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

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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.

1. Introduction

A multitude of pharmaceuticals from different therapeutical classes is used in 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 co-workers, for example, have just published an extensive monitoring study on the occurrence of 56 pharmaceuticals and seven 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 had already been found by Andreozzi et al. [2] who analysed pharmaceutical mixtures in European effluent streams in 2003 and found, on average, 18 of the 26 pharmaceuticals that were included in the analysis.

In particular, human pharmaceuticals predominantly enter the aquatic environment via effluents from sewage treatment plants (STPs). As a consequence, complex pharmaceutical mixtures are also found in STP sludge. For example, Jelić et al. [3] found 21, 24 and 30 pharmaceuticals from different classes in sludge from three different Spanish STP plants, and McClellan et al. [4] found 38 pharmaceuticals and personal care products in at least one composite sample from archived US biosolids. Exposure patterns in surface waters are equally complex. Vulliet & Cren-Olivé [5], for instance, found between six and 21 pharmaceuticals when monitoring drinking water supplies in the Rhône-Alpes region, and Proia et al. [6] 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.

The available evidence points to less complex exposures in soil. For example, Vazquez-Roig et al. [7] analysed soils from a Spanish wetland for the presence of 17 pharmaceuticals, and found, on average, only two compounds per sample (maximum six). Whether this points to a more general pattern, driven by the specific exposure and fate pattern of pharmaceuticals, or whether currently available techniques for extracting complex mixtures of pharmaceutical residues from...
soil are a limiting factor, cannot be concluded with any certainty, given the comparable scarcity of empirical data.

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 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 [10–12]). 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 (see also [13,14]).

Unfortunately, the compilation and presentation of the analytical data in published papers often does not allow us 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 set-up 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 [15]. 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 top 20’ of the big questions concerning pharmaceuticals in the environment [16].

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

(i) the ecotoxicity of a pharmaceutical mixture is typically higher than the effects of each individual component, and, consequently,

(ii) 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.

2. 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 for describing 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 [17].

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 [18,19].

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 interlinked but 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 at a given site or 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. Task (i) can be approached by directly testing the mixture of interest, for example, in the context of applying for market approval for a new medicinal product. Task (ii), however, basically analyses future scenarios, and might therefore use to a large extent component-based, predictive approaches. Additionally, task (ii) would require site- or compartment-specific information such as information on the species expected to occur. Finally, task (iii) would require more detailed mechanistic information on how various chemicals actually act jointly in a mixture.

(a) 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, as 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, whereas component-based approaches (see below) necessitate putting 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 (e.g. histopathological data). 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 [20–22]. In particular, the study by Richards et al. [21] points to some of the limitations of such experiments with whole mixtures: strong and unexpected fish mortalities were observed after exposure to a three component mixture of fluoxetine, ibuprofen and ciprofloxacin. Although the authors hypothesize 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. [23], showing substantial impacts on chlorophyll a content, primary production and biofilm respiration. Unfortunately, the use of pharmaceutical-diffusing substrata in the study, while providing a convenient means for exposing biofilms to constant concentrations in situ, does not allow an easy back-calculation to the actual concentrations that the biofilms were exposed to.

Galus et al. [24] analysed the impact of two concentrations (0.5 and 10 μg l⁻¹ in total) of a mixture of acetaminophen, carbamazepine, gemfibrozil and venlafaxine on zebrafish fecundity, with both exposures having significant effects on cumulative embryo production. Madureira et al. [25] described the effect of two concentrations (maximum field-observed concentrations, plus a 10,000 times elevated concentration) of a mixture of carbamazepine, fenofibrate acid, propranolol, sulfamethoxazole and trimethoprim on various biomarkers and histopathological parameters in zebrafish. Interestingly, both mixture concentrations, although four orders of magnitude apart, caused very similar effects on hepatocyte nuclear volumes, as a non-specific biomarker for toxic stress. This seems to indicate that the endpoint plateau already at the lower of the two tested concentrations, which is already in the μg l⁻¹ range. The pattern furthermore demonstrates that complete concentration–response information is needed for defining critical ecotoxicological thresholds.

