



Review

Environmental side effects of pharmaceutical cocktails: What we know and what we should know



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HIGHLIGHTS

- Designing strategies for pharmaceutical mixture testing.
- Mixtures of pharmaceuticals with emerging environmental concern.
- Methods of analysis of results relevant to mixtures of pharmaceuticals.
- Gaps of knowledge and future perspectives with regard to pharmaceutical mixtures effects.

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ABSTRACT

Cocktails of pharmaceuticals are released in the environment after human consumption and due to the incomplete removal at the wastewater treatment plants. Pharmaceuticals are considered as contaminants of emerging concern and, a plethora of journal articles addressing their possible adverse effects have been published during the past 20 years. The emphasis during the early years of research within this field, was on the assessment of acute effects of pharmaceuticals applied singly, leading to results regarding their environmental risk, potentially not realistic or relevant to the actual environmental conditions. Only recently has the focus been shifted to chronic exposure and to the assessment of cocktail effects. To this end, this review provides an up-to-date compilation of 57 environmental and human toxicology studies published during 2000–2014 dealing with the adverse effects of pharmaceutical mixtures. The main challenges regarding the design of experiments and the analysis of the results regarding the effects of pharmaceutical mixtures to different biological systems are presented and discussed herein. The gaps of knowledge are critically reviewed highlighting specific future research needs and perspectives.

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Abbreviations: CA, concentration addition; IA, independent action; CI, combination index; EC, effective concentration; TU, toxic unit; MIC, minimal inhibitory concentration; MoA, mode of action; LC, lethal concentration; NOEC, no observed effect concentration; PVL, Pantone-Valentine leukocidin; NSAID, non-steroidal anti-inflammatory drug; SSRI, selective serotonin reuptake inhibitor; NF, nuclear factor; AP, activator protein.

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1. The importance of investigating the side effects of mixtures of pharmaceutical residues in the environment

The pollution of the environment with regard to the occurrence of pharmaceutical residues in mixtures is an area of increasing concern, with various open questions referring to their adverse effects towards non-target organisms [1]. The exposure of the latter to pharmaceuticals as multi-component mixtures (i.e. parent compounds, metabolites and transformation products) is a result of (i) the consumption of various medicinal products, (ii) their metabolism, which in some cases is very poor, (iii) their incomplete removal at the urban wastewater treatment plants and, (iv) their transformation either during transport in the sewage pipes, treatment or when in the natural environment. It is not an easy task to predict and fully characterize potential effects by modelling, as the effects can be altered, depending on the components of the mixtures, as well as the individual concentrations of the pharmaceutical residues (real-life scenarios are unlimited in number), but also due to a variety of natural stressors. The pharmaceutical compounds when in mixtures may interact biochemically in the same way with molecules such as, a protein receptor or an enzyme, hence activating the same specific target in an additive way. This is a mere assumption, which however cannot be neglected. Furthermore, many effects can result from mechanisms that are more complex than simply binding to a receptor. The various compounds may act through a combination of mechanisms, such as altering gene expression of cellular regulators, changing levels of intracellular concentrations of ions, or altering cellular metabolism. Each of these mechanisms can be affected at different levels depending on the mixtures involved. As a consequence, mixtures may have different effects on different tissues and organs [2] and, thus on different biological systems or organisms.

In a number of recent studies, it has been shown that pharmaceutical residues in the environment from a wide range of therapeutic groups such as, antibiotics, analgesics, anticancer drugs, contraceptives and anti-depressants have clear toxic effects [3–5]. Pharmaceuticals, unlike most other chemical compounds that enter the environment, are designed to alter physiological functions. More specifically, pharmaceuticals are designed to induce effects in humans and therefore there is a high probability of being biologically active towards wildlife species as well. The most frequently detected pharmaceutical compounds fall among others within the classes of analgesics, antibiotics, diuretics, beta-blockers, hormones, antidepressants, psychiatric, hormones, and lipid regulators. It has to be noted though that the results obtained from the various studies performed are biased by the capability of each laboratory's multi-residue method to analyze such

compounds, and therefore the information provided on the type of the classes of pharmaceuticals present in mixtures in environmental matrices most often is not fully delineated.

When taken up by organisms, they may undergo metabolic detoxification, with the resultant metabolites excreted via the urine and/or faeces in the environment. The degree of metabolism varies, with some compounds not metabolized at all and excreted as parent compounds. Before excretion by organisms, pharmaceuticals, (besides the transformation that takes place during metabolism), are also susceptible to further biological transformation by microorganisms that live symbiotically in their intestinal tracks. Furthermore, biotic and abiotic transformation continues to take place during wastewater treatment, and also after the release of the pharmaceuticals in the environment. When in the environment, abiotic processes are usually the main route of degradation of pharmaceuticals, like photolysis and hydrolysis [6].

According to the scientific literature, most research on the effects of pharmaceuticals on biological systems is conducted so far using only one pharmaceutical compound at a time [7,8]. However, as mentioned above, pharmaceuticals do not only occur as isolated, pure substances in the environment. This is now acknowledged by current risk assessment and characterization strategies [9]. Despite this fact, the possible effects of pharmaceuticals to the environment are still evaluated individually according to the EMEA guidelines for the risk assessment of human pharmaceuticals [10], and of veterinary pharmaceuticals [11]. The exposure to mixtures of other chemical compounds e.g. pesticides, polycyclic aromatic hydrocarbon, trihalomethanes, are usually regulated by the summation of the concentration of individual compounds. The risk assessment of such mixtures, for example pesticides, is usually evaluated by the application of safety factors on the results obtained from assessing the effects of individual compounds [12]. This approach however, is of limited relevance to the mixtures of pharmaceuticals, mainly due to the fact that they do not have the same mode of action (MoA).

The assessment of the toxicity of pharmaceutical mixtures is both an urgent need and a great challenge to achieve more progressive and proactive risk assessment. Variation in the mixtures and the great number of potential adverse effects to human health and the environment makes it difficult though to design uniform guidelines. Regulation is a stringent necessity, since in environmental compartments only mixtures of compounds are present, and not isolated substances. At the European Commission level, there are some efforts to establish regulations for the risk assessment of chemical mixtures emitted in the environment [9]. Guidelines on mixtures by the WHO and US EPA, are already available, but they focus on the possible adverse effects on human health only [13]. In the publication "State of the Art Report on Mixture toxicity" [14] the

Table 1
Research projects dealing with the assessment of effects of pharmaceutical mixtures.

Research project title	Main focus
CYTOTHREAT Fate and effects of cytostatic pharmaceuticals in the environment and identification of biomarkers for an improved risk assessment on environmental exposure (www.cytothreat.eu) (2011–2015)	To explore combined effects of mixtures of cytostatic pharmaceuticals, their excreted metabolites and transformation products formed in the environment and/or waste water treatment
PHARMAS <i>Ecological and human health risk assessments of antibiotics and anti-cancer drugs found in the environment</i> (www.pharmas-eu.org) (2011–2014) Developing Rapid Assessment Tools to Evaluate the Biological Effects of Complex and Biologically Active Chemical Mixture (www.epa.gov/ppcp/projects) (2005–2009)	To investigate the toxicity of realistic mixtures involving antibiotics and anti-cancer drugs in several dedicated case studies To develop applicable rapid assessment tools to determine biologically relevant effects of exposure to mixtures of endocrine disrupting chemicals (including pharmaceuticals). A three-phase approach is employed to complete this objective: • survey concentrations of relevant chemicals in the effluents of two wastewater treatment plant effluents • expose aquatic organisms to relevant concentrations of these mixtures, determine their effect on multiple reproductive endpoints, and correlate these effects with alterations in biochemical pathways • test the applicability of the biochemical pathway endpoints in two field exposure studies using wastewater effluents from the same treatment plants that were sampled in phase 1. To evaluate the potential impacts of pharmaceuticals and their multi-component mixtures to humans and the ecosystem
TOMIXX Development of novel methods for the toxicity assessment of multi-component mixtures to humans and the ecosystem (www.eng.uci.ac.cy/tomixx) (2010–2013)	To evaluate the potential impacts of pharmaceuticals and their multi-component mixtures to humans and the ecosystem

scientific and regulatory current status concerning the toxicity of chemical mixtures is discussed, highlighting the necessity of developing relevant guidelines for assessing mixture toxicity. This has led to the publication of a Communication on combination effects by the European Commission [15]. Few relevant research projects towards this direction are being or have been carried out recently, as can be seen in Table 1.

It is important to note that the number of active pharmaceutical ingredients continuously increases [16]. The importance of studying the effects of mixtures rather than of single pharmaceuticals as these may have different MoA and different direct effects against other targets, compared to the single compounds, is obvious [17]. Single pharmaceuticals can be present in concentrations that individually can provoke only low, non-significant effects [7]. Long-term exposure to low concentrations of pharmaceuticals is unlikely to elicit acute toxic effects, but this, may have subtle effects with impact on growth, fertility, sex ratios or reproductive behaviour mainly in the aquatic life [18]. The necrosis and/or apoptosis of cells, the mutation of DNA in ways that may lead to cancer and, the disruption of chemical signalling mechanisms controlling cellular development, are the most often toxicological endpoints for chemicals that the scientific community should consider [1].

The behaviour of each substance in a multi-component mixture may vary, depending on the composition, concentration and the bioassay applied to evaluate the effects. Furthermore, the duration and frequency of exposure could alter the toxicological effects of pharmaceutical mixtures. Combinations of compounds can have unfavourable joint outcomes that may be synergistic, antagonistic or additive. In specific, the three major characteristics of their effects are: (a) the toxicity of mixtures can be higher than the effects of their individual components [19–21], (b) a mixture can have considerable toxicity effects even if all components are present in low concentrations that do not induce toxic effects singly [22,23] or, (c) a mixture of chemical compounds can have lower effects, e.g. enzyme induction than the effect of the single compounds [24].

The goal of this review is to critically examine a set of questions concerning the experimental design, the analysis of results and the effects of pharmaceutical mixtures in the environment. A synthesis of the most recent studies available in the literature (i.e. 57 journal publications from 2000 to 2014) was carried out. An effort is made herein to discuss the most relevant studies focusing on the assessment of the toxicity of pharmaceutical mixtures at environmentally

relevant concentration levels. Furthermore, the in vitro and in vivo bioassays applied the most in the various studies available in the literature, are presented. The various experimental designs applied for the bioassays, the parameters they consider, and the methods and models used for the analysis and/or prediction of results, are discussed. Relevant gaps and needs in order to progress the current state-of-the-art in the field are identified.

To the authors' knowledge, studies dealing with mixtures of metabolites and transformation products only recently have been published [25–28] and the information available so far is not sufficient to draw conclusions. Therefore, the emphasis herein is given on mixtures of parent compounds of pharmaceuticals, as substantial information already exists, and no other similar analysis has been carried out so far.

2. Experimental designs for assessing the toxicological effects of pharmaceutical cocktails

Studies with mixtures of pharmaceuticals mainly refer to “component-based approaches”, in the sense that the exact components of the mixture are known; i.e. the pharmaceutical compounds present in the mixture and the ingredients of the matrix used. Table 2 provides an overview of the two main types of studies identified during the preparation of this review. Tables 3 and 4 are a compilation of the studies for which significant mixture effects were identified for pharmaceuticals at environmental concentrations; hence intrinsic risks for the environment cannot be excluded. Special attention should be paid on these compounds/mixtures therefore, based on these findings. Studies on pharmaceutical mixtures in which effects were observed for concentrations higher than the environmentally relevant ones are included in the supplementary Tables S1 and S2. The behaviour of the mixture has been coded in the Tables for mixtures that can be modelled by the CA model (CA) and by the IA model (IA). Synergistic (SG), antagonistic (AG) and other mixture effects (+) are also identified.

The experimental design usually follows the main objective of the study, which may be to understand conceptually the behaviour of the individual compounds when in mixtures, or to explain/predict the effects of the whole mixtures in the environment. Depending on the objective of the study, the composition and the concentration of the compounds in the mixture, the bioassays applied and even the handling of data, differ.

