Chemosphere 92 (2013) 1253–1259

Contents lists available at SciVerse ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Aquatic toxicity of four veterinary drugs commonly applied in fish farming and animal husbandry

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HIGHLIGHTS

- Ecotoxicity of four veterinary drugs to four aquatic organisms was evaluated.
- New data about their ecotoxicological potential has been presented.
- All the ecotoxicological tests were supported by chemical analyses.
- DOR was found to be highly toxic toward *Daphnia magna.*

ARTICLE INFO

Article history:
Received 14 January 2013
Received in revised form 18 April 2013
Available online 18 May 2013

Keywords:
Ecotoxicity
Oxytetracycline
Florfenicol
Doramectin
Metronidazole
Aquatic organisms

ABSTRACT

Doramectin (DOR), metronidazole (MET), florfenicol (FLO), and oxytetracycline (OXT) are among the most widely used veterinary drugs in animal husbandry or in aquaculture. Contamination of the environment by these pharmaceuticals has given cause for concern in recent years. Even though their toxicity has been thoroughly analyzed, knowledge of their ecotoxicity is still limited. We investigated their aquatic toxicity using tests with marine bacteria (*Vibrio fischeri*), green algae (*Scenedesmus vacuolatus*), duckweed (*Lemna minor*) and crustaceans (*Daphnia magna*). All the ecotoxicological tests were supported by chemical analyses to confirm the exposure concentrations of the pharmaceuticals used in the toxicity experiments, since deviations from the nominal concentration can result in underestimation of biological effects. It was found that OXT and FLO have a stronger adverse effect on duckweed (EC_{50} = 3.26 and 2.96 mg L^{-1} respectively) and green algae (EC_{50} = 40.4 and 18.0 mg L^{-1}) than on bacteria (EC_{50} = 108 and 29.4 mg L^{-1}) and crustaceans (EC_{50} = 114 and 337 mg L^{-1}), whereas MET did not exhibit any adverse effect in the tested concentration range. For DOR a very low EC_{50} of 6.37 × 10^{-5} mg L^{-1} towards *D. magna* was determined, which is five orders of magnitude lower than values known for the toxic reference compound $K_2Cr_2O_7$. Our data show the strong influence of certain veterinary drugs on aquatic organisms and contribute to a sound assessment of the environmental hazards posed by commonly used pharmaceuticals.

1. Introduction

Large quantities of veterinary pharmaceuticals (VPs) are in use worldwide. As animals do not completely metabolize these compounds, a large proportion of them are excreted unchanged in feces and urine. Therefore, both the drugs and their metabolites are released into the environment, either directly from aquaculture and by grazing animals, or indirectly during manure spreading (Reemtsma and Jekel, 2006).

Of the various pharmaceuticals commonly used in veterinary medicine, special attention has been paid to four of them in the present work: doramectin, metronidazole, florfenicol and oxytetracycline, which differ in their activity and physicochemical properties (Table 1).

OXT, FLO and MET are antibiotics and have a similar mode of action at the DNA/RNA-level. OXT is commonly used because of its broad-spectrum efficacy in the treatment of infections caused by Gram-positive and Gram-negative bacteria, mycoplasma and large viruses. It inhibits protein synthesis by preventing the association of aminoacyl-tRNA with bacterial ribosomes (Reemtsma and Jekel, 2006). FLO is a fluorinated derivative of thiamphenicol, inhibits transpeptidation in the bacterial protein synthesis, and is effective

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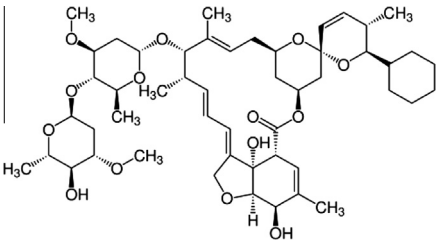
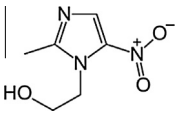
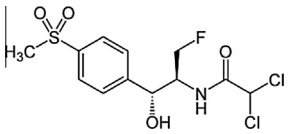
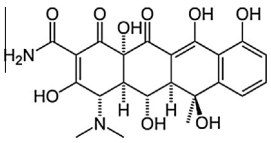
0045-6535/$ - see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.chemosphere.2013.04.057

Table 1
Structures and physicochemical properties of the investigated veterinary drugs.

