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Toxicity study of antibiotics to the common duckweed *(Lemna minor)*

The following paper demonstrates the results of the scientific study of toxicity effect of five antibiotics as amoxicillin, clarithromycin, azithromycin, sulfamethoxazole and oxytetracycline hydrochloride to the growth of common duckweed Lemna minor. It was defined that the half maximal effective concentration of five previously mentioned antibiotics was equal to 27.8, 15.29, 28.77, 3.67 and 13.51 mg/L respectively. Overall, the results of the study showed that the duckweed was sensitive to all selected compounds, however sulfamethoxazole was the most toxic to these species. The present study is significant, as currently people consume antibiotics in huge amount. Their occurrence on the surface was found around the world and their effect to the environment and living organisms are not fully studied yet. The recent studies show that in most cases they have adverse effect to the aquatic microorganisms.

Keywords: antibiotics, Lemna minor, ecotoxicology, amoxicillin, clarithromycin, azithromycin, sulfamethoxazole, oxytetracycline hydrochloride, surface water.

Introduction

An antibiotic is the substance that has tendency to demolish or suppress the growth of bacteria, fungi, protozoa and other microorganisms and helps to defeat viruses [1]. Over the 50 year antibiotics have been using for treatment of public health. However, currently they led to another issue such as overuse of them. The consumption of antibiotics varies among 100,000–200,000 ton per year [2]. When antibiotics enter to the environment, they can have negative impacts on living organisms. It is not well known yet, if antibiotics stimulate antibiotic resistance in microorganisms [1].

The interaction of drugs with the natural environment and biological species are poorly studied. In most cases, they have common properties as dangerous pollutants, because they can enter to the membranes and be persistant and in some cases their impact to the environment can be worther than agricultural pollutants [3, 4]. Active pharmaceutical ingredients (APIs) can be easily absorbed and interact with living organisms. As a result they can have unintetial adverse effect to the wildlife [5]. The environmental effect of pharmaceuticals can be related to any level of biological hirarchy as cells-organs-populations-ecosystems. Moreover, drugs can have impacts on function of organisms, endocrine distruption, genotoxicity, metabolism or it can change nutrition cycle of ecosystem [6].

Effects of pharmaceuticals on aquatic organisms have been reported in many papers [7, 8]. Chronic toxicity studies can have effects in low concentration on fish, daphnia, algae and bacteria. For example, the range of wastewater concentration of diclofenac was enough to have adverse impact on aquatic organisms, while maximally measured sewage treatment plant (STP) effluent concentration of propranolol and fluoxetine had negative effect on zooplankton and benthic organisms [8]. Another harmful effect of pharmaceuticals is endocrine disruption. It effects to the function of hormones and can be permanent even at low level [7]. Also, estrogens that were detected in aquatic environment had an adverse impact on fish reproduction and could lead to the population decline [8].

Amoxicillin is a widely spread β-lactam antibiotic, that used in human and veterinary medicine. It suppresses peptidoglycan synthesis in bacterial cell wall [9]. Currently, this substance is one most popular product in sale in some European and Asian countries. Furthermore, amoxicillin is not stable and therefore it is hard to detect its concentration in natural water [10].

Another compound is sulfamethoxazole, that is a bacteriostatic broad-spectrum antibiotic, that is wide spread in the pharmacy. Its acute bacterial toxicity is low, because it has biosynthesis-related mechanisms of action [11]. Azithromycin is a macrolide antibiotic and it has a wide spectrum. It is consumed to treat and prevent diseases as toxoplasmosis, pediatric infections and respiratory tract infections [12]. Oxytetracycline hydrochloride is a tetracycline broad-spectrum antibiotic that has a bacteriostatic action. This action works against different gram-positive and gram-negative bacteria [13]. Clarithromycin is a macrolide antibacterial and its structure is common to erythromycin. The highest concentration of clarithromycin occurs in tissues rather than in the blood [14].

According to Gonzalez-Pleiter et al. [15] investigation, there are a large number of publications on antibiotic pollution in Europe, Asia, North America countries. They were found in various concentration in river water, seawater, sediments, soils, manure and STP effluents and detected in low concentration in waters. Nevertheless, because they enter to the environment continuously, antibiotics regarded as «pseudopersistant» pollutants [15].

