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ORIGINAL ARTICLE

Ecotoxicity of selected antibiotics for organisms of aquatic and terrestrial ecosystems

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Abstract

OBJECTIVES: The aim of this study was to assess the ecotoxicity of selected antibiotics (i.e. penicillin G, vancomycin and tetracycline) using ecotoxicological tests. Tests were conducted on organisms representing all trophic levels of the aquatic ecosystem, namely producers (green freshwater algae *Pseudokirchneriella subcapitata*), consumers (water fleas *Daphnia magna*) and decomposers (bacteria *Vibrio fischeri*). The effect of antibiotics on the representative of edaphon was measured by testing the inhibition of the reproduction of springtails *Folsomia candida* and earthworms *Eisenia fetida*.

DESIGN: Methodologically, the procedure was carried out in accordance with the following standards: OECD 201 (Fresh water algal growth inhibition test), OECD 202 (Inhibition of the mobility of *Daphnia magna*), ISO 11348-2 (Inhibitory effect of antibiotics on the light emission of *Vibrio fischeri*), OECD 232 (Inhibition of reproduction of Collembola *Folsomia candida*) and OECD 222 (Inhibition of reproduction of *Eisenia fetida*).

RESULTS: In aquatic organisms the highest level of toxicity was shown by tetracycline to algae (72hEC50 = 1.82 mg.l^{-1}) and daphnia (48hEC50 = 8.16 mg.l^{-1}). The least toxic for all test organisms was penicillin G. The results of the tests performed on the representative of edaphon, Folsomia candida, showed that its reproduction was most inhibited by penicillin G (28dEC50 = 328 mg.kg^{-1}) and least by tetracycline (28dEC50 = 2 560 mg.kg^{-1}). Similar results were observed in Eisenia fetida (56dEC50 = 348 mg.kg^{-1} for penicillin G and 56dEC50 = 2 735 mg.kg^{-1} for tetracycline.

CONCLUSION: The ecotoxicity of antibiotics differed significantly depending on the test organism and testing conditions.

Abbreviations:

CI - confidence interval
CV - coefficient of variation
EC50 - medium effective concentration is the concentration that causes an effect in test organisms amounting to 50% within a given exposure period when compared with the control
LC50 - median lethal concentration is the concentration of test substance that kills 50% of exposed test organisms within a given time period
SD - standard deviation

INTRODUCTION

Pharmaceuticals, both human and veterinary, are foreign substances which pose a risk to the individual components of the environment. Water biocenoses in particular can be significantly influenced by the presence of pharmaceuticals. Veterinary antibiotics are used both for therapeutic purposes and as a means of disease prevention, which can affect not only animal health but also the development of resistance and the safety of food of animal origin. Antibiotics were found in all components of the environment. Antibiotic residues may affect different trophic levels of aquatic biota, as they do in the case of the terrestrial ecosystem on representatives of the edaphon (Daneshvar *et al.* 2010; Panditi *et al.* 2013; Stuart *et al.* 2012; Valcárcel *et al.* 2013). The contributing factors include the frequent use of antibiotics, their production and their incomplete removal in wastewater treatment plants.

As reported by Wise (2002), global consumption of antibiotics is between 100 to 200 thousand tons per year. According to Van Boeckel *et al.* (2015) more than 63,200 tons of antibiotics were consumed by livestock in 2010. In addition, it was found that ß-lactam antibiotics, including the sub-groups of penicillin, make up the largest share of human use antibiotics in most countries. They account for approximately 50–70% of total antibiotic use. Vancomycin, for example, is heavily used in the USA, whereas in Germany it is only used in cases in which all other possible compounds have proven to be ineffective due to resistance. Data on the country-specific use for groups of antibiotics are available from different sources (Kümmerer 2009).

