mixture toxicity	González-Pleiter et al. (2013)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Levofloxacin+ Tetracyclin	*R. subcapitata*	Algae	Growth rate inhibition / 72 h	Acute*	A fixed constant ratio (1:1) was used based on the individual EC_{50} values of the components	CI-isobologram equations and the CA and IA models	The mixture was nearly additive at very low effect levels, becoming clearly synergistic at effect levels above 0.1, according to the CI model. Both CA and IA underestimated the mixture toxicity, except at the lowest effect level	González-Pleiter et al. (2013)
Norfloxacin+ Tetracyclin	*R. subcapitata*	Algae	Growth rate inhibition / 72 h	Acute*	A fixed constant ratio (1:1) was used based on the individual EC_{50} values of the components	CI-isobologram equations and the CA and IA models	The mixture showed antagonism effects at very low to low effect levels, which changed into synergism at higher effect levels, according to the CI model. Both CA and IA overestimated the mixture toxicity at the lowest and intermediate effect levels and underestimated it at the higher effect level	González-Pleiter et al. (2013)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Erythromycin+ Levofloxacin+ Norfloxacin+ Tetracycline	*R. subcapitata*	Algae	Growth rate inhibition / 72 h	Acute*	A fixed constant ratio (1:1) was used based on the individual EC_{50} values of the components	CI-isobologram equations and the CA and IA models	The mixture was nearly additive at very low effect levels and clearly synergistic at effect levels above 0.1, according to the CI model. The IA model predicted better than CA the mixture toxicity at intermediate and higher effect levels. At the lowest effect level, the three methods CA, IA and CI offered very similar predictions.	González-Pleiter et al. (2013)
Sulfaquinoxaline+ Sulfaguanidine	*R. subcapitata*	Algae	Growth rate inhibition / 96 h	Acute*	Concentrations used in the mixture test were based on the individual EC_{50} values, comprising 5 exposition levels and different combination ratios. Three selected combination ratios equidistantly distributed on the additivity line were analyzed	The isobologram method was used to evaluate the mixture effects. Deviation from additivity was considered if the confidence intervals for the effect concentrations of the combined pharmaceuticals did not overlap the confidence belt of the additivity line	N/A. The interaction showed to be ratio-dependent, but a less than additive interaction was predominant	De Liguoro et al. (2010)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Sulpiride+ Clarithromycin+ Diphenhydramine+ Benzafibrate+ Acetaminophen+ Ketoprofen+ Phenytoin+ Etodolac+ Crotamiton+ Epinastine	*R. subcapitata*	Algae	Growth inhibition / 72 h	Chronic*/Acute	A fixed ratio design was used, based on the maximum detected concentrations of each pharmaceutical found in effluent samples and in the Tama River (Tokyo, Japan)	2-parameter log-logistic models were used to calculate de concentration-response curves. The observed mixture toxicities were statistically compared to the CA and IA models	Both the CA and the IA models predicted equally the mixture toxicity	Watanabe et al. (2016)
Sulfathiazole+ Sulfamerazine+ Sulfadimidine+ Sulfamethoxazol+ Sulfadimethoxine+ Sulfapyridine	*Scenedesmus vacuolatus*	Algae	Growth inhibition / 24h (corresponding to the 96 h standard test)	Acute*	A fixed ratio design was used, comprising 1/6 of the EC_{50} values of the single compounds	The best fit model among logit, linlogit, probit and Weibull was selected. The CA and the IA models were used to predict the mixture toxicity	Better predict by the CA model, although a less than additive effect was observed	Białk-Bielińska et al. (2017)
Sulfathiazole+ Sulfamerazine+ Sulfadimidine+ Sulfamethoxazol+ Sulfadimethoxine+ Sulfapyridine+ Sulfanilamide (photodegradation product)	*Scenedesmus vacuolatus*	Algae	Growth inhibition / 24h (corresponding to the 96 h standard test)	Acute*	A fixed ratio design was used, comprising 1/7 of the EC_{50} values of the single compounds	The best fit model among logit, linlogit, probit and Weibull was selected. The CA and the IA models were used to predict the mixture toxicity	Better predict by the CA model, although a less than additive effect was observed	Białk-Bielińska et al. (2017)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Clotrimazole+ Triclosan+ Zinc-pyrithione+ Fluoxetine+ Propranolol	Microalgae from marine periphyton	Algae	Total pigment content and specific pigments (Chlorophyll a, Diadinoxanthin, Diatoxanthin, Fucoxanthin, Prasinoxanthin, Zeaxanthin, and β-carotene) / 96 h	Chronic*	A fixed-ratio design was used based on the NOEC values of the compounds	Regression models (Biquadratic and Generalized Logit 2) were fitted to the observed data. The CA and the IA models were used to predict the mixture toxicity	Better predicted by the IA model at higher effect concentrations such as on the EC_{50} level. The CA model slightly overestimated the mixture toxicity at that same higher concentration levels	Backhaus et al. (2011)
Propranolol+ Losartan	*Lemna minor*	Macrophyte	Growth inhibition based on frond number/ 7 d	Acute/ Chronic*	The compounds were combined in five effect concentration levels, using half of the effect concentrations at 10, 20, 50, 70 and 80 % of each pharmaceutical, based on previous experiments with the individual compounds	A three-parameter logistic function was used to describe the concentration-response curves for the mixture effects. The observed effects were compared to the predicted ones calculated using the CA and the IA equations. The effect residual ratio method was used to calculate the deviations percentages of the observed effects compared to the predicted ones.	Both the CA and the IA models overestimated the mixture toxicity. The authors reported that an antagonistic interaction occurred regarding both the reference models adopted.	Godoy et al. (2015)
Sulfathiazole+ Sulfamerazine+ Sulfadimidine+ Sulfamethoxazol+ Sulfadimethoxine+ Sulfapyridine	*L. minor*	Macrophyte	Growth inhibition / 7d	Acute*	A fixed ratio design was used, comprising 1/6 of the EC_{50} values of the single compounds	The best fit model among logit, linlogit, probit and Weibull was selected. The CA and the IA models were used.	Better predict by the CA model, although a less than additive effect was observed	Białk-Bielińska et al. (2017) (To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Sulfathiazole+ Sulfamerazine+ Sulfadimidine+ Sulfamethoxazol+ Sulfadimethoxine+ Sulfapyridine+ Sulfanilamide (photodegradation product)	*L. minor*	Macrophyte	Growth inhibition / 7d	Acute*	A fixed ratio design was used, comprising 1/7 of the EC_{50} values of the single compounds	The best fit model among logit, linlogit, probit and Weibull was selected. The CA and the IA models were used to predict the mixture toxicity	Better predict by the CA model, although a less than additive effect was observed	Białk-Bielińska et al. (2017)
Atorvastatin+ Acetaminophen+ Caffeine+ Sulfamethoxazol+ Carbamazepine+ Levofloxacin+ Sertraline+ Trimethoprim	*Myriophyllum sibiricum* and *Lemna gibba*	Macrophyte	Shoot growth, wet and dry mass, root number, primary root lengths, number of nodes, chroprophyll a and b and carotenoid content for *M. sibiricum*/ 35 d and Frond number, growth rate, chlorophyll a and b and carotenoid content for *L. gibba*/ 14 d	Unclassified	Four different concentrations of each coumpound, comprising four dose levels, from low dose to ultra-high dose were selected based on available data for surface water concentrations. For some of the pharmaceuticals, the reason for the choice of the concentrations was not explained	Regression models (Linear, Logistic, Gompertz, Exponential or Hormetic) were fitted to the data. Significant effects compared to the controls were analyzed by analysis of variance.	N/A. The authors reported that the toxicity of the mixture was probably via response addition, and that sulfamethoxazol, levofloxacin and atorvastatin were found to be the active phytotoxic agents in the mixture.	Brain et al. (2004)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Oxytetracycline+ Chlortetracycline+ Tetracycline+ Doxytetracycline	*M. sibiricum* and *L. gibba*	Macrophyte	Shoot growth, wet and dry mass, root number, primary root lengths, number of nodes, chroprophyll a and b and carotenoid content for *M. sibiricum*/ 35 d Frond number, growth rate, chlorophyll a and b and carotenoid content for *L. gibba*/ 14 d	Unclassified	Four different concentrations of each compound (10; 30; 100 and 300 μgL^{-1}), comprising four exposure levels, were summed up. The concentrations were selected in order to address environmental realism, as well as to consider measurable toxic responses.	Regression models (Linear, Logistic, Logistic 4 parameters, Gompertz, Exponential) were fitted to the data. Significant effects compared to the controls were analyzed by analysis of variance. Individual effects were not compared to the mixture ones	N/A. The authors reported that the mixture toxicity was most likely a result of the CA model, due to the similar mode of action of the antibiotics used. However, they were not able to elucidate which compounds contributed greatest to the mixture toxicity	Brain et al. (2005)
Diclofenac+ Ibuprofen	*Tetrahymena pyriformis*	Protozoa	Growth inhibition / 24 h	Acute	A full factorial design was used, in which concentrations of the mixture components corresponded to 0.25, 0.50, 0.75 and 1.0 TU determined based on the individual EC_{50} values	Four parameter logistic curves were fitted to the observed data. A one-way analysis of variance was performed for comparisons between the mixture and the individual effects of the two constituents. Synergistic/ Antagonistic interaction was considered when the combined toxic effect was significantly higher/lower than the sum of the individual toxicity values	Additivity occurred at the sum of 0.25 TU of each pharmaceutical. Antagonism was observed at all the other three effect levels evaluated	Láng and Kőhidai (2012)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Diclofenac+ Metoprolol	*T. pyriformis*	Protozoa	Growth inhibition / 24 h	Acute	A full factorial design was used, in which concentrations of the mixture components corresponded to 0.25, 0.50, 0.75 and 1.0 TU determined based on the individual EC_{50} values	Four parameter logistic curves were fitted to the observed data. A one-way analysis of variance was performed for comparisons between the mixture and the individual effects of the two constituents	Additivity was the predominant form of combined effects. However, synergism was observed at the following combined concentrations: 1 TU diclofenac + 0.25 TU metoprolol; 0.5 TU diclofenac + 1 TU metoprolol; 0.75 TU diclofenac + 0.75 TU metoprolol; 1 TU diclofenac + 0.5 TU metoprolol. Antagonism was observed at 1 TU of both pharmaceuticals	Láng and Kőhidai (2012)
Diclofenac+ Propranolol	*T. pyriformis*	Protozoa	Growth inhibition / 24 h	Acute	A full factorial design was used, in which concentrations of the mixture components corresponded to 0.25, 0.50, 0.75 and 1.0 TU determined based on the individual EC_{50} values	Four parameter logistic curves were fitted to the data. A one-way analysis of variance was performed for comparisons between the mixture and the individual effects of the two constituents	Additivity was observed at the lowest mixture concentrations (0.5 and 0.75 TU); Additivity alterned with antagonism at the intermediate mixture concentrations (1, 1.25 and 1.5 TU); Antagonism was observed at the highest mixture concentrations (1.75 and 2 TU)	Láng and Kőhidai (2012)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Ibuprofen+ Metoprolol	*T. pyriformis*	Protozoa	Growth inhibition / 24 h	Acute	A full factorial design was used, in which concentrations of the mixture components corresponded to 0.25, 0.50, 0.75 and 1.0 TU determined based on the individual EC_{50} values	Four parameter logistic curves were fitted to the observed data. A one-way analysis of variance was performed for comparisons between the mixture and the individual effects of the two constituents.	Additivity was observed at the lowest mixture concentrations (0.5 and 0.75 TU); Additivity alterned with antagonism at the intermediate mixture concentrations (1 and 1.25 TU); Antagonism was observed at the highest mixture concentrations (1.5, 1.75 and 2 TU)	Láng and Kőhidai (2012)
Ibuprofen+ Propranolol	*T. pyriformis*	Protozoa	Growth inhibition / 24 h	Acute	A full factorial design was used, in which concentrations of the mixture components corresponded to 0.25, 0.50, 0.75 and 1.0 TU determined based on the individual EC_{50} values	Four parameter logistic curves were fitted to the observed data. A one-way analysis of variance was performed for comparisons between the mixture and the individual effects of the two constituents	Additivity and antagonism were observed at the lowest mixture concentrations (0.5 and 0.75 TU); Additivity was more frequent at intermediate mixture concentrations (1 and 1.25 TU); Antagonism was observed at the highest mixture concentrations (1.5, 1.75 and 2 TU)	Láng and Kőhidai (2012)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Metoprolol+ Propranolol	*T. pyriformis*	Protozoa	Growth inhibition / 24 h	Acute	A full factorial design was used, in which concentrations of the mixture components corresponded to 0.25, 0.50, 0.75 and 1.0 TU determined based on the individual EC_{50} values	Four parameter logistic curves were fitted to the observed data. A one-way analysis of variance was performed for comparisons between the mixture and the individual effects of the two constituents	Antagonism was observed in almost all cases, except for the following ones: 0.25 TU of both pharmaceuticals; 0.5 TU of both pharmaceuticals; 0.25 TU propranolol + 0.5 TU metoprolol	Láng and Kőhidai (2012)
Diclofenac+ Ibuprofen	*Atyaephyra desmarestii*	Arthropoda (Crustacea)	Lethality at 20 and 25 ºC / 96 h	Acute	Pharmaceuticals were added in mixture at proportions corresponding to half of the lethal concentrations obtained from acute toxicity tests (LC_5, LC_{10}, LC_{20}, LC_{50} and LC_{80})/2	Concentration-responses relationships were modeled using generalized linear model and non-linear regression, sigmoidal equation logistic with 3 parameters. Mixture toxicities were predicted using the CA and IA equations	Neither of the CA and IA models was able to predict well the observed toxicity	Nieto et al. (2016)
Diclofenac+ Carbamazepine	*A. desmarestii*	Arthropoda (Crustacea)	Lethality at 20 and 25 ºC / 96 h	Acute	Pharmaceuticals were added in mixture at proportions corresponding to half of the lethal concentrations obtained from acute toxicity tests (LC_5, LC_{10}, LC_{20}, LC_{50} and LC_{80})/2	Concentration-responses relationships were modeled using generalized linear model and non-linear regression, sigmoidal equation logistic with 3 parameters. Mixture toxicities were predicted using the CA and IA equations	Better predicted by the IA model at low concentrations at 25ºC, but at 20ºC neither of the two models predicted well the mixture toxicity	Nieto et al. (2016)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Diclofenac+ Ibuprofen+ Carbamazepine	*A. desmarestii*	Arthropoda (Crustacea)	Lethality at 20 and 25 ºC / 96 h	Acute	Pharmaceuticals were added in mixture at proportions corresponding to a third of the lethal concentrations obtained from acute toxicity tests (LC_5, LC_{10}, LC_{20}, LC_{50} and LC_{80})/3	Concentration-responses relationships were modeled using generalized linear model and non-linear regression, sigmoidal equation logistic with 3 parameters. Mixture toxicities were predicted using the CA and IA equations	Better predicted by the CA model for both low and high concentrations at 25ºC and also predicted by the IA model at higher concentrations at 20ºC	Nieto et al. (2016)
Fluoxetine+ Sertraline+ Paroxetine+ Citalopram	*Ceriodaphnia dubia*	Arthropoda (Crustacea)	Immobilization / 48 h	Acute	The relative amount of each pharmaceutical, determined by their individual median lethal concentration (LC_{50}), was kept constant in the mixture. Concentrations were chosen so that each pharmaceutical would contribute equally to the toxic effects of the mixture	Logistic regression (generalized logistic model) were fitted to the data. The CA and the IA models were used to predict the mixture toxicity	Better predicted by the CA model, however mixture toxicity was significantly higher than predicted by the CA or IA model	Henry and Black (2007)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Sertraline+ Fluoxetine	*C. dubia*	Arthropoda (Crustacea)	Immobilization / 48 h	Acute	The relative amount of each pharmaceutical, determined by their individual median lethal concentration (LC_{50}), was kept constant in the mixture. Concentrations were chosen so that each pharmaceutical would contribute equally to the toxic effects of the mixture	Logistic regression (generalized logistic model) was fitted to the observed data. The CA and the IA models were used to predict the mixture toxicity	Better predicted by the CA model	Henry and Black (2007)
Sertraline+ Paroxetine	*C. dubia*	Arthropoda (Crustacea)	Immobilization / 48 h	Acute	The relative amount of each pharmaceutical, determined by their individual median lethal concentration (LC_{50}), was kept constant in the mixture. Concentrations were chosen so that each pharmaceutical would contribute equally to the toxic effects of the mixture	Logistic regression (generalized logistic model) was fitted to the observed data. The CA and the IA models were used to predict the mixture toxicity	Better predicted by the CA model	Henry and Black (2007)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Sertraline+ Citalopram	*C. dubia*	Arthropoda (Crustacea)	Immobilization / 48 h	Acute	The relative amount of each pharmaceutical, determined by their individual median lethal concentration (LC_{50}), was kept constant in the mixture. Concentrations were chosen so that each pharmaceutical would contribute equally to the toxic effects of the mixture	Logistic regression (generalized logistic model) was fitted to the observed data. The CA and the IA models were used to predict the mixture toxicity	Better predicted by the CA model	Henry and Black (2007)
Fluoxetine+ Paroxetine	*C. dubia*	Arthropoda (Crustacea)	Immobilization / 48 h	Acute	The relative amount of each pharmaceutical, determined by their individual median lethal concentration (LC_{50}), was kept constant in the mixture. Concentrations were chosen so that each pharmaceutical would contribute equally to the toxic effects of the mixture	Logistic regression (generalized logistic model) was fitted to the observed data. The CA and the IA models were used to predict the mixture toxicity	Better predicted by the CA model	Henry and Black (2007)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Fluoxetine+ Citalopram	*C. dubia*	Arthropoda (Crustacea)	Immobilization / 48 h	Acute	The relative amount of each pharmaceutical, determined by their individual median lethal concentration (LC_{50}), was kept constant in the mixture. Concentrations were chosen so that each pharmaceutical would contribute equally to the toxic effects of the mixture	Logistic regression (generalized logistic model) was fitted to the observed data. The CA and the IA models were used to predict the mixture toxicity	Better predicted by the CA model	Henry and Black (2007)
Paroxetine+ Citalopram	*C. dubia*	Arthropoda (Crustacea)	Immobilization / 48 h	Acute	The relative amount of each pharmaceutical, determined by their individual median lethal concentration (LC_{50}), was kept constant in the mixture. Concentrations were chosen so that each pharmaceutical would contribute equally to the toxic effects of the mixture	Logistic regression (generalized logistic model) was fitted to the observed data. The CA and the IA models were used to predict the mixture toxicity	Better predicted by the CA model	Henry and Black (2007)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Cisplatin+ 5-Fluorouracil	*C. dubia*	Arthropoda (Crustacea)	Inhibition of reproduction / 7d	Chronic	The concentrations applied of each pharmaceutical in mixture were obtained by the isobologram method. Two predicted effect levels were then tested based on the data from individual drugs: 44.6 and 89.2% offspring reduction	A log logistic function was fitted to the data for determining reduction of *D. similis* offspring. Deviation from expected results under the IA model was tested by ANOVA. Significant deviations ($p<0.05$) from equality between combined and single effects were considered antagonism/ synergism.	Predicted by the IA model at the two effect concentrations tested	Parrella et al. (2014)
Imatinib+ 5-Fluorouracil	*C. dubia*	Arthropoda (Crustacea)	Inhibition of reproduction / 7d	Chronic	The concentrations applied of each pharmaceutical in mixture were obtained by the isobologram method. The predicted effect level tested (44.6% offspring reduction) was based on the data from individual drugs	A log logistic function was fitted to the data for determining reduction of *D. similis* offspring. Deviation from expected results under the IA model was tested by ANOVA. Significant deviations ($p<0.05$) from equality between combined and single effects were considered antagonism/ synergism.	Predicted by the IA model	Parrella et al. (2014)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Etoposide+ 5-Fluorouracil	*C. dubia*	Arthropoda (Crustacea)	Inhibition of reproduction / 7d	Chronic	The concentrations applied of each pharmaceutical in mixture were obtained by the isobologram method. Two predicted effect levels were then tested based on the data from individual drugs: 44.6 and 89.2% offspring reduction	A log logistic function was fitted to the data for determining reduction of *D. similis* offspring. Deviation from expected results under the IA was tested by ANOVA. Significant deviations ($p<0.05$) from equality between combined and single effects were considered antagonism/ synergism.	Predicted by the IA model at the lower effect concentration. At the higher effect level, an antagonistic interaction was observed regarding the IA model	Parrella et al. (2014)
Etoposide+ Cisplatin	*C. dubia*	Arthropoda (Crustacea)	Inhibition of reproduction / 7d	Chronic	The concentrations applied of each pharmaceutical in mixture were obtained by the isobologram method. Two predicted effect levels were then tested based on the data from individual drugs: 44.6 and 89.2% offspring reduction	A log logistic function was fitted to the data for determining reduction of *D. similis* offspring. Deviation from expected results under the IA was tested by ANOVA. Significant deviations ($p<0.05$) from equality between combined and single effects were considered antagonism/ synergism.	Predicted by the IA at the lower effect concentration. At the higher effect level, an antagonistic interaction was observed regarding the IA model	Parrella et al. (2014)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Imatinib+ Etoposide	*C. dubia*	Arthropoda (Crustacea)	Inhibition of reproduction / 7d	Chronic	The concentrations applied of each pharmaceutical in mixture were obtained by the isobologram method. The predicted effect level tested (44.6% offspring reduction) was based on the data from individual drugs	A log logistic function was fitted to the data for determining reduction of *D. similis* offspring. Deviation from expected results under the IA was tested by ANOVA. Significant deviations ($p<0.05$) from equality between combined and single effects were considered antagonism/ synergism.	Predicted by the IA model	Parrella et al. (2014)
Imatinib+ Cisplatin	*C. dubia*	Arthropoda (Crustacea)	Inhibition of reproduction / 7d	Chronic	The concentrations applied of each pharmaceutical in mixture were obtained by the isobologram method. The predicted effect level tested (44.6% offspring reduction) was based on the data from individual drugs	A log logistic function was fitted to the data for determining reduction of *D. similis* offspring. Deviation from expected results under the IA was tested by ANOVA. Significant deviations ($p<0.05$) from equality between combined and single effects were considered antagonism/ synergism.	A tendency to synergism was observed regarding to the IA model	Parrella et al. (2014)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Sulpiride+ Clarithromycin+ Diphenhydramine+ Benzafibrate+ Acetaminophen+ Ketoprofen+ Phenytoin+ Etodolac+ Crotamiton+ Epinastine	*C. dubia*	Arthropoda (Crustacea)	Inhibition of reproduction / 6 - 8 d	Chronic	A fixed ratio design was used, based on the maximum detected concentrations of each pharmaceutical found in effluent samples and in the Tama River (Tokyo, Japan)	2-parameter log-logistic models were used to calculate de concentration-response curves. The observed mixture toxicities were statistically compared to the CA and IA models	Both the CA and the IA models slightly underestimated the observed toxicity in the lower mixture concentrations. A synergy was suspected but could not be confirmed by the authors	Watanabe et al. (2016)
Clofibrinic acid + Carbamazepine	*Daphnia magna*	Arthropoda (Crustacea)	Immobilization / 48 h	Acute	Half of the calculated effect concentrations from the single components (EC_5, EC_{10}, EC_{20}, EC_{50}, EC_{80}, EC_{90}) were used in the mixture test	Nonlinear curve fitting (four-parameter logistic function) was used to describe the observed data. The CA and the IA models were used to predict the mixture toxicity	Better predicted by the CA model	Cleuvers (2003)
Ibuprofen+ Diclofenac	*D. magna*	Arthropoda (Crustacea)	Immobilization / 48 h	Acute	Half of the calculated effect concentrations from the single components ($EC_5/2$, $EC_{10}/2$, $EC_{20}/2$, $EC_{50}/2$, $EC_{80}/2$) was used in the mixture test	Nonlinear curve fitting (four-parameter logistic function) was used to describe the observed data. The CA and the IA models were used to predict the mixture toxicity	Both the CA and IA models underestimated the mixture toxicity	Cleuvers (2003)
Propranolol+ Metoprolol+ Atenolol	*D. magna*	Arthropoda (Crustacea)	Immobilization / 48 h	Acute	A third of the calculated effect concentrations from the single components ($EC_5/3$, $EC_{10}/3$, $EC_{20}/3$, $EC_{50}/3$, $EC_{80}/3$) used	Nonlinear curve fitting (four-parameter logistic function) was used to describe the observed data. The CA and the IA models were used.	Better predicted by the CA model	Cleuvers (2005)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Sertraline+ Citalopram	*D. magna*	Arthropoda (Crustacea)	Immobilization / 48 h	Acute	Ray design consisting on seven different mixture ratios based on the single-compound effect concentration (100:0; 77:23; 63:37; 50:50; 37:63; 23:77; 0:100)	Isobologram was built from calculated EC_{50} values from concentration-response curves. The CA and the IA models were used in the analysis of chemical mixtures	Better predicted by the CA model. An additive effect was observed	Christensen et al. (2007)
Sertraline+ Fluoxetine	*D. magna*	Arthropoda (Crustacea)	Immobilization / 48 h	Acute	Ray design consisting on seven different mixture ratios based on the single-compound effect concentration (100:0; 77:23; 63:37; 50:50; 37:63; 23:77; 0:100)	Isobologram was built from calculated EC_{50} values from concentration-response curves. The CA and the IA models were used in the analysis of chemical mixtures	Better predicted by the CA model. An additive effect was observed	Christensen et al. (2007)
Fluoxetine+ Citalopram	*D. magna*	Arthropoda (Crustacea)	Immobilization / 48 h	Acute	Ray design consisting on seven different mixture ratios based on the single-compound effect concentration (100:0; 77:23; 63:37; 50:50; 37:63; 23:77; 0:100)	Isobologram was built from calculated EC_{50} values from concentration-response curves. The CA and the IA models were used in the analysis of chemical mixtures	Better predicted by the CA model. An additive effect was observed	Christensen et al. (2007)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Carbamazepine+ Diclofenac+ 17α-Ethinylestradiol+ Metoprolol	*D. magna*	Arthropoda (Crustacea)	Body length, reproduction and sex ratio / Multigenerationa l study (6 generations)	Chronic	Components were added in environmentally relevant concentrations, considering the maximum concentrations found in rivers and streams in Germany. Nominal concentrations tested in mixture were the following ($\mu g L^{-1}$): 0.50 carbamazepine; 0.36 diclofenac; 1.20 metoprolol and 0.10 ng L^{-1}ethinylestradiol	One-way analysis of variance and nested analysis of variance were used to compare individual effects of each compound to the mixture toxicity	N/A. Pharmaceutical mixture did not provoke stronger effects on the test organisms than the single drugs	Dietrich et al. (2010)
Fluoxetine+ Clofibrinic acid	*D. magna*	Arthropoda (Crustacea)	Survival, morphology, adult length, resting egg production, fecundity, sex ratio / 6 d	Acute	Components were added in predetermined fixed concentrations (10 or 100 μg L^{-1} for clofibrinic acid and 36 μg L^{-1} for fluoxetine) similar to environmental detection levels reported in the literature	The Fisher method was used to compare individual effects of each compound to the mixture toxicity	N/A. Significant effects such as mortality were caused by the mixture in concentrations that yielded no apparent effects when tested individually. A suspected additivity or synergism remains to be investigated	Flaherty and Dodson (2005)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Erythromycin+ Triclosan+ Trimethoprim	*D. magna*	Arthropoda (Crustacea)	Reproduction; Sex ratio / 6d	Acute	Components were added in predetermined fixed concentrations (1-100 µg L^{-1}) similar to environmental detection levels reported in the literature	The Fisher method was used to compare individual effects of each compound to the mixture toxicity	N/A. The mixture elicited a significant decrease in sex ratio, not observed when the components were tested individually. A suspected additivity or synergism remains to be investigated	Flaherty and Dodson (2005)
Erythromycin+ Triclosan+ Trimethoprim+ Lincomycin+ Sulfamethoxazole	*D. magna*	Arthropoda (Crustacea)	Number of males produced (sex ratio)/ 6d	Acute	Components were added in predetermined fixed concentrations (1-100 µg L^{-1}) similar to environmental detection levels reported in the literature	The Fisher method was used to compare individual effects of each compound to the mixture toxicity	N/A. The mixture significantly increased the number of males produced, which was not predictable from results of single pharmaceuticals bioassays. A possible additivity or synergism remains to be investigated.	Flaherty and Dodson (2005)
Erythromycin+ Triclosan+ Trimethoprim+ Lincomycin+ Sulfamethoxazole	*D. magna*	Arthropoda (Crustacea)	Decrease in sex ratio/ 30 d	Chronic	Components were added in predetermined fixed concentrations (1-100 µg L^{-1}) similar to environmental detection levels reported in the literature	The Fisher method was used to compare individual effects of each compound to the mixture toxicity	N/A. The mixture led to a significant decrease in sex ratio of the first brood, which was not predictable from results of single pharmaceuticals bioassays. A possible additivity or synergism remains to be investigated.	Flaherty and Dodson (2005)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Sulfamethazine+ Sulfamerazine	*D. magna*	Arthropoda (Crustacea)	Immobilization / 48 h	Acute	Concentrations used in the mixture test were based on the individual EC_{50} values, comprising 5 exposition levels. Three selected combination ratios equidistantly distributed on the additivity line were analyzed	The isobologram method was used to evaluate the mixture effects. Deviation from additivity was considered if the confidence intervals for the effect concentrations of the combined pharmaceuticals did not overlap the confidence belt of the additivity line	N/A. A less than additive interaction was observed for the mixture effects	De Liguoro et al. (2009)
Sulfamethazine+ Sulfadimethoxine	*D. magna*	Arthropoda (Crustacea)	Immobilization / 48 h	Acute	Concentrations used in the mixture test were based on the individual EC_{50} values, comprising 5 exposition levels. Three selected combination ratios equidistantly distributed on the additivity line were analyzed	The isobologram method was used to evaluate the mixture effects. Deviation from additivity was considered if the confidence intervals for the effect concentrations of the combined pharmaceuticals did not overlap the confidence belt of the additivity line	N/A. A less than additive interaction was observed for the mixture effects	De Liguoro et al. (2009)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Sulfamethazine+ Sulfaquinoxaline	*D. magna*	Arthropoda (Crustacea)	Immobilization / 48 h	Acute	Concentrations used in the mixture test were based on the individual EC_{50} values, comprising 5 exposition levels. Three selected combination ratios equidistantly distributed on the additivity line were analyzed	The isobologram method was used to evaluate the mixture effects. Deviation from additivity was considered if the confidence intervals for the effect concentrations of the combined pharmaceuticals did not overlap the confidence belt of the additivity line	N/A. The interaction was mixture-ratio dependent, since high doses of sulfaquinoxaline combined with low doses of sulfamethazine showed a more than additive interaction; however, with higher doses of sulfamethazine, sulfaquinoxaline showed additivity and subadditivity	De Liguoro et al. (2009)
Sulfamethazine+ Trimethoprim	*D. magna*	Arthropoda (Crustacea)	Immobilization / 48 h	Acute	Concentrations used in the mixture test were based on the individual EC_{50} values, comprising 5 exposition levels. Three selected combination ratios equidistantly distributed on the additivity line were analyzed	The isobologram method was used to evaluate the mixture effects. Deviation from additivity was considered if the confidence intervals for the effect concentrations of the combined pharmaceuticals did not overlap the confidence belt of the additivity line	N/A. A simple additivity was detected for the mixture effects	De Liguoro et al. (2009)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Sulfamethazine+ Sulfaguanidine	*D. magna*	Arthropoda (Crustacea)	Immobilization / 48 h	Acute	Concentrations used in the mixture test were based on the individual EC_{50} values, comprising 5 exposition levels. Three selected combination ratios equidistantly distributed on the additivity line were analyzed	The isobologram method was used to evaluate the mixture effects. Deviation from additivity was considered if the confidence intervals for the effect concentrations of the combined pharmaceuticals did not overlap the confidence belt of the additivity line	N/A. A less than additive interaction was observed for the mixture effects	De Liguoro et al. (2009)
Sulfamethazine+ Sulfadiazine	*D. magna*	Arthropoda (Crustacea)	Immobilization / 48 h	Acute	Concentrations used in the mixture test were based on the individual EC_{50} values, comprising 5 exposition levels. Three selected combination ratios equidistantly distributed on the additivity line were analyzed	The isobologram method was used to evaluate the mixture effects. Deviation from additivity was considered if the confidence intervals for the effect concentrations of the combined pharmaceuticals did not overlap the confidence belt of the additivity line	N/A. A less than additive interaction was observed for the mixture effects	De Liguoro et al. (2009)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Sulfaquinoxaline+ Sulfaguanidine	*D. magna*	Arthropoda (Crustacea)	Immobilization / 48 h	Acute	Concentrations used in the mixture test were based on the individual EC_{50}, comprising 5 exposition levels. Three selected combination ratios equidistantly distributed on the additivity line were analyzed	The isobologram method was used to evaluate the mixture effects. Deviation from additivity was considered if the confidence intervals for the effect concentrations of the combined pharmaceuticals did not overlap the confidence belt of the additivity line	N/A. A less than additivity was observed for the mixture effects	De Liguoro et al. (2010)
17 α - Ethinylestradiol + Fluoxetine	*D. magna*	Arthropoda (Crustacea)	Survival, reproduction and population growth rates/ 40 d	Chronic	Concentrations in mixture were applied at low, medium and high levels, based on environmental and literature data (Low: 0.01 µg L^{-1} EE2 + 0.01 µg L^{-1} fluoxetine; Medium: 0.1 µg L^{-1} EE2 + 1.0 µg L^{-1} fluoxetine; High: 1.0 µg L^{-1} EE2 + 100 µg L^{-1} fluoxetine	Relative population growth rates (normalized to the control) were estimated for the individual and the mixture effects and the respective 95% confidence intervals were compared. Differences were considered significant when the confidence intervals did not overlap	N/A. The authors reported that synergistic effects occurred on time to first reproduction and on population growth rate endpoints	Luna et al. (2015)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Cisplatin+ 5-fluorouracil	*D. magna*	Arthropoda (Crustacea)	Inhibition of reproduction/ 21d	Chronic	The concentrations applied of each pharmaceutical in mixture were obtained by the isobologram method. Two predicted effect levels were then tested based on the data from individual drugs: 31.6 and 63.3% offspring reduction	A log logistic function was fitted to the data for determining reduction of *D. similis* offspring. Deviation from expected results under the IA model was tested by ANOVA. Significant deviations ($p<0.05$) from equality between combined and single effects were considered antagonism/ synergism	Predicted by the IA model at the two effect concentrations tested	Parrella et al. (2014)
Imatinib+ 5-fluorouracil	*D. magna*	Arthropoda (Crustacea)	Inhibition of reproduction/ 21d	Chronic	The concentrations applied of each pharmaceutical in mixture were obtained by the isobologram method. Two predicted effect levels were then tested based on the data from individual drugs: 31.6 and 63.3% offspring reduction	A log logistic function was fitted to the data for determining reduction of *D. similis* offspring. Deviation from expected results under the IA was tested by ANOVA. Significant deviations ($p< 0.05$) from equality between combined and single effects were considered antagonism/ synergism.	Predicted by the IA model at the two effect concentrations tested	Parrella et al. (2014)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Etoposide+ 5-fluorouracil	*D. magna*	Arthropoda (Crustacea)	Inhibition of reproduction/ 21d	Chronic	The concentrations applied of each pharmaceutical in mixture were obtained by the isobologram method. Two predicted effect levels were then tested based on the data from individual drugs: 31.6 and 63.3% offspring reduction	A log logistic function was fitted to the data for determining reduction of *D. similis* offspring. Deviation from expected results under the IA model was tested by ANOVA. Significant deviations ($p<0.05$) from equality between combined and single effects were considered antagonism/ synergism.	Predicted by the IA model at the two effect concentrations tested	Parrella et al. (2014)
Etoposide+ Cisplatin	*D. magna*	Arthropoda (Crustacea)	Inhibition of reproduction/ 21d	Chronic	The concentrations applied of each pharmaceutical in mixture were obtained by the isobologram method. Two predicted effect levels were then tested based on the data from individual drugs: 31.6 and 63.3% offspring reduction	A log logistic function was fitted to the data for determining reduction of *D. similis* offspring. Deviation from expected results under the IA model was tested by ANOVA. Significant deviations ($p< 0.05$) from equality between combined and single effects were considered antagonism/ synergism.	Predicted by the IA model at the two effect concentrations tested	Parrella et al. (2014)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Imatinib+ Etoposide	*D. magna*	Arthropoda (Crustacea)	Inhibition of reproduction/ 21d	Chronic	The concentrations applied of each pharmaceutical in mixture were obtained by the isobologram method. Two predicted effect levels were then tested based on the data from individual drugs: 31.6 and 63.3% offspring reduction	A log logistic function was fitted to the data for determining reduction of *D. similis* offspring. Deviation from expected results under the IA model was tested by ANOVA. Significant deviations ($p< 0.05$) from equality between combined and single effects were considered antagonism/ synergism.	Predicted by the IA model at the lower effect concentration. For the higher concentration, an antagonistic interaction regarding the IA model was observed.	Parrella et al. (2014)
Imatinib+ Cisplatin	*D. magna*	Arthropoda (Crustacea)	Inhibition of reproduction/ 21d	Chronic	The concentrations applied of each pharmaceutical in mixture were obtained by the isobologram method. Two predicted effect levels were then tested based on the data from individual drugs: 31.6 and 63.3% offspring reduction	A log logistic function was fitted to the data for determining reduction of *D. similis* offspring. Deviation from expected results under the IA model was tested by ANOVA. Significant deviations ($p<0.05$) from equality between combined and single effects were considered antagonism/ synergism.	Predicted by the IA model at the lower effect concentration. For the higher concentration, antagonistic interaction regarding the IA model was observed.	Parrella et al. (2014)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Caffeine+ Sulfamethoxazole+ Carbamazepine+ Trimethoprim+ Acetaminophen+ Diltiazem+ Ciprofloxacin+ Levofloxacin+ Atorvastatin+ Sertraline+ Fluoxetine	*D. magna*	Arthropoda (Crustacea)	Inhibition of reproduction/ 21d	Chronic	The pharmaceuticals were applied in the mixture at concentrations corresponding to 1x, 10x, 25x, 50x, 75x, 100x and 1000x the maximum values detected in the upper Tennessee River	A one-way analysis of variance followed by Tukey test was performed for comparisons between treatments and control and between individual and mixture effects	N/A. The mixture of pharmaceuticals presented a lower LOEC for reproduction than any of the individual pharmaceuticals separately. The authors considered that there were possible interactions of the mixture components but they did not evaluate the nature of the interactions	Wolfe et al. (2015)
Acetaminophen+ Diclofenac+ Gemfibrozil+ Ibuprofen+ Naproxen+ Salicylic acid+ Triclosan	*Hyalella azteca*	Arthropoda (Crustacea)	Survival, number of mating pairs, body size, reproduction, sex ratio / 8 weeks	Chronic	About 200 ng L^{-1} of each component was applied in the mixture test, based on a worst-case scenario in most Canadian fresh waters	A two-way analysis of variance was performed for the analysis of the mixture effects. The mixture effects were not statistically compared to the individual ones of each compound	N/A. The effect of the pharmaceutical mixture was not significant in comparison to the control, except for a 17% increase in percent males.	Borgmann et al. (2007)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Clofibrinic acid+ Diclofenac	*Palaemon longirostris*	Arthropoda (Crustacea)	Larval survival, duration of the larval development and growth rate / 47 days	Chronic	Mixture of the pharmaceuticals were carried out at low doses (40 μg L^{-1} of diclofenac plus 17 μg L^{-1} of clofibric acid) and at high doses (750 μg L^{-1} of diclofenac plus 361 μg L^{-1} of cloficbric acid), based on threshold concentrations leading to sublethal effects	Survival was evaluated by nominal variable with a binomial distribution and through the Kaplan-Meier log-rank test; the number of stages, duration of development and growth were tested by generalized linear mixed model. The individual effects of each compound were statistically compared to the mixture effects	The authors suggested that the mixture toxicity followed the IA model, although they did not use this model to predict the combined effects. Effects caused by clofibrinic acid alone were comparable to the mixture effects, while no similar effect of diclofenac alone was observed	González-Ortegón et al. (2016)
Diclofenac+ Ibuprofen+ Paracetamol	*Dreissena polymorpha*	Mollusca	Lysosomal membrane stability; activity of the enzimes CAT, SOD, GPx and GST; genotoxicity evaluated by apoptotic frequency and the micronucleus test / 96 h	Unclassified	The pharmaceuticals were mixed at three different environmental concentrations, corresponding to concentrations of the pharmaceuticals measured in European surface waters and in the outflow of wastewater treatment plants	Analysis of variance followed by the Bonferroni post-hoc test was performed to evaluate significant differences between treatments and controls, and the Pearson´s correlation test was performed to investigate correlations between biological responses. The percent mixture effects were compared to the individual effects posed by each pharmaceutical, as observed in a previous study	N/A. The authors suggested that the genotoxic action of the mixture follows the concept of CA, since the toxic effects obtained with the mixture were significantly higher than that observed for individual exposures. However, the data were not modeled regarding the CA model	Parolini and Binelli (2012)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Venlafaxine+ Carbamazepine+ Diazepam+ Atenolol+ Furosemide+ Hydrochlorothiazide+· Lisinopril+ Atorvastatin+ Gemfibrozil+ Benzafibrate+ Ciprofloxacine+ Erythromycine+ Novobiocin+ Oxytetracycline+ Sulfamethoxazole+ Trimethoprim	*Lymnaea stagnalis*	Mollusca	Immunological parameters and gene expression/ 3 d	Acute	Intermediate median reported concentrations found in municipal effluents were applied for the pharmaceuticals in the mixture (200 ng L^{-1} for venlafaxine, carbamazepine and oxytetracycline; 10 ng L^{-1} for diazepam; 500 ng L^{-1} for atenolol; 300 ng L^{-1} for furosemide and hydrochlororthiazide; 50 ng L^{-1} for lisinopril, atorvastatin, erythromycine, sulfamethoxazole and trimethoprim; 100 ng L^{-1} for gemfibrozil, benzafibrate, ciprofloxacine and novobiocin)	One-way analysis of variance followed by the least-square difference test was performed for comparisons of treatment groups. Correlations analyses were evaluated by a Pearson-moment test. Individual pharmaceutical effects were not tested.	N/A. The global mixture was closely associated with the antibiotic mixture. However, there was not a comparison between single pharmaceutical effects with the ones caused by the global mixture	Gust et al. (2013)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Propranolol+ Diclofenac	*Mytilus edulis trossulus*	Mollusca	Respiration, absorption efficiency, consumption and energy budget / 14 d	Unclassified	The following combinations of the pharmaceuticals were used in mixtures: 75% prop. plus 25% diclof.; 50% of each pharmaceutical and 25% prop. plus 75% diclof. (Total concentration of 1000 µ g L^{-1}). The reason of the choice of the concentrations was not specified	A one-way analysis of variance followed by Tukey´s test was used to analyze differences among the treatments. The mixture treatments were statistically compared with each other. However, no mathematical/statistical model/approach was used to a direct comparison between individual and mixture effects	N/A. The authors reported that a possible synergistic effect occurred from higher concentrations of diclofenac and lower concentrations of propranolol, although they did not test any mathematical model to confirm their statement	Ericson et al. (2010)
Propranolol+ Fluoxetine	*Mytilus galloprovincialis*	Mollusca	Cyclic adenosine monophosphate (cAMP) levels, protein kinase A activity, levels of serotonin (5-HT_1) mRNA expression and levels of ABCB mRNA expression in digestive glands and mantle/gonads expression / 7 d	Unclassified	Environmental concentrations of 0.3 ng L^{-1} of each pharmaceutical were used in the mixture, based on the lower range of environmental levels for the compounds	A factor analysis using the Principal Component Analysis was performed to detect significant different effects of the mixture compared to the single pharmaceutical exposures	N/A. The mixture lowered the detrimental effects of the single pharmaceuticals	Franzellitti et al. (2013)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Ibuprofen+ Diclofenac+ Fluoxetine	*M. galloprovincialis*	Mollusca	Activities of the enzymes SOD, CAT, GR, GST and AChE; lipid peroxidation; endocrine disruption (vitellogenin-like protein concentrations) / 15 d	Unclassified	Fixed environmental concentrations, based on previous studies were applied in the mixture (250 ng L^{-1} ibuprofen+ 250 ng L^{-1} diclofenac +75 ng L^{-1} fluoxetine)	Principal component analysis was applied for comparison of biomarker responses of single pharmaceuticals exposure with the mixture	N/A. There was different time and tissue dependent biomarker responses from the selected mixtures compared to the single pharmaceutical effects. The authors highlighted several possible synergistic, antagonistic and potentiation interaction effects among the evaluated pharmaceuticals	Gonzalez-Rey et al. (2014)
17 α - ethynylestradiol+ Fluoxetine	*Physa pomilia*	Mollusca	Survival, reproduction and population growth rates / 12 weeks	Chronic	Environmentally relevant concentrations (except for the high level) of each pharmaceutical were applied in the mixture, at three levels: Low: 0.01 µg L^{-1} EE2 + 0.01 µg L^{-1} fluoxetine; Medium: 0.1 µg L^{-1} EE2 + 1.0 µg L^{-1} fluoxetine; High: 1.0 µg L^{-1} EE2 + 100 µg L^{-1} fluoxetine	One-way analysis of variance followed by Tuckey´s test and Mantel-Cox test were performed for reproduction analysis; a nonparametric bootstrapping approach was used in population growth estimates. Differences were considered significant if the 95% confidence intervals between the control and each treatment did not overlap	N/A. Different effects were observed between single pharmaceuticals and mixture exposures. The authors considered that there was a potential important interaction between the two pharmaceuticals	Luna et al. (2013)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Carbamazepine+ Acridine+ Acridone+ Acridine-9-carbaldehyde+ Carbamazepine photoproduct IV+ Carbamazepine photoproduct V	*Scrobicularia plana*	Mollusca	Survival and biochemical responses / 96 h	Acute	The organisms were exposed to irradiated carbamazepine at environmental concentrations (0.00 - 9.00 µg L^{-1})	Hypothesis testing using permutation of multivariate analysis of variance and the t-statistic for pairwise comparisons were used for statistical comparisons between irradiated (mixture) and non- irradiated carbamazepine assays. However, effects of each individual compound were not compared to the mixture effects	N/A. The exposure to the mixture of carbamazepine plus its photoproducts did not result in higher acute toxicity compared to the non-irradiated carbamazepine. Biochemical changes suggested the occurrence of an antagonistic effect between photoproducts and the parental compound	Almeida et al. (2017)
Ibuprofen+ Naproxen+ Gemfibrozil+ Benzafibrate+ Carbamazepine+ Sulfapyridine+ Oxytetracycline+ Novobiocin+ Trimethoprim+ Sulfamethoxazole+ Caffeine	*Hydra attenuata*	Cnidarian	Survival, feeding behavior, hydranth number and attachment (regeneration degree) / 96 h	Acute	Pharmaceuticals were applied in mixture at 1x and up to 10000x concentrations found in a primary treated effluent from a WWTP	Effect mixtures were analyzed using Trimmed Spearman-Karber. Development was analyzed based on the mean score plot technique. The toxicity threshold value was calculated for the mixture and for each individual pharmaceutical effect, based on the respective NOEC and LOEC values. These values were then compared with each other	N/A. The pharmaceuticals in the mixture showed toxic effects at concentrations lower than the NOEC for each substance acting alone. Thus, the authors concluded that additive effects occurred, but only at concentrations above those found in the effluent considered (10000x).	Quinn et al. (2009)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Roxithromycin+ Fluoxetine	*Carassius auratus*	Fish	Bioaccumulation; liver EROD, BFCOD and SOD activities; concentration of MDA; CYP1A and CYP3A mRNA expression / 7 d	Unclassified	Three different binary combinations of the components were tested, comprising each one a fixed concentration of roxithromycin in dietary exposure (100 μg Kg^{-1}) and aqueous exposure of fluoxetine at 4, 20 or 100 μg L^{-1}. The reason for the choice of the concentrations was not specified	Two-way ANOVA followed by Tukey´s post-hoc test was applied to test differences across treatments. Principal component analysis was performed to evaluate the variability associated with each biomarker factorial weight.	N/A. The bioaccumulation of roxithromycin in the liver seemed to be significantly increased by addition of fluoxetine; the mixture induced stronger antioxidant responses than the single pharmaceuticals exposures in fish livers	Ding et al. (2016)
Propranolol+ Fluoxetine	*C. auratus*	Fish	Bioaccumulation; liver EROD, BFCOD and SOD activities; concentration of MDA; CYP1A and CYP3A mRNA expression / 7 d	Unclassified	Three different binary combinations of the components were tested, comprising each one a fixed concentration of propranolol in dietary exposure (100 μg Kg^{-1}) and aqueous exposure of fluoxetine at 4, 20 or 100 μg L^{-1}. The reason for the choice of the concentrations was not specified	Two-way ANOVA followed by Tukey´s post-hoc test was applied to test differences across treatments. Principal component analysis was performed to evaluate the variability associated with each biomarker factorial weight.	N/A. The bioaccumulation of propranolol in the liver seemed to be significantly increased by addition of fluoxetine; the mixture induced stronger antioxidant responses than the single pharmaceuticals exposures in fish livers	Ding et al. (2016)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Sulfamethoxazole+ Tetracycline+ Caffeine+ Pentoxifylline+ Acetaminophen+ Ciprofloxacin+ Ofloxacin+ Cephalexin+ Cephradine+ Cephapirin+ Cefazolin+ Naproxen+ Ketoprofen+ Diclofenac+ Piroxicam+ Gemfibrozil+ Salbutamol+ Propranolol+ Atenolol	*Cyprinus carpio*	Fish	Survival / 96 h	Acute	3.19 mg L^{-1} of each pharmaceutical was applied in the mixture. The reason for the choice of this concentration was not specified	Median lethal concentration (LC_{50}) was determined by the Probit or Trimmed Spearman-Karber Methods. The mixture effects were compared to the single pharmaceutical toxicity, by using the same concentrations of each pharmaceutical both in mixture and in individual test	N/A. Individual pharmaceuticals at the same concentrations as applied in the mixture showed no toxicity; however, as a mixture, these concentrations caused the death of half of the fish. The authors suggested a synergistic toxicity for the mixture components	Li and Lin (2015)
Acetaminophen+ Carbamazepine+ Gemfibrozil+ Venlafaxine	*Danio rerio*	Fish	Reproduction, histopathology, development / 6 weeks	Chronic*	Equal concentrations of the compounds (0.5 and 10 µg L^{-1}), based on prior studies of single compound exposures	One-way ANOVA, analysis of co-variance and student's *t*-test for comparisons with the negative controls. The results observed for the mixture were compared to the ones observed for the single pharmaceutical effects, as obtained in a previous test. However, no statistical/mathematical model was used for this.	N/A. The mixture caused similar responses as most of those observed for the compounds individually. However, the additive model is not applicable to some endpoints measured.	Galus et al. (2013)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
17α - Ethinylestradiol+ Levonorgestrel	*D. rerio*	Fish	Expression of the zebrafish *cyp19a1b* gene, using *cyp19a1b* - green fluorescent protein (GFP) transgenic zebrafish line / 96 h (4 d post-fertilisation)	Unclassified	A ray design was used, comprising three different mixture ratios (3:1; 1:1: 1:3), based on the EC_{50} from individual concentration-response curves	Individual concentration-response surfaces were modeled with the Hill model and mixture concentration-responses were modeled with the CA model, added to interaction terms for simple antagonism/synergy, dose-ratio dependent interactions and dose-level dependent interactions	Observed responses were in agreement with the CA model. No deviation of the EC_{50} isobole was observed. The pharmaceuticals exerted additive effects in mixture	Hinfray et al. (2016)
Megestrol acetate+ 17α - ethinylestradiol	*D. rerio*	Fish	Egg production (reproduction); plasma concentrations of 17β - estradiol and testosterone in females and 11-ketotestosterone in males; histological alterations in the ovaries and in testes; transcription of genes involved in steroid production, maturation and ovulation / 21 d	Chronic*	One fixed concentration of 17α - ethinylestradiol (10 ng L^{-1}) was tested in combination with three concentrations of megestrol acetate (33; 100; 333 ng L^{-1}), based on environmentally relevant concentrations	Single and mixture effects were compared by means of analysis of variance followed by Tukey´s test	N/A. Overall, the mixture was reported to induce additive impairment of reproduction function. However, a synergistic effect was suggested by the authors regarding the inhibition of oocyte maturation in the ovary and the reduction of proportions of late vitellogenic/mature oocytes	Hua et al. (2016)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
17α - ethinylestradiol+ Norgestrel	*D. rerio*	Fish	Transcriptional expression profiles of target genes along the HPG axis and circadian rhythm signaling / from 2 - 4 to 96 hours post fertilization	Unclassified	Concentrations of pharmaceuticals used in combination were 50 + 50; 50 + 500; 50 + 5000; 500 + 50; 500 + 500; 500 + 5000 ng L^{-1} of 17α - ethinylestradiol+ norgestrel. The reasons for choosing the concentrations were not detailed in the text	The significance in mRNA expression was analyzed by ANOVA followed by Tukey´s multiple comparison tests.	N/A. Strong transcriptional alterations mainly occurred for the binary mixtures, but not for single 17α - ethinylestradiol and norgestrel groups. The authors suggested that a synergistic interaction was observed for most of gene transcripts in embryonic zebrafish.	Liang et al. (2017)
17β - trenbolone+ 17α - ethinylestradiol	*D. rerio*	Fish	Vitellogenin production, sex ratio and gonad maturation of larvae / From 20 to 60 d post-hatch	Unclassified	Six different mixture concentrations of 17α - ethinylestradiol (2 and 5 ng L^{-1}) combined with 17β - trenbolone (1, 10 and 50 ng L^{-1}) were tested. Concentrations were chosen based on environmental relevance and on potential for inducing adverse effects	For vitellogenin and sex ratio data analysis, analysis of variance followed by Dunnett´s test was performed for multiple comparisons of each treatment with controls. Kruskal-Wallis test followed by the Mann-Whitney U test with Bonferroni-Holm p value adjustments were performed for gonad maturation analysis (compared to the control group)	N/A. The mixture combinations of the pharmaceuticals resulted in significant differences in sex ratios compared with the control group. Severe cases of intersex fish were observed after exposure to 50 ng L^{-1} of trenbolone in combination with 2 ng L^{-1} of ethinylestradiol. Single and mixture effects were not statistically compared.	Örn et al. (2016)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Drospirenone+ Progesterone	*D. rerio*	Fish	Transcriptional changes of up to 14 selected target genes in embryos, including those encoding hormone receptors, a steroidogenic enzyme and estrogenic markers/ from 2-4 h post fertilization until 48 h post fertilization (embryos) and until 96 h and 144 h post fertilization (eleuthero-embryos)	Unclassified	Equal concentrations of both pharmaceuticals were combined in mixture, based on previous studies that showed effects on the reproductive and transcriptional level. Low concentrations were environmentally relevant and highest concentrations were pharmacologically relevant	Significant differences in transcript levels were determined by analysis of variance and Tukey´s test	N/A. Overall, the expressional pattern of the mixture indicated a non-additive interaction	Rossier et al. (2016)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Drospirenone+ 17α - ethinylestradiol	*D. rerio*	Fish	Transcriptional changes of up to 14 selected target genes in embryos, including those encoding hormone receptors, a steroidogenic enzyme and estrogenic markers/ from 2-4 h post fertilization until 48 h post fertilization (embryos) and until 96 h and 144 h post fertilization (eleuthero-embryos)	Unclassified	A fixed-ratio (10:1 drospirenone: 17α - ethinylestradiol) design was used. The mixture was also evaluated at equi-effective concentrations of EC_{02}, EC_{10}, EC_{25}, EC_{30} and EC_{50}.	The CA model and the toxic unit approach were used to predict/ assess the mixture effects. Significant differences in transcript levels were determined by analysis of variance and Tukey´s test.	Overall, the expressional pattern of the mixture indicated a non-additive interaction. Antagonistic interactions were observed for the equi-effective mixtures, based on progesterone receptor transcripts, both by the predicted CA and the Toxic Unit approach. An antagonistic interaction or independent action was suggested regarding the transcriptional responses of some other genes.	Rossier et al. (2016)
Chlormadinone acetate+ 17α - ethinylestradiol	*D. rerio*	Fish	Transcriptional alterations of 15 selected genes of different signaling pathways in embryos/at 2-4 h post fertilization for up to 96 and 144 h post fertilization	Unclassified	The pharmaceuticals were combined at four different concentrations (10+100; 100+1000; 1000+10,000; 100+100 ng L^{-1}), based on previous studies that showed reproductive and transcriptional effects	The data were analyzed using analysis of variance with subsequent Bonferroni test for comparison between treatment and control groups	N/A. The pattern of expressional changes was very similar to 17α - ethinylestradiol alone, suggesting that the mixture acted following the independent action model. However, this model was not effectively tested.	Siegenthaler et al. (2017)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Cyproterone acetate+ 17α - ethinylestradiol	*D. rerio*	Fish	Transcriptional alterations of 15 selected genes belonging to different signaling pathways in eleuthero-embryos /at 2-4 h post fertilization for up to 96 and 144 h post fertilization	Unclassified	The pharmaceuticals were combined at four different concentrations (10+100; 100+1000; 1000+10,000; 100+100 ng L^{-1}), based on previous studies that showed reproductive and transcriptional effects	The data were analyzed using analysis of variance with subsequent Bonferroni test for comparison between treatment and control groups	N/A. An additive interaction was suggested by the authors.	Siegenthaler et al. (2017)
Drospirenone+ Progesterone	*D. rerio*	Fish	Transcriptome and ovarian histological alterations; vitellogenin protein levels / 14 d exposure	Acute	Nominal concentrations (fixed-ratio design) of 50 + 4; 500 + 40 and 5000 + 400 ng L^{-1} of drospirenone and progesterone, respectively, were combined in mixtures. Concentrations chosen were either environmentally and/or pharmacologically relevant	The CA model was used to predict the mixture effects. Principal Component Analysis was performed for differentially expressed gene analysis	The observed effects on transcriptional changes, vitellogenin down-regulation and ovarian histology indicated that mixtures of these two pharmaceuticals acted additively (according to the CA model). The principal component analysis showed that the transcriptional response of the mixture resembles more to that of drospirenone than progesterone	Zucchi et al. (2014)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Medroxyprogesterone acetate+ Dydrogesterone	*D. rerio*	Fish	Reproduction (egg production); histological alterations of ovaries and testes of breeding pairs; transcriptional alterations of 28 genes belonging to different pathways (steroid hormone receptors; steroidogenesis enzymes, circadian rhythm genes) of adult fish and eleuthero-embryos/ adult fish : 14 d pre-exposure; 1 d interval for chemical-dosing; 21 d exposure / at 2-4 h post fertilization for up to 144 h post fertilization for eleuthero-embryos	Chronic*	The pharmaceuticals were combined at concentrations of 50 + 500 and 500 + 5000 ng L-1 medroxyprogesterone acetate + dydrogesterone in the adult fish evaluation. In the embryos exposure, the pharmaceuticals were combined at equal concentrations of 5, 50 and 500 ng L^{-1}. Concentrations chosen were environmentally and/or pharmacologically relevant	The CA model was used to predict the mixture effects. Significant differences between the controls and treatments were evaluated by analysis of variance followed by Tukey´s test	Additive (according to the CA model) or less than/greater than additive effects were observed depending on the transcript and concentration of exposure. The CA model was strongly supported for some transcriptional responses, such as circadian rhythm genes and for other responses such as egg production (at low mixture concentration). However, for other responses, the mixture effect deviated from the CA prediction.	Zhao et al. (2015)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Sulpiride+ Clarithromycin+ Diphenhydramine+ Benzafibrate+ Acetaminophen+ Ketoprofen+ Phenytoin+ Etodolac+ Crotamiton+ Epinastine	*D. rerio*	Fish	Survival of larvae / 9 d	Acute	A fixed ratio design was used, based on the maximum detected concentrations of each pharmaceutical found in effluent samples and in the Tama River (Tokyo, Japan)	2-parameter log-logistic models were used to calculate de concentration-response curves. The observed mixture toxicities were statistically compared to the CA and IA models	The CA model slightly overestimated while the IA model marginally underestimated the observed mixture toxicity. However, the differences were not considered significant by the authors (the differences between observed and predicted values were less than a factor of 2)	Watanabe et al. (2016)
Fluoxetine+ Venlafaxine	*Moronesaxatilis*x *Moronechrysops* (hybrid striped bass)	Fish	Brain serotonin levels decrease and time to capture prey / 6 d exposure plus 6 d of recovery	Unclassified	Fixed ratio design was used based on the LOEC of the single effects of each pharmaceutical. 1 toxic unit, 2 toxic units and 4 toxic units of each pharmaceutical were tested in mixtures	The mixture effects were compared to the effects obtained in the individual pharmaceutical exposures. The authors considered the mixture effects as additive if the predicted additivity, calculated from the effects caused by 1 TU for each individual component, fell within the standard error of the mean for each mixture. A two factors analysis of variance was used	Additive effects of both endpoints analyzed were only predicted at low concentrations (1 TU). At higher concentrations (2 and 4 TU), additivity was unable to be predicted probably because of saturation of serotonin depression in the brains of the exposed fish	Bisesi Jr. et al. (2016)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
17 β - estradiol+ 17α - ethynylestradiol	*Oncorhynchus mykiss*	Fish	Vitellogenin induction in juvenile female - 14 d	Unclassified	A fixed-ratio of 17 β - estradiol: 17α - ethynylestradiol (25:1) was used in the mixture based on the median effect concentrations derived for each pharmaceutical in single experiments. Therefore, estradiol+ ethynylestradiol were applied at the concentrations of 5.0 + 0.20 ng L^{-1}, 12.5 + 0.50 ng L^{-1} and 87.5 +3.5 ng L^{-1} respectively	A four-parameter logit regression model was used to model the mixture data. The CA model was used to model the theoretical concentration-effect relationship of the binary mixture	The CA model predicted the mixture effects only at low effect levels, i.e., at vitellogenin concentrations below 10000 ng L^{-1}, but it was not able to predict the effect mixture at concentrations above this value	Thorpe et al. (2003)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Citalopram+ Cyamemazine+ Fluoxetine+ Oxazepam+ Sertraline+ Valproate	*Oryzias latipes*	Fish	Survival and disruption of larval locomotor behavior / 72 h	Unclassified	Four different concentrations were tested in mixtures corresponding to 1x, 10x, 100x and 1000x the concentrations of each compound measured in the Isle River (France). The following concentrations corresponded to 1x (ng L^{-1}): 15.1 citalopram; 30.3 cyamemazine; 3 fluoxetine; 500 oxazepam; 1.5 sertraline; 400 valproate	Locomotion data were compared between treatments with one-way analysis of variance followed by Tuckey´s test. Larval velocities were tested using repeated measure analysis of variance followed by the Newman-Keuls post hoc test. Lethal and effective concentrations were tested with a non-linear curve fitting based on a sigmoid model. The effects caused by the single pharmaceuticals were compared to the mixture effects. However, no mathematical model or a direct statistical test was used for this comparison	N/A. The mixture induced hypolocomotion and thigmotaxis at concentrations 10 to 100 times lower than with single compounds. The authors assumed that interactions between the pharmaceutical compounds could potentiate the toxicity of individual compounds in the mixture.	Chiffre et al. (2016)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Levonorgestrel+ 17α-ethinylestradiol	*Pimephales promelas*	Fish	Pair breeding assay (reproductive performance): vitellogenin induction; cumulative egg production; morphometric data (lenght; weight; condition factor; abdominal girth; liver somatic index; gonad-somatic index; ovipositor lenght); secondary sexual characteristics (tubercle number; tubercle prominence; fin spots; fatpad index and height); and sex steroid hormone levels (11 ketotestosterone and 17β - estradiol) / 45 days	Chronic*	A fixed-ratio design was used (1:1), over three nominal concentrations (0.25; 2.5 and 12.5 ng L^{-1}) of each individual pharmaceutical. Environmentally relevant concentrations used in mixture were based on expected individual potencies. Mixture effects were reported for both nominal and measured concentrations	The predictive power of the CA model was evaluated by comparing the predicted and observed effects of the mixture on the egg production. The Logit and Weibull regression functions were employed for single concentration-responses regarding egg reproduction	The mixture induced an additive effect (in good agreement with the CA prediction) regarding the egg production. Also for the other endpoints assessed, there was no evidence of any interaction effect, i.e., all the positive responses to the mixture could be explained by the observed effects of one or other (or both) of the individual pharmaceuticals	Runnalls et al. (2015)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Naproxen+ Carbamazepine+ Sulfamethoxazole	*Limnodynastes peronii*	Amphibia	Loss of tactile response / 96 h	Acute	Naproxen and Carbamazepin were applied in the mixture at concentrations ranging from 6.25 to 50 µg L^{-1} while Sulfamathoxazole was applied at concentrations ranging from 25 to 200 µg L^{-1}. The choice of the ratios of the concentrations of the compounds applied in the mixture was not well specified	Repeated-measures analysis of variance were performed to analyze the interactive effects between the mixture components	N/A. An acute increased toxicity was observed with exposure of the organisms to the mixture compared to exposures to the individual pharmaceuticals	Melvin et al. (2014)
Naproxen+ Carbamazepine+ Sulfamethoxazole	*L. peronii*	Amphibia	Tadpole development, snout-vent length, weights and liver weights of tadpoles / 21 d	Chronic	All pharmaceuticals were used each at 10 and 100 µg L^{-1}. The choice of the concentrations of the compounds applied in the mixture was not well specified	Repeated-measures analysis of variance were performed to analyze the interactive effects between the mixture components	N/A. No significant interactive effect was observed for the pharmaceutical mixtures	Melvin et al. (2014)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Diclofenac+ Naproxen+ Atenolol+ Gemfibrozil	*L. peronii*	Amphibia	Detoxification pathways, energy storage, growth, development and swimming performance of larva / 30 d	Unclassified	Equal nominal concentrations of 0.1, 1.0, 10, 100 and 1000 µg L^{-1} were applied of each pharmaceutical. The reason for the choice of the concentrations of the compounds was not specified 1	One-way analysis of variance followed by Tuckey´s test and regression analysis were performed to compare the mixture effects with the control. The single pharmaceutical effects were not compared to the mixture ones	N/A. The author did not compare the effects caused by single pharmaceuticals with the ones caused by the mixture, although this last one caused significant effects at concentrations as low as 10 µg L^{-1} of each pharmaceutical in mixture	Melvin (2016)
Levonorgestrel+ 17α - Ethinylestradiol	*Xenopus tropicalis*	Amphibia	Estrogenic biomarkers: gonadal sex ratio; hepatic mRNA levels of genes encoding vitellogenin and quantification of estrogen, progesterone and androgen receptors / exposure period from freely swimming tadpoles stage until complete metamorphosis	Unclassified	One fixed concentration of ethinylestradiol (29.6 ng L^{-1}) and three concentrations of levonorgestrel were tested in combination (3.12; 31.2 ng L^{-1} and 312 ng L^{-1}). Concentrations used in mixture studies were based on environmental relevant concentrations and on effective concentrations observed from previous studies	The frequencies of phenotypic females in the co-exposure groups were compared to that of the control group and the ethinylestradiol-alone group using one-sided Fisher´s exact test. mRNA levels of all groups were compared using one-way analysis of variance with Tukey´s post-hoc-test for females. mRNA levels of the control and the levonorgestrel-alone group were compared using the unpaired *t*-test for males.	N/A. The mixture did not significantly alter the response of the estrogenic biomarkers: sex ratio and *vtg beta1* expression compared to exposure to ethinylestradiol alone. An antagonistic effect of ethinylestradiol and levonorgestrel was observed on mRNA expression of genes encoding hepatic steroid hormone receptors	Säfholm et al. (2015)

