Medicines, shaken and stirred: a critical review on the ecotoxicology of pharmaceutical mixtures

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1 Abstract

Analytical monitoring surveys routinely confirm that organisms in the environment are exposed to complex multi-component pharmaceutical mixtures. We are hence tasked with the challenge to take this into consideration when investigating the ecotoxicology of pharmaceuticals. This review first provides a brief overview of the fundamental approaches for mixture toxicity assessment, which is then followed by a critical review on the empirical evidence that is currently at hand on the ecotoxicology of pharmaceutical mixtures. It is concluded that, while the classical concepts of Concentration Addition and Independent Action (Response Addition) provide a robust scientific footing, several knowledge gaps remain. This includes in particular the need for more and better empirical data on the effects of pharmaceutical mixtures on soil organisms as well as marine flora and fauna, and exploring the quantitative consequences of toxicokinetic, toxicodynamic and ecological interactions. Increased focus should be put on investigating the ecotoxicology of pharmaceutical mixtures in environmentally realistic settings.

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35 **Competing Interests:** I have no competing interests.

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2 Introduction

A multitude of pharmaceuticals from different therapeutical classes is used human and veterinary medicine in any given region. Pharmaceuticals hence never occur as isolated contaminants, but organisms in the environment are exposed to multi-component pharmaceutical mixtures. Kostich and coworkers for example just published an extensive monitoring study on the occurrence of 56 pharmaceuticals and 7 of their metabolites in the effluents of US sewage treatment plants [1], in which they detected an average of 24 pharmaceuticals (min=6, max=59). Similar results were found by Andreozzi and coworkers, who analysed pharmaceutical mixtures in European effluent streams already in 2003 and found in average 18 of the 26 pharmaceuticals that were included in the analysis [2].

Especially human pharmaceuticals predominantly enter the aquatic environment via STP effluents. As a consequence, complex pharmaceutical mixtures are also found in STP sludge. For example, Jelic at al. found 21, 24 and 30 pharmaceuticals from different classes in sludge from three different Spanish STP plants [3], and McClellan et al. found 38 pharmaceuticals and personal care products in at least one composite sample from archived US biosolids [4]. Exposure patterns in surface waters are equally complex. Vulliet and Cren-Olivé for instance found between 6 and 21 pharmaceuticals when monitoring drinking water supplies in the Rhône-Alpes region [5], and Proia and coworkers recently published a report on the occurrence of pharmaceuticals in the Llobregat river in Spain, demonstrating the occurrence of 57 pharmaceutical compounds from 14 different therapeutic groups [6].

The available evidence points to less complex exposures in soil. For example Vazquez-Roig et al analyzed soils from a Spanish wetland for the presence of 17 pharmaceuticals [7], and found in average only 2 compounds per sample (maximum 6). Whether this points to a more general pattern, driven by the specific exposure and fate pattern of pharmaceuticals, or whether monitoring studies of more polluted sites are simply not yet conducted, cannot be concluded with any certainty from the few available monitoring studies.

Exposure to pharmaceutical mixtures in the aquatic environment is often highly dynamic, e.g. [8], differs even between closely related emission sources, e.g. [9], and most pharmaceuticals

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will either be transformed by physical and chemical processes and/or are biotransformed to a broad mélange of chemicals with often unknown ecotoxicological properties, see reviews e.g. in [10][11][12]. All these factors add additional layers of chemical complexity, which in total poses a formidable challenge for the hazard and risk assessment of pharmaceuticals for environmental organisms, usually exposed over their whole life cycle.

Unfortunately, the compilation and presentation of the analytical data in published papers often does not allow to elucidate how many compounds were present concurrently in a given analytical sample. This often limits the use of reported monitoring surveys as a basis for subsequent mixture toxicity assessments, although the setup of the sampling campaign itself and the recorded primary data would allow for such an analysis. Valuable knowledge on the type and composition of pharmaceutical mixtures actually occurring in the environment is hence lost or not easily accessible.

Stakeholders from government, industry and academia ranked potential mixture effects already in 2006 as one of the major sources of uncertainty, hampering appropriate management strategies [13]. The question on how mixture effects can be adequately considered in order to increase the realism of current risk assessment approaches was also highlighted during an effort to identify "the top20" of the big questions concerning pharmaceuticals in the environment [14].

Two characteristics in particular make the joint toxic effect of a pharmaceutical mixture a major issue for hazard and risk assessment:

1. The ecotoxicity of a pharmaceutical mixture is typically higher than the effects of each individual component, and, consequently,
2. such a mixture can have a considerable ecotoxicity, even if all individual pharmaceuticals are present only in low concentrations that do not provoke significant toxic effects if acting singly on the exposed organisms.

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3 Approaches for Mixture Toxicity Analysis, Prediction and Assessment

The analysis of combination effects of pharmaceuticals has a long standing in medicine where mixture studies are implemented for studying the biological function of various cell components and describe their interactions in order to map the underlying biochemical / physiological networks. Mixture studies are also commonly used to uncover mechanisms of drug action and novel therapeutically valuable combinations, e.g. [15].

Such work uses defined, simple combinations of only a few pharmaceuticals whose concentrations are tightly controlled and whose primary mechanisms of action are often well understood in the employed test system. In contrast, the environmental assessment of pharmaceutical mixtures faces the challenge to describe and model the joint action of complex multi-component mixtures of pharmaceuticals from unrelated therapeutical classes, acting on an ecological system which comprises a multitude of different species with different life cycles and diverse, often poorly characterized, biochemical, physiological and genetic make-up. In addition, ecological interactions such as predator-prey relationships introduce a completely new level of biological complexity.

