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Study of the effect of residues of pharmaceuticals on the environment on the example of bioassay Microtox®

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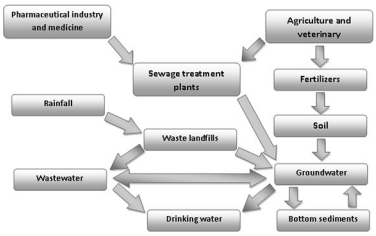
Abstract Residues of pharmaceuticals present in the aqueous environment can be found in a mixture of different substances wherein drugs not remain inert with respect to each other. In such mixtures, a phenomenon of synergism or antagonism may occur, which can contribute to increase or decrease the overall toxicity of the mixture of drugs. Pharmaceuticals, namely, diclofenac (sodium salt), oxyteracycline hydrochloride, fluoxetine hydrochloride, chloramphenicol, progesterone, estrone, androstendione, ketoprofen, and gemfibrozil, were mixed with each other at different ratios of EC_{50} (effective concentration) and tested using MICROTOX®. Attempt was also made to determine whether a pH change of the sample containing pharmaceuticals affects the total toxicity of the sample. The most toxic mixtures of selected pharmaceuticals proved to be those containing diclofenac and chloramphenicol, reaching up to 90 % of the toxicity against the test organism. Based on results obtained, it can be concluded that the change of pH has a significant effect on the toxicity of androstenedione, gemfibrozil, oxytetracycline hydrochloride, diclofenac, and progesterone.

Graphical abstract



Keywords Drugs · Drug research · Pharmaceuticals' interaction · Ecotoxicity · Ecology · MICROTOX®

Introduction

The average citizen in Poland buys and consumes almost 30 packs of different drugs annually, which puts Poland in fifth place in Europe. Sale of the most popular drugs reaches as much as several hundred tons—for example, annual consumption of ibuprofen, the popular representative of NSAIDs (Non-Steroidal Anti-Inflammatory Drugs), in Germany in 2000 reached 300 tons, 162 tons in England, 58 tons in Poland, and 25 tons in Switzerland [1, 2].

Unfortunately, pharmaceuticals are not completely eliminated in conventional wastewater treatment plants (Fig. 1). The main sources of pharmaceuticals in the environment are medical, veterinary, and utilities waste, which may penetrate into the groundwater due to infiltration and leakage from landfills [3].

Toxic effects caused by the current environment drug residues depend on the affected organism. Evidences

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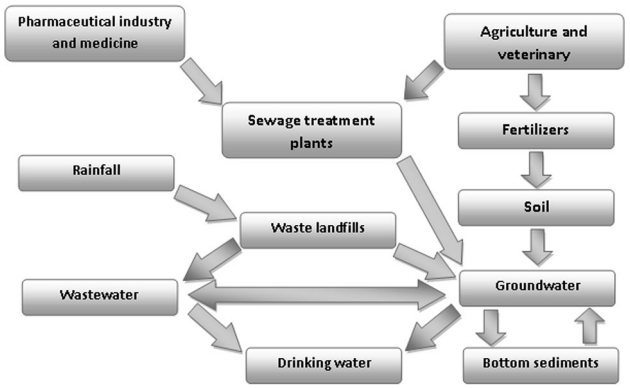

Fig. 1 Pathways of drugs in the environment [3]

indicate that, for example, ibuprofen can inhibit the growth of Gram-positive bacteria at the same time not affecting the growth of colonies of Gram-negative bacteria and can indicate antifungal properties [4, 5].

One of the many serious consequences of drug abuse and their accumulation in the environment is drug resistance and the emergence of pathogens resistant to antibiotics, which seriously hampers the treatment of the diseases [4]. The presence of drug residues in the environment drew attention of the scientific community when it became clear that the presence of drugs at even low concentration levels in the environment (often several ng/dm^3 or mg/dm^3) may result in prolonged adverse effects (e.g., hormones can cause endocrine disruption effects) [5]. Even such low concentrations of drugs in the mixture make them interfere with each other. Action off mixture of compounds can result in amplification (synergism) and masking (antagonism) of total toxic effect with respect to individual compounds [1].

In this study, the effect of the mixture of nine drugs against *Vibrio fischeri* bacteria was measured to identify potential interactions which may occur between the selected pharmaceuticals in contact with the living bacterial organisms.

