Chapter 6

Selected Pharmaceuticals and Musk Compounds in Wastewater

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Additional information is available at the end of the chapter

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1. Introduction

In order to achieve sustainable development, environmental protection shall constitute an integral part of the development process and cannot be considered in isolation from it [1]. The environment, especially water ecosystem, is continuously loaded with foreign organic chemicals (xenobiotics) released by urban communities and industries. Water is not a commercial product like any other but, rather, a heritage which must be protected, defended and treated as such [2]. In the 20th century, many organic compounds, such as polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) have been produced and, in part, released into the environment [3]. The ultimate sink for many of these contaminants is the aquatic environment, either due to direct discharges or to hydrologic and atmospheric processes [4]. In the 21st century „new" pollutants namely pharmaceuticals, cosmetics and endocrine disrupting chemicals (EDCs) have become a source of concern. Collectively, they are referred to as PPCPs (Pharmaceuticals and Personal Care Products) and are now viewed as emerging contaminants. A wide range of pharmaceutical and personal care products (PPCPs) is available on the market. From this range various classes, e.g., antibiotics, antiphlogistics, antiepileptics, beta-blockers, lipid regulators, vasodilators, and sympathomimetics, have been detected in drinking water, groundwater, wastewater, sewage, and manure [5]. In last time there is increasing evidence that some of these compounds are persistent in the environment, impacting nontarget organisms in various ways including changes in sex ratios of higher organisms [6,7]. The presence of a xeno-

biotic compound in a segment of an aquatic ecosystem does not, by itself, indicate injurious effects. Traditional chemical measurements alone are an insufficient basis for ecotoxicity assessments. In general, both basic and advanced analytical chemical instruments such as ICP/MS, GC/MS, HPLC/MS etc. are used for water quality analysis. However, it is difficult to distinguish accurately the diverse and complex chemicals, even when using those advance chemical instruments. Furthermore, it is also almost impossible to detect the impact on living organisms in the receiving environment due to their bioavailability and the interaction caused by the synergistic and antagonistic effect of different chemicals. Therefore a new approach of identifying viable and ecologically relevant invertebrate toxicity testing models seems very promising to assess the biological effects and ecological risk of exotic chemicals when released into the environment as a battery of single species bioassays [8].

1.1. Pharmaceuticals

Pharmaceuticals (also drugs, medicaments, medications, medicines etc.) are biologically active substances designated for use in the medical diagnosis, cure, treatment, or prevention of disease [9]. These compounds improve the quality of human life, but due to their increasing production and consumption resulting in their growing input into the environment there is increasing impact of these compounds on the natural ecosystems, caused either by the active compounds contained in medicaments or by their metabolites and transformation products [10]. These compounds are sometimes called as pseudo-persistent pollutants, because in many cases their persistence is not high, but due to continual input their levels in the environment are kept less or more constant. The discharges from waste water treatment plants represent one important source of pharmaceuticals in the water ecosystem, because most of drugs is incompletely removed in waste water treatment plants (WWTP) [11-13]; they could be partially removed by sorption on the sewage sludge or degraded by microorganisms in activated sludge. The removal efficiency depends on many factors like drug properties, type and parameters of the cleaning process, age of the activated sludge. The sludge activity could be also negatively influenced by the presence of antibiotics in treated waste water.

Another important source of pharmaceuticals in the water ecosystem is agriculture, especially livestock production, where growth stimulants are used to increase production and antibiotics are administered as prophylactic medication to animals. Biotransformation of drugs during animal digestion is not very effective; from 30 to 90 % of administered active compounds is excreted unchanged [10,14] and enter the environment directly via urine or faeces, or in manure and suds used as fertilizers.

Non-steroidal anti-inflammatory drugs (NSAIDs) with analgesic, antipyretic and anti-inflammatory effects are one group of the most frequently used medicaments. Ibuprofen and paracetamol followed by diclofenac, ketoprofen and naproxen are the most well-known members of this group. Their extensive use is caused by the fact, that many drugs in this group do not require medical prescription. These compounds also belong to the most frequently detected pharmaceuticals in the European waters. E.g, for ibuprofen the concentra-

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tions of units to tens of $ng.L^{-1}$ in surface water, tens of $ng.L^{-1}$ in raw waste water and from tenths to units of $ng.L^{-1}$ in discharged water were found in the Czech Republic [15].

Antibiotics are another important group of pharmaceuticals. This term originally denoted "any substance produced by a microorganism that is antagonistic to the growth of other microorganisms in high dilution" [16]. Nowadays also synthetic compounds are included in this group. Antibiotics have been recently classified as a priority risk compounds due to their high toxicity to algae and bacteria. Hence, these compounds in surface water have the potential to disrupt the key bacterial cycles and/or processes critical to aquatic ecology (nitrification/denitrification), agriculture (soil fertility) and animal production (rudimentary processes) [17,18]. Under long-term exposition the resistance of some pathogenic organisms could develop [11,12].

Macrolide antibiotics are a group of drugs frequently used in human and veterinary medicine. These primarily bacteriostatic antibiotics with a broad antibacterial spectrum are probably the largest group of natural medicines. Macrolides have acquired its name by macrocyclic lactone ring with 14, 15 or 16 carbon atoms, substituted with alkyl, aldehyde, ketone or hydroxyl groups, and with one or more neutral or basic amino sugars bonded to the ring by glycosidic bond. The first macrolide antibiotic, erythromycin, was isolated in 1952 from the metabolic product of fungus *Streptomyces erythreus* [19].

Macrolide antibiotics can be classified into four groups [20]:

1. Natural macrolides of 1st generation have a short half-life; therefore they must be administered in relatively high and frequent doses. There is a potential of interactions with some other drugs.
2. Synthetic macrolides of 2nd generation have more favourable pharmacokinetic properties, applications are therefore less frequent and doses are lower than at first -generation macrolides. There is also a lower incidence of drug interactions.
3. Azalides are formed by incorporating nitrogen into the 14-member lactone ring. From other macrolides they differ with high half-life and very slow release from tissues.
4. Ketolides are the newest and so far little studied group of macrolide antibiotics. These drugs were prepared by replacing sugar cladinose in the 14-member lactone ring by keto-group and by attaching a cyclic carbamate group in the lactone ring. Due to these modifications ketolides have much broader antimicrobial spectrum than other macrolides; besides, they are also effective against macrolide-resistant bacteria, due to their ability to bind at two sites at the bacterial ribosome.

1.2. Musk Compounds

Musk compounds - synthetic fragrances – are substances with pleasant smell which are present in personal hygiene products (perfumes, cosmetics, soaps, and shampoo), in cleaning and disinfection products, industrial cleaning products, air fresheners, etc. to give them characteristic and pleasant scent. These compounds have been marketed since the begin-

ning of 20th century and their industrial production has significantly increased during the last 50 years [21]. Nowadays, four major classes of synthetic fragrances could be met: nitromusks, polycyclic musks, macrocyclic musks and alicyclic (or linear) musks. Nitromusks were the first produced compounds of this type; structure of these compounds is based on two- or threefold nitrated benzene with additional substitution by alkyl-, methoxy- or keto- groups. Musk xylene (MX), musk ketone (MK) and musk ambrette (MA) are the most important members of this group. These compounds show musk-like odour in spite of the fact that their structure is very different from natural musk compounds. They are partially soluble in water (0.15 $ng.L^{-1}$ for MX; 0.46 $ng.L^{-1}$ for MK), but their relatively high octanol-water partition coefficients (log Kow = 4.4, 3.8 and 4.0 for MX, MK and MA, respectively) [22] indicate high bioaccumulation potential in water biota. These compounds are also relatively persistent. According to data published till now, nitro musks show low or none acute toxicity to aquatic organisms, but they are potentially toxic over long time period [23,24]. It has been suggested that their transformation products are potentially highly toxic [25]. The worldwide production of MX and MK (which are the only two nitromusks of industrial importance today) in 2000 was estimated to 200 metric tons and it shows decreasing tendency [26].