It is hence not surprising, that our understanding of the ecotoxicology of pharmaceutical mixtures is still in its infancy and that its exploration is based for the most part on simple mixture toxicity concepts that are rooted in general pharmacological principles.

A holistic, mixture-aware environmental assessment of pharmaceuticals has at least three distinct purposes:

(i) to quantify and assess the hazard and risk that a given pharmaceutical mixture poses for the environment.

(ii) to predict which pharmaceutical mixtures, in terms of composition and concentration, can be tolerated in a given environmental compartment.

(iii) to identify which compounds are the ecotoxicological drivers at a given site.

Different conceptual, experimental and statistical approaches are used for each aim, which will be briefly outlined in the following.

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3.1 Whole-mixture approaches

Whole-mixture approaches are based on the direct ecotoxicological assessment of a given pharmaceutical mixture, either in the form of extracts from environmental samples and biota, or specifically blended in a laboratory, after which the mixture is then assessed using standard ecotoxicological tools, methods and bioassays. This approach is appealing, as it does not require specific methods and approaches. However, whole-mixture studies lack generalizability, results are usually only applicable to the specifically tested mixture. Any extrapolation to a different exposure situation, especially when different compounds might be present, is almost impossible.

A major strength of whole-mixture studies is the focus of the available experimental power on the mixture itself, while component-based approaches (see below) require to put a major effort on the study of individual compounds. Whole mixture approaches are therefore frequently used in studies with complex test systems (higher organisms, micro- and mesocosms), and/or demanding endpoints, as such studies often have limited experimental capacity, or in studies that employ semi-quantitative endpoints. The ecological consequences of an exposure to selected pharmaceutical mixtures have been documented for example in a series of papers from the University of Guelph, based on controlled exposures in mesocosms [16][17][18]. In particular the by Richards and coworkers [17] points to some of the limitations of such whole-mixture studies: strong and unexpected fish mortalities were observed after exposure to a three component mixture of fluoxetine, ibuprofen and ciprofloxacin. Although the authors hypothesise that it could be either an unexpected high single substance toxicity of fluoxetine or synergistic mixture effects, the actual reasons for the observed high mixture toxicity remain to be elucidated.

Responses of complex microbial biofilms to a six-compound mixture of caffeine, cimetidine, ciprofloxacin, diphenhydramine, metformin and ranitidine were analysed by Rosi-Marshall et al. [19], showing substantial impacts on *chl a* content, primary production and biofilm respiration. Unfortunately, the use of pharmaceutical-diffusing substrata in the study, while providing a

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144 convenient means for exposing biofilms to constant concentrations *in situ*, does not allow an
145 easy back-calculation to the actual concentrations that the biofilms were exposed to.

146 Galus at al analysed the impact of two concentrations (0.5 and 10 µg/L in total) of a mixture of
147 acetaminophen, carbamazepine, gemfibrozil, and venlafaxine on zebrafish fecundity [20], with
148 both exposures having significant effects on cumulative embryo production. Madureira and
149 colleagues described the effect of two concentrations (maximum field-observed
150 concentrations, plus a 10 000 times elevated concentration) of a mixture of carbamazepine,
151 fenofibric acid, propranolol, sulfamethoxazole and trimethoprim on various biomarkers and
152 histopathological parameters in zebrafish [21]. Interestingly, both mixture concentrations,
153 although 4 orders of magnitude apart, caused very similar effects on hepatocyte nuclear
154 volumes, as a non-specific biomarker for toxic stress. Vannini et al. evaluated the toxicity of
155 mixture of 13 pharmaceuticals at a total concentration that simulated their occurrence in Italian
156 wastewaters to the green algae *Pseudokirchneriella subcapitata* [22] and found impacts on a
157 range of biochemical markers, such as chl *a*/*b* ratio, or the amounts of glutamine synthase. In
158 particular, the expression of chloroplast proteins was affected, a finding that might offer an
159 additional explanation on why algae are often the most sensitive organism to pharmaceutical
160 mixtures ([9,23,24]).

161 Quinn and colleagues provided one of the few datasets on the toxicity of pharmaceutical
162 mixtures on non-standard organisms when they analyzed the overall toxicity of an 11-
163 compound mixture of pharmaceuticals to the cnidarian, *Hydra attenuate* [25]. They observed a
164 bi-phasic response of the organism to the mixture, with effects already visible at $1/10^{th}$ of
165 typical effluent concentrations found in the effluent of the Montréal STP.

166 Almost the only study that compared individual effects of pharmaceuticals with the effects of
167 their mixture and found no elevated toxicity has been published by Dietrich and coworkers in a
168 multi-generational study with daphnids exposed to a four-compound mixture of
169 carbamazepine, diclofenac, metoprolol and ethinylestradiol [26]. They found, however specific
170 mixture effects that were not observed in the individual exposures, such as e.g. significantly
171 increased sizes of the daphnids at the time of first reproduction. A systematic exploration of the

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relationship between single substance and mixture effects were difficult, as, in the author's own words, "the influence of the pharmaceutical mixture was very inconsistent". The same mixture was also assessed by the same authors for its effects on the amphipod *Gammarus*, in which moulting behavior was impacted at the maximum concentrations of those compounds found in rivers and streams of southern Germany [27].