(To be continued)

Table A.1 (continued)

In vitro tests								
Pharmaceutical mixture	**Species**	**Taxonomic group**	**Endpoint/ Duration of the test**	**Type of test**	**Experimental design of the mixture test**	**Model/ software used to assess combination effects**	**Model/ Deviation that better explained the mixture effect**	**Reference**
Furosemide+ 17 β estradiol	*Saccharomyces cerevisiae*	Yeast	Estrogenic activity assessed in recombinant yeast cells containing the human estrogen receptor / 72 h	Unclassified	Equipotent mixtures of the components were tested in various ratios, at the concentration at which 50% of the maximal effect for the standard 17 β estradiol was reached	A non-linear regression using the four-parameter logistic equation was fitted to the various concentrations of the mixture. Isoboles were also used for analysis of the mixture effects. Results were compared to the CA and IA models by a one sample *t*-test.	Better predicted by the CA model, but a trend to synergism was observed depending on the effect level applied.	Fent et al. (2006)
Furosemide+ Phenazone	*Saccharomyces cerevisiae*	Yeast	Estrogenic activity assessed in recombinant yeast cells containing the human estrogen receptor / 72 h	Unclassified	Equipotent mixture concentrations were used by applying each compound at the 20% and 25% of the maximal induction concentration of the standard 17 β estradiol effects.	A non-linear regression using the four-parameter logistic equation was fitted for the various concentrations of the mixture. Isoboles were also used for analysis of the mixture effects. Results were compared to the CA and IA models by a one sample *t*-test	Better predicted by the CA model, but at lower concentrations the mixture effects were higher than the predicted values according to both CA and IA.	Fent et al. (2006)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Furosemide+ Fenofibrate	*Saccharomyces cerevisiae*	Yeast	Estrogenic activity assessed in recombinant yeast cells containing the human estrogen receptor / 72 h	Unclassified	Equipotent mixture concentrations were used by applying each compound at the 1% and 10% of the maximal induction concentration of the standard 17 β estradiol effects.	A non-linear regression using the four-parameter logistic equation was fitted for the various concentrations of the mixture. Isoboles were also used for analysis of the mixture effects. Results were compared to the CA and IA models by a one sample *t*-test	Equally predicted by both the CA and IA models at the 10% effect level, but a lower activity than the predicted by both CA and IA models was observed at the 01% effect level	Fent et al. (2006)
Furosemide+ Cimetidine	*Saccharomyces cerevisiae*	Yeast	Estrogenic activity assessed in recombinant yeast cells containing the human estrogen receptor / 72 h	Unclassified	Equipotent mixture concentrations were used by applying each compound at the 1% and 10% of the maximal induction concentration of the standard 17 β estradiol effects.	A non-linear regression using the four-parameter logistic equation was fitted for the various concentrations of the mixture. Isoboles were also used for analysis of the mixture effects. Results were compared to the CA and IA models by a one sample *t*-test	Equally predicted by both the CA and IA models at the two effect levels evaluated	Fent et al. (2006)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Furosemide+ Paracetamol	*Saccharomyces cerevisiae*	Yeast	Estrogenic activity assessed in recombinant yeast cells containing the human estrogen receptor / 72 h	Unclassified	Equipotent mixture concentrations were used by applying each compound at the 1% and 10% of the maximal induction concentration of the standard 17 β estradiol effects.	A non-linear regression using the four-parameter logistic equation was fitted for the various concentrations of the mixture. Isoboles were also used for analysis of the mixture effects. Results were compared to the CA and IA models by a one sample *t*-test	Equally predicted by both the CA and IA models at the 10% effect level, but better predicted by the CA model at the 01% level	Fent et al. (2006)
Cimetidine+ Fenofibrate	*Saccharomyces cerevisiae*	Yeast	Estrogenic activity assessed in recombinant yeast cells containing the human estrogen receptor / 72 h	Unclassified	Equipotent mixture concentrations were used by applying each compound at the 1% and 10% of the maximal induction concentration of the standard 17 β estradiol effects.	A non-linear regression using the four-parameter logistic equation was fitted for the various concentrations of the mixture. Isoboles were also used for analysis of the mixture effects. Results were compared to the CA and IA models by a one sample *t*-test	Equally predicted by both the CA and IA models at the 10% effect level, but at the 01% effect level, both CA and IA underestimated the mixture effects	Fent et al. (2006)
Furosemide+ Cimetidine+ Fenofibrate	*Saccharomyces cerevisiae*	Yeast	Estrogenic activity assessed in recombinant yeast cells containing the human estrogen receptor / 72 h	Unclassified	Equipotent mixture concentrations were used by applying each compound at the 10% of the maximal induction concentration of the standard 17 β estradiol effects.	A non-linear regression using the four-parameter logistic equation was fitted to the mixtures. Isoboles were also used for analysis of the mixture effects. Results were compared to the CA and IA models by a one sample *t*-test	Both the CA and IA models underestimated the mixture effects	Fent et al. (2006)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Furosemide+ Phenazone+ Fenofibrate	*Saccharomyces cerevisiae*	Yeast	Estrogenic activity assessed in recombinant yeast cells containing the human estrogen receptor / 72 h	Unclassified	Equipotent mixture concentrations were used by applying each compound at the 10% of the maximal induction concentration of the standard 17 β estradiol effects.	A non-linear regression using the four-parameter logistic equation was fitted for the various concentrations of the mixture. Isoboles were also used for analysis of the mixture effects. Results were compared to the CA and IA models by a one sample *t*-test	Both the CA and IA models underestimated the mixture effects	Fent et al. (2006)
Phenazone+ Cimetidine+ Fenofibrate	*Saccharomyces cerevisiae*	Yeast	Estrogenic activity assessed in recombinant yeast cells containing the human estrogen receptor / 72 h	Unclassified	Equipotent mixture concentrations were used by applying each compound at the 10% of the maximal induction concentration of the standard 17 β estradiol effects.	A non-linear regression using the four-parameter logistic equation was fitted for the various concentrations of the mixture. Isoboles were also used for analysis of the mixture effects. Results were compared to the CA and IA models by a one sample *t*-test	Both the CA and IA models underestimated the mixture effects	Fent et al. (2006)
Phenazone+ Cimetidine+ Furosemide	*Saccharomyces cerevisiae*	Yeast	Estrogenic activity assessed in recombinant yeast cells containing the human estrogen receptor / 72 h	Unclassified	Equipotent mixture concentrations were used by applying each compound at the 10% of the maximal induction concentration of the standard 17 β estradiol effects.	A non-linear regression using the four-parameter logistic equation was fitted to the mixtures. Isoboles were also used for analysis of the mixture effects. Results were compared to the CA and IA models by a one sample *t*-test	Both the CA and IA underestimated the mixture effects	Fent et al. (2006)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Furosemide+ Phenazone+ Cimetidine+ Fenofibrate	*Saccharomyces cerevisiae*	Yeast	Estrogenic activity assessed in recombinant yeast cells containing the human estrogen receptor / 72 h	Unclassified	Equipotent mixture concentrations were used by applying each compound at the 1 % and at the 0.05% (NOEC) of the maximal induction concentration of the standard 17 β estradiol effects.	A non-linear regression using the four-parameter logistic equation was fitted for the various concentrations of the mixture. Isoboles were also used for analysis of the mixture effects. Results were compared to the CA and IA models by a one sample *t*-test	Better predicted by the CA model at the 01% effect level, but at the NOEC level, both the CA and IA models underestimated the mixture effect	Fent et al. (2006)
Furosemide+ Phenazone+ Cimetidine+ Fenofibrate+ Paracetamol	*Saccharomyces cerevisiae*	Yeast	Estrogenic activity assessed in recombinant yeast cells containing the human estrogen receptor / 72 h	Unclassified	Equipotent mixture concentrations were used by applying each compound at the 1 % and at 10% of the maximal induction concentration of the standard 17 β estradiol effects.	A non-linear regression using the four-parameter logistic equation was fitted for the various concentrations of the mixture. Isoboles were also used for analysis of the mixture effects. Results were compared to the CA and IA models by a one sample *t*-test	Both the CA and IA models underestimated the mixture effects at both the effect levels evaluated	Fent et al. (2006)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Drospirenone+ Progesterone	*Saccharomyces cerevisiae*	Yeast	Transactivation of the progesterone, androgen and estrogen receptors assessed in recombinant yeast cells expressing the human progesterone, androgen or estrogen receptor / 72h	Unclassified	The pharmaceuticals were combined at equi-effective concentrations, based on the EC_{50}, EC_{25} and EC_{10}, calculated from individual concentration-response curves of each compound	The CA model, (including the isobole method) and the toxic unit approach were used to assess the combination effects. The inverse function of the Hill equation was used in individual concentration-response curves	An overall additivity was observed according to all three methods for progestagenic, androgenic and anti-estrogenic activity. However, at the EC_{10} level, an antagonism was reported according to the Toxic Unit approach for progestagenic activity and according to CA (and isoboles) for androgenic activity	Rossier et al. (2016)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Dydrogesterone+ Medroxyprogesterone acetate	*Saccharomyces cerevisiae*	Yeast	Transactivation of the progesterone, androgen and estrogen receptors assessed in recombinant yeast cells expressing the human progesterone, androgen or estrogen receptor / 72h	Unclassified	The pharmaceuticals were combined at equieffective concentrations, based on the EC_{50}, EC_{25} and EC_{10}, calculated from individual concentration-response curves of each compound	The CA model, (including the isobole method) and the toxic unit approach were used to assess the combination effects. The inverse function of the Hill equation was used in individual concentration-response curves	An overall antagonism was observed according to all three methods for progestagenic and anti-estrogenic activity. For androgenic activity, the approaches suggested different activities, varying from synergism at low doses and antagonism at high doses (CA model), to an overall additivity (Toxic Unit) or overall synergism (isoboles)	Rossier et al. (2016)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Drospirenone+ 17α - ethinylestradiol	*Saccharomyces cerevisiae*	Yeast	Transactivation of the progesterone, androgen and estrogen receptors assessed in recombinant yeast cells expressing the human progesterone, androgen or estrogen receptor / 72h	Unclassified	The pharmaceuticals were combined at equieffective concentrations, based on the EC_{50}, EC_{25} and EC_{10}, calculated from individual concentration-response curves of each compound	The CA model, (including the isobole method) and the toxic unit approach were used to assess the combination effects. The inverse function of the Hill equation was used in individual concentration-response curves	An overall additivity was observed according to all three methods for progestagenic and androgenic activity at the EC_{25} and EC_{50} levels. However, at the EC_{10} level, an antagonism was reported according to the Toxic Unit approach for progestagenic activity and according to all the three methods for androgenic activity	Rossier et al. (2016)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Chlormadinone acetate+ Cyproterone acetate	*Saccharomyces cerevisiae*	Yeast	Transactivation of the progesterone receptor assessed in recombinant yeast cells expressing the human progesterone receptor / 72h	Unclassified	The pharmaceuticals were combined at equieffective concentrations of EC_{50}, EC_{25} and EC_{10} for progestogenic activity, based on their individual concentration-response curves	The CA model, (including the isobole method) and the toxic unit approach were used to assess the combination effects. The inverse function of the Hill equation was used in individual concentration-response curves	At the EC_{50} level, deviation from the CA model was observed. An antagonistic interaction was confirmed by the isobole and the toxic unit approach. At the EC_{10} and EC_{25} levels, an additive behavior was observed following the isobole method, but antagonism was observed following the toxic unit approach	Siegenthaler et al. (2017)
Chlormadinone acetate+ 17α - ethinylestradiol	*Saccharomyces cerevisiae*	Yeast	Transactivation of the progesterone receptor assessed in recombinant yeast cells expressing the human progesterone receptor / 72h	Unclassified	The pharmaceuticals were combined at equieffective concentrations of EC_{50}, EC_{25} and EC_{10} for progestogenic activity, based on their individual concentration-response curves	The CA model, (including the isobole method) and the toxic unit approach were used to assess the combination effects. The inverse function of the Hill equation was used in individual concentration-response curves	At the EC_{50} and EC_{25} levels, antagonism was observed for all the three approaches employed. At the EC_{10} level, additivity was observed.	Siegenthaler et al. (2017)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Ciprofloxacin+ Erythromycin+ Novobiocin+ Oxytetracycline+ Sulfamethoxazole+ Trimethoprim	*Elliptio complanata*	Mollusca	Immune parameters (hemocyte viability; thiols; ROS; phagocytosis; lysozyme; nitric oxide; cyclooxygenase) observed from exposition of hemolymph / 24 h	Unclassified	Antibiotics in the mixture were tested at environmentally relevant concentrations and proportions (40, 200, 1000 and 5000 ng/L for oxytetracycline; 20, 100, 500 and 2500 ng/L for ciprofloxacin and novobiocin; and 10, 50, 250 and 1250 ng/L for erythromycin, sulfamethoxazole and trimethoprim)	One-way analysis of variance followed by Dunnett test was performed for comparisons of treatment groups, including the mixture. Correlations analyses were evaluated by a Pearson - moment test. A discriminant function analysis was performed to find out the principal components responsible for the mixture toxicity	N/A. No additive effect of the antibiotics was observed. However, opposite effects between the mixture and the antibiotics alone were revealed by the drastic reduction of the cyclooxygenase activity caused only by the mixture. Furthermore, the immunotoxicity of the antibiotic mixture was different from that of novobiocin, oxytetracycline and ciprofloxacin alone.	Gust et al. (2012)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Atenolol+ Benzafibrate+ Carbamazepine+ Cyclophosphamide Ciprofloxacin+ Furosemide+ Hydrochlorothiazide Ibuprofen+ Lincomycin+ Ofloxacin+ Ranitidine+ Salbutamol+ Sulfamethoxazole	*Danio rerio*	Fish	Zebrafish liver cells proliferation, gene expression / 72 h	Unclassified	Concentrations (ng L^{-1}) of each pharmaceutical applied in mixture were similar to the combination of drugs detected in northern Italian rivers (environmental levels). Moreover, the mixture was also tested at 0.1, 10 and 100 times the environmental levels	Proliferation data were analyzed by comparing the effects of increasing levels of the pharmaceutical mixture in cells with respect to the controls. Relevance interaction networks and cluster analysis were also performed. The effects posed by the single pharmaceutical components were not compared to the mixture ones	N/A. The mixture effects were not compared to the individual expositions. Nonetheless, the pharmaceutical mixture inhibited cells proliferation and caused transcriptional changes of genes involved in primary metabolism, regulation of the cell cycle, inflammation processes, DNA-damage and related signal transduction and caused changes in receptors and proteins linked to endocrine disrupting effects	Pomati et al. (2007)

(To be continued)

Table A.1 (continued)

Pharmaceutical mixture	Species	Taxonomic group	Endpoint/ Duration of the test	Type of test	Experimental design of the mixture test	Model/ software used to assess combination effects	Model/ Deviation that better explained the mixture effect	Reference
Ibuprofen+ Naproxen+ Ketoprofen+ Diclofenac	*Oncorhynchus mykiss*	Fish	Cytotoxicity for the rainbow trout liver cell line RTL-W1, evaluated by monitoring metabolic activity and cell membrane integrity / 24 h	Unclassified	A fixed ratio design was used, based on the EC_{50} of each mixture constituent, comprising 0.1, 0.5, 1, 2 and 5 TU	The allosteric decay model was used to fit the data obtained for the mixture. Predicted values were determined considering the CA and IA models	Accurately predicted by the CA model	Schnell et al. (2009)
Fenofibrate+ Clofibrate+ Benzafibrate+ Gemfibrozil	*Oncorhynchus mykiss*	Fish	Cytotoxicity for the rainbow trout liver cell line RTL-W1, evaluated by monitoring metabolic activity and cell membrane integrity / 24h	Unclassified	A fixed ratio design was used, based on the EC_{50} of each mixture constituent, comprising 0.1, 0.5, 1, 2 and 5 TU	The allosteric decay model was used to fit the data obtained for the mixture. Predicted values were determined considering the CA and IA models	Accurately predicted by the CA model	Schnell et al. (2009)
Fluoxetine+ Paroxetine+ Fluvoxamine	*Oncorhynchus mykiss*	Fish	Cytotoxicity for the rainbow trout liver cell line RTL-W1, evaluated by monitoring metabolic activity and cell membrane integrity / 24 h	Unclassified	A fixed ratio design was used, based on the EC_{50} of each mixture constituent, comprising 0.1, 0.5, 1, 2 and 5 TU	The allosteric decay model was used to fit the data obtained for the mixture. Predicted values were determined considering the CA and IA models	More accurately predicted by the IA model	Schnell et al. (2009)

* According to the Technical Guidance nº 27 of the European Commission (2011), the EC_{50} from the 72-h algae test or from the 7 d Lemna sp. test is considered as acute, while the NOEC or EC_{10} of the same test is regarded as a chronic value. Therefore, in this review, the algae and macrophyte test was classified as acute and/or chronic according to the point estimates (EC_{50}, EC_{10} and/or NOEC) assessed in the respective experimental designs, as proposed by the European Commission (2011). According to this same protocol, tests with cyanobacteria are considered as additional algal data and thus cyanobacteria data were classified according this same scheme. Reproduction studies with fish can be considered as chronic toxicity studies, according to the European Commission (2011).

EC_x - Effect concentration at x %
NOEC - Non effect concentration
CA - Concentration addition
IA - Independent action
CI - Combination Index
TU - Toxic Unit
N/A - Not applicable
EROD - 7-ethoxyresorufin O-deethylase
BFCOD - 7-benzyloxy-4-trifluoromethyl-coumarin O-dibenzyloxylase
SOD - superoxide dismutase
MDA - malondialdehyde
CAT - catalase
GR - Glutathione reductase
GST -Glutathione-S-transferase
AChE -Acetylcholinesterase
ROS - Reactive oxygen species
LOEC – lowest effect concentration

Source: Godoy and Kummrow (2017)

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4.2 Article II

Ecotoxicological effects, water quality standards and risk assessment for the anti-diabetic metformin

Aline Andrade Godoy[a,b], Inês Domingues[c], António José Arsénia Nogueira[c], Fábio Kummrow[a,d*]

[a]Department of Clinical and Toxicological Analyses, Faculty of Pharmaceutical Sciences, University of São Paulo (USP), Avenida Professor Lineu Prestes, 580, São Paulo, SP, 05508-000, Brazil.

[b]Science and Technology Institute, Federal University of Alfenas (Unifal-MG), Rodovia José Aurélio Vilela, 11,999, Poços de Caldas-MG, 37715-400, Brazil.

[c]Department of Biology & CESAM, University of Aveiro, *Campus* Universitário de Santiago, 3810-193, Aveiro, Portugal.

[d]Institute of Environmental, Chemical and Pharmaceutical Sciences, Federal University of São Paulo (Unifesp), Rua São Nicolau, 210, 09972-270, Diadema, SP, Brazil.

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ABSTRACT

Metformin (MET) is among the most consumed pharmaceuticals worldwide. This compound has been frequently detected in fresh surface water. However, ecotoxicological information for MET is still too limited, particularly regarding chronic and behavioral data. This study aimed to help filling these knowledge gaps, by carrying out both acute and chronic studies with four different test organisms from three different trophic levels. We assessed different endpoints, including the swimming behavior of *Danio rerio* larvae. We also derived both short-term and long-term environmental quality standards (EQS) for the protection of freshwater pelagic biota towards MET adverse effects. A risk quotient (RQ) was calculated for MET in fresh surface water, considering a worst-case scenario. *Daphnia similis* was by far the most sensitive species evaluated. An EC_{10} of 4.4 mg L^{-1} was obtained from the reproduction test with *D. similis*. A long-term EQS of 88 µg L^{-1} was derived and a RQ of 0.38 was obtained. An ecological risk is not expected for the chronic exposure of pelagic freshwater species to MET, considering the endpoints and the standard bioassays usually recommended in standard protocols. However, endocrine disruptive effects and potential interactive effects of MET with other co-occurring contaminants cannot be ruled out. To the best of our knowledge, this study presents the first data related with MET effects on population endpoints of *D. similis* and *Hydra attenuata*, as well as on the locomotor activity of *D. rerio*.

Keywords: Biguanide; CRED; Ecological risk; Locomotor activity; Population relevance; Sensitive endpoint

1. Introduction

Active Pharmaceutical Ingredients (APIs) are among the contaminants considered of emerging concern by the scientific community due to their potential environmental risk (Godoy and Kummrow, 2017). Of special environmental relevance are the pharmaceuticals sharing the properties of (1) high production volume; (2) environmental persistence; and (3) biological activity, especially considering long-term exposure (Fent et al., 2006). The anti-diabetic metformin (MET) appears to be a potential candidate to meet these requirements.

The biguanide MET is the first-line oral therapy and the most widely used oral agent prescribed for type 2 diabetes (Foretz et al., 2014; Rena et al., 2013). This drug also presents one of the highest consumption rates of all pharmaceuticals worldwide (Scheurer et al., 2012). Rena et al. (2013) estimate that over 100 million patients are prescribed MET annually worldwide. Moreover, it is considered to be one of the APIs with the largest emissions into the environment on a mass basis from wastewater treatment plants (WWTP) (Crago et al., 2016; Dong et al., 2013; Kosma et al., 2015; Scheurer et al., 2009). These findings are partly due to the increasing number of people affected by diabetes mellitus. Projection from the International Diabetes Federation points out that the number of people with diabetes worldwide will increase from 415 million in 2015 to 642 million by 2040 (IDF, 2015).

It must be also highlighted the large quantities of MET required for therapeutic effects, with daily dosage varying from 500 to up to 2500 mg L^{-1} (Rena et al., 2013; Trautwein and Kümmerer, 2011). In addition to its high consumption, MET is excreted unaltered in the urine (Bailey and Turner, 1996), which makes the emissions of this pharmaceutical after consumption relevant. Trautwein et al. (2014) highlighted the high rates of removal of MET in WWTP (93 to 97 %), mainly due to its microbiological transformation into guanylurea. Despite of these high removal rates, this compound has been detected at relatively high concentrations in effluent and surface waters due to its high influent load (Oosterhuis et al., 2013). Moreover, since MET lacks functional groups that hydrolyze under environmental conditions, hydrolysis is not likely to occur with this compound (ter Laak and Baken, 2014). As a consequence, the aquatic organisms may be exposed to considerable concentrations of this API. In fact, unexpected high concentrations of this hydrophilic pharmaceutical (27.8 ng g^{-1}) were observed in sculpin fishes (*Leptocottus armatus*) from the Nisqually estuary (Meador et al., 2016).

Regarding the biological activity, MET acts by inhibiting complex I in the mitochondrial electron transport chain in humans, leading to adenosine triphosphate (ATP) depletion and an increase in adenosine monophosphate (AMP) levels (Foretz et al., 2014; Rena et al., 2013).

Although these same mechanisms remain relatively unexplored on non-target organisms, the crucial involvement of mitochondria in the molecular mechanism of action of MET could raise an alert on its potential effects also on this organelle of aquatic invertebrates and vertebrates, following the concept of evolutionary conserved molecular drug targets. In fact, Pinho et al. (2013) showed that zebrafish and mammalian mitochondria display high genetic and functional homology and proved that mitochondrial inhibitors, such as antimycin, myxothiazol, rotenone and oligomycin, induced developmental and cardiovascular dysfunctions in this fish species. Therefore, the possible adverse effects of MET on non-target organisms deserve special investigation.

However, despite its high production, prescription, environmental load and persistence, information on ecotoxic effects of MET is still scarce, especially taking into account long-term effects, which hampers interpretation of its risk (ter Laak and Baken, 2014). Therefore, ter Laak and Baken (2014) recommend filling this knowledge gap in order to allow that proper interpretation of monitoring results and environmental risk assessment (ERA) for this pharmaceutical can be achieved.

In this sense, the present study aimed to assess the ecotoxicological effects of MET, in short and long-term studies with organisms of different trophic levels and including different endpoints of proved population relevance. The swimming behavior was also considered in our study, by monitoring the effects of MET on the locomotor activity of *Danio rerio* (zebrafish). Behavior has been demonstrated to give rise to very sensitive measures of stress exposure (Andrade et al., 2016; Henriques et al., 2016). Moreover, locomotor behavior has a relevant connection with survival of populations (Scott and Sloman, 2004). In the *D. rerio* case, we hypothesized that possible physiological dysfunctions via ATP depletion, already shown to be induced by mitochondrial complex I inhibitors such as rotenone (Pinho et al., 2013), could ultimately impact locomotor activity of zebrafish exposed to MET.

Moreover, by assessing several endpoints from standard (*Lemna minor*, *Daphnia similis* and *D. rerio*) as well as a non-standard species (*Hydra attenuata*), we aimed to enlarge the ecotoxicological database regarding MET adverse effects. Based on our results and on reports from the literature, we also aimed to derive environmental quality standards (EQS) for the protection of freshwater pelagic biota towards MET, based on relevant and reliable ecotoxicity tests. We finally aimed to assess the environmental risk posed by MET, considering a worst-case scenario.

2. Materials and methods

2.1 Chemicals

Metformin hydrochloride (1,1-Dimethylbiguanide hydrochloride; CAS number 115-70-4), was provided by Abhilasha Pharma (India), with 99.2 % purity. Stock solutions and tested concentrations were achieved by dissolving MET in the appropriate test medium for each organism without using any solvent. All compounds used for composition of the respective test medium were of high purity (> 98 %), supplied by Sigma-Aldrich (Brazil) or by Merck (Germany).

2.2 Analytical determination of MET in the test medium

In order to confirm the nominal concentrations of MET used in the chronic tests, chemical analyses were performed using a double beam UV-visible spectrophotometer (Cintra 6, GBC scientific equipment). The methodology used for the analyses was in accordance with the procedure described in the U.S. Pharmacopoeia (USP, 2015). Prior to the analysis, spectral scans were performed at the concentration of 10 mg L^{-1} of MET, dissolved in each culture medium as well as in distilled water, in order to confirm the maximum absorption peak established by the U.S. Pharmacopoeia for distilled water. The peak of absorbance of 232 nm for MET was used for quantification, using the respective test medium as a blank. The limit of detection (LOD) and limit of quantification (LOQ) for the analyses of MET were determined, respectively, by the following mathematical formulas:

$$\text{LOD} = (3.3\ s)/\text{S} \quad (1)$$

$$\text{LOQ} = (10\ s)/S \quad (2)$$

in which s = the estimate of the standard deviation of the blank samples (n = 10) and S = the slope of the calibration curve.

The parameters obtained for the calibration curves (linearity, determination coefficient and equations) as well as the analytical results are described in Appendix A of Supplementary material. The quantification of MET in Steinberg medium was not possible, due to the noise of the baseline observed for the analysis of the Steinberg medium at the 232 nm. However,

previous stability tests were carried out for the MET in distilled water, at the same conditions of exposure time, temperature and luminosity as those used in the *L. minor* toxicity tests (Table A.5 of Appendix A of Supplementary material) and proved the stability of this pharmaceutical during the 7-d exposure time.

2.3 Test organisms

L. minor, *D. similis* and *H. attenuata* test organisms were provided by the Laboratory of Ecotoxicology and Genotoxicity (LAEG), State University of Campinas, Unicamp (Brazil). *L. minor* plants were maintained in Steinberg medium (OECD, 2006), pH 5.5 ± 0.2, conductivity 900 ± 50 µS cm^{-1}, at 24 ± 2°C and under continuous cool white fluorescent lighting with light intensity of 6500 lux. Young plants without visible lesions or discoloration (chlorosis) were selected for the tests using a magnifying glass.

D. similis were cultivated in MS medium (ABNT NBR 12713, 2016), hardness 40 - 48 mg L^{-1} $CaCO_3$, conductivity 200 ± 20 µS cm^{-1}, at 20 ± 2 °C and under a photoperiod of 16:8 h light/dark. The organisms were fed three times a week with the algae *Raphidocelis subcapitata*. Neonates less than 24 h old, derived from a healthy culture, were used for the tests.

H. attenuata were cultivated in *Hydra* medium (Trottier et al., 1997), pH 7.0 ± 0.1. The *H. attenuata* organisms designated to acute tests were maintained at 22 ± 2°, under a 16:8 h light-dark photoperiod, according to Trottier et al. (1997). The organisms were fed three times a week with newly hatched nauplii of *Artemia salina*. Animals were unfed 24 h prior to testing. Only *H. attenuata* organisms without buds were selected for the acute tests. *H. attenuata* organisms designated to chronic tests were cultivated at 25 ± 0.5 °C, under a 12h photoperiod, according to Holdway (2005). Stock animals were fed twice a week up until one week prior to a test when they were fed daily to achieve maximal budding rates. Only *H. attenuata* organisms representing a hydroid (one animal with one tentacled bud) were selected for the chronic tests.

Zebrafish (*D. rerio*) eggs were supplied by the facility established at the Department of Biology of the University of Aveiro (Portugal). About 30 min after natural mating of adult fish, the eggs were collected and rinsed in fish system water. Using a stereomicroscope (Stereomicroscope Zoom Microscope-SMZ 1500, Nikon Corporation), the unfertilized eggs and the injured embryos were screened and excluded. The adult zebrafish are cultivated in carbon-filtered water, pH 7.5 ± 0.5, conductivity of 750 ± 50 µS cm^{-1}, dissolved oxygen at 95 % saturation, at 26 ± 1°C and photoperiod cycle of 16:8 h light/dark. The fishes were fed twice a day with commercial artificial diet (ZM 500 Granular).

2.4 Ecotoxicity tests

2.4.1 L. minor growth inhibition test

L. minor toxicity tests were performed according to the OECD guideline 221 (OECD, 2006). Twelve fronds (using only colonies with three fronds each and with similar total area), were assigned to each of the 250 mL glass beakers containing 100 mL of the following concentrations of MET dissolved in Steinberg medium: 0, 6.2, 12.5, 25.0, 50.0, 100.0, 200.0 and 400.0 mg L^{-1}. Stock concentrations (10000 mg L^{-1}) were prepared immediately before each test. Three control and treatment replicates were used for each tested concentration. The tests were carried out at the same conditions of luminosity and temperature as described for the maintenance of the macrophytes. Three independent tests were carried out. Test duration was 7 days. Despite the verified stability of MET in the test conditions, a semi-static method was adopted, with renewal of the test solutions each 48 h, in order to allow a better background transparency for taking pictures for frond area evaluation. After exposure, the specific average growth rates (μ) were determined based on the endpoints frond number, total frond area and fresh weight. The evaluation of each endpoint was performed according to Godoy et al. (2015). The pH and dissolved oxygen were measured at the beginning and at the end of the tests (Appendix B of Supplementary Material)

2.4.2 D. similis acute and chronic toxicity test

D. similis acute toxicity tests were performed according to the ABNT NBR 12.713/2016 (ABNT, 2016) and OECD guideline 202 (OECD, 2004). Five neonates < 24 h old, from 2 to 3-week-old mothers, were transferred to each of the 15 mL vials containing 10 mL of the following concentrations of MET dissolved in MS medium: 0, 5.0, 8.0, 12.5, 20.0, 30.0 and 50.0 mg L^{-1}. Stock concentrations (500 mg L^{-1}) were prepared immediately before each test. Four control and treatment replicates were used for each tested concentration. The photoperiod and temperature conditions used in the tests were the same ones as for the maintenance of the daphnids. Three independent tests were carried out. After 48 h, the number of immobile daphnids was recorded. The pH, dissolved oxygen and conductivity were measured at the end of the tests (Appendix B of Supplementary Material).

D. similis chronic toxicity tests were performed according to the OECD guideline 211 (OECD, 2012), with the modification of the exposure time to 14 days for this species, according to Vacchi et al. (2016). 1 neonate < 24 h old, from the third progeny, was transferred to each of the 50 mL recipients containing 40 mL of the following concentrations of MET dissolved in MS medium: 0, 1.0, 3.0, 5.0, 8.0 and 11.0 mg L^{-1}. These concentrations were prepared from a stock concentration of 1000 mg L^{-1}, which was prepared immediately before each test. Ten control and treatment replicates were used for each tested concentration. The test organisms were fed daily with *R. subcapitata*. The test medium was renewed every two days. The photoperiod and temperature conditions of exposure were the same ones as used for the maintenance of the daphnids culture. Three independent tests were carried out. The number of living offspring produced by each parent animal was counted daily and summed up for matters of assessing reproduction inhibition. The pH, dissolved oxygen, conductivity and hardness were measured once a week, in fresh and old media (Appendix B of Supplementary Material).

2.4.3 H. attenuata acute and chronic toxicity test

H. attenuata acute toxicity tests were performed according to Trottier et al. (1997). Three *Hydras* without buds were introduced into each of the wells of 12-well plates containing 5 mL of the following concentrations of MET dissolved in *Hydra* medium: 2000; 2300; 2700; 3100; 3600; 4200 and 5000 mg L^{-1}. Stock concentrations (20000 mg L^{-1}) were prepared immediately before each test. Control and treatments were performed in triplicate. The photoperiod and temperature conditions were the same ones as used for the cultivation of the organisms. Three independent assays were performed. After 96 h of exposure, the morphological changes were recorded. The clubbed and shortened tentacle stages were selected as endpoints of sub-lethality, while the tulip and disintegrated stages (considered irreversible) were the endpoints recorded for lethality. The pH and conductivity were measured at the end of the tests (Appendix B of Supplementary Material).

The chronic toxicity tests with *H. attenuata* were carried out according to the Holdway (2005) protocol. Five hydroids were placed into each of the glass dishes filled with 35 mL of the following concentrations of MET, dissolved in *Hydra* medium, from a stock solution daily prepared (10000 mg L^{-1}): 0; 200; 360; 650; 1200 and 2000 mg L^{-1}. Control and treatments were performed in triplicate. The photoperiod and temperature used were the same ones as used for the maintenance of the organisms. Three independent assays were carried out. The test organisms were fed daily with *A. salina* nauplii and the test medium was renewed daily after

feeding period (2 h). The number of hydroids in each replicate was counted daily during a period of 7 days in order to calculate the mean relative population growth rate (K) for each concentration. The pH, conductivity and dissolved oxygen were evaluated at the beginning and at the end of each test (Appendix B of Supplementary Material).

2.4.4 Fish embryo acute toxicity (FET) test

FET-tests with *D. rerio* were performed following the OECD guideline 236 (OECD, 2013). One newly fertilized egg (3 hours post-fertilization (hpf)) was transferred to each of the 24 wells of the plate, filled with 2 mL of the following concentrations of MET, dissolved in zebrafish water system, from a stock solution of 2000 mg L^{-1}: 0; 100; 180; 330; 600; 1100; 1500 and 2000 mg L^{-1}. One 24-well plate was used for each test concentration. Each of these plates contained 20 eggs, each one in a well containing 2 mL of a determined test concentration (replicates) and 4 eggs, each one in a well containing 2 mL of dilution water (internal plate control). One 24-well plate was used for negative control (24 eggs in dilution water). The tests were performed under the same photoperiod and temperature conditions as used for the cultivation of the adult fishes. A total of three independent tests were carried out. Every 24 h, lethal and sub-lethal endpoints such as coagulation of fertilized eggs, lack of somite formation, lack of tail detachment, hatching success, pigmentation failures, edema (heart and yolk), spinal deformation (scoliosis) and heart beat rate (beats/20 s) were observed and reported using a stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon Corporation). The pH, conductivity and dissolved oxygen were evaluated at the end of each test (Appendix B of Supplementary Material).

2.5 Behavioral assessment

MET concentrations not inducing any abnormalities or mortality in the FET-test (0; 0.05; 0.5; 5.0; 50.0; 100.0; 180.0; 330.0 and 600.0 mg L^{-1}) were selected for the behavioral assay. Because of the high variability of behavioral responses and in order to increase the statistical power of the experiment, three independent sets of dilution water control groups were used, called CT1, CT2 and CT3, in accordance with Margiotta-Casaluci et al. (2014). The locomotor activity of zebrafish was evaluated at 120 hpf. A total of 20 embryos per concentration (each embryo individually placed in each well) had their activity tracked using

the system Zebrabox-Zeb (Viewpoint Life sciences, Lyon, France). Recordings were made directly on the 24-well plates used for exposure. Distances and time moving were recorded for each 60 s integration period, during light (25 % light intensity) - dark intervals over a period of 25 min, by alternating 10 min dark and 10 min light, after a 5 min light adaptation period. A transparent background mode with a detection threshold of 20 was set. Behavioral endpoints measured were total swimming distance (TSD) and total swimming time (TST).

2.6 Reference substances and control charts

Test procedure was checked using NaCl (Sigma-Aldrich, 99 % purity) as a reference substance (positive control) for *L. minor*, *D. similis* and *H. attenuata*. The control charts for the tests using NaCl are shown in Fig. C1, C2 and C3 (Appendix C of Supplementary Material). The 3,4-dichloroaniline (Sigma-Aldrich, 98 % purity) at 4 mg L^{-1} was tested as a reference substance in order to check the test procedure regarding the *D. rerio* bioassays (results are shown in Table C.1 of Appendix C of Supplementary Material).

2.7 Data analysis

The results obtained in each independent test with the respective test organisms were grouped for building the respective concentration-response curves and calculating the ecotoxicity data. Median effective/lethal concentrations (E/LC_{50}) and the respective 95 % confidence intervals (95 % C.I.) were calculated by non-linear regression analysis (allosteric decay model), using an automated Excel spreadsheet (ToxCalcMix, v. 1.0). This automated spreadsheet was developed at the University of Aveiro & CESAM, Portugal (available at https://pydio.bio.ua.pt/public/toxcalcmix), according to the equations and models described elsewhere (Barata et al., 2006). The EC_{10} values (corresponding to the non-observed effect concentration, NOEC) and the respective 95 % C.I. were determined using regression analysis, by applying the models that showed the best fit to the data, considering the residual analysis and the coefficient of determination (R^2). In the case of *H. attenuata* and *L. minor*, a four-parameter-logistic-fit (sigmoidal dose-response model) was the model that best fit to the data, while a three-parameter-logistic-fit (sigmoidal logistic model) was better applied to the *D. similis* data. The OriginPro *software* (v. 9.4.0.220, USA) was used for these regression analyses. A one-way analysis of variance (ANOVA) was used to infer statistically significant differences between treatments and controls in the behavioral tests. Prior analysis, ANOVA assumptions

were verified. Normality was tested using histogram analysis and the Shapiro-Wilk test. Homogeneity of variances was performed using the Bartlett test. When the ANOVA assumptions were not met, data were log-transformed or a Kruskal-Wallis test was performed. The Box and Whiskers charts, including the statistical analysis for identifying outliers in the behavioral tests, were performed on Microsoft Excel (version 2016).

2.8 Evaluation of the reliability and relevance of the ecotoxicity test

We aimed to provide a complete and adequate reporting regarding the methodology and presentation of the results of our ecotoxicity data, aiming to guide risk assessors in performing unbiased and transparent evaluations, as it was recommended by Moermond et al. (2016). Therefore, the Criteria for Reporting and Evaluating Ecotoxicity Data (CRED) method (Moermond et al., 2016) was applied for reporting the acute and the chronic ecotoxicity data used in the PNEC and EQS derivation (Tables D.1 and D.2 of Appendix D of Supplementary Material). Raw data are presented in Appendix E of Supplementary Material.

2.9 EQS derivation and risk assessment

EQS values for protecting freshwater pelagic community from adverse effects of MET were derived according to the Technical Guidance for Deriving Environmental Quality Standards (TGD EQS) on the Water Framework Directive (2000/60/EC) (European Commission, 2011). EQS values were derived considering both a long-term and a short-term exposure. The long-term EQS, expressed as an annual average concentration (AA-EQS), was based on chronic ecotoxicity data while the short-term EQS, referred to as a maximum acceptable concentration (MAC-EQS), was derived based on acute ecotoxicity data. Although the aquatic biota is usually continuously exposed to pharmaceuticals such as metformin, we decided to also derive the short-term standard value in order to take into account possible incidental concentration peaks. The deterministic approach was used for deriving the AA-EQS and the MAC-EQS values, by applying an adequate assessment factor (AF) to the lowest reliable and relevant ecotoxicity data from the dataset. It must be highlighted that the EC_{50} from the *L. minor* tests were considered acute values, while the EC_{10} from these same tests were regarded as chronic values, according to the European Commission (2011) guideline. All the values used for deriving the EQS were expressed in terms of the pharmacological base content (MET) present in the hydrochloride salt.

PNEC value for ERA was derived according to the European Commission (2003) and EMEA (2006) protocols, by applying an adequate AF to the lowest relevant EC_{10}/NOEC value from the available dataset. In our study, the PNEC derivation for ERA purposes was based on long-term rather than on short-term toxicity data. This is because it is supposed a continuous exposure of the aquatic organisms to pharmaceuticals, via WWTP effluents discharge (EMEA, 2006). The highest measured environmental concentration (MEC) value of MET in fresh surface water used for risk assessment was retrieved from the literature, from a previous analysis of reliable data (Table F.1 of Appendix F of Supplementary Material). From Table F.1, only analytical studies reporting the LOD and LOQ or the Laboratory Reporting Limit (which is based on the detection limit of the method) were considered for composing reliable $MEC_{surface\ water}$. A risk quotient (RQ) was calculated by dividing the highest $MEC_{surface\ water}$ by the PNEC estimated based on long-term toxicity data, in order to allow for a realistic worst-case scenario.

It is worth mentioning that although a PNEC estimated as a part of a risk assessment is an important precursor in the derivation of an EQS, PNEC and EQS are not the same. While a PNEC is a tool used in the risk assessment, an EQS is a legally binding limit value (Merrington et al. 2018). The TGD EQS (European Commission, 2011) also highlights conceptual differences between EQS derivation and the estimation of a PNEC. Among those differences, it is pointed out that the EQS is required to protect a higher proportion of waterbodies compared to the PNEC estimated as part of a risk assessment (European Commission, 2011). Therefore, although the process of deriving EQS is similar to that used in the estimation of a PNEC, we decided to calculate both EQS and PNEC in this paper, following the respective guidelines.

The updated review tables containing the acute and the chronic toxicity data for metformin reported in the literature are described in Appendix G of Supplementary material. Only the data validated according to the CRED method and/or the Klimisch et al. (1997) scheme were used for directly deriving the EQS and for estimating the PNEC (see Appendix G of Supplementary Material).

3. Results and discussion

3.1 MET ecotoxicity and quality of the data

As the concentration of MET was satisfactorily maintained within the 80-120 % interval of the nominal concentration throughout the chronic tests (Table A.1 of Appendix A of Supplementary Material), the analyses of the results were always based on nominal values. In

the case of the *L. minor* test (Steinberg medium), the reported results were also based on nominal values. The validation criteria of the acute tests with *L. minor*, *D. similis*, *H. attenuata* and *D. rerio* were met, which confirm the validity of the results (raw data available in Appendices B and D of Supplementary Material). The EC_{50} values and respective 95 % C.I. for the acute ecotoxicity data obtained in this study are shown in Table 1. Validation criteria were also met for the chronic assays (raw data available in Appendices B and D of Supplementary Material). The respective concentration-response curves are available in Appendix H of Supplementary Material. The EC_{50} and EC_{10} values and respective 95 % C.I. for the chronic ecotoxicity data obtained in this study are shown in Table 2.

Table 1 Lethal/effect concentration at 50 % (L/EC_{50}) values obtained in the acute tests carried out for evaluating the toxicity of metformin. In brackets are indicated the 95 % confidence limits

Test organism	Toxicological endpoint	Ecotoxicity data (95 % C.I.) mg L^{-1}
Lemna minor	$EC_{50\text{-}7d}$ – growth inhibition (frond number)	58.9 (56.7 - 61.0)
L. minor	$EC_{50\text{-}7d}$ –growth inhibition (total frond area)	53.7 (51.7 – 55.6)
L. minor	$EC_{50\text{-}7d}$ – growth inhibition (fresh weight)	58.7 (56.4 – 61.1)
Daphnia similis	$EC_{50\text{-}48h}$ - immobilization	14.3 (13.8 - 14.8)
Hydra attenuata	$LC_{50\text{-}96h}$ – lethality	3918.0 (3905.0 – 3931.0)
H. attenuata	$EC_{50\text{-}96h}$ – morphological changes	2709.0 (2215.0 – 3203.0)
Danio rerio	$LC_{50\text{-}96h}$ – lethality	1315.5 (1278.3 – 1352.7)

Source: Godoy et al. (2018)

Table 2 Effect concentration at 10 % (EC_{10}) values obtained in the chronic tests carried out for evaluating the toxicity of metformin. In brackets are indicated the 95 % confidence limits

Test organism	Toxicological endpoint	Ecotoxicity data (95 % C.I.) mg L^{-1}
Lemna minor	$EC_{10\text{-}7d}$ – growth inhibition (frond number)	24.2 (21.6 – 26.8)
L. minor	$EC_{10\text{-}7d}$ – growth inhibition (total frond area)	31.9 (29.2 – 34.8)
L. minor	$EC_{10\text{-}7d}$ – growth inhibition (fresh weight)	31.6 (28.0 – 35.6)
Daphnia similis	$EC_{10\text{-}14d}$ - reproduction	4.4 (3.0 – 5.6)
Hydra attenuata	$EC_{10\text{-}7d}$ – reproduction	701.8 (610.6 – 790.8)

Source: Godoy et al. (2018)

The MET acute and chronic toxicity ranged up to 2 orders of magnitude among the test species (Tables 1 and 2), *D. similis* being by far the most sensitive species. The EC_{50} and EC_{10} values obtained in this study for the acute and chronic effects of MET on *D. similis* (Tables 1 and 2) were in the same concentration range but slightly lower than the results reported in the literature for the species *Daphnia magna*. For instance, Cleuvers (2003) reported a 48h-EC_{50} of 64 mg L^{-1} and Moermond and Smit (2016) calculated a 21 d - NOEC geometric mean of 11.5 mg L^{-1} regarding the effects of MET on the survival and reproduction of *D. magna*, respectively.