Polycyclic musks with several cycles in their structure were discovered in 1950s [27]. Chemically they are indane, tetraline or coumarine derivatives and tricyclic compounds. Currently, these musks are the most widely used. Galaxolide (HHCB) and tonalide (AHTN) in recent years are the most important commercial synthetic musks [21,28] followed by celestolide (ADBI), phantolide (AHMI), and traesolide (ATII). Total worldwide use of polycyclic musks in year 2000 was approximately 4000 tons [26]. These compounds are more resistant against light and bases and bind well to fabric. Nevertheless, HHCB and AHTN are toxic to aquatic invertebrates at concentration levels of ppb to low ppm, but they are almost non-toxic to fish; similar situation occurs during longer exposition [29]. The first report about the presence of these compounds in water and fish appeared in 1984, one year later these compounds were found in human samples [27].

Macrocyclic musk compounds were discovered in 1926 by Austrian chemist Leopold Ruzicka [30,31] who characterized natural musks muscone and civetone as cyclic macromolecules and proposed the method of their synthesis. Since then, many other compounds of this type has been characterized and synthesized. It was found that natural macrocyclic musks are 15- or 17-membered rings, musks of animal origin are mainly ketones, whilst those of plant origin are lactones. These compounds show excellent stability to light and alkaline condition and very good fixation to fabric, nevertheless their synthesis is difficult and usually multi-step procedure, and therefore their production costs are high. Due to this fact the use of these compounds is limited, but they are expected to be of increasing importance in future [27].

Alicyclic musks, known also as linear musks or cycloakyl esters, represent the youngest group of synthetic musks. Their structure is formed by modified cykloalkyl esters. The first compound of this group – cyclomusk - was introduced in 1975 [32]. In 1990, the first commercially successful linear musk – helvetolide – was launched, another linear musk – Ro-

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mandolide – was described ten years later [33]. Due to relative novelty there is lack of information describing their occurrence in the environment and their ecotoxicity. A wide range of musk compounds of this group are produced and marketed by the Czech company Aroma Prague Ltd.

2. Environmental Analysis

2.1. Target Compounds

For this study six frequently used acidic non-steroid anti-inflammatory drugs (NSAIDs) were selected. Figure 1 shows their structures and Table 1 summarizes their physical-chemical properties.

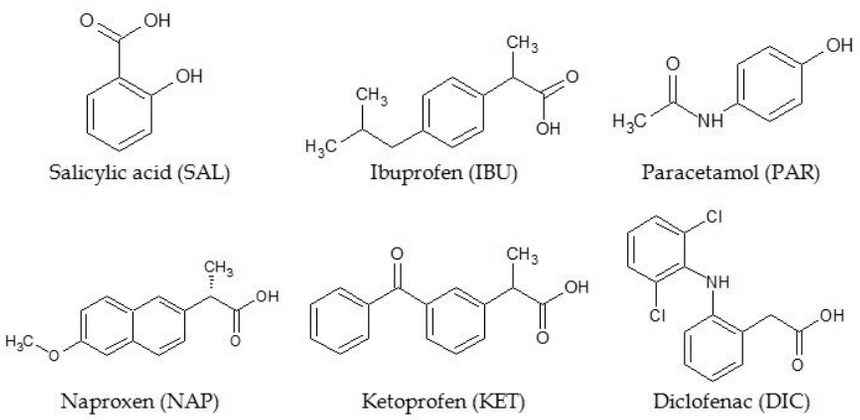

Figure 1. Structures of selected NSAIDs.

Compound	CAS No.	Molecular mass ($g.mol^{-1}$)	pK_a	log K_{ow}
Salicylic acid	69-72-2	138.1207	2.97	2.4
Ibuprofen	15687-27-1	206.2808	4.91	3.6
Paracetamol	103-90-2	151.1626	9.38	0.4
Naproxen	22204-53-1	230.2592	4.15	2.8
Ketoprofen	22071-15-4	254.2806	4.45	3.2
Diclofenac	15307-86-5	296.149	4.15	3.9

Table 1. Physical-chemical properties of selected NSAIDs [34-37].

From the group of macrolide antibiotics following drugs were selected:

Erythromycin is a mixture of macrolide antibiotics that are produced by the microorganism *Streptomyces erythreus*. The main ingredient is erythromycin A. Erythromycin is a white to pale yellow powder or form a colourless to pale yellow crystals. It is slightly hygroscopic, poorly soluble in water, soluble in ethanol and methanol. It is metabolized in the acidic environment of the stomach to inactive by-products (ketones, alcohols, ethers), which are responsible for its low bioavailability and gastrointestinal side effects. This bacteriostatic macrolide antibiotic is used for treatment of respiratory infections caused mainly mycoplasma, chlamydia, staphylococci or streptococci, as well as of infections of the skin or urinary tract.

Clarithromycin is used for treating of respiratory infections caused mainly by mycoplasma, chlamydia, staphylococci or streptococci, as well as of infections of the skin or urinary tract. Clarithromycin has strong antibacterial properties and is more resistant against acidic environment than erythromycin; it has also improved pharmacokinetic properties and is better tolerated in the GIT. Clarithromycin is a white crystalline powder, practically insoluble in water but soluble in acetone.

Roxithromycin is a newer macrolide antibiotic with better tolerance than that of erythromycin. It is used to treat the same diseases as erythromycin and also to treat isosporiases.

Chemical structures of selected macrolide antibiotics are in Figure 2.

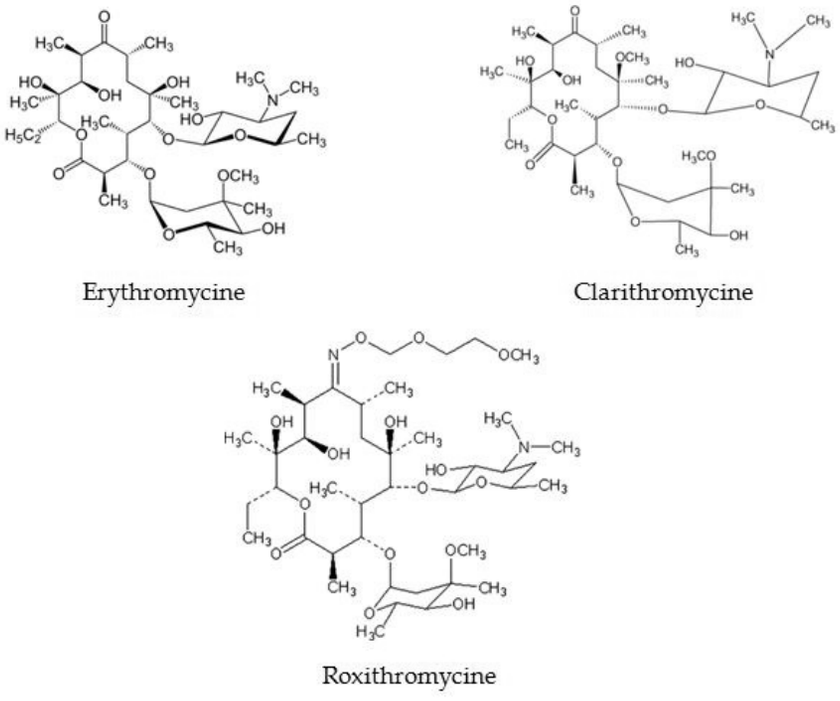

Figure 2. Structures of selected macrolide antibiotics.

