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The toxic effect of oxytetracycline and trimethoprim in the aquatic environment

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ABSTRACT

The objective of our study was the investigation of the toxic properties of two antimicrobial drugs: oxytetracycline (OTC) and trimethoprim (TMP) in the aquatic environment. The toxic effects were tested according to the OECD guidelines for the testing of chemicals, on the cyanobacteria *Anabaena flos-aque*, on the alga *Pseudokirchneriella subcapitata*, on the daphnid *Daphnia magna* as well as on the activated sludge. We discussed the short term and long term results of tests on cyanobacteria and microalgae. Both experiments were concluded in 72 h allowing direct comparison of sensitivity of the two tested species. The results of our study showed toxic effect in the same range for both groups. In the test on the toxicity of OTC to *P. subcapitata* we obtained the 72 h ErC_{50} of 1.04 mg L^{-1} (72 h ErC_{10} 0.47 mg L^{-1}) which are lower in comparison to the results on the toxicity to *A. flos-aque* of ErC_{50} of 2.7 mg L^{-1} (72 h ErC_{10} 1.5 mg L^{-1}). TMP is less toxic to both photosynthetic plankton species. Similar to the test results on OTC, the *P. subcapitata* is more sensitive to TMP (ErC_{50} 129 mg L^{-1}; ErC_{10} 65 mg L^{-1}) than *A. flos-aque* (72 h ErC_{50} 253 mg L^{-1}; 72 h ErC_{10} 26 mg L^{-1}). OTC is toxic to the activated sludge (3 h EC_{50} 17.9 mg L^{-1}), while the calculated 3 h EC_{50} value for TMP exceeded solubility for the compound. In comparison to other species, both tested antimicrobials showed low toxicity to daphnids.

1. Introduction

Antimicrobial agents are extensively used in human and veterinary medicine and in aquaculture. Worldwide estimation of antimicrobial agents consumption lies between 100,000 and 200,000 ton per year (Wise, 2002). According to the European Federation of Animal Health report, in the year 1999, 65% of antimicrobials were used in human medicine (Kümmerer, 2009). In the survey by the European medical agency (EMA), the sales of veterinary antimicrobial agents was compared among 10 European countries, resulting from 18 to 188 mg kg^{-1} of antimicrobials per kilogram of biomass of food producing animals (Grave et al., 2010). The EMA report On Sales of veterinary antimicrobial agents in 2011 reveals a total EU sell of 82 tons. Data on the usage of human antimicrobials in the EU are not available, however, due to their substantial use, scattered distribution and direct exposure, the effect of biologically active molecules to the environmental compartments should not be overlooked.

Since VICH (International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Products) has released The Guidance for environmental impact assessment for veterinary medicinal products (VICH 6, 2000) (VICH 38, 2005) and the EMA (European Medical Agency) has released the Guideline On The Environmental Risk Assessment Of Medicinal Products For Human Use (CHMP, 2006), the increased demand for new ecotoxicological data on active substances is created. The European Union legislation set the obligation to take environmental risk into account at the registration of veterinary medicinal products (Directive, 2004/28/EC). The applicant is required to provide data on environmental fate and behaviour and ecotoxicological properties that enable an assessment of the potential risks posed by the medicinal product to the environment. When the potential risk is identified, regulators should reduce it to the acceptable level by mitigation measures or even by preventing the registration of the product (Montforts et al., 2004).

The objective of our study was to investigate the environmental toxicity properties of two antimicrobial substances, oxytetracycline (OTC) and trimethoprim (TMP), in the aquatic environment. Both of the substances are commonly used in human medicine and for the treatment of animals reared in terrestrial environment and in aquaculture. OTC belongs to the broad-spectrum tetracycline group, widely used in human and veterinarian medicine. As a drug, it undergoes minimal metabolism and is mainly excreted

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via urine as an unchanged drug (CVMP, 1995). Urinary recovery of OTC within 72 h post-treatment ranges between 42–62% of the administered dose in pigs and between 62–88% in cows (Mevius et al., 1986). TMP belongs to the class of chemotherapeutic agents acting on dihydrofolate reductase, inhibiting the synthesis of tetrahydrofolic acid. In veterinary medicine the most often-used combination is TMP with sulphonamide. In humans, pigs and poultry, 46% of the applied dose is excreted through urine and feces, 22% of which as unchanged TMP (CVMP, 1997).

In line with the Guidance for environmental impact assessment for veterinary medicinal products (VICH Expert Working Group, 2005), published data can only be used to substitute studies, if the publication contains a sufficient amount of data and sufficient details on the design and conduct of the study to allow a full and independent assessment (EMA, 2012).

In support to the assessment of the effect of OTC and TMP on the aquatic environment we selected four toxicity tests and performed experiments following the OECD guidelines for the testing of chemicals. In addition to the acute toxicity test on daphnids and the multigeneration test on eukaryotic single cell green algae, we chose the multigeneration test on prokaryotic cyanobacteria as recommended in the VICH guidance, Phase II (VICH 38, 2005). Additionally, we conducted the test on the inhibition of respiration on activated sludge according to the Guideline On The Environmental Risk Assessment Of Medicinal Products For Human Use (CHMP, 2006). In the tests, we met the validity criteria which enable the comparison of the results from our study with the published data.