On the other hand, *H. attenuata* was the least sensitive test organism to both the acute and chronic MET effects. This finding runs counter to the observation that *Hydras* (diploblastic organisms) are sensitive environmental indicators to a variety of toxicants, including pharmaceuticals (Quinn et al., 2008; 2009).

The producer species *L. minor* showed intermediate sensitivity to the adverse effects posed by MET compared to the consumers test species evaluated in this study. There was not a statistically significant difference among the three evaluated endpoints ($\alpha = 0.05$). The EC_{50} values obtained for the MET effects on the growth inhibition of *L. minor* (Table 1) were lower than the $EC_{50} > 320$ mg L^{-1} reported for the algae *Desmodesmus subspicatus* by Cleuvers (2003). Similarly, the EC_{10} values obtained in our study were equally lower than the NOEC $\geq$ 78 mg L^{-1} reported for the algae *R. subcapitata* by EMEA (2011).

Regarding the effects induced on *D. rerio* in the FET-test, lethality, malformations and changes in the number of heart beats in relation to the control groups were not statistically significant ($\alpha = 0.05$) in larvae exposed to up to 600 mg L^{-1} of MET. The $NOEC_{malformations}$ obtained in the FET-test was of 600 mg L^{-1}. Malformations such as scoliosis and abnormal pigmentation appeared significant only at concentrations from 1100 mg L^{-1}. Similarly, regarding the swimming behavior, *D. rerio* larvae showed no statistically significant difference ($\alpha = 0.05$) compared to the control groups in MET exposure concentrations up to 600 mg L^{-1}, at 120 hpf (Fig. 1), considering both the dark and the light cycles. Therefore, although mitochondrial respiratory chain inhibitors may affect zebrafish development and cardiovascular function (Pinho et al., 2013), those effects were observed in *D. rerio* larvae only at MET exposure concentrations far above the ones in which this pharmaceutical is usually reported in fresh surface water (see Appendix F of Supplementary Material). Likewise, the swimming behavior of zebrafish larvae seems not to be disrupted at concentrations in which MET is usually detected in fresh surface water. Therefore, although some studies have shown that behavioral endpoints seem to be more sensitive if compared to the classical ones usually

recommended in standardized protocols (Andrade et al., 2016; Henriques et al., 2016), this finding was not observed for the acute exposure of zebrafish to MET.

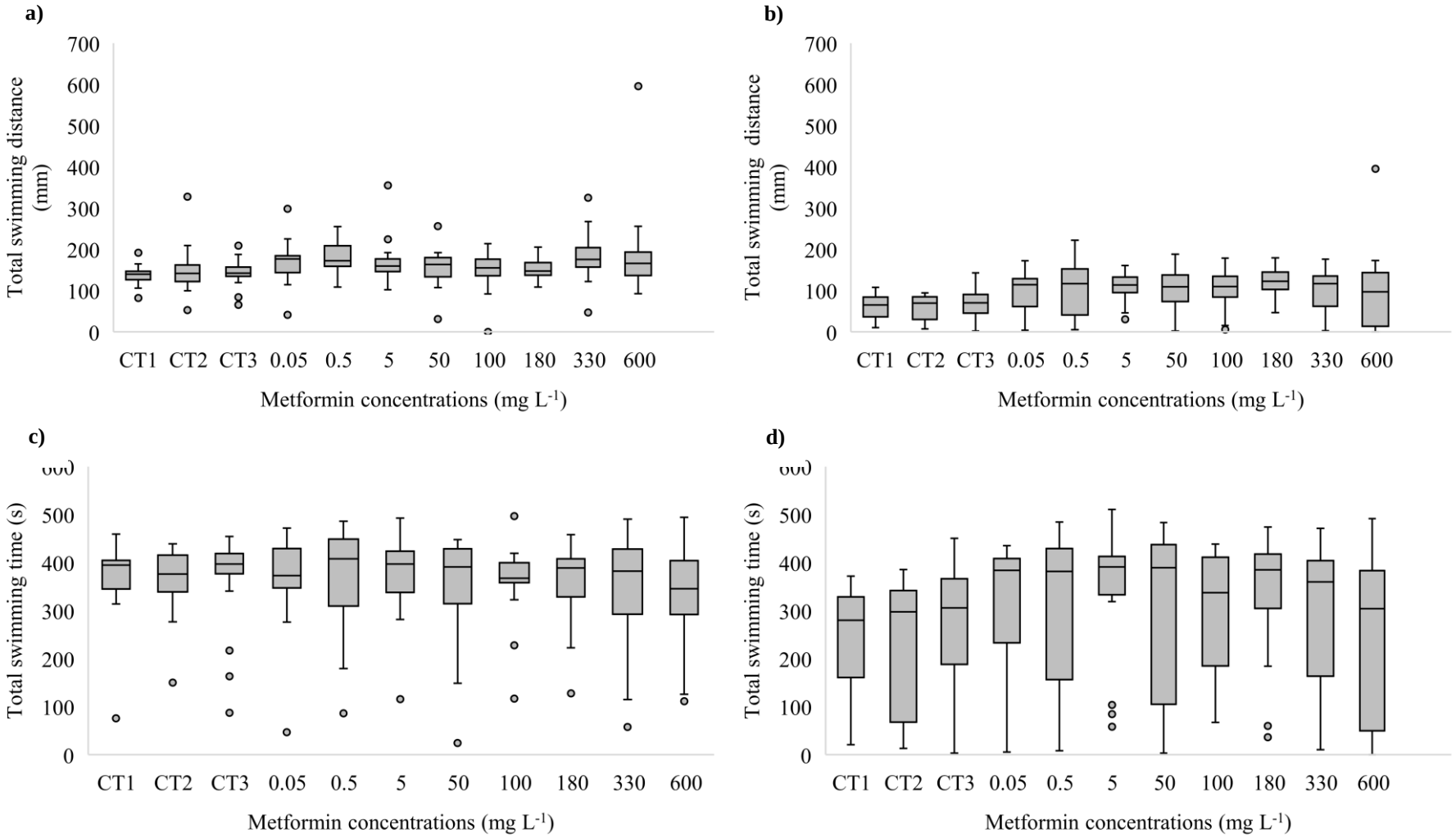

Fig. 1 Effect of metformin on the *Danio rerio* larvae locomotor behavior quantified after 120 h post-fertilization. A) Total swimming distance at the dark cycle (10 min); B) Total swimming distance at the light cycle (10 min); C) Total swimming time at the dark cycle (10 min); D) Total swimming time at the light cycle (10 min). CT1, CT2 and CT3 indicate control group 1, control group 2 and control group 3, respectively. Boxes represent medians (full line), with 5th and 95th percentiles (n = 24 for controls and n = 20 for treatments) and the respective outliers. Source: Godoy et al. (2018)

Overall, according to the 48h-EC_{50} and the 168h-EC_{50} acute values obtained, respectively, for the crustacea and the macrophyte (Table 1), MET can be classified as hazardous to the aquatic environment, in the categories acute and chronic III, following the Harmonized Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures, proposed by the OECD (2002).

As both acute and chronic endpoints evaluated in the *D. similis* tests were the most sensitive ones regarding the MET effects, they were selected for the PNEC estimation and the EQS derivation. Our studies fulfilled 19 of the 20 reliability criteria (except for being strictly no GLP study) and 11 of the 13 relevance criteria (the other two not being applicable) (Tables D.1 and D.2 of Appendix D of Supplementary Material).

3.2 EQS derivation for aquatic life protection and risk assessment

The AA-EQS and MAC-EQS were derived for protection of the freshwater pelagic community, using data from this study and from the literature. The ecotoxicity data directly used for deriving the acute and the chronic standards are shown respectively in Tables 3 and 4. (For the complete review of the toxicity data reported in the literature for MET, see Appendix G of Supplementary Material).

Table 3 Ecotoxicity data regarding the acute effects induced by metformin, used for deriving environmental quality standard – maximum acceptable concentration (MAC-EQS) for the protection of freshwater pelagic organisms

Taxonomic group	Trophic level	Species	Endpoint	L/EC_{50} ($mg\ L^{-1}$)	Reference
Algae	Producer	*Desmodesmus subspicatus*	Growth inhibition – 72 h	>320	Cleuvers (2003)
Algae	Producer	*Raphidocelis subcapitata*	Growth rate – 72 h	>77.2	Confidential data reported by Moermond and Smit (2015)
Macrophyte	Producer	*Lemna minor*	Growth inhibition – 7 d (frond area)	53.7*	This study
Crustacean	Primary consumer	*Daphnia magna*	Immobilization – 48 h	64.0	Cleuvers (2003)
Crustacean	Primary consumer	*Daphnia similis*	Immobilization – 48 h	14.3	This study
Cnidarian	Secondary consumer	*Hydra attenuata*	Morphological alterations – 96 h	2709.0**	This study
Fish	Secondary consumer	*Danio rerio*	Lethality – 96 h	1315.5	This study

*Value obtained for the most sensitive endpoint (total frond area) from studies with *Lemna minor*.

** Value obtained for the most sensitive endpoint (morphological alterations) from this study with *Hydra attenuata*

Source: Godoy et al. (2018)

Table 4 Ecotoxicity data regarding the chronic effects induced by metformin, used for deriving environmental quality standard – annual average (AA-EQS) for the protection of freshwater pelagic organisms.

Taxonomic group	Trophic level	Species	Endpoint	EC_{10}/NOEC (mg L^{-1})	Reference
Algae	Producer	*Raphidocelis subcapitata*	Growth inhibition – 96 h	≥ 78	EMEA (2011)
Macrophyte	Producer	*Lemna minor*	Growth inhibition (frond number) – 7 d*	24.2*	This study
Crustacean	Primary consumer	*Daphnia similis*	Reproduction – 14 d	4.4	This study
Crustacean	Primary consumer	*Daphnia magna*	Reproduction – 21 d	Geometric mean = $(17 \times 7.8)^{1/2}$ = 11.5	EMEA (2011) and Janssen confidential data reported by Moermond and Smit (2015)
Cnidarian	Secondary consumer	*Hydra attenuata*	Reproduction – 7 d	701.8	This study
Fish	Secondary consumer	*Danio rerio*	Hatching rate, time to hatch, survival, length, weight – 30 d post-hatch	≥ 10	EMEA (2011)

*Value obtained for the frond number (most sensitive endpoint considering the effect concentration at 10 % - EC_{10}) assessed in this study with *Lemna minor*.

Source: Godoy et al. (2018)

From Table 3, we can observe that at least one short-term L(E)C_{50} from each of the three trophic levels of the base set is available. Because of the unbounded values reported for algae, it is not possible to assess whether the standard deviation of the log transformed L/EC_{50} values from the Table 3 is lower than 0.5. Anyway, considering only the bounded acute data from Table 3, this standard deviation is higher than 0.5. Moreover, until the moment, there is no certainty that MET has a specific mode of action (Moermond and Smit, 2016). Therefore, following the TGD EQS (European Commission, 2011), an AF of 100 was applied to the EC_{50} obtained in our acute study with *D. similis* (14.3 mg L^{-1}), resulting in a MAC-EQS of 0.143 mg L^{-1} (143 µg L^{-1}). Similarly, the complete data set was available for the chronic effects of MET (Table 4). From Tables 3 and 4, it is possible to see that *D. similis* showed to be the most sensitive species to both the short-term and the long-term exposure. Therefore, an AF of 10 would be normally applied. However, the TGD EQS (European Commission, 2011) also establishes that a larger AF may be needed when there are indications that a substance may induce adverse effects via endocrine disruption. This seems to be the case of MET. Niemuth and Klaper (2015) showed that MET at 40 µg L^{-1} induced the development of intersex gonads in adult males of *Pimephales promelas*, as well as reduced size of treated male fish and reduced fecundity of treated pairs. Niemuth et al. (2015) also demonstrated that MET at this same concentration induced significant up-regulation of messenger ribonucleic acid (mRNA) encoding the egg-protein vitellogenin in adult male *P. promelas*, indicating possible endocrine disruption. Finally, Crago et al. (2016) observed a significant increase of mRNA expression of vitellogenin, estrogen receptor-alpha (ERα), cytochrome P450 3A4-like isoform (CYP3A126) and gonadotropin releasing hormone (GnRH3) in juvenile *P. promelas* exposed to MET at 1, 10 and 100 µg L^{-1}. In view of these indications that MET may induce adverse effects via disruption of the endocrine system of fish, an increase of the AF from 10 to 50 is reasonable, following the dossier proposed for MET and its transformation product guanylurea by the Oekotoxzentrum, Centre Ecotox (2016). Therefore, the EC_{10} of 4.4 mg L^{-1}, obtained in our study with *D. similis*, was divided by an AF of 50, resulting in an AA-EQS of 0.088 mg L^{-1} (88 µg L^{-1}).

The values derived in our study for MET are lower than the MAC-EQS = 640 µg L^{-1} and the AA-EQS = 780 µg L^{-1} values obtained by Moermond and Smit (2016). Likewise, our EQS values are lower than the ones proposed by the Oekotoxzentrum, Centre Ecotox (2016), which derived a MAC-EQS = 640 µg L^{-1} and an AA-EQS = 156 µg L^{-1} for MET. This is due to the more pronounced MET adverse effects observed on the immobilization of *D. similis* (this study) compared to *D. magna* (Cleuvers, 2003), as well as the higher effects obtained in our

study for the inhibition of reproduction of *D. similis* compared to the unbounded NOEC value reported for *Pimephales promelas* (≥ 7.8 mg L^{-1}) by Moermond and Smit (2016). This shows that deriving EQS is a continuous, dynamic process and that EQS values must be revised as new ecotoxicity data are generated. In addition, our study contributes in generating only bounded values for deriving EQS for MET, which decreases the degree of uncertainty generated in studies such as the performed by Moermond and Smit (2016), who used some unbounded values for deriving the standard values.

It is worth mentioning that the results at the mRNA level obtained for the exposure of *P. promelas* to MET (Niemuth et al., 2015; Crago et al., 2016) cannot yet be directly used for deriving EQS, since their relevance at population level is still unclear (European Commission, 2011). However, as these studies represent a potential indication of the MET endocrine disrupting effects, these endpoints might be reconsidered when a definitive correlation or a causal relationship with population sustainability can be settled (European Commission, 2011). Likewise, the results obtained by Niemuth and Klaper (2015) cannot also be directly included in the EQS proposal, since that study was classified as not clearly valid for this purpose (effects were assessed at the only one tested concentration, 40 µg L^{-1}), according to the CRED method (Oekotoxzentrum, Centre Ecotox, 2016).

A long-term PNEC was estimated for MET by using the same ecotoxicity data set as described for AA-EQS derivation (Table 4). Like the TGD EQS (European Commission, 2011), the European Commission (2003) protocol for risk assessment also mentions that an increase of the AF would be appropriate when there is evidence of endocrine disrupting effects. Therefore, although the EMEA (2006) protocol establishes that an AF of 10 should be applied to the lowest NOEC/EC_{10} from the base set, an increase of the AF from 10 to 50 is reasonable in the case of MET, for the reasons previously discussed. The MEC, AF and EC_{10} values used for calculating the PNEC and the RQ are shown in Table 5.

Table 5 Risk quotient (RQ) based on maximum measured environmental concentrations (MEC) reported in the literature for the detection and quantification of metformin in fresh surface water. The predicted non-effect concentration (PNEC) value was estimated by the application of an assessment factor (AF) to the lowest EC_{10}/NOEC value obtained from chronic tests

MEC (µg L^{-1})	Reference	EC_{10} (µg L^{-1})	Reference	AF	PNEC	MEC/PNEC (RQ)
33.60	Elliott et al. (2017)	4400.00	This study with *Daphnia similis*	50	88.00	0.38

Source: Godoy et al. (2018)

The RQ obtained for MET was lower than 1, thus indicating that an ecological risk is not expected for the chronic exposure of freshwater pelagic species to MET, considering the endpoints and the standard bioassays usually recommended for standard protocols. However, it is worth mentioning that existing standard toxicity assays may underestimate the adverse effects of substances acting via disruption of the endocrine system. Niemuth and Klaper (2015) performed a non-standard toxicity test and showed that MET induced adverse effects on fish using a sensitive endpoint of population relevance (sex score). The adverse effects found by these authors occurred at a concentration (40 µg L^{-1}) that is more than 100-fold lower than the lowest EC_{10} value (4400 µg L^{-1}) obtained in our study from standard toxicity tests. In addition, the concentration at which Niemuth and Klaper (2015) found the adverse effects on *P. promelas* is only slightly above the highest MEC value of MET quantified in fresh surface water (33.6 µg L^{-1}) by Elliott et al. (2017). Moreover, it must be highlighted that the number of prescriptions of MET has been increasing annually, due to the epidemic levels of people diagnosed with diabetes (Briones et al., 2016; Chen et al., 2012). Since MET is excreted 100 % unaltered in the urine and has a relatively high stability in aqueous solutions (Sharma et al., 2010), its increasing consumption may ultimately result in increased concentrations of this pharmaceutical in aquatic environments. Therefore, a long-term environmental risk for aquatic organisms exposed to MET cannot yet be ruled out.

Moreover, it is worth mentioning that MET, like other pharmaceuticals, does not occur isolated in aquatic environments. Backhaus et al. (2016) highlighted that since the toxicity of mixtures is higher than of that observed for each individual pharmaceutical (at the concentration at which it is present in the mixture), compliance with individual environmental quality standards does not necessarily guarantee protection against adverse mixture effects. Therefore, the possible individual contribution of MET to the overall toxic potential of the complex mixture of compounds found in the environment should also be considered. This is especially concerning since MET may act as an endocrine disruptor in fish (Overturf et al., 2015) and thus this pharmaceutical could induce potential interactive effects with other co-occurring endocrine disruptors of environmental concern.

4. Conclusion

This study presented effective concentrations calculated based on several relevant and reliable endpoints, from standard as well as non-standard tests with aquatic organisms exposed to MET. To the best of our knowledge, this study presents the first data related with MET effects

on population endpoints of *D. similis* and *H. attenuata*, as well as on the locomotor activity of *D. rerio*.

This study also contributed generating and using only bounded values for deriving EQS values for the protection of freshwater pelagic communities against adverse effects from MET, which decreases the uncertainties from other studies. In addition, this study helps to fill the knowledge gaps regarding the effects of contaminants of emerging concern on behavior of non-target organisms, since studies employing this type of endpoint are still scarce in the literature.

RQ calculated according to standard current protocols showed that MET is not expected to pose an ecological risk to the aquatic organisms, at the current maximum concentrations it is detected in fresh surface water. However, a long-term environmental risk cannot yet be ruled out for this pharmaceutical, considering its tendency of increasing consumption, as well as its potential endocrine disrupting effects and its contribution to the toxic potential of mixtures of environmental concern. Additional chronic tests aiming to evaluate other relevant endpoints are demanded in order to refine the EQS derivation and the risk assessment presented in our study. For this purpose, future studies should focus on searching for a concentration-response pattern and establishing a clear correlation of sensitive endpoints with population sustainability.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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SUPPLEMENTARY MATERIAL

Ecotoxicological effects, water quality standards and risk assessment for the anti-diabetic metformin

APPENDIX A - Analytical parameters and measurements assessed during chronic tests with metformin

Table A.1 - Analytical parameters for the calibration curves obtained in the spectrophotometric analyses of metformin (λ = 232 nm) in the test medium of *Daphnia similis*

Test number	Week	Limit of Detection	Limit of quantification	Equation of calibration curve	Determination coeficiente (R^2)
1	1	0.0076	0.0232	y = 0.100x + 0.006	0.999
	2	0.0091	0.0277	y = 0.100x + 0.001	0.999
2*	1	0.0058	0.0177	y = 0.101x + 0.001	1.000
3	1	0.0040	0.0123	y = 0.102x	0.995
	2	0.0055	0.0166	y = 0.103x	0.999

*In the test number 2, calibration curve and measurements were made only in the first week, due to instrumental problems

Source: Godoy et al. (2018)

Table A.2 - Measured concentrations of metformin in the test medium of *Daphnia similis*, obtained for the two weeks of analyses for each of the three chronic toxicity tests performed

Chronic toxicity test number 1					
		Fresh medium		Old medium	
Week	Nominal concentration (mg L^{-1})	Measured concentration (mg L^{-1})	Percentage of the measured concentration in relation to the nominal one	Measured concentration	Percentage of the measured concentration in relation to the nominal one
1	1.0	1.0	101.0	0.9	90.0
	3.0	3.0	100.3	2.7	89.7
	5.0	5.0	100.4	4.6	91.6
	8.0	8.1	101.1	7.6	95.0
	11.0	11.0	100.0	10.6	96.5
2	1.0	0.9	91.0	1.2	120.0
	3.0	2.9	97.0	3.2	106.7
	5.0	4.9	97.2	5.2	104.0
	8.0	7.8	98.1	8.2	102.5
	11.0	10.8	98.5	11.2	101.8
Chronic toxicity test number 2					
		Fresh medium		Old medium	
Week	Nominal concentration (mg L^{-1})	Measured concentration (mg L^{-1})	Percentage of the measured concentration in relation to the nominal one	Measured concentration	Percentage of the measured concentration in relation to the nominal one
1	1.0	1.0	102.0	1.0	102.0
	3.0	3.0	100.0	3.0	99.7
	5.0	5.0	100.6	4.9	99.6
	8.0	8.1	101.1	8.0	100.5
	11.0	11.0	100.0	11.0	99.8
Chronic toxicity test number 3					
		Fresh medium		Old medium	
Week	Nominal concentration (mg L^{-1})	Measured concentration (mg L^{-1})	Percentage of the measured concentration in relation to the nominal one	Measured concentration	Percentage of the measured concentration in relation to the nominal one
1	1.0	0.9	98.0	0.9	94.0
	3.0	2.9	98.7	2.9	97.3
	5.0	4.9	99.0	4.9	99.8
	8.0	7.9	99.0	7.9	99.1
	11.0	10.9	99.0	10.9	99.2
2	1.0	1.3	129.0	1.3	120.0
	3.0	3.3	109.7	3.2	107.7
	5.0	5.3	106.8	5.3	105.4
	8.0	8.3	104.0	8.2	103.1
	11.0	11.3	102.8	11.2	102.2

Source: Godoy et al. (2018)

Table A.3 - Analytical parameters for the calibration curves obtained in the spectrophotometric analyses of metformin (λ = 232 nm) in the test medium of *Hydra attenuata*

Test number	Limit of detection	Limit of quantification	Equation of calibration curve	Determination coeficiente (R^2)
1	0.0060	0.0183	y = 0.101x + 0.006	0.999
2	0.0040	0.0122	y = 0.101x	0.999
3	0.0092	0.0280	y = 0.101x + 0.002	1

Source: Godoy et al. (2018)

Table A.4 - Measured concentrations of metformin (λ = 232 nm) in the test medium of *Hydra attenuata*

Chronic test number 1		
Nominal concentration (mg L^{-1})	Measured concentration (mg L^{-1})	Percentage of the measured concentration in relation to the nominal one
200	178.0	89.0
360	340.7	94.6
650	627.6	96.5
1200	1202.5	100.2
2000	2004.6	100.2
Chronic test number 2		
Nominal concentration (mg L^{-1})	Measured concentration (mg L^{-1})	Percentage of the measured concentration in relation to the nominal one
200	188.4	94.2
360	321.1	89.2
650	526.6	81.0
1200	1059.9	88.3
2000	1891.5	94.6
Chronic test number 3		
Nominal concentration (mg L^{-1})	Measured concentration (mg L^{-1})	Percentage of the measured concentration in relation to the nominal one
200	199.7	99.9
360	347.2	96.4
650	633.8	97.5
1200	1168.7	97.4
2000	2005.1	100.2

Source: Godoy et al. (2018)

Table A.5 – Stability analysis of metfomin dissolved in distilled water, at the same conditions of exposure time, temperature and luminosity as those used in the *Lemna minor* toxicity tests

Calibration curve			
Limit of detection	Limit of quantification	Equation of the calibration curve	Determination coefficient (R^2)
0.0111	0.034	y = 0.102x – 0.003	0.9999
Stability analyzes			
Day	Nominal concentration (mg L^{-1})	Measured concentration (mg L^{-1})	Percentage of the measured concentration in relation to the nominal one
0	0.5	0.6	110.1
	2.0	2.0	98.8
	3.5	3.4	97.6
	5.0	5.0	100.4
	6.5	6.5	100.0
	9.5	9.5	99.9
	11.0	11.0	99.8
3	0.5	0.5	108.6
	2.0	2.0	98.0
	3.5	3.4	97.4
	5.0	5.0	99.8
	6.5	6.5	99.9
	9.5	9.5	100.1
	11.0	11.0	100.1
7	0.5	0.5	108.4
	2.0	2.0	99.8
	3.5	3.5	99.3
	5.0	5.1	101.5
	6.5	6.6	101.2
	9.5	9.5	100.3
	11.0	11.0	99.8

Source: Godoy et al. (2018)

APPENDIX B – Physical-chemical parameters assessed during the ecotoxicological tests

Table B.1 Physical-chemical parameters measured at the end of the acute test with *Daphnia similis*

Concentration (mg L^{-1})	pH	Dissolved oxygen (mg L^{-1})	Conductivity (µS cm^{-1})
0.0	6.97	7.79	216.3
5.0	6.69	7.36	221.1
8.0	6.89	7.32	231.5
12.5	6.89	7.31	227.4
20.0	6.92	7.32	232.8
30.0	6.90	7.30	239.8
50.0	6.98	7.26	253.8

Source: Godoy et al. (2018)

Table B.2 Physical-chemical parameters measured at the end of the acute test with *Hydra attenuata*

Concentration (mg L^{-1})	pH	Conductivity (µS cm^{-1})
0	6.81	276.4
2,000	6.70	942.0
2,300	6.72	1114.0
2,700	6.70	1493.0
3,100	6.70	1498.0
3,600	6.71	1535.0
4,200	6.70	1974.0
5,000	6.68	2599.0

Source: Godoy et al. (2018)

Table B.3 Physical-chemical parameters measured at the end of the acute test with *Danio rerio*

Concentration (mg L^{-1})	pH	Dissolved oxygen (mg L^{-1}) (Percentage of saturation)	Conductivity (µS cm^{-1})
0	7.7	7.8 (84 %)	950
100	7.9	7.8 (84 %)	930
1,500	7.4	8.0 (87 %)	1705
2,000	7.9	8.0 (88 %)	2250

Source: Godoy et al. (2018)

Table B.4 Physical-chemical parameters measured during the performance of the chronic tests with *Daphnia similis.* DO = Dissolved oxygen. The terms new and old refer, respectively, to the freshly prepared media and old media (after 48 h of exposure).

		Test n° 1				Test nº 2				Test nº 3			
Concentration (mg L^{-1})	Parameters	Week 1		Week 2		Week 1		Week 2		Week 1		Week 2	
		New	Old	New	Old	New	Old	New	Old	New	Old	New	Old
Control	Conductivity (µS cm^{-1})	216.2	211.9	225.3	218.9	210.1	211.3	212.4	213.9	221.8	227.2	222.8	224.2
	DO (mg L^{-1})	7.10	6.87	6.98	6.90	6.73	7.55	7.69	7.94	8.43	-	8.12	8.48
	pH	6.51	7.09	6.55	6.86	6.41	6.62	7.14	6.72	6.91	6.91	6.54	6.66
	Hardness (mg L^{-1} $CaCO_3$)	45	42	43	45	41	42	43	42	46	43	44	43
1	Conductivity (µS cm^{-1})	214.8	213.1	224.0	219.4	210.8	211.4	213.0	215.7	220.6	226.5	224.0	224.3
	DO (mg L^{-1})	7.05	6.92	6.93	6.72	6.61	7.42	6.74	7.96	8.48	-	8.15	8.41
	pH	6.60	7.14	6.46	6.92	6.41	6.70	6.89	6.82	6.57	6.90	6.59	6.54
	Hardness (mg L^{-1} $CaCO_3$)	46	44	45	46	40	41	44	41	44	45	44	43
3	Conductivity (µS cm^{-1})	216.0	214.2	225.7	222.5	211.5	213.0	212.3	218.2	221.9	226.7	225.4	226.9
	DO (mg L^{-1})	6.94	7.01	6.94	6.72	6.68	7.61	6.79	8.42	8.47	-	8.14	8.50
	pH	6.49	7.22	6.54	6.77	6.48	7.11	6.76	7.32	6.58	6.95	6.63	6.69
	Hardness (mg L^{-1} $CaCO_3$)	45	44	42	44	40	44	45	41	44	43	44	45
5	Conductivity (µS cm^{-1})	220.6	216.9	228.4	225.7	214.9	212.7	214.8	213.6	225.6	232.4	227.0	228.0
	DO (mg L^{-1})	6.89	7.16	6.95	6.99	6.56	7.78	6.71	8.25	8.47	-	8.01	8.43
	pH	6.57	7.25	6.53	6.94	6.48	7.13	6.66	7.01	6.58	6.93	6.66	6.65
	Hardness (mg L^{-1} $CaCO_3$)	42	43	45	48	41	41	42	41	44	44	42	44
8	Conductivity (µS cm^{-1})	205.9	216.3	231.1	228.1	217.3	216.0	217.4	219.2	225.2	231.7	230.0	226.0
	DO (mg L^{-1})	6.93	7.02	6.90	6.47	6.60	7.65	6.77	7.98	8.50	-	8.08	7.79

(To be continued)

Table B. 4 (continued)

Concentration (mg L^{-1})	Parameters	Week 1		Week 2		Week 1		Week 2		Week 1		Week 2	
8	pH	6.54	7.15	6.52	6.85	6.50	7.03	6.67	6.86	6.60	6.99	6.64	6.70
	Hardness (mg L^{-1} $CaCO_3$)	44	42	45	48	41	42	43	41	45	44	43	42
11	Conductivity (µS cm^{-1})	217.3	220.9	232	231.6	218.6	216.5	221.4	221	228.5	234.3	234.3	229.1
	DO (mg L^{-1})	7.07	6.86	6.94	6.46	6.58	7.59	6.79	7.96	8.42	-	7.42	8.67
	pH	6.58	7.14	6.56	6.81	6.49	7.08	6.67	6.81	6.59	6.94	6.60	6.73
	Hardness (mg L^{-1} $CaCO_3$)	43	43	48	46	40	41	42	42	45	44	43	44

Source: Godoy et al. (2018)

Table B.5 Physical-chemical parameters measured during the performance of the chronic tests with *Hydra attenuata.* DO = dissolved oxygen

		Test n° 1		Test nº 2		Test nº 3	
Concentration (mg L^{-1})	Parameters	Initial day	Final day	Initial day	Final day	Initial day	Final day
Control	DO (mg L^{-1})	7.39	7.21	7.93	7.66	7.67	7.35
	pH	6.92	6.64	6.80	6.60	6.85	6.57
	Conductivity (µS cm^{-1})	246.3	266.0	267.9	287.4	253.1	275.0
200	DO (mg L^{-1})	7.77	7.12	7.87	7.30	7.92	7.28
	pH	6.93	6.67	6.88	6.70	6.95	6.68
	Conductivity (µS cm^{-1})	377.0	410.6	420.0	456.8	389.0	437.0
360	DO (mg L^{-1})	7.79	7.09	7.81	7.07	7.83	7.06
	pH	6.90	6.72	6.88	6.70	6.90	6.69
	Conductivity (µS cm^{-1})	493.0	578.6	524.0	602.1	480.0	592.0
650	DO (mg L^{-1})	7.69	7.04	7.75	7.06	7.79	7.00
	pH	6.91	6.80	6.91	6.81	6.89	6.82
	Conductivity (µS cm^{-1})	703.0	807.6	739.0	834.5	696.0	810.0
1.200	DO (mg L^{-1})	7.73	7.13	7.83	7.25	7.74	7.11
	pH	6.86	6.69	6.89	6.71	6.92	6.70
	Conductivity (µS cm^{-1})	1089.0	1200.3	1142.0	1234.9	1027.0	1166.0
2.000	DO (mg L^{-1})	7.70	6.99	7.82	6.88	7.80	6.83
	pH	6.85	6.83	6.84	6.83	6.88	6.85
	Conductivity (µS cm^{-1})	1631.0	1878.9	1705.0	1928.5	1475.0	1721.0

Source: Godoy et al. (2018)

Table B.6 Physical-chemical parameters measured during the performance of the chronic tests with *Lemna minor*. DO = dissolved oxygen. Godoy et al. 2018

		Test n° 1		Test nº 2		Test nº 3	
Concentration (mg L^{-1})	Parameters	Initial day	Final day	Initial day	Final day	Initial day	Final day
Control	DO (mg L^{-1})	8.50	8.19	7.45	6.85	8.20	8.39
	pH	5.64	6.27	5.35	6.77	5.46	6.35
	Conductivity (µS cm^{-1})	932	939	892	831	933	889
6.2	DO (mg L^{-1})	7.88	8.17	7.48	7.53	8.17	8.38
	pH	5.63	6.17	5.68	6.84	5.30	6.79
	Conductivity (µS cm^{-1})	956	941	905	934	942	889
12.5	DO (mg L^{-1})	7.75	8.15	7.42	7.78	8.16	8.32
	pH	5.63	6.44	5.62	6.87	5.25	6.70
	Conductivity (µS cm^{-1})	961	946	909	840	946	915
25.0	DO (mg L^{-1})	7.84	8.10	7.39	7.67	8.06	8.30
	pH	5.59	6.23	5.35	6.88	5.24	6.70
	Conductivity (µS cm^{-1})	963	967	914	838	957	932
50.0	DO (mg L^{-1})	7.76	8.28	7.40	7.61	8.18	8.16
	pH	5.70	5.92	5.38	6.69	5.27	6.77
	Conductivity (µS cm^{-1})	986	995	936	905	981	979
100.0	DO (mg L^{-1})	7.67	8.32	7.38	7.79	8.02	8.01
	pH	5.63	5.86	5.45	6.31	5.26	6.48
	Conductivity (µS cm^{-1})	1024	1021	964	1026	1024	1018
200.0	DO (mg L^{-1})	7.73	8.16	7.47	7.78	7.75	8.05
	pH	5.62	5.01	5.80	6.35	5.34	6.35
	Conductivity (µS cm^{-1})	1095	1096	1039	1031	1063	1090
400.0	DO (mg L^{-1})	7.73	8.14	7.27	7.54	8.11	7.90
	pH	5.77	4.91	5.41	6.23	5.52	6.15
	Conductivity (µS cm^{-1})	1240	1240	1176	1158	1229	1235

Appendix C - Control charts and results obtained for the *Lemna minor*, *Daphnia similis*, *Hydra attenuata* and *Danio rerio* test procedure, using NaCl as a reference substance.

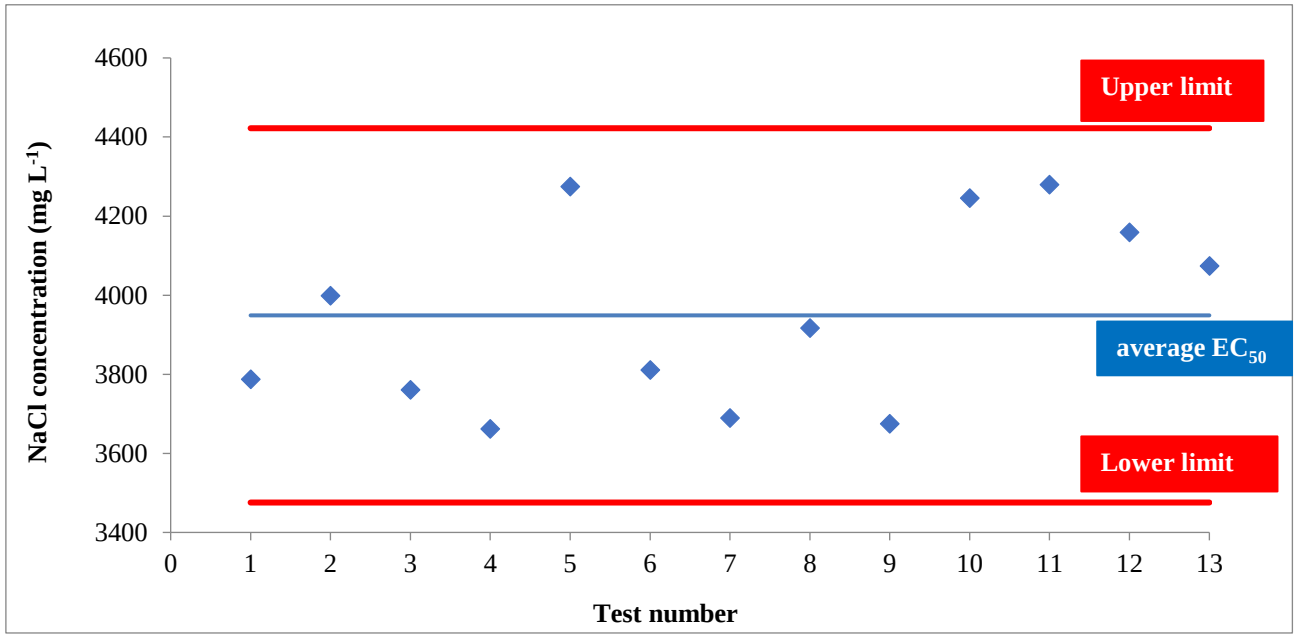

Figure C.1 - Control chart for the *Lemna minor* test procedure. Results refer to the endpoint frond number, which was evaluated in the macrophytes exposed to NaCl during 7 d

Source: Godoy et al. (2018)

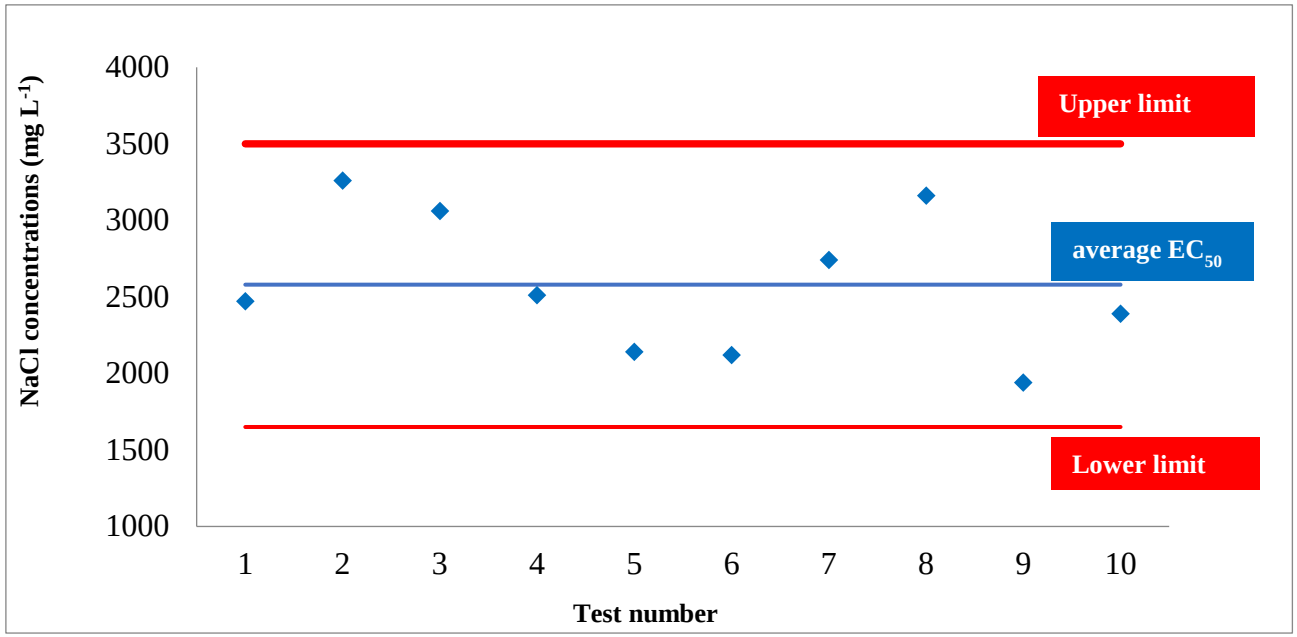

Figure C.2 - Control chart for the *Daphnia similis* test procedure. Results refer to the endpoint immobilization, which was evaluated in the crustaceans exposed to NaCl during 48 h

Source: Godoy et al. (2018)

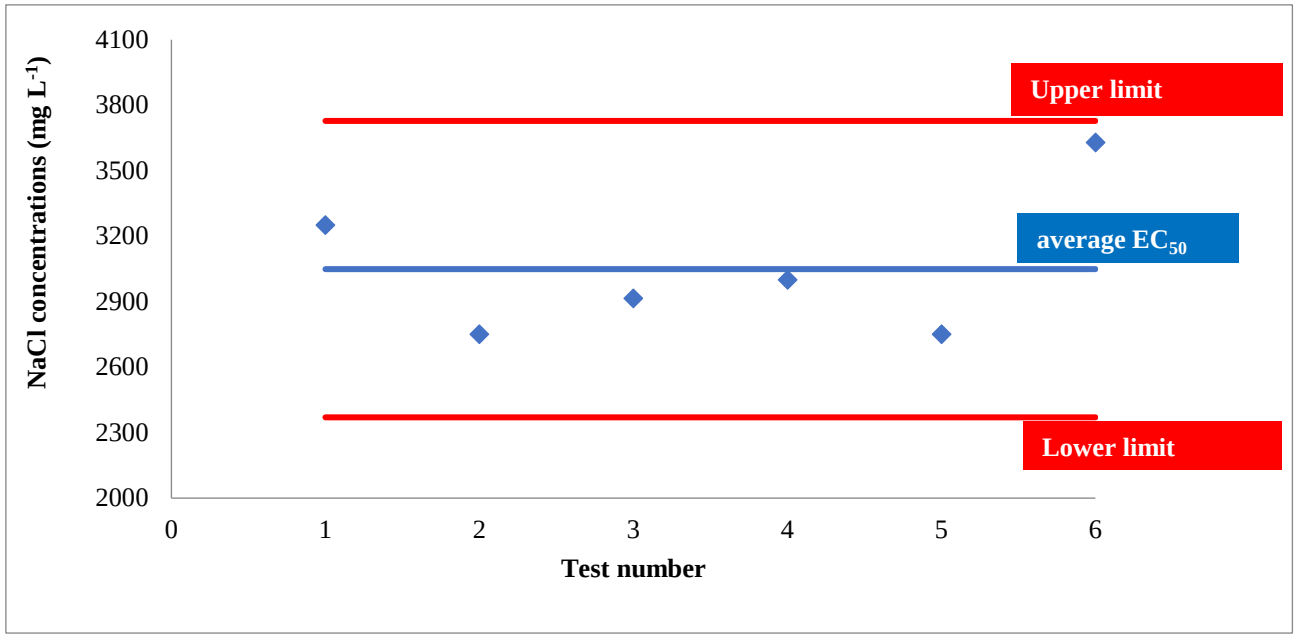

Figure C.3 - Control chart for the *Hydra attenuata* test procedure. Results refer to the endpoint lethality, which was evaluated in the cnidarians exposed to NaCl during 96 h

Source: Godoy et al. (2018)

Table C.1 – Results obtained for the exposure of *Danio rerio* embryos to the concentration of 4 mg L^{-1} of the reference substance 3,4 – dichloroaniline, according to the OECD 236 (2013)

Test	Percentage of mortality at the end of 96 h exposure
1	100 %
2	100 %
3	100 %

Source: Godoy et al. (2018)

Appendix D – CRED evaluation method for reliability and relevance of the acute and chronic ecotoxicological studies performed, whose obtained results were used for estimating the predicted no-effect concentration (PNEC) and for deriving the environmental quality standards (EQS's) for the pharmaceutical metfomin, according to Moermond et al. (2016)

Table D.1 – Reliability criteria evaluation

Number	**General Information**	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
1	Is a standard method (e.g, OECD/ISO) or modified standard used?	X				Yes. The toxicity tests were performed according to OECD guidelines for *Lemna minor* (n. 221 (2006); for *Daphnia similis* n. 202 (2004) and n. 211 (2012), modified according to Vacchi et al. (2016) regarding the exposure duration (14 d); and for *Danio rerio* n. 236 (2013). Tests with *Hydra attenuata* were performed following the standard method described in Trottier et al. (1997) and in Holdway et al. (2005).
2	Is the test performed under GLP conditions?		X			No. However, the quality management system of the laboratory where the tests were performed follows the requirements of ISO/IEC 17025/2005.
3	If applicable, are validity criteria fulfilled (e.g. control survival, growth)?	X				Yes, validity criteria were fulfilled according to the respective guidelines, as it was shown in the Appendices B (physical-chemical parameters) and E (raw data) of the Supplementary Material.

(To be continued)

Table D.1 (continued)

Number	**General Information**	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
4	Are appropriate controls performed (e.g. solvent control, negative and positive control)?	X				Yes. Negative controls were performed for each of the toxicity tests and positive control were performed using NaCl as a reference substance. Control charts were built and they are shown in the Appendix C of the Supplementary Material
	*These criteria are of minor importance for study reliability, but may support study evaluation					
	Test compound					
5	Is the test substance identified clearly with name or CAS-number? Are test results reported for the appropriate compound?	X				Yes. Metformin hydrochloride was the tested substance (CAS 115-70-4). Results were reported for the base content (metformin) and this was accordingly described in the paper.
6	Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?	X				Yes. The purity of the test substance is 99.2 %, reported according to chemical analysis described in chemical report.
7	If a formulation is used or if impurities are present: Do other ingredientes in the formulation exert an effect? Is the amount of test substance in the formulation known?	X				The test substance is not a formulation; it is a pharmaceutical substance with high purity (>99 %). Therefore, effects from other ingredients are not expected to occur.
	Test organism					
8	Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	X				Yes. All the organisms used in the tests are well described and the adequate age/life stage was used for each test, according to the respective guidelines/methods.

(To be continued)

Table D.1 (continued)

Number	**General Information**	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
9	Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	X				Yes. *L. minor* plants were donated by prof. Odete Rocha, from the Federal University of São Carlos, SP, Brazil. *D. similis* organisms were donated by Prof. Clarice Botta from University of São Paulo, SP, Brazil. *H. attenuata* were donated by prof. Regina Monteiro, from the University of São Paulo, SP, Brazil, who in turns obtained them from Dr. Christian Blaise, Canada. The *D. rerio* have been cultivated in the facility of the University of Aveiro, Portugal, since 2007 and are from the ABwt strain. None of the organisms have been pre-exposed to test compound or other stressors.
	Exposure conditions					
10	Is the experimental system appropriate for the test substance, taking into account its physical-chemical characteristics?	X				Yes. Metformin hydrochloride is stable under the conditions of the tests, as it was showed by the chemical analysis.
11	Is the experimental system appropriate for the test organism (e.g., choice of medium or test water, feeding, water characteristics, temperature, light/dark conditions, pH, oxygen content)? Have conditions been stable during the test?	X				Yes, all the tests were performed under experimental conditions described in OECD guidelines and/or methodologies published and well recognized in the international literature. The conditions have been stable during the tests, as it was shown in the Appendix B of the Supplementary Material.

(To be continued)

Table D. 1 (continued)

Number	**General Information**	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
12	Were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	X				Yes, exposure concentrations were always below the limit of water solubility, which is of 1000 g L^{-1} metformin hydrochloride. Thus, no solvent was needed.
13	Is a correct spacing between exposure concentrations applied?	X				Yes. Concentrations were always arranged in a geometric series with separation factories not exceeding the recommendations of the respective OECD guidelines.
14	Is the exposure duration defined?	X				Yes. The exposure duration for all the testes was defined according to the respective methodologies described in the literature.
15	Are chemical analyses adequate to verify substance concentrations over the duration of the study?	X				Chemical analyses were performed for *D. similis* and *H. attenuata* chronic tests. The respective validation parameters are described in the Appendix A of the Supplementary Material. However, only nominal values were used for *L. minor*
16	Is the biomass loading of the organisms in the test system within the appropriate range (e.g. < 1 g/L)?	X				Yes. The biomass loading of the organisms in each of the test system was within the appropriate range, as it was recommended by the respective methodologies reported in the literature and OECD guidelines.
	Statistical Design and Biological Response					
17	Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	X				Yes. Replicates and respective number of organisms were always used in a sufficient number, following the recommendations of the OECD guidelines and/or international literature. (continued)

Table D.1 (continued)

Number	**General Information**	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
18	Are appropriate statistical methods used?	X				Yes. Statistical methods used were appropriate for the analysis of the data, following OECD recommendations. Statistical analyses are described in detail in the section 2.7 of the paper.
19	Is a dose-response curve observed? Is the response statistically significant?	X				Yes. Concentration-response curves were observed for all the tests performed, and they are shown in the Appendix H of the Supplementary Material. The responses were statistically significant ($p < 0.05$)
20	Is sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, dose-response curves)?	X				Yes, raw data are available in the Appendix E of the Supplementary Material.

Source: Godoy et al. (2018)

Table D.2 – Relevance criteria evaluation

Number	Biological relevance	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
1	Is the species tested relevant for the compartment under evaluation?	X				Yes. The four test organisms evaluated are representative of three different trophic levels existing in freshwater ecosystems. Moreover, tests with plants, daphnids and fishes are required for risk assessment of freshwater compartments, according to technical guidance for deriving environmental quality standards.
2	Are the organisms tested relevant for the tested compound?	X				Yes. Metformin is a water-soluble compound, with a pKa of <1. It has been detected and quantified in surface waters. Thus, the use of organisms representing the freshwater pelagic communities is relevant for evaluating the possible toxic effects and risk posed by metformin.
3	Are the reported endpoints appropriate for the regulatory purpose?	X				Yes. The test endpoints relate to the effects at the population level of the species, according to guidances for deriving environmental quality standards. Thus, they are appropriate for regulatory purposes.
4	Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	X				Yes. Since the mode of action of metformin in non-target organisms is not well known, it is assumed that the use of several endpoints (lethality, inhibition of reproduction, inhibition of growth rate, as well as evaluation of malformations and alterations in the morphology), assessed in different organisms representative of different trophic levels, are representative of acute and chronic toxicity and cover various possible mode of action of the test substance.
5	Is the effect relevant on a population level?	X				Yes, all the endpoints reported relate to the effects at the population level.
6	Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC_{10}, EC_{50})?	X				Yes. The EC_{10} derived from chronic tests were used for deriving a long-term environmental quality standard (EQS), and the EC_{50} derived from acute tests were used for deriving a short-term EQS, following the technical guidance that usually support regulatory decision-making.

(To be continued)

Table D.2 (continued)

Number	Biological relevance	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
7	Are appropriate life-stages studied?	X				Yes, the life-stages studied were appropriate to each test, according to the OECD guidelines and/or methodologies reported in the international literature.
8	Are the experimental conditions relevant for the tested species?	X				Yes, the experimental conditions were appropriately used according to the respective OECD guidelines and/or methodologies reported in the international literature for each test organism.
9	Is the time of exposure relevant and appropriate for the studied endpoints and species?	X				Yes, the time of exposure were always according to the methodologies recommended in standard guidelines/international literature for performing acute and chronic tests with each test species.
10	If recovery is studied, is this relevant for the framework for which the study is evaluated?			X		Not applicable.
11	In case of a formulation, other mixture, salts or transformation products: Is the substance tested representative and relevant for the substance being assessed?			X		Yes, the metformin hydrochloride is a salt form, however the results were reported regarding the base content (metformin), that is the form usually found in aquatic environments from chemical analysis.
12	Is the tested exposure scenario relevant for the substance?	X				Yes. Both acute and principally chronic toxicity may be expected for metformin, as a result of both intermittent and continuous exposure. However, when it comes to pharmaceuticals, it is assumed that a long-term exposure is more probable.
13	Is the tested exposure scenario relevant for the species?	X				Yes, the exposure scenarios used are relevant for the species, following standard methodologies reported in the international literature.