Vannini et al. [26] evaluated the toxicity of a mixture of 13 pharmaceuticals at a total concentration that simulated their occurrence in Italian wastewaters to the green algae Pseudokirchneriella subcapitata and found impacts on a range of biochemical markers, such as chl a/b ratio, or the amounts of glutamine synthase. In particular, the expression of chloroplast proteins was affected, a finding that might offer an additional explanation why algae are often the most sensitive organism to pharmaceutical mixtures [9,27,28].

Quinn et al. [29] provided one of the few datasets on the toxicity of pharmaceutical mixtures on non-standard organisms when they analysed the overall toxicity of an 11-compound mixture of pharmaceuticals to the cnidarian, Hydra attenuata. They observed a bi-phasic response of the organism to the mixture, with effects already visible at 1/10th of typical concentrations found in the effluent of the Montréal STP.

Almost the only study that compared individual effects of pharmaceuticals with the effects of their mixture and found no elevated toxicity has been published by Dietrich et al. [30] in a multi-generational study with daphnids exposed to a four-compound mixture of carbamazepine, diclofenac, metoprolol and ethinylestradiol. They found, however, specific mixture effects that were not observed in the individual exposures, such as, significantly increased sizes of the daphnids at the time of first reproduction. A systematic exploration of the relationship between single substance and mixture effects was 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 fossarum, in which moultng behaviour was impacted at the maximum concentrations of those compounds found in rivers and streams of southern Germany [31].

Additional studies that analysed mixtures at assumedly environmentally realistic concentrations of the individual pharmaceuticals include the report by Pomati et al. [32] in which the effects of a mixture of 13 human pharmaceuticals on human embryonic cells was analysed. At assumed environmental exposure levels, cell growth was significantly inhibited. And, finally, Borgmann et al. [30] analysed the effects of a seven-compound pharmaceutical mixture on the amphipod Hyalella. 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, using experimental approaches. This allows the determination of standard descriptors such as EC₅₀ values or no observed effect concentrations (NOECs), if sufficient data were recorded for describing the concentration–response relationship, and comparison of them 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.

(b) 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, for example, to explore the question of 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), provide such a link and are practically applicable for the assessment of pharmaceutical mixtures in the environment.

(c) Concentration addition

In the field of human toxicology and pharmacology, the median effect principle has found widespread application [33], which is a special case of the more general concept of 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 curve is usually captured within only one parameter, rendering it a quite rigid approach. It has previously been demonstrated that a more flexible approach is needed in order to capture the different shapes of the concentration–response curves with sufficient precision and accuracy [34].

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

$$\frac{c_{\text{mix}}}{EC_{x_{\text{mix}}}} = \sum_{i=1}^{n} \frac{c_i}{EC_{x_i}}$$

where n denotes the number of mixture components, cₙ and cₙ give the individual concentrations in the mixture and the mixture concentration, respectively, and x is a common effect level, which is provoked by an exposure to a single substance or mixture concentration ECₙ mix respectively ECₙ.

The fraction c/ECₙ is often termed a ‘toxic unit’, and CA is hence also known as ‘toxic unit summation’. It follows from equation (2.1) 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 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 random events [35]. In case the biological response increases with increasing concentrations (e.g. when mortality is analysed), IA is mathematically expressed as

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E(c_{\text{mix}}) = 1 - \prod_{i=1}^{n} [1 - E(c_i)],
\]

where \(E(c_{\text{mix}})\) denotes the mixture effect at a concentration \(c_{\text{mix}} = \sum_{i=1}^{n} c_i\). \(E(c)\) 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 [36,37]. 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. That is, inert compounds do not add to the toxicity of a mixture. Furthermore, both concepts 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. Finally, CA as well as IA build on the notion that the components of a mixture do not interact, neither chemically nor during their toxicokinetic and toxicodynamic phases.

