Eesti Maaülikool
Estonian University of Life Sciences

RESIDUES OF SOME PHARMACEUTICALS IN SEWAGE SLUDGE IN ESTONIA, THEIR STABILITY IN THE ENVIRONMENT AND ACCUMULATION INTO FOOD PLANTS VIA FERTILIZING

MÕNEDE RAVIMIJÄÄKIDE SISALDUS EESTI REOVEESETTES,
NENDE STABIILSUS KESKKONNAS
JA AKUMULEERUMINE KOMPOSTVÄETISEST TOIDUTAIMEDESSE

MERIKE LILLENBERG

PhD Thesis
in Environmental Protection

Väitekiri
filosoofiadoktori kraadi taotlemiseks keskkonnakaitse erialal

Tartu 2011

EESTI MAAÜLIKOOL
ESTONIAN UNIVERSITY OF LIFE SCIENCES

Eesti Maaülikool
Estonian University of Life Sciences

RESIDUES OF SOME PHARMACEUTICALS IN SEWAGE SLUDGE IN ESTONIA, THEIR STABILITY IN THE ENVIRONMENT AND ACCUMULATION INTO FOOD PLANTS VIA FERTILIZING

MÕNEDE RAVIMIJÄÄKIDE SISALDUS EESTI REOVEESETTES, NENDE STABIILSUS KESKKONNAS JA AKUMULEERUMINE KOMPOSTVÄETISEST TOIDUTAIMEDESSE

MERIKE LILLENBERG

PhD Thesis
in Environmental Protection

Väitekiri
filosoofiadoktori kraadi taotlemiseks keskkonnakaitse erialal

Tartu 2011

Institute of Agricultural and Environmental Sciences
Estonian University of Life Sciences

According to verdict No 83 of July 20, 2011, the Doctoral Commitee of the Agricultural and Natural Sciences has accepted the thesis for the defence of the degree of Doctor of Philosophy in Environmental Protection.

Opponent: Prof. Sergey V. Mikhalovsky, PhD, CChem, FRSC
School of Pharmacy and Biomolecular Sciences
University of Brighton, UK

Supervisors: Prof. Lembit Nei, PhD
Department of Environmental Protection
Tartu College of Tallinn University of Technology, Estonia

Prof. Kalev Sepp, PhD
Department of Landscape Management and Nature Conservation
Institute of Agricultural and Environmental Sciences
Estonian University of Life Sciences, Estonia

Defence of the thesis:
Estonian University of Life Sciences, Karl Ernst von Baer House, Veski St 4, Tartu
on September 16, 2011, at 12:00.

The English in the current thesis was revised by Villem Aruoja and the Estonian by Jaagup Alaots.

The publication of this dissertation is granted by the Estonian University of Life Sciences and by the Doctoral School of Earth Sciences and Ecology created under the auspices of European Social Fund.

© Merike Lillenberg, 2011

ISBN 978-9949-484-02-7

To my son Lembit Lillenberg

CONTENTS

ABSTRACT

Environmental pollution caused by sewage sludge has become a global problem. Most wastewater treatment processes produce sludge that has to be disposed of. Therefore, novel efficient technologies of re-use of this waste are developed with the aim of reducing its impact on the environment. Sewage sludge can be used as an alternative to the fossil fuels. Unfortunately, incineration is costly and contributes to air pollution, and landfill space is becoming scarce.

Sewage sludge contains useful organic matter and nutrients for plants. "Intelligent" treatment technology could transform this hazardous waste into an excellent fertilizer. Composting or aerobic biological treatment of organic wastes is an ancestral way to reduce wastes and to reuse organic matter. The usefulness of compost depends on its environmental compatibility and the correspondence to market necessities. These features involve the absence of toxic inorganic and organic substances, which may enter the food chain through the plant uptake. Among these, pharmaceuticals are frequently present in sewage sludge. Their quantities rise from year to year. In spite of the fact that very low drug levels in the environment can have undesirable ecological and health effects, until now the problems related to the presence of pharmaceuticals in sewage sludge and its compost have received little attention. There exist no trigger values for drug residues in sewage compost in the European Union. Drug residues may accumulate into plants. As a result, concentrations of drug residues in food plants may exceed the maximum residue limits (MRL) for meat and milk. No limits have been set for drug residues in plant products at present.

The aim of the current work was to study the presence of some widely used pharmaceuticals in Estonian sewage sludge and its compost and the uptake of these pharmaceuticals from fertilized soils by some food plants. The selection of pharmaceuticals was made considering the level of consumption, stability in soil and potential accumulation into plants. These drugs include fluoroquinolones (ciprofloxacin $C_{17}H_{18}FN_3O_3$, norfloxacin $C_{16}H_{18}FN_3O_3$ and ofloxacin $C_{18}H_{20}FN_3O_4$), sulfonamides (sulfadimethoxine $C_{12}H_{14}N_4O_4S$ and sulfamethoxazole $C_{10}H_{11}N_3O_3S$) and tetracyclines (tetracycline $C_{22}H_{24}N_2O_8$ and doxycycline $C_{22}H_{24}N_2O_8$). It was presumed that the antimicrobials, marketed in Estonia in recent

years, should be present in sewage sludge, and their migration from fertilized soils into plants depends on their adsorption to soil particles.

The present work involved the following:

- Comparison of the efficiency of the sewage sludge composting technologies used in Tallinn and Tartu.

- Assessment of the safety of sewage sludge compost as a fertilizer.

- Recommendations concerning sewage sludge composting technologies and compost application.

The presence and content of the studied pharmaceuticals was determined in sewage sludge and in its compost of the two Estonian largest cities, Tartu and Tallinn. In Tartu the sewage sludge compost is made by mixing the raw sludge with tree bark. In Tallinn the methane fermentation and mixing with peat are used.

A new scheme for the quantitative determination of traces of fluoroquinolones, tetracyclines, and sulfonamides in sewage sludge and compost was developed: the compounds were simultaneously extracted from the matrix. In all samples the residues of fluoroquinolones and sulfonamides were present, but tetracyclines were not detected. The highest contents of fluoroquinolones were: 442 μg/kg (ciprofloxacin) and 439 μg/kg (norfloxacin) in Tartu; 1520 μg/kg (ciprofloxacin) and 580 μg/kg (norfloxacin) in Tallinn. All these concentrations exceed the trigger values (100 μg/kg) for manure. The contents of sulfonamides remained below the trigger value for manure. The highest concentrations in Tartu were 32 μg/kg (sulfadimethoxine) and 16 μg/kg (sulfamethoxazole); in Tallinn 73 μg/kg (sulfadimethoxine) and 22 μg/kg (sulfamethoxazole).

This study showed that the concentrations of fluoroquinolones and sulfonamides sufficiently varied both in sewage sludge and in compost. The concentrations of the studied pharmaceuticals in compost were significantly lower, if compared to the relevant concentrations in sewage sludge. This is partly caused by adding peat or tree bark to sewage sludge. Still, the main reason of the decrease in pharmaceutical concentrations during composting is the applied sludge treatment technology. The safest

way to prevent the exposure of plants to pharmaceuticals is to ensure that these substances are adequately degraded before sewage sludge compost is applied onto arable land. The decomposition of pharmaceuticals was faster in the case of anaerobic digesting and mixing with peat, used in Tallinn.

Humans may be exposed to residues of drugs in the environment by a number of routes including the consumption of crops that have accumulated substances from fertilized soils. As the compost made from sewage sludge contains detectable amounts of pharmaceutical residues, there was a need to determine the significance of uptake into plants from soil under "real" conditions as a potential migration route for pharmaceuticals in the environment. Therefore, the current study was conducted to determine the potential for fluoroquinolones and sulfonamides to be taken up by food plants from soil fertilized with sewage sludge or its compost. The results of this work clearly show that pharmaceuticals are able to accumulate in plants. This phenomenon remarkably depends on the nature and concentration of pharmaceuticals and the soil type. Before using the sewage sludge compost as a fertilizer, it should be carefully tested against the content of different pharmaceuticals. The content of pharmaceuticals in the compost made from sewage sludge may easily lead to the elevated concentrations in food plants, if the compost is used as a fertilizer.

This work should be continued by the development of novel and more efficient sewage sludge treatment technologies, leading to intelligent solutions of environmental problems related to sewage sludge exploitation. The current work is very timely, as drug residue analysis is a big topic in environmental chemistry, but only in recent years has the technology been developed sufficiently to quantify low levels in difficult matrices. It is novel for Estonia and unique in the world, being the first attempt to study the uptake of human medicine pharmaceuticals by food plants, grown in soils having "real" drug concentrations.

LIST OF ORIGINAL PUBLICATIONS

The present thesis is based on the following original articles referred to in the text by the Roman numerals I to VII:

I **Lillenberg, M.**, Roasto, M., Püssa, T. (2003). Drug residues in environment. Estimation of fluoroquinolones in soil and food plants. *Journal of Agricultural Science,* 14(1), 13-26;

II **Lillenberg, M.**, Yurchenko, S., Kipper, K., Herodes, K., Pihl, V., Sepp, K., Lõhmus, R., Nei, L. (2009). Simultaneous determination of fluoroquinolones, sulfonamides and tetracyclines in sewage sludge by pressurized liquid extraction and liquid chromatography electrospray ionization-mass spectrometry. *Journal of Chromatography A,* 1216, 5949-5954;

III Nei, L. and **Lillenberg, M.** (2009). Mackereth oxygen sensor: measurement uncertainty. *The Electrochemical Society Transactions,* 19(22), 55-63;

IV **Lillenberg, M.**, Yurchenko, S., Kipper, K., Herodes, K., Pihl, V., Lõhmus, R., Ivask, M., Kuu, A., Kutti, S., Litvin, S.V., Nei, L. (2010). Presence of fluoroquinolones and sulfonamides in urban sewage sludge and their degradation as a result of composting. *International Journal of Environmental Science and Technology,* 7(2), 307-312;

V **Lillenberg, M.**, Herodes, K., Kipper, K., Nei, L. (2010). Plant uptake of some pharmaceuticals from fertilized soils. *Proceedings of 2010 International Conference of Environmental Science and Technology, Bangkok, Thailand, 23-25 April, 2010*, 161-165;

VI Kipper, K., Herodes, K., **Lillenberg, M.**, Nei, L., Haiba, E.; Litvin, S.V. (2010). Plant uptake of some pharmaceuticals commonly present in sewage sludge compost. Proceedings of 2nd International Conference on Chemical, Biological and Environmental Engineering, Cairo, Egypt, 2-4 November, 2010, 261-264;

VII **Lillenberg, M.**, Litvin, S.V., Nei, L., Roasto, M., Sepp, K. (2010). Enrofloxacin and ciprofloxacin uptake by plants from soil. *Agronomy Research*, 8(1), 807-814.

The original articles have been reprinted with kind permission from the *Journal of Agricultural Science* (I), *Journal of Chromatography A* (II), *The Electrochemical Society Transactions* (III), *International Journal of Environmental Science and Technology* (IV), *Proceedings of 2010 International Conference of Environmental Science and Technology* (V), *Proceedings of 2nd International Conference on Chemical, Biological and Environmental Engineering* (VI) *and Agronomy Research* (VII).

Merike Lillenberg´s contribution to the original articles:

I sampled the soil and plant material, in co-operation with the third author elaborated the microbiological agar-diffusion method, analyzed the samples using the elaborated method, extracted the antibiotics form plant samples for HPLC analyzes, evaluated the results, wrote the manuscript.

II planned the studies, sampled the material, in co-operation with other authors elaborated the PLE method, SPE method and evaluated the results of LC-MS, wrote the manuscript.

III studied and assessed the reliability of oxygen measurement procedures used in sewage treatment, in cooperation with the first author wrote the manuscript.

IV planned the studies, sampled the material, in co-operation with other authors extracted antibiotics from samples by PLE, cleaned up the extracts by SPE and evaluated the results of LC-MS, wrote the manuscript.

V-VI planned the studies, prepared the soils and grew the plants, prepared the plant samples. In co-operation with other authors extracted the antibiotics by LE, cleaned the extracts by SPE, evaluated the results of LC-MS, wrote the manuscript.

VII sampled the soil and plant material, elaborated the microbiological agar-diffusion method, analyzed the samples by microbiological method, extracted the antibiotics form plant samples for HPLC analyzes, evaluated the results, wrote the manuscript.

ABBREVIATIONS

AM	antimicrobials
ADI	acceptable daily intake
ADI $_{mic}$	microbiological ADI
ADI $_{tox}$	toxicological ADI
CIP	ciprofloxacin
DOX	doxycycline
dm	dry matter
ELISA	enzyme-linked immunosorbent assay
ENR	enrofloxacin
EU 15	EU 15 member states before the enlargement of EU in 2004
FQs	fluoroquinolones
HLB	hydrophilic-lipophilic balance
HPLC	high performance liquid chromatography
LC-MS	liquid chromatography - mass spectrometry
LE	liquid extraction
LOD	limit of detection
LOQ	limit of quantification
MEC	minimum effect concentration
MIC	minimum inhibitory concentration
MRL	maximum residue level
n.d.	not detected
NOR	norfloxacin
OFL	ofloxacin
PLE	pressurized liquid extraction
SAs	sulfonamides
SCX	strong cation-exchange
SD	standard deviation
SDM	sulfadimethoxine
SMX	sulfamethoxazole
SPE	solid phase extraction
STPs	sewage treatment plants
TCs	tetracyclines
TCL	tetracycline
ww	wet weight

1. INTRODUCTION

Sewage sludge is the residue from the treatment of domestic and industrial wastewater. Sewage sludge contains useful organic matter and nutrients for plants (Kaonga *et al.*, 2010). The contents of potassium, nitrogen and phosphorus are 10-100 times higher in sewage sludge and its compost, if compared to common Estonian agricultural soils (Lillenberg *et al.*, 2010[1]). Composting or aerobic biological treatment of organic wastes is an ancestral way to reduce wastes and to reuse organic matter. Sewage sludge composting enables the production of a quality product that may be used as a soil conditioner or as an organic fertilizer (Tremier *et al.*, 2005), since its organic matter content may vary from 50% to 70% of the total solids content (Banegas *et al.*, 2007). Compost is a relatively stable material similar to humus. It is fine textured and has low moisture. Still, its use as a fertilizer is restricted due to a large number of biological and chemical pollutants found in it (EU Council Directive 86/278/EEC, 1986).

Environmental pollution caused by sewage sludge has become a very serious problem (Lu *et al.*, 2009). According to the EU legislation (EU Council Directive 86/278/EEC, 1986) the sewage sludge is allowed to be used as an agricultural fertilizer, if it is treated to be safe to surface and ground water, soil, plants, animal and human health. It is prohibited to use untreated raw sewage sludge in agriculture. The sludge can be made safe by different treatment technologies such as anaerobic digestion (methane fermentation) or aerobic stabilization (composting). As a result of these treatments, all biological and chemical contaminants are believed to be removed or decreased to the level that does not present any danger for the environment (Decree of Estonian Minister of the Environment No 78, 2004). The treated sewage sludge is considered to be safe, if it does not contain heavy metals, fecal coliforms and eggs of helminths over the allowed trigger value (EU Council Directive 86/278/EEC, 1986). Analyses for the named contaminants are required. Some random samples during a year are recommended for analyses of pathogenic bacteria. The directive does not prescribe analyses for pharmaceuticals. There exist no trigger values for residues of human pharmaceuticals in sewage sludge or its compost neither in Estonia nor in European Union. The most closely related act is EU directive establishing trigger values for veterinary medicines in manure (EMEA/

CVMP/055/96, 1996). The content of drugs should not exceed 100 μg/kg in manure, and 10 μg/kg in soil fertilized with manure. However, the EU Scientific Steering Committee considers these trigger values non-scientific and recommends a value considerably lower – 1 μg/kg for the soil compartment. Only such concentration can be safe for all soil organisms. However, the antibiotic resistance in soil bacteria can develop even at lower drug concentration in soil. This would push the soil concentration trigger further down to 0.01-0.1 μg/kg (Montforts, 2005). The antibiotic resistance can be transferred from soil bacteria to pathogens via horizontal gene transfer (Knapp *et al.*, 2010).

Pharmaceuticals are frequently present in sewage sludge (Ingerslev and Halling-Sørensen, 2000; Golet *et al.,* 2002; Göbel *et al.,* 2005; Lindberg *et al.*, 2005; Büyüksönmez and Sekeroglu, 2005; Jones-Lepp and Stevens, 2007; Li and Zhang, 2010). In spite of the fact that very low drug levels in the environment can have undesirable ecological and health effects, until now the problems related to the presence of pharmaceuticals in sewage sludge and its compost have received little attention (Carballa *et al.*, 2007). Although the usage of sewage sludge compost as a fertilizer widely takes place in the Eastern Europe, the presence of pharmaceuticals in it has been ignored.

Most wastewater treatment processes produce sludge that has to be disposed of. The novel effective technologies of re-use of this waste are developed with the aim of reducing its impact on the environment (Lu *et al.*, 2009). Sewage sludge can be used to generate energy as an alternative to the fossil fuels (Babel *et al.*, 2009), but unfortunately incineration is costly and contributes to air pollution and landfill space is becoming scarce (Mahzuz *et al.*, 2009). The quality of the sewage sludge compost depends on its environmental compatibility and the correspondence to market necessities. These features involve the absence of toxic inorganic and organic substances, which may pollute the environment, create multiresistant bacteria or enter the food chain through the plant uptake (Migliore *et al.*, 1995; Lazzari *et al.*, 2000; O´Connor *et al.,* 2001; Knapp *et al.*, 2010). The degradation of human pharmaceuticals as a result of sewage sludge composting and their possible accumulation into food plants via fertilizing needs to be studied. No systematic research concerning these problems has been published before.

2. REVIEW OF THE LITERATURE

2.1. Sewage sludge: problems, challenges, solutions

Due to the rapid increase in world population the amount of sewage sludge has increased dramatically in the past two decades (Lu *et al.*, 2009). The management of sewage sludge from sewage treatment plants represents one of the major challenges in sewage treatment today (Odegaard, 2003).

Historically, sewage sludge has been disposed of by incineration, landfilling or ocean disposal (Bridle and Skrypski-Mantele, 2000). Composting is also recognized as one of the sludge recycling options (Hara and Mino, 2008). From 1999 ocean disposal of sewage sludge is forbidden in the European Union (Council of the European Communities, 1991). In some countries land application has not been widely accepted because of the fear of possible land contamination caused by heavy metals (Taruya *et al.*, 2002). Guibelin (2002) compared sludge incineration with agricultural use in terms of toxicity criteria, greenhouse gas emission and energy consumption. Lundin *et al.* (2004) compared different sludge recycling and disposal options – agricultural application, co-incineration with waste, incineration combined with phosphorous recovery and fractionation including phosphorous recovery – in terms of environmental and economic aspects.

For the EU-15 the following routes of disposal were expected in 2010 for approximately 9 Mt/yr: landfill 18%; other 7%; thermal 23%; composting 7%; agriculture 45%. With the accession of the new 12 members, the production was likely to rise by about 25%, with a bias towards use on land, particularly in agriculture. The total figure should be taken as an indication rather than an absolute figure. If composting is included in the land use category, there is more than a 50% chance that the sludge in a European city would be treated and used on land as biosolids (Global Atlas of Excreta …, 2008).

Data from different sources indicate that in the United States of America 6.5 dry Mt of biosolids were beneficially used or disposed of in the fifty states in 2004 (Global Atlas of Excreta …, 2008). Overall, current data suggest little change nationwide, since the late 1990s, in the rate of biosolids recycling to soils, and half of state biosolids coordinators

report that the amounts of biosolids applied to soils are not increasing in their states (Global Atlas of Excreta …, 2008). According to federal and state regulations, before they are used as soil amendments or fertilizers, biosolids must be tested for regulated pollutants (e.g. heavy metals); in addition, the rate of application is usually limited by the nutrient needs of the crop being grown. The following disposal practises were used in 2004 in USA: landfilling – 63%; incineration – 33%; surface disposal – 4% (Global Atlas of Excreta …, 2008).

In Estonia there is no reliable database on the quantity of sludge generated in sewage treatment. According to waste statistics, over 300 000 tons of sewage sludge was generated in 2000 (National Waste Management Plan, 2002). As the solid content of this amount is uneven, it is only a tentative figure. One of the main reasons for non-reliability of data is the lack of uniform requirements for solid content. In the data received, the solid content ranges between 3% and 85%. Therefore the total quantity of the sewage sludge is only an estimate. It can be estimated that 360 000 - 500 000 tons of sewage sludge is generated in Estonia annually. At the same time, biological treatment of sewage and also phosphorus removal is increasing, which increases also the amount of sludge generated. In 2000, 38–46 thousand tons of sludge was deposited (National Waste Management Plan, 2002).

Sewage sludge landfilling has frequently been the least expensive option, in monetary terms and/or in terms of "hassle." In developing countries, dumping untreated excreta, septage, and wastewater sludge on land is common. Sometimes it is dumped in a hole, sometimes just on the surface. Such dumps, if they grow large enough, have environmental and public health impacts. This leads to development of managed landfills. Modern landfills are not as cheap and easy methods of disposal (Global Atlas of Excreta …, 2008).

As a rule, sewage sludge must be dewatered to at least 15-20% solids before landfilling, to avoid excessive generation of leachate and for landfill stability. While dewatering is costly, it is often the only requirement for placing sewage sludge in a landfill. Sewage sludge disposed in landfills is generally not treated further, nor is tested for contaminants (Global Atlas of Excreta …, 2008). Energy consumption of the sewage treatment plants without incineration and melting processes are low, but their

greenhouse gas emissions are high because of CH_4 and N_2O emissions from sludge cake at the landfill site (Soda *et al.*, 2010).

Incineration greatly reduces the volume of sewage sludge by oxidizing the organic matter, and it can take advantage of the energy in these materials. Incineration, however, requires a large capital investment in infrastructure and requires fuel – usually fossil fuel – to create the burn. Incinerators are subject to strict air pollution control standards, which require increased complexity and costs. The nitrogen content of the sludge is considerably higher than that of other fuels, such as coal and wood. Thus the emissions of NO_x and N_2O are anticipated to be high. The global warming potential of N_2O is 310 times that of CO_2, so the emission of N_2O is a big problem (Murakami *et al.*, 2009). Despite these disadvantages, incineration of sewage sludge has become standard practice in large, densely populated areas of some technologically advanced countries (Global Atlas of Excreta …, 2008). Japan incinerates more than 70% of its wastewater sludge. As fossil fuel prices have risen dramatically in recent years, the interest in bio-solids as an alternative fuel has also increased. This has pushed many incineration facilities to begin to recover heat to generate electricity and provide heat to facility processes like digestion (Global Atlas of Excreta …, 2008). The ash resulting from incineration of sewage sludge is usually disposed of in landfill or used as fill material in construction projects or as an ingredient in cement. Sewage sludge ashes have been used in cement mortars (Monzó *et al.*, 2003), in concrete mixtures (Tay and Show, 1991) and in asphaltic paving mixes (Al Sayed *et al.*, 1995). An enhancement of the strength of mortars in which 15% of Portland cement is replaced by sewage sludge ash has been attributed to the properties of ashes (Monzó *et al.*, 1996). Presently, two European standards forbid the use of ash from co-firing of coal and municipal sewage sludge as an additive to cement or concrete (Cenni *et al.*, 2001). The use of wastewater sludge ashes for obtaining ceramic materials has been suggested recently (Endo *et al.*, 1997). The production of bricks by mixing ash and clay has been studied (Anderson, 2002). In the above mentioned studies it was recommended to use limited amounts of ash in mixes. Glass-ceramic has been prepared by melting a blend of limestone and ash at 1450 °C and further reheating at lower temperatures (Suzuki *et al.*, 1997). Incineration and melting are methods of disposing of waste by burning at 800–900 °C and 1300–1800 °C, respectively (Hong *et al.*, 2009). The most serious

environmental concern related to the incineration of sewage sludge is the production of significant amounts of dioxin, furan and fly ash (Hong *et al.*, 2009). The fly ash itself contains toxic metals, dioxins and furans. Although several types of filters are used in incineration processes for removing dioxin and heavy metals, complete removal of these substances is difficult to achieve. Also, the filters are quite expensive. However, incineration is particularly popular in Japan. Melting processes can be advantageous for the following reasons: (1) less production of dioxins due to their crystallizability at high temperature, (2) recyclable slag and metal production and (3) significant volume reduction for final landfilling. However, an increase in the running cost can be expected with increases in temperature. During the last few years, the investment in the above-mentioned treatment methods has been strongly debated (Hong *et al.*, 2009).