Source: Godoy et al. (2018)

Appendix E – Raw data regarding the acute and chronic ecotoxicity tests performed with metformin

Table E.1 Raw data obtained in the acute tests with *Daphnia similis*

Concentration (mg L^{-1})	Number of immobilized organisms Test 1					Number of immobilized organisms Test 2					Number of immobilized organisms Test 3				
	1	2	3	4	Total	1	2	3	4	Total	1	2	3	4	Total
0.0	0/5	0/5	0/5	0/5	0/20	1/5	0/5	0/5	0/5	1/20	0/5	0/5	0/5	0/5	0/20
5.0	0/5	0/5	0/5	0/5	0/20	0/5	0/5	0/5	0/5	0/20	0/5	0/5	0/5	0/5	0/20
8.0	0/5	0/5	0/5	1/5	1/20	0/5	1/5	0/5	0/5	1/20	0/5	0/5	1/5	1/5	2/20
12.5	1/5	1/5	0/5	0/5	2/20	1/5	3/5	3/5	0/5	7/20	2/5	3/5	3/5	2/5	10/20
20.0	2/5	4/5	5/5	4/5	15/20	3/5	5/5	5/5	5/5	18/20	5/5	5/5	5/5	5/5	20/20
30.0	5/5	5/5	5/5	5/5	20/20	5/5	5/5	5/5	5/5	20/20	5/5	5/5	5/5	5/5	20/20
50.0	5/5	5/5	5/5	5/5	20/20	5/5	5/5	5/5	5/5	20/20	5/5	5/5	5/5	5/5	20/20

Source: Godoy et al. (2018)

Table E.2 Raw data obtained in the acute tests with *Hydra attenuata*. The letters refer to the stages of morphological alterations, according to Trottier et al. (1997): N: normal; C: clubbed tentacles; S: shortened tentacles; T: tulip, D: disintegrated

Concentration ($mg\ L^{-1}$)	Stages of morphological alterations Test 1					Stage of morphological alterations Test 2					Stage of morphological alterations Test 3				
	1	2	3	Total lethal	Total sublethal	1	2	3	Total lethal	Total sublethal	1	2	3	Total lethal	Total sublethal
0	NNN	NNN	NNN	0/9	0/9	NNN	NNN	NNN	0/9	0/9	NNN	NNN	NNN	0/9	0/9
2,000	NNN	NNN	NNN	0/9	0/9	NNN	NNN	NNN	0/9	0/9	NNN	NNN	NNN	0/9	0/9
2,300	NNN	NNN	NNN	0/9	0/9	NNN	NNN	NNN	0/9	0/9	NNN	NNN	NNN	0/9	0/9
2,700	NNN	NNN	NNN	0/9	0/9	CCC	CNN	NNS	0/9	5/9	CCC	NCC	NCC	0/9	7/9
3,100	SSS	SSS	SSS	0/9	9/9	CCC	CCC	CCC	0/9	9/9	SSC	CCS	CCC	0/9	9/9
3,600	SSS	SSS	SSS	0/9	9/9	SSS	SSS	TSS	1/9	9/9	CSS	SSD	SCC	1/9	9/9
4,200	DDD	SDT	DDT	8/9	9/9	TDD	DDS	DTT	8/9	9/9	DDS	DDT	DDT	8/9	9/9
5,000	DDD	DDD	DDD	9/9	9/9	DDD	DDD	DDD	9/9	9/9	DDD	DDD	DDD	9/9	9/9

Source: Godoy et al. (2018)

Table E.3 Raw data obtained in the acute tests performed with *Danio rerio*. Abbreviation letters: N = normal; C = coagulated; S = scoliosis; PE = pericardial edema; PF = pigmentation failure; TM = tail malformation; DH = delayed hatch; D = disequilibrium; LH = lack of heartbeat

	Test n. 1																						
	24 hours post-fertilization																						
	Replicate																						
Concentration (mg L^{-1})	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Control	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
100	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
250	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
625	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
1,500	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	C	N	N	N	N	N
4,000	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	48 hours post-fertilization																						
Control	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	PE	N	N	N	N	N
100	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
250	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
625	N	N	N	N	PF	N	N	PF	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
1,500	N	N	PF	LH	LH	N	N	N	PF	N	PF	N	N	N	PF	N	N	N	LH	N	N	LH/ PF	N	N
4,000	LH PF	LH PF	LH PF	LH PF	LH PF	N	LH PF	LH PF	N	LH PF	LH PF	N	LH PF	LH PF	LH PF	LH PF	LH PF	N	LH PF	LH PF	LH PF	LH PF	LH PF	LH PF
	72 hours post-fertilization																						
Control	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	PE/ S	N	N	N	N	N
100	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
250	N	N	N	S	N	N	N	N	S	N	N	N	N	N	N	S	LH	N	S	N	S	N	N	N
625	N	N	N	N	LH	N	N	PF	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
1,500	N	LH	LH	LH	LH	N	N	N	LH	PF	LH	N	N	N	LH	DH	LH	N	LH	S	S	LH	S	N
4,000	LH PF	LH PF	LH PF	LH PF	LH PF	N	LH PF	LH PF	LH PF	LH PF	LH PF	N	LH PF	LH PF	LH PF	LH PF	LHPF	N	LH PF	LH PF	LH PF	LH PF	LH PF	N

(To be continued)

Table E.3 (continued)

	Test n. 1																						
	96 hours post-fertilization																						
Control	N	N	N	N	N	N	N	N	N	N	N	N	N	N	LH	N	N	N	PE/S	N	N	N	N
100	N	N	N	N	N	N	N	N	N	N	N	N	D	N	N	N	N	N	N	N	N	N	N
250	N	N	S	S	N	N	N	N	S	N	N	N	N	N	N	S	LH	N	S	N	S	N	N
625	N	N	N	N	LH	N	N	LH	N	N	N	N	D	N	N	N	D	N	N	N	N	N	N
1,500	LH	LH	LH	LH	LH	N	N	N	LH	LH	LH	N	LH	LH	LH	LH	LH	N	LH	S/D	S/D	LH	LH	TM
4,000	LH PF	LH PF	LH PF	LH PF	LH PF	N	LH PF	LH PF	LH PF	LH PF	LH PF	N	LH PF	LH PF	LH PF	LH PF	LHPF	N	LH PF	LH PF	LH PF	LH PF	LH PF	N
	Test n. 2																						
	24 hours post-fertilization																						
	Replicate																						
Concentration (mg L^{-1})	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Control	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
100	N	N	N	N	N	N	N	N	N	C	N	N	N	N	N	N	N	N	N	C	N	N	N	N
180	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
330	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
600	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
1,100	C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
2,000	N	N	N	N	C	N	N	N	N	N	N	N	N	N	N	N	N	N	C	N	N	N	N	N
	48 hours post-fertilization																						
Control	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
100	N	N	N	N	N	N	N	N	N	C	N	N	N	N	N	N	N	N	N	C	N	N	N	N
180	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	TM	N	N	N	N	N	N	N
330	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
600	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
1,100	C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
2,000	C	LH	LH	LH	LH	N	N	LH	LH	LH	N	N	N	LH	LH	LH	LH	N	LH	PF	LH	N	PF	N

(To be continued)

Table E.3 (continued)

	Test n. 2																						
	72 hours post-fertilization																						
Control	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
100	N	N	N	N	N	N	N	N	N	C	N	N	N	N	N	N	N	S	N	C	N	N	N	N
180	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	S TM	N	N	N	N	N	N	N
330	N	N	N	S	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
600	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
1,100	C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
2,000	C	LH	LH	LH	LH	N	S	LH	LH	LH	LH	N	N	LH	LH	LH	LH	N	LH	LH	LH	N	LH	N
	96 hours post-fertilization																							
Control	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
100	N	N	N	N	N	N	N	N	N	C	N	N	N	N	N	N	N	N	N	C	N	N	N	N
180	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	S/TM	N	N	N	N	N	N	N
330	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
600	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
1,100	C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	D	N
2,000	LH	LH	LH	LH	LH	N	LH	LH	LH	LH	LH	N	D	LH	LH	LH	LH	N	LH	LH	LH	D	LH	N
	Test n. 3																							
	24 hours post-fertilization																							
	Replicate																							
Concentration (mg L^{-1})	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Control	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
100	N	N	N	N	N	N	N	N	N	N	N	N	N	N	C	N	N	N	N	N	N	N	N	N
180	N	N	N	N	N	C	C	N	N	N	N	N	N	C	N	C	N	N	N	N	N	N	N	N
330	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	C	N	N	N	N	C	N
600	N	N	N	N	N	N	N	N	N	T M	C	N	N	N	N	N	N	N	N	N	N	N	N	N
1,100	N	N	N	N	C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
2,000	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

(To be continued)

Table E.3 (continued)

	Test n. 3																						
	48 hours post-fertilization																						
Control	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
100	N	N	N	N	N	N	N	N	N	N	N	N	N	N	C	N	N	N	N	N	N	N	N
180	N	N	N	N	N	N	C	N	N	N	N	N	N	C	N	C	N	N	N	N	N	N	N
330	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	C	N	N	N	N	C	N
600	N	N	N	N	N	N	N	N	N	LH	C	N	N	N	N	N	N	N	N	N	N	N	N	N
1,100	N	N	N	N	N	N	N	N	N	PF	PF	N	N	N	N	N	N	N	N	N	N	N	N	N
2,000	LH	LH	LH	LH	LH	N	LH	LH	LH	N	PF	N	PF	LH	LH	LH	LH	N	LH	LH	LH	PF	LH	N
	72 hours post-fertilization																						
Control	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
100	N	N	N	N	N	N	N	N	N	N	N	N	N	N	C	N	N	N	N	N	N	N	N	N
180	N	N	N	N	N	N	C	N	N	N	N	N	N	C	N	C	N	N	N	N	N	N	N	N
330	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	C	N	N	N	N	C	N
600	N	N	N	N	N	N	N	N	N	LH	C	N	N	N	N	N	N	N	N	N	N	N	N	N
1,100	N	N	S	N	N	N	N	S	N	PF LH	PF LH	N	N	N	N	S	N	N	N	LH	N	N	N	N
2,000	LH	LH PE	LH	LH	LH	N	LH PF	LH	LH	S	S PF	N	LH	LH	LH	LH	LH	N	S PF	LH	LH	PF	LH	N
	96 hours post-fertilization																						
Control	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
100	N	N	N	N	N	N	N	N	N	N	N	N	N	N	C	N	N	N	N	N	N	N	N	N
180	N	N	N	N	N	C	C	N	N	N	N	N	N	C	N	C	N	N	N	N	N	N	N	N
330	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	C	N	N	N	N	C	N
600	N	N	N	N	N	N	N	N	N	LH PE	C	N	N	N	N	N	N	N	N	N	N	N	N	N
1,100	N	LH	S	N	N	N	N	LH	N	LH PF	LH PF	N	N	N	N	LH	N	N	N	LH	N	N	N	N
2,000	LH	LH PE	LH	LH	LH	N	LH PF	LH	LH	S/ D	S/ D/ PF	N	LH	LH	LH	LH	LH	N	LH PF	LH	LH	LH PF	LH	N

Source: Godoy et al. (2018)

Table E.4 Raw data obtained in the chronic test number 1 performed in order to evaluate the toxic effects of metformin in the reproduction of *Daphnia similis*

Concentration (mg L^{-1})	Number of living offsprings per replicate										
	Day	1	2	3	4	5	6	7	8	9	10	Total
Control	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	0	0	0	0	0	0	
	4	0	0	0	0	0	0	0	0	0	0	
	6	16	0	0	0	13	0	9	18	14	10	
	8	16	12	13	16	0	13	16	22	19	14	
	10	25	12	25	25	47	21	19	25	33	26	
	12	19	21	28	26	31	22	28	26	36	28	
	14	9	25	31	32	29	33	39	36	14	12	
	Total	85	70	97	99	120	89	111	127	116	90	1004
1.0	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	0	0	0	0	0	0	
	4	0	0	0	0	0	0	0	0	0	0	
	6	17	0	0	0	0	12	9	14	6	12	
	8	12	5	22	17	13	19	0	27	12	17	
	10	17	0	29	21	22	21	46	31	20	29	
	12	21	8	35	32	0	25	38	30	22	29	
	14	27	26	22	35	35	17	22	25	29	24	
	Total	94	39	108	105	70	94	115	127	89	111	952
3.0	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	0	0	0	0	0	0	
	4	0	+	+	0	0	0	0	0	0	0	
	6	15	-	-	13	13	11	10	11	0	12	
	8	18	-	-	22	21	19	22	20	11	23	
	10	23	-	-	22	26	29	28	25	20	32	
	12	30	-	-	33	33	33	0	27	24	25	
	14	24	-	-	44	25	30	0	51	29	32	
	Total	110	0	0	134	118	122	60	134	84	124	886

(To be continued)

Table E.4 (continued)

Concentration (mg L^{-1})	Number of living offsprings per replicate										
	Day	1	2	3	4	5	6	7	8	9	10	
	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	0	0	0	0	0	0	
	4	0	0	0	0	0	+	0	0	0	0	
	6	0	0	19	0	0	-	0	12	14	0	
	8	15	11	32	16	10	-	14	20	2	0	
	10	21	20	34	21	13	-	8	30	45	0	
	12	32	33	42	23	22	-	48	40	37	0	
	14	26	30	29	27	32	-	35	45	27	0	
	Total	94	94	156	87	77	0	105	147	125	0	885
8.0	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	0	0	0	0	0	0	
	4	0	0	0	0	+	0	+	0	0	0	
	6	9	0	0	0	-	0	-	0	0	0	
	8	23	0	8	6	-	8	-	12	8	9	
	10	24	8	14	14	-	21	-	19	13	17	
	12	37	19	24	22	-	20	-	25	16	32	
	14	20	30	27	27	-	37	-	30	31	33	
	Total	113	57	73	69	0	86	0	86	68	91	643
11.0	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	+	+	0	0	0	0	
	4	0	0	+	0	-	-	0	+	+	0	
	6	0	0	-	+	-	-	+	-	-	0	
	8	2	0	-	-	-	-	-	-	-	0	
	10	16	0	-	-	-	-	-	-	-	0	
	12	23	13	-	-	-	-	-	-	-	4	
	14	18	4	-	-	-	-	-	-	-	10	
	Total	59	27	0	0	0	0	0	0	0	14	100

Table E.5 Raw data obtained in the chronic test number 2 performed in order to evaluate the toxic effects of metformin in the reproduction of *Daphnia similis*

Concentration (mg L^{-1})	Number of living offsprings per replicate											
	Day	1	2	3	4	5	6	7	8	9	10	Total
Control	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	0	0	0	0	0	0	
	4	0	0	0	0	0	0	0	0	0	0	
	6	0	0	0	0	0	0	0	0	0	0	
	8	7	5	6	0	4	0	6	8	8	6	
	10	9	3	0	30	3	0	1	4	7	5	
	12	6	7	18	25	5	7	9	7	9	4	
	14	4	2	0	13	2	5	9	4	2	3	
	Total	26	17	24	65	14	12	25	23	26	18	250
1.0	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	0	0	0	0	0	0	
	4	0	0	0	0	0	0	0	0	0	0	
	6	0	0	0	0	0	0	0	0	0	0	
	8	0	0	0	0	5	0	6	0	0	0	
	10	6	6	11	9	0	7	12	4	0	0	
	12	10	5	10	12	0	6	10	10	11	0	
	14	11	8	1	6	17	9	11	1	7	13	
	Total	27	19	22	27	22	22	39	15	18	13	224

(Continued)

Table E.5 (continued)

Concentration (mg L^{-1})	Number of living offsprings per replicate											
	Day	1	2	3	4	5	6	7	8	9	10	Total
3.0	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	0	0	0	0	0	0	
	4	0	0	+	0	0	0	0	0	0	+	
	6	0	0	-	8	6	0	0	0	0	-	
	8	0	11	-	9	8	6	7	0	0	-	
	10	2	15	-	8	5	8	3	4	5	-	
	12	0	18	-	10	+	11	0	8	10	-	
	14	10	21	-	3	-	12	+	10	7	-	
	Total	12	65	0	38	19	37	10	22	22	0	225
5.0	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	0	0	0	0	0	0	
	4	+	0	0	0	+	0	0	0	0	0	
	6	-	+	0	2	-	0	0	0	7	0	
	8	-	-	6	3	-	0	11	0	17	6	
	10	-	-	0	0	-	0	0	6	0	0	
	12	-	-	5	8	-	9	13	+	18	12	
	14	-	-	0	9	-	11	19	-	8	15	
	Total	0	0	11	22	0	20	43	6	50	33	185

(To be continued)

Table E.5 (continued)

Concentration (mg L^{-1})	Number of living offsprings per replicate											
	Day	1	2	3	4	5	6	7	8	9	10	Total
8.0	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	0	0	+	0	0	0	
	4	0	0	0	+	0	0	-	0	0	0	
	6	0	0	0	-	0	0	-	+	0	+	
	8	0	5	0	-	4	8	-	-	0	-	
	10	0	15	9	-	7	12	-	-	0	-	
	12	0	20	14	-	10	13	-	-	6	-	
	14	6	3	16	-	7	11	-	-	7	-	
	Total	6	43	39	0	28	44	0	0	13	0	173
11.0	0	0	0	0	0	0	0	0	0	0	0	
	2	0	+	0	0	0	0	+	+	0	+	
	4	+	-	0	0	0	0	-	-	+	-	
	6	-	-	+	+	+	0	-	-	-	-	
	8	-	-	-	-	-	0	-	-	-	-	
	10	-	-	-	-	-	10	-	-	-	-	
	12	-	-	-	-	-	17	-	-	-	-	
	14	-	-	-	-	-	26	-	-	-	-	
	Total	0	0	0	0	0	53	0	0	0	0	53

Source: Godoy et al. (2018)

Table E.6 Raw data obtained in the chronic test number 3 performed in order to evaluate the toxic effects of metformin in the reproduction of *Daphnia similis*

Concentration (mg L^{-1})	Number of living offsprings per replicate										
	Day	1	2	3	4	5	6	7	8	9	10	Total
Control	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	0	0	0	0	0	0	
	4	0	0	0	0	0	0	0	0	0	0	
	6	6	7	9	10	0	9	0	5	8	6	
	8	8	7	10	10	9	4	9	8	12	8	
	10	10	8	7	7	16	9	29	10	9	8	
	12	8	6	2	13	0	10	12	12	8	10	
	14	10	13	10	3	0	13	12	12	0	10	
	Total	42	41	38	43	25	45	62	47	37	42	422
1.0	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	0	0	0	0	0	0	
	4	0	0	0	0	0	0	0	0	0	0	
	6	0	8	9	11	6	5	10	0	10	0	
	8	12	7	12	9	4	7	10	9	8	13	
	10	8	6	12	16	+	5	10	19	7	14	
	12	0	6	9	9	-	2	13	10	10	9	
	14	0	0	15	14	-	2	13	0	2	0	
	Total	20	27	57	59	10	21	56	38	37	36	361
3.0	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	0	0	0	0	0	0	
	4	0	0	0	0	0	0	0	0	0	0	
	6	0	0	10	0	5	+	7	10	0	0	
	8	4	7	17	11	11	-	11	16	20	5	
	10	15	11	5	29	16	-	15	14	13	20	
	12	17	4	3	19	12	-	15	12	15	15	
	14	0	0	14	22	8	-	17	7	18	0	
	Total	36	22	49	81	53	0	65	59	66	40	471

(To be continued)

Table E.6 (continued)

Concentration (mg L^{-1})	Number of living offsprings per replicate											
	Day	1	2	3	4	5	6	7	8	9	10	Total
5.0	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	0	0	0	0	0	0	
	4	0	0	0	0	0	0	0	0	0	0	
	6	0	0	10	12	0	11	10	8	0	0	
	8	0	+	16	8	7	7	12	7	4	+	
	10	17	-	15	30	11	16	11	7	16	-	
	12	17	-	14	16	9	11	12	10	2	-	
	14	0	-	17	0	14	8	15	17	0	-	
	Total	34	0	72	66	41	53	60	49	41	0	416
8.0	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	0	0	0	0	0	+	
	4	0	0	0	0	0	+	0	0	0	-	
	6	0	0	0	0	0	-	0	0	0	-	
	8	6	+	6	3	0	-	0	+	+	-	
	10	13	-	26	35	0	-	15	-	-	-	
	12	0	-	26	28	4	-	16	-	-	-	
	14	13	-	0	0	0	-	25	-	-	-	
	Total	32	0	58	66	4	0	56	0	0	0	216
11.0	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	+	0	+	0	+	+	0	
	4	+	+	+	-	0	-	+	-	-	+	
	6	-	-	-	-	0	-	-	-	-	-	
	8	-	-	-	-	0	-	-	-	-	-	
	10	-	-	-	-	5	-	-	-	-	-	
	12	-	-	-	-	3	-	-	-	-	-	
	14	-	-	-	-	0	-	-	-	-	-	
	Total	0	0	0	0	8	0	0	0	0	0	8

Source: Godoy et al. (2018)

Table E.7 Raw data obtained in the chronic tests performed in order to evaluate the toxic effects of metformin in the reproduction of *Hydra attenuata*. The K value refers to the mean relative population growth rate, calculated according to Holdway (2005).

		Test 1			Test 2			Test 3		
		Number of hydras per replicate			Number of hydras per replicate			Number of hydras per replicate		
Concentration (mg L^{-1})	Day	A	B	C	A	B	C	A	B	C
Control	1	13	12	12	13	13	14	14	13	13
	2	14	16	15	16	15	17	18	19	16
	3	22	28	28	-	-	-	22	25	20
	4	27	34	34	31	34	31	40	41	29
	5	44	48	44	42	34	32	-	-	-
	6	-	-	-	52	45	40	75	64	61
	7	88	86	92	66	64	60	75	69	74
K value		0.32	0.31	0.32	0.27	0.26	0.26	0.29	0.28	0.29
200	1	13	12	13	13	15	14	13	14	12
	2	14	16	16	16	15	18	18	19	15
	3	24	30	31	-	-	-	26	25	19
	4	30	34	36	30	31	29	40	40	30
	5	42	44	46	30	31	39	-	-	-
	6	-	-	-	40	42	56	69	78	56
	7	94	98	100	53	51	70	78	94	60
K value		0.32	0.33	0.33	0.24	0.23	0.28	0.29	0.32	0.26

(To be continued)

Table E.7 (continued)

		Test 1			Test 2			Test 3		
		Number of hydras per replicate			Number of hydras per replicate			Number of hydras per replicate		
Concentration (mg L^{-1})	Day	A	B	C	A	B	C	A	B	C
360	1	12	11	14	14	12	14	12	13	14
	2	15	14	16	17	14	16	16	15	17
	3	21	24	33	-	-	-	22	18	27
	4	29	32	33	32	29	25	32	30	36
	5	32	42	36	33	29	29	-	-	-
	6	-	-	-	48	44	35	63	53	60
	7	61	90	84	68	53	43	79	61	63
K value		0.26	0.31	0.30	0.27	0.24	0.21	0.29	0.26	0.26
650	1	12	12	13	12	12	12	14	11	11
	2	17	14	14	13	17	15	17	16	16
	3	33	26	26	-	-	-	20	22	18
	4	38	30	28	26	29	29	32	31	32
	5	42	38	32	27	31	35	-	-	-
	6	-	-	-	36	40	40	59	57	53
	7	88	65	68	46	49	41	56	65	54
K value		0.31	0.27	0.27	0.22	0.23	0.20	0.25	0.27	0.24

(To be continued)

Table E.7 (continued)

		Test n. 1			Test n. 2			Test n. 3		
		Number of hydras per replicate			Number of hydras per replicate			Number of hydras per replicate		
Concentration (mg L^{-1})	Day	A	B	C	A	B	C	A	B	C
1,200	1	11	12	10	12	13	14	13	13	12
	2	14	14	15	12	13	16	17	15	14
	3	22	23	21	-	-	-	17	17	19
	4	28	20	27	18	21	23	25	29	25
	5	31	22	33	18	22	25	-	-	-
	6	-	-	-	21	25	24	42	41	31
	7	41	36	48	22	27	25	38	47	31
K value		0.20	0.18	0.22	0.11	0.14	0.13	0.19	0.22	0.16
2,000	1	10	12	11	12	12	12	11	10	14
	2	13	14	11	13	11	13	16	14	14
	3	16	21	14	-	-	-	19	15	17
	4	13	17	11	16	16	14	21	14	21
	5	11	13	10	16	9	15	-	-	-
	6	-	-	-	12	6	13	17	10	18
	7	11	7	7	6	7	10	11	8	9
K value		0.01	-0.05	-0.05	-0.07	-0.05	0	0.01	-0.03	-0.02

Source: Godoy et al. (2018)

Table E.8 Raw data for the endpoint frond number, obtained in the chronic tests performed in order to evaluate the toxic effects of metformin in the population growth of *Lemna minor*. The μ value refers to the average specific growth rate, calculated according to OECD (2006)

		Test 1			Test 2			Test 3		
		Number of frondes per replicate			Number of frondes per replicate			Number of frondes per replicate		
Concentration (mg L^{-1})	Day	A	B	C	A	B	C	A	B	C
Control	0	12	12	12	12	12	12	12	12	12
	3	36	37	37	37	37	39	38	36	38
	5	89	91	92	92	101	101	86	90	91
	7	140	172	176	160	190	200	142	129	145
μ value		0.35	0.38	0.38	0.37	0.39	0.40	0.35	0.34	0.36
6.2	0	12	12	12	12	12	12	12	12	12
	3	36	36	36	37	38	37	38	38	38
	5	86	91	89	98	100	99	92	90	88
	7	162	172	177	186	192	185	140	129	136
μ value		0.37	0.38	0.38	0.39	0.40	0.39	0.35	0.34	0.35
12.5	0	12	12	12	12	12	12	12	12	12
	3	36	36	37	39	39	39	38	39	40
	5	94	90	96	98	100	96	91	94	97
	7	181	169	160	193	207	201	147	150	148
μ value		0.39	0.38	0.37	0.40	0.41	0.40	0.36	0.36	0.36

(To be continued)

Table E.8 (continued)

		Test n. 1			Test n. 2			Test n. 3		
		Number of frondes per replicate			Number of frondes per replicate			Number of frondes per replicate		
Concentration (mg L^{-1})	Day	A	B	C	A	B	C	A	B	C
25.0	0	12	12	12	12	12	12	12	12	12
	3	34	36	36	37	36	37	36	38	39
	5	81	90	92	87	80	89	73	88	83
	7	134	145	146	157	139	156	115	135	128
μ value		0.34	0.35	0.36	0.37	0.35	0.37	0.32	0.35	0.34
50.0	0	12	12	12	12	12	12	12	12	12
	3	29	26	27	30	29	31	33	32	32
	5	36	35	37	39	40	48	48	43	42
	7	44	42	44	56	55	65	56	50	52
μ value		0.18	0.18	0.18	0.22	0.22	0.24	0.22	0.20	0.21
100.0	0	12	12	12	12	12	12	12	12	12
	3	20	19	20	19	19	16	23	18	19
	5	20	21	21	26	23	23	25	21	24
	7	19	20	21	26	26	24	26	20	23
μ value		0.06	0.07	0.08	0.11	0.11	0.10	0.11	0.07	0.09

(To be continued)

Table E.8 (continued)

		Test n. 1			Test n. 2			Test n. 3		
		Number of frondes per replicate			Number of frondes per replicate			Number of frondes per replicate		
Concentration (mg L^{-1})	Day	A	B	C	A	B	C	A	B	C
200.0	0	12	12	12	12	12	12	12	12	12
	3	17	16	16	15	17	16	16	18	15
	5	15	16	17	18	20	19	18	17	14
	7	15	17	17	16	20	21	17	17	17
µ value		0.03	0.05	0.05	0.04	0.07	0.08	0.05	0.05	0.05
400.0	0	12	12	12	12	12	12	12	12	12
	3	15	14	13	16	15	15	13	13	14
	5	15	14	12	16	15	16	14	16	14
	7	15	12	13	16	16	17	15	15	14
µ value		0.03	0.00	0.01	0.04	0.04	0.05	0.03	0.03	0.02

Source: Godoy et al. (2018)

Table E.9 Raw data for the endpoint total frond area, obtained in the chronic tests performed in order to evaluate the toxic effects of metformin in the population growth of *Lemna minor*. The μ value refers to the average specific growth rate, calculated according to OECD (2006)

		Test 1			Test 2			Test 3		
		Frond area (mm^2) per replicate			Frond area (mm^2) per replicate			Frond area (mm^2) per replicate		
Concentration (mg L^{-1})	Day	A	B	C	A	B	C	A	B	C
Control	0	78.29	84.07	82.31	77.25	79.28	83.41	95.36	81.03	97.58
	3	234.24	258.81	263.41	232.51	259.51	264.90	317.02	287.91	332.68
	5	587.40	660.33	650.86	561.38	592.10	673.72	612.61	553.12	580.31
	7	1111.21	1294.56	1347.80	1200.49	1310.88	1466.75	1102.12	1211.46	1079.95
μ value		0.38	0.39	0.40	0.39	0.40	0.41	0.35	0.39	0.34
6.2	0	74.76	77.02	80.82	82.26	74.86	71.16	89.11	89.06	92.54
	3	224.91	244.25	261.51	248.94	235.24	230.28	297.02	273.50	264.81
	5	593.84	502.43	528.99	596.62	567.26	608.49	586.60	577.70	556.05
	7	1217.20	1264.73	1277.81	1440.36	1345.68	1317.64	1049.25	1073.44	1112.50
μ value		0.40	0.40	0.39	0.41	0.41	0.42	0.35	0.35	0.35
12.5	0	79.35	76.39	73.32	81.06	81.93	79.47	88.56	92.29	97.65
	3	253.14	244.97	240.41	249.85	261.37	255.94	281.57	258.45	330.95
	5	466.41	465.95	439.41	657.20	602.49	598.06	541.04	612.79	611.80
	7	1307.16	1232.76	1130.63	1305.87	1336.46	1168.37	1098.43	1152.04	1081.50
μ value		0.40	0.40	0.39	0.40	0.40	0.38	0.36	0.36	0.34

(To be continued)

Table E.9 (continued)

		Test 1			Test 2			Test 3		
		Frond area (mm^2) per replicate			Frond area (mm^2) per replicate			Frond area (mm^2) per replicate		
Concentration (mg L^{-1})	Day	A	B	C	A	B	C	A	B	C
25.0	0	77.07	82.32	81.12	78.83	73.20	75.63	80.53	94.14	86.65
	3	219.42	229.91	241.78	228.59	210.88	223.66	217.48	231.14	279.19
	5	453.78	446.36	340.29	523.28	494.82	527.43	468.60	556.17	524.27
	7	1006.05	1085.83	1035.60	1239.44	1041.86	1196.84	953.47	1012.70	980.98
μ value		0.37	0.37	0.36	0.39	0.38	0.39	0.35	0.34	0.35
50.0	0	79.63	76.06	77.98	71.97	78.38	76.51	94.04	100.18	94.93
	3	156.56	148.17	151.85	149.88	148.84	164.41	168.95	205.11	221.29
	5	180.00	183.96	176.27	212.86	226.23	263.07	275.56	265.52	285.31
	7	305.41	303.67	280.15	330.02	316.61	409.42	318.82	390.12	376.30
μ value		0.19	0.20	0.18	0.22	0.20	0.24	0.17	0.19	0.20
100.0	0	78.39	81.87	74.74	76.04	78.94	74.89	89.70	84.97	93.52
	3	118.94	118.21	108.47	109.95	115.49	105.54	142.64	111.78	102.63
	5	102.98	94.19	88.90	125.50	123.77	110.75	125.65	133.98	125.17
	7	113.98	114.33	121.88	136.45	120.19	140.75	136.59	141.10	132.13
μ value		0.053	0.050	0.070	0.083	0.060	0.090	0.060	0.072	0.049

(To be continued)

Table E.9 (continued)

		Test n. 1			Test n. 2			Test n. 3		
		Frond area (mm^2) per replicate			Frond area (mm^2) per replicate			Frond area (mm^2) per replicate		
Concentration (mg L^{-1})	Day	A	B	C	A	B	C	A	B	C
200.0	0	75.96	81.11	85.16	74.69	76.54	76.91	87.29	94.39	89.59
	3	107.41	112.26	116.43	98.64	102.25	106.70	102.11	107.35	89.79
	5	101.58	105.10	103.48	100.05	95.21	98.82	124.49	120.09	114.57
	7	95.29	114.90	125.10	113.54	121.65	102.86	118.48	108.92	121.58
µ value		0.032	0.050	0.055	0.060	0.066	0.041	0.043	0.020	0.044
400.0	0	79.51	70.91	70.70	77.44	77.60	76.85	89.20	90.83	93.16
	3	104.34	99.75	92.78	110.68	111.16	111.57	125.09	99.94	113.35
	5	105.98	81.80	89.43	107.04	104.13	104.17	123.77	116.20	109.31
	7	105.32	98.13	110.38	107.27	83.94	111.44	132.74	124.62	131.67
µ value		0.040	0.046	0.064	0.046	0.011	0.053	0.057	0.045	0.050

Source: Godoy et al. (2018)

Table E.10 Raw data for the endpoint fresh weight, obtained in the chronic tests performed in order to evaluate the toxic effects of metformin in the population growth of *Lemna minor*. The μ value refers to the average specific growth rate, calculated according to OECD (2006)

		Test 1			Test 2			Test 3		
		Fresh weight (mg L^{-1}) per replicate			Fresh weight (mg L^{-1}) per replicate			Fresh weight (mg L^{-1}) per replicate		
Concentration (mg L^{-1})	Day	A	B	C	A	B	C	A	B	C
Control	0	10.2	10.2	10.2	10.6	10.6	10.6	10.4	10.4	10.4
	7	153.1	184.7	182.6	157.6	169	191.7	164	169.2	168
μ value		0.39	0.41	0.41	0.38	0.39	0.41	0.39	0.40	0.40
6.2	0	10.2	10.2	10.2	10.6	10.6	10.6	10.4	10.4	10.4
	7	168.3	174.3	181.9	175.7	176.8	187	161.2	164.3	162.7
μ value		0.40	0.40	0.41	0.40	0.40	0.41	0.39	0.39	0.39
12.5	0	10.2	10.2	10.2	10.6	10.6	10.6	10.4	10.4	10.4
	7	186.2	172.5	152.9	181.6	188.6	189.8	159.2	173.7	174.4
μ value		0.41	0.40	0.39	0.40	0.41	0.41	0.39	0.40	0.40
25.0	0	10.2	10.2	10.2	10.6	10.6	10.6	10.4	10.4	10.4
	7	138.9	169.5	154	160.7	151.3	163.3	126.1	158.1	151.3
μ value		0.37	0.40	0.39	0.39	0.38	0.39	0.36	0.39	0.38
50.0	0	10.2	10.2	10.2	10.6	10.6	10.6	10.4	10.4	10.4
	7	42.4	43.9	42.8	53.9	42.9	43.9	53.9	54.4	59.8
μ value		0.20	0.21	0.20	0.23	0.20	0.20	0.23	0.24	0.25

Table E.10 (continued)

		Test 1			Test 2			Test 3		
		Fresh weight (mg L^{-1}) per replicate			Fresh weight (mg L^{-1}) per replicate			Fresh weight (mg L^{-1}) per replicate		
Concentration (mg L^{-1})	Day	A	B	C	A	B	C	A	B	C
100.0	0	10.2	10.2	10.2	10.6	10.6	10.6	10.4	10.4	10.4
	7	16.7	19.4	16.7	19.9	20.4	17.9	22.2	23	23.9
µ value		0.070	0.092	0.074	0.09	0.09	0.07	0.11	0.11	0.12
200.0	0	10.2	10.2	10.2	10.6	10.6	10.6	10.4	10.4	10.4
	7	13	15.5	17.8	17.1	15	15.1	17.2	19.5	18.7
µ value		0.035	0.06	0.08	0.07	0.05	0.05	0.07	0.09	0.08
400.0	0	10.2	10.2	10.2	10.6	10.6	10.6	10.4	10.4	10.4
	7	13.8	12	14.2	13.9	13.7	13.1	15.6	18.1	17.3
µ value		0.04	0.02	0.05	0.04	0.04	0.03	0.06	0.08	0.07

Source: Godoy et al. (2018)

Appendix F – Concentrations of metformin reported in the literature for fresh surface water

Table F.1 Reported concentrations (µg L^{-1}) of metformin in surface waters in several countries. Only values accompanied by the respective detection and quantification limits of the methods or the reporting limit (derived based on the method detection limit) were considered for the calculation of the preliminary risk quotient. The value in bold was used to estimate the MEC/PNEC ratio. N.I. = not informed

Sample	Country	Reported concentration (µg L^{-1})	Detection limit of the method (µg L^{-1})	Quantification limit of the method/ Laboratory reporting limit (µg L^{-1})	Reference
Lake Michigan	USA	Up to 9.24	0.0005	0.0015	Blair et al. (2013)
Rivers Rhine, Danube, Main, Neckar and Elbe	Germany	Up to 1.7	0.01	0.04	Scheurer et al. (2009)
German rivers and small creeks	Germany	Mean of 3.1	N.I.	0.01	Scheurer et al. (2012)
Surface waters from Tianjin	China	Up to 20.015	N.I.	N.I.	Kong et al. (2015)
Surface waters of four Midwestern parks	USA	Up to 0.903	N.I.	0.01	Elliott and VanderMeulen (2017)
Surface waters from the tributaries to the Great Lakes	USA	Up to **33.6**	N.I.	0.013	Elliott et al. (2017)
Lake Constance and rivers Elbe, Rhine and Weser	Germany	Up to 0.643	N.I.	0.005	Trautwein et al. (2014)
Surface waters from Rhône-Alpes region	France	Up to 0.735	0.015	N.I.	Vulliet and Cren-Olivé (2011)
River Meuse basin	France, Belgium, Netherlands	Up to 1.3	0.07	N.I.	Houtman et al. (2013)
River Rhine	Switzerland, Austria, Germany, France, Belgium, Netherlands	Up to 1.314	N.I.	0.02	Ruff et al. (2015)
Surface waters downstream from sewage treatment plants and rivers located on Annapolis Valley, South Shore and Metropolitan Halifax	Canada	Up to 1.487	0.012	N.I.	Ghoshdastidar et al. (2015)

(To be continued)

Table F.1 (continued)

Sample	Country	Reported concentration (µg L^{-1})	Detection limit of the method (µg L^{-1})	Quantification limit of the method/ Laboratory reporting limit (µg L^{-1})	Reference
Surface waters from Danube river	Austria	Mean of 0.104	N.I.	0.014	Martín et al. (2012)
Langat river in Bangi town	Malaysia	0.293	0.281	N.I.	Al-Odaini et al. (2010)
Streams across the USA	USA	Up to 0.15	N.I.	0.003	Kolpin et al. (2002)

Al-Odaini, N.A., Zakaria, M.P., Yaziz, M.I., Surif, S., 2010. Multi-residue analytical method for human pharmaceuticals and synthetic hormones in river water and sewage effluents by solid-phase extraction and liquid chromatography–tandem mass spectrometry. J. Chromatogr. A. 1217, 6791-6806. DOI: 10.1016/j.chroma.2010.08.033

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APPENDIX G – Update of ecotoxicity data for metformin reported in the literature using aquatic test-organisms.

Table G.1 Acute ecotoxicity data reported for metformin. Data are reported considering metformin base concentrations (data reported for metformin hydrochloride were recalculated into metformin base). Only the values in bold have been used for deriving environmental quality standard (MAC-EQS). The validity of the toxicity data generated from our study has been evaluated by us according to the Criteria for Reporting and Evaluating Ecotoxicity Data (CRED) (Moermond et al., 2016). The validity of the other data reported in the literature was evaluated by Moermond and Smit (2016) and/or by the Swiss Ecotox Centre (Oekotoxzentrum, Centre Ecotox, 2016), according to Klimisch et al. (1997) and the CRED criteria.

Taxonomic group	Trophic level	Species	Endpoint	Parameter	Value (mg L^{-1})	Reference	Observation
Algae	Producer	*Desmodesmus subspicatus*	Growth inhibition – 72 h	EC_{50}	**> 320**	Cleuvers (2003)	Validated by Moermond and Smit (2016)
Algae	Producer	*Raphidocelis subcapitata*	Growth inhibition – 72 h	EC_{50}	**>77.2**	Confidential data reported by Moermond and Smit (2016)	Validated by Moermond and Smit (2016)
Macrophyte	Producer	*Lemna minor*	Growth inhibition (frond area) – 7d	EC_{50}	**53.7**	This study	Validated by us according to the CRED method
Macrophyte	Producer	*L. minor*	Growth inhibition (frond number) – 7 d	EC_{50}	58.9*	This study	Validated by us according to the CRED method
Macrophyte	Producer	*L. minor*	Growth inhibition (frond number) – 7 d	EC_{50}	110*	Cleuvers (2003)	Validated by Moermond and Smit (2016)
Macrophte	Producer	*L. minor*	Growth inhibition (fresh weight) – 7 d	EC_{50}	58.7*	This study	Validated by us according to the CRED method
Crustacean	Primary consumer	*Daphnia similis*	Immobilization – 48 h	EC_{50}	**14.3**	This study	Validated by us according to the CRED method
Crustacean	Primary consumer	*Daphnia magna*	Immobilization – 48 h	EC_{50}	**64**	Cleuvers (2003)	Validated by Moermond and Smit (2016)
Crustacean	Primary consumer	*D. magna*	Immobilization – 48 h	EC_{50}	>86***	Confidential data reported by Moermond and Smit (2016)	Validated by Moermond and Smit (2016)
Crustacean	Primary consumer	*Daphnia sp.*	Immobilization – 48 h	EC_{50}	101	FDA-CDER (2002)	Moermond and Smit (2016) attributed a quality code of 4 according to the Klimisch et al. (1997) scheme

(To be continued)

Table G.1 (continued)

Taxonomic group	Trophic level	Species	Endpoint	Parameter	Value (mg L^{-1})	Reference	Observation
Crustacean	Primary consumer	*Daphnia sp.*	Immobilization – 48 h	EC_{50}	60**	Landesumweltamt Brandenburg (2002). Original data from Merck Lipha (1997)	Moermond and Smit (2016) attributed a quality code of 4 according to the Klimisch et al. (1997) scheme
Cnidarian	Secondary consumer	*Hydra attenuata*	Mortality – 96 h	LC_{50}	3918.0*	This study	Validated by us according to the CRED method
Cnidarian	Secondary consumer	*H. attenuata*	Morphological alterations- 96 h	EC_{50}	**2709.0**	This study	Validated by us according to the CRED method
Fish	Secondary consumer	*Danio rerio*	Mortality – 96 h	LC_{50}	**1315.5**	This study	Validated by us according to the CRED method
Fish	Secondary consumer	*D. rerio*	Mortality – 96 h	LC_{50}	>86****	Confidential data reported by Moermond and Smit (2016)	Validated by Moermond and Smit (2016)
Fish	Secondary consumer	*D. rerio*	Locomotor behavior and malformations – 120 h	NOEC	600	This study	Validated by us according to the CRED method
Fish	Secondary consumer	*Lepomis macrochirus*	Mortality – 96 h	NOEC	≥766	FDA-CDER (2002)	Moermond and Smit (2016) attributed a quality code of 4 according to the Klimisch et al. (1997) scheme
Fish	Secondary consumer	*Pimephales promelas* (juveniles)	Increase of mRNA expression of vitellogenin, estrogen receptor-alpha, cytochrome CYP3A126 and gonadotropin releasing hormone – 7 d	LOEC	≤0.001	Crago et al. (2016)	The Oekotoxzentrum-Centre Ecotox (2016) attributed the quality score R2/C3 to this study, according to the CRED method

(To be continued)

Table G.1 (continued)

Taxonomic group	Trophic level	Species	Endpoint	Parameter	Value (mg L^{-1})	Reference	Observation
Fish	Secondary consumer	*P. promelas* (adults)	Increase of mRNA expression of vitellogenin, estrogen receptor-alpha, cytochrome CYP3A126 and gonadotropin releasing hormone – 7 d	NOEC	≥0.001	Crago et al. (2016)	The Oekotoxzentrum-Centre Ecotox (2016) attributed the quality score R2/C3 to this study, according to the CRED method

[*] Multiple toxicity values (from different endpoints) are available for this same species. Therefore, the most-sensitive EC_{50} value obtained in our study was selected for directly deriving the MAC-EQS.

[**] Not specified if the endpoint is based on metformin hydrochloride or metformin base.

[***] Multiple toxicity values are available for this same species (*Daphnia magna*). The bounded value was chosen instead of this unbounded one for directly deriving the EQS.

[****] Multiple toxicity values are available for this same species (*Danio rerio*). The bounded value was chosen instead of this unbounded one for directly deriving the EQS.

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Table G.2 Chronic ecotoxicity data reported for metformin. Data are reported considering metformin base concentrations (data reported for metformin hydrochloride were recalculated into metformin base). Only the values in bold have been used for deriving environmental quality standard (AA-EQS). The validity of the toxicity data generated from our study has been evaluated by us according to the Criteria for Reporting and Evaluating Ecotoxicity Data (CRED) (Moermond et al., 2016). The validity of the other data reported in the literature was evaluated by Moermond and Smit (2016) and/or by the Swiss Ecotox Centre (Oekotoxzentrum, Centre Ecotox, 2016), according to Klimisch et al. (1997) and the CRED criteria

Taxonomic group	Trophic level	Species	Endpoint	Parameter	Value ($mg\ L^{-1}$)	Reference	Observation
Algae	Producer	*Raphidocelis subcapitata*	Growth inhibition – 96 h	NOEC	**≥78**	EMEA (2011)	Validated by Moermond and Smit (2016)
Macrophyte	Producer	*Lemna minor*	Growth inhibition (frond number) – 7d	EC_{10}	**24.2**	This study	Validated by us following the CRED method
Macrophyte	Producer	*L. minor*	Growth inhibition (frond area) – 7d	EC_{10}	31.9*	This study	Validated by us following the CRED method
Macrophyte	Producer	*L. minor*	Growth inhibition (fresh weight) – 7d	EC_{10}	31.6*	This study	Validated by us following the CRED method
Crustacean	Primary consumer	*Daphnia similis*	Reproduction – 14 d	EC_{10}	**4.4**	This study	Validated by us following the CRED method
Crustacean	Primary consumer	*D. magna*	Reproduction – 21 d	NOEC	**17**	EMEA (2011)	Validated by Moermond and Smit (2016)
Crustacean	Primary consumer	*D. magna*	Mortality – 21 d	LC_{50}	38	EMEA (2011)	Validated by Moermond and Smit (2016)
Crustacean	Primary consumer	*D. magna*	Mortality – 21 d	LC_{100}	55	EMEA (2011)	Validated by The Oekotoxzentrum- Centre Ecotox (2016), according to the Klimisch et al. (1997) scheme
Crustacean	Primary consumer	*D. magna*	Reproduction – 21 d	NOEC	**7.8**	Confidential data reported by Moermond and Smit (2016)	Validated by Moermond and Smit (2016)

(To be continued)

Table G.2 (continued)

Taxonomic group	Trophic level	Species	Endpoint	Parameter	Value (mg L^{-1})	Reference	Observation
Cnidarian	Secondary consumer	*Hydra attenuata*	Reproduction inhibition – 7 d	EC_{10}	**701.8**	This study	Validated by us following the CRED method
Fish	Secondary consumer	*Danio rerio*	Hatching rate, time to hatch, survival, length, weight – 30 d post-hatch	NOEC	**≥10**	EMEA (2011)	Validated by Moermond and Smit (2016)
Fish	Secondary consumer	*Pimephales promelas*	Expression of mRNA encoding vitellogenin in male fish – 28 d	LOEC	≤0.04	Niemuth et al. (2015)	The Oekotoxzentrum-Centre Ecotox (2016) attributed the quality code R4/C4 to this study, following the CRED method
Fish	Secondary consumer	*P. promelas*	Intersex in male, size of male and fecundity of treated pairs – 360 d	LOEC	≤0.04	Niemuth and Klaper (2015)	The Oekotoxzentrum-Centre Ecotox (2016) attributed the quality code R4/C1 to this study, following the CRED method

[*] Multiple toxicity values (from different endpoints) are available for this same species (*Lemna minor*). Therefore, the most-sensitive EC_{10} value obtained in our study (for frond number) was selected for directly deriving the AA-EQS.

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APPENDIX H - Concentration-response curves for the acute and chronic tests performed with metformin

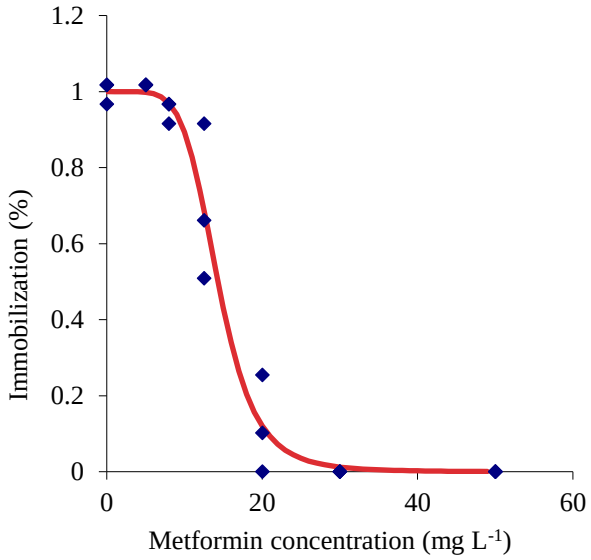


Fig. H.1 Concentration-curve for the immobilization (%) of *Daphnia similis* exposed to metformin during 48 h.

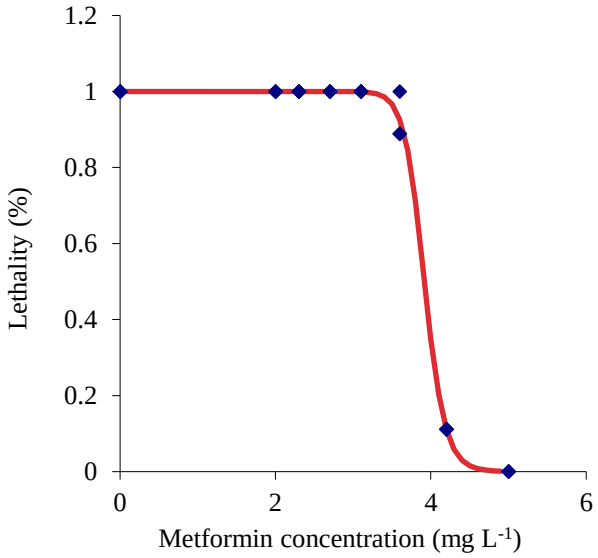


Fig. H.2 Concentration-curve for the lethality (%) of *Hydra attenuata* exposed to metformin during 96 h.

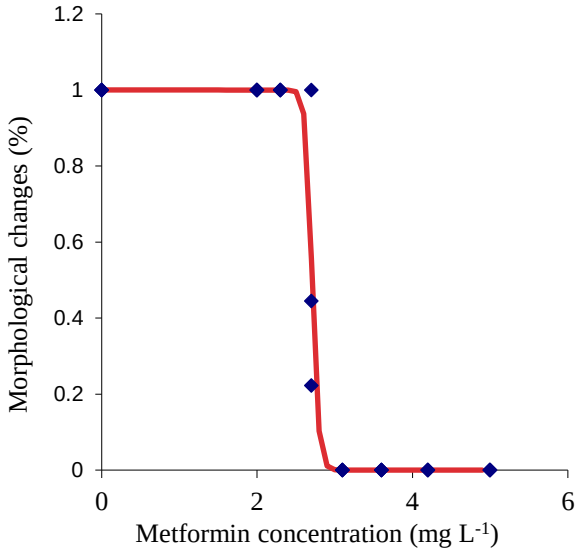


Fig. H.3 Concentration-curve for the morphological changes in relation to the control group (%) of *Hydra attenuata* exposed to metformin during 96 h.

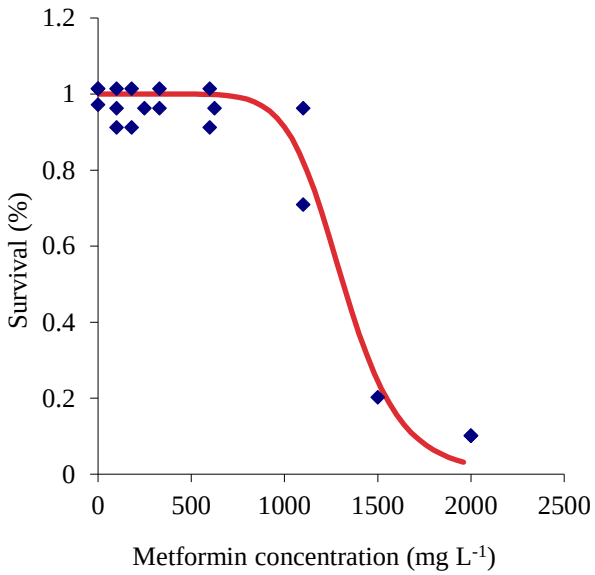


Fig. H.4 Concentration-curve for the lethality (%) of *Danio rerio* larvae exposed to metformin during 96 h.

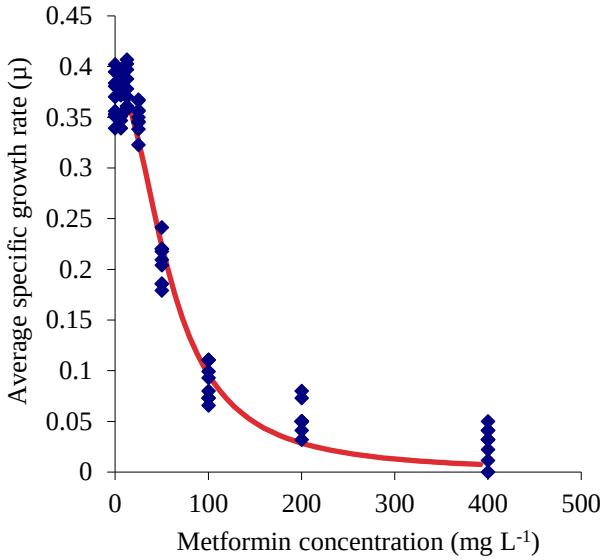


Fig. H.5 Concentration-curve for the average specific growth rate inhibition of *Lemna minor*, exposed to metformin during 7 d, based on the frond number endpoint

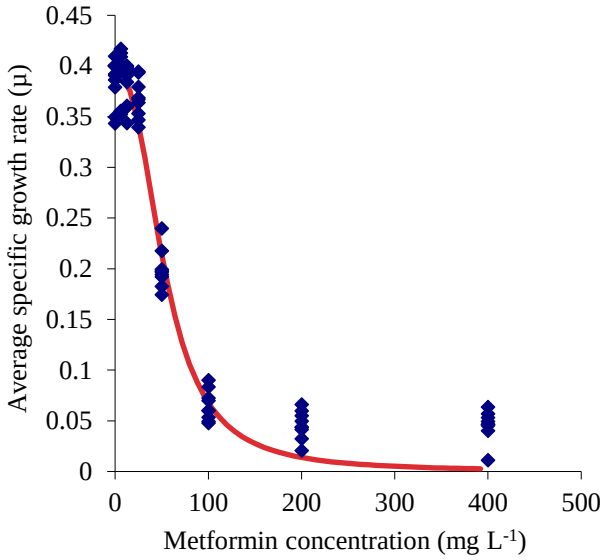


Fig. H.6 Concentration-curve for the average specific growth rate inhibition of *Lemna minor*, exposed to metformin during 7 d, based on the total frond area endpoint

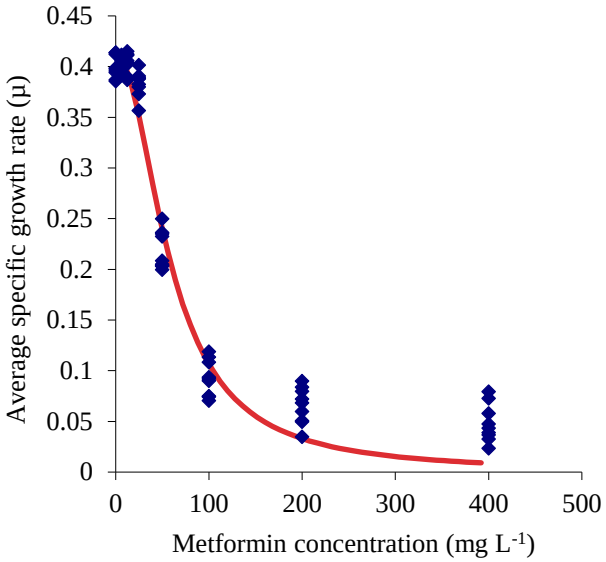


Fig. H.7 Concentration-curve for the average specific growth rate inhibition of *Lemna minor*, exposed to metformin during 7 d, based on the fresh weight endpoint

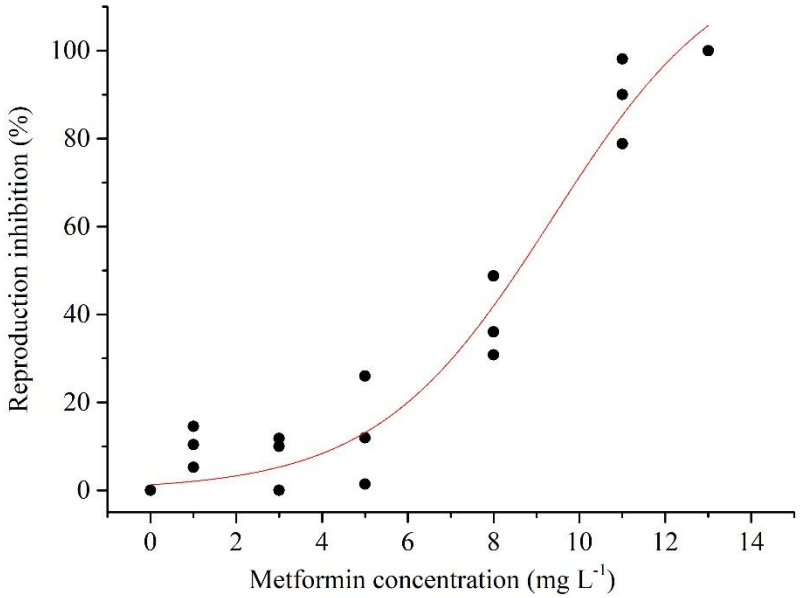


Fig. H.8 Concentration-curve for the reproduction inhibition of *Daphnia similis*, exposed to metformin during 14 d

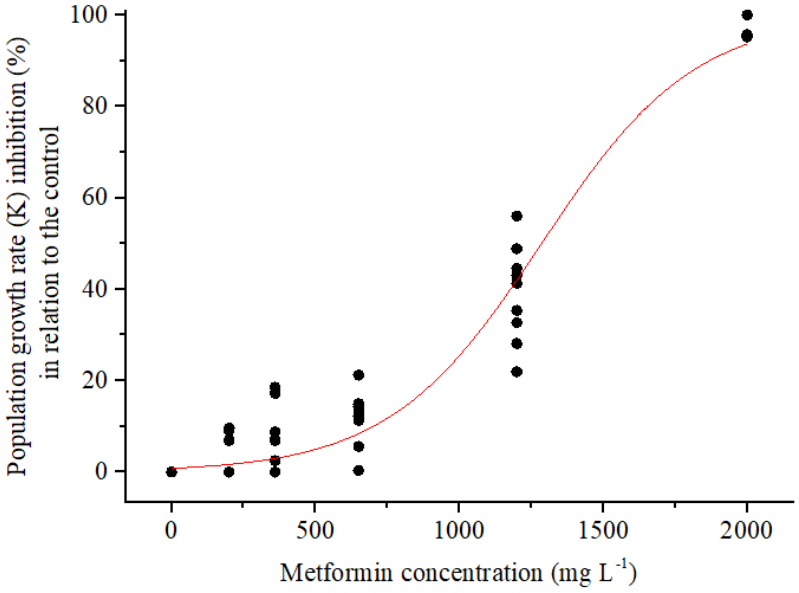


Fig. H.9 Concentration-curve for the population growth rate (K) inhibition of *Hydra attenuata*, exposed to metformin during 7 d

4.3 Article III

Assessment of the ecotoxicity of the pharmaceuticals bisoprolol, sotalol and ranitidine using standard and behavioral endpoints

Aline Andrade Godoy[a,b], Inês Domingues[c], Luciano Bastos de Carvalho[d], Ádria Caloto Oliveira[e], Carina Cristina de Jesus Azevedo[e], Jeniffer Marins Taparo[e], Patrícia Kushima[e] Assano, Vivien Mori[e], António José Arsénia Nogueira[c], Fábio Kummrow[a,f*]

[a]Department of Clinical and Toxicological Analyses, Faculty of Pharmaceutical Sciences, University of São Paulo (USP), Avenida Professor Lineu Prestes, 580, São Paulo, SP, 05508-000, Brazil.

