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Application of advanced instrumentation for analysis of environmental pollutants

Olga Koba

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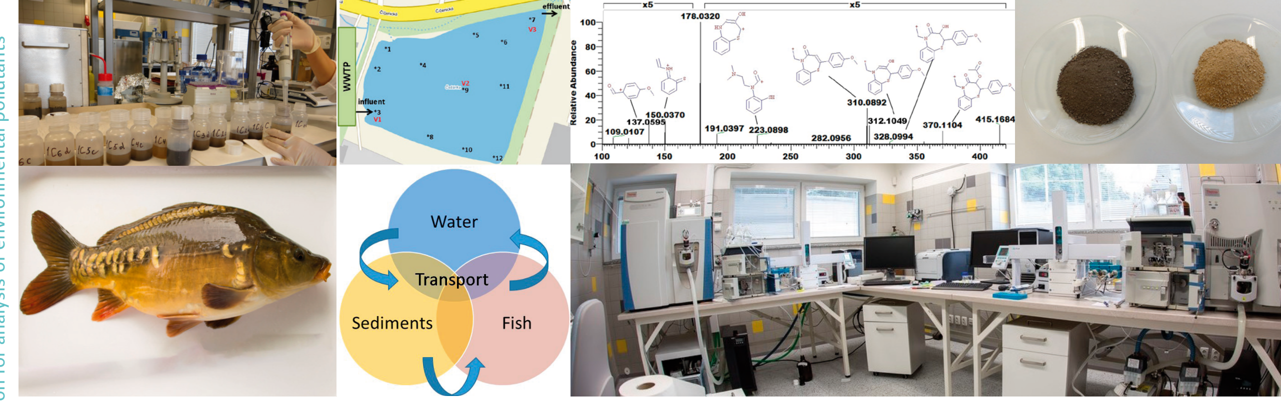

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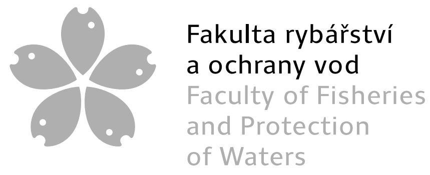

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Supervisor:
Assoc. Prof. Roman Grabic
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses (CENAKVA)
Zátiší 728/II, 389 25 Vodňany, Czech Republic

Head of Laboratory of Environmental, misspell Chemistry and Biochemistry, USB, FFPW, RIFCH:
Assoc. Prof. Tomáš Randák

Dean of Faculty of Fisheries and Protection of Waters:
Prof. Otomar Linhart

Board of doctorate study defence with referees:
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Assoc. prof. Jerker Fick Ph.D. – Umeå University, Department of Chemistry, Sweden – thesis reviewer
Prof. William Shelton – language proofreading

Date, hour and place of Ph.D. defence:
14th September 2017, at 9:00, in USB, FFPW, RIFCH, Vodňany, Czech Republic

Name: Olga Koba

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CHAPTER 1

GENERAL INTRODUCTION

Environmental contaminants: their origin, occurrence and further implementation for environment

Rapid urbanization, industrial development, and improvements in life quality lead to increasing consumption and development of new chemical substances. Unfortunately, uncontrolled utilization of these compounds results in their appearance in all environment compartments.

Environmental pollutants are represented by several groups, such as heavy metals, chlorinated pesticides, flame retardants, plasticizers, cleaning agents, illicit drugs, hormones, pharmaceuticals and personal care products (PPCPs), metabolites and transformation products (TPs) of these substances, antibiotic resistant genes, etc. All of these contaminants were investigated in the different aquatic environmental matrices during the last decade (Aymerich et al., 2016; McGrory et al., 2017; Stayner et al., 2017; Wang et al., 2016; Wang et al., 2015; Woudneh et al., 2015), soils and sediments (Huber et al., 2016; Styszko, 2016; Svahn and Björklund, 2015; Tan et al., 2016), and different biological matrices (de Solla et al., 2016; Du et al., 2016; Grabicova et al., 2015; McGoldrick et al., 2014).

Industry and agriculture are the main sources of "traditional" contaminants like heavy metals and pesticides. Nowadays, compounds emitted by consumption and use in global population are in the spotlight.

Among all contaminants, PPCPs constitute a large part of environmental pollutants. These chemical substances are continuously entering the aquatic environment due to incomplete removal by wastewater treatment plants (WWTPs) (Figueroa-Nieves et al., 2014; Grabicova et al., 2015; Roberts et al., 2016; Zaibel et al., 2016). It is well known that WWTPs were initially designed to remove solids, dissolved organic matter and nutrients. Although, it has been shown that most of conventional technologies for wastewater treatment cannot remove all PPCPs from wastewater (Golovko et al., 2014a; Kasprzyk-Hordern et al., 2009; Liang and Liu, 2016), novel tertiary treatments, such as advanced oxidation processes, are considered to be quite promising (Barbosa et al., 2016; Lonappan et al., 2016). Soils may be contaminated via direct sludge and manure application to fertilize agriculture fields or by irrigation with effluent wastewater (Paltiel et al., 2016). It has been proved that PPCPs may cause several adverse effects on aquatic organisms (Brodin et al., 2013; Hargitai et al., 2016; Henriques et al., 2016; Osorio et al., 2016), since these compounds are widespread in the environmental water bodies.

Due to a high consumption and possible harmful effects on the ecosystem, PPCPs have been identified as "emerging organic contaminants". In addition, pharmaceutical's metabolites and TPs originated from the human or bacterial metabolisms (during sewage treatment) or different environmental processes may preserve biological activity. Detailed literature analysis showed an increasing trend in research related to different aspects of pharmaceutical metabolites and TPs (Fig. 1).

Although PPCPs, their metabolites and TPs are frequently detected in the environment and have potential to be harmful for living organisms, no restriction for their trace levels in any environmental matrix have been reported, , with the exception of maximum residue limits for veterinary drugs in foods (WHO, FAO, 2015). For example, European Commission has been regulating maximum residue levels of pesticides in food and feed of plant and animal origin since 2005 (European Commission, 2005). In addition, pesticide and their metabolite levels are regulated in different environmental waters worldwide (Hamilton et al., 2003).

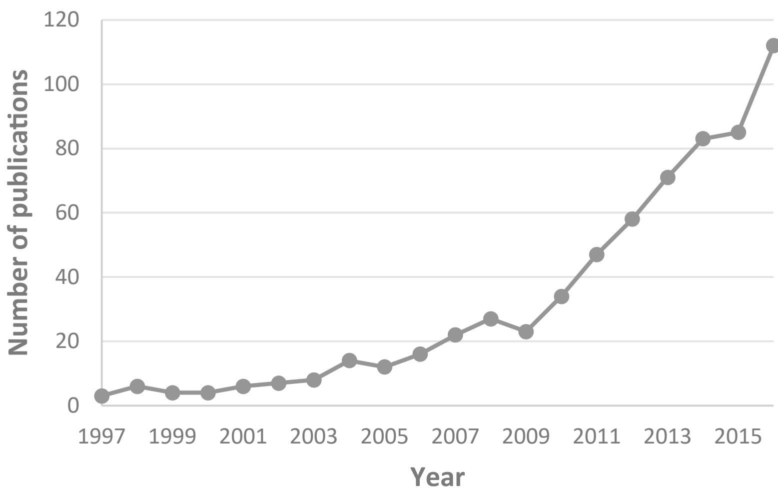

Figure 1. *Number of scientific publications on pharmaceuticals and their metabolites and TPs, which can be found through a Web of Science. Search entry parameters: TOPIC: (metabolites and TPs of pharmaceuticals) AND YEAR PUBLISHED: (1997–2016).*

Increasing environmental concern regarding PPCPs occurrence in the environment forced European Parliament to pay attention to this problem. Recently, a few pharmaceuticals, which belong to different therapeutic groups, such as anti-inflammatory drugs (diclofenac (DIC)), macrolide antibiotics (azithromycin (AZI), erythromycin (ERY) and clarithromycin (CLA)), and oral contraceptives (17-Alpha-ethinylestradiol (EE2)) have been included in the "watch list" of priority substances under the Water Framework Directive (European Commission, 2015). Unfortunately, this legislation only sets a maximum acceptable methods detection limit for these mentioned compounds and forces national regulatory bodies to monitor.

The literature survey showed a great interest in the occurrence and fate in the environment and removal of these micropollutant compounds. Although several review papers have been published in the last decade (Barbosa et al., 2016; Barreiros et al., 2016; Bu et al., 2013; Lonappan et al., 2016; Quadra et al., 2017; Zhang et al., 2008); more studies should be performed to better understand and evaluate adverse effects on living organisms. A brief overview regarding different environmental characteristics of selected "watch list" pharmaceuticals is presented in Table 1.

Table 1. *Removal efficiency (RE), occurrence and effect of selected pharmaceuticals that belong to "watch list".*

Compound	RE, %	Occurrence in the environment			Effects (Mode of action, tested concentrations, organism)
		Water [ng L^{-1}]	Sediments [ng g^{-1}]	Biota [ng g^{-1}]	
AZI	69 (1)	< LOQ–8 (5) nd–29.6 (6) nd–67 (7) 0.6–1620 (21) nd–4.7 (22)	35.62–265.1(12) < LOQ–44 (13)	Hydropsyche < LOQ–85 (5)	Cause macrolide-resistance genes appearance, 128 mg L^{-1}, marine *Vibrio* and *Photobacterium* (16)
CLA	40 (1)	< LOQ–120 (5) < LOQ–616 (8) nd–26.8 (6) 0.01–778(21) nd–180(22)	1.61–2.65 (12) 28–130 (14)	Hydropsyche < LOQ–2.9(5)	Cause macrolide-resistance genes appearance, > 512 mg L^{-1}, marine Vibrio and Photobacterium (16); growth inhibition, 6.9 µg L^{-1}, algae Pseudokirchneriella subcapitata (17)
DIC	17 (2) 21.8–65.8 (3) 20-> 93(25)	< LOQ–94 (5) < LOQ–324 (8) nd–64 (7) 620 (9) nd–17.6 (10) 0.8-1043 (21) nd–717(22) 8.1-5900(23)	234 (15) nd–5.55 (10)	*Erpodella octoculata.* 12–33(5) *Bellamya* 0.45–11.7 Corbiculidae 1.41–5.42 *Cyprinus carpio* 0.58–8.07 *Coilia ectenes* nd–13.8, *Carassius auratus* 1–6.22 (10)	Oxidative stress with increased superoxide dismutase and glutathione peroxidase activities and lipoperoxidation in liver, testosterone levels reduce, 0.2–20 µg kg^{-1}, food exposure of Hoplias malabaricus (18), adverse affect on the growth in egg phase and significant reduction of hatchability and delay in hatching, 0.001–10 mg L^{-1}, Japanese medaka; a heavy damage of gill, liver and kidney, 50 µg L^{-1}, brown trout, induction of lipid peroxidation, order ng L^{-1}, mussels, adverse affect on the metabolism and growth, 0.001–10 mg L^{-1}, blue mussels (25)

Table 1 continues

Compound	RE, %	Occurrence in the environment	Effects (Mode of action, tested concentrations, organism)		
		Water [ng L^{-1}]	Sediments [ng g-1]	Biota [ng g^{-1}]	
ERY	-30 (1) 25.2–43 (3) 2.8–61.7 (4)	12ʹ336 (11) 19 (9) nd–10.4(10) 0.28–2246(21) nd–1540(22)	nd–67.5 (11) nd–0.78 (10)	*Lepomis megalotis* 1.6, *Menidia beryllina* 1.6 , *Ictiobus bubalus* 3.5, *Ictalurus punctatus* 3.2 (9), Bellamya nd–0.34, Corbiculidae nd–5.21, *Cyprinus carpio* nd–1.93, *Carassius auratus* nd–7.64, *Coilia ectenes* nd–0.78 (10)	Cause macrolide-resistance genes appearance, > 512 mg L^{-1}, marine Vibrio and Photobacterium (16); inhibition of acetylcholinesterase activity and the induction of EROD and SOD activities, 3.5–70.5 µg L^{-1}, crucian carp (19); DNA damage,0.05–0.8 µg L^{-1}, rainbow trout (20); cause immobility, order mg L^{-1}, D. magna (23)
EE2	70–99(21)	0.2–230 (21) nd–3500(23)	< LOD-0.7(24)	*Carassius auratus* 1.35–8.43 (24)	Embryos/larvae mortality increase, significantly retardation of hatching time, teratogenesis induction, 0.05 ng L^{-1} – 1 mg L^{-1}, clearhead icefish(26), anxiety and shoal cohesion increase, alteration in gene expression in brain tissue, effects on pathways connected to the circadian rhythm, cytoskeleton and motor proteins and synaptic proteins, 2.14 and 7.34 n g L^{-1}, zebrafish(27)

(1) (Golovko et al., 2014b), (2) (Golovko et al., 2014a), (3) (Radjenović et al., 2009), (4) (Zhang et al., 2015), (5) (Grabicova et al., 2015), (6) (Paíga et al., 2016), (7) (Zhou et al., 2016), (8) (Petrovic et al., 2014), (9) (Du et al., 2016), (10) (Xie et al., 2015), (11) (Dong et al., 2016), (12) (Feitosa-Felizzola and Chiron, 2009), (13) (Gibs et al., 2013), (14) (Blair et al., 2013), (15) (Koumaki et al., 2017), (16) (Nonaka et al., 2015), (17) (Watanabe et al., 2016), (18) (Guiloski et al., 2015), (19) (Liu et al., 2014), (20) (Rodrigues et al., 2016), (21)(Barbosa et al., 2016), (22)(Bu et al., 2013), (23)(Quadra et al., 2016), (24)(Barreiros et al., 2016), (25) (Lonappan et al., 2016), (26) (Hu et al., 2017), (27) (Porseryd et al., 2017); LOQ – limit of quantification; nd – not detected.

Advances in analytical and detection methods

The increase of concern and frequently reported occurrence of pharmaceuticals in various environmental matrices is mainly related to technological progress in the sensitivity and accuracy of detection equipment and analytical methods. Gas chromatography with mass spectrometry (GC-MS) or tandem mass spectrometry (GC-MS/MS) and liquid chromatography with mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) are the most common techniques for detection of pharmaceutical compounds (Fatta et al., 2007). So far, based on physical and chemical properties of targeted analytes, LC methods are suitable for polar and highly water soluble compounds, whereas GC are better for lipophilic or volatile substances.

Recent advances in mass spectrometers are primarily devoted to development of high resolution mass spectrometers (HRMS) (time-of-flight and orbital trap instruments) for untargeted analysis, screenings and identification of new contaminants or metabolites/TPs (Pico and Barcelo, 2015) and modern triple quadrupoles (QqQ) for improving limits of detection and quantitation for routine analysis (Fig. 2).

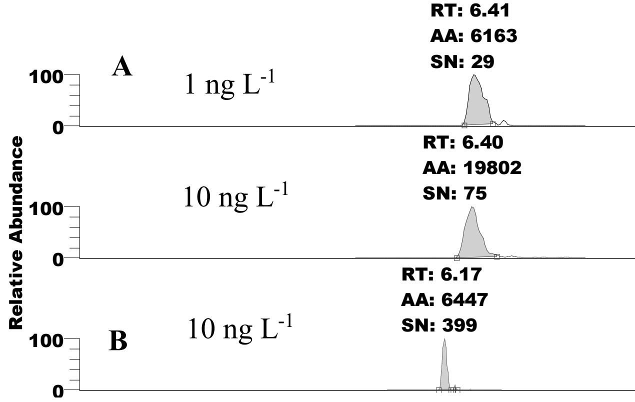

Figure 2. *Comparison of chromatograms obtained for different azithromycin concentrations spiked into tap water detected with different triple quadruple generations. A – a triple-stage quadrupole MS/MS TSQ Quantum Ultra, B – TSQ Quantiva™ Triple Quadrupole Mass Spectrometer. Extracted transition is 749 → 591 m/z. The same chromatographic gradient, analytical column type, and ion source set up were used for both devices.*

High resolution mass spectrometry has a few great advantages for screening and identification of new environmental pollutants. Usage of full scan and data-independent acquisition (DIA) mode gives a possibility to search for other compounds of interest without additional measurements, since these data contain all measured ions and possible MS/MS products. Unfortunately, this cannot be done using target analysis with QqQs (Pico and Barcelo, 2015). HRMS instrumentation is also more sensitive and selective, compare with quadrupoles, and it provides less time-consuming sample analysis for identification and determination, because with help of DIA it is possible to obtain full scan data and MS/MS fragmentation for tentative identification in one step. This is not possible using QqQs, where several additional steps should be done, such as direct injection of samples containing possible metabolites, development of analytical method, targeted analysis of possible "candidates", etc (Acena et al., 2015).

Metabolites/TPs identification and quantification methods

To our knowledge, environmentally related metabolite studies are mainly represented by *in vitro* and *in vivo* approaches applied for different mammalian and fish species (Koba et al., 2016; Liu et al., 2012; Scheidweiler and Huestis, 2014). TPs may be formed by different processes, such as ozonation, chlorination, UV and solar photolysis, advanced oxidation processes, etc. in laboratory studies and under real environmental conditions (Blum et al., 2017; Koba et al., 2017; Li et al., 2014; Matsushita et al., 2016; Michael et al., 2012; Olmez-Hanci et al., 2015; Salgado et al., 2013; Svan et al., 2016).

Unambiguous metabolites and TPs identity confirmation may be carried out by using analytical standards or nuclear magnetic resonance mass spectroscopy. Unfortunately, a limited number of metabolites and TPs are commercially available nowadays and their chemical synthesis is time consuming and expensive. Consequently, a tentative identification using high resolution mass spectrometry has become a popular technique for identification and screening of new environmental contaminants (Acena et al., 2015; Bu et al., 2014; Gomez-Ramos et al., 2011; Ibáñez et al., 2017; Li et al., 2014; Svan et al., 2016).

Based on literature analysis, several specific steps for metabolites and TPs identification may be highlighted. Initially, optimization of analytical method, such as selection a proper ionization technique and chromatographic conditions, should be done based on parent compound from which metabolites or TPs may be originated. The second step is to use full scan mode in high resolution. Full scan data are later filtered based on 5 ppm difference from accurate mass of possible candidates, isotopic pattern, double bond equivalent, predicted retention time (RT). As a next step, MS/MS conformation data should be obtained from a list of candidates coming from HRMS full scan fanalysis. A spectra interpretation softwares, databases with MS/MS spectra, available in the internet or in the laboratory, similarities with parent compound fragmentation pathway are commonly applied for the last step in metabolites and TPs tentative identification.

Due to high interest in metabolite and TPs identification, lots of commercial and free available software for database search, generation of inclusions list for MS/MS confirmation based on applied filters, blank subtraction, RTs and collision energies prediction, spectra interpretation may be used to simplify identification (Gomez-Ramos et al., 2011; Ibáñez et al., 2017; Li et al., 2014; Pico and Barcelo, 2015).

To evaluate dynamic metabolic profiles, tissue distribution, excretion and actual level in the environment of tentatively identified metabolites and TPs, a semi-quantitative approach is frequently used, since majority of tentatively identified metabolites and TPs standards are not available on market. Semi-quantification is a method based on using the standard curve of the parent drug instead of metabolite/TP due to the similar structure, fragmentation pathway and same mass spectrometry or UV response (Koba et al., 2016; Ravindran et al., 2014; Vikingsson et al., 2016).

Aims and outcomes of the thesis

Since PPCPs, their metabolites and TPs have become frequently occurring pollutants in the different environmental compartments and little is known about their fate in the environment, this study aimed:

- to develop a method to detect, identify and quantify metabolites formed in fish exposed to diltiazem;
- to develop an analytical method to evaluate a fate of several pharmaceuticals in the laboratory conditions in the soil environment by means of identification and determination of formed degradation products and study their behaviour in soil matrix;
- to develop and validate several analytical methods to study pharmaceuticals and their metabolites/TPs behaviour via complex approach using pond affected with treated wastewater for contaminants transport assessment between water, sediments and fish.

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CHAPTER 2

INVESTIGATION OF DILTIAZEM METABOLISM IN FISH USING A HYBRID QUADRUPOLE/ORBITAL TRAP MASS SPECTROMETER

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Investigation of diltiazem metabolism in fish using a hybrid quadrupole/orbital trap mass spectrometer

Olga Koba*, Christoph Steinbach, Hana Kocour Kroupová, Kateřina Grabicová, Tomáš Randák and Roman Grabic

University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Zatisi 728/II, 389 25 Vodnany, Czech Republic

RATIONALE: Diltiazem, a calcium channel blocker drug, is widespread in the environment because of its incomplete elimination during water treatment. It can cause negative effects on aquatic organisms; thus, a rapid and sensitive liquid chromatography/mass spectrometry (LC/MS) method to detect its presence was developed. Our approach is based on accurate mass measurements using a hybrid quadrupole-orbital trap mass spectrometer that was used to measure diltiazem and its metabolites in fish tissue.

METHODS: Blood plasma, muscle, liver, and kidney tissues of rainbow trout (*Oncorhynchus mykiss*), exposed for 42 days to 30 μg L^{-1} diltiazem, were used for the method development. No metabolite standards were required to identify the diltiazem biotransformation products in the fish tissue.

RESULTS: Overall, 17 phase I diltiazem metabolites (including isomeric forms) were detected and tentatively identified using the MassFrontier spectral interpretation software. A semi-quantitative approach was used for organ-dependent comparison of the metabolite concentrations.

CONCLUSIONS: These data increase our understanding about diltiazem and its metabolites in aquatic organisms, such as fish. These encompass desmethylation, desacetylation and hydroxylation as well as their combinations. This study represents the first report of the complex diltiazem phase I metabolic pathways in fish. Copyright © 2016 John Wiley & Sons, Ltd.

The calcium channel blocker diltiazem is used mainly in the treatment of cardiovascular diseases, such as angina pectoris and hypertension.[1] It is also prescribed to prevent migraines and, as an ointment, treat chronic anal fissures.[2,3] Due to its widespread use and incomplete elimination in sewage treatment plants (STPs), diltiazem has been frequently detected in STP effluent and in recipient surface waters.[4–7] Diltiazem metabolites are excreted the same way as diltiazem and can also be found in aquatic environments surrounding STPs. Recently, concentrations of acetyl diltiazem higher than its parent compound have been found in effluent waste water in India.[8] In the same study, it was also reported that acetyl diltiazem has a lower removal efficiency compared with diltiazem.[8]

Diltiazem is bioconcentrated in fish tissue.[9,10] For example, diltiazem was detected in the liver and muscles of white suckers (*Catostomus commersonii*) and johnny darters (*Etheostoma nigrum*) living downstream of a local STP in the USA.[9] Under laboratory conditions,[10] whole homogenates of the eastern mosquitofish (*Gambusia holbrooki*) demonstrated rapid uptake (rate = 0.033 mL g^{-1} h^{-1}) and a relatively long half-life (117 h) of diltiazem. However, details about the presence and behavior of diltiazem metabolites in fish are currently missing. Therefore, providing this information would improve our understanding of the the metabolic pathways and chronic effects of diltiazem on fish.

In mammals, diltiazem metabolism is well documented.[11–18] In humans, it is metabolized into three major products, *O*-desacetyl diltiazem, *N*-desmethyl diltiazem, and *N*-desmethyl-*O*-desacethyl diltiazem,[19,20] which are all found in blood plasma. *O*-Desacethyl and *N*,*O*-didesmethyl diltiazem are pharmacologically active and their effects differ from the parent compound. For example, those metabolites are more potent inhibitors of blood cell aggregation and adenosine transport by erythrocytes.[21] Similarly, *N*-desmethyl and *N*,*N*-didesmethyl diltiazem are stronger inhibitors of CYP450 3A compared with diltiazem.[16,18] In contrast, it has been shown in mammals[22] that some diltiazem metabolites might affect non-target organisms with a higher probability of the same mode of action as the parent compound.

Most studies focused on diltiazem metabolites have generally dealt with mammalian and human tissues. Recent studies have presented analytical methods for only a limited number of diltiazem metabolites (*N*-desmethyl diltiazem and desacetyl diltiazem).[20,23] To the best of our knowledge, only one study has described a method for determining 11 phase I metabolites of diltiazem in human plasma.[24]

* *Correspondence to:* O. Koba, University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Zatisi 728/II, 389 25 Vodnany, Czech Republic.
E-mail: okoba@frov.jcu.cz

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Metabolic pathways and tissue-specific metabolite distribution can help to explain diltiazem effects on tested fish as well as be used for investigating diltiazem (and its metabolites) bioaccumulation, transformation and excretion in wild fish. Since there is limited information on diltiazem metabolites in fish tissues, we developed a new approach for diltiazem metabolite detection and tentative identification based on accurate mass measurements using the hybrid quadrupole-orbital trap mass spectrometer.

EXPERIMENTAL

Chemicals

Diltiazem [(2*S*,3*S*)-5-[2-(dimethylamino)ethyl]-2-(4-methoxyphenyl)-4-oxo-2,3-dihydro-1,5-benzothiazepin-3-yl] acetate (CAS No. 42399-41-7) and the internal standard verapamil hydrochloride (CAS No. 152-11-4) were purchased from Sigma-Aldrich (Steinheim, Czech Republic). The LC/MS grade acetonitrile (LiChrosolv Hypergrade) was obtained from Merck (Darmstadt, Germany). Formic acid was used to acidify the mobile phases and was purchased from Labicom (Olomouc, Czech Republic). Ultrapure water was prepared using an Aqua-MAX-Ultra system (Younglin, Gyeonggi-do, South Korea).

Instrumentation

A TissueLyser II homogenizer (Quiagen, Germany) and Micro 200R centrifuge (Hettich Zentrifugen, Germany) were used for tissue extraction. An Accela 1250 liquid chromatography (LC) pump (Thermo Fisher Scientific, San Jose, CA, USA) coupled with a hybrid quadrupole/orbital trap Q-Exactive mass spectrometer (Thermo Fisher Scientific) and a HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland) were used to separate and detect target analytes. An analytical Hypersil Gold column (50 mm length, 2.1 mm i.d., 3 µm particles; Thermo Fisher Scientific) preceded with the same phase pre-column (10 mm length, 2.1 mm i.d., and 3 µm particles) was used for chromatographic separation of the target analytes.

Fish exposure setup

Juvenile rainbow trout (*Oncorhynchus mykiss*; mean body length 23.1 ± 1.5 cm, mean body weight 163.8 ± 27.5 g) were obtained from a local commercial hatchery (Husinec, Czech Republic). The fish were kept in aquaria with 200 L of fresh water in a semi-static system. The aquaria were equipped with continuous aeration, and the temperature was held at 15.4 ± 1.4°C with a photoperiod of 12:12 h (light:dark). During the acclimation time and the experimental period, the fish were fed a commercial fish food (Bio Mar, Aarhus, Denmark) at a ratio of 1% body weight on a daily basis. The experimental bath (150 L) was refreshed daily with new diltiazem-fortified water. The fish were exposed to 30 µg L^{-1} diltiazem for 42 days. Kidney, liver, muscle and blood plasma samples were taken and stored at –20°C until further analysis. All samples were coded to ensure traceability among different tissue samples, analyzed parameters and corresponding individual fish.

Extraction

Two extraction methods were used in this study: one specific for blood plasma and another for the other tissues (muscles, liver, and kidney). A general extraction method for 32 antibiotics in fish muscles was published previously,[25] and further investigations have modified this method to make it tissue-specific.[26] We essentially used this tissue-specific extraction method, but modified it for blood plasma because no homogenization step was needed for this matrix. Generally, these two approaches represent the same solvent extraction technique. The method has been validated for all tissue types with satisfactory recovery.[27]

Approximately 0.5 g of tissue was weighed in an Eppendorf vial, and 20 ng of the internal standard (verapamil) and 1 mL of acetonitrile, acidified with 0.1% formic acid, were added. The samples were homogenized (TissueLyser II, Qiagen, Hilden, Germany) and extracted using a stainless steel ball at 1800 oscillations/min for 10 min. The extracts were then centrifuged at 9500 *g* for 10 min. The supernatant was frozen overnight (–20°C) and then filtered through a syringe filter (regenerated cellulose, 0.45 µm pore size). A 100-µL aliquot was used for LC/MS analysis.