Table 2
Objectives of studies performed for the assessment of effects of pharmaceutical mixtures.

Objective of the study	Conceptual questions	Environmental relevance
Selection of pharmaceuticals	Selection usually based on physicochemical characteristics, mode-of-action	Resemble environmental situation
Number of compounds in the mixture	Usually less than 4	Usually greater than four and can reach up to 12 compounds
Concentration of pharmaceuticals	Usually non-environmentally relevant concentrations of mg L ⁻¹ or higher	Environmentally relevant concentrations of ng to µg L ⁻¹
Bioassays	Standard bioassays or bioassays selected depending on the mode-of-action of pharmaceuticals	Bioassays related to the exposure sites
Data analysis	Concentration Addition (CA) model and Independent mode of Action (IA) usually applicable	Difficult to model and predict due to interactions at low levels and number of compounds in the mixture

In order to design a toxicological study for a compound it is very useful to assess [54]:

- the exposure conditions which include the concentration of the compound to which the biological system is exposed, the duration and frequency. The exposure assessment is usually performed indirectly based on the environmental concentrations (occurrence) of the compound and not on the concentrations to which each individual is actually exposed. This practice is considered of little relevance, since the actual exposure depends on many other factors such as, the fate of chemicals, sorption effects, degradation and transformation processes [55],
- the bioavailability and toxikokinetics of the substance,
- the relevant biological system to study. In the case of organisms, the life stage and any behavioural patterns should also be taken into account,
- the routes (pathways) and target sites in the biological system,
- the dose which is the amount of the compound that ultimately reaches the sites of action taking into account possible absorption, distribution and elimination mechanisms (it is noted that the kinetics, dynamics and the metabolism of a substance influence the dose) [54],

- the hazard due to the intrinsic toxicity of the substance according to its chemical properties,
- the MoA which describes key events and processes leading to molecular and functional effects.
- the risk as the estimated or measured probability of adverse effects resulting from exposure to the chemical.

In the case of designing a study with multiple compounds, in principle, all the above mentioned should be considered. Furthermore, the interactions between substances should be taken into account. These may affect the bioavailability, uptake and toxicity of the mixture. Tables 3 and 4 (as well as Tables S1 and S2) summarize the most significant aspects currently taken into consideration when designing experimental studies with mixtures of pharmaceuticals.

2.1. Selection of type, number and concentration of compounds

The component-based approach is usually implemented based on information on the chemicals present in the mixture, in order to explain or predict possible effects in the environment. The vast majority of the studies have been up to date performed using combinations of pharmaceuticals from different groups, with quite different MoA, making it more difficult to predict their combined effects. Fig. 1 shows which pharmaceuticals are mainly included

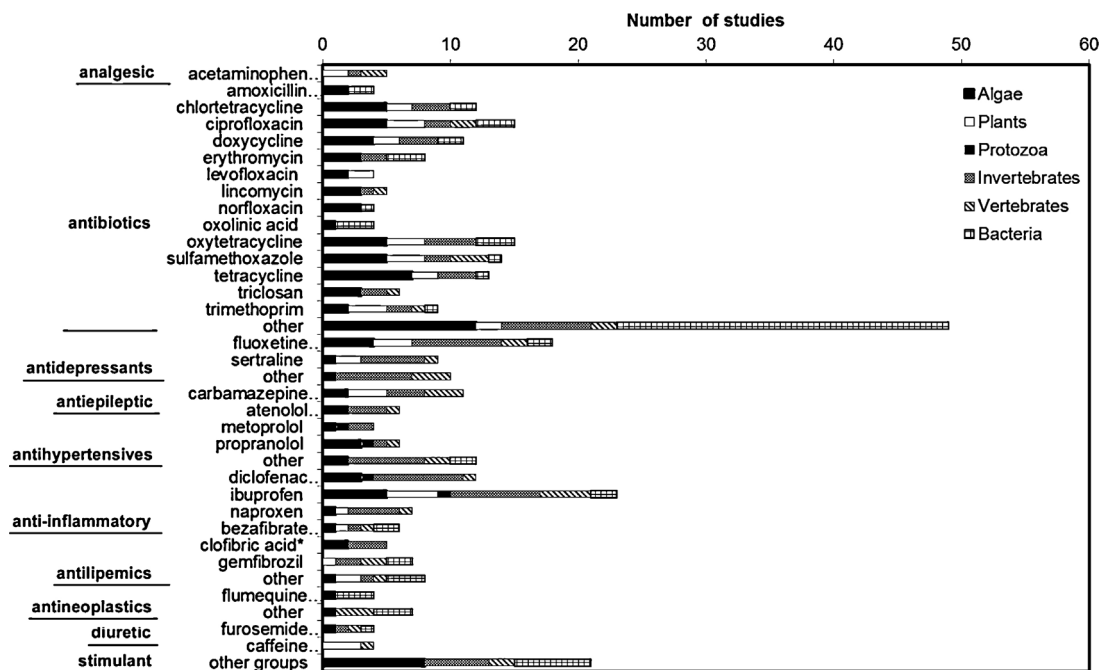


Fig. 1. Pharmaceuticals and bioassays used to assess mixture effects. Pharmaceutical groups and components present in more than three studies are presented from a total of 48 studies (published 2000–2014).

Table 3

Ecotoxicological effects of the mixture of pharmaceuticals at environmentally relevant concentrations.

Pharmaceutical group	Mixture composition[x:lowest concentration tested(μg L ⁻¹)]	Species (exposure time)	Endpoint (mixture behaviour) ^a /other information	Ref.	
Antibiotics	amoxicillin[25.7], cefazolin[4.5], cefuroxime[4.5], ceftazidime[4.5], ciprofloxacin[1.6], doxycycline[0.65], erythromycin[1.7], penicillin V[7.5], sulfamethoxazole[133.6], trimethoprim[26.7], vancomycin[0.36]	Multi-resistant <i>A. baumannii</i> Synthetic sewage inoculum (27–55 d at ×, 55–84 d at 10×)	DOC (Biodegradation performance)[=], Colony Forming Units (CFUs)[=], Chemotaxonomic parameters[+]	- Concentrations are 10-fold (×) the calculated annual average input of antibiotics in municipal influents in Germany. 100-fold (10×) also tested. - No selective advantage for the resistant bacteria was noted. 20 polyamines, ubiquinones, and menaquinones were evaluated as chemotaxonomic parameters. - Addition of <i>A. baumannii</i> resulted in an increase of ubiquinone Q9. - The concentration of menaquinone M7 was altered.	[29]
Antibiotics	chlortetracycline[0.001], ciprofloxacin[0.001], clarithromycin[0.001], norfloxacin[0.001], roxithromycin[0.001], sulfamethoxazole[0.001], sulfamethazine[0.001], triclosan[0.001], triclocarban[0.001], tylosin[0.001], tetracycline[0.001], trimethoprim[0.001]	<i>P. subcapitata</i> (72 h)	Growth inhibition[+]	0.01, 0.1, 1, 10 μg L⁻¹ of each component were also tested. M used as solvent. - Additive effects for the binary mixtures of sulfonamides and most tylosin, triclosan, triclocarban and fluoroquinones, as well as combined drugs such as trimethoprim and sulfonamides or tylosin and tetracyclines. - Potential AG with the mixtures of tylosin and triclocarban, triclosan and norfloxacin, and triclocarban and norfloxacin. - Average growth rate inhibited 23% from exposure to the 12 antibiotics even when present at 0.1 μg L⁻¹. At 1 or 10 μg L⁻¹ AG observed.	[30]
Antibiotics	amoxicillin[10], erythromycin[1] levofloxacin[10], norfloxacin[10], tetracycline[10]	<i>Anabaena</i> sp. (72 h) <i>P. subcapitata</i> (72 h)	Bioluminescence inhibition[AG] Chlorophyll[AG]	- Binary and quarternary mixtures were also assessed. - Antagonistic effects were observed at the low concentrations evaluated. - Binary mixtures had antagonistic or synergistic effects depending on the composition of the mixtures.	[31]
Antibiotics + microcystins	amoxicillin[0.1]+microcystins spiramycin [0.1]+microcystins	<i>V. fischeri</i> (15 min)	Bioluminescence inhibition[+]	- Synergistic effect of both mixtures when antibiotics were applied at their EC₅₀ values - Antagonistic effect of the mixture of amoxicillin and microcystins and synergistic effect of the mixture of spiramycin and microcystin at lower concentrations	[32]
Antibiotics (Quinolones)	cinoxacin[26.22], enoxacin[2.88], flumequine[2.64], lomefloxacin[1.99], nalidixic acid[72.46], norfloxacin[10.38], ofloxacin[1.13], oxolinic acid[0.73], pipemidic acid[394.33], piromitic acid[13.55]	<i>V. fischeri</i> (24 h)	Bioluminescence inhibition[CA]	- Concentrations are the calculated NOECs values. Mixtures with the EC₀₁s and EC₅₀s of each component were also tested. M used as solvent. - Similar MoA present.	[22]
Antibiotics (Tetracyclines)	chlortetracycline[1], doxycycline[1], tetracycline[1], oxytetracycline[1]	<i>M. sibiricum</i> (35 d) <i>L. gibba</i> (14 d, 35 d)	Shoot growth[+], Biomass[+], Root number[+], Primary root lengths[+], Number of nodes[+], Number of fronds[+], Chromophyll -a,-b and carotenoid contents[=]	- Mixtures with 10, 30, 100, 300 μg L⁻¹ of each component were also tested. - The microcosms were treated every second day. <i>L. gibba</i> showed no significant concentration response relationship for any endpoint investigated, whereas <i>M. sibiricum</i> was several orders of magnitude more sensitive.	[33]

Table 3 (Continued)

Pharmaceutical group	Mixture composition[x:lowest concentration tested($\mu\text{g L}^{-1}$)]	Species (exposure time)	Endpoint (mixture behaviour) ^a /other information	Ref.	
Antibiotics (Tetracyclines)	chlortetracycline[10], doxycycline[10], oxytetracycline[10], tetracycline[10]	<i>Chlorophyta</i> <i>Cryptophyta/Dinophyta</i> <i>Cyanobacteria</i> <i>Heterokont</i> <i>Cladocera</i> , <i>Copepoda</i> <i>Rotifera</i> <i>Small macroinvertebrates</i> <i>Large macroinvertebrates</i> (35 d)	Chlorophyll- α production[+], Oxygen concentration[+], Biomass[+], Number of taxa[+]	• 30, 100 and 300 $\mu\text{g L}^{-1}$ of each component were also tested. • Total phytoplankton abundance (sum of counts of all four groups) and species richness showed a significant treatment dependent reduction. • Sporadic significant responses were observed among the zooplankton groups, but these were inconsistent and not treatment related. • Rate of biomass production and respiration increased. Community metabolism decreased. • Oxygen concentration decreased.	[34]
Antidepressants	fluoxetine[0.0022], sertraline[0.0013], venlafaxine[0.117], bupropion[0.0074]	<i>P. promelas</i> (21 d)	Secondary sex characteristics[+], gonadosomatic index[+], hepatosomatic index[+], interstitial cell prominence[+], abundance of spermatogonia and spermatozoa[+], sexual maturity[+], proliferation of vacuolization of hepatocytes[+], vitellogenin level[+], paternal nest care[+]	• High concentrations were tested: fluoxetine[0.028], sertraline[0.022], venlafaxine[0.798], bupropion[0.466] • No additive effect were observed	[35]
SSRIs	fluoxetine[12.5], fluvoxamine[12.5], sertraline[12.5]	<i>Rotifera</i> <i>Cladocera</i> <i>Copepoda</i> <i>Microcosm studies</i> 4 d (acute) 35 d (chronic)	Abundance[+], Species richness[+]	• Mixtures with 25, 50 and 100 $\mu\text{g L}^{-1}$ of each component were also tested. • At 25 $\mu\text{g L}^{-1}$ an increase of rotifer abundance was noticed on day 4. No difference in species richness observed. • No differences in cladoceran abundance and species richness were observed. • Copepod abundance increased in dose-response manner. No difference in species richness observed.	[36]
Various	acetaminophen[0.2], diclofenac[0.2], gemfibrozil[0.2], ibuprofen[0.2], naproxen[0.2], salicylic acid[0.2], triclosan[0.2]	<i>H. azteca</i> (56 d)	Survival mating[=], body size[=], Reproduction[=], Sex ratio[+]	• DMSO used as solvent. • Multi-generation experiment. • The sex ratio changed slightly to 17% more males.	[37]
Various	acetaminophen, atorvastatin, caffeine, carbamazepine, levofloxacin, sertraline, sulfamethoxazole, trimethoprim[1] ^a	<i>M. sibiricum</i> (35 d) <i>L. gibba</i> (14 d, 35 d)	Shoot growth[+], Biomass[+], Root number[+], Primary root lengths[+], Number of nodes[+], number of fronds[+], Chromophyll -a,-b and carotenoid contents[=]	• Mixtures with 10, 100, 300 $\mu\text{g L}^{-1}$ of each component were also tested. • The microcosms were treated on Days 1, 3, 7, 10, 14, 17, 21, 24, 28, 31, 35. • Concentration-response relationships found. Both organisms displayed similar sensitivity with phytotoxic injury evident in both species. Somatic endpoints were found to be more sensitive than pigment ones.	[38]
Various	carbamazepine[0.5], diclofenac[0.36], 17 α -ethinylestradiol[0.0001], metoprolol[1.2]	<i>D. magna</i> (Multigeneration experiment F_0 – F_6)	Age at first reproduction[+], Body length of egg-carrying females and neonates[+], Number of neonates[+], Sex ratio[=]	• M used as solvent. • A significant decrease in the age at first reproduction in the F_0 and F_2 only was observed. • An increase of body length of egg-carrying females was noticed in the F_0, F_3, F_4 generations. Neonates at F_1 were larger and smaller in F_5. • An increase in the number of offspring was also observed in the F_3 generation.	[39]