[b]Science and Technology Institute, Federal University of Alfenas (Unifal-MG), Rodovia José Aurélio Vilela, 11,999, Poços de Caldas-MG, 37715-400, Brazil.

[c]Department of Biology & CESAM, University of Aveiro, *Campus* Universitário de Santiago, 3810-193, Aveiro, Portugal.

[d] Faculty of Economics, Administration and Accounting at Ribeirão Preto – University of São Paulo (USP), Avenida Bandeirantes, 3900, Ribeirão Preto, SP, 14040-905, Brazil

[e] School of Technology, State University of Campinas (Unicamp), Rua Paschoal Marmo, 1888 – Jardim Esmeralda, Limeira-SP, 13484-461, Limeira-SP, Brazil

[f]Institute of Environmental, Chemical and Pharmaceutical Sciences, Federal University of São Paulo (Unifesp), Rua São Nicolau, 210, 09972-270, Diadema, SP, Brazil.

ABSTRACT

The pharmaceuticals bisoprolol (BIS), sotalol (SOT) and ranitidine (RAN) are among the most consumed pharmaceuticals worldwide and are frequently detected in different aquatic ecosystems. However, very few ecotoxicity data are available in the literature for them. To help filling these data gaps, toxicity tests with the algae *Raphidocelis subcapitata*, the macrophyte *Lemna minor*, the cnidarian *Hydra attenuate*, the crustacean *Daphnia similis* and the fish *Danio rerio* were performed for assessing the ecotoxicity of these pharmaceuticals. Standard as well as non-standard endpoints were evaluated, including the locomotor behavior of *D. rerio* larvae. Results obtained for SOT and RAN showed that acute adverse effects are not expected to occur on aquatic organisms at the concentrations at which these pharmaceuticals are usually found in fresh surface waters. On the other hand, BIS was classified as hazardous to the environment in the acute III category. Locomotor behavior of *D. rerio* larvae was not affected by BIS and RAN. A disturbance on the total swimming distance at the dark cycle was observed only for larvae exposed to the highest test concentration of 500 mg L^{-1} of SOT. *D. similis* reproduction was affected by BIS with an EC_{10} of 3.6 (0.1 – 34.0) mg L^{-1}. A risk quotient (RQ) of 0.04 was calculated for BIS in fresh surface water, considering a worst-case scenario. An ecological risk is not expected for the chronic exposure of pelagic freshwater species to BIS, considering the endpoints and the bioassays performed in this study. To the best of our knowledge, this study presents the first chronic toxicity data with BIS on non-target organisms.

Keywords: Ecological risk; β-receptors; Locomotor activity; Reproduction; Zebrafish; Chronic toxicity

1. Introduction

The increased worldwide consumption of drugs and the observed medicinal development progress have made pharmaceuticals one of the top issues of environmental concern (Stankiewicz et al. 2015). Antagonists of beta-adrenergic receptors (betablockers) are among the most consumed pharmaceuticals all over the world (Maszkowska et al. 2014a). They are prescribed for the treatment of hypertension, ischemic heart disease, congestive heart failure and certain arrhythmias (Westfall and Westfall 2012). They are also among the most frequently detected therapeutical classes in the environment (Santos et al. 2010; Godoy at al. 2015).

Within the betablockers therapeutic class, atenolol, metoprolol and principally propranolol are the most studied components from the ecotoxicological prespective (Godoy et al. 2015), mainly due to their common presence in the environment as a result of their high consumption, high resistance to hydrolysis, bioavailability, mobility (Maszkowska et al. 2014a; Maszkowska et al. 2014b) and their insufficient removal from wastewater (Ternes 1998; Maurer et al. 2007; Radjenovic et al. 2007). However, these same characteristics are also present in other betablockers frequently detected in aquatic environments but much less studied regarding their potential ecotoxicity effects. This is the case of the betablockers bisoprolol (BIS) and sotalol (SOT).

BIS is a highly selective antagonist of the β1 receptors (Westfall and Westfall 2012). This pharmaceutical is highly consumed in countries such as Germany, Russia, Canada, Slovenia and Sweden (Scheurer et al. 2010; DSM GROUP 2014; IMS HEALTH 2015; Klančar et al. 2016; Lindim et al. 2016). Moreover, 50 – 60 % of BIS is excreted unaltered in the urine (Bühring et al. 1986). SOT is a non-selective antagonist of β-receptors (Sampson and Kass 2012), also highly consumed worldwide, especially in Germany, Netherlands and Sweden (Scheurer et al. 2010; Oosterhuis et al. 2013; Lindim et al. 2016). 80 – 90 % of SOT is excreted unaltered in the urine (Stankiewicz et al. 2015). Regarding the abiotic degradation of these pharmaceuticals, BIS and SOT only absorb in the UV-C range and therefore the photodegradation by direct photolysis of them is not probable in environmental waters (Piram et al. 2008). Moreover, BIS and SOT are stable under UV radiation in pure water (Piram et al 2008). It is also worth mentioning the incomplete removal of BIS and SOT in wastewater treatment plants (WWTP). Removal rates for SOT in WWTP employing conventional activated sludge, advanced membrane bioreactor and oxidation ditch treatments have been reported to be of 0 to 59 % (Radjenović et al. 2009; Scheurer et al. 2010; Lara-Martín et al. 2014). For BIS,

these removal rates vary from 36 % using activated sludge treatment (Scheurer et al. 2010) to 40 – 70 % using secondary and tertiary composed treatments (Gabet-Giraud et al. 2010). Lee et al. (2007) confirmed that betablockers, including BIS and SOT, are stable enough to resist to the conventional wastewater treatment techniques. As a result, BIS and SOT have been frequently detected in fresh surface waters (Gonçalves et al. 2013; Houtman et al. 2013; UBA et al. 2013; Jauković et al. 2014; Ruff et al. 2015), effluents from WWTP (Gabet-Giraud et al. 2010; Huerta-Fontela et al. 2010; Loos et al. 2013; Oliveira et al. 2015; Klančar et al. 2016), hospital effluents (Verlicchi et al. 2012; Santos et al. 2013; Oliveira et al. 2015), groundwater (López-Serna et al. 2013; Reh et al. 2013) and even in drinking water (Huerta-Fontela et al. 2011; Petrović et al. 2014).

Another therapeutic class of environmental concern is of the drugs called histamine H2-receptor antagonists, of which ranitidine (RAN) is an important representant. Ranitidine (RAN) is a very popular and also one of the most consumed pharmaceuticals all over the world (Savci 2013; Bojić et al. 2015). It is prescribed for pharmaceuticals. RAN was in the top 10 list of pharmaceuticals most consumed in volume in European hospitals in 2007 (Ferrer et al. 2011). This pharmaceutical was classified as a drug of high environmental concern because of its widespread occurrence in aquatic environments (Bergheim et al. 2012). In Italy, Castiglioni et al. (2006) calculated the load of 87-520 mg/day/1000 inhabitants of RAN in sewage treatment plants (STP) influents, while in STP effluents, this range was of 21-266 mg/day/1000 inhabitants. The average daily output loads of RAN, i.e., the quantities of this pharmaceutical discharged into the environment for Rubí WWTP (Barcelona, Spain) was calculated to be of 0.55 – 5.30 g day^{-1} for the treated effluent in a study performed by Radjenović et al. (2007). For the Terrassa WWTP (Barcelona, Spain), this output load was calculated to be of 4.4 – 9.1 g day^{-1} (Radjenović et al. 2009). Therefore, RAN release into aquatic environments is expressive. RAN is eliminated mainly as unchanged drug. Its percentage of excretion in the unaltered form vary between 30 – 70 % after oral administration and between 70 – 80% after intravenous dosage (Vediappan and Lee 2011). The removal of RAN in WWTP Terrassa (Spain) employing conventional activated sludge (CAS) treatment was considered poor (around 25 %) in a study conducted by Radjenović et al. (2009). These percentages ranged from around 29.5 % to 44 % using pilot-scale membrane bioreactors treatments operating in parallel with the CAS treatment. Therefore, RAN is resistant to conventional biological treatment. In addition, RAN stability in water is considered prolonged, being of 160 h at pH 6.18 and 65°C (Ferrari et al. 2011). Thus, these factors contribute to the presence and persistence of RAN in aquatic environments such as fresh surface water (Ginebreda et al. 2010; López-Roldan et al. 2010; Ruff et al. 2015),

marine and estuarine water (Gros et al. 2012, Meador et al. 2016), WWTP effluents (Gros et al. 2012, Oliveira et al. 2015, Meador et al. 2016), hospital effluents (Verlicchi et al. 2012, Santos et al. 2013, Oliveira et al. 2015), industrial effluents (Larsson et al. 2007), groundwaters (López-Serna et al. 2013) and drinking water (Gros et al. 2012, Kot-Wasik et al. 2016).

In addition to their presence in aquatic matrices, BIS and RAN have been recently reported to be present also in the aquatic invertebrate biota from six streams near Melbourne, Australia (Richmond et al. 2018). Richmond and collaborators (2018) quantified the pharmaceuticals BIS and RAN, in the order of ng g^{-1} dry weight, in filter-feeding caddisfly larvae of the Hydropsychidae family, which is dominant in invertebrate communities from streams receiving effluent from a wastewater treatment facility (Brushy Creek, Australia) with tertiary treatment and disinfection. The authors mentioned that the consequences for fish and wildlife of such chronic exposures to the pharmaceuticals found in the affected biota are still unknown.

Therefore, despite their common presence in several aquatic environments, acute and principally chronic toxicity effects on non-target organisms are still poorly understood for the pharmaceuticals SOT, BIS and RAN (Besse and Garric 2008; Isidori et al. 2009; Bergheim et al. 2012; Godoy et al. 2015). Lahti and Oikari (2011) reported that BIS may pose risks to aquatic organisms based on microbial transformation studies but they recognized the difficult of assessing the realism of this risk in view of the lack of experimental data concerning the ecotoxicity of this betablocker. In fact, apart from the acute ecotoxicity studies of Guo et al. (2005) with the algae *R. subcapitata* and of Minguez et al. (2014) with *R. subcapitata* and the crustacean *D. magna*, no study was found in the literature for the ecotoxic effects induced by BIS on fish. Likewise, no chronic toxicity data was found in the literature for this betablocker. Databases such as USEPA Ecotox Knowledgebase (https://cfpub.epa.gov/ecotox/) and Wikipharma (http://www.wikipharma.org/api_data.asp) do not show ecotoxicity data for BIS. Regarding SOT, a long-term test carried out by Feiner et al. (2014) showed that this betablocker can alter the reproductive period of the mudsnail *Potamopyrgus antipodarum*. However, more ecotoxicity studies on organisms belonging to other trophic levels and using different endpoints are still needed in order to conclude about the ecological risk posed by SOT (Godoy et al. 2015). Regarding RAN, Besse and Garric (2008) included this pharmaceutical in a list of priority pharmaceuticals regarding their environmental relevance, based on predicted environmental concentration (PEC) values for this compound (>100 ng L^{-1} in surface waters). These authors also highlighted the need to build ecotoxicological data for this pharmaceutical (Besse and Garric 2008).

In addition, β-receptors have been reported to be present in fishes (Steel et al. 2011). Orthologs for human drug targets such as β1 and β2-adrenergic receptors (target of BIS and SOT), potassium voltage-gated channel (target of SOT) and Histamine H_2-receptor (target of RAN) have been predicted to exist in daphnids and especially in fishes (Gunnarsson et al. 2008). Therefore, possible toxic effects of these pharmaceuticals on these non-target organisms must be investigated.

In order to fill some of these data gaps, this study aimed to evaluate the ecotoxicity of the betablockers BIS and SOT and of the H2-receptor antagonist RAN using five test organisms from three different trophic levels and different endpoints, including the locomotor behavior of *Danio rerio*. In addition, a preliminary ecological risk assessment was performed for the pharmaceutical BIS, based on the chronic data generated in this study.

2. Materials and Methods

2.1 Pharmaceuticals

Bisoprolol hydrochloride (1-[4-[[2-(1-Methylethoxy)ethoxy]methyl]phenoxy]-3-[(1-methylethyl)amino]-2-propanol fumarate salt; CAS number 104344-23-2) and Sotalol hydrochloride (N-[4-[1-Hydroxy-2-(isopropylamino)ethyl]phenyl]methanesulfonamide hydrochloride; CAS number 959-24-0) were provided by Moehs Catalana (Spain) with 98.8 and 99.8 % purity, respectively. Ranitidine hydrochloride (Dimethyl[(5-{[2-{[(E)-1-(methylamino)-2-nitroethenyl]amino}ethyl)sulfanyl]methyl}furan-2-yl)methyl]amine hydrochloride; CAS number 66357-59-3) was provided by Fagron (India), with 100.8 % purity. Stock solutions and test concentrations were achieved by dissolving the pharmaceuticals in the test medium of each organism, without using any solvent. All chemicals used for preparing the test medium were of high purity (> 98 %), supplied by Sigma-Aldrich (Brazil) or by Merck (Germany). Stock solutions and test concentrations were prepared just before each experiment.

2.2 Test-organisms

The algae *Raphidocelis subcapitata*, the macrophyte *Lemna minor*, the crustacean *Daphnia magna* and the cnidarian *Hydra attenuate* were cultivated and tested at the Laboratory of Ecotoxicology and Genotoxicity (LAEG), State University of Campinas, Unicamp (Brazil). The *Danio rerio* eggs were supplied by the facility located at the Department of Biology of the University of Aveiro (Portugal), where the toxicity testes with this species were performed.

R. subcapitata was cultivated in Oligo medium, according to ABNT NBR 12648 (2018). Prior to the toxicity tests, algal culture was maintained during 72 h under continuous agitation (100 – 175 rpm) and under continuous fluorescent light (> 4000 lux) in order to achieve the logarithmic phase of growth. The conditions of cultivation of *L. minor*, *D. similis*, *H. attenuate* and *D. rerio* were the same as described in Godoy et al. (2018).

2.3 Toxicity tests

2.3.1 R. subcapitata growth inhibition test

Toxicity tests with the algae *R. subcapitata* were performed according to the ABNT NBR 12648 (2018) and OECD n. 201 (2011) protocols. Algal inoculum was obtained from a 3-day old culture in logarithmic phase of growth. Test-inoculum had an initial cell density varying between 3.51 to 5.81 x 10^7 cells/mL. The tests were performed in erlenmeyers filled with the test sample (pharmaceuticals dissolved in the test medium) plus the algal inoculum at the final volume of 50 mL (for BIS) or 20 mL (for SOT and RAN). The erlenmeyers were maintained under continuous agitation (100 – 175 rpm) and under continuous fluorescent light (4000 ± 400 lux), at 24 ± 2 °C. Tests duration was of 72 h. Each test concentration was performed in triplicate. The endpoint evaluated was the growth rate inhibition, measured by the correlation of the number of algal cells with the spectrophotometric absorbance at 440 nm.

The definitive test concentrations of BIS, SOT and RAN were defined based on the results of preliminary experiments. For evaluating the effects of BIS on the algae, the concentrations used were of 10, 30, 50,100, 300 and 500 mg L^{-1}, obtained from a stock solution of 5000 mg L^{-1}. For assessing the toxicity of RAN, the test concentrations were the same ones as used for BIS plus 1000 mg L^{-1}, which were prepared from a stock solution of 10000 mg L^{-1}. For SOT, the concentrations of 50, 100, 300, 500, 1000 and 3000 mg L^{-1} were tested, prepared from a stock solution of 30000 mg L^{-1}.

2.3.2 L. minor growth inhibition test

L. minor growth inhibition tests were performed according to the OECD n. 221 guideline (2006). Glass beakers were filled at the final volume of 100 mL with the following concentrations of each of the single pharmaceuticals BIS, SOT or RAN dissolved in Steinberg medium: 25, 50, 85, 160, 300, 540 and 1000 mg L^{-1}. Stock solutions of each pharmaceutical were of 15000 mg L^{-1}. A total of four *L. minor* colonies with three fronds each (with similar frond area) was assigned to each of the beakers containing the test samples. Each test concentration was performed in triplicate. The tests were carried out at 24 ± 2°C, under continuous cool white light intensity of 6500 lux. Test duration was 7 days. Tests were semi-static, with renewal of the test solutions each 48 h, in order to allow for possible degradations of the pharmaceuticals and a better background transparency for frond area evaluation. The endpoint assessed was the specific average growth rates (μ), determined based on the frond number, total frond area and fresh weight, according to the method described in Godoy et al. (2015).

2.3.3 D. similis immobilization and reproduction tests

D. similis acute toxicity tests were carried out following the ABNT NBR n. 12.713/2016 (2016) and the OECD n. 202 (2004) guidelines. The acute tests consisted of the exposition of five neonates < 24 h (2 to 3-week-old mothers) to 10 mL each of the following test concentrations of BIS: 50, 60, 70, 80, 90, 100 and 120 mg L^{-1}, prepared from a stock solution of 500 mg L^{-1}. For SOT and RAN, test concentrations were of 100, 150, 200, 300, 400 and 500 mg L^{-1}, obtained from the respective stock solutions of 1000 mg L^{-1}. Four treatment replicates were performed for each test concentration. The tests were carried out in vials maintained in climatic chambers at 20 ± 2 °C and 16 h light: 8 h dark photoperiod. Acute toxicity test duration was of 48 h. The endpoint evaluated was the number of daphnids immobilized.

Chronic toxicity tests using *D. similis* were performed according to the OECD n. 211 (2012) guideline. A modification was made regarding the exposure time, which was of 14 d (Godoy et al. 2018). 1 neonate < 24 h old (from the third progeny) was assigned to each of the plastic recipients filled with 40 mL of the test concentrations of BIS dissolved in MS medium. From a stock solution of 5000 mg L^{-1}, the following concentrations of BIS were prepared: 0.1; 0.6; 1.0; 3.0; 10.0; 30.0; 60.0 and 100.0 mg L^{-1}. Ten treatment replicates were performed for each test concentration. 80 μL of *R. subcapitata* was used to feed each of the test mother daily. The tests

were performed in semi-static conditions, with medium renewal every two days. Tests were incubated at 20 ± 2 °C and 16 h light: 8 h dark photoperiod. The reproduction compared to the controls was the endpoint evaluated in the chronic toxicity tests, by the counting of the number of living offsprings produced by each parent daphnid.

2.3.4 H. attenuata morphological effects and reproduction tests

H. attenuata acute toxicity tests were carried out according to Trottier et al. (1997). Three hydras without buds were addressed to each well of a 12-well plate filled with 5 mL of the test concentrations of BIS or SOT dissolved in the appropriate medium. From stock solutions of 5000 mg L^{-1} of BIS, the following concentrations, calculated in geometric scale, were prepared: 20.0; 40.0; 75.0; 140.0; 265.0 and 500.0 mg L^{-1}. For SOT and RAN, besides these concentrations, the additional concentration of 1000 mg L^{-1} was also tested. Control and treatments were tested in triplicate. The tests were incubated at 22 ± 2 °C, under a 16:8 h light-dark photoperiod. *H. attenuata* were not fed 24 h prior the tests. Test duration was 96 h. The endpoint evaluated were the morphological changes observed in the test organisms, comprising 4 stages: clubbed tentacle, shortened tentacle, tulip and disintegrated, according to Trottier et al. (1997). The first two stages were considered as endpoints of sub-lethality, while the last two ones were recorded endpoints of lethality.

The *H. attenuata* chronic toxicity tests were performed following Holdway (2005). To each of the glass dishes filled with 35 mL of test concentrations of BIS were assigned five hydroids (hydra with one tentacled bud). From a stock solution of 5000 mg L^{-1} of BIS, the following test concentrations were prepared in geometric scale: 10.0; 16.0; 25.0; 40.0; 62.0 and 100.0 mg L^{-1}. Three replicates were performed for each of the test concentrations. Controls were also performed in triplicate. The tests were incubated at 25 ± 0.5° C, at 12:12 h photoperiod. The test organisms were fed daily with nauplii of *Artemia salina*. Just after feeding period (2 h), the test medium was renewed. Test duration was 7 days. The endpoint evaluated was the mean relative population growth rate (K), compared to the controls and calculated based on the number of hydroids in each replicate, which were counted daily.

2.3.5 D. rerio embryo-larval development and behavior tests

Fish embryo acute toxicity (FET) tests with *D. rerio* were carried out according to the OECD n. 236 (2013) guideline. One newly fertilized egg (3 h post-fertilization, hpf) was assigned to each well of 24-well plates filled with 2 mL of the test concentrations of BIS or SOT, dissolved in zebrafish water system. The following test concentrations of BIS were prepared from a stock solution of 1000 mg L^{-1}: 25.0; 40.0; 60.0; 100.0; 160.0; 250.0 and 400.0 mg L^{-1}. For SOT and RAN, besides these test concentrations, the additional concentration of 1000 mg L^{-1} was also tested. Twenty eggs individually exposed in each well filled with 2 mL of each test concentration composed the replicates. In addition, four eggs individually placed in each well were used as internal plate control. 24 eggs individually placed into each well filled with 2 mL of dilution water were used as negative control. Tests were performed in climatized room, at 26 ± 1 ° C, at 16 h light: 8 h dark photoperiod. FET-test duration was of 96 h. Every 24 h, the lethal and sub-lethal following endpoints were observed using a stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon Corporation): coagulation of fertilized eggs, lack of somite formation, lack of tail detachment, hatching success, pigmentation failures, edema (heart and yolk) and spinal deformation. In addition, at 48 hpf, the number of heart beats was visually counted and recorded for each embryo during 20 s.

For evaluating the effects of BIS, SOT and RAN on the locomotor behavior of *D. rerio*, 20 larvae individually placed in each well of 24-well plates were exposed to each of the test concentrations of each of the single betablockers. Test concentrations not inducing any abnormalities or mortality in the FET-tests were used in the behavior assay. For BIS, these test concentrations were the following ones: 0.04; 0.4; 4.0; 40.0; 60.0 and 100.0 mg L^{-1}. For assessing the SOT and the RAN effects, the concentrations selected were of 0.05; 0.5; 5.0; 50.0 and 500 mg L^{-1}. In order to increase the statistical power of the experiments, considering the high variability of behavioral responses, three sets of independent negative controls were tested, called CT1, CT2 and CT3. The locomotor activity of the larvae was tracked at 120 hpf using the system Zebrabox – Zeb (Viewpoint Life sciences, Lyon, France). The recordings were made directly on the 24-well plates used for exposure, over a period of 25 min, by alternating 10 min dark and 10 min light (25 % light intensity), after a 5 min light adaptation period. The locomotor behavioral endpoints assessed were the Total swimming time (TST) and Total swimming distance (TSD).

2.4 Statistical analyses

All toxicity tests were performed at least twice in independent tests. Data from independent tests were grouped for calculating the toxicological endpoints. Effective and lethal concentrations at 50 % (EC_{50} and LC_{50}) and their respective 95 % confidence intervals (95 % C.I.) were calculated using an automated Excel spreadsheet (ToxCalcMix v. 1.0) developed at the University of Aveiro & CESAM, Portugal (available at https://pydio.bio.ua.pt/public/toxcalcmix). Equations and models used in the development of this spreadsheet, including the allosteric decay model adjusted to our data, are described in Barata et al. (2006) and Jonker et al. (2005). The effective concentrations at 10 % (EC_{10}) were calculated using non-linear regressing analyses with the software OriginPro v. 2015 (OriginLab Corporation, USA). The sigmoidal models that respectively showed the best fit to the chronic data obtained for BIS were applied considering the R^2 coefficient and the residues analysis. Following these prerogatives, the logistic model was used for algae data; the logistic type 3 model was applied to the *H. attenuata* data; the dose-response model was fitted to the data obtained for *L. minor*, while for *D. similis*, the Hill type 1 model showed the best fit to the data. A one-way analysis of variance (ANOVA) was used to analyze statistically significant differences between treatments and controls and to determine the non-observed effect concentrations (NOEC) in the locomotor behavior tests and in the heart beat analyses. ANOVA assumption of normality was verified using histogram analysis and the Shapiro-Wilk test. The homogeneity of variances was verified using the Bartlett test. When normality and/or homogeneity of variances were not verified, a Kruskal-Wallis test was performed. Box and whisker charts, including the analysis of outliers for the behavioral tests, were performed on Microsoft Excel v. 2016. The the concentration-response curves obtained for the pharmaceuticals and test organisms used in this study are shown in Supplementary Material.

2.5 Ecological risk assessment of BIS

Predicted non-effect concentration (PNEC) was estimated for BIS according to the European Commission (2003) and the EMEA (2006) protocols. Since it is supposed a continuous exposure of the aquatic organisms to BIS via WWTP effluents, the PNEC was derived based on long-term toxicity data. The deterministic approach was used, by applying an adequate assessment factor (AF) to the lowest relevant EC_{10} value obtained from our study. The maximum measured environmental concentrations (MEC) in fresh surface waters ($MEC_{surface\ water}$) value used for risk assessment of BIS was retrieved from the database of the German Environmental Agency (Umwelt Bundesamt), available at:

https://www.umweltbundesamt.de/dokument/database-pharmaceuticals-in-the-environment-excel. This database gathers MEC data of human and veterinary pharmaceuticals reported worldwide (Africa, Asia-Pacific, Eastern Europe, Latin America and Caribbean States, Western Europe, North America, Australia and New Zealand) for environmental matrices including fresh surface waters. For the ecological risk assessment, a risk quotient (RQ) was calculated by dividing the $MEC_{surface\ water}$ by the PNEC estimated for BIS. Thus, a realistic worst-case global scenario was considered in our study.

3. Results and Discussion

3.1 Ecotoxicity of BIS, SOT and RAN

The results reported in this study are based on nominal concentrations. The EC_{50} values and the respective 95 % C.I. obtained for the toxicity effects induced by BIS, SOT and RAN on the evaluated test organisms are shown in Table 1.

Table 1 Effect concentration at 50 % (EC_{50}) with the respective 95 % confidence interval (95 % C.I.) and non-effect concentration (NOEC) obtained in the acute toxicity tests performed for assessing the effects induced by the pharmaceuticals sotalol (SOT), bisoprolol (BIS) and ranitidine (RAN) on aquatic test organisms from three trophic levels

Pharmaceutical	Test organism	Toxicological endpoint	Ecotoxicity data (95 % C.I.) mg L^{-1}
RAN	*R. subcapitata*	$EC_{50\text{-}72\ h}$ – growth inhibition	613. 9 (544.5 – 707.7)
	L. minor	$EC_{50\text{-}7d}$ - growth inhibition (frond number, total frond area and fresh weight)	>1000
	D. similis	$EC_{50\text{-}48\ h}$ - immobilization	247.3 (239.7 – 254.9)
	H. attenuata	$LC_{50\ -\ 96\ h}$ - lethality	>1000
	H. attenuata	$EC_{50\ -\ 96\ h}$ – morphological changes	>1000
	D. rerio	$LC_{50\ -\ 96\ hpf}$ - lethality	>1000
	D. rerio	$NOEC_{96hpf}$ – malformations	>1000
	D. rerio	$NOEC_{48hpf}$ – heart beat rate	>1000
SOT	*R. subcapitata*	$EC_{50\text{-}72\ h}$ – growth inhibition	620.7 (552.7 – 667.2)
	L. minor	$EC_{50\text{-}7d}$ - growth inhibition (frond number, total frond area and fresh weight)	>1000
	D. similis	$EC_{50\text{-}48\ h}$ - immobilization	325.2 (319.5 – 330.8)
	H. attenuata	$LC_{50\ -\ 96\ h}$ - lethality	>1000
	H. attenuata	$EC_{50\ -\ 96\ h}$ – morphological changes	>1000
	D. rerio	$LC_{50\ -\ 96\ hpf}$ - lethality	>1000
	D. rerio	$NOEC_{96hpf}$ – malformations	>1000
	D. rerio	$NOEC_{48hpf}$ – heart beat rate	>1000
BIS	*R. subcapitata*	$EC_{50\text{-}72\ h}$ – growth inhibition	92.1 (84.3 – 100.0)
	L. minor	$EC_{50\text{-}7d}$ - growth inhibition (frond number)	338.7 (316.4 – 361.0)
	L. minor	$EC_{50\text{-}7d}$ - growth inhibition (total frond area)	313.1 (286.4 – 339.7)
	L. minor	$EC_{50\text{-}7d}$ - growth inhibition (fresh weight)	345.1 (319.3 – 370.9)
	D. similis	$EC_{50\text{-}48\ h}$ - immobilization	93.1 (91.5 – 94.8)
	H. attenuata	$LC_{50\ -\ 96\ h}$ - lethality	192.8 (191.8 – 193.7)
	H. attenuata	$EC_{50\ -\ 96\ h}$ – morphological changes	115.2 (111.2 – 119.2)
	D. rerio	$LC_{50\ -\ 96\ h}$ - lethality	213.0 (206.2 – 219.8)
	D. rerio	$NOEC_{96h}$ - malformations	160.0
	D. rerio	$NOEC_{48hpf}$ – heart beat rate	160.0

From Table 1, we can observe that acute toxicity data for SOT and RAN showed $L(E)C_{50}$ >100 mg L^{-1} for algae, macrophyte, daphnid and fish. Therefore, the toxicity induced by these pharmaceuticals is considered insufficient to warrant classification in the Harmonised

Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures (OECD 2002).

Our results obtained for SOT are in accordance with the data reported by Hernando et al. (2004) (EC_{50-48h} > 300 mg L^{-1}) and Minguez et al. (2014) (EC_{50-48h} > 100 mg L^{-1}) using immobilization tests with *Daphnia magna* exposed to SOT. Regarding algae data, the EC_{50-72h} obtained in our tests with *R. subcapitata* were in accordance with the value of > 100 mg L^{-1} of SOT reported by Minguez et al. (2014) for this toxicological endpoint assessed on this algae species. Compared to other algae species, our EC_{50} value for *R. subcapitata* was lower than the EC_{50-24h} > 3000 mg L^{-1} reported by Escher et al. (2006) for *Desmodesmus subspicatus* in tests with SOT. Besides those data reported in the literature for the toxicity of this betablocker on non-target organisms, an $EC_{50-30min}$ > 1000 mg L^{-1} was calculated from bioluminescent inhibition experiments carried out using the bacteria *Aliivibrio fischeri* (Escher et al. 2006).

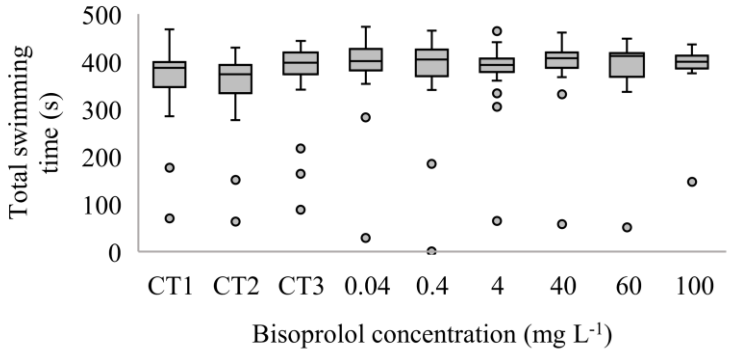
Regarding the acute toxicity data obtained for RAN, no $L/EC_{50} \leq 100$ mg L^{-1} was verified for any of the test organisms. Likewise, in acute toxicity studies performed by Isidori et al. (2009), RAN did not have any acute effect on the crustaceans *Thamnocephalus platyurus* and *Ceriodaphnia dubia* and the rotifer *Brachionus calyciflorus* at the highest concentration tested of 100 mg L^{-1}. In addition, an EC_{50-48h} value of 650 mg L^{-1} was reported for the acute exposition of *D. magna* to RAN (Webb 2001). Therefore, acute toxicity induced on aquatic organisms by SOT and RAN is unlikely to occur at the concentrations at which they are usually reported in aquatic ecosystems.

On the other hand, according to the OECD (2002) Harmonised Classification System, BIS can be classified as hazardous for the aquatic environment, in the category of acute III, which comprises 48 h EC_{50} values for crustacea and 72 h EC_{50} values for algae >10 - ≤ 100 mg L^{-1}. Our EC_{50} values obtained from *D. similis* and *R. subcapitata* tests were lower than the results obtained by Minguez et al. (2014), who reported EC_{50-48h} and EC_{50-72h} > 100 mg L^{-1} for *D. magna* and *R. subcapitata*. On the other hand, the EC_{50-72h} of 11.5 mg L^{-1} reported by Guo et al. (2015) from tests with the algal species *Desmodesmus subspicatus* corroborated with our results for BIS, reinforcing the warning of hazardous that this betablocker presents to the aquatic environment in acute expositions. Besides the acute toxicity shown to algae and daphnid in this study, this pharmaceutical is not rapidly degradable (Lahti and Oikari 2011). Therefore, these data justified the additional tests performed with BIS in order to assess their chronic toxicity.

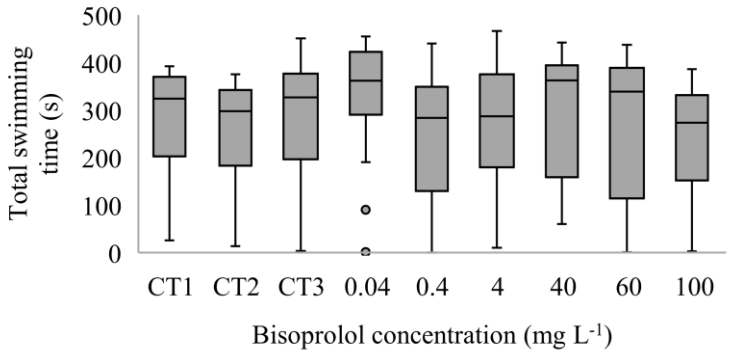
D. rerio has been described to possess β-adrenergic receptors (Wang et al. 2009, Steele et al. 2011), which are target of betablockers such as BIS and SOT. Based on ortholog prediction, Gunnarsson et al. (2008) have shown that the similarity of the β1 and β2- adrenergic receptors, between human and *D. rerio*, is of almost 46 and 53 %, respectively. Based on these factors, a significant reduction in heart rate, which is the main pharmacological action of betablockers in human, could be also expected to occur in the *D. rerio* embryos exposed to BIS and SOT. However, a statistically significant reduction in heart beat rate (20 s) was observed only in BIS concentrations > 160 mg L^{-1} and SOT concentrations > 1000 mg L^{-1}. Therefore, these toxic effects are only observed at concentrations far above those ones in which the betablockers are currently found in aquatic environments. This may be due to the protective action of the chorion or to the immaturity of the potential molecular targets of the pharmaceuticals in *D. rerio* embryos (Oliveira et al. 2016; Sanches et al. 2018).

The locomotor behavior of the *D. rerio* larvae exposed to the pharmaceuticals BIS, SOT and RAN is shown in Figures 1, 2 and 3 (a-d).

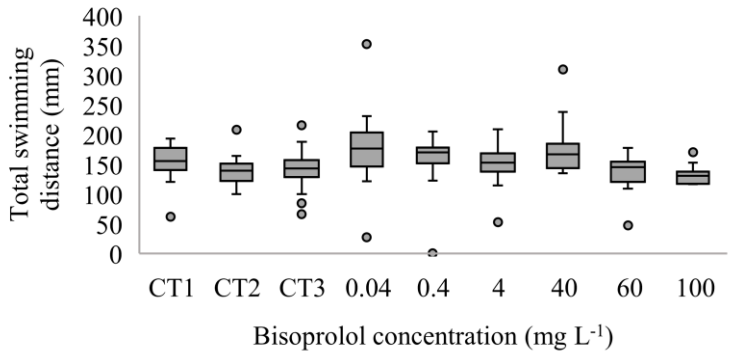
A



B



C



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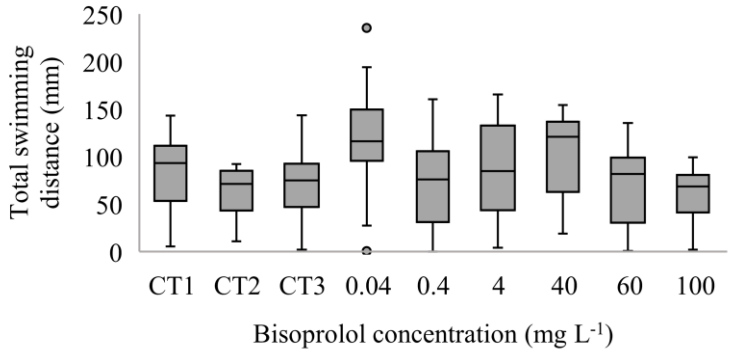


Fig. 1 Effect of the pharmaceutical bisoprolol (BIS) on the *Danio rerio* locomotor behavior quantified after 120 h post-fertilization. A) Total swimming time at the dark cycle (10min); B) Total swimming time at the light cycle (10min); C) Total swimming distance at the dark cycle (10 min); D) Total swimming distance at the light cycle (10 min). CT1, CT2, CT3 are the three independent control groups. Boxes represent medians (full line), with 5th and 95th percentiles (n=24 for controls and n=20 for treatments) and the respective outliers.

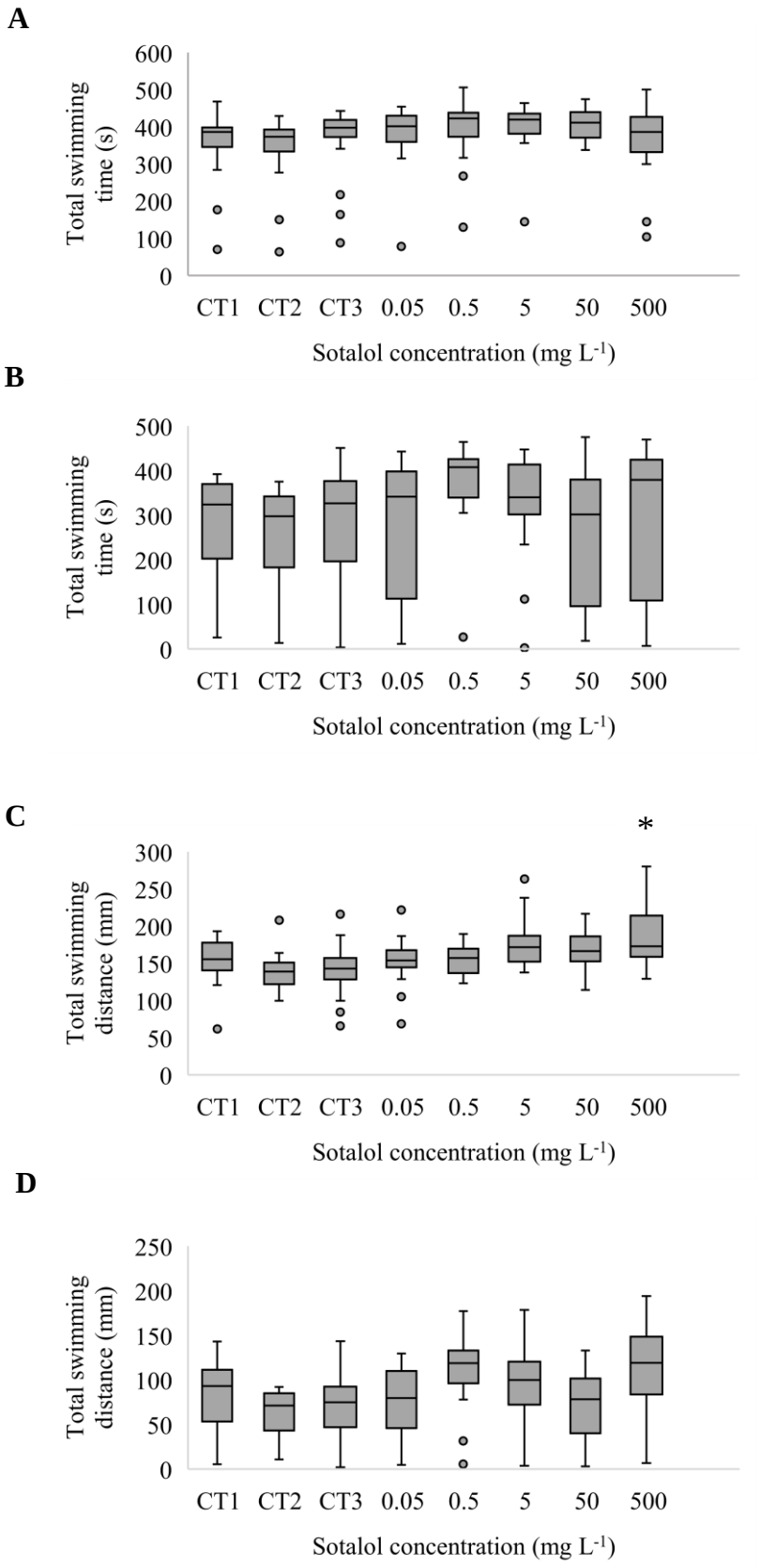
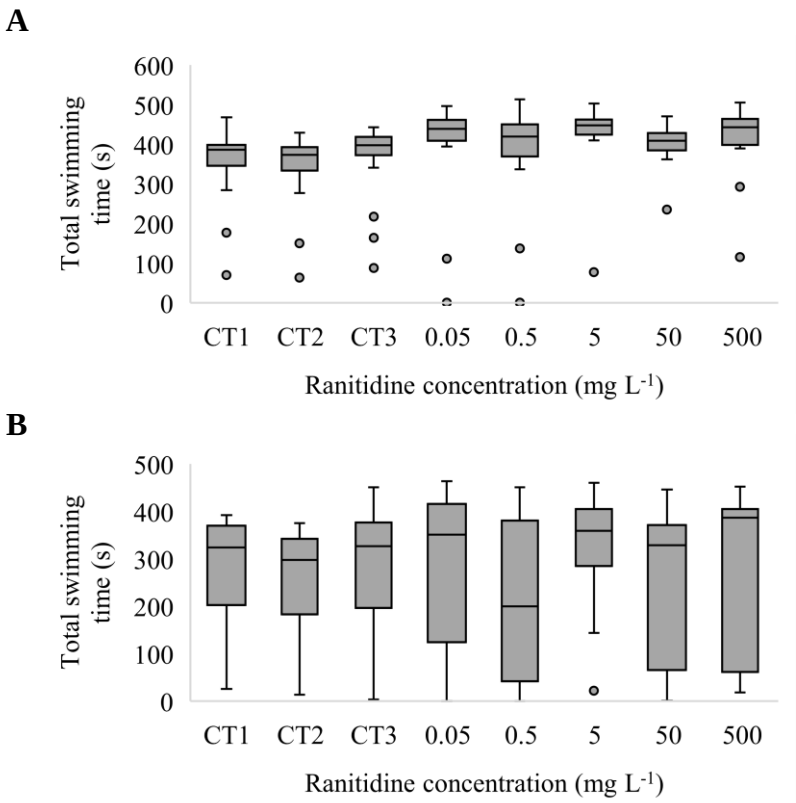


Fig. 2 Effect of the pharmaceutical sotalol (SOT) on the *Danio rerio* locomotor behavior quantified after 120 h post-fertilization. A) Total swimming time at the dark cycle (10min); B) Total swimming time at the light cycle (10min); C) Total swimming distance at the dark cycle (10 min); D) Total swimming

distance at the light cycle (10 min). CT1, CT2, CT3 are the three independent control groups. Boxes represent medians (full line), with 5th and 95th percentiles (n=24 for controls and n=20 for treatments) and the respective outliers. *Statistically significant difference in comparison to the control groups.



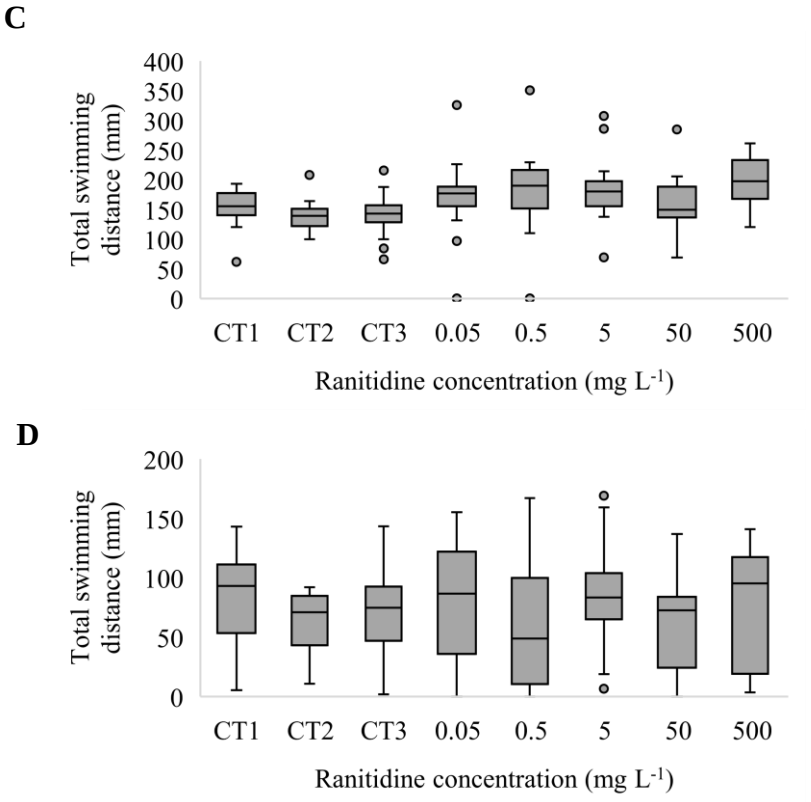


Fig. 3 Effect of the pharmaceutical ranitidine (RAN) on the *Danio rerio* locomotor behavior quantified after 120 h post-fertilization. A) Total swimming time at the dark cycle (10min); B) Total swimming time at the light cycle (10min); C) Total swimming distance at the dark cycle (10 min); D) Total swimming distance at the light cycle (10 min). CT1, CT2, CT3 are the three independent control groups. Boxes represent medians (full line), with 5th and 95th percentiles (n=24 for controls and n=20 for treatments) and the respective outliers.

From Figs. 1, 2 and 3, we can observe that the locomotor behavior of *D. rerio* larvae is not adversely impacted by BIS and RAN in any of the test concentrations. With regard the SOT effects, significant statistically difference in relation to the controls was observed only for the total swimming distance at the dark cycle for the larvae exposed to the highest concentration tested (500 mg L^{-1}). Locomotor behavior is a sensitive endpoint to assess stress exposure (Andrade et al. 2016; Oliveira et al. 2016; Sanches et al. 2018) and has important connection with population survival (Scott and Sloman, 2004). Given the considerable similarity between human and *D. rerio* target proteins for the pharmaceuticals evaluated in our study (Gunnarsson et al., 2008), possible physiological disfunctions induced by the action of the pharmaceuticals in the referred drug targets could ultimately impact locomotor activity of *D. rerio* larvae. Those findings were confirmed in our study with the pharmaceutical SOT, for which a disruption in the locomotor behavior was observed in concentrations 10-fold lower than the lethal ones. Nonetheless, even for this more sensitive endpoint, toxicity induced by the single

pharmaceuticals evaluated are observable only at concentrations far above the ones in which BIS, SOT and RAN are usually detected in aquatic ecosystems.

The EC_{10} and the respective 95 % C.I. for data obtained from chronic toxicity tests performed with BIS are shown in Table 2.

Table 2 Effect concentration at 10 % (EC_{10}) with the respective 95 % confidence interval (95 % C.I.) obtained in the chronic toxicity tests performed for assessing the effects induced by the betablocker bisoprolol (BIS) on different aquatic test organisms

Test organism	Toxicological endpoint	EC_{10} (95 % C.I.)
Raphidocelis subcapitata	$EC_{10\text{-}72\ h}$ – Growth inhibition	30.5 (23.0 – 38.5)
Lemna minor	$EC_{10\text{-}7d}$ - Growth inhibition (frond number)	118.2 (99.1 – 140.1)
L. minor	$EC_{10\text{-}7d}$ - Growth inhibition (total frond area)	114.6 (90.1 – 144.1)
L. minor	$EC_{10\text{-}7d}$ - Growth inhibition (fresh weight)	136.1 (112.1 – 164.2)
Daphnia similis	$EC_{10\text{-}14d}$ - Reproduction	3.6 (0.1 – 34.0)
Hydra attenuata	$EC_{10\text{-}7d}$ - Reproduction	43.0 (15.3 – 64.1)

From Table 2, we can observe that the sensibility of the two species representing the first trophic level, the algae *R. subcapitata* and the macrophyte *L. minor* to the toxic effects of BIS differs in a magnitude of almost 4-fold between the two species. Guidelines for deriving environmental quality standards such as the Technical Guidance of the European Commission (European Commission 2011) establishes that chronic data from algae or macrophyte studies can be used interchangeably. However, in the case of pharmaceuticals, for which the mode of action on non-target organisms is usually unknown, both algae and macrophyte should be used for testing, since their sensibilities can significantly vary.

D. similis reproduction showed to be the most sensitive population endpoint evaluated to the BIS toxic effects. Similar results were obtained in our studies with the pharmaceutical metformin (Godoy et al. 2018). It was not possible to compare our chronic results obtained for BIS with data from the literature because of the absence of reported chronic ecotoxicity data for this betablocker. The data generated in our study contribute to fill this knowledge gap and allowed us to perform a preliminary ecological risk assessment for the presence of BIS in fresh surface waters.

3.2 Ecological risk assessment for BIS

The highest value of $MEC_{surface\ water}$ reported in the Database of the German Environmental Agency for BIS was of 2.9 µg L^{-1}. This maximum concentration was quantified in German rivers and streams by Ternes (1998). The limit of detection of the method used by Ternes was of 0.010 µgL^{-1}.

For the $PNEC_{fresh\ water}$ estimation, an AF of 50 was applied to the lowest EC_{10} obtained from our chronic toxicity studies, i.e., from *D. similis* tests. The choice of this AF was made considering that long-term results from studies with fish are missing. In addition, the two long-term EC_{10} values considered for risk assessment in this study, i.e., from algae and daphnia species (Table 2), were generated covering that level showing the lowest EC_{50} in the short-tests (Table 1).

The MEC, AF, and EC_{10} value used for determining the PNEC and the RQ for the pharmaceutical BIS are shown in Table 3.

Table 3 – Values of maximum measured environmental concentration (MEC), assessment factor (AF) and effect concentration at 10 % (EC_{10}) used for estimating the predicted non-effect concentration (PNEC) and the risk quotient (RQ) for the pharmaceutical bisoprolol.

MEC (µg L^{-1})	Reference	EC_{10} (µg L^{-1})	AF	PNEC (µg L^{-1})	RQ
2.9	Ternes (1998)	3600.0	50	72.0	0.04

According to the RQ calculated considering a real worst-case global scenario (Table 3), an ecological risk to pelagic freshwater organisms exposed to BIS is not expected. However, this betablocker does not occur isolated in aquatic ecosystems and the toxicity of pharmaceuticals in mixtures can be higher than the toxicity of these compounds individually (Godoy et al. 2015; Godoy and Kummrow 2017). Therefore, the contribution of BIS to the overall risk posed by complex mixtures of environmental contaminants present in aquatic ecosystems cannot be ruled out and the joint toxicity of this pharmaceutical with other co-occurring environmental pharmaceuticals must also be investigated.

Conclusion

To the best of our knowledge, our study presents the first toxicity data on *L. minor* growth, *D. rerio* development and behavior and on the reproduction of *D. similis* and *H. attenuata* exposed to BIS. Acute toxicity induced by the pharmaceuticals SOT and RAN is not expected to occur on aquatic non-target organisms at the concentrations at which these

compounds are usually found in aquatic ecosystems. On the other hand, BIS was classified as hazardous to the environment considering the acute toxicity induced on the algae and on the daphnid. However, the risk quotient calculated based on a worst-case scenario and using the chronic data from the bioassays performed in our study showed that an ecological risk is not expected for the chronic exposure of pelagic freshwater species to BIS.

The locomotor behavior of *D. rerio* larvae was not affected by the exposition to BIS, RAN and SOT at the concentrations of environmental concern. Nonetheless, the contribution of these pharmaceuticals to the overall toxicity of complex mixtures present in aquatic ecosystems cannot be ruled out and must be investigated. Our results contributed to fill knowledge gaps and provided ecotoxicity data that add up in order to advance in the scientific knowledge about the ecological risk posed by pharmaceuticals of environmental concern.

Acknowledgments

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SUPPLEMENTARY MATERIAL

Assessment of the ecotoxicity of the pharmaceuticals bisoprolol, sotalol and ranitidine using standard and behavioral endpoints

Figures S1-S15 Concentration-response curves for the ecotoxicity tests performed with the pharmaceuticals sotalol, bisoprolol and ranitidine.