A simplified method was used for the blood plasma samples. Verapamil (5 ng) was added to 200 µL of plasma in an Eppendorf vial; the samples were then mixed with 200 µL of acetonitrile acidified with 0.1% formic acid. The extracts were handled in the same way as the tissue samples.

LC/HRMS-HRPS analysis

A heated electrospray ionization interface (HESI-II) was used to ionize the target compounds. The spray voltage was set to 3.5 kV. Nitrogen (purity >99.999%) was used as the sheath gas (40 arbitrary units), auxiliary gas (10 arbitrary units) and collision gas. The vaporizer was heated to 250°C and the capillary to 325°C. There were two acquisition methods: full scan high-resolution mass spectrometry (HRMS) and high-resolution product scan (HRPS). The general properties of the full scan MS included a resolution of 70,000 FWHM, AGC target of 1e6, maximum filling time of 100 ms and a scan range from 50 to 550 *m/z*. A MS^2 experiment was performed using the mass inclusion list. Key parameters of the HRPS were optimized and used an isolation window at the quadrupole of 1 *m/z*, orbital trap resolution of 17,500 FWHM, AGC target of 1e6 and a maximum filling time of 30 ms.

RESULTS AND DISCUSSION

Metabolite detection

Diltiazem was measured as a protonated molecule ($[M+H]^+$) and determined as 415.1686 *m/z* with a retention time (RT) of 7.30 min. This corresponds to a chromatographic gradient as presented in Supplementary Table S1 (see Supporting Information). A list of all prospective metabolites of diltiazem was prepared for further investigation based on the literature and general principles of drug metabolism in different living tissues.[20,24,28] Generally, the products of different drug biotransformations and decomposition are assumed to be more hydrophilic, so they should elute before the parent compound. Targeted screening in full scan mode showed all

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Table 1. Diltiazem and its possible selected metabolites

Compound (metabolite name used in current study)	Formula	Structure	Exact mass (Da)	Measured mass (Da)[a]	Mass error (mDa)	Mass error (ppm)	RT (min)
diltiazem	$C_{22}H_{26}N_2O_4S$		415.1686	415.1679	−0.7	1.69	7.30
N-desmethyl diltiazem (M1)	$C_{21}H_{24}N_2O_4S$		401.1530	401.1523	−0.7	1.74	7.19
O-desmethyl diltiazem	[b]		[b]	[b]	[b]	[b]	-
O-desacetyl diltiazem (M2)	$C_{20}H_{24}N_2O_3S$		373.1580	373.1574	−0.6	1.61	6.51
N-desmethyl, *O*-desacetyl diltiazem (M3)	$C_{19}H_{22}N_2O_3S$		359.1424	359.1418	−0.6	1.67	6.46
O-desmethyl, *O*-desacetyl diltiazem	[b]		[b]	[b]	[b]	[b]	-
N,*O*-didesmethyl, *O*-desacetyl diltiazem (M4a)	$C_{18}H_{20}N_2O_3S$		345.1267	345.1336	−0.6	1.74	5.55
N,*N*-didesmethyl, *O*-desacetyl diltiazem (M4b)	[b]		[b]	[b]	[b]	[b]	6.27
N,*O*-didesmethyl diltiazem (M5a)	$C_{20}H_{22}N_2O_4S$		387.1373	387.1365	−0.8	2.07	6.20
N,*N*-didesmethyl diltiazem (M5b)	[b]		[b]	[b]	[b]	[b]	7.05

(*Continues*)

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Table 1. (Continued)

Compound (metabolite name used in current study)	Formula	Structure	Exact mass (Da)	Measured mass (Da)[a]	Mass error (mDa)	Mass error (ppm)	RT (min)
hydroxy diltiazem (M6a,b,c,d)	C22H26N2O5S		431.1635	431.1336	0.1	0.23	5.62 5.89 6.52 7.46
N-desmethyl, hydroxy diltiazem (M7a,b,c)	C21H24N2O5S		417.1479	417.1477	−0.2	0.48	5.57 5.76 6.38
O-desmethyl, hydroxy diltiazem	b		b	b	b	b	-
O-desacetyl, hydroxy diltiazem (M8a,b,c)	C20H24N2O4S		389.1530	389.1525	−0.5	1.28	4.17 4.71 5.78

[a]Diltiazem and its transformation products were measured as $[M + H]^+$ ions.
[b]Isomeric form (all the parameters are written in the previous line).

possible metabolites present in the blood plasma, muscle, kidney, and liver of the fish. Due to the complexity of the matrices, the full scan data was not suitable for further analysis, especially for minor metabolites (Supplementary Fig. S1, see Supporting Information). Hence, peaks in extracted chromatograms (XICs) corresponding to the exact masses of those metabolites (calculated from their element composition) that matched the 5 ppm window from the exact mass were selected for the next analysis step (Table 1). The HRPS mode was then used to confirm the selected structures. Finally, eight groups of metabolic transformation products were detected (Table 1). These included desmethyl diltiazem (M1), desacetyl diltiazem (M2), desacetyl desmethyl diltiazem (M3), desacetyl didesmethyl diltiazems (M4), didesmethyl diltiazems (M5), hydroxy diltiazems (M6), desmethyl hydroxy diltiazems (M7) and desacetyl hydroxy diltiazems (M8). No phase II diltiazem metabolites were found in the current study.

Identification of metabolites and their isomers

MassFrontier software was used to predict all possible MS^2 products from proposed structures where HRPS with data processing confirmed or disproved the suggestions based only on accurate mass measurements. Diltiazem (Supplementary Fig. S2, see Supporting Information) and its metabolites have the same fragmentation patterns in the collision cell, and the major product ion at m/z 178.0320 was present in all compounds of interest. Product ions with lower relative abundance values were observed in almost all of the metabolites (Supplementary Fig. S2) along with minor fragments.

The fragment ions 137.0595, 150.0370, 178.0320, 223.0898, 310.0892, 312.1049, 328.0994, and 370.1104 m/z of diltiazem were identified by the accurate mass measurements with errors of –1.46, 1.33, –0.56, –0.9, –1.29, –1.28, –2.44, and –1.08 ppm, respectively (Supplementary Table S2, see Supporting Information). This enabled us to exclude possible interference and made identification easier.

The data suggest that the metabolites can be divided into three groups: (1) metabolites M1, M2, and M3 appeared as a single peak despite the possible existence of structural isomers (*N*- or *O*-desmethyl) for M1 and M3 (Table 1); (2) only two isomeric forms were found for M4 and M5 because of different mechanisms during the second desmethylation; (3) M6, M7, and M8 might have a higher number of isomers then previous metabolites due to the hydroxylation process that can occur at up to eight sites on the diltiazem molecule (Table 1).

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M1 and M2 have been the most frequently identified and measured in a wide range of matrices.[20,29] Metabolites present as single isomers (M1, M2, and M3) eluted at 7.19, 6.51, and 6.46 min, respectively. All metabolites in this group have fragmentation pathways similar to diltiazem (Fig. 1). M1 and M3 were identified as desmethylation products of the methyl group located at the *N* atom based on spectra and with using software and theoretical knowledge based on precise fragment mass shifts. The specific fragment structures are proposed in Fig. 1. The fragment of 209.0739 *m/z* (–1.91 ppm) corresponds to loss of $C_{12}H_{13}O_3$ and it represents the *N*-monomethyl moiety of diltiazem while product ions of 219.0647 and 326.0840 *m/z* (–2.28 and –1.53 ppm) correspond to the loss of $C_9H_{12}N_2OS$ and $C_4H_{10}NO$, respectively. Both proposed structures indicate the presence of an *O*-methyl moiety of the diltiazem metabolite molecule. Comparison of loss composition and the diltiazem structure provided us with molecular structures of the fragments, which ensured *N*-desmethylation. M3 had values of 209.0747, 284.0736, and 341.1297 *m/z* with errors of –1.91, –1.41, and –6.16, respectively (Supplementary Table S2, see Supporting Information). M2 was much easier to identify because the measured spectra matched the acetyl diltiazem spectra available on the *m/z* Cloud database.[30]

M4 and M5 (didesmethyl desacetyl diltiazem and didesmethyl diltiazem) were present in two isomeric forms. The second desmethylation may take place at two different sites of the molecule, *N* or *O*, based on the structure of diltiazem. *N*,*O*-Didesmethyl and *N*,*N*-didesmethyl diltiazem were identified based on possible reaction mechanisms and fragmentation patterns. The MS^2 spectra and specific fragments used for identification of both metabolites are given in Figs. 2 and 3. A few particular fragments, which were identified in the same way as for M1, helped to confirm the identity of metabolites: 209.0747 (1.91 ppm) and 314.0838 (–2.23 ppm) *m/z* for M4a; 195.0589 (1.03 ppm) *m/z* for M4b. As shown above, the fragment of 209.0747 *m/z* represents the *N*-desmethyl moiety, whereas 314.0838 *m/z* corresponds to the *O*-desmethyl moiety of the molecule. An accurate mass of 195.0590 *m/z* for the M4b fragment represents double *N*-desmethylation at the same moiety, as illustrated in Fig. 2. Similar specific product ions were found in the spectra of M5a and M5b. The presence of a specific product ion of 209.0739 (–1.9 ppm) in the spectrum of M5a corresponds to the *N*-desmethyl moiety while 296.0735 (–1.69 ppm) and 356.0942 (–2.53 ppm) *m/z* indicate the presence of only a hydroxyl group instead of a methoxy group. The metabolite M5a can be

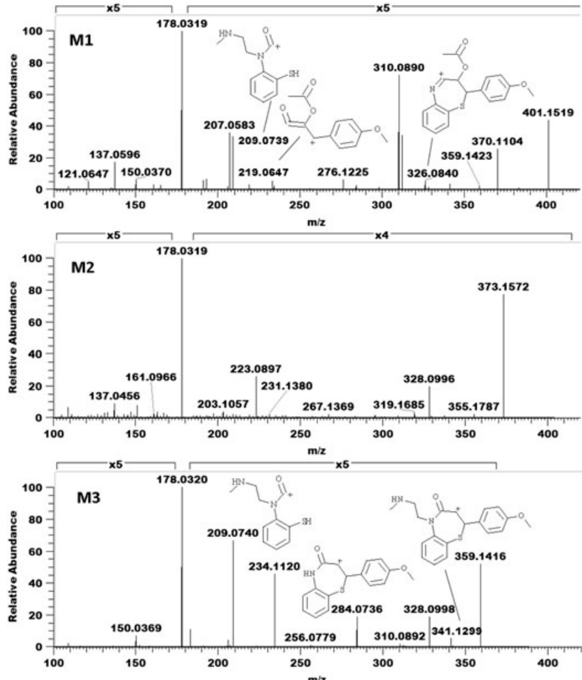

Figure 1. HRPS spectra of M1–M3: mass range 100–177 was amplified 5 times and 179–410 was amplified 4 or 5 times to improve readability.

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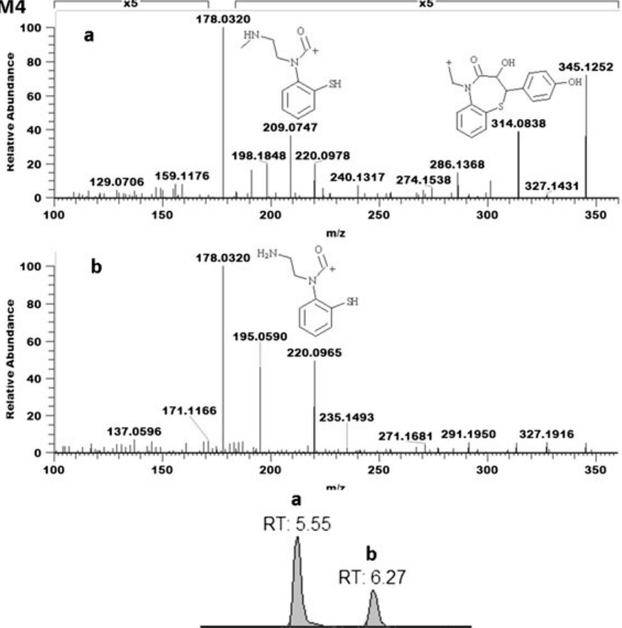

Figure 2. HRPS spectra of M4 in fish liver: (a) *N*,*O*-didesmethyl *O*-desacetyl diltiazem and (b) *N*,*N*-didesmethyl *O*-desacetyl diltiazem). Mass ranges 100–177 and 179–410 were amplified 5 times to improve readability (a).

with high probability identified as *N*,*O*-didesmethyl diltiazem. Consequently, the presence of 195.0587 (0 ppm) and 327.1153 –2.75 ppm) *m*/*z* in the product ion spectrum of M5b indicates the presence of a doubly desmethylated amino group in the *N*,*N*-didesmethyl diltiazem molecule. All fragment masses with corresponding errors are summarized in Supplementary Table S2 (see Supporting Information).

The behavior of isomers M4 and M5 in LC was identical where the *N*,*O*-isomer (M4a and M5a) eluted before the *N*,*N*-isomer (M4b and M5b).

The importance of correct identification of the corresponding isomers can be demonstrated by the extreme differences observed among tissue samples from different individuals. The kidney and liver data were particularly indicative. For example, M4a,b and M5a,b were present in both tissues, but the amount of M5a was lower than M5b in both tissues and this difference was greater in the kidney samples. The amount of M4a in the kidney samples was much lower than M4b, but the opposite was observed in the liver samples.

Identification of the third isomer group was the most complex. To the best of our knowledge, the hydroxyl metabolites M6–M8 have not been previously detected in any biological sample. Specific fragments corresponding to hydroxylation at different diltiazem moieties are formed during the MS^2 experiment of M6 (Table 1). When the hydroxyl group is situated at the methoxyphenyl ring (hereafter described as the first ring), the same specific fragment for M6 and diltiazem is present in the HRPS spectra (i.e., 178.0320 *m*/*z*). The presence of the hydroxyl group in the core diltiazem structure (hereafter referred to as the second ring) generated new specific fragments at 194.0272 (1.03 ppm), 177.0546 (0 ppm), 328.0996 (–2.33 ppm), 166.0321 (0 ppm), 219.0653 (0.46 ppm), and 344.0968 (4.94 ppm) *m*/*z*. The masses are shown according to the order of their relative abundance in the mass spectrum and their respective errors are given in brackets (Supplementary Table S2, see Supporting Information).

The hydroxyl diltiazem isomers of M6 were found mainly in the liver samples. Isomers that eluted earlier with RTs of 5.62 min (M6a) and 5.89 min (M6b) correspond to hydroxylation at the second ring. The remaining isomers with RTs of 6.52 min (M6c) and 7.46 min (M6d) are related to hydroxylation at the first ring. M6a and M6b were not detected in kidney samples, possibly as a result of fast phase II metabolic biotransformation with subsequent excretion via bile. Another potential isomer (M6e) that eluted at 8.07 min was only found in the liver samples but it was identified by HRPS as interference because of a completely different fragmentation pattern.

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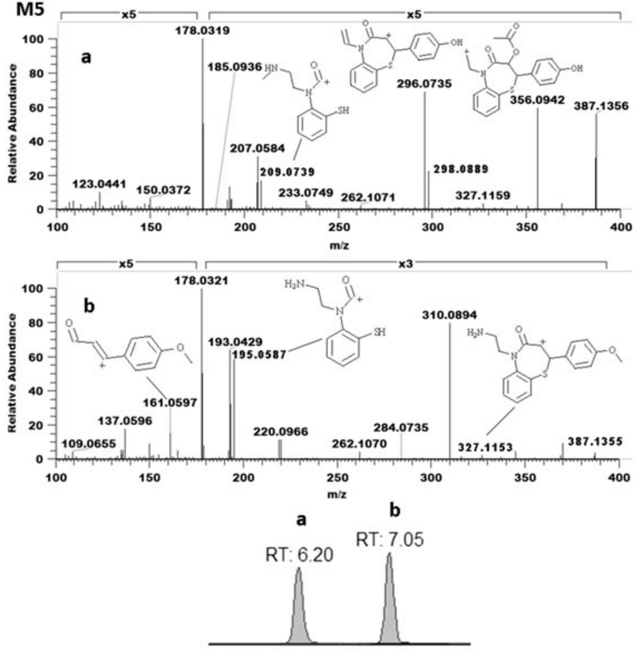

Figure 3. HRPS spectra of M5 in fish liver: (a) *N*,*O*-didesmethyl diltiazem and (b) *N*,*N*-didesmethyl diltiazem. Mass range 100–177 was amplified 5 times and 179–410 was amplified 5 or 3 times, respectively, to improve readability.

The same pattern from M6 was observed for the M7 group. The M7 metabolite family was identified as hydroxylation and *N*-desmethylation products (*N*-form because of specific fragmentation). There were three isomers (a, b, c) of M7 in the liver tissue that eluted at 5.57, 5.76, and 6.38 min, respectively. Specific MS^2 products at 161.0596 (–0.62 ppm), 177.0546 (0 ppm), and 194.0272 (1.03 ppm) m/z (Supplementary Table S2, see Supporting Information) indicated hydroxylation at the second ring for M7a and M7b whereas M7c had the same fragmentation as diltiazem. This suggests that hydroxylation of M7c in the liver occurred in the first ring. Only M7c was present in the kidney samples. Another possible isomeric form (M7d) appeared in all tissues, but the fragmentation pathway was the same as M6e. It could not be matched with proposed fragmentation mechanisms and overall only three of the four M7 metabolites found in HRPS were identified as diltiazem products.

Identification of desacetyl hydroxyl (M8) metabolites was the most interesting and the most difficult part of the study. First, the highest number (six) of possible isomers (M8a, M8b, M8c, M8d, M8e, and M8f) eluted at 4.17, 4.71, 5.78, 6.11, 6.64, and 7.04 min, respectively, was detected in full scan mode of kidney and liver (five samples) extracts. Metabolite M8f had the same fragmentation pattern as those compounds previously identified as interference (M6e and M7d). It was immediately excluded from the list of isomers. In the kidney samples, M8c, M8d, and M8e exhibited a specific fragment for diltiazem, the prevailing product ion at 178.0320 m/z, but, after checking the mass error (ranging from 89 to 254 ppm for different isomers) for the precursor ion at 389.1530 m/z, M8d and M8e were also excluded as obvious interferences.

In the liver samples, the same isomers were present with the exception of M8c. Other prospective metabolites (M8d and M8e) could not be identified as compounds related to diltiazem. M8a and M8b were present in both tissues, but the corresponding HRPS spectra both contained fragments at 178.0320 and 194.0272 m/z. Accordingly, the hydroxyl groups would need to be present in both diltiazem rings simultaneously, which is not reflected in the corresponding MS^2 spectra. Simulated fragmentation pathways of different positional isomers of desacetyl hydroxyl diltiazem explained this observation. Four first ring and four second ring M8 isomers may exist, but only those substituted at the first ring resulted in the presence of a 194.0272 m/z fragment in MS^2 spectra. This ion, which further fragmented to 178.0320 m/z, had the same elemental composition as the MS^2 fragment of the M6 and M7 metabolites, which had hydroxylation at the

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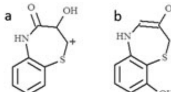

Figure 4. Mass Frontier predicted structure of the 194.0272 m/z fragment for M8 (a), M6, and M7 (b).

second ring, but a different structure (Fig. 4(a)). Consequently, M8a and M8b were identified as hydroxylation products on the first ring.

Semi-quantification of metabolites in fish tissue

Fish are a non-target organism for pharmaceuticals and, consequently, fish tissue is not a common matrix for metabolite analysis. Most studies have focused on mammalian tissue to investigate therapeutic effects and the pharmacokinetics of drugs. One study on techniques for drug and drug metabolite discovery described a semi-quantitative approach that could be used to perform quantification.[31] There have been a couple of subsequent studies where this technique was applied for the quantification of metabolites formed *in vitro* in rat blood plasma[32] and in a liver homogenate containing all the metabolizing enzymes present in the human liver (instead of human liver microsomes).[33]

Only a few reference standards are commercially available for diltiazem metabolites; we used a semi-quantitative approach for characterizing the differences among the various analyzed tissues. The full scan data did not show sufficiently high selectivity, particularly at low concentrations, so we decided to use the more selective HRPS data. In the first step, we assumed that the HRPS signals at the 178.0320 m/z fragment from protonated molecular ions $[M(1\text{-}8)\text{–}H]^+$ have equal intensities. Consequently, we used the corresponding peak area to calculate the metabolite concentration and to determine the metabolic rate in the different fish tissues (Fig. 5). Finally, we were able to semi-quantify diltiazem and its metabolites at the ng g^{-1} level in each of the tissue type samples.

The blood plasma and muscle samples did not contain all of the possible metabolites (Figs. 5(A) and 5(B)). These tissues did not have as many isomeric forms (Supplementary Fig. S1, see Supporting Information) as the kidney and liver samples. The highest total metabolite concentration was found in the kidney samples but the greatest number of isomeric forms was found in the liver (Fig. 5(C) and 5(D)).

Biotransformation of diltiazem in fish

Seventeen diltiazem metabolites and their isomeric forms were detected and tentatively identified (Scheme 1). In mammals, diltiazem undergoes extensive metabolism in hepatic and extra-hepatic tissues. *N*-Desmethyl diltiazem (M1) and desacetyl diltiazem (M2) are the two main diltiazem metabolites that retain pharmacological activity.[34] M1 and M2 were also the main metabolites of diltiazem detected in fish tissues in this study. While CYP2D6 performs *O*-desmethylation of diltiazem in mammals,[12] the CYP2D sub-family is not present in fish,[35] which explains why the M1 and M3 metabolites were identified as products of desmethylation at the *N* atom only. M4 and M5 are products of double desmethylation and desacetylation. This might be explained by the fact that enzymes other than CYP2D6 are involved in the formation of *N*,*O*-didesmethyl diltiazem in fish. Molden *et al*. proposed that, in addition to CYP2D6, other enzymes might be involved in the formation of *N*,*O*-didesmethyl diltiazem in humans.[13]

The hydroxylated diltiazem metabolites (M6, M7, and M8) were identified in this study for the first time and indicated that the hydroxylation of diltiazem is unique to fish. It was possible to discriminate between metabolite isomers from desmethylation and hydroxylation via hybrid quadrupole-orbital trap HRMS. We determined if the desmethylation or hydroxylation took place at the first or second ring of the

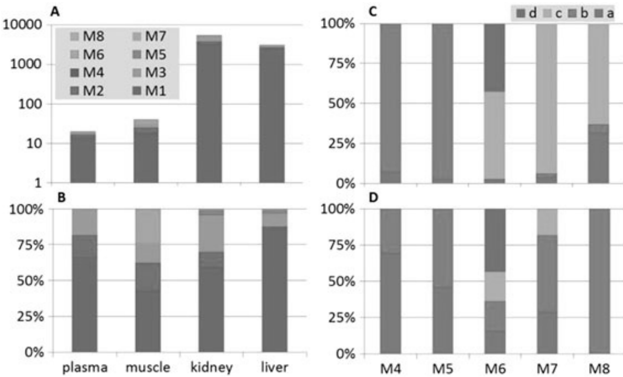

Figure 5. (A) Metabolite concentrations (in ng g^{-1}, logarithmic scale with base 10); (B) distribution of total amount of metabolites in all studied tissues; (C) isomer composition of diltiazem metabolites in the kidney; and (D) isomer composition of diltiazem metabolites in the liver (a, b, c, and d are different isomeric forms).

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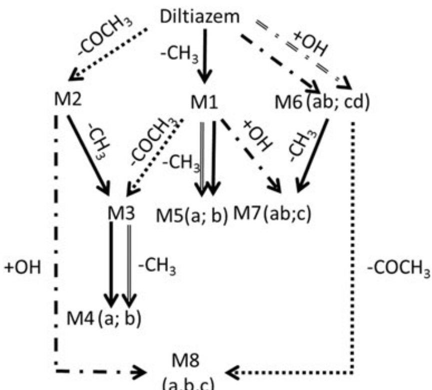

Scheme 1. Isomeric forms of diltiazem metabolites in rainbow trout. Solid line – N-desmethylation; solid line double – *O*-desmethylation; dotted line – desacetylation; dashed dotted line – hydroxylation at the core diltiazem structure; double dashed dotted line – hydroxylation at the methoxyphenyl-ring. M1 – *N*-desmethyl diltiazem; M2 – desacetyl diltiazem; M3 – desacetyl, *N*-desmethyl diltiazem; M4 – desacetyl, (a – N,O; b – N;N) – didesmethyl diltiazem; M5 – (a – *N,O*; b – *N,N*)-didesmethyl diltiazem M6 – (ab – core diltiazem structure; cd – methoxyphenyl-ring) – hydroxy diltiazem; M7 – *N*-desmethyl (ab-core diltiazem structure; c-methoxyphenyl-ring)-hydroxy diltiazem; M8 – desacetyl, hydroxy (a, b, c – hydroxylation products at methoxyphenyl ring) diltiazem.

diltiazem molecule. To the best of our knowledge, there are no published studies that use this advanced instrumentation for detection and identification of diltiazem metabolites. The method presented here extends our knowledge on diltiazem biotransformation processes in fish and might be applied to mammal tissue analyses in the future.

CONCLUSIONS

This study demonstrated the possibilities of a hybrid quadrupole-orbital trap mass spectrometer and modern spectral interpretation software (MassFrontier) for identifying the complex diltiazem metabolites in fish tissue. In the study, more metabolites were detected compared with previously published methods. Consequently, tandem mass spectrometry was successfully used for metabolite identity confirmation and the elimination of false positive results. Finally, a method based on liquid chromatography with HRPS mass spectrometry was developed for identifying diltiazem and semi-quantification of its phase I metabolites in fish tissue. Phase II metabolites were not found in fish tissues. This method facilitates the study of organ-dependent formation of diltiazem metabolites via a semi-quantitative approach when reference standards are not available. Finally, this method could be further modified for further application in mammalian samples or other types of environmental matrices.

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CHAPTER 3

STUDY OF PHARMACEUTICALS AND THEIR METABOLITES IN THE SOIL ENVIRONMENT

3.1. Koba, O., Golovko, O., Kodešová, R., Fér, M., Grabic, R., 2017. Antibiotics degradation in soil: A case of clindamycin, trimethoprim, sulfamethoxazole and their transformation products. Environ. Pollut. 220, 1251–1263.

3.2. Koba, O., Golovko, O., Kodešová, R., Klement, A., Grabic, R., 2016. Transformation of atenolol, metoprolol, and carbamazepine in soils: The identification, quantification, and stability of the transformation products and further implications for the environment. Environ. Pollut. 218, 574-585.

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Antibiotics degradation in soil: A case of clindamycin, trimethoprim, sulfamethoxazole and their transformation products☆

Olga Koba [a, *], Oksana Golovko [a], Radka Kodešová [b], Miroslav Fér [b], Roman Grabic [a]

[a] University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Zátiší 728/II, 389 25 Vodňany, Czechia

[b] Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Dept. of Soil Science and Soil Protection, Kamýcká 129, 16521 Prague 6, Czechia

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ABSTRACT

Twelve different soil types that represent the soil compartments of the Czech Republic were fortified with three antibiotics (clindamycin (CLI), sulfamethoxazole (SUL), and trimethoprim (TRI)) to investigate their fate. Five metabolites (clindamycin sulfoxide (CSO), hydroxy clindamycin sulfoxide (HCSO), *S*-(SDC) and *N*-demethyl clindamycin (NDC), N_4-acetyl sulfamethoxazole (N_4AS), and hydroxy trimethoprim (HTR)) were detected and identified using HPLC/HRMS and HRPS in the soil matrix in this study. The identities of CSO and N_4AS were confirmed using commercially available reference standards.

The parent compounds degraded in all soils. Almost all of the metabolites have been shown to be persistent in soils, with the exception of N_4AS, which was formed and degraded completely within 23 days of exposure. The rate of degradation mainly depended on the soil properties.

