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POTENTIAL EFFECTS OF PHARMACEUTICALS AND THEIR RESIDUES IN AQUATIC ENVIRONMENT

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Abstract: The presence of pharmaceuticals, their metabolites and transformation products in the aquatic environment has become an increased concern in the last decades. The widespread presence of medicines in the environment can be explained by extensive use in medical practices and incomplete removal in Waste Water Treatment Plant. This paper provides a summary of knowledge about occurrence, fate and effects of more frequently used pharmaceuticals and some experimental data about accumulation properties of oxytetracycline in environmental and fish samples.

Keywords: pharmaceuticals, tetracycline, effects, bioaccumulation, elimination half-life.

INTRODUCTION

Occurrence and fate of emerging environmental pollutants

Increasing quantities of pollutants are released into nature due to industrial production, modern plant protection and intensive use of medicines. The effect on the homeostasis of different artificial substances (xenobiotics) has been demonstrated even in the thirteen's of last century (Dodds and Lawson, 1938). Among others, the contaminant effect of medicines and endocrine-disrupting chemicals has become evident only in the last decades (Colborn et al., 1996), when it was disclaimed to a have potential danger to human.

Human and veterinary pharmaceuticals have become a class of emerging environmental contaminants. Literature often treats medicines and household products as one group (Pharmaceutical and Personal Care Products, PPCP) because of the similarity of the pollution source. In the European Union about 3000 different substances are used in human medicine such as analgesics and anti-inflammatory drugs, contraceptives, antibiotics, beta-blockers, lipid regulators, neuroactive compounds and many others. Also large numbers of pharmaceuticals are used in veterinary medicine, among them antibiotics and anti-inflammatories. The amount of produced antibiotics is 100,000-200,000 tons/year in the world; sales of non steroid anti-inflammatory drugs are also relatively large (Fent et al., 2006), in the range of hundreds of tons per year. For example in 2004 in Hungary 7764 kg of diclofenac were sold (Záray, 2006).

Pharmaceuticals are excreted in their native form or as metabolites after application and enter into aquatic system via different ways. The main pathway from humans is ingestion and disposal via wastewater. The pharmaceuticals not readily degraded in the sewage treatment plant (STP) are being discharged in treated effluents resulting in the contamination of rivers (Pouliquen, 2009; Minh, 2009), lakes, estuaries and rarely groundwater and drinking water. Where sewage sludge is applied to agricultural fields, contamination of soil, run-off into surface water, but also drainage may occur. In addition, veterinary pharmaceuticals may enter aquatic system via manure application to field, but also via direct application in aquaculture (fish farming).

These compounds are present in the environment as persistent compounds (Daughton, 2003) because might accumulate in the different organisms trough bioaccumulation and biomagnifications. Monitoring of these contaminants, - that were previously neglected, often considered harmless, however, have significant effects - has become possible due to the up-to-date analytical methods and instruments. In the last decade in monitoring studies of aquatic environment around hundred of pharmaceuticals and their metabolites have been detected (Jones et al., 2002). Some the most frequently measured substances are listed in the Table 1. where metabolites and by-products of pharmaceuticals also have to be considered. Unfortunately, analysis of occurrence and toxicity of all active pharmaceuticals is impractical. Occurrence studies are complicated by the large number of substances, many of which are biologically degraded to active metabolites that should be accounted for. Current analytical tools can quantify several analytes, however, availability of suitable standards, especially for drug metabolites, is limited.

Table 1. Levels of some pharmaceuticals detected in surface water monitoring studies (from Boxall ABA, 2004)

Medicine class	Substances detected	Maximum concentrations (ng L^{-1})
Antibiotics	Chloramphenicol	355
	Chlortetracycline	690
	Oxytetracycline	140
	Tetracycline	110
	Ciprofloxacin	30
	Sulphametazine	220
	Norfloxacin	120
Analgesic	Codeine	1000
	Acetylsalicilic acid	340
	Carbamezapine	1100
	Diclofenac	1200
	Ketaprofen	120
	Naproxen	190
	Phenazone	950
	Indomethacine	200
Antidepressant	Fluoxetine	12
Antihyperlipidemic	Gemfibrozil	790
Antidiabetic	Metfarmin	150
Anti-inflammatory	Ibuprofen	3400
Antiseptic	Triclosan	150
Beta blockers	Carazolol	110
	Metoprolol	2200
Contraceptive	17a-Athinylestradiol	4.3
Lipid regulator	Benzafibrate	3100