Results and discussion

The first step to measure the toxicity of a mixture of drugs was the evaluation of EC_{50} parameters, which has been designated by the two research protocols (Figs. 2–10). EC_{50} results obtained for each drug against *Vibrio fischeri* are shown in Table 1.

Mixture of diclofenac and fluoxetine hydrochloride exhibited the highest toxicity towards *Vibrio fischeri* when compared to controls of individual compounds prepared at 100, 66, and 33 % of the EC_{50} (0.037, 0.024, and 0.012 mmol/dm^3 for fluoxetine hydrochloride and 0.72,

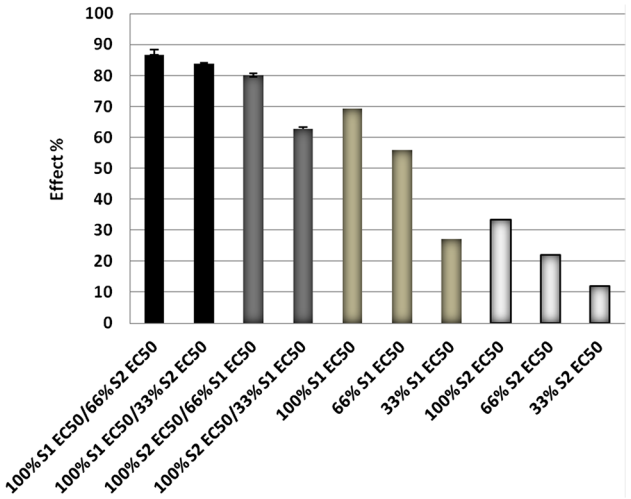

Fig. 2 Results obtained for the mixture of diclofenac (sodium salt) (S1) and oxyteracycline hydrochloride (S2)

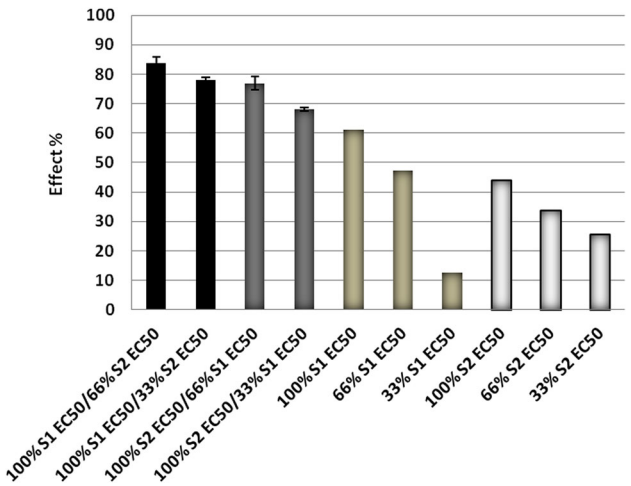

Fig. 3 Results obtained for the mixture of diclofenac (sodium salt) (S1) and chloramfenicol (S2)

0.47, and 0.24 mmol/dm^3 for diclofenac, respectively). The most toxic combination was proven to take place at the ratio of 100 % EC50 of diclofenac and 66 % EC_{50} of fluoxetine. Diclofenac and chloramphenicol belong to four of the nine toxic mixtures, next to mixtures of gemfibrozil and progesterone.

In Figs. 11 and 12, the influence of pH changes on the toxicity of pharmaceuticals in the sample is shown.

Changes of pH contribute to the increase in the toxicity of oxytetracycline and diclofenac, at a pH of 6.5 followed decrease by toxicity within the range of 7–8.5. A significant decrease in toxicity was observed for gemfibrozil in the range of pH 7–8.5. A slight fluctuation of toxicity was observed for ketoprofen, oxytetracycline hydrochloride, and chloramphenicol, but in these cases, it is difficult to