Additional studies that analysed mixtures at assumedly environmentally realistic concentrations of the individual pharmaceuticals include the report by Pomati and coworkers, in which the effects of a mixture of 13 human pharmaceuticals to human embryonic cells was analysed [28]. At assumed environmental exposure levels, cell growth was significantly inhibited. And, finally, Borgmann and coworkers analysed the effects of a seven compound pharmaceutical mixture on the amphipod *Hyalella* [30]. At environmentally realistic concentrations a significant change in sex ratio as well as small, non-significant reductions in survival and number of offspring were observed.

All these studies show that a mixture can be analysed as if it were a single chemical, allowing to compare ecotoxicological estimates such as EC50 values or NOECs to be estimated and compared with environmental concentrations. However, they also show that such whole mixture approaches are limited when it comes to establishing causal links to individual mixture components.

3.2 Component-based approaches

A conceptually sound link between the toxicity of the individual components and the effects of the mixture would allow the prediction of mixture toxicities, enabling if-then-else analyses, e.g. to explore the question what would happen if an STP plant is improved for the advanced treatment of micropollutants. Such a link is also the basis for any hazard- or risk-based ranking and prioritization of the components in a mixture. Two principal concepts, Concentration Addition (CA) and Independent Action (IA) that provide such a link and that are practically applicable for the assessment of pharmaceutical mixtures in the environment have been described in the literature.

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3.3 Concentration Addition

In the field of human toxicology and pharmacology, the median-effect principle has found widespread application [29], which is a special case of the more general concept of Concentration Addition (CA). The median effect principle is rooted in the law of mass action, but its application in ecotoxicology is limited by the fact that the shape of the individual concentration-response curves is usually captured within only one parameter, rendering it a quite rigid approach. It has previously demonstrated, that a more flexible approach is needed in order to capture the different shapes of the concentration-response curves with a sufficient precision and accuracy [30].

The general form of CA is independent of any specific concentration-response model and is mathematically formulated as:

$$\frac{c_{mix}}{ECx_{Mix}} = \sum_{i=1}^{n} \frac{c_i}{ECx_i} \qquad \text{(eq 1)}$$

with n denoting the number of mixture components, c_i and c_{mix} gives the individual concentrations in the mixture, respectively the mixture concentration and x is a common effect level, which is provoked by an exposure to a single substance or mixture concentration ECx_{Mix} resp. $ECx_{i.}$

The fraction c/ECx is often termed a “toxic unit”, and CA is hence also known as “Toxic Unit Summation”. It follows from eq. 1 that each component i of the mixture can be replaced totally or in part by another compound with the same toxic unit. This interchangeability is commonly interpreted as the compounds sharing a similar mode or mechanism of action.

The conceptual counterpart of CA is called “Independent Action” (IA) or “Response Addition” and assumes that the components in a mixture have completely unrelated, dissimilar mechanisms of action. By activating differing effector chains, every component of a mixture of dissimilarly acting chemicals is thought to provoke effects independent of all other agents that might also be present. The resulting combined effect can be calculated from the effects caused by the individual mixture components by following the statistical concept of independent

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random events [31]. In case the biological response increases with increasing concentrations (e.g. when mortality is analysed), IA is mathematically expressed as:

$$E(c_{Mix}) = 1 - \prod_{i=1}^{n} [1 - E(c_i)] \qquad \text{(eq. 2)}$$

where $E(c_{Mix})$ denotes the mixture effect at a concentration $c_{Mix} = \sum_{i=1}^{n} c_i$ and $E(c_i)$ describes the effects that the individual components would cause if applied singly at the concentration at which they are present in the mixture.

Both concepts share a number of important assumptions [32][33]. First and foremost, both are using information on the individual components and are hence only applicable to mixtures whose composition is known. Both concepts assume that each individual component is toxic if applied singly (although perhaps only at higher concentrations) and they both predict that the mixture toxicity is higher than the toxicity of the individual pharmaceuticals. Qualitatively new effects of the mixture are not considered by either concept.

Although the notion that the total effect of a mixture simply equals the arithmetic sum of the effects of its components is intuitively appealing and is used even in modern publications e.g. [15], it should be emphasized that such an “Effect Summation” lacks a pharmacologically sound basis unless all the concentration-response curves are strictly linear. In case of the typical sigmoidal concentration response curves that are commonly observed in ecotoxicological and toxicological investigations, Effect Summation would lead to the obviously nonsensical conclusion that an individual pharmaceutical acts synergistically or antagonistically with itself [33,34]. Effect summation would also predict that 10 pharmaceuticals, each present at a concentration that causes 15% mortality, would cause 150% mortality if present as a mixture.