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. [17]), it should be emphasized that such an ‘effect summation’ lacks a pharmacologically sound basis unless all the concentrations–response curves are strictly linear. In the 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 [37,38]. 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.

3. Empirical evidence on the applicability of concentration addition and independent action for estimating the ecotoxicity of pharmaceutical mixtures

Owing to its general pharmacological basis, CA has been found widespread application to study the ecotoxicology of pharmaceutical mixtures. Early studies have been published by, for example, Backhaus et al. for a mixture of 10 quinolone antibiotics, [39] and for a mixture of dissimilarly acting pharmaceuticals [40]; and Cleuvers [41] for mixtures of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen and acetylsalicylic acid in a study with daphnids and algae as well as for mixtures of the β-blockers propranolol, atenolol and metoprolol [42]. All these studies demonstrated a high predictive power of CA. In addition, studies with binary mixtures of selective serotonin re-uptake inhibitors citalopram, fluoxetine, furoxamine, paroxetine and sertraline did not find any significant deviations from CA-expected mixture toxicities in studies with algae and daphnids [43]. Oestrogenic mixture effects of furosemide and 17β-oestradiol, as well as furosemide and phenazole, followed CA expectations closely in a study by Fent et al. [44] employing the yeast oestrogen screen. 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 [45], and the effects of a five-compound mixture of the protein-biosynthesis-inhibiting antibiotics chlortetracycline, rifampicin, fusidic acid, chloramphenicol and streptomycin followed the CA prediction in studies with natural planktonic bacterial communities [46].

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, whereas CA slightly overestimated the observed mixture toxicity [40]. An algal toxicity study with the five dissimilar pharmaceuticals propranolol, sulamethoxazole, ethinyloestriadiol (EE2), diclofenac and 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 [47]. This was explained by the fact that four of the components (sulamethoxazole, EE2, diclofenac, ibuprofen) were classified as acting primarily as baseline toxicants in algae and hence sharing 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-pyrihthione and clotrimazole) to marine benthic microalgal communities (periphyton) was investigated by Backhaus et al. [48], describing an almost identical mixture toxicity prediction by CA and IA. The observed mixture toxicity 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.

(a) 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 (equation (2.1)). 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 (equation (2.2)). 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
Low-dose effects of pharmaceuticals have been experimentally demonstrated in two earlier studies by Backhaus et al. [39,40] for a mixture of 10 quinolone antibiotics and a 12 compound mixture of dissimilarly acting pharmaceuticals (see also the analysis in [49]). Significant mixture effects from low-effect individual concentrations (EC05) were also observed (see also the analysis in [49]). Significant mixture effects from compound mixture of dissimilarly acting pharmaceuticals [39,40] for a mixture of 10 quinolone antibiotics and a 12 compound mixture of dissimilarly acting pharmaceuticals is present only at their NOEC. 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 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 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 et al. [39,40] for a mixture of 10 quinolone antibiotics and a 12 compound mixture of dissimilarly acting pharmaceuticals (see also the analysis in [49]). Significant mixture effects from low-effect individual concentrations (EC05) were also observed in a study by Fent et al. [44] for a mixture of cimetidine, fenofibrate, furosemide and phenazone. 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 [50]. 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. In addition, two binary mixtures of clofibric acid and carbamazepine as well as diclofenac and ibuprofen showed clear mixture effects in acute daphnia tests, although each individual component was present in a concentration below its individual NOEC [41]. Eguchi et al. [51] demonstrated in a study with algae that trimethoprim shifts the concentration–response curve of sulfamethoxazole and sulfadiazine by a factor of 4 to 5 towards higher toxicities, even if present only at its NOEC.

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 (figure 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, after the entirety of the components present has been assessed. Figure 1 shows the result of such an analysis. It depicts the toxic unit distribution of the pharmaceuticals found in the sewage effluent stream from the STP plant in Gothenburg (from [28], 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.