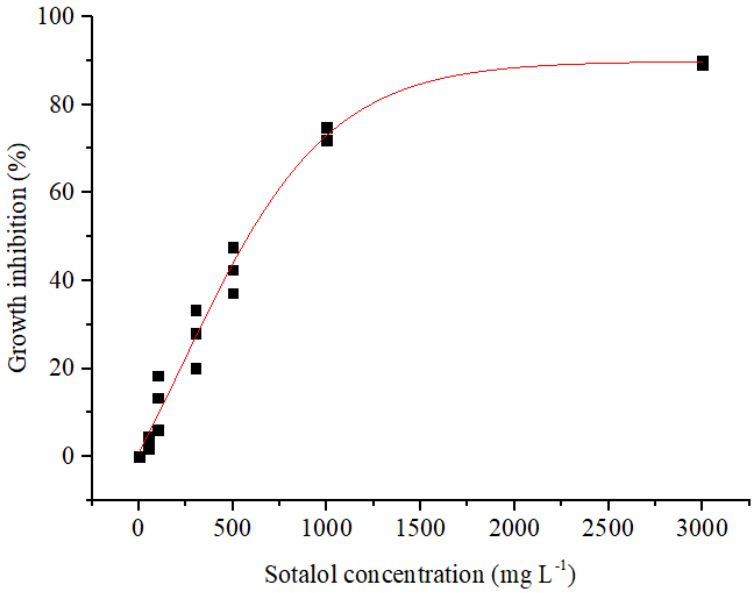

Fig S1 Concentration-response curve for the growth inhibition (% relative to the control) of *Raphidocelis subcapitata* induced by the exposition to sotalol during 72h

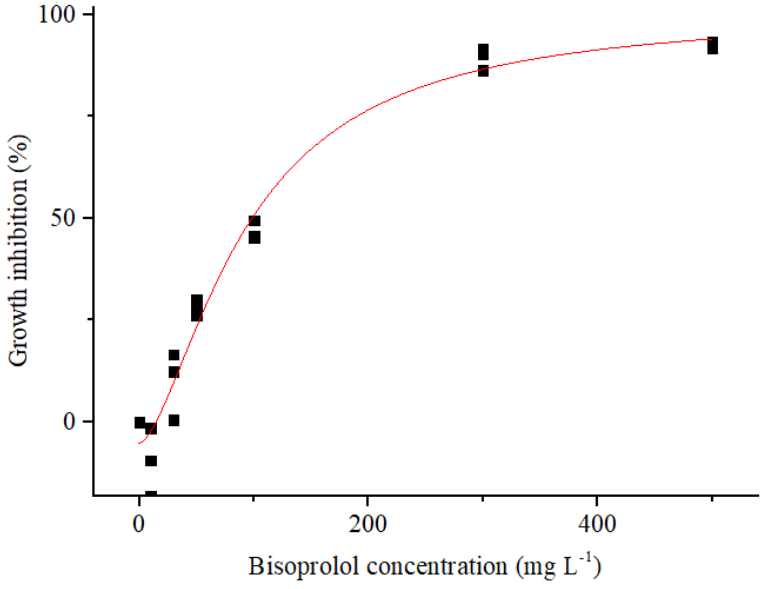

Fig S2 Concentration-response curve for the growth inhibition (% relative to the control) of *Raphidocelis subcapitata* induced by the exposition to bisoprolol during 72h

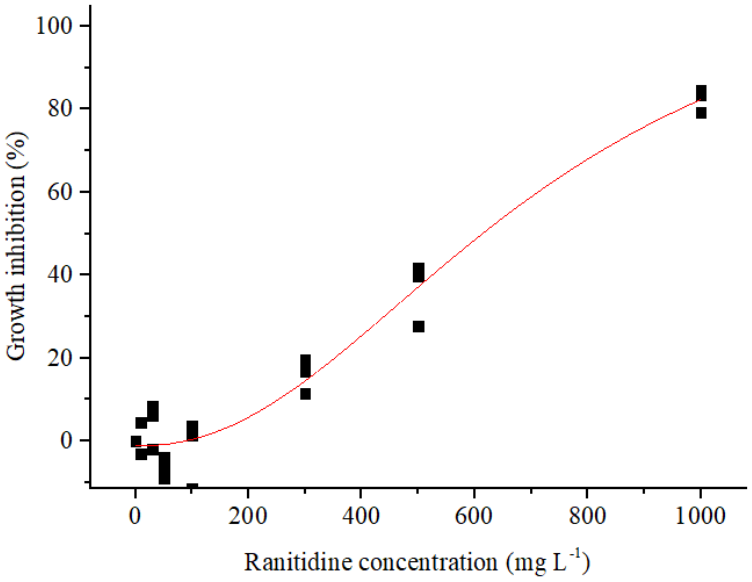

Fig S3 Concentration-response curve for the growth inhibition (% relative to the control) of *Raphidocelis subcapitata* induced by the exposition to ranitidine during 72h

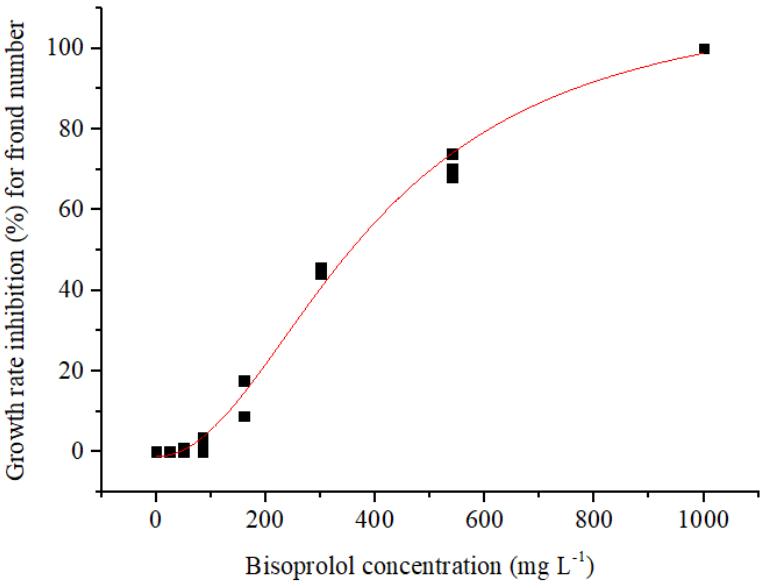

Fig. S4 Concentration-response curve for the average specific growth rate inhibition of *Lemna minor*, exposed to bisoprolol during 7 d, based on the frond number endpoint

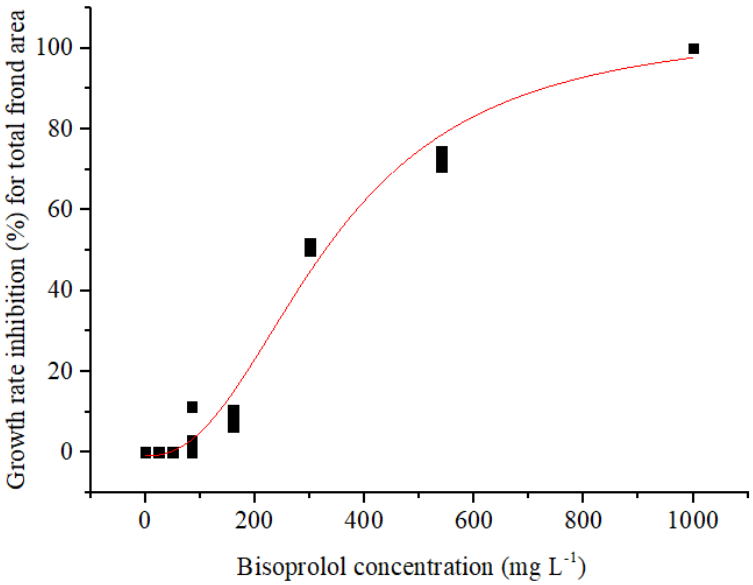

Fig. S5 Concentration-response curve for the average specific growth rate inhibition of *Lemna minor*, exposed to bisoprolol during 7 d, based on the total frond area endpoint

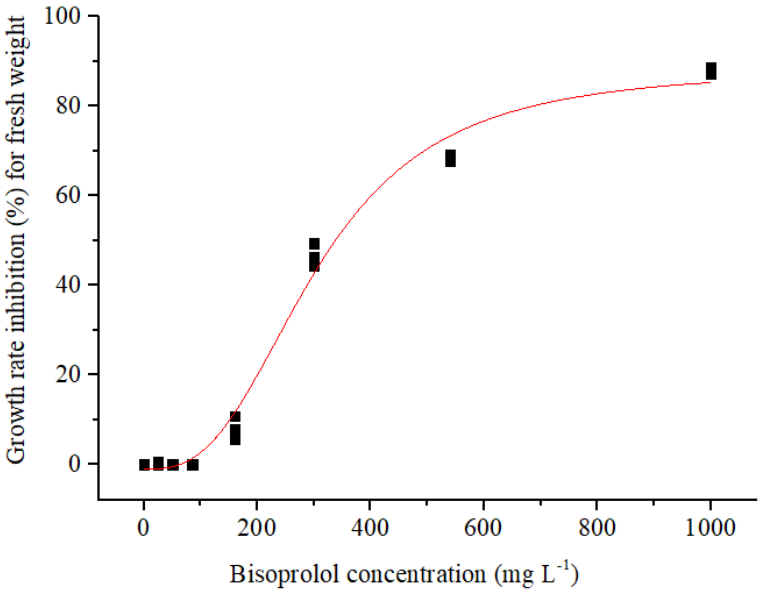

Fig. S6 Concentration-response curve for the average specific growth rate inhibition of *Lemna minor*, exposed to bisoprolol during 7 d, based on the fresh weight endpoint

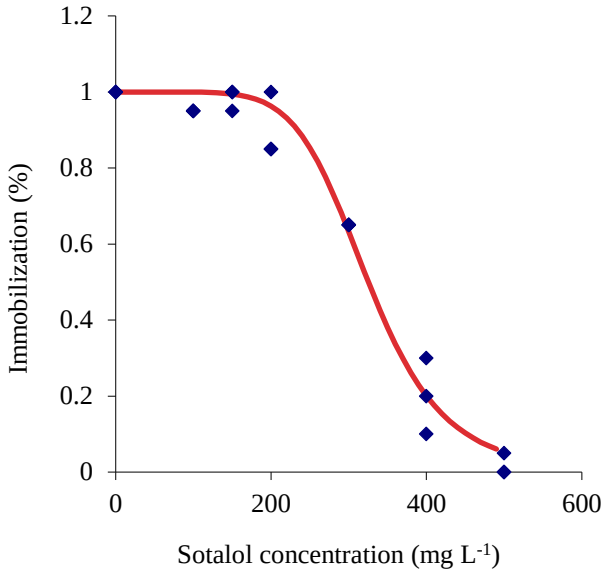

Fig. S7 Concentration-response curve for the immobilization (% relative to the control) of *Daphnia similis* exposed to sotalol during 48 h

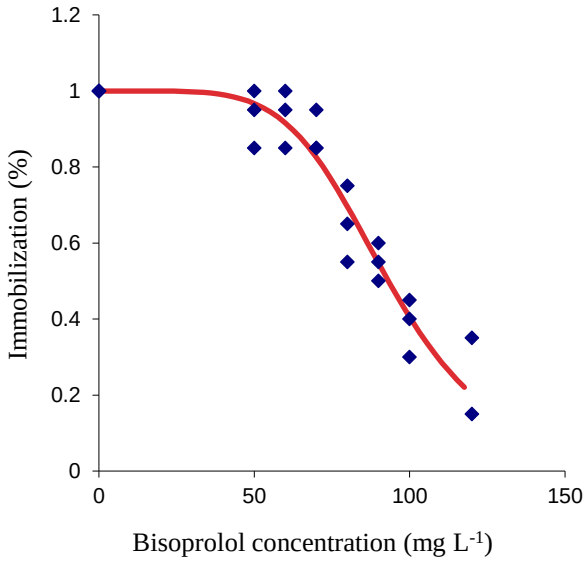

Fig. S8 Concentration-response curve for the immobilization (% relative to the control) of *Daphnia similis* exposed to bisoprolol during 48 h.

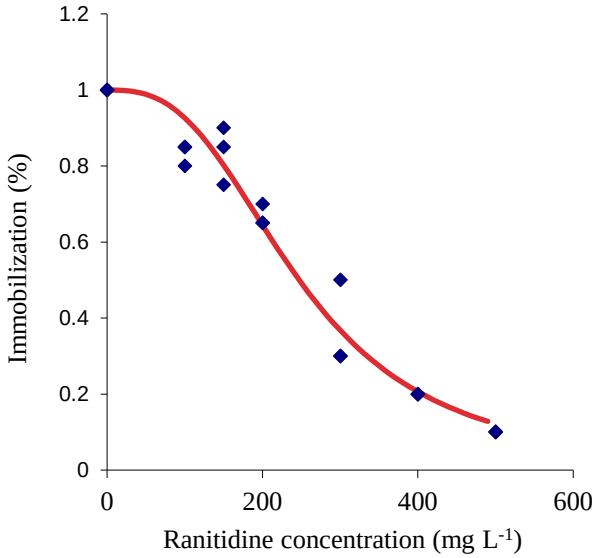

Fig. S9 Concentration-response curve for the immobilization (% in relation to the control) of *Daphnia similis* exposed to ranitidine during 48 h.

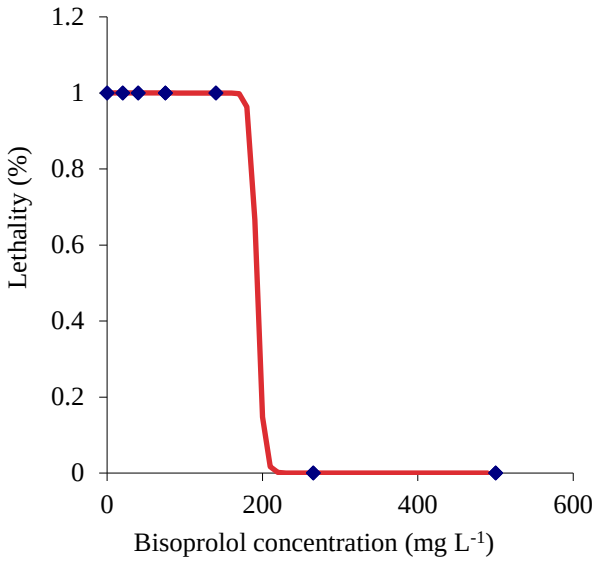

Fig. S10 Concentration-response curve for the lethality (% in relation to the control) of *Hydra attenuata* exposed to bisoprolol during 96 h.

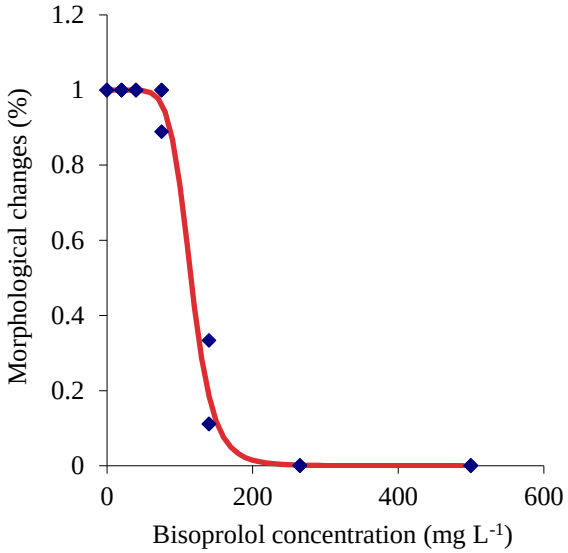

Fig. S11 Concentration-response curve for the morphological changes in relation to the control group (%) observed in *Hydra attenuata* exposed to bisoprolol during 96 h

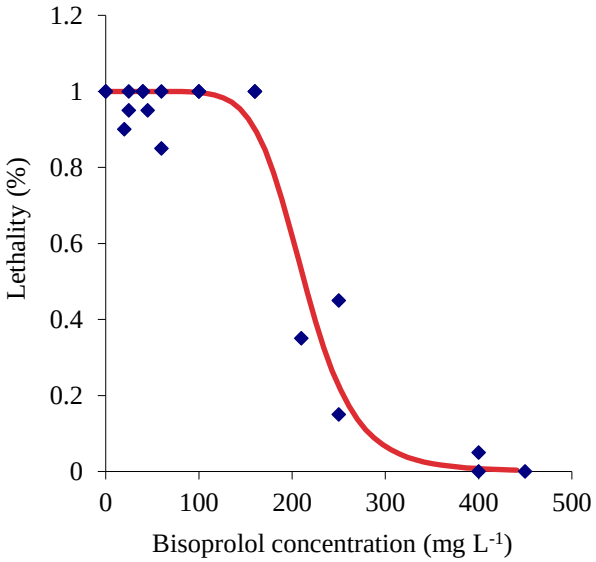

Fig. S12 Concentration-response curve for the lethality (% relative to the control) of *Danio rerio* larvae exposed to bisoprolol during 96 h.

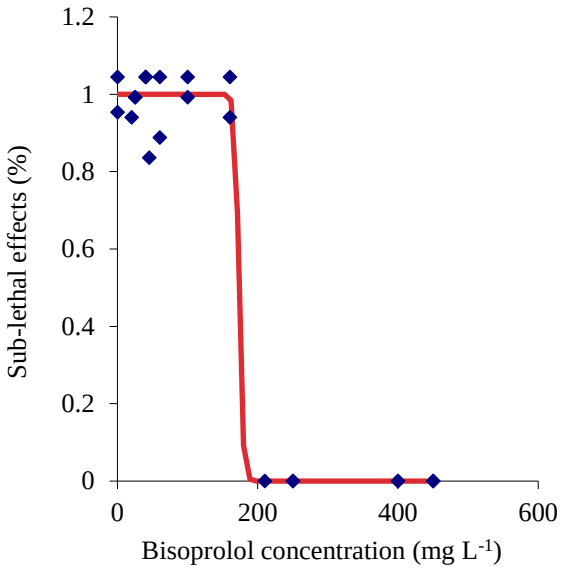

Fig. S13 Concentration-response curve (% relative to the control) for the sub-lethal effects induced on *Danio rerio* larvae exposed to bisoprolol during 96 h

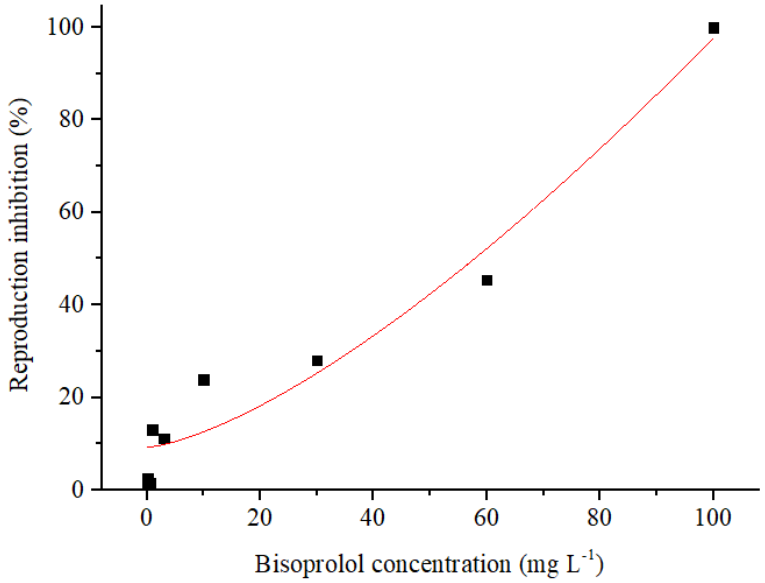

Fig. S14 Concentration-response curve for the reproduction inhibition (% relative to the control) of *Daphnia similis* exposed to bisoprolol during 14 d

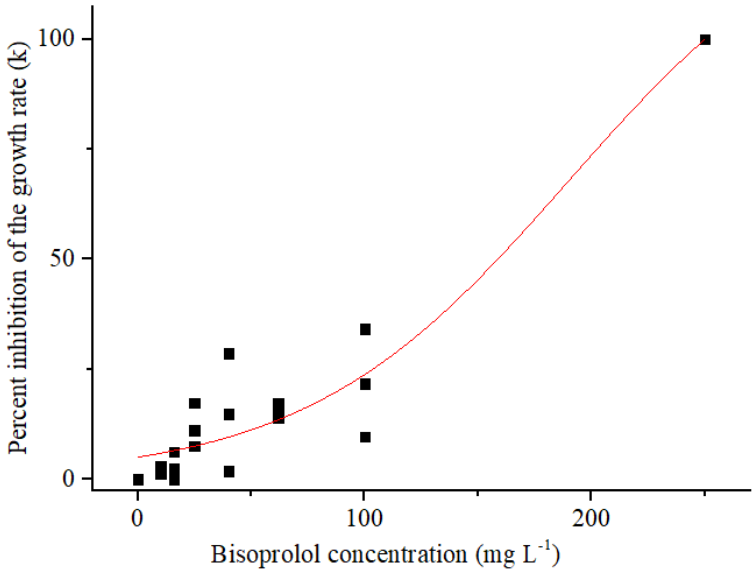

Fig. S15 Concentration-response curve for the population growth rate (K) inhibition of *Hydra attenuata*, exposed to bisoprolol during 7 d

4.4 Article IV

Single and mixture toxicity of four pharmaceuticals of environmental concern to aquatic organisms, including a behavioral assessment

Aline Andrade Godoy[a,b], Ádria Caloto de Oliveira[c], João Gabriel Mesquita Silva[c], Carina Cristina de Jesus Azevedo[c], Inês Domingues[d], António José Arsénia Nogueira[d], Fábio Kummrow[a,e*]

[a]Department of Clinical and Toxicological Analyses, Faculty of Pharmaceutical Sciences, University of São Paulo (USP), Avenida Professor Lineu Prestes, 580, São Paulo, SP, 05508-000, Brazil.

[b]Science and Technology Institute, Federal University of Alfenas (Unifal-MG), Rodovia José Aurélio Vilela, 11,999, Poços de Caldas-MG, 37715-400, Brazil.

[c] Faculty of Technology, State University of Campinas (Unicamp), Rua Paschoal Marmo, 1888, Limeira-SP, 13484-332, Brazil

[d]Department of Biology & CESAM, University of Aveiro, *Campus* Universitário de Santiago, 3810-193, Aveiro, Portugal.

[e]Department of Pharmaceutical Sciences, Institute of Environmental, Chemical and Pharmaceutical Sciences, Federal University of São Paulo (Unifesp), Rua São Nicolau, 210, 09972-270, Diadema, SP, Brazil.

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ABSTRACT

Pharmaceuticals are frequently detected in aquatic environments as mixtures and can cause toxic effects to non-target organisms. We aimed to evaluate the single and mixture effects of the pharmaceuticals metformin, bisoprolol, ranitidine and sotalol using *Daphnia similis* and *Danio rerio*. In addition, we aimed to test the predictive accuracy of the mathematical models concentration addition and independent action and to evaluate the nature of the possible toxicological interactions among these pharmaceuticals using the combination index-isobologram model. The acute toxicity of these four pharmaceuticals individually and of their binary mixtures were evaluated using the *D. similis* tests. Developmental and behavioral effects induced by the pharmaceuticals in quaternary mixtures were evaluated using *D. rerio* embryos. We observed that most of the binary mixture effects were in the zone between the effects predicted by the concentration addition and the independent action model. The combination index-isobologram model showed to be adequate to describe the nature of possible interactions occurring between the combined pharmaceuticals. Developmental and behavioral acute adverse effects seem not to be induced by the joint action of the quaternary mixture of the evaluated pharmaceuticals on *D. rerio* embryos, at the concentrations at which they are usually found in surface fresh waters. However, from the results obtained with *D. similis,* we can conclude that assessing the ecological risk based on the effects of individual pharmaceuticals can underestimate the risk level posed by these environmental contaminants.

Keywords: Joint toxicity**;** Betablockers; Antidiabetics; Locomotor activity; Synergism

1. Introduction

The worldwide contamination of aquatic environments by active pharmaceutical ingredients is an issue of growing concerning by the scientific community**.** Despite the knowledge gained over the past two decades regarding the effects and risks of pharmaceuticals in the environment, major research gaps still remain. One of these key issues is regarding the effects induced by pharmaceutical mixtures and the methods to assess them (Boxall et al., 2012).

The classical mathematical models of concentration addition and independent action have been extensively used for predicting the mixture effects of chemicals of environmental relevance, including pharmaceuticals (Godoy and Kummrow, 2017). However, the suitability in terms of accuracy of these two models in predicting the joint action effects of environmental compounds is still a controversial issue. Some studies have pointed out that the concentration addition and/or the independent action model are able to adequately predict the effects of mixtures of environmental contaminants (Cleuvers, 2005; Faust et al., 2003), while others have pointed that this accuracy is limited (Godoy and Kummrow, 2017; Rodea-Palomares et al., 2010; Yang et al., 2017). At the same time, the combination index-isobologram model (Chou, 2006) has been pointed out as a useful tool in ecotoxicological assessment, capable of predicting and describing toxic interactions between contaminants of environmental relevance with adequate accuracy (González-Pleiter et al., 2013; Mo et al., 2016; Wang et al., 2015; Yang et al., 2017). In view of this, there is a need for additional ecotoxicity data regarding mixture effects of contaminants of environmental concern in order to improve the knowledge and the methodologies that enable the accurate prediction of those responses.

Several prioritization approaches have been proposed for selecting active pharmaceutical ingredients that are likely to pose the greatest risk in certain situations (Boxall et al., 2012). In this study, we focused on pharmaceuticals of high and frequent consumption worldwide, growing use, incomplete metabolization, incomplete removal at wastewater treatment plants and that have been frequently detected simultaneously in aquatic environments. Within these criteria, we looked for pharmaceuticals for which ecotoxicological data are still scarce and for which the need of filling these knowledge gaps have been pointed out by many authors. This is the case of the pharmaceuticals metformin, ranitidine, sotalol and bisoprolol (Bergheim et al., 2012; Godoy et al., 2015; Lahti and Oikari, 2011; Ter Laak and Baken, 2014).

Metformin, an anti-diabetic, is one of the most consumed pharmaceuticals worldwide and one of the active pharmaceutical ingredients with the largest release in mass basis into the environment from wastewater treatment plants (Godoy et al., 2018). Besides its high consumption, metformin is excreted unaltered in the urine (Bailey et al., 1996). Although metformin presents relatively high removal rates in wastewater treatment plants (Trautwein et al., 2014), it is frequently detected in aquatic environments (see Table S1 of Supplementary Material) due to its high influent load (Oosterhuis et al, 2013).

Pharmaceuticals from the betablockers class are also extensively consumed worldwide (Cleuvers, 2005; Oosterhuis et al., 2013; Scheurer et al., 2010) for the treatment of various cardiovascular diseases, including hypertension and heart rhythm disturbances. Because of their widespread use and incomplete metabolism (Bühring et al., 1986; Maszkowska et al., 2014; Singh et al., 1987), betablockers are often detected in aquatic environments (Godoy et al., 2015). Among the betablockers, bisoprolol and sotalol have been pointed out to be incompletely removed during the treatment in wastewater treatment plants (Gabet-Giraud et al., 2010; Lara-Martín et al., 2014; Scheurer et al., 2010) and are frequently detected in several aquatic compartments (see Table S1 of Supplementary Material).

Ranitidine is a popular pharmaceutical (Bojić et al., 2015) that belongs to a class of drugs called H2 (histamine 2)-receptor blockers, which is often prescribed for treating gastric disturbances, including those caused by the frequent use of other pharmaceuticals. Its incomplete metabolism (Vediappan and Lee, 2011) and poor removal in wastewater treatment plants (Gros et al., 2012; Radjenović et al., 2009) make ranitidine also a pharmaceutical of environmental concern (Table S1 of Supplementary Material).

These 4 pharmaceuticals have been detected simultaneously in influents and effluents of WWTPs, in hospital effluents and even in surface waters (see Table S2 of Supplementary Material). It is known that the toxicity induced by mixtures may be higher than that presented by each single pharmaceutical (Backhaus, 2016; Smith et al., 2013). Therefore, the ecotoxicity of their combined action must be investigated.

The aim of this study was to determine the single and mixture toxicity of four pharmaceuticals of environmental concern in order to fill important knowledge gaps in the ecotoxicity field. For this purpose, we aimed to test the predictive accuracy of the classical mathematical models usually employed in mixture ecotoxicity studies in acute toxicity tests with the cladoceran *Daphnia similis*, and to evaluate the nature of the possible toxicological interactions among the investigated pharmaceuticals using the combination index-isobologram model. We also aimed to evaluate the toxicity of the quaternary mixture of the pharmaceuticals

in concentrations of environmental relevance (in the µg L^{-1} order) using tests with the fish species *Danio rerio.*

We intended to provide data that might aid in the prediction of the effects of pharmaceuticals mixtures on aquatic organisms and that consequently could help improving the knowledge and the methodologies proposed for environmental risk assessment of pharmaceutical mixtures. For this purpose, mixture data from testing with organisms from different trophic levels are required. In view of this, the *D. similis* and the *D. rerio* were chosen for this study because of their relevance for the aquatic compartment, besides being species from different trophic levels recommended in standard protocols for environmental risk assessment purposes. This study contributes to a scientific basis for improving the ecological risk assessment of pharmaceutical mixtures in aquatic ecosystems.

2. Materials and Methods

2.1 Test pharmaceuticals

Metformin hydrochloride (1,1 – Dimethylbiguanide hydrochloride; CAS number 115-70-4) was provided by Abilasha Pharma (India), with 99.2 % purity. Bisoprolol fumarate (1-[4-[[2-(1-Methylethoxy)ethoxy]methyl]phenoxy]-3-[(1-methylethyl)amino]-2-propanol fumarate salt; CAS number 104344-23-2) and sotalol hydrochloride (N-[4-[1-Hydroxy-2-(isopropylamino)ethyl]phenyl]methanesulfonamide hydrochloride; CAS number 959-24-0) were provided by Moehs Catalana (Spain), with 98.8 % and 99.8 % purity, respectively. Ranitidine hydrochloride (Dimethyl[(5-{[2-{[(E)-1-(methylamino)-2-nitroethenyl]amino}ethyl)sulfanyl]methyl}furan-2-yl)methyl]amine hydrochloride; CAS number 66357-59-3) was provided by Fagron (India), with 100.8 % purity. Stock solutions and test concentrations were prepared just before each experiment. They were prepared in 200 mL volumetric glass flasks by dissolving each pharmaceutical in the respective exposure medium recommended for each test-organism. No additional solvent was used. The negative controls consisted only of the exposure medium, without the addition of any pharmaceutical. *D. similis* test procedure was checked using NaCl (Sigma-Aldrich, 99 % purity) dissolved in the exposure medium for positive control. For *D. rerio* bioassays, the test procedure was checked using 3,4-dichloroaniline (Sigma-Aldrich, 98 % purity) dissolved in the exposure medium at 4 mg L^{-1} for positive control.

2.2 Test organisms

Daphnia similis test organisms were supplied by the Laboratory of Aquatic Ecotoxicology, University of São Paulo, São Carlos (Brazil) and were cultivated at the Laboratory of Ecotoxicology and Genotoxicity (LAEG), State University of Campinas, Unicamp (Brazil). The organisms were cultivated in MS medium, at 20 ± 2°C and under a photoperiod of 16:8h light/dark, following the protocols OECD 202 (2004) and ABNT NBR 12713 (2016). The crustaceans were fed five times at a week with the algae *Raphidocelis subcapitata*. Three times a week, the cultivation medium was changed.

Danio rerio test organisms were supplied by the facility of the Department of Biology of the University of Aveiro (Portugal). The adult zebrafish were cultivated at that facility, in carbon-filtered water (pH 7.5 ± 0.5, dissolved oxygen at 95 % saturation), at 26 ± 1 °C and under a photoperiod cycle of 16:8 h light/dark, following the protocol of OECD 236 (2013). The fishes were fed twice a day with commercial artificial diet (ZM 500 Granular).

2.3 *Acute toxicity tests with D. similis and D. rerio* and behavioral assessment

Acute single and mixture toxicity tests with *D. similis* were performed according to the ABNT NBR 12.713/2016 (ABNT, 2016) and OECD guideline 202 (OECD, 2004). Five neonates <24 h old, from 2 to 3-week-old mothers, were transferred to each of the vials containing 10 mL of the test concentrations of each pharmaceutical and/or of their binary mixtures. The photoperiod and temperature were the same as used in the cultivation of the organisms. During the test, the organisms were not fed. Four control and replicates were used for each test concentration/mixture. Three independent tests were carried out for the single toxicity evaluation of each pharmaceutical. Each binary mixture toxicity test was performed twice. The endpoint evaluated was the immobilization of the daphnids after 48 h. The parameters pH, conductivity and dissolved oxygen were measured at the end of each test.

Quaternary mixture toxicity tests with *D. rerio* embryos (Fish embryo acute toxicity - FET test) were performed following standard protocols (see the detailed description in the Supplementary Material). All experimental procedures involving fish were performed following the International Guiding Principles for Biomedical Research Involving Animals (EU 2010/63) and are in accordance with Portuguese laws on animal safety. Animal handling was performed by accredited researchers.

2.4 Experimental design of the mixture toxicity tests

The prediction of the acute binary mixture toxicity of the tested pharmaceuticals to *D. similis* was performed in relation to the concentration addition and the independent action models and deviations of each model (synergism/antagonism; dose level-dependent or dose ratio-dependent), using an automated Excel spreadsheet (ToxCalcMix v. 1.0), developed at the University of Aveiro & CESAM, Portugal (available at https://pydio.bio.ua.pt/public/toxcalcmix). This automated spreadsheet was developed according to the models and equations described elsewhere (Barata et al., 2006; Jonker at al., 2005). From the parameters (EC_{50} and slope) obtained for the respective concentration-response curves for the single effects of each pharmaceutical on the *D. similis* immobilization, an experimental design was performed for each binary mixture, considering a factorial design including 5 x 5 treatments. This experimental design allowed us to cover several possible interactions at various mixture ratios and effect levels, while making it possible to reduce the number of test organisms used. According to the respective factorial designs, the pharmaceuticals were combined at 0.4; 0.6; 0.8 and 1.2 toxic units. A total of 8 different binary mixture treatments was performed for each toxicity test. This same experimental design was used for the analysis of possible interactions between the pharmaceuticals using the combination index-isobologram model. Single and mixture toxicity tests were performed simultaneously.

For evaluating the toxicity effects of the quaternary mixture to the embryo larval development and the locomotor behavior of *D. rerio*, the pharmaceuticals were combined at 0.1, 1.0, 10 and 100 µg L^{-1} each. This range of concentrations was chosen in order to cover the mean values at which the pharmaceuticals metformin, ranitidine, bisoprolol and sotalol have been detected in aquatic environments (Table S1 of Supplementary Material). Besides, three independent negative controls were also tested, i.e., three 24-well plates containing only the reconstituted water used for maintenance of the fishes.

2.5 Data analysis

The data obtained in each independent test were grouped for obtaining the concentration-response curves for the single toxicity and the mixture toxicity parameters. Data regarding the single toxicity effects of each pharmaceutical to *D. similis* were analyzed using the automated Excel spreadsheet ToxCalcMix v.1.0. The allosteric decay model was fit to the

data from single toxicity tests for obtaining the median lethal/effective concentrations (L/EC_{50}) and the respective 95 % confidence intervals. A one-way analysis of variance (ANOVA) was used to infer statistically significant differences between treatments and controls in the FET and in the behavioral tests. The ANOVA assumptions of normality and homogeneity were verified by using the Shapiro-Wilk and the Bartlett tests, respectively. When the ANOVA assumptions were not met, a Kruskal-Wallis test was performed. The software Statistica v. 7.0 (StatSoft Inc., USA) was used for performing ANOVA and their assumptions.

Box and whisker charts obtained for the behavioral analysis data, including the statistical analysis for identifying outliers, were performed using Microsoft Excel (version 2016). Data from mixture toxicity tests with *D. similis* were analyzed using the ToxCalcMix spreadsheet for comparisons with the concentration addition, independent action models and their eventual deviations synergistic, antagonistic, dose-ratio dependent or dose-level dependent effects (Jonker et al., 2005). The free computer program CompuSyn (Chou and Martin, 2005) was used to obtain the concentration-effect curve parameters and the CI values for the mixture toxicity analysis considering this model.

3 Results and discussion

3.1 Toxicity of single pharmaceuticals to D. similis

The LC_{50} and their respective 95 % confidence interval values obtained from the tests with *D. similis* in order to assess the single toxicity of the pharmaceuticals metformin (previously published in Godoy et al., 2018), bisoprolol, ranitidine and sotalol are described in Table 1.

Table 1 - Median lethal concentrations (LC_{50}) and the respective 95 % confidence interval (C.I.) values obtained from the acute toxicity tests with *Daphnia similis* exposed to each of the pharmaceuticals metformin (Godoy et al., 2018), ranitidine, sotalol and bisoprolol individually

Pharmaceutical	LC_{50} (95 % C.I.) mg L^{-1}
Metformin	14.3 (13.8 – 14.8)
Bisoprolol	93.1 (91.5 – 94.8)
Ranitidine	247.3 (239.7 – 254.9)
Sotalol	325.2 (319.5 – 330.8)

Source: Godoy et al. (2019)

The results shown in Table 1 allow us to classify the pharmaceuticals metformin and bisoprolol as hazardous for the aquatic environment, in the category of acute III, which comprises 48h EC_{50} values for crustacea >10 - ≤ 100 mg L^{-1}, according to the Harmonised Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures (OECD, 2002). Those results justify a need for additional studies with these two pharmaceuticals, employing chronic toxicity tests with different endpoints and test organisms. For metformin, we have already published some of these required results (Godoy et al., 2018). Regarding the betablockers sotalol and bisoprolol, studies have pointed out for their high degree of persistence in aquatic environments as a result of their slightly biodegradation and high hydrolytic and photochemical stability (Feiner et al., 2014; Piram et al., 2008). Nonetheless, ecotoxicological studies with sotalol and bisoprolol are still scarce (Godoy et al., 2015), which hampers that an accurate environmental risk assessment can be implemented for them. Thus, our study contributes to fill this data gap.

3.2 Joint toxicities of the pharmaceuticals to D. similis

The comparison between the acute toxicity induced by the single pharmaceuticals and their respective binary mixtures is shown in Figure 1a-f and in Table S3 of Supplementary Material.

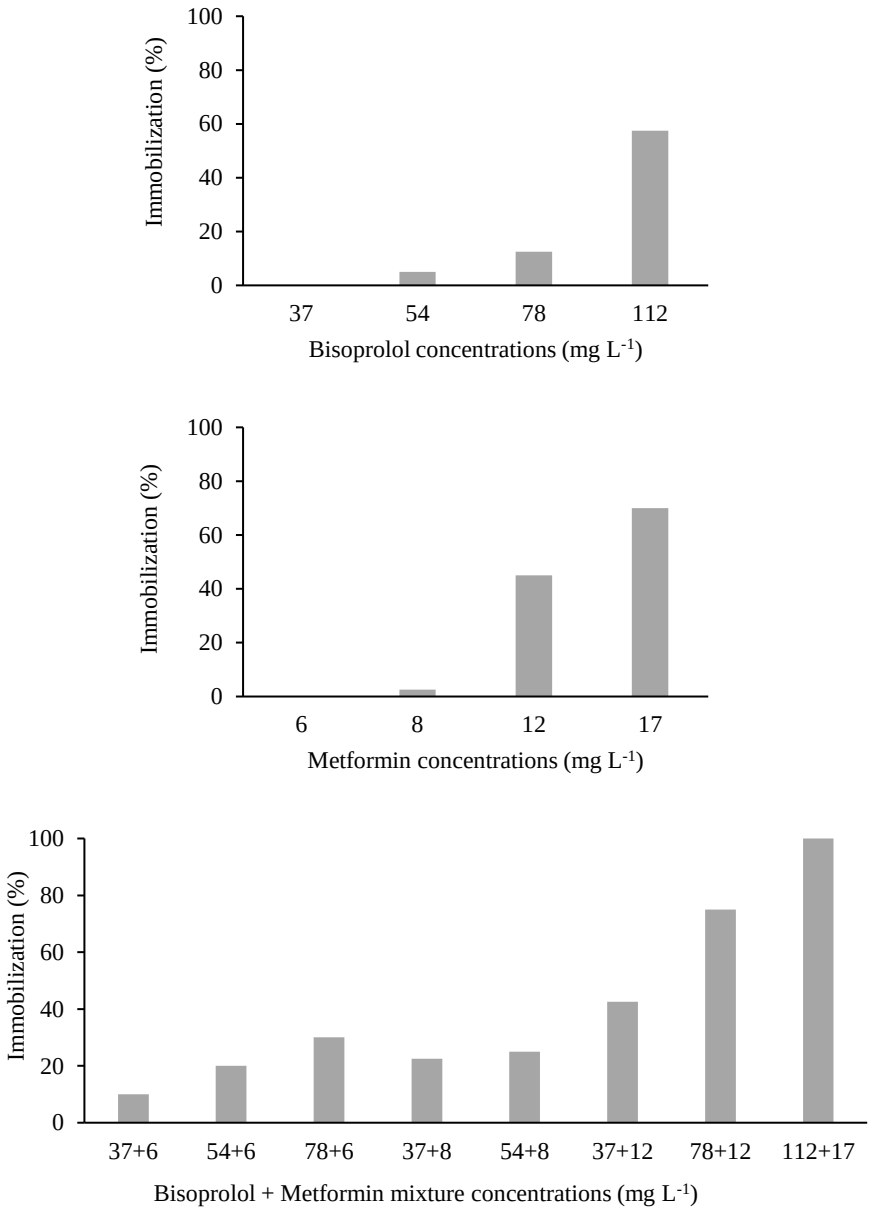


Fig. 1a Comparison between the percent of immobilization of *Daphnia similis* exposed to bisoprolol and metformin individually and in mixtures

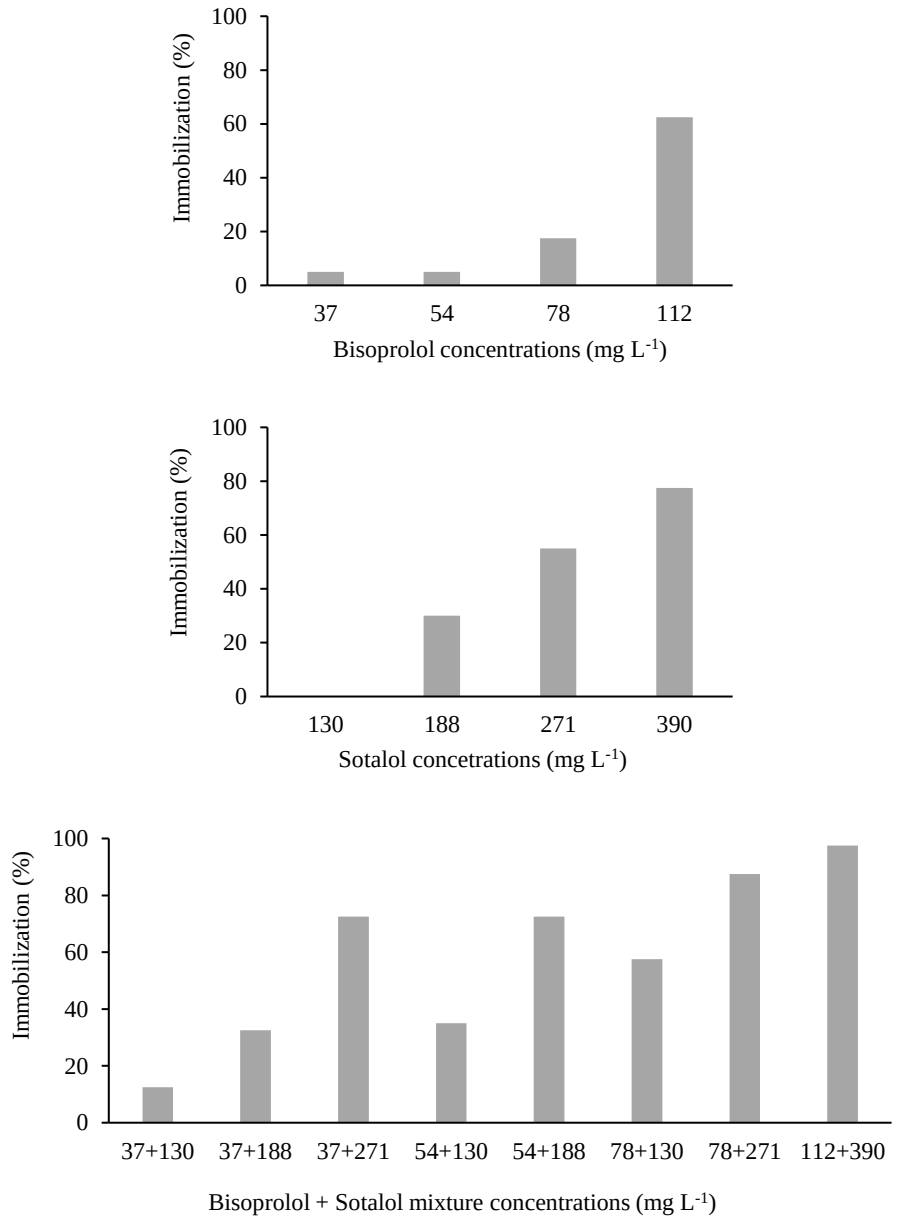


Fig. 1b Comparison between the percent of immobilization of *Daphnia similis* exposed to bisoprolol and sotalol individually and in mixtures

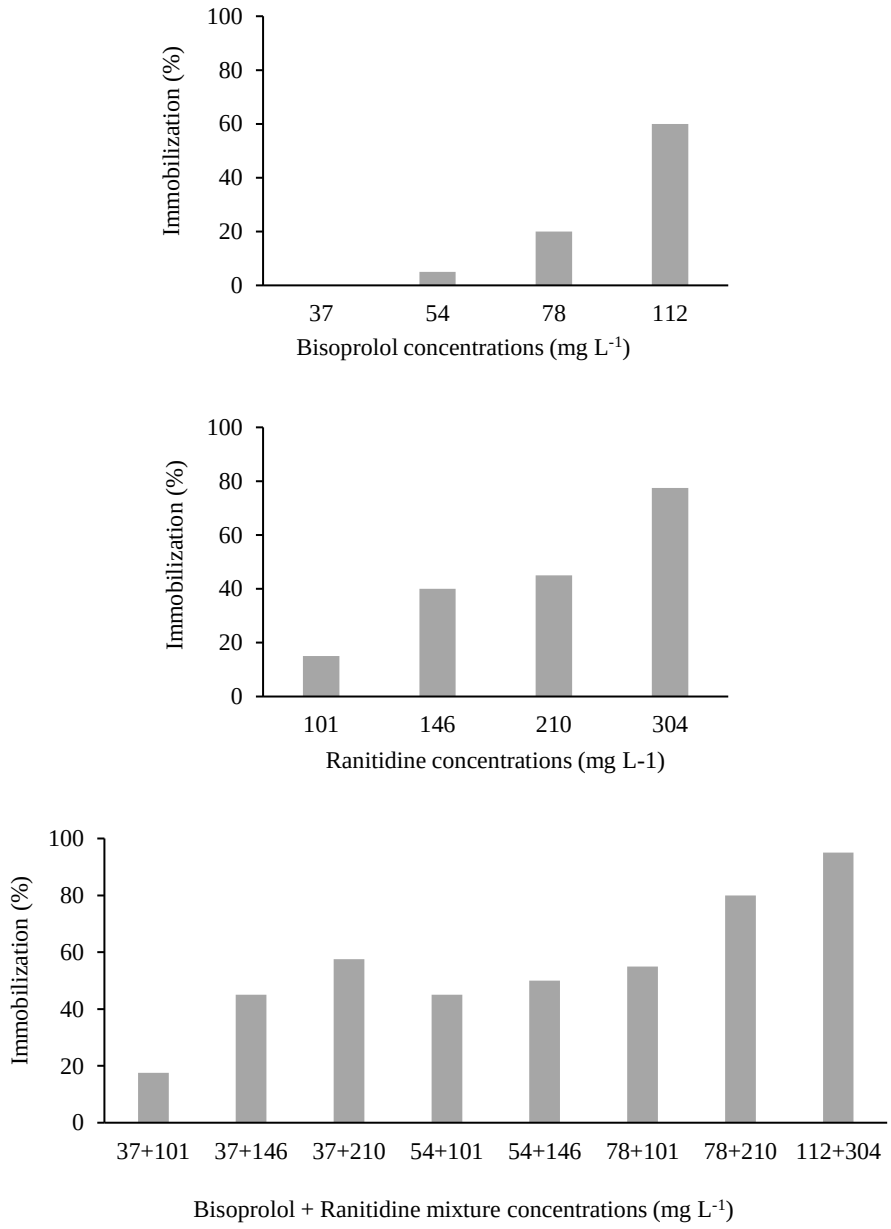


Fig. 1c Comparison between the percent of immobilization of *Daphnia similis* exposed to bisoprolol and ranitidine individually and in mixtures

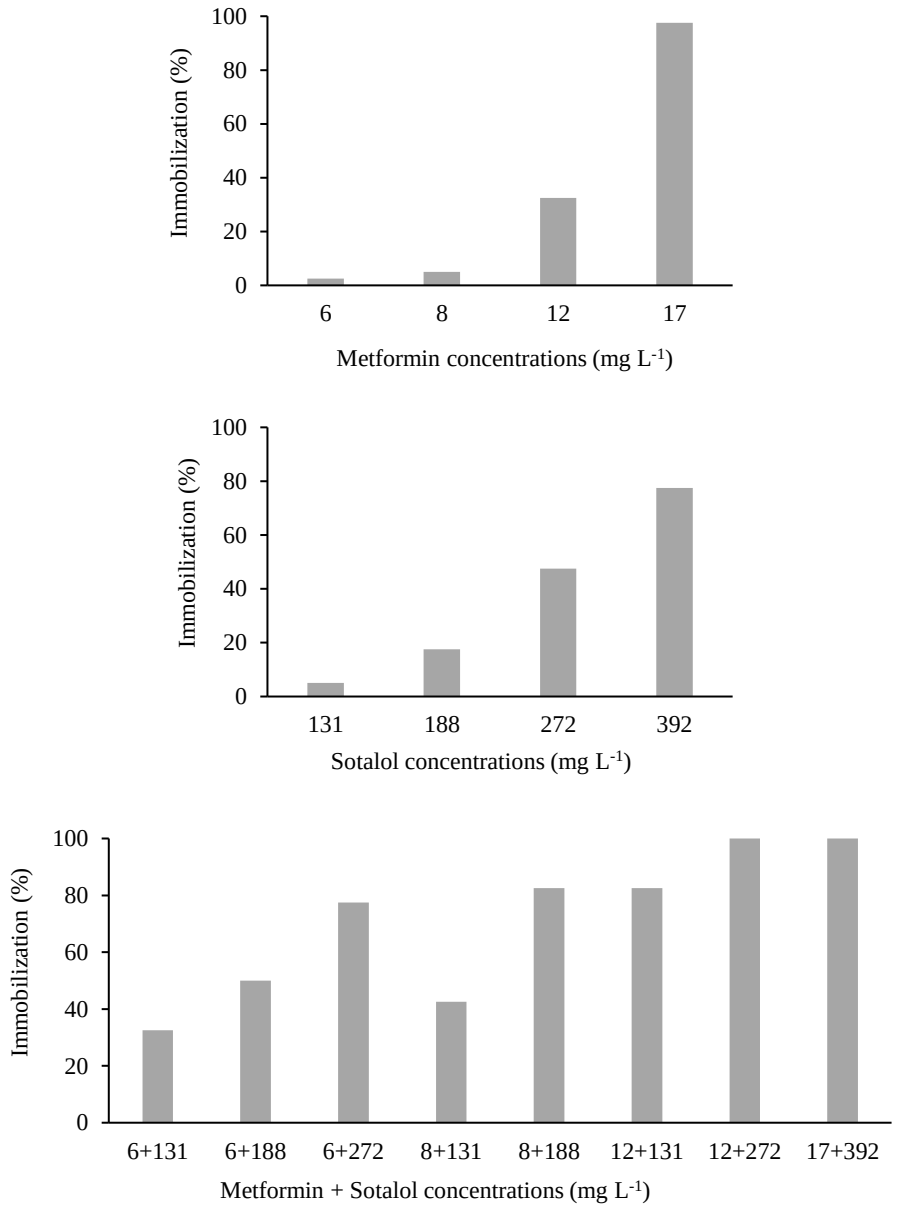


Fig. 1d Comparison between the percent of immobilization of *Daphnia similis* exposed to metformin and sotalol individually and in mixtures

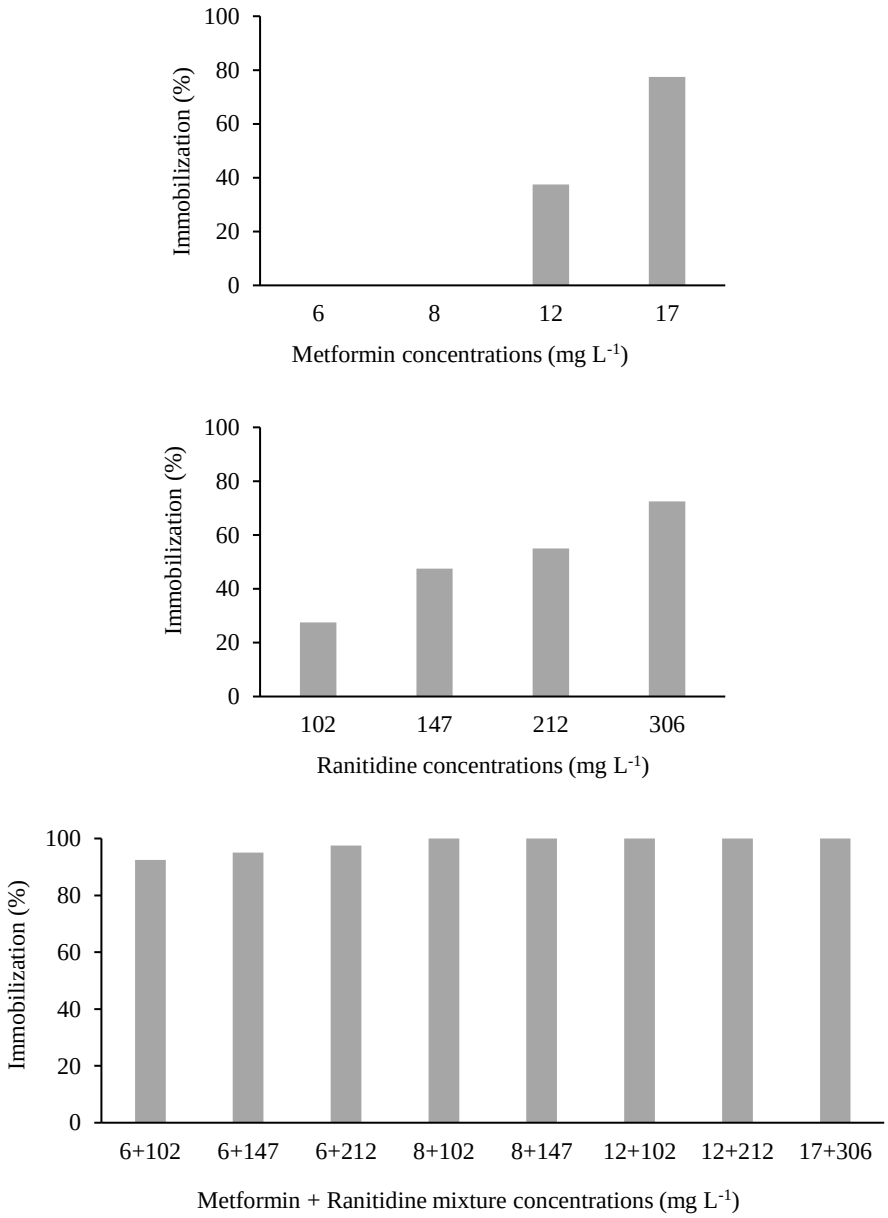


Fig. 1e Comparison between the percent of immobilization of *Daphnia similis* exposed to metformin and ranitidine individually and in mixtures

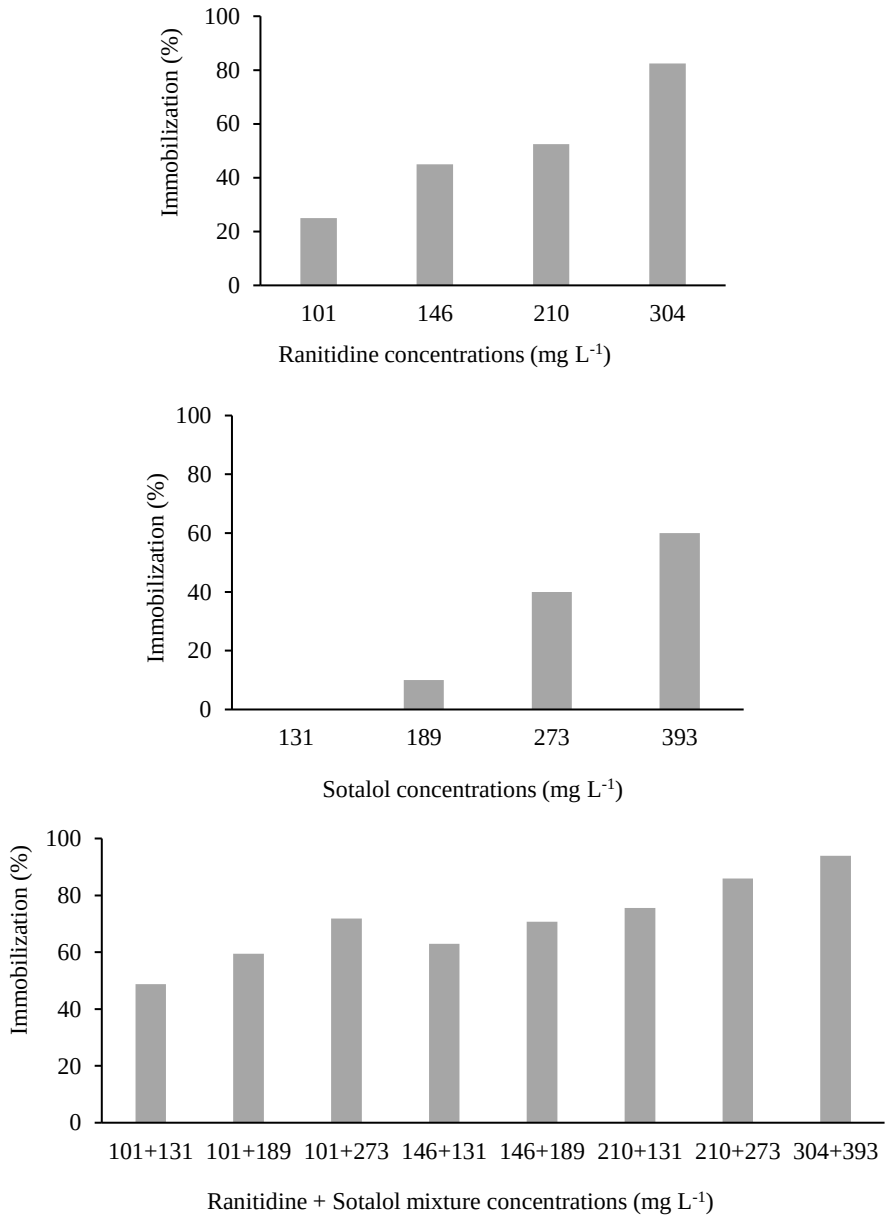


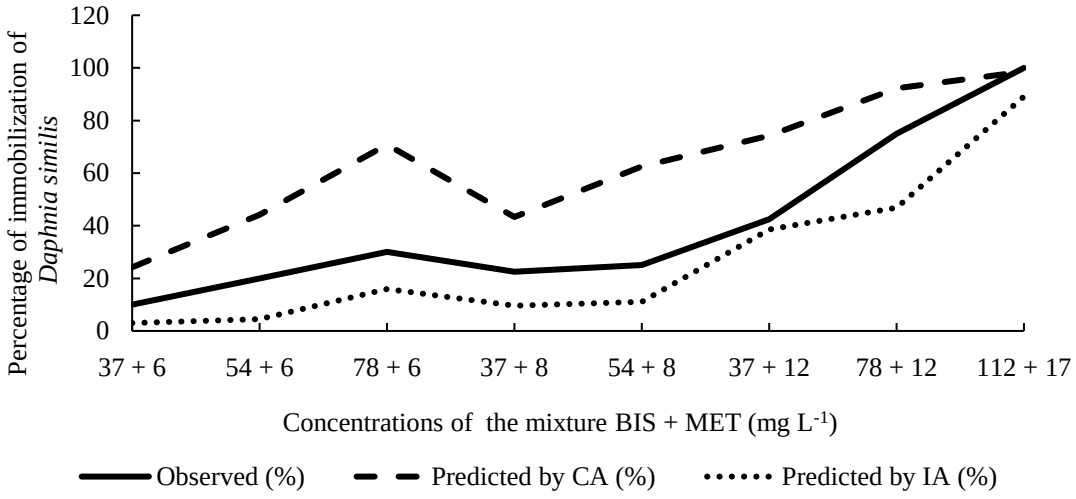
Fig. 1f Comparison between the percent of immobilization of *Daphnia similis* exposed to ranitidine and sotalol individually and in mixtures

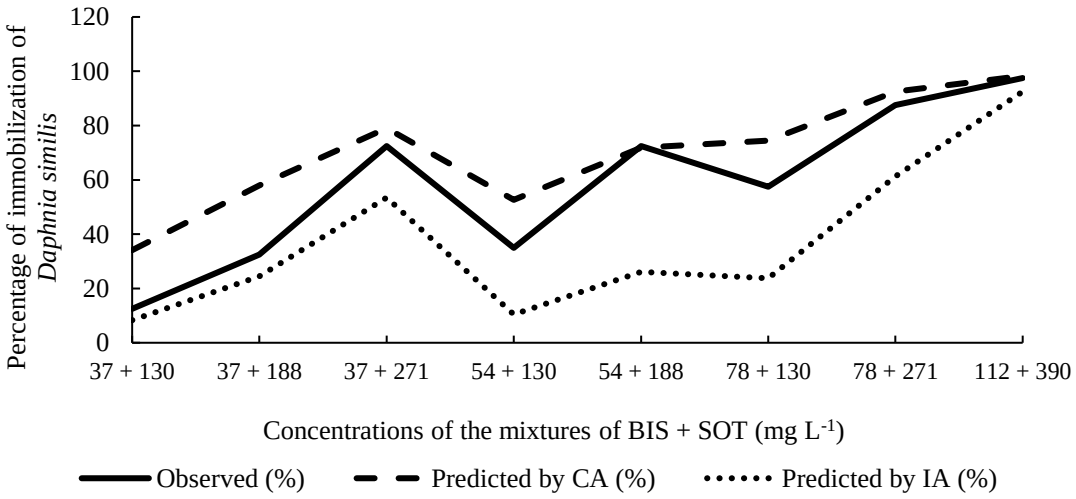
Source: Godoy et al. (2019)

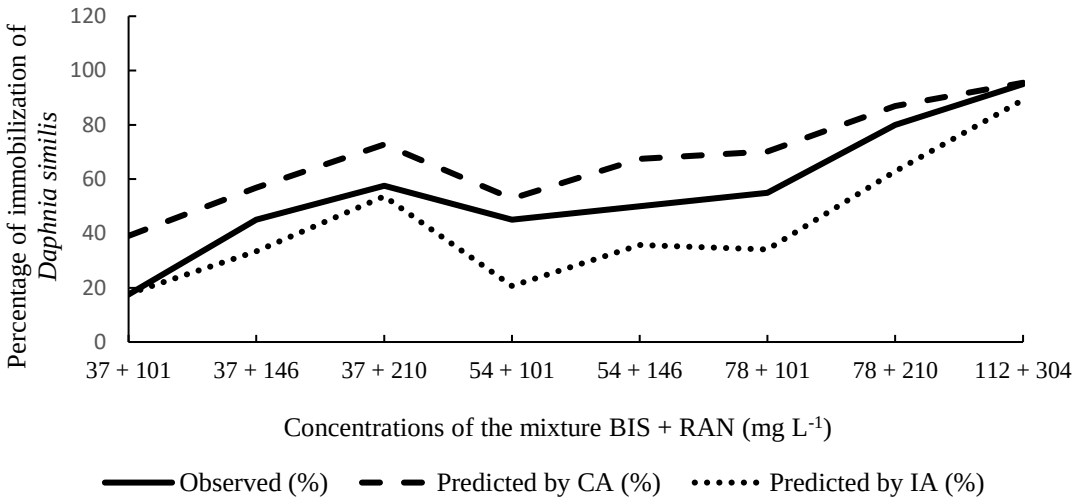
From Fig.1a-f, we can observe that even non-effect concentrations of metformin and bisoprolol (6 mg L^{-1} and 37 mg L^{-1}, respectively) induced 10 % immobilization in the daphnids exposed to the binary mixture. This phenomenon was described in the literature as 'something from nothing' (Thrupp et al., 2018). We can also observe that immobilization was induced in the whole of the daphnids exposed at concentrations of the binary mixture of metformin and ranitidine which affect the daphnids mobilization only to a minor degree when each pharmaceutical is applied individually. This other mixture phenomenon was termed 'a lot from

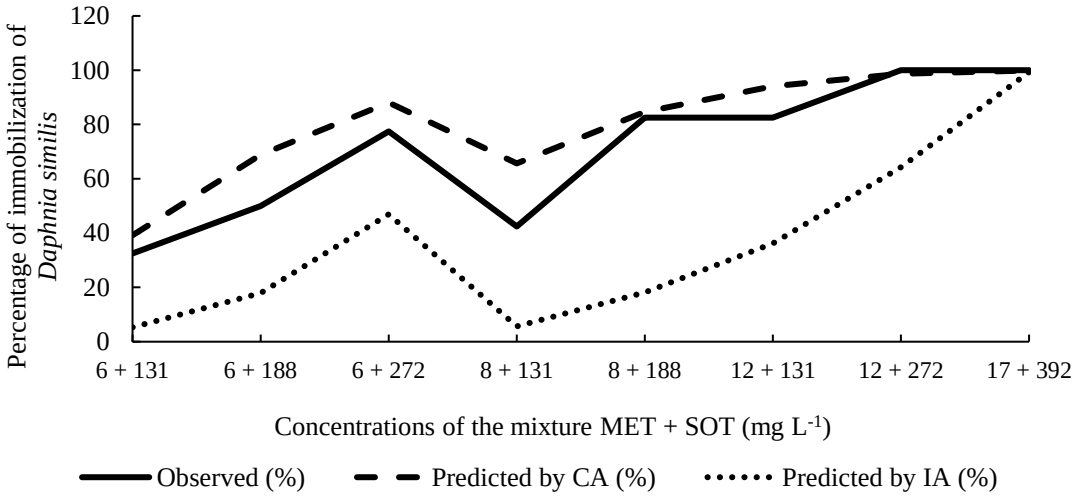
a little' (Thrupp et al., 2018). These results demonstrate that small toxic effects can nevertheless add up to reach a statistically significant response when the daphnids are exposed to combined effects of pharmaceuticals. Therefore, ecological risk assessments based on the toxicity of single pharmaceuticals can underestimate the real impact of these compounds on aquatic ecosystems.