Antibiotics were detected not only in waste water but also in surface and ground waters and sediments. Environmental concentrations are relatively low, e.g. in surface waters they range from $ng.l^{-1}$–$g.l^{-1}$ (Daughton & Ternes 1999; Jarova *et al.* 2015). Likewise, available data show levels of human pharmaceuticals in biosolids in the range of $ng.kg^{-1}$ to $\mu g.kg^{-1}$. However, some antibiotics seem to reach higher ranges, e.g. tetracycline can be detected at a maximum concentration of 2.7 $mg.kg^{-1}$ in U.S. biosolids (Pino *et al.* 2015).

Most antibiotics are water soluble and excreted with urine as parent compounds (e.g. tetracyclines and some β-lactam antibiotics). The water soluble antibiotics or antibiotic residues may therefore leach to surface water in the vicinity of fields and affect the ecosystem e.g. microalgae or bacteria (Halling-Sørensen 2000).

Kemper (2008) states that penicillins and tetracyclines are not usually expected to be found in the aquatic environment due to the easy hydrolysation of penicillins and the precipitation and accumulation of tetracyclines. The structure of β-lactams such as penicillin, consisting of the β-lactam-ring, contribute to the poor stability of this group in the environment: the ring can be opened by β-lactamase, a widespread enzyme in bacteria, or by chemical hydrolysis. Thus, intact penicillins are usually not found in the environment. Nor were tetracyclines detected in any water sample. Data concerning the concentrations and biological fate of selected antibiotics, especially vancomycin in the environment are sporadic and insufficient.

Scientific articles state that tetracycline remains in soil and accumulates in the environment. Several studies conducted in Beijing indicate that concentrations of tetracycline in agricultural soils reached values of up to 307 $mg.kg^{-1}$. These soils were irrigated with wastewater and pig faeces. Normally, however, there is a much smaller concentration of tetracycline in soil (Dong *et al.* 2012). According to Xie (2011), the average concentration of tetracycline in agricultural soils is in the range from 450 to 900 $\mu g.kg^{-1}$. However, there are no regulations for antibiotic concentration limits in soil or sewage water (Thiele-Bruhn 2003).

The toxicity of antibiotics depends strongly on their fate in the environment. The sorption behaviour of an antibiotic in soil, sediment and activated sludge may affect the bioavailability of the antibiotic. The degree of sorption can vary greatly across soil, sediment and sludge types implying that toxicity can be highly variable between different soils and different antibiotics (Brandt *et al.* 2015).

The widespread use of antibiotics, along with their subsequent release into the environment, leads to an increase in resistance among bacterial strains. Continuous exposure could lead to changes in microbial communities in nature and affect food chains (Daughton & Ternes 1999). The issue of resistance has recently been dealt with in many studies (Nayarit-Ballesteros *et al.* 2016; Oteo & Aracil 2015; Tammer *et al.* 2016).

The aim of the work is to assess the ecotoxicity of selected antibiotics used in human and veterinary medicine using ecotoxicology tests on aquatic and terrestrial organisms as well as to compare the sensitivity of selected aquatic and soil organisms to the test substance.

MATERIAL & METHODS

Test organisms

Test organisms (*Daphnia magna*, *Folsomia candida, Eisenia fetida*) originated in cultures from the Ecotoxicological laboratory of the University of Veterinary and Pharmaceutical Sciences Brno (Czech Republic), except for the liquid-dried bacteria *Vibrio fischeri* which were obtained from producer Hach-Lange GmbH (Dusseldorf, Germany) and a culture of green algae *Pseudokirchneriella subcapitata* (Korshikov) Hindak, which was obtained from the collection of autotrophic organisms of the Botanical Institute of the Academy of Sciences (Trebon, Czech Republic).

