Desalination and Water Treatment
www.deswater.com

doi: 10.5004/dwt.2018.21964

117 (2018) 15–20
June

Mixture toxicity of pharmaceuticals present in wastewater to aquatic organisms

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Received 13 December 2017; Accepted 21 January 2018

ABSTRACT

The aim of this study is the assessment of the impact of mixture of three pharmaceutical substances (ciprofloxacin, 17α-ethinylestradiol and 5-fluorouracil) on aquatic animals, cyanobacteria and plants. Based on previous work and literature data, three concentrations of each substance were used to prepare mixtures: predicted or measured environmental concentrations (PEC/MEC), predicted no effect concentrations (PNEC), concentrations that induced a response of 10% in bioindicators (EC_{10}). Immobilization tests with crustaceans (*Daphnia magna* and *Artemia salina*), growth tests with cyanobacteria (*Cyanosarcina* sp.), algae (*Desmodesmus quadricauda*, *Raphidocelis subcapitata*) and plants (*Lemna minor*), enzymatic test Fluotox and reproduction test with *D. magna* were performed. The results of this work confirm the importance of low concentration mixture exposure. Effect in PEC/MEC concentrations of the mixture of tested compounds was equal 15% in *R. subcapitata* growth test. As expected, effects obtained for mixtures of pharmaceuticals in their EC_{10} concentrations were frequently higher than 10%. The obtained results were compared with the concept of independent action, which either underestimated or overestimated the effects in concentrations used. The results obtained in this study suggest that the exposure to tested mixtures of pharmaceuticals even in low concentrations of components, that individually cause no harm to organisms, may trigger adverse effects in aquatic environment.

Keywords: Mixture ecotoxicity; Pharmaceuticals; Ciprofloxacin; 17α-Ethinylestradiol; 5-Fluorouracil

1. Introduction

The extensive use of pharmaceuticals in health care of people and farm animals results in getting of medicines' residues and metabolites of their biotransformation to wastewater, surface waters and potable water [1,2]. Medicines are detected in aquatic environment in concentrations ranging from ng/L to μg/L, with the highest concentrations in pharmaceutical and hospital effluents and lower in surface waters and in water intended for consumption [3]. Ecotoxicological data show that most of drugs do not exert significantly negative effects on organisms tested in acute, short-term tests, when tested as single substances [4]. It is assumed that chronic toxicity is more likely, that pharmaceuticals may trigger long-term effects on bioindicators, which additionally may differ significantly in their sensitivity to these contaminants [5]. A specific feature of most pharmaceuticals among other chemicals is that they are designed to be biologically active at low concentrations, and therefore there is a particular concern that they may affect aquatic wildlife [5].

Pharmaceuticals do not occur in environmental compartment as isolated, pure substances, but as multi-component mixtures. Kostich et al. [6] reported the occurrence from 6 to 59 pharmaceuticals and their metabolites in the effluents of US wastewater treatment plants. In another study, out of 26 analyzed pharmaceuticals, an average of 18 occurred in European effluent streams [7]. Traditional ecotoxicological risk assessments do not address the emerging question of

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Presented at the 13th Conference on Microcontaminants in Human Environment, 4–6 December 2017, Czestochowa, Poland.

what are the effects of mixtures of pharmaceuticals [8–10]. It is claimed that knowledge of the ecotoxicity of individual pharmaceuticals is the first step, but insufficient alone to assess the environmental risk of drug residues in water [10]. Although some effect data have been generated for single substances, corresponding data for mixtures of pharmaceuticals are unavailable [10].

The ecotoxicity of a mixture is almost always higher than the effects of its individual components [4]. A mixture can be considerably ecotoxic, even if all components are present only in low concentrations that do not provoke significant toxic effects if acting separately on the exposed organisms [1,4,11,12]. Even mixtures of comparatively few compounds often show a similar pattern [11].

Effects of mixtures cannot be calculated by simply adding the effects of the mixture of components when applied alone, especially if the components have differently shaped dose–response curves. Two competing fractional additivity models are widespread in literature: the concept of concentration addition (CA) (the effect of the mixture is the addition of the effects of the single compounds in proportion to their relative fraction in the mixture) and the concept of independent action (IA) (the effect of one compound is independent of the others) [4]. The necessary assumption of both concepts is that the mixtures components do not interact and their toxicities are not influenced by other substances in the mixture [13]. However, interactions between pharmaceuticals are well documented in the literature [14,15] and called synergism (when toxicity is higher than predicted), potentiation (when one chemical, not toxic itself at the exposure concentration, enhance the toxicity of another chemicals in a mixture) and antagonism (when toxicity is lower than predicted) [16].