Substance (Abbreviation) [CAS]	Structure	M.w. (g mol^{-1})	pK_{a1}	pK_{a2}	pK_{a3}	Log P	Water solubility (mg L^{-1})
Doramectin (DOR) [117704-25-3]		899.1	12.4	–	–	4.44	0.025
Metronidazole (MET) [443-48-1]		171.2	2.4	–	–	−0.1	10000
Florfenicol (FLO) [73231-34-2]		358.2	9.3	–	–	−0.12	1320
Oxytetracycline (OXT) [96310-42-8]		460.4	3.3	3.7	9.1	−1.22	>100000 (HCl salt)

against many Gram-negative and Gram-positive bacteria (Christensen et al., 2006). The nitroimidazole MET is an antibiotic effective against anaerobic bacteria, protozoans and certain parasites. It acts by entering bacterial and protozoan cells and interfering with DNA (Lanzky and Halling-Sørensen, 1997). DOR is an antiparasitic drug which is a one of the most popular compound for curing anthelmintic disease. DOR binds to receptors that increase membrane permeability to chloride ions. This inhibits the electrical activity of nerve cells in nematodes and muscle cells in arthropods, paralyzing and ultimately killing the parasites (Horvat et al., 2012; Lumaret et al., 2012). In many countries these pharmaceuticals are registered as medical premixes so they can be used in feedstuffs for the treatment or prevention of animal diseases. For example the usage of OXT in the UK in 2000 was 8.5 t and in the US, just in aquacultures in 2003, was 15 t (Sarmah et al., 2006; Schmidt et al., 2007). Furthermore, in accordance to the IMS Health Market Prognosis (2012) the global pharmaceutical market has grown twice during the last decade. However the real amounts of drugs entering the environment can be much more higher due to lack of control with type and amounts of pharmaceuticals usage in developing countries e.g. India, Thailand, Indonesia (Sarmah et al., 2006). The relatively low cost and the broad spectrum of activity of these pharmaceuticals means they are very commonly used not only in animal husbandry (DOR, MET, FLO, OXT) but also in aquaculture (MET, FLO, OXT) (Christensen et al., 2006; Ferreira et al., 2007; Lai et al., 2009; Lumaret et al., 2012; Horvat et al., 2012).

It must be pointed out that the veterinary medicines used in aquaculture are commonly administered as a medicated feed mainly as a bath formulation (Hekoten et al., 1995; Boxall, 2010). Hence a considerable proportion of these drugs administered in an intensive fish farm was found to have been released into the aquatic environment via urinary and fecal excretions and in unconsumed medicated food (Hekoten et al. 1995). For example, Ferreira et al. (2007) highlighted that when OXT was administrated orally, fish took up only about 10–30% of the total amount administered, while 70–90% of it entered the environment and was available for distribution to other compartments.

Disregarding the different routes by which these pharmaceuticals enter the environment, their presence in its different compartments has already been determined (Kolpin et al., 2002; Kay et al., 2005). Despite their quite low concentrations in environmental samples at the μg L^{-1} or ng L^{-1} level (MET – 30 ng L^{-1}, OXT – 340 μg L^{-1}, FLO – 2.4 μg L^{-1}) (Kolpin et al., 2002; da Silva et al., 2011; Wei et al., 2012) and their different stabilities in the environment (FLO is resistant to abiotic degradation but easily biodegradable; OXT is susceptible to photodegradation; MET cannot be biodegraded, photodegradation under UV light is also less effective; DOR is quite susceptible to both biodegradation or photodegradation), they are continuously being released into ecosystems (Jacobsen and Berglind, 1988; Oka et al., 1989; Lunestad, 1992; Pfizer Inc., 1996; Doi and Stoskopf, 2000). Consequently, these compounds may be considered pseudo-persistent. Therefore, the kind of exposure organisms may be subjected to will resemble that of traditional pollutants (e.g. pesticides, detergents).

This may result in adverse ecological effects, including, for example, the development of resistant bacterial populations or direct toxicity to microflora and microfauna. Since these compounds (like FLO and MET) are polar pollutants with quite a low sorption potential and/or are directly introduced into aquatic environment (like OXT), it can be assumed that aquatic organisms may be the species most endangered by the presence of pharmaceuticals used in aquaculture and animal husbandry. Kolar and Kožuh Eržen (2006) reported that DOR is excreted mainly with the feces: almost 98% of the drug is excreted as the non-metabolized

Table 2
Aquatic toxicity data of investigated veterinary drugs from literature.

