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Ecotoxicity of chosen pharmaceuticals in relation to micro-organisms—risk assessment

Monika Załęska-Radziwiłł[a,*], Katarzyna Affek[a], Justyna Rybak[b]

[a]*Faculty of Environmental Engineering, Department of Biology, Warsaw University of Technology, Nowowiejska 20, 00-653 Warsaw, Poland*
Tel. +48 22 2347903, +48 22 6212979; email: monika.radziwill@is.pw.edu.pl
[b]*Institute of Meteorology and Water Management—National Research Institute, Podleśna 61, 01-673 Warsaw, Poland*

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ABSTRACT

The aim of this study is to assess the impact of three pharmaceutical substances from the group of antibacterial drugs (ciprofloxacin), estrogens (17α-ethinylestradiol) and cytostatic drugs (5-fluorouracil) on micro-organisms. Enzymatic tests (bioluminescence tests with *Vibrio fischeri*, dehydrogenase and hydrolytic activity of activated sludge organisms) and growth tests (with *Pseudomonas fluorescens* and microbial assay for toxic risk assessment with 10 species of bacteria and 1 species of fungi) were performed. The obtained values of concentrations of EC_{50}-t and NOEC showed a different sensitivity of the organisms to the examined active substances. According to the EU criteria, ciprofloxacin was extremely toxic and very toxic to nine species and toxic to three species of bacteria. 17α-ethinylestradiol was extremely toxic and toxic to five species and also harmful to five species. 5-fluorouracil proved to be extremely toxic to seven micro-organisms and very toxic and toxic to five. All active ingredients were non-toxic to activated sludge micro-organisms. The lowest NOEC values for ciprofloxacin and 17α-ethinylestradiol equalled 0.0015 μg/L (*V. fischeri*), and for 5-fluorouracil 0.08 μg/L (*K. gibsonii*). The risk assessment, conducted on the basis of the PEC/PNEC quotient, showed a significant risk in relation to micro-organisms caused by the presence of ciprofloxacin and 17α-ethinylestradiol in concentrations detected in surface waters.

Keywords: Ecotoxicity; Ciprofloxacin; 17α-Ethinylestradiol; 5-Fluorouracil; Micro-organisms; Risk assessment

1. Introduction

The last three decades have been characterized by rapid development of the pharmaceutical industry and have showed a significant increase in the use of drugs in the treatment of humans and animals. It is estimated that the number of pharmaceuticals produced worldwide is about 200,000, and in Poland in 2001, about 2,500 pharmaceutical products were produced [1]. In Germany, out of 50,000 registered pharmaceuticals 2,700 are used and they cover 90% of the consumption of all drugs. They contain about

*Corresponding author.

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900 different active substances, which corresponds to the production of 38,000 ton/y [2]. In the UK, Germany and Austria the annual production of pharmaceuticals is over 100 tons [3].

Consumption of particular groups of pharmaceuticals throughout the year varies in different countries from several dozen to several hundred tons [4]. The annual consumption of antibiotics worldwide amounts to 200,000 ton [5], in Europe—10,000 ton, half of which are antibiotics used in animal breeding. In 2003, a statistical pole bought 29 packages of drugs [1].

Increasing production, large availability and widespread use of medicinal products resulted in the appearance of pharmaceuticals in the elements of the environment. It is alarming that an increase in concentrations of pharmaceuticals was recorded in surface waters [6–8], and their detection in groundwater [9].

Concentration of pharmaceuticals' active substances in surface waters ranges from ng/L to μg/L [10]. In the environment, there are also present metabolites, products of pharmaceuticals' degradation and chemical compounds formed in reactions which occur, among others, in the process of water treatment—formation of halogenated drugs during chlorination of drinking water, including salicylic acid [11].

Some of these (including metabolites of carbamazepine, diclofenac and acetaminophen) are still biologically active, and frequently, as it is in the case of products of chlorination of acetaminophen, have a higher toxicity than the matrix [12]. Certain drugs are resistant to the impact caused by abiotic and biotic elements. They are not removed during wastewater treatment and water self-purification, and are detected in surface waters and water intended for consumption [13,14]. Drugs used in veterinary medicine are often introduced directly into surface waters, for instance, the antibiotics given in food in fish farming. Washed from fields fertilized with animal manure or as a result of the use of wastewater sludge, they also reach surface waters.