The PCA results showed a high dependence between the soil type and behaviour of the pharmaceutical metabolites.

The mentioned metabolites can be formed in soils, and the most persistent ones may be transported to the ground water and environmental water bodies. Because no information on the effects of those metabolites on living organism are available, more studies should be performed in the future to predict the risk to the environment.

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1. Introduction

Antibiotics (ATBs) are a large group of pharmaceuticals that are used to treat and prevent bacterial infections. They have frequently been detected in different matrices in the last decade (Fedorova et al., 2014; Grabicova et al., 2015; Johnson et al., 2015). Some antibiotics were found to be relatively persistent and were not completely removed during waste water treatment (Golovko et al., 2014; Zhang et al., 2015). Due to facts, mentioned above, in some countries TWW usage is regulated and the situations where it may be applied are restricted by law (Del Re et al., 2007), because an agriculture field irrigation with effluent waste water may be a source of soil contamination by pharmaceuticals, including ATBs. A negative impact on the environment has been shown for this group of compounds due to the compounds causing microorganism resistance (Bebora et al., 1994; Heelan et al., 2004; Kristiansson et al., 2011), which can lead to problems in the treatment of human diseases. Furthermore, antibiotic usage for veterinary medicine introduces constant field contamination through animal or land application of manure.

According to a recent global tendency, the demand for ATBs continues to rise. Trimethoprim (TRI) is an anti-infective agent that is used to treat urinary (McCarthy et al., 1999; van Merode et al., 2005) and respiratory tract (Franks and Gleiner, 1984) infections. Sulfamethoxazole (SUL) is an antibacterial and an anti-infective agent that is used to treat urinary tract infections (McCarthy et al., 1999), bronchitis (Franks and Gleiner, 1984), and prostatitis (Kurzer and Kaplan, 2002). TRI and SUL are often used in combination for encephalomyelitis (Sakiyama et al., 2015), ocular toxoplasmosis (Berkani et al., 2015), brucellosis (Teker et al., 2014), and acute pyelonephritis (Carrie et al., 2004) therapy. SUL and TRI are also widely used in veterinary medicine (Bottari et al., 2015; Compiani et al., 2015; Matozzo et al., 2015; McClure et al., 2015;

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* Corresponding author.
E-mail address: okoba@frov.jcu.cz (O. Koba).

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Phu et al., 2015). Clindamycin (CLI) is an anti-bacterial drug that is used to treat a number of diseases, such as acute bacterial skin and skin-structure infections (Wargo et al., 2015), bacterial vaginosis (Hantoushzadeh et al., 2012), osteoarticular infections (El Samad et al., 2008), and aspiration pneumonia (Talaie et al., 2008).

The facts mentioned above have shown that ATBs have the potential to be a significant contamination source for a soil environment. It is already known that ATBs can be transported from soil to plants (Boxall et al., 2006). Consumption of antibiotic contaminated plants represents a chronic human exposure pathway to antibiotics and may cause problems in terms of human health. Because this group of compounds can cause adverse effects to living organisms and the environment, their fate needs to be investigated.

Several studies have reported information about the fate of mentioned ATBs in different soil types (Kodesova et al., 2015; Kodešová et al., 2016; Li et al., 2015; Liu et al., 2010; Siemens et al., 2010; Srinivasan and Sarmah, 2014; Wu et al., 2012). Those studies presented specific issues, such as the influence of soil sterilization (Liu et al., 2010) and environmental factors (Srinivasan and Sarmah, 2014), on dissipation, land application of biosoils or compost on mineralization (Li et al., 2015), sorption (Kodesova et al., 2015), transport in soil columns (Siemens et al., 2010), and so on.

Different transformation products may be formed while the ATBs interact with a soil compartment. It has been shown that antibiotic metabolites may keep their pharmacological activity (Johne et al., 2005). Consequently, antibiotic transformation products may be potentially harmful to the environment and living organisms.

Due to a lack of information related to antibiotic degradation in soil, transformation product formation, and further degradation, this study aimed to investigate the fate of the selected ATBs and their possible metabolites in the soil matrix of twelve soils from different localities of the Czech Republic.

2. Materials and methods

2.1. Chemicals

All of the analytical standards were high purity (mostly 99%). The native standards TRI and CLI were obtained from Sigma-Aldrich (St. Louis, MO, USA). SUL was purchased from Riedel-de Haen (Germany). The internal standards (ISs) TRI ($^{13}C_3$), CLI (D_3) and SUL ($^{13}C_6$) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Metabolite standards of clindamycin sulfoxide (CSO), N_1-acetyl sulfamethoxazole (N_1AS), and N_4-acetyl sulfamethoxazole (N_4AS) were purchased from Labicom (Olomouc, Czech Republic). A stock solution of each pharmaceutical was prepared in methanol at a concentration of 1 mg mL^{-1}. A spiking mixture of each pharmaceutical was prepared by diluting the stock solution with methanol to a final concentration of 1 μg mL^{-1}. All stock and spiking solutions were stored at −20 °C. Liquid chromatography-mass spectrometry (LC-MS) grade acetonitrile (LiChrosolv Hypergrade) was obtained from Merck (Darmstadt, Germany). Formic acid was used to acidify the mobile phases and was purchased from Labicom (Olomouc, Czech Republic). Ultrapure water was prepared by the Aqua-MAX-Ultra System (Younglin, Kyounggi-do, Korea).

2.2. Instrumentation

An Accela 1250 LC pump (Thermo Fisher Scientific, San Jose, CA, USA) coupled with a hybrid quadrupole/orbital trap Q-Exactive mass spectrometer (Thermo Fisher Scientific) and a HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland) was used to detect and quantify the target analytes. An analytical Hypersil Gold column (50-mm length, 2.1 mm i.d., 3-μm particles; Thermo Fisher Scientific) preceded by the same phase pre-column (10-mm length, 2.1 mm i.d., and 3-μm particles) and a Cogent Bidentate C18 column (50 mm × 2.1 mm i.d., 4-μm particle size from MicroSolve Technology Corporation Eatontown, NJ, USA) were used to chromatographically separate the target analytes. The soil samples were extracted using an ultrasonic bath (DT 255, Bandelin electronic, Sonorex digitec, Berlin, Germany).

2.3. Sample collection and experimental set-up

Twelve different soil types, including Stagnic Chernozem Siltic developed on marlite (X), Haplic Chernozem on loess A (I), Haplic Chernozem on loess B (D), Chernozem Arenic on gravely sand (L), Haplic Luvisol on loess (S), Haplic Cambisol on orthogneiss A (H), Haplic Cambisol on syenite B (P), Haplic Cambisol on quartzite C (J), Dystric Cambisol on paragneiss (W), Arenosol Epieutric on sand (E), two soil substrates loess (U) and sand (Q), were sampled from several locations in the Czech Republic. The soil properties were described in detail elsewhere (Kodesova et al., 2015). The samples were collected from the surface horizon (0–25 cm), except loess and sand, which were taken from the subsurface (50–80 cm). Then, the soils were air-dried, ground, and sifted through a 2-mm sieve. The basic chemical and physical soil properties were obtained using standard laboratory procedures under a constant laboratory temperature of 20 °C and are presented elsewhere (Kodesova et al., 2015). A summary of the soil properties is presented in Table A1.

Each air-dried soil (50 g) was placed into an incubation plastic flask and spiked with different pharmaceutical solutions. The amount of the spiked solution and its concentration were calculated individually to reach 2 μg g^{-1} dry soil. The field water holding capacity (soil water content for a pressure head of 150 cm) was taken into account during the calculations mentioned above. The flasks (16 including duplicates) were prepared for each soil and pharmaceutical. In total, 576 samples were incubated in the dark at a constant temperature of 20 °C. The samples were removed from the incubator 1, 2, 5, 12, 23, 40 and 61 days after applying the antibiotics, as well as on the day of application (initial point). The soil moisture content was regularly controlled, and losses were compensated for by adding water at 2 week intervals.

2.4. Sample preparation and validation method for metabolites

An ultrasonic based solvent approach was used to extract the selected compounds from the soil matrix. A slightly modified method (Golovko et al., 2016) was used to achieve the best extraction efficiency and reduce the number of sample preparation steps. Briefly, 125 ml of solvent (acetonitrile and water (1/1, v/v, 0.1% formic acid)) were used for three consecutive steps (60, 35, and 30 ml) of ultrasonic bath extraction with duration 15 min each. The samples were extracted in the degradation bottles. Three supernatants were collected and mixed well, and a 10-ml aliquot was filtered using a regenerated cellulose syringe filter (0.45-μm pores). The internal standards were added to the sample before analysis on the LC-MS/MS system.

Because twelve different soils were used for the experiment, matrix matched calibration curves were prepared from the lyophilized soil extracts. The extracts for the calibration curves were fortified to 5, 10, 50, 100, 500 and 1000 ng ml^{-1} of the targeted analytes.

All of the available analytical standards (CSO, N_1AS, and N_4AS) of the metabolite were purchased for precise identification. Detailed analysis of the soil properties (Kodesova et al., 2015; Kodešová et al., 2016), matrix effect values, and extraction efficiency of the parent

compounds (Golovko et al., 2016) helped to select four representative soils (P, X, S, and Q), which had high variable soil textures and covered extreme soil properties that affect the compound sorption (organic carbon content between 0.08 and 3.2%, pH_{H2O} between 5.3 and 8.07, cation exchange capacity between 10.6 and 315 $mmol^{+}$ kg^{-1}) to check metabolite recovery. The extraction efficiency for the available metabolites was checked at concentrations of 10 and 100 ng g^{-1} using the method described above.

2.5. LC-HRMS/HRPS analysis

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods were used to obtain the concentrations of the target analytes. One millilitre of the filtered extract was placed in an autosampler vial with 100 ng of the IS per aliquot of sample. The samples were analysed using a Hypersil Gold column for CLI and its metabolites and a Cogent Bidentate C18 column for TRI, SUL and their metabolites. A heated electrospray ionization (HESI-II) source in positive and negative ion modes was used to ionize the target compounds. The operating parameters were set as follows: spray voltage, 3.5 kV (positive mode) and 2.8 kV (negative mode); sheath gas, 40 arbitrary units; auxiliary gas, 10 arbitrary units; capillary temperature, 325 °C; and vaporizer temperature, 250 °C. The soil extracts were measured in full scan mode at a resolution of 70,000 FWHM, an AGC target of 5e6, and a maximum filling time of 100 ms in a scan range of 50–800 *m/z* with optimized LC conditions (Table A2). The high resolution product scan (HRPS) mode was used to obtain the MS^2 fragmentation of the possible metabolites. The HRPS parameters were optimized for both the positive and negative modes and set as follows: an isolation window at the quadrupole of 1 *m/z*, an orbital trap resolution of 17,500 FWHM, an AGC target of 1e6 and a maximum filling time of 40 ms. MS^2 parameters for target analytes are presented in the Supplementary materials (Table A3).

2.6. Data quantification and statistical analysis

All of the soil samples were measured, and our target analytes were quantified based on the internal standard addition method and semi-quantification approach (Ravindran et al., 2014) in case no reference standards were commercially available. Final concentrations of each target analytes were obtained in ng g^{-1} units. They were later recalculated to molar concentrations (pmol g^{-1} units obtained by dividing weigh concentration with corresponding molecular mass of studied compounds) for easier comparison, which was not dependent on molecular mass of each analyte. Based on the results, metabolite kinetic curves (i.e., metabolite concentrations versus time describing the cumulative formation and following degradation of the metabolites in time) were constructed. Metabolite kinetic curves were used to calculate C-Integrals, which are actually the areas under the kinetic curves of each metabolite normalized by initial concentration (C_{P0}) of parent compounds (Table A5). . A detailed explanation of C-Integrals calculation is described in the Appendix (Fig. A4). C-Integrals (Fig. 1) and kinetic curves of each metabolite (Figs. 3–5), were applied to explain each pharmaceuticals behaviour in the soil matrix. The C-Integral values were also used to determine the formation level for each metabolite (10, 1- major metabolites, 0.1, 0.01- minor metabolites) (Fig. 1).

Principal component analysis (PCA) was applied to find the relationship between the fate of metabolites and soil properties. PCA is a tool that is used to search the variation to find patterns in a dataset. It is often used to make data easy to explore and visualize. The software STATISTICA was used for PCA.

3. Results and discussion

3.1. Detection of parent compounds and their behaviour in all of the soils

First, the parent compounds CLI, SUL, and TRI were measured at 425.1869 (−0.59 ppm), 254.0593 (−0.35 ppm), and 291.1445 (−2.30 ppm) *m/z* (mass error values are presented in brackets) with retention times (RT) of 5.74, 7.04, and 5.78 min, respectively, in full scan mode. The MS^2 fragmentation spectra of CLI, SUL, and TRI were obtained during the second analysis step using optimized conditions (Fig. A1).

All of the soil extracts were measured, and the logarithmic dissipation curves for the parent compounds of interest were constructed to evaluate a degradation rate by means of slope values (Fig. A2). One more parameter for parent compounds behaviour analysis in the soils, such as degradation efficiency (D_e), which represent relationship between initial and final concentration of parent compounds, was calculated (Table A5).

CLI was almost completely degraded in all of the soils by the end of the exposure, the fastest degradation was observed in J and W soils. Parent compound concentration was eliminated for 97% (Table A5). The concentration of the target compound was almost stable in U soil (loess), just 44% of CLI initial amount was degraded (Table A5). The most interesting behaviour for CLI was observed in Q soil (sand) because the reaction did not fit the 1st order kinetic model (Fig. A2). No degradation was observed during the first 41 days of exposure, while the degradation process was extremely accelerated beyond 41 days of exposure. Sand (Q) contained the lowest amount (0.08%) of organic carbon compared to the rest of the soils. Consequently, the bacterial community population and diversity may be affected. A faster degradation rate might also be explained by antibiotic resistance development during the exposure time. It had been shown earlier that the dissipation half-life time, under the same conditions as used in our experiment for CLI in sewage sludge (biosolids) was 1–1.6 days (Chenxi et al., 2008), whereas in loamy sand, sandy loam, and slay, degradation was slower, with a calculated half-life time of 21 ($r^2 = 0.95$), 33 ($r^2 = 0.89$), and 84 ($r^2 = 0.79$) days for the given soils, respectively (Wu et al., 2010). Based on our data and the literature, we can conclude that CLI degradation depends on soil properties. However, CLI was shown to be less persistent in soils.

With the exception of Q and U (soil substrates), for which 25 and 44% of SUL amount was shown to be degraded under current experimental conditions (Table A5), SUL degraded rapidly in all of the soils (Fig. A2). According to our data, all of the mentioned soils can be divided into two groups. The first group contained the H, J, W, L, P, S, and E soils, in which degradation was relatively slow and with low D_e values less than 98% compared to the second soil group that included the X, D, and I soils. A relatively high degradation rate in this group of soils may be explained by the high quality soil types, i.e., Chernozems (X, D, and I), which developed on marlite and loess.

TRI degradation in soils occurred similarly to that of SUL, but with a much lower degradation efficiency ranging from 13 to 71% (Table A5). Slow degradation with a relatively high final concentration compared to the initial concentration was observed in the Q, E, J, U, H, P, L, and W soils (Table A5). The S, D, I, and X soils showed a better degradation capacity with D_e values ranging from 50 to 71% (Table A5; Fig. A2). The better degradation capacity can be attributed to the same causes that were described above for SUL.

Degradation of SUL and TRI under aerobic conditions has been described in sand and medium loam. The half-lives of SUL in the mentioned soils were 11.4 and 9.0 d, respectively, while the value for TRI in sand was 26 days and the amount remaining in the

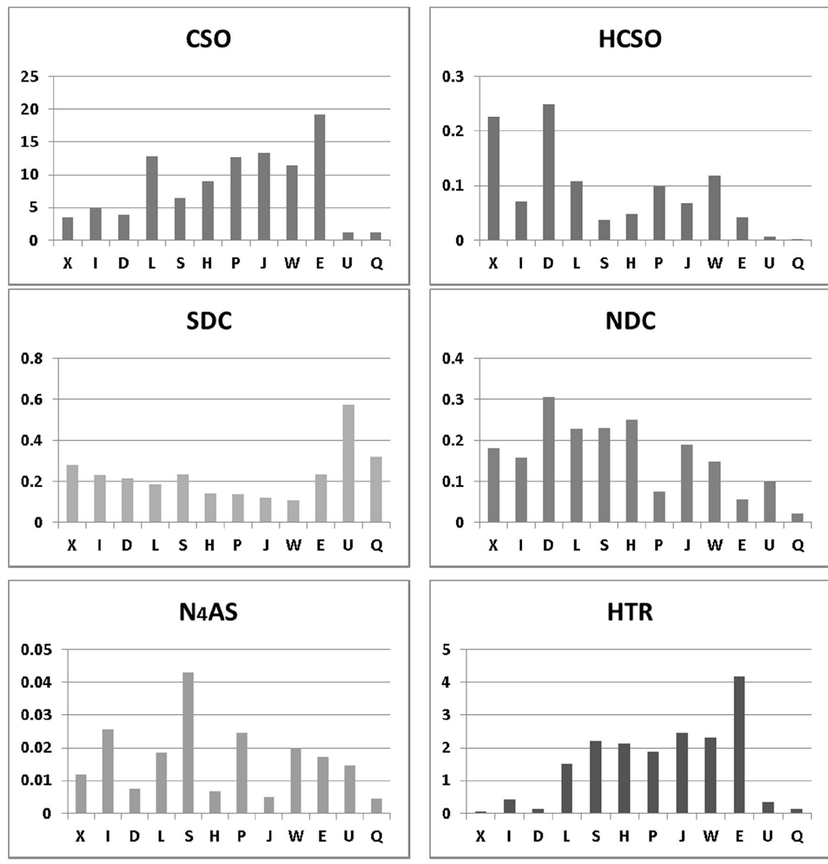


Fig. 1. C-Integrals for all of the detected metabolites and all of the soils: Stagnic Chernozem Siltic developed on marlite (X), Haplic Chernozem on loess A (I), Haplic Chernozem on loess B (D), Chernozem Arenic on gravely sand (L), Haplic Luvisol on loess (S), Haplic Cambisol on orthogneiss A (H), Haplic Cambisol on syenite B (P), Haplic Cambisol on quartzite C (J), Dystric Cambisol on paragneiss (W), Arenosol Epieutric on sand (E), loess (U) and sand (Q); horizontal axis is a soil type, vertical is C-Integral values.

medium loam fluctuated from 68% to 84% between 7 and 84 days (Lin and Gan, 2011). Faster degradation was observed in the non-sterile clay loam with a total organic carbon content of 13.5 g kg^{-1} under aerobic conditions, with SUL and TRI having half-lives of 2 and 4 days, respectively (Liu et al., 2010). SUL and TRI dissipated more rapidly in non-sterile soil than in sterile soil in both of the mentioned studies. Hence, biodegradation played a major role in the dissipation of SUL and TRI in the soil (Lin and Gan, 2011; Liu et al., 2010).

To conclude, SUL was less persistent compared to TRI, which may lead to TRI having more adverse effects on the environment.

Few more parameters should be mentioned in terms of pharmaceuticals fate in soils. A quite significant one is a sorption of pharmaceuticals. Detail data describing sorption affinity of studied pharmaceutical to all studied soils were reported previously (Kodesova et al., 2015). A sorption was evaluated using the Freundlich coefficients and it was shown to be increasing in the following order SUL (1.89 $cm^{3/n}$ $\mu g^{1-1/n}$ g^{-1}), CLI (5.43 $cm^{3/n}$ $\mu g^{1-1/n}$ g^{-1}), and TRI (22.46 $cm^{3/n}$ $\mu g^{1-1/n}$ g^{-1}) with the average value for all soils presented in the brackets. Based on this data, we can conclude that the higher sorption of the compound lead to lower available amount for degradation, as in case of TRI, which exhibits the highest sorption affinity to soils (Kodesova et al., 2015) and the lowest D_e compare with the rest of compounds (Table A5). However, any significant correlation was found between compounds dissipation rate and soils properties (Kodešová et al., 2016).

A biodegradation should be considered as the major degradation pathway for pharmaceuticals dissipation in soil. It has been exhibited above that SUL and TRI were faster degraded in non-sterile soil (Liu et al., 2010; Srinivasan and Sarmah, 2014). Based on mentioned studies we can suggest that biodegradation may be sufficient for the pharmaceuticals removal from the soil. To our knowledge, there were no studies published related to characterization of microbial population in pharmaceuticals exposed to soils.

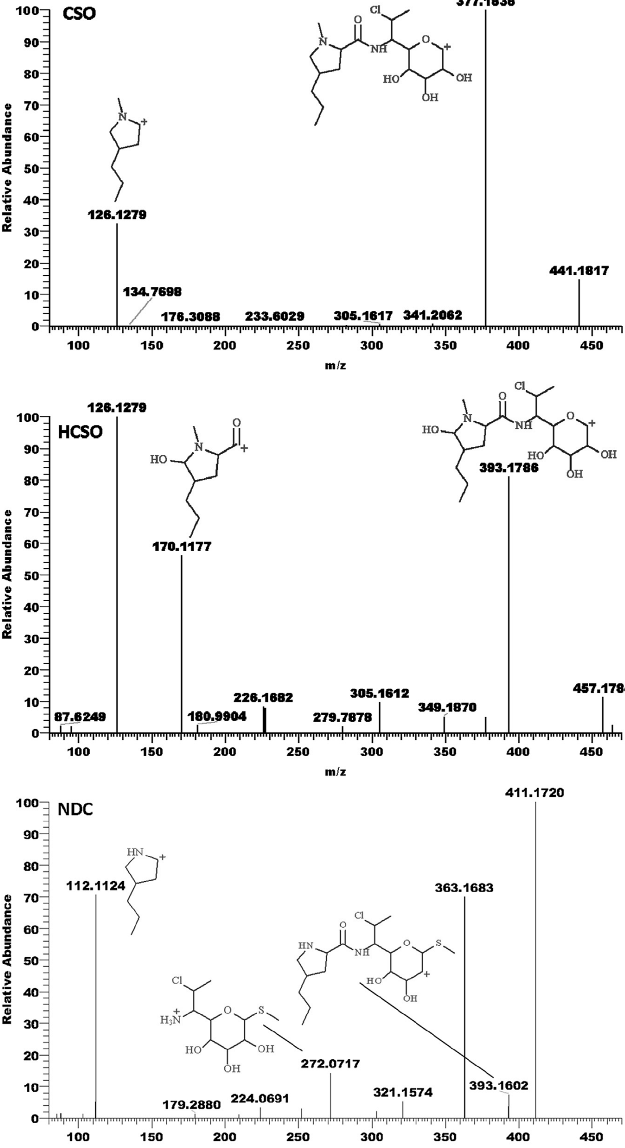


Fig. 2. MS^2 fragmentation spectra of CSO, HCSO, and NDC.

Unfortunately, no microbial population analysis or biodegradation contribution by means of sterile soil approach were used in the current study. Since microbial degradation processes play an important role in pharmaceutical and their metabolites behaviour in the soils, complex studies related to this issues should be performed.

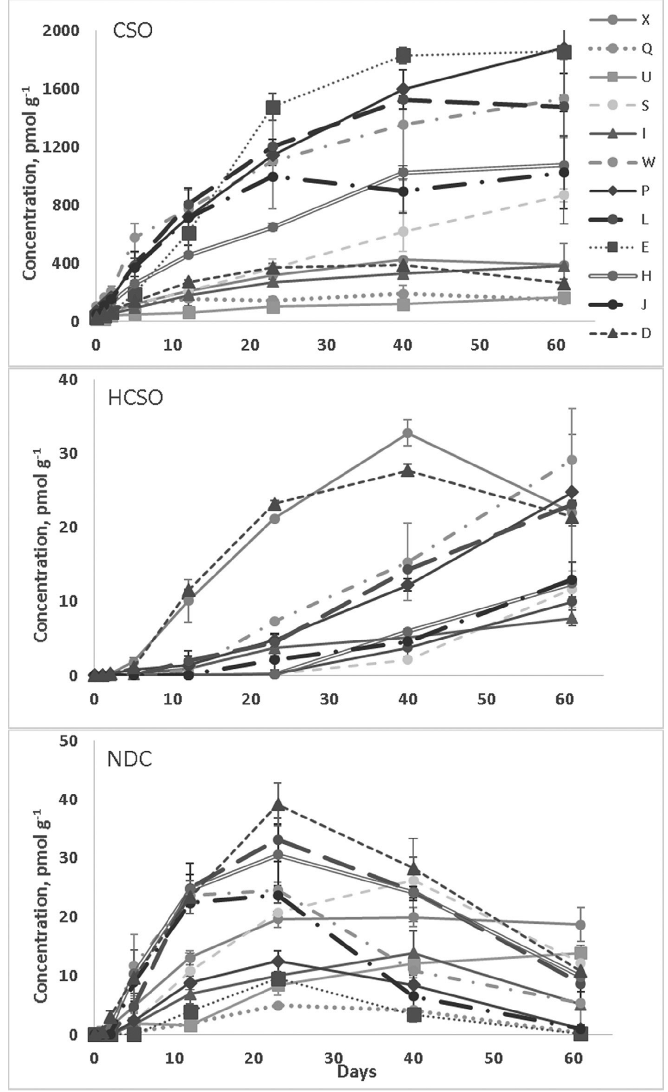


Fig. 3. Formation and concentration changes of CLI metabolites in the soils: Stagnic Chernozem Siltic developed on marlite (X), Haplic Chernozem on loess A (I), Haplic Chernozem on loess B (D), Chernozem Arenic on gravely sand (L), Haplic Luvisol on loess (S), Haplic Cambisol on orthogneiss A (H), Haplic Cambisol on syenite B (P), Haplic Cambisol on quartzite C (J), Dystric Cambisol on paragneiss (W), Arenosol Epieutric on sand (E), loess (U) and sand (Q). Error bars are expressed as the absolute difference between the duplicates divided by the average value.

3.2. Metabolite identification

The full scan mode data were further analysed using a specific software for differential analysis (SIEVE™), the literature and basic chemical knowledge to obtain a list of possible metabolites. The candidates that fit the 5 ppm window from the exact mass that were obtained from the elemental composition were selected for the next analysis step. HRPS optimized for each compound

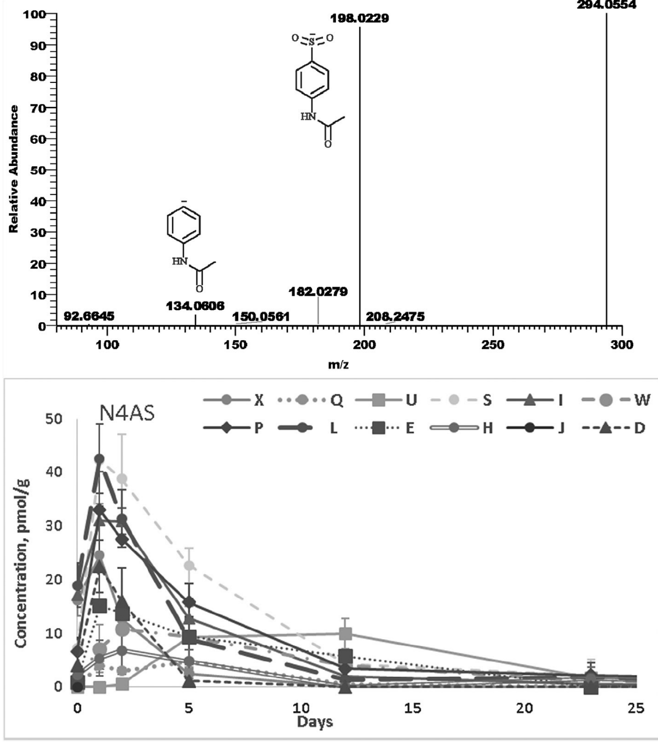


Fig. 4. MS^2 fragmentation spectra and behaviour over 25 days of the experiment for N4-acetyl sulfamethoxazole in soils: Stagnic Chernozem Siltic developed on marlite (X), Haplic Chernozem on loess A (I), Haplic Chernozem on loess B (D), Chernozem Arenic on gravely sand (L), Haplic Luvisol on loess (S), Haplic Cambisol on orthogneiss A (H), Haplic Cambisol on syenite B (P), Haplic Cambisol on quartzite C (J), Dystric Cambisol on paragneiss (W), Arenosol Epieutric on sand (E), loess (U) and sand (Q). Error bars are expressed as the absolute difference between the duplicates divided by the average value.

condition was applied to obtain the MS^2 fragmentation spectra for the possible metabolites. A final list of the compounds of interest was prepared (Table 1) after examination of the fragmentation spectra. A summary of the MS^2 fragments for all of the compounds presented in this study with mass errors between the theoretical and measured *m/z* values are presented in the supporting information (Table A4).