Several EU countries and Japan are exploring other high-technology thermal treatments, such as gasification and pyrolysis, with the hope of obtaining more net energy from wastewater sludge than standard incineration yields (Global Atlas of Excreta …, 2008). These treatments produce a gas that is rich in H_2 and/or CH_4, which can in turn be used in a gas engine to generate electricity. In other words, the purpose of these technologies is to convert the organic material in the sludge into energy. However, gasification produces tar simultaneously with gas, so this processing is complex and costly (Adegoroye *et al.*, 2004; Manya *et al.*, 2006; Murakami *et al.*, 2006; Murakami *et al.*, 2007). On the other hand, the heating value of the digested sludge after fermentation is so low that a large amount of supplementary fuel is needed for incineration (Worden *et al.*, 1991).

The following alternative sewage treatment technologies – (1) anaerobic digestion of mixed raw sludge with subsequent cogeneration of obtained biogas, (2) incineration of mixed raw sludge utilizing energy contained in flue gas, and (3) incineration of anaerobic digested sludge utilizing energy contained in biogas and flue gas – have been compared in Houdková *et al.*, 2008. Heat balances showed that the highest amount of excess energy is generated by incineration of mixed raw sludge and the lowest amount in the system of anaerobic digestion of mixed raw sludge. The energy aspect cannot be the only criterion in the selection of the most convenient sludge management for a specific sewage treatment plants. When economic aspects are taken into account the most suitable

is anaerobic digestion of mixed raw sludge with incineration of digested sludge. The reason is that biogas is generated during digestion and the price of electric power generated from biogas is subsidized by governments. Biogas is also the product in the case of mixed raw sludge digestion, but this option includes high disposal costs of dewatered sludge (if incinerated, the amount is notably reduced). In a simplified balance the least efficient, from an economic aspect, is direct combustion of mixed raw sludge (Houdková *et al.*, 2008).

The results of a model set up to determine carbon footprints for sludge treatment solutions with and without standard or advanced anaerobic digestion are presented in Barber, 2009. Generally, raw sludge treatment has a higher carbon footprint than any option involving anaerobic digestion, regardless of the end point of the sludge. It may not be sustainable in the long term regarding carbon emissions. The benefits of standard anaerobic digestion when compared with raw treatment are lost due to emissions of methane from secondary digestion. However, flaring these emissions can significantly reduce the carbon footprint. The lowest carbon footprints were associated with the addition of advanced pretreatment before digestion. Regarding carbon alone, it is suggested that all new digestion plants should be designed with pretreatment technology as a standard.

Sewage sludge is a residue resulting from the treatment of wastewater released from various sources including homes, industries, medical facilities, street runoff and businesses. It contains nutrients and organic matter that can provide soil benefits and are widely used as soil amendments. Sewage sludge contains contaminants including metals, pathogens, and organic pollutants (Harrison *et al.*, 2006). The high content of organic matter, nitrogen and phosphorus suggest the use of sewage sludge as a soil conditioner and fertilizer (Kaonga *et al.*, 2010). Composted sludge returns carbon, nitrogen, phosphorus and other essential elements back to the soil. Pathogens and heavy metals can still limit the reuse of composted sludge (Hong *et al.*, 2009). Many thousands of industrial and domestic chemicals are possibly present in sewage sludge (Rockefeller, 2002). Despite of that the sewage sludge is not classified as hazardous waste. The usage of different sewage treatment technologies is believed to eliminate or reduce chemical and biological contaminants in it. Analyses for the content of heavy metals, fecal coliforms, helminth's eggs and some pathogenic bacteria in sewage

sludge compost are considered to ensure the safety of this matter (EU Council Directive 86/278/EEC, 1986).

The application of treated sewage sludge as fertilizer is practiced in many countries (Rockefeller, 2002; Giger *et al.,* 2003). Regulations in the United States of America and in the European Union share the same objective of controlling pathogens and pollutants in sewage sludge (EPA 530-R-99.009, 1999; EU Council Directive 86/278/EEC, 1986). Sewage sludge management is probably a more urgent issue in the EU, since Europe produces more sewage sludge and has less agricultural area available for its disposal (Iranpour *et al.,* 2004). Despite regulations to reduce the risk from sewage sludge, public opposition to sewage sludge land application is growing in the EU, just as it is in the USA (Jones-Lepp and Stevens, 2007). In Switzerland the disposal of sewage sludge into agricultural fields has been forbidden since January 2003 (Giger *et al.,* 2003). Wastewater treatment facilities and sewage sludge producers face increasing difficulty in using and disposing of sewage sludge. Due to fertilizing with sewage sludge, heavy metals are known to pollute the arable lands (Al-Enezi *et al.,* 2001; Al-Muzaini and Al-Obied, 2010). The term "treated sewage sludge" does not always mean that the sludge is surely composted. Sometimes the treatment process consists of mechanical screening, extended aeration, chemical treatment (chlorination), thickening and drying (Al-Muzaini *et al*., 1991). In developed European and American countries the anaerobic digestion (methane fermentation) is usually added (Golet *et al.,* 2003, Lindberg *et al*., 2005; Jones-Lepp and Stevens, 2007). The digestion of sewage sludge before application to agricultural soil is a meaningful activity, because it reduces the environmental impact associated with the pollutants present in the sludge (Hospido *et al.,* 2010).

A significant number of sewage treatment plants (STP-s) send de-watered sewage sludge to compost operations (Jones-Lepp and Stevens, 2007). Composting is only one of the possible ways of aerobic treatment and it is not unconditionally required (EU Council Directive 86/278/EEC, 1986). The recent works have shown the importance of composting in treatment of sewage sludge. There is general consensus in the scientific literature that heavy metals are strongly bound to the compost matrix and organic matter, limiting their solubility and potential bioavailability in soil (Smith, 2009). It is well known, that some heavy metals are able to accumulate from sewage sludge treated soil into crops (Davis, 1984).

As it is complicated and time-consuming to investigate all kinds of pollutants in sewage sludge, this work focuses on the content of pharmaceuticals. Numerous studies have shown that a wide variety of pharmaceutically active compounds are present in wastewater effluents, surface waters, and ground waters (GWRC, 2008), and the STPs are unable to remove all these substances. The removal rates of individual drugs during passage through a STP have varied from 12 to 90% (Stumpf *et al.,* 1999). The fate of medical substances may be divided into three principal possible routes (Richardson and Bowron, 1985):

1. The substance is ultimately mineralized to carbon dioxide and water;
2 The substance is lipophilic and not readily degradable, so part of the substance will be retained in the sludge. These substances are able to contaminate the soil if the sludge is dispersed onto fields;
3. The substance is metabolised to a more hydrophilic form of the parent lipophilic substance, but is still persistent and therefore will pass the STP, ends up in the receiving waters (rivers, seas) and may therefore affect the aquatic organisms, if the metabolites are biologically active.

Presence of different pharmaceuticals in sewage sludge is apparent, but there is still a lack of knowledge about the fate of pharmaceutical residues in the environment (Kümmerer, 2008). Different antimicrobials are often not readily degradable (Gavalchin and Katz, 1994; Richardson and Bowron, 1985; Marengo *et al.*, 1997; Hamscher *et al.*, 2002; Halling-Sørensen *et al.*, 2002; Carballa *et al.*, 2004*)*. Still, remarkable amounts of pharmaceuticals enter the soil via fertilizing with sewage sludge (Golet *et al.*, 2002). As long as such a disposal practice is widely applied, a better knowledge on the fate and effects of chemicals in sludge treated soils is needed.

2.2. Pharmaceuticals in the environment

Pharmaceuticals play an important role in the treatment and prevention of disease in humans and animals. The side effects on human and animal health have been intensively studied, but only recently the occurrence, fate and effects of medicines in the environment have been considered (Daughton and Ternes, 1999; Boxall *et al.*, 2003; Boxall *et al.*, 2004). The

residues of pharmaceuticals may enter the environment by different routes via several different nonpoint sources, such as manufacturing plants, effluents of sewage treatment plants, household waste, and landfill effluent. Pharmaceuticals applied in veterinary medicine as growth promoters and for other purposes are excreted by the animals and reach soil. If they are not bound to soil constituents, they may reach groundwater. In the case of heavy rain events, some may also be transported to surface water from runoff. Usually, it is assumed that emissions from pharmaceutical manufacturing and production are low in Europe and North America. However, it has been found only recently that pharmaceutical manufacturing facilities can be a significant source of pharmaceuticals in the environment (Kümmerer, 2010).

The primary route of entry of human pharmaceuticals into the environment is through sewage point sources. Regardless of the route of entry into the environment, the fate of pharmaceuticals can be divided into three categories: transport, sequestration and degradation. Pharmaceuticals may be transferred without degradation and stored, at least temporarily, in other matrices or compartments through processes such as bio-concentration, sorption and deposition of particles (Glassmeyer *et al.*, 2008).

Veterinary medicines are released to land either directly in feces or urine or indirectly through the application of manure as a fertilizer (Boxall *et al.,* 2002). Veterinary medicines, such as antibiotics, hormones, and parasiticides, have been detected in soil, surface and ground water (Hirsch *et al.,* 1999; Kolpin *et al.,* 2002; Yan and Carlson, 2003; Meyer, 2004). Although the reported concentrations are generally low, some pharmaceuticals may persist in the environment for a long time (Kay *et al.*, 2004). A large number of pharmaceuticals have been detected in ambient waters, wastewater, and drinking water at very low levels (USGS, 2002; Benotti *et al.,* 2009), and the use of pharmaceuticals is expected to increase (Conerlya and Ohaniana, 2010). In the environment, pharmaceutical residues are commonly found in the ng/L to μg/L range (Halling-Sørensen *et al.*, 1998; Kümmerer, 2001; Loos *et al.*, 2009). When pharmaceuticals are eliminated from the human body they can be excreted in their native form or as metabolites (Ternes, 1998; Fent *et al.*, 2006). Since pharmaceuticals are developed to have a specific mode of action, even low levels of pharmaceuticals are able to cause

effects in organisms (Fent *et al.*, 2006). Industrial chemicals on the other hand, although not designed to have a specific mode of action, can still interact with biological systems (Jin *et al.*, 2009). The molecules of pharmaceuticals are generally stable in order to reach their target site in the body before being degraded (Fent *et al.*, 2006), which means that they also are of some persistence in the environment (Lundström *et al.*, 2010). Although the residues of pharmaceuticals may be degraded, their continuous release from sewage treatment plants and other sources results in pseudo-persistence in the environment (Daughton and Ternes, 1999; Loos *et al.*, 2009).

Humans may be exposed to residues of veterinary medicines in the environment by a number of routes including the consumption of (1) crops that have accumulated substances from soils as a result of exposure to contaminated manure and slurry; (2) livestock that have accumulated veterinary medicines through the food chain; (3) fish exposed to treatments used in aquaculture; and (4) ground water and surface water containing veterinary medicines. Exposure to pharmaceuticals of humans via plant-derived foodstuffs is usually low and effects on human health are unlikely. This route of exposure may, however, be more significant for the small number of highly toxic medicines or in situations when long-term low-level exposure could elicit subtler effects (e.g., promotion of antibacterial resistance or endocrine disruption) (Boxall *et al.*, 2006). A chemical can undergo different structural changes by a variety of biotic and non-biotic processes after its introduction into the environment. Structural transformations may also be a result of effluent treatment (Qiting and Xiheng, 1988; Ravina *et al.*, 2002; Schröder, 2002; Ternes *et al.*, 2003; Zühlke *et al.*, 2004; Lee *et al.*, 2007; Trautwein *et al.*, 2008; Méndez-Arriaga *et al.*, 2008).

Many pharmaceuticals are bio-transformed by organisms such as bacteria and fungi in the environment (Haiß and Kümmerer, 2006; Gröning *et al.*, 2007). They undergo structural changes in the body of humans and animals (Kümmerer, 2009). This could be due to micro-organisms in the gut or by human enzymes such as cytochromes. Metabolites are the result of such a process. However, the meaning of "metabolite" is somewhat confusing. The term metabolite is used for compounds resulting from the structural change of pharmaceuticals within the human body, not differentiating biochemical processes performed by

human enzymes from the ones due to bacterial activity in the alimentary system and the ones present on skin or non-biotic processes such as hydrolysis in the stomach. The term is also used for molecules resulting from structural change by fungi and bacteria in the environment and sometimes even for structural changes that are the result of non-biotic processes such as oxidation, hydrolysis and photolysis (Méndez-Arriaga *et al.,* 2008) in different environmental compartments such as surface water, soil or sewage treatment (Kümmerer, 2009). Such a structural change of pharmaceuticals results in a change in their physicochemical and pharmaceutical properties. It is normally assumed that metabolism and other transformation processes lead to decreased toxicity. In some cases however, metabolism leads to more active compounds. The same has been found for phototransformation and other oxidizing processes (Burhenne *et al.*, 1997). Possible sources and pathways for the occurrence of pharmaceutical residues in soil and in the aquatic environment are presented in Halling-Sørensen *et al.* (1998) and Heberer (2002).

Although antibiotics have been used in large quantities for some decades, until recently the existence of these substances in the environment has received little notice (Kümmerer, 2009[1]). In contrast to the properties and effects desired from the therapeutic application of antibiotics, these same properties are often disadvantageous for those target and non-target organisms present in the environment. The fate of antibiotics in soil or surface water run-off after application of manure is discussed in Kreuzig and Höltge, 2005. A review of pharmaceuticals, including antibiotics for veterinary use and related environmental issues on a global scale, was recently published by Sarmah *et al.* (2006).

An antibiotic in a broader sense is a chemotherapeutic agent that inhibits or abolishes the growth of microorganisms, such as bacteria, fungi, or protozoa (Kümmerer, 2009[1]). Antibiotics are used extensively in human and veterinary medicine, as well as in aquaculture, for the purpose of preventing or treating microbial infections. Several hundred different antibiotic and antimycotic substances are used in human and veterinary medicine, e.g. more than 250 in Germany (Kümmerer and Henninger, 2003). Internationally comparable data on antibiotic consumption is scarce, and whatever information is available is heterogeneous. Usage patterns may be different in different countries (Kümmerer, 2008). Wise (2002) estimated antibiotic consumption worldwide to lie between 100,000 and 200,000 ton per annum. In 1996, about 10,200 ton of

antibiotics were used in the EU, of which approximately 50% was applied in veterinary medicine and as growth promoters. According to data supplied by the European Federation of Animal Health (FEDESA, 2001), in 1999 there were a total of 13,216 ton of antibiotics used in the European Union and Switzerland, 65% of which was applied in human medicine. In the United States, one estimate is that 50% of the 22,700 metric tons of all antimicrobials prescribed annually are for humans and 50% for use in animals, agriculture and aquaculture (Kümmerer, 2009[1]). US livestock producers use approximately 11,200 metric tons of antimicrobials for non-therapeutic purposes primarily to promote the growth of cattle, hogs, and poultry. Clinical uses are estimated at about 10% of total antimicrobial use (Mellon *et al.*, 2001).

The application of biosolids onto agricultural fields is a farming practice common in many countries such as the USA, Canada, and within Europe (Angin and Yaganoglu, 2009; Carballa *et al.*, 2009; Mantovi *et al.,* 2005). A national survey on biosolids regulations, quality, end use, and disposal conducted in 2004 reported an annual U.S. production of approximately 6.5 million dry metric tons of sewage sludge of which approximately 49% was applied to soils (NEBRA, 2007). Three quarters of the total mass of land applied biosolids was used on farmlands for agricultural purposes (NEBRA, 2007). Although it is well known that digested sewage sludge is laden with organic wastewater contaminants, the fate and behavior of micropollutants in biosolids-amended agricultural soils are still unclear (Walters *et al.*, 2010), and the effects of these compounds on terrestrial ecosystems remain relatively uninvestigated (Liu *et al.*, 2009).

Over the past decade, the scientific community has become increasingly interested in the impacts of pharmaceutical contaminants to the environment and human health. Recent studies have shown (Büyüksönmez and Sekeroglu, 2005), that the degradation of some pharmaceuticals (ibuprofen, galaxolide) and personal care products (phthalate esters) may take place during bio-solid composting. Biosolids were amended with straw, and composted for up to 45 days using a laboratory-scale composting system. For all spiked and most unspiked compounds degradation efficiencies surpassed 85% at the end of the 45 days of composting study (Büyüksönmez and Sekeroglu, 2005). It is considered to be very useful to learn the degradation of pharmaceuticals, if diverse sewage sludge treatment technologies are to be applied (Jemba, 2002).

Analytical methods have been developed and applied for the determination of different pharmaceuticals in sewage sludge, biosolids and sludge treated soil (Golet *et al.,* 2002; Stevens *et al.,* 2003; Lindberg *et al.,* 2005; Göbel *et al.,* 2004; Göbel *et al.,* 2005[1]; Kinney *et al*., 2006; Jones-Lepp and Stevens, 2007; Heidler *et al*., 2006; Kaleta *et al*., 2006; Okuda *et al*., 2009). A number of pharmaceuticals, known to be persistent in soil, are able to accumulate into food plants (Migliore *et al*., 1995; Migliore *et al*., 1996; Brambilla *et al*., 1996; Jjemba, 2002; Migliore *et al*., 2003; Lillenberg *et al.,* 2003; Boxall *et al.,* 2006; Dolliver *et al.,* 2007), but no systematic work concerning the degradation of pharmaceuticals during sewage sludge composting has been published.

2.3. Fate of pharmaceuticals in sewage, sewage sludge, compost and soil

Several studies have been performed to investigate the occurrence and fate of pharmaceuticals in sewage treatment plants or surface water (Steger-Hartmann *et al*., 1997; Hirsch *et al.,* 1998; Alder *et al*., 2001; Golet *et al.,* 2001; Lindsey *et al*., 2001; Kolpin *et al*., 2002; Carballa *et al*. 2004). Many of the pharmaceuticals applied in human medical care are not completely eliminated in the human body (Heberer, 2002). Pharmaceuticals have been measured in the effluent of medical care units, sewage and the effluent of sewage treatment plants, in surface water, ground water, and in drinking water (Heberer, 2002). In general, the concentrations of pharmaceuticals are in the higher µg-per-litre range in hospital effluent, in the lower µg-per-litre range in municipal waste water, and in the higher and lower µg-per-litre range in different surface waters (Kümmerer, 2008). Seasonal variations have been studied in sewage and reclaimed wastewater, as well as in final effluent (Loraine and Pettigrove, 2006; Alexy *et al*., 2006).

Macrolide antibiotics (clarithromycin, dehydro-erythromycin [metabolite of erythromycin], roxithromycin, lincomycin), sulfonamides (sulfamethoxazole, sulfadimethoxine, sulfamethazine, and sulfathiazole), fluoroquinolones (ciprofloxacin, norfloxacin, and enrofloxacin), chloramphenicol, tylosin and trimethoprim have been found up to the low µg/l-levels in sewage and surface water samples (Hirsch *et al*., 1999). Golet *et al*. (2001) analyzed fluoroquinolone antibiotics in primary and tertiary wastewater effluents. In these samples, ciprofloxacin and

norfloxacin occurred at concentrations between 249 and 405 ng/L and from 45 to 120 ng/L, respectively. Antibiotics have also been identified at high concentrations in hospital effluents: between 3 and 87 μg/L of the fluoroquinolone antibiotic ciprofloxacin was detected in hospital effluents (Hartmann *et al.*, 1998; Alder *et al.,* 2001).

Sacher *et al.* (2001) reported the occurrence of sulfamethoxazole (up to 410 ng/L) and dehydro-erythromycin (up to 49 ng/L) in groundwater samples. Sulfamethoxazole and sulfamethazine have also been detected at low concentrations in a few groundwater samples in other investigations (Hartig *et al.*, 1999; Hirsch *et al.*, 1998; Lindsey *et al.*, 2001). Pharmaceuticals have been detected in the effluent from landfill sites. There is evidence of the occurrence of some 160 different drugs in sewage treatment plant effluent, surface water and groundwater (Kümmerer, 2009). Holm *et al.* (1995) found residues of different sulfonamides at high concentrations in groundwater samples collected downgradient of a landfill.

Sewage treatment facilities do not remove pharmaceutical residues completely. A considerable amount of pharmaceuticals reaches surface water and can end up in drinking water (Halling-Sørensen *et al.,* 1998). Within sewage treatment plants, pharmaceuticals may be removed by degradation (Carballa *et al.*, 2004; Daughton and Ternes, 1999). Compounds that are not degraded will either pass through the sewage treatment process and are released in sewage effluents or adsorb to biosolids (Golet *et al.*, 2002; Xia *et al.*, 2005; Miao *et al.*, 2005; Ternes *et al.,* 2002; Göbel *et al.*, 2005). Several investigations have shown some evidence that substances of pharmaceutical origin are often not eliminated during waste water treatment and also not biodegraded in the environment (Ternes, 1998; Daughton and Ternes, 1999; Zwiener *et al.*, 2000; Heberer and Stan, 1996). The sewage sludge containing pharmaceutical residues is used as a fertilizer in the fields (Radjenović *et al.,* 2009; Lapen *et al.*, 2008). This is the way how pharmaceuticals reach the soil where they can affect microorganisms and accumulate in plants.

Growth stimulators and medicines used in animal breeding reach manure either unaltered or as metabolites and finally get to the fields. On pastures, they go through the cattle organism and are excreted. In this manner, extremely high concentrations of pharmaceutical residues are concentrated locally in soil, and they essentially have strong impact on

soil organisms and plants. Pharmaceuticals and their metabolites, which have reached soil, are either mineralised by soil organisms or they reach groundwater unaltered (Halling-Sørensen *et al.,* 1998). The lifetime of drugs in the environment depends on the structure of their molecules. The microorganisms of soil decompose drugs either into organic metabolites or carbon dioxide and water. The ability to produce antibiotics is a long-term evolutionary process, and it represents an important factor in the struggle for existence (Tshervjakova and Terezova, 1986). At the same time the pathways of biodegradation have evolved in nature to mineralise natural antibiotics. Synthetic and semi-synthetic antibacterial substances are currently in wide use. They are „stangers" to nature and might be hard to degrade (Lillenberg *et al.,* 2003).

Hirsch *et al.* (1999) did not detect penicillins or tetracyclines from various sewage, surface and groundwater samples. This result is no surprise as penicillins are easily hydrolyzed and tetracyclines readily precipitate with cations such as calcium and accumulate in sewage sludge or sediments (Daughton and Ternes, 1999; Stuer-Lauridsen *et al.*, 2000). Nevertheless, Lindsey *et al.* (2001) and Kolpin *et al.* (2002) detected tetracycline drugs (chlortetracycline, oxytetracycline, and tetracycline) in surface water samples.

The use of biosolids as fertilizer on agricultural fields is a common practice since it recycles organic waste materials and is a valuable source of nutrients. A number of pharmaceuticals have been detected in biosolids destined for land application (Kinney *et al.,* 2006; Metcalfe *et al.*, 2003) and in biosolid-amended soils (Golet *et al.*, 2002; Kinney *et al.*, 2008). Concerns have therefore been raised over the potential impacts of biosolid-associated pharmaceuticals on terrestrial systems and associated groundwater and surface water (Monteiro and Boxall, 2009). Recent studies have investigated the transport of pharmaceuticals from soils to surface water and groundwater (Topp *et al.,* 2008; Oppel *et al.*, 2004).