The majority of studies on toxicity of antibiotics are concerned mostly on bacterial resistance. However, there is deficit data on their impact to higher plants. For instance, macrophytes and phytoplankton are main biomass in marine environment and main carbon source for the aquatic biosphere. There were not many investigations done on duckweeds. Moreover, available data is not enough for regulation purposes. Nevertheless, it is believed that primary producers are more sensitive to antibiotics in comparison with algae species [16, 17].

Duckweeds are basically ubiquitous in nature. They can be meet on relatively fresh water as ponds, lakes and quite streams. *Lemna minor* is the most widespread duckweed species [18]. Duckweeds are well known test organisms since the 1930, toxicity of phemoxy-herbicides on plants were found by using them [19]. There are many advantages on selecting duckweeds *Lemna minor* as the object for the toxicity study. They do not need the big laboratory equipments and can be easily observed without usage of microscope. Growth inhibition test on *Lemna minor* is not a long term and does not require expensive vessels, disposable vessels can be used [18].

The aim of the current study was to assess the toxicity of five antibiotics and to the duckweed *Lemna minor*.

Methods

All antibiotics were purchased from Sigma Aldrich UK. Table 1 provides information about their physicochemical properties. *Lemna minor* species were kindly presented from Food and Environment Research Agency UK. Table 1 provides information about the present compounds used for the toxicity test.

Table 1

Properties of the five study antibiotics

	Amoxicillin	Clarithromycin	Azithromycin	Sulfamethoxazole	Oxytetracycline hydrochloride
CAS-no	26787-78-0 [20]	81103-11-9 [20]	83905-01-5 [20]	723-46-6 [20]	2058-46-0 [20]
Molecular formula	$C_{16}H_{19}N_3O_5S$ [20]	$C_{38}H_{69}NO_{13}$ [20]	$C_{38}H_{72}N_2O_{12}$ [20]	$C_{10}H_{11}N_3O_3S$ [20]	$C_{22}H_{25}ClN_2O_9$ [21]
Molecular weight, g/mol	365.40416 [21]	747.953 [21]	748.98448 [21]	253.27764 [21]	496.897 [21]
pKa	3.23 [20]	8.99 [20]	8.74 [20]	6.16 [20]	3.27 [20]
Solubility in water, mg/L	3430 [20]	1.693 [21]	2.37 [21]	610 [20]	1000 [21]
LogKow	0.87 [20]	3.16 [20]	4.02 [21]	0.89 [20]	-0.90 [21]

Toxicity test on *Lemna minor* was made as suggested by OECD 221 Guidelines [22]. Two to four frond colonies were put in 15 mL Petri dishes with 10 mL of duckweed nutrition solution (Swedish SIS medium). The toxicity test was set up with five selected concentrations (from 10 to 100 mg/L) and controls.

Duckweeds with two or three fronds were selected for the test and three replicates were used for controls and test samples. All samples were incubated to environmental growth room with temperature 24±2 °C and the light intensity 85–135 $\mu E{*}m^2s^{-1}$ for 7 days. The pH was measured at the beginning of the test and after 7 days. The number of fronds were counted at the start, then after 3 days and after 7 days of toxicity test. Based on number of fronds, the growth rate of *Lemna minor* was calculated following the OECD guideline Equation (1) [22]:

$$\mu_{i-j} = \frac{\ln(N_j) - \ln(N_i))}{t}, \qquad (1)$$

where μ_{i-j} — mean growth rate from time i to j; N_i — measurement variable in the test or control vessel at time I; N_j — measurement variable in the test or control vessel at time j; t — time period from i to j.

In addition, the following test detected the total frond area. Total frond area was found by using ImageJ software. Then, based on the value of total frond area the growth inhibition was calculated by the following equation (2) [22]:

$$\% I_r = \frac{(\mu c - \mu t)}{\mu c} * 100, \quad (2)$$

where I_r — the percentage of inhibition in average specific growth rate; μc — mean value for μ in the control; μt — mean value for μ in the treatment group.

The half maximal effective concentration (EC_{50}) was found with non-linear regression analysis. Each test compound was tested for identification of significant effects ($p < 0.05$) with using a one-way analysis of variance (ANOVA).