Antibiotics present in wastewater and surface waters create favourable conditions for an increase in drug resistance of micro-organisms since they interfere with wastewater treatment processes and the ecological balance in surface waters [15].

Despite an increasing number of studies on the effects of drugs on aquatic organisms in recent years, the knowledge regarding their harmful effect is still limited. Data sheets of drugs' active substances most often include scarce toxicological data, and there are no results of ecotoxicity studies. Available data from relevant literature relate to a small percentage of pharmaceutical substances. It usually includes a small set of test organisms, often insufficiently sensitive to drugs. Particularly, there is a lack of information about chronic ecotoxicological effects caused by long-term and multigenerational exposure of aquatic organisms to active substances [16]. For the majority of pharmaceutical substances, there is still no reliable ecological risk assessment. Most of the available ecotoxicological data relates to animal studies; particularly limited is the information on the effects of medicinal products on the physiological processes of micro-organisms.

European Medicines Agency (EMEA) guidelines from 1998 to 2006 including a set of toxicological analyses to assess the risk posed by the presence of medicinal products in surface waters suggest conducting chronic tests on protozoa, crustaceans and fish [17,18]. Tests with the use of micro-organisms are recommended in relation to antibiotics. It seems that the lack of ecotoxicological data regarding decomposers in relation to other medicinal substances prevents the development of a full environmental risk profile associated with the presence of drugs in aquatic ecosystems.

Micro-organisms play an important role in the processes of biological treatment of wastewater and water self-purification. Thus, micro-organisms may be in fact sensitive to many other pharmaceuticals, not only antibiotics.

The aim of this study was to assess the risk posed by the effects of chosen antibacterial drugs (ciprofloxacin), cytostatics (5-fluorouracil) and estrogens (17α-ethinylestradiol) on micro-organisms.

Ciprofloxacin belongs to the group of fluoroquinolones—antibiotics, whose antibacterial effect is based on DNA topoisomerase (gyrase) inhibition, involved in biosynthesis of DNA. Ciprofloxacin is the most active drug among the fluoroquinolones, which are mainly used in gram-negative bacterial infections. It produces genotoxic effect in genetic material and induces bacterial resistance to fluoroquinolones, which may be transmitted in the process of horizontal gene transfer [19,20].

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 common use in contraceptive pills. The conducted studies have shown that even trace concentrations of estrogens have an impact on fish reproduction. In the presence of ethinylestradiol 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 [21–23].

Cytostatics are drugs used in anticancer therapy. They inhibit cell proliferation, mainly by interacting

with DNA. Some of them inhibit synthesis of nucleotides, other ones intercalate with 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 tumour cell proliferation [24].

2. Materials and methods

2.1. 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 deionized water or in a buffered saline solution, and further diluted with corresponding test media. Appropriate solvent controls were tested.

2.2. Toxicological tests with the use of bacteria and fungi

Enzymatic and growth tests (acute and chronic) were performed with 12 species of bacteria and 1 species of fungi (yeast) as well as activated sludge micro-organisms. Lyophilized strains of bacteria and fungi came from kits supplied by the manufacturers of the tests. *Pseudomonas fluorescens* bacteria and activated sludge micro-organisms came from the own laboratory culture of the Department of Biology, Faculty of Environmental Engineering, Warsaw University of Technology.

2.2.1. Bioluminescence test with V. fischeri—LUMIStox

LUMIStox test was performed in accordance with the methodology included in the implementing instruction provided by Dr Lange (Germany) [25]. The assessment of the bioluminescence inhibition was conducted after 15, 30 min and 24 h of bacteria incubation with toxic substances. Calculation of the bioluminescence inhibition was performed with the use of LUMISsoft II software.

In addition in a 24-h test, an assessment of *V. fischeri* bacterial growth inhibition was conducted in accordance with LUMIS.24.tox test instruction on the basis of initial and final values of the optical density of bacteria suspension measured in a spectrophotometer with the wave length 585 nm.

Growth inhibition (I) was calculated according to the following equation:

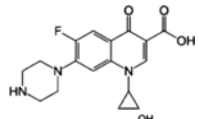
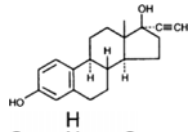
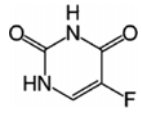
$$I = \frac{B_c - B_n}{B_c - B_0} \times 100\% \quad (1)$$

where B_c—optical density of suspension in control sample after time t; B_n—optical density of suspension in the sample examined after time t; B_0—optical density of suspension in control in time 0.