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Compound	CAS No.	Molecular mass ($g.mol^{-1}$)	log K_{ow}
Erythromycine	114-07-8	733.93	3.06
Clarithromycine	81103-11-9	747.95	3.16
Roxithromycine	80214-83-1	837.05	2.75

Table 2. Physical-chemical properties of selected macrolides [34,35,37].

Musk compounds selected for this study are from the group of linear musks produced and marketed in the Czech Republic by Aroma Prague Company. They are used for preparation of various fragrances and perfume compositions. Their structures are given in Figure 3 and physical-chemical properties in Table 3.

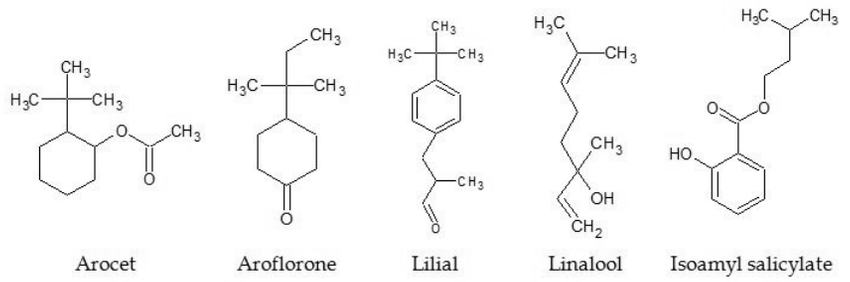

Figure 3. Structures of selected linear musks.

Compound	CAS No.	Molecular mass ($g.mol^{-1}$)	log K_{ow}
Arocet	88-41-5	198.30	4.42
Aroflorone	16587-71-6	168.26	3.40
Lilial	80-54-6	204.31	4.36
Linalool	126-91-0	154.25	3.38
Isoamyl salicylate	87-20-7	208.25	4.49

Table 3. Physical-chemical properties of selected linear musks.

2.2. Sampling locality

The presence of target compounds was monitored in the wastewater from municipal waste water treatment plant (WWTP) Brno – Modřice (catchment region for population of about

500,000 people). This facility was launched in 1961 as classic two-stage plant with anaerobic sludge stabilization. In the period between 2001 – 2003 the overall reconstruction and extension of the WWTP was realized with the main objective to meet the treated wastewater effluent limits set by Czech and European standards and regulations, and to ensure sufficient capacity of the facility to accommodate the growing demand of the city of Brno with almost 500 thousand of inhabitants and several industrial facilities, and also increasing number of the surrounding agglomerations successively connecting to the Brno sewerage system. Nowadays, the technology in WWTP Brno-Modřice corresponds to the EU parameters. Waste water cleaning process includes mechanical removal of rough solid particles – mechanical treatment, which is followed by fat removal. Water is then directed to the sedimentation tanks for removal of fine particles. The next step is biological treatment under anaerobic conditions where dephosphatation and denitrification occurs, followed by biological degradation under aerobic conditions. The rest of the non-biodegradable phosphorus is subsequently removed by chemical precipitation with ferric sulphate. Activated sludge is removed from the water in sedimentation tank, water is then discharged into the recipient and sludge is thickened and decayed. Produced bio-gas is used for the combined generation of heat and electricity. The residence time (technological delay) between inlet and comparable outlet in Brno WWTP is 24 hours.

Figure 4. Sampling locality – waste water treatment plant Brno - Modřice.

Composite 24-hour samples were collected at inflow and outflow of the WWTP by automatic sampling device in 2-hours intervals. Individual portions were collected in the dark glass sample containers with a capacity of 1 L. Samples for analysis of NSAIDs were collected at inflow and outflow of WWTP during July and August 2011, for determination of macrolides

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and musk compounds from 11th to 20th of April 2011. Samples were picked up from the WWTP daily and transported to laboratory, where they were either analysed immediately or stored in a refrigerator at 5 °C and analysis was initiated within 24 hours.

2.3. Analysis of Pharmaceuticals

Solid phase extraction (SPE) was applied for the isolation of target compounds from waste water. The suspended particles were removed by filtration using Büchner funnel and filter paper Munktell Filtrak No 388 and No 390 for inflow samples and 390 for outflow samples and pH of the samples was adjusted to a value of 2 by addition of hydrochloric acid (NSAIDs) or formic acid (antibiotics). 300 mL of waste water was then subjected to solid phase extraction using Oasis HLB cartridges (volume 3 mL, 60 mg of sorbent, Waters, USA), which were previously activated by 6 mL of methanol and washed with 6 mL Milli-Q water at pH = 2. After loading of sample the cartridge was again washed by 6 mL Milli-Q water at pH = 2, dried for 5 minutes under flow of nitrogen and then the target compounds were eluted by 6 mL (NSAIDs) or 10 ml (antibiotics) of methanol. The eluate was then evaporated to dryness under gentle stream of nitrogen. For the analysis of NSAIDs the residue was dissolved in 300 μL of BGE and analysed by capillary zone electrophoresis with UV detection. For analysis of macrolides the residue was dissolved in 1 ml of acetonitrile and analysed by HPLC/MS.

2.3.1. Analysis of NSAIDs by capillary zone electrophoresis:

Agilent CE instrument equipped with UV-VIS detector of DAD type was used. Analytical conditions were as follows.

- Separation capillary: fused silica uncoated, ID = 75 μm, L = 83.5 cm, l = 75.4 cm
- Background electrolyte (BGE): 25 mmol.L^{-1} $Na_2B_4O_7$ in Milli-Q water (before each injection, the capillary was treated successively with alkaline solution of 0.1 M NaOH, water and BGE
- Separation voltage: 30 kV, positive polarity
- Temperature of separation capillary: 25 °C
- Detection: 210 nm (bandwith of 40 nm), 200 nm (bandwith of 20 nm), 230 nm (bandwith of 10 nm)
- Sample injection: hydrodynamic, pressure pulse at capillary inlet 5 kPa for 5 s
- Analysis time: 25 min

Fig. 5 shows an example of electrophoregram.

Obtained results together with removal efficiency and limits of detection are presented in Table 4.

Compound	Concentration		Removal efficiency (%)	LOD [μg.L^{-1}]
	influent [μg.L^{-1}]	effluent [μg.L^{-1}]		
Salicylic acid	5.58–44.15	0.47–3.53	97	0.46
Ibuprofen	10.94–42.32	1.18–2.75	96	1.17
Paracetamol	1.00–14.61	0.52–1.65	97	0.46
Naproxen	0.61–14.48	0.51–2.35	78	0.50
Ketoprofen	2.15–28.21	1.34–6.46	92	1.28
Diclofenac	1.09–9.46	1.02–2.17	92	0.98

Table 4. Concentrations of NSAIDs at inflow and outflow, removal efficiency and limits of detection.

The ranges of concentrations of selected drugs in the influent and effluent and average removal efficiency of the WWTP for each drug are listed in Table 4. All selected drugs were detected in analysed samples of wastewater. Salicylic acid (average concentration 28.21 μg.L^{-1}) and ibuprofen (average concentration 23.11 μg.L^{-1}), were detected at highest concentrations and almost in all samples. It is caused by the fact, that these compounds are contained in the majority of the most frequently used drugs in the Czech Republic. The levels of other monitored analgesics were below 10 μg.L^{-1}. Relatively low concentration of favourite painkiller – paracetamol – was surprising, but the reason could be partial decomposition of this compound in waste water before the inflow to the WWTP. Ketoprofen, diclofenac and naproxen were detected in wastewater only in some cases.