Table 3 (Continued)

Pharmaceutical group	Mixture composition[x:lowest concentration tested($\mu\text{g L}^{-1}$)]	Species (exposure time)	Endpoint (mixture behaviour) ^a /other information	Ref.
Various	clofibric acid[10], fluoxetine[36], erythromycin, triclosan, trimethoprim[10], erythromycin, lincomycin, sulfamethoxazole, triclosan, trimethoprim[100]	<i>D. magna</i> (6 d, 30 d)	Immobilization[+], Morphology of adults and neonates[+], Adult length[=], Number of epiphipia[=], Neonates size[=], Number of males[+]	[40]
Various	aspirin[0.2], atenolol[0.035], diclofenac[0.38], ibuprofen[0.6], naproxen[0.185], metformin[0.735], acetaminophen[36,000]	<i>C. fluminea</i> (3 d, 14 d)	Changes in protein expression profile[+]	[41]
Various	atenolol[1], bezafibrate[0.1], carbamazepine[0.1], ciprofloxacin[0.1], cyclophosphamide[0.01], furosemide[1], hydrochlorothiazide[1], ibuprofen[0.1], lincomycin[0.1], ofloxacin[0.1], ranitidine[0.1], salbutamol[0.01], sulfamethoxazole[0.1]	<i>D. rerio</i> liver cells (24 h)	Proliferation[+], Gene expression[+], Apoptosis[=], Morphology[=], Phases of the cell-cycle[+]	[42]
Various	acetaminophen[0.01], diclofenac[0.01], gemfibrozil[0.01], ibuprofen[0.01], naproxen[0.01], salicylic acid[0.01], triclosan[0.01]	<i>P. promelas</i> (109 d)	Number of egg hatching[=], Fish weight[=], liver/gonad weight[=], sex ratio[=], Number of eggs[=], fertilized eggs[=], dead/mutant eggs[=], egg size[=], number of egg hatching[=], Larval development[+], Secondary female/male characteristics monitored[=], Testosterone, 17 β -estradiol, 11-ketotestosterone released into media [=]	[43]
Various	bezafibrate[0.072], caffeine[22.18], carbamazepine[0.033], gemfibrozil[0.059], ibuprofen[11.91], naproxen[0.217], novobiocin[0.33], oxytetracycline[0.44], sulfamethoxazole[0.099], sulfapyridine[0.046], trimethoprim[0.063]	<i>H. attenuate</i> (96 h)	Morphology (lethal)[+], Morphology[+], feeding behaviour[+], hydranth number[+], attachment (sublethal)[+], Ability to regenerate (teratogenic)[+]	[44]

Table 3 (Continued)

Pharmaceutical group	Mixture composition[x:lowest concentration tested(μg L ⁻¹)]	Species (exposure time)	Endpoint (mixture behaviour) ^a /other information	Ref.	
Various	ciprofloxacin[10], fluoxetine[10], ibuprofen[6]	<i>L. gibbosus</i> <i>Phytoplankton</i> <i>Zooplankton</i> <i>M.spicatum</i> <i>M. sibiricum</i> <i>L. gibba</i> Bacteria (35 d)	Mortality[+], Diversity decrease[+], Abundance increase[+], Plant length[=], Wet mass[=], Root number[=], Primary root lengths[=], Number of nodes[=], Abundance[=]	• 10 and 100× concentrations also tested. • Increased mortality at 10 and 100×. • Phytoplankton and zooplankton increased in abundance and decreased in diversity (number of taxa) in the 100×. Same trend in 10×. • Increased mortality on <i>Myriophyllum</i> species and <i>L. gibba</i>.	[45]
Various	17β-Estradiol[0.2], letrozole[10] or tamoxifen[10] Binary mixtures	<i>O. latipes</i> (21 d)	Number of eggs[+], Ratio of fertilized eggs[+], Number of hatching eggs[+], Time to hatch[+], Gross abnormalities, Sex ratio[+], Mortality[=], Body length[=], Body weight[=], Liver and gonads weight[=], Plasma vitellogenin levels[+]	• 50 and 250 μg L⁻¹ letrozole or tamoxifen was also tested. A was used as a solvent. • Both fecundity and fertility decreased significantly.	[46]
Various	atenolol[0.241], bezafibrate[0.057], carbamazepine[0.033], ciprofloxacin[0.026], cyclophosphamide[0.01], furosemide[0.255], hydrochlorothiazide[0.256], ibuprofen[0.092], lincomycin[0.249], ofloxacin[0.15], ranitidine[0.039], salbutamol[0.0046], sulfamethoxazole[0.046]	<i>P. subcapitata</i> (72 h)	Growth inhibition[=], Concentration of pharmaceuticals in the cells[=], DNA damage[=], Protein production[=], Concentration of chlorophyll α, b and carotenoids[+]	• A mixture of 10× was also tested. M was used as a solvent. • Quantitative changes in proteins involved in metabolism and photosynthesis. • Increase of chlorophyll b.	[47]
Various	clotrimazole[1], fluoxetine[2], propranolol[10], triclosan[1], zinc-pyriothone[0.1]	<i>Periphyton</i> (96 h)	Total pigment model, Chlorophyll a, Diadinoxanthin, Diatoxanthin, Fucoxanthin, Prasinoxanthin, Zeaxanthin, β-carotene	• Concentration is expressed as ratio based on the NOEC values ranging from 0.001 to 10 μmol L⁻¹ • DMSO for Zn-pyridoxide M for all other compounds were used as solvents for stock solutions • Lack of strong synergistic or antagonistic effects • Mixture effects more accurately predicted by the IA model • Hormetic effects were observed at concentrations <0.2 μmol L⁻¹ • Different composition of the periphyton in a repeated experiment caused a shift towards higher concentrations	[48]
Various	acetaminophen[0.5], carbamazepine[0.5], gemfibrozil[0.5], venlafaxine[0.5]	<i>D. rerio</i> (32 d)	Number of embryos[+], mortality, hatching developmental abnormalities of embryos[=], estradiol and 11-keto testosterone production[=], histopathological alterations in liver, kidney and gonad[+], apoptotic cell death	• 10 μg L⁻¹ was also tested • DMSO for carbamazepine and gemfibrozil was used as solvent • The number of viable embryos produced decreased. • The survival, hatching success or incidence of developmental abnormalities was not affected by the exposure to the mixture and only by the exposure to the single compounds, indicating non-additive mixture toxicity • Elevated oocyte atresia in the ovaries of females and histological alterations to the kidneys was observed	[49]

Table 3 (Continued)

Pharmaceutical group	Mixture composition[x:lowest concentration tested(μg L ⁻¹)]	Species (exposure time)	Endpoint (mixture behaviour) ^a /other information	Ref.	
Various	Phychiatrics: carbamazepine[0.2], diazepam[0.01], venlafaxine[0.2] Antihypersentives: atenolol[0.5], furosemide[0.3], hydrochlorodiazine[0.3], lisinopril[0.05] Hypolipemics: atorvastatin[0.05], gemfibrozil[0.1], benzafibrate[0.1] Antibiotics: ciprofloxacin[0.1], erythromycin[0.5], novobiocin[0.1], oxytetracycline[0.2], sulfamethoxazole[0.05], trimethoprim[0.05]	<i>L. stagnalis</i> (3 d)	Hemocyte count[+], viability[=], intracellular ROS and thiols[+], phagocytosis[+], immunocapacity[+], immunoefficiency[+], gene expression	• The different pharmaceutical groups were tested separately and in combination • The intracellular ROS and thiols increased and decreased significantly • Phagocytosis • Immunocapacity and immunoefficiency increased • Overexpression of TLR4 mRNA and underexpression of AIF1 and CAT mRNA was observed • The effects of the different pharmaceutical groups differed from the ones of the total mixture	[50]
Various	diuron[5], ibuprofen[5], isoproturon[5]	<i>C. gigas</i> (6 h)	Cellular mortality[=], phagocytosis[+], laccase-type phenoloxidase activity[+]	• Phagocytosis inhibition by more than 50% • An effect of 20% was observed in the activity of laccase-type phenoloxodase	[51]
Various	carbamazepine[0.178], fenofibric acid[0.070], propranolol[0.003], sulfamethoxazole[0.053] trimethoprim[0.016]	<i>D. rerio</i> (21 d)	Liver histology[+], hepatosomatic index[+], nuclear size[+], CYP1A induction[+], vitellogenin production[+]	• 10× were evaluated • Ethanol was used as the solvent for stock solutions • Male specific reactions were observed. • Increase of vitellogenin production, increased hepatic mass and increase in metabolic activity were observed	[52]

CA: The ecotoxicity of the mixture can be predicted by CA model.

SG: Values greater than predicted by CA or substances act synergistically.

AG: Substances act antagonistically.

IA: The ecotoxicity of the mixture can be predicted by IA model.

MoA: Mode of Action.

=: No effect after the mixture exposure.

+: Any other effects which are further analyzed in the column of other information.

^a x: lowest concentration tested ($\mu\text{g L}^{-1}$).

when assessing the effects of mixtures based on the current literature. Amongst the non-steroidal anti-inflammatory drugs (NSAIDs) ibuprofen, diclofenac and naproxen are quite popular, whereas from the antibiotics, tetracyclines, ciprofloxacin, sulfamethoxazole, trimethoprim, erythromycin and triclosan are used the most. Regarding β -blockers, atenolol is the most popular one, whereas propranolol and metoprolol are tested in a lower extent. Selective serotonin reuptake inhibitors (SSRIs) are also among the most studied classes of pharmaceutical mixtures; especially fluoxetine and sertraline. The lipid regulators bezafibrate and gemfibrozil, and the antiepileptic carbamazepine are also frequently studied in mixtures. Amongst others, psychiatric drugs, X-ray contrast media, and antihistamines only scarcely have been investigated in mixtures and their inclusion in future studies, is therefore suggested.