EFFECTS AND RISK

Potential effect of pharmaceuticals and their residues in aquatic environment are in most cases restricted to short-term acute responses such as lethality in algae, invertebrates and fish (Webb, 2001). Chronic effects of pharmaceuticals are scarcely investigated. However, long-term exposures are needed for an accurate environment risk assessment. Toxicity testing, based on acute endpoint, such as EC50 bacterial luminescence (30min), algae growth (96h) or Daphnia magna mortality (48h) suggests a rather low acute toxicity of some pharmaceuticals (Ferrari et al., 2003, 2004), but results can be found about lethality and teratogenicity in diclofenac exposed zebra fish embryos after 96h exposure to 480 ± 50 µg/L. (Dietrich and Prietz (1999), or estrogenic and carcinogenic effect of diclofenac to Japanese medaka fish juveniles in a 4 days experiment (Hong, et al., 2007). Brooks et al. (2005) describe accumulation of antidepressants in fish and other aquatic organisms residing in effluent dominated or influenced water bodies. Some literature data (Schwaiger et al, 2004; Brown et al., 2007, Oaks et al. 2004) has shown elevated accumulation of diclofenac in aquatic tissues, (up to 2700x in fish organs) despite its low lipid solubility. Han et al. (2010) explain in their paper, that chronic effect of ibuprofen could have interaction with oestrogen homeostasis of medaka fish. Mixture interactions of PPCPs could not be ignored. In some cases elevated effects were observed using of medicine mixture than single drug (Cleuvers, et al., 2004), in other experiments was not possible to draw conclusions from the mixture toxicity (Dietrich, et al., 2010).

The most dangerous environmental pollutants of the medicines are the antibiotics, which may contribute to the evolution of resistant pathogen bacteria. An increasing number of studies also have docu-

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mented elevated levels of bacterial antibiotic resistance in around aquaculture production environments. Bacterial antibiotic resistance arises and is maintained trough mutations in bacterial DNA or trough horizontal gene transfer mechanisms including conjugation with other bacteria, transduction with bacteriophage, and the uptake of free DNA via transformation (Fuhrman, 1999; Bushman, 2002; Casas et al., 2005). It was also demonstrated that development of bacterial antibiotic resistance in aquaculture environments could contribute to or influence bacterial antibiotic resistance occurring among human populations. A study by Rhodes et al. (2000) investigated the transfer of oxytetracycline resistance plasmids between aquaculture and hospital isolates, and found direct evidence that resistance plasmids have been transferred between Aeromonas spp. (fish isolates) and E. coli (human isolates) in specific geographic locations.

In addition to selecting for bacterial antibiotic resistance, the great use of antibiotics in aquaculture environments can lead to elevated antibiotic residues in pond, marine sediments, aquaculture products, wild fish and natural aquatic environments that are impacted by aquaculture facilities. Many antibiotics are quite persistent, most part of the antibiotics are excreted without modification (e.g. Amoxicillin: 80-90%); in addition, their conjugates can turn into the original compound in the nature or in wastewater treatment plants. Significant amounts of antibiotics are adsorbed on wastewater sludge, and thus, in anaerobic conditions, they become less susceptible to degradation than in water where they are degraded as a result of solar radiation. They can also damage the flora of biological wastewater treatment systems, which reduces the removal efficiency of other substances. Many authors have described the adsorption and accumulation of antibiotics in sediment, and it was found measurable residues in soils following the application of manure containing antibiotics (Table 2.). Some antibiotics are taken up by vegetables from manure treated soils such as carrot roots, lettuce leaves (Boxall et al., 2006), and corn (Kumar et al., 2005). The tetracycline and sulfamethazine concentrations measured in plant tissues were small (2-17 µg kg -1 fresh weight), but these concentrations increased with increasing amount of antibiotics present in the manure (Kumar et al., 2005). These exposure levels, in summary, do not present appreciable risk but may elicit subtle effects over prolonged periods or when exposure is occurring via a number at routes at once (Boxell et al., 2006). Some antibiotics often used in fish farming were detected also in the river's flora. For example, bryophyte Fontinalis antipyretica Hedw. was found to strongly accumulate OTC in freshwater (Pouliquen et. al., 2009, Delépée at al., 2009). Several results are available on the toxicity of antibiotics for aquatic plants (Migliore, et al 2000, Hanson et al, 2006, Brain et al., 2004), but these studies are based on acute toxicity tests. For example 160 mg kg -1 oxytetracycline is phytotoxic to Phaseolus vulgaris L. under laboratory conditions (Jjemba, 2002). In short, regardless of the amount of research that have been carried out about presence and risk of different pharmaceuticals, metabolites and residues, more information is necessary to estimate the potential risks of these dangerous compounds.