2.2.2. Microbial assay for toxic risk assessment (MARA) growth test

MARA test with the use of lyophilized strains of bacteria and fungi (Table 2) was performed in accordance with the methodology included in the implementing instruction provided by NCIMB (UK, 2008) [26]. The assessment of the micro-organism growth inhibition was conducted after 18 h of incubation with

Table 1
Basic information about the tested chemicals

Compound	CAS no.	Molar mass (g/mol)	Molecular formula	Structural formula
Ciprofloxacin	85721-33-1	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	$C_4H_3FN_2O_2$	

Note: CAS—Chemical Abstracts Services.

Table 2
List of species of bacteria and fungi used in MARA test

MARA No.	Species
1	*Microbacterium* sp.
2	*Brevundimonas diminuta*
3	*Citrobacter freundii*
4	*Comamonas testosteroni*
5	*Enterococcus casseliflavus*
6	*Delftia acidovorans*
7	*Kurthia gibsonii*
8	*Staphylococcus warneri*
9	*Pseudomonas aurantiaca*
10	*Serratia rubidaea*
11	*Pichia anomala*

toxic substances on the basis of measurement of the surface area of the sludge in the wells of the microplate, in which tetrazolium red contained in the culture medium was reduced.

Image analysis was performed with the use of MARA Software ver. 2.01.

2.2.3. Growth test with P. fluorescens

Growth test with *P. fluorescens* bacteria was performed in accordance with the methodology contained in ISO 107122-1994 for *Pseudomonas putida* [27]. The assessment of growth inhibition was conducted on the basis of measurement of the optical density of samples with $\lambda = 610$ nm at the beginning and at the end of the 16-h test.

Growth inhibition (I) was calculated according to Eq. (1).

2.2.4. Dehydrogenase specific activity test of activated sludge micro-organisms with TTC

A test with TTC was performed in accordance with the methodology contained in PN-C-04616-8:2008 [28]. The assessment of dehydrogenase specific activity inhibition was conducted after 30 min and additionally after 24 h of micro-organisms' contact with toxic compounds.

Inhibition of dehydrogenase specific activity (I_A) was calculated according to the following equation:

$$I_A = \frac{A_K - A_B}{A_K} \times 100\% \quad (2)$$

where A_K—specific enzymatic activity in control sample after time t; A_B—specific enzymatic activity in tested sample after time t.

2.2.5. Hydrolytic activity test of activated sludge micro-organisms with FDA

A hydrolytic activity test of activated sludge micro-organisms with fluoresceine diacetate (FDA) was performed in accordance with the methodology provided by Schnürer and Rosswall [29]. The assessment of hydrolases specific activity inhibition was conducted after 30 min and after 24 h of micro-organisms' incubation with pharmaceuticals' active substances.

Inhibition of hydrolases specific activity (I_A) was calculated according to Eq. (2).

2.3. Calculation of EC_{50} and NOEC

Effective concentrations (EC_{50}) in acute and chronic tests were calculated using probit analysis, determining 95% confidence intervals [30].

No observed effect concentrations (NOEC-t) were determined using single factor analysis of variance Anova ($p < 0.05$) and Tukey's test [31].

2.4. Toxicity assessment of compounds

The assessment of toxicity of the test pharmaceuticals in relation to bioindicators was performed on the basis of the EU criteria—Directive 93/67/EEC (Table 3) [32].

2.5. Risk assessment—risk quotient RQ

RQ was calculated for predicted or measured concentrations of PEC (MEC) of pharmaceuticals according to the equation:

$$RQ = \frac{PEC}{PNEC} \quad (3)$$

where PEC—predicted (or measured) environmental concentration; PNEC—predicted no effect concentration in the environment.

Table 3
Assessment of toxicity of chemicals in relation to the criteria of their harmfulness to aquatic biocenoses according to EU

EC_{50} (μg/L)	Assessment of toxicity of chemical
<100	Extremely toxic
100–1,000	Very toxic
>1,000–10,000	Toxic
>10,000–100,000	Harmful
>100,000	Non-toxic

The results were interpreted according to the following criteria:

RQ ≥ 1—high risk;
RQ < 1—low risk.