Different experimental and conceptual approaches are used in research concerning mixtures of chemical substances, for example, whole-mixture and component-based approaches and using of different concentrations of components in the mixture, for example, effective or environmentally relevant concentrations. Most of the studies include tests on high concentration mixtures (close to $LC(EC)_{50}$) [15,17–19]. However, some authors confirmed the difficulty of concentration extrapolation in mixture toxicity data [20]. Methods for assessing mixture toxicity using high concentrations seem to be unjustified as interaction between chemical in a mixture may be completely different at low, environmentally relevant concentrations [17].

Therefore, to imitate the possible situation in the environment, the aim of this study was to assess the effects of low concentration mixtures of selected drugs (ciprofloxacin, 17α-ethinylestradiol and 5-fluorouracil) on aquatic animals, cyanobacteria and plants. Based on previous work three concentrations of each substance were used to prepare mixtures: predicted or measured environmental concentrations (PEC/MEC), predicted no effect concentrations (PNEC), effective concentration for 10% of bioindicators (EC_{10}) [21–24].

Ciprofloxacin is an antibiotic from the group of fluoroquinolones. Its antibacterial effect is based on inhibition of enzymes involved in DNA biosynthesis. Estrogens belong to sex hormones. They pose a threat to the environment, mainly due to their common use in contraceptive pills and hormone replacement therapy. Cytostatics, including 5-fluorouracil, are drugs that inhibit cell proliferation during anticancer therapy. The focus on these substances is because they have been detected in wastewater, surface water and also in water intended for human consumption [25–28] and they are representatives of different types of pharmaceuticals.

2. Materials and methods

2.1. Chemicals

Ciprofloxacin (Fluka, Poland), 17α-ethinylestradiol (Sigma-Aldrich, Poland) and 5-fluorouracil (Fluka, Poland) of purity over 98% were purchased from Sigma-Aldrich (Poland). The compounds were initially dissolved in deionized water, sonicated for 30 min using an ultrasonic disintegrator of MDM-10 type (0.4 kW at a frequency of 20 kHz) and further diluted with corresponding test media. Nominal concentrations of stocks of pharmaceuticals equaled 1 and 10 mg/L.

2.2. Mixtures

For assessing mixture toxicity three concentrations of single substances in the mixture were used:

- PEC/MEC (predicted or measured environmental concentration)
- PNEC (predicted no effect concentration)
- EC_{10} (effective concentration for 10% of bioindicators)

PEC/MEC values originated from the literature and PNEC and EC_{10} values were taken from previous research of the authors (Table 1).

2.3. Ecotoxicological tests

Immobilization, growth, enzymatic and reproduction tests (acute and chronic) were performed with two species of crustaceans, one species of cyanobacteria, two species of algae and one species of higher plants. Crustaceans *A. salina* (Linnaeus, 1758) were obtained from the dormant eggs in the hatching procedure, according to the appropriate test protocol [29]. Cyanobacteria *Cyanosarcina* sp. (CCALA 058) and green algae *D. quadricauda* (CCALA 463), *R. subcapitata* (CCALA 433) came from Institute of Botany, Academy of Science in Czech Republic. Higher plants *L. minor* and neonates of *D. magna* (Straus, 1820) came from the own laboratory culture of Department of Biology, Faculty of Building Services, Hydro and Environmental Engineering, Warsaw University of Technology.

Crustacean immobilization assay Artoxkit M™ (MicroBioTests, Belgium) was performed according to the protocols provided with each test kit [29]. The organisms were incubated with mixtures of compounds for 24 h in the temperature of 25°C. Then, immobilized organisms were counted.

Daphnia magna acute immobilization test was performed according to OECD 202 [30]. The organisms were incubated with mixtures of compounds for 48 h in the temperature of 22°C. Then, immobilized organisms were counted.

Fluotox fluorescence inhibition assay (IQ toxicity test) was conducted according to the methodology developed by Espiritu et al. [31]. Organisms showing no fluorescence were counted after 1 h of exposure to the mixtures of pharmaceuticals.