Results

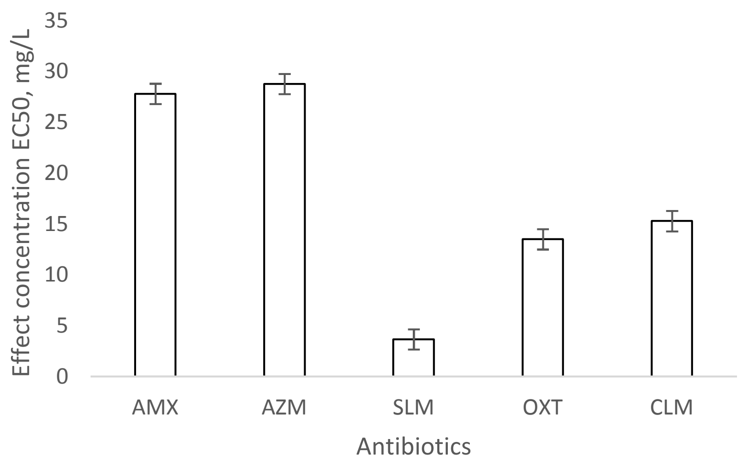
According to EU-Directive 93/67/EEC chemical compounds can be categorized based on the half maximal effective concentration (EC_{50}) value (Table 2) [23]. Figure illustrates dose effect parameters of selected compounds. Overall, EC_{50} values ranged from 3.8 mg/L to 28.8 mg/L.

Table 2

Classification of substances according to their EC50 value (Adopted from EU-Directive 93/67/EEC [23])

Concentration	Classes
< 1 mg/L	Very to aquatic life
1–10 mg/L	Toxic to aquatic life
10–100 mg/L	Harmful to aquatic life

EC_{50} value considering as a toxic to aquatic organisms were detected for azithromycin, sulfamethoxazole and oxytetracycline. Sulfamethoxazole showed the highest toxicity with EC_{50} = 3.67 mg/L. Azithromycin showed less toxicity in comparison with other substances, its EC_{50} was 28.77 mg/L.



APIs — active pharmaceutical compounds; EC_{50} — half maximal effective concentration;
CLM — clarithromycin; OXT — oxytetracycline hydrochloride;
SLM — sulfamethoxazole; AZM — azithromycin; AMX — amoxicillin

Figure. The comparison of effect concentration parameters of five antibiotics ($p < 0.001$)

Discussion

The results of growth inhibition test showed that *Lemna minor* is sensitive to antibiotics. In most cases, EC_{50} values were lower than 10 mg/L, which was considered as toxic to aquatic organisms. The selected compounds in the present study were detected in various concentration around the world. For instance, sulfamethoxazole was detected in range of 0.05-0.09 μg/L concentration in effluents of European countries [24]. The concentration of azithromycin was found in Germany surface water and ranged from few ng/L to 13 ng/L [25]. The high concentration (2.20 μg/L) of oxytetracycline was detected in China [26]. Amoxicillin was also found in high concentration above 70 ng/L to 300 ng/L in South Wales of the UK [27]. Clarithromycin concentration in Glatt river in Switzerland reached 75 ng/L [28].

In most cases the following antibiotics showed toxicity to aquatic environment in previous studies. Pan et al. [9] investigation showed that amoxicillin had a toxic effect on the photosystem II of *Synechocystis sp* and inhibit the transport of donor side and acceptor side. Sulfamethoxazole has a toxic effect to Gram-negative bacterium *P.putida*, its half maximal inhibition concentration (IC_{50}) was 256 μg/L [29]. Pro et al. [30] found that oxytetracycline is toxic to *Lemna minor* in concentration 4.92 mg/L. Cleuvers [31] study of *Lemna* species to various active pharmaceutical ingredients such as carbamazepine, diclofenac, naproxen, ibuprofen, metformin, propranolol and metoprolol, showed that it is very sensitive test species, its EC_{50} values was around 7.5-320 mg/L. Furthermore, higher plants *Lemna* was sensitive to five compounds of sulfonylurea herbicides [32]. Orvos et al. [33] study states that triclosan can have impact on higher plant species as *Lemna*. Sulfamethoxazole was the most toxic antibiotic to *Lemna* species in various studies, as its EC_{50} ranged from 146 to 7800 μg/L [34].