Different pharmaceuticals are transported to different extents and one of the key factors affecting the amount transported is the persistence of the pharmaceutical in the soil environment (Monteiro and Boxall, 2009). The degradation rates of pharmaceutical compounds in soils vary widely, with half-lives ranging from days to years (Boxall, 2008). Within the same

therapeutic class, half-lives can still be significantly different (Schlüsener and Bester, 2006). These differences can be explained by differences in soil properties such as moisture content, organic carbon, pH, and soil bioactivity; climate (temperature); and physico-chemical properties of the compound such as degree of dissociation and lipophilicity (Collucci *et al.,* 2001; Kah *et al.*, 2007; Topp *et al.*, 2008[1]; Topp *et al.*, 2006).

The persistence of organics in soils is determined by a number of processes taking place in it. These processes include (1) photodecomposition; (2) microbial metabolism; (3) chemical reactions; (4) volatilization; (5) adsorption by clay minerals and organic colloids and (6) leaching and plant uptake (Evangelou, 1998). The form in which pharmaceuticals reach the soil environment may also affect persistence. The major route of entry is via the application of biosolids to agricultural fields. As biosolid application increases organic carbon and phosphorus content, increases microbial activity, and significantly influences pH (Gascó *et al.*, 2004; Tsadilas, *et al.*, 1995; Garcia-Gil *et al.*, 2004; Furczak *et al.*, 2007), it is possible that the presence of biosolids will affect persistence in the natural environment (Monteiro and Boxall, 2009). The formation of bound residues of the pharmaceutical with the biosolids may also reduce the potential for degradation (Monteiro and Boxall, 2009).

Pharmaceuticals never occur in the environment on their own and are likely to co-occur with other pharmaceuticals and also with contaminants from other classes. Interactions between pharmaceuticals and other contaminants may affect the fate of the pharmaceutical, and its degradation may be significantly slower in mixtures (Monteiro and Boxall, 2009). Antibiotics are likely to be present in biosolids and since they have been designed to affect microorganisms, they may influence microbial degradation. The presence of biosolids significantly reduces degradation rates compared to soil alone (Monteiro and Boxall, 2009).

Little information is available on degradation of pharmaceuticals in the environment. It has been shown that antibacterial drugs can bind strongly to solid particles, which could be an additional reason for their slow degradation (Marengo *et al.*, 1997; Carmosini and Lee, 2008). Some antibiotics tend to accumulate in the soil and do not reach groundwater. The persistence of antibiotic residues in sediment or soil mostly depends on a number of parameters as such as sorption capability, degradation

rate, and mobility in water (Avisar *et al.*, 2010). Cation exchange, cation-bridging, complexation, and H-bonding can all contribute to the sorption of amphoteric antimicrobials by soils and soil components, thus transfer to water is generally not predicted (Carmosini and Lee, 2008; Nowara *et al.*, 1997; Figueroa *et al.*, 2004; Gu and Karthikeyan, 2005; Sassman and Lee, 2005). Nevertheless, certain strongly sorbing antimicrobials, such as tetracyclines and quinolones, are routinely detected in surface water monitoring studies (Golet *et al.*, 2002; Kolpin *et al.*, 2002).

Various studies have concentrated on the sorption of antibiotics onto clays, as clay minerals imbedded within soils are well known for their ability to modulate organic molecules delivery in soils. Studies of the mobility and sorption of sulfonamides, tetracyclines, and fluoroquinolones have determined that sorption rates are highly dependent on soil particle size, pH conditions, and the presence of other ions in addition to the polarity and binding strength of the compounds (Thiele-Bruhn *et al.,* 2004; Figueroa *et al.*, 2004; Golet *et al.,* 2003; Kulshrestha *et al.,* 2004; Parolo *et al.,* 2008). Sulfonamide antibiotics show increased sorption capacities at lower pH values (2–7). In environmental pH conditions, the adsorption capacity of sulfamethoxazole (SMX) and sulfadimethoxine (SDM) to clay is expected to be negligible. In comparison, the adsorption of tetracyclines to clay was high under environmental conditions, suggesting that this group of antibiotics will accumulate in soil and will be less mobile (Avisar *et al.*, 2010).

It is inevitable that pharmaceuticals will be released to the soil environment. Studies with single substances in different soil types indicate that degradation rates are variable but it is not yet possible to correlate persistence with soil properties or soil bioactivity (Monteiro and Boxall, 2009). The effect of biosolids is probably highly dependent on the source and nature of the biosolids used (Monteiro and Boxall, 2009). Degradation of pharmaceuticals in the environment is a very complex issue and a lot more data on the degradation behavior of pharmaceuticals in a range of well-characterized soils with different properties are needed in order to understand what happens to a pharmaceutical in the real soil environment (Monteiro and Boxall, 2009).

2.4. Plant uptake of pharmaceuticals from soil

The potentially hazardous organic compounds that may be present in sewage and sewage sludge number in the thousands (O'Connor, 1996). Fortunately, the concentrations of toxic organic chemicals are usually low (Rogers *et al.*, 1989; Webber and Lesage, 1989), and for most of them their plant bioaccumulation factors are small (O'Connor, 1996). Still, compounds with strong sorption and recalcitrant to degradation remain in surface soils and have the potential to subsequently be uptaken by plants (Wu *et al.*, 2010). However, very limited information is currently available about plant uptake of pharmaceuticals from soil. Previous research has focused primarily on plant uptake of veterinary pharmaceuticals that are associated with animal waste, that is, manures, and demonstrated their potential to accumulate in plants (Boxall *et al.*, 2006; Kumar *et al.*, 2005; Dolliver *et al.*, 2007). Recently, uptake of human pharmaceuticals in plants grown hydroponically or in nutrient solution has also been reported (Redshaw *et al.*, 2008; Herklotz *et al.*, 2010). In some cases these pharmaceuticals can have phytotoxic effects (Farkas *et al.*, 2009). It was demonstrated by Herklotz *et al.* (2010) that human pharmaceuticals commonly present in treated wastewater and biosolids can be actively taken up by various species of plants grown using a nutrient solution fortified with pharmaceuticals under ideal hydroponics conditions.

A study was performed (Boxall *et al.*, 2006) to investigate the potential for a range of veterinary medicines to be taken up from soil by plants used for human consumption and to assess the potential significance of this exposure route in terms of human health. Soil analyses indicated that, for selected substances, measurable residues were likely to occur in soils for at least 5 months following application of manure containing these compounds. Experimental studies carried out by Boxall *et al. (*2006) on the uptake of veterinary medicines into carrot roots and lettuce leaves showed that only florfenicol, levamisole, and trimethoprim were taken up by lettuces, whereas diazinon, enrofloxacin, florfenicol, and trimethoprim were detected in carrot roots. Measured concentrations in plant material were used to model potential adult human exposure to these compounds. Although exposure concentrations were appreciable in a few instances, accounting for – 10% of the acceptable daily intake values (ADI), all were lower than the ADI values, indicating that, at least for compounds with properties similar to those considered in Boxall

et al. (2006), there is little evidence of an appreciable risk. This exposure route may, however, be important when veterinary medicines have a very low ADI, at which they elicit subtle effects over prolonged periods, or when exposure is occurring via a number of routes at once (Boxall *et al.*, 2006). Although degradation products (produced in the soil or the plant) were not measured in this work, it was considered to be possible for some substances that these could increase the risks to consumers.

The introduction of pharmaceuticals through the food-chain pathway is within the same magnitude or even higher than via drinking water (Shenkera *et al.*, 2011). Therefore the combined effects should be investigated especially in areas using intensive irrigation of crops with reclaimed wastewater. Further, the significantly higher uptake in leaves versus fruits found by Shenkera *et al.* (2011) may imply a need for greater concern in crops such as lettuce, whose edible parts are the leaves.

Paterson *et al.* (1991), Paterson *et al.* (1991[1]), Trapp *et al.* (1990), and Riederer (1990) have developed models to describe the transport and distribution of chemicals between soil, water, and plant tissues. The role of terrestrial vegetation in transferring chemicals from soil and air into specific plant tissues (e.g., stems, leaves, and roots) is still not well characterized (McKone and Maddalena, 2007).

2.5. Structure and properties of fluoroquinolones, sulfonamides and tetracyclines and their fate in the environment

2.5.1. Fluoroquinolones

The fluoroquinolones (FQs) are a class of compounds that comprise a large group of synthetic antimicrobial agents. Structurally, all FQs contain a fluorine at the 6-position of the basic quinolone nucleus (Figure 1).

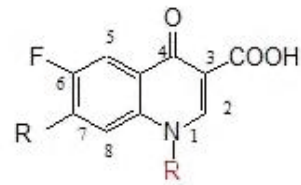


Figure 1. The basic structure of the fluoroquinolones (Schaumann and Rodloff, 2007).

Despite the basic similarity in the core structure of these molecules, their physicochemical properties and microbial activities can vary markedly across compounds. FQs exhibit bactericidal activity by inhibiting the activity of DNA gyrase and topoisomerase, enzymes essential for bacterial DNA replication, that leads to fragmentation of the chromosome and cell death. Because of their chemical structure, FQs are zwitterions, containing two ionizable moieties. The lowest pKa value is associated with the acidic carboxylic acid moiety while the second is attributable to the basic tertiary amine (Barbosa *et al.*, 1999). Examples of pKa values are provided in Table 1. The pKa or ionisation constant is defined as the negative logarithm of the equilibrium coefficient of the neutral and charged forms of a compound. This allows the proportion of neutral and charged species at any pH to be calculated, as well as the basic or acidic properties of the compound to be defined. pKa values are temperature dependent. Standard practice is to measure pKa´s at 25 °C (Earll, 1999-2006). pKa is important because of its effect on other physicochemical properties, notably on lipophilicity, solubility and permeability (Sirius Analytical Instruments, 2008). It should be noted that pKa estimates can vary, depending upon the buffer system used and the experimental conditions (Barbosa *et al.*, 1999).

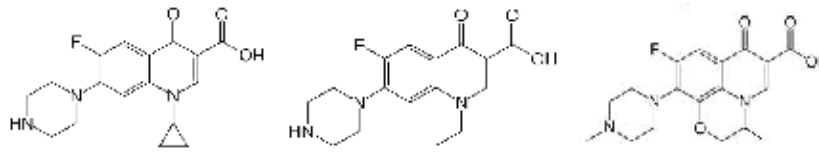
With few exceptions, FQs exhibit poor water solubility between pH 6 and 8. For this reason at pH values between the pKa_1 and pKa_2, these zwitterions have a net neutral charge. Accordingly, they can freely diffuse across biological membranes. When pH values are lower than the pKa_1, FQs have a net positive charge. At pH values exceeding pKa_2, the FQs have a net negative charge (Martinez *et al.*, 2006). FQs have their greatest lipid solubility within the pH range 6.0-8.0. This lipophilicity facilitates diffusion into biological tissues, including bacterial cells. Maximal transfer of FQs from the aqueous to the lipid phase (octanol/water partitioning) occurs at pH 7 (Lizondo *et al.*, 1997). Octanol/water partitioning coefficient K_{ow} is defined as:

$$K_{ow} = c_o / c_w$$

where c_o is the concentration of a compound in octanol phase at equilibrium, c_w is the concentration of a compound in aqueous phase at equilibrium. Log K_{ow} is the logarithm of the octanol-water partitioning coeficient. K_{ow} varies between different compounds (Table 1). The higher

K_{ow} the more lipophilic is the compound. K_{ow} of FQ-s depends also on temperature, increasing with the increase of temperature (Zhang and Wang, 2010).

Ciprofloxacin (CIP) is a faintly yellowish to light yellow crystalline substance. Its empirical formula is $C_{17}H_{18}FN_3O_3$ and its molecular weight is 331.4 g/mol (Tabel 1; Figure 2). CIP is used in the treatment of human´s urinary tract infections, cystitis, chronic bacterial prostatitis, lower respiratory tract infections, acute sinusitis (inflammation of the paranasal sinuses), skin and skin structure infections, bone and joint infections, infectious diarrhea, typhoid fever caused by *Salmonella typhi,* and gonorrhea. The main administration of the drug is oral and parenteral, but CIP is also used in the treatment of eye and ear infections caused by susceptible bacteria. It is a broad-spectrum antibiotic that is active against both gram-positive and gram-negative bacteria. CIP is eliminated primarily by renal excretion. However, the drug is also metabolized and partially cleared through the liver and the intestine.



Ciprofloxacin Norfloxacin Ofloxacin

Figure 2. The structure formulas of fluoroquinolones (Golet *et al.*, 2001).

In general, CIP is well tolerated. The serious adverse effects that may occur as a result of ciprofloxacin therapy include irreversible peripheral neuropathy, spontaneous tendon rupture, acute liver failure or serious liver injury (hepatitis), toxic epidermal necrolysis, and severe central nervous system disorders. Psychotic reactions and confusional states, acute pancreatitis, bone marrow depression, interstitial nephritis and hemolytic anemia may also occur during ciprofloxacin therapy (Merck Manuals[1]; RxList[1]).

Norfloxacin (NOR) is a white to pale yellow crystalline powder with a molecular weight of 319.34 g/mol. Its empirical formula is $C_{16}H_{18}FN_3O_3$ (Figure 2). NOR is occasionally used to treat human´s urinary tract infections, sexually-transmitted diseases (eg, uncomplicated urethral and cervical gonorrhea) and prostatitis due to *Escherichia coli*. NOR is active

against both gram-positive and gram-negative bacteria. Unlike CIP and OFL, NOR is prescribed only for oral use (Merck Manuals[1]). In ophthalmology, NOR is used for the treatment of conjunctival infections caused by susceptible bacteria. NOR ear drops are used for treating ear infections caused by certain bacteria. The uses for NOR are quite limited as it is considered a drug of last resort when all other antibiotics have failed. The NOR´s limitation is associated with a number of serious and life threatening adverse reactions as well as spontaneous tendon ruptures and irreversible peripheral neuropathy. Such reactions may manifest long after therapy has been completed and in severe cases may result in lifelong disabilities. Hepatoxicity resulting in fatalities has also been reported with the use of NOR. Serious visual complications have been reported to occur with therapy with norfloxacin eye drops, especially corneal perforation, but also evisceration and enucleation. This drug is known to be substantially excreted by the kidney. Allergic nephropathy is associated with norfloxacin as well as other serious kidney problems. Renal failure, neutropenia-thrombopenia, agranulocytosis, nephrotic syndrome, eosinophilia and acute interstitial nephritis are all being associated with norfloxacin therapy (RxList[2]).

Ofloxacin (OFL) is an off-white to pale yellow crystalline powder with a molecular weight of 361.4 g/mol. Its empirical formula is $C_{18}H_{20}FN_3O_4$ (Figure 2). As CIP and NOR, OFL is active against both gram-positive and gram-negative bacteria. OFL is recommended for treatment of acute bacterial exacerbations of chronic bronchitis, pneumonia, skin structure infections, urethritis and cervicitis, cystitis, complicated urinary tract infections, prostatitis and acute gonorrhea. The treatment routes are oral, parenteral or topical, as eye and ear drops (Merck Manuals[1]).

Table 1. Physicochemical properties of fluoroquinolones.

Compound	Mol wt g/mol	pKa_1 (25 °C)	pKa_2 (25 °C)	Log K_{ow} (25 °C)
Ciprofloxacin	331.40	6.0 [1]	8.8 [1]	1.1 [2]
Norfloxacin	319.34	6.4 [1]	8.7 [1]	-1.4 [2]
Ofloxacin	361.40	6.1 [1]	8.2 [1]	0.4 [2]

Log K_{ow} – logarithm of the octanol-water partitioning coefficient; pKa_1, pKa_2 – the two ionisation constants of the compound; [1] – Martinez *et al.*, 2006; [2] – Zhang and Wang, 2010.

Like other fluoroquinolones, OFL has been associated with a significant number of serious adverse reactions, such as tendon damage (including spontaneous tendon ruptures) and peripheral neuropathy, such reactions may manifest long after therapy had been completed, and, in severe cases, may result in life-long disabilities. It has also been associated with severe psychiatric adverse reactions. OFL is eliminated primarily by renal excretion. However, the drug is also metabolized and partially cleared through the liver. Hepatotoxicity and hepatitis has been reported with the use of ofloxacin (RxList[3]).

In two decades the fluoroquinolones moved from a relatively small and unimportant group of drugs, used predominantly for treatment of urinary tract infections, to a class with worldwide sales (Katzung, 2000). These compounds have now been used in human therapy and veterinary treatment for over a decade, and during this time their input into the environment has been continuous.

Ciprofloxacin is the most widely prescribed fluoroquinolone in the world; the second is ofloxacin, followed by levofloxacin, lomefloxacin and norfloxacin (Katzung, 2000). Norfloxacin is very common in Europe but is no longer used in the US (Katzung, 2000). The major human-use FQs consumed in Switzerland are ciprofloxacin and norfloxacin (Boxall *et al.,* 2003). Most important veterinarian-used FQs are enrofloxacin, danofloxacin, sarafloxacin, orbifloxacin, marbofloxacin, and difloxacin.

Examination of the adsorption of FQs in mineral soil (mainly clays) was conducted by Nowara *et al*., (1997). FQ acid derivatives appear in soil solution, partly as anions, and interact with exchangeable cations bound to the negatively charged mineral surfaces (Stern layer). The maximum amount of substrate will be adsorbed when the molecules are oriented as flatly as possible. Soils with a variety of different pedological properties readily absorb nearly 100% of the FQs, and desorption is low. This behavior is attributed to the ability of clay minerals to preferentially adsorb plane anionic substrates between the mineral layers and at outer surfaces via Coulombic interactions. These results, together with the fact that FQs are relatively polar compounds, suggest that several hydrophobicity-independent mechanisms, such as cation exchange, cation bridging at clay surfaces, surface complexation, and hydrogen bonding are involved (Picó and Andreu, 2007). Another important aspect

that should be considered is biotic and abiotic (by phototransformation) degradation in soils. Experimental studies have revealed, however, that if any biodegradation (or phototransformation) of FQs occurs in soil, it is not complete, and residual FQs persist in agricultural soils (Golet *et al.*, 2003). NOR, OFL, and CIP are rapidly eliminated from sewage by adsorption. The half-lives for the three fluoroquinolones were up to 67−112 h, suggesting that the biodegradation is insignificant in sewage treatment plants (Li and Zhang, 2010). Walters *et al.* (2010) in their outdoor studies showed extremely long persistence of FQs in biosolid-amended soils: their environmental half lives were 2310 ± 1155 days (CIP), 1155 ± 198 days (NOR) and 1386 ± 434 days (OFL). CIP and OFL were initially found at the highest concentrations (542 and 470 μg/kg dry weight, respectively). After 994 days of weathering, both antibiotics were still present at over 390 and 267 μg/kg dray weight, respectively.

Boxall *et al.* (2006) studied the potential for a range of veterinary medicines, including enrofloxacin, to be taken up from soil by plants used for human consumption, and to assess the potential significance to human health of this route of exposure. Soil analysis indicates that measurable residues of enrofloxacin are likely to persist for at least five months after application of manure containing this FQ. Experimental studies on the uptake of veterinary medicines by carrot (root tubers) and lettuce leaves showed that enrofloxacin was detected in carrots but not in lettuces (Boxall *et al.*, 2006).

2.5.2. Sulfonamides

The sulfonamides (SAs) are synthetic bacteriostatic antibiotics that competitively inhibit conversion of *p*-aminobenzoic acid to dihydropteroate, which bacteria need for folate synthesis and ultimately purine and DNA synthesis. Humans do not synthesize folate but acquire it in their diet, so their DNA synthesis is less affected (Merck Manuals[2]). All SAs contain the sulfonamide group – SO_2NH_2 (Figure 3) and are poorly water soluble. SAs show an amphotheric behavior. They are characterized by two pKa values indicating protonation of the amino group at a pH value near pKa_1 and deprotonation of the $R_1SO_2NHR_2$ moiety at pH value near pKa_2. SAs behave as weak acids and form salts in strong acids as well as in strong bases (Thiele-Bruhn, 2003). The

cationic species as SA^+ dominates at low pH values, the neutral form (SA^0) is the principal species at pH values between pKa_1 and pKa_2 and the anionic species SA^- is the main form at higher pH values (Gao and Pedersen, 2005). The physicochemical properties of SAs selected for the current study are shown in Table 2. Unlike the FQs, the octanol/water partition coefficient of SA-s decreases with the increase of temperature (Zhang *et al.,* 2007).

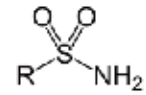


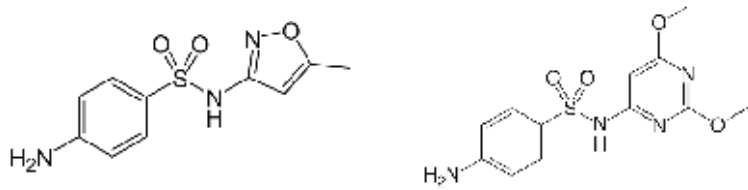
Figure 3. The structure of the sulfonamide group (Avisar *et al.*, 2010).

SAs are used in both human and veterinary medicine. Only few sulfonamide derivates are still in clinical use because resistance to these drugs has become widespread (Avisar *et al.*, 2010). SAs are eliminated from the organism by kidneys. Because SAs concentrate in the urine before being excreted, treating urinary tract infections is one of their most common uses. SAs are recommended for the treatment of infections caused by gram-positive and gram-negative bacteria, chlamydias, and protozoa (Hamscher, 2006).

Sulfamethoxazole (SMX) is a white or yellowish white powder with a molecular weight of 253.28. Its empirical formula is $C_{10}H_{11}N_3O_3S$ (Figure 4). SMX is commonly used in treatment of urinary tract infections, can be used to treat sinusitis and toxoplasmosis. The adverse effects of the treatment with SMX are most often nausea, vomiting, and rash. Photosensitivity, insomnia, headache, and rarely severe hepatic necrosis can occur. Renal failure has occurred in persons with renal insufficiency (Merck Manuals[3]).

Sulfadimethoxine (SDM) is a white powder with a molecular weight of 310.33 and empirical formula $C_{12}H_{14}N_4O_4S$ (Figure 4). SDM is used to treat many infections including treatment of respiratory, urinary tract, enteric, and soft tissue infections, predominantly in veterinary medicine. It is the most common drug for treatment of coccidiosis in many animal species. Side effects of treatment with SDM in animals include redness of the eye, bleeding, vomiting and diarrhoea. Most common is increased thirst (Pet Education.com). In recent years SDM is not recommended

in human medicine, except in Russia, where it is approved for use in humans, including children. In Russia SDM is a drug of choice for the treatment of urinary tract infections, pneumonia, tonsillitis, otitis, soft tissue infections, dysentery and malaria. Described side effects of SDM in humans are headache, fever, rash, indigestion and leucopenia (Pamba.ru. Справочник лекарств).



Sulfamethoxazole Sulfadimethoxine

Figure 4. The structural formulas of sulfonamides (Perez *et al.*, 2005; Thiele-Bruhn *et al.*, 2004).

Sulfonamides are water-soluble and have negligible adsorption to activated sludge (Batt *et al.*, 2007; Ingerslev and Halling-Sørensen, 2000; Pérez *et al.*, 2005). The half-lives of SMX and sulfadiazine (SDZ) are so long (42-133 h) that the biodegradation of sulfonamides in sewage treatment plants is negligible.

Table 2. Physicochemical properties of sulfonamides.

Compound	Mol wt g/mol	pKa_1 (25 °C)	pKa_2 (25 °C)	$logK_{ow}$ (25 °C)
Sulfadimethoxine	310.33	2.1 [3]	6.1 [3]	1.6 [1]
Sulfamethoxazole	253.28	1.9 [3]	5.6 [3]	0.9 [2]

Log K_{ow} – logarithm of the octanol-water partitioning coefficient; pKa_1, pKa_2 – the two ionisation constants of the compound; [1] – Hamscher, 2006; [2] – Zhang *et al.*, 2007; [3] – Qiang and Adams, 2004.