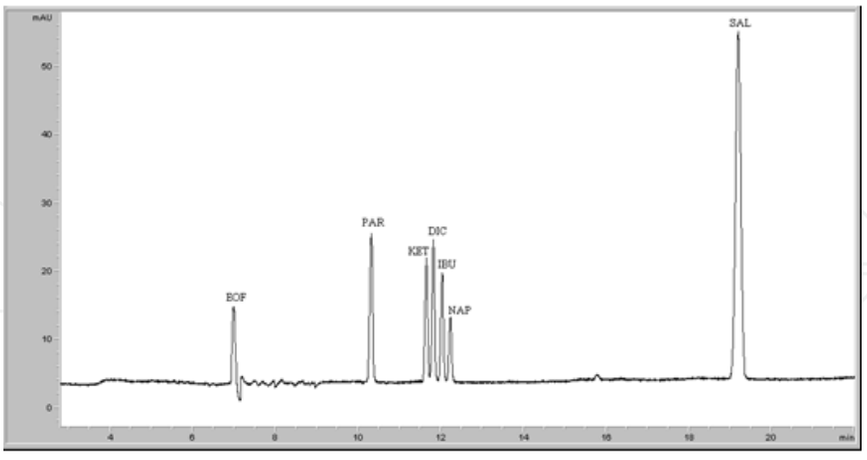

Figure 5. Electrophoregram of NSAIDs standards: EOF – Mesityl oxid (marker of electroosmotic flow); PAR – paracetamol; KET – ketoprofen; DIC – diclofenac; IBU – ibuprofen; NAP – naproxen; SAL - salicylic acid.

Average removal efficiency of all analysed compounds was above 90 %, except for naproxen with an average removal efficiency of 78 %.

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2.3.2. Analysis of antibiotics by HPLC/MS

Analysis of samples was performed using high performance liquid chromatography with mass spectrometric detection (HPLC/MS). Agilent 1100 Series liquid chromatograph with Agilent 6320 spherical ion trap mass spectrometer and electrospray ionization were employed. Zorbax Eclipse XDB - C18 column (2.1 x 150 mm, particles 3.5 μm) protected by Zorbax Eclipse XDB - C18 precolumn (2.1 x 20 mm, particles 3.5 μm) was used for separation, binary mobile phase consists from 10mM ammonium acetate (A) and acetonitrile (B), gradient started from 25 % B to 55 % B in 3 min, then 90 % B in 10 min. Flow rate was 150 $\mu L.min^{-1}$. Conditions for electrospray: pressure of nebulizing gas (N_2) 20 psi, flow and temperature of drying gas (N_2) 10 $L.min^{-1}$ and 350 °C, respectively. Positive ions were scanned within the range m/z 100 – 900. Individual compounds were identified by the combination of retention time and quasi-molecular ion detection (erythromycin: t_R =11.2 min, m/z = 734.8; clarithromycin: t_R = 13.0 min, m/z = 748.3; roxithromycin: t_R = 13.4 min, m/z = 837.4), external standard method based on the response on fragmentograms corresponding to the quasi-molecular peaks of individual compounds was used. Metrological parameters of used analytical method are presented in Table 5.

Parameter	Compound		
	Erythromycin	Clarithromycin	Roxithromycin
Coefficient of determination (R^2)	0.9962	0.9993	0.9971
LOD [$\mu g.L^{-1}$]	0.1440	0.2428	0.0970
LOQ [$\mu g.L^{-1}$]	0.4305	0.7251	0.2903

Table 5. Metrological parameters of HPLC/MS method.

In real samples the presence of macrolide antibiotics was proved only exceptionally and at levels close to limits of detection (erythromycin 17., 18. and 19. 4. 2011 at inflow at levels of 0.274 $\mu g.L^{-1}$, roxithromycin 12.4.2011 at outflow 0.1 $\mu g.L^{-1}$). Clarithromycin was not detected at concentrations exceeding LOD at all. Therefore it could be concluded that macrolide antibiotics don't represent any serious risk for the receiving water.

2.4. Analysis of musk compounds

Solid Phase Microextraction (SPME) in head-space mode was used for the isolation of target analytes from waste water. Fibre with 65 μm mixed layer polydimethylsiloxane – divinylbenzene was selected as optimal on the base of previous studies realized in our laboratory. 22 mL glass vials closed with Teflon-lined silicon septum were used. 14 mL of raw sample was placed into the vial, 3.75 g NaCl was added, after inserting of magnetic stirrer vial was closed and heated up to the temperature of 80 °C in water bath. Magnetic stirrer was set to 900 rpm. Equilibration time was 5 minutes, followed by 40 minute sorption. Blank samples were treated by the same method using de-ionized water.

Compound	Quantification ion (m/z)	Qualifier ions (m/z)		t_R (min)	
Linalool	93	71	121	9.12	
Arocet (2 isomers)	82	123	57	13.483	14.044
Aroflorone	98	168	71	15.330	
Lilial	189	204	147	20.675	
Isoamyl salicylate	120	138	208	20.825	

Table 6. Experimental parameters for the GC/MS analysis of linear musks.

Isolated compounds were analysed by GC/MS using Agilent 6890N GC and Agilent 5973 MS equipped with quadrupole analyser and electron ionization @ 70 eV. Separation column was DB-5MS (20 m x 0.18 mm x 0.18 μm) (J&W), helium 6.0 (SIAD, Czech Republic) at a flow rate of 0.8 $mL.min^{-1}$ (constant flow mode) was the carrier gas. Desorption from SPME fibre was realized in split/splitless injector of the GC in splitless mode for 3 min at a temperature of 250 °C. Column temperature program was as follows: 50 °C for 3 min, then 10°/min to 90 °C, then 5°/min to 120 °C, hold 4 min, then 10°/min to 160 °C, 5°/min to 185 °C, 20°/min to 285 °C, final isotherm 2 min. GC/MS interface temperature was set to 285 °C, temperature of ion source was 250 °C. Mass spectrometer was operated in SIM mode; parameters are summarized in Table 6.

Date	Linalool [μg.L^{-1}]		Arocet [μg.L^{-1}]		Aroflorone [μg.L^{-1}]		Lilial [μg.L^{-1}]		Isoamyl salicylate [μg.L^{-1}]	
	Inflow	Outflow	Inflow	Outflow	Inflow	Outflow	Inflow	Outflow	Inflow	Outflow
11.4.11	61.31	ND	2.633	ND	3.442	ND	1.222	0.049	0.975	NQ
12.4.11	42.33	NQ	2.406	ND	1.342	ND	0.406	0.049	0.922	ND
13.4.11	33.28	NQ	2.847	ND	1.413	ND	0.439	0.017	0.328	ND
14.4.11	36.75	ND	1.388	ND	0.809	NQ	0.429	0.060	0.589	NQ
15.4.11	25.92	0.199	0.473	ND	0.369	ND	0.197	0.042	0.121	NQ
16.4.11	66.72	0.139	1.399	ND	1.427	ND	0.404	0.049	0.403	NQ
17.4.11	39.57	NQ	0.546	ND	0.727	ND	0.307	0.033	0.202	ND
18.4.11	90.81	0.114	3.223	ND	5.336	ND	0.391	0.058	0.492	NQ
19.4.11	75.67	ND	4.294	ND	2.419	NQ	0.684	0.065	0.734	NQ
20.4.11	84.79	ND	4.406	ND	0.925	ND	0.433	0.047	0.495	ND
Average	55.72	0.046	2.361	0.0002	1.821	0.0007	0.491	0.047	0.526	0.0003
LOD	0.0012		0.0004		0.0011		0.0002		0.0004	
LOQ	0.0041		0.0014		0.0037		0.0008		0.0012	

Table 7. Concentrations of selected linear musks in waste water. For the calculation of average compound concentrations following values were used: ND = 0.5 • LOD and NQ = LOD.