Algal growth inhibition test

The experiments were carried out as described in OECD Guideline 201 (CSN EN ISO 8692 – Fresh water

algal growth inhibition test with unicellular algae *Pseudokirchneriella subcapitata*) with minor changes to fit our experimental conditions (Bednarova *et al.* 2012). The test endpoint was the algal culture growth rate inhibition over a 72±2-hour period compared with a control. Value of 72hEC50 (effect concentration) was calculated using growth rate as an endpoint (EC50 = concentration that caused a 50% reduction in growth rate). For the test to be valid, we followed the criteria stated in the guideline.

Inhibition of the mobility of Daphnia magna

The experiments were carried out as described in the OECD Guideline OECD 202 (CSN EN ISO 6341 – Determination of the inhibition of the mobility of *Daphnia magna*) with minor changes to fit our experimental conditions (Beklova *et al.* 2010). Juvenile water fleas (*Daphnia magna*) aged less than 24 hours were exposed to different concentrations of the tested substance under precisely defined temperature conditions (20 ± 2 °C) and light regime (photoperiod of 16 hours of light and 8 hours of dark) for 48 hour. At the end of the test the numbers of immobilized specimens were counted and we calculated the value of 48hEC50 (effect concentration) using the probit analysis. For the test to be valid, we followed the criteria stated in the guideline.

Luminescence inhibition of the Vibrio fischeri

The experiments were carried out as described in ISO 11348-2 (CSN EN ISO 11348-2 – Determination of the inhibitory effect of antibiotics on the light emission of *Vibrio fischeri*) with minor changes to fit our experimental conditions (Beklova *et al.* 2010). Liquid-dried luminescent bacteria of the strain *Vibrio fischeri* NRRL-B-11177, Reactivation solution and Standard solution (7.5% NaCl) were obtained from Hach-Lange GmbH (Dusseldorf, Germany), and stored at –18 °C until use. LUMIStox 300 (Hach-Lange GmbH) with the thermostat unit was used for measuring. This bioassay measures the emitted light from bioluminescent marine bacteria. The inhibition of cellular activity causes a decrease in respiration with the consequence of a decrease in luminescence. We used glass cuvettes with the reactivated bacteria and the prepared dilution series. The time period for measuring was 15 and 30 minutes. The 20% effective concentration (EC20) and the 50% effective concentration (EC50) values were determined according to the valid standard. For a test to be valid the value of the correction factor (f_{kt}) for 15 or 30 min incubation must be between 0.6–1.3.

Inhibition of reproduction of Folsomia candida

The experiments were carried out as described in the OECD Guideline 232 (CSN EN ISO 11267 – Inhibition of reproduction of Collembola *Folsomia candida*) with minor changes to fit our experimental conditions (Nemcova *et al.* 2012, 2013). The results were evaluated as the inhibition of reproduction and compared to the control. The EC50 (median effective concentration) was determined after 28 days. For the test to be valid, we followed the criteria stated in the guideline.

Inhibition of reproduction of Eisenia fetida

The experiments were carried out as described in the OECD Guideline 222 (CSN EN ISO 11268-2 Determination of effects on reproduction to *Eisenia fetida*) with minor changes to fit our experimental conditions (Heger *et al.* 2015). The results were evaluated as the inhibition of reproduction and compared to the control. The EC50 (median effective concentration) was determined after 56 days. For the test to be valid, we followed the criteria stated in the guideline.

Antibiotics

The effects of Tetracycline (Chlortetracycline HCl – Duchefa biochemie B.V, > 95.0%), Vancomycin (Duchefa biochemie B.V, > 95.0%) and Penicillin G (Penicillin G sodium – Duchefa biochemie B.V, > 96.0%), in particular, were examined.

All the tested antibiotics are water soluble. Therefore, for the preparation of the stock solutions, dilution water of composition described in the relevant standards was used. In the test with *Vibrio fischeri* the dilution water was 2% NaCl solution; in the algae test standard nutrient solution and in the test with *Daphnia magna* dilution water (CSN EN ISO 6341) were used. In tests with *Folsomia candida* and *Eisenia fetida* the soil was contaminated by dissolved antibiotics in an adequate amount of deionised water to achieve soil moisture equal to 50% of maximum water-holding capacity.