All those mentioned above compounds were included to the European Union (EU) watch list. EU watch list is the technical report that presents data on concentration of chemicals which pollute aquatic environment. The key objective of this report is to detect substances that can pollute the environment and put them in the list of under regulation of Water Framework Directive [35].

In conclusion, antibiotics cause toxicity to aquatic environment. Their effect to microorganisms are unpredictable. Including the fact that the consumption of pharmaceuticals in Kazakhstan is growing, tt is recommended to conduct further studies with other aquatic species as daphnia, algae in order to assess the toxic effects of antibiotics.

References

1 *Kummerer K.* Antibiotics in the aquatic environment. — A review. — P. I // Chemosphere. — 2009. — Iss. 75. — № 4. — P. 417–434.

2 *Wise R*. Antimicrobal resistance: priorities for action // Journal of Antimicrobial Chemotherapy. — 2002. — Iss. 49. — № 4. — P. 585–586.

3 *Cooper E., Siewicki T., Phillips K.* Preliminary risk assessment database and risk ranking of pharmaceuticals in the environment // Science of The Total Environment. — 2008. — Iss. 398. — № 1–3. P. 26–33.

4 *Sanderson H. et al.* Ranking and prioritization of environmental risks // Regulatory Toxicology and Pharmacology. — 2004. — Iss. 39. — P. 158–183.

5 *Sangion A., Gramatica P.* PBT assessment and prioritization of contaminants of emerging concern: Pharmaceuticals // Environmental Researc. — 2016. — Iss. 147. — P. 207–306.

6 *Jorgensen S., Halling-Sorensen B*. Drugs in the environment // Chemospher. — 2000. — Iss. 40. — № 7. — P. 691–699.

7 *Daughton C., Ternes T.* Pharmaceuticals and personal care products // Environmental Health Perspective. — 1999. — Iss. 107. — № 6. — P. 907–938.

8 *Kummerer K.* Pharmaceuticals in the Environment // Annual Review of Environment and Resources. — 2010. — Iss. 35. — P. 57–75.

9 *Pan X., Deng C., Zhang D., Wang J., Mu G., Chen Y.* Toxic effects of amoxicillin on the photosystem II of Synechocystis sp. characterized by a variety of in vivo chlorophyll fluorescence tests // Aquatic Toxicology. — 2008. — Iss. 89. — № 4. — P. 207–213.

10 *Andreozzi R., Caprio V., Cinglia C., Champdore M., Lo Giudice R., Marotta R., Zuccato E.* Antibiotics in the Environment: Occurrence in Italian STPs, Fate, and Preliminary Assessment on Algal Toxicity of Amoxicillin // Environmental Science and Technology. — 2004. — Iss. 38. — № 24. — P. 6832–6838.

11 *Johansson C., Janmar L., Backhaus T.* Toxicity of ciprofloxacin and sulfamethoxazole to marine periphytic algae and bacteria // Aquatic Toxicology. — 2014. — Iss. 156. — P. 248–258.

12 *Zubata P., Ceresole R., Rosasco M., Pizzorna M.* A new HPLC method for azithromycin quantitation // Journal of Pharmaceutical and Biomedical Analysis. — 2002. — Iss. 27. — P. 833–836.

13 *Zounkova R., Klimesova Z., Nepejchalova L., Hilscherova K., Blaha L.* Complex Evalution of Ecotoxicity and Genotoxicity of Antimicrobials Oxytetracycline and Flumequine Used in Aquaculture // Environmental Toxicology and Chemistry. — 2011. — Iss. 30. — № 5. — P. 1184–1189.

14 *Fraschini F., Scaglione F., Demartini G.* Clarithromycin clinical pharmacokinetics // Clinical Pharmacokinetics. — 1993. — Iss. 25. — № 3. — P. 189–204.

15 *Gonzalez-Pleiter M., Gonzalo S., Rodea-Palomares I., Leganes F., Rosal R., Boltes K., Marco E., Fernandez-Pinas F.* Toxicity of five antibiotics and their mixtures towards photosynthetic aquatic organisms: implications for environmental risk assessment // Water Research. — 2013. — Iss. 47. — № 6. — P. 2050–2064.

