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Single and mixture toxicity of pharmaceuticals and chlorophenols to freshwater algae *Chlorella vulgaris*

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ABSTRACT

Organisms in the aquatic environment are exposed to a variety of substances of numerous chemical classes. The unintentional co-occurrence of pharmaceuticals and other contaminants of emerging concern may pose risk to non-target organisms. In this study, individual and binary mixture toxicity experiments of selected pharmaceuticals (ibuprofen and ciprofloxacin) and chlorophenols (2.4-dichlorophenol (2,4-DCP) and 3-chlorophenol (3-CP)) have been performed with freshwater algae *Chlorella vulgaris*. All experiments have been carried out according to the 96-h algal growth inhibition test OECD No. 201. Binary mixture tests were conducted using proportions of the respective IC_{50}s in terms of toxic unit (TU). The mixture concentration-response curve was compared to predicted effects based on both the concentration addition (CA) and the independent action (IA) model. Additionally, the Combination Index (CI)-isobologram equation method was used to assess toxicological interactions of the binary mixtures.

All substances individually tested had a significant effect on *C. vulgaris* population density and revealed IC_{50} values $< 100\ mg\ L^{-1}$ after exposure period of 96-h. The toxic ranking of these four compounds to *C. vulgaris* was 2,4-DCP > ciprofloxacin > 3-CP > ibuprofen. Generally, it can be concluded from this study that toxic mixture effects of all tested chemicals to *C. vulgaris* are higher than the individual effect of each mixture component. It could be demonstrated that IC_{50} values of the tested mixtures predominately lead to additive effects. The CA model is appropriate to estimate mixture toxicity, while the IA model tends to underestimate the joint effect. The CI-isobologram equation method predicted the mixtures accurately and elicited synergism at low effect levels for the majority of tested combinations.

1. Introduction

Pharmaceuticals and Personal Care Products (PPCPs) are widely discharged into ecosystems and comprise numerous chemical classes. Human pharmaceuticals predominantly enter the environment through effluents from wastewater treatment plants, and to a minor extent from emissions from manufacturing sites. Pharmaceuticals are designed to produce a biological and therapeutic effect on the human body through interaction of specific pathways in target humans and are usually active at very low concentrations. If these targets are found in other organisms in the environment, exposure of pharmaceuticals can cause adverse biological effects in non-target organisms (Henschel et al., 1997). Therefore, determination of the toxicity to non-target species such as algae is beneficial to understand the impact to ecosystems. Most of bioactive PPCPs remain active after being released into surface and groundwater, because removal in waste water treatment plants is not sufficient (Hohem and Santos, 2011). The effect caused by drugs varies according to the chemical structure (Wiegel et al., 2004).

Concentrations of single compounds in the environment might be too low to show an effect individually, but substances following a similar mode of action are expected to induce effects through joint toxicity at concentrations below the individual No Observed Effect Concentrations (NOEC) (Kortenkamp, 2008; Backhaus et al., 2011). The effect of mixtures of PPCPs on algae have been widely studied (DeLorenzo and Fleming, 2008; Backhaus et al., 2011; Brezovsek et al., 2014; Peterson et al., 2014). However, little is known about the effect of PPCPs that co-occur with other relevant contaminants such as chlorophenolic compounds originated from the agricultural, pharmaceutical, biocide or dye industry. Risks resulting from the unintentional co-occurrence of pharmaceuticals

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and chlorophenols in the aquatic environment could be a matter of concern under the Water Framework Directive (Directive 2000/60/EC).

Generally, the biochemical mode of action (MoA) of the substances needs to be known, that determines the basic concepts of mixture toxicity. Mixtures can be based on similar or dissimilar MoA. Moreover, the substances can interact with each other, and therefore exert an effect on the respective MoA of each chemical, or work in a non-interactive way and do not influence each other´s MoA. Basically, two different concepts are available for that purpose, and are termed concentration addition (CA) and independent action (IA) which are introduced by Loewe and Muischnek (1926) and Bliss (1939), respectively. Both, the CA and IA concepts, have been suggested as default models in regulatory risk assessment in order to predict the toxicity of chemical mixtures. Pharmaceuticals with various MoA in complex mixtures are detectable in the environment (Kolpin et al., 2002), and particularly found in effluent-dominated ecosystems (Brooks et al., 2006). In most cases the joint effects of PPCPs to green algae revealed additive toxicities (Cleuvers, 2004; DeLorenzo and Fleming, 2008; Backhaus et al., 2011; Peterson et al., 2014).

