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ENVIRONMENTAL RISK ASSESSMENT OF SELECTED PHARMACEUTICALS PRESENT IN SURFACE WATERS IN RELATION TO ANIMALS

MONIKA ZAŁĘSKA-RADZIWIŁŁ*, MARIA ŁEBKOWSKA, KATARZYNA AFFEK, AGNIESZKA ZARZECZNA

Warsaw University of Technology, Faculty of Environmental Engineering,
Department of Biology
Nowowiejska 20, 00-653 Warsaw, Poland
*Corresponding author's e-mail: monika.radziwill@is.pw.edu.pl

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Abstract: This paper addresses the issue of antibacterial drugs, estrogens and cytostatic drugs' presence in surface waters and their influence on animals. The ecotoxicity and the impact of three active compounds: ciprofloxacin, 17α-ethinylestradiol and 5-fluorouracil on protozoa, crustaceans and fish were examined. Acute tests (crustaceans' immobilization test, fish survival test, enzymatic test on *Daphnia magna*) and chronic tests (growth test on protozoa, reproduction test on crustaceans and juvenile growth test on two species of fish) were performed. Acute toxicity studies revealed diversified species – sensitivity to the tested compounds. Crustaceans *Artemia salina* were the most resistant to all three pharmaceuticals. Fish also demonstrated low sensitivity to ciprofloxacin and 5-fluorouracil ($LC(EC)_{50}$-96h > 100 mg/l). In the survival tests, the greatest harm in respect to fish and crustaceans was demonstrated by 17α-ethinylestradiol, and in the enzymatic tests – by ciprofloxacin. In all chronic tests, the toxic effects of drugs were proven. Tested compounds limited reproduction of crustaceans and growth of protozoa and fry. The risk assessment, conducted on the basis of the PEC/PNEC quotient, showed a significant risk in relation to aquatic animals caused by the presence of 17α-ethinylestradiol and 5-fluorouracil in concentrations detected in surface waters.

INTRODUCTION

The occurrence of various pharmaceuticals in the environment is associated with their ever-increasing production and wide applicability in human and livestock treatment. Drugs residues are transferred to hospital, municipal and industrial sewage [16]. Many pharmaceuticals are not metabolized by the humans or animals. A certain part of medicines is biotransformed to intermediate metabolites, and therefore not only drugs, but also products of their biochemical transformation, may be present in sewage [8, 14].

Many drugs are resistant to chemical degradation and biodegradation. They accumulate in tissues and organs, which increases hazard to organisms inhabiting surface water. The elimination of pharmaceuticals during the processes of wastewater treatment and water self-purification occurs only to a little extent, or does not occur at all, due to their high persistence [22]. The concentration of pharmaceuticals in surface water ranges from ng/l to μg/l [16]. Using surface water as a source of drinking water leads to the presence

of drugs in water intended for human and livestock consumption. Moreover, the presence of pharmaceuticals in sewage and surface water increases the number of drug-resistant microorganisms [18].

The actions undertaken within the European Union oblige pharmaceutical companies to take into account environmental risk assessment (ERA) in the procedures associated with drug registration and launching. The requirements for testing ecotoxicity of veterinary and human drugs were included in the Directives: 92/18 EEC, 2001/83 EEC and EMEA (*European Medicines Agency*) guidelines, dated 1998 and 2006 [6, 7]. The basic set of ecotoxicological tests proposed within the EMEA guidelines, including tests on protozoa, crustaceans and fish, is not sufficient to develop a complete profile of environmental risks associated with the presence of pharmaceuticals in aquatic ecosystems. In fact, pharmaceuticals may have adverse impact on many other groups of organisms, with different physiologies, bioavailability of drugs and their biotransformation.

The aim of this study was to assess the risk posed by the effects of active substances, representing three groups of pharmaceuticals used in human treatment, on aquatic animals. The study comprises the effects of antibacterial agents (ciprofloxacin), estrogens (17α-ethinylestradiol) and cytostatics (5-fluorouracil). All these compounds are detected in wastewater and surface water.