16 *Brain R., Johnson D.J., Richards S.M., Sanderson H., Sibley P.K., Solomon K.R.* Effects of 25 pharmaceutical compounds to Lemna gibba using a seven-day static-renewal test // Environmental Toxicology and Chemistry. — 2004. — Iss. 23. — № 2. — P. 371–382.

17 *Ebert I. Bachmann J., Kuhnen U., Kuster A., Kussatz C., Maletzki D., Schlüter C.* Toxicity of the fluoroquinolone antibiotics enrofloxacin and ciprofloxacin to photoautotrophic aquatic organisms // Environmental Toxicology and Chemistry. — 2011. — Iss. 30. — № 12. — P. 2786–2792.

18 *Moody M., Jennifer M.* Lemna minor growth inhibition test.

19 *Blackman G., Roberson-Cuninghame R.* Interrelationships between light intensity, temperature, and the physiological effects of 2:4-dichlorophenoxyacetic acid on the growth of Lemna minor // Journal of Experimental Botany. — 1955. — Issue 6. — P. 156–176.

20 *Wishart D.S., Knox C., Guo A.C., Shrivastava S., Hassanali M., Stothard P., Chang Z., Woolsey J.* DrugBank: a comprehensive resource for in silico drug discovery and exploration // Nucleic Acids Res. — 2006. — Iss. 34. — P. 668–672.

21 *Kim S., Thiessen P.A., Bolton E.E., Chen J., Fu G., Gindulyte A., Han L., He J., He S., Shoemaker B.A., Wang J., Yu B., Zhang J., Bryant S.H.* PubChem Substance and Compound databases // Nucleic Acids Res. — 2016. — Iss. 44. — P. 1202–1213.

22 OECD. Test No. 221: Lemna sp. Growth Inhibition Test. B: OECD Guidelines for the Testing of Chemicals. — Paris: OECD Publishing. — 2006. — P. 1–22.

23 Communities C. o. t. E. Methods for determination of ecotoxicity; Annex V, C.2, Daphnia, acute toxicity to Daphnia. L 383A. // EC Directive 92/ 69/EEC. — 1992.

24 *Andreozzi R., Raffaele M., Nicklas P.* Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment // Chemosphere. — 2003. — Iss. 50. — № 10. — P. 1319–1330.

25 *Christian T., Schneider R., Farber H., Skutlarek D., Meyer M., Goldbach H.* Determination of Antibiotic Residues in Manure, Soil, and Surface Waters // Acta hydrochimica et hydrobiologica. — 2003. — Iss. 31. — № 1. — P. 36–44.

26 *Ji K., Kim S., Han S., Seo J., Lee S., Park Y., Choi K., Kho Y.L., Kim P.G., Park J., Choi K.* Risk assessment of chlortetracycline, oxytetracycline, sulfamethazine, sulfathiazole, and erythromycin in aquatic environment: are the current environmental concentrations safe? // Ecotoxicology. — 2012. — Issue 21. — P. 2031–2050.

27 *Kasprzyk-Hordern B., Dinsdale R., Guwy A.* The occurrence of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs in surface water in South Wales, UK // Water Research. — 2008. — Iss. 42. — № 13. — P. 3498–3518.

28 *Giger W., Alder A., Golet E.M., Suter M.* Occurrence and Fate of Antibiotics as Trace Contaminants in Wastewaters, Sewage Sludges, and Surface Waters // CHIMIA International Journal for Chemistry. — 2003. — Issue 57. — № 9. — P. 485–491.

29 *Al-Ahmad A., Daschner F., Kummerer K.* Biodegradability of Cefotiam, Ciprofloxacin, Meropenem, Penicillin G, and Sulfamethoxazole and Inhibition of Waste Water Bacteria // Arch Environ Contam Toxicol. — 1999. — Iss. 37. — № 2. — P. 158–163.

30 *Pro J. Ortiz J.A., Boleas S., Fernandez C., Carbonell G., Tarazona J.V.* Effect Assessment of Antimicrobial Pharmaceuticals on the Aquatic Plant Lemna minor // The Bulletin of Environmental Contamination and Toxicology. — 2003. — Iss. 70. — P. 290–295.