Concentrations were chosen on the basis of the range finding test. Based on the results a concentration range was determined which enabled us to set the values of EC20/EC50. Concentrations of stock solutions and dilutions of selected antibiotics used for individual tests are given in Table 1. Five replicates were used per the tested concentration.

Statistical analysis

The test results were evaluated using the software TOXICITA 3.1 (VÚV Ostrava, Czech Republic), by means of regression analysis of data with 95% confidence interval (CI) which were based on squared deviations of experimental values from the selected approximation function.

RESULTS & DISCUSSION

Ecotoxicity of antibiotics was determined in three aquatic and two terrestrial tests. The influence of selected antibiotics (i.e., penicillin G, tetracycline and vancomycin) on the observed effect in *P. subcapitata, D. magna, V. fischeri*, *F. candida* and *E. fetida* is shown in Figures 1–5, respectively. A summary of the results of the toxicity tests is shown in Table 2. The results of these

Tab. 1. Overview of the stock solutions and dilutions of selected antibiotics for individual test organisms.

Antibiotics		Test organisms				
		Daphnia magna	*Pseudokirchneriella subcapitata*	*Vibrio fischeri*	*Folsomia candida*	*Eisenia fetida*
PENICILLIN G	s.s.	10000 mg.l^{-1}	100000 mg.l^{-1}	100 mg.l^{-1}	10 g.l^{-1}	–
	c.t.	1200, 1400, 1600, 1800, 2000 mg.l^{-1}	6000, 7000, 8000, 9000, 10000, 11000, 12000 mg.l^{-1}	0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 mg.l^{-1}	10,100, 200, 300, 400 mg.kg^{-1}	10, 100, 200, 300, 400 mg.kg^{-1}
TETRACYCLINE	s.s.	100 mg.l^{-1}	100 mg.l^{-1}	100 mg.l^{-1}	100 g.l^{-1}	–
	c.t.	1, 3, 5, 7, 10 mg.l^{-1}	1.2, 1.5, 1.8, 2.1, 2.4, 2.7 mg.l^{-1}	0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 mg.l^{-1}	1500, 2000, 2500, 3000, 3500 mg.kg^{-1}	1500, 2000, 2500, 3000, 3500 mg.kg^{-1}
VANKOMYCIN	s.s.	10000 mg.l^{-1}	10000 mg.l^{-1}	100 mg.l^{-1}	20 g.l^{-1}	–
	c.t.	400, 600, 800, 1000, 1200 mg.l^{-1}	300, 350, 400, 450, 500 mg.l^{-1}	0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 mg.l^{-1}	250, 500, 750, 1000, 1250 mg.kg^{-1}	

s.s. – stock solution, c.t. – concentrations tested

Tab. 2. Summary of the test results (confidence interval is calculated for normal distribution and a level of 95%).

Antibiotics	Test organisms					
	Daphnia magna 48hEC50 (mg.l^{-1}) (95% CI)	*Pseudokirchneriella subcapitata* 72hEC50 (mg.l^{-1}) (95% CI)	*Vibrio fischeri* EC20 15', EC20 30' (mg.l^{-1})	*Vibrio fischeri* EC50 15', EC50 30' (mg.l^{-1})	*Folsomia candida* 28dEC50 (mg.kg^{-1}) (95% CI)	*Eisenia fetida* 56dEC50 (mg.kg^{-1}) (95% CI)
PENICILLIN G	1496.9	7114.3	– [a]	– [a]	328	348
	(1422.7–1574.9)	(6581.6–7646.9)	– [a]	– [a]	(89–568)	(208–487)
TETRACYCLINE	8.16	1.82	1.72 *fkt 1.08*	17.58 *fkt 1.16*	2560	2735
	(6.61–10.10)	(1.71–1.93)	0.22 *fkt 1.08*	6.70 *fkt 1.16*	(1760–3360)	(1352–4119)
VANKOMYCIN	686.9	370.8	– [a]	– [a]	546	– [b]
	(597.14–790.10)	(266.9–481.3)	4.36 *fkt 1.14*	– [a]	(354–739)	