2. Materials and methods

In the study, antimicrobial substances were tested according to the OECD guidelines for the testing of chemicals on the toxicity to the cyanobacteria *Anabaena flos-aque*, to the alga *Pseudokirchneriella subcapitata*, to the daphnid *Daphnia magna* and to the microbial community of active sludge.

Test compounds of pharmacopeia purity were donated by the pharmaceutical company Krka d.d., the chemicals used for the growth medium were all of analytical grade and purchased from Merck. The physicochemical properties of OTC and TMP are included in Table 1.

2.1. Chemical measurements

In order to provide information on exposure concentrations to antimicrobials during the tests, the concentrations of OTC and TMP in toxicity tests to cyanobacteria, algae, daphnids and the microbial community of active sludge were determined by the LC–MS–MS analysis.

An Agilent 1200 HPLC system coupled with an AB Sciex API 4000 tandem mass spectrometer with electrospray ionization (ESI) interface operating in positive Multiple Reaction Monitoring (MRM) mode was used for both analytes. For OTC, the transition m/z 461 → 426 and m/z 461 → 443 were chosen. For TMP the transition m/z 291 → 230 and m/z 291 → 123 were chosen. The column used for separation was Luna C18(2) 100 × 2 mm with 3 μm particles. The mobile phase A consisted of 0.1% formic acid and acetonitrile (95/5) and the mobile phase B consisted of 0.1% formic acid in acetonitrile. The gradient elution conditions were initially A–B (95–5) programming to 40% B over 8 min. The flow rate was 0.3 ml min^{-1} with oven temperature 40 °C and the injection volume of 20 μl.

The limit of detection (LOD) was 0.01 mg L^{-1} and the limit of quantification (LOQ) was 0.025 mg L^{-1} for both analytes. The average recovery for OTC was 95% and for TMP 96% with the corresponding RSD of 9.8% for OTC and 15.7% for TMP.

The WTW Multiline P4 multimeters with WTW Cell Oxi 325 and the pH probe WTW Sen Tix ORP for oxygen and pH measurements were used during tests.

Table 1
Physicochemical properties of two tested antimicrobial agents.

Substance	CAS	WS at 25 °C (mg L^{-1})	pK_a	Log K_{ow}
Oxytetracycline	79-57-2	313	9.5	−0.90
Trimethoprim	738-70-5	400	7.12	0.91

WS = water solubility.
Data were obtained from the TOXNET, Hazardous Substances Data Bank http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB.

2.2. Toxicity to the freshwater alga P. subcapitata and to the cyanobacteria A. flos-aque

A 72 h toxicity test was performed on two photosynthetic plankton species: green alga *P. subcapitata* (formerly known as *Selenastrum capricornutum*) and the cyanobacteria *A. flos-aque* to determine the effects of the given substance on the growth of freshwater photosynthetic plankton organisms. The test was performed according to the OECD TG 201 "Freshwater Alga and Cyanobacteria, Growth Inhibition Test" (OECD, 2011). Both test species are recommended in these guidelines.

2.2.1. Preparation of test solutions

Green algae and cyanobacteria were cultivated and tested in the OECD TG 201 medium and in the growth medium BG 11(Stanier et al., 1971) respectively.

The reference substance 3,5-dichlorophenol was tested as a means of verifying the test procedure.

2.2.2. Tested strains

The algal species *P. subcapitata*, strain CCAP 278/4 and the cyanobacteria *A. flos-aque* strain CCAP 1403/13A were obtained from the supplier SAMS Research Service Ltd. Scottish Marine Institute, Dunbeg, Argyll, PA 37 1QA, UK. The strains were cultivated in in the appropriate growth medium for at least 10 d prior to the test.

2.2.3. The test

Exponentially growing algae and cyanobacteria were exposed to the test substance in batch cultures over a period of 72 h. The test endpoint was inhibition of growth, expressed as the ErC_{50} and ErC_{10}. Five concentrations arranged in a geometric series were prepared for each test. In the test with cyanobacteria the geometric series for OTC and TMP were 2 and 1.3 respectively. For algae exposed to OTC and TMP the geometric series were 3 and 2 respectively. The tests on algae were conducted in 250 mL Erlenmeyer test flasks in 3 replicates, incubated for 72 h at 20 ± 1 °C in the growth chamber under continuous illumination and the light intensity of 7000 lux. The cyanobacteria were incubated at 24 ± 2 °C under continuous light at 3500–4000 lux. Each vessel was filled with 100 mL of the test medium. Algae were constantly shaken at approximately 120 rpm, while cyanobacteria were stirred just before sampling. Algae and cyanobacteria showed typical form without any inclusions seen under 500-fold magnifications.

The cell concentration in each flask was determined 24, 48 and 72 h after the start of the test by using fluorometric measurements (440–680 nm), Perkin Elmer Victor 3, 1420 Multilabel Counter (Perkin Elmer, Singapore, Republic of Singapore). Fluorometry as a surrogate measurement was validated by the measurement of cyanobacteria dry biomass and by counting the cell number of algae .The pH was measured at the beginning of the test and after 72 h of exposure.