Therefore, the elimination of sulfonamides in sewage treatment plants by activated sludge treatment is insignificant (Li and Zhang, 2010). SMX is not biodegradable in sewage treatment tanks (Richardson and Bowron, 1985). Nevertheless, Carballa *et al.,* (2004) reported the removal efficiency of 60% for SMX within STPs.

The objective of the study carried out by Dolliver *et al.* (2007) was to evaluate plant uptake of a sulfonamide-class antibiotic, sulfamethazine, in corn (*Zea mays L.*), lettuce (*Lactuca sativa L.),* and potato (*Solanum tuberosum L.)* grown in a manure-amended soil. Results from the 45-d greenhouse experiment showed that sulfamethazine was taken up by all three crops, with concentrations in plant tissue ranging from 0.1 to 1.2 mg kg^{-1} (dm). Sulfamethazine concentrations in plant tissue increased with corresponding increase of sulfamethazine in manure. Highest plant tissue concentrations were found in corn and lettuce, followed by potato. Total accumulation of sulfamethazine in plant tissue after 45 d of growth was less than 0.1% of the amount applied to soil in manure (Dolliver *et al.*, 2007). The established ADI value for sulfamethazine is 5 mg kg^{-1} body weight (JEFCA, 2006). It has been estimated that a typical adult consumes approximately 0.6 kg of fresh and processed cereal, pulse, and vegetable crops on a daily basis (WHO, 2003). Assuming a strictly plant-based diet and using the maximum fresh weight concentration for sulfamethazine observed in plant tissue in this study (0.1 mg kg^{-1}), daily intake is considerably below the ADI value. However, these ADI values do not account for issues such as development and spread of antibiotic resistance, which is a major problem globally (Dolliver *et al.*, 2007).

Brambilla *et al.* studied the accumulation of SDM from soil into barley. The content of SDM was approximately 4 times higher in roots than in leaves and stems, being 79 and 18 μg/g accordingly (content of SDM in soil was 100 μg/g). The study concludes that maximum residue limit (MRL) of veterinary medicine residues in plants should be imposed (Brambilla *et al.*, 1996).

2.5.3. Tetracyclines

Tetracyclines (TCs) are a group of broad-spectrum bacteriostatic antibiotics. They are so named for their four ("tetra-") hydrocarbon rings ("-cycl-") (Figure 5). The first discovered TCs were the natural products of soil bacteria of Genus *Streptomyces.* Chlortetracycline was isolated in the late 1940s from *Streptomyces aureofaciens,* oxytetracycline from *Streptomyces rimosus* in the early 1950s. Tetracycline itself is a semisynthetic antibiotic produced from natural oxytetracycline. Later many chemically altered synthetic and semisynthetic antibiotics were developed. TCs are protein synthesis inhibitors, inhibiting the binding of aminoacyl-tRNA

to the mRNA-ribosome complex. Thus, they prevent introduction of new amino acids to the nascent peptide chain. TCs are characterized by three pKa values. They exist as cations at pH values below pKa_1, as zwitterions between pKa_1 and pKa_2, and as anions between pKa_2 and pKa_3 (Table 3).

TCs have been used extensively in the prophlylaxis and therapy of human and animal infections and also at subtherapeutic levels in animal feed as growth promoters (Chopra and Roberts, 2001). However, their use for these indications is now less popular due to widespread resistance development in the causative organisms. Despite of this, they remain the treatment of choice for some specific indications. Administration of TC-s can be oral, parenteral or external. Most of TCs are eliminated in urine (60% of dose) and in the feces (40% of dose) (Riviere and Spoo, 2001). TCs are effective in treatment of infections of the respiratory tract, sinuses, middle ear, urinary tract, gonorrhoea and intestines. The use of drugs of TC class during tooth development (last half of pregnancy, infancy, and childhood up to the age of 8 years) may cause permanent discoloration of the teeth (yellow-gray-brown).

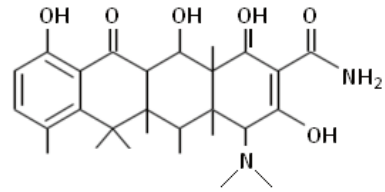
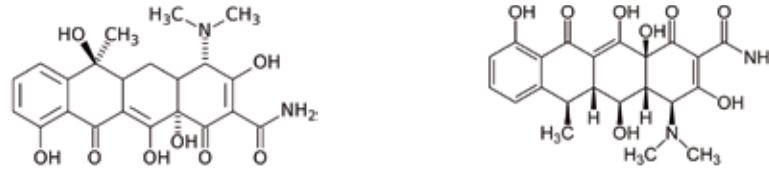


Figure 5. The basic tetracycline structure (Chopra and Roberts, 2001).

Results of animal studies indicate that TCs cross the placenta, are found in fetal tissues, and can cause retardation of skeletal development of the developing fetus. TC class drugs are known to cause hyperpigmentation in many organs, including nails, bone, skin, eyes, oral cavity. They have been associated with the development of autoimmune syndromes with symptoms of fever, rash, and malaise (RxList[4])

Tetracycline (TCL) is a yellow crystalline powder, the derivate of oxytetracycline. Its empirical formula is $C_{22}H_{24}N_2O_8$ and molecular weight 444.45 g/mol. TCL is slightly soluble in water. Log K_{ow} of TCL is negative (Figure 6, Table 3).

Doxycycline (DOX) is a light-yellow crystalline powder, slightly soluble in water. It is a semi-synthetic long-acting antibiotic, derived from oxytetracycline in the early 1960s. The formula of DOX $C_{22}H_{24}N_2O_8$ and molecular weight 444.45 g/mol are identical to TCL. The lipofilic parameter K_{ow} of DOX is higher, than K_{ow} TCL, therefore DOX can better penetrate the biomembranes (Table 3).



Tetracycline Doxycycline

Figure 6. The structural formulas of tetracyclines (Copra and Roberts, 2001).

DOX is used to treat non-gonococcal urethritis and cervicitis, bronchitis in patients with chronic obstructive pulmonary disease and adult periodontitis, chronic prostatitis, sinusitis, syphilis, chlamydia, pelvic inflammatory disease, acne, rosacea, Rickettsial infections, Legionellosis, and also in prophylaxis against malaria (Riviere and Spoo, 2001).

Table 3. Physicochemical properties of TCs.

Compound	Mol wt g/mol	pKa_1 (25 °C)	pKa_2 (25 °C)	pKa_3 (25 °C)	$logK_{ow}$ (25 °C)
Tetracycline	444.43	3.3 [2]	7.8 [2]	9.6 [2]	-1.3 [1]
Doxycycline	444.45	3.0 [2]	8.0 [2]	9.2 [2]	-0.02 [1]

Log K_{ow} – logarithm of the octanol-water partitioning coefficient. pKa_1, pKa_2 pKa_3 – the three ionisation constants of the compound. [1] – Hamscher, 2006; [2] – Qiang and Adams, 2004.

TCL adsorbs significantly and rapidly onto activated sludge with no biodegradation (Li and Zhang, 2010), being consistent with earlier studies (Batt *et al.*, 2007; Kim *et al.*, 2005). Tetracyclines do not decompose in soil during 7 months period (Hamscher *et al.,* 2002). The slow degradation is explained with their strong adsorption to solid particles (Porubcan *et al.*, 1978; Carmosini and Lee, 2008).

3. AIMS OF THE STUDY

The aims of the present work were:

1. To study the presence of some widely used pharmaceuticals in Estonian sewage sludge and its compost and the uptake of these pharmaceuticals from fertilized soils by some food plants (I-VII).
2. To compare the efficiency of different sludge treating technologies (III, IV).
3. To assess the safety of sewage sludge compost as a fertilizer (II, V, VI, VII).
4. To give recommendations concerning sewage sludge compost application.

4. MATERIAL AND METHODS

4.1. Chemicals and equipment (II, IV, V, VI)

Antibiotics were purchased from Riedel-de-Haën (Seelze, Germany) - three fluoroquinolones: ciprofloxacin (CIP, purity 99.8%), norfloxacin (NOR, purity 99.9%) and ofloxacin (OFL, purity 99.3%); two tetracyclines: tetracycline hydrochloride (TCL, purity 97.3%) and doxycycline hyclate (DOX, purity 99.5%); two sulfonamides: sulfadimethoxine (SDM, purity 99.4%) and sulfamethoxazole (SMX, purity 99.9%). Strong cation-exchange (SCX) cartridges (Strata SCX (55 m, 70 Å) 500 mg/6 mL) were supplied by Phenomenex (Torrance, CA, USA); Hydrophilic-lipophilic balanced (HLB) cartridges (Oasis HLB (60 m), 500 mg/6 mL) by Waters (Milford, MA, USA). Acetonitrile and methanol were obtained from J.T.Baker (Deventer, The Netherlands), phosphoric acid from Lachema (Brno, Czech Republic), citric acid monohydrate from Fisher Scientific (Pittsburgh, PA, USA), formic acid from Riedel-de-Haën, ammonium acetate from Fluka (Buchs, Germany). All solvents were of reagent grade or higher quality.

4.2. Collection of the sewage sludge and compost samples (II, IV)

The samples were taken from anaerobically digested sludge (before mixing with peat) in Tallinn and from untreated sludge (before composting with tree bark) in Tartu. Approximately 200 g of sludge (content of dry matter was 28% in Tallinn and 25% in Tartu) was placed into a 500 mL glass jar and mixed thoroughly. The jar was covered hermetically with a lid. The samples were stored at +4 °C in the dark to avoid photodegradation of antimicrobials. The samples were analyzed as soon as possible, typically within a week. Alternatively they were stored in polypropylene vials frozen at temperature –80 °C.

4.3. Method for determination of antimicrobials from sewage sludge and compost (II, IV)

4.3.1. Pressurized liquid extraction

Pressurized liquid extraction (PLE) was performed using an in-house designed system schematically depicted in Figure 7. The extractor was designed using ultra high vacuum components. In order to withstand high pressure the stainless steel chamber cylinder wall thickness was 10 mm and copper gaskets were used for sealing flanges. Volume of the pressure chamber was 55 mL. Standard HPLC valves and stainless steel tubing were used. 9±1 g (wet weight, ww) of sewage sludge sample was mixed 1:1 with sand, and 9±1 g of sludge/sand blend was packed into cellulose filter and placed into the extraction cell mounted in an oven. Extraction was performed with 0.35% phosphoric acid and acetonitrile mixture (1:1, v/v) adjusted to pH 2.50 with 0.01 M citric acid monohydrate. For one extraction cycle approximately 30 mL of solvent was pumped into the extraction cell with static valve D1 open. The system was pressurized with argon using valve D3; subsequently the cell was heated. The operating conditions were the following: temperature in the range of 100–110 °C with a 30 min heat-up time, pressure in the range of 100–110 atm, static extraction 10 min, 5 cycles and 60% solvent flush volume.

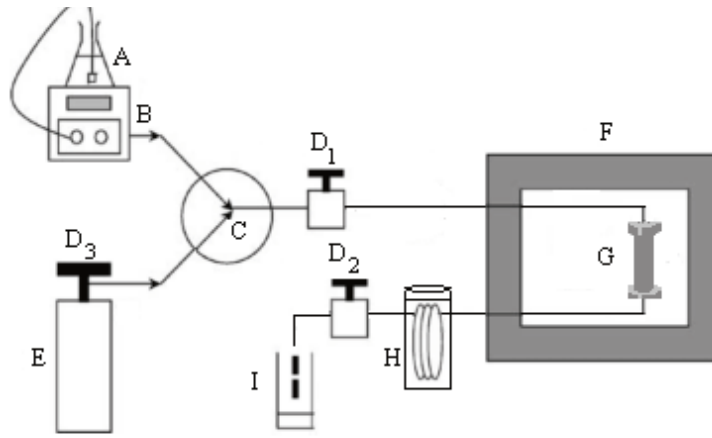


Figure 7. The diagram of laboratory-assembled pressurized liquid extraction (PLE) system: A – extraction solvent; B – HPLC pump; C – three-way switching valve; D1 and D2 – static valves; D3 – the valve of argon gas; E – argon tank; F – oven; G – extraction cell; H – cooling coil; I – extract collection vial.

The extracted analytes were purged from the sample cell using pressurized argon for 40 s. The solvent used for flushing of the extraction cell was collected with static valve D2 open after the first cycle of extraction. Subsequent cycles of extraction were carried out using the same operating conditions. The extract cooling was accomplished by stainless steel tubing in cold water. The total volume of the extract collected was in the range of 150–160 mL.

4.3.2. Solid phase extraction

The extracts collected by PLE were cleaned up by solid phase extraction (SPE). Antibiotics such as CIP, NOR, OFL, TCL, DOX, SDM and SMX were extracted using SCX and HLB cartridges. Two different cartridges were tested with the aim of securing the best possible recoveries. For SPE procedure the vacuum manifold, supplied by Agilent Technologies, was used. For extraction with SCX cartridges the cartridges were preconditioned with 6 mL of methanol and 6 mL of buffer solution (1 mM ammonium acetate and 0.1% formic acid, pH 2.8). A portion (80 mL) of sludge PLE extract was diluted to 500 mL with H_2O (pH adjusted to 2.0) and then percolated through the cartridge at a flow rate 1.5 mL/min using the vacuum manifold. After extraction, the compounds were eluted from cartridges using 20 mL of 20% ammonia water solution in 40% methanol. For extraction with HLB cartridges the cartridges were preconditioned with 20 mL of methanol and 10 mL of Milli-Q water. Dilution of PLE extract was preformed as for SCX cartridges. Flow rate of sample loading was 6 mL/min. After extraction, the compounds were eluted from cartridges using 12 mL of methanol. The SPE extracts were concentrated in polypropylene vials in N2 stream. Polypropylene vials were used to avoid sorption to glass walls and samples were not evaporated to complete dryness. Residues were dissolved in 1 mL of 1:1 solution of methanol with buffer solution (1 mM ammonium acetate and 0.1% formic acid, pH 2.8).

4.3.3. LC–MS method for detection of antimicrobials from sewage sludge and compost

The SPE extracts were analyzed by LC–MS method (liquid chroma-to-graphy electrospray ionization – mass spectrometry), Agilent Series 1100 LC-MSD Trap XCT (Santa-Clara, CA, USA) equipped with a

binary pump, a degasser, an auto-sampler and a column thermostate. Antibiotics were chromatographed using a Phenomenex Synergi Hydro-RP column (250 mm × 4.6 mm, 4 μm) equipped with a Phenomenex SecurityGuard cartridge AQ 4 mm × 2 mm. Electrospray interface (ESI) was used in positive ionmode for ionization. Selected reaction monitoring was used. For instrument control and data analysis Agilent ChemStation for LC Rev. A. 10.02; MSD Trap Control version 5.2 and Data Analysis for LC-MSD Trap 3.2. software were used. Gradient elution with methanol and ammonium buffer solution (1 mM ammonium acetate and 0.1% formic acid, pH 2.8) was used. The linear gradient with a flow rate 0.4 mL/min started at 35% methanol for 20 min and was raised to 80% within 20 min, after that methanol concentration was lowered to 35% in 5 min. Column temperature was set to 30 °C and the injection volume was 5 μL.

Under these chromatographic conditions the separation of seven antibiotic drugs was successfully performed. Antibiotics were detected using electrospray ionization in the positive ion mode which ensured better sensitivity. Stock solutions of 1 mg/mL in the appropriate solvent were prepared. Stock solution for SDM was 0.5 mg/mL due to its poor solubility. The working standard contained 7 antibiotics at 0.1 mg/mL. Solutions were stored at −20 °C.

For calibration the antibiotic solutions were prepared in eluent (35% methanol). The calibration graphs with peak area versus concentration were composed. In order to take into account possible matrix effects standard addition experiments were carried out at least once for each batch of samples. Recovery was calculated from standard addition experiment data. The results presented are corrected with recoveries.

4.3.4. Method validation

For calibration standards appropriate dilutions from working standard in the concentration range of 0.5–500 ng/mL (n = 7) for NOR, CIP, OFL, 0.1–500 ng/mL (n = 8) for SMX, SDM and 10–5000 ng/mL (n = 6) for TCL and DOX were made. The calibration graphs showed excellent linearity in the studied concentration ranges ($r^2 \geq 0.9994$). An unweighted linear regression analysis of representative calibration curves resulted in a slope 966,229.6 (OFL), 163,082.8 (NOR), 652,880.7 (CIP), 399,829.1 (SDM), 74,338.5 (SMX), 414,634.6 (DOX), 11,211.1 (TCL).

The intercepts of the calibration curves were not statistically significant at 95% confidence level. The described method was validated for the simultaneous determination of 7 antibiotics belonging to three different antibiotic groups – FQs, SAs and TCs – from sewage sludge. The average recovery rates for the studied antibiotics by using SCX cartridges were: SMX - 96% (standard deviation, SD = 1.0%), SDM - 43% (SD = 7.4%), OFL - 55% (SD = 5.9%), NOR - 49% (SD = 10.6%), CIP - 44% (SD = 10.2%), DOX - 11% (SD = 8.4%), TCL - 3% (SD = 0.9%).

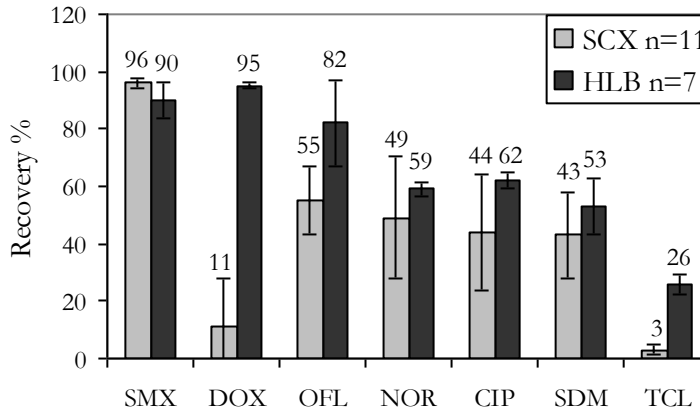


Figure 8. Recovery percentage of antibiotics from sewage sludge by using SCX and HLB cartridges, error bars are 2 x standard deviation.

CIP – ciprofloxacin; NOR – norfloxacin; OFL – ofloxacin; SDM – sulfadimethoxine; SMX – sulfamethoxazole.

By using HLB cartridges the average recovery rates were: SMX - 90% (SD = 3.0%), SDM - 53% (SD = 4.8%), OFL - 82% (SD = 7.6%), NOR - 59% (SD = 1.3%), CIP - 62% (SD = 1.4%), DOX - 95% (SD = 0.6%), TCL - 26% (SD = 1.7%) (Figure 8). An excellent recovery for SMX was obtained when using SCX cartridges. However, SCX cartridges could be utilized only for cleaning up the extracts of 5 antibiotics: 3 FQs and 2 SAs, whereas the recovery of TCs was too low. Much better recovery and repeatability for all the antibiotics were obtained when using the HLB cartridges but the recovery of SMX was smaller than in case of SCX. Therefore, SPE by using HLB cartridges is more efficient and can be applied for determination of 6 antibiotics from sewage sludge: CIP, NOR, OFL, SDM, SMX and DOX. Determination of TCL needs to be improved.

Limits of quantification (LOQ) were estimated (as 10 times the standard deviation) from five replicate analyses of unspiked and spiked sludge samples using HLB cartridges. For the antibiotics present in the sludge, standard deviation of the determinations was used; for the rest of pharmaceuticals results obtained from spiked samples were used. Method precision was estimated as relative standard deviation from five replicate analyzes. The validation data is presented in table 4.

Table 4. Validation data of 7 antimicrobials in sewage sludge (n=5) using SPE with HLB cartridge.

Compound	Unfortified sludge sample		Fortified sludge sample (spiked)			Rec %	LOQ ng/g
	Conc. ng/g	SD	Conc. ng/g	SD	RSD %		
TCL	n.d.	–	310.66	15.72	5.06	26	160
DOX	n.d.	–	682.37	7.67	1.12	95	80
NOR	2.59	0.13	42.58	0.80	1.89	59	1.3
CIP	6.55	0.18	50.65	0.39	0.77	62	1.8
OFL	0.54	0.08	12.34	0.11	0.92	82	0.8
SMX	0.11	0.01	1.40	0.01	0.43	90	0.1
SDM	n.d.	–	0.45	0.01	2.01	53	0.1

RSD – relative standard deviation; Rec – average recovery of antimicrobials; n.d. – not detected; SD – standard deviation; LOQ – limit of quantification.

4.4. Plant experiments (V, VI)

Potato *(Solanum tuberosum L)*, carrot *(Daucus carota L)*, lettuce *(Lactuca sativa L)* and wheat *(Triticum vulgare L)* were grown in the presence of five antimicrobials, found in Estonian sewage sludge (CIP, NOR, OFL, SDM, and SMX). The potato tubers or seeds of plants were planted into the pots, one tuber or 35 seeds in every pot. The plants were cultivated in greenhouse under natural light conditions for 120 days from planting (lettuce 70 days).

Two different soils were used for experiments – loamy and loamy sand (Annex 1). The soil was weighted and aqueous solutions of the studied pharmaceuticals were mixed with soil. The final concentration of each

pharmaceutical was 0.01; 0.1; 0.5; 1 and 10 mg/kg (dry weight). To assure better dissolution of the studied pharmaceuticals fluoroquinolones were dissolved in 2 ml of 0.1 mM ammonium acetate buffer solution with pH=2.8 and sulfonamides were dissolved in 2 ml of 0.3 M NaOH. Three parallel pots were used for each concentration of antimicrobials in both soils, and for control plants grown in antimicrobial-free soil. The plants were collected, washed carefully, dropped, dried in the dark and milled for analyses. The milled samples were dried in a thermostate at 45 °C and held in hermetic plastic bags at –80 °C before analysis.

4.5. Determination of antimicrobials from plants (V, VI)

4.5.1. Liquid extraction

Method for liquid extraction was modified from Palmada *et al.,* 2000. 250 mg of dried plant sample was extracted with 10 mL of 1:1 (v/v) mixture of acetonitrile and 1% acetic acid, then homogenized with laboratory homogenizer DIAX 900 (Heidolph Instruments, Germany) 25 000 rpm, sonicated (5'), vortexed (1') and centrifuged at 8000 rpm. The supernatant was then separated and dried by nitrogen stream. Approximately 15 mL of 1% acetic acid was added to the 1 mL of evaporation residue.

4.5.2. Solid phase extraction

The extract collected by liquid extraction was cleaned up by solid phase extraction (SPE). Antibiotics – CIP, NOR, OFL, SDM and SMX – were extracted using HLB cartridges. For SPE procedure the vacuum manifold, supplied by Agilent Technologies, was used. HLB cartridges were preconditioned with 20 mL of methanol and 10 mL of Milli-Q water. The sample was loaded at a rate of 6 mL/min. After extraction, the compounds were eluted from cartridges using 12 mL of methanol. The SPE extracts were concentrated in polypropylene vials in N_2 stream. Residue was dissolved in 1 mL of 10% methanol with a buffer solution (5 mM 1,1,1,3,3,3-hexafluoro-2-propanol, pH adjusted to 9.0 with NH_4OH).

4.5.3. LC-MS method for detection of antimicrobials from plants

The SPE extracts were analyzed by liquid chromatography–mass spectrometry (LC–MS). Antimicrobials were chromatographed using a Waters XBridge C18 column (150 mm × 3 mm, 3.5 μm) equipped with a Waters Guard Cartridge 4.6 mm × 20 mm. Gradient elution was carried out with methanol and hexafluoroisopropanol (HFIP) buffer solution (5 mM 1,1,1,3,3,3-hexafluoro-2-propanol, pH adjusted to 9.0 with NH_4OH). The linear gradient started at 10% methanol and was raised to 100% within 50 min, after that methanol concentration was 100% for 5 min, then lowered to 10% in 5 min and kept in 10% for 5 min. The eluent flow rate was 0.3 mL/min, the column temperature was set to 30 °C and the injection volume was 10 μL.

