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Toxicity of antibiotics mixture to the aquatic biota

Nowadays, the occurrence of pharmaceuticals in various concentrations in surface waters and effluents were reported in many papers worldwide. The studies of developed countries showed that many pharmaceutical ingredients can have the negative effect on the growth and reproduction of environmental species. Nevertheless, pharmaceutical pollution has not been considered as environmental issue in Kazakhstan. Previous studies on pharmaceuticals were focused on the effect of single pharmaceutical ingredient exposure to living organisms. Nevertheless, in most cases pharmaceuticals occur in the environment in mixture forms. Therefore, the aim of the following paper was to perform ecotoxicological test for the effect of the mixture antibiotics to the growth of *Lemna minor*. The combination of five antibiotics as amoxicillin, clarithromycin, azithromycin, sulfamethoxazole and oxytetracycline was used as objects for assessment study. Two measurement variables were used for the evaluation: growth rate and growth inhibition. The results of the experiment showed that the mixture of studied compounds is highly toxic to macrophyte, as the half-maximal effect concentration was below 1 mg/L (0.13 mg/L).

Keywords: amoxicillin, clarithromycin, azithromycin, sulfamethoxazole, oxytetracycline, *Lemna minor*, ecotoxicology, environment, pharmaceuticals, pharmaceutical pollution.

Introduction

In the last three decades of the studies, pharmaceuticals were classified as environmental pollutants and it was concluded that they can lead to environmental contamination and even cause risk to human health. Likely, the production and usage of drugs will grow in the future, as people live longer and consume more pharmaceutical products when they become older. Moreover, the living standards are becoming higher and the number of budget friendly drugs are increasing. As a result, it can lead to the increase of pharmaceutical pollution worldwide [1].

Currently, above 3000 active pharmaceutical compounds as antibiotics, beta-blockers, painkillers, contraceptives, lipid regulators, tranquilizers and impotence drugs are consumed in the world. After usage pharmaceuticals released as parent or metabolized form. The majority of drugs are well soluble in water and therefore they easily enter to the aquatic system. The consequences of such releases and effect of pharmaceuticals to the environment and human are not well studied. Medical substances do not release only in specific location, they are spread worldwide everywhere. Furthermore, drugs do not meet individually, they occur in mixtures [2]. Thus, pharmaceutical products can be included to the uncontrolled chemical stressors that enter to the environment [3].

According to research by scientists at the University of Gothenburg in Sweden, clotrimazole pollutes the ecosystem of the ocean. The community of natural microalgae *Periphyton* were used for their study. The species were exposed to different concentrations of clotrimazole during 4 days. The results demonstrated that clotrimazole violates algae metabolism. The fact that single-celled microalgae are the fundamental basis of the food chain in the ocean, and the use of clotrimazole could affect the whole ecosystem of the ocean [4].

The well-known case of the negative impact of pharmaceuticals has been registered in South Asia. In these countries, the population of vulture species as *Gyps bengalensis*, *Gyps indicus*, *Gyps tenuirostris* and *Sarcogyps bald* declined sharply in 2000–2007. The reason for the extinction of this population became a pharmaceutical formulation of diclofenac. This compound was used for the treatment of tumors and injuries of cattle. After the introduction of the pharmaceutical compound, 0.3 mg of diclofenac were found in cattle liver. Birds fed on the carcasses of those cattle and accumulated in their body in concentration up to 0.1 mg/kg. As a result, there was decline of more than 90 % of vulture population and this led to the increase of wild dog population and consequently spread rabies [5–7].

Photoautotrophs as algae and macrophytes play an important role in total biomass in the aquatic system. Moreover, algae and higher aquatic plants are the major carbon sources for the aquatic environment. However, there have not been performed many toxicity test of antibiotics on macrophytes and algae. It can be noted, that risk assessment results pay a big attention representatives of aquatic organisms [8].

The aim of the following research was to assess the effect of mixture five antibiotics as amoxicillin, clarithromycin, azithromycin, sulfamethoxazole and oxytetracycline to the growth of duckweed. *Lemna minor* was selected as object of the study. Overall, duckweeds occur in the majority of surface waters worldwide. The most well-known duckweed is *Lemna minor*. This macrophyte is very convenient for ecotoxicity study, as it is budget friendly and time saving. Moreover, there have not been conducted many studies on effects of pharmaceuticals to duckweeds. Mostly, ecotoxicity studies are focused on impacts of drugs to fish and algae [9].

Materials and methods

Antibiotics were supplied from Sigma Aldrich UK. *Lemna minor* were collected from Food and Environment Research Agency. Table represents data about the study substances used for the ecotoxicity test.