The spectra interpretation software Mass Frontier was used to tentatively identify the metabolites. Purchasing commercially available standards was the last step required to confirm the metabolites in this study.

3.3. CLI metabolites

The highest number of metabolites was detected and identified for CLI. The identity of CSO was confirmed by the available reference standard. CSO was the main metabolite of CLI, which was apparent from the C-Integral values (Fig. 1). CSO was formed in all of the soils at a high concentration, with the maximum value found in the E soil (Fig. 1). All of the soils can be divided into three groups based on the C-Integral values of CSO. The E and L soils (soils with a higher fraction of sand) represented the high concentration group, whereas J, P, W, H and S belonged to the middle concentration group (cambisols and luvisol) (Fig. 1). The lowest concentrations were found in the X, I, D (Chernozems, better quality soils with higher organic carbon content), Q, and U (soil substrates) soils. We can conclude that the organic carbon content was not the key parameter that effected degradation because the lowest amount of CSO was formed in Chernozems (X, I, and D) or the metabolite was extremely rapidly transformed into some minor metabolites in these soils. According to the results, the sorption complex saturation (SCS) was the only parameter that effected CSO formation. The higher the value of SCS for a soil (Table A1), the lower the amount of metabolite formed. This result may be explained by the sorption process (Kodesova et al., 2015) related to the SCS value, the higher sorption, and the lower amount of the parent compound being available for degradation, lead to a lower detected concentration of the metabolite.

CSO formation was almost the same for all of the studied soils. The concentration of the metabolite started to increase after the second day of exposure and reached the maximum value at the end of the experiment (Fig. 3). The metabolite was shown to be

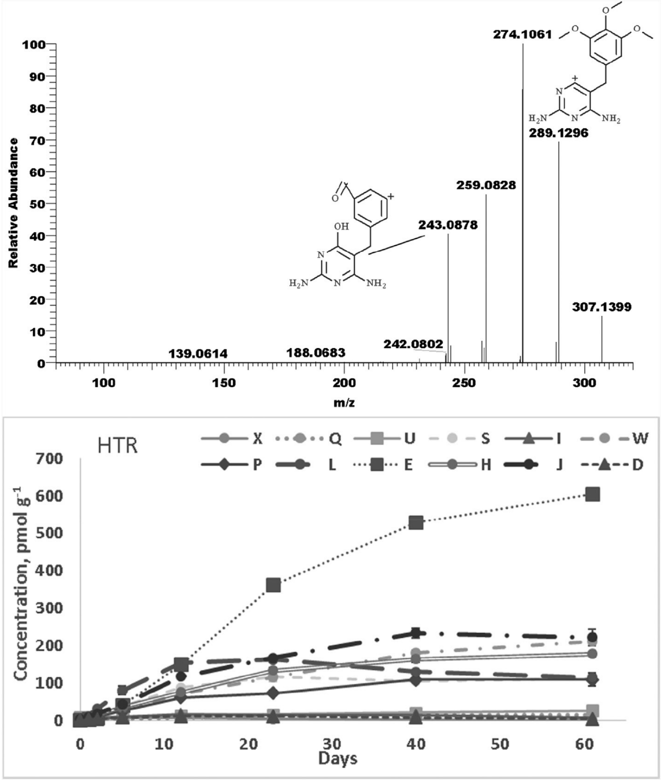


Fig. 5. MS^2 fragmentation spectra of hydroxyl trimethoprim and its formation kinetics in 12 studied soils: Stagnic Chernozem Siltic developed on marlite (X), Haplic Chernozem on loess A (I), Haplic Chernozem on loess B (D), Chernozem Arenic on gravely sand (L), Haplic Luvisol on loess (S), Haplic Cambisol on orthogneiss A (H), Haplic Cambisol on syenite B (P), Haplic Cambisol on quartzite C (J), Dystric Cambisol on paragneiss (W), Arenosol Epieutric on sand (E), loess (U) and sand (Q). Error bars are expressed as the absolute difference between the duplicates divided by the average value.

persistent during the studied period of time. Therefore, CSO formed in the environment may be transported from the soil to ground water.

Hydroxy clindamycin sulfoxide (HCSO) was identified using specific fragments at 170.1177 and 393.1786 *m/z* measured with errors of 0.59 and −0.25 ppm, respectively (Fig. 2). The mass shifts corresponding to the loss of CH_4OS at 393.1786 *m/z* and the loss of $C_9H_{16}O_2N$ at 170.1177 *m/z* helped us to create a structure that confirmed the hydroxylation of CSO (Fig. 2). Unfortunately, spectra data cannot clarify the position at which hydroxylation occurs, which makes the existence of a two isomeric form of this metabolite possible (Table 1). The metabolite was present in 10 soils with the exception of Q and U (i.e., soil substrates). The behaviour of HCSO was not the same in all of the soils as with CSO. HCSO is a minor metabolite of CLI with the highest formed concentration in the D soil (Fig. 1). The highest amount of HCSO was formed in the X and D soils, where it started to degrade after 41 days of exposure (Fig. 3). No HCSO degradation was observed for the rest of the soils where higher concentrations were formed for the W, P, L, and I soils and lower concentrations were present for H, J, S, and E (Figs. 1 and 3).

There are several possible demethyl metabolites of CLI based on its structure. Two chromatographic peaks with RTs of 5.12 and 5.83 min were present in all of the soil extracts. Different MS^2 fragmentation spectra enabled the identification of a parent compound site where demethylation took place. Those metabolites were identified as products of demethylation at the *N*- and *S*-moieties.

A metabolite, identified as *S*-demethyl clindamycin (SDC), was an impurity of CLI because it was present in the calibration curve points at which it was not added. SDC was almost completely degraded in all of the soils during the 61 days of exposure. The MS^2 spectra with specific fragments and degradation curves are presented in the supporting information (Fig. A3).

N-demethyl clindamycin (NDC) was identified using the specific MS^2 fragment measured at 112.1124 *m/z* (2.85 ppm), which made it possible to create a structure of *N*-demethylation; 272.0717 *m/z* (−0.29 ppm) confirmed the presence of the CH_3 group at the *S*-moiety, and 393.1602 *m/z* confirmed both of the above mentioned CLI transformations (Fig. 2). The NDC metabolite was present in all

Table 1
CLI, SUL and TRI and their selected metabolites.

Compound	Formula	Structure	Exact mass (Da)	Measured mass (Da)[a]	Mass error (mDa)	Mass error (ppm)	RT (min)
CLI	C18H33ClN2O5S		425.1872	425.1869	−0.25	−0.59	5.74
CSO	C18H33ClN2O6S		441.1821	441.1817	−0.4	−0.91	4.90
HCSO	C18H33ClN2O7S		457.1770	457.1784	1.4	3.06	5.18
SDC	C17H31ClN2O5S		411.1715	411.1713	−0.2	−0.49	5.12
NDC	C17H31ClN2O5S		411.1715	411.1720	0.5	1.22	5.83
SUL	C10H11N3O3S		254.0594	254.0593	−0.09	−0.35	7.04
N_1AS	C12H13N3O4S		296.0700	296.0696	−0.35	−1.18	7.04
N_4AS	C12H13N3O4S		294.0554	294.0554[b]	0	0	6.27
TRI	C14H18N4O3		291.1452	291.1445	−0.67	−2.30	5.78

(*continued on next page*)

Table 1 (*continued*)

Compound	Formula	Structure	Exact mass (Da)	Measured mass (Da)[a]	Mass error (mDa)	Mass error (ppm)	RT (min)
HTR	C14H18N4O4		307.1401	307.1399	−0.18	−0.59	5.29

[a] Compounds were measured as $[M+H]^+$ ions.
[b] Compound was measured as an $[M+H]^-$ ion.

12 soils. The highest C-Integral values of NDC were obtained for the D, L, S and H soils; middle values were obtained for the X, J, I, and W soils; and the lowest values were observed in the P, E, U, and Q soils (Fig. 1). Formed NDC was almost completely eliminated during the 61 days of the experiment (Fig. 3). Almost identical degradation curve shapes were exhibited in the D, L, and H soils. W and J behaved similarly, with lower concentrations (Fig. 3). According to our data, there was no soil parameter that could explain the formation/degradation of NDC.

3.4. SUL metabolites

Only one metabolite of SUL was found and identified in this study. Despite the possible existence of two acetyl sulfamethoxazole metabolites (Table 1), N_1 and N_4, N_4AS was detected as a deprotonated ion at 294.0554 *m/z* in all of the soil extracts. The presence of specific fragments in the MS^2 spectra (Fig. 4) and the available reference standard made identification and further confirmation easier. A completely different behaviour was observed for N_4AS compared to the rest of the metabolites. N_4AS was formed in all of the soils, but the formation process started immediately after spiking the soil with the SUL solution (Fig. 4). The highest N_4AS C-Integral values were observed in the S, P, and I soils, and the middle values were in the L, W, E, and U soils (Fig. 1). The metabolite was found at low concentration levels in the rest of the soils. The highest amounts of N_4AS were formed in the better developed soils with relatively high organic carbon contents. N_4AS was degraded rapidly during the first 12 days of exposure in all of the soils, with the exception of the U soil, where complete degradation was not finished until 23 days of exposure (Fig. 4).

SUL and its metabolite N_4AS were measured in a real field study in soils from rural areas and also along the Ebro River basin (Garcia-Galan et al., 2013). The maximum detected amounts of SUL and N_4AS were 2.58 and 1.38 ng g^{-1} (dry weight), respectively. Although there was fast degradation and low concentrations of the parent compound and metabolite in the soil compartment, it has been reported that SUL can change the bacterial diversity in the soils (Liu et al., 2012).

3.5. TRI metabolites

Only one metabolite was identified for TRI. The MS^2 fragmentation spectra with high values of relative abundance for ions simplified the identification process. Specific fragments, such as 243.0878 *m/z* (0.41 ppm) and 289.1296 *m/z* (0.35 ppm), and analysis of the loss composition based on the mass difference between the precursor and product ions made it possible to identify the metabolite as a hydroxy trimethoprim (Fig. 5). The metabolite was not degraded during the exposure time. The maximum C-Integral values of hydroxy trimethoprim (HTR) were calculated in the E soil (Fig. 1). The rest of the soils can be divided into two groups. S, J, W, H, L, and P represent the first group, with values in the middle. The second group consisted of the X, Q, U, I and D soils, where amounts of HRT low were present (Fig. 1). The amount of HTR formed was strongly correlated to the SCS value for each soil. Thus, the maximum and minimum concentrations were observed for the E and X soils, respectively, which inversely corresponded to SCS values of 56.5 and 98.9%. Our data indicated that as the concentration increased (Fig. 5) the SCS value decreased (Table A1).

HTR (α-form) has been previously detected in a few studies dealing with activated sludge (Eichhorn et al., 2005; Jewell et al., 2016). HTR has been exhibited as a major metabolite produced by nitrifying activated sludge bacteria (Eichhorn et al., 2005). It has also been shown that the TRI transformation pathway depends on the concentration value (demethylation – low concentration of 5 μg L^{-1}, hydroxylation – high concentration of 500 μg L^{-1}) due to the limitation of specific enzymes or processes or to toxic/inhibition or enzyme activation effects of TRI to the sludge bacterial community at higher concentrations (Jewell et al., 2016). Unfortunately, no α-form of HTR was detected based on tentative spectra identification in our study to enable further comparisons to the available literature.

3.6. Matrix effect evaluation

Because twelve different soils were used for the experiment, the matrix effect needed to be evaluated to precisely quantify the results. Co-extracted compounds usually affect (suppress or enhance) ionization and the consequent ion handling processes in the mass spectrometer. A comparison of the calibration curves (based on analyte peak area) prepared in the extraction solvent and real matrix extracts was made to evaluate the ion suppression/enhancement value evaluation. The calibration curve slopes of the compounds of interest in the soil extracts were compared to the slope calculated for the extraction solvent. The results are presented in Table 2. According to the results, the studied compounds showed a mostly low matrix effect (<20%) in all of the soils. There were a few exceptions. The signal was enhanced by 23, 27, 28, and 30% for N_1AS in the Q, U, D, and I soils, respectively. Extracts from the I and U soils effected the signal by 23 and 28% for SUL. A signal suppression of 23% was observed for CSO in the E soil, while ion enhancement was exhibited for CLI in the Q soil.

3.7. Metabolite method validation

As mentioned above, the extraction efficiency of the parent compounds was checked during the initial step of the experiment. Analytical standards for some of the tentatively identified metabolites were used to evaluate their extraction efficiency. A recoveries summary is presented in Table 3. Other method validation parameters, such as method detection limits (MDLs) and method quantification limits (MQLs) are presented in Table A6. According to

Table 2
Matrix effect (suppression/enhancement) for all of the tested soils (values are given in %): Stagnic Chernozem Siltic developed on marlite (X), Haplic Chernozem on loess A (I), Haplic Chernozem on loess B (D), Chernozem Arenic on gravely sand (L), Haplic Luvisol on loess (S), Haplic Cambisol on orthogneiss A (H), Haplic Cambisol on syenite B (P), Haplic Cambisol on quartzite C (J), Dystric Cambisol on paragneiss (W), Arenosol Epieutric on sand (E), loess (U) and sand (Q).

Compound	P	J	H	W	X	L	E	I	D	S	Q	U
CLI	−5[a]	−6	0	−12	6	8	−2	1	15	19	23	22
CSO	−18	−18	−8	−15	−10	−7	−23	−9	9	2	1	16
SUL	7[b]	2	2	1	0	4	9	23	16	−3	20	28
N_1AS	11	19	16	6	8	19	17	30	28	−8	23	27
N_4AS	−5	−2	−2	−10	−6	−2	−6	0	0	−7	5	9
TRI	−2	2	−4	−12	−14	−1	−4	0	0	−20	−1	4

[a] Suppression, value is < 0.
[b] Enchantment, value is > 0.

Table 3
Average recoveries (ARF) of the target analytes in all of the tested soils fortified to 10 and 100 ng/g (values are given in %): Stagnic Chernozem Siltic developed on marlite (X), Haplic Luvisol on loess (S), Haplic Cambisol on syenite B (P), and sand (Q). RSD – relative standard deviation.

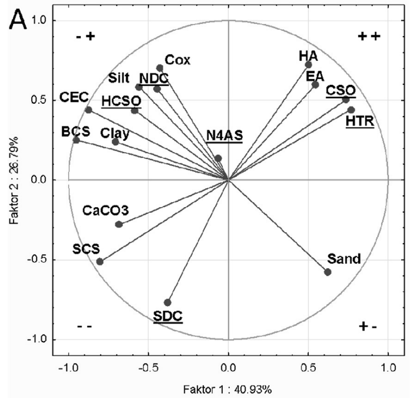
	Soil type							
	P				X			
	10 (n = 5)		100 (n = 5)		10 (n = 5)		100 (n = 5)	
	ARF	RSD	ARF	RSD	ARF	RSD	ARF	RSD
CLI	116	6	131	4	111	8	135	3
CSO	76	8	93	8	69	11	100	5
SUL	103	17	103	11	82	6	109	4
N_1AS	83	6	115	9	78	11	111	5
N_4AS	99	9	102	9	101	8	120	5
TRI	88	10	114	11	85	3	93	5
	Soil type							
	S				Q			
	10 (n = 5)		100 (n = 5)		10 (n = 5)		100 (n = 5)	
	ARF	RSD	ARF	RSD	ARF	RSD	ARF	RSD
CLI	110	4	124	8	99	8	124	6
CSO	66	11	91	5	76	8	102	3
SUL	85	7	120	5	94	6	111	4
N_1AS	90	8	122	5	97	6	110	1
N_4AS	104	3	123	4	109	9	121	1
TRI	81	11	89	3	90	8	100	8

our data, all of the metabolites had a recovery efficiency that ranged from 66 to 123% in the soils with extreme properties (Table 3). This step helped to clarify that degradation products CSO, N_1AS, and N_4AS had satisfactory recovery values. Thus, the absence of or low concentrations for some of the metabolites was related to the fact that they were not formed or they rapidly transformed under the exposure conditions and were not affected by the extraction procedure.

3.8. *Principal component analysis (PCA)*

Because no simple relationship among the soil properties and metabolite fates were found, PCA was applied for the dataset. PCA is a technique that is used to emphasize variation and bring out strong patterns in a dataset. It is often used to make data easy to explore and visualize. The software STATISTICA was used for PCA. Factors 1 (40.93%) and 2 (26.79%) were the most valuable for the data.

The representation of variables shows that most of them are not highly correlated for both factors because active components for each soil were placed in all (++, +⁻, ⁻+, and ⁻⁻) of the quarters (Fig. A6). Factor 1 represents a high correlation to "HTR" and "CSO" (based on their coordinate value), which may be explained by the fact that they were the main metabolites that were present in all of the soils, with a moderate correlation to sand content, which had a negative correlation to Factor 2. (Fig. 6A). The variables that were



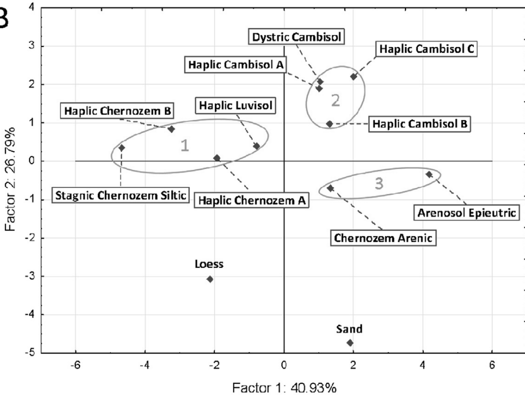


Fig. 6. Scatter plot of the loading factors (A) based on soil properties and normalized integrals for metabolite concentrations (metabolites names are underlined) and scores scatterplot (B), represented by soil type, for the principal component selection.

most correlated to the second factor were “HA”, “EA”, “Cox”, “Silt”, and “NDC” (the last three were negatively correlated to Factor 1). According to the cases plot in Fig. 6B, we can conclude that “Sand” and “Loess”, which represent substrates, are strong outlier soils in terms of their soil properties and the total metabolite concentration. The rest of the soils can be divided into three groups (Fig. 6B), where better quality soils, formed on loess and marlite, represent the first group, soils belonging to the same Cambisol type formed a second group, and the third group consisted of two soils formed on different sandy material.

Even if almost no correlation to the soil properties for each metabolite was found, based on the PCA results, we can assume that the soil properties and, consequently, the fate of the pharmaceuticals and their metabolites are strongly related to the soil type (Fig. 6B). More detailed analysis of each soil coordinate can clarify the importance of the variables (soil properties and total amount of formed metabolite) to which Factors 1 and 2 are correlated, as described above. For example, Arenosol Epieutric, highly correlated to Factor 1 (Fig. 6B), which was represented mostly by “HTR” and “CSO” (Fig. 6A), was the soil with the highest amount of HTR and CSO metabolites (Fig. 1), among others.

4. Conclusions

The fate of three ATBs, CLI, SUL, and TRI was investigated in 12 different soil types. The parent compounds degraded in all of the soils. The degradation rate mainly depended on the soil properties. Furthermore, five metabolites, CSO, HCSO, NDC, N_4AS and HRT, were detected and identified in the soil matrix in this study. HRPS data and the specific spectra interpretation software Mass Frontier enabled the identification of the metabolites when no analytical standards were available. The identities of CSO and N_4AS were confirmed using reference standards. The PCA results showed a high dependence between the soil type and behaviour of the studied pharmaceutical metabolites (Fig. 6B).

Almost all of the metabolites were persistent in the soils, with the exception of N_4AS, which formed and degraded completely within 23 days of exposure. The mentioned metabolites can be formed in soils and transported to ground water and environmental water bodies. Because no information on the effects of those metabolites on living organism is available, more studies should be performed in the future to predict their risk to the environment.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.11.007.

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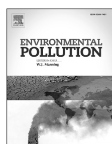

Transformation of atenolol, metoprolol, and carbamazepine in soils: The identification, quantification, and stability of the transformation products and further implications for the environment☆

Olga Koba [a, *], Oksana Golovko [a], Radka Kodešová [b], Aleš Klement [b], Roman Grabic [a]

[a] University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Zatisi 728/II, 389 25 Vodnany, Czech Republic

[b] Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Dept. of Soil Science and Soil Protection, Kamýcká 129, 16521 Prague 6, Czech Republic

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A B S T R A C T

Pharmaceuticals are a large group of substances that have been recognized as environmental contaminants in recent years. Research on the pharmaceutical fate in soils is currently limited or missing. In this study, three pharmaceuticals (atenolol (ATE), carbamazepine (CAR), and metoprolol (MET)) were introduced to soils and exposed for 61 day under aerobic conditions. Thirteen different soils were used in the study to increase the understanding of pharmaceutical behaviour in the soil matrix. Ten metabolites were detected and tentatively identified. Some of them, such as atenolol acid (AAC), carbamazepine 10,11-epoxide (EPC), 10,11-dihydrocarbamazepine (DHC), *trans*-10,11-Dihydro-10,11-dihydroxy carbamazepine (RTC), and metoprolol acid (MAC), were consequently confirmed using commercial reference standards. It was concluded that the aerobic conditions of the experiment determined the pharmaceutical degradation pathway of studied compounds in the soils. The different amounts/rates and degradation of the transformation products can be attributed to differences in the soil properties. ATE degraded relatively quickly compared with CAR, whereas MET degradation in the soils was unclear. The persistence of CAR and its metabolites, in combination with low CAR sorption, enable the transportation of CAR and its metabolites within soils and into the ground water. Thus, CAR may cause adverse effects on the environment and humans.

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1. Introduction

Pharmaceuticals are a widely used group of compounds that are present in various environmental compartments. Due to their high consumption and possible harmful effect on ecosystems, pharmaceuticals have been identified as “emerging organic contaminants” (Brodin et al., 2013; Li, 2014). ATE is used as the treatment of cardiovascular disorders (Wadworth et al., 1991) and may be used as adrenergic beta-antagonists, anti-arrhythmia, and antihypertensive agents. Currently, ATE is also used for infantile hemangioma treatment (Abarzúa-Araya et al., 2014). MET is related to the same group of pharmaceuticals as ATE. Recently, MET replaced ATE in clinical practice for the treatment of hypertension (van den Born et al., 2005). MET has been frequently used for ventricular arrhythmia (Gao et al., 1997), severe heart failure (Goldstein et al., 2001), etc. CAR has been shown to be effective as an analgesic, non-narcotic, and anticonvulsant drug. CAR has been used to treat certain psychiatric disorders, such as schizophrenia (Leucht et al., 2002), epilepsy (Shakespeare and Simeon, 1998), bipolar mood disorders (Zhang et al., 2007), and other illnesses, such as fibromyalgia (Wiffen et al., 2014), acute and chronic pain (Wiffen et al., 2005), and aggression (Yatham and McHale, 1988). Additionally, MET and CAR have been included as generic medications in the World Health Organization's List of Essential Medicines (Organization, October 2013).

It has been reported that CAR exhibits a low removal efficiency, and in some cases, even a negative removal efficiency (Collado et al., 2014) with no seasonal variation (Golovko et al., 2014b) in concentration, whereas the behaviour of MET and ATE are more complicated. The MET removal efficiency has been

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* Corresponding author.
E-mail address: okoba@frov.jcu.cz (O. Koba).

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reported as medium (Collado et al., 2014) or low (Kasprzyk-Hordern et al., 2009). ATE exhibits a low removal efficiency of 10% during winter and 50% in summer (Golovko et al., 2014a), but a high (>70%) removal efficiency has been documented in some cases (Collado et al., 2014; Kasprzyk-Hordern et al., 2009). All of the mentioned pharmaceuticals constantly enter the environment via WWTPs. This led to their frequent detection in surface water (Collado et al., 2014; Grabicova et al., 2015; Li, 2014), which may be used for irrigation of agricultural fields. Agriculture field fertilization with sewage sludge may be another possible way of environmental contamination (Poulsen et al., 2013) (in the case of soil and ground water). It has been also exhibited that CAR and MET were persistent in soils when introduced via treated wastewater and that the pre-exposure of the soils to pharmaceuticals via irrigation did not enhance their biodegradation (Grossberger et al., 2014).

Several studies have describe stability of selected compounds under different conditions, such as solar-simulator irradiation (Neamtu et al., 2014), photocatalytic ozonation (Rey et al., 2012), the direct UV photolysis (Calza et al., 2013), indirect photolysis in the presence of fulvic acids (Chen et al., 2012), and UV/H_2O_2 oxidation (Shu et al., 2013) etc. To conclude, CAR photodegradation was slow, compound was shown to be relatively persistent towards direct photolysis, while ATE and MET had a moderate photostability.

The fate of these compounds in soil has not been sufficiently studied. No information on the ATE and MET transformation in soil is available. A few studies have focused on CAR degradation in soils (Li et al., 2013; Monteiro and Boxall, 2009; Salvia et al., 2014; Yu et al., 2013). Those studies included small amounts of soils and biosoils (5 is max), and primarily focused on specific issues, such as the degradation in soil columns (Salvia et al., 2014), the impact of sterilization on dissipation (Yu et al., 2013), etc.

Those compounds were quite well studied in the different kind of sludge. A few research were focused on sorption coefficient determination, such as distribution coefficients for linear (K_d) isotherms. Thus, it has been shown that ATE has 4.6×10^2 l kg^{-1} values for K_d in a primary sludge, while 1.6×10^3 L kg^{-1} for secondary sludge long sludge age (Horsing et al., 2011) and average value of 0.038 L g^{-1} in wastewater sludge for another study (Maurer et al., 2007). The solid-water distribution coefficient (Kd) has also been obtained for CAR in mesophilic (35.4 L kg^{-1}) (Carballa et al., 2008), thermophilic (20.2 L kg^{-1}) (Carballa et al., 2008), and secondary (1.2 L kg^{-1}) (Ternes et al., 2004) sludge. An average K_d value of 0.001 L g^{-1} has been found for MET in wastewater sludge (Maurer et al., 2007). According to literature data, adsorption of studied compounds was hardly depended on type of matrix, which was investigated. Several studies have reported the harmful effect of these compounds on aquatic organisms during different developmental stages (Almeida et al., 2014; Contardo-Jara et al., 2010; Kim et al., 2007; Lamichhane et al., 2013; Steinbach et al., 2014; Triebskorn et al., 2007). There is no information available on the effects of these pharmaceuticals on organisms living in soils. During various degradation processes, pharmaceuticals form different metabolites and degradation products (Abramovic et al., 2011; Rubirola et al., 2014; Salgado et al., 2013; Tay et al., 2011). The impact of these compounds on the environment is still not known (Kim et al., 2007; Triebskorn et al., 2007).

The first step to studying the influence of various substances (i.e., pharmaceuticals and their metabolites) on soil organisms is their identification and behaviour in a soil environment. To our knowledge, there is only one published study on the degradation of CAR and its metabolite identification in soils (Li et al., 2013). According to the study, two loam and clay soils were exposed for 120 days and five metabolites were formed: 10,11-dihydro-10-hydroxycarbamazepine, carbamazepine-10,11-epoxide, acridone-N-carbaldehyde, 4-aldehyde-9-acridone, and acridine (Li et al., 2013).