95% CI – 95% confidence interval, f_{kt} – correction factor for 15 or 30 min incubation must be between 0.6–1.3, [a] Not obtainable [b] Not available

tests showed significant differences in the sensitivity of aquatic and soil organisms. The biggest differences were reflected in the sensitivity of aquatic and terrestrial organisms to tetracycline and penicillin G (β-lactam). While tetracycline showed the highest toxicity to aquatic organisms: algae (72hEC50 = 1.82 mg.l^{-1}), bacteria (EC50 30' = 6.70 mg.l^{-1}) and daphnia (48hEC50 = 8.16 mg.l^{-1}), penicillin G was the least toxic for aquatic organisms: algae 72hEC50 = 7114.3 mg.l^{-1}, daphnia 48hEC50 = 1496.9 mg.l^{-1} and even in bacteria luminescence inhibition was not observed as compared to control. Similar results were seen in other studies, e.g. the EC50 value for tetracycline *Pseudokirchneriella subcapitata* was 3.31 ± 0.96 mg.l^{-1} (Gonzales-Pleiter *et al.* 2013) and the value of NOEC (no observed effect concentration) for *Daphnia magna* was 340 mg.l^{-1} (Wollenberger *et al.* 2000). Halling-Sørensen (2000) determined the toxicity of tetracycline and penicillin G for the green alga *Selenastrum capricornutum* to be EC50 = 2.2 mg.l^{-1} = NOEC 100 mg.l^{-1} and for cyanobacteria *Microcystis aeruginosa* as EC50 = 0.09 mg.l^{-1} and 0.0006 mg.l^{-1}, respectively. Data from various authors differ only slightly, mainly because of the diversity of experimental conditions.

From the above-mentioned results, it is evident that whilst penicillin G is highly toxic for cyanobacteria, its toxicity for green algae is lower. One explanation for this species difference in sensitivity to β-lactams may be the fact that at pH between 5 and 7 the compound exhibits the lowest possible hydrolyses of the β-lactam ring in aqueous solutions. Due to a lower growth rate *M. aeruginosa* exhibits almost no increase in pH during the test period (remains around a pH of 7), where as in the test with green alga pH may increase up to 1.5 pH units during the 3 day test period. This may enhance the abiotic degradation of the compound (Halling-Sørensen 2000).

High toxicity of tetracycline for the green algae is probably related to their metabolism. Since green algae are eukaryotic organisms and the chloroplast belongs

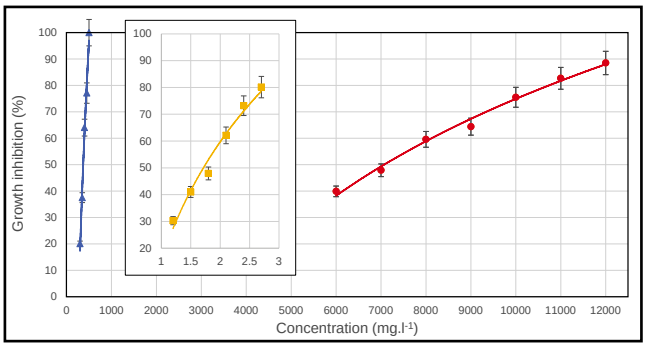

Fig. 1. Dose–response curves of tetracycline (■; inset), vancomycin (▲) and penicillin G (●) for *Pseudokirchneriella subcapitata*. Error bars correspond to 95% confidence intervals. Dotted lines represent the fitting to the median effect equation.