Table

Physico-chemical properties of study compounds

Antibiotic	Molecular formula	Molecular weight, g/mol	Solubility in water, mg/L
Amoxicillin	$C_{16}H_{19}N_3O_5S$ [10]	365.40416 [11]	3430 [10]
Clarithromycin	$C_{38}H_{69}NO_{13}$[10]	747.953 [11]	1.693 [11]
Azithromycin	$C_{38}H_{72}N_2O_{12}$ [10]	748.98448 [11]	2.37 [11]
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$ [10]	253.27764 [11]	610 [10]
Oxytetracycline	$C_{22}H_{25}ClN_2O_9$ [11]	496.897 [11]	1000 [11]

Lemna minor growth inhibition test was performed according to OECD Guideline for the testing of chemicals 221 [12]. In order to conduct *Lemna minor* ecotoxicity study, 15 mL petri dishes were used and filled with 10 mL of Swedish SIS medium nutrition solution. Due to low solubility of macrolides in deionized water, the stock solution of was made in ethanol. The mixture of antibiotics was made in ratio 2:1:1:6:5 (amoxicillin, clarithromycin, azithromycin, sulfamethoxazole, oxytetracycline respectively). The following ratio was selected due to their exposure indices in surface waters of Kazakhstan [13]. Each petri dish included 2–4 frond colonies with total 6–8 fronds. Ecotoxicological experiment was set up with three replicates test compounds and six replicates of controls. The toxicity of antibiotics was assessed in concentrations from 0.2 to 1 mg/L. Fronds of *Lemna minor* were calculated at the beginning of the test, then on day 3 and day 7. The area of the colonies in each test sample was detected using the image analysis software Image J. The effect of test samples on growth of *Lemna minor* was assessed using response endpoints: growth inhibition and mean specific growth rate of frond numbers [12].

Results and discussion

According to results of half maximal effect concentrations (EC_{50}) of five compounds to *Lemna minor* was 0.13±0.02 mg/L. Figure demonstrates the growth inhibition and growth rate of tested duckweed to the mixture of the compounds. Initially, tested concentrations ranged from 0.2 mg/L to 1 mg/L. However, at the end of the test, the fronds in the samples with concentration 0.8 mg/L and 1 mg/L died and in concentration 0.6 mg/L almost total growth inhibition was recorded. The concentration value 0.6 mg/L led growth rate till 0.01 d^{-1}, while the value of controls in growth rate was 0.17 d^{-1}.

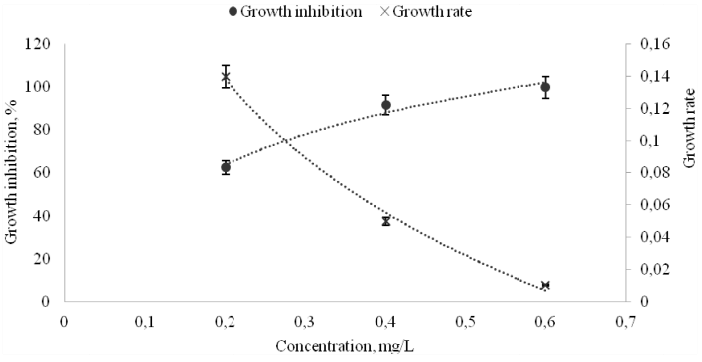

Figure. The growth inhibition and growth rate of the mixture five antibiotics: amoxicillin, clarithromycin, azithromycin, sulfamethoxazole and oxytetracycline

The value of EC_{50} of combined compounds to duckweed is lower than 1 mg/L. According to EU-Directive 93/67/EEC classification, this mixture is very toxic to aquatic life [14].

Overall, the results of the performed ecotoxicolgical test are persistent with previous with results of previous research. In 2002, Cleuvers carried out investigation on ecotoxicological potential of prescription drugs to aquatic organisms. For the research, he used pharmaceuticals as clofibric acid, carbamazepine, ibuprofen, diclofenac, naproxen, captopril, metformin, metoprolol and propranolol and aquatic organisms as crustacean *Daphnia magna*, green algae *Desmodesmus subspicatus* and macrophytes *Lemna minor*. The results showed immobilization in 24 and 48 hours. During the test, it was found that *Desmodesmus subspicatus* and *Lemna minor* are sensitive to the most of drugs as they showed a high growth inhibition percentage. In addition, in analysis of effect of drugs to aquatic organisms, Cleuvers identified that the mixture of medications had a stronger effect to tested organisms rather than individual exposure of substances. In view of this study it may suppose that in most cases pharmaceutical compounds affect in combination to environmental species [15].

A recent study by Aubakirova et al. found that amoxicillin, clarithromycin, azithromycin, sulfamethoxazole and oxytetracycline individually also have a toxic effect to Lemna minor. Aubakirova et al. highlighted EC_{50} of sulfamethoxazole below 10 mg/L and concluded that this antibiotic is highly toxic to aquatic species [9].