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Table 7 presents the concentrations of selected linear musks in raw and cleaned waste water. Linalool was found in highest concentrations at inflow ranging from 33 to 91 $\mu g.L^{-1}$, followed by arocet and aroflorone with levels in low units of $\mu g.L^{-1}$. Inflow concentrations of lilial and isoamyl acetate were in tenths of $\mu g.L^{-1}$. These concentrations are lower than that of polycyclic musks at the same locality – levels found for galaxolide and tonalide were in hundreds and tens of $\mu g.L^{-1}$, respectively [38]. For all linear musks except of lilial, high removal efficiencies were attained, usually more than 99.5 %. Lilial removal efficiency was between 78.68 and 96.13 % (average 88.7 %). These results are very satisfactory.

3. Ecotoxicology

Our generation has recently stepped over a threshold of the new millennium. The growth of the human population coupled with increasing consumption and overuse of natural resources brings with it also growing impact on the total environment. The human activities that have accelerated since the 18th century with the beginning of the industrial revolution led in many cases to long-term consequences which disturbed the natural balance and gathered an irreversible and uncontrollable character [39]. Effects of above mentioned human activities, mainly uncontrolled release of various manmade chemicals, is not without adverse consequences. These negative effects are studied within the discipline of ecotoxicology, which was firstly defined around 1969 by Dr. René Truhaut, a member of the French Academy of Sciences. This new field of science "Ecotoxicology" he defined as "the study of adverse effects of chemicals with the aim of protecting natural species and populations." Thus ecotoxicology deals with potentially harmful effects of countless man-made chemicals and wastes released into biosphere on organisms. Ecotoxicity involves the identification of chemical hazards to the environment. "Ecotoxicity studies measure the effects of chemicals on fish, wildlife, plants, and other wild organisms" [40,41]. Bioassays are one of the main tools in ecotoxicological assessments. Ecotoxicology has the task to examine effects of chemicals or environmental samples on species, biocenoses and ecosystems. Results of ecotoxicological research constitute the main scientific background for setting immission standards for the protection of the environment. The Water Policy Directive [2] of the European Union (EU) strives for a good ecological and chemical status for surface waters. This Directive is to contribute to the progressive reduction of emissions of hazardous substances to water. However, the Directive is aimed especially at a monitoring of the state of the waters and is based on a combined approach using control of pollution at source (substance-specific assessment) through the setting of emission limit values and of environmental quality standards instead of an assessing threats to the waters from effluent discharges [2,42]. The solution is the whole effluent assessment (WEA), which can be defined as the assessment of the whole effluents by using a range of biological methods or techniques in order to reveal (potential) effects. It focuses on toxicity (acute and chronic), genotoxicity (including mutagenicity), bioaccumulation and persistence. Therefore WEA increases the understanding of the combined effect of all known and unknown substances, especially in complex mixtures [43]. Global evaluation of wastewaters should include ecotoxicological tests to complete the chemical

characterization. The integrated assessment of biological effects of wastewater discharges in the ecosystems is relevant and ecotoxicity tests are referred as extremely useful tools for the identification of environmental impacts [44]. On the other hand there exist some ways how to partially prevent environment and water ecosystem. On 1st June 2007 EU regulation REACH entered into force. The law is the European Community Regulation on chemicals and their safe use [45]. It deals with the Registration, Evaluation, Authorisation and Restriction of Chemical substances. The aim of REACH is to improve the protection of human health and the environment through the better and earlier identification of the intrinsic properties of chemical substances. Three specific properties of a chemical are used to describe its potential hazard to the aquatic environment [46,47]:

- Aquatic toxicity: The hazard of a substance to living organisms, based on toxicity tests to aquatic animals and plants.
- Degradability: The persistence of the substance in the environment, based on molecular structure or analytical testing.
- Bioaccumulation/bioconcentration: The accumulation of a substance in living organisms (from water sources for bioconcentration), which may or may not lead to a toxic effect; based on calculations or bioconcentration factor (BCF) studies using fish.

Aquatic toxicity is determined using internationally harmonized test methods, which are preferred; in practice, data from national methods may also be used where they are considered as equivalent. Data are preferably to be derived using OECD Test Guidelines, US Environmental Protection Agency (EPA) or equivalent according to the principles of Good Laboratory Practices (GLP). For ecotoxicity evaluation of chemicals fish, crustacean, algae and freshwater plant (*Lemna minor*) are used. On the base of obtained results from tests the hazards to the aquatic environment which they present is identified and chemical substances are classified into categories and they are assigned risk phrases [46,48,49].

3.1. Ecotoxicity testing of chemical compounds

To assess the effect of chemical compounds on various aquatic organisms the ecotoxicity tests, biotests, bioassays using organisms from various trophic levels are used. The goal of the ecotoxicological tests is the determination of effective concentration (EC), eventually lethal concentration (LC) or inhibition concentrations (IC) [40]. These parameters refer to the concentration of toxic substance that results in 50% reduction of end-point relative to control at a given period of time [50]. These concentrations of tested compounds cause the mortality of 50 % testing organisms or 50% inhibition growth rate in relation to control group. Lower values of LC (EC, IC)50 means higher toxicity of the tested chemical compounds. In accordance with testing regulation the limit test, preliminary tests and definitive test were conducted with single compounds. In limit test concentration 100 $mg.L^{-1}$ of tested compound is used. Preliminary tests (range finding test) are used to find approximate toxicity of the chemical compounds if it is unknown. In this case the dilution series is following: 100 $mg.L^{-1}$, 10 $mg.L^{-1}$, 1 $mg.L^{-1}$, 0.1 $mg.L^{-1}$ and 0.01 $mg.L^{-1}$. The results of preliminary tests are

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used to determine the range of dilution series of the final test. From obtained experimental endpoints (mortality, immobility, growth inhibition etc.) in ecotoxicity tests the ecotoxicological values EC50, IC50, LC50 are calculated

3.1.1. Daphnia magna – acute toxicity test

Daphnia magna is a common component of freshwater zooplankton. It refers to the group of *Arthropoda, Branchiopoda, Daphnidae*. Daphnia are small arthropods of 1–5 mm in size. They live in various aquatic environments. Ontogenesis of individual is direct without larval stages. During the year there is one or several biological cycles in which parthenogenetic generations are alternated by bisexual generations which enclose the cycle. Species *D. magna* is the largest species of *Daphnia* group. Thus it is vulnerable to fish predation that it is excluded from fish-inhabiting lakes. It occurs mainly in ephemeral habitats like small ponds and rockpools where vertebrate predators are rare. *D. magna* is most commonly used species in aquatic toxicity testing because of many characters that make it easy and economical to culture it in the laboratory. It is relatively small but bigger than other daphnids, thus manipulation with it is easy. It has short life cycle, high fecundity, and parthenogenetic reproduction. On the other hand in a few comparative studies *D. magna* tended to be less sensitive to toxic substances than other cladorerans, and this may be due in part to life-history and size differences [51,52]. Daphnids are integral part of water biocenosis and food chain; this is the reason why their using in ecotoxicity testing is important. There exist many national and international standard methods which use this organism for acute or chronic ecotoxicity assessment [53-59].

Alternative small scale method Daphtoxkit F™ (purchased from MicroBioTests Inc., Gent, Belgium) for the determination of EC50 value was used for our purposes. The Standard Operational Procedure of the Daphtoxkit F™ is in accordance with the OECD and ISO test protocols for the acute *Daphnia magna* toxicity tests [54,55]. Standard Freshwater was prepared with the concentrated salt solutions included in the kit. This medium, which has the composition recommended by the ISO for acute toxicity tests with *D. magna*, is used as a hatching medium and as a dilution medium for the preparation of the toxicant dilution series. Because of low water solubility of tested substances DMSO as solvent for preparation of 100 $mg.L^{-1}$ stock solutions of tested compounds was used. Maximal concentration of DMSO used for dilution series preparation in tests was 3 %. This concentration doesn't exhibit any negative influence on testing organisms in control group. Ephippia were hatched in Petri dishes with Standard Freshwater (ISO) medium three days before test at temperature 20 - 22 °C under continuous illumination of 6 000 lux. Pre-feeding of neonate with suspension of spirulina powder was done two hours before the test to prove them energetic reserve. Daphnids (aged less than 24 hours) were exposed to dilution series of tested compounds in preliminary and final tests. Experiments were conducted at temperature 20 °C in darkness incubator. After 24 and 48 h the endpoint - immobility was observed. The values of 24hEC50 and 48hEC50 were calculated by probit analysis. The test was considered valid if the number of dead organisms in the control did not exceeded 10 %.