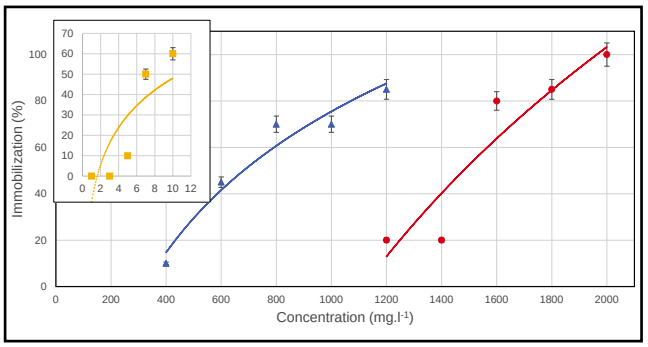

Fig. 2. Dose–response curves of tetracycline (■; inset), vancomycin (▲) and penicillin G (●) for *Daphnia magna*. Error bars correspond to 95% confidence intervals. Dotted lines represent the fitting to the median effect equation.

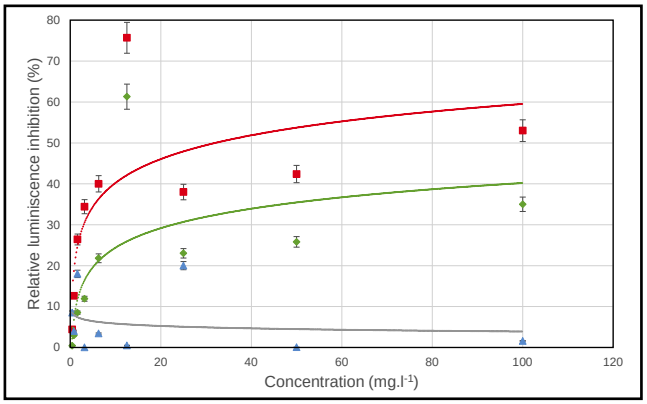

Fig. 3. Dose–response curves of tetracycline after 15 minutes incubation (◆), tetracycline after 30 minutes incubation (■) and vancomycin (▲) for *Vibrio fischeri*. Error bars correspond to 95% confidence intervals. Dotted lines represent the fitting to the median effect equation.

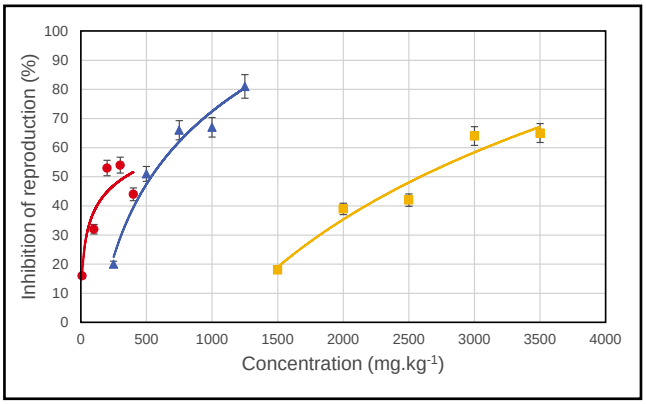

Fig. 4. Dose–response curves of tetracycline (■), vancomycin (▲) and penicillin G (●) for *Folsomia candida*. Error bars correspond to 95% confidence intervals. Dotted lines represent the fitting to the median effect equation.

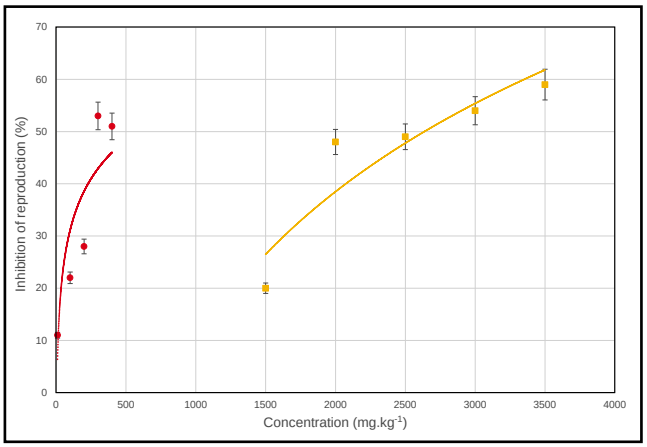

Fig. 5. Dose–response curves of tetracycline (■) and penicillin G (●) for *Eisenia fetida*. Error bars correspond to 95% confidence intervals. Dotted lines represent the fitting to the median effect equation.