Ciprofloxacin belongs to the group of fluoroquinolones – antibiotics, whose antibacterial effect is based on DNA topoisomerase (gyrase) inhibition, involved in biosynthesis of DNA. Second-generation fluoroquinolones are mainly used in gram-negative bacterial infections. They also act on atypical pathogens (*Chlamydia*, *Mycoplasma* and *Legionella*). Ciprofloxacin is the most active drug among the fluoroquinolones. It produces genotoxic effect in genetic material and induces bacterial resistance to fluoroquinolones, which may be transmitted in the process of horizontal gene transfer [10, 11].

Estrogens are a group of chemical compounds of steroid structure, belonging to sex hormones. They pose a threat to the environment, mainly due to their vast use in contraceptive pills. The conducted studies have shown that even trace concentrations of estrogens have impact on fish reproduction. In the presence of ethinylestradiol (EE2) with concentration of 0.32 ng/l, male fish did not develop secondary sexual characteristics. The negative impact of this compound on fish fertilization was also observed [17, 21, 26].

Cytostatics are drugs used in anticancer therapy. They inhibit cell proliferation, mainly by interaction with DNA. Some of them inhibit synthesis of nucleotides, the other intercalate in DNA, which prevents from transcription and translation. One of the cytostatic drugs used in anticancer therapy is 5-fluorouracil. It acts as a biosynthesis inhibitor of thymidine monophosphate (TMP). A low level of TMP leads to disruption of DNA replication and inhibition of tumor cell proliferation [36].

MATERIALS AND METHODS

Chemicals

Ciprofloxacin (Fluka), 17α-ethinylestradiol (Sigma) and 5-fluorouracil (Fluka) of purity over 98% were purchased from Sigma-Aldrich. For detailed information see Table 1. With the exception of 17α-ethinylestradiol (ethanol used as carrier, 1% v/v), the compounds were initially dissolved in the buffered saline solution, and further diluted with corresponding test media. Appropriate solvent controls were tested.

Table 1. Basic information about the tested chemicals

Compound	CAS no.*	Molar mass [g/mol]	Molecular formula	Structural formula
ciprofloxacin	8571–33–1	331.35	$C_{17}H_{18}FN_3O_3$	
17α-ethinylestradiol	57–63–6	296.41	$C_{20}H_{24}O_2$	
5-fluorouracil	51–21–8	130.08	$C_4H_3FN_2O_2$	

*CAS no. – Chemical Abstracts Services

Ecotoxicological tests

Acute and chronic tests were performed on protozoa, crustaceans and fish. Ciliates *Tetrahymena thermophila*, crustaceans *Artemia salina* (Linnaleus, 1758) and neonats of *Daphnia magna* (Straus, 1820) were obtained from the dormant eggs in the hatching procedure, according to the appropriate test protocol [1, 4, 28]. Fish species *Danio rerio* (Hamilton, 1822) and *Lebistes reticulatus* (Peters, 1859) came from the own laboratory culture of the Department of Biology, Faculty of Environmental Engineering, Warsaw University of Technology.

Acute tests

- Crustacean immobilization assays Artoxkit M™ and Daphtoxkit F™ (Microbiotests) were performed according to the protocols provided with each kit. The organisms were incubated with toxic compounds for 24 and 48 hours, respectively, in the temperature of 25°C. Then, immobilized organisms were counted.
- Fluotox fluorescence inhibition assay (IQ toxicity test) was conducted according to the methodology developed by Espiritu *et al*. [5]. Organisms showing no fluorescence were counted after one hour of exposure.
- *Danio rerio* and *Lebistes reticulatus* survival tests were performed according to the Polish norm PN-C-04610-04:1990 [27]. Fish were exposed to different concentrations of chemicals at 20–22°C. Dead specimens were counted after 24, 48, 72 and 96 hours, and removed from the test vessels.

Chronic tests

- Protozoa growth assay Protoxkit F™ (Microbiotests) was performed according to the protocol provided with the kit. Ciliates *Tetrahymena thermophila* were incubated in test vessels, with tested compounds and food suspension, in the temperature of 30°C. Growth inhibition was determined on the basis of turbidity changes (OD at λ = 440 nm), at the beginning and at the end of the test.
- Reproduction test with *D. magna* crustaceans was conducted according to the OECD methodology 211 [24], in semistatic conditions with daily solution exchange (28 days, 20–22°, 8/16-hr dark/light photoperiod). Crustaceans were fed with a unicellular algae suspension. Juveniles were counted daily and removed from the test vessels.