4.5.4. Method validation

The described method was validated for the simultaneous determination of CIP, NOR, OFL, SDM, and SMX from plants. For calibration antimicrobials standard solutions were prepared in eluent (hexafluoroisopropanol and 10% methanol). The calibration graphs with peak area versus concentration were composed on concentration range 1-10 000 ng/mL and were linear with r^2>0.9998. Recovery was calculated from standard addition experiments. Recoveries for all detected pharmaceuticals in all matrices varied from 54 to 98%, the average recoveries are shown in figure 9. The method validation was performed in the matrix, which showed the lowest recovery – carrot roots in loamy soil (recovery ranges 54 -78 %, average recovery 66%) (Table 5).

The average recoveries of antimicrobials from carrot roots were 73% (CIP), 69% (NOR), 76% (OFL), 55% (SDM), 70% (SMX). Standard deviations for the recoveries were 1% (CIP), 2% (NOR), 2% (OFL), 1% SDM and 1% SMX (Table 5, Figure 10). The limits of quantification (LOQ) were as follows: CIP 108.3; NOR 162.2; OFL 22.9; SDM 71.2 and SMX 130.6 μg/kg. The standard deviations were accordingly 2.7; 4.1; 0.6; 1.8 and 3.3 (Table 5). LOQ was estimated as 10 times of the standard deviation from five replicate analysis of unspiked and spiked plant samples using HLB cartridges.

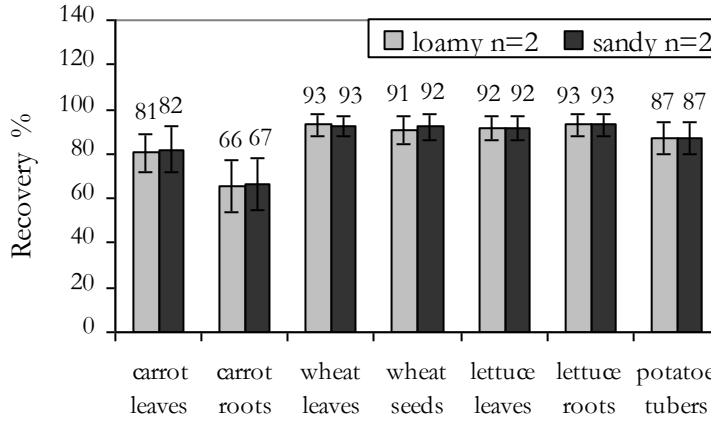


Figure 9. Average recoveries of 5 antimicrobials (CIP, NOR, OFL, SDM, SMX) from different parts of food plants grown in different soils using LE and SPE. Error bars show the recovery ranges.

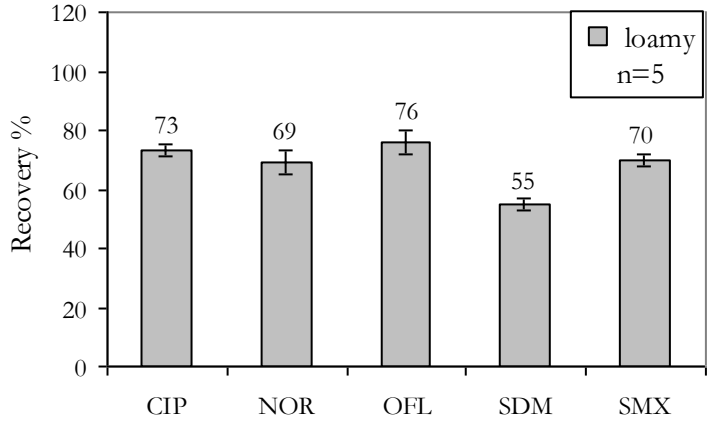


Figure 10. Average recoveries for 5 antimicrobials from carrot roots using LE and SPE. Matrix: carrot roots grown in loamy soil. Error bars are 2 x standard deviation. CIP – ciprofloxacin; NOR – norfloxacin; OFL – ofloxacin; SDM – sulfadimethoxine; SMX – sulfamethoxazole.

Table 5. Validation data of antimicrobials in carrot roots using LE and SPE (n=5).

Plant samples	Recovery %				
carrot roots in loamy soil	SMX	SDM	NOR	CIP	OFL
sample 1	71%	55%	72%	73%	73%
sample 2	71%	54%	68%	75%	78%
sample 3	73%	56%	68%	72%	77%
sample 4	69%	55%	68%	72%	75%
sample 5	70%	54%	70%	72%	78%
Average recovery	**70%**	**55%**	**69%**	**73%**	**76%**
SD	**1%**	**1%**	**2%**	**1%**	**2%**
Concentration of antimicrobials in fortified (spiked) samples μg/g					
carrot roots in loamy soil	SMX	SDM	NOR	CIP	OFL
sample 1	0.1627	0.1265	0.1658	0.1683	0.0225
sample 2	0.1621	0.1248	0.1564	0.1712	0.0239
sample 3	0.1667	0.1284	0.1565	0.1644	0.0235
sample 4	0.1577	0.1273	0.1572	0.1653	0.0231
sample 5	0.1607	0.1241	0.1608	0.1664	0.0237
Average conc.	0.1620	0.1262	0.1593	0.1671	0.0234
SD	**0.0033**	**0.0018**	**0.0041**	**0.0027**	**0.0006**
LOQ (μg/mL)	0.0326	0.0178	0.0406	0.0271	0.0057
LOQ (μg/g)	**0.1306**	**0.0712**	**0.1622**	**0.1083**	**0.0229**
LOD ((μg/mL)	0.0098	0.0053	0.0122	0.0081	0.0017
LOD (μg/g)	**0.0392**	**0.0214**	**0.0487**	**0.0325**	**0.0069**

SD – standard deviation; LOQ – limit of quantification; LOD – limit of detection

5. RESULTS

5.1. Content of antimicrobials in sewage sludge (II)

A novel reliable method for simultaneous determination of fluoroquinolones, sulfonamides and tetracyclines in sewage sludge was developed. The compounds were extracted from sewage sludge by PLE. The solution of 0.35% phosphoric acid and acetonitrile (1:1, v/v) with 0.01 M citric acid monohydrate was used as an extraction solvent. Extracts were cleaned up by SPE using two different cartridges, SCX and HLB, from which HLB was more efficient and can be applied for determination of 6 antibiotics from sewage sludge.

Different samples of sewage sludge were analyzed. The sewage sludge from sewage treatment plants of the two Estonian largest cities, Tallinn and Tartu, was used. The highest contents of antibiotics were found in Tallinn: CIP 1520 μg/kg and NOR 580 μg/kg (dm). Maximum content of CIP exceeds the trigger value for manure (100 μg/kg) over four times. Contents of OFL (134 μg/kg), SDM (73 μg/kg) and SMX (22 μg/kg) were lower (Table 6). The average contents of antibiotics in January were: CIP 737 μg/kg, NOR 279 μg/kg, OFL 80 μg/kg SDM 2 μg/kg and SMX 18 μg/kg (dm) (Figure 11).

As a rule, the concentrations of antimicrobials in the sewage sludge from Tallinn were relatively low. Still, in some cases the contents of CIP, NOR and OFL were over the trigger value (Table 6). In Tartu, contrarily, the content of CIP and NOR was mostly over the trigger value, the high content of OFL was detected only in August, September and October. TCL and DOX were not detected in Tallinn nor in Tartu.

The content of SAs was quite low in both cities, under the trigger value set for drug residues in manure (100 μg/kg) (Table 6, Table 7; Figure 11; Figure 12). In Tartu at least one of SAs was present in every sludge sample (Table 7). The contents of SMX were in the range of 0.0 – 22 μg/kg, and SDM 0.0 – 73 μg/kg (dm) in Tallinn. In Tartu contents of SMX were between 0.0 – 16 μg/kg, and SDM 0.0 – 32 μg/kg (dm) (Table 8). The highest concentrations of antimicrobials in sewage sludge from Tartu were: NOR – 439 μg/kg and CIP – 442 μg/kg (dm). OFL was present in every sludge sample from Tartu and the highest concentration was

157 μg/kg (dm) (Table 7). TCs were absent in sludge and compost. For screening TCs presence in sewage, some samples of Tartu sewage were taken. The content of tetracycline in Tartu sewage varied from not detected (n.d.) to 0.69 ng/mL. Doxycycline was not detected in sewage (Table 8). Surprisingly CIP and OFL were not detected in these sewage samples, despite of their content in sludge and compost.

Table 6. The highest contents of antimicrobials in sewage sludge in Tallinn μg/kg (dm).

Month	CIP	NOR	OFL	SDM	SMX
January	**1520**	**580**	**134**	3	22
February	67	67	17	73	5
March	58	31	8	3	1
April	58	33	3	n.d.	2
May	**150**	**215**	7	0.4	n.d.
June	**206**	**163**	17	n.d.	4
July	39	37	4	n.d.	n.d.
August	11	26	5	n.d.	4
September	0.4	0.4	n.d.	n.d.	n.d.
November	42	16	9	3	3
December	53	85	37	4	7

CIP – ciprofloxacin; NOR – norfloxacin; OFL – ofloxacin; SDM – sulfadimethoxine; SMX – sulfamethoxazole; n.d. – not detected.

The average concentrations of antimicrobials in sewage sludge from Tartu were: CIP – 359 μg/kg, NOR – 291 μg/kg, OFL – 107 μg/kg, SDM – 27 μg/kg and SMX – 8 μg/kg (dm). Average contents of NOR and OFL were slightly higher than in the case of the sludge from Tallinn (Figure 12). The results showed that antibiotics are present in Estonian sewage sludge and their content may exceed the relevant trigger values for manure. The sewage and sewage sludge are not homogeneous. The variability of the content of antimicrobials during the year is large: from „not detected" to hundreds and thousands μg/kg (Table 8). The concentrations of FQs were especially high in Tallinn (Figure 13). The chromatogram of sewage sludge sample from Tallinn is shown in figure 14.

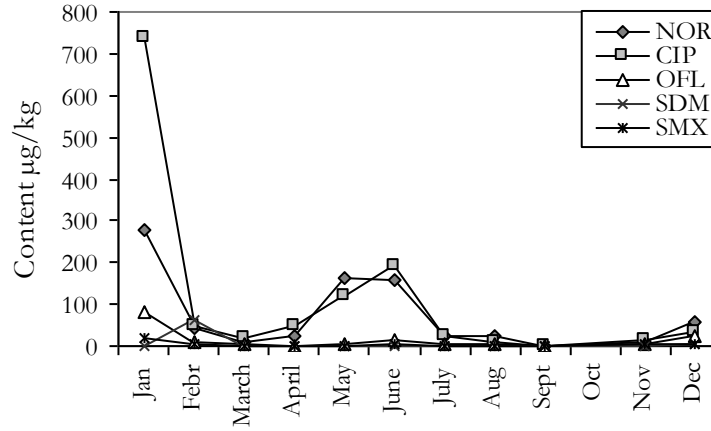


Figure 11. The average content of antimicrobials in sewage sludge in Tallinn µg/kg (dm).

NOR – norfloxacin; CIP – ciprofloxacin; OFL – ofloxacin; SMX – sulfamethoxazole; SDM – sulfadimethoxine.

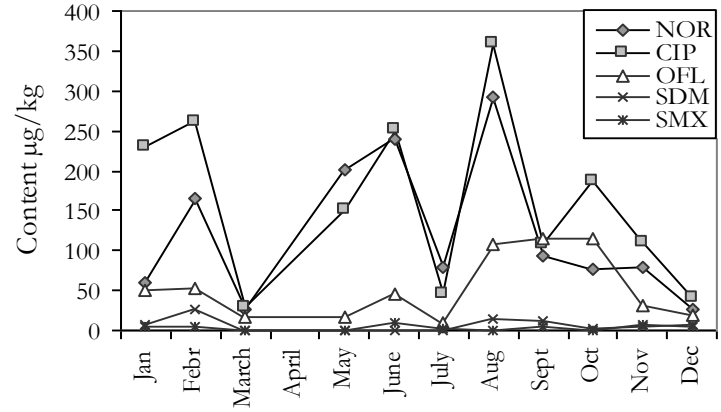


Figure 12. The average content of antimicrobials in sewage sludge in Tartu µg/kg (dm).

NOR – norfloxacin; CIP – ciprofloxacin; OFL – ofloxacin; SMX – sulfamethoxazole; SDM – sulfadimethoxine.

Table 7. The highest contents of antimicrobials in sewage sludge in Tartu μg/kg (dm).

Month	CIP	NOR	OFL	SDM	SMX
January	**315**	82	86	8	6
February	**423**	**263**	68	32	7
March	89	60	26	0.4	1
May	**174**	**264**	22	1	n.d.
June	**265**	**264**	47	n.d.	16
July	67	**104**	19	n.d.	6
August	**442**	**439**	**111**	24	n.d.
September	**231**	**188**	**157**	22	9
October	**259**	**126**	**149**	4	n.d.
November	**134**	**105**	33	6	11
December	71	40	32	9	6

CIP – ciprofloxacin; NOR – norfloxacin; OFL – ofloxacin; SDM – sulfadimethoxine; SMX – sulfamethoxazole; n.d. – not detected.

Table 8. Variability of the content of antimicrobials in sewage (ng/mL), sewage sludge and compost stack (μg/kg dm).

City	Sample	CIP	NOR	OFL	SDM	SMX	TCL	DOX
Tartu	sewage **	n.d.	0.05–0.08	n.d.	0.011–0.014	0.41–0.42	n.d.–0.69	n.d.
	sewage sludge	10–442	n.d–439	2–157	n.d.–32	n.d.–16	n.d.	n.d.
	compost	n.d.–70	n.d.–64	n.d.–11	n.d.–15	n.d.–3	n.d.	n.d.
Tallinn	sewage sludge	n.d.–1520	n.d.–580	n.d.–134	n.d.–73	n.d.–22	n.d.	n.d.
	compost	n.d.–19	n.d.–17	n.d.–8	n.d.–0.5	n.d.–1	n.d.	n.d.

CIP – ciprofloxacin; NOR – norfloxacin; OFL – ofloxacin; SDM – sulfadimethoxin; SMX – sulfamethoxazole; TCL – tetracycline; DOX – doxycycline; n.d. – not detected; ** only three random samples were taken from sewage.

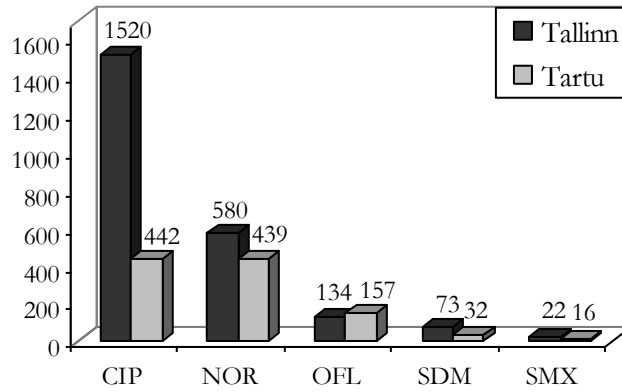


Figure 13. The highest content of antimicrobials in sewage sludge μg/kg (dm). CIP – ciprofloxacin; NOR – norfloxacin; OFL – ofloxacin; SDM – sulfadimethoxin; SMX – sulfamethoxazole.

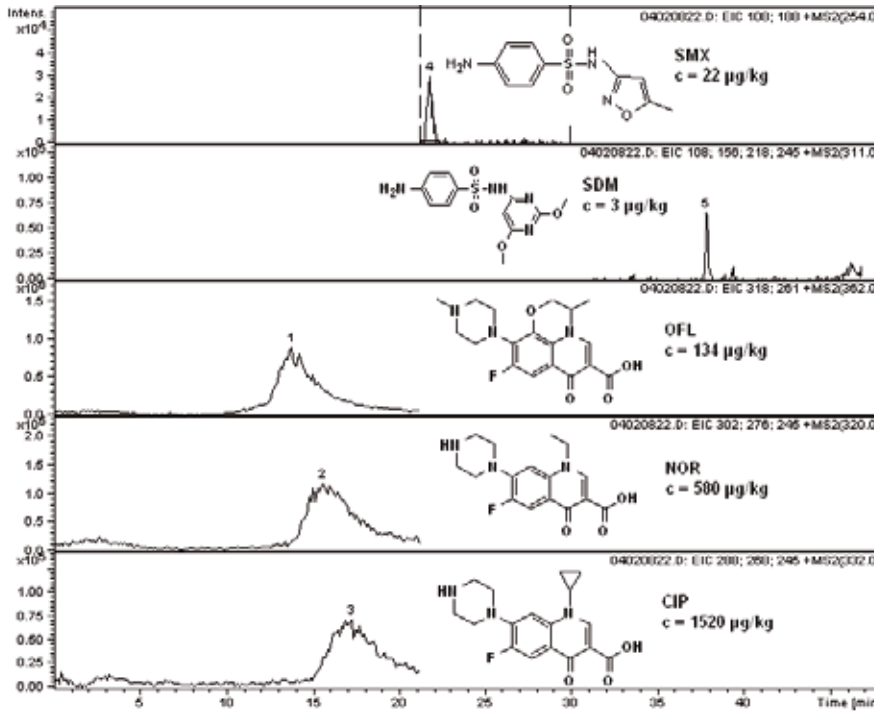


Figure 14. The chromatogram of the sewage sludge sample from Tallinn. 1 – ofloxacin; 2 – norfloxacin; 3 – ciprofloxacin; 4 – sulfamethoxazole; 5 – sulfadimethoxine.

5.2. Degradation of antimicrobials as a result composting (IV)

As it can be seen from table 9, the concentrations of the studied antimicrobials decreased remarkably during composting. The major decrease can be seen in the case of the stacks stored for 2 months. In Tallinn the antimicrobials were almost absent in compost stacks that had been formed 12 months earlier. However, in the compost stored for 6 month the content of NOR was over and the content of CIP was near the recommended trigger value for soil. In Tartu the degradation rate of antibiotics in compost was lower (see table 10). The antibiotics were not completely degraded even after 12 months of storage of the compost stack. The contents of CIP and NOR were high enough to exceed the trigger values for soil. The contents of OFL and SMX were lower, but still exceeded the trigger of 1 μg/kg. SDM was not detected in compost stored for 12 months.

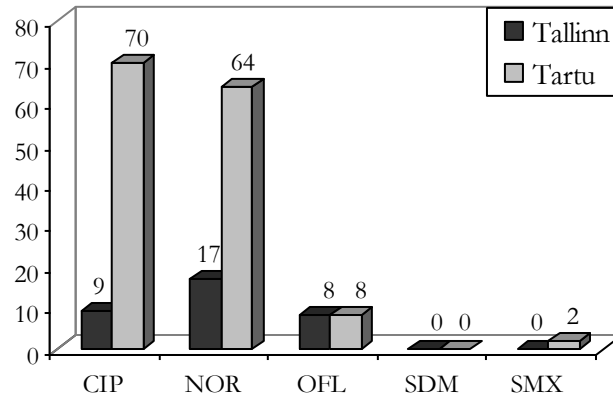


Figure 15. The highest contents of antimicrobials μg/kg in compost ready for utilization.

CIP – ciprofloxacin; NOR – norfloxacin; OFL – ofloxacin; SDM – sulfadimethoxine; SMX – sulfamethoxazole.

Table 9. The highest contents of antimicrobials in sewage sludge and compost in Tallinn.

AM	Content μg/kg (dm)				trigger value for soil
	sewage sludge	2 months stored compost	6 months stored compost	12 months stored compost	
CIP	1520	19	9	0.3	
NOR	580	15	17	0.1	10*
OFL	134	n.d.	8	0.03	
SDM	73	0.5	n.d.	n.d.	1**
SMX	22	0.8	n.d.	0.01	

AM – antimicrobial; CIP – ciprofloxacin; NOR – norfloxacin; OFL – ofloxacin; SDM – sulfadimethoxine; SMX – sulfamethoxazole; * recommended by EMEA/CVMP/055/96; ** recommended by EU Scientific Steering Commitee; n.d. – not detected.

Time dependence of the average concentrations of antibiotics in the same compost stacks in Tallinn and Tartu are presented in tables 11 and 12. In Tallinn the 6-months stored compost is ready for application, in Tartu the storage time must be at least one year. The results of the comparison of the contents of antimicrobials in 6-month stored compost from Tallinn and in 1-year stored compost from Tartu are presented in Figure 15.

Table 10. The highest contents of antimicrobials in the sewage sludge and compost from Tartu.

AM	Content μg/kg (dm)				trigger value for soil
	sewage sludge	2 months stored compost	6 months stored compost	12 months stored compost	
CIP	442	n.d.	44	70	
NOR	439	21	40	64	10*
OFL	157	11	9	8	
SDM	32	15	1	n.d.	1**
SMX	16	3	2	2	

AM – antimicrobial; CIP – ciprofloxacin; NOR – norfloxacin; OFL – ofloxacin; SDM – sulfadimethoxine; SMX – sulfamethoxazole; * recommended by EMEA/CVMP/055/96; ** recommended by EU Scientific Steering Commitee; n.d. – not detected.

Table 11. Degradation rate of antimicrobials during sewage sludge composting in Tallinn.

AM	Average content of antimicrobials μg/kg (dm)				Degradation rate during 12 months (%)
	sewage sludge	2 months stored compost	6 months stored compost	12 months stored compost	
CIP	737	3	5	0.3	99.9
NOR	279	7	7	0.1	99.9
OFL	80	n.d.	3	0.03	99.9
SDM	2	0.4	n.d.	n.d.	100
SMX	18	0.3	n.d.	0.003	99.9

AM – antimicrobial; CIP – ciprofloxacin; NOR – norfloxacin; OFL – ofloxacin; SDM – sulfadimethoxine; SMX- sulfamethoxazole; n.d. – not detected.

Table 12. Degradation rate of antimicrobials during sewage sludge composting in Tartu.

AM	Average content of antimicrobials μg/kg (dm)				Degradation rate during 12 months (%)
	sewage sludge	2 months stored compost	6 months stored compost	12 months stored compost	
CIP	359	n.d.	36	47	87
NOR	291	21	34	42	86
OFL	107	11	7	6	94
SDM	27	15	0.6	n.d.	100
SMX	8	3	1	1	88

AM – antimicrobial; CIP – ciprofloxacin; NOR – norfloxacin; OFL – ofloxacin; SDM – sulfadimethoxine; SMX – sulfamethoxazole; n.d. – not detected.

The chromatogram for the 12-months stored Tartu compost shows remarkable content of fluoroquinolones (Figure 16).

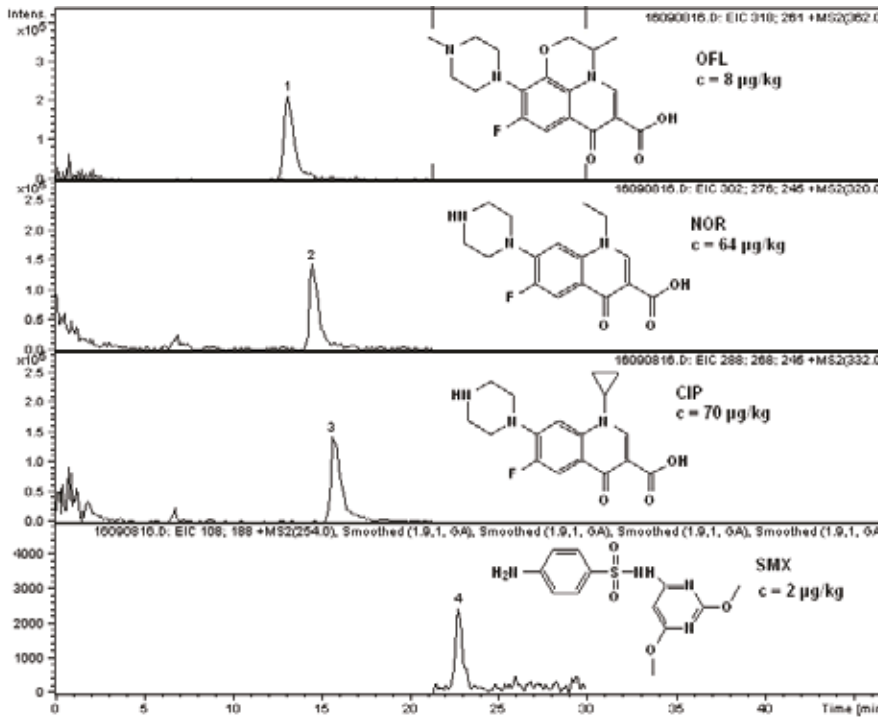


Figure 16. The chromatogram of 12 months stored compost sample from Tartu. 1 – ofloxacin; 2 – norfloxacin; 3 – ciprofloxacin; 4 – sulfamethoxazole.