Concerning the available studies so far, it should be highlighted that it still remains unclear *how* each component in a mixture should be selected, and also *of how many* components a mixture should consist of. When the selection of the components of a mixture is justified, the main reasons provided so far in the various published studies, include prescription or consumption numbers [29,56], similar MoA [19,22], and the concurrent uptake or exposure [3,38]. Another obvious reason is the capability of each laboratory to perform the chromatographic analysis of the target compounds. The amount of components may vary from binary [53,57], ternary [21,36], quaternary [39,58], to multi-component mixtures [29,47]. It should be stressed that the criteria for using a specific mixture are not always in-depth and convincingly explicated and therefore,

most often studies focus on various mixtures with no solid justification with regard to their selection. It can be then noted that future studies should clearly explain the criteria for selecting a specific mixture.

As already mentioned, no uniform regulation is available for risk assessment for combination effects from chemical substances. Therefore a variety of strategies have been used during the last decade to study the effects of mixtures of pharmaceutical residues. The selection of the concentrations of each pharmaceutical in a mixture usually follows one of the different strategies presented below based on the single compounds dose–response concentrations or the environmentally relevant concentration levels.

One approach deals with the concentration of each pharmaceutical in the mixture, taking into account their toxicity as single compounds (Table S1). The effective concentration (EC_x), which refers to the concentration of the pharmaceutical that induces a specific response to the x percentage of the population after a defined exposure time, is used extensively; in particular the EC_1 , EC_5 and EC_{50} [19,20,22,59]. The EC_{50} is one of the four parameters that define a graded or quantal dose–response curve between the baseline and maximum response after a specific exposure time and the slope of the curve. It is usually calculated using regression analysis such as, the logit and probit regression models or other methods. The median lethal concentration (LC_{50}), which is the statistically derived concentration expected to kill 50% of organisms in a given population under a defined set of conditions [60] and the Toxic Unit (TU) which is calculated based on the EC_x have been used

Table 4

Effects of mixtures of pharmaceuticals to humans using in vitro assays/animal models at environmentally relevant concentrations.

Pharmaceutical group	Mixture composition[x:lowest concentration tested($\mu\text{g L}^{-1}$)]	Mammalian cells	Solvent	Endpoint	Exposure time	Results	Ref.
Various	atenolol[1], bezafibrate[0.1], carbamazepine[0.01], cyclophosphamide[0.1], ciprofloxacin[0.01], furosemide[1], hydrochlorothiazide[1], ibuprofen[0.1], lincomycin[0.1], ofloxacin[0.1], ranitidine[0.1], salbutamol[0.01], sulfamethoxazole[0.1]	Human embryonic cells HEK293	All in methanol	Cell proliferation Apoptosis Necrosis Activation of cell signalling Cell morphology	0, 1, 4, 24 h	• 30% decrease in cell proliferation affecting physiology and morphology. • Activation of ERK1/2 pathways. • Induced overexpression of glutathione-S-transferase P1 gene. • No evidence of apoptosis or necrosis.	[4]
Various	ethynylestradiol[1], trenbolone[25] ethynylestradiol[1], cyproterone[62] ethynylestradiol[1], tylosin[3000] cyproterone[62], salbutamol[0.5] Binary mixtures	H295R cell bioassay human adrenocortical carcinoma cells	DMSO ^a /methanol	Cell viability/cytotoxicity, Steroidogenic gene expression, Hormone production	48 h	• Decrease in CYP19 expression. • Increase of estradiol production. • Increase of progesterone production. • Decrease in CYP19 expression.	[53]
Various	acetaminophen[0.12], dextro-propoxyphene[0.0031], diclofenac[0.018], ketoprofen[0.05], naproxen[0.08], erythromycin[0.012], sulfamethoxazole[0.006], trimethoprim[0.008], atenolol [0.28], metoprolol[0.043], hydrochlorothiazide[0.05], furosemide[0.2], citalopram[0.015], oxazepam[0.028] salbutamol[0.0013], ranitidine[0.016] Exposure to 50 and 100% of the mixture	T24 human bladder epithelial cells Jurkat T-cells		Inflammatory responses (NF- κ B activity), Immune cell responses (transcription factor AP-1)	6 h 12 h	• Reduction of the NF-κB. • Reduced AP-1 activity.	[3]
Various	atenolol[5×10^{-3}] bezafibrate[5×10^{-4}] carbamazepine [5×10^{-3}], ciprofloxacin[0.5], cyclophosphamide[0.05], furosemide[5], hydrochlorothiazide[5], ibuprofen[0.5], lincomycin[0.5], ofloxacin[0.5], ranitidine[0.5], salbutamol[0.05], sul- famethoxazole[0.0005] Each concentration at various rates 0.02, 1, 2x	Human embryonic cell HEK 293 Oestrogen-responsive OVCAR3 cells	Methanol	Absorbance, Optical density at 492 nm and 620 nm	3 h 48 h 48 h	• Antagonistic-synergistic interactions and dose dependency of effects may hamper the modelling and prediction of mixture toxicity with pharmaceuticals.	[5]

^a Dimethyl sulfoxide.

to a lesser extent. The main restriction of using the aforementioned parameters is that they do not reflect environmental conditions. In many cases (as presented in Table S1), these parameters are much higher than environmental ones.

The no observed effect concentration (NOEC), indicating the highest concentration in a test with a mean response not differing statistically significantly from the mean response of the control may also be used (Table S1). The NOEC value is usually used to

complement studies performed with different EC_x values [56,61]. The main reason for this could be the constraints of the NOEC value per se. Crane and Newman [62] stated that its use has been heavily criticized, partly because it is a poor estimator of “safe” chemical concentrations. They concluded that NOEC is not considered as a consistent global statistic value due to the following reasons: (i) the use of post-ANOVA testing to identify a concentration at which there is no effect is considered a fundamentally invalid interpretation of hypothesis testing; (ii) the generally accepted α of 0.05 used to identify statistical significance is considered a reasonable but arbitrary number and, (iii) the NOEC value is considered highly influenced by the specific experimental concentrations selected and hence no confidence limits can be calculated. Even though NOEC values have some limitations, their use is attractive as they are easy to calculate and understand, and consequently are a key element of current risk assessment procedures. Backhaus et al. [9] also indicate that the NOEC values are not true no-effect levels and the incapacity of detecting a statistically significant effect does not prove a ‘no effect’.

Regarding the study of mixtures of pharmaceuticals from an environmental aspect, the selection of concentrations based on the individual acute EC_{50} values of the components is usually of limited relevance due to the elevated values compared to the realistic concentrations present in the environment. At present, the majority of acute EC_{50} values regarding pharmaceuticals lay in the range of $mg\ L^{-1}$, whereas the environmental concentrations in the range of ng to $\mu g\ L^{-1}$ [63]. The popularity of the use of EC_{50} values relies on that they provide a direct correlation with the effect of each mixture component separately, which helps interpreting the experimental results. The use of EC_{50} values seems to be more appropriate for conceptual studies. NOEC values may be used too; however the limitations of these values should always be taken into account when interpreting data, as already explained.

The concentrations of each component in the mixture may be selected in such a way (i.e. keeping the concentrations of the rest of the compounds constant while altering the concentration of a specific compounds) in order to understand (i) the effect of each component and (ii) the effects of the potential interactions between the components towards a specific response. However only a limited number of studies have tackled this issue via fixed-ratio [59,64], isoboles [65] and fractional factorial designs [5].

Another option is to use the environmental concentrations to which organisms are exposed [3,39,41]. In Tables 3 and 4 relevant studies are presented in order to provide a better understanding of which mixtures have been shown to cause effects at environmental concentrations and hence, are of most concern. It can be seen that hazard effects may be expected even at low μg to $ng\ L^{-1}$. The disadvantage of this approach is that it is usually space- and time-specific. The great number of combinations that result from this type of studies lessens their universality and applicability.

As literature on the presence of pharmaceuticals in the environment has increased enormously during the past decade, the concern followed the same trend. This is reflected in the number of studies devoted to assessing various exposure scenarios. However, a uniform or standardized methodology regarding the number and type of components in a mixture is currently absent. More concrete actions into this direction in the near future should direct research efforts into convergence. For instance, uniform-monitoring programmes of pharmaceuticals should be applied, in order to establish knowledge regarding their occurrence, seasonality and, consequently the composition of the pharmaceutical mixtures relevant to the environment.

2.2. Bioavailability and toxicokinetics of mixtures of pharmaceuticals

Apart from the exposure, the bioavailability of the pharmaceuticals when present in mixtures, as the truly dissolved fraction of the amount of the compounds, should be investigated. As expected, the interactions between chemicals in the mixture may influence their bioavailability and as a result, the potential effects on the biological system being studied. Studies concerning the bioavailability of pharmaceuticals in mixtures are scarce, with the main focus being on how to increase bioavailability of pharmaceuticals during human treatment purposes [66].

In the field of environment and ecology, only recently some relevant studies have been published. For instance, Lahti et al. [67] were able to identify only diclofenac, ibuprofen and naproxen from a total of 15 pharmaceuticals monitored in the bile and plasma of trout exposed to wastewater effluents. Interestingly, when the concentrations in the bile and plasma were compared to the concentrations of the exposure, derived from the use of passive samplers, it was found that the latter tended to overestimate the bioavailable fraction. This finding indicates that some of the available indirect methods for assessing bioavailability are still not accurate sufficiently to be able to replace fish or molluscs, which are usually used for such type studies.

It has been shown, that the bioavailability of pharmaceuticals is influenced by various factors such as, the presence of colloids, which are particles of the order of 5 kDa and 0.7 μm [68]. Colloids may interact with organic compounds and thus affect the environmental behaviour of the latter [69,70]. Some pharmaceuticals have been found to strongly sorb onto colloids, making colloids act as a “sink” of pharmaceuticals, increasing potentially their persistence in the environment, whereas reducing their bioavailability [69].

Toxicokinetic and toxicodynamic studies, by means of investigating the metabolism, elimination and the mechanisms of toxicity in the organisms are also needed to complement the understanding of the different exposure scenarios, during the implementation of studies with pharmaceutical mixtures. For instance, a 10-day exposure of rainbow trout was necessary for establishing a steady-state uptake and elimination for a mixture of diclofenac, naproxen, ibuprofen, bisoprolol, and carbamazepine [71]. Furthermore, long-term effects might occur at much lower concentrations and follow different toxicodynamic mechanisms than those extrapolated from short-term studies [72]. As a conclusion, the toxicokinetics including absorption, distribution, metabolism and excretion and, the toxicodynamics including the interaction with the receptor sites should be investigated in greater detail in future studies.

2.3. Biochemical pathways, bioassays, endpoints and aqueous matrices applied

A classification of the organisms most frequently used to assess the effects of pharmaceutical mixtures has been performed herein based on the trophic level. In vitro and in vivo assays, acute and chronic, have been categorized under each trophic level, respectively. In general, the criteria for selecting an organism for testing include various parameters the most important of which are ecosystem-relevance, sensitivity, reproducibility, standardization, practicability, suitability, speed of response after exposure, and animal ethics. Tables 3, 4, S1 and S2 Tables S1 and S2 provide a detailed description of the experimental settings applied so far in the various relevant studies, while the main results are discussed below. A summary of the number of studies performed for each ecotoxicity bioassay and the endpoints evaluated are presented in Figs. 2 and 3, respectively. Both figures include studies published during 2000–2014.