– Juvenile growth test with *Danio rerio* and *Lebistes reticulatus* was carried out according to the OECD methodology 215 [23]. Juvenile fish were in exponential growth phase weighing 0.05–0.1 g (the total weight of fish in 1l of water was 0.2–1.0 g). The study was performed in semistatic conditions with daily solution exchange. The exposure period was 28 days, the temperature was 20–22°C, and the light exposure lasted 12–16 hours. Fish were weighed at the beginning and at the end of the test, and specific juvenile growth rate was calculated for each concentration of chemicals.

Calculation procedures

– Lethal and effect concentrations ($LC(EC)_{50}$) were calculated using probit analysis with 95% confidence intervals [35].
– No observed effect concentrations (NOEC) were determined using ANOVA and Tukey's test [2].

Risk and toxicity assessment

– Toxicity assessment of compounds was performed according to the European Union Directive 93/67 [3] and the U.S. Environmental Protection Agency [32].
– Assessment of the risk due to the presence of active substances in surface waters in relation to animals was based on the risk quotient RQ:

$$RQ = \frac{PEC}{PNEC},$$

where:
PEC – Predicted Environmental Concentration in the environment;
PNEC – Predicted No Effect Concentration in the environment;
$RQ \geq 1$ – high risk; $RQ < 1$ – low risk.

PEC was assumed equal to the concentrations detected in surface waters (MEC – Measured Environmental Concentration), or calculated according to the EMEA (2006) (Table 5) [6]. PNEC was calculated on the basis of chronic toxicity data (NOEC) by obligatory safety factor method (assessment factor AF = 10, due to the quality and quantity of toxicological data) [3].

RESULTS

The results of ecotoxicological tests are presented in Tables 2–4. The $LC(EC)_{50}$ values obtained in the study are listed in decreasing order.

Toxicity profiles include toxicity assessment of pharmaceuticals according to the European Union Directive (93/67/EEC) [3] and the U.S. Environmental Protection Agency (U.S. EPA) criteria [32].The results of acute tests revealed diversified sensitivity of organisms to the tested active compounds. In the immobilization tests of crustaceans, the most resistant to the effects of all compounds was *Artemia salina*. In all cases, EC_{50}-24h was higher than 100 mg/l. Similar low sensitivity to the effects of ciprofloxacin and 5-fluorouracil (Tables 2 and 4) revealed fish (*Danio rerio* and *Lebistes reticulatus)* in survival tests and crustacean *Daphnia magna* in immobilization test. However, 17α-ethinylestradiol showed different effects on fish and crustaceans (Table 3). This compound was toxic to bioindicators. Fish species (*Danio rerio* $LC(EC)_{50}$ – 4.77 mg/l, *Lebistes reticulatus* – 6.55 mg/l)

Table 2. Ecotoxicity of ciprofloxacin – study results

Compound	Tested organism	Toxicity assessment criteria	Exposure time [h]	$LC(EC)_{50}$-t (95% confidence interval) [mg/l]	NOEC [mg/l]	Toxicity assessment	
						EU Directive 93/67/EEC	US EPA
Ciprofloxacin	*Tetrahymena thermophila*	growth	24	> 100	0.195	nontoxic	slightly toxic
	Artemia salina	immobillization	24	> 100	-		
	Daphnia magna	immobillization	48	> 100	-		
	Danio rerio	survival	96	> 100	-		
		juvenile growth	672	-	< 0.780	-	-
	Lebistes reticulatus	survival	96	> 100	-	nontoxic	slightly toxic
		juvenile growth	672	-	0.780	-	-
	Daphnia magna	reproduction	672	14.4 (11.2–17.7)	0.156	harmful	moderately toxic
		fluorescence	1	3.77 (3.05–4.53)	-	toxic	