5.3. Uptake of antimicrobials by food plants (I, V, VI, VII)

5.3.1. Antimicrobials in edible parts of food plants

At soil concentrations of 10 mg/kg antimicrobials accumulated in edible parts of plants (except wheat seeds) in amounts, which exceeded their maximum residue levels (MRL) set for food of animal origin - milk and meat (EMEA/MRL/026/95; EMEA/MRL/820/02). The highest concentrations of antimicrobials accumulated in plants are shown in table 14 and the average concentrations in table 15. Plants accumulated antimicrobials from soil, even at soil concentration of

0.01 mg/kg (CIP, OFL). The drug residues were detected in leaves, roots and tubers of plants. CIP, OFL and SDM were detected also in wheat seeds (Table 13, 14; Figure 19, 20, 21). The accumulation range depended on chemical properties of the compound, soil type, plant species and part (overground or underground). As a rule, the higher concentrations of antimicrobials were detected in underground parts of the plants grown in sandy soil (Figure 26, 27).

Table 13. The highest contents of antimicrobials detected in edible parts of food plants μg/kg.

AM	AM conc. in soil mg/kg (dm)	Carrot roots		Potato tubers		Lettuce leaves		Wheat seeds		MRL for milk and meat μg/kg
		loamy	sandy	loamy	sandy	loamy	sandy	loamy	sandy	
CIP	10	-	**740**	**170**	**160**	**220**	-	-	†	100
	1	-	50	20	10	40	-	-	-	
	0.5	-	70	-	50	-	-	40	-	
	0.1	-	-	40	6	20	-	-	-	
	0.01	-	-	-	3	-	-	-	-	
NOR	10	-	**990**	**180**	**260**	-	-	-	†	-
	1	-	80	40	-	-	-	-	-	
	0.5	-	-	-	-	-	-	-	-	
	0.1	-	-	40	-	-	-	-	-	
OFL	10	40	**830**	**110**	**240**	**140**	**130**	-	†	-
	1	-	**160**	60	50	50	24	-	9	
	0.5	30	80	30	90	-	20	30	-	
	0.1	5	10	6	20	10	7	15	5	
	0.01	3	10	3	5	-	4	-	-	
SDM	10	**100**	**660**	**340**	**1750**	**540**	**240**	50	†	SDM + SMX 100
	1	**130**	**20**	**120**	40	70	-	-	-	
	0.5	**40**	10	-	10	40	-	36	-	
SMX	10	**480**	**4910**	**580**	**5150**	**390**	**750**	-	†	
	1	**120**	**290**	-	-	-	-	-	-	
	0.5	**60**	**110**	-	-	-	-	-	-	
	0.1	-	20	-	-	-	-	-	-	

AM – antimicrobial; CIP – ciprofloxacin; NOR – norfloxacin; OFL – ofloxacin; SDM – sulfadimethoxine; SMX – sulfamethoxazole; MRL – maximum residue level; † – at soil AM concentration 10 mg/kg the wheat plants wilted before flowering.

The average contents of antimicrobials in edible parts of plants grown at lower soil concentration (1 mg/kg) were higher than MRL in case of OFL, SDM and SMX in carrot roots. The MRL for SAs 100 μg/kg is set for the sum of all SAs in meat and milk (EMEA/MRL/026/95). In carrot roots the sum of average concentrations of SDM and SMX was ower the MRL (100 μg/kg) (Table 14).

Table 14. The average contents of antimicrobials detected in edible parts of food plants μg/kg.

AM	AM conc. in soil mg/kg (dm)	Carrot roots		Potato tubers		Lettuce leaves		Wheat seeds		MRL for milk and meat μg/kg
		loamy	sandy	loamy	sandy	loamy	sandy	loamy	sandy	
CIP	10	-	**473**	**115**	**140**	93	-	-	†	100
	1	-	40	7	3	27	-	-	-	
	0.5	-	40	-	23	-	-	13	-	
	0.1	-	-	23	2	7	-	-	-	
	0.01	-	-	-	1	-	-	-	-	
NOR	10	-	**633**	**145**	**233**	-	-	-	†	-
	1	-	27	13	-	-	-	-	-	
	0.5	-	-	-	-	-	-	-	-	
	0.1	-	-	23	-	-	-	-	-	
OFL	10	30	**820**	**103**	**147**	**103**	70	-	†	-
	1	-	**127**	43	33	30	11	-	8	
	0.5	10	57	20	47	-	10	12	-	
	0.1	2	10	5	11	3	2	6	4	
	0.01	1	3	1	2	-	1	-	-	
SDM	10	**67**	**403**	**173**	**1477**	**180**	**117**	17	†	SDM + SMX 100
	1	**70**	17	43	37	40	-	-	-	
	0.5	13	3	-	3	23	-	12	-	
SMX	10	**413**	**3400**	**393**	**3897**	**130**	**447**	-	†	
	1	**60**	**207**	-	-	-	-	-	-	
	0.5	20	80	-	-	-	-	-	-	
	0.1	-	7	-	-	-	-	-	-	

AM – antimicrobial; CIP – ciprofloxacin; NOR – norfloxacin; OFL – ofloxacin; SDM – sulfadimethoxine; SMX – sulfamethoxazole; MRL – maximum residue level; † – at soil AM concentration 10 mg/kg the wheat plants wilted before flowering.

The quite high content of antimicrobials was detected in lettuce leaves. At soil antimicrobial concentration of 10 mg/kg the average contents of OFL, SDM and SMX in lettuce leaves were over the MRL for milk and meat. The sum of SA-s in lettuce gown in loamy soil was 310 μg/kg, which is more than three times over the MRL (Table 14). In lettuce leaves grown in the sandy soil at antimicrobial concentration of 10 mg/kg the sum of average contents of SAs was 564 μg/kg, exceeding the MRL over five times.

OFL was detected in lettuce leaves grown in the sandy soil at every soil antimicrobial concentration, including the lowest (0.01 mg/kg). CIP and NOR were not detected in lettuce leaves grown in the sandy soil (Table 13, 14). The highest contents of antimicrobials accumulated in lettuce leaves from loamy soil are shown in figure 17. The chromatogram of the sample of the lettuce leaves grown in loamy soil at antimicrobial concentration of 10 mg/kg is shown in figure 18. CIP, OFL and SDM were detected in wheat seeds grown in loamy soil, however, in seeds grown in sandy soil only OFL was found (Table 13, 14; Figure 19, 20, 21). Wheat plants grown in sandy soil at antimicrobial concentration of 10 mg/kg wilted before flowering and did not grow seeds. In wilted wheat leaves high concentrations of antimicrobials were detected (Figure 29, 30).

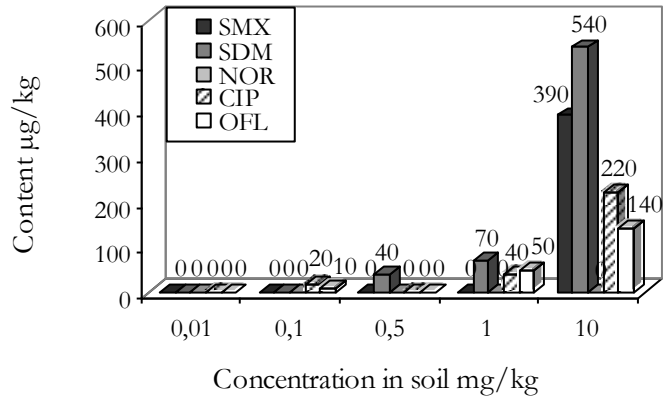


Figure 17. The highest contents of antimicrobials μg/kg (dm) in lettuce leaves grown in loamy soil.

CIP – ciprofloxacin; NOR – norfloxacin; OFL – ofloxacin; SDM – sulfadimethoxine; SMX – sulfamethoxazole.

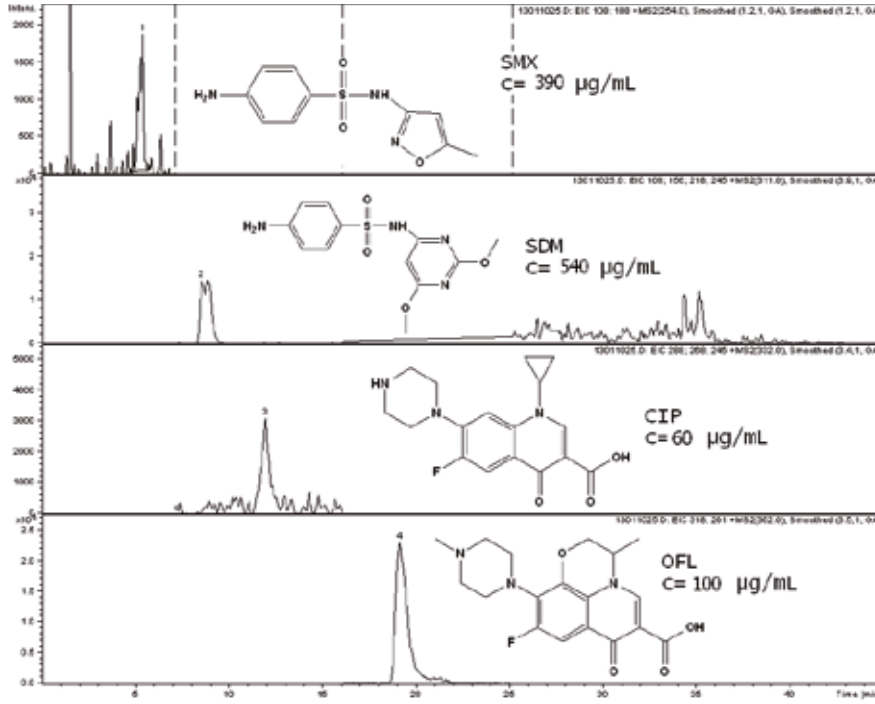


Figure 18. The chromatogram of the sample of lettuce leaves, grown in loamy soil at antimicrobials concentration of 10 mg/kg (dm).

1 – sulfamethoxazole; 2 – sulfadimethoxine; 3 – ciprofloxacin; 4 – ofloxacin.

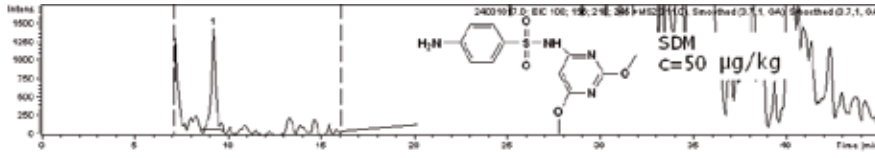


Figure 19. The chromatogram of the sample of wheat seeds cultivated in loamy soil at antimicrobials concentration of 10 mg/kg (dm).

1 – sulfadimethoxine.

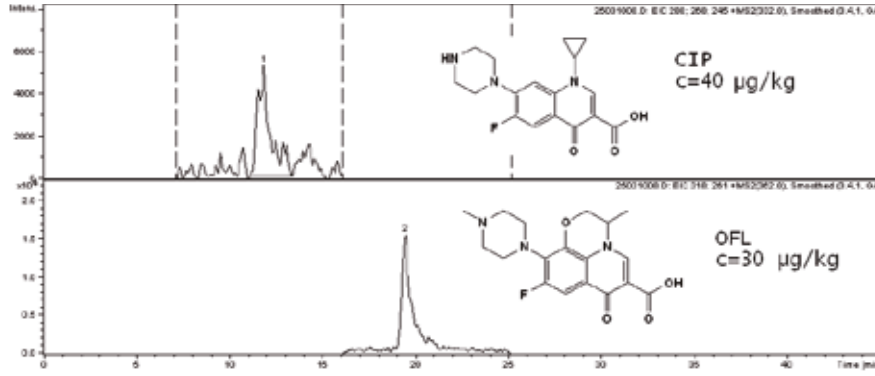


Figure 20. The chromatogram of the sample of wheat seeds cultivated in loamy soil at antimicrobial concentration of 0.5 mg/kg (dm).

1 – ciprofloxacin; 2 – ofloxacin.

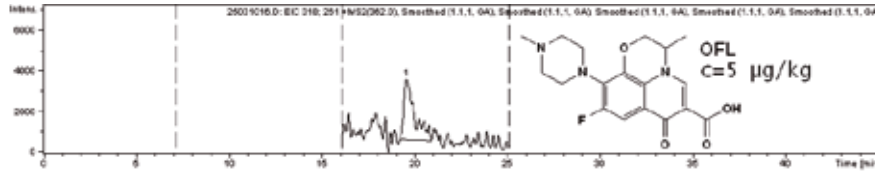


Figure 21. The chromatogram of the sample of wheat seeds cultivated in sandy soil at antimicrobial concentration of 0.1 mg/kg (dm).

1 – ofloxacin.

In carrot roots and potato tubers most of the studied antimicrobials were detected, except CIP and NOR in carrots grown in loamy soil. The chromatograms of the samples of carrot roots and potato tubers grown in sandy soil at antimicrobial concentration of 10 mg/kg are shown in figures 22 and 24. OFL accumulated into carrots and potatoes from soils with lowest antimicrobial concentration – 0.01 mg/kg. (Table 13, 14; Figure 23, 25). The content of CIP was found only in potatoes grown in sandy soil at antimicrobial concentration of 0.01 mg/kg (Table 13, 14; Figure 25).

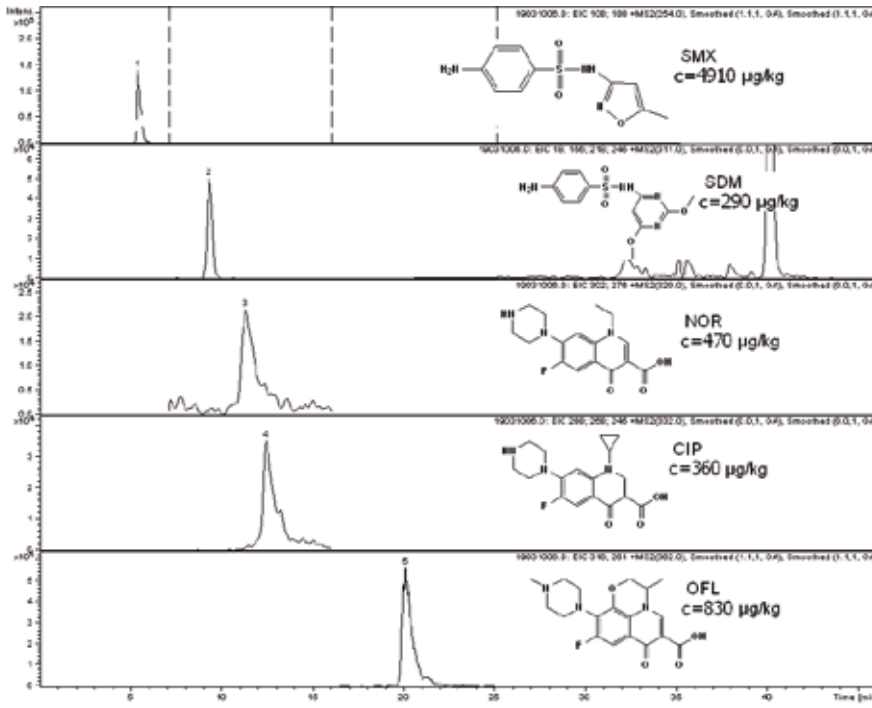


Figure 22. The chromatogram of the sample of carrot roots, grown in sandy soil at antimicrobials concentration of 10 mg/kg (dm).

1 – sulfamethoxazole; 2 – sulfadimethoxine; 3 – norfloxacin; 4 – ciprofloxacin; 5 – ofloxacin.

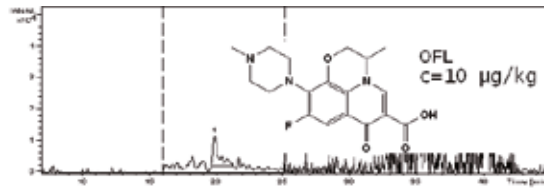


Figure 23. The chromatogram of the sample of carrot roots, grown in sandy soil at antimicrobials concentration of 0.01 mg/kg (dm).

1 – ofloxacin.

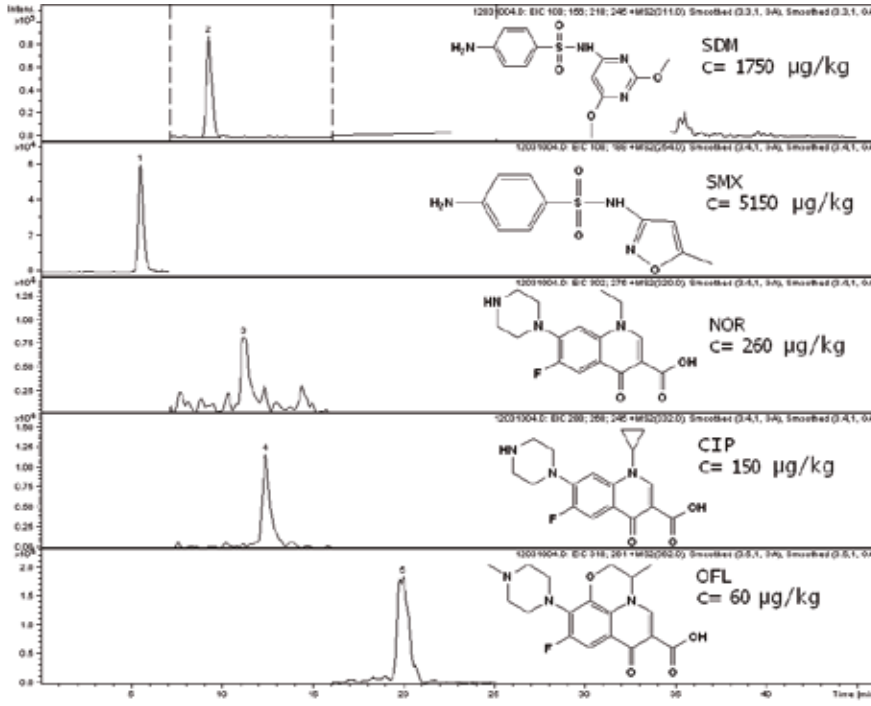


Figure 24. The chromatogram of the sample of potato tubers, grown in sandy soil at antimicrobials concentration of 10 mg/kg (dm).

1 – sulfamethoxazole; 2 – sulfadimethoxine; 3 – norfloxacin; 4 – ciprofloxacin; 5 – ofloxacin.

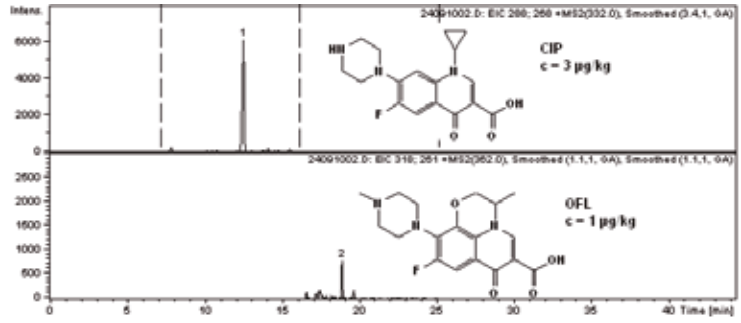


Figure 25. The chromatogram of the sample of potato tubers grown in sandy soil at antimicrobial concentration of 0.01 mg/kg (dm).

1 – ciprofloxacin; 2 – ofloxacin.

5.3.2. Antimicrobials in different parts of plants

Comparing the content of accumulated antimicrobials in leaves and roots of lettuce and carrot, it is noticeable, that in underground parts of plants the content of antimicrobials is much higher (Table 15, 16).

Table 15. Comparison of average contents of antimicrobials μg/kg (dm) in lettuce leaves and roots grown in loamy soil.

	Conc. of AM in soil mg/kg (dm)	Content of antimicrobials in lettuce μg/kg (dm)				
		CIP	NOR	OFL	SDM	SMX
leaves	10	93 (220)*	–	103 (140)*	181 (540)*	129 (390)*
	1	27	–	30	40	–
	0.5	–	–	–	23	–
	0.1	20	–	10	–	–
	0.01	–	–	–	–	–
roots	10	890 (1220)*	785 (1070)*	2355 (3530)*	3845 (3990)*	3630 (3650)*
	1	20	–	50	400	443
	0.5	20	–	30	103	110
	0.1	27	–	10	37	63
	0.01	–	–	3	–	–

AM – antimicrobial; CIP – ciprofloxacin; NOR – norfloxacin; OFL – ofloxacin; SDM – sulfadimethoxine; SMX – sulfamethoxazole; * maximum contents.

Tabel 16. Comparison of average contents of antimicrobials μg/kg (dm) in carrot leaves and roots grown in sandy soil.

	Conc. of AM in soil mg/kg (dm)	Content of antimicrobials in lettuce μg/kg (dm)				
		CIP	NOR	OFL	SDM	SMX
leaves	10	557 (1040)*	380 (590)*	467 (910)*	140 (150)*	163 (180)*
	1	27	–	30	40	–
	0.5	–	–	–	23	–
	0.1	20	–	10	–	–
	0.01	–	–	–	–	–
roots	10	473 (740)*	633 (990)*	820 (830)*	403 (660)*	3400 (4910)*
	1	40	27	127	16	206
	0.5	40	–	57	3	80
	0.1	–	–	10	–	7
	0.01	–	–	3	–	–

AM – antimicrobial; CIP – ciprofloxacin; NOR – norfloxacin; OFL – ofloxacin; SDM – sulfadimethoxine; SMX – sulfamethoxazole; * maximum contents.

The content of antimicrobials in plants cultivated in sandy soil was usually higher than in plants grown in loamy soil. Potato tubers and carrot roots grown in sandy soil at highest drug concentration of 10 mg/kg contained hundreds and thousands micrograms of antimicrobials per kg. The content of antimicrobials in potatoes and carrots grown in loamy soil was considerably lower (Figure 26, 27).

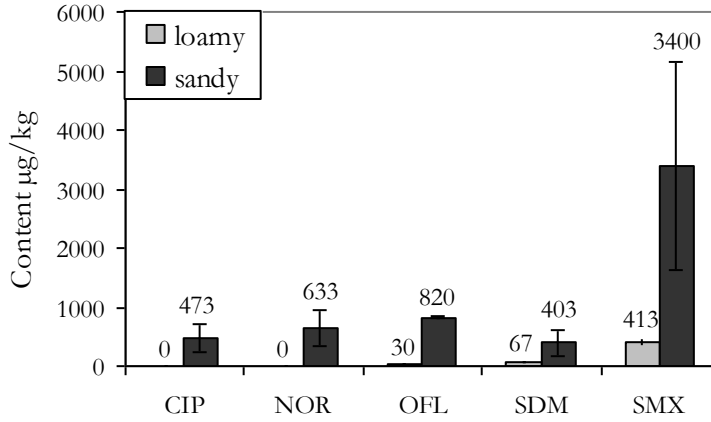


Figure 26. Average contents of antimicrobials in carrot roots grown in different soils at drug concentration of 10 mg/kg. CIP – ciprofloxacin; NOR – norfloxacin; OFL – ofloxacin; SDM – sulfadimethoxine; SMX – sulfamethoxazole. Error bars show standard deviations.