PNEC was calculated from chronic toxicity data (NOEC) by obligatory safety factor method, using (assessment factor AF = 10, due to the quality and quantity of toxicological data [33].

PEC was assumed to be equal to the concentrations detected in surface waters (MEC) or calculated according to EMEA guidelines—Table 4.

Table 4
Predicted or measured concentrations of tested pharmaceuticals in surface waters

Compound	PEC (MEC) in surface waters (μg/L)	Reference
Ciprofloxacin	0.03 (MEC USA)	[34]
	0.06 (MEC Germany)	[35]
	0.67 (PEC*)	[36]
17α-Ethinylestradiol	0.043 (MEC UE)	[34]
	0.831 (MEC USA)	
5-Fluorouracil	0.005 (PEC[a])	[37]
	0.00064 (PEC[b])	

[a]PEC calculated according to the EMEA guidelines—with consideration of daily use only (not taking into account biodegradation, metabolism).
[b]PEC calculated according to the EMEA guidelines—with consideration of market penetration factor *Fpen* (not taking into account biodegradation, metabolism).

3. Results and discussion

Obtained values of EC_{50}-t and NOEC-t showed different sensitivity of micro-organisms to the tested active substances (Tables 5–7). In acute enzymatic tests, both the stimulation and inhibition of enzymatic processes were observed. Ciprofloxacin stimulated bioluminescence of bacteria, 5-fluorouracil inhibited the process in 19% in the highest tested concentration. EC_{50}-30 min was in both cases > 100,000 μg/L (Tables 5 and 7). Hernando et al. in a standard acute test with *V. fischeri* obtained in the case of ciprofloxacin EC_{50} > 5,900 μg/L [38].

17α-ethinylestradiol visibly limited the bioluminescence of micro-organisms (Table 6). EC_{50}-30 min amounted to 19,009 μg/L and it was an order of magnitude lower than the one defined by Escher et al. —296,410 μg/L [39]. The compound also caused a ~20% inhibition of dehydrogenase activity and a ~15% inhibition of hydrolytic enzymes activity of activated sludge micro-organisms in the highest tested

Table 5
Ecotoxicity of ciprofloxacin in relation to micro-organisms

Ciprofloxacin						
No.	Tested organism	Test character	Test duration	EC_{50}-t (μg/L) (95% confidence interval)	NOEC (μg/L)	Toxicity assessment UE Directive 93/67/EEC
1	Activated sludge micro-organisms	Enzymatic (dehydrogenase activity)	30 min/24 h	>100,000	–	Non-toxic
2	Activated sludge micro-organisms	Enzymatic (hydrolytic activity)	30 min/24 h	>100,000	–	
3	*Vibrio fischeri*	Enzymatic (bioluminescence)	15 min/30 min	>100,000	–	
4	*Pichia anomala*	Growth	18 h	>10,000	1,250	Harmful
5	*Brevundimonas diminuta*	Growth	18 h	5057.0 (4731.1–5337.2)	156.3	Toxic
6	*Enterococcus casseliflavus*	Growth	18 h	5023.6 (4631.3–5423.2)	156.3	
7	*Staphylococcus warneri*	Growth	18 h	1528.6 (1410.6–1685.1)	9.8	
8	*Kurthia gibsonii*	Growth	18 h	370.4 (341.5–398.8)	4.9	Very toxic
9	*Serratia rubidaea*	Growth	18 h	265.6 (242.3–281.3)	39.1	
10	*Pseudomonas aurantiaca*	Growth	18 h	150.0 (142.1–156.4)	39.1	
11	*Microbacterium* sp.	Growth	18 h	144.1 (141.1–148.3)	19.5	
12	*Comamonas testosteroni*	Growth	18 h	56.1 (53.0–58.9)	2.4	Extremely toxic
13	*Delftia acidovorans*	Growth	18 h	6.2 (5.1–7.1)	1.2	
14	*Citrobacter freundii*	Growth	18 h	4.6 (3.4–5.8)	0.04	
15	*Vibrio fischeri*	Growth	24 h	1.4 (1.3–1.6)	0.0015	
16	*Pseudomonas fluorescens*	Growth	16 h	0.175 (0.158–0.185)	0.005	
17	*Vibrio fischeri*	Enzymatic (bioluminescence)	24 h	0.0137 (0.0119–0.0149)	0.0015	