Table 2. Oxytetracycline values detected in sediment and soil monitoring studies

type of the samples	concentration values µg kg $^{-1}$	literature
marine sediment near fish farm	max 900 (w.w.)	Capone, et al., 1996
river sediment	max 200 (w.w.)	Pouliquen et al., 2009
sediment from fish farms	min 0.1 max 246 (d. w.)	Lalumera et al., 2004
manure from animal farm	min 50 max 450 (w.w.)	Karci and Balcioglu, 2009
soil from agricultural field	min 20 max 500 (w.w.)	Karci and Balcioglu, 2009

Improving our knowledge, antibiotic contents of composted water sludge was determined in a Hungarian waste water treatment plant and some medicinal treatment experiments was carried out with common carp.

The aim of our measurements was to study the accumulation and biodegradation of oxytetracycline in composted waste water sludge, moreover in the fish tissues after drug administration.

MATERIAL AND METHODS

In the first part, some month old composted sludge was collected in waste water treatment plants, which are re-used in agriculture practises. Two waste water plants were sampled, which are slightly different in number of population of the cities. The samples were kept in refrigerator at -30°C until measurements.

Healthy common carp weighing 300-500 g were selected and set in 500 l recirculation tanks, supplied with aerated freshwater at a mean 23.5°C temperature. The fish were divided into two thanks and were acclimatized during one week. OTC was given to common carp through medicated feed for 7 consecutive days followed by a period of feeding without antibiotic for another 7 days. The diet type was Scretting diet spiked with OTC, (doses: 120 mg/kg body weight/day). Feeding rate was 2.5 % body weight. Three carp samples were randomly selected from the thanks and sampled at each sampling time during the feeding period and withdrawal period. Muscle +skin and liver samples were collected 21 times during the experiment and frozen at -30°C. Control fish were sampled before the initiation of the experiment.

In the second fish experiment 30 pieces of common carp were selected with body weight 1500-2200 g. 133 mg/kg body weight/day of OTC single dose was applied to fish with intraperitoneal injection method. Water temperature in the fish tanks during the experiment was 20.9 °C. Blood serum was collected from three different fish in each sampling time during 9 days.

Chemicals

Oxytetracycline hydrochloride analytical standard was supplied from Vetranal FLUKA. The ANITETRA with 48% OTC content and TETRAWET L.A. injection were obtained form veterinary pharmacy. Citric acid monohydrate, EDTA sodium salt, sodium diphosphate, calcium dichloride dehydrate, sodium acetate was purchased from SIGMA. The HPLC grade distillate water was produced by Millipore Milli-Q academic system.

Sample analysis

OTC residues in common carp muscle plus skin tissues and liver were determined by the method Maia et al., 2008 with slight modification. The Waters 2695 HPLC system comprised a Waters 470 Fluorescence detector, an automatic injection system, vacuum degasser, reversed-phase analytical column Nova-Pak C-18 (Waters Co., USA), 4 µm; 3,9 x 300 mm with guard column Nova-Pak C-18. The mobile phase was: 70 % water, with 0,035 M CaCl2*2H20 , 0,025 M EDTA, 0,075 M CH3COONa, and 30% methanol, adjusted to pH=7,3. The excitation wavelength was at 390 nm, the emission at 512 nm. The column temperature was 20 °C, injection volume 20 µl, flow rate 0,5 ml/min in isocratic mode. Under these conditions, the retention time of OTC was 8.7 min and the detection limit of OTC ≥ 5 ng/ml.

Tissue samples (1g) were homogenized with 10 ml McIlvaine buffer (citric acid monohydrate, Na2HPO4, EDTA sodium salt at pH 3,8) and centrifuged for 10 min at 4000 rpm. The supernatant was retained and the procedure repeated. To the combined supernatants, 10 ml hexane was added and the solutions centrifuged at 4000 rpm for 5 min. The aquatic layer containing the OTC, was evaporated to dryness under nitrogen at 40 °C and resuspended in 1 ml of HPLC mobile phase solvent, vortexed and filtered (0.45 µm).