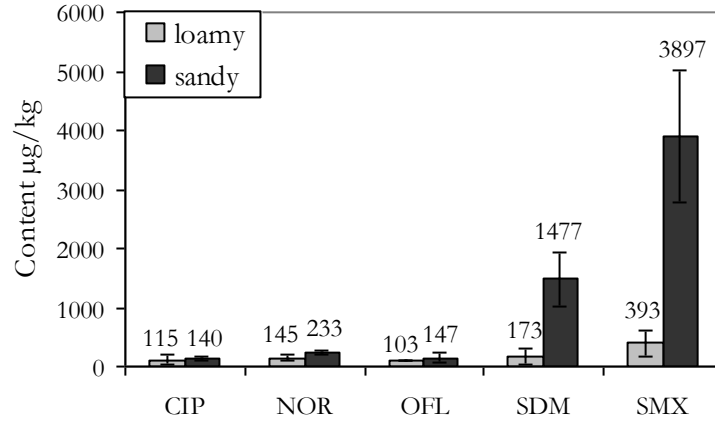


Figure 27. Average contents of antimicrobials in potato tubers grown in different soils at drug concentration of 10 mg/kg. CIP – ciprofloxacin; NOR – norfloxacin; OFL – ofloxacin; SDM – sulfadimethoxine; SMX – sulfamethoxazole. Error bars show standard deviations.

Remarkable contents of antimicrobials were detected in wheat leaves grown at drug concentration of 10 mg/kg in loamy soil. In wheat plants grown at soil concentrations 0.5 and 1 mg/kg only the content of OFL was detected (Figure 28). Much higher concentrations of antimicrobials were discovered in wheat leaves grown in sandy soil.

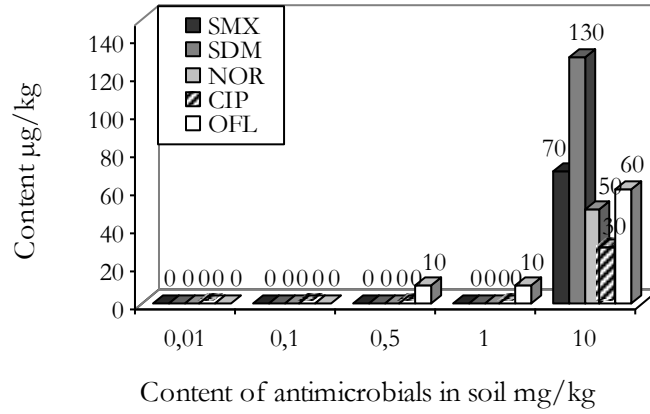


Figure 28. The highest contents of antimicrobials in wheat leaves grown in loamy soil. CIP – ciprofloxacin; NOR – norfloxacin; OFL – ofloxacin; SDM – sulfadimethoxine; SMX – sulfamethoxazole.

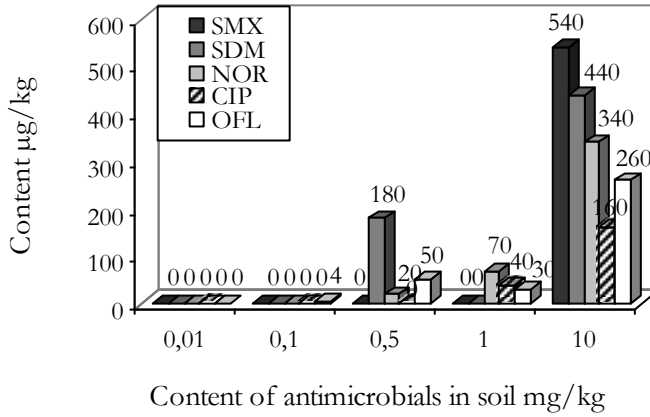


Figure 29. The highest contents of antimicrobials in wheat leaves grown in sandy soil. CIP – ciprofloxacin; NOR – norfloxacin; OFL – ofloxacin; SDM – sulfadimethoxine; SMX – sulfamethoxazole.

Wheat plants cultivated in sandy soil at drug concentration of 10 mg/kg wilted. From the samples of wilted wheat leaves especially high concentrations of antimicrobials were detected (Figure 29, 30). The content of SAs was the highest: 540 μg/kg (SMX) and 440 μg/kg (SDM). Quite high content of SDM was detected in wheat leaves grown in sandy soil at drug concentration of 0.5 mg/kg.

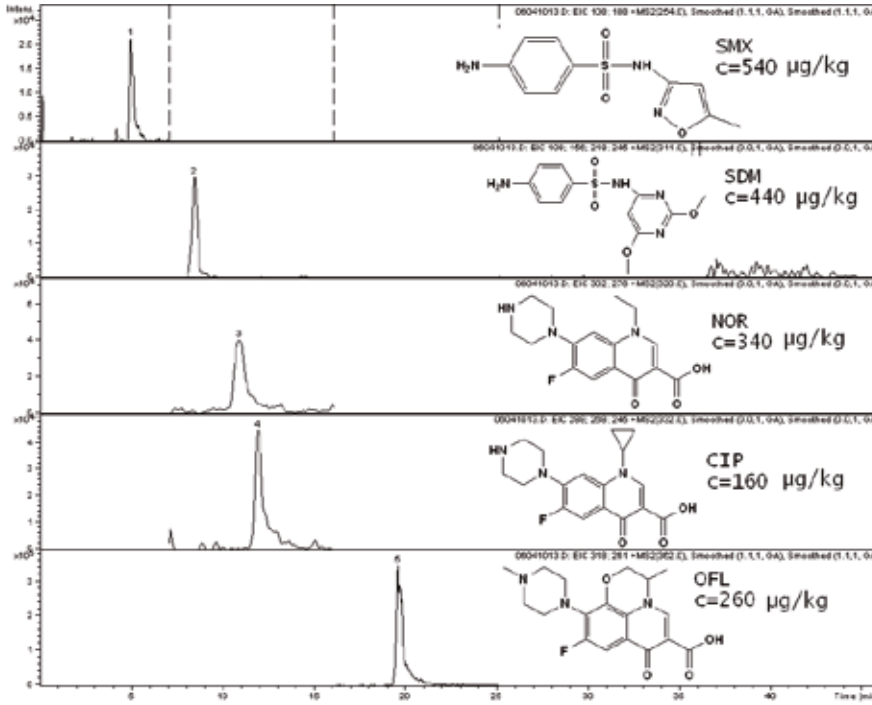


Figure 30. The chromatogram of the sample of wheat leaves, grown in sandy soil at antimicrobials concentration of 10 mg/kg (dm).

1 – sulfadimethoxine; 2 – sulfamethoxazole; 3 – norfloxacin; 4 – ciprofloxacin; 5 – ofloxacin.

6. DISCUSSION

6.1. Determination of fluoroquinolones, sulfonamides and tetracyclines

Analytical tools have been developed for different media, e.g. food products (Toussaint *et al.*, 2002; Toussaint *et al.*, 2005; Posyniak *et al.*, 2005), environmental water samples (Lindsey *et al.*, 2001; Lindberg *et al.,* 2004; Nakata *et al.*, 2005; Choi *et al.*, 2007), soil (Golet *et al.,* 2002; Lillenberg *et al.,* 2003; Christian *et al.*, 2003; Jacobsen *et al.,* 2004; Stoob *et al.*, 2006) and manure (Christian *et al.,* 2003; Jacobsen and Halling-Sørensen, 2006), while only few methods have been designed for extraction of antibacterial agents from sewage sludge (Golet *et al.,* 2002; Göbel *et al.*, 2005[1]). The existing methods (Christian *et al.,* 2003) cannot be used for simultaneous extraction of FQs, TCs and SAs from sewage sludge.

Several extraction techniques have been applied for the determination of antibiotics from solid phase, such as ultrasonic-assisted extraction (USE) (Lindberg *et al.,* 2005; Blackwell *et al.*, 2004; Turiel *et al.*, 2006), microwave-assisted extraction (MAE) (Hermo *et al.,* 2005), pressurized liquid extraction (PLE) and accelerated solvent extraction (ASE) (Golet *et al.*, 2002; Christian *et al.,* 2003). For extracts clean-up liquid-liquid extraction (LLE) (Haller *et al.*, 2002; Jacobsen and Halling-Sørensen, 2006) and solid-phase extraction (SPE) (Lindsey *et al.,* 2001; Golet *et al.,* 2002; Lindberg *et al.*, 2004; Posyniak *et al.*, 2005) were used. ASE or PLE have clear advantages over other methods such as higher precision, smaller amounts of extraction solvent and reduced sample preparation time (Giergielewicz-Mozajska e*t al.,* 2001). Sludge extraction is usually followed by pre-concentration and clean-up of the PLE extracts using SPE with different cartridges (Golet *et al.,* 2002; Jacobsen *et al.,* 2004; Göbel *et al.*, 2005). Most of the relevant analytical methods reported are based on liquid chromatography – mass spectrometry (LC–MS) (Niessen, 1998; Diaz-Cruz and Barceló, 2005), liquid chromatography – tandem mass spectrometry (LC–MS/MS) (Toussaint *et al.*, 2002; Ternes *et al.,* 2005) and HPLC-UV (Nakata *et al.*, 2005; Hermo *et al.*, 2005).

The developed method is based on the combination of PLE, SPE, LC–MS and its subsequent application to perform screening of sewage sludge for a total of 7 structurally and chemically diverse antibiotics (CIP, NOR,

OFL, SDM, SMX, TCL and DOX) using the same extraction process. The variables optimized were the extraction solvent and pH, time, temperature, pressure and the number of extraction cycles. Methanol and acetonitrile aqueous solutions showed lower extraction efficiencies for most of the studied antibiotics. The mixture of 0.35% phosphoric acid and acetonitrile (1:1, v/v) with 0.01 M citric acid monohydrate was finally chosen as an extraction solvent. During PLE optimization it was established that 10 min extraction times were insufficient, and with times >15 min the recoveries for TCs decreased. The decrease in recovery was also observed at temperatures exceeding 120 °C, resulting either from thermal degradation or from a loss in method selectivity due to the more efficient extraction of interfering matrix components. The effect of pressure on extraction efficiency of antibiotics was studied in the range from 70 to 110 atm (7,091 to 11,143 kPa), and no significant impact on the extraction efficiency was seen in this range.

The next variable studied was the number of extraction cycles. Five consecutive simple extractions were applied to the same sample with identical extraction conditions. The recoveries were considered negligible (lower than 2%) during the fourth cycle. The fifth cycle showed no antibiotics. In the current study, five extraction cycles were applied when treating the samples. Spiking and recovery experiments with quartz sand were preformed during the method development. Recoveries for TCL, DOX, NOR, CIP and SMX ranged from 55 to 100%. During the PLE method development a serious carryover was observed, especially after the extraction of spiked samples. Repetitive cleaning with different solvents appeared to be of low efficiency. For cleaning the PLE vessel an original simple solution was proposed: a small volume of ethanol was burnt in the extraction vessel and subsequently the vessel was rinsed with the extraction solvent. Optimum conditions with regard to extraction solvent and number of extraction cycles were established.

For the PLE extracts clean-up procedure two types of SPE cartridges – SCX (strong cation-exchange) and HLB (hydrophilic-lipophilic balance) – were used. To optimize the SPE procedure, SCX cartridges with various sorbents based on silica (supplied by Phenomenex) and polymer (supplied by Alltech) were applied. From different types of SCX cartridges the most reliable results were obtained when using Phenomenex SCX modified silica cartridges due to better isolation of antibiotics from the

sample. Different solvents, such as 1:4 NH_4OH/water in methanol or in acetonitrile were tested for eluting antibiotics from the cartridges. The comparison of the tested solvents showed that the higher content of organic modifier resulted in higher recoveries. Best results with SCX cartridges were obtained when using 1:4:1 NH_4OH/water/methanol. Good repeatability was achieved using SCX cartridges.

To increase the accuracy of the method, the HLB cartridges were used due to their ability to retain both hydrophilic and hydrophobic compounds (Lindsey *et al.*, 2001). The sample pH was adjusted to 2.0 and methanol was used for the elution. The pH of the PLE extracts was adjusted to pH 2.0 before SPE analysis using concentrated hydrochloric acid. Higher pH values resulted in lower recoveries.

Identification and quantification of the pharmaceuticals were carried out by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) using electrospray ionization (ESI). The best recovery of FQs and TCs and SDM was obtained using HLB cartridges, recoveries ranged 59% for NOR to 82% for OFL and 95% for DOX. As an exception, SMX had better recovery (96%) and smaller standard deviation with SCX cartridges (Figure 8).

Comparison of the two cartridges showed that HLB cartridges were more efficient and reliable: the standard deviation of the results ranged from 0.01 to 0.18 and the recoveries ranged from 26% to 95% (overall recovery from standard addition experiments). Limit of quantification ranged from 0.1 ng/g for SAs to 160 ng/g for TCL (Table 5). Therefore, SPE by using HLB cartridges can be applied for determination of 6 antibiotics from sewage sludge CIP, NOR, OFL, SDM, SMX and DOX. However, determination of TCL needs to be improved.

6.2. Antimicrobials in sewage sludge and its compost

Current national and international regulations governing the application of sewage sludge ignore the possible presence and fate of pharmaceuticals in this medium. For example, sulfonamides have been detected in raw sewage sludge (Thomsen *et al.*, 2003) and in treated sewage (Lindberg *et al.*, 2005). Sulfamethoxazole is not biodegradable in sewage treatment tanks (Richardson and Bowron, 1985). Elevated concentrations of

fluoroquinolones have been found in treated sewage and in soil fertilized with sewage sludge (Golet *et al.*, 2002). Tetracyclines do not decompose in soil during a 6 month period (Sczesny *et al.*, 2003). The slow degradation is explained by their strong adsorption to solid particles (Marengo *et al.*, 1997; Christian *et al.*, 2003; Carmosini and Lee, 2008).

The results of this work showed, that antibiotics are present in Estonian sewage sludge. The highest antibiotic concentration in sewage sludge was detected in the case of ciprofloxacin in Tallinn: 1520 μg/kg (dm). It exceeds the trigger value for manure (100 μg/kg; dm) over fifteen times. The highest content of NOR, also over the trigger value, was found in Tallinn: 580 μg/kg. The content of OFL in Tallinn was lower – 134 μg/kg, but still exceeding the trigger value for manure. The content of SAs was quite low in both cities, under 100 μg/kg, but at least one of them was present in every sludge sample. In Tartu the highest concentrations of antibiotics were NOR 439 and CIP 442 μg/kg. OFL was present in every sludge sample from Tartu, the highest content was 157 μg/kg. TCs were not found in the sewage sludge, neither in Tallinn nor in Tartu.

Detected fluoroquinolones in sewage sludge are in good correlation with antimicrobials sold in recent years, except SMX and DOX. (Table 18). SMX was sold at quite large amounts, but its detected content in sewage sludge and compost was always low. Probably sulfonamides do not absorb in solid sewage sludge, but end up in surface water.

Comparing with other studies the highest CIP content in Tallinn sludge 1.52 mg/kg was comparable with its content in cities of Sweden: Gothenburg 1.4–3.4 mg/kg and Floda 0.5–0.9 mg/kg. The highest content of NOR in Tallinn sludge 0.58 mg/kg was comparable with its content in Floda 0.1-0.4 mg/kg. The highest content of OFL in Tallinn sludge 0.13 mg/kg was comparable with its content in Gothenburg 0.00–0.1 mg/kg (Lindberg *et al.*, 2005).

In most of the studied sewage sludge and compost samples the concentrations of pharmaceuticals were clearly extremely low, but in some sludge samples the content of pharmaceuticals still exceeded 100 μg/kg, and in some sewage compost samples the drug concentrations were above the trigger value for soil – 10 μg/kg. Remarkable concentration variations could be observed due to the heterogeneity of the studied

matter. CIP and OFL were not present in the randomly taken sewage samples in Tartu. In spite of this their presence in sludge and compost was obvious.

A large variability of concentrations of antimicrobials in sewage and sewage sludge in both cities was apparent. As a result of this heterogeneity the concentration of pharmaceuticals varies noticeably within the same compost stack. For example, the content of fluoroquinolones differed up to 1.8 times within the same stack in Tartu. The determined concentrations of sulfonamides were much lower in compost, if compared to the concentrations of fluoroquinolones. SDM content in the 6-month stored compost stacks was under the detection limit (0.01 μg/kg) in Tallinn and very low, from n.d. to 1 μg/kg in Tartu. Concentrations of SMX in all compost samples of Tallinn (2-, 6-, and 12-month stored) varied from n.d. to 1 μg/kg, in Tartu from n.d. to 3 μg/kg. Surprisingly, SDM was present in most sludge and 2-month stored compost samples from both cities, although this antimicrobial was not marketed in the years 2007 and 2008 in Estonia (Table 17). It is possible that old supplies were put to use or small amounts of this chemical were imported from other countries.

Table 17. Amounts of human medicine antimicrobials (kg) marketed in Estonia 2001–2008 (State Agency of Medicines[1,2]).

AM	2001	2002	2003	2004	2005	2006	2007	2008
CIP	202.1	217.8	260.3	280.5	276.6	293.4	305.9	311.8
NOR	69.3	69.9	73.4	86.2	95.0	110.1	118.2	122.5
OFL	61.7	20.2	16.0	28.5	36.9	37.7	27.3	45.4
SDM	39.2	27.4	19.1	11.5	4.7	0.8	0	0
SMX	882.9	707.8	619.4	349.2	441.1	446.6	420.0	400.4
TCL	341.1	292.2	133.9	87.0	84.6	59.5	56.2	50.2
DOX	141.7	135.1	137.9	122.8	116.2	112.3	112.6	106.1

AM – antimicrobial; CIP – ciprofloxacin; NOR – norfloxacin; OFL – ofloxacin; SDM – sulfadimethoxine; SMX – sulfamethoxazole; TCL – tetracycline; DOX – doxycycline.

It is known that 90% of fluoroquinolones are eliminated in conventional sewage treatment with sorption to sewage sludge being the main process responsible (Golet *et al.*, 2002; 2003). For sulfonamides, sorption to sewage sludge has been found to be of minor importance (Göbel *et al.*, 2005, 2007). Distribution coefficients for the adsorption of antimicrobials to soil material and aquatic sediments vary for SAs from 0.6 to 4.9, for TCs from 290 to 1620, and for FQs from 310 to 6310 (Tolls, 2001). The residues of TCs and FQs, strongly adsorbed on solid particles, may not decompose during the sewage sludge treatment. It is known that fluoroquinolones are also thermally stable (Golet *et al.*, 2002; Lillenberg, 2003). Fluoroquinolone residues 2.13 – 2.42 mg/kg (that exeeds trigger values for manure) have been found in anaerobically treated sewage sludge (Golet *et al.*, 2002). Even 21 months after fertilization the residues of fluoroquinolones have been found: in average 0.27 mg/kg of CIP and 0.3 mg/kg of NOR (Golet *et al.*, 2002), i.e. again over the trigger value for soil. Walters *et al.* (2010) showed the persistence of FQs in biosolid-amended soils: after 994 days of weathering CIP and OFL were still present at over 390 and 267 μg/kg dray weight, respectively. This demonstrates extremely long stability of FQ-s in soil.

Heterogeneity of the compost may be the result of adsorption of the pharmaceuticals to solid particles. The content of pharmaceuticals in the compost made from sewage sludge may easily lead to elevated concentrations in food plants, if the compost is used as a fertilizer.

It is inevitable that pharmaceuticals are released to the soil environment. Studies with single substances in different soil types indicate that degradation rates are variable but it is not yet possible to correlate persistence with soil properties or soil bioactivity (Monteiro and Boxall, 2009). Pharmaceuticals enter the environment associated with biosolids and presence of biosolids may significantly reduce degradation rates compared to soil alone. The effect of biosolids is probably highly dependent on the source and nature of the biosolids used (Monteiro and Boxall, 2009). Degradation of pharmaceuticals in the environment is a very complex issue and a lot more data on the degradation behavior of pharmaceuticals in a range of well-characterized soils with different properties are needed in order to understand what happens to a pharmaceutical in the real soil environment (Monteiro and Boxall, 2009).

Pharmaceuticals never occur in the environment on their own and are likely to co-occur with other pharmaceuticals and also with contaminants from other classes. Interactions between pharmaceuticals and other contaminants may affect the fate of the pharmaceutical, and its degradation may be significantly slower in mixtures (Monteiro and Boxall, 2009). Antimicrobials are likely to be present in biosolids and since they have been designed to affect microorganisms, they may influence microbial degradation. It has been shown that the presence of biosolids significantly reduces degradation rates compared to soil alone (Monteiro and Boxall, 2009).

In the current work the concentrations of the studied pharmaceuticals in the compost were up to one order of magnitude lower, if compared to the relevant concentrations in sewage sludge. This effect is partly caused by the addition of peat or tree bark to the sewage sludge. Still, the main reason of the decrease in pharmaceutical concentrations during composting is the applied sludge treatment technology.

The decomposition of pharmaceuticals was faster in the case of the composting technology used in Tallinn (compost was made by mixing the anaerobically treated sewage sludge with peat), if compared to the results obtained in Tartu (pressed raw sewage sludge was mixed with tree bark). Nevertheless, the concentrations of FQ-s in the compost ready for land application reached 70 μg/kg (CIP), 64 μg/kg (NOR) and 8 μg/kg (OFL) in Tartu, in Tallinn the contents of FQ-s were 9 μg/kg, 17 μg/kg and 8 μg/kg respectively.

No trigger values exist for drug residues in sewage compost in the European Union. Their content should not exceed 100 μg/kg in manure and 10 μg/kg in soil (EMEA/CVMP/055/96). However, the EU Scientific Steering Committee considered the soil trigger value as non-scientific. It is required that the trigger values should be protective for all substances. Thus, scientifically proved content of a pharmaceutical in soil should not exceed 1 μg/kg (Montforts, 2005).

Large-scale production and use of antimicrobials have taken place for over 60 years. At the same time, the resistance to antibiotics is increasing both in benign and pathogenic bacteria. Exposure of living organisms to antimicrobials might create reservoirs of resistance traits in soil organisms

that may have broader consequences to public health (Knapp *et al.*, 2010). There is evidence that resistant bacteria in the environment potentially can confer resistance to pathogens via horizontal gene transfer and other mechanisms (Davies, 1994). Bacteria that survive the sewage sludge treatment processes, are resistant to several pharmaceuticals (Reinthaler *et al.* 2003; Sahlström *et al.,* 2009). These findings allow to presume that both sewage sludge and manure are reservoirs of the antibiotic resistant bacteria and fertilization is the way to transfer them into the environment. As the sewage sludge may contain tens of different antimicrobials in low concentrations, it is the perfect matter for developing multidrug resistant bacteria. High prevalence of SA-resistance has been observed in bacteria from animals and humans all over the world (Bean *et al.,* 2009; Hammerum *et al.*, 2006; Wu *et al.,* 2010). Resistance to FQs is still low, but is increasing with time (Boyd *et al.*, 2008).

Pharmaceutical antibiotics can exert a temporary selective pressure on soil microorganisms even at environmentally relevant concentrations (Thiele-Bruhn and Beck, 2005). Resistance development occurs already at the Minimum Effect Concentration (MEC) at which bacterial growth is reduced, that is tenfold below the Minimum Inhibitory Concentration (MIC), the endpoint used to derive the soil concentration trigger (O`Reilly and Smith, 1999). If it is required that the trigger values should be protective for all substances, these trigger values should be set at no higher than 1 μg/kg for soil and no higher than 0.4 ng/L for water. Since effects may occur at sub-therapeutic levels, a safety factor of 10 for this aspect would push the soil concentration trigger further down to 0.01 – 0.1 μg/kg (Montforts, 2005). To avoid exceeding these concentrations in soil, content of pharmaceutical in sewage sludge compost (or manure) should not exceed 1 μg/kg.

The concentrations of fluoroquinolones detected in sewage sludge compost from Tallinn and Tartu were in several samples around or more than 10 μg/kg. In some sewage sludge compost samples the concentrations of sulfonamides exceeded 1 μg/kg. The safest way to exclude exposing plants to pharmaceuticals is to ensure that these substances are adequately degraded before sewage sludge compost is applied onto arable land.