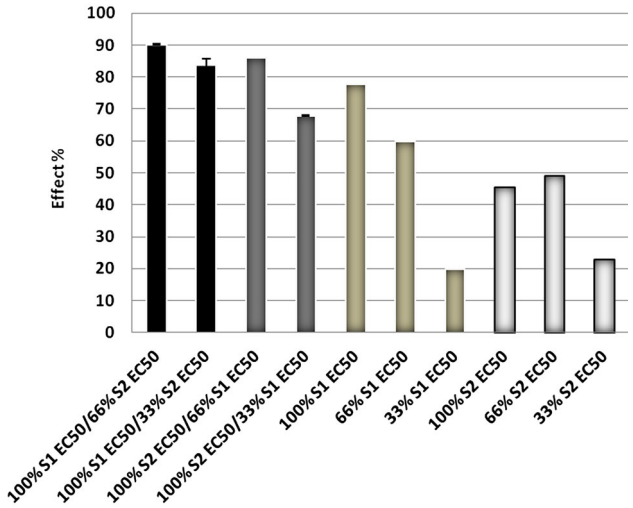

Fig. 4 Results obtained for the mixture of diclofenac (sodium salt) (S1) and fluoxetine hydrochloride (S2)

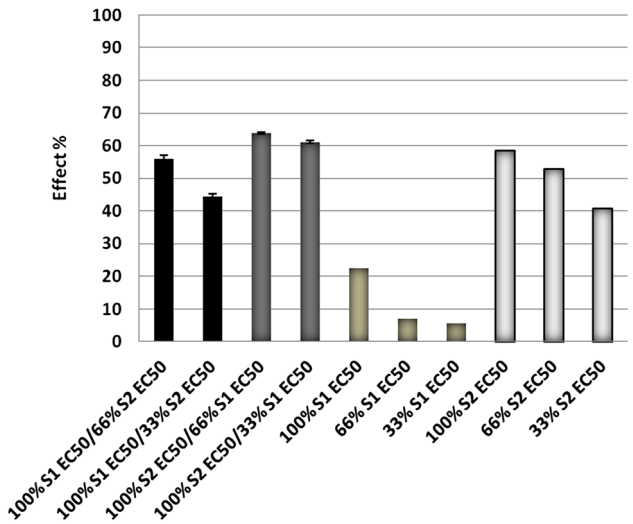

Fig. 5 Results obtained for the mixture of androstenedione (S1) and chloramfenicol (S2)

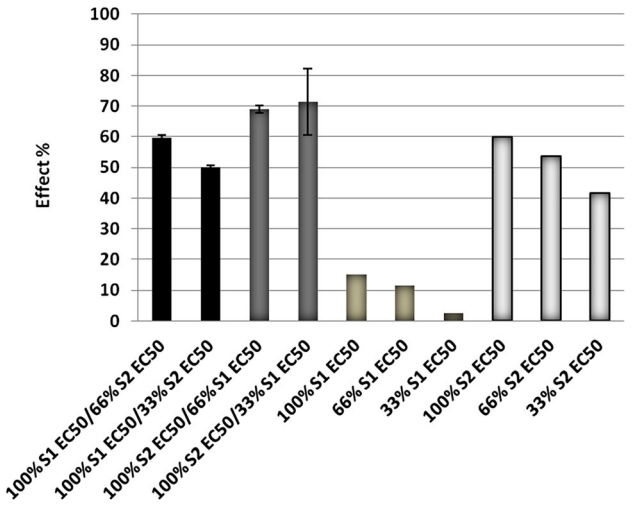

Fig. 6 Results obtained for the mixture of estrone (S1) and chloramfenicol (S2)

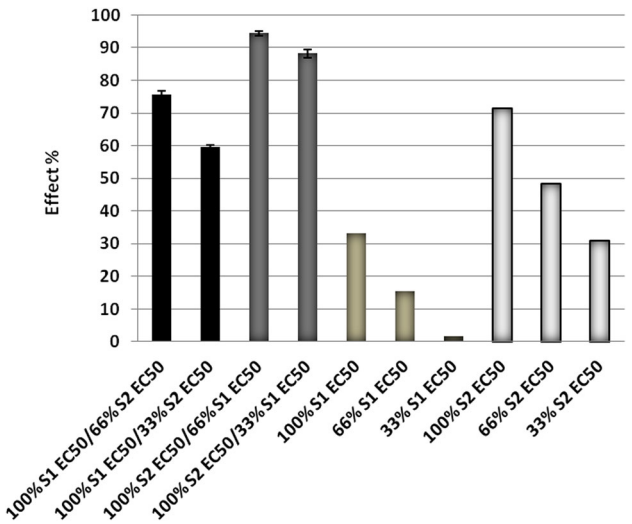

Fig. 7 Results obtained for the mixture of gemfibrozil (S1) and diclofenac (sodium salt) (S2)

conclude about a clear reduction or increase in toxicity. A major decline of toxicity was observed for all examined hormones that initially exhibited toxicity of approx. 10 % of the initial concentration values.