The blood serum samples (250µl) were homogenized with 5 ml McIlvaine buffer on tube rotator still 3 minutes, and in ultrasound homogenizer 20 minutes more incubation was applied. Degreasing step was evaluated in 5 ml hexane and after centrifugation at 4000 rpm for 5 min. The aqueous layer was used for the analysis. The sludge samples were extracted in the same way as muscle samples, only the extraction step with hexane was left.

Data acquisition and peak integration was performed by the software Millennium Chromatography Manager (Waters). A matrix standard calibration curve at concentrations 0, 5, 10, 25, 50, 100, 250, 500 µg kg-1 was prepared for OTC muscle plus skin samples. The matrix calibration curve characteristics were

R2 =0.999, the mean method validated recovery was between 72 and 112 %.

The total tetracycline (parent drug + metabolite) amount in muscle samples and sludge was determined by ELISA assay method using R-Biopharm Ridascreen® Tetracycline (Art. No.: R3501) test kit. This is a competitive enzyme immunoassay for tetracycline, minocycline, rolitetracycline, chlortetracycline in milk, honey and meat samples. All reagents required for enzyme immunoassay are contained in the test kit. Sample extraction was carried out with McIlvaine buffer, using same method described above. For sample purification Rida ® C18 column was used. A microtiter plate spectrophotometer (ELISA READER) was used for quantification of absorbance at 450 nm.

RESULTS AND DISCUSSION

In the composted waste water sludge samples we measured min 8.3 max 280 µg kg-1 fresh weight OTC concentration and in each sub-sample detectable amount were found (Table 3.). Differences between sub-samples are due to the inhomogeneity of the samples, and to the difference in the adsorption and degradation of antibiotics with the structure and microbial activity of the sludge.

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Table 3. OTC levels (µg kg-1 fresh weight) in the waste water composted sludge

Sample code	Concentration µg kg^{-1} fresh weight HPLC	Concentration µg kg^{-1} fresh weight ELISA
BCs 1	16.40	14.87
BCs 2	25.95	12.46
BCs 3	128.26	19.55
BCs 4	27.29	15.40
BCs 5	21.70	20.83
Sz 16	18.83	19.84
Sz 17	8.95	14.75
Sz 18	8.34	21.05
Sz 19	280.00	23.82
Sz 20	140.24	19.42

Figures 1.and 2 show elimination of drug residues from muscle and liver of common carp. The samples have been taken before daily feeding. On the first diagram it can be seen that elimination of OTC is very slowly in muscle, after eight days of post treatment period the concentration values are decreased to the half of starting concentration level. In the liver samples a constant level of OTC is observed in the 15 to 20 mg kg -1 range after drug administration. In the control samples of liver we measured a mean 10 mg kg -1 OTC concentrations. These quantity possible remains from an earlier drug treatment of fish about two month before our experiment.

The elimination processes in the living organism follow first order kinetics based on exponential equations. From these equation the elimination half-lives of drug were calculated (Table 4). Comparing these elimination half-lives values the shortest value can be seen for the blood, followed by muscle and liver. Concentration values of OTC in blood samples are shown on Figure 3.

Concentration data were determined for total active tetracycline molecules with ELISA technique in the muscle plus skin samples. Detectable amount of active components was found in the control samples, as well.