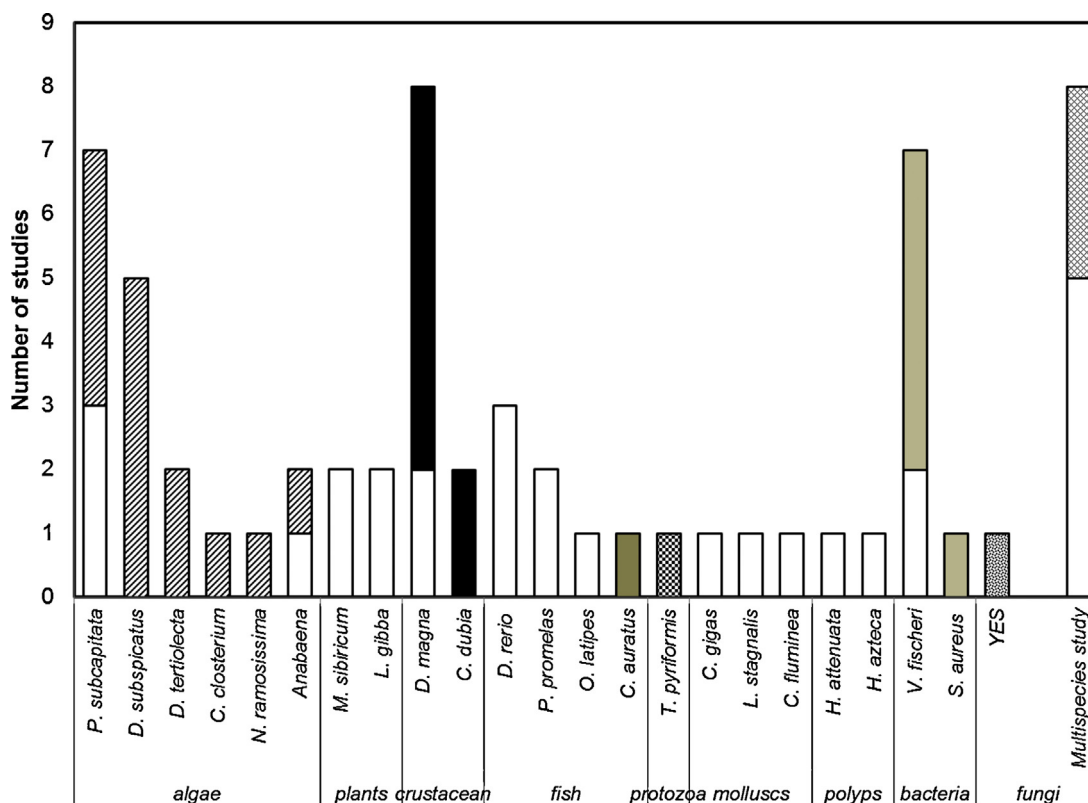


Fig. 2. Bioassays used for the ecological assessment of mixtures of pharmaceuticals. Each group has a different colour/pattern. In white colour the number of studies performed at environmentally relevant concentrations are marked. The total number of studies was 48 (published 2000–2014).

2.3.1. Producers

Among the producers, green algae are the most frequently studied. *Pseudokirchneriella subcapitata* and *Desmodesmus subspicatus* are the most popular. Usually, the 72 h growth inhibition test is applied, which is considered as a chronic test. For example, sulfa-antibiotics ($217\text{--}784\text{ }\mu\text{g L}^{-1}$) in combination with trimethoprim and pyrimethamine have been found to exhibit an additive effect on the growth inhibition to *P. subcapitata* [56]. In addition, when a greater number of antibiotics (i.e. chlortetracycline, ciprofloxacin, clarithromycin, norfloxacin, roxithromycin, sulfamethoxazole, sulfamethazine, triclosan, triclocarban, tylosin, tetracycline, trimethoprim) at concentrations from 0.001 to $10\text{ }\mu\text{g L}^{-1}$ were present, synergistic or antagonistic behaviour was observed indicating different behaviour at different concentrations [30]. In the chronic test with *D. subspicatus*, an additive effect was observed, when a binary (i.e. ibuprofen and diclofenac) and a quaternary (i.e. acetylsalicylic acid, diclofenac, ibuprofen, naproxen)

mixture of NSAIDs at concentrations from 18200 to $157500\text{ }\mu\text{g L}^{-1}$ [19,20] and a ternary mixture of β -blockers (i.e. atenolol, metoprolol, propranolol) at concentrations of $3579\text{--}1304457\text{ }\mu\text{g L}^{-1}$ [73] were studied. A binary mixture of lipid-lowering agents (clofibrate acid at $224180\text{ }\mu\text{g L}^{-1}$ and simvastatin at $22800\text{ }\mu\text{g L}^{-1}$) did not seem to have a clear additive effect when used to evaluate effects towards *D. tertiolecta*, despite having a similar MoA [57]. Worth noting is a recent study by Vannini et al. [47] with *P. subcapitata*, in which, not only the average growth was monitored, but also endpoints regarding metabolism, DNA damage and protein production. Concentrations even at ng L^{-1} for each of the components caused an effect when a mixture of various pharmaceuticals (i.e. atenolol, bezafibrate, carbamazepine, ciprofloxacin, cyclophosphamide, furosemide, hydrochlorothiazide, ibuprofen, lincomycin, ofloxacin, ranitidine, salbutamol, sulfamethoxazole) was present. It is noted that the global concentration of the mixture was also at the ng L^{-1} range. Specifically, an increase of chlorophyll-b, and

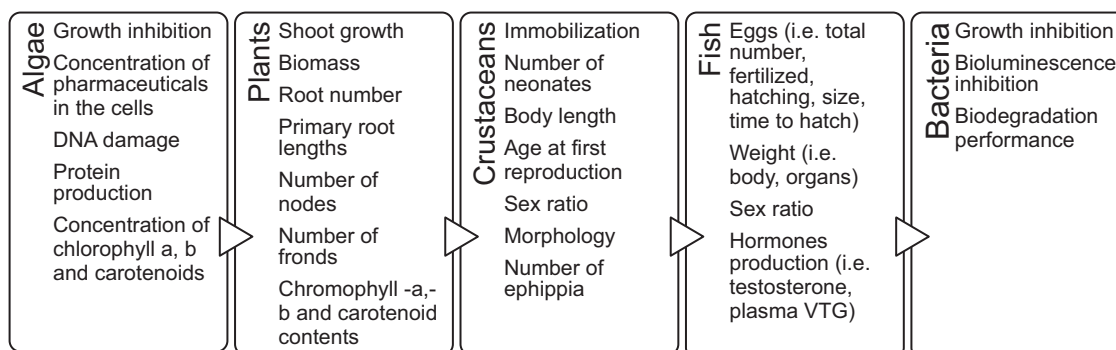


Fig. 3. Endpoints evaluated in the most popular ecotoxicity bioassays used for assessing acute and chronic effects of pharmaceutical mixtures.

quantitative changes in the occurrence of proteins involved in metabolism and photosynthesis were observed.

Among plants, aquatic angiosperms such as, the monocot *Lemna gibba* and the eudicot *Myriophyllum sibiricum* and *Myriophyllum spicatum* have been studied. The exposure usually spans for approximately 35 days, hence the tests are considered chronic. Somatic endpoints such as shoot growth, biomass, root number, primary root lengths, number of nodes and pigment endpoints such as production of chlorophyll-a and -b, and carotenoids are used. When the plants were exposed to a mixture of various pharmaceuticals (i.e. atorvastatin, acetaminophen, caffeine, carbamazepine, levofloxacin, sertraline, sulfamethoxazole, trimethoprim), these species displayed similar sensitivity at concentrations 1–300 $\mu\text{g L}^{-1}$ [38]. *L. gibba* showed signs of chlorosis and necrosis by day 7, whereas *M. sibiricum* by day 14, when the concentration of each component of the mixture was 100 $\mu\text{g L}^{-1}$. When the plants were exposed to a mixture of tetracyclines (i.e. chlortetracycline, doxycycline, tetracycline, oxytetracycline) at the same concentrations, *M. sibiricum* was found to be more sensitive than *L. gibba* [33]. This fact was mainly attributed to indirect toxic effects related to the significant reduction (99.8% at 70 cm depth) in the surface irradiance penetrating the water column that occurred due to the presence of the mixture [50]. In general, increases in solution colour result in decreases in light transmission, and the degree of light attenuation is a function of the concentration of analyte or substrate (colourant) in the medium. Oxytetracycline has been documented to modify the colouration of aqueous solutions, which turn cloudy due to the liberation of oxytetracycline base. This fact demonstrates that indirect toxic effects may occur relating to the specific niche (i.e. light penetration, direct photolytic potential of the compounds, floating or submerged in a water column organism tested, etc.).

In summary, as far as algae is concerned, it can be concluded, that mixtures of antibiotics inhibit growth to a greater extent than other groups of pharmaceuticals. Endpoints at the cellular level such as, the amount of chlorophyll-b and protein production have been found to be more sensitive since detrimental effects at environmental concentrations of ng L^{-1} were observed. In general chloroplasts have been found to be relatively sensitive to antibiotics [31,74], reinforcing the endosymbiosis theory in which bacteria and chloroplasts have common ancestors [75]. Relatively few studies have been performed using plants, as shown in Fig. 2, and effects have been observed at concentrations of several $\mu\text{g L}^{-1}$. It is worth noting that effects to producers may be caused due to changes of the characteristics of the matrix, such as water transparency and not due to the exposure to the pharmaceutical residues per se.

2.3.2. Consumers

Among crustaceans, *Daphnia magna* and *Ceriodaphnia dubia* are the most widely used species in the bioassays. The acute 24/48 h immobilization test is usually applied. When SSRIs (i.e. chlortetracycline, doxycycline, tetracycline, oxytetracycline) in binary mixtures were used to evaluate the immobilization on *D. magna* and the growth inhibition on *P. subcapitata*, similar results were found at concentrations ranging at the low mg L^{-1} range [59]. *C. dubia* was found to be more sensitive than *D. magna* for assessing SSRIs [60]. It should be noted that a strong additive effect on *D. magna* (acute test) was observed when tested with binary (ibuprofen, diclofenac [19]) and quaternary (i.e. acetylsalicylic acid, diclofenac, ibuprofen, naproxen [20]) NSAIDs, at concentrations from 34 to 54 mg L^{-1} . This strong additive effect was also observed when the reproduction test with *Daphnia* was used, at concentrations 10-fold lower, in which both reproduction and body size were affected [58]. Amongst the endpoints studied for *D. magna*, the most sensitive endpoint was sex ratio, which shifted with exposure to a mixture of antibiotics

(i.e. erythromycin, triclosan, and trimethoprim) at concentrations from 10 to 100 $\mu\text{g L}^{-1}$ [40].

The effects at environmental concentrations have been documented in a lesser extent for other aquatic species. For instance, Quinn et al. [44] have used the freshwater hydroid *Hydra attenuata* for evaluating the effects of pharmaceutical mixtures from different classes (i.e. bezafibrate, caffeine, carbamazepine, gemfibrozil, ibuprofen, naproxen, novobiocin, oxytetracycline, sulfamethoxazole, sulfapyridine, trimethoprim). Lethal, sublethal and teratogenic endpoints were selected and the organism was exposed for 96 h at concentrations quantified in effluents and their multiples in the ng and lower $\mu\text{g L}^{-1}$ range. The effects were clear for the teratogenic endpoints with a decrease in the ability of regeneration. A clear negative effect was observed at the highest concentration studied, whereas the serially diluted concentrations posed contradictory results. It was proposed that a detoxifying mechanism could be activated when higher concentrations of toxicants were present.

Fish, as second order consumer species have also been used to study the chronic effects of pharmaceutical mixtures at environmental concentrations with no clear effect trends. The freshwater *Pimephales promelas* (Fathead minnow) and the amphidromous *Oryzias latipes* (also known as Medaka) have been tested the most. Parrot and Bennie [43] used *P. promelas* to assess a mixture of seven pharmaceuticals (i.e. acetaminophen, diclofenac, gemfibrozil, ibuprofen, naproxen, salicylic acid, triclosan), in which the magnitude of deformities observed were low after an exposure of 109 d at concentrations up to 1 $\mu\text{g L}^{-1}$. Sun et al. [46] used *O. latipes* to evaluate binary mixtures of 17 β -estradiol at 0.2 $\mu\text{g L}^{-1}$ with letrozole or tamoxifen at concentrations up to 250 $\mu\text{g L}^{-1}$, and found that both fecundity and fertility decreased significantly after an exposure of 21 d. An in vitro test with *Danio rerio* liver cells was applied to assess the effects of a 13-component mixture (i.e. atenolol, bezafibrate, carbamazepine, ciprofloxacin, cyclophosphamide, furosemide, hydrochlorothiazide, ibuprofen, lincomycin, ofloxacin, ranitidine, salbutamol, sulfamethoxazole) [42]. After an exposure of 24 h and at concentrations up to 100 $\mu\text{g L}^{-1}$ different effects were observed: (a) strong growth reduction of cells, (b) transcriptional repression of primary metabolism and regulation of the cell cycle, (c) up-regulation of genes for protein signalling pathways, (d) induction of DNA-repair mechanisms, and (e) overexpression of oestrogen receptor beta and responsive protein GREB1. It was interpreted that the identified transcripts could be associated with general toxicity, while others, could be possibly linked to the effects of specific pharmaceuticals.