Table 3. Ecotoxicity of 17α-ethinylestradiol – study results

Compound	Tested organisms	Toxicity assessment criteria	Exposure time [h]	$LC(EC)_{50}$-t (95% confidence interval) [mg/l]	NOEC [mg/l]	Toxicity assessment	
						EU Directive 93/67/EEC	US EPA
17 α - ethinylestradiol	*Artemia salina*	immobillization	24	> 100	-	nontoxic	slightly toxic
	Daphnia magna	fluorescence	1	> 100	-		
		immobillization	48	14.28 (11.18–16.20)	-	harmful	moderately toxic
	Lebistes reticulatus	survival	96	6.55 (5.05–7.70)	-	toxic	
		juvenile growth	672	-	0.0065	-	-
	Danio rerio	survival	96	4.77 (4.37–5.17)	-	toxic	moderately toxic
		juvenile growth	672	-	0.00038	-	-
	Daphnia magna	reproduction	672	1.89 (1.55–2.24)	0.0780	toxic	moderately toxic
	Tetrahymena thermophila	growth	24	0.084 (0.064–0.104)	0.0031	extremely toxic	highly toxic

Table 4. Ecotoxicity of 5-fluorouracil – study results

Compound	Tested organism	Toxicity assessment criteria	Exposure time [h]	$LC(EC)_{50}$-t (95% confidence interval) [mg/l]	NOEC [mg/l]	Toxicity assessment	
						UE Directive 93/67/EEC	US EPA
5 – fluorouracil	*Artemia salina*	immobillization	24	> 100	-	nontoxic	slightly toxic
	Daphnia magna	immobillization	48	> 100	-		
		fluorescence	1	> 100	-		
	Danio rerio	survival	96	> 100	-		
		juvenile growth	672	-	< 1.56	-	-
	Lebistes reticulatus	survival	96	> 100	-	nontoxic	slightly toxic
		juvenile growth	672	-	< 1.56	-	-
	Tetrahymena thermophila	growth	24	44.02 (36.3–53.2)	0.195	harmful	moderately toxic
	Daphnia magna	reproduction	672	0.0015 (0.0011–0.0019)	0.000006	extremely toxic	highly toxic

showed higher sensitivity than *Daphnia magna* – 14.28 mg/l. Toxicity of pharmaceuticals in *Daphnia magna* enzymatic test (Fluotox) differed from the one determined in immobilization tests. After one-hour exposure to the chemicals, crustaceans revealed the strongest response to the action of ciprofloxacin (EC_{50}-1h = 3.77 mg/l). Other compounds did not limit hydrolysis of *4-methylumbelliferyl-β-D-galactoside* to 4-*methylumbelliferone,* even in the highest concentrations.

The results derived from chronic tests (growth test on protozoa, reproduction test on *Daphnia magna* and juvenile growth test on two species of fish), lasting from 24 hours for the *Tetrahymena thermophila,* up to 28 days for crustaceans and fish, showed toxic effects of tested pharmaceuticals. Ciprofloxacin inhibited reproduction of *Daphnia magna* by 50% at the concentrations of 4.46 mg/l, 5.8 mg/l and 14.4 mg/l after 7, 14 and 28 days, respectively, and inhibited growth of protozoa by about 26% at the concentration of 100 mg/l.

17α-ethinylestradiol inhibited reproduction of crustaceans by 50% at the concentrations of 0.23 mg/l, 0.60 mg/l and 1.89 mg/l after 7, 14 and 28 days, respectively, and revealed 50% growth inhibition of ciliates at the concentration of 0.084 mg/l. EC_{50} in *Daphnia magna* reproduction test was 1.03 mg/l, 0.060 mg/l, 0.0015 mg/l after 7, 14, and 28 days, respectively, while EC_{50}-24h for protozoa was 44.02 mg/l. The lowest NOEC was obtained in the reproduction test on *Daphnia magna* for 5-fluorouracil – 0.000006 mg/l and 17 α-ethinylestradiol in the Protoxkit F™ assay – 0.0031 mg/l.

All tested pharmaceuticals inhibited the growth of juvenile fish *Danio rerio* and *Lebistes reticulatus*. 17α-ethinylestradiol induced the strongest growth inhibition. *Danio rerio* (NOEC = 0.00038 mg/l) proved to be more sensitive to estrogen than *Lebistes reticulatus* (NOEC = 0.0065 mg/l). 5-fluorouracil inhibited growth of fish at the concentrations of 12.5 mg/l and 6.25 mg/l, whereas at lower concentrations, stimulation of growth was observed. Juvenile growth stimulation was proven for all tested concentrations of ciprofloxacin. It was particularly effective in the case of *Danio rerio,* for which the specific growth rate was between 3 and 7-fold greater than in control. Such fish response made the NOEC determination impossible in the chosen concentration range. It should be noticed that lethal effects of the hormone and cytostatic drug were observed within 28 days at the concentrations which did not cause fish mortality in the 96-hour acute test.