Table 6
Ecotoxicity of 17α-ethinylestradiol in relation to micro-organisms

17α-Ethinylestradiol						
No.	Tested organism	Test character	Test duration	EC_{50}-t (μg/L) (95% confidence interval)	NOEC (μg/L)	Toxicity assessment UE Directive 93/67/EEC
1	Activated sludge micro-organisms	Enzymatic (dehydrogenase activity)	30 min/ 24 h	>100,000	–	Non-toxic
2	Activated sludge micro-organisms	Enzymatic (hydrolytic activity)	30 min/ 24 h	>100,000	–	
3	*Enterococcus casseliflavus*	Growth	18 h	>100,000	390.6	
4	*Brevundimonas diminuta*	Growth	18 h	>100,000	1562.5	
5	*Pseudomonas aurantiaca*	Growth	18 h	>100,000	6,250	
6	*Serratia rubidaea*	Growth	18 h	66170.2 (61121.1–71356.9)	781.3	Harmful
7	*Microbacterium* sp.	Growth	18 h	51880.0 (46156.1–55920.3)	6,250	
8	*Kurthia gibsonii*	Growth	18 h	32336.2 (27448.1–37562.3)	1562.5	
9	*Vibrio fischeri*	Enzymatic (bioluminescence)	15 min	23581.8 (21279.9–2556.7)	–	
10	*Pichia anomala*	Growth	18 h	20458.3 (18468.4–21856.1)	1562.5	
11	*Vibrio fischeri*	Enzymatic (bioluminescence)	30 min	19009.0 (17501.5–21010.7)	–	
12	*Staphylococcus warneri*	Growth	18 h	17849.3 (16931.1–18348.3)	48.8	
13	*Delftia acidovorans*	Growth	18 h	7307.7 (6801.3–7631.4)	97.7	Toxic
14	*Comamonas testosteroni*	Growth	18 h	4,364 (3,921–4,665)	48.8	
15	*Citrobacter freundii*	Growth	18 h	1088.5 (993.1–1166.3)	6.1	
16	*Pseudomonas fluorescens*	Growth	16 h	1037.1 (1026.1–1066.1)	24.4	
17	*Vibrio fischeri*	Growth	24 h	2.7 (2.3–3.1)	0.095	Extremely toxic
18	*Vibrio fischeri*	Enzymatic (bioluminescence)	24 h	0.391 (0.351–0.443)	0.0015	

concentrations. Ciprofloxacin also caused a slight inhibition of activity of those enzymes—EC_{50}-30 min for both compounds was > 100,000 μg/L (Tables 5 and 6), whereas 5-fluorouracil caused an increase in the enzymes' activity, especially in the highest concentrations.

Chronic tests proved a much greater impact of pharmaceuticals on micro-organisms. In a 24-h bioluminescence test it was ciprofloxacin that was most toxic to *V. fischeri*—it inhibited luminescence of the bacteria in 50% at the concentration of 0.0137 μg/L, and then 17α-ethinylestradiol, for which EC_{50} equalled 0.391 μg/L. EC_{50}-24 h for 5-fluorouracil equalled 16.0 μg/L and it was several times lower than the one published by Backhaus and Grimme (0.122 mg/L) [40]. Ciprofloxacin and 5-fluorouracil also inhibited dehydrogenase activity of activated sludge micro-organisms in 24.85 and 42.53%, respectively, in the highest tested concentrations, and EC_{50}-24 h in both cases equalled > 100,000 μg/L (Tables 5 and 7). In research by Halling-Sørensen et al. EC_{50}-t of ciprofloxacin for activated sludge bacteria was determined as 610 μg/L [36].