Substance	Type of test	Species	Critical effect	Exposure time	Toxicity (mg L^{-1})	Ref.
DOR		*Daphnia magna* (Crustacean)	Immobilization	48 h	EC_{50} = 0.0001	Pfizer inc. (1996)
MET	ISO 8692 (1989)	*Chlorella sp.* (Green algae)	Growth inhibition	72 h	EC_{50} = 38.8	Lanzky and Halling-Sørensen (1997)
		Selenastrum capricornutum (Freshwater green algae)		72 h	EC_{50} = 39.1	
	ISO/CD 14669	*Acartia tonsa (Crustacea)*	Immobilization	72 h	NOEC = 100	Lanzky and Halling-Sørensen (1997)
	OECD, *D. Magna* Reproduction Test (1996)	*Daphnia magna* (Crustacean)	Reproduction	21 d	NOEC = 250	Wollenberger et al. (2000)
	ISO 6341 (1989)		Immobilization	48 h	LOEC = 1000	Wollenberger et al. (2000)
FLO	ISO 11348-3	*Vibrio fischeri* (Bacteria)	Luminescence	15 min	EC_{50} > 65	Christensten et al. (2006)
	Miniaturized international standard method	*Pseudokirchneriella subcapitata* (Green algea)	Growth inhibition	48 h	EC_{50} = 2.3–9.3	Christensten et al. (2006)
	OECD 201 (1984)	*Chlorella pyrenoidosa* (Freshwater green algea)	Growth inhibition	96 h	EC_{50} = 215	Lai et al. (2009)
		Isochrysis galbana (Marine algea)		96 h	EC_{50} = 8	
		Tetraselmis chui (Marine algea)		96 h	EC_{50} = 1.3	
	OECD 201 (1984)	*Tetraselmis chuii* (Microalgea)	Growth inhibition	72 h	EC_{50} = 11.31	Ferreira et al. (2007)
				96 h	EC_{50} = 6.06	
	ArToxKit test protocol	*Artemia parthenogenetica* (Crustacean)	Immobilization	24 h	EC_{50} > 889	Ferreira et al. (2007)
OXT	Microtox Model 500 analyzer	*Vibrio fischeri* (Bacteria)	Luminescence	15 min 5 min	EC_{50} = 87.0 EC_{50} = 235.4	Park and Choi (2008)
	According to Microtox Manual (1995)		Luminescence	30 min	EC_{50} = 64.50	Isidori et al. (2005)
	ISO 11348-3		Luminescence	15 min	EC_{50} = 66	Christensten et al. (2006)
	ISO 11348 (1994)		Luminescence	30 min	EC_{50} = 132.3	Lalumera et al. (2004)
	ISO 11348-3		Luminescence	30 min	EC_{50} = 21	Zounková et al. (2011)
	OECD 201 (1993)	*Chlorella vulgaris* (Green algae)	Growth inhibition	72 h	EC_{50} = 7.05	Eguchi et al. (2004)
	OECD 201 (1984)		Growth rate inhibition	48 h	EC_{50} = 6.4	Pro et al. (2003)
	ISO/DIS 8692 (1989)	*Pseudokirchneriella subcapitatata* (Green algae)	Growth inhibition	72 h	EC_{50} = 0.17	Isidori et al. (2005)
	Miniaturized international standard method		Growth inhibition	48 h	EC_{50} = 0.47–2.0	Christensten et al. (2006)
	ISO/DIS 8692 (1989)		Growth inhibition	96 h	EC_{50} = 3.1	Zounková et al. (2011)
	OECD 201 (1993)	*Selenastrum capricornutum* (Freshwater green algae)	Growth inhibition	72 h	EC_{50} = 0.342	Eguchi et al. (2004)
	ISO 8692 (1989), modified version		Growth inhibition	72 h	EC_{50} = 4.5	Lützhøft et al. (1999)
	OECD 201 (1984)	*Tetraselmis chuii* (Microalgea)	Growth inhibition	72 h	EC_{50} = 13.16	Ferreira et al. (2007)
				96 h	EC_{50} = 11.18	
	ASTM, static-relevant toxicity test with *Lemna gibba* G-3, E 1415-91 (1998)	*Lemna gibba* (Duckweed)	Growth inhibition	7 d	EC_{50} = 1.01	Brain et al. (2004)
	not defined	*Lemna minor* (Duckweed)	Growth inhibition	7 d	EC_{50} = 4.92	Pro et al. (2003)
	ISO 20079 (2005)		Growth inhibition	7 d	EC_{50} = 2.1	Zounková et al. (2011)
	US EPA-821-R-02-012 (2002)	*Moina macrocopa* (Crustacean)	Immobilization	24 h 48 h	EC_{50} = 137.1 EC_{50} = 126.7	Park and Choi (2008)
	US EPA-821-R-02-012 (2002)	*Daphnia magna* (Crustacean)	Immobilization	24 h 48 h	EC_{50} = 831.6 EC_{50} = 621.2	Park and Choi (2008)
	ISO 6341 (1989)		Immobilization	48 h	LOEC = 100	Wollenberger et al. (2000)
	ISO 6341 (1996)		Immobilization	48 h	EC_{50} = 86	Zounková et al. (2011)
	OECD, *D. Magna* Reproduction Test (1996)		Reproduction	21 d	EC_{50} = 46.2	Wollenberger et al. (2000)
	ISO/6341 (1996)	*Daphnia magna Straus* (Crustacean)	Immobilization	24 h	EC_{50} = 22.64	Isidori et al. (2005)
	ArToxKit test protocol	*Artemia parthenogenetic* (Crustacean)	Immobilization	24 h	EC_{50} = 870.47	Ferreira et al. (2007)
				48 h	EC_{50} = 805.99	
	USEPA, EPA-600-4-90-027F (1993)	*Ceriodaphnia dubia* (Crustacean cladocera)	Immobilization	48 h	EC_{50} = 18.65	Isidori et al. (2005)
	ISO/CD 20665 (2001)		Population growth inhibition	7 d	EC_{50} = 0.18	Isidori et al. (2005)