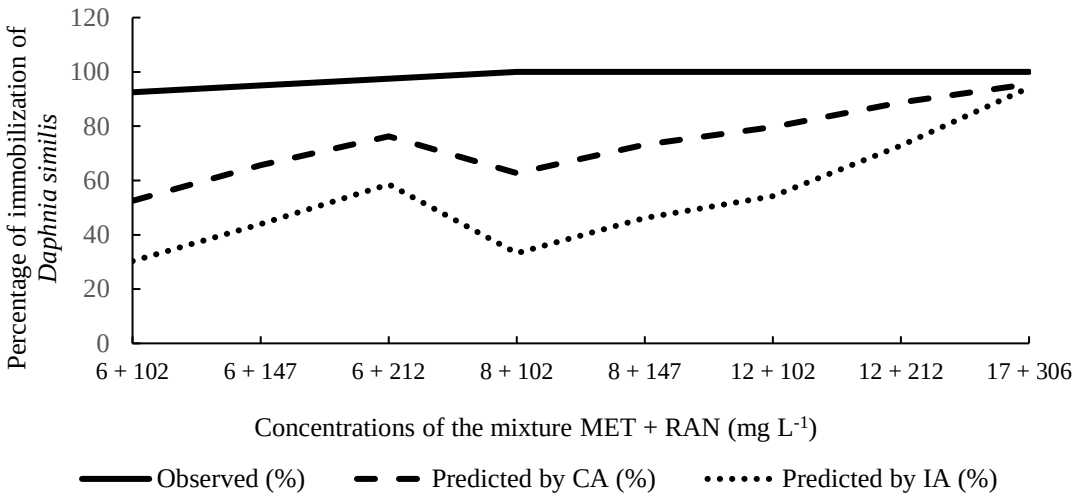
The comparisons between the observed percentage of immobilization in relation to the control induced on *D. similis* by the binary mixtures and those predicted by the models concentration addition and independent action are shown in Fig. 2. The raw percentual data regarding the Fig. 2 are described in the Supplementary Material (Table S4).






Percentage of immobilization of *Daphnia similis*
120
100
80
60
40
20
0
37 + 101
37 + 146
37 + 210
54 + 101
54 + 146
78 + 101
78 + 210
112 + 304
Concentrations of the mixture BIS + RAN (mg L-1)
Observed (%)
Predicted by CA (%)
Predicted by IA (%)


Percentage of immobilization of *Daphnia similis*
120
100
80
60
40
20
0
6 + 131
6 + 188
6 + 272
8 + 131
8 + 188
12 + 131
12 + 272
17 + 392
Concentrations of the mixture MET + SOT (mg L-1)
Observed (%)
Predicted by CA (%)
Predicted by IA (%)


Percentage of immobilization of *Daphnia similis*
120
100
80
60
40
20
0
6 + 102
6 + 147
6 + 212
8 + 102
8 + 147
12 + 102
12 + 212
17 + 306
Concentrations of the mixture MET + RAN (mg L-1)
Observed (%)
Predicted by CA (%)
Predicted by IA (%)

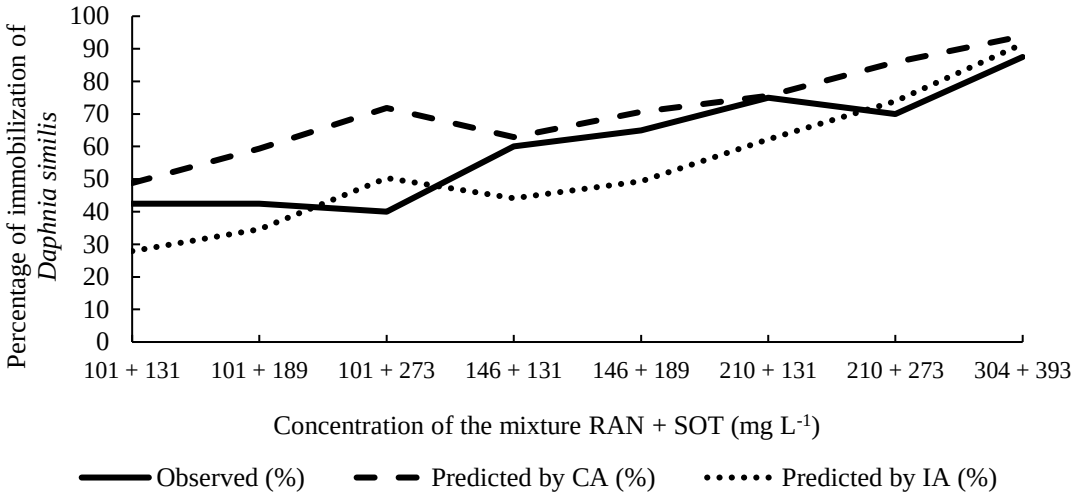


Fig. 2 Observed toxicity values (%) and predicted concentration-response curves of the binary mixtures of the pharmaceuticals metformin (MET), bisoprolol (BIS), ranitidine (RAN) and sotalol (SOT) based on Concentration addition (CA) and Independent action (IA) models for *Daphnia similis* acute toxicity tests.

Source: Godoy et al. (2019)

From Fig. 2, we can observe that most of the observed effects induced by the binary mixtures are between the values predicted by the concentration addition and the independent action models. In the case of the binary mixture metformin + ranitidine, both models underestimated the effects induced by the mixture. Such limitations of the concentration addition and the independent action models in predicting the mixture effects have been reported in the literature for compounds such as other pharmaceuticals, hormones and pesticides (Fent et al., 2006; González-Pleiter et al., 2013; Yang et al., 2017). It is worth mentioning that neither concentration addition nor independent action take into account the complexity of the target biological system (Backhaus, 2014). Moreover, additive predictions depend on the types or shapes of the individual concentration-response curves of each component of a mixture (Berthoud, 2013; Godoy and Kummrow, 2017). In this sense, the concentration addition model shows severe limitations when the concentration-response curves of the individual pharmaceuticals display different shapes (Rodea-Palomares et al., 2010). This could explain the limitation of both models in accurately predicting the observed effects induced by the binary mixtures tested in this study.

On the other hand, the combination index-isobologram method considers not only the potency but also the shape of the individual concentration-response curves (Chou, 2006). The combination index-isobologram values obtained for each of the combinations of the

pharmaceuticals in the binary mixtures and the respective toxicological interaction interpreted according to Chou and Martin (2005) and Chou (2006) are described in Table S5 of Supplementary Material. The classical model (concentration addition or independent action) and respective deviations that best described the mixture effects, according to the ToxCalcMix analyses, are also shown in Table S5 of Supplementary Material.

3.2.1 Bisoprolol + metformin mixtures

For the mixture of bisoprolol + metformin, the concentration addition model best described the mixture effects compared to the independent action one, i.e., the dose addition model explained more variability of acute toxic responses to the exposed daphnids. In relation to the concentration addition model, an antagonistic effect was pointed out as the deviation function that best fitted to the observed responses regarding the immobilization of *D. similis.* Those results were confirmed by the combination index-isobologram method, which showed that slight to moderate antagonism predominated along the range of combined concentrations tested of bisoprolol and metformin.

3.2.2 Bisoprolol + sotalol mixtures

The concentration addition model described slightly better the effects of the binary mixture of the β- blockers bisoprolol and sotalol on the immobilization of *D. similis* when compared to the independent action model. Synergism in relation to the independent action model and a consequent antagonism dose level and dose ratio-dependent in relation to the concentration addition model were identified. The combination index-isobologram method pointed out that additivity was the predominant response when the daphnids were exposed to the various combinations of these two β- blockers.

3.2.3 Bisoprolol + ranitidine mixtures

For the combinations of bisoprolol and ranitidine, the concentration addition model was the approach that best explained the variability of data for *D. similis* immobilization. An antagonism dose ratio-dependent mainly due to ranitidine and dose level-dependent was observed in relation to the concentration addition model for this binary mixture effects. The

combination index-isobologram method identified that slight to moderate antagonism predominated in the range of combined concentrations tested for these pharmaceuticals.

3.2.4 Metformin + sotalol mixtures

The independent action model described slightly better the effects of immobilization of the daphnids exposed to the binary mixture of the pharmaceuticals metformin and sotalol in comparison to the concentration addition model. Synergism in relation to the independent action model was pointed out as the deviation model that best explains the responses of the test organisms exposed to the combinations of this binary mixture. As a consequence, antagonism was identified in relation to the concentration addition model. The slight to moderate antagonism was the predominant deviation observed when using the combination index-isobologram approach for this binary mixture.

3.2.5 Metformin + ranitidine mixtures

For the combinations of the pharmaceuticals metformin and ranitidine, neither the concentration addition nor the independent action model showed to be statistically significant to explain the acute toxicity effects on *D. similis*. Synergism dose level and dose ratio dependent due mainly to metformin in relation to both models was identified. Those observations were confirmed by the combination index-isobologram approach, which pointed out that synergism between the pharmaceuticals occurred in all the combined concentrations tested.

3.2.6 Sotalol + ranitidine mixtures

For the daphnids exposed to the binary mixture of sotalol and ranitidine, the concentration addition model best explained the acute toxicity effects when compared to the independent action model. Antagonism dose ratio-dependent due mainly to sotalol was identified. The combination index-isobologram method also pointed out that antagonism predominated in the range of combinations of concentrations of these two pharmaceuticals, but with different degrees depending on the proportions of each pharmaceutical in the mixture.

From Table S5 and from the analysis of each binary combination above described, we can conclude that the nature of the interaction between the compounds of a mixture depends on the effect level and on the ratio in which each one is applied. Our findings are in accordance

with the studies of De Liguoro et al. (2009, 2010), who found that the interactions among antimicrobials to the algae *R. subcapitata* and to the crustacean *Daphnia magna* were mixture-ratio dependent. In view of this, it is very important to consider an experimental design able to cover several possible interactions at various mixture ratios, such as the fractional factorial design employed in this study, instead of the constant mixture ratios used in most of the studies in this field.

We can also observe that synergism/antagonism or additivity do not depend on the similarity/dissimilarity of the mode of action of the compounds of a mixture. In fact, Rodea-Palomares et al. (2010) concluded that previous knowledge of the mechanism of toxic action of a compound is not useful enough to predict their patterns of interaction when it is combined with other compounds with different or similar toxic mechanism. In addition, these authors showed that different species will show completely different responses to a same mixture of toxicants. Our results also showed that the concept of concentration addition or independent action are not dependent on a similar/dissimilar mode of action of the components of a mixture, differently from their basic assumptions. The binary mixtures of bisoprolol + metformin, bisoprolol + ranitidine and sotalol + ranitidine, thus containing pharmaceuticals with different modes of action, at least in human, were best described by the concentration addition model.

Special attention should be paid to synergistic interactions such as those observed in this study for the binary mixture of metformin plus ranitidine. According to Cedergreen (2014), synergism may be a result of interactions around one or more of the following six processes that occur as a result of the toxicity of a chemical towards an organism: bioavailability, uptake, internal transportation, metabolization, binding at the target site and excretion. The exact underlying mechanisms for the observed synergism in the mixture toxicity test with metformin and ranitidine using *D. similis* may only be elucidated with studies at the molecular level. However, Yang et al. (2017) draws attention to the fact that one of the major reasons for synergistic effects has been assumed to be in the metabolization level, i.e., by alterations in enzymes such as cytochrome P450, in which one of the compounds can decrease the detoxification of the other (enzymatic inhibition). Cedergreen (2014) also stated that it is likely that the majority of severe synergistic interactions can be due to interactions on metabolism. We speculate that this may be a possible mechanism by which the binary mixture of metformin + ranitidine showed a synergistic interaction in the test with *D. similis*. In fact, H2-receptor antagonist pharmaceuticals such as ranitidine have a potential to bind to cytochrome P450 and may inhibit the metabolism of drugs metabolized by the mixed function oxygenase system (Rendiæ, 1999; Smith and Kendall, 1988).

3.2.7 Possible modes-of-action of the acute toxicity of the pharmaceuticals

Our study focuses on acute toxicity, where it is likely that unspecific narcosis is the mode-of-action driving the effects induced by the pharmaceuticals. Based on quantitative structure-activity relationships, Sanderson and Thomsen (2009) analyzed if the mode-of-action of 275 pharmaceutical compounds from different therapeutic classes (including the H2-histamine receptor antagonist ranitidine) is specific or not, i.e., if unspecific narcosis is the mechanism driving the acute toxicity of the majority of pharmaceuticals to algae, daphnia and fish. The authors analyzed a total of 5691 acute effect data points from seven publicly available databases containing experimental ecotoxicological data. Sanderson and Thomsen (2009) found that almost 70 % of the pharmaceutical acute ecotoxicological mode-of-action is non-specific narcosis. In addition, Huggett et al. (2002) identified that beta-blocker acute toxicity to the cladocerans *Daphnia magna* and *Ceriodaphnia dubia* was dependent to the partition coefficient (log P) values. According to Brausch et al. (2012), this observation suggests that the narcosis is the mechanism driving the acute toxicity of beta-blockers to invertebrates.

On the other hand, Sanderson and Thomsen (2007) identified that the anti-diabetic metformin may have some kind of specific acute ecotoxicological mode-of-action, since an excess toxic ratio (Te > 7) between predicted and measured EC_{50} for fish and daphnid was calculated for this pharmaceutical. To date, the ecotoxicological mode-of-action of metformin is uncertain and remains underexplored on non-target organisms (Godoy et al., 2018; Moermond and Smit, 2016). However, it is known that metformin induces inhibition of the complex I in the mitochondrial electron transport chains, leading to adenosine triphosphate (ATP) depletion in humans (Foretz et al., 2014; Rena et al., 2013). In addition, Rena et al. (2013) stated that metformin stimulates 5'- AMP activated-protein kinase (AMPK), an enzyme that acts as a critical cellular energy sensor and regulator of energy homeostasis. Pinho et al. (2013) showed that there is a high genetic and functional homology between *D. rerio* and mammalian mitochondria. Gunnarsson et al. (2008) predicted that the similarity between the AMPK between humans and *D. rerio* is of almost 80 % and between daphnia and humans this similarity is of almost 50 %. From these observations, we can speculate that the involvement of mitochondria and specifically of the enzyme AMPK in the molecular mode-of-action of metformin may exist also in non-target aquatic invertebrates and vertebrates.

Finally, it is also worth remembering that most pharmaceuticals are weak acids or bases and therefore they are ionizable depending on the pKa (negative log of the acid dissociation constant) value and on the pH of the medium. The octanol-water partition coefficient (Kow)

changes with the pH value in the aqueous phase, thus influencing the narcotic toxicity of pharmaceuticals (Sanderson and Thomsen, 2009). Therefore, in the environment the modes-of-action of pharmaceuticals could differ because of the variations in the pH (Sanderson and Thomsen, 2009).

3.3 Quaternary mixture toxicity to D. rerio

No mortality was observed at any of the quaternary mixture concentrations at which the *D. rerio* embryos were exposed in the FET test. Likewise, no visible sub-lethal effect was observed in the embryos up to the maximum exposure concentration. Regarding the behavioral assay, no statistically significant difference was observed for any of the pharmaceutical mixture concentrations at which the embryos were exposed (Figure S1 of Supplementary Material).

In view of the results obtained in this study, developmental and behavioral acute adverse effects seem not to be induced by the joint action of the pharmaceuticals metformin, sotalol, bisoprolol and ranitidine on *D. rerio* embryos, at the concentrations at which they are usually found in surface fresh waters. It is important to note that the range of concentrations employed in the tests with *D. rerio* (μg L^{-1}) were lower than the concentrations used in the *D. similis* tests (mg L^{-1}). This could explain why the acute toxicity observed in the cladoceran species exposed to the pharmaceutical mixtures was not observed in the fish species. In other words, the non-observed effect concentrations of the single pharmaceuticals employed in the quaternary mixture were not high enough to interact resulting in any adverse effect that could be observed by the endpoints evaluated in the tests with *D. rerio* embryos. In addition, it must be mentioned that the protective action of the chorion of the embryos could serve as a barrier to the pharmaceuticals penetration, especially considering the hydrophilicity and ionization of these pharmaceuticals at the test pH. This may have resulted in a toxicity reduction, as it has been speculated with other chemicals (Oliveira et al., 2016; Sanches et al., 2018). In addition, the incomplete development of the metabolic pathways capable of activating potential toxicants in *D. rerio* embryos (Embry et al., 2010) may also have contributed to the apparent lack of observed toxicity of the pharmaceutical mixtures.

Nonetheless, pharmaceuticals from other therapeutic classes both individually and as mixtures have been shown to alter the behavior of fish in adult and larval forms. Brodin et al. (2013) showed that 7 days of exposure to the anxiolytic drug oxazepam altered behavioral traits boldness, activity, sociality and feeding rate of juvenile wild European perch (*Perca fluviatilis*) at the environmental concentration as low as 1.8 μg L^{-1}. In a study recently published, Zhou et

al. (2019) showed that there was a significant decrease in the swimming speed of 118 hpf *D. rerio* larvae exposed during 48 h to a mixture of 8 pharmaceuticals (diclofenac, triclosan, carbamazepine, bezafibrate, sulfamethoxazole, ibuprofen, caffeine and clarithromycin). This mixture was composed by the referred pharmaceuticals at the highest concentration at which they are usually found in European surface waters (from 0.2 to 40 μg L^{-1}) (Zhou et al., 2019). These results show that fish behavior can act as sensitive endpoint for screening of pharmaceuticals that adversely affect the nervous system and thus can pose an ecological risk to aquatic populations.

It is also worth mentioning that the results obtained in this study are based on nominal concentrations. Nonetheless, significant losses are not expected to occur with the pharmaceuticals used in this study. Polystyrene of tubes and well plates used in our ecotoxicity tests is an amorphous plastic with a surface highly hydrophobic and negatively charged, which indicates that lipophilic and positively charged drugs tend to adsorb to the surface of these recipients (Lammer et al., 2009). Basic drugs such as the pharmaceuticals metformin, ranitidine, sotalol and bisoprolol are partially or fully positively charged at the pH ~ 7.0 ± 0.5 (daphnia and FET test medium) (Palmgrén et al., 2006). Nonetheless, they are not lipophilic pharmaceuticals. Instead, they are hydrophilic compounds with relatively low Kow values (Metformin Kow = -2.64; Bisoprolol Kow = 1.87; Sotalol Kow = 0.24 and Ranitidine Kow = 0.27, at 25 °C and pH = 7) (PubChem, 2019). Therefore, hydrophobic interactions with polystyrene surface of the test recipients are not expected to occur. Significant losses via adsorption to polystyrene have been shown to occur with lipophilic and positively charged drugs such as propranolol but not with hydrophilic and positively charged drugs such as atenolol (Palmgrén et al., 2006). Therefore, the lipophilicity of the pharmaceuticals explains the different adsorption profiles (Palmgrén et al., 2006). In fact, according to OECD n. 236 (2013), adsorption to polystyrene of the test recipients is suspected for non-polar, planar compounds with high Kow, which is not the case of the pharmaceuticals used in our study, as previously demonstrated. In addition, the higher the concentration in the test solution the lower the proportional drug loss, since polystyrene possibly contains a limited number of binding sites (Palmgrén et al., 2006). Thus, the surface of plastic tubes/well plates can interact with only a limited amount of drugs (Palmgrén et al., 2006). Therefore, the possible losses in our daphnia tests were possibly not significant also in view of the relatively high pharmaceutical concentrations used (in the mg L^{-1} order). In addition, losses by volatilization are also not expected to occur because of the low Henry's law constant of the pharmaceuticals metformin (7.64×10^{-16} atm/m^3/M), sotalol (2.7×10^{-14} atm/m^3/M), bisoprolol (9.54×10^{-9} atm/m^3/M) and

ranitidine (3.4 x 10^{-15} atm/m^3/M) (Domènech et al., 2011; PubChem, 2019). Significant losses by hydrolysis are also not likely to occur to metformin (ter Laak and Baken, 2014), sotalol (Feiner et al., 2014), ranitidine (Ferrari et al., 2011) and bisoprolol (Kasagić-Vujanović et al., 2017).

4 Conclusions

Our study provides the first data about the toxicity of the binary mixtures of metformin, sotalol, bisoprolol and ranitidine as pharmaceuticals of environmental concern to *D. similis*, and about the developmental and behavioral effects induced by an acute exposure of *D. rerio* embryos to environmentally relevant concentrations of mixtures of these pharmaceuticals. In addition, the data generated in this study contribute to improve the knowledge regarding the interaction profiles of pharmaceutical compounds in mixtures.

Our results confirmed other studies on mixture toxicity reported in the literature regarding the predictive accuracy of the concentration addition and the independent action models and the utility of the combination index-isobologram method as an important tool to define toxicological interactions in mixtures of environmental contaminants. Our results also confirmed that even statistically significant non-effect concentrations can nonetheless add up to elicit significant mixture responses. We reinforced the concern that ecological risk assessment based on single toxic effects of pharmaceuticals of environmental concern can lead to an underestimation of the real impact of these compounds to the aquatic ecosystems.

We also showed that abnormal development or behavioral disturbance are unlikely to occur on *D. rerio* embryos exposed to environmental concentrations of these pharmaceuticals in joint action. However, additional long-term studies with fishes are required in order to conclude about possible chronic adverse effects induced by this pharmaceutical mixture.

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SUPPLEMENTARY MATERIAL

Single and mixture toxicity of four pharmaceuticals of environmental concern to aquatic organisms, including a behavioral assessment

Methodology and results obtained from the behavior tests with *Danio rerio* larvae

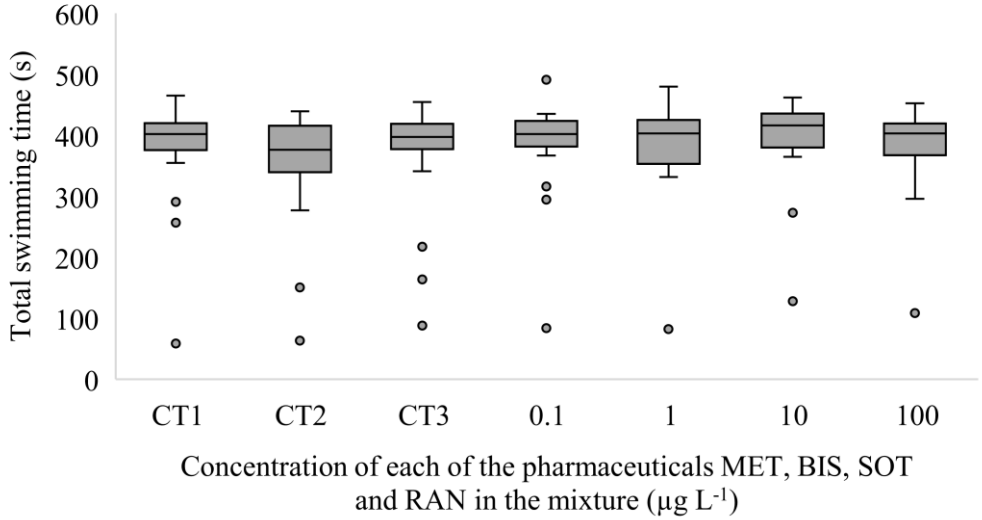
Methodology of the tests with *Danio rerio*

Testing with *Danio rerio* was carried out following the OECD guideline 236 (OECD, 2013). One 3 h post-fertilization (hpf) egg was placed into each well of 24-well plates. Each egg was exposed to 2 mL of the test concentrations of each of the pharmaceuticals and/or of their quaternary mixtures. In each plate, twenty eggs were exposed to the test concentrations while four eggs were exposed only to the reconstituted water used for fish maintenance (internal plate controls). One 24-well plate was used for each test concentration/mixture. The conditions for temperature and photoperiod were the same as for the cultivation of the adult fishes. Three independent toxicity tests were carried out for each single pharmaceutical. The quaternary mixture toxicity tests were performed twice. Every 24 h, the lethal and sub-lethal endpoints evaluated were coagulation of fertilized eggs, lack of somite formation, lack of tail detachment, hatching success, pigmentation failure, edema (heart and yolk) and spinal deformation. In addition, at 48 hpf, the heart beat rate (beat/20 s) was assessed in the embryos. Those observations were made until 96 hpf, using a stereomicroscope (Stereoscopic Zoom Microscope – SMZ 1500, Nikon Corporation). The parameters pH, conductivity and dissolved oxygen were measured at the end of each test.

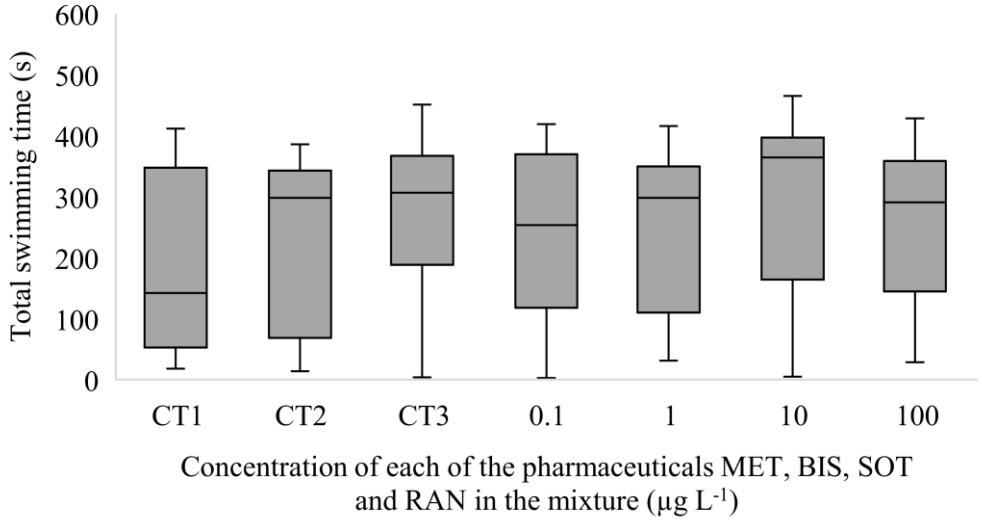
The locomotor activity of zebrafish was evaluated at 120 hpf, according to the protocol described in our previous study (Godoy et al., 2018). Briefly, the 120 hpf-larvae had their locomotor activity tracked by the system Zebrabox-Zeb (View-Point Life Sciences, Lyon, France). The behavioral endpoints total swimming distance (TSD) and total swimming time (TST) were measured in alternating light/dark intervals of 10 min each, after a 5 min light adaptation period. Three independent negative controls, i.e., three 24-well plates containing eggs exposed to only the reconstituted water were used for the behavior assessment (C1, C2 and C3), in order to increase the statistical power of the experiments. The treatments were replicated twice.

Results obtained with *Danio rerio* larvae

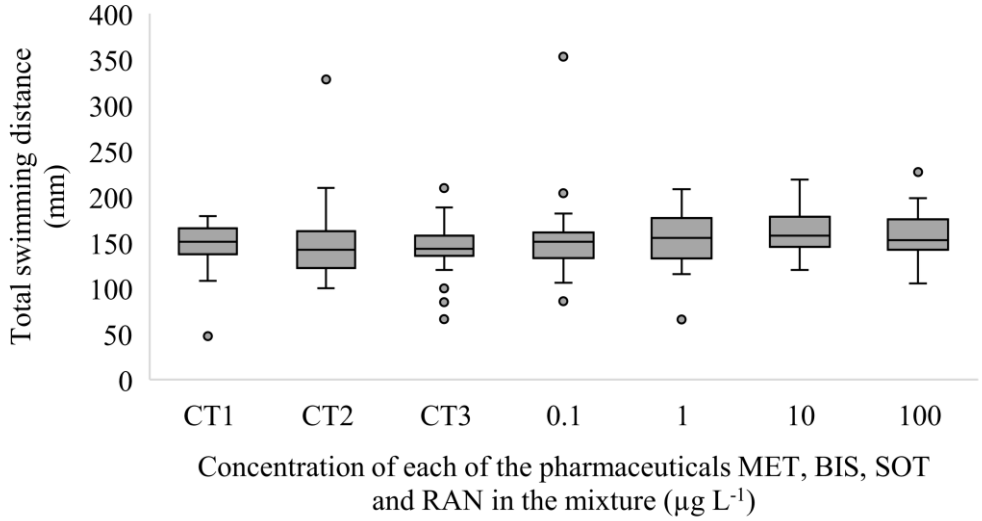
A)



B)



C)



D)

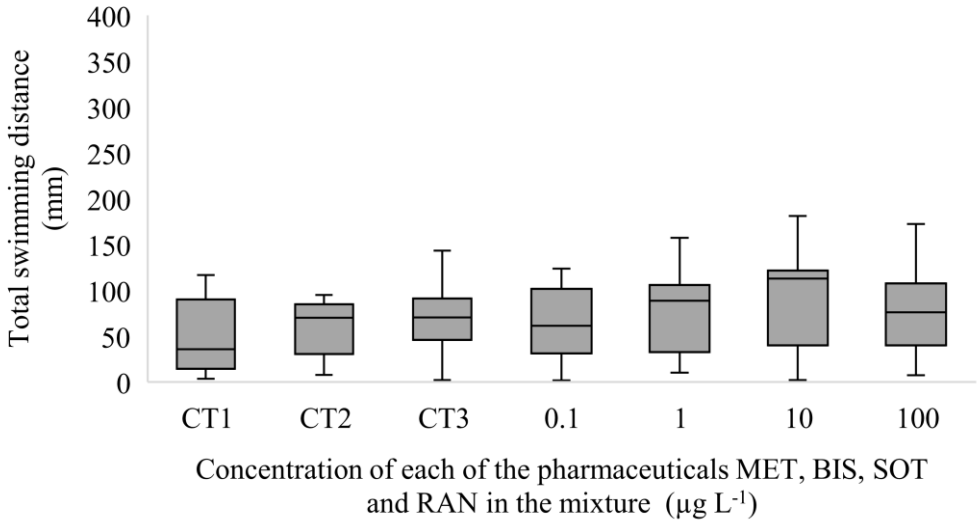


Fig. S1 Effect of the quaternary mixtures of the pharmaceuticals metformin (MET), bisoprolol (BIS), sotalol (SOT) and ranitidine (RAN) on the *Danio rerio* locomotor behavior quantified after 120 h post-fertilization. A) Total swimming time at the dark cycle (10min); B) Total swimming time at the light cycle (10min); C) Total swimming distance at the dark cycle (10 min); D) Total swimming distance at the light cycle (10 min). CT1, CT2, CT3 are the three independent control groups. Boxes represent medians (full line), with 5^{th} and 95^{th} percentiles (n=24 for controls and n=20 for treatments) and the respective outliers.

SUPPLEMENTARY MATERIAL

Single and mixture toxicity of four pharmaceuticals of environmental concern to aquatic organisms, including a behavioral assessment

Table S1- Reported concentrations (µg L^{-1}) of the pharmaceuticals bisoprolol, sotalol, ranitidine and metformin in aquatic matrices in several countries. WWTP = wastewater treatment plant

Pharmaceutical	Sample	Country	Reported concentration (µg L^{-1})	Reference
		Fresh surface water		
Bisoprolol	Lakes and rivers receiving effluents from WWTP	Sweden	0.0001 – 0.15	Fick et al. (2011)
	Rivers and streams	Germany	Up to 2.9	Ternes (1998)
	Surface waters	Germany	<0.003 – 0.36	UBA (2013)
	Leça River	Portugal	Up to 0.026	Gonçalves et al. (2013)
	Velika Morava River	Serbia	0.0072	Jauković et al. (2014)
	Surface waters along the Maas river and the Albert channel	Belgium	0.017 – 0.023	Vergeynst et al. (2014)
Sotalol	Glatt River	Switzerland	Up to 0.052	Alder et al. (2010)
	Llobregat	Spain	Up to 1.82	Ginebreda et al. (2010)
	Surface waters	Germany	Up to 1.3	UBA database (2019)
	Waterbodies receiving effluents (Po Valley)	Italy	0.373 – 0.504	Aukidy et al. (2012)
	Llobegrat River basin	Spain	0.0019 – 0.7876	López-Roldán et al. (2010)
	Ebro River basin	Spain	Up to 0.423	López-Serna et al. (2011)
	Rivers in Madrid	Spain	0.123 – 0.864	Valcárcel et al. (2011a)
	Meuse river basin (The Netherlands	The Netherlands	0.004 – 0.0802	Houtman et al. (2013)
	Rhine river	Switzerland. Austria. Germany. France. Belgium and Netherland	0.018 – 0.040	Ruff et al. (2015)
	Surface waters	Germany	<0.005 – 1.3	UBA (2013)
	Rivers Vantaa and Luhtajoki	Finland	Up to 0.052	Vieno et al. (2006)
Ranitidine	Lakes and rivers receiving effluents	Sweden	0.0054 – 0.110	Fick et al. (2011)
	Llobregat river	Spain	Up to 0.57	Ginebreda et al. (2010)
	Llobegrat river basin	Spain	0.0023 – 0.0696	López-Roldán et al. (2010)

(To be continued)

Table S1 (continued)

Pharmaceutical	Sample	Country	Reported concentration ($\mu g\ L^{-1}$)	Reference
		Fresh surface water		
Ranitidine	Ebro river basin	Spain	Up to 0.084	Silva et al. (2011)
	Ebro river basin	Spain	Up to 0.109	López-Serna et al. (2011)
	Rivers in Madrid	Spain	0.105 – 1.944	Valcárcel et al. (2011b)
	Rhine River	Switzerland. Austria, Germany, France., Belgium and The Netherlands	0.010 - 0.011	Ruff et al. (2015)
	Surface waters of national parks (recreation area)	USA	Up to 0.0331	Elliott and VanderMeulen (2017)
Metformin	Michigan Lake	USA	Up to 9.2	Blair et al. (2013)
	Surface Waters of national parks (Indiana Dunes National Lakeshore)	USA	Up to 0.903	Elliott and VanderMeulen (2017)
	Rhine, Elbe, Danube, Main and Neckar Rivers	Germany	0.130 – 1.7	Scheurer et al. (2009)
	Rhine, Neckar, Ruhr, Main, Danube, Körsch, Lein and Schwarzbach rivers	Germany	0.06 – 3.1	Scheurer et al. (2012)
	Constance Lake and Rhine river	Germany	0.035 – 0.216	Trautwein et al. (2014)
	Surface waters from Rhône-Alpes	France	0.1006	Vulliet and Cren-Olivé (2011)
	Meuse River basin (The Netherlands)	The Netherlands	Up to 1.699	Houtman et al. (2013)
	Rhine River	Switzerland, Austria, Germany, France, Belgium and The Netherlands	0.172 - 1.314	Ruff et al. (2015)
	Surface waters receiving effluents (100 to 200 m downstream (Southwest Nova Scotia)	Canada	0.012 - 1.487	Ghoshdastidar et al. (2015)
	Surface waters from Tianjin	China	Up to 20.015	Kong et al. (2015)
	Surface waters from Danube river	Austria	0.104 (mean)	Martín et al. (2012)
	Langat river in Bangi town	Malaysia	0.293	Al-Odaini et al. (2010)
	Streams across the USA	USA	Up to 0.15	Kolpin et al. (2002)
		WWTP effluent		
Bisoprolol	WWTP effluents from the Lyon urban area	France	Up to 2.838	Miège et al. (2006)
	WWTP effluents	Sweden	0.059 – 0.250	Fick et al. (2011)

(To be continued)

Table S1 (continued)

Pharmaceutical	Sample	Country	Reported concentration (μg L^{-1})	Reference
		WWTP effluent		
Bisoprolol	WWTP effluents (Catalonia)	Spain	0.059 – 0.114	Huerta-Fontela et al. (2010)
	WWTP effluents	Germany	Up to 0.37	Ternes (1998)
	WWTP effluents (European Union)	18 countries from European Union	Up to 0.423	Loos et al. (2013)
	WWTP effluents (Ljubljana)	Slovene	0.036 - 0.2164	Klančar et al. (2016)
	Municipal WWTP effluents	Portugal	24.256	Sousa et al. (2013)
	WWTP effluents	Serbia	0.048	Jauković et al. (2014)
Sotalol	WWTP effluents (Dübendorf, Kloten-Opfikon and Niederglatt)	Switzerland	0.21 - 0.33	Alder et al. (2010)
	WWTP effluent	Finland	Up to 0.3	Vieno et al. (2006)
	WWTP effuents	Germany	Up to 6.5	UBA database (2019)
	WWTP effluents (Po Valley)	Italy	0.152 – 0.366	Aukidy et al. (2012)
	WWTP effluents (Catalonia)	Spain	0.011 – 0.168	Huerta-Fontela et al. (2010)
	WWTP effluents (Stony Brook)	USA	0.246	Lara-Martín et al. (2014)
	WWTP effluents (New York)	USA	0.025 - 0.755	Oliveira et al. (2015)
	WWTP effluents	Germany	1.314 ± 0.130	Nödler et al. (2010)
	WWTP effluents from Enschede and Ootmarsum	The Netherlands	0.88 – 1.29	Oosterhuis et al. (2013)
	WWTP effluents (Coimbra)	Portugal	0.0831 – 0.186	Santos et al. (2013)
	WWTP effluents (Po Valley)	Italy	0.21 – 0.47	Verlicchi et al. (2012)
Ranitidine	WWTP effluents	Sweden	0.0068 – 0.150	Fick et al. (2011)
	WWTP effluents	Italy	Up to 0.610	Castiglioni et al. (2005)
	WWTP effluents (Girona)	Spain	0.118 – 0.179	Gros et al. (2012)
	WWTP effluents from Tricity (Gdańsk)	Poland	0.274 - 0.9825	Kot-Wasik et al. (2016)
	WWTP effluents (Stony Brook)	USA	0.030	Lara-Martín et al. (2014)
	WWTP effluents (New York)	USA	0.289 - 3.0	Oliveira et al. (2015)
	WWTP effluents (Bremerton and Tacoma Rivers)	USA	0.494	Meador et al. (2016)
	WWTP effluents (Coimbra)	Portugal	0.0317 – 0.313	Santos et al. (2013)
	WWTP effluents (Po Valley)	Italy	0.04 – 0.10	Verlicchi et al. (2012)
Metformin	WWTP effluents	Sweden	0.290 – 0.370	Fick et al. (2011)
	WWTP effluents (Enschede and Ootmarsum)	The Netherlands	1.22 – 1.82	Oosterhuis et al. (2013)
	WWTP effluents	Germany	2.2 - 21	Scheurer et al. (2009)

(To be continued)

Table S1 (continued)

Pharmaceutical	Sample	Country	Reported concentration (μg L^{-1})	Reference
		WWTP effluent		
Metformin	WWTP effluents	Germany	1.2 - 26	Scheurer et al. (2012)
	WWTP effluents	Germany	3.4 – 6.4	Trautwein et al. (2014)
	WWTP effluents from Tricity (Gdańsk)	Poland	0.0075 - 0.0629	Kot-Wasik et al. (2016)
	WWTP effluents	Greek	Up to 0.026	Kosma et al. (2015)
	WWTP effluent	Faroe Islands, Iceland and Greenland	0.234 - 7.420	Huber et al. (2016)
	WWTP effluents (Southwest da Nova Scotia)	Canada	0.067 - 10.608	Ghoshdastidar et al. (2015)
	WWTP effluents (New York)	USA	0.401 - 58.9	Oliveira et al. (2015)
	WWTP effluents (Bremerton and Tacoma cities)	USA	29.3 - 82.7	Meador et al. (2016)
		Hospital effluents		
Sotalol	Hospital effluents (Coimbra)	Portugal	0.0237 – 0.345	Santos et al. (2013)
	Hospital effluents	Italy	0.35 – 6.7	Verlicchi et al. (2012)
	Hospital effluents (Nova Iorque)	USA	0.035 - 1.1	Oliveira et al. (2015)
Ranitidine	Hospital effluents (Coimbra)	Portugal	0.0162 – 19.840	Santos et al. (2013)
	Hospital effluents (Po Valley)	Italy	0.24 – 4.1	Verlicchi et al. (2012)
	Hospital effluents (New York)	USA	0.145 - 6.090	Oliveira et al. (2015)
Metformin	Hospital effluents (Coimbra)	Portugal	0.0161 – 4.04	Santos et al. (2013)
	Hospital effluents (New York)	USA	0.009 - 630	Oliveira et al. (2015)
	Hospital effluents	Faroe Islands, Iceland and Greenland	3.580 - 7.950	Huber et al. (2016)
		Industrial effluents		
Ranitidine	Industrial effluents in Patancheru	India	90 - 160	Larsson et al. (2007)
		Groundwaters		
Bisoprolol	Groundwaters from Germany	Germany	Up to 0.0540	UBA database (2019)
Sotalol	Urban groundwaters underlying the metropolis of Barcelona	Spain	Up to 0.0201	López-Serna et al. (2013)
	Groundwater samples from Baden-Württemberg	Germany	Up to 0.560	Sacher et al. (2001)
	Groundwaters from an aquifer system	Germany	0.0059 – 0.0543	Reh et al. (2013)
Ranitidine	Urban groundwaters underlying the metropolis of Barcelona	Spain	Up to 0.0176	López-Serna et al. (2013)

(To be continued)

Table S1 (continued)

Pharmaceutical	Sample	Country	Reported concentration (μg L^{-1})	Reference
		Groundwaters		
Metformin	Groundwaters from the Rhône-Alpes region	France	0.0099 (media)	Vulliet and Cren-Olivé (2011)
		Drinking water		
Sotalol	Drinking water (postchlorinated) from a treatment plant	Spain	Up to 0.003	Huerta-Fontela et al. (2011)
	Raw and chlorinated drinking water	Serbia	0.0004	Petrović et al. (2014)
Ranitidine	Tap water in Girona	Spain	0.0006	Gros et al. (2012)
	Drinking water post treatment in Gdańsk	Poland	0.0049 - 0.0056	Kot-Wasik et al. (2016)
Metformin	Drinking water post treatment in Stuttgart	Germany	0.002 – 0.061	Trautwein et al. (2014)
	Drinking water post treatment in Gdańsk	Poland	0.0017 - 0.0080	Kot-Wasik et al. (2016)
		Estuarine and sea waters		
Sotalol	Mediterranean Sea, at Gola de Ter beach	Spain	0.002	Gros et al. (2012)
	Estuary of the Jiulong River,	China	Up to 0.0008	Lv et al. (2014)
	Baltic sea, Aegean & Dardanelles and Venice	Germany,Greek, Turkey, Italy and	Up to 0.067	Nödler et al. (2014)
Ranitidine	Mediaterranean sea, at Gola de Ter Beach	Spain	0.0008	Gros et al. (2012)
	Estuary waters (Sinclair Inlet, estuary of the Puyallup river and Nisqually estuary)	USA	0.00075	Meador et al. (2016)
Metformin	German Bight and North Sea	Germany	Up to 0.033	Trautwein et al. (2014)
	Estuary waters (Sinclair Inlet, estuary of the Puyallup river and Nisqually estuary)	USA	0.105 - 0.832	Meador et al. (2016)

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Table S2 – Concentrations (µg L^{-1}) of the pharmaceuticals metformin (MET), bisoprolol (BIS), sotalol (SOT) and ranitidine (RAN) simultaneously detected in several aquatic matrices worldwide. WWTP = wastewater treatment plant

Sample	Country	Pharmaceuticals detected simultaneously	Concentrations (µg L^{-1})	Reference
Influent wastewater samples from WWTP	Belgium	MET BIS RAN	20.331 – 94.311 0.001 – 0.169 Up to 0.963	van Nujis et al. (2010)
Effluent of Vidy, Lausanne	Switzerland	MET SOT	1.027 0.260	De La Cruz et al. (2012)
Inffluent of WWTP Enschede and Ootmarsum	The Netherlands	MET SOT	73.73 – 84.41 1.06 – 1.70	Oosterhuis et al. (2013)
Effluent from WWTP Terrassa	Spain	RAN SOT	0.347 0.509	Radjenović et al. (2009)
Hospital effluents in Coimbra	Portugal	RAN SOT MET	0.0162 – 19.840 Up to 0.345 Up to 3.836	Santos et al. 2013
Surface Waters from Katrineholm, Vallentuna, Skövde and Uppsala	Sweden	BIS RAN	Up to 0.150 Up to 0.110	Fick et al. (2011)
Surface Waters from the Llobregat River	Spain	RAN SOT	0.01 – 0.57 0.11 – 1.82	Ginebreda et al. (2010)

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Table S3 - Percentage of immobilization of *Daphnia similis* exposed to the designed concentrations (mg L^{-1}) of the single pharmaceuticals metformin (MET), bisoprolol (BIS), ranitidine (RAN) and sotalol (SOT) and of their respective binary mixtures

Individual pharmaceuticals	Concentration (mg L^{-1})	Effect (%)	Mixture	Combined concentrations (mg L^{-1})	Effect (%)
Bisoprolol	37	0		37 + 6	10
	54	5		54 + 6	20
	78	12.5		78 + 6	30
	112	57.5		37 + 8	22.5
Metformin	6	0	BIS + MET	54 + 8	25.0
	8	2.5		37 + 12	42.5
	12	45		78 + 12	75.0
	17	70		112 + 17	100.0
Individual pharmaceuticals	Concentration (mg L^{-1})	Effect (%)	Mixture	Combined concentrations (mg L^{-1})	Effect (%)
Bisoprolol	37	5.0		37 + 130	12.5
	54	5.0		37 + 188	32.5
	78	17.5		37 + 271	72.5
	112	62.5		54 + 130	35.0
Sotalol	130	0.0	BIS + SOT	54 + 188	72.5
	188	30.0		78 + 130	57.5
	271	55.0		78 + 271	87.5
	390	77.5		112 + 390	97.5
Individual pharmaceuticals	Concentration (mg L^{-1})	Effect (%)	Mixture	Combined concentrations (mg L^{-1})	Effect (%)
Bisoprolol	37	0.0		37 + 101	17.5
	54	5.0		37 + 146	45.0
	78	20.0		37 + 210	57.5
	112	60.0		54 + 101	45.0
Ranitidine	101	15.0	BIS + RAN	54 + 146	50.0
	146	40.0		78 + 101	55.0
	210	45.0		78 + 210	80.0
	304	77.5		112 + 304	95.0
Individual pharmaceuticals	Concentration (mg L^{-1})	Effect (%)	Mixture	Combined concentrations (mg L^{-1})	Effect (%)
Metformin	6	2.5		6 + 131	32.5
	8	5.0		6 + 188	50.0
	12	32.5		6 + 272	77.5
	17	97.5		8 + 131	42.5
Sotalol	131	5.0		8 + 188	82.5
	188	17.5	MET + SOT	12 + 131	82.5
	272	47.5		12 + 272	100.0
	392	77.5		17 + 392	100.0
Individual pharmaceuticals	Concentration (mg L^{-1})	Effect (%)	Mixture	Combined concentrations (mg L^{-1})	Effect (%)
Metformin	6	0		6 + 102	92.5
	8	0		6 + 147	95.0
	12	37.5		6 + 212	97.5
	17	77.5		8 + 102	100.0
Ranitidine	102	27.5	MET + RAN	8 + 147	100.0
	147	47.5		12 + 102	100.0
	212	55.0		12 + 212	100.0
	306	72.5		17 + 306	100.0

(To be continued)

Table S3 (continued)

Individual pharmaceuticals	Concentration (mg L^{-1})	Effect (%)	Mixture	Combined concentrations (mg L^{-1})	Effect (%)
Ranitidine	101	25.0		101 + 131	48.8
	146	45.0		101 + 189	59.4
	210	52.5		101 + 273	71.8
	304	82.5		146 + 131	62.9
Sotalol	131	0.0	RAN + SOT	146 + 189	70.7
	189	10.0		210 + 131	75.6
	273	40.0		210 + 273	85.9
	393	60.0		304 + 393	93.9

Table S4 - Comparison between the observed percentage of immobilization in relation to the controls and those predicted by the Concentration addition (CA) and Independent action (IA) models for the exposition of *Daphnia similis* to the binary mixtures of the pharmaceuticals metformin (MET), bisoprolol (BIS), sotalol (SOT) and ranitidine (RAN).