to the semi-autonomous organelle, the toxic effects of antibiotics on green algae are related to the inhibition and interference of chloroplast metabolism such as photosynthesis procedures and interrelated protein synthesis, which disturb the function of the photosynthetic apparatus and finally affect cell growth (Magdaleno *et al.* 2015). The toxic effect data (EC50) of antibacterial agents on various aquatic species found in the literature shows values in the mg/l range.

On the contrary, the reproduction of soil organisms was most inhibited by penicillin G (28dEC50 = 328 $mg.kg^{-1}$ for springtails and 56dEC50 = 348 $mg.kg^{-1}$ for earthworms). The reproductive parameters were least affected by tetracycline; its 28dEC50 had a value of 2,560 $mg.kg^{-1}$ for *F. candida* and 2,735 $mg.kg^{-1}$ for *E. fetida*. No significant effect on mortality was found.

The low toxicity of tetracycline assessed in 28/56 day long reproduction tests is probably related to its relatively rapid biodegradation and photosensitivity. As stated in Pino *et al.* (2015), in the acute toxicity test with *Eisenia fetida*, tetracycline was completely degraded after 14 days. In the Pino *et al.'s* test the LC50 value of tetracycline was set at >2,000 $mg.kg^{-1}$.Similar results were obtained by Baguer *et al.* (2000) who tested the effects of antibiotics on three species of soil fauna: earthworms, springtails and enchytraeids and the lowest observed effect concentration was 3,000 $mg.kg^{-1}$

and in many cases no effect was seen even at the highest test concentration of 5,000 mg.kg^{-1}. Pan & Chu (2016) tested adsorption and degradation properties of several antibiotics on farm land. He found out that tetracycline was adsorbed the most in soil. However, data about concentrations of these drugs in soils are very scarce (Pino *et al.* 2015).

Likewise, information from different authors differ, mainly because of the diversity of experimental conditions. For instance, Kim *et al.* (2005) found no evidence of tetracycline biodegradation over the test duration.

The effective concentration of vancomycin, which caused 50% inhibition of the reproduction of springtails *F. candida* in 28 days was 546 mg.kg^{-1}. There was a significant effect on mortality. The LC50 of vancomycin was 914.4 mg.kg^{-1}. All CV values were less than 40% for each concentration of vancomycin. Mean adult survival rates ranged from 1 to 10 out of 10 collembolans per vessel. The 72hEC50 and 48hEC50 of vancomycin for *P. subcapitata* and *D. magna* amounted to 370 and 686.9 mg.l^{-1}, respectively. While in a study by Magdaleno *et al.* (2015), the EC50 value of vancomycin for *P. subcapitata* was found at 724 ± 20 mg.l^{-1}.

Tests with *Vibrio fischeri* confirmed the results of a study by Brandt *et al.* (2015) that *Vibrio fischeri* is insensitive to antibiotics as the inhibition of luciferase activity (bioluminescence) after short-term exposure is not related to the mode of toxic action of most antibiotics.

CONCLUSION

To conclude, the ecotoxicity of antibiotics differed significantly depending on the test organism and the variety of experimental conditions. In our study the biggest differences were manifested in the sensitivity of aquatic and terrestrial organisms to tetracycline and penicillin G. Data concerning the concentrations and biological fate of selected antibiotics, especially vancomycin, in the environment are sporadic and insufficient.

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