pharmaceutical. DOR's mobility is very low which is related to the very high soil organic carbon-water partitioning coefficient (K_{oc} >1000). The K_{oc} value is very useful in predicting the mobility of organic soil contaminants; generally the higher K_{oc} values the less mobile organic chemicals. However, studies have shown that the parent form of this drug can be present in the aquatic environment at concentration of 0.24–0.42 ng L^{-1} (Pfizer Inc., 1996).

Several studies have investigated the aquatic toxicity of some of these compounds (summarized in Table 2). While hardly any data is available for DOR; OXT, FLO and MET have been more extensively investigated. Even so, systematic studies investigating these compounds in different test systems and linking ecotoxicological research with instrumental analysis are still lacking. The aim of the present work is to enrich our limited knowledge so far of the potentially deleterious effects on the environment of commonly used VPs in aquacultures or animal husbandry. To this end we used a battery of ecotoxicological tests comprising organisms of different trophic levels and complexity: luminescent marine bacteria (*Vibrio fischeri*), limnic unicellular green algae (*Scenedesmus vacuolatus*), duckweed (*Lemna minor*) and crustaceans (*D. magna*). Although EC_{50} values for investigated compounds are available in literature they are highly different. Hence, the objective of our work was also to evaluate the water soluble fraction of the test compounds in different test media performed via HPLC–UV measurements. Such experiment is an essential tool to evaluate trustworthy EC_{50} values and it enhances much reliability of obtained data.

2. Experimental

All the data generated in our study were obtained according to internationally accepted test guidelines (e.g. OECD, ISO) or on specific (e.g. national) testing guidelines (e.g. DIN). Hence, according to the European Medicines Agency (Klimisch et al. (1997) and EMEA, 2008 our results can be classified under "reliability index 1".

According to Klimisch et al. (1997) different terms are being used synonymously to characterize the quality of the data of toxicological and ecotoxicological studies: validation/validity, reliability and adequacy. These terms describe not only procedures to define the quality of test results (data), but also test methods are validated to prove their relevance and reproducibility. The RI category includes studies or data from the tests generated according to generally valid and/or internationally accepted testing guidelines or in which the test parameters documented are based on a specific (national) testing guideline (preferably performed according to Good Laboratory Practice) or in which all parameters described are closely related/comparable to a guideline method. Therefore, all the ecotoxicological tests used in our study fulfill the validation criteria (for example for ISO 8692 validity criteria includes: the average control growth, variability of control growth rates (C.V.%), changes of medium pH) stated in respective Standard Protocols Procedures (SOP) and according to ENV/JM/MONO(2008)28.