Our study investigates the possible formation of degradation products from the selected compounds and their stability in a soil matrix. Thirteen different soil types, which represent different regions of the Czech Republic, were selected to determine the pharmaceutical degradation in soils under near natural conditions. To our knowledge, no other studies on pharmaceuticals and their metabolite fates in many different soil types have been reported to date.

2. Materials and methods

2.1. Chemicals

ATE, CAR, and metoprolol tartrate salt were purchased from Sigma-Aldrich (Steinheim, Czech Republic). An isotope-labelled internal standard (IS) of CAR (D10) was acquired from CDN Isotopes (Pointe-Claire, Quebec, Canada), whereas ATE (D6) and metoprolol hydrochloride (D7) were purchased from Alsachim (Strasbourg, France). Metabolite standards of AAC,MAC, EPC, OXC, DHC, and RTC were purchased from Labicom (Olomouc, Czech Republic). A stock solution of each pharmaceutical was prepared in methanol at a concentration of 1 mg mL^{-1}. A spiking mixture of each was prepared by diluting the stock solution with methanol to a final concentration of 1 μg ml^{-1}. All stock and spiking solutions were stored at −20 °C. The LC-MS grade acetonitrile (LiChrosolv Hypergrade) was obtained from Merck (Darmstadt, Germany). Formic acid was used to acidify the mobile phases and was purchased from Labicom (Olomouc, Czech Republic). The ultrapure water was prepared via an Aqua-MAX-Ultra System (Younglin, Kyounggi-do, Korea).

2.2. Instrumentation

An Accela 1250 LC pump (Thermo Fisher Scientific, San Jose, CA, USA) coupled with a hybrid quadrupole/orbital trap Q-Exactive mass spectrometer (Thermo Fisher Scientific) and a HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland) were used to separate and detect target analytes. An analytical Hypersil Gold column (50-mm length, 2.1 mm i.d, 3-μm particles; Thermo Fisher Scientific) preceded the same phase pre-column (10-mm length, 2.1 mm i.d., and 3-μm particles) and the Cogent Bidentate C18 column (50 mm × 2.1 mm i.d., 4-μm particle size from MicroSolv Technology Corporation Eatontown, NJ, USA), which were used for the chromatographic separation of the target analytes. The soil samples were extracted using an ultrasonic bath (DT 255, Bandelin electronic, Sonorex digitec, Berlin, Germany).

2.3. Sample collection and experimental set-up

Thirteen different soil types, including Stagnic Chernozem Siltic developed on marlite (X), Haplic Chernozem on loess A (I), Haplic Chernozem on loess B (D), Chernozem Arenic on gravely sand (L), Greyic Phaeozem on loess (C), Haplic Luvisol on loess (S), Haplic Cambisol on orthogneiss A (H), Haplic Cambisol on syenite B (P), Haplic Cambisol on quartzite C (J), Dystric Cambisol on paragneiss (W), Arenosol Epieutric on sand (E), loess (U), and sand (Q), were collected from several locations in Czech Republic. These soils were described in detail previously (Kodesova et al., 2015). Samples were collected from the surface horizon (0–25 cm), except loess and sand, which were taken from the subsurface (50–80 cm). The soils were then air-dried, ground, and sieved through a 2 mm sieve. The

basic chemical and physical soil properties were obtained using standard laboratory procedures under a constant laboratory temperature of 20 °C and are described elsewhere (Kodesova et al., 2015). Additionally, all soil characteristics are presented in Table A.1.

Fifty grams of each air-dried soil were placed in a plastic incubation flask and spiked with different pharmaceutical solutions. The amount of spiked solution and its concentration were calculated individually to reach 2 μg g^{-1} per dry soil. The field water holding capacity (soil water content for a head pressure of 150 cm) was taken into account during the abovementioned calculations. Sixteen flasks (including duplicates) were prepared for each soil and pharmaceutical. In total, 624 samples were incubated in the dark at a constant temperature of 20 °C. Samples were removed from the incubator 1, 2, 5, 12, 23, 40 and 61 days after the pharmaceutical application, which included the day of application (initial point). The soil moisture content was regularly controlled, and losses were compensated for by adding water in 2 week intervals.

2.4. Sample preparation and validation method for the metabolites

Selected compounds were extracted from soil matrices via ultrasonic-based extraction in a solvent mixture. An extraction method (Golovko et al., 2016) was modified to achieve the best extraction efficiency and to reduce the expense of organic solvents. Briefly, 125 ml of solvent ((acetonitrile and water (1/1, v/v, 0.1% formic acid), respectively)) was used for three extraction steps (60, 35, and 30 ml) in an ultrasonic bath with a duration of 15 min for each. Three supernatants were combined into one, and it was mixed and filtered using a regenerated cellulose syringe filter (0.45-μm pores). It was then analysed via LC-MS/MS. The ISs were added prior analysis.

Since 13 different soils were used for the experiment, matrix matched calibration curves were prepared from lyophilised soil extracts. The extracts were fortified to concentrations 5, 10, 50, 100, 500 and 1000 ng ml^{-1} of targeted analytes.

All of the available analytical standards (AAC/MAC, EPC, OXC, DHC, and RTC) of the target compound metabolites were purchased for their authentication and precise quantification. Detailed analysis of the soil properties (Kodesova et al., 2015; Kodešová et al., 2016), matrix effect values and extraction efficiencies of the parent compounds were used to select four soils (P, X, S, and Q) that represent a variable soil texture and have border soil properties that affect the sorption of the compound (organic carbon content between 0.08 and 3.2%, pH_{H2O} between 5.3 and 8.07, cation exchange capacity between 10.6 and 315 $mmol^{+}$ kg^{-1}) for the recovery verification of the metabolites. The extraction efficiency of available metabolites was verified at concentrations of 10 and 100 ng g^{-1} using the above method.

2.5. LC-HRMS/HRPS analysis

One millilitre of filtrated extract was transferred into an autosampler vial that contained 100 ng of the IS for each sample aliquot. The samples were analysed using a Hypersil Gold column (50-mm length, 2.1 mm i.d, 3-μm particles) for CAR and a Cogent Bidentate C18 column (50 mm × 2.1 mm i.d., 4-μm particles) for ATE and MET. A heated electrospray ionization (HESI-II) source in positive ion mode was used to ionize the target compounds. The operating parameters were set as follows: a spray voltage of 3.5 kV, a sheath gas of 40 arbitrary units, an auxiliary gas of 10 arbitrary units, a capillary temperature of 325 °C and a vaporizer temperature of 250 °C. Soil extracts were measured in full scan mode at a resolution of 70,000 FWHM, The AGC target was 5×10^6, the maximum C-trap filling time was 100 ms for a scan range of 50–800 *m/z* under optimized LC conditions (Table A.2). The high resolution product scan (HRPS) mode was used to obtain the MS^2 fragmentation of the possible metabolites. The HRPS parameters were optimized and set as follows: an isolation window at the quadrupole of 1 *m/z*, an orbital trap resolution of 17,500 FWHM, an AGC target of 1×10^6 and a maximum filling time of 40 ms.

3. Results and discussion

3.1. Detection of parent compounds and their metabolites

First, the analytical method for ATE, CAR, and MET was developed using an earlier published study for similar difficult matrices (Grabicova et al., 2015). Parent pharmaceuticals were detected as protonated molecular ions with 267.1700 *m/z* for ATE, 237.1020 *m/z* for CAR, and 268.1902 *m/z* for MET. Second, the soil extracts were measured in full scan mode. All chromatograms were checked using SIEVE™ Software for Differential Analysis (Thermo Scientific) and Compound Discoverer 1.0 software (Thermo Scientific). A list of the possible metabolite candidates, which matched the 5-ppm window from the exact mass, were selected for further identification (Table 1). MS^2 spectra were used for metabolite confirmation and obtained in HRPS mode with a 1 *m/z* isolation window. The MS^2 parameters for the targeted analytes are presented in Appendix A (TableA.3).

3.2. Matrix effect evaluation

Soil extracts are possibly a difficult matrix for LC-MS analysis. Extracts may contain substances that do not belong to targeted compound groups. Co-extracted compounds usually affect (suppress or enhance) the ionization and, consequently, the ion handling processes in a mass spectrometer (Fedorova et al., 2013). Ion suppression/enhancement values were obtained from the differences between calibration curve slopes that were obtained from the extraction solvent and real matrix extracts (based on analyte peak area). The results are presented in Table 2. Based on our data, we conclude that there was no significant change in the response for the studied compounds, and almost all of the ion suppression/enhancement values ranged from 0 to 20%. The exceptions were observed for ATE (−28%) and AAC/MAC (−22%) in the S soil, and for EPC in the D (21%) and C (26%) soils in which the values were greater than 20%.

3.3. Metabolite method validation

Correct metabolite identification should be accomplished using LC-MS in combination with a confirmatory method such as NMR spectrometry or a reference standard application. Tentative identification may also be possible with the use of special spectral interpretation software and the knowledge of fundamental reaction chemistry. Unfortunately, the tentative approach cannot be applied in the case of isomers that have almost identical MS^2 fragmentation spectra (EPC and OXC case). According to our results, some metabolites were found in extremely low concentrations that were close to the limit of quantification (LOQ). This may be explained by unsatisfactory recovery values for the metabolites, a low formation rate and/or fast degradation under our experimental conditions. A summary of the recoveries of the metabolites with available standards is presented in Table 3. The metabolite recovery values of the studied compounds were satisfactory and differed as a function of soil type. The lowest extraction efficiency was noticed for RTC, which ranged from 56 to 91% depending on

Table 1
ATE, CAR and MET and their selected metabolites; A1, A2, C1, C2 and C3 are tentatively identified metabolites of ATE and CAR, respectively.

Compound	Formula	Structure	Exact mass (Da)	Measured mass (Da)[a]	Mass error (mDa)	Mass error (ppm)
ATE	$C_{14}H_{22}N_2O_3$		267.1703	267.1700	−0.3	−1.12
AAC	$C_{14}H_{21}NO_4$		268.1543	268.1542	−0.1	−0.37
A1	$C_{13}H_{19}NO_4$		254.1387	254.1388	0.1	0.39
A2	$C_{11}H_{15}NO_4$		226.1074	226.1074	0	0
CAR	$C_{15}H_{12}N_2O$		237.1022	237.1020	−0.2	−0.84
EPC	$C_{15}H_{12}N_2O_2$		253.0972	253.0975	0.3	1.19

(*continued on next page*)

Table 1 (*continued*)

Compound	Formula	Structure	Exact mass (Da)	Measured mass (Da)[a]	Mass error (mDa)	Mass error (ppm)
OXC	$C_{15}H_{12}N_2O_2$		253.0972	–	–	–
DHC	$C_{15}H_{14}N_2O$		239.1179	239.1178	−0.1	−0.42
RTC	$C_{15}H_{14}N_2O_3$		271.1077	No parent	–	–
C1	$C_{13}H_9NO$		196.0757	196.0760	0.3	1.53
C2	$C_{14}H_9NO_2$		224.0706	224.0703	−0.3	−1.34
C3	$C_{15}H_{10}N_2O_3$		267.0764	267.0767	0.3	1.12
MET	$C_{15}H_{25}NO_3$		268.1907	268.1902	−0.5	−1.86
MAC	$C_{14}H_{21}NO_4$		268.1543	268.1542	−0.1	−0.37

[a] compounds were measured as $[M+H]^+$ ions.

Table 2
Matrix effects (suppression/enhancement) for all of the tested soils (values are given in %): Stagnic Chernozem Siltic developed on marlite (X), Haplic Chernozem on loess A (I), Haplic Chernozem on loess B (D), Chernozem Arenic on gravely sand (L), Greyic Phaeozem on loess (C), Haplic Luvisol on loess (S), Haplic Cambisol on orthogneiss A (H), Haplic Cambisol on syenite B (P), Haplic Cambisol on quartzite C (J), Dystric Cambisol on paragneiss (W), and Arenosol Epieutric on sand (E), loess (U), and sand (Q).

Compound	P	J	H	W	X	L	E	I	D	S	Q	U	C
ATE	−5[a]	−4	−3	−10	−14	−6	−4	−1	10	−28	1	12	11
AAC/MAC	−12	2	−8	−11	−19	−6	−6	−4	6	−22	7	3	7
MET	1[b]	5	3	−1	−7	6	4	9	18	−6	9	14	16
CAR	20	6	8	2	2	−1	1	6	16	12	9	14	12
EPC	14	7	5	−6	4	6	1	11	21	16	7	15	26
OXC	−5	−9	−11	−12	−11	−6	−8	−2	10	11	3	8	15
DHC	5	−4	−2	−7	−9	−12	−17	−9	6	1	−3	1	3
RTC	1	−8	−4	−12	−9	−4	−10	−3	−1	−1	−5	−2	0

[a] suppression, value is < 0.
[b] enhancement, value is > 0.

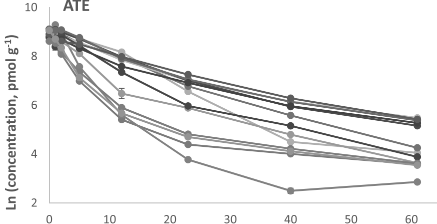
Table 3
Average recoveries (ARF) of analytes with available standards in four soils fortified to 10 and 100 ng g^{-1} (values are given in %). RSD – relative standard deviation.

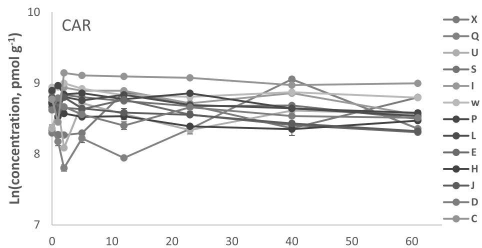
	Soil type							
	P				X			
	10 (n = 5)		100 (n = 5)		10 (n = 5)		100 (n = 5)	
	ARF	RSD	ARF	RSD	ARF	RSD	ARF	RSD
ATE	112	9	131	9	106	3	120	4
AAC/MAC	108	10	131	6	80	7	112	4
MET	110	12	114	11	105	3	108	4
CAR	97	10	130	13	111	10	144	8
EPC	91	10	128	12	85	12	119	9
OXC	83	13	100	10	79	13	88	11
DHC	97	11	134	11	118	15	143	9
RTC	78	16	78	12	67	23	91	13
	Soil type							
	S				Q			
	10 (n=5)		100 (n=5)		10 (n=5)		100 (n=5)	
	ARF	RSD	ARF	RSD	ARF	RSD	ARF	RSD
ATE	97	4	115	4	99	48	107	4
AAC/MAC	90	4	123	4	82	8	108	5
MET	99	10	104	3	94	5	98	5
CAR	96	4	124	6	97	5	115	4
EPC	82	6	133	6	86	4	103	6
OXC	61	4	71	7	81	4	66	3
DHC	106	6	125	6	108	7	102	6
RTC	60	8	75	6	56	5	64	5

the soil. Thus, the absence or low concentration of some metabolites was not induced by the low extraction efficiency of this method.

3.4. Degradation related to soil properties

Differences in the chemical structures of the studied compounds lead to different behaviours in the soils that had different properties (Kodesova et al., 2015; Kodešová et al., 2016). The largest sorption was found for MET, followed by ATE, and CAR for the soils that were used in our experiment (Kodesova et al., 2015). The CAR (a neutral form in the present systems) sorption was mainly controlled by the organic carbon content, whereas MET and ATE (cation forms) sorption, mostly depended on the base cation saturation (Kodesova et al., 2015). Although the sorption affinities to the different soils could be related to particular soil properties, the degradation of each compound is related to the overall soil conditions (Kodešová et al., 2016). ATE exhibited faster degradation in all soils compared to MET and CAR (Fig. 1). ATE was almost completely metabolized by the end exposure time (61 days), whereas MET had the same behaviour for the X, C, D, and I soils that were better quality (soils with well-developed structures, high nutrition contents and associated biological conditions,





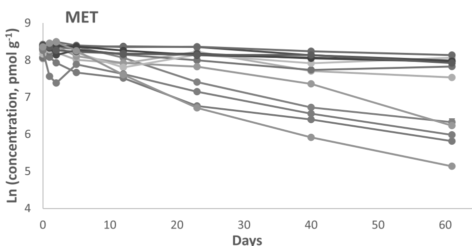


Fig. 1. Degradation of parent compounds (logarithmic scale) in soils: Stagnic Chernozem Siltic developed on marlite (X), Haplic Chernozem on loess A (I), Haplic Chernozem on loess B (D), Chernozem Arenic on gravely sand (L), Greyic Phaeozem on loess (C), Haplic Luvisol on loess (S), Haplic Cambisol on orthogneiss A (H), Haplic Cambisol on syenite B (P), Haplic Cambisol on quartzite C (J), Dystric Cambisol on paragneiss (W), and Arenosol Epieutric on sand (E), loess (U), and sand (Q). Concentrations were expressed in pmol g^{-1}. Error bars are expressed as the difference between duplicates divided by the average value.

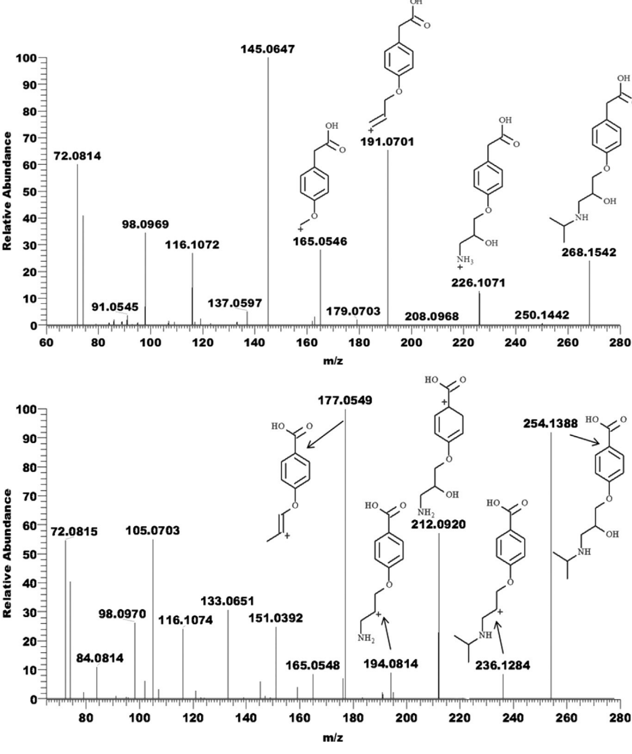


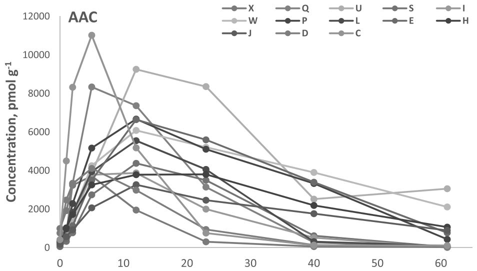
Fig. 2. HRPS spectra of AAC/MAC (upper spectra) and A1 (lower spectra).

such as Chernozems). Thus, there were likely a better microbial activity (Kodešová et al., 2016), and in sand (Q), the reasons for degradation were not obvious (Fig. 1). The CAR concentration did not decrease significantly during the experiment. Taking into account the extended uncertainty of the analysis, it was almost on the border of significance. This is in agreement with another study in which the experiment was performed over 30 days (Salvia et al., 2014). Looking at the concentration trend as a function of time, the CAR behaviour in all of the soils was unclear. The CAR concentrations during the exposure time were almost constant for the L, E, H, J, and D soils, whereas there was a slight concentration decrease observed in the S, I, W, and P soils. There was a high standard deviation between some duplicates of the X, Q, and U soils and an irregular degradation curve shape for the first two soils (Fig. 1).

3.5. Atenolol metabolites

The major ATE metabolite that was identified was AAC. AAC was measured at 268.1542 *m/z* in positive mode. The obtained MS^2 spectrum of this compound showed several fragments that were identical to the ATE product ion spectra (Fig. A.1) and that match spectra measured for the AAC analytical standard. MassFrontier software was used to predict all possible MS^2 products of the compounds of interest. Fragment ions at 98.0967, 116.1071, 133.0646, 145.0644, 162.0910, 178.0859, 190.086, 208.0964, and 225.1227 *m/z* for ATE were identified via accurate mass measurements with errors that were between the measured and theoretical values of 3.06, 0.86, −1.50, −2.76, −1.85, −2.25, −1.58, −1.92, and −3.11 ppm, respectively. The specific AAC fragments that represent the replacement of the amide group with oxygen in AAC were 165.054, 191.0703, and 226.1074, and they were measured with errors of −0.61, 1.05, and 1.33 ppm, respectively (Fig. 2).

AAC was found to be a major atenolol metabolite in sediment systems (Svan et al., 2016). The analysis of the AAC concentration for different degradation times showed that the formation of AAC is followed by its consequent degradation. The largest amount of metabolite was formed in C, U, Q, E, P, W, and L soils, whereas for the rest of the soils, the concentration did not exceed 4000 pmol g^{-1}. AAC was completely degraded in almost all of the soils after 40 days. A slower AAC transformation rate was observed for P and E (soils developed on syenite and sand), whereas the degradation was almost completed by the end of exposure (61 day), and for the W, H, and J soils (i.e., Cambisols),



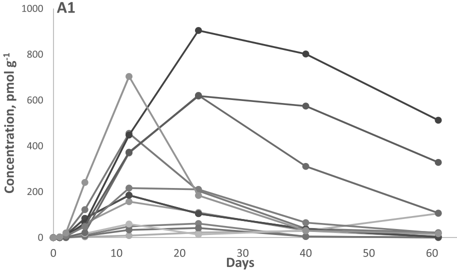


Fig. 3. Kinetics of the atenolol metabolites, AAC and A1, in the following soils: Stagnic Chernozem Siltic developed on marlite (X), Haplic Chernozem on loess A (I), Haplic Chernozem on loess B (D), Chernozem Arenic on gravely sand (L), Greyic Phaeozem on loess (C), Haplic Luvisol on loess (S), Haplic Cambisol on orthogneiss A (H), Haplic Cambisol on syenite B (P), Haplic Cambisol on quartzite C (J), Dystric Cambisol on paragneiss (W), and Arenosol Epieutric on sand (E), loess (U), and sand (Q).

the AAC concentration at the end of the experiment was still relatively high.

A1 was the second metabolite that was identified in all of the soils. Identical lower molecular weight fragments (below 133.0646 *m/z*), as for ATE, were present in the A1 spectra. This was determined due to the specific fragmentation pattern, which included ions at 151.0392, 177.0549, 194.0814, 212.0920, and 236.1284 *m/z*, with mass errors of 1.32, 1.69, 1.03, 1.41, and 1.27 ppm, respectively. These fragments tentatively confirmed the predicted structure of the A1 metabolite (Fig. 2).

All of the soils may be divided into four groups, according to the transformation kinetics, maximum amount formed, and concentration of A1 at the end of the experiment (Fig. 3). The first group contained the H, J, and E soils, where the largest amount of A1 was formed and A1 was not completely degraded until the end of the experiment. An interesting behaviour was observed for the J and E soils (i.e., soils developed on quartzite and quartzite sand, respectively), whereas the transformation kinetics was the same until 23 day, and it significantly changed after this point. The A1 concentration in the J soil was 575 pmol g^{-1}, whereas for E, it was just 311 pmol g^{-1}, where the speed of the degradation increased for the next sampling point (40 days). The C and X soils represent the second group, whereas the smaller amount of A1 was formed quickly and then rapidly degraded during the 40 days of the experiment. The third group that contained the largest number of soils, D, L, P, I, Q, and S, can be characterized with a low amount of A1 that formed (ranging from 42 pmol g^{-1} for S to 211 pmol g^{-1} for D). Almost complete elimination of A1 occurred in the third soil group until the end of the experiment. Additionally, the behaviour (kinetics of transformation) of some soils, such as S, Q and I, with L was quite similar, but there was a small difference in concentration. W and U formed the fourth group in which no degradation pattern for A1 was observed. The concentration was low during all of the exposure times and was almost constant. The behaviour of A1 in the soils of the second, third and fourth group is not related to particular soil types or substrates.

The A2 structure was identified using two specific fragments, 137.0602 (3.65 ppm) and 179.0702 (−0.56 ppm) (Fig. A.2). For this metabolite detection, a problem appeared due to the presence of higher intensity interference that matched the 5 ppm window in the chromatogram. A peak was present in the full scan, but it was lost after MS^2 fragmentation. Using further precursor ion extraction from the total ion content and the 145.0648 *m/z* fragment (identical to the fragment in the ATE HRPS spectra), we were able to detect it. The more intensive peak from the full scan was due to interference. The correct detection of A2 was possible via the MS^2 experiment (Fig. A.3).

We observed 191.0705 *m/z* (2.09 ppm) as a major fragment in the spectra for A2 and AAC. It was observed in only some soil samples. For A2, we did not observe the degradation as a function of time because of the issue mentioned above.

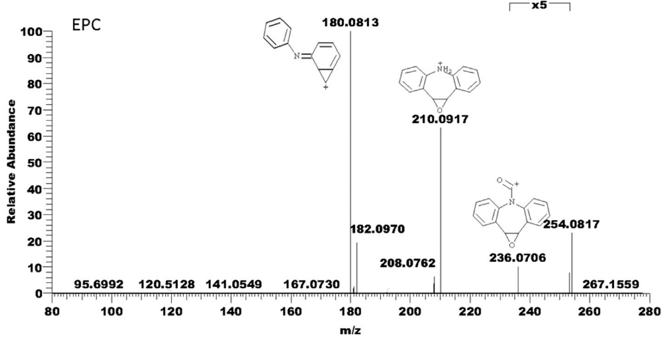
3.6. Carbamazepine metabolites

Although the half-life time of CAR was determined to be 46.2 days for sandy clay loam (Li et al., 2013), the CAR degradation was obviously slower in all of soils in our experiment. We did not expect to achieve a high number of transformation products for this reason. Despite our expectations, six CAR transformation products were identified.

The major CAR metabolite found in our degradation experiments was measured at 253.0975 *m/z*, which was present in all of the soils. There are two possible compounds with a corresponding element composition: EPC and OXC. Both compounds are commercially available as reference standards; thus, the metabolite was subsequently identified as EPC. The MS^2 fragmentation spectrum of EPC is shown in Fig. 4. The specific fragments were measured at 180.0613, 210.0917, and 236.0706 *m/z* with mass errors of 2.78, 1.9, and 0 ppm, respectively.

The EPC behaviour in all of the 13 soils was almost identical; the metabolite was formed in low concentrations. L soil was different compared with the others with an amount of EPC of 681 pmol g^{-1}, which was the highest concentration for this metabolite. EPC exhibited increasing concentrations in all soils until the end of exposure (Fig. 5). Thus, the formation rate of this transformation product was higher than its degradation rate under the studied conditions.