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4 Empirical evidence on the applicability of CA and IA for estimating the ecotoxicity of pharmaceutical mixtures

Due to its general pharmacological basis, CA has been found widespread application for studying the ecotoxicology of pharmaceutical mixtures. Early studies have been published by e.g. Backhaus et al. for a mixture of 10 quinolone antibiotics, [35] and for a mixture of dissimilarly acting pharmaceuticals [36], Cleuvers for mixtures of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen and acetylsalicylic acid in a study with daphnids and algae [37], as well as for mixtures of the ß-blockers propranolol, atenolol and metoproplol [38]. All these studies demonstrated a high predictive power of CA. Also studies with binary mixtures of selective serotonin re-uptake inhibitors citalopram, fluoxetine, fuvoxamine, paroxetine and sertraline did not find any significant deviations from CA-expected mixture toxicities in studies with algae and daphnids [39]. Estrogenic mixture effects of furosemide and 17ß-estradiol as well as furosemide and phenazone followed CA-expectations closely in a study by Fent and workers, employing the yeast estrogen screen [40]. Finally, even investigations in multi-species tests show a similar pattern: in tests with sewage sludge bacteria, the toxicity of a binary mixture of the two quinolone antibiotics oxolinic acid and flumequine followed the predictions made by CA [41], and the effects of a five-compound mixture of the protein-biosynthesis inhibiting antibiotics chlortetracycline, rifampicin, fusidic acid, chloramphenicol, streptomycin followed the CA-prediction in studies with natural planktonic bacterial communities (Brosche, et al., 2010).

Fewer studies with mixtures of dissimilar pharmaceuticals that explored the usefulness of IA have been published. The results from a 14-compound mixture demonstrated that IA in fact provided a good prediction of the experimentally observed toxicity, while CA slightly overestimated to observed mixture toxicity [36]. An algal toxicity study with the 5 dissimilar pharmaceuticals propranolol, sulfamethoxazole, ethinylestradiol (EE2), diclofenac, ibuprofen and the herbicide diuron resulted in a mixture toxicity that followed IA expectations in the lower tested concentration range and CA in the region of higher concentrations [42], which was explained by the fact that four of the components (sulfamethoxazole, EE2, diclofenac, ibuprofen) were classified as acting primarily as baseline toxicants in algae and hence sharing

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an identical mode of action in this organism – although they belong to different chemical groups and therapeutical classes. The mixture toxicity of five pharmaceuticals and personal care products (fluoxetine, propranolol, triclosan, zinc-pyrithione, and clotrimazole) to marine benthic microalgal communities (periphyton) was investigated by Backhaus and colleagues [43], describing an almost identical mixture toxicity prediction by CA and IA. The observed a mixture toxicity that was largely in line with the conceptual expectations in the upper effect range. Substantial hormetic effects of the mixture were observed in the lower effect range and limited the applicability of either concept.

4.1 Mixture effects from non-significantly toxic individual concentrations

CA implies that every pharmaceutical in the mixtures contributes to the joint toxicity of the mixture, in strict proportion to its toxic unit (eq. 1). According to this concept it is hence irrelevant whether the individual concentrations in the mixture are high enough to cause toxicity.

IA on the other hand, is effect-based (eq. 2). This implies that any pharmaceutical present at a non-toxic concentration, i.e. at zero effect levels, does not add to the joint effect of the mixture. Consequently, a combination of dissimilarly acting pharmaceuticals in which each compound is present only at low, individually non-toxic concentrations, is sometimes assumed to also be non-toxic as a mixture. However, two issues challenge the real-world relevance of this line of reasoning: first of all it is questionable, whether a true zero-effect concentration actually exists on a molecular level, where each compound entering a cell will interact sooner or later with a biomolecule. And secondly, the fundamental assumption of IA – that the individual pharmaceuticals are completely independently acting and that they hence do not influence each other's toxicity in any way – will never be entirely fulfilled in reality, if chemicals are simultaneously present in a cell, organ, or tissue.

Apart from these fundamental considerations, it might also be important to highlight that the standard ecotoxicological measure for non-significant effects, the No Observed Effect Concentration (NOEC), is simply the highest concentration tested that does not provoke any statistically significant toxicity. But, obviously, absence of proof for a toxic effect is in fact no

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proof of its absence. A NOEC hence only indicates a grey area of concentrations where toxic effects cannot be statistically demonstrated, given the statistical power of the test. It does not describe a “safe” concentration or a “no effect” concentration. IA-compliant mixture effects therefore cannot be ruled out, even if all components of a mixture of dissimilarly acting pharmaceuticals is present only at their individual NOECs.

Low-dose effects of pharmaceuticals have been experimentally demonstrated in two earlier studies by Backhaus and colleagues, for a mixture of 10 quinolone antibiotics and a 12 compound mixture of dissimilarly acting pharmaceuticals [35][36], see also the analysis in [44]. Significant mixture effects from low-effect individual concentrations (EC05) were also observed in a study by Fent and coworkers for a mixture of cimetidine, fenofibrate, furosemide and phenazone [40]. Even mixtures of only comparatively few compounds often show a similar pattern. A mixture of fluoxetine and clofibric acid killed more than 50% of a daphnia population after an exposure of 6 days, although the individual components were only present at concentrations that did not provoke significant effects [45]. In the same study, a significant shift in sex ratio was observed after an exposure to a three component mixture of erythromycin, triclosan and trimethoprim – again at a mixture concentration at which all components were present at concentrations that did not provoke significant individual effects. Also two binary mixtures of clofibric acid and carbamazepine as well as diclofenac and ibuprofen show clear mixture effects in acute daphnia tests, although each individual component was present in a concentration below its individual NOEC [37]. Eguchi and colleagues demonstrated in a study with algae that trimethoprim shifts the concentration-response curve of sulfamethoxazole and sulfadiazine by a factor of 4-5 towards higher toxicities, even if present only at its NOEC [46].