It can be thus concluded, that among the chronic endpoints in reproduction tests with *Daphnia*, sex ratio has been found to be the most sensitive one, affected by a mixture of antibiotics at 10 $\mu\text{g L}^{-1}$. Genetic and molecular endpoints such as changes in the protein expression levels were also found to be sensitive at the same range of concentrations. The data gathered and analyzed in this review, indicate the importance of examining changes in the cells, in particular the potential genotoxic effects of mixtures of pharmaceuticals.

2.3.3. Decomposers

The Gram-negative marine luminescent bacterium *Vibrio fischeri* has been extensively tested and is most often found not to be relatively sensitive towards acute exposure. From the available literature, both acute (15–30 min) and chronic (24 h) exposure can be found. For instance, a mixture of 10 quinonoles (i.e. cinoxacin, enoxacin, flumequine, lomefloxacin, nalidixic acid, norfloxacin, ofloxacin, oxolinic acid, pipemidic acid, piromidic acid) showed an additive effect on bioluminescence inhibition based on their NOEC or EC₁ values ranging from 0.73 to 394 $\mu\text{g L}^{-1}$ [22], while when antibiotics with a different MoA at concentrations selected based on their EC₁ values, ranging from 0.2 to 78939 $\mu\text{g L}^{-1}$ were used, the trend followed the IA model [61]. A recent study by

Rodea-Palomares et al. [65] using acute tests demonstrated how the behaviour of a mixture of lipid regulators (i.e. bezafibrate, gemfibrozil, fenofibric acid) at concentrations of their EC_{50} values ($mg\ L^{-1}$) could be shifted from antagonism at low effects levels, to synergism at higher effect levels, indicating that the concentrations of the components can affect the mixture behaviour as well. Effects of binary mixtures of antibiotics at their MIC values ($mg\ L^{-1}$) were assessed with *Staphylococcus aureus* addressing the induction of the cytotoxin Panton-Valentine leukocidin (PVL), associated with infections like for example necrotizing pneumonia and osteomyelitis [76]. The main results showed that in combination with oxacillin, subinhibitory concentrations of clindamycin or rifampicin inhibited PVL induction significantly, whereas linezolid was less inhibitory. Fusidic acid did not inhibit PVL induction in combination with oxacillin. This finding reinforces the general belief that combinations of components at their sub-inhibitory concentrations in mixtures may cause a significant effect like the one observed in xenoestrogen mixtures [77].

V. fischeri has been extensively used for the study of pharmaceutical mixtures providing a rapid screening tool particularly useful for unravelling particularly conceptual questions. One of its greatest limitations, however, has to do with the microorganism itself and its environmental relevance. Several studies tried to find correlations between *V. fischeri* and other organisms using single substances [78]. However, this has not been performed for pharmaceutical mixtures up to date.

A limited number of studies tried also to assess the effects of mixtures of pharmaceuticals to a variety of decomposers exposed simultaneously. In a study by Al-Ahmad et al. [29], bacterial inoculum from secondary treated wastewaters was evaluated using a mixture of antibiotics at the average annual input of these compounds in the municipal influents for an exposure period of 84 d. Regarding biodegradation performance and number of Colony Forming Units, the presence of an antibiotic mixture (i.e. amoxicillin, cefazolin, cefuroxime, cefazidime, ciprofloxacin, doxycycline, erythromycin, penicillin V, sulfamethoxazole, trimethoprim, vancomycin), even at microbiologically effective concentration levels, did not markedly influence the bacterial populations present in the sewage treatment plant. The chemotaxonomic parameters evaluated were found to be affected to a limited extent [79].

The effects of mixtures however, should be accounted not only from the side of the chemicals. In the environment as well as in biodegradability tests, mixtures of bacteria, fungi and other microorganisms, are also present. The effects against these organisms might be masked by the fact that there are mixed populations. That is, if a certain species is affected, its function may be overtaken by others that are normally out-competed by the originally dominating ones. Therefore, it has to be stressed that the number of a certain species as well as the number of different species present are of high importance. Furthermore, it has to be noted, that the density of bacteria taken from a natural environment such as surface water (with relatively low bacterial density), or sewage sludge (with high bacterial density) is different. Therefore, the number of species (i.e. biodiversity) as well as the number of individuals belonging to a certain species is different, and may be of relevance for toxicity and biodegradability of pharmaceutical mixtures as well. Higher diversity of species means higher diversity in degrading or detoxifying enzymes. Higher number of individuals within a species could indicate a higher amount of a specific enzyme capable to degrade a certain substance. The number of environmental bacterial species amounts to at least several 100000 to millions of species, depending on the environmental media [80], and as the bacteria present in different habitats are still not well-known, the significance of these issues cannot be accurately assessed. For example it is sometimes observed in biodegradability testing, that

chemicals are not biodegraded in a test with low bacterial density but are sometimes degraded in a test using higher bacterial density. Bacterial population studies are scarce and should be reinforced. Moreover, the need to increase understanding with regard to biochemical endpoints is apparent.

2.3.4. Microcosms studies

A more ecological approach has also been used for the study of mixtures of pharmaceutical residues, in which a variety of organisms (different taxonomic classes) living in their natural environment are included. This is quite important, since studies of this type, may provide information on the behaviour of various species when exposed to the same pharmaceutical mixtures. For example, a study to evaluate the effects of a ternary mixture of SSRIs (i.e. fluoxetine, fluvoxamine, sertaline) was performed, in which each component was tested at a maximum concentration of $100\ \mu g\ L^{-1}$ after acute and chronic exposures using *Rotifera*, *Cladocera* and *Copepoda*. The abundance and species richness were monitored. Specifically, an increase of abundance of *Rotifera* on day 4 followed by a decrease in *Copepoda* abundance and species richness on day 35 was observed [36]. Furthermore, a more complex study was performed by Richards et al. [45] in which, a greater variety of organisms was studied. *Lepomis gibbosus*, *phytoplankton*, *zooplankton*, *M. spicatum*, *M. sibiricum*, *L. gibba* and bacteria were assessed chronically to a mixture of ciprofloxacin, fluoxetine and ibuprofen at concentrations from 6 to $100\ \mu g\ L^{-1}$. An increased mortality of *L. gibbosus*, *M. spicatum*, *M. sibiricum* and *L. gibba* was observed, whereas an increase in abundance and a decrease in diversity (number of taxa) of phyto- and zoo-plankton were obtained, respectively. Similarly, a study performed by Wilson et al. [34] on *Chlorophyta*, *Cryptophyta/Dinophyta*, *Cyanobacteria*, *Heterokont*, *Cladocera*, *Copepoda*, *Rotifera*, small and large macro-invertebrates, to assess the effects of a mixture of tetracyclines (i.e. chlortetracycline, doxycycline, oxytetracycline, tetracycline) in chronic tests (in total 34 d), indicated a reduction in the total phytoplankton abundance (sum of counts of all four groups) and species richness. However, among the zooplankton groups, sporadic significant responses were observed, but these were found to be inconsistent and not treatment-related. Finally, relatively more general endpoints were affected, as the rate of biomass production and respiration increased as soon as the community metabolism and the oxygen concentration decreased. These studies therefore can provide information on also on indirect effects e.g. predator-prey relationships, as well as ecosystem functions to be included and assessed as well.

Laboratory microcosms are considered as a good compromise between field experiments, most often characterized by high environmental variability and cost, and standard laboratory tests, the results of which only rarely are representative of the real world, since the experimental conditions and the organism tested are not site-specific. Moreover, microcosms studies are very useful, as it is possible to produce many replicates at the same time, by varying the experimental conditions. Thus, it is possible to establish relationships between a toxicant and its effects on microbial communities under different conditions [81]. Microcosms studies can address a wider range of ecological issues than laboratory tests, and they provide a more realistic physical environment, taking into consideration natural conditions and stressors.

2.3.5. In vitro studies with human cell lines

Traditionally, the toxicological evaluation of environmental chemicals has largely relied on animal models that have been used to extrapolate to potentially harmful events in humans. These models have been developed to evaluate specific toxicological endpoints such as oral, dermal and ocular toxicity; immunotoxicity; genotoxicity; reproductive and developmental toxicity; and

carcinogenicity. While these animal models have provided useful information on the safety of chemicals, they are relatively expensive, low-throughput, and sometimes inconsistently predictive of human biology and pathophysiology. During the last years, several studies have begun to utilize in vitro methods and a variety of new technologies to develop in vitro signatures [82]. Furthermore, various studies have tried to address the adverse effects of mixtures of pharmaceuticals in human and mammalian cell lines in vitro (presented in Table 4). It is noted, that specifically for pharmaceutical mixtures only a handful studies have been performed using mammalian cell lines as shown in Table S2; however in environmentally irrelevant concentrations.

Cell culture can be used to screen for toxicity both by estimation of the basal functions of the cell (i.e. those processes common to all types of cells) or by tests on specialized cell functions.

For example, by using standard human cell lines such as human embryonic cell line HEK293 and oestrogen-responsive tumour cell line OVCAR3, specific, statistically significant and reproducible effects have been observed, pointing at pharmaceuticals of toxicological concern at environmentally relevant exposure levels [4,5].

Atenolol, bezafibrate, ciprofloxacin and lincomycin were found to decrease the proliferation of HEK293 cells, activate the stress-response signalling protein kinases (ERK1/2) and lead to the overexpression of glutathione-S-transferase P1 gene encoded for the detoxifying enzyme GSTP1. Morphological changes were also observed. In addition, these pharmaceuticals were identified as potential hazard for specific groups of people, such as pregnant women or infants, in the case of chronic exposure via contaminated drinking water [4].

Furthermore, evidence was found that some pharmaceuticals could interact at environmentally relevant exposure levels in a dose-dependent manner and that combined effects may represent a statistically significant component in mixture toxicity [4,5].

Various pharmaceuticals such as NSAIDs, β -blockers, immunosuppressants and steroid hormones have been shown to induce liver injury and hepatotoxicity in human liver cells [83,84]. According to Schoonen et al. [83] for example, evaluation up to dosages of 3.2×10^{-4} or even 3.2×10^{-3} M for some of the compounds for these four assays in Hep G2 cells demonstrated toxicity for 45 of the 60 (75%) reference compounds with known toxicity in these assays. With a new set of 50 compounds, among which there were estrogens, androgens, progestagens and antiestrogens, 18 (36%) were identified as toxic up to a concentration of 3.2×10^{-5} M. Gracia et al. [53] tested the effects of antibiotics, corticosteroids, anti-cancer and anti-inflammatory drugs, contraceptives and β -blockers on human adrenocortical carcinoma cell line H295R for the expression of genes, encoding enzymes that control the production of hormones. Although compounds with the same therapeutic mechanism of action may show similar gene expression profiles, their effects on hormone production may be different, perhaps due to differences in the particular effects that each compound can exert directly or indirectly into the steroidogenic pathway, independently of their genomic effects [53].

The nuclear factor (NF)- κ B and activator protein (AP)-1 play a pivotal role in inflammatory responses and have the potential to be used as indicators of inflammation as well as an environmental marker after exposure with pharmaceuticals. NSAIDs at environmentally relevant concentration levels and surface water samples were tested for potential human risk by evaluating the NF- κ B and AP-1 response using human T24 bladder epithelial cells and Jurkat T-cells [3]. Reduction in the activity of NF- κ B and AP-1 indicates the ability of pharmaceuticals to interfere with the regulation of inflammatory responses. As a result, incorrect regulation of NF- κ B and AP-1 can be linked to susceptibility to cancer, inflammatory autoimmune diseases, septic shock, viral infection, and improper immune developments.