Five more metabolites, RTC, DHC, C1, C2, and C3, were detected and identified in some soil samples. The concentrations of these metabolites were low, and in some samples, the values were lower than the corresponding LOQs. Thus, it was possible to investigate the transformation kinetics for only RTC, C1, and C3, which were present in larger amounts. The specific fragments of RTC were detected at 182.0971, 210.0917, and 254.0815 *m/z* with errors of 3.84, 1.9, and 1.18 ppm, respectively (Fig. 4). In the MS^2 spectra of DHC, C1, C2, and C3 were present, as shown in Appendix A (Fig A.4). The RTC metabolite began to appear later than the previously mentioned metabolites after 5 days, but it was mainly present after 12–23 days from the beginning of the experiment. A slightly higher concentration (57 pmol g^{-1}) of RTC was found in soil E. The TP concentration vs. time was plotted for 8 soils, E, L, H, W, P, S, J, and D (Fig. 5). The C1 metabolite was formed in 6 soils, E, W, H, J, U, and S. (Fig. 6). This metabolite requires a longer formation time, and its



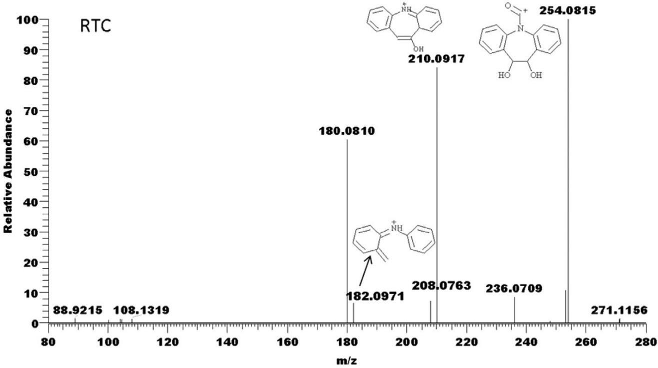


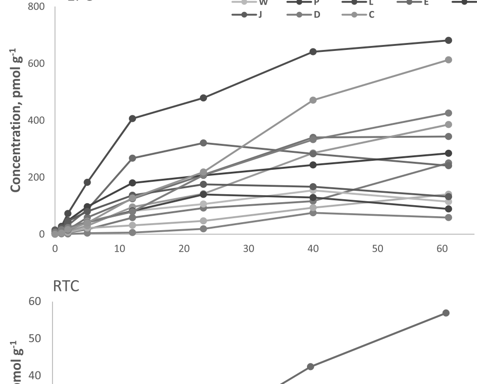
Fig. 4. HRPS spectra of EPC and RTC that were formed from carbamazepine for the mass range 230–255, which was amplified to improve readability.

soil concentration kept increasing in all soils, indicating a faster formation than degradation under the given conditions. All of the soils where C1 was formed were divided into 3 groups according to the maximum formed concentration: (1) E and W (soils developed on sand and paragneiss) represent soils with a high concentration of C1, (2) H and J (Cambisols on orhogneiss and quartzite) are soils with a medium concentration of TP, and (3) U and S (loess and soil developed on loess) have C1 present in low concentrations (<10 pmol g^{-1}) (Fig. 6). C3 was present in more soils, such as in E, H, W, L, J, P, and S. The metabolite was formed in a low amount with the highest concentration being 12 pmol g^{-1}, which was measured for E (Fig. 6).

All of the CAR metabolites were formed in low amounts due to a low degradation rate of the parent compound. Completely different intermediates, with the exception of EPC, were found in a previous study (Li et al., 2013). The transformation products exhibited approximately the same persistency as CAR in our experiment. Assuming a similar sorption to the soil as for the parent compound, we expected a high mobility of all CAR transformation product in the soils. Based on its persistency and transport ability in soils and water, we consequently anticipate that carbamazepine can significantly contribute to soil pollution, e.g., via reclaimed wastewater irrigation (Paltiel et al., 2016).

3.7. *Metoprolol metabolites*

There were two metabolites, AAC/MAC and A1, in the soil extracts, which were the same as for ATE. Previously published data reported the appearance of metoprolol acid, α-hydroxymetoprolol, *O*-desmethyl metoprolol and two tentatively identified transformation products under aerobic degradation in activated sludge (Rubirola et al., 2014). Recently, some additional metabolites were identified in wetland sediments, *N*-de-isopropyl metoprolol and deaminated metoprolol (Svan et al., 2016). All of these specific metabolites were not found for MET in our experimental LC/HRMS data. The AAC/MAC amount was an order of magnitude lower compared with that formed during ATE degradation. This may be explained by the different structures of ATE and MET. Whereas the amido moiety in ATE is hydrolysed by a specific bacterial enzyme to AAC (Fig.A.5) (Helbling et al., 2010; Sathyamoorthy et al., 2013), different enzymes must be involved in the *O*-desmethylation and consequent oxidation of desmethyl MET. Thus, a) the MET degradation pathway is different from those published for sludge/sediments, b) the formed metabolites were impossible to detect under our LC and ionization conditions, and c) the formed metabolites were rapidly transformed into low-mass organic compounds. MAC was present in all of the



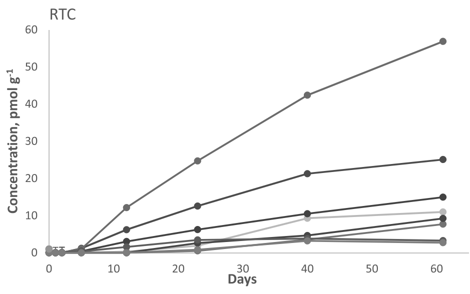
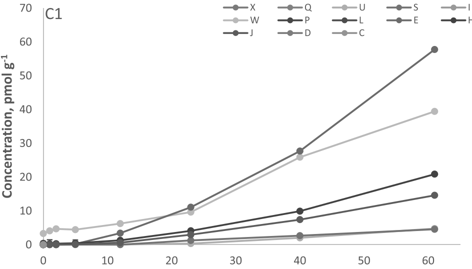


Fig. 5. The occurrence of the carbamazepine metabolites of EPC and RTC in the following soils: Stagnic Chernozem Siltic developed on marlite (X), Haplic Chernozem on loess A (I), Haplic Chernozem on loess B (D), Chernozem Arenic on gravely sand (L), Greyic Phaeozem on loess (C), Haplic Luvisol on loess (S), Haplic Cambisol on orthogneiss A (H), Haplic Cambisol on syenite B (P), Haplic Cambisol on quartzite C (J), Dystric Cambisol on paragneiss (W), and Arenosol Epieutric on sand (E), loess (U), and sand (Q). Error bars are expressed as the difference between duplicates divided by the average value.



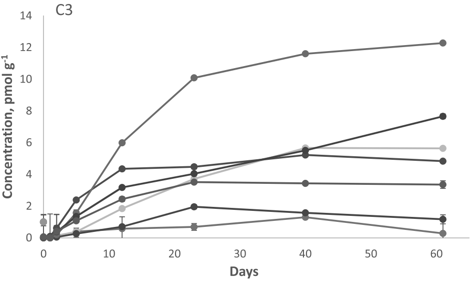


Fig. 6. Kinetics of the carbamazepine metabolites, C1 and C3, in the following soils: Stagnic Chernozem Siltic developed on marlite (X), Haplic Chernozem on loess A (I), Haplic Chernozem on loess B (D), Chernozem Arenic on gravely sand (L), Greyic Phaeozem on loess (C), Haplic Luvisol on loess (S), Haplic Cambisol on orthogneiss A (H), Haplic Cambisol on syenite B (P), Haplic Cambisol on quartzite C (J), Dystric Cambisol on paragneiss (W), and Arenosol Epieutric on sand (E), loess (U), and sand (Q). Error bars are expressed as the difference between duplicates divided by the average value.

studied soils but in a low abundance (Fig. 7). The metabolite was almost completely degraded at the end of exposure with the exception of the loess substrate (U). The maximum concentration of MAC that was formed in the U soil was 82 pmol g^{-1}.

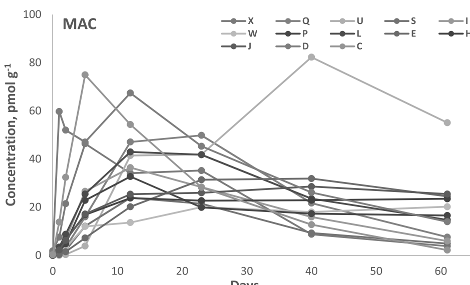


Fig. 7. Occurrence of the metoprol metabolite, MAC, in the following soils: Stagnic Chernozem Siltic developed on marlite (X), Haplic Chernozem on loess A (I), Haplic Chernozem on loess B (D), Chernozem Arenic on gravely sand (L), Greyic Phaeozem on loess (C), Haplic Luvisol on loess (S), Haplic Cambisol on orthogneiss A (H), Haplic Cambisol on syenite B (P), Haplic Cambisol on quartzite C (J), Dystric Cambisol on paragneiss (W), and Arenosol Epieutric on sand (E), loess (U), and sand (Q).

4. Conclusions

ATE and MET degraded relatively quickly compared with CAR. Major metabolites were only observed for ATE and CAR, whereas MET degradation in the soil was unclear. Since the same metabolites were formed in all 13 soils, we can conclude with high probability that the aerobic condition of the experiment is the most important factor for pharmaceutical degradation pathways of studied compounds (ATE, CAR, and MET) in soils. The different amounts/rates of formed products and further transformation product degradation can be attributed to differences in the soil properties.

Although ATE and its transformation product AAC were almost completely degraded in all soils after 61 days, CAR and its metabolites were persistent under the studied conditions. This fact in addition to the low CAR sorption enable the transportation of CAR and its metabolites into ground water and, as a result, may cause adverse effects on the environment and humans.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.07.041.

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CHAPTER 4

DETERMINATION OF PHARMACEUTICALS AND THEIR METABOLITES THAT PARTITION BETWEEN WATER AND SEDIMENTS AS A FURTHER POTENTIAL EXPOSURE FOR AQUATIC ORGANISMS

Koba, O., Grabicova, K., Cerveny, D., Turek, J., Kolarova, J., Randak, T., Zlabek, V., Grabic, R. Determination of pharmaceuticals and their metabolites that partition between water and sediments as a further potential exposure for aquatic organisms. J. Hazard. Mater. 10.1016/j.jhazmat.2017.08.039 (accepted)

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DETERMINATION OF PHARMACEUTICALS AND THEIR METABOLITES THAT PARTITION BETWEEN WATER AND SEDIMENTS AS A FURTHER POTENTIAL EXPOSURE FOR AQUATIC ORGANISMS

Olga Koba*, Katerina Grabicova, Daniel Cerveny, Jan Turek, Jitka Kolarova, Tomas Randak, Vladimir Zlabek, Roman Grabic

University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Zátiší 728/II, 389 25 Vodňany, Czech Republic

**Corresponding author, +420387774715, okoba@frov.jcu.cz, Zátiší 728/II, 389 25 Vodňany, Czech Republic*

Abstract

Although pharmaceuticals are frequently studied contaminants, their fate in the environment is still not completely clear. During one year study, complex approach including water, sediments and fish sampling was used to describe pharmaceuticals and their metabolites (PTMs) behaviour in the environment.

Eighteen pharmaceuticals and seven of their metabolites were determined in the pond, used for tertiary treatment of wastewater effluent. Liquid chromatography tandem mass spectrometry methods were applied to determine PTMs concentrations in all matrices. Seasonal variations in concentrations were evaluated. Contaminants partition between pond compartments was estimated by means of solid water distribution coefficient for sediments (Kd) and bioaccumulation factors (BAF) for fish liver. Kd values were almost stable during a year, that may be a sign of continuous transport of PTMs between water and sediment under experimental conditions. Almost all studied compounds, with exception of sertraline (BAF of 6200), were found to be not bioaccumulative in fish liver.

Pond removal efficiency was calculated for all PTMs and favourable conditions for a natural pharmaceuticals removal were proposed. We can also assume that cyprinid fish farming in ponds, dominated by treated wastewater did not affect growth of fish. Further aspects of pharmaceuticals exposure on fish are needed to be studied.

Key words: *Drugs; wastewater; fish; biological pond; fate; solid water distribution coefficient; bioaccumulation factor*

Introduction

Increasing human life quality requires the development of new chemical compounds, such as pharmaceuticals, personal care products, cleaning agents, and hormones, which may later become organic pollutants. Wastewater treatment plants (WWTPs) were initially designed to remove solid and dissolved organic matter, mainly fats and nutrients; however, currently common WWTPs cannot completely eliminate newer contaminants (Golovko et al., 2014a, Zhang et al., 2015). Treated wastewater (TWW) is discharged into different water bodies, such as channels, pond, rivers, and seas, and reaches the aquatic ecosystem with concentrations of pharmaceuticals and their metabolites (PTMs) that range from ng L^{-1} to μg L^{-1} levels (Aymerich et al., 2016; Grabicova et al., 2015).

Since pharmaceuticals are designed to be biologically active, they may also affect non-target organisms and cause adverse effects (Brodin et al., 2013; Burkina et al., 2015; Steinbach et al., 2016). Consequently, the anti-inflammatory drug diclofenac (DIC) and antibiotics (azithromycin, erythromycin (ERY), and clarithromycin (CLA)) have recently been included on the "watch list" of priority substances under the Water Framework Directive (European Commission, 2015).

Because of their widely variable chemical-physical properties, some pharmaceuticals in water bodies may also accumulate in sediments and aquatic organisms (Grabicova et al., 2015; Svahn and Björklund, 2015).

A complex approach that includes water, sediment, and fish interactions should be studied to better understand the fate of PTMs and their effects on the aquatic environment. A few studies on this question were recently published (Maier et al., 2015; Wagil et al., 2015; Wei et al., 2014), but they showed a limited range of compounds. Some studies intended to follow the fate of pharmaceuticals along the chain of wastewater-TWW-recipient water-sediments (Huber et al., 2016; Maier et al., 2015; Zaibel et al., 2016), but this approach cannot examine all of the processes in the aquatic environment, e.g., the interactions of contaminants with biota.

Thus, the current study only focuses on a specific discharge scenario to describe the fate and transformation processes in three essential compartments. The water, sediment and biota of a biological pond that only receives TTW were used to determine the behaviour of PTMs in a complex frame. Our goal is to cover a wide range of relevant compounds that can enter aquatic organisms and adsorb onto solids.

Materials and methods

Chemicals

Standards of the native pharmaceuticals were purchased from Sigma-Aldrich (Steinheim, Czech Republic). An isotope-labelled internal standard (IS) of carbamazepine (D10) was obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada). Atenolol (D_6) and metoprolol hydrochloride (D7) were purchased from Alsachim (Strasbourg, France). Trimethoprim ($^{13}C_3$) and sulfamethoxazole ($^{13}C_6$) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Clarithromycin-N-methyl (D_3) was obtained from Toronto Research Center (North York, Canada). Methamphetamine (D_6), venlafaxine (D_6), and tramadol (D_3) were purchased from Lipomed AG (Arlesheim, Switzerland).

The standards of all metabolites were purchased from Labicom (Olomouc, Czech Republic). A stock solution of each pharmaceutical was prepared in methanol at a concentration of 1 mg ml^{-1}. A spiking mixture of each was prepared by diluting the stock solution with

methanol to a final concentration of 1 μg ml^{-1}. All of the stock and spiking solutions were stored at -20 °C. LC-MS grade acetonitrile (LiChrosolv Hypergrade) was obtained from Merck (Darmstadt, Germany). Formic acid was purchased from Labicom (Olomouc, Czech Republic) to acidify the mobile phases. Ultrapure water was prepared via an Aqua-MAX-Ultra System (Younglin, Kyounggi-do, Korea).

Experimental setup

Cezarka pond was selected as the experimental ecosystem. Cezarka pond (2.6 ha) is a biological pond that was designed to retain and treat effluent from Vodnany WWTP, Czech Republic. Thus, all of the inflow water of the pond consists of only TWW and natural precipitations. This WWTP involves primary mechanical filtration, sedimentation and activated sludge treatment. Cezarka is the tertiary treatment of the WWTP processes and the first pond in a cascade of aquaculture ponds that are connected to the Blanice River. This arrangement is often used for the tertiary treatment of effluent wastewater in the Czech Republic.

Sampling was performed in April, May (only water (2 samplings) and fish), July, August (only water (2 samplings)), September (only water), October and February (only water and fish), March (only water and sediments), and April (2 samplings of water) during 2015–2016. Sampling was designed to cover a wide range of possible contaminants according to seasonal changes in consumption during a year.

Twelve points (Fig. A.1, marked in black colour as numbers with stars) were selected for the initial sediment sampling, when the pond was not filled with water. Three sites (inflow-V1, middle-V2, and outflow-V3) were used in the other cases to collect water and sediment (Fig. A.1). One-thousand tagged Common carps (0.066 ± 0.016 kg) (*Cyprinus carpio* L.) were stocked in the pond in April 2015. Twelve fish were caught during each sampling campaign.

Sample preparation

The water samples were filtered using syringe filters (0.45 μm, regenerated cellulose, Labicom, Olomouc, Czech Republic) into 10-ml glass vials, frozen and kept at -18 °C until analysis. The thawed samples were spiked with ISs and analysed using an LC-LC/MS^2 instrument.

The sediment samples were collected using a standard Ekman-Birk sampler, placed in 100-ml plastic bottles and stored in a freezer at -18 °C. Then, the thawed sediment samples were centrifuged (Refrigerated Centrifuge 4-16 K, Sigma, Osterode am Harz, Germany) with 13131 RCF for 10 min and extracted via ultrasonic (DT 255, Bandelin electronic, Sonorex digitec, Berlin, Germany)-based solvent extraction. Finally, the dry weight of the sediments was evaluated (Samples dryer, BMT Medical Technology, Brno, Czech Republic).

Fish were caught by electrofishing and transported to the lab, where they were sacrificed and liver samples from 12 fish were collected during each sampling campaign and kept at -18 °C. The experimental animals were handled according to national and institutional guidelines for the protection of human subjects and animal welfare (European Parliament and Council, 2010).

Analytical procedures

A hybrid quadrupole/orbital trap Q-Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) (biota analysis) and a TSQ Quantiva triple-stage quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) (water and sediment analysis), both of which were coupled with Accela 1250 LC and Accela 600 LC pumps (Thermo Fisher

Scientific, San Jose, CA, USA) and HTS XT-CTC autosamplers (CTC Analytics AG, Zwingen, Switzerland), were used to separate and detect our target analytes. An analytical Hypersil GOLD aQ column (50 mm length, 2.1 mm i.d, 5-µm particles; Thermo Fisher Scientific) (biota analysis) and Accucore aQ column (50-mm length, 2.1 mm i.d, 2.6-µm particles; Thermo Fisher Scientific) were used to chromatographically separate the target analytes.

The water analysis method was validated for all compounds of interest. Heated electrospray ionization in positive mode was used to ionize the target compounds. The spray voltage was set as 3.5 kV. Nitrogen (purity >99.99%) was used as the sheath (42 arbitrary units), auxiliary (12 arbitrary units) and sweep (1 arbitrary unit) gases. Argon was used as the collision gas at a pressure of 2 mTorr. The vaporizer and capillary temperatures were set to 338 °C. Chromatographic separation of all compounds was provided with acidified by formic acid acetonitrile/ultrapure water gradient, as presented in Table A.1. The water sample analysis method was adopted from a previous publication (Lindberg et al., 2014) with a different mass spectrometer. The method shows good linearity (R2 = 0.98–1.0) over the concentration range of 1–1000 ng L^{-1}. The trueness of the method for the pond water samples was 68–130% (unpublished).

The sediment extracts were analysed with the LC-MS2 method. The method is described in detail in another study(Golovko et al., 2016).

The modified method was used to extract fish liver tissues with a mixture of acidified acetonitrile and isopropanol (3:1 v/v with 0.1% formic acid), which was found to be the most suitable mixture according to a validation study (Grabicova et al., 2015). The limit of the quantification (LOQ) values for all of the matrices are shown in the Appendix (Table A. 2).

Results and discussion

Occurrence of pharmaceuticals in the pond

In total, eighteen pharmaceuticals (DIC, tramadol (TRA), atenolol (ATE), irbesartan (IRB), metoprolol (MET), cetirizine (CET), fexofenadine (FEX), meclozine (MEC), CLA, clindamycin (CLI), ERY, sulfamethoxazole (SMX), carbamazepine (CAR), oxcarbamazepine (OXC), citalopram (CIT), methamphetamine (MTP), sertraline (SER), and venlafaxine (VEN)) and seven of their metabolites/transformation products (metoprolol/atenolol acid (MAC), clindamycin sulfoxide (CSO), N4-acetyl sulfamethoxazole (N4AS), 10,11-epoxycarbamazepine (EPC), trans-10,11-dihydro-10,11-dihydroxy carbamazepine (RTC), N-desmethylcitalopram (NDC), and O-desmethylvenlafaxine (ODV)) were detected and monitored in different matrices of the pond during the year. These pharmaceuticals belong to different therapeutic classes, such as analgesics/anti-inflammatory, antihypertensive/cardiovascular, antihistamine, antibiotics, psychoactive and illicit drugs (Table A.3).

Water samples

The PTM concentrations for each sampling campaign are presented as the average of all samples per month. In total, 22 PTMs were found to be above the LOQ in the pond water. Most PTMs were determined in all samples, except N4AS, CLA, OXC, and SER (Table A.4). The occurrence of N4AS, CLA, OXC, and SER may be affected by seasonal consumption (CLA; (Golovko et al., 2014b)), high sorption to sediments (SER; (Hörsing et al., 2011)) or low prescription and significant removal in WWTP (OXC; (Kaiser et al., 2014), N4AS; (Zhang et al., 2015), respectively). The concentrations of several metabolites (MAC, RTC, and EPC) and parent compounds (CAR and CET) were almost stable over the year of investigation, with

a relative standard deviation below 30%. This fact may indicate the high persistence of some compounds in the aquatic environment, as in the case of CAR (Calza et al., 2013).

The concentrations of all of the studied compounds were compared with previously published data (Table A.4). The PTM levels were notably similar, except that IRB was found to be up to 2 orders of magnitude higher in our study. This difference may be the result of the higher consumption rate or different removal efficiency of the local WWTP.

The seasonal variation in the concentration of different drugs was also investigated in this study (Fig. 1). The total month average concentration of each therapeutic group was calculated for the pond inflow. These concentrations represent the dependence on consumption of different groups and their removal efficiency during the wastewater treatment since the removal efficiency is shown to vary in different seasons (Golovko et al., 2014a,b). For example, antibiotics were present at higher concentration during February-April 2016 because of their higher consumption during the cold seasons of the year and insufficient removal by WWTP during this period (Golovko et al., 2014b).

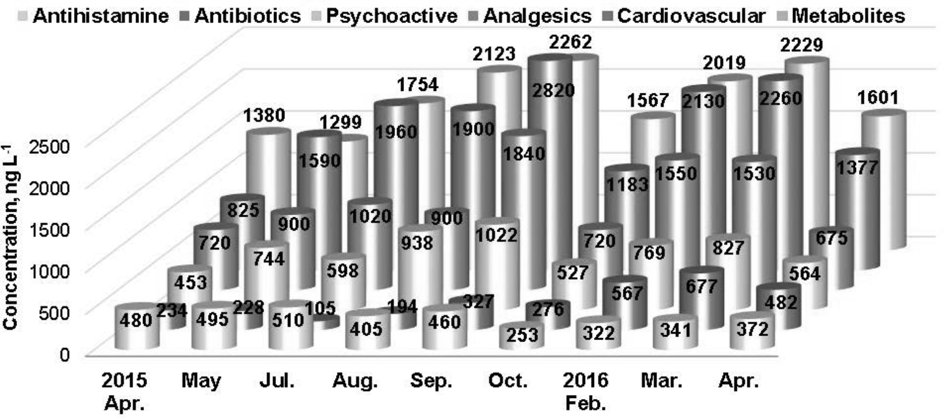

Figure 1. *Seasonal variation of the concentrations of different pharmaceutical groups detected in pond inflow .*

Sediments samples

Sixteen PTMs were found in the pond sediment samples (Table A.5). The monthly concentration was obtained as the average value of the V1, V2, and V3 pond sites, except the April 2015 (n=12) value, which was the initial pond bottom sampling. The initial sampling, when the pond was not filled with water, helped us to evaluate the sorption potential of each target analyte. We assume that compounds with higher sediment concentrations should have a higher affinity to sediments, since no water was present in the pond. Based on the measured concentration, the pharmaceuticals are ordered as follows: ODV > CIT > CLA > NDC > CET > MEC > MET > VEN > TRA > SER > FEX > IRB > CLI > DIC > MTP > CAR (Fig. A.2).

Several compounds were present in all of the measured samples, such as MET, CET, MEC, CLI, CIT, NDC, SER, VEN, and ODV (Table A.5). MEC is the only compound that was only found in the sediment samples and not in fish, despite its high bioconcentration potential because the Log P (Kow) value was 5.87 (Table A.3). The concentration of most PTMs significantly decreased after filling the pond with TWW, but MET, CLI, CAR, SER, and ODV showed low variations in concentration during the year, with a relative standard deviation below 30% (Table A.5). The decreasing tendency of the PTM concentrations in the sediments after pond refill may indicate

the transport of these compounds between liquid and solid phases. Unfortunately, limited up-to-date information is available regarding the levels of the studied compounds in the sediment compared with water matrices. A concentration level comparison with previously published studies is shown in Table A.5.

Solid-water distribution coefficient calculation

The solid-water distribution coefficient (Kd) is an important parameter for evaluating the fate and mobility of PTMs in water-sediment systems. Kd highly depends on the properties of solid particles, such as sewage sludge and sediments (Al-Khazrajy and Boxall, 2016; Hörsing et al., 2011). Thus, the distribution coefficient can vary with different surrounding conditions and may be used to evaluate the sorption/desorption processes in aquatic environments.

In our study, Kd was calculated as the ratio of the measured concentrations in the sediment and water samples for the same sampling points (July, October 2015; March, April 2016). A detailed analysis of the average Log Kd values proves its high stability during the year with low relative standard deviations (Table 1), which indicates a continuous partitioning between water and sediments in the pond during the year. This dynamic equilibrium may explain the reduction in PTM concentrations in the sediments after the pond refill with water, as described above.

To our knowledge, few studies have reported Kd values, but only for a limited number of compounds, which makes comparison with our results difficult. Kd was calculated as a concentration ratio between sediments and water in the published studies, where the water and sediments concentrations were simultaneously monitored (Table 1).

Our experimental Kd values were similar to previously published data for some compounds. Some exceptions were observed, e.g., the published Kd values of TRA, CET, CIT, SER, and VEN were lower than those in our study, whereas the CLA distribution coefficients were higher in the published studies.

Table 1. *Comparison of the Log Kd values calculated from the pond water and sediments in 2015–2016 with previously reported data.*

Compound	**Log Kd**						
	2015		**2016**		**Average**	**RSD**	**Reference**
	Jul.	**Oct.**	**Mar.**	**Apr.**			
DIC	1.26	1.09	1.24	1.48	1.27	13%	0.84–2.03 (Agunbiade and Moodley, 2016) 1.58–2.72 (Zhou and Broodbank, 2014) -0.7–0.15 (Dobor et al., 2012) 1.56–2.48 a (Osorio et al., 2016) 0.68–0.74 (Styszko, 2016)
TRA	1.62	1.63	1.92	2.04	1.80	12%	0.38–0.89 (Stein et al., 2008)
IRB	1.07	1.02	1.30	1.34	1.18	14%	0.84–2.67 a (Osorio et al., 2016)
MET	2.55	2.61	2.54	3.12	2.70	10%	0.22–2.57 a (Osorio et al., 2016)
CET	1.99	2.46	2.49	2.31	2.31	10%	1.42–2 a (Aminot et al., 2015)
FEX	1.91	2.47	2.64	2.69	2.43	15%	–
CLA	3.07	2.15	2.78	2.72	2.68	14%	3.65–4.87 a (Osorio et al., 2016)
CLI	2.44	2.39	2.44	2.54	2.45	3%	–
CAR	1.38	1.32	1.39	1.44	1.38	4%	0.23–1.09 (Stein et al., 2008) 1–1.3 (Williams et al., 2009) 0.46–0.74 (Styszko, 2016)
CIT	3.67	3.28	3.40	4.01	3.59	9%	1.37–2.58 a (Osorio et al., 2016)
NDC	3.52	3.42	3.49	3.87	3.57	6%	–
MTP	2.25	2.66	n.c.	2.87	2.59	12%	–
SER	4.04	3.95	3.67	4.50	4.04	9%	1.97–3.04 a (Osorio et al., 2016)
VEN	2.66	2.64	2.64	3.10	2.76	8%	1.21–2.19 a (Osorio et al., 2016)
ODV	2.81	2.80	2.63	2.88	2.78	4%	–

a – the value was calculated based on published results as Kd=Cs/Cw, where Cs is the concentration determined in sediments and Cw is the concentration determined in water samples;

n.c. – the value was not calculated; Cs was below the LOQ.