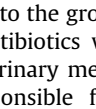
In this study, two pharmaceuticals (ibuprofen and ciprofloxacin) and two chlorophenols (3-chlorophenol and 3,4-dichlorophenol) were chosen for toxicological assessment considering their widespread use and environmental significance. The knowledge about MoA of mixture components is of paramount importance to assess joint effects. The selected chlorophenols may act by a non-specific narcotic MoA, also denoted as baseline toxicity, while the MoA of the chosen pharmaceuticals remains unkown. In this study, the observed algal toxicities should help to understand if the assumption of baseline toxicity also applies to the selected pharmaceuticals or if indications for an excess toxicity over baseline toxicity can be examined. Table 1 presents the properties of the tested compounds in this study including concentrations found in the environment.

Ibuprofen ((*RS*)-2-(4-(2-methylpropyl) phenyl) propanoic acid) is used as a nonsteroidal anti-inflammatory drug (NSAID), known for its anti-inflammatory, antipyretic and analgesic properties and with an estimated annual production of several kilotons (Cleuvers, 2004). The exact mode of action of ibuprofen is unknown; however it is a non-selective inhibitor of cyclooxygenase, an enzyme involved in prostaglandin synthesis.

Ciprofloxacin belongs to the group of fluoroquinolones, which form a major class of antibiotics world-wide. This substance is used for human and veterinary medicine against most strains of bacterial pathogens responsible for urinary tract, respiratory, gastrointestinal and abdominal infections. Fluoroquinolones become an emerging area of concern, as they are widely used and not readily biodegradable by microorganisms (Al-Ahmad et al., 1999). The antibiotic residues detected in some effluent waters originating from hospitals and effluents can be very high (Table 1). To green algae, the toxic effects of antibiotics are mostly attributed to the inhibition of the pathways involved in photosynthetic metabolism and finally affect the cell growth (Halling-Sorensen, 2000).

Chlorophenols have been used in the production of pesticides, perfumes, dyes, synthetic resins, pharmaceuticals, synthetic tanning agents, lubricating oils and solvents since 1860 (Rayne et al., 2009). 2,4-dichlorophenol (2,4-DCP) is a chlorinated derivative of phenol and an important intermediate in the industrial manufacture of 2,4-dichloro-phenoxyacetic acid (2,4-D), the well-known industrial commodity herbicide used in the control of broadleaf weeds. 3-chlorophenol (3-CP) is a halophenol with antifungal activity and is commonly used as a building block or intermediate in the preparation of variety of biologically active compounds. A majority of toxic substances including

Table 1
Estimated chemical properties of tested compounds (3-chlorophenol, 2,4-dichlorophenol, ciprofloxacin HCl and ibuprofen) retrieved from EPISuite, version 4.11 and concentration detected in the environment.

Chemical	CAS#	Chemical class	Log *Kow*	Structure	Formula weight [g/mol]	Mode of Action (MoA)	Amounts detected in environment
2,4-DCP	120-83-2	Phenol	3.06		163	Polar narcotic	In drinking water 2,4-DCP have been found in the range of < 8–30 ng L^{-1}, in groundwater and surface water in the µg L^{-1} range (HSDB, 2016)
3-CP	108-43-0	Phenol	2.5		128.56	Polar narcotic	2–51 µg L^{-1} in surface waters and 0.5–93 µg L^{-1} in effluents have been found to contain 3-CP (HSDB, 2016)
ciprofloxacin-HCl	93107-08-5	fluoroquinolone antibiotic	0.28		367.8	Inhibition of enzymes topoisomerase II and IV (DNA gyrase)	0.02 µg L^{-1} in 26% US streams (Kolpin et al., 2002); 0.06 µg L^{-1} in surface water in Germany (UBA, 2011); 31 mg L^{-1} in WWTP in India (Larsson et al., (2007); 0.7–124.5 µg L^{-1} in WW of Swiss hospital and 249–405 ng L^{-1} in Swiss sewage WWTP (Fink et al., 2012).
ibuprofen	15687-27-1	nonsteroidal anti-in-flammatory agent	3.97		206.28	Inhibitor of cyclooxygenase	0.1 µg L^{-1} detected in Berlin waterways (Heberer, 2002), 0.2 µg L^{-1} in US streams (Kolpin et al., 2002) 2.4 µg L^{-1} in surface water in Germany (UBA, 2011)