Diverse reactions of micro-organisms to pharmaceuticals were also observed in growth tests. Ciprofloxacin inhibited growth of all the bacteria. The highest sensitivity was shown in the case of *P. fluorescens*—EC_{50}-16 h equalled 0.175 μg/L. An experiment conducted by Kűmmerer et al. also showed some inhibition of multiplication of *Pseudomonas putida* in the presence of ciprofloxacin with EC_{50} equalling 80 μg/L [35]. In addition they noticed an impact of the antibiotic on DNA of *Escherichia coli* mutants at concentrations 0.2–0.4 μg/L (SOS-Chromotest). The values EC_{50}-18 h obtained in the course of this study in MARA test ranged from 4.6–5,057 μg/L, for *Citrobacter freundii* and *Brevundimonas diminuta*, respectively. It was *Pichia anomala* (yeast) which was most resistant to

Table 7
Ecotoxicity of 5-fluorouracil in relation to micro-organisms

5-Fluorouracil						
No.	Tested organism	Test character	Test duration	EC_{50}-t (μg/L) (95% confidence interval)	NOEC (μg/L)	Toxicity assessment UE Directive 93/67/EEC
1	Activated sludge micro-organisms	Enzymatic (dehydrogenase activity)	30 min/ 24 h	>100,000	–	Non-toxic
2	Activated sludge micro-organisms	Enzymatic (hydrolytic activity)	30 min/ 24 h	>100,000	–	
3	*Vibrio fischeri*	Enzymatic (bioluminescence)	15 min/ 30 min	>100,000	–	
4	*Vibrio fischeri*	Growth	24 h	10421.8 (9542.1–10967.9)	6,100	Harmful
5	*Brevundimonas diminuta*	Growth	18 h	>10,000	625	
6	*Comamonas testosteroni*	Growth	18 h	2648.3 (2550.3–2710.6)	312.5	Toxic
7	*Staphylococcus warneri*	Growth	18 h	1681.5 (1620.1–1730.3)	9.8	
8	*Pseudomonas aurantiaca*	Growth	18 h	695.6 (665.3–721.4)	19.5	Very toxic
9	*Citrobacter freundii*	Growth	18 h	326.9 (301.3–338.9)	4.9	
10	*Pichia anomala*	Growth	18 h	242.7 (228.7–256.6)	39.1	
11	*Serratia rubidaea*	Growth	18 h	82.2 (74.4–89.2)	9.8	Extremely toxic
12	*Delftia acidovorans*	Growth	18 h	45.7 (41.6–49.3)	9.8	
13	*Microbacterium* sp.	Growth	18 h	37.3 (34.4–39.9)	9.8	
14	*Vibrio fischeri*	Enzymatic (bioluminescence)	24 h	16.0 (14.4–17.3)	1.5	
15	*Enterococcus casseliflavus*	Growth	18 h	11.8 (10.9–12.4)	0.08	
16	*Pseudomonas fluorescens*	Growth	16 h	8.3 (7.5–8.9)	1.3	
17	*Kurthia gibsonii*	Growth	18 h	3.6 (3.2–4.0)	0.08	

Table 8
Risk assessment for ciprofloxacin, 17α-ethinyloestradiol and 5-fluorouracil in relation to micro-organisms

Compound	PNEC (μg/L)	PEC (MEC) in surface waters (μg/L)	RQ (PEC/PNEC)	Risk assessment
Ciprofloxacin	0.00015	0.03	200	High risk
		0.06	400	
		0.67	4,467	
17α-Ethinylestradiol	0.00015	0.043	287	High risk
		0.831	5,540	
5-Fluorouracil	0.008	0.005	0.625	Low risk
		0.00064	0.08	

the antibiotic—EC_{50}-18 h was > 10,000 μg/L (Table 5). Nałęcz-Jawecki et al. examined different antibiotics with MARA test and noticed some growth of inhibition of sensitive strains of micro-organisms caused by ciprofloxacin at concentrations 12–75 μg/L [41]. The most sensitive species, like in this study, appeared to be *Citrobacter freundii* strain. A detrimental effect of the antibiotic on natural microbial communities was observed in the study of mineralization of pyrene in natural marine sediments (EC_{50} = 560 μg/L) [42].

17α-Ethinylestradiol proved to be less toxic to most micro-organisms than other pharmaceuticals. The lowest value of concentration inhibiting growth by 50% compared to the control was obtained in test with *P. fluorescens*—EC_{50}-16 h = 1037.1 μg/L. A similar sensitivity was shown by species *C. freundii*. For three species of bacteria in MARA test EC_{50}-18 h were > 100,000 μg/L (Table 6).