Toxicity assessment on the basis of $LC(EC)_{50}$ showed that 17α-ethinylestradiol causes the greatest harm in relation to most bioindicators. According to EU criteria, this medicine was toxic to the crustacean *Daphnia magna*, both species of fish and extremely toxic to protozoa (Table 3). 5-fluorouracil was harmful to *Tetrahymena thermophila* and extremely toxic to *Daphnia magna* in the reproduction test (Table 4). Ciprofloxacin was harmful to the crustacean *Daphnia magna* in the reproduction test, and was toxic in the Fluotox bioassay (Table 2).

The risk assessment in relation to aquatic animals, based on the PEC/PNEC quotients, indicates high risk associated with the presence of 17α-ethinylestradiol and 5-fluorouracil, and low risk associated with the presence of ciprofloxacin in surface waters (Table 5).

However, considering the results obtained in juvenile growth test (indicating stimulation of the growth of both fish species), it should be concluded that ciprofloxacin may also have adverse effects on aquatic organisms at the concentrations detected in surface waters.

Table 5. Risk assessment for ciprofloxacin 17α-ethinyloestradiol and 5-fluorouracil

Compound	PNEC [mg/l]	PEC (MEC) in surface waters [mg/l]	RQ (PEC/ PNEC)	Risk assessment
ciprofloxacin	0.0156	0.00003 (MEC USA) [30] 0.00006 (MEC Germany) [15] 0,00067 (PEC*) [9]	0.002 0.004 0.042	Low risk
17α-ethinylestradiol	0.000038	0.000043 (MEC EU) [30] 0.000831 (MEC USA) [30]	1.13 21.80	High risk
5-fluorouracil	0.0000006	0.000005 (PEC*) [31] 0.00000064 (PEC**) [31]	8.30 1.06	High risk

PEC* calculated according to the EMEA guidelines – with consideration of daily use only (not taking into account biodegradation, metabolism)

PEC** calculated according to the EMEA guidelines – with consideration of market penetration factor *Fpen* (not taking into account biodegradation, metabolism)

DISCUSSION

Ecotoxicological research on three pharmaceuticals (ciprofloxacin, 17α-ethinylestradiol, and 5-fluorouracil) carried out in this study showed their harmful effect on aquatic animals. Acute test results of all tested active compounds confirmed previous reports, which indicated low risk to aquatic biota at short-term exposure, even at high concentrations [19]. The values of $LC(EC)_{50}$ were even several orders of magnitude higher than EC_{50} derived from chronic tests. In 48-hour *Daphnia magna* immobilization test, EC_{50} was higher than 100 mg/l for ciprofloxacin and 5-fluorouracil. For 17α-ethinylestradiol, EC_{50} was 14.28 mg/l. In 28-day reproduction test, EC_{50} results were 14.4 mg/l, 1.89 mg/l and 0.0015 mg/l, respectively.

The literature data on acute toxicity of tested pharmaceuticals are diversified. Khetan and Collins (ciprofloxacin), Webb *et al.* (17α-ethinylestradiol) and Zounková *et. al.* (5-fluorouracil) reported EC_{50}-48h values: 10 mg/l, 6.4 mg/l and 36 mg/l, respectively, in *D. magna* immobilization test [13, 33, 36]. Our study demonstrated lower values. According to the study published by Halling-Sørensen *et al.,* NOEC for ciprofloxacin from 48-hour acute test was 60 mg/l. The results of fish survival tests (exposure to ciprofloxacin and estrogen) were comparable with those obtained by other authors [9].