There are few in vitro studies available, in which pharmaceutical mixtures with classical genotoxic and mutagenic endpoints (e.g. formation of micronuclei, induction of repair mechanisms, chromosome aberrations or mutations) in mammalian cells are analyzed. Studies have been carried out to assess the effect of mixtures from other environmental pollutants like polycyclic aromatic hydrocarbons, heavy metals or pesticides, but only a limited number concern combination effects [14]. For instance, a mixture of benzo[a]pyrene, benz[a]anthracene, and dibenz[a,c]anthracene was used by Lutz et al. [85] in the Ames test with *Salmonella typhimurium* TA100. They used doses of the lowest observable effect levels (LOEL), which should produce a doubling of revertants. The chemical mixtures delivered responses in good agreement with dose additivity [85].

It is then apparent, that very specific endpoints and pathways in humans can be assessed using in vitro assays. Furthermore, in vitro toxicity tests performed in human-derived cell lines may provide important biomarkers of exposure, which can be directly tested in human populations [86]. Human risk assessment from some in vitro studies may prove difficult, thus a step-wise approach implementing carefully designed tissue cell-based assays will provide a stronger and mechanistically predictive approach for human risk assessment.

2.3.6. Aqueous matrices

The pharmaceutical mixtures in their vast majority are assessed in ultra-pure water or the specific medium needed for each bioassay. In some cases solvents are used to prepare stock solutions. The most popular solvents are methanol, ethanol, dimethyl sulfoxide and acetone. The reasons for selecting a specific solvent or if the solvent causes any toxicity on its own are not always explained clearly and thoroughly. This fact is quite important, as the toxicity of a solvent may be greater for some species than others. For instance Kilroy and Gray [87] compared the toxicity of various organic solvents and found that methanol was less toxic than acetone to activated sludge. Methanol was also found to be two orders of magnitude less toxic than acetonitrile when tested on daphnids and algae [88]. It should also be mentioned that from an environmental point of view, solvents such as acetone should be reduced or even substituted as it has been shown that they cause the greatest impacts when summing the environmental impacts, the health hazards and the resource and energy needed for production [89]. Also, their use in ecotoxicity studies should be minimized, because they should not be present in the aquatic environment.

The solubility of the components in a mixture of pharmaceutical compounds may be affected by the coexistence of various compounds and/or by the environmental matrix itself [90]. A distinctive scenario was studied by Rodea-Palomares et al. [65], in the framework of which the pharmaceutical mixture was assessed using secondary treated effluents. The effects of a mixture of lipid regulators (i.e. bezafibrate, gemfibrozil, fenofibric acid) in a real matrix at concentrations based on the EC₅₀ values of the components shifted from antagonism to synergism and vice versa. In particular *V. fischeri* exposed for 30 min to the mixture showed antagonism at low concentration levels that turn into synergism at higher ones, whereas *Anabaena* CPB4337 exposed for 1 h had a strong synergism at low levels and strong antagonism at high levels. Furthermore, the wastewater stimulated growth when assessed with *V. fischeri*, whereas when investigated with *Anabaena* CPB4337, a synergistic effect with the mixture of pharmaceuticals was observed. These findings highlight the need of taking into account real matrices when evaluating such mixtures.

In summary, concerning the experimental design of the bioassays, further studies should consider different matrices such as stormwater runoffs, drinking water, groundwater, and urban wastewater from different treatments processes. Relevant abiotic processes such as the interaction with soil, sediment and humic

acids, should be evaluated as well. The effects of mixtures of pharmaceuticals should also be investigated before and after different treatment processes (e.g. photocatalysis, ozonation) as it has been shown that transformation products, may be formed and limited knowledge is available in the literature concerning the transformation in actual matrices and when in environmentally relevant concentrations. The transformation products may pose greater risks than their parent compounds [25,28,91], and this may still hold true when tested in mixtures.

3. Analysis of the results obtained from studies on the exposure to mixtures of pharmaceutical residues

As already mentioned, most real-world environmental exposures include low doses of chemical compounds and a more complex range of chemicals [92]. Different methodologies can be applied to calculate the effects of mixtures that can either be conducted using complex environmental samples or laboratory-generated mixtures. These are largely mathematical rather than biological in nature, and are described in detail below.

The approaches that have been suggested so far are based on developing a quantitative relationship between the chemical composition of a mixture and the individual toxicities of its constituents. Two predictive models are often used to describe the behaviour of pharmaceutical mixtures. For the non-interactive or independent types of joint action, the Concentration Addition (CA) of chemicals with the same toxic MoA and the Independent Action (IA) for chemicals with dissimilar toxic MoA are available. The approaches of CA and IA for ecotoxicological assessments of chemical mixtures have been applied in both retrospective as well as prospective assessments, and the concept can be expressed using a mathematical formula [23]. The CA is expressed using a mathematical equation where for a mixture of n components, the sum of the ratio of the concentration of the component which elicits $x\%$ total effect over the concentration of that substance which provokes $x\%$ effect if applied singly must be equal to 1 [93]. In contrast to CA, the concept of IA (also known as response addition, Bliss independence, or effect multiplication) assumes that the mixture components act dissimilarly. The IA concept can be mathematically formulated as the effect that the total mixture at a concentration provokes and the effects that the individual components would cause, if applied singly at the same concentration present in the mixture. One criteria of the model is that the combined mixture proportion of the effect (P_{mix}) added with the product (Π) of the index of multiplication ($i = 1$) for the proportion of effect of the individual components [$1 - P(X_i)$] must equal 1 [92].

Cedergreen et al. [94] compared the two models to determine which describes best data for 158 data sets. The data sets represented 98 different mixtures of pesticides and pharmaceuticals. Their analyses showed that approximately 20% of the mixtures were adequately predicted only by IA, 10% were adequately predicted only by CA, and both models could predict the outcome of another 20% of the experiment. Half of the experiments could not be correctly described with either of the two models. When quantifying the maximal difference between modelled synergy or antagonism and the reference model predictions at a 50% effect concentration, neither of the models performed significantly better than the other. Thus, the authors concluded that neither model could be selected over the other, on the basis of accuracy alone [94].

According to Chen et al. [95] synergistic effects can also occur in mixtures containing reactive toxicants with different mechanisms of toxicity [95]. In the same study, synergistic and antagonistic effects were observed for chemically dissimilar organic pollutants with flat-dose response curves and very steep dose response curves, respectively. The fact that synergism can occur when toxicants

have flat-dose response curves, suggests the existence of response addition or response multiplication joint actions. They explain that different organisms may respond to the stresses from mixtures of toxicants in quite different manners. Other classes have also shown that combined toxic effects are additive, whilst other dissimilar chemicals with very steep concentration–response curves generally, have shown less-than additive effects.

Isoboles as isoeffective lines through the mixture–response surface [96] can be used to interpret the toxicity of pharmaceutical mixtures [59,64,97]. The number of tests groups required for this experimental design has limited its application only to binary pharmaceutical mixtures.

Another model is the Chou-Talalay method for drug combination. It is based on the median-effect equation derived from the mass-action law principle, where the resulting combination index (CI) theorem of Chou-Talalay offers both qualitative and quantitative definition for additive ($CI = 1$), synergistic ($CI < 1$), and antagonistic effects ($CI > 1$) in drug combinations [98,99]. This method is considered as advantageous as it helps answer questions such as: (a) is there any synergism? (b) how much synergism? (c) synergism at what dose level? (d) synergism at what effect level? (e) what is the response for each drug as a result of synergism?

Of course statistical analysis is needed to validate quantitative risk assessment models and to support model assumptions. These may include testing for similar shapes of chemical dose–response curves, determining whether additivity assumptions are applicable or not for describing mixture risk, and using algorithms to form groups of similar mixtures [100].

3.1. Use of predictive models of IA and CA

Backhaus et al. [22,61] explain the effects of pharmaceutical mixtures using IA and CA. In these studies the effects of pharmaceuticals with similar [22] and dissimilar MoA [61] were examined using the chronic test with *V. fischeri*. It was found that toxicity at NOEC, EC_1 and EC_{50} values could be predicted by CA for pharmaceuticals with similar MoA, and IA for the pharmaceuticals with dissimilar MoA. The CA underestimated the EC_{50} of the mixture only by a factor of less than three. A more recent study by Brosche and Backhaus [101] using a mixture of antibiotics with different MoA, confirmed previous findings that CA might slightly underestimate the observed EC_{50} by a factor of 1.5 and might predict up to 69% of the effects. It should be noted that when the IA model was applied, the predicted higher mixture toxicity obtained in comparison to the results obtained by the CA model, was attributed to the flatness of the individual concentration–response curves. Apart from bacteria, the CA model could perform better than the IA, in tests carried out with *P. subcapitata*, *D. magna* and *C. dubia* to assess SSRIs [59,60]. In another study, the toxicity of a binary mixture of carbamazepine and clofibrinic acid could be predicted by CA or IA model using *D. magna* or *D. subspicatus*, respectively [19]. This fact stresses the different response of a mixture to different organisms. The same trend was observed when NSAIDs mixtures were tested, in which toxicity for *D. magna* was predicted by CA to be higher, whereas toxicity for *D. subspicatus* was accurately predicted by CA [19,20]. Furthermore, CA could also be used to explain the effects of β -blockers (atenolol, metoprolol, propranolol) to *D. subspicatus*, indicating a mutual specific non-target effect on algae, probably having an indirect effect on photosynthesis [73].

In some cases, the mixture toxicity can be predicted by both IA and CA. This fact however, indicates some level of weakness since the two models in principle consider very different criteria in their analysis and prediction. For instance, when β -blockers (i.e. acebutolol, atenolol, metoprolol, nadolol, oxprenolol, propranolol) were tested on *C. dubia*, depending on the mixture synthesis, immobilization could be predicted by both models [102]. Another example is

given by DeLorenzo and Fleming, [57] in which the growth inhibition on *D. tertiolecta* for two binary mixtures (i.e. fluoxetine, triclosan and clofibric acid, simvastatin) was evaluated. The former mixture behaviour could be predicted by IA, whereas the latter by both IA and CA. Moreover, Escher et al. [103,104] investigated the effects of an antiviral agent and its metabolite to *V. fischeri* showing that luminescence inhibition was lower than predicted by CA, but higher than predicted by IA (sub-additivity), and that the effects of each mixture varied in slope depending on their composition, whereas the effects on *P. subcapitata* could be predicted by IA. In order to overcome these limitations, an interesting approach was implemented to a multi-component pharmaceutical mixture with dissimilar compounds, by Escher et al. [104], in which a two-stage model improved the prediction at low effects levels.

Furthermore, an innovative approach towards the development of an effect-based water quality trigger values for baseline toxicity in which the mixture toxicity of up to 56 compounds having different MoAs (including some pharmaceuticals) has been recently developed [105]. Surprisingly, the mixture effect of these chemicals mixed at various fixed concentration ratios (equipotent mixture ratios) was adequately described by concentration addition model of mixture toxicity.

It can also be suggested that from the literature review, with the exemptions of the studies by Christensen et al. [59] and Cleuvers [19,20], effects by pharmaceutical mixtures on algae are more difficult to predict by the use of the CA model.

Finally, a particular scenario takes place when the dose–response curves of the components are flat, as IA usually predicts higher toxicity than CA. For this reason the toxicity of each one of the components of a mixture should be taken into consideration. The concentration of each component may lead to different mixture behaviour and, uncertainty increases if the mixture is complex, with unknown chemicals present in it (e.g. actual wastewater).

In general, when similar MoA is present among the compounds under examination, then the effect of the mixture tends to follow the CA model, even for organisms different than the one initially tested. However, the behaviour is differentiated when mixtures with dissimilar MoA are present. As a conclusion though, it can be said that neither of the models can be distinguished for its accuracy against the other and, the use of both give a more holistic approach. Furthermore, little evidence is provided that enables the prediction of the behaviour of a pharmaceutical mixture, especially if the mixture is complex and is constituted by compounds of dissimilar MoA. Greater deviations from the IA and CA models are expected when the MoA is not fully understood [106]. In principle, even identifying the MoA of a compound – acting at the same molecular target or causing the same biochemical effect – in the environment, is quite challenging due to the complexity and plethora of endpoints and biological systems that may be affected. The investigation at molecular level, at gene/protein (e.g. the CYP enzymes) and/or pathway levels should be strengthened to overcome these difficulties.