3.1.2. Thamnocephalus platyurus - acute toxicity test

Ecotoxicological evaluation of selected musk substances was done also via freshwater crustaceans *Thamnocephalus platyurus*. It refers to class *Branchiopoda* orders Anostraca, originated from North America. For calculation value of 24LC50 alternative test Thamnotoxkit F™ was used (purchased from MicroBioTests Inc., Gent, Belgium). The *T. platyurus* assay has already been incorporated in some countries in regional or national regulations for toxicity testing but requests have also been formulated from various sides to propose this microbiotest to "international" organisations for endorsement as a "standard toxicity test", for specific applications in a regulatory framework. On the base of proposal to the International Standardisation Organisation (ISO) for consideration the *T. platyurus* microbiotest as a new ISO standard ecotoxicological test committee draft ISO/CD 14380 was in 2010 prepared. This test is often used to toxicity assessing in freshwater, waste water and determination of acute toxicity of chemicals [60-62]. Thamnotoxkit F™ is similar to Daphtoxkit F™ - it also contains all the materials to perform six complete acute (24-hour) toxicity tests (range-finding or definitive) based on mortality of testing organisms. Larvae of the fairy shrimp *T. platyurus* hatched from cysts are used. The test procedure followed the Standard Operational Procedure manual of the Thamnotoxkit F™ microbiotest. Standard freshwater was prepared by diluting of the concentrated salt solutions included in the kit to obtain 1 L of medium, which served for hatching of the cysts and for preparation of the toxicant dilution series. In case of organisms *T. platyurus* acetone as solvent for preparation of 100 $mg.L^{-1}$ stock solution of tested compounds was used. Maximal concentration of acetone used for dilution series preparation in tests was 3 %, which have no negative effect on testing organisms in control group. Before testing the eggs of *T. platyurus* were hatched 24 hours at a temperature of 25 °C under continuous illumination at 4 000 lux. The assays were carried out in the multiwell test plates provided in the kits in the darkness at temperature of 25 °C. Larvae were exposed to dilution series of tested compounds in preliminary and final tests. Lethality (endpoint for effect calculation) was observed after 24 h. The values of 24hLC50 were calculated by probit analysis. The test was considered valid if the number of dead organisms in the control did not exceed 10 %.

3.2. Ecotoxicity of linear musk compounds

In our study four selected synthetic linear musk compounds were evaluated via alternative ecotoxicity tests on freshwater crustaceans *T. platyurus* and *D. magma*: Arocet (2-*tert*-butylcyclohexylacetate, Aroflorone (4-*tert*-amylcyclohexanone), Lilial [3-(4-*tert*-butylphenyl)-2-methylpropanal] and Linalool (3,7-dimethylocta-1,6-diene-3-ol). All substances were obtained from their producer Aroma Praha Company Ltd. Information on the occurrence of these substances in waste water and surface water as well as information concerning their ecotoxicity is absent in scientific literature. Material safety data sheet (MSDS), if available, gives only data concerning their toxicity. The Globally Harmonized System for Classification and Labelling of Chemicals (GHS) describes testing for hazards to the aquatic environment in Part 4, Chapter 4.1 [47]. The purpose of obtaining aquatic toxicity data for chemicals

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is to classify them to their acute or chronic toxicity in the hazard classification in different classes. Ecotoxicological values obtained on the most sensitive of testing organisms (fish, crustacean algae or other aquatic plant) in acute toxicity tests serve to classification in three acute classification categories; ecotoxicological value < 1 $mg.L^{-1}$, (class I-very toxic to aquatic organisms); 1 - 10 $mg.L^{-1}$ (class II-toxic to aquatic organisms); 10 - 100 $mg.L^{-1}$ (class III-harmful to aquatic organisms). Substances with value EC50 above 100 $mg.L^{-1}$ would not be classified. Results obtained in test of acute toxicity on *D. magna* and *T. platyurus* are summarized in Table 8. To compare toxicity of linear musk compounds with other musks in Table 9 are summarized results obtained in our laboratory on the same testing organisms via the same testing procedure [38].

Tested compounds	***Thamnocephalus platyurus***	***Daphnia magna***	
	24hLC50 [$mg.L^{-1}$]	24EC50 [$mg.L^{-1}$]	48EC50[$mg.L^{-1}$]
Lilial	11.98	4.4	2.13
Arocet	54.52	63.68	40.23
Arofloron	68.34	53.63	40.42
Linalool	53.94	156.26	124.59

Table 8. Results of acute toxicity tests of linear musks on *Thamnocephalus platyurus* and *Daphnia magna.*

Group	**Compound**	***Thamnocephalus platyurus***	***Daphnia magna***	
		24h LC50 [$mg.L^{-1}$]	24h EC50 [$mg.L^{-1}$]	48h EC50 [$mg.L^{-1}$]
Nitromusks	Musk xylene	6.15	2.39	2.22
	Musk ketone	6.14	2.33	2.13
Polycyclic musks	Galaxolide (HHCB)	1.14	1.22	1.12
	Tonalide (AHTN)	1.58	1.51	1.33

Table 9. Results of acute toxicity tests of nitromusks and polycyclic musks on *Thamnocephalus platyurus* and *Daphnia magna*.

From compounds tested in our study lilial was found as the most toxic to testing organisms. Although we have ecotoxicological values only on one organism defined for chemicals water ecotoxicity assessment, on the base of 48EC50 values obtained for *D. magna* we could try to classify them as follows: all substances except linalool and lilial were harmful to aquatic organisms (class-III). Lilial was found to be toxic to aquatic organisms (class-II). From results obtained on a limited number of species it seems that linalool is not hazardous to aquatic environment. In comparison with results obtained in our similar study on the same testing organism for polycyclic and nitro musk (see Table 9) we can conclude that linear

musk compounds are more friendly to the environment than polycyclic and nitro musks. The 48EC50 values for tonalide, galaxolide, musk ketone and musk xylene on *D. magna* were 1.33 $mg.L^{-1}$, 1.12 $mg.L^{-1}$, 2.13 $mg.L^{-1}$ and 2.22 $mg.L^{-1}$, respectively. In this case they could be classified as toxic to aquatic organisms (II-class). As seems the linear musk compounds (exception lilial) are in our case in the order of ten times less toxic to the testing organism *T. platyurus* and *D. magna* than polycyclic and nitro musks. As mentioned above this finding is very positive in view of prevention of environmental pollution because the production of linear musk compounds in the Czech Republic is on the rise and replaces the use polycyclic and nitro musk compounds. Equally important is the finding that the concentration at the outlet of the WWTP was mostly below the detection limit as in this article published. Exception is only lilial, but its levels detected at the WWTP outflow (mean value 0.047 $\mu g.L^{-1}$, see Table 7), are much lower than in our case the value of 24LC50 found in our experiments.

4. Conclusions

As a consequence of increasing living standard of mankind the environment is loaded with increasing number of various chemicals. Pharmaceuticals and personal care products (PPCPs) belong to the group with increasing use, but these compounds also attract increasing interest as new or emerging environmental contaminants. Negative effects of these compounds or formulations are caused not only by parent compounds, but also their degradation or transformation products could show in some cases even stronger negative effects than their precursors.