Table 1
Concentration of pharmaceuticals in mixtures for each bioindicator and test

Bioindicator	Type of the test/duration (d – days; h – hours)	Concentration in mixtures	Concentration of compound in the mixture (mg/L)			Reference
			Ciprofloxacin	17α-Ethinylestradiol	5-Fluorouracil	
		PEC/MEC	0.00006[a]	0.000043[b]	0.000064[c]	[25][a] [27][b] [28][c]
		PNEC	0.00000015	0.00000015	0.0000006	[25]
Cyanosarcina sp.	Growth/72 h	EC_{10}	0.03*	0.4*	7.65*	[21–24]
Desmodesmus quadricauda	Growth/72 h		0.02	0.03	0.57	
Raphidocelis subcapitata	Growth/72 h		0.52	0.008	0.26	
Lemna minor	Growth/72 h		0.03	0.004	0.01	
Artemia salina	Survival/24 h		139.3	50	150.6	
Daphnia magna	Survival/48 h		122.3	4.2	214.1	
Daphnia magna	Enzymatic/1 h		1.3	105.2	70.7	
Daphnia magna	Reproduction/ 21 d		0.53	0.17	0.00000095	

[a]MEC Germany.
[b]MEC European Union.
[c]PEC.
*Values for *Microcystis* sp.

Reproduction test with *D. magna* was performed according to modified OECD 211 in semi-static conditions with replacement of solutions (every 2–3 d) [32]. Test was performed in six vessels for each mixture and for the control. Exposure of organisms to the mixtures of compounds lasted 21 d. The offspring was counted daily and removed from the test vessels.

Growth test with cyanobacteria and algae was performed according to modified PN-EN ISO 8692:2012 [33]. Test was performed in three vessels for each mixture and for the control. Assessment of growth inhibition of organisms was made by measuring the density of the cells after 72-h contact with the mixtures of compounds on ISO mineral medium [33].

Growth test using *L. minor* was performed according to the methodology contained in PN-EN ISO 20079:2006 [34]. Test was performed in three vessels for each mixture and for the control. Evaluation of growth inhibition was based on measuring the area and number of leaves at the beginning and the end of the 7-d test. The measurements were carried out using computer software for digital image analysis (UTHSCSA ImageTool version 3.0).

2.4. Analyses of ecotoxicity data

As the studied pharmaceuticals have potentially dissimilar action in organisms, their EC_{10} concentrations vary 1–6 orders of magnitude and concentrations used in the study gave small or zero effect, comparison with IA mathematical concept was carried out solely. In this model, the effect of a mixture comprised of *n* compounds is calculated by the formula [35]:

$$E_{(\mathrm{Cmix})} = 1 - \prod_{i=1}^{n} [1 - E_{(Ci)}]$$

where $E_{(Ci)}$ is the effect of compound *i*, if applied alone at concentration C_i – the concentration in mixture. This concept predicts effects of mixture. According to the above equation, any substance for which $E_{(\mathrm{Ci})}$ is equal to zero is not expected to contribute to the joint effect of the mixture.

3. Results and discussion

The very important question raised by experts in the field is whether exposures to mixtures at level assumed to be safe for the environment and in environmentally relevant concentrations may produce adverse effects [10]. In this study, low concentration mixtures were analyzed and their effects on bioindicators obtained in comparison with the control samples are presented in Fig. 1.

In most cases, individual, as well as mixture, effects in PEC/MEC concentrations of tested compounds was equal to 0. The only exception was *R. subcapitata* test, in which algal growth was inhibited in 15%. In this case, the effect was also higher than predicted and consistent with effects for PNEC mixture for this bioindicator. The results suggest that the interaction of tested compounds in PEC and PNEC concentrations for *R. subcapitata* is via potentiation. Consequently, even if PEC/MEC and PNEC effects for the components are equal to 0, hazard of population and community effects caused by mixture exposure cannot be excluded. Reports of similar observations may be found in the literature. Pomati et al. [20] noticed that a mixture of 13 different pharmaceuticals at environmentally relevant concentrations triggered adverse consequences on human and zebra fish cells in vitro. González-Pleiter et al. [36] showed that binary erythromycin and tetracycline mixture in wastewater effluents showed a strong synergism at low effect levels in cyanobacteria and green alga