BIS + MET			
Combined concentrations (mg L^{-1})	**Observed experimentally (%)**	**Predicted by CA (%)**	**Predicted by IA (%)**
37 + 6	10.0	24.2	3.0
54 + 6	20.0	44.2	4.5
78 + 6	30.0	70.8	15.9
37 + 8	22.5	43.3	9.6
54 + 8	25.0	62.5	11.0
37 + 12	42.5	74.2	38.6
78 + 12	75.0	92.2	46.8
112 + 17	100.0	98.6	89.1
BIS + SOT			
Combined concentrations (mg L^{-1})	**Observed experimentally (%)**	**Predicted by CA (%)**	**Predicted by IA (%)**
37 + 130	12.5	34.1	8.3
37 + 188	32.5	57.9	24.4
37 + 271	72.5	79.1	53.5
54 + 130	35.0	52.6	10.4
54 + 188	72.5	71.9	26.1
78 + 130	57.5	74.4	23.8
78 + 271	87.5	92.5	61.3
112 + 390	97.5	98.3	92.5
BIS + RAN			
Combined concentrations (mg L^{-1})	**Observed experimentally (%)**	**Predicted by CA (%)**	**Predicted by IA (%)**
37 + 101	17.5	39.1	17.8
37 + 146	45.0	56.8	33.4
37 + 210	57.5	72.8	53.7
54 + 101	45.0	52.8	20.7
54 + 146	50.0	67.5	35.7
78 + 101	55.0	70.2	34.1
78 + 210	80.0	86.9	62.9
112 + 304	95.0	95.5	89.2
MET + SOT			
Combined concentrations (mg L^{-1})	**Observed experimentally (%)**	**Predicted by CA (%)**	**Predicted by IA (%)**
6 + 131	32.5	39.2	5.3
6 + 188	50.0	68.9	17.9
6 + 272	77.5	88.1	46.9
8 + 131	42.5	65.7	5.6
8 + 188	82.5	84.7	18.2
12 + 131	82.5	94.1	36.2
12 + 272	100.0	98.7	64.3
17 + 392	100.0	99.9	99.3

(To be continued)

Table S4 (continued)

MET + RAN			
Combined concentrations (mg L^{-1})	**Observed experimentally (%)**	**Predicted by CA (%)**	**Predicted by IA (%)**
6 + 102	92.5	52.5	30.3
6 + 147	95.0	65.7	43.9
6 + 212	97.5	76.3	58.6
8 + 102	100.0	62.7	33.2
8 + 147	100.0	73.2	46.2
12 + 102	100.0	79.7	54.2
12 + 212	100.0	88.8	72.8
17 + 306	100.0	95.5	94.2
RAN + SOT			
Combined concentrations (mg L^{-1})	**Observed experimentally (%)**	**Predicted by CA (%)**	**Predicted by IA (%)**
101 + 131	42.5	48.8	27.9
101 + 189	42.5	59.4	34.6
101 + 273	40.0	71.8	50.3
146 + 131	60.0	62.9	44.1
146 + 189	65.0	70.7	49.3
210 + 131	75.0	75.6	62.2
210 + 273	70.0	85.9	73.9
304 + 393	87.5	93.9	91.5

Table S5 – Values of combination index (CI) obtained for each combination of the pharmaceuticals metformin (MET), bisoprolol (BIS), ranitidine (RAN) and sotalol (SOT) by the use of the software CompuSyn, the respective toxicological interactions interpreted according to Chou and Martin (2005) and Chou (2006) and the classical model/respective deviations that best described the observed effects of each binary mixture. CA = concentration addition; IA = independent action.

BIS + MET			
Combined concentrations (mg L^{-1})	**CI value**	**Toxicological interaction**	**Model/deviation best describing the effects**
37 + 6	1.17	Slight antagonism	Antagonism from the CA model
54 + 6	1.21	Moderate antagonism	
78 + 6	1.34	Moderate antagonism	
37 + 8	1.14	Slight antagonism	
54 + 8	1.31	Moderate antagonism	
37 + 12	1.25	Moderate antagonism	
78 + 12	1.24	Moderate antagonism	
112 + 17	0.90	Nearly additive	
BIS + SOT			
Combined concentrations (mg L^{-1})	**CI value**	**Toxicological interaction**	**Model/deviation best describing the effects**
37 + 130	1.30	Moderate antagonism	Antagonism dose level and dose ratio-dependent from the CA model mainly due to bisoprolol
37 + 188	1.21	Moderate antagonism	
37 + 271	1.06	Nearly additive	
54 + 130	1.12	Slight antagonism	
54 + 188	0.92	Nearly additive	
78 + 130	1.08	Nearly additive	
78 + 271	1.05	Nearly additive	
112 + 390	1.01	Nearly additive	
BIS + RAN			
Combined concentrations (mg L^{-1})	**CI value**	**Toxicological interaction**	**Model/deviation best describing the effects**
37 + 101	1.39	Moderate antagonism	Antagonism dose ratio-dependent from the CA model due mainly to RAN and dose level-dependent
37 + 146	1.16	Slight antagonism	
37 + 210	1.27	Moderate antagonism	
54 + 101	1.08	Nearly additive	
54 + 146	1.25	Moderate antagonism	
78 + 101	1.18	Slight antagonism	
78 + 210	1.18	Slight antagonism	
112 + 304	1.07	Nearly additive	
MET + SOT			
Combined concentrations (mg L^{-1})	**CI value**	**Toxicological interaction**	**Model/deviation best describing the effects**
6 + 131	1.13	Slight antagonism	Synergism from the IA model
6 + 188	1.18	Slight antagonism	
6 + 272	1.15	Slight antagonism	
8 + 131	1.22	Moderate antagonism	
8 + 188	1.02	Nearly additive	
12 + 131	1.16	Slight antagonism	
12 + 272	0.87	Slight synergism	
17 + 392	1.25	Moderate antagonism	

(To be continued)

Table S5 (continued)

MET + RAN			
Combined concentrations (mg L^{-1})	**CI value**	**Toxicological interaction**	**Model/deviation best describing the effects**
6 + 102	0.43	Synergism	Both CA and IA models failed to describe the mixture data. Synergism was observed from both models
6 + 147	0.43	Synergism	
6 + 212	0.39	Synergism	
8 + 102	0.33	Synergism	
8 + 147	0.35	Synergism	
12 + 102	0.47	Synergism	
12 + 212	0.52	Synergism	
17 + 306	0.74	Moderate syergism	
RAN + SOT			
Combined concentrations (mg L^{-1})	**CI value**	**Toxicological interaction**	**Model/deviation best describing the effects**
101 + 131	1.08	Nearly additive	Antagonism dose ratio-dependent from the CA model due mainly to SOT
101 + 189	1.26	Moderate antagonism	
101 + 273	1.58	Antagonism	
146 + 131	1.08	Nearly additive	
146 + 189	1.16	Slight antagonism	
210 + 131	1.10	Slight antagonism	
210 + 273	1.55	Antagonism	
304 + 393	1.59	Antagonism	

5 FINAL CONSIDERATIONS

- Important data gaps pointed in the literature regarding the ecotoxicity of the pharmaceuticals investigated in this study were filled, which expanded the knowledge about their adverse effects on non-target organisms. These results can help in prioritization schemes for monitoring campaigns and risk assessment for these pharmaceuticals as well as can subsidize possible regulatory actions;
- Scientific knowledge about the behavior of a vertebrate species exposed to pharmaceuticals was also increased and behavioral endpoints showed to be more sensitive than developmental endpoints in some cases, as in the sotalol case in this study;
- Environmental Quality Standard (EQS) values were updated for metformin with bounded values obtained from this study. The lower values (more protective) derived for metformin in this study compared to the existing in the literature showed that deriving EQS is a continuous dynamic process that can benefit from new ecotoxicity information;
- Environmental risk assessment could be performed for the pharmaceutical bisoprolol for the first time, using experimental chronic data generated in this study; This allowed to increase the realism of the possible risk posed by this pharmaceutical to aquatic biota, a demand that had been pointed out in the literature;
- Knowledge regarding mixture toxicity of pharmaceuticals was expanded with the new data generated. The phenomenon of ‘something from nothing’ and ‘a lot from a little’ showed to be also present in binary mixtures of pharmaceuticals from different therapeutic classes, which raises concerning about these important combined effects;
- These results reinforced the concerning that assessing risk based on the effects of individual pharmaceuticals can significantly underestimate their degree of risk. This is especially important from a regulatory perspective, since mixtures constitute the typical environmental exposure situation with respect to pharmaceuticals in the environment;
- Results reported in this study also reinforced the need that current guidelines for the environmental risk assessment and regulatory frameworks for pharmaceuticals explicitly address mixture effects. The mixture toxicity experimental data generated in this study add up to help in this task that is still in its very beginning.

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ATTACHMENTS

Attachment I

Scientific activities

Aline Andrade Godoy held a sandwich doctorate internship at the Department of Biology of the University of Aveiro, under the supervision of the Professor Dr. António José Arsénia Nogueira. At that University, Aline helped in the husbandry of *Danio rerio*, performed tests with fish embryos, learned about mathematical modelling of mixtures and attended the discipline *Biomarkers in Ecotoxicology: user-friendly approach* and the advanced course on *Fundamentals of animal cell culture*. Aline also contributed to the studies performed by the Ph.D. student Ana Letícia Madeira Sanches at the University of Aveiro and is co-author of the article '*Lethal and sublethal toxicity of abamectin and difenoconazole (individually and in mixture) to early life stages of zebrafish'*, Chemosphere, v. 210, p. 531 – 538, 2018. https://doi.org/10.1016/j.chemosphere.2018.07.027.

During the sandwich doctorate internship, Aline attended the *SETAC Europe 27th Annual Meeting*, held in Brussels (Belgium) in May 2017, where she presented the work entitled '*A critical overview of the mixture ecotoxicity data with pharmaceuticals and personal care products (PPCP) reported in the last 16 years*' in the poster form. In addition to this international meeting, during the period of the doctorate course, the contents of this thesis were presented in the oral or poster form in the *XIV Brazilian Congress Of Ecotoxicology* (Curitiba, Brazil, September 7 – 10, 2016), *XXI Pharmaceutical Week of Science and Technology of the University of São Paulo* (São Paulo, Brazil, September 26 – 30, 2016), *XVIII International Symposium on Toxicity Assessment (*Limeira, Brazil, July 16 - 21, 2017*), 12th SETAC Latin America Biennial Meeting* (Santos, Brazil, September 7 – 10, 2017), *III International Symposium on Pathophysiology and Toxicology – ISPAT and VIII Symposium of Post-Graduation in Clinical Analyses – SIMPAC* (São Paulo, Brazil, April 26 - 27, 2018), and in the *XV Brazilian Congress of Ecotoxicology* (Aracaju, Brazil, September 1 - 4, 2018).

Attachment II

Aline Andrade Godoy

Endereço para acessar este CV: http://lattes.cnpq.br/6763739135622460
ID Lattes: **6763739135622460**
Última atualização do currículo em 08/07/2019

Graduada em Farmácia com habilitação em Farmácia Industrial pela Universidade Federal de Alfenas (2008). Especialista em Farmacologia Clínica com ênfase em Assistência Farmacêutica pela Universidade Federal de Alfenas (2009). MBA em Gestão Ambiental pela Faculdade Pitágoras (2013). Mestre em Ciência e Engenharia Ambiental pela Universidade Federal de Alfenas (2014). Doutoranda em Farmácia (Fisiopatologia e Toxicologia) pela Faculdade de Ciências Farmacêuticas da Universidade de São Paulo (USP-SP), com período sanduíche na Universidade de Aveiro, Portugal. Servidora do quadro de técnicos-administrativos em educação da Universidade Federal de Alfenas desde 2008. **(Texto informado pelo autor)**

Identificação

Nome	Aline Andrade Godoy
Nome em citações bibliográficas	GODOY, A.A.;GODOY, ALINE A.;GODOY, A. A.;GODOY, ALINE ANDRADE

Endereço

Endereço Profissional	Universidade Federal de Alfenas, Instituto de Ciência e Tecnologia. Rodovia José Aurélio Vilela, 11999 Cidade Universitária 37715400 - Poços de Caldas, MG - Brasil Telefone: (035) 36974744 URL da Homepage: www.unifal-mg.edu.br

Formação acadêmica/titulação

2015 - 2019	Doutorado em Farmácia (Fisiopatologia e Toxicologia) (Conceito CAPES 7). Universidade de São Paulo, USP, Brasil. com **período sanduíche** em Universidade de Aveiro (Portugal) (Orientador: António José Arsénia Nogueira). Título: Assessment of the single and mixture ecotoxicity of pharmaceuticals of environmental concern using aquatic test organisms, Ano de obtenção: 2019. Orientador: Fábio Kummrow. Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES, Brasil. Palavras-chave: Mixture modelling; Synergism; Behavior locomotor; Chronic toxicity; Risk assessment. Grande área: Ciências da Saúde Grande Área: Ciências da Saúde / Área: Farmácia / Subárea: TOXICOLOGIA.
2012 - 2014	Mestrado em CIÊNCIA E ENGENHARIA AMBIENTAL (Conceito CAPES 3). Universidade Federal de Alfenas, UNIFAL/MG, Brasil. Título: Avaliação ecotoxicológica dos fármacos cloridrato de propranolol e losartana potássica, em ação individual e combinada, na macrófita Lemna minor L. (1753),Ano de Obtenção: 2014. Orientador: Paulo Augusto Zaitune Pamplin. Coorientador: Fábio Kummrow. Palavras-chave: Lemna minor; Fármacos anti-hipertensivos; Fitotoxicidade de fármacos; Toxicidade de mistura; Critérios de qualidade da água. Grande área: Engenharias
2011 - 2013	Especialização em Gestão Ambiental. (Carga Horária: 360h). Faculdades Integradas Pitágoras, PITÁGORAS, Brasil. Título: Novo perfil de aulas práticas: mais sustentáveis e menos poluentes. Proposta de substituição e/ou de redução de quantidades de reagentes e de soluções químicas utilizados nos laboratórios da Universidade Federal de Alfenas, campus Poços de Caldas-MG. Orientador: Alexandre Ferreira Machado.
2008 - 2009	

Especialização em Farmacologia Clínica. (Carga Horária: 360h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.
Título: Monitoramento da qualidade de cápsulas de anti-hipertensivos em Farmácia.
Orientador: Dra. Magali Benjamim de Araújo.

2003 - 2008 Graduação em Farmácia Industrial.
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2009 - 2009 Curso técnico/profissionalizante em Técnico em Farmácia.
Instituto Técnico Ana Nery, ANANERY, Brasil.

Formação Complementar

2018 - 2018 Habilidades sociais na Universidade: quais são e como desenvolver?. (Carga horária: 2h).
Universidade Estadual de Campinas, UNICAMP, Brasil.

2017 - 2017 Biomarkers in Ecotoxicology: user-friendly approach. (Carga horária: 40h).
Universidade de Aveiro, UA, Portugal.

2017 - 2017 Advanced course on fundamentals of animal cell culture. (Carga horária: 40h).
Universidade de Aveiro, UA, Portugal.

2016 - 2016 Análise de Dados em Ecotoxicologia. (Carga horária: 36h).
Sociedade Brasileira de Ecotoxicologia, SETAC BRASIL, Brasil.

2016 - 2016 Método Lógico para Redação Científica. (Carga horária: 8h).
Universidade Estadual de Campinas, UNICAMP, Brasil.

2016 - 2016 Tratamento de dados em Ecotoxicologia. (Carga horária: 4h).
XIV Congresso Brasileiro de Ecotoxicologia, XIV ECOTOX, Brasil.

2016 - 2016 Auditoria Interna. (Carga horária: 24h).
Universidade Estadual de Campinas, UNICAMP, Brasil.

2016 - 2016 Criteria for reporting and evaluating ecotoxicity data (CRED). (Carga horária: 15h).
Universidade Estadual de Campinas, UNICAMP, Brasil.

2015 - 2015 Princípios da Toxicologia Forense. (Carga horária: 8h).
Faculdade de Ciências Farmacêuticas - USP (SP), USP, Brasil.

2015 - 2015 Toxicidade de Misturas. (Carga horária: 21h).
Sociedade Brasileira de Ecotoxicologia/EESC/USP, SBE/EESC/USP, Brasil.

2015 - 2015 Aplicações da Toxicologia Forense. (Carga horária: 8h).
Faculdade de Ciências Farmacêuticas - USP (SP), USP, Brasil.

2015 - 2015 How to answer reviwer´s comments. (Carga horária: 1h).
Faculdade de Ciências Farmacêuticas - USP (SP), USP, Brasil.

2014 - 2015 Toxicologia Laboratorial. (Carga horária: 80h).
Programa de Educação Continuada do Portal Educação, PORTAL EDUCAÇÃO, Brasil.

2013 - 2013 Ecotoxicologia. (Carga horária: 2h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2013 - 2013 Propriedades físico-químicas na farmacocinética.... (Carga horária: 4h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2013 - 2013 Avaliação in vitro da segurança de compostos. (Carga horária: 3h).
XVIII Congresso Brasileiro de Toxicologia, CBTOX, Brasil.

2013 - 2013 Biotecnologia. (Carga horária: 100h).
Programa de Educação Continuada do Portal Educação, PORTAL EDUCAÇÃO, Brasil.

2013 - 2013 Uso de GC-MS em Toxicologia Analítica e Forense. (Carga horária: 7h).
XVIII Congresso Brasileiro de Toxicologia, CBTOX, Brasil.

2012 - 2013 Toxicologia Ambiental. (Carga horária: 40h).
Programa de Educação Continuada do Portal Educação, PORTAL EDUCAÇÃO, Brasil.

2012 - 2012 Ecotoxicologia Aquática. (Carga horária: 90h).
Universidade Federal de São Carlos, UFSCAR, Brasil.

2011 - 2011 Reagentes e resíduos químicos. (Carga horária: 2h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2011 - 2011 Segurança e mapa de risco de laboratórios. (Carga horária: 2h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2011 - 2011 Química Orgânica. (Carga horária: 90h).
Formação web, FORMAÇÃO WEB, Brasil.

2008 - 2008 Controle de qualidade no laboratório clínico. (Carga horária: 30h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2008 - 2008 Biossegurança no laboratório. (Carga horária: 30h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2008 - 2008 Organização do processo de trabalho no laboratório. (Carga horária: 40h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2008 - 2008 Ética e serviço público. (Carga horária: 20h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2006 - 2006 Biossensores: construção e aplicação em química. (Carga horária: 8h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2006 - 2006 Plantas da Amazônia com atividade antitumoral. (Carga horária: 4h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2006 - 2006 Química Medicinal: Estereoquímica de fármacos. (Carga horária: 8h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2006 - 2006 Aplicação de injeções. (Carga horária: 10h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2005 - 2005 Extensão universitária em Atenção Farmacêutica na visita domiciliar. (Carga horária: 76h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2005 - 2005 Avaliação laboratorial da função tireoidiana. (Carga horária: 8h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2005 - 2005 Toxinfecções alimentares. (Carga horária: 8h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2005 - 2005 Bioquímica da beleza. (Carga horária: 8h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2005 - 2005 Atenção Farmacêutica a pacientes geriátricos. (Carga horária: 8h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2004 - 2004 Extensão universitária em Assistência Farmacêutica. (Carga horária: 48h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2004 - 2004 Nutrição e esporte: uma abordagem bioquímica. (Carga horária: 8h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2004 - 2004 Quimioterapia e Antineoplásicos. (Carga horária: 3h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2004 - 2004 Atenção Farmacêutica em distúrbios Menores. (Carga horária: 4h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2004 - 2004 Obesidade, estilo de vida e exercício físico. (Carga horária: 3h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2004 - 2004 Fitoterapia e uso clínico de fitoestrógenos. (Carga horária: 8h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2004 - 2004 Conhecendo o paciente. (Carga horária: 4h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2004 - 2004 Controle de poluição ambiental. (Carga horária: 3h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

2003 - 2003 Bioquímica do Câncer. (Carga horária: 8h).
Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

Atuação Profissional

Universidade de Aveiro, UA, Portugal.

Vínculo institucional

2017 - 2017 Vínculo: Bolsista, Enquadramento Funcional: Estagiário de Doutorado, Carga horária: 40, Regime: Dedicação exclusiva.

Outras informações Doutorado sanduíche, com bolsa CAPES, realizado no Departamento de Biologia da Universidade de Aveiro, Portugal, no cultivo e realização de testes toxicológicos letais, sub-letais e comportamentais com embriões da espécie de peixe Danio rerio (zebrafish), para avaliação da toxicidade de fármacos de ocorrência ambiental. Treinamento em modelagem de efeitos toxicológicos de misturas, sob orientação do professor Dr. António José Arsénia Nogueira. Ainda nesse mesmo departamento, participou-se, como aluna, dos seguintes cursos avançados: 'Biomarkers in Ecotoxicology: user-friendly approach' e 'Advanced course on fundamentals of animal cell culture'.

Universidade de São Paulo, USP, Brasil.

Vínculo institucional

2015 - Atual Vínculo: Aluna de Pós Graduação, Enquadramento Funcional: Doutoranda, Regime: Dedicação exclusiva.

Atividades

08/2015 - Atual Pesquisa e desenvolvimento , Faculdade de Ciências Farmacêuticas, .
Linhas de pesquisa
Toxicologia Ambiental (Ecotoxicologia)
Toxicologia de efeitos de misturas de fármacos

Universidade Estadual de Campinas, UNICAMP, Brasil.

Vínculo institucional

2016 - Atual Vínculo: Pesquisadora, Enquadramento Funcional: Doutoranda, Carga horária: 40, Regime: Dedicação exclusiva.

Outras informações Pesquisa de doutorado realizada no Laboratório de Ecotoxicologia e Genotoxicidade da Faculdade de Tecnologia da Unicamp (campus Limeira-SP), envolvendo cultivo e testes ecotoxicológicos com organismos aquáticos (Daphnia similis, Hydra attenuata, Raphidocelis subcapitata e Lemna minor).

Universidade Federal de Alfenas, UNIFAL/MG, Brasil.

Vínculo institucional
2010 - Atual Vínculo: , Enquadramento Funcional: Técnico de laboratório generalista, Carga horária: 40
Outras informações Montagem e acompanhamento de aulas práticas e suporte e auxílio em atividades de ensino, pesquisa e extensão dos cursos de Bacharelado Interdisciplinar em Ciência e Tecnologia, Engenharia Química e Engenharia Ambiental do Instituto de Ciência e Tecnologia da Unifal-MG campus Poços de Caldas.

Vínculo institucional
2008 - 2010 Vínculo: Servidor Público, Enquadramento Funcional: Farmaceutica, Carga horária: 40
Outras informações Atuação como farmacêutica concursada, analista de controle de qualidade do Núcleo Controle de Qualidade/ Centro de Equivalência Farmacêutica (Cefar/NCQ) da Universidade Federal de Alfenas- Unifal-MG, em análises físico-químicas e microbiológicas de água, fármacos e medicamentos.

Vínculo institucional
2006 - 2006 Vínculo: Monitor, Enquadramento Funcional: Monitor da disciplina de Análise Farmacêutica, Carga horária: 4
Outras informações Monitora da disciplina de Análise/Química Farmacêutica, tendo auxiliado no preparo de soluções e de aulas práticas no laboratório, além de auxílio aos alunos no processo de aprendizagem da disciplina.

Atividades
08/2012 - Atual Pesquisa e desenvolvimento , Pró-Reitoria de Pesquisa e Pós-Graduação, .
Linhas de pesquisa
Monitoramento Ambiental

Universidade Federal de São Carlos, UFSCAR, Brasil.

Vínculo institucional
2012 - 2012 Vínculo: Estagiário, Enquadramento Funcional: Estagiário, Carga horária: 20
Outras informações Treinamento realizado no laboratório de Ecotoxicologia Aquática, no Departamento de Ecologia e Biologia Evolutiva, no cultivo de organismos-teste e em ensaios ecotoxicológicos.

Pró - Federal, PRÓ-FEDERAL, Brasil.

Vínculo institucional
2009 - 2009 Vínculo: Colaborador, Enquadramento Funcional: Professora, Carga horária: 4
Outras informações Professora do cursinho pré-vestibular, tendo ministrado aulas de Língua Portuguesa (Gramática e Interpretação de Textos).

União Química Farmacêutica Nacional, União Química, Brasil.

Vínculo institucional
2007 - 2008 Vínculo: estagiária, Enquadramento Funcional: estagiária, Carga horária: 40
Outras informações Atuação na área de Garantia da Qualidade Operacional, em acompanhamento de não-conformidades de lotes da produção de medicamentos injetáveis e embalagem de produtos, orientações de Boas Normas Produção aos funcionários da empresa e em análise e correção de histórico de produção de lotes.

Hospital Maternidade e Pronto-Socorro Santa Lúcia, SANTA LÚCIA, Brasil.

Vínculo institucional
2007 - 2007 Vínculo: estagiária, Enquadramento Funcional: estagiária, Carga horária: 40
Outras informações Estágio em fracionamento e dispensação de medicamentos no hospital.

Artpharma Farmácia de Manipulação, ARTPHARMA, Brasil.

Vínculo institucional
2006 - 2006 Vínculo: estagiária, Enquadramento Funcional: estagiária, Carga horária: 40
Outras informações Estágio em homeopatia e manipulação de cápsulas de medicamentos.

Farmácia Central do SUS de Alfenas, SUS, Brasil.

Vínculo institucional
2006 - 2006 Vínculo: estagiária, Enquadramento Funcional: estagiária, Carga horária: 40
Outras informações Estágio em dispensação de medicamentos e prestação de atenção farmacêutica em ambulatórios do SUS de Alfenas-MG.

Linhas de pesquisa

1.	Monitoramento Ambiental
2.	Toxicologia Ambiental (Ecotoxicologia)
3.	Toxicologia de efeitos de misturas de fármacos

Projetos de pesquisa

2014 - 2015	CARACTERIZAÇÃO AMBIENTAL E AVALIAÇÃO DA QUALIDADE/QUANTIDADE DA ÁGUA NA BACIA DO CÓRREGO DA ARIRANHA, POÇOS DE CALDAS - MG Descrição: A bacia do Córrego da Ariranha está localizada na região Oeste do município de Poços de Caldas (MG) e possui um uso do solo diversificado com reflorestamentos, culturas anuais, mineração, pastos, matas nativas além da Universidade Federal de Alfenas (UNIFAL), Campus avançado de Poços de Caldas (MG). Junta-se a isso, um contexto geológico e geomorfológico com relevos e declividades contrastantes além de desníveis marcantes, propensos ao transporte de sedimentos e/ou cargas difusas. O exutório da bacia situa-se na represa Bortolan, importante manancial que movimenta as turbinas da Central Geradora Hidrelétricas (CGH de Bortolan) e frequentada por praticantes de esportes náuticos e turistas em geral. Outro fato importante, é que esta bacia apresenta uma facilidade muito grande em relação à coleta de dados pluviométricos e fluviométricos num grande intervalo de tempo, bem como uma boa caracterização climática, geológica e pedológica, sendo sempre de fácil acesso os pontos de coleta. Assim, o presente trabalho visa à caracterização ambiental da bacia hidrográfica, seguido dos aspectos de qualidade/quantidade de água de forma a considerar os aspectos qualitativos e quantitativos do Córrego da Ariranha em escoar suas águas a jusante para o reservatório Bortolan.. Situação: Concluído; Natureza: Pesquisa. Alunos envolvidos: Graduação: (1) . Integrantes: Aline Andrade Godoy - Integrante / Diego de Souza Sardinha - Coordenador / Fabiana Gonçalves Carvalho - Integrante.
2013 - 2014	Dinâmica populacional de Lemna minor L., 1753 (Araceae, Angiospermae) e sua relação com metais pesados Situação: Concluído; Natureza: Pesquisa. Alunos envolvidos: Graduação: (1) / Mestrado acadêmico: (1) . Integrantes: Aline Andrade Godoy - Integrante / Pamela Almeida Oliveira - Integrante / Paulo Augusto Zaitune Pamplin - Coordenador.
2012 - 2014	Avaliação ecotoxicológica dos fármacos cloridrato de propranolol e losartana potássica, em ação individual e combinada, na macrófita Lemna minor L. (1753) Descrição: O projeto visa, como objetivo final, avaliar os efeitos da toxicidade, em ação individual e combinada, dos fármacos cloridrato de propranolol e losartana potássica, os quais possuem mecanismos de ação distintos e são princípios-ativos de medicamentos comumente prescritos na terapêutica da hipertensão arterial. O organismo-alvo é a macrófita Lemna minor, comumente conhecida como lentilha- d´água, indicada pelo Guideline da OECD (2006), cujos ensaios de inibição do crescimento com os fármacos acima citados permitirão avaliar possíveis interações toxicológicas dos mesmos para a macrófita.. Situação: Concluído; Natureza: Pesquisa. Alunos envolvidos: Mestrado acadêmico: (1) . Integrantes: Aline Andrade Godoy - Coordenador / Paulo AugustoZaitune Pamplin - Integrante / Fábio Kummrow - Integrante.
2012 - 2013	Avaliação do crescimento populacional de Lemna minor L., 1753 (Araceae, angiospermae) em diferentes meios de cultivo Descrição: As macrófitas aquáticas desempenham um importante papel no funcionamento e na dinâmica de ecossistemas aquáticos, são as principais produtoras de biomassa, sendo fundamentais na ciclagem e estocagem de nutrientes. Lemna minor é uma macrófita aquática flutuante de pequeno porte, popularmente conhecida como lentilha d?água, e que apresentam crescimento populacional acelerado. A reprodução da L. minor se dá por brotamento, no qual, uma fronde (= folha) dá origem a outras. Esta macrófita aquática tem sido utilizada em testes ecotoxicológicos e como tratamento alternativo de águas residuais. O presente projeto tem como objetivo avaliar o crescimento populacional de Lemna minor em diferentes condições de cultivo. Para isto, exemplares de L. minor foram coletados no laboratório de limnologia do departamento de ecologia e biologia evolutiva.. Situação: Concluído; Natureza: Pesquisa. Alunos envolvidos: Graduação: (1) / Mestrado acadêmico: (1) . Integrantes: Aline Andrade Godoy - Integrante / Paulo AugustoZaitune Pamplin - Coordenador / Pamela Almeida Oliveira - Integrante / Patrícia Neves Mendes - Integrante / Daniel Juliano Pamplona Silva - Integrante. Financiador(es): Fundação de Amparo à Pesquisa do Estado de Minas Gerais - Auxílio

financeiro.

Projetos de extensão

2005 - 2005

Viva a Vida - Atenção Farmacêutica na Visita Domiciliar
Descrição: Prestação de assistência farmacêutica em visita domiciliar a pacientes da rede pública de saúde do município de Alfenas-MG, incluindo acompanhamento do tratamento farmacológico..
Situação: Concluído; Natureza: Extensão.
Alunos envolvidos: Graduação: (22) .

Integrantes: Aline Andrade Godoy - Integrante / Olinda Maria Gomes da Costa Vilas Boas - Coordenador / Olivina Maria Carneiro Vieira - Integrante / Ana Tereza Silva Gonçalves - Integrante / Marla de Souza Zampar - Integrante / Júlio César Souza Silva - Integrante / Nara Alvarenga Mendes - Integrante / Gisele Augusto Rodrigues - Integrante / Fernando Roberto de Souza - Integrante / Pammela Araújo Lacerda - Integrante / Hugo Henrique da Silva - Integrante / Bárbara Araújo Catallane Mellen Kairala - Integrante / Olímpia Maria Martins Santos - Integrante / André Luiz Machado Vianna - Integrante / Raquel Sintra Brandão - Integrante / Nádya Gislene de Melo - Integrante / Carla de Alencar Mota - Integrante / Andriny Mendes - Integrante / Ângela Nascimento de Oliveira - Integrante / Daniela Mayra de Oliveira - Integrante / Gabriela C. Fonseca - Integrante / Juliana Kushima - Integrante / Marina Bertonha Pinotti - Integrante / Alessandro Rocha Cardoso - Integrante.

2004 - 2004

Assistência Farmacêutica
Descrição: Assistência farmacêutica dispensada a mães de alunos de cheches e a alunos de escolas públicas do município de Alfenas, incluindo a ministração de palestras acerca de assuntos diversos referentes à saúde e ao uso de medicamentos, aferição de pressão arterial, glicemia e avaliação de possíveis interações medicamentosas e usos inadequados de medicamentos..
Situação: Concluído; Natureza: Extensão.

Integrantes: Aline Andrade Godoy - Integrante / Olinda Maria Gomes da Costa Vilas Boas - Coordenador.

Revisor de periódico

2016 - Atual Periódico: International Journal of Marine Science
2016 - Atual Periódico: Environmental Engineering and Management Journal (Print)
2017 - Atual Periódico: SCIENCE OF THE TOTAL ENVIRONMENT
2018 - Atual Periódico: ECOTOXICOLOGY
2018 - Atual Periódico: TOXICOLOGY REPORTS
2018 - Atual Periódico: ECOTOXICOLOGY AND ENVIRONMENTAL CONTAMINATION
2019 - Atual Periódico: ENVIRONMENTAL POLLUTION

Áreas de atuação

1. Grande área: Ciências da Saúde / Área: Farmácia / Subárea: Avaliação e analises toxicológicas.
2. Grande área: Ciências da Saúde / Área: Farmácia / Subárea: Farmácia clínica, assistência e atenção farmacêuticas.

Idiomas

Inglês Compreende Bem, Fala Bem, Lê Bem, Escreve Bem.

Produções

Produção bibliográfica

Artigos completos publicados em periódicos

Ordenar por

Ordem Cronológica ▼

1. **GODOY, ALINE ANDRADE**; CALOTO DE OLIVEIRA, ÁDRIA ; MESQUITA SILVA, JOÃO GABRIEL ; CRISTINA DE JESUS AZEVEDO, CARINA ; DOMINGUES, INÊS ; NOGUEIRA, ANTÓNIO JOSÉ ARSÉNIA ; KUMMROW, FÁBIO . Single and mixture toxicity of four pharmaceuticals of environmental concern to aquatic organisms, including a behavioral assessment. CHEMOSPHERE JCR , v. 235, p. 373-382, 2019.
2. SANCHES, ANA LETÍCIA MADEIRA ; DAAM, MICHIEL ADRIAAN ; FREITAS, EMANUELA CRISTINA ; **GODOY, ALINE ANDRADE** ; MEIRELES, GABRIELA ; ALMEIDA, ANA RITA ; DOMINGUES, INÊS ; ESPÍNDOLA, EVALDO LUIZ GAETA . Lethal and sublethal toxicity of abamectin and difenoconazole (individually and in mixture) to early life stages of zebrafish. CHEMOSPHERE JCR , v. 210, p. 531-538, 2018.
3. **GODOY, ALINE ANDRADE**; DOMINGUES, INÊS ; ARSÉNIA NOGUEIRA, ANTÓNIO JOSÉ ; KUMMROW, FÁBIO . Ecotoxicological effects, water quality standards and risk assessment for the anti-diabetic metformin. ENVIRONMENTAL POLLUTION JCR , v. 243, p. 534-542, 2018.
4. **GODOY, ALINE A.**; KUMMROW, FÁBIO . What do we know about the ecotoxicology of pharmaceutical and personal care product mixtures? A critical review. CRITICAL REVIEWS IN ENVIRONMENTAL SCIENCE AND TECHNOLOGY JCR , v. 47, p. 1453-1496, 2017.
5. **GODOY, ALINE ANDRADE**; DE CARVALHO, LUCIANO BASTOS ; KUMMROW, FÁBIO ; ZAITUNE PAMPLIN, PAULO AUGUSTO . Sodium chloride as a reference substance for the three growth endpoints used in the Lemna minor L. (1753) test. Revista Ambiente & Água, v. 12, p. 8-16, 2016.
6. **GODOY, ALINE A.**; KUMMROW, FÁBIO ; PAMPLIN, PAULO AUGUSTO Z. . Ecotoxicological evaluation of propranolol hydrochloride and losartan potassium to Lemna minor L. (1753) individually and in binary mixtures. Ecotoxicology (London) JCR , v. 24, p. 1112-1123, 2015.

   Citações: WEB OF SCIENCE 4 | SCOPUS 6
7. **GODOY, ALINE A.**; KUMMROW, FÁBIO ; PAMPLIN, PAULO AUGUSTO Z. . Occurrence, ecotoxicological effects and risk assessment of antihypertensive pharmaceutical residues in the aquatic environment - A review. Chemosphere (Oxford) JCR , v. 138, p. 281-291, 2015.

   Citações: WEB OF SCIENCE 11 | SCOPUS 24

Trabalhos completos publicados em anais de congressos

1. **GODOY, A.A.**; REIS, E. A. ; PEREIRA JUNIOR, J. A. ; ALMEIDA, M. S. ; LIMA, M. K. T. ; VILLAR, R. P. ; SOUZA, A.D.G. ; SILVEIRA, A. . Balanço Hídrico da Bacia do Ribeirão de Poços, calculado a partir da estimativa da evapotranspiração potencial, segundo métodos baseados na temperatura. In: XX Simpósio Brasileiro de Recursos Hídricos, 2013, Bento Gonçalves-RS. XX Simpósio Brasileiro de Recursos Hídricos 2013, 2013. p. 1.

Resumos publicados em anais de congressos

1. **GODOY, A. A.**; NOGUEIRA, A. J. A. ; KUMMROW, F. . Modeling of the joint toxicity of two pharmaceuticals of environmental relevance. In: III International Symposium on Pathophysiology and Toxicology - ISPAT and VIII Simpósio de Pós-Graduação em Análises Clínicas, 2018, São Paulo - SP. ISPAT and SIMPAC da FCF / USP. São Paulo - SP: FCF / USP, 2018. p. 50-50.
2. **GODOY, A. A.**; DOMINGUES, INÊS ; NOGUEIRA, A. J. A. ; KUMMROW, F. . Developmental and behavioral assessment of zebrafish embryos exposed to a mixture of pharmaceuticals at environmentally relevant concentrations. In: XV Congresso Brasileiro de Ecotoxicologia, 2018, Aracaju-SE. XV Congresso Brasileiro de Ecotoxicologia. Campinas-SP: Sociedade Brasileira de Ecotoxicologia, 2018.
3. **GODOY, A.A.**; KUMMROW, F. . A critical overview of the mixture ecotoxicity data with pharmaceuticals and personal care products reported in the last 16 years. In: SETAC Europe 27th Annual Meeting in Brussels, Belgium, 2017, Bruxelas, Bélgica. SETAC Europe 27th Annual Meeting. Brussels, Belgium, 2017. p. TH151.
4. **GODOY, A.A.**; AZEVEDO, C.C.J ; OLIVEIRA, A.C ; KUMMROW, F. . Derivation of water quality criteria and environmental risk assessment for the pharmaceutical metformin. In: 18th International Symposium on Toxicity Assessment (ISTA 18), 2017, Limeira - SP. Applied Research in Toxicology. São Paulo: SBTox, 2017. v. 2. p. 45-45.
5. **GODOY, A. A.**; CANDIDO, J.C.S. ; KUMMROW, F. . Assessment of the growth inhibition induced by three beta-blockers of environmental concern in the macrophyte Lemna minor L. (1753). In: SETAC Latin America 12th Biennial Meeting, 2017, Santos. SETAC Latin America 12th Biennial Meeting, 2017. v. 1. p. 61-61.
6. **GODOY, A. A.**; MEIRELES, G. ; NOGUEIRA, A. J. A. ; KUMMROW, F. . Effects of the acute exposure of aquatic organisms to the beta-blocker bisoprolol , including a behavioural evaluation. In: SETAC Latin America 12th Biennial Meeting, 2017, Santos. SETAC Latin America 12th Biennial Meeting, 2017. v. 1. p. 63-63.
7. **GODOY, A. A.**; CARVALHO, L. B. ; PAMPLIN, P. A. Z. ; KUMMROW, F. . Uso do modelo do Índice de Combinação para avaliar as interações na toxicidade da mistura dos fármacos propranolol e losartana para Lemna minor L.. In: XIV Congresso Brasileiro de Ecotoxicologia, 2016, Curitiba - PR. Ecotox - XIV Congresso Brasileiro de Ecotoxicologia. São Paulo-SP: Sociedade Brasileira de Ecotoxicologia, 2016. p. 1-1827.
8. **GODOY, A. A.**; KUMMROW, FÁBIO . Asssessment of the acute ecotoxicity of pharmaceuticals using Daphnia similis. In: XXI Semana Farmacêutica de Ciência e Tecnologia e LI Semana Universitária Paulista de Farmácia e Bioquímica, 2016, São Paulo. Brazilian Journal of Pharmaceutical Sciences. São Paulo: USP, 2016.
9. **GODOY, A.A.**; KUMMROW, F. ; PAMPLIN, P.A.Z. . Avaliação dos efeitos ecotoxicológicos do fármaco cloridrato de propranolol para a macrófita Lemna minor. In: XIII Congresso Brasileiro de Ecotoxicologia - ECOTOX 2014, 2014, Guarapari-ES. ECOTOX 2014, 2014. p. 456.
10. **GODOY, A.A.**; KUMMROW, F. ; PAMPLIN, P.A.Z. . Cálculos de critérios preliminares de qualidade da água para a proteção da vida aquática para o fármaco losartana potássica. In: XIII Congresso Brasileiro de Ecotoxicologia - ECOTOX 2014, 2014, Guarapari-ES. ECOTOX 2014, 2014. p. 699.
11.

GODOY, A.A.; KUMMROW, F. ; PAMPLIN, P.A.Z. . Uso do cloreto de sódio como substância de referência para avaliação da sensibilidade de Lemna minor em testes ecotoxicológicos. In: XVIII Congresso Brasileiro de Toxicologia, 2013, Porto Alegre - RS. XVIII Congresso Brasileiro de Toxicologia, 2013.

12. OLIVEIRA, P.A. ; PAMPLIN, P.A.Z. ; **GODOY, A.A.** ; MENDES, P.N ; SILVA, D.J.P. . Avaliação do crescimento populacional de Lemna minor L., 1753 (Araceae, angiospermae) em diferentes meios de cultivo. In: XX Congresso de Iniciação Científica e Tecnológica, 2013, São Carlos-SP. XX CIC UFSCar, 2013.

Apresentações de Trabalho

1. **GODOY, A. A.**; DOMINGUES, INÊS ; NOGUEIRA, A. J. A. ; KUMMROW, F. . Reproductive, developmental and behavioral assessment of aquatic organisms exposed to two pharmaceuticals of environmental concerning and their mixture acute toxicity. 2018. (Apresentação de Trabalho/Congresso).
2. **GODOY, A. A.**; AZEVEDO, C.C.J ; OLIVEIRA, A.C ; KUMMROW, F. . Comparação entre as sensibilidades da Hydra attenuata e da Daphnia similis para a avaliação da toxicidade dos fármacos metformina, bisoprolol, sotalol e ranitidina. 2016. (Apresentação de Trabalho/Congresso).
3. **GODOY, A. A.**; KUMMROW, F. ; PAMPLIN, PAULO AUGUSTO Z. . Cálculo de critérios preliminares de qualidade da água para a proteção da vida aquática para o fármaco losartana potássica. 2014. (Apresentação de Trabalho/Congresso).
4. **GODOY, A. A.**; KUMMROW, F. ; PAMPLIN, PAULO AUGUSTO Z. . Avaliação dos efeitos ecotoxicológicos do fármaco cloridrato de propranolol para a macrófita Lemna minor. 2014. (Apresentação de Trabalho/Congresso).
5. **GODOY, A.A.**; VIEIRA, O. M. C. ; VILAS BOAS, O. M. G. C. ; GONCALVES, A. T. S. ; ZAMPAR, M. S. ; SILVA, J. C. S. ; MENDES, N. A. ; RODRIGUES, G. A. ; SOUZA, F. R. ; LACERDA, P. A. ; SILVA, H. H. ; KAIRALA, B. A. C. M. ; SANTOS, O. M. M. ; VIANNA, A. L. M. ; BRANDAO, R. S. ; MELO, N. G. ; MOTA, C. A. ; CARDOSO, A. R. ; MENDES, A. ; OLIVEIRA, A. N. ; OLIVEIRA, D. M. ; FONSECA, G. C. ; KUSHIMA, J. ; PINOTTI, M. B. . Viva a Vida - Atenção Farmacêutica na visita domiciliar. 2005. (Apresentação de Trabalho/Outra).
6. **GODOY, A.A.**; VILAS BOAS, O. M. G. C. ; GONCALVES, A. T. S. ; MELO, N. G. ; MENDES, N. A. ; BRANDAO, R. S. ; SANTOS, O. M. M. ; ZAMPAR, M. S. ; MOTA, C. A. ; ALEXANDRE, M. M. ; VIEIRA, O. M. C. ; CASTRO, R. C. ; COELHO, J. G. B. ; SILVA, G. T. M. ; SILVA, M. E. D. ; RODRIGUES, J. L. ; ROCHA, P. R. ; PELOSO, A. G. F. ; RODRIGUES, S. R. S. . Atenção Mães - Orientação as mães do SARAI e creche dos Santos Reis. 2004. (Apresentação de Trabalho/Outra).
7. **GODOY, A.A.**. Reciclar para preservar. 2003. (Apresentação de Trabalho/Outra).

Bancas

Participação em bancas de trabalhos de conclusão

Trabalhos de conclusão de curso de graduação

1. **GODOY, A.A.**; SILVA, D. J. P.; PAMPLIN, P.A.Z.. Participação em banca de Pamela Almeida Oliveira.Dinâmica populacional de Lemna minor L., 1753 (Araceae, Angiospermae) e sua relação com metais pesados. 2014. Trabalho de Conclusão de Curso (Graduação em Engenharia Ambiental e Urbana) - Universidade Federal de Alfenas.
2. **GODOY, A.A.**; SOUZA, A.D.G.; SARDINHA, D.S. Participação em banca de Fabiana Gonçalves Carvalho.Avaliação de cargas difusas com base no uso do solo da Bacia do Córrego da Ariranha, Poços de Caldas-MG. 2014. Trabalho de Conclusão de Curso (Graduação em Engenharia Ambiental e Urbana) - Universidade Federal de Alfenas.
3. **GODOY, A.A.**; Brucha, G.; SOUZA, A.D.G.. Participação em banca de Danilo Mazer Pignata e outros.Avaliação da influência da ocupação urbana sobre a qualidade da água na Bacia Hidrográfica do Córrego Vai e Volta, manancial de Poços de Caldas-MG. 2014. Trabalho de Conclusão de Curso (Graduação em Interdisciplinar em Ciência e Tecnologia) - Universidade Federal de Alfenas.

Eventos

Participação em eventos, congressos, exposições e feiras

1. XV Congresso Brasileiro de Ecotoxicologia. Reproductive, developmental and behavioral assessment of aquatic organisms exposed to two pharmaceuticals of environmental concerning and their mixture acute toxicity. 2018. (Congresso).
2. Ensaios Ecotoxicológicos com organismos aquáticos atendimento à legislação ambiental. 2017. (Seminário).
3. SETAC Europe 27th annual meeting. A critical overview of the mixture ecotoxicity data with pharmaceuticals and personal care products reported in the last 16 years. 2017. (Congresso).
4. 51ª Semana Universitária Paulista de Farmácia e Bioquímica. 2016. (Outra).
5. XIV Congresso Brasileiro de Ecotoxicologia. Uso do modelo do Índice de Combinação para avaliar as interações na toxicidade da mistura dos fármacos propranolol e losartana para Lemna minor L.. 2016. (Congresso).
6. Atuação do Centro de Biologia e Química de Proteínas Quinases da Unicamp. 2015. (Outra).
7. I International Symposium on Pathophysiology and Toxicology. 2015. (Simpósio).
8. Seminário internacional: Inovações analíticas para avaliação da contaminação ambiental. 2015. (Seminário).
9. Workshop SciFinder. 2015. (Outra).
10.

XIV Simpósio de Biossegurança e Descartes de Produtos Químicos Perigosos e Organismos Geneticamente Modificados em Instituições de Ensino e Pesquisa. 2015. (Simpósio).

11. International Conference on Reproductive Biology and Toxicology. 2014. (Encontro).
12. XIII Congresso Brasileiro de Ecotoxicologia - ECOTOX 2014. Avaliação dos efeitos ecotoxicológicos do fármaco cloridrato de propranolol para a macrófita Lemna minor e Cálculo de critérios preliminares de qualidade da água para a proteção da vida aquática para o fármaco losartana potássica. 2014. (Congresso).
13. II Jornada Científica do Instituto de Ciência e Tecnologia. 2013. (Outra).
14. X Congresso Nacional de Meio Ambiente. 2013. (Congresso).
15. XVIII Congresso Brasileiro de Toxicologia - CBTOX 2013. Uso do cloreto de sódio como substância de referência para avaliação da sensibilidade de Lemna minor em testes ecotoxicológicos. 2013. (Congresso).
16. XX Simpósio Brasileiro de Recursos Hídricos.Balanço Hídrico da Bacia do Ribeirão de Poços, calculado a partir da estimativa da evapotranspiração potencial, segundo métodos baseados na temperatura. 2013. (Simpósio).
17. VIII Congresso Nacional de Meio Ambiente. 2011. (Congresso).
18. 2ª Semana da Química e 41ª Semana Farmacêutica. 2006. (Outra).
19. Seminário do PET Odontologia "Medo - Ansiedade e Fobia". 2006. (Seminário).
20. Seminário do PET Odontologia "O Câncer". 2006. (Seminário).
21. Seminário do PET Enfermagem " Considerações sobre Alzheimer". 2005. (Seminário).
22. Seminário do PET Enfermagem " Questões atuais sobre células-tronco". 2005. (Seminário).
23. Seminário PET Farmácia "Distúrbios alimentares e obesidade". 2005. (Seminário).
24. Seminário PET Farmácia " Inteligências múltiplas e síndrome de Savant". 2005. (Seminário).
25. Seminário PET Farmácia " Radicais livres". 2005. (Seminário).
26. VI Seminário Internacional de Farmacêuticos e Expofar. 2005. (Seminário).
27. XIV Congresso Paulista de Farmacêuticos. 2005. (Congresso).
28. XL Semana Farmacêutica. 2005. (Outra).
29. 39ª Semana Farmacêutica da Efoa/Ceufe. 2004. (Outra).
30. Seminário do PET Enfermagem " Ansiedade e seus transtornos". 2004. (Seminário).
31. Seminário do PET Farmácia "DNA Forense: Investigação de paternidade e criminal". 2004. (Seminário).
32. Seminário do PET Farmácia "Musicoterapia". 2004. (Seminário).
33. 38ª Semana Farmacêutica da Efoa/Ceufe. 2003. (Outra).
34. II Mostra do Conhecimento : Graduação, Pesquisa e Extensão e II Jornada Científico-Cultural do PET. 2003. (Outra).

Organização de eventos, congressos, exposições e feiras

1. SOUZA, A. D. G. ; **GODOY, A.A.** . Exposição de experimentos. 2014. (Exposição).
2. SOUZA, A. D. G. ; **GODOY, A.A.** . Conhecendo a Unifal-MG - Atividades Integradas no Campus Poços de Caldas. 2014. (Outro).
3. SOUZA, A.D.G. ; **GODOY, A.A.** . Exposição de Experimentos de Ciências Naturais. 2012. (Exposição).

Outras informações relevantes

Primeira colocada no vestibular do curso de Farmácia da Universidade Federal de Alfenas (2003/2). Primeira colocada no concurso público para a carreira de Farmacêutica na Universidade Federal de Alfenas (DOU 01/07/2008, p.45-seção3). Primeira colocada no concurso público para a carreira de técnico de laboratório generalista da Universidade Federal de Alfenas campus Poços de Caldas (DOU 11/11/2009, p.64-seção3). Primeira colocada no processo seletivo para ingresso no Programa de Pós-Graduação (Mestrado) em Ciência e Engenharia Ambiental da Universidade Federal de Alfenas. Primeira colocada no processo seletivo para ingresso no Programa de Pós-Graduação (Doutorado) em Toxicologia e Análises Toxicológicas da Universidade de São Paulo (USP).

ATTACHMENT III

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial
FICHA DO ALUNO

9143 - 4936213/1 - Aline Andrade Godoy

Email:	alineandradegodoy@usp.br
Data de Nascimento:	12/10/1984
Cédula de Identidade:	RG - MG-13.977.679 - MG
Local de Nascimento:	Estado de Minas Gerais
Nacionalidade:	Brasileira
Graduação:	Farmacêutico - Universidade Federal de Alfenas - Minas Gerais - Brasil - 2008
Mestrado:	Mestra em Ciência e Engenharia Ambiental (1) - Universidade Federal de Alfenas - Minas Gerais - Brasil - 2014

Curso:	Doutorado
Programa:	Farmácia (Fisiopatologia e Toxicologia)
Área:	Toxicologia
Data de Matrícula:	07/07/2015
Início da Contagem de Prazo:	07/07/2015
Data Limite para o Depósito:	10/07/2019
Orientador:	Prof(a). Dr(a). Fábio Kummrow - 13/12/2017 até 05/07/2019. Email: fkummrow@gmail.com
Proficiência em Línguas:	Inglês, Aprovado em 07/07/2015
Data de Aprovação no Exame de Qualificação:	Aprovado em 20/02/2017
Data do Depósito do Trabalho:	30/04/2019
Título do Trabalho:	"Avaliação da ecotoxicidade individual e das misturas de fármacos de preocupação ambiental usando organismos-teste aquáticos"
Data Máxima para Aprovação da Banca:	14/06/2019
Data de Aprovação da Banca:	08/05/2019
Data Máxima para Defesa:	21/08/2019
Data da Defesa:	05/07/2019
Resultado da Defesa:	Aprovado

Acesso à dissertação/tese: **'Banco de Teses da USP'**

A titulação é: Somente USP

Histórico de Ocorrências:	Primeira Matrícula em 07/07/2015 Titulado em 05/07/2019

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 6542 em vigor de 20/04/2013 até 28/03/2018).
Última ocorrência: Titulado em 05/07/2019

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial

FICHA DO ALUNO

9143 - 4936213/1 - Aline Andrade Godoy

Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
SEA5907-1/1	Avaliação de Risco Ambiental de Agrotóxicos: Modelos de Ecossistemas em Condições Tropicais (Escola de Engenharia de São Carlos - Universidade de São Paulo) (2)	20/08/2013	26/08/2013	30	2	100	A	N	Concluída
6045840-2/1	Tópicos Avançados em Toxicologia (Faculdade de Ciências Farmacêuticas de Ribeirão Preto - Universidade de São Paulo) (2)	10/11/2014	16/11/2014	30	2	100	A	N	Concluída
FBA5897-2/2	Nutrigenômica do Câncer	03/08/2015	09/08/2015	30	2	100	A	N	Concluída
FBC5802-3/7	Tópicos Avançados em Toxicologia I	04/08/2015	16/11/2015	15	1	100	A	N	Concluída
FBC5803-3/6	Sistemas de Garantia da Qualidade em Laboratórios de Ensaio	18/08/2015	31/08/2015	30	2	100	A	N	Concluída
FBC5814-6/1	Toxicologia Aplicada aos Alimentos	15/09/2015	05/10/2015	75	5	100	A	N	Concluída
FBC5737-3/3	Farmacocinética Avançada	01/10/2015	09/12/2015	120	8	100	A	N	Concluída
SAS5706-1/3	Toxicologia Ambiental (Faculdade de Saúde Pública - Universidade de São Paulo)	21/10/2015	02/12/2015	60	4	100	A	N	Concluída
FBA5728-4/1	Aprimoramento Pedagógico	03/11/2015	30/11/2015	60	4	100	A	N	Concluída
FBC5784-3/8	Tópicos Avançados em Toxicologia II	08/03/2016	20/06/2016	15	1	90	A	N	Concluída

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito de tese	
Disciplinas:	0	20	31
Estágios:			
Total:	0	20	31

Créditos Atribuídos à Tese: 167

Observações:

1) Curso com validade nacional, de acordo com a Portaria nº 1.324, de 08.11.2012. .

2) Disciplina(s) cursada(s) isoladamente e aceita(s) pelo(a) orientador(a) do(a) candidato(a).

Conceito a partir de 02/01/1997:

A - Excelente, com direito a crédito; B - Bom, com direito a crédito; C - Regular, com direito a crédito; R - Reprovado; T - Transferência.

Um(1) crédito equivale a 15 horas de atividade programada.

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial
FICHA DO ALUNO

9143 - 4936213/1 - Aline Andrade Godoy

Comissão julgadora da tese de doutorado:

NUSP	Nome	Vínculo	Função
3082961	Fábio Kummrow	UNIFESP(FCF)	Presidente
3072406	Camilo Dias Seabra Pereira	UNIFESP - Externo	
3228906	Maria Tereza Pepe Razzolini	FSP - USP	
9769821	Luciane Alves Maranho	UNIFESP - Externo	