Fish liver samples

Common carp exposure to TWW did not affect fish growth since a fast increase in body weight of the fish from 0.066 ± 0.016 kg to 2.0 ± 0.1 kg was observed during the year of exposure (Fig. A.3). Eleven PTMs were found in the fish liver samples (Table A.6). The highest individual concentrations were observed for SER (61 ng g^{-1}), MAC (26 ng g^{-1}), NDC (24 ng g^{-1}), and CIT (22 ng g^{-1}), which indicates a higher tendency to accumulate.

Limited information is available on the PTM concentrations in fish collected from areas that are affected by discharges from WWTPs. For example, several PTMs, such as VEN (1.2 ng g^{-1}), NDC (2.84 ng g^{-1}), CIT (2.9 ng g^{-1}), and SER (3.83 ng g^{-1}), were present in the full-body homogenate of fathead minnows (*Pimephales promelas*) that were exposed in cages approximately 10 m below the discharge point for 2 weeks (Metcalfe et al., 2010). Grabicova et al. found psychoactive compounds in liver of rainbow trout (*Oncorhynchus mykiss*) that were exposed to an effluent for 13 days: CIT (12 ng g^{-1}), SER (4.5 ng g^{-1}) and VEN (21 ng g^{-1}) (Grabicova et al., 2014). These measured values are similar to the measured concentrations in

our study (May 2015, Table A.6). SER, at maximum value of 105 ng g^{-1}, was also found in the liver of common carp (*Cyprinus carpio*) from an effluent-dominated river that received direct discharge from wastewater treatment facilities in Phoenix, Arizona, USA (Ramirez et al., 2009). SER in the range of 75–110 ng g^{-1} and CAR in the range of < LOQ-1.1 ng g^{-1} were detected in the liver tissues of brown trout (*Salmo trutta*) collected downstream from East Canyon Creek, Park City, UT, USA (Du et al., 2012). These reported values for SER are much higher than those in our study, whereas the CAR concentration level was notably similar (Table A.6).

Bioaccumulation factor calculation

Bioconcentration is a process of chemical-compound accumulation in biota from a contaminated aquatic environment. In our study, the environment is a pond that was only fed with TWW. Because fish were exposed in the real condition, we cannot exclude the uptake of detected PTMs from other sources, such as food. Thus, the bioaccumulation factor (BAF), but not bioconcentration, was used in this study. The BAF was calculated as the ratio of the contaminant concentration detected in the fish liver to the concentration in the surrounding environment for eleven PTMs (Table 2). Eight pharmaceuticals (CET, CAR, CLI, ODV, MET, TRA, MAC, and VEN) exhibited low BAFs under the current experimental conditions. The order is shown with an increasing average BAF value (Table 2). CIT and its metabolite NDC were reportedly more significant in terms of bioaccumulation, but the values could not reach the BAF threshold of 2000 (European Chemicals Agency, 2016). SER bioaccumulation in the fish liver was the highest under the current conditions. An average SER BAF of 6200 was obtained, which is three times higher than the set limit for a compound to be bioaccumulative. However, there were large differences between cold and warm periods, which can be attributed to the changes in the feeding activity of carp. Based on the evidence of pharmaceutical accumulation in benthic organisms (Grabicova et al., 2015), which are an important part of the carp diet, we assume that for PTMs with a higher BAF, such as SER, CIT, and NDC, the bioaccumulation process includes uptake from water and food, whereas the remaining compounds represent only the steady-state bioconcentration, which is a direct uptake from water.

One additional point must be included to clarify PTM bioaccumulation in living organisms. Some compounds enter an animal body only via direct uptake, whereas others can be formed by in vivo metabolism, which has been shown in the case of rainbow trout (*Oncorhynchus mykiss*) exposed to diltiazem (Koba et al., 2016). Since a vast majority of decomposition of different contaminants occurs in the liver, it is impossible to suggest which amount of metabolites originates from the external environment and which is formed inside the organism.

Since the BAFs depend on the animal species and their specific biology, different environmental conditions, and other factors (de Solla et al., 2016; Miguel-Queralt and Hammond, 2008; Nakamura et al., 2008), any comparison with other published BAFs is pointless.

Despite the low BAF values, with the exception of SER, additional parameters should be considered to examine the effect of PTMs on aquatic organisms. Exposure to industrial and hospital effluent has been reported to induce geno-, cytotoxicity and oxidative stress in the gill, liver and brain of common carp (*C. carpio* L.) (Neri-Cruz et al., 2015; SanJuan-Reyes et al., 2015), etc.

Table 2. *BAFs for the selected compounds in common carp liver.*

Compound	2015			2016		Average
	May	Jul.	Oct.	Feb.	Apr.	
CAR	1.2	0.8	1.1	2.2	1.4	1.3
CET	1.4	0.41	0.62	0.38	0.64	0.69
CIT	510	870	140	170	710	480
CLI	2.6	2.8	4.9	2.0	3.8	3.2
MET	1.4	5.8	8.1	2.3	11	5.7
MAC	7.3	13	11	16	13	12
NDC	460	1100	340	250	1700	770
ODV	1.9	4.2	5.6	1.3	3.7	3.3
TRA	3.2	7.7	2.6	8	16	7.5
SER	4700	11000	850	430	> 14000a	6200
VEN	22	28	17	15	51	27

a – a minimum possible BAF; since SER was not detected in the water, the LOQ value was used for calculation.

Principal component analysis (PCA)

It is difficult to find relationship and time trends among the concentrations of the studied compounds in all matrices. Therefore, principal component analysis (PCA) was applied to highlight the variation and elucidate the strong patterns in the dataset. STATISTICA software was used for the PCA. The first four factors represent most correlations.

The two most significant factors contribute approximately 70% to the total score. Factor 1 (44.3%) shows a high negative correlation (based on their coordinate value) to the PTM concentration in water, whereas Factor 2 (24.5%) is correlated with the PTM concentration in sediments (Fig. 2.A). Unfortunately, almost no correlation was found for the PTM concentration in fish and Log P(Kow) values, except the concentration in fish liver in October and February shows a moderately negative correlation to Factor 1 (Fig. 2.A). However, PCA helped us divide all of the PTMs into several groups based on their presence in the studied matrices (Fig. 2.B).

The positions of SER, NDC, CIT, VEN and TRA in the PCA plot of Factor 3 vs. 4 correspond to the variability of BAF with the feeding activity, whereas the opposite position of MAC is difficult to explain (Figs. 2.C–D). Nevertheless, the relatively high concentration of MAC in fish appears to reflect a lower steady state with a high water concentration of MAC and MET (as parent compounds). All of the other compounds are scattered around the centre with no apparent trend.

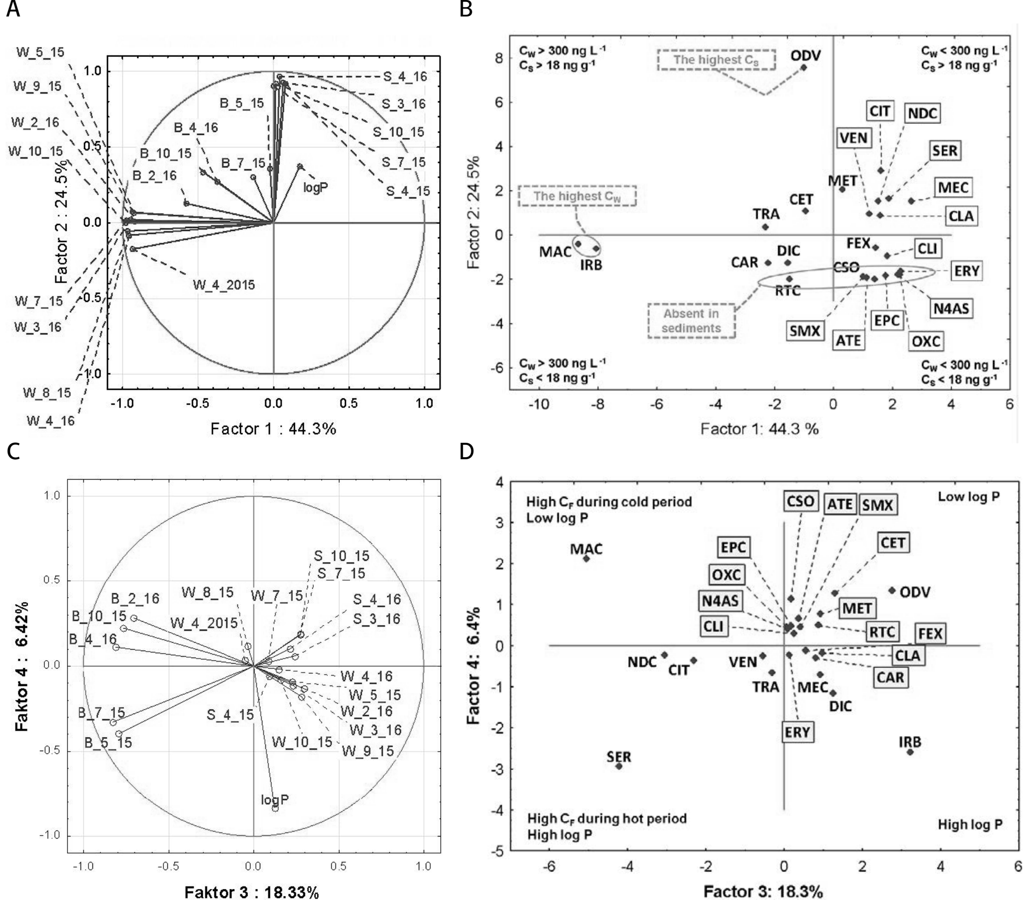

Figure 2. *Scatter plot of the loading factors (A, C) based the PTM concentrations in three matrices and the Log P values and score scatterplot (B, D) of the PTMs for principal component selection; CW, S, F are the concentrations in water, sediment and fish, respectively.*

Pond removal efficiency (PRE)

As previously mentioned, Cezarka pond is used as an additional treatment of effluent from the local WWTP. This pond may be considered to be a natural treatment system, where different processes occur, such as photodegradation, interaction between water and the atmosphere, sedimentation processes, and biodegradation. An experimental design for pond water sampling enabled us to evaluate the changes in the concentration of the inflow (V1) and the outflow (V3) of the pond. Using this assumption, the PRE values were calculated for all of the target analytes (Table A.7).

Based on our data, we selected compounds with a high average removal efficiency (> 50%) during a year, such as DIC (51%), OXC (55%), CLA (56%), MET (59%), NDC (59%), VEN (62%), CIT (70%) and SER (89%); the PRE values are presented in brackets. A negative year-average PRE was reported for several compounds, including N4AS (-37%), SMX (-12%), CET (-7%), MAC (-5%), and EPC (-1%) (Table A.7).

Almost all of the studied compounds were effectively removed (more than 90%) from July to August, except that some metabolites showed a negative or notably low removal efficiency

(Table A.7). This finding may be explained by the highest metabolic activity of bacteria and high uptake to plants. Many compounds showed decreasing PRE values during sampling in October (Table A.7). This time corresponds to the post-growing season and decay of aquatic plants. Degraded plants may return some compounds to the pond water.

Based on our observation and hydro meteorological data (http://portal.chmi.cz), we can suggest favourable conditions for the natural treating system, such as an air temperature of 20–25 °C, water temperature of 15–23 °C, and sunshine duration 250–300 h $month^{-1}$. The pond should also have a sufficient amount of silty sediments and a corresponding ecosystem that is rich in vegetation and aquatic organisms, such as fish, invertebrates and microorganisms.

Conclusions

Eighteen different pharmaceuticals and seven of their metabolites were found in a TWW-affected pond in a one-year experiment. The seasonal variations were evaluated in three matrices: water, sediments and fish liver. Several pharmaceuticals were characterized as having a high affinity to the pond sediments. However, partitioning between sediments and water occurred for most target pharmaceuticals. Thus, the sediment represents a secondary source of these compounds in water when the hydrological conditions are changed.

In addition, the fish were exposed to the pond conditions during the experimental time. The BAFs for fish liver tissue were obtained for all seasons of the year. Almost all of the studied compounds except SER (BAF of 6200) were not bioaccumulative under the current conditions.

Based on our results, we assume that cyprinid fish farming in TWW-dominated ponds does not affect the growth of fish. Further aspects of pharmaceutical exposure on fish, such as changes in behaviour and reproduction as well as histopathological alterations must be studied.

In addition, the PRE values for all of the PTMs were calculated, and favourable conditions for natural pharmaceutical removal were proposed. Regarding the pharmaceuticals, the pond ecosystem is convenient for use as an additional treatment of municipal wastewater effluents. A high removal efficiency was found for most of the target compounds, particularly in summer.

Acknowledgements

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Appendix A. Supplementary material

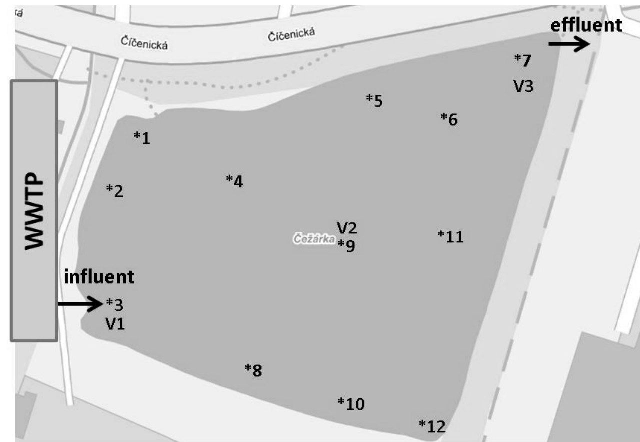


Figure A.1. *Map for sediments and water sample sites collection.*

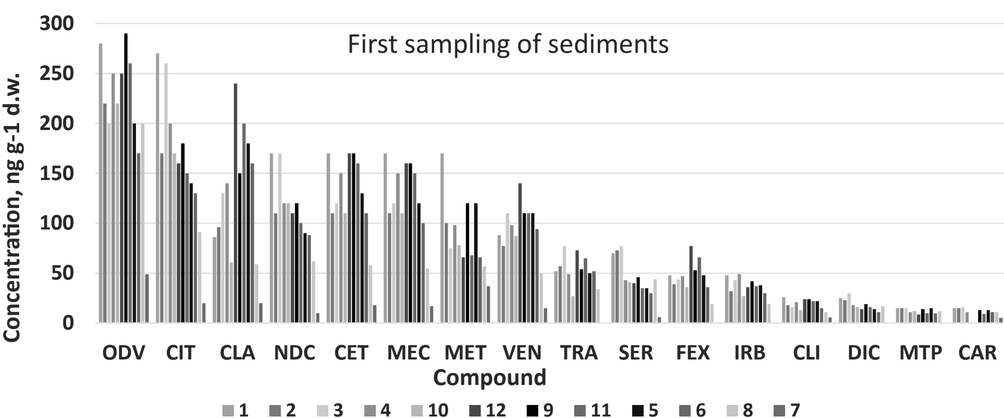
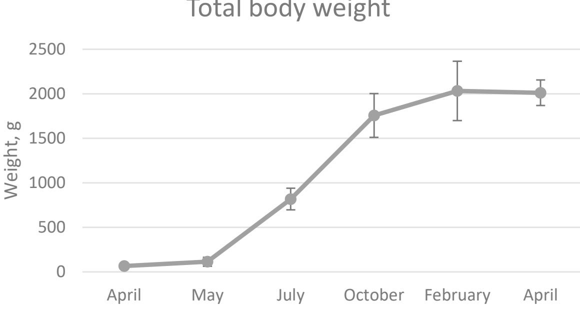


Figure A.2. *Concentrations of all target analytes detected in sediment samples during first sampling event, numbers represent locations.*



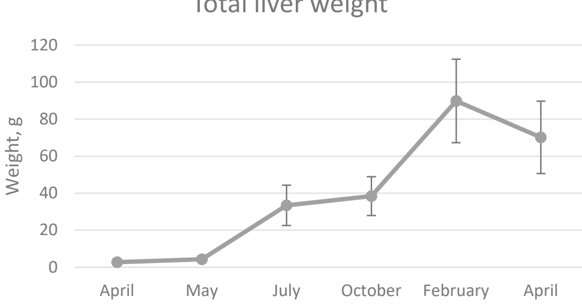


Figure A.3. *Experimental fish body parameters.*

Table A.1. *Gradient for the elution of target compounds.*
Pump 1

Time (min)	Mobile phase composition/vol. %		Flow rate, (µL min^{-1})
	Ultrapure water*	Acetonitrile*	
0.00	95	5	300
1.00	95	5	350
3.00	75	25	400
8.00	0	100	400
10.00	0	100	400
10.01	95	5	350
13.00	95	5	350

Pump 2

Time (min)	Mobile phase composition/vol. %		Flow rate, (μL min^{-1})
	Ultrapure water*	Acetonitrile*	
0.00	100	0	1100
1.05	100	0	1100
1.07	0	100	100
9.00	0	100	100
10.00	100	0	1500
10.01	100	0	1000
13.00	100	0	1000

*Constituents acidified with 0.1 vol. % of FA.

Table A.2. *LOQs for all studied matrices: pond water [ng L^{-1}], sediments [ng g^{-1}, dry weight], and fish liver [ng g^{-1}, wet weight].*

Compound	Water			Sediments			Fish liver		
	Average	Min	Max	Average	Min	Max	Average	Min	Max
DIC	2.5	2.2	3	4.6	1.6	8.9	8.4	4.2	14
TRA	0.53	0.43	0.64	8.4	4.1	17	0.31	0.16	0.45
ATE	0.18	0.12	0.25	3.8	2.1	8	0.022	0.011	0.036
IRB	0.48	0.41	0.57	6.3	2.2	12	0.11	0.054	0.18
MET	0.41	0.35	0.49	5	3.1	9	0.011	0.007	0.016
MAC	0.19	0.12	0.27	4.2	2.6	7.5	0.26	0.13	0.43
CET	5.1	4.3	6	5.7	1.9	11	0.049	0.024	0.079
FEX	5.1	4.5	6.1	5.9	2	11	0.28	0.14	0.46
MEC	0.57	0.49	0.68	5.9	2	11	0.12	0.059	0.19
CLA	5	4.5	5.5	5.9	3.1	11	9.8	7.2	13
CLI	0.73	0.65	0.8	4.3	2.3	7.4	0.032	0.022	0.045
CSO	0.64	0.57	0.7	4.2	2.2	7.1	0.13	0.087	0.18
ERY	0.15	0.14	0.17	7.2	3.8	12	0.35	0.24	0.49
SMX	0.2	0.17	0.23	4.5	2.3	6.4	0.39	0.17	0.63
N4AS	0.59	0.51	0.7	3.5	1.8	5	13	5.5	21
CAR	0.73	0.64	0.87	6.3	2.1	12	0.24	0.12	0.39
EPC	0.43	0.37	0.52	6.4	2.2	12	0.82	0.41	1.3
OXC	0.49	0.43	0.59	5.8	2	11	0.06	0.03	0.099
RTC	0.34	0.29	0.4	5.7	1.9	11	0.86	0.43	1.4
CIT	0.42	0.37	0.5	5.9	2	11	0.19	0.097	0.32
NDC	0.4	0.35	0.48	6.6	2.2	13	0.23	0.12	0.38
MTP	0.62	0.53	0.69	5.2	2.7	9.9	0.35	0.19	0.55
SER	0.24	0.21	0.29	6	2	11	0.075	0.038	0.12
VEN	0.45	0.37	0.54	14	6.7	30	0.15	0.088	0.2
ODV	0.51	0.42	0.62	14	7.1	33	0.13	0.077	0.18

Table A.3. *Compound list*

Compounds	Structure [a]	Formula [a]	Log P (Kow)
Analgesics/anti-inflammatory drug			
Diclofenac (DIC)		$C_{14}H_{11}Cl_2NO_2$	4.02 [b]
Tramadol (TRA)		$C_{16}H_{25}NO_2$	3.01 [b]
Anti-hypertensive/cardiovascular drugs			
Atenolol (ATE)		$C_{14}H_{22}N_2O_3$	-0.03 [b] 0.16 [c]
Irbesartan (IRB)		$C_{25}H_{28}N_6O$	5.31 [b]
Metoprolol (MET)		$C_{15}H_{25}NO_3$	1.69 [b] 1.88 [c]
Metoprolol acid (MAC)		$C_{14}H_{21}NO_4$	-2.34 [b]

Antihistamine

Cetirizine (CET)	$C_{21}H_{25}ClN_2O_3$	-0.61 [b] 1.70 [c]
Fexofenadine (FEX)	$C_{32}H_{39}NO_4$	2.94 [e]
Meclozine (MEC)	$C_{25}H_{27}ClN_2$	5.87 [b]

Antibiotics

Clarithromycin (CLA)	$C_{38}H_{69}NO_{13}$	3.16[d]
Clindamycin (CLI)	$C_{18}H_{33}ClN_2O_5S$	2.01 [b] 2.16[c]
Clindamycin sulfoxide (CSO)	$C_{18}H_{34}Cl_2N_2O_6S$	-0.98 [e]

Erythromycin (ERY)		$C_{37}H_{67}NO_{13}$	3.06 [d]
Sulfamethoxazole (SMX)		$C_{10}H_{11}N_3O_3S$	0.48[b] 0.89[c]
N4-Acetyl sulfamethoxazole (N4AS)		$C_{12}H_{13}N_3O_4S$	1.21[b]
Psychoactive and illicit drugs			
Carbamazepine (CAR)		$C_{15}H_{12}N_2O$	2.25 [b]
Carbamazepine 10,11-epoxide (EPC)		$C_{15}H_{12}N_2O_2$	0.95 [b] 1.97 [e]
Oxcarbazepine (OXC)		$C_{15}H_{12}N_2O_2$	1.11 b
trans-10,11-Dihydro-10,11-dihydroxy carbamazepine (RTC)		$C_{15}H_{14}N_2O_3$	-0.21 b 0.81 e
Citalopram (CIT)		$C_{20}H_{21}FN_2O$	3.74 [b]
N-Desmethyl citalopram (NDC)		$C_{19}H_{19}FN_2O$	3.53 [b]
Methamphetamine (MTP)		$C_{10}H_{15}N$	2.22 [b] 2.07 [c]

Sertraline (SER)		$C_{17}H_{17}Cl_2N$	5.29 [b]
Venlafaxine (VEN)		$C_{17}H_{27}NO_2$	3.28 [b]
O-Desmethyl venlafaxine (ODV)		$C_{16}H_{25}NO_2$	2.72 [b]

a – http://www.chemspider.com
b– predicted, KOWWIN v1.67 estimate, http://www.chemspider.com (EPI Suite KowWin program (http://www.epa.gov/oppt/exposure/pubs/episuitedl.htm))
c – experimental, (Hansch et al., 1995)
d – experimental, (McFarland et al., 1997)
e – predicted, http://www.chemicalize.org

Table A.4.

PTMs concentrations [ng L^{-1}] measured in the pond water during 2015-2016 years and previously published.

	2015						2016			Min	Max	DF[b]	Reference
Compound	**Apr.**	**May**	**Jul.**	**Aug.**	**Sep.**	**Oct.**	**Feb.**	**Mar.**	**Apr.**				
Analgesics/anti-inflammatory drug													
DIC	510 ± 7.1	190 ± 140	250 ± 190	190 ± 170	400 ± 42	310 ± 17	660 ± 150	570 ± 230	290 ± 59	22	870	35/35	94 (Grabicova et al., 2015) 4–39 (Zaibel et al., 2016) < LOQ-324 (Petrovic et al., 2014) 20-330 (Gracia-Lor et al., 2012)
TRA	230 ± 17	580 ± 53	480 ± 200	250 ± 210	490 ± 440	420 ± 15	400 ± 280	440 ± 210	210 ± 110	71	970	36/36	230 (Grabicova et al., 2015)
Anti-hypertensive/cardiovascular drugs													
ATE	82 ± 11	130 ± 29	63 ± 63	57 ± 79	78 ± 83	62 ± 1.2	230 ± 51	230 ± 67	120 ± 47	0.9	310	36/36	95 (Grabicova et al., 2015)
IRB	640 ± 25	970 ± 120	860 ± 660	710 ± 620	1200 ± 840	950 ± 35	1200 ± 150	1200 ± 290	950 ± 140	100	2100	36/36	79 (Grabicova et al., 2015) 15(Petrovic et al., 2014) 57 (Gracia-Lor et al., 2012)
MET	74 ± 12	340 ± 43	240 ± 100	130 ± 150	240 ± 270	170 ± 5.8	180 ± 220	210 ± 210	60 ± 58	12	550	36/36	210 (Grabicova et al., 2015)
MAC	910 ± 5.8	620 ± 150	830 ± 95	920 ± 66	640 ± 170	730 ± 50	730 ± 46	830 ± 140	790 ± 90	480	990	36/36	c
Antihistamine													
CET	330 ± 5.8	350 ± 9.8	410 ± 10	320 ± 59	270 ± 61	210 ± 26	230 ± 15	240 ± 29	320 ± 42	190	420	36/36	9 (Kosonen and Kronberg, 2009)
FEX	160 ± 5.8	160 ± 9.8	110 ± 10	110 ± 46	85 ± 31	61 ± 3.8	65 ± 7	60 ± 9.6	47 ± 4.5	41	180	36/36	11 (Kosonen and Kronberg, 2009) 15 (Grabicova et al., 2015)
Antibiotics													
CLA	110 ± 12	79 ± 37	11 ± 3.6	47 ± 64	71 ± 84	78 ± 17	140 ± 91	200 ± 150	98 ± 38	8.9	370	31/36	120 (Grabicova et al., 2015) < LOQ–616 (Petrovic et al., 2014)
CLI	38 ± 1	33 ± 2.5	58 ± 8	55 ± 22	33 ± 6.7	57 ± 6.7	71 ± 10	62 ± 3.6	55 ± 5	26	99	36/36	1.2–16.5 (Wu et al., 2014)
CSO	55 ± 2.1	43 ± 6.9	93 ± 41	140 ± 130	41 ± 17	87 ± 7.8	160 ± 5.8	100 ± 6.4	61 ± 15	30	410	36/36	c
ERY	22 ± 2.3	26 ± 5.6	8.6 ± 2.3	8.4 ± 7.3	9.1 ± 10	8.1 ± 1	21 ± 12	35 ± 9.5	16 ± 4.2	1.3	46	36/36	23 (Grabicova et al., 2015) 292 (Petrovic et al., 2014) 808 (Wu et al., 2014)

	2015						2016			Min	Max	DF[b]	Reference
Compound	**Apr.**	**May**	**Jul.**	**Aug.**	**Sep.**	**Oct.**	**Feb.**	**Mar.**	**Apr.**				
SMX	73 ± 4.2	58 ± 6.4	33 ± 9.5	45 ± 9.2	120 ± 23	130 ± 15	200 ± 5.8	200 ± 0	280 ± 31	22	340	36/36	32 (Grabicova et al., 2015) 19 (Petrovic et al., 2014) 4.4–14.5 (Wu et al., 2014)
N4AS	19 ± 4.6	11 ± 2.2	6.6 ± 3.3	9.6 ± 3.4	8.3 ± 2.6	7.2 ± 2.4	43 ± 5.3	54 ± 2.6	36 ± 8	4.3	56	35/36	c
Psychoactive pharmaceuticals and illicit drugs													
CAR	370 ± 5.8	420 ± 24	350 ± 30	440 ± 90	430 ± 96	390 ± 12	430 ± 30	390 ± 10	440 ± 19	290	560	36/36	n.d.–2 (Wu et al., 2014) < LOQ–35.5 (Petrovic et al., 2014) 110 (Grabicova et al., 2015) 3–176 (Gracia-Lor et al., 2012) 65–889 (Kaiser et al., 2014) 278–1500 (Zaibel et al., 2016)
EPC	47 ± 1.5	46 ± 3.3	59 ± 9.3	59 ± 9.5	63 ± 6.2	58 ± 2.5	67 ± 5.2	57 ± 0.58	62 ± 3.4	42	71	36/36	< LOQ–932 (Petrovic et al., 2014)
OXC	< LOQ	12 ± 3.4	12 ± 10	17 ± 7.2	28 ± 18	15 ± 1.5	16 ± 10	16 ± 11	6.9 ± 2.3	1.5	40	28/36	< LOQ–234 (Kaiser et al., 2014)
RTC	290 ± 0	290 ± 12	360 ± 47	370 ± 63	340 ± 50	330 ± 5.8	400 ± 25	370 ± 35	370 ± 21	270	490	36/36	131–2320 (Kaiser et al., 2014)
CIT	11 ± 1.8	18 ± 17	8 ± 5.3	22 ± 33	33 ± 52	22 ± 6.7	18 ± 26	32 ± 43	8.4 ± 6.7	2.2	93	36/36	< LOQ–96 (Schlüsener et al., 2015) 29 (Grabicova et al., 2015) 2.59 (Huber et al., 2016)
NDC	7.1 ± 1.1	12 ± 6.2	6.1 ± 2.6	9.1 ± 11	12 ± 16	9.5 ± 2.3	10 ± 10	14 ± 12	5.8 ± 3.3	0.77	31	36/36	78 (Schlüsener et al., 2015) 110 (Metcalfe et al., 2010)
MTP	21 ± 2.5	120 ± 23	56 ± 7.1	17 ± 17	31 ± 34	14 ± 1.5	25 ± 30	53 ± 59	9 ± 8	1.3	140	36/36	58.2 (Li et al., 2016)
SER	< LOQ	2.4 ± 0.93	1.9a	6 ± 4.4	8.5a	2.8 ± 1.4	2.8a	8.4a	1.2 ± 0.07	1.1	9.1	16/36	5(Grabicova et al., 2015) < LOQ–1.72 (de Solla et al., 2016)
VEN	44 ± 12	130 ± 31	87 ± 43	150 ± 220	120 ± 140	84 ± 5.6	75 ± 82	93 ± 84	32 ± 28	8.9	580	36/36	74 (Grabicova et al., 2015) 28 (Petrovic et al., 2014)
ODV	53 ± 17	310 ± 58	280 ± 160	210 ± 220	440 ± 390	320 ± 32	520 ± 97	470 ± 95	280 ± 69	15	870	36/36	38 (Aymerich et al., 2016) < LOQ–400 (Schlüsener et al., 2015)

a – rest of values were below LOQ;
b – determination frequency, amount of samples > LOQ/total sample number;
c – no data was found in the published literature.