2.1. Chemicals and reagents

DOR, MET, FLO and OXT (HCl salt), acetonitrile (ACN) and salts (see Table A1, Appendix A) used for the culturing media were purchased from Sigma–Aldrich (Steinheim, Germany). The standard stock solution of DOR was prepared by dissolving it in acetonitrile at a concentration of 500 mg L^{-1}; it was then stored at −18 °C in the dark. All standard stock solutions of OXT (HCl salt), MET and FLO used in the assays were prepared in the proper medium (buffer) to obtain the desired concentration. For DOR 0.2% of ACN was added to the control samples in the toxicity test to offset the influence of this solvent on the test organisms.

2.2. Luminescent inhibition assay with marine bacteria

The toxicity test based on *Vibrio fischeri* was done using the LCK 482 test kit (Dr Lange GmbH, Germany). The 30-min standard bioluminescence inhibition assay was carried out according to a modified DIN 38412-L34 protocol (1991).

The tests were carried out at least twice for each substance. At least four controls (2% NaCl solution, phosphate-buffered) were used during each test.

The tests were performed at 15 °C using thermostats (LUMIStherm, Dr. Lange GmbH, Germany). The luminescence was measured with a luminometer (LUMIStox 300, Dr Lange GmbH, Germany). The lyophilized bacteria were rehydrated according to the test protocol; then, 500 μL aliquots of the bacteria solution were pre-incubated for 15 min at 15 °C. After the initial luminescence had been measured, 500 μL of the diluted samples were added to the bacteria. The bioluminescence was measured again after an incubation time of 30 min. The relative toxicity of the samples was expressed as a percentage inhibition compared to the controls.

2.3. Reproduction inhibition assay with limnic green algae

A synchronized culture of the green algae *S. vacuolatus* (strain 211-15, SAG (Culture Collection of Algae), Universität Göttingen, Germany) was used for this assay. The stock culture was grown under photoautotrophic conditions at 28 °C (±0.5 °C) in an inorganic, sterile medium (pH 6.4) with saturating white light (22–33 klx, Lumilux Daylight L 36W-11 and Lumilux Interna L 36W-41, Osram, Berlin, Germany). The cells were aerated with 1.5 vol% CO_2 and synchronized using a 14–10 h light – darkness cycle. The stock culture was diluted every day to a cell density of 5×10^5 cells mL^{-1}. This test is a modified version of the assay described by Altenburger et al. (1990), and its sensitivity is comparable to the standardized 72 h test (ISO 8692, 1989).

The toxicity tests started with autospores. The algae were exposed to the test substances for one growth cycle (24 h). The endpoint of this assay is the inhibition of algal reproduction, measured as the inhibition of population growth. Cell numbers were determined with a Coulter Counter Z2 (Beckmann, Nürnberg, Germany). The tests were performed in sterilized glass tubes, the algae were stirred throughout the 24 h test period, and the test conditions were the same as for the stock culture except for the CO_2 source. Here, 150 μL of $NaHCO_3$ solution was added to each test tube. The methods of stock culturing and testing are described in detail by Faust et al. (2001). Growth inhibition was calculated using the cell counts of the treated samples in relation to the untreated controls. At least six controls were used for each assay. The tests were performed using six different concentrations in two replicates of every compound and each test was carried out at least twice.

2.4. Growth inhibition assay with duckweed

The growth inhibition assay with *L. minor* was performed according to a modified version of the test protocol described in detail by Drost et al. (2007). The plants were grown in open Erlenmeyer flasks in sterilized Steinberg medium (pH 5.5 ± 0.2) in a climate chamber with a constant temperature of 25 ± 2 °C. To exclude pH effects on plant growth, the pH was checked at the beginning and end of the test. Based on control samples evaluation the pH changes did not affect growth inhibition. The chamber was illuminated continuously with a maximum of 6 klx. The assays were performed on six-well cell culture plates (Greiner Bio-One GmbH, Frickenhausen, Germany). All compounds were tested twice in three replicates, with a minimum of six controls in each test. The test started with one plant consisting of three duckweed fronds,

and the measured endpoint was the inhibition of the growth rate determined by the frond area (mm^2), which was calculated for the treated plants in relation to the untreated controls. The frond area was detected using a Scanalyzer from Lemnatec GmbH (Würselen, Germany).