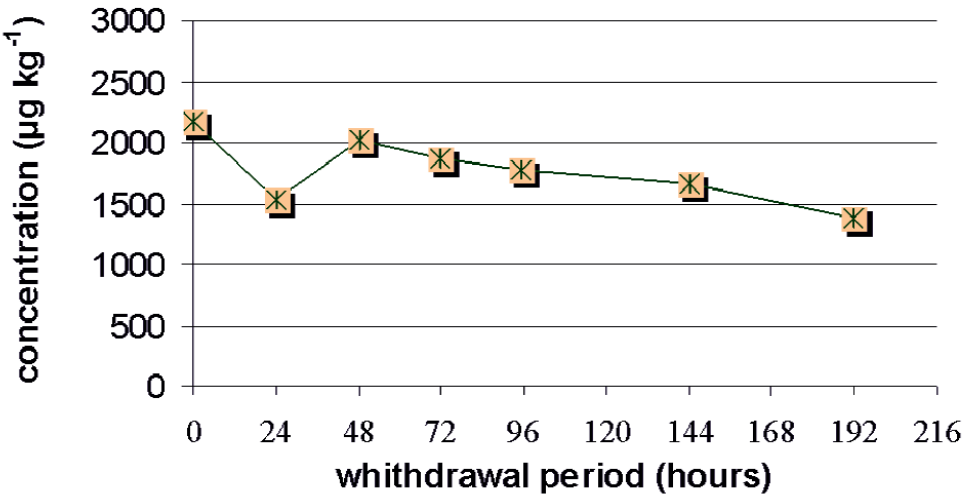

Figure 1. Elimination of OTC from muscle after drug administration to fish

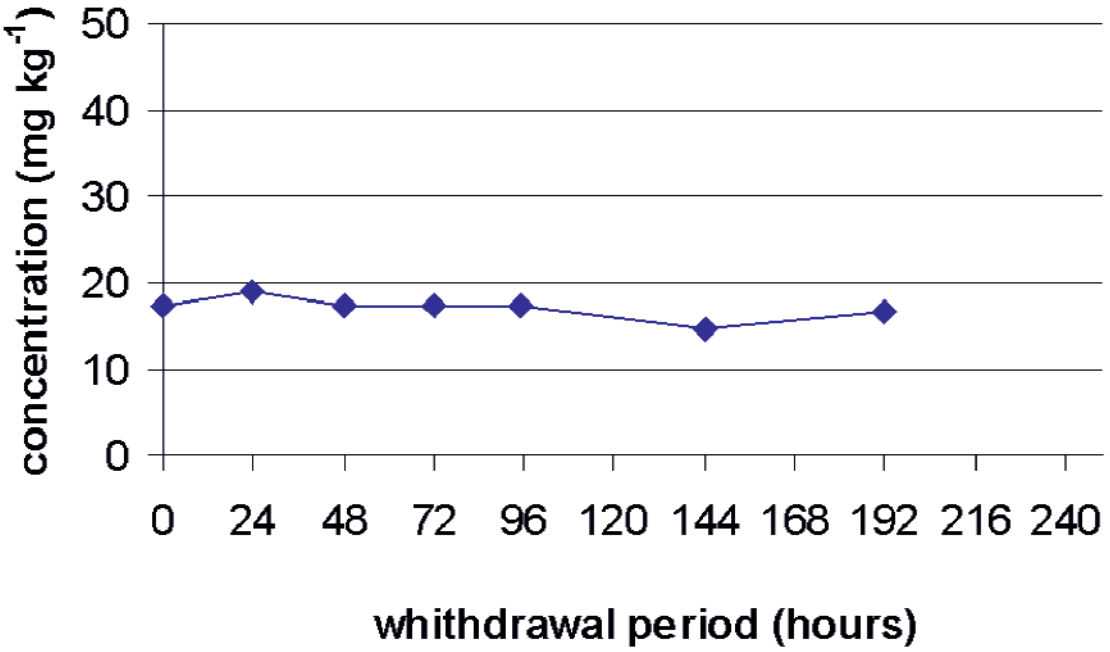

Figure 2. Elimination of OTC from liver after drug administration to fish

Figure 3. Elimination of OTC from blood after a single dose administere d intraperitoneally to fish

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Table 4. Elimination half-lives and maximum concentrations of OTC in fish blood, muscle and liver

Type of sample	Elimination half-life $t_{½}$	Maximum concentrations
Liver	32 days	22000 µg kg^{-1} fresh weight
Muscle	11.6 days	2400 µg kg^{-1} fresh weight
Blood	6.4 days	149 µg mL^{-1}

CONCLUSION

The concentration values and elimination half-lives measured in tissues and blood confirm the accumulation of persistent OTC and its metabolites in drug-treated animals. After some months of the treatments, residues of OTC can be detected in the liver of common carp and active OTC molecules in the muscle plus skin. These measurements confirm a longer residence of this antibiotic in the body of fish and sediment that was supposed before.

Finding of other research studies are in good correlation with our results. High amount of antibiotics might accumulate in the water sludge and their degradation is more slower under these anaerobe conditions than it is in the water where effect of sunshine might increase the decomposition of these compounds. So thus residues of medicines, like antibiotics might not decreased trough the treatments of waste water treatment plants.

Based on these observations we would like to call your attention to the fact that presence of different pharmaceuticals in the ecosystem is highly risky.

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