4. Deviations from concentration addition and/or independent action: 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, in 1995, Greco et al. [52] listed 13 different definitions of those terms, inconclusive and not supporting each other. 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, that is, the situation in which a pharmaceutical without biological activity if applied singly increases 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, whereas it had an intermediate toxicity to algae [41]. 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) from the predictions by CA and IA were also observed by Schnell et al. [53] for an equitoxic mixture of diclofenac, bezafibrate, fluvoxamine, musk ketone and galaxolide. Clear synergistic effects to algae were observed for mixtures of flumequine + erythromycin and oxytetracycline + flumequine by Christensen [45].

Brezovšek et al. [54] observed deviations from the CA- and IA-predicted mixture toxicities in binary combinations of antineoplastics (5-fluorouracil, cisplatin, etoposide and imatinib) in algae growth assays using *Pseudokirchneriella* and *Synechococcus*. 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, whereas 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 *P. subcapitata* are described [55]. 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), whereas 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 (equation (2.1)) 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.

Rodea-Palomares et al. [56] studied the joint action of lipid regulators (fibrates) in *Vibrio fischeri* and *Anabaena* CPB4337, also using the median effect principle. 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 that 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 figure 2, where it is assumed that one of the pharmaceuticals of the STP mixture analysed in figure 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 five 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 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 a strength and weakness of the concepts. On the one hand, this simplicity allows general rules for mixture toxicity assessment to be established, 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 or 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 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.
5. Eco-epidemiology and field impacts of pharmaceutical mixtures

A critical issue for any 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 the compartment that is targeted in an investigation—usually selected a priori—in view of, for example, known emission sources or chemical–analytical capacities—and field-observed impacts. An early study by Schallenberg et al. [57], for example, investigated the toxicity of drainage water, which was suspected to be contaminated by veterinary antibiotics, on a bacterial community from an uncontaminated lake. 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 accompanying the experiments with reliable chemical analysis.

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 past decades to indicate the presence and effects of compounds with oestrogenic effects, such as ethinylestradiol [58,59]. Similarly, the prevalence of resistance genes is supposed to indicate and/or confirm the presence of antibiotics at a polluted site [60]. However, modes of action are usually not specific for a certain pharmaceutical group (e.g. vitellogenin is also induced by alkyl-phenols and phthalate plasticizers [61]), and the prevalence of antibiotic resistance genes is also increased by the presence of certain metals [62]. An analysis of the toxic units of the different compounds, as outlined in figure 1, might be helpful for developing hypotheses of which compounds are present in toxic units that are sufficiently high 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 were published over the past few years which analysed the pollution situation in the Llobregat river in northeastern Spain with respect to pharmaceuticals and accompanying chemical contaminants. A first study was published in 2009 by Muñoz et al. [63], who investigated the correlation between the occurrence of 21 pharmaceuticals and benthic community structure. 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 on 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 was caused by pesticides, whereas pharmaceuticals contributed less than 5% [64]. It should be mentioned, though, that the authors calculated the total risk of the pesticide/pharmaceutical cocktails by multiplying the toxic units (c/predicted no effect concentration) 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 unaffected fractions (1 − E(c)), see equation (2.2)), and not toxic units.

In contrast, Cinebreda et al. [27] based their mixture risk assessment on an addition of hazard indices, which basically follows the idea of CA, 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. [65] 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. 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 less than 1 ng l$^{-1}$ and 3 μg l$^{-1}$ [6]. 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 analogues (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 [66]. Furthermore, physical and biological factors might impose additional environmental stress upon chemically exposed organisms and populations [67].

6. Knowledge gaps and the next steps

The fact that CA and IA have been proved 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, daphnids or zebrafish, 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 behaviour. Such interactions can play a role on the level of individuals (toxicokinetic and toxicodynamic interactions) as well as on an ecological level of biological complexity.