2.5. Reproduction inhibition assay with D. magna

The 48 h acute immobilization test with *D. magna* was assessed using the commercially available Daphtoxkit F (MicroBioTest Incorporation, Gent, Belgium), referred to OECD guideline 202 (2004). The detailed description of this assay is given in the supplier's standard operational procedure (MicroBioTest Inc., 1996). The tests with neonates less than 24 h old, obtained by the hatching of ephippia, were performed at 20 °C in the dark. 5 pre-fed animals were incubated with the toxicants in a volume of 10 mL of mineral medium. In each test five different concentrations of the test substance were investigated in five parallels and five controls. All the experiments were performed twice. The numbers of immobilized organisms were checked after 24 and 48 h. The sensitivity of the organisms to $K_2Cr_2O_7$ was checked routinely once a new batch of organisms was obtained.

2.6. Effect data modeling

Dose-response curve parameters and plots were obtained using the drift package (version 0.05-95) for the R language and environment for statistical computing (www.r-project.org) (R Development Core Team, 2005).

2.7. Instrumental analysis

In order to determine the soluble fraction of the investigated compound in biological media (see in Table A1, Appendix A) HPLC analysis was performed using different conditions depending on the analyte (see in Appendix A, Table A2). The stock solution concentrations of DOR, MET, FLO and OXT in media solutions used in four ecotoxicity tests were estimated by fitting chromatographic peak areas to the calibration curve.

3. Results and discussion

3.1. Instrumental analysis

The results presented in Table 3 show that the concentration of the soluble fraction of MET, FLO and OXT in biological media did not differ from the nominal concentration of these drugs prepared in media solution. The accuracy of the results was from 94.4% to 105.6%. It can therefore be assumed that the solubility of the test compounds in biological media does not affect EC_{50}s.

Because of the very low solubility of DOR in water, solutions with nominal concentrations were prepared in biological media with the addition of 0.2% ACN. Although, it is recommended not to use more than 0.1% of ACN, it was assessed during control samples examination that amount of 0.2% did not affect the organisms used in the tests. This is also supported by the data presented in Table 4. However, in the presence of ACN, DOR is generally poorly soluble in different media (<0.34 mg L^{-1}), and is even less so in *V. fischeri* media (<0.24 mg L^{-1}), which can be explained by the high ionic strength of this buffer.

3.2. Toxicity testing

All the EC_{50} values and confidence intervals (CI) for the drugs investigated here and the EC_{50}s for the toxic reference compounds (atrazine, potassium dichromate, acetonitrile and 3,5-dichlorophenol) are presented in Table 4.

3.2.1. Luminescence inhibition assay with marine bacteria

The results listed in Table 4 show that none of the tested drugs displayed a high level of acute toxicity toward *V. fischeri*. The lowest EC_{50} was found for FLO (29.4 mg L^{-1}); the values for OXT and MET were >100 mg L^{-1}. No effect up to 0.24 mg L^{-1} could be determined for DOR. This concentration is, however, well above environmentally relevant concentrations, which range from ng L^{-1} to hundreds of μg L^{-1} (Boxall et al., 2006). The 30 min EC_{50} obtained for OXT (108 mg L^{-1}) is comparable to the results reported by Lalumera et al. (2004) (121–139 mg L^{-1}) (Table 2). However, no clear correlation between available ecotoxiciy data for OXT to *V. fischeri* and exposure time can be observed.

Isidori et al. (2005) highlighted that most of the toxicity data available for *V. fischeri* using short exposure times (between 5 and 30 min) rather than a 24 h exposure show that most pharmaceuticals have a low toxic potential in this respect, because these compounds interfere only slightly with biosynthetic pathways. For example, the low sensitivity of *V. fischeri* to OXT can be explained by its mode of action on protein synthesis, which is not of importance during short-term bioluminescence testing (Zounková et al., 2011).

3.2.2. Reproduction inhibition assay with green algae

The toxicity of veterinary drugs toward *S. vacuolatus* is reported for the first time. Our results indicate that this limnic green alga was generally more sensitive than *V. fischeri* for FLO and OXT. FLO exhibited the strongest growth inhibition against *S. vacuolatus*, followed by OXT and MET. The toxicity of DOR to green algae has not been calculated yet, but our study found its EC_{50} to be >0.34 mg L^{-1}. The exact value was not determined because of the low water solubility of this compound and the necessity of performing the test with an additional concentration of acetonitrile. Nevertheless, *S. vacuolatus* was found to be less sensitive to OXT, MET and FLO than *C. vulgaris, P. subcapitata* or *S. capricornatum*, as shown in Table 2. The results for FLO (EC_{50} = 18.0 mg L^{-1}) are comparable to those given by Ferreira et al. (2007) and Lai et al. (2009), who evaluated the toxicity of FLO to different algae (*Isochrysis galbana* and *Tetraselmis chui*) (Table 2), widely used as food sources in early larval cultures of mollusks, fish and crustaceans. Ferreira et al. (2007)) compared the toxicity of FLO and OXT to

Table 3
Concentrations of soluble fraction of examined compounds in biological media.