31 *Cleuvers M.* Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects // Toxicology Letters. — 2003. — Iss. 142. — P. 185–194.

32 *Mohammad M., Itoh K., Suyama K., Yamamoto H.* Recovery of Lemna sp. after Exposure to Sulfonylurea Herbicides // Bulletin of Environmental Contamination and Toxicology. — 2006. — Iss. 76. — P. 256–263.

33 *Orvos D., Versteeg D.J., Inauen J., Capdevielle M., Rothenstein A., Cunningham V.* Aquatic toxicity of triclosan // Environmental Toxicology and Chemistry. — 2002. — Iss. 21. — № 7. — P. 1338–1349.

34 *Brain R., Hanson M., Solomon K., Brooks B.* Aquatic Plants Exposed to Pharmaceuticals: Effects and Risks // Reviews of Environmental Contamination and Toxicology. — 2008. — Iss. 192. — P. 67–115.

35 Joint Research Centre. Development of the first watch list under the Environmental Quality Standards Directive // Luxembourg: Publications Office of the European Union. — 2015.

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Антибиотиктардың кіші балдыршөптерге *(Lemna minor)* улылығын зерттеу

Мақалада амоксициллин, кларитромицин, азитромицин, сульфаметоксазол және окситетрациклин гидрохлориді антибиотиктардың *Lemna minor* кіші балдыршөбіне улылығы зерттелген ғылыми жұмыс нәтижелері көрсетілген. Жұмыстың мақсаты — зерттелетін фармацевтикалық препараттардың кіші балдыршөп өсуіне токсикалық әсерін бағалау. Амоксициллин, кларитромицин, азитромицин, сульфаметоксазол және гидрохлорид окситетрациклиннің осы өсімдікке жартылай максималды әсер ету концентрациясы 27.8, 15.29, 28.77, 3.67 және 13.51 мг/л нәтижелеріне сәйкес болды. Зерттеу нәтижелері бойынша *Lemna minor* барлық зерттелген заттарға сезімталдығы жоғары, алайда сульфаметоксазол кіші балдыршөбіне ең улы антибиотик болып табылды. Қазіргі кезде бұл зерттеу өзекті, себебі адамдар антибиотиктерді үлкен көлемде тұтынады. Оның мөлшері беткей суларда кездеседі және олардың қоршаған орта мен тірі ағзаларға әсері әлі толығымен зерттелмеген. Соңғы зерттеулер нәтижелеріне сәйкес, антибиотиктардың су микроағзаларына жағымсыз әсері дәлелденіп жатыр.

Кілт сөздер: антибиотиктар, кіші балдыршөп, экотоксикология, амоксициллин, кларитромицин, азитромицин, сульфаметоксазол, окситетрациклин гидрохлориді, беткі сулар.

Б.Н. Аубакирова, А.Б.А. Боксал, Р.Р. Бейсенова

Изучение токсического воздействия на рост ряски малой *(Lemna minor)*

Данная статья является результатом исследований экотоксикологического воздействия пяти антибиотиков, таких как амоксициллин, кларитромицин, азитромицин, сульфаметоксазола и гидрохлорид окситетрациклина, на рост ряски малой *Lemna minor*. Целью данный работы было определение токсического воздействия данных фармацевтических препаратов на рост ряски малой. Выявлено, что полумаксимальная эффективная концентрация была равна 27.8, 15.29, 28.77, 3.67 и 13.51 мг/л для амоксициллина, кларитромицина, азитромицина, сульфаметоксазола и гидрохлорида окситетрациклина соответственно. На сегодняшний день люди употребляют антибиотики в большом количестве. Результаты исследования показали высокую чувствительность *Lemna minor* ко всем исследуемым веществам, однако сульфаметоксазол оказался наиболее токсичным из исследуемых антибиотиков. В настоящее время данное исследование особенно актуально, поскольку антибиотики были обнаружены на поверхностных водах и их влияние на окружающую среду и живые организмы еще не до конца изучено. Недавние исследования показывают, что в большинстве случаев они воздействуют неблагоприятно на водные микроорганизмы.

Ключевые слова: антибиотики, ряска малая, экотоксикология, амоксициллин, кларитромицин, азитромицин, сульфаметоксазол, гидрохлорид окситетрациклина, поверхностные воды.