It is worth pointing out in the context of this low-dose discussion, that the application of CA certainly does not imply that each and every pharmaceutical mixture is of ecotoxicological relevance, nor that every compound present is actually worth considering (Fig 1). But neither CA nor IA provide an *a priori* cut-off value below which a mixture component or the total mixture does not warrant attention – although this might very well be the final result of the mixture toxicity assessment, i.e. *after* the entirety of the components present has been

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assessed. Fig 1 shows the result of such an analysis. It depicts the toxic unit distribution (eq. 1) of the pharmaceuticals found in the sewage effluent stream from the STP plant in Gothenburg (from [24], exposure data from [2]). It can be clearly seen that ofloxacin alone is responsible for 50% of the expectable joint toxicity and that the first five compounds explain more than 90% of the sum of toxic units. The contribution of more than half the detected compounds is negligible for all intents and purposes, even under the assumption of a concentration-additive mixture behaviour. That is, the analysis of the toxic unit distribution of the compounds in a mixture can actually be used as a tool to prioritize and rank the ecotoxicological importance of pharmaceuticals in a complex mixture.

5 Deviations from CA and/or IA: synergisms and antagonisms in pharmaceutical mixtures

Several studies have observed mixture toxicities that deviate from the conceptual expectations of CA and/or IA. For such patterns the terms “synergism” and “antagonism “are frequently used. However, already in 1995 Greco listed 13 different definitions of those terms, inconclusive and not supporting each other [47]. Additionally, special care has to be taken to account for the fact that CA and IA usually predict different mixture toxicities, and a mixture whose toxicity is perfectly predictable by CA is hence “synergistic” in relation to IA, and *vice versa*. Explicitly specifying the frame of reference against which a mixture is evaluated is hence critically important. Synergisms should also be differentiated from a potentiation, i.e. the situation in which a pharmaceutical without biological activity if applied singly is increasing the effect of a second compound.

Several publications describe deviations from CA- and/or IA-predicted mixture toxicities, in particular studies that investigated two-compound mixtures. A mixture of diclofenac and ibuprofen was slightly more toxic to *Daphnia* than predicted by both of the concepts, while it had an intermediate toxicity to algae [37]. Slight deviations (predicted EC50 values lower than predicted by a factor of roughly 2, unfortunately, the authors do not provide numerical values for the observed and predicted EC50’s) from the predictions by CA and IA were also observed

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by Schnell and colleagues for an equitoxic mixture of diclofenac, bezafibrate, fluvoxamine, musk ketone and galaxolide [48]. Clear synergistic effects to algae were observed for mixtures of flumequine+erythromycin and oxytetracycline+flumequine by [41].

Brezovšek et al. observed deviations from the CA- and IA-predicted mixture toxicities in binary combinations of antineoplastics (5-fluoruracil, cisplatin, etoposide and imatinib) in algal growth assays using *Pseudokirchneriella* and *Synechococcus* [49]. The most pronounced antagonistic interaction resulted in the complete abolishment of the cisplatin-induced algal toxicity by etoposide. Synergistic (more than predicted by CA as well as IA) mixture toxicities were observed for *Pseudokirchneriella* exposed to the combination of 5-fluorouracil and imatinib, while the same combination was antagonistic in the assay with *Synechococcus.* Such results certainly highlight the importance of species-dependent interactions between pharmaceuticals in a mixture.

Similarly complex results are described in a recent paper by González-Pleiter et al., in which the results from a series of binary mixture experiments in growth inhibition assays with the blue-green algae *Anabaena* CPB4337 and the green algae *Pseudokirchneriella subcapitata* are described [50]. Several combinations were consistently more toxic than predicted by the median effect principle (levofloxacin+tetracycline, amoxicillin+norfloxacin in the test with *Anabaena*, levofloxacin+norfloxacin, erythromycin+tetracycline in the *Vibrio* assay), while the joint toxicity was well approximated by the median effect principle for other combinations, e.g. the joint toxicity of erythromycin and norfloxacin to *Anabaena*. Interestingly the authors also apply CA in its more general form (eq 1) and conclude that the median effect principle has superior predictive power. The cause of these discrepancies between CA and the median effect principle remain largely unexplained in the paper. However, as mentioned earlier, the median effect principle is just one slightly restricted incarnation of CA. Differences between the more general form of CA and calculations based on the median effect principle are hence most likely caused by systematic biases in the description of the concentration-response curves of the individual compounds.

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Rodea-Palomares et al. studied the joint action of lipid regulators (fibrates) in *Vibrio fischeri* and *Anabaena* CPB4337, also using the median effect principle [51]. The study results depict a complex effect pattern of the mixture, in which especially the combination of fenofibric acid and bezafibrate showed a strong, effect-level dependent antagonism in the assay with *Anabaena*. On the EC50 level, where the inflexibility of the median effect principle matters least, the combination effects could be approximated well, within a factor of less than 2, for all mixtures.

It is interesting to note that the observations on synergisms and antagonisms are often limited to a mere phenomenological description of modelled and observed effects. Usually, the underlying causes, be it on a molecular, physiological or ecological level of complexity, are not identified. This is a shortcoming which hampers the broader assessment of interactions for the ecotoxicology of pharmaceutical mixtures.

From the available evidence it can furthermore be concluded that synergisms and antagonisms are more often than not concentration- and effect-level dependent and rather specific for the tested mixture and bioassay. In particular, synergistic mixtures seem to be largely confined to mixtures of only a few compounds, usually not more than two or three, which seems to be an apparent contradiction to the results from multi-component mixtures, in which synergistic or antagonistic mixture effects are rarely, if ever, observed. This might on the one hand be simply caused by the lack of published studies on multi-component pharmaceutical mixtures.