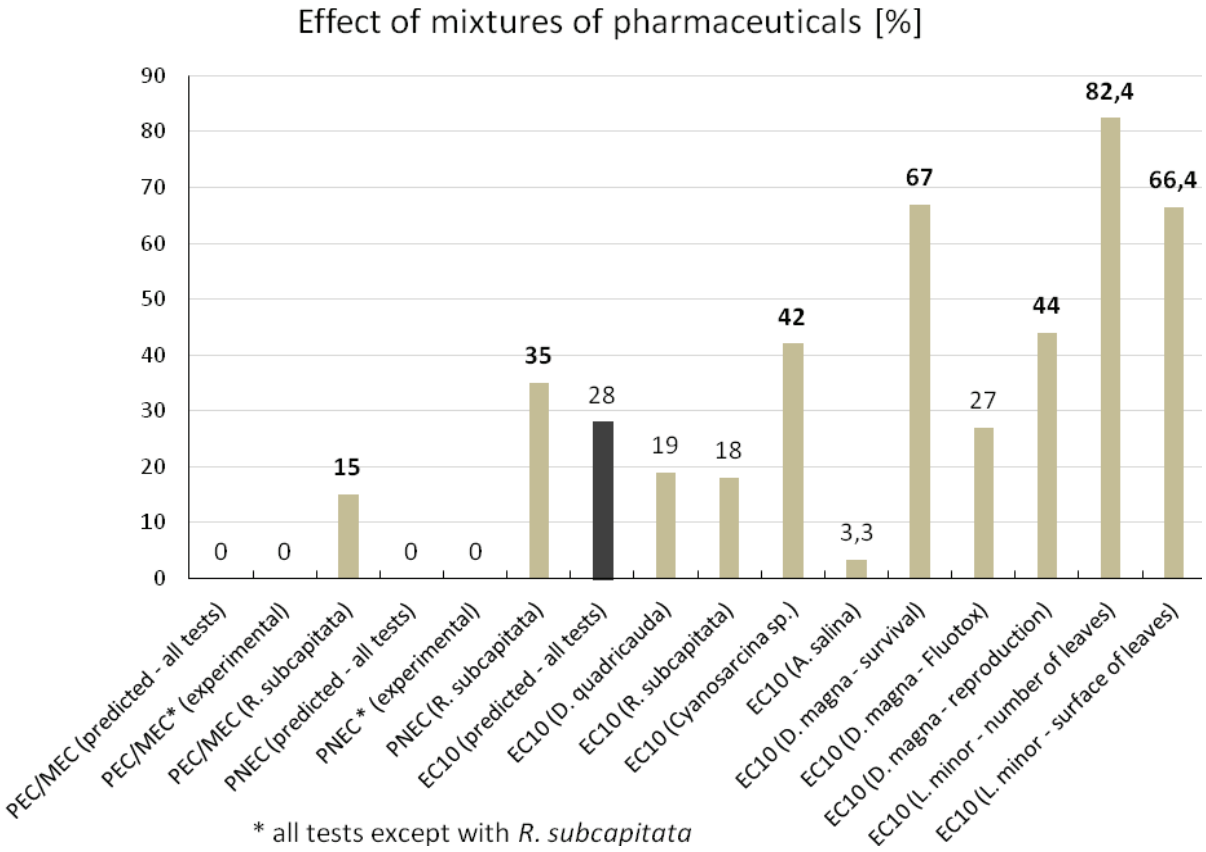

Fig. 1. Comparison of effect (%) of mixtures of pharmaceuticals (predicted by IA model and experimental).

and concluded that certain specific combinations of pharmaceuticals may pose a potential ecological risk for aquatic ecosystems in the environmentally measured concentrations.

Mixtures of pharmaceuticals in their EC_{10} concentrations repeatedly triggered higher effects in bioindicators. In 10 out of 11 tests, inhibition of growth, reproduction and enzymatic activity, as well as the influence on survival of bioindicators was higher or much higher than the established 10%.

EC_{10} concentrations were used in this work instead of NOECs (no observed effect concentrations). The main reason is that NOECs derived from experimental studies are often associated with effect levels in the range of 5% to 20% and consequently NOEC exposures may contribute to mixture effects for dissimilarly acting substances. Similar conclusions were drawn by Cleuvers [19]. Binary combinations of clofibric acid and carbamazepine as well as diclofenac and ibuprofen showed clear mixture effects in acute *D. magna* tests, although each individual component was present in a concentration below its individual NOEC. In another study, a mixture of fluoxetine and clofibric acid killed more than 50% of *D. magna* population after 6-d exposure, although the components were present in concentrations that did not provoke significant effects individually [37]. Simple additive effects were observed also in binary mixtures of sulfonamides [18]. In another study, five PPCPs (pharmaceuticals and personal care products mixed at the NOEC of each substance provoked a significant effect of 28% [13]. Significant joint effects of dissimilarly acting toxicants at or below individual NOECs for four studies were also reported by Krotenkamp et al. [38].