This study was focused on three groups of chemicals belonging to PPCPs: non-steroid anti-inflammatory drugs, which are used widely, macrolide antibiotics which gain wider importance due to their therapeutical properties, and linear musk compounds which represent the most modern synthetic fragrances with great perspectives. The levels of these compounds at the inflow and outflow of waste water in municipal waste water treatment plant in Brno-Modřice were determined. From the group of non-steroid anti-inflammatory drugs ibuprofen was the compound with the highest concentration in the raw waste water reaching more than 40 $\mu g.L^{-1}$, followed by salicylic acid and ketoprofen. The removal efficiency of the cleaning process was found to be very good for all compounds under study with the exception of naproxen – its removal efficiency was 78 %, in all other cases it was better than 90 %.

The levels of macrolide antibiotics (erythromycin, clarithromycin and roxithromycin) were found to be very low in raw waste water (in several samples erythromycin and roxithromycin were found in sub- $\mu g.L^{-1}$, their levels in cleaned waste water were below the limits of detection of used analytical procedure. It could be stated that these compounds due to low concentrations don't represent any serious risk for the receiving water.

The concentrations of linear musks produced in the Czech Republic in the raw waste water ranged from tens of $\mu g.L^{-1}$ for linalool, units of $\mu g.L^{-1}$ for arocet and aroflorone to sub- $\mu g.L^{-1}$ levels for lilial and isoamylacetate. Removal efficiencies were in common better than 99.5 % with exception of lilial with average removal efficiency of 88.7 %. The last compound also

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exhibited the highest ecotoxicity from all tested linear musk compounds with 24EC50 value 4.4 $mg.L^{-1}$. Nevertheless, this value significantly exceeds the concentrations found in real samples.

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References

[1] UN- United Nations. (1992). Report of the United Nations Conference on Environment and Development. Resolutions Adopted by the Conference. A/CONF.1/26/Rev. 1, United Nations, 3-14 June, Rio de Janeiro; CONF.151/26/Rev.1(Report)., 1.

[2] Directive 2000/60/EC of European Parliament and of the Council of 23 October. (2000). establishing a framework for Community action in the field of water policy. 2000/60 (Directives).

[3] Van der Oost, R., Beyer, J., & Vermeulen, N. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology*, 13(2), 57-149.

[4] Stegeman, J. J, & Hahn, M. E. (1994). Biochemistry and molecular biology of monooxydase: current perspective on forms, functions and regulation of cytochrome P450 in aquatic species. In: Malins DC, Ostrander GK, editors. Aquatic toxicology, Molecular, Biochemical and Cellular Perspectives1st ed. Boca Raton Lewis Publishers, CRC press , 87-206.

[5] Jjemba, P. K. (2006). Excretion and ecotoxicity of pharmaceuticals and personal care products in the environment. *Ecotoxicology and Environmental Safety*, 63(1), 113-130.

[6] Pascoe, D., Karntanut, W., & Muller, C. T. (2003). Do pharmaceuticals affect freshwater invertebrates? A study with the cnidarian Hydra vulgaris. *Chemosphere*, 51(6), 521-528.

[7] Krahn, M. M, Hanson, M. B, Schorr, G. S, Emmons, C. K, Burrows, D. G, Bolton, J. L, et al. (2009). Effects of age, sex and reproductive status on persistent organic pollutant concentrations in "Southern Resident" killerwhales. *Marine Pollution Bulletin*, 58(10), 1522-1529.

[8] Tapaneeyakul, N. (2008). Perspective of Ecotoxicological Conduction for Water Quality. *Monitoring in Thailand. Interdisciplinary Studies on Environmental Chemistry- Biological Responses to Chemical Pollutants. 1st ed.*, Thailand, TERRAPUB, 31-35.

[9] Directive 2004/27/EC of the European Parliament and of the Council of 31 March 2004 amending Directive 2001/83/EC on the Community code relating to medicinal products for human use. Article 1. Published March 31, 2004. Accessed 17 August 2008. 2004

[10] Halling-Sorensen, B., Nielsen, S., Lanzky, P., Ingerslev, F., Lutzhoft, H., & Jorgensen, S. (1998). Occurrence, fate and effects of pharmaceutical substances in the environment- A review. *Chemosphere*, 36(2), 357-394.

[11] Beausse, J. (2004). Selected drugs in solid matrices: a review of environmental determination, occurrence and properties of principal substances. *Trac-Trends in Analytical Chemistry*, 23(10-11), 753.

[12] Gomez, M., Petrovic, M., Fernandez-Alba, A., & Barcelo, D. (2006). Determination of pharmaceuticals of various therapeutic classes by solid-phase extraction and liquid chromatography-tandem mass spectrometry analysis in hospital effluent wastewaters. *Journal of Chromatography A*, 1114(2), 224-233.

[13] Ramirez, A. J., Mottaleb, M. A., Brooks, B W., & Chambliss, C K.. (2007). Analysis of pharmaceuticals in fish using liquid chromatography-tandem mass spectrometry. *Analytical Chemistry*, 79(8), 3155-3163.

[14] Hirsch, R., Ternes, T., Haberer, K., & Kratz, K. (1999). Occurrence of antibiotics in the aquatic environment. *Science of Total Environment*, 225, 1-2, 109-118.

[15] Lacina, P. (2012). Appplication of gas chromatography with mass spectrometric detection for the assessment of drug-residue contamination of wastewaters and surface waters. *PhD thesis. Faculty of Chemistry, Brno University of Technology*.

[16] Waksman, S. A. (1947). What Is an Antibiotic or an Antibiotic Substance? *Mycologia*, 39(5), 565-569.

[17] Watkinson, A. J., Murby, E. J., & Costanzo, S. D. (2007). Removal of antibiotics in conventional and advanced wastewater treatment: Implications for environmental discharge and wastewater recycling. *Water Research*, 41(18), 4164-4176.

[18] Watkinson, A. J., Murby, E. J., Kolpin, D. W., & Costanzo, S. D. (2009). The occurrence of antibiotics in an urban watershed: From wastewater to drinking water. *Science of Total Environment*, 407(8), 2711-2723.

[19] Lilly, E., Bunch, R. L., & McGuire, J. M. Erythromycin, its salts, and method of preparation. *U.S.A. patent 2,653,899*.

[20] Dang, V., Nandan, N., Cooper, T. W., Greenfield, R. A., & Bronze, M. S. (2007). Part VII. Macrolides, azalides, ketolides, lincosamides, and streptogramins. *Journal of the Oklahoma State Medical Association*, 100(3), 75-81.

[21] Luckenbach, T., & Epel, D. (2005). Nitromusk and polycyclic musk compounds as long-term inhibitors of cellular xenobiotic defense systems mediated by multidrug transporters. *Environmental Health Perspectives*, 113(1), 17-24.

[22] Schramm, K., Kaune, A., Beck, B., Thumm, W., Behechti, A., Kettrup, A., et al. (1996). Acute toxicities of five nitromusk compounds in Daphnia, algae and photoluminescent bacteria. *Water Research*, 30(10), 2247-2250.

[23] Sousa, J. V., & Suprenant, D. C. (1984). Acute Toxicity of 1618.02 (musk xylene) to Bluegill (Lepomis macrochirus). Report #BW-84-2-1549.

[24] Tas, J., Balk, F., Ford, R., & van de Plassche, E. (1997). Environmental risk assessment of musk ketone and musk xylene in the Netherlands in accordance with the EU-TGD. *Chemosphere*, 35(12), 2973-3002.

[25] Daughton, C., & Ternes, T. (1999). Pharmaceuticals and personal care products in the environment: Agents of subtle change? *Environmental Health Perspectives*, 107, 907-938.