Conclusions

Pharmaceuticals are known to be newly emerging contaminants in the environment; therefore, studies on the environmental fate of pharmaceuticals are both very important ad complicated issue. Interactions between them, such as synergism or antagonism, are again almost not studied in modern environmental science.

In the present study, an effort was undertaken to assess the toxicity interaction of drug mixtures consisting of two drugs. Data obtained with the Microtox® studies justify the statement that drugs in mixture with hormones show mainly antagonistic or synergistic actions, e.g., ketoprofen in mixture with androstenedione and estrone proved to be synergic toxic in its nature, gemfibrozil with progesterone exhibited synergism, while antagonism with estrone, and diclofenac with progesterone exhibited synergic properties and antagonistic ones with estrone.

The continuously increasing concentration of different pharmaceuticals in the environment may contribute to irreversible changes in ecosystems such as described in the literature feminization of male fish or masculinization of

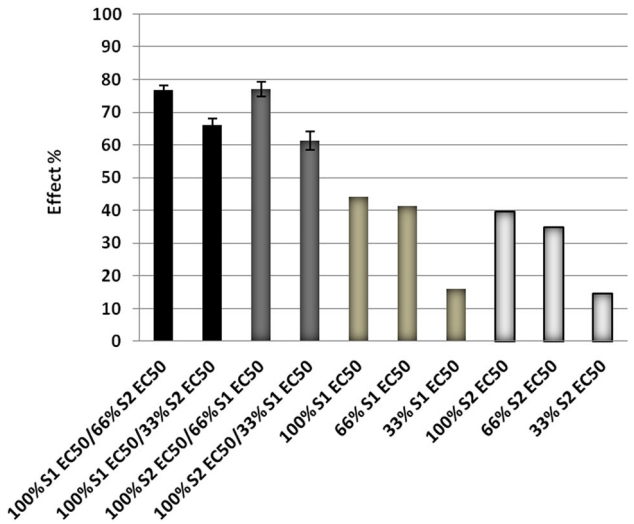

Fig. 8 Results obtained for the mixture of gemfibrozil (S1) and fluoxetine hydrochloride (S2)

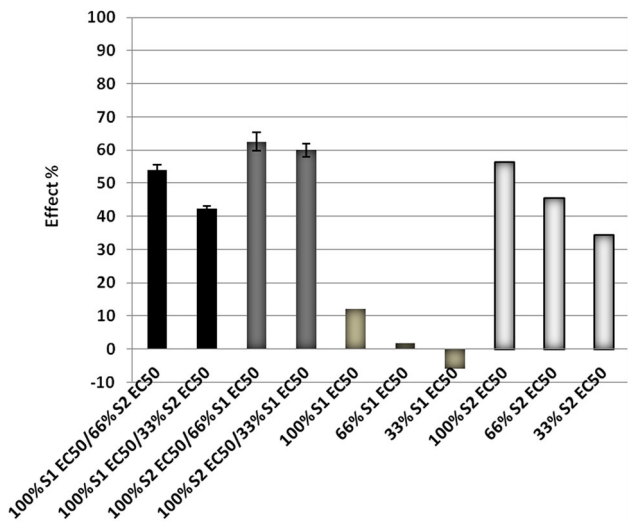

Fig. 9 Results obtained for the mixture of progesterone (S1) and chloramphenicol (S2)

females. Accumulation of different pollutants in particular of compounds exhibiting biological activity may lead to the extinction of sensitive organisms in ecosystems and, consequently, to the total destruction of that ecosystem. Research described in this paper shows that diclofenac (sodium salt) and chloramphenicol indicated the highest toxicity levels against *Vibrio fischeri*. The pH of the environmental compartment also affects the toxicity of the pharmaceuticals and their environmental fate. This study did not aim to find answers on possible mechanism of the combined toxicity of a mixture of drugs plausibly present in the environmental sample, but it can serve as a useful tool for the selection of ecotoxicity tests for assessing the operation of binary drugs mixtures.