Pharmaceuticals tested in this study belong to different groups and are considered to have different mode of action in humans. Therefore, for the prediction of effects IA model was used. Although the empirical evidence on the performance of IA is much more limited, the effects of mixtures are sometimes better described by this model, than by other model described in the literature CA [11,39,40] or both models predict similar mixture toxicities. CA usually predicts higher toxicity of the mixture than IA, and considering the worst case environmental scenario – it is sometimes claimed to be better concept to use [41]. However, there are no grounds to use CA concept, basing on the assumptions of this model, when chemicals in the mixtures have different mode of action and their effect concentrations vary several orders of magnitude. That is why both concepts have been suggested as default approaches in regulatory risk assessment of chemical mixtures [10].

The concept of IA proved to be relevant only in *D. magna* Fluotox test. It failed to predict mixture toxicity in case of low concentrations (PEC/MEC, PNEC of *R. subcapitata*, EC_{10} of *Cyanosarcina* sp., *D. magna*) in this study, but it also overestimated the effect in case of EC_{10} of algae, *A. salina*. Percent of effects obtained for mixtures of pharmaceuticals in their EC_{10} concentrations was in five cases higher than predicted for the mixture, which is in line with expectations. However, in four cases the effects were lower. This may imply interactions (synergism, antagonism, potentiation) depending on the bioindicator and the end point of the test method. Interactions among

components of mixtures usually occur at medium or high concentrations level. At low exposure levels (near and below NOEC) they are unlikely to occur. They may vary also according to the duration of exposure and the biological targets. However, Boobis et al. [14] reported examples for low concentration synergy. Among 90 toxicity studies, only 6 provided "quantitative estimates of synergy" and the magnitude of synergy was within a factor of 4 of the levels predicted by additive models. The presumption that some drugs can interact at environmentally relevant exposure levels, giving potentially subtle effects, was confirmed in this work. The results, given also PEC/MEC and PNEC mixture exposure of *R. subcapitata*, may also be an example of changing interactions between components in mixture in dependence of concentration of exposure and species sensitivity. In the literature, there may be found another concept to study ecotoxicological interactions of pharmaceuticals named combination index – Isobologram equation [42]. This model was also used to study the ecotoxicological interactions of pharmaceuticals using several aquatic organisms and its main advantage is that it offers a framework where interactions among compounds in mixtures are not ignored [42].

Results of Melvin et al. [15] demonstrated increased toxicity – loss of tactile response of striped marsh frog tadpoles exposed to a mixture of naproxen, carbamazepine and sulfamethoxazole, compared with exposures to the individual compounds. In conclusion they proposed the assessment of physiological and metabolic endpoints in the future studies. The high obtained % of effects for growth, reproduction and enzymatic tests for mixture of pharmaceuticals in EC_{10} concentrations are consistent with this statement and demonstrate that not-survival endpoints are much more accurate, when testing mixtures.

4. Conclusions

There is scientific evidence that when organisms are exposed to a number of different chemical substances, these substances may act in a way that affects the overall level of toxicity. Current assessment methods do not take proper account of these joint actions and there are advantages and disadvantages of the different approaches. There are no generally applicable guidelines as to when assessment of combinations of chemicals should be carried out. Testing of combined effect of mixtures is not required by most regulations in European Union. Scientists agree mostly that mixtures should be tested at exposure levels that are representative for the environment or significant levels (close to PNECs for components), but still there is a problem with composition of mixtures that is always changing.

The major knowledge gaps with regard to the assessment of the toxicity of mixtures are detection data, data from experimental studies and data concerning mode of action. Interactions of chemicals in mixtures are really difficult to predict, if possible [10]. There is a need for improving the current methodologies and development of holistic approaches for environmental risk assessment of pharmaceuticals under realistic conditions.

The results obtained in this study clearly imply that the presence of the tested mixtures of medicines in surface waters may have ecological significance and may result in an unacceptable change in the environment. Current regulations on environmental risk assessment of pharmaceuticals do not protect ecosystem function and species diversity, as PNECs mixture gave 35% effect in relation to algae. Further studies are needed including testing of binary mixtures and determining which compound is responsible for joint action of tested mixtures.

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