	V. fischeri		*S. vacuolatus*		*L. minor*		*D. magna*	
	Nominal concentration (mg L^{-1})	Concentration of soluble fraction (mg L^{-1})±SD	Nominal concentration (mg L^{-1})	Concentration of soluble fraction (mg L^{-1})±SD	Nominal concentration (mg L^{-1})	Concentration of soluble fraction (mg L^{-1})±SD	Nominal concentration (mg L^{-1})	Concentration of soluble fraction (mg L^{-1})±SD
DOR	1	0.24 ± 0.02	1	0.34 ± 0.02	1	0.31 ± 0.01	1	0.31 ± 0.01
MET	250	258 ± 1	2000	1920 ± 5	25	25.3 ± 0.1	2500	2550 ± 23
FLO	125	123 ± 2	–	nd	–	nd	500	494 ± 6
OXT	125	118 ± 2	1000	983 ± 13	5	5.5 ± 0.1	500	508 ± 9

Table 4
Experimentally obtained EC_{50} values for the selected veterinary drugs and the toxic reference compounds ([a]Palma et al., 2008, [b]Faust et al., 2001; [c]Teodorović et al., 2012, [d]Kaiser and Palabrica, 1991;[e]ISO 6341, [f]EC, 2007).

Substance	EC_{50} (mg L^{-1}) (Confidence Interval (mg L^{-1})			
	V. fischeri	*S. vacuolatus*	*L. minor*	*D. magna*
DOR	nr[1]	nr[2]	nr[3]	6.37×10^{-5} (5.72×10^{-5} – 7.09×10^{-5})
MET	243 (231–257)	705 (600–827)	>25	211 (170–258)
FLO	29.4 (18.7–49.6)	18.0 (14.8–24.8)	2.96 (2.63–3.35)	337 (261–447)
OXT	108 (86–181)	40.4 (30.6–54.4)	3.26 (2.82–3.86)	114 (96–138)
Atrazine	69.4[a]	0.039[b]	0.188[c]	35.5[a]
$K_2Cr_2O_7$				0.6 – 2.1[e]
ACN	24 175[d]		3663[f]	>1000[f]
3,5-Dichlorophenol	3.91[d]			

na – not available.
nr – not reached (due to low solubility).
[1] The maximum investigated concentration – 0.24 mg L^{-1}.
[2] The maximum investigated concentration – 0.34 mg L^{-1}.
[3] The maximum investigated concentration – 0.31 mg L^{-1}.

Tetraselmis chui. As a result, despite having the same general mode of action, FLO was found to be more toxic (EC_{50} = 6.06 mg L^{-1}) than OXT (EC_{50} = 11.18 mg L^{-1}), a situation backed up by our findings.

3.2.3. Growth inhibition assay with duckweed

No toxic effect toward *L. minor* was found for the maximal soluble fraction of MET (>25 mg L^{-1}) and DOR (>0.31 mg L^{-1}). Effects were found for OXT and FLO – both EC_{50} values were around 3 mg L^{-1} – but they were one order of magnitude higher than the values determined for the toxic reference compound atrazine.

Pro et al. (2003) and Zounková et al. (2011) determined the toxicity of OXT toward *L. minor* in a 7-d test, obtaining EC_{50}s of 4.92 mg L^{-1} and 2.1 mg L^{-1}, respectively. Brain et al. (2004), however, assessed the phytotoxicity of OXT to *Lemna gibba* in 7-d static-renewal tests at the level of EC_{50} = 1.01 mg L^{-1} (calculated for the frond number). Halling-Sørensen (2000) suggested that the toxic effect may have been underestimated in static tests because, for example, of photodegradation of compounds with short half-lives like OXT. Other authors, however, have confirmed the negligible influence of photodegradation on the results under the conditions of a 7-d static test (Zounková et al., 2011). Photodegradation can also be presumed insignificant in the context of the current study, because the EC_{50} for OXT calculated in this study is generally comparable with the results obtained by both static and static-renewal tests (Brain et al. 2004; Zounková et al., 2011). Brain et al. (2004) also suggested that the ability of antibiotics (like MET, OXT and FLO) to affect plants might be understood by examining the chloroplast, which originated from the engulfment of an endosymbiotic *Cyanobacteria* – like a prokaryotic cell by a larger eukaryotic cell. However, no information on the toxic effect of DOR toward aquatic higher plants can be found in the literature. This is probably due to the assumption that this compound, having a high Koc, will have the potential to sorb to soil or other solid matrices. As knowledge of its environmental fate and mobility in the environment is very limited, however, its effects on aquatic organisms should not be excluded from an evaluation of its ecotoxicity potential (Kolar and Kožuh Eržen, 2006.).