[26] Salvito, D. (2005). Synthetic musk compounds and effects on human health? *Environmental Health Perspectives*, 113(12), A802-A803.

[27] Sommer, C. (2004). The role of musk and musk compounds in the fragrance industry. Rimkus GG, editor, *Synthetic musk fragrances in the environment* (1st ed.), Berlin-Heidelberg-New York, Springer-Verlag, 1-16.

[28] Tanabe, S. (2005). Synthetic musks - arising new environmental menace? *Marine Pollution Bulletin*, 50(10), 1025-1026.

[29] Dietrich, D. R., & Chou, Y. (2001). Ecotoxicology of musks. Daughton CG, Jones-Lepp TL, editors. Pharmaceuticals and Personal Care Products in the Environment: Scientific and Regulatory Issues: American Chemical Society , 156-167.

[30] Ruzicka, L. (1926). Zur Kenntnis des Kohlenstoffringes I. Über die Konstitution des Zibetons. *Helv Chim Acta*, 9, 230-248.

[31] Ruzicka, L. (1926). Zur Kenntnis des Kohlenstoffringes VII. Über die Konstitution des Muscons. *Helv Chim Acta*, 9, 715-729.

[32] Kraft, P. (2004). 'Brain aided' musk design. *Chemical Biodiversity*, 1(12), 1957-1974.

[33] Eh, M. (2004). New alicyclic musks: The fourth generation of musk odorants. *Chemical Biodiversity*, 1(12), 1975-1984.

[34] Schroeder, H. F., Tambosi, J. L., Sena, R. F., Moreira, R. F. P. M., Jose, H. J., & Pinnekamp, J. (2012). The removal and degradation of pharmaceutical compounds during membrane bioreactor treatment. *Water Science and Technology*, 65(5), 833-839.

[35] Yoon, Y., Westerhoff, P., Snyder, S. A., Wert, E. C., & Yoon, J. (2007). Removal of endocrine disrupting compounds and pharmaceuticals by nanofiltration and ultrafiltration membranes. *Desalination*, 16 -23 .

[36] Narvaez, J. F. V., & Jimenez, C. C. (2012). Pharmaceutical Products in the Environment: Sources, Effects and Risks. *Vitae Revista De La Facultad De Quimica Farmaceutica*, 19(1), 93-108.

[37] Kim, J. W., Jang, H. S., Kim, J. G., Ishibashi, H., Hirano, M., Nasu, K., et al. (2009). Occurence of pharmaceutical and personal care products (PPCPs) in surface water from Mankyung river South Korea. *Journal of Health Science*, 55(2), 249-258.

[38] Zlámalová Gargošová, H., Vávrová, M., Doležalová Weissmannová, H., Mravcová, L., Vydrová, L., & Zouhar, L. (2011). Waste Water Quality Monitoring: The Use of Methods of Environmental Analysis and Ecotoxicological Tests in the Evaluation of Wastewater. In: Einschlag FSG, editor, *Waste Water Evaluation and Management*, 3-30.

[39] Twardowska, I. (2004). Ecotoxicology, environmental safety, and sustainable development- challenges of the third millennium. *Ecotoxicology and Environmental Safety*, 58(1), 3-6.

[40] Hoffman, D., Rattner, B. A., Burton Jr, G. A., & Cairns, J., Jr. (2003). Handbook of ecotoxicology. 2nd ed., Boca Raton, Lewis Publishers.

[41] US EPA ECOTOX database. (2007). http://cfpub.epa.gov/ecotox/.

[42] Pessala, P., Schultz, E., Nakari, T., Joutti, A., & Herve, S. (2004). Evaluation of wastewater effluents by small-scalebiotests and a fractionation procedure. *Ecotoxicology and Environmental Safety*, 59(2), 263-272.

[43] OSPAR-PRAM. (2000). Whole Effluent Assessment within the Framework of OSPAR, PRAM 00/5/5-E. *2000 Pram 00/5/5-E(OSPAR-PRAM)*.

[44] Mendonca, E., Picado, A., Paixao, S. M., Silva, L., Cunha, M. A., Leitao, S., et al. (2009). Ecotoxicity test in the environmental analysis of wastewater treatment plants: Case study in Portugal. *Journal of Hazardous Materials*, 665 -670.

[45] Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. 2006;1907/2006(REACH).

[46] Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH). 2008;440/2008(REACH).

[47] Globally Harmonized System of classification and labeling of chemicals (GHS). (2011). Annex 8, Guidance on hazards to the aquatic environment. 4 st edition(http://www.unece.org/trans/danger/publi/ghs/ghs_rev04/04files_e.html).

[48] Commission Regulation (EC) No 761/2009 of 23 July 2009 amending, for the purpose of its adaptation to technical progress, regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registrarion, Evaluation, Autorization and Restriction of Chemicals (REACH). 2009;761/2009(REACH).

[49] Regulation (EC) no 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. 2008.

[50] ISO/TS 20281. (2006). Water quality- Guidance on statistical interpretation of ecotoxicity data.

[51] Enserink, L., de la Haye, M., & Maas, H. (1993). Reproductive strategy of *Daphnia magna*: Implications for chronic toxicity tests. *Aquatic Toxicology*, 25(1-2), 111.

[52] Koivisto, S. (1995). Is Daphnia magna an ecologically representative zooplankton species in toxicity tests? *Environmental Pollution*, 90(2), 263-267.

[53] ASTM. (2000). Standard Guide for Conducting Three-Brood, Renewal Toxicity Test with Ceriodaphnia dubia, Method E 1295-89. *Annual Book of ASTM Standards*, 11.05, ASTM, Philadelphia, PA.

[54] ISO 6341. (1996). Water quality - Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) - Acute toxicity test. Edition 3, (monolingual), 9.

[55] OECD. (2004). Guidelines for the testing of Chemicals/Section 2: Effects on Biotic Systems: Test 202 *Daphnia* sp. *Acute Immobilisation Test*, OECD Code: 979920201E1, OECD Publishing.

[56] OECD. (1998). Guideline for the Testing of Chemicals. *Daphnia magna* Reproduction Test 211. OECD, Paris, France.

[57] ASTM. (2000). Standard Guide for Conducting *Daphnia magna* Life-Cycle Toxicity Tests, Method E 1193-97. *Annual Book of ASTM Standards*, 11.05, AST, Philadelphia, PA.

[58] U.S. EPA. (1996). Daphnid chronic toxicity test. Ecological Effects Test Guidelines, OPPTS 850.1300. *U.S. Environmental Protection Agency, Washington, OC.*

[59] ISO. (2000). Water Quality. Determination of Long Term Toxicity of Substances to *Daphnia magna* Straus (Cladocera, Crustacea). *Intemational Standard ISO*, 10706, Geneva, Switzerland.

[60] Palma, P., Alvarenga, P., Palma, V., Matos, C., Fernandes, R. M., Soares, A., et al. (2010). Evaluation of surface water quality using an ecotoxicological approach: a case study of the Alqueva Reservoir (Portugal). *Environmental Science and Pollution Research*, 17(3), 703-716.

[61] Palma, P., Palma, V. L., Fernandes, R. M., Soares, A. M. V. M., & Barbosa, I. R. (2008). Acute toxicity of atrazine, endosulfan sulphate and chlorpyrifos to *Vibrio fischeri*, *Thamnocephalus platyurus* and *Daphnia magna*, relative to their concentrations in surface waters from the Alentejo region of Portugal. *Bull Environ Contam Toxicol*, 81(5), 485-489.

[62] Daniel, M., Sharpe, A., Driver, J., Knight, A., Keenan, P., Walmsley, R., et al. (2004). Results of a technology demonstration project to compare rapid aquatic toxicity screening tests in the analysis of industrial effluents. *Journal of Environmental Monitoring*, 6(11), 855-865.