There are few studies on drugs ecotoxicity referring to chronic tests. The reported 7-day EC_{50} value in the juvenile growth test for ciprofloxacin was 10 mg/l [13], while in our study NOEC for *Lebistes reticulatus* was 0.760 mg/l. In the same test, 120-hour EC_{50} for 5-fluorouracil equaled 400 mg/l (LOEC - 20 mg/l) [36], while in our study, after 28 days, NOEC was < 1.56 mg/l. Moreover, very low NOEC value (0.000006 mg/l) was observed in the 28-day reproduction test with *Daphnia magna*. A higher value (0.0028 mg/l) was presented by Jergentz *et al.* [12]. Such a difference may occur due to the longer exposure in our study.

There is no information in the literature about the impact of the tested compounds on protozoa. In this study, they proved to be very sensitive, especially to the action of

17α-ethinylestradiol and 5–fluorouracil (just as *D. magna*). Therefore, it seems reasonable to include unicellular animals to the battery of tests used in ERA.

The NOEC values determined in chronic toxicity tests are several orders of magnitude lower than $LC(EC)_{50}$ obtained in acute tests. This fact makes it impossible to extrapolate acute to chronic toxicity with the application of ACR (Acute to Chronic Ratio) factor = 10, commonly used and accepted by the EU and OECD [3, 25]. The analysis of available data showed that ACR values are often much higher than 10, and show high volatility in relation to different compounds (e.g. 77 and 2820 for effects of acetylsalicylic acid and clofibric acid on *Daphnia magna*, respectively) [20, 34]. Therefore, calculation of PNEC, based on NOEC values determined in chronic tests, is recommended. This parameter is essential for risk assessment.

The conducted assessment revealed high risk to aquatic animals for 17α-ethinylestradiol and 5-fluorouracil, and low risk for ciprofloxacin. A similar result for 5-fluorouracil was reported by Straub (RQ = 5) [31]. The hazard assessment performed by Robinson *et al*. [29] on fish and crustaceans (*Pimephales promelas*, *D. magna*) revealed low hazard for ciprofloxacin at environmental concentrations (100 μg/l and 1μg/l).

The study revealed that long-term presence of pharmaceuticals in aquatic ecosystems may adversely affect the animals, and consequently lead to changes in biodiversity. Therefore, reliable evaluation of real hazard requires constant monitoring of these pollutants, development of analytical methods, and modeling for accurate PEC determination, as well as investigation of chronic toxicities of pharmaceuticals in respect to aquatic organisms, particularly in relation to changes in the tissues and organs, and in immunology.

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OCENA RYZYKA WYWOŁANEGO OBECNOŚCIĄ WYBRANYCH FARMACEUTYKÓW W WODACH POWIERZCHNIOWYCH W STOSUNKU DO ZWIERZĄT

W pracy podjęto problem obecności leków antybakteryjnych, estrogenów i cytostatyków w wodach powierzchniowych i ich oddziaływania na organizmy zwierzęce. Zbadano ekotoksyczność trzech substancji aktywnych: cyprofloksacyny, 17 α-etynyloestradiolu i 5-fluorouracylu w odniesieniu do pierwotniaków, skorupiaków i ryb. Przeprowadzono testy ostre: immobilizacji ze skorupiakami, przeżywalności z rybami, enzymatyczny z *Daphnia magna*, oraz chroniczne: wzrostowy z pierwotniakami, reprodukcji ze skorupiakami i wzrostu narybku z dwoma gatunkami ryb. Badania toksyczności ostrej wykazały zróżnicowaną wrażliwość organizmów – najbardziej oporne na działanie wszystkich badanych związków były skorupiaki *Artemia salina*, małą wrażliwość na działanie cyprofloksacyny i 5-fluorouracylu wykazały również ryby – ($LC(EC)_{50}$-96h > 100mg/l). Największą szkodliwością w stosunku do ryb i skorupiaków w badaniach przeżywalności charakteryzował się 17 α-etynyloestradiol, a w badaniach enzymatycznych cyprofloksacyna. We wszystkich testach chronicznych stwierdzono działanie toksyczne badanych leków. Związki te ograniczały reprodukcję skorupiaków, wzrost pierwotniaków i narybku. Ocena ryzyka dokonana na podstawie ilorazu PEC/PNEC wykazała duże ryzyko w stosunku do zwierząt wodnych wywołane obecnością 17 α-etynyloestradiolu i 5-fluorouracylu w wykrywanych stężeniach w wodach powierzchniowych.