On the other hand, this phenomenon might be explained by the fact that a multi-component mixture would buffer against the impact of a few synergistic or antagonistic interactions. This is visualized in Fig. 2 where it is assumed that one of the pharmaceuticals of the STP-mixture analysed in Fig. 1 is five times more toxic in the mixture. That is, the corresponding toxic unit is assumed five times bigger when present in the mixture than as an individual substance. Figure 2 shows that, even if ofloxacin, the compound that is vastly dominating the mixture, is 5 times more toxic than expected, the overall toxicity of the mixture only increases by a factor of 3.1. If any of the other compounds is synergized, the overall mixture toxicity becomes increasingly less affected. In fact, the mixture toxicity is not measurably impacted if any of the compounds

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that contributes less than 10% to the sum of toxic units is synergized. This buffering against synergistic interactions in multi-component mixtures might be a major reason why the toxicity of this mixture type is well predictable by either CA or IA.

Neither CA nor IA make any assumption on the targeted biological system nor do they consider any specific properties of mixture components beyond their pharmacological (dis)similarity. This is both strength and weakness of the concepts. On the one hand, this simplicity allows to establish general rules for mixture toxicity assessment, which is essential for considering the joint action of chemicals in regulatory guidelines. On the other hand, it cannot be assumed that these concepts actually describe biological reality, except perhaps in biologically extremely simple systems. Even if all the pharmaceuticals of a mixture have strictly similar, respectively dissimilar primary mechanisms of action, differences in toxicokinetics, biotransformation pathways and additional unspecific binding sites will provide a complex mixture toxicity pattern, if the employed biological assay has the sufficient resolution and statistical power. The crucial question when applying CA and/or IA for describing the ecotoxicology of pharmaceutical mixtures is therefore not whether deviations between simple concepts and complex biological realities can be observed, but whether their predictive power is sufficient for a certain purpose.

6 Eco-epidemiology and field-impacts of pharmaceutical mixtures

Critical issue for any of study that strives to analyse the impact of pharmaceutical mixtures in the field is to establish a causal link between the subset of compounds present in a compartment that are targeted in an investigation –usually selected *a priori* in view of e.g. known emission sources or chemical-analytical capacities – and field-observed impacts. An early study by Schallenberg et al. for example investigated the toxicity of drainage water that was suspected to be contaminated by veterinary on a bacterial community from an uncontaminated lake [52]. Sporadic effects of the drainage water were indeed observed, but could not be linked back to specific veterinary antibiotics, in particular because the actual contamination of the different drainage water samples was not determined. The study hence clearly highlights the limits of investigating complex real world exposures under uncontrolled conditions, without an accompanying highly performant chemical analytics.

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Mode-of-action specific biomarkers might help to pinpoint the presence and effects of certain chemical groups in a complex mixture. Vitellogenin induction for example has been widely employed over the last decades to indicate the presence and effects of compounds with estrogenic effects, such as ethinylestradiol see e.g. [53,54]. Similarly, the prevalence of resistance genes is supposed to indicate and/or confirm the presence of antibiotics at a polluted site, e.g. [55]. However, modes of action are usually not specific for a certain pharmaceutical group, i.e. vitellogenin is also induced by alkyl-phenols and phthalate plasticizers [56], and the prevalence of antibiotic resistance genes is also increased by the presence of certain metals e.g. [57]. An analysis of the toxic units of the different compounds, as outlined in Fig 1 might be helpful for developing hypotheses, which compounds are present in sufficient toxic units to actually contribute to field-observed effects. Such an approach, however, relies on the availability of reliable ecotoxicity estimates for each compound included in the study and for each exposed (group of) species.

Another means to unravel causal links between the presence of pharmaceutical mixtures and field observed ecotoxicological effects is the use of correlation based methods, employing translocation experiments and advanced chemical-analytical surveys. A series of studies was published over the last years which analyzed the pollution situation in the Llobregat river in North-Eastern Spain with respect to pharmaceuticals and accompanying chemical contaminants. A first study was published in 2009 by Muñoz et al. who investigated the correlation between the occurrence of 21 pharmaceuticals and benthic community structure [58]. The authors found an impacted diatom diversity at one of the polluted sites, but no significant overall correlation between diatom biodiversity and pharmaceutical concentrations. Such a correlation, however, was found between the occurrence of indomethacin, propranolol, atenolol and ibuprofen and the abundance and biomass of several benthic invertebrates (*Chironomus* and *Tubifex*). Damasio et al then comparatively assessed the impact of the pharmaceutical and pesticide mixtures present in the Llobregat, based in investigations with field collected and transplanted invertebrate species. It was concluded in this study that more than 95% of the observed overall toxicity to the invertebrate communities in the Llobregat is caused by pesticides, while pharmaceuticals contributed less than 5% [59]. It should be

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mentioned, though, that the authors calculated the total risk of the pesticide/pharmaceutical cocktails by multiplying the toxic units (=c/PNEC) of the individual compounds. The idea behind this calculation rule seems to be rooted in an assumed independent action. However, the original IA-concept is based on the multiplication of *effects*, and not toxic units, see equations 3 and 4.