Table A.5. *Concentration range [ng g^{-1}, dry weight] of PTMs expressed as year mean ± SD in pond sediments during 2015–2016 years.*

Compound	2015			2016		Min	Max	DF[b]	Reference
	Apr.	**Jul.**	**Oct.**	**Mar.**	**Apr.**				
Analgesics/anti-inflammatory drug									
DIC	25 ± 7.8	4.6 ± 0.14	3.8 ± 1.3	10 ± 5.9	8.7 ± 3.2	2.6	30	22/24	1.04 (Huber et al., 2016) < LOQ–3.36(da Silva et al., 2011) 7.6 (Golovko et al., 2016) 9.5 (Azzouz and Ballesteros, 2012) 262 (Darwano et al., 2014)
TRA	66 ± 16	20 ± 2.1	18 ± 9.9	37 ± 25	23 ± 7	11	77	20/24	57 (Golovko et al., 2016)
Anti-hypertensive/cardiovascular drugs									
IRB	43 ± 0.71	10 ± 2.1	10 ± 3.6	24 ± 17	21 ± 2.6	4.7	49	23/24	0.05–1.63 (Osorio et al., 2016)
MET	77 ± 42	85 ± 23	70 ± 15	72 ± 14	79 ± 14	37	170	24/24	0.04–0.6 (Osorio et al., 2016) 62.8 (Huber et al., 2016) 83 (Golovko et al., 2016)
Antihistamine									
CET	100 ± 77	40 ± 25	61 ± 31	74 ± 32	66 ± 20	18	170	24/24	–
FEX	49 ± 6.4	9 ± 7.3	18 ± 12	26 ± 12	23 ± 23	2.7	77	23/24	12 (Golovko et al., 2016)
MEC	99 ± 74	38 ± 24	58 ± 31	72 ± 33	62 ± 20	17	170	24/24	–
Antibiotics									
CLA	100 ± 70	13 ± 5	11 ± 0.71	120 ± 7.1	51 ± 12	6.8	240	22/24	15 (Golovko et al., 2016)
CLI	15 ± 9.1	16 ± 6.9	14 ± 0.58	17 ± 4.6	19 ± 4.4	5.8	26	24/24	4.9 (Golovko et al., 2016)
Psychoactive and illicit drugs									
CAR	11 ± 5.6	8.4 ± 1.3	8.1 ± 0.76	9.5 ± 1.8	12 ± 2.9	5.1	16	22/24	2.84 (da Silva et al., 2011) 11 (Golovko et al., 2016) 20 (Zhou and Broodbank, 2014) 32 (Azzouz and Ballesteros, 2012)
CIT	150 ± 120	37 ± 27	42 ± 22	80 ± 39	86 ± 30	18	270	24/24	0.23–7.79 (Osorio et al., 2016) 44.2 (Huber et al., 2016) 100 (Golovko et al., 2016)
NDC	100 ± 82	20 ± 15	25 ± 13	43 ± 22	43 ± 18	9.7	170	24/24	–
MTP	14.5 ± 0.71	10 ± 3.4	6.4 ± 2.7	< LOQ	6.6 ± 0.85	4.2	15	19/24	–
SER	43 ± 36	21 ± 11	25 ± 11	39 ± 31	38 ± 27	6	77	24/24	1.15–119.28(Osorio et al., 2016)
VEN	78 ± 5528	40 ± 23	37 ± 17	41 ± 28	40 ± 8.4	14	140	24/24	0.05–1.94 (Osorio et al., 2016) 49 (Golovko et al., 2016) 73.6 (Huber et al., 2016)
ODV	180 ± 120	180 ± 55	200 ± 40	200 ± 74	210 ± 35	49	290	24/24	–

a rest of values were below LOQ; b – determination frequency, samples > LOQ/all measured samples

Table A.6. *Summary of measured concentration [ng g^{-1}, wet weight] of PTMs in fish liver samples.*

Compound	**2015**			**2016**		**Min**	**Max**	**DF**[a]
	May	**Jul.**	**Oct.**	**Feb.**	**Apr.**			
Analgesics/anti-inflammatory drug								
TRA	1.9 ± 0.32	3.7 ± 2	1.1 ± 0.76	3.2 ± 1	3.6 ± 1.1	0.51	9.3	60/60
Anti-hypertensive/cardiovascular drugs								
MET	0.5 ± 0.34	1.4 ± 3.4	1.4 ± 2.6	0.42 ± 0.23	0.65 ± 1	0.11	11	42/60
MAC	3.8 ± 1.1	11 ± 7.4	8.1 ± 4.3	12 ± 5.2	11 ± 6.4	0.7	26	60/60
Antihistamine								
CET	0.48 ± 0.25	0.17 ± 0.1	0.13 ± 0.059	0.089 ± 0.043	0.18 ± 0.12	0.043	0.95	52/60
Antibiotics								
CLI	0.083 ± 0.038	0.16 ± 0.083	0.28 ± 0.42	0.14 ± 0.078	0.2 ± 0.14	0.031	1.4	47/60
Psychoactive and illicit drugs								
CAR	0.47 ± 0.12	0.28 ± 0.054	0.41 ± 0.14	0.95 ± 0.29	0.6 ± 0.22	0.18	1.6	44/60
CIT	4.6 ± 1.6	6.9 ± 5.1	3 ± 2.1	3.1 ± 2.9	5.1 ± 5.5	0.68	22	60/60
NDC	3.8 ± 1.4	6.4 ± 5.1	3.2 ± 6.9	2.6 ± 1.4	9 ± 5.6	0.34	24	59/60
SER	9.9 ± 3.6	20 ± 17	2.4 ± 0.98	1.2 ± 0.48	3.3 ± 2.6	0.45	61	60/60
VEN	2.6 ± 0.74	2.4 ± 0.81	1.4 ± 0.57	1.1 ± 0.43	1.8 ± 0.71	0.47	3.9	60/60
ODV	0.63 ± 0.33	1.2 ± 0.71	1.8 ± 1.4	0.69 ± 0.49	1.1 ± 0.73	0.12	5.2	54/60

a – determination frequency, amount of positive samples/total amount.

Table A.7. *Pond removal efficiency values.*

	Sampling events												PREa
	27.04.15	5.05.15	11.05.15	9.07.15	5.08.15	19.08.15	24.09.15	8.10.15	23.02.16	10.03.16	4.04.16	18.04.16	
DIC	n.d.b	52%	8%	89%	95%	89%	92%	-10%	35%	52%	28%	30%	51%
TRA	0%	20%	-9%	60%	83%	83%	88%	-2%	69%	57%	66%	52%	47%
ATE	15%	37%	0%	97%	98%	100%	94%	0%	34%	39%	30%	34%	48%
IRB	8%	25%	0%	87%	94%	90%	80%	-4%	21%	33%	27%	20%	40%
MET	26%	21%	-6%	61%	93%	91%	91%	-6%	89%	83%	93%	73%	59%
MAC	1%	-51%	-20%	-22%	-4%	14%	35%	6%	-9%	25%	-13%	-17%	-5%
CET	-3%	-6%	-3%	-5%	-13%	-82%	29%	-26%	8%	19%	3%	0%	-7%
FEX	0%	0%	-14%	9%	46%	62%	49%	-2%	19%	25%	0%	15%	17%
CLA	1%	48%	25%	36%	100%	100%	100%	16%	68%	70%	61%	45%	56%
CLI	-5%	-3%	-17%	14%	65%	6%	-50%	6%	22%	-8%	-24%	2%	1%
CSO	-8%	26%	9%	63%	86%	19%	50%	-13%	0%	9%	-6%	14%	21%
ERY	0%	35%	4%	41%	93%	83%	92%	6%	59%	37%	41%	35%	44%
SMX	11%	-15%	0%	-73%	-75%	0%	27%	-18%	5%	0%	0%	0%	-12%
N4AS	-20%	0%	-58%	n.d.	-165%	18%	-86%	-87%	-22%	7%	-2%	10%	-37%
EPC	-2%	-14%	-19%	26%	13%	-30%	14%	-9%	13%	-2%	9%	-5%	-1%
RTC	0%	-4%	-11%	23%	27%	-17%	26%	-3%	12%	17%	10%	-3%	6%
OXC	n.d.	45%	-50%	93%	100%	100%	100%	-7%	64%	69%	42%	44%	55%
CIT	14%	75%	25%	60%	93%	96%	98%	31%	94%	92%	87%	74%	70%
NDC	7%	54%	1%	54%	93%	91%	95%	24%	80%	78%	66%	61%	59%
MTP	10%	35%	-8%	-10%	50%	59%	84%	-7%	88%	88%	83%	79%	46%
SER	n.d.	100%	48%	100%	100%	100%	100%	37%	100%	100%	n.d.	100%	89%
VEN	39%	37%	15%	66%	98%	89%	95%	-5%	85%	79%	86%	62%	62%
ODV	22%	32%	18%	74%	97%	87%	89%	-20%	30%	29%	39%	19%	43%

a – pond removal efficiency, $PRE = \frac{Concentration(V_1) - Concentration(v2)}{Concentration(V_1)}, \%$

b – compound was not detected at V1 and V3 sites.

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CHAPTER 5

GENERAL DISCUSSION
ENGLISH SUMMARY
CZECH SUMMARY
ACKNOWLEDGMENTS
LIST OF PUBLICATIONS
TRAINING AND SUPERVISION PLAN DURING THE STUDY
CURRICULUM VITAE

GENERAL DISCUSSION

In the first part of this dissertation (Chapter 2), we aimed to investigate metabolism and effect of diltiazem, a calcium channel blocker drug, on a non-target organism, rainbow trout (*Oncorhynchus mykiss* L.). To our knowledge, past researches were dedicated to metabolism of this drug in different mammal species and humans, but nothing is known about poikilothermic organisms e.g. fish (Homsy W, 1995; Sutton et al., 1997; Yan et al., 2013). For this reason, juvenile rainbow trout were exposed for 21 and 42 days to three nominal concentrations of diltiazem: 0.03 μg L^{-1}, 3 μg L^{-1}, and 30 μg L^{-1} (Steinbach et al., 2016b). Fish exposed to the longest period and highest concentration, which corresponded to the predictable critical environmental concentration, was used to discover diltiazem effect and metabolism, since drug has a quite low Log P value of 2.7 and it does not accumulate much in fish tissue (Fick et al., 2010; Steinbach et al., 2016b). HRMS and spectral interpretation software (MassFrontier) were used to detect and tentative identify diltiazem metabolites in the fish tissues, such as blood plasma, muscles, liver and kidney (Koba et al., 2016b). As result, 17 phase I diltiazem metabolites were identified in all fish tissues. Since these tentatively identified metabolites were not available on market, a semi-quantification approach was applied, where parent compound response factor was used for calculation of metabolite concentrations. These data helped to evaluate metabolites distribution in fish tissues and propose a metabolic pathway for their formation (Koba et al., 2016b). Some isomeric forms identification was impossible, this was the only disadvantage of using HRMS, for example, in case of all hydroxy diltiazem metabolites. In addition, it was shown that diltiazem causes adverse effects on the biochemical profile of the blood and histopathological alteration in liver, heart and kidneys. An elevated creatine kinase level was present in exposed fish as the important indicator of muscular tissue damage. The calculated bioconcentration factor in fish can be characterized as relatively low (with the highest values of 194 found in the kidneys) (Steinbach et al., 2016a,b). Moreover, our study proved that diltiazem may be metabolized in a different way compared to mammals by non-target organisms, since a specific the CYP2D sub-family is not present in fish (Kirischian et al., 2011).

The second part of the thesis (Chapter 3) is related to using HRMS in the investigation the fate of pharmaceuticals in the soil environment. Potential usage of sludge and TWW for fertilizing agriculture fields and irrigation and continuous field contamination through animals and manure land application are among the main sources of soil contamination by pharmaceuticals (Del Re et al., 2007; Grossberger et al., 2014). Thirteen different soil types representing the majority of soils in the Czech Republic, in combination with six pharmaceuticals, such as atenolol, metoprolol, carbamazepine, clindamycin, sulfamethoxazole, and trimethoprim were used in this research. These pharmaceuticals were selected based on their frequent use in the human and veterinary medicine and on their presence in wastewater and sludge. The soil samples were air-dried, ground, passed through a 2-mm sieve and spiked with each individual pharmaceuticals to reach a concentration of 2 μg g^{-1} per dry soil. Samples were incubated in darkness at a constant temperature of 20 °C for 1, 2, 5, 12, 23, 40 and 61 days. After extraction, soil samples were analysed using LC/HRMS methods. Eventually, 15 metabolites were tentatively identified (Koba et al., 2017; Koba et al., 2016a). Moreover, identity of 6 metabolites were confirmed by commercially available analytical standards. Analysis of degradation kinetics of parent drugs exhibited high persistence of carbamazepine in all soils, whereas atenolol was shown to be almost completely eliminated during 61 days (Koba et al., 2016a). Rest of pharmaceuticals were transformed differently depending on the soil type (Koba et al., 2017, 2016a). In addition to parent compounds, formation and degradation kinetics of metabolites were determined. We created a useful parameter, such as C-Integral

that helped to determine a formation level of each metabolite. This parameter was calculated as total area under each metabolite kinetic curve normalized by parent compound amount from which the metabolite originated (Koba et al., 2017). To the best of our knowledge, this is the first study on such a large number of pharmaceutical metabolites and TPs fate in the soil environment. Based on our data, we demonstrated that pharmaceuticals can be transformed in the soils. Some of their metabolites/TPs were shown to be notably persistent. Almost identical metabolite profile was observed in all soils with exception of formed amount that, probably, was related to different soils properties and microbial community, which enhances or suppress metabolites formation. Unfortunately, microbial community composition was not analyzed and we cannot comment that the same major metabolites were determined in all studied samples. Since most of the compounds in our study did not completely degrade in the soils, it could have a serious implication for environment and humans, such as contamination of ground water and plants, due to transport from contaminated soils that can consequently cause a chronic exposure to pharmaceutical and their metabolites/TPs (Malchi et al., 2014; Paltiel et al., 2016; Radovic et al., 2015).

In the Chapter 4, a combination of HRMS and a new generation of QqQ was applied to clarify interactions of pharmaceuticals and their metabolites/TPs within an actual aquatic environment, represented by a pond that is supplied exclusively by TWW. HRMS was used to analyse biological samples (fish liver) to avoid interferences in the signal because of complexity of the matrix, whereas new generation QqQ was used to detect all contaminants in the water and sediments due to improved detection limits compare to old device. Occurrence and fate of 18 pharmaceuticals and 7 of their metabolites are presented in this study. We mainly focused on transport of pharmaceuticals between water, sediments and fish. Pond water, sediments and fish liver samples were collected several times during one year period and concentrations of selected pharmaceuticals and their metabolites/TPs were determined. In this study, solid water distribution coefficients (Kd) and bioaccumulation factors (BAF) were calculated, as quantitative indicators of contaminant transport between all studied pond compartments. Our investigation showed that Kd values were almost stable during the one year observation, which may indicate a continuous partition between water and sediments under experimental conditions. Seasonal variations in BAFs, with higher values in spring and summer, suggests that pharmaceutical uptake from food can play an important role for more lipophilic compounds, such as sertraline, citalopram, and N-desmethyl citalopram. It was previously shown that benthic organisms, a main common carp diet, are also able to accumulate pharmaceuticals (Grabicova et al., 2015). A high content of metoprolol acid in the fish liver tissues is more likely corresponds to its formation from a parent drug, metoprolol in a fish liver than metoprolol acid accumulation from surrounding environment due to low Log P value of -2.34 and absence in liver of another parent drug with similar properties (atenolol, Log P – 0.03). Since pond is used for the tertiary treatment of effluent wastewater, we also evaluated the removal efficiency for all compound of interest. Unique experimental design enabled to evaluate differences in concentration for all pharmaceuticals and their metabolites/TPs between inflow and outflow of the pond. Vast majority of the compound were effectively removed during summer, whereas several compounds, such as DIC (51%), OXC (55%), CLA (56%), MET (59%), NDC (59%), VEN (62%), CIT (70%) and SER (89%) were shown to be successfully eliminated during the year, average removal efficiency value is presented in brackets.

The same types of biological ponds are also used for cyprinid fish farming in Czech Republic. Based on our results, we can assume that cyprinid fish farming in ponds, effected by TWW is quite safe in terms of pharmaceutical bioconcentration, since muscles, used for human consumption, usually contain much lower amount of contaminants compare with liver tissue (Grabicova et al., 2014; Koba et al., 2016b). However, further aspects of pharmaceutical

exposure to fish, such as changes in the behaviour, reproduction, histopathological alteration remain unexplored.

Conclusions

There is limited amount of information available on fate of a wide range of compounds, especially metabolites, in the different compartments of the environment. Advanced analytical instruments, such as a hybrid quadrupole/orbital trap Q-Exactive and TSQ Quantiva triple-stage quadrupole mass spectrometers can be successfully used to fulfill such knowledge gaps as it is presented in this thesis. The application of HRMS brought a new insight into in vivo metabolisation of pharmaceuticals in fish as non-target organism; behavior of pharmaceuticals and their metabolites in the soil environment with their further implementation for the environment; transport among water, sediments and fish in the aquatic environment, efficiency of pharmaceuticals removal by natural treating system and interesting aspects of pharmaceuticals and their metabolites/TPs bioaccumulation.

As it was shown in this work, advanced instrumentation and specific software are helpful and necessary tools that enable to exploit large scale experiments that had a significant impact on improvement of understanding contaminates interactions with the different environmental compartments.

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ENGLISH SUMMARY

Application of advanced instrumentation for analysis of environmental pollutants

Increasing life comfort and safety in the human population requires development and application of new chemical substances for health and personal care. These pharmaceuticals and personal care products (PPCPs) are released in wastewater after use. Unfortunately, they cannot be completely removed by WWTPs. Thus, these compounds, their metabolites and TPs formed during human/animals/bacteria metabolism, wastewater treatment and natural environmental processes contaminate all the environmental compartments.

Appearance of advanced analytical instrumentation and techniques enable research on the fate of emerging environmental contaminants. This thesis was focused on application of high resolution mass spectrometer (HRMS) and new generation of triple quadrupole for analysis of the environmental pollutants. All studies were mainly devoted to such a group of contaminants as pharmaceuticals because of their high consumption, frequent occurrence in all environmental matrices and potential to cause adverse effects for non-targeted organism due to their biological activity.

The first part of the thesis is related to method development for identification and quantification of metabolites in the different tissues of rainbow trout exposed to diltiazem. As a result, 17 diltiazem metabolites were tentatively identified by HRMS and spectral interpretation software. The complex metabolic pathway in metabolic phase I for diltiazem and tissue specific bioconcentration in fish were proposed in the study.

The second part of the work is mainly focused on investigation of pharmaceuticals fate in the soil environment. The transformation of several pharmaceuticals, such as atenolol, metoprolol, carbamazepine, sulfamethoxazole, trimethoprim, and clindamycin in 13 different soils was studied under laboratory conditions. The parent compounds were analyzed in all studied soils and dissipation curves were plotted. Finally, 15 known and new metabolites were detected and identified. Moreover, identity of 6 of them was confirmed by available analytical standards. It was shown that almost the same metabolite profile was observed in all soils, but formed amount and degradation rate of metabolites were soil-type dependent. Several parent compounds and metabolites were shown to be persistent in the soils. Consequently, pharmaceuticals can be transported via ground water to plants. Further fate of pharmaceuticals must be studied as it can have adverse effects on crop consumers.

The third part of the dissertation deals with an important issue of pharmaceutical transport between different aquatic environmental compartments. Water, sediments and fish from a pond affected with TWW from a local WWTP were analyzed within this project; 18 pharmaceuticals and 7 their metabolites were determined in all studied matrices. Solid water distribution coefficients for sediments and bioaccumulation factors for fish liver were obtained under real conditions. Seasonal variations in all parameters were investigated for a one-year period. This study helps to extend a limited available information on fate of wide range of compound, especially metabolites in the aquatic environment affected by treated wastewater.

CZECH SUMMARY

Aplikace pokročilých přístrojových technik pro analýzu polutantů životního prostředí

Zvyšování kvality života velké části populace vede k vývoji a používání stále nových chemických látek. Důležitou skupinou těchto sloučenin jsou farmaka a prostředky denní péče. Značná část z nich je po aplikaci vyloučena do odpadní vody, ze které však nejsou úplně odstraněny v čistírnách odpadních vod. Tyto sloučeniny, jejich metabolity i transformační produkty pak znečišťují životní prostředí.

Komerční dostupnost pokročilé analytické techniky umožnila v poslední době studium osudu nových typů znečišťujících látek v prostředí. Tato doktorská práce je zaměřena na aplikaci vysokorozlišující hmotností spektrometrie (HRMS) a nové generace trojitých kvadrupólů na analýzu těchto nových skupin polutantů. Všechny prezentované práce jsou zaměřeny na léčiva vzhledem k jejich vysoké spotřebě ve společnosti, časté přítomnosti ve všech složkách prostředí a potenciálu negativně působit na necílové organizmy.

První část práce popisuje vývoj metody pro identifikaci a kvantifikaci metabolitů vznikajících v orgánech pstruhů duhových exponovaných kardiovaskulárnímu léčivu diltiazem. Celkem bylo identifikováno 17 metabolitů, byla navržena komplexní metabolická dráha pro fázi I metabolizmu a také byla vyhodnocena jejich biokoncentrace v jednotlivých orgánech.

Druhá část práce se zabývá výzkumem osudu farmak v půdním prostředí. V laboratorních podmínkách jsme studovali transformaci šesti léčiv (atenololu, metoprololu, karbamazepinu, sulfamethoxazolu, trimethoprimu a clindamycinu). Z analýz mateřských sloučenin jsme získali rychlosti odstranění těchto látek z půdy. Dále jsme detekovali a identifikovali 15 známých i nových metabolitů. Identita šesti z nich byla potvrzena srovnáním s dostupnými analytickými standardy. Ukázali jsme, že profil metabolitů byl téměř stejný ve všech půdách, ale množství vzniklých metabolitů a také rychlost jejich transformace závisela na typu půdy. Některá studovaná léčiva i jejich metabolity jsou v půdě perzistentní, což umožňuje jejich následný transport do podzemní vody a plodin, které se na dané půdě pěstují. Z důvodů možných nežádoucích efektů na konzumenty takových plodin je nutné sledovat další osud jak farmak, tak i jejich transformačních produktů.

Třetí část disertační práce se zabývá důležitou oblastí transportu farmak a jejich metabolitů mezi složkami vodního prostředí. V rámci tohoto projektu byly analyzovány vzorky vody, sedimentů a ryb z rybníka napájeného pouze vyčištěnou odpadní vodou. Ve studovaných matricích jsme nalezli 18 léčiv a 7 jejich transformačních produktů. Z těchto dat jsme vypočítali distribuční konstanty v reálných podmínkách – distribuční koeficienty pro vodu a sediment, a bioakumulační faktory pro rybí játra. Sledovali jsme sezónní změny v průběhu jednoho roku. Tato studie pomohla zvýšit znalost (zatím pouze omezenou) o osudu širokého spektra farmak a zejména pak jejich metabolitů v reálném systému ovlivněném odpadní vodou.

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TRAINING AND SUPERVISION PLAN DURING STUDY

Name	Olga Koba	
Research department	2013–2017 – Laboratory of Enviromental Chemistry and Biochemistry of FFPW	
Daily supervisor	Assoc. Prof. Roman Grabic	
Supervisor	Assoc. Prof. Roman Grabic	
Period	3rd October 2013 until 30th September 2017	

Ph.D. courses	**Year**
Pond aquaculture	2013
Applied hydrobiology	2013
Basic of scientific communication	2014
Ichthyology and fish taxonomy	2015
English language	2015
Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2014 2015 2016 2017
International conferences	**Year**
Koba, O., Golovko, O., Fedorova, G., Randak, T., Grabic, R., 2014. Application of a Q-exactive hybrid high-resolution mass spectrometr for identification and semiquantification of pharmaceuticals biodegradation products in soil and sludge. In: European Chemistry Congress – 5th EuCheMS, August 31 – September 4, 2014, Turkey, Istanbul. p. 1454	2014
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Foreign stays during Ph.D. study at RIFCH and FFPW	**Year**
Hanna Söderström Lindström, Ph.D., Department of Chemistry, Umeå University, Umeå, Sweden (2 months)	2015
Prof. İdil Arslan-Alaton, Water Chemistry, Chemical Treatment Processes and Ecotoxicological Impacts Research Group, Environmental engineering department, Istanbul Technical University, Istanbul, Turkey (2 months)	2017
Project leader	**Year**
Grand agency of the University of South Bohemia in České Budějovice: "Determination of pharmaceuticals partitioning between water and sediments using LC/hybrid HRMS Q-Exactive".	2016

CURRICULUM VITAE

Surname Koba
First name Olga
Title M.Sc.
Born 24th July, 1991
Nationality Ukranian
Martial status Single

EDUCATION

2013 – present Ph.D. student, University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, Laboratory of Enviromental Chemistry and Biochemistry

2012 – 2013 M.Sc., V.N.Karazin Kharkov National University, Ukraine (Biophysics, Teacher of Mathematics and Informatic)

2008 – 2012 B.Sc., V.N.Karazin Kharkov National University, Ukraine (Applied Physics)

RESEARCH INTERESTS Fate of emerging pollutants in the aquatic and soil environment. Targeted and untargeted screening, metabolites and transformation products identification. Advanced oxidation processes and wastewater treatment.

PH.D. COURSES Pond aquaculture, Basic of scientific communication, Applied hydrobiology, Ichthyology and fish taxonomy, English language

LEADING OF PROJECTS Grand agency of the University of South Bohemia in České Budějovice: "Determination of pharmaceuticals partitioning between water and sediments using LC/hybrid HRMS Q-Exactive"

LANGUAGE SKILLS Ukrainian (native), Russian (native), English (IETLS certificate), Czech (Intermediate)