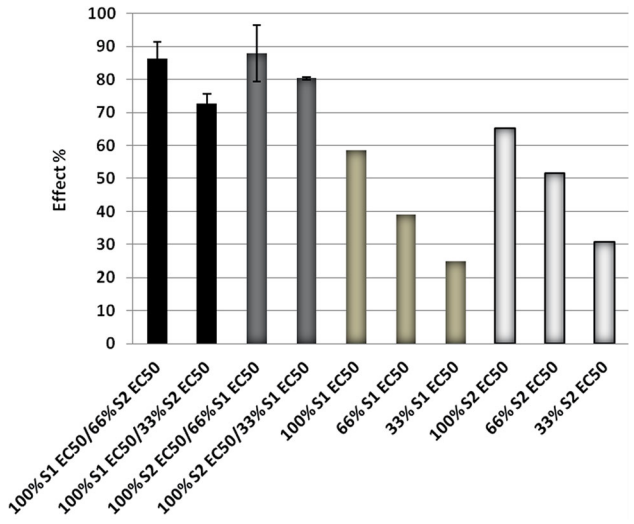

Fig. 10 Results obtained for the mixture of progesterone (S1) and ketoprofen (S2)

Experiment

Vibrio fischeri is a Gram-negative bacilli residing in salty waters. Bioluminescence is a natural result of their metabolic processes. In the Microtox® study, the decrease or increase in the bioluminescence of bacterial suspension after a period of incubation with sample is measured. After incubation period, the reading of the level of bioluminescence and calculating the EC_{50} parameter takes place [6].

Table 2 provides information on the tested pharmaceuticals, namely, diclofenac (sodium salt), oxyteracycline hydrochloride, fluoxetine hydrochloride, chloramphenicol, ketoprofen, gemfibrozli, progesterone, estrone, and androstenedione, are widely used in various therapeutic treatments. The collated data indicate that there is a risk of adverse effects of those compounds' presence.

Reagents and model substances

The MICROTOX® test reagent (freeze dried *Vibrio fischeri*), osmotic adjustment solution (OAS), reconstitution solution (RS), and diluent were purchased from Modern Water (GB).

Model substances selected for the study: diclofenac (sodium salt) (CAS no. 15307-79-6), chloramphenicol (CAS no. 56-75-7), oxytetracycline hydrochloride (CAS no. 2058-46-0), fluoxetine hydrochloride (CAS no. 56296-78-7), ketoprofen (CAS no. 22071-15-4), progesterone (CAS no. 57-83-0), gemfibrozil (CAS no. 25812-30-0), and androstenedione (CAS no. 63-05-8) were purchased from Sigma Aldrich (Germany).

Table 1 EC_{50} values for tested pharmaceuticals

Analyte	EC_{50}/mmol dm^{-3}
Oxytetracycline hydrochloride	0.12
Fluoxetine hydrochloride	0.035
Diclofenac (sodium salt)	0.072
Chloramphenicol	0.92
Ketoprofen	0.45
Gemfibrozil	0.099
Progesterone	0.13
Estrone	0.40
Androstenedione	0.11

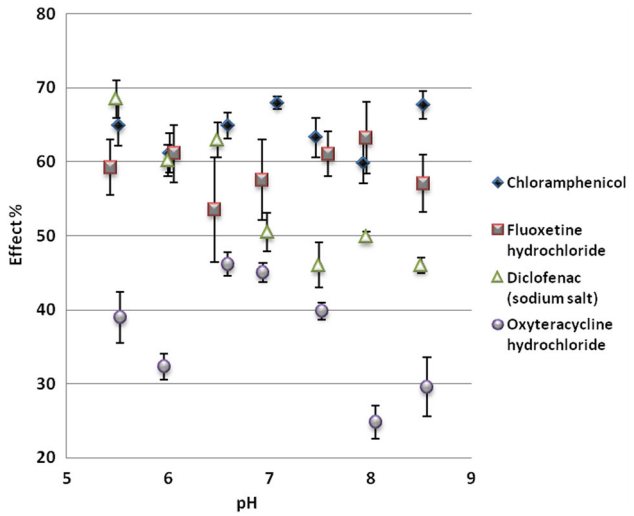

Fig. 11 Effect of pH changes to the toxicity of the substance chloramphenicol, oxytetracycline, diclofenac, and fluoxetine