In contrast, Ginebreda et al. based their mixture risk assessment on an addition of hazard indices, which basically follows the idea of CA [23], and found a good correlation between *in situ* invertebrate biodiversity and the sum of hazard quotients for daphnids. Such results indicate the possibility to use a combination of CA and laboratory-based toxicity data to provide an ecotoxicological assessment of chemical-analytical fingerprints (see also Figure 1).

Proia et al. focused their first study on the ecological impact of antibiotic contamination in the Llobregat river and found a significant correlation between the concentrations of 16 antibiotics and impacts on microbial biodiversity, bacterial mortality and activity of extracellular enzymes in biofilms translocated from pristine sites [60]. In a follow-up study, the group then broadened the scope of chemicals considered and included monitoring data from 57 pharmaceuticals from different classes and 16 pesticides that were present in concentrations between < 1ng/L to 3 µg/L [61]. Mainly effects on periphytic algae were analysed in this study, which demonstrated that the pesticide-pharmaceutical cocktail present at polluted sites negatively impacted the photosynthetic efficiency of the biofilms while increasing autotrophic biomass. Redundancy analyses showed that analgesics (mainly diclofenac, ibuprofen and paracetamol), barbiturates, triazines and organophosphates were the ecotoxicologically most important compound groups. These results also demonstrate that the issue of mixture occurrences is not restricted to pharmaceuticals in the environment, as site-specific exposures almost always will also contain chemicals from other use classes. A more holistic exploration of the ecological consequence of mixtures of emerging pollutants in the environment is the aim of the recently started EU project SOLUTIONS [62].

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7 Knowledge gaps and the next steps

The fact that CA and IA have been proven to be quite accurate and precise predictive instruments for the ecotoxicological assessment of pharmaceutical mixtures in several experimental studies should not blind us to the fact that the body of empirical evidence is still threadbare in many aspects. Perhaps it might be hardly worthwhile to test yet another mixture of pharmaceuticals of the same mode of action class in a standard aquatic single-species assay with, say, algae or daphnids, in order to check whether CA applies. But knowledge on the impact of pharmaceutical mixtures on non-standard test species, especially from the terrestrial and marine environment is still severely lacking. Additionally, we know very little about the joint ecotoxicity of pharmaceuticals under environmentally more realistic conditions – i.e. in situations where several interconnected populations of different species are exposed to a multitude of pharmaceuticals from different classes in very uneven mixture ratios.

One of the main challenges will be to systematically explore how far CA and IA provide reasonable mixture estimates under these circumstances. This implies the collection of evidence on the importance of interactions that might lead to synergisms or antagonisms, or even to a qualitatively unexpected mixture behavior. Such interactions can play a role on the level of individuals (toxikokinetic and toxicodynamic interactions) as well as on an ecological level of biological complexity.

Toxikokinetic and toxicodynamic interactions, i.e. interactions on the level of uptake and biotransformation, respectively at the receptor site, are well known and well investigated confounders for the use of pharmaceuticals in human and veterinary medicine. Their assessment is hence an integral part of the safety evaluation of pharmaceuticals for human health [63]. Such interactions will be dependent on the genetics, physiology and ecology of the exposed species, the exposure conditions, and the pharmaceuticals present. Both the Food and Drug Administration [http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm080499.htm, accessed 11.July 2014] as well as the European Medicines Agency [64] have developed guidance documents for studying interactions for their impact on

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human health, which might also provide a suitable starting point for their investigation in an ecotoxicological setting. Both organizations for example provide detailed approaches for studying toxicokinetic interactions on the level of uptake (e.g. interactions with transporter proteins such as PGPs) and metabolisation (e.g. cytochrome P450 driven metabolization), processes that are also well known to play a major role for organisms in the environment.

However, ecological interactions such as the competition for nutrients and space, predator-prey relationships, pollution-driven evolutionary processes (adaptation, tolerance development), or parasitism and symbiosis introduce a new layer of complexity for the environmental assessment of pharmaceutical mixtures. Some critical questions to be answered in future studies are: (i) how much do such ecological interactions affect the ecotoxicity of pharmaceutical mixtures? (ii) under which conditions do they occur, what are the underlying causes? (iii) what are the quantitative consequences, i.e. to which extent do those processes hamper the predictive power of the very simple mixture concepts of Concentration Addition and Independent Action? First evidence on the potential importance of taking the ecological level into consideration can be gained from studies on pesticide interactions, such as e.g. reported by van Brink et al. [65], who analysed the ecotoxicity of a herbicide-insecticide mixture in aquatic microcosms and found that, while macroinvertebrates were seriously affected, several phytoplankton species actually increased in their abundance, due to reduced grazing pressure.

8 Summary and conclusions

Knowledge on the ecotoxicology of pharmaceuticals has tremendously increased over the last decade, although empirical evidence is still biased towards the freshwater environment and data on terrestrial organisms and marine flora and fauna are still scarce. Solid ecotoxicological information is critical for drafting adequate risk assessments and for developing solid risk management and mitigation measures. However, given that the environmental exposure is characterized by the presence of multi-component pharmaceutical mixtures, investigating the ecotoxicology of individual pharmaceuticals has to be considered as a necessary but not sufficient first step – it has to be followed up by studying the ecotoxicology of pharmaceutical

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mixtures. This endeavor requires a deeper connection to environmental exposure assessments and monitoring studies, in order to focus experiments on those mixtures that are either proven or likely to occur. Otherwise, the number of possible mixtures (in terms of nature and number of compounds and mixture ratios) becomes overwhelming. Interactions studies, taking into consideration toxikokinetic and –dynamic interactions for in exposed individuals as well as ecological interactions, should be increasingly implemented to further increase the realism of ecotoxicological investigations of pharmaceutical mixtures.