3.2.4. Growth inhibition assay with D. magna

D. magna is usually regarded as the least sensitive organism, toward which, for example, OXT demonstrated no toxicity up to 400 mg L^{-1} in an acute test and only low toxicity in a chronic reproduction test (Zounková et al., 2011). Similar results were also obtained by Wollenberger et al. (2000) for both OXT and MET; these observations are in agreement with our results for these two compounds. No data about the toxicity of FLO toward *D. magna* are available to date. Our investigation demonstrated the very low toxicity of this compound toward *D. magna* – 337 mg L^{-1}. However, we obtained strong effects (EC_{50} = 0.0637 μg L^{-1}) with DOR, which shows that this compound is five orders of magnitude more toxic than $K_2Cr_2O_7$, the reference substance for this test. This was also stated by Pfizer Inc. (1996), who reported an EC_{50} of 0.1 μg L^{-1} for DOR. In their experiments, however, the authors did not consider determining the water-soluble fraction of this drug in biological media, which can be lower than in pure water. Hence the amount of compound which can cause harmful effect on the tested organisms can be lower than suspected. Therefore the EC_{50} of DOR that they determined was almost twice as high as our value (Pfizer Inc., 1996). These results highlight the importance of combining ecotoxicity tests with instrumental analysis in order to obtain reliable data, and to confirm the exposure concentrations of pharmaceuticals used in toxicity experiments, especially of compounds with a very low water solubility: if this is not done, chemical hazards may be underestimated as a consequence.

Such a high toxicity of DOR to *D. magna* reveals a serious threat when this compound reaches the aquatic environment. But so far only limited data have been available concerning the occurrence and fate of anthelmintics (including DOR) in the environment (Horvat et al., 2012). Pfizer Inc. (1996) calculated the maximum expected concentrations of DOR in a surface water body to range from 0.24 to 0.42 ng L^{-1}, values backed up by Boxall et al. (2006). Even though these estimated concentrations are lower than observed EC_{50}s, it must be pointed out that organisms are typically exposed to multi-component chemical mixtures. It is possible that DOR, in predicted environmental concentrations and in combination with residues of veterinary products and/or other pharmaceuticals/pollutants, will cause adverse effects to biota.

4. Conclusions

As a result of such a comparative ecotoxicological analysis, the potential effects of four selected veterinary drugs, most commonly used in livestock or in aquacultures, on aquatic organisms were defined. DOR was found to be highly toxic toward *D. magna*. Even if environmental concentrations and "local hot spots" during the application of these pharmaceuticals are still much lower that the EC_{50}s of these pharmaceuticals vis-à-vis certain organisms, it must always be borne in mind that these drugs occur in natural media usually together with others of the same family or type. Therefore, mixture effects need to be considered, as in such situations toxic thresholds could already be reached. Moreover, ecotoxicological hazard assessments of these pharmaceuticals may be misleading if only acute toxicity assays are done. Therefore, a further research is necessary. This includes ecotoxicological testing in combination with analytical methods in order to acquire a complete picture of the exposure to and the risk posed by these pollu-

tants, as we have done in our research. Despite these reservations, the ecotoxicological test battery used in this study is a suitable tool for compiling a comprehensive hazard profile for environmentally relevant contaminants.

Acknowledgements

The authors would like to acknowledge the financial support of the German Academic Exchange Service (DAAD), Polish National Science Centre under grants DEC-2011/03/B/NZ8/03009 and DEC-2011/03/B/NZ8/03010. The authors are grateful to Prof. Dr. Juliane Filser, Andrea Böschen and Ulrike Bottin-Weber for their support.

Appendix A. Supplementary material

Detailed information about chromatographic measurements is presented in Appendix A. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2013.04.057.

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