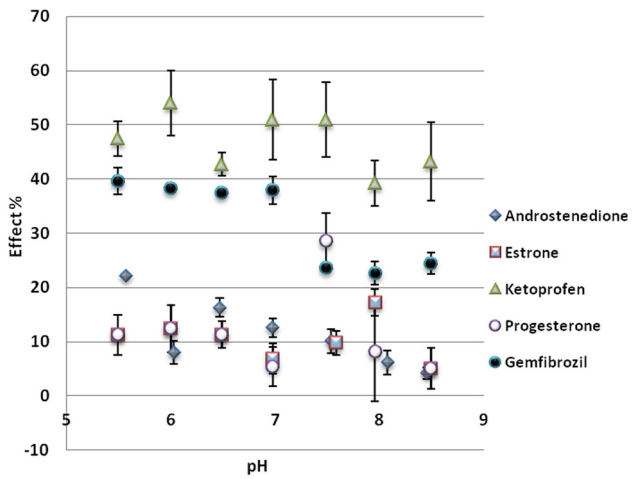

Fig. 12 Effect of pH changes to the toxicity of the substance androstenedione, estrone, progesterone, gemfibrozil, and ketoprofen

Instrumentation

MICROTOX® analyzer model 500 (M500) was used to conduct the study. Apparatus is equipped with 30 incubation wells as well as reagent (bacterial suspensions) and read wells. Temperatures are assigned to the corresponding type of performed test (in this case, acute toxicity test) and the internally maintained at 5.5 ± 1 °C for reagent well and 15 ± 0.5 °C for both the incubator part and the read well.

MICROTOX® acute toxicity assay

To determine the EC_{50} values for four selected drugs, the range screening tests were performed according to two protocols, one for water-soluble substances and the other for insoluble chemicals. Lyophilized reagent was hydrated with RS and maintained at 5.5 ± 1 °C. 100 mm^3 of cell suspension prepared in diluent were added into the cuvettes in every row. The EC_{50} for water-soluble pharmaceuticals (namely, diclofenac (sodium salt), chloramphenicol, oxytetracycline, and fluoxetine) was determined with the standard Acute Toxicity Test Basic 81.9 % protocol using the Model 500 Analyzer Microtox® and serial dilutions. To determine EC_{50} of insoluble compounds, the modification of the assay protocol was necessary, as the samples were treated as solid ones—the appropriate volume of sample was added directly to the vials of bacterial suspension, OAS, and diluent. Subsequently, a pre-made standard dilution of the samples was added into vials with bacterial suspension.

Each test was performed with 14 dilutions of each standard. After narrowing the search range, additional tests were performed for each standard in three replicate in four dilutions. To determine whether the addition of one substance to another would change the toxic effect, concentrated solutions of the compounds were prepared. Test mixtures were prepared in such a way that the compounds were present in an appropriate ratio, respectively, 100 % of EC_{50} of the first model substance and 66 and 33 % of EC_{50} of the second substance tested and vice versa.

To induce a suitable osmotic pressure (above 2 %), OAS was added to each test vial. Sample incubation time was set to 30 min according to the research protocol. After determining the EC50 parameter, the samples having the concentration of pharmaceuticals were subjected to pH adjustment with sodium hydroxide and hydrochloric acid, and the pH was studied in the range from 5.5 to 8.5, with pH increment at every 0.5.

Table 2 Literature study on th occurrence and concentration of selected pharmaceuticals in the environment

Analyte	Effect on living organisms	Concentration in environment/ ng dm^{-3}	Sample matrix/sample location	References
Diclofenac	NSAID	10–55	Surface water samples (river Vantaa), Finland	[7]
Chloramphenicol	Antibiotic	<2–15	River water samples (Taff and Ely rivers), South Wales, UK	[8]
Oxytetracycline	Antibiotic	377,000 ± 142,000	Waste water, China	[9]
Fluoxetine	Used to treat depression and obsessive–compulsive disorders	0.5–43.2	Stream water samples, USA	[10]
Gemfibrozil	Regulates lipids concentration in the plasma	20–60	Wastewater, Whitby, Peterborough, Canada	[11]
Estrone	Estrogen	0.75–1.68	Waste, drinking water samples (Llobregat River), Barcelona, Spain	[12]
Androstenedione	Testosterone precursor	>100	River water samples (Fenholloway River), USA	[13]
Progesterone	Used to prevent miscarriages and menstrual disorders	66 ± 36	Wastewater samples (influents and effluents), Beijing, China	[14]
Ketoprofen	Anti-inflammatory drug	0–180	Waste water effluents samples (Aa Uster and Aabach Moenchaltorf rivers), lake Greifensee, Switzerland	[15]

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