5-Fluorouracil inhibited growth both of bacteria and fungi; the bacteria affected most was *Kurthia*

gibsonii in MARA test (EC_{50}-18 h = 3.6 μg/L) and *P. fluorescens* (EC_{50}-16 h = 8.3 μg/L) (Table 7). Zaunková et al. in turn obtained in growth test with *P. putida* the value EC_{50} equal 27 μg/L [24].

The lowest NOEC values were 0.0015 μg/L (*V. fischeri*) for ciprofloxacin and 17α-ethinylestradiol, and 0.08 μg/L (*K. gibsonii).*

According to the EU criteria, ciprofloxacin was extremely and very toxic to nine species and toxic to three species of bacteria. It showed weak toxicity only in relation to *P. anomala* (Table 5).

17α-Ethinylestradiol, which is characterized by a lower toxicity with respect to bioindicators, was extremely toxic and toxic to five species and harmful to five species of micro-organisms as well (Table 6).

5-Fluorouracil proved to be extremely toxic to seven species of micro-organisms, and very toxic and toxic to five (Table 7).

All the active substances were non-toxic for activated sludge micro-organisms.

To assess the risk posed by the presence of the researched active substances in surface waters their PNEC concentrations were calculated by dividing the lowest NOEC value by AF (assessment factor) = 10. As PEC concentrations of the examined pharmaceuticals detected in surface waters worldwide, or calculated in accordance with EMEA guidelines (Table 4), were adopted. This demonstrated a significant risk associated with the presence in surface waters of ciprofloxacin and 17α-ethinylestradiol and low risk for 5-fluorouracil (Table 8). Backhaus and Grimme while studying toxicity of antibiotics with respect to *V. fischeri* in a 24-h bioluminescence test compared concentrations detected in the environment with EC_{10} values from tests and found that antibiotics may have a harmful effect on natural microbial communities [40]. They also proved disruptions to the process of nitrification in concentrations of antibiotics detected in the sediments of ponds for fish farming [43].

The results of this study showed that the harmful impact of pharmaceuticals' active substances on micro-organisms depends not only on the type of drug, but also on the species of micro-organisms, and on the duration of the compounds activity. Kümmerer et al. found that even within a group of pharmaceuticals having the same mode of action, toxicity testing should be carried out separately for each substance, since micro-organisms may differ in sensitivity [43]. The enzymatic tests conducted in the study, bioluminescence test, dehydrogenase activity test and hydrolytic activity test, show that proper assessment of the impact of pharmaceuticals' active substances on micro-organisms' enzymatic processes requires a longer contact with drugs' active substances. A similar regularity was observed in the studies by Kümmerer et al. in which they used OECD 209 respiratory test to assess the ecotoxicity of antibiotics, disinfectants and cytotoxins [43]. Also Backhaus et al. while comparing the results of drugs research in a 30-min and 24-h bioluminescence test with *V. fischeri* found that values EC_{50} from the chronic test were even 1,000-fold lower than those obtained in the acute test [44].

This study also shows that even in the long term, activated sludge micro-organisms are less sensitive to drugs than pure strains. Ricco et al. while comparing OECD 209 respiratory method used for the assessment of toxicity of several chemical substances with the use of activated sludge micro-organisms with Microtox method with *V. fischeri* also proved that the bioluminescence test is more sensitive than the respirometric test [45]. It results from a higher sensitivity of *V. fischeri* compared to that of activated sludge micro-organisms.

4. Conclusions

The results obtained in this study clearly indicate a need to conduct an ecotoxicological risk assessment due to the presence of pharmaceuticals in surface waters in relation to micro-organisms for all active substances contained in drugs. The compounds tested —antibiotic, oestrogen and cytostatic, posed a major threat to many species of bacteria, especially in the long term. The obtained concentration values EC_{50} and NOEC exclude the possibility of extrapolating the results of acute toxicity to those of chronic toxicity with the commonly used AF equals 10.

Analysing the results obtained at work and the data from the relevant literature, it should be noted that OECD 209 respiratory test with activated sludge micro-organisms recommended for risk assessment in EMEA guidelines is not sufficient to protect the biodiversity of natural microbial communities. In order to protect them much broader studies should be conducted on individual strains of micro-organisms, not only the conventional, but also the molecular ones, which would provide explanation of the mechanisms behind pharmaceuticals' impact on micro-organisms, including dangerously growing resistance to drugs.

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