3.2. Use of concentration–response relationships

The behaviour of a mixture, often, cannot be predicted using the CA and IA models due to the complex interactions between molecules; hence concentration–response relationships are investigated. For example, Brain et al. [38] investigated the effects of a mixture of various pharmaceuticals (i.e. atorvastatin, acetaminophen, caffeine, carbamazepine, levofloxacin, sertraline, sulfamethoxazole, trimethoprim) using the aquatic plant *M. sibiricum*. In this study, concentration–response relationships were observed and somatic endpoints were found to be more sensitive than pigment ones. A later study by Brain et al. [33] using the same organism but exposing it to a mixture of tetracyclines showed

that the mixture significantly reduce the amount of surface irradiance penetrating the water column in a concentration-dependent way by as much as 99.8% relative to controls. Furthermore, concentration–response relationships were found for only six of the ten endpoints. Cleuvers [58] examined the effects of a mixture of diclofenac, ibuprofen, naproxen to *D. magna* after chronic exposures and, an influence on the body length was observed. The total number of offspring decreased in a dose dependent manner, and in the highest treatment group, the reduction of offspring was almost 100% compared to the control. Moreover, Dietrich et al. [39] performed a multi-generation experiment of various pharmaceuticals (i.e. carbamazepine, diclofenac, 17 α -ethinylestradiol, metoprolol) using *D. magna* and a decrease in the age of the first reproduction was observed in F_0 and F_2 generations. The body length of females and neonatal changed in the different generations, e.g. they increased in F_0 and F_3 of females. The number of offspring increased in F_3 generation and no difference in sex ratio was observed. The study concluded that mixtures might behave partly in a contrary way to single substances.

It is therefore obvious that this testing does not reveal any potential interactions among the components of the mixture. It is very case-specific, and thus cannot be used for predicting effects (as the conclusion of each study are not general and cannot be transferred to other species and conditions).

Hormesis, as the stimulation of growth by low levels of inhibitors, has been reported for mixtures of pharmaceuticals [38,48,107]. The prospect of hormesis (which has been described for a range of substances and in a range of organisms for more than a century [108]), has highly controversial implications within the areas of environmental and medical toxicology, as it questions the ways we set limit values for pollutants and toxins [109]. The implication here is not only to set limit values for single substances, but also to consider complex mixtures of chemicals, especially within the framework of the hormetic dose response. As hormesis is a central concept in toxicology and pharmacology Calabrese [109] claimed a regular inclusion in dose–response assessments, including that of mixtures. However, as Belz et al. [109] discuss, what happens in the low concentration range when all, or some, of the substances in a mixture induce biphasic concentration–response relationships? Can the mixture effects then still be predicted, especially in the low concentration range? Can the size and concentration range of hormesis in the mixture be predicted from knowledge of the concentration–response relationship of the individual compounds? And how much does it affect joint effect predictions if the hormetic response is ignored?

3.3. Synergistic and antagonistic effects

There are some studies available, in which the presence of a component augments or diminishes the effect of another component; hence the combinations of compounds might act synergistically or antagonistically, respectively. A few examples are already given in Section 2.1. For example, a mixture of NSAIDs and lipid lowering-agent metabolite (i.e. diclofenac, ibuprofen, clofibric acid) was found to cause a synergistic effect to *D. magna* after acute and chronic exposure [21]. For lipid regulators, antagonism at low effects levels that turn into synergism at higher effect levels to *V. fischeri* acute tests, was demonstrated by Rodea Palomares et al. [65]. In the same study a strong synergism at low levels and strong antagonism at high levels was observed for *Anabaena* CPB4337. Yang et al. [30] observed additive effects for the binary mixtures of sulfonamides and tylosin, triclosan, triclocarban and fluoroquinolones, as well as combined drugs such as trimethoprim and sulfonamides or tylosin and tetracyclines. However, potential antagonistic effects with mixtures of tylosin and triclocarban, triclosan and norfloxacin, or triclocarban and norfloxacin were also

observed. The average growth rate was inhibited by 23% after exposure to the 12 antibiotics, even when present at $0.1 \mu\text{g L}^{-1}$ whereas at 1 or $10 \mu\text{g L}^{-1}$ antagonistic effects were observed. In a study with *Carassius auratus* (goldfish), similar biological responses for a mixture of caffeine and sulfamethoxazole were found. The effect of caffeine/sulfamethoxazole was found to be additive with regard to induction of acetylcholinesterase (AChE) and activation of glutathione S-transferase (GST) and antagonistic with regard to 7-ethoxyresorufin O-deethylase (EROD) and superoxide dismutase (SOD) induction [24].

It can be concluded that greater effects than predicted can be distinguished when analysing mixture data results of pharmaceuticals with both, similar and dissimilar MoA. The use of IA/CA for the explanation of results cannot be considered, as catholic and specific criteria for assessing a common MoA should be defined by experts on mixture toxicity. To the authors' knowledge, the prediction of synergistic and antagonistic effects is still very difficult based on the available models. Additional methods to evaluate the combined risk of multi-chemical exposure are urgently needed, given the complex mixtures existing in the environment. Exploring the toxicological risks associated with the presence of mixtures of various chemical classes of micropollutants should be considered as a prime concern for the health of aquatic organisms and humans, since the possibility that the presence of other chemicals that may alter potentially in a currently unforeseeable manner the behaviour of pharmaceuticals, exists. In addition, one needs to consider that synergism is comparative [82], as it describes a mixture toxicity that is substantially higher than expected. Its use therefore, requires a definition of the expected response of a mixture first and foremost.

4. Future perspectives: what are the main gaps of knowledge?

4.1. Uniform criteria for designing the testing of pharmaceutical mixtures

Considering that the mixture toxicity is a very complex problem to solve and understand, it is quite a challenge to integrate this issue into regulatory frameworks. Understanding the interactions and the mechanisms involved will be the first step towards the solution of this puzzle.

One of the most concerning issues regarding the design of mixture effects testing is setting the criteria for choosing the components of the mixture. It is generally accepted that when a similar MoA is present, the mixture effect tends to behave additively. The most significant question with an environmental perspective is how the mixture behaves when substances with a dissimilar MoA are present. Studies should primarily focus on the effects of mixtures of pharmaceutical residues present in the environment, as well as on those in which synergistic effects are expected. However, it is generally accepted that currently, to assess the synergies of chemical mixtures with environmental relevance, considering also natural stressors, is a big challenge.

Full and factorial designs represent a good practice. Herein the interaction between a relatively high number of pharmaceuticals is also investigated and not only as a summation of individual components. In this context, it would be very beneficial to study interactions between pharmaceuticals and other chemicals present in the environment to design more realistic scenarios. In specific, whether significant mixture effects are to be expected by low exposure to these dissimilar chemicals is an important question. The phrase "something from nothing", implying that concurrent exposure to a variety of chemicals at very low concentrations, with no measurable effect of each component, should be considered as a prerequisite for the modern risk assessment strategies.

Of utmost importance is the need to use actual environmental aqueous matrices like for example treated urban wastewater, drinking water and freshwater, so that the potential effects of each matrix can be considered.

Up to now, the existence of transformation products of pharmaceuticals, such as biodegradation, phototransformation products and metabolites from humans and animals barely are addressed in toxicity studies. Exposure studies should however consider relevant transformation products and metabolites. The interactions between the mixtures of pharmaceuticals and other natural stressors such as, heat stress, low temperature, increased salinity, decreased oxygen concentration and starvation, and also the existence of compounds of varying chemical origin, should also be evaluated. Important also is to note that all these interactions may affect differently each species and endpoint.

The use of more environmental relevant assays such as chronic microcosms studies and natural populations studies should be enhanced, as these supplement and provide an integral approach to the classic ecotoxicity assessment. Bioassays using terrestrial organisms should also be encouraged as the effects of pharmaceutical mixtures could be observed in regions with water scarcity, which depend on the reuse of treated wastewater to fulfil their water needs.

Very important is to note also that no matter how accurate a combined effect prediction might be, this is still constrained by the fact that static exposure is taken into account, while in nature perhaps this not a valid scenario. Most probably the static scenario simulates the worst-case scenario but the opposite cannot be excluded as a possibility. Towards this end, process-based models are suggested; however currently no sufficient evidence exists to support such efforts. Altenburger et al. [110], discuss also this issue.

4.2. Calculating and understanding the effects of pharmaceutical mixtures

The use of predicting models such as CA and IA provides a comprehensive way of understanding the behaviour of pharmaceutical mixtures. There is a general consensus that CA is suitable for the prediction of the toxicity of mixtures of similarly acting substances, whereas IA assumes that all substances in a mixture exert their effects completely independently of each other. The main weakness of the CA modelling, as Kortenkamp et al. [14] stated, is that deviations can be observed for several mixtures containing pharmaceuticals for which only low effects are observed. These deviations can be partly explained to the calculation of the CA, which is based mainly on extrapolations. For pharmaceuticals with different MoA, a hybrid or two-tiered approach in which both CA and IA are used should potentially be further studied.

Regarding the form of presentation of results, two different types of comparisons can be found in the literature: *mixture effects* are evaluated or predicted, and observed *mixture effect concentrations* such as EC_{50} values are compared. The latter, provides a uniform way of presentation, allowing for comparison studies and thus, should be encouraged. As Kortenkamp notes [14] the difference ratio between predicted and observed effect concentrations (e.g. EC_{50} values) seem to be lower than a factor of 5, with the vast majority of studies showing a clearly lower ratio, confirming that the use of both models, i.e. CA and IA, give reasonable safety levels and therefore they are valuable tools for estimating mixture effects.

4.3. Future perspectives

Modelling chemical perturbations in test animals on pathways and networks that can be compared to human pathways and networks will bring human risk assessment one step closer to

tethering pathway perturbations directly to human disease instead of basing the mechanism of action on animal models; an issue that is also discussed by Burgess-Herbet and Euling [111]. Developing quantitative interspecies differences would facilitate risk assessment methodologies. In addition, key metabolic pathways and biomarkers should be highly considered. State-of-the-art technologies that have emerged from molecular biology and analytical chemistry, such as genomics, transcriptomics, proteomics, metabolomics and metabolomics, hold great potential for the characterization of new biomarkers at fundamental level. These as discussed by Dorne et al. [112], may also assist in quantifying changes in overall and specific CYP expression after exposure to mixtures and to quantify the toxicological consequences that these may have.

Another important aspect that is related to ecotoxicogenomics is that it can answer questions regarding the MoA of compounds. In their intended species, pharmaceuticals have usually been designed with a specific biochemical target in mind, and when they reach the environment, the biochemistry of the exposed species may not recognize the molecular target. In these cases, determining the MoA may be difficult. In some cases, a particular compound with a known specific MoA in the target species will show only baseline toxicity linked to membrane disruption while these effects may be specific in another species through interaction with the intended molecular target. For risk assessment of mixtures in particular, knowledge concerning MoA is vitally important. This is because the choice of which of the two main models for mixture toxicity to apply is driven by prior knowledge of MoA.

Environmentally relevant concentrations tested in human cells studies only recently have started to be applied. In vitro tests in mammalian cells and in vivo chronic tests in animals should be considered as an essential part of ecotoxicological testing. Multiple parameters such as gene expression, detoxification metabolism, mitochondrial activity and damages against the genetic material represent endpoints for toxicity testing and inference. They allow reproducible events of pharmaceuticals with diverse MoA and the detection of unknown subtherapeutic side effects. As Backhaus and Kaslrsen concludes in a very recent study [113] the absence of data on in vivo chronic toxicity for fish, is a major knowledge gap.

Therefore it is apparent that there are still important open questions and the research towards answering them can and should be intensified. This is specifically important when one considers the widespread wastewater reuse and disposed practices applied in many parts of the world, through which, compounds such as pharmaceutical residues are released in the environment on a continuous basis.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhazmat.2014.06.069>.

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