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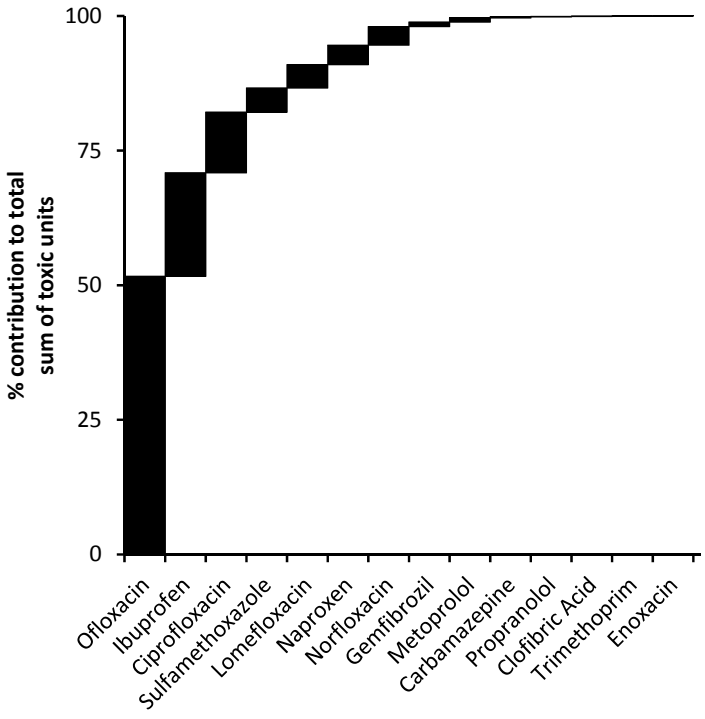
742 **Figure 1:**



743

744 **Distribution of relative toxic units for algal toxicity in a mixture of pharmaceuticals found in**
745 **the STP effluent of Ryaverket (Gothenburg, Sweden).**

746 The uneven contribution of the different pharmaceuticals to the CA-expected mixture toxicity is
747 evident. Ofloxacin alone contributes more than 50%, while, on the other end, Trimethoprim or
748 Enoxacin do not contribute to a significant extent. For further details see [24]

749

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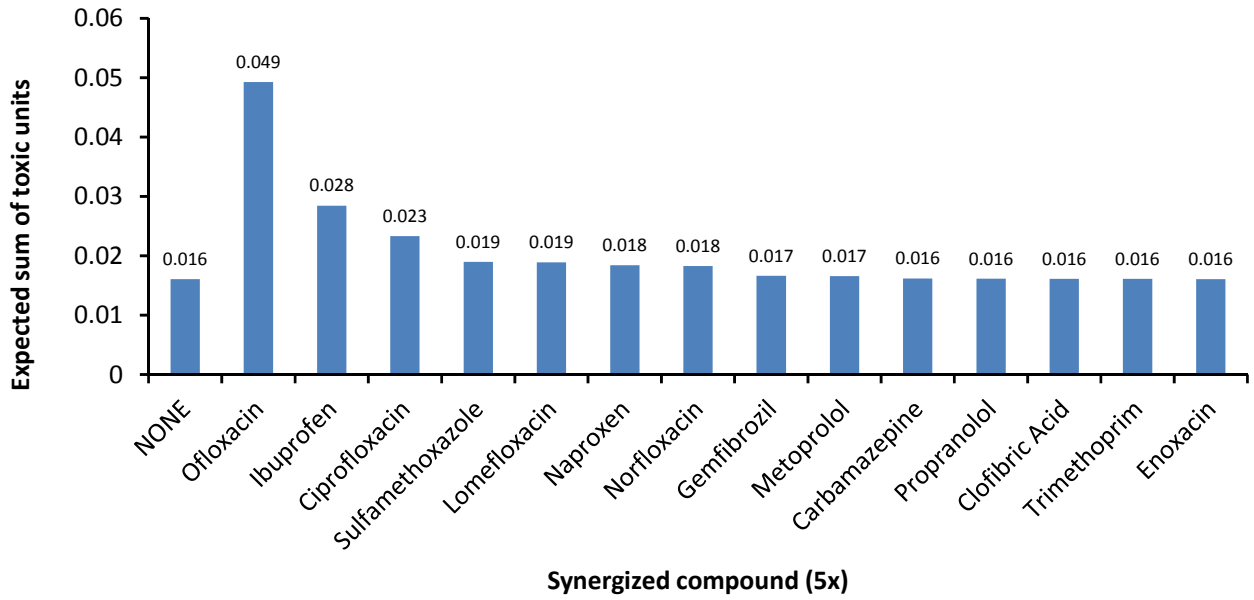
Figure 2:



The quantitative impact of a synergistic interaction on the total CA-expected toxicity

The same mixture as in figure 1 is visualized here. Given are the sums of toxic units under the assumption that one compound is synergized, i.e. is five times more toxic in the mixture than as a single compound. The sum of toxic units is 0.016 (without using any safety factor), which increases by a factor of 3.1 if the most important compound, ofloxacin, is synergized by a factor of 5. If any other pharmaceutical in the mixture is synergized, the overall toxicity of the mixture is hardly impacted.

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