In the same vein, in 2017 Bialk-Bielinska et al. have performed investigation on effects of the combination six antimicrobial sulfonaimides and their degradation compounds sulfanic acid and sulfanilamides to green algae *Scenedesmus vacuolatus* and macrophyte *Lemna minor*. The study also included the assessment of toxicity of individual substances as sulfanic acid and sulfanilamides to selected species. According to the results, sulfanilamides are toxic to the species in the aquatic biota, whereas sulfanic acid does not cause risk to aquatic organisms. However, the mixtures of sulfonaimides and sulfanilamides showed a high risk, as their effect concentration (EC_{50}) value to *Lemna minor* was lower than 1 mg/L and to *Scenedesmus vacuolatus* was around 1–1,5 mg/L. These outcomes demonstrate that single pharmaceutical compound can be not toxic, but their combination is harmful to environmental species [16].

Conclusion

To sum up, over the last 20 years, there has been increasing interest in the occurrence, fate, effects and risk of pharmaceuticals in the natural environment. However, we still have only limited or no data on ecotoxicological risks of many of the pharmaceutical ingredients currently in use. Consequently, it is essential to conduct ecotoxicity studies of pharmaceutical substances on various environmental species. Nowadays, the most studies are focusing on individual impact of drug to aquatic biota. However, pharmaceuticals occur on surface waters in the combination. Therefore, we believe it is significant to carry on research on the effect of mixture pharmaceutical compounds to living organisms.

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Су биотасына антибиотиктер қоспасының улылығы

Қазіргі уақытта дүниежүзі бойынша фармацевтикалық препараттардың алуан түрлі концентрацияда беткей және ағын суларда таралуы туралы көптеген мақалаларда ақпарат бар. Дамыған елдердің зерттеулеріне сәйкес, фармацевтикалық ингредиенттер экологиялық тірі ағзалардың өсуіне және көбеюіне жағымсыз әсер етеді. Дегенмен, Қазақстанда фармацевтикалық ластану экологиялық мәселе ретінде қарастырылмаған. Дәрілік препараттар бойынша алдыңғы зерттеулер жалғыз фармацевтикалық ингредиенттің тірі ағзаға әсерін қарастырған. Алайда қоршаған ортада көп жағдайда фармацевтикалық препараттар қоспа түрінде кездеседі. Сол себепті берілген мақаланың мақсаты антибиотиктер қоспасының *Lemna minor* өсуіне әсерін зерттеуде экотоксикологиялық тест жүргізу болды. Балдыршөптің өсуін бағалау үшін зерттеу нысана ретінде амоксициллин, кларитромицин, азитромицин, сульфаметоксазол және окситетрациклин антибиотиктер қоспасы қолданылды. Бағалауда екі өлшем пайдаланылды: өсу жылдамдығы мен өсу тежелуі. Зерттеу нәтижелеріне сәйкес зерттелген ингредиенттер қоспасы макрофит үшін аса улы болып табылады, себебі оның жартылай максималды әсер ету концентрациясы 1 мг/л-ден төмен (0,13 мг/л).

Кілт сөздер: амоксициллин, кларитромицин, азитромицин, сульфаметоксазол, окситетрациклин, *Lemna minor*, экоулану, қоршаған орта, фармацевтикалық препараттар, фармацевтикалық ластану.

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Токсичность смесей антибиотиков к водной биоте

На сегодняшний день имеются данные о распространении фармацевтических препаратов в различных концентрациях в поверхностных и сточных водах по всему миру. Исследования развитых стран показали, что многие фармацевтические ингредиенты могут оказывать негативное влияние на рост и размножение живых существ. Однако фармацевтическое загрязнение не рассматривается в качестве экологической проблемы в Казахстане. Предыдущие исследования по загрязнению фармацевтическими препаратами были сосредоточены на влиянии одного фармацевтического ингредиента на живые организмы. Тем не менее в большинстве случаев фармацевтические препараты встречаются в окружающей среде в виде смесей. Таким образом, целью данный работы было проведение экотоксикологического теста на воздействие смесей антибиотиков на рост *Lemna minor*. В качестве объектов для оценки использовалась комбинация из пяти антибиотиков: амоксициллин, кларитромицин, азитромицин, сульфаметоксазол и окситетрациклин. Для оценки использовались две переменные измерения: скорость роста и ингибирование роста. Результаты эксперимента показали, что смесь исследуемых соединений высоко токсична для макрофита, так как его полумаксимальная эффектная концентрация была ниже 1 мг/л (0,13 мг/л).

Ключевые слова: амоксициллин, кларитромицин, азитромицин, сульфаметоксазол, окситетрациклин, *Lemna minor*, экотоксикология, окружающая среда, фармацевтические препараты, фармацевтическое загрязнение.

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