Toxicokinetic and toxicodynamic interactions, that is, 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 [68]. Such interactions will be dependent on the genetics, physiology and ecology of the exposed species (e.g. [69]), the exposure conditions and the pharmaceuticals present. Both the Food and Drug Administration (http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentRe sources/DrugInteractionsLabeling/ucm080499.htm, accessed 11 July 2014) as well as the European Medicines Agency [70] have developed guidance documents for studying interactions for their impact on 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 PGP) and metabolism (e.g. cytochrome P450-driven metabolism), 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, (iii) what are the underlying causes? and (iv) what are the quantitative consequences, that is, to what extent do those processes hamper the predictive power of the very simple mixture concepts of CA and IA? First evidence on the potential importance of taking the ecological level into consideration can be gained from studies on pesticide interactions, such as those reported by van Brink et al. [19], 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, owing to reduced grazing pressure.

The necessity to verify nominal concentrations has been recently highlighted in a discussion paper on ‘principles of sound ecotoxicology’ [71], and many of the experimental mixture studies discussed in this review do not fulfil this criterion. There is hence certainly room for improving the quality of mixture studies in this aspect. However, I would also argue that it depends strongly on the specific aims of a given mixture study, the conclusions drawn from it, the employed biotest and the composition of the investigated mixture whether analytical exposure verifications are possible in the first place, whether they are merely ‘nice to have’ or whether they are ‘absolutely essential’ (or something in between). For example, analytical exposure verifications might be straightforward in experimental systems that are sufficiently large, but might be impossible in miniaturized biotests that are conducted in a volume of just a few microlitres, for example, using microtiter plates. Analytical exposure verifications also become especially challenging when testing pharmaceuticals that are already active at low concentrations (e.g. ethinylestradiol).

Given that the resources for conducting a particular study are always limited, it should be carefully considered whether it is, indeed, advisable to spend a most likely substantial proportion of the available resources on analytical chemistry—or whether the resources are better spent on actual ecotoxicological work. A more in-depth exploration of the underlying concentration–response curves or an increase in the number of included mixture components might provide information that is more valuable for the study interpretation. The almost inevitable trade-off between
investments in chemical-analytical work versus ecotoxicological work needs to be carefully evaluated for each study, depending on its specific aim and design.

Any component-based study that explores whether CA and/or IA apply for a given mixture inherently assumes that the concentration–response curves of all individual substances are reproducible per se, which also has been discussed as a hallmark for ‘sound ecotoxicology’ [71]. Unfortunately, almost none of the studies analysed for this review provides information on the reproducibility of the experimental study results. Although this would certainly improve the inherent reliability of a given study, the lack of study repeats is hardly surprising given the empirically extremely challenging nature of mixture studies. In particular, if a study is exploring the usefulness of component-based approaches, ecotoxicological data on each and every component of the mixture need to be recorded prior to the mixture experiment itself. However, given this inherent complexity, every opportunity should be taken to cross-validate the study results for the individual components and the mixture with existing data and, if available, modelling approaches (for example, quantitative structure–activity relationships).

As in any other scientific field, extraordinary results require extraordinary proof. In the context of mixture studies, increased demands should hence be put to any study that demonstrates strong synergistic (more than concentration-additive) or antagonistic mixture effects. Such results might be of major relevance for our understanding of the general importance of pharmaceuticals in the environment and might guide specific risk-management measures. The retraction of the study by Arnold et al. [72] on synergistic interactions of environmental oestrogens was a major blow to the field of mixture (eco)toxicology in general, an experience that certainly must be avoided in the future.

It should be finally noted in this context that biotests that make use of natural populations (and not specifically bred strains of standard laboratory animals and clones of microorganisms) as well as biotests on an ecological level of complexity inherently contain an element of non-repeatability, which is fundamentally different from the non-reproducibility of an experimental outcome for technical reasons. At the same time, such studies are inherently more relevant to our understanding of the ecotoxicology of chemicals in the real world. So far, it is largely unclear how these partly conflicting demands (high repeatability versus high ecological realism) should be dealt with.

7. Summary and conclusion

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 mixtures. This endeavour 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 toxicokinetic and toxicodynamic interactions 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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