This work shows that studies of the sewage sludge used for making compost and the development of novel sewage sludge treatment technologies with the aim of solving environmental problems related to sewage sludge exploitation are needed. The possible danger resulting from the content of pharmaceuticals in sewage sludge can not be ignored.

6.3. Uptake of antimicrobials by food plants

Antimicrobials have many properties that are neccessary for bio-accumulation and provoking changes in ecosystems. They are often lipophilic in order to pass biomembranes and persistent in order to avoid the inactivation before having a curative efect (Halling-Sørensen *et al.*, 1998). Pharmaceuticals are poorly biodegraded by the bacterial community present in sewage sludge amended soils (Redshaw *et al.*, 2008). Therefore, undesirable effects, such as intake by plants, leaching into the groundwater and negative impact on the terrestrial organisms are not excluded. Fertilizing fields with sewage sludge or manure, containing antimicrobials, expose these compounds to plants. Due to adsorption of antimicrobials to solid sewage sludge (or manure) particles, the concentration of drugs may be locally higher, than average in soil (Lillenberg, 2003). Plants unlike animals have no excretion, therefore, drug residues may accumulate into plants. As a result, concentrations of drug residues in food plants may exceed the maximum residue limits (MRL) for meat and milk (EMA/EPMARs). No limits have been set for drug residues in plant products at present. Antimicrobials consumed with everyday food in amounts below MRL_{mic} may generate strains of resistant bacteria in human and animal organisms.

Only a relatively small number of investigations have been published on the mobility and bioavailability of pharmaceuticals in the environment. Uptake of antimicrobials into vegetation is a major route for these substances into the food chain. In the current study uptake of ciprofloxacin, norfloxacin, ofloxacin, sulfadimethoxine and sulfamethoxazole was demonstrated in the case of lettuce, potato, carrot and wheat.

Measureable residues of the studied compounds occured in food plants. The uptake of fluoroquinolones and especially sulfonamides by plants

like potato and carrot might pose health risk, as the detected levels of the studied pharmaceuticals were of considerable magnitude, if compared to their soil concentrations. Sulfonamides are among the most commonly used antibiotics in veterinary medicine and to a lesser extent in human medicine (García-Galán *et al.*, 2009). They are both fairly water-soluble and polar (Thiele-Bruhn *et al.*, 2004). The low adsorption of SAs on soil particles is known (Brambilla *et al.*, 1996) and due to this phenomenon they are "free" to migrate into plants. Nevertheless, García-Galán *et al.*, (2009) have pointed out that in loamy soil the molecules of sulfonamides attach to clay particles, reducing their uptake by plants. This behavior is more characteristic to fluoroquinolones. It has been shown that more than 90% of applied ciprofloxacin and ofloxacin is adsorbed on different soils (Brambilla *et al.*, 1996). For this reason no significant migration of fluoroquinolones from soil into plants takes place, however, the uptaken amounts of FQs from the highest soil antimicrobial concentration (10 mg/kg) exceeded the MRL for milk and meat (220 μg/kg CIP and 140 μg/kg OFL in lettuce leaves; 170 μg/kg CIP, 260 μg/kg NOR and 240 μg/kg OFL in potato tubers; 740 μg/kg CIP, 990 μg/kg NOR and 830 μg/kg OFL in carrot roots). In carrot roots the content of OFL (160 μg/kg) exceeded the MRL at soil antimicrobial concentration of 1 mg/kg. As a rule, the uptake of antimicrobials was more efficient from sandy soil into underground parts on plants - roots and tubers. In wheat seeds the concentrations of studied antimicrobials were always below the MRL. The average concentration of SMX in lettuce roots was of the same magnitude if compared to the edible parts of potato and carrot – 3630 μg/kg, 3897 μg/kg and 3400 μg/kg, respectively. The average content of SDM in plants was highest in the case of lettuce roots - 3845 μg/kg.

Once released into the environment, sulfonamides become very mobile, and as a consequence they may affect many non-target organisms. As there are usually several pollutants of the same family or the same type that coexist in soil, synergic or antagonistic effects should be considered (Thiele-Bruhn, 2003). The average concentrations of CIP, NOR and OFL in lettuce roots – 890,

785 and 2355 μg/kg, respectively – were lower than the concentrations of sulfonamides – SDM 3845 and SMX 3630 μg/kg – but they still exceeded the concentrations that were detected from potato tubers and

carrots roots (140 μg/kg CIP, 233 μg/kg NOR and 147 μg/kg OFL in potato tubers; 473 μg/kg CIP, 633 μg/kg NOR and 820 μg/kg OFL in carrot roots).

Accumulation of antimicrobials in food plants may pose a danger, as very small amounts of these drugs in everyday food, being clearly under the levels of MRL for meat and milk, may generate the strains of resistant bacteria in humans. Eating of different food products (potato, meat, milk, lettuce, carrot, etc) together in regular diet may lead to intake of elevated amounts of antimicrobials, exceeding the value of ADI_{mic}.

The concentrations of antimicrobials in plants exceeding the MRL values for meat and milk were seen only at antimicrobial soil concentrations equal to 0,5 mg/kg, 1 mg/kg and 10 mg/kg. Such concentrations might be present in soil if sewage sludge treated by anaerobic digestion is used as a fertilizer. In Estonia the soil concentrations of antimicrobials are presumably lower, as only sewage sludge compost may be used for fertilization.

Different MRLs for drug residues in animal products are set, depending on animal species, target tissues and pharmacological properties of the drug (EMA/EPMARs). There are still no limits set for drug residues in plant products at present time. Therefore, further studies are needed to determine the uptake of different pharmaceuticals and other organic pollutants from different types of soils by various crop plants. Further studies should be conducted to determine the uptake of different types of pharmaceuticals and other organic pollutants by various crop plants, and sorption kinetics at the soil-root interface has to be studied.

7. CONCLUSIONS

1. Pharmaceuticals were present in sewage sludge and its compost from both Tallinn and Tartu and in several samples their concentrations exceeded the relevant trigger values for manure. The highest concentrations of antibiotics were found in the sewage sludge from Tallinn: CIP 1520 μg/kg and NOR 580 μg/kg (dm). The maximum concentration of CIP exceeded the trigger value for manure (100 μg/kg) over fifteen times. The concentrations of OFL (134 μg/kg), SDM (73 μg/kg) and SMX (22 μg/kg) were lower. As a rule, the concentrations of antimicrobials in the sewage sludge from Tallinn were relatively low. Still, in some cases the contents of CIP, NOR and OFL were over the trigger value. In Tartu, contrarily, the content of CIP and NOR was mostly over the trigger value, the high content of OFL was detected only in August, September and October. TCL and DOX were not detected in Tallinn nor in Tartu. The content of SAs was quite low in both cities, under the trigger value set for drug residues in manure. In Tartu at least one of them was present in every sludge sample. The highest concentrations of antimicrobials in sewage sludge of Tartu were: NOR – 439 μg/kg and CIP – 442 μg/kg (dm). OFL was present in every sludge sample from Tartu and the highest concentration was 157 μg/kg (dm).

2. Degradation of pharmaceuticals took place as a result of composting. The concentrations of the studied antimicrobials decreased remarkably during composting both in Tallinn and in Tartu. A major decrease was seen in the case of 2-month stored stacks. In Tallinn the antimicrobials were almost absent in compost stacks that had been formed 12 months earlier. However, in 6-months stored compost the content of NOR exceeded and the content of CIP was near the recommended trigger value for soil. In Tartu the degradation rate of antimicrobials in compost was lower.

3. The main reason of the decrease in pharmaceutical concentrations during composting was the applied sludge treatment technology. The safest way to avoid exposing plants to pharmaceuticals is to ensure that these substances are adequately degraded before sewage sludge compost is applied onto arable land. The decomposition of pharmaceuticals was faster in the case of the composting technology used in Tallinn.

4. The uptake of the studied pharmaceuticals by food plants was obvious. As a rule, the uptake of antimicrobials was more efficient from sandy soil into underground parts of plants – roots and tubers. In wheat seeds the concentration of antimicrobials was always below the MRL for meat and milk. In lettuce leaves the concentrations of antimicrobials exceeded the MRL only in plants grown at the highest soil antimicrobial concentration – 10 mg/kg. Such concentrations might be present in soil if sewage sludge treated by anaerobic digestion is used as a fertilizer. In Estonia the soil concentrations of antimicrobials are presumably lower, as only sewage sludge compost may be used for fertilization of soil. The uptake of fluoroquinolones and sulfonamides by plants like potato and carrot might pose a health risk, as the detected levels of the studied pharmaceuticals were of considerable magnitude.

5. The application of sewage sludge compost as a fertilizer and the following uptake of pharmaceuticals by food plants may cause contamination of these plants. The results of the current work show, that the uptake antimicrobials may cause the contamination of these plants if the concentration of antimicrobials in soil is 10 mg/kg. The uptake of ciprofloxacin and ofloxacin by plants may take place from the soil at antimicrobial concentration of 0.01 mg/kg. Antimicrobials consumed in very small amounts with everyday food may generate strains of resistant bacteria in human and animal organisms. For prevention of development of microbial resistance in human/animal organism and in soil the concentration of antimicrobials in compost must be clearly below 1 μg/kg, securing the relevant soil concentrations at 0.01–0.1 μg/kg. The application of sewage sludge compost as a fertilizer may take place only after careful testing against possible toxic pollutants.

6. Further work should be conducted to determine the uptake of different types of pharmaceuticals and other organic pollutants by various crop plants, and sorption kinetics at the soil-root interface has to be studied.

8. SUMMARY IN ESTONIAN

MÕNEDE RAVIMIJÄÄKIDE SISALDUS EESTI REOVEESETTES, NENDE STABIILSUS KESKKONNAS JA AKUMULEERUMINE KOMPOSTVÄETISEST TOIDUTAIMEDESSE

8.1. Sissejuhatus

Maakera rahvastiku kasv toob kaasa üha uusi lahendamist vajavaid keskkonnaprobleeme. Intensiivistuva toiduainete tootmise tingimustes on vaja üha enam tähelepanu pöörata toiduohutuse tagamisele. Käesoleva doktoritöö autor on uurinud toidutekkelisi parasitaar- ja viirushaigusi (Lillenberg ja Järvis, 2005), toiduainete saastumist patogeensete bakteritega (Roasto *et al*., 2009; Meremäe *et al*., 2010; Roasto *et al*., 2011), tegelenud mullas ja toidutaimedes sisalduvate ravimijääkide määramismeetodite väljatöötamisega (Lillenberg, 2003; Lillenberg *et al*., 2003) ning reoveepuhastusalase probleemistikuga (Nei ja Lillenberg, 2009[1]). Põhiline osa uurimistööst on aga seotud reoveesette kasutamisvõimaluste uurimisega mullaviljakuse tõstmisel. Reoveesete on väga toiteaineterikas, kuid lisaks toitainetele sisaldab see kahjuks mitmesuguseid saasteaineid, olles seega oma olemuselt ohtlik jääde. Vaatamata sellele on reoveesetet lubatud kasutada väetisena haljastuses, metsanduses ja ka põllumajanduses tingimusel, et see on muudetud ohutuks keskkonnale ning inimeste ja loomade tervisele. Rahvusvaheliselt ja siseriiklikult kehtivad regulatsioonid nõuavad raskemetallide, fekaalsete *coli*-laadsete bakterite ja helmintide munade sisalduse määramist, mis on aga sette ohutuse hindamise seisukohalt selgelt ebapiisav, sest selles sisalduvate bioloogiliste ja keemiliste kontaminantide hulk võib-olla mitmeid kordi suurem. Seetõttu leiab reoveesete julgemat kasutamist haljastuses, metsanduses ja prügilate katmisel kui põllumajanduses. Üha enam on hakatud reoveesetet koos muude kütustega põletama või sellest biogaasi tootma.

Viimasel aastakümnel on pööratud varasemast suuremat tähelepanu keskkonna saastumisele ravimijääkidega. Kanalisatsiooni ja sealt edasi reoveesettesse satub ravimeid, mille liikumine ahelas reovesi – reoveesete – kompost – muld – taim – inimene (või loom) võib ohustada ahela viimast lüli. Reoveesettes sisalduvate ravimijääkide lagunemiskiiruse sõltuvust komposti valmistamise tehnoloogiast ei ole seni maailmas uuritud, see

on uudne ja oluline teema. Piisavalt ei ole uuritud ka ravimite liikumist väetatud mullast taimedesse. Teaduspublikatsioonides rõhutatakse ravimite taimedesse akumuleerumise väljaselgitamise tähtsust.

Mõned laialdaselt kasutatavad antibiootikumid, näiteks tetratsükliinid ja fluorokinoloonid, säilivad mullas kaua. Laborkatsed on kinnitanud, et sulfoonamiidid ja fluorokinoloonid akumuleeruvad taimedesse, sh. toidutaimedesse. Erinevalt loomorganismist puudub taimedel väljutusmehhanism ning seetõttu võivad ravimid taimedes kontsentreeruda. Kasvuperioodi lõpuks võib taime ravimisisaldus olla suurem kui kasvumullas ja ületada loomsele toidutoormele kehtestatud piirnormi, millega kaasneb oht inimese tervisele (Lillenberg *et al.*, 2003). Tuginedes teadaolevatele andmetele, uuriti keskkonnas kauapüsivate ja potentsiaalselt taimedesse akumuleeruvate ravimijääkide sisaldust Eesti suuremate linnade, Tallinna ja Tartu, reoveesettes, nende lagunemist sette töötlemise käigus ja akumuleerumist mullast toidutaimedesse. Uuritavate ravimite valikul lähtuti ka eelnevatel aastatel Eestis müüdud ravimite kogustest (Eesti ravimistatistika 2002–2006; Eesti ravimistatistika 2006–2008). Ravimijääkide sisaldust reoveesettes ja kompostis ei ole Eestis varem uuritud. Esmakordselt uuriti ravimite akumuleerumist mullast toidutaimedesse madalate ravimikontsentratsioonide korral, millised võiksid reoveesettega väetatud mullas esineda.

Reoveesette ja komposti proovid võeti AS Tallinna Vesi ja AS Tartu Veevärk reoveepuhastusjaamadest. Katsemullad valmistati ette Eesti Maaülikooli PKI mullateaduse ja agrokeemia osakonnas. Taimkatsed viidi läbi Luunjas, AS Grüne Fee kasvuhoones. Kromatograafilised analüüsid teostati Tartu Ülikooli Keemiainstituudis. Taimkatseteks valiti lehtsalat *(Lactuca sativa L.)*, porgand *(Daucus carota L.),* kartul *(Solanum tuberosum L.)* ja nisu *(Triticum vulgare L.)*. Käesolevas töös kasutatud uurimismetoodikate väljatöötamine ja töö tulemused on leidnud kajastamist *ISI* teadusartiklites (Lillenberg *et al.*, 2009; Lillenberg *et al.*, 2010[1]; Lillenberg *et al.*, 2010[2]; Kipper *et al.*, 2010). Töö tulemusi on esitletud ka mitmetel rahvusvahelistel konverentsidel (*SETAC* Varssavi, 2008; *SETAC* Tampa, 2008; *CNSSS* Tallinn, 2009; *ICEST* Bangkok, 2010; *SETAC* Sevilla, 2010; *ICBEE* Kairo, 2010) ning populaarses vormis ajakirjas „Keskkonnatehnika" (Nei ja Lillenberg, 2009[2]).

8.2. Kirjanduse ülevaade

Keskkonna saastumine ravimitega on muutunud oluliseks uurimisvaldkonnaks. Läbinud inimese või looma organismi, väljuvad ravimid kas muundumata kujul või metaboliitidena keskkonda. Neid on leitud sõnnikus ja reovees, reoveesettes ja pinnavees, kompostväetises ja väetatud mullas. Ravimid võivad keskkonnas kahjulikeks osutuda, kuna nad on loodud eesmärgiga mõjutada bioloogilisi objektide. Neil on sageli biostruktuuridega sarnased füüsikalis-keemilised omadused nagu lipofiilsus, mis võimaldab läbida biomembraane ja stabiilsus, mis hoiab ära nende inaktiivseks muutumise enne raviefekti tekitamist. Nii on ravimitel olemas vajalikud omadused, et akumuleeruda organismides ja kutsuda esile muutusi vee ja pinnase ökosüsteemides (Halling-Sørensen *et al.*, 1998). Sõnniku või reoveesette kompostväetise koostises jõuavad ravimid põllumajandusmaadele. Osa neist lagundatakse mulla mikroorganismide poolt mõne päeva või nädala jooksul (Thiele-Bruhn, 2003), stabiilsemad võivad mullas muutumatuna säilida isegi üle aasta (Golet *et al.*, 2002).

Reoveesette kasutamine põllumajandusväetisena on globaalne probleem. Seoses maailma rahvastiku kiire kasvuga suurenevad ka reovee töötlemisel tekkiva sette kogused, mis tuleb puhastitest eemaldada. Reoveepuhastite territooriumitele kuhjuvad kompostihunnikud, mille utiliseerimine ei ole kerge. Kuigi reoveesette kompost on kahtlemata hea orgaaniline väetis, võib see osutuda keskkonnale, inimesele ja loomadele ohtlikuks nende keemiliste või bioloogiliste kontaminantide sisalduse tõttu, mille regulaarset kontrollimist reoveesette kasutamise määrus ette ei näe (ravimijäägid, patogeensed bakterid, seened, viirused). Komposti kasutamine haljastusväetisena või rekultiveerimiseks Eestis probleeme ei tekita, põllumajandusväetisena leiab kompost kasutamist palju harvemini.

Ravimeid fluorokinoloonide, sulfoonamiidide ja tetratsükliinide rühmadest on leitud mitmel pool maailmas reovees, reoveesettes ja puhastatud reovees (Golet *et al.*, 2002; Lindberg *et al.*, 2005; Göbel *et al.*, 2005; Okuda *et al.*, 2009; Spongberg and Witter, 2008; Gros *et al.*, 2007) Reoveesette töötlemise tehnoloogiad on erinevad, kuid kõik need peaksid tagama sette ohutuse keskkonnale ning inimeste ja loomade tervisele. Töötlemata reoveesette kasutamine põllumajanduses on keelatud. Euroopa Liidus, sh. Eestis kehtiv reoveesette kasutamise määrus lubab

töödeldud reoveesetet kasutada põllumajandusväetisena, kui see ei sisalda üle normi fekaalseid *coli*-laadseid baktereid, raskemetallide jääke ega helmintide mune. Teiste bioloogiliste või keemiliste kontaminantide sisalduse kontrollimine ei ole kohustuslik (Riigi Teataja I, 2004).

Kuigi on andmeid, et ravimid jõuavad mullast taimedesse, piirnormid ravimite jääkidele taimses toidutoormes puuduvad. Loomsele toidutoormele kehtestatud MRL (*maximum residue limit* – ravimijäägi maksimaalne lubatud sisaldus*)* sõltub ravimi farmakoloogilistest omadustest, looma liigist ja koest (EMA/EPMARs). Osa allikaid väidab, et ravimijääkide „omastamine" mullast on tühine (Boxall *et al.*, 2006; Thiele-Bruhn, 2003). Teised autorid, vastupidi, peavad ravimite akumuleerumist mullast toidutaimedesse sedavõrd tõsiseks probleemiks, et on teinud ettepaneku kehtestada MRL ka taimsele toidutoormele (Brambilla *et al.*, 1996). Artiklis (Jjemba, 2002) rõhutatakse ravimite taimedesse akumuleerumise uurimise olulisust. Vajalikuks peetakse ka ravimite degradatsiooni uurimist reoveesette erinevate töötlemistehnoloogiate korral. Vähestes töödes on uuritud ravimijääkide sisalduse vähenemist sõnniku kompostimisel (Dolliver *et al.*, 2008). Antibiootikumide degradatsiooni reoveesette kompostimise käigus ei ole maailmas seni uuritud. Euroopa Liidus puuduvad normatiivid ravimijääkide sisalduse kohta reoveesette kompostis (EU Council Directive 86/278/EEC, 1986). Soovitatavad veterinaarravimite sisalduse piirnormid sõnnikus on 100 µg/kg ja sõnnikuga väetatud mullas 10 µg/kg (EMEA/CVMP/055/96, 1996). Kuid need normid on tänaseks seatud kahtluse alla kui liiga kõrged. Euroopa Liidu Teaduskomitee toksikoloogia, ökotoksikoloogia ja keskkonna küsimustes (EU ESCTEE) peab antud piirnorme mitteteaduslikeks, kuna need ei välista ohtu kõigile mulla mikroorganismidele. Uueks, keskkonnale ohutuks ravimisisalduse piirnormiks mullas pakutakse 1 µg/kg. See on arvutatud arvestades erinevate ravimite MIC-i *(Minimum Inhibitory Concentration* – minimaalne inhibeeriv kontsentratsioon) väärtusi mulla mikroorganismidele. MIC-l põhinev ravimisisalduse piirnorm ei välista aga ravimresistentsuse arenemist mullamikroobidel. Selleks piisab palju väiksemast ravimikontsentratsioonist mullas – MEC (*Minimum Effect Concentration* - minimaalse mõju kontsentratsioon), mille juures mikroobide kasv aeglustub (O´Reilly and Smith, 1999). Ravimresistentsuse teket välistav ravimisisaldus mullas ei tohiks ületada 0,01–0,1 µg/kg (Montforts, 2005).

Anaeroobse töötlemisega ohutuks muudetud reoveesettest on leitud fluorokinoloonide jääke kontsentratsioonides 2130-2420 μg/kg (Golet *et al.*, 2002), mis ületab ravimisisalduse piirnormi sõnnikus 100 μg/kg (EMEA/CVMP/055/96, 1996) üle kahekümne korra. Reoveesettega väetatud mullast on leitud fluorokinoloonide jääke 21 kuu pärast: tsiprofloksatsiini keskmiselt 270 μg/kg ja norfloksatsiini 300 μg/kg kohta (Golet *et al.*, 2002). Walters *et al.* (2010) näitasid fluorokinoloonide pikaajalist säilimist reoveesettega väetatud mullas. Vahetult pärast reoveesette laotamist oli tsiprofloksatsiini sisaldus mullas 542 ja ofloksatsiini sisaldus 470 μg/kg (kuivaine kohta). 994 päeva pärast ei olnud kumbki antibiootikum mullas lõplikult lagunenud, nende sisalduseks saadi 390 μg/kg (tsiprofloksatsiin) ja 267 μg/kg (ofloksatsiin) (Walters *et al.*, 2010). Sulfoonamiide on leitud reoveesetetest (Göbel *et al.*, 2005*)* ja puhastatud reoveest (Göbel *et al.*, 2004; Lindberg *et al.*, 2005), sulfametoksasool ei ole reoveepuhastis biodegradeeritav (Richardson and Bowron, 1985).

Reoveesette kompostimise käigus ei pruugi laguneda fluorokinoloonide ja tetratsükliinide jäägid. Nende aeglast degradeerumist põhjendatakse tugeva seondumisega tahketele osakestele (Marengo *et al.*, 1997; Carmosini and Lee, 2008). Kaks nädalat pärast väetamist seasõnnikuga tuvastati tetratsükliini sisaldus erinevatelt sügavustelt võetud mullaproovides 195 ja 254 μg/kg (Sczesny *et al.*, 2003). Seitse kuud pärast väetamist võetud mullaproovides oli keskmine tetratsükliini sisaldus 65,5 μg/kg (Hamscher *et al.*, 2002). Kõik eeltoodud ravimijääkide sisaldused mullas ületavad mullale kehtestatud ravimite piirnormi 10 μg/kg kümneid kordi, teaduslikult põhjendatud mullaorganismidele ohutu piirnormi 1 μg/kg sadu kordi ja mullamikroobide ravimresistentsuse arenemist ennetava piirnormi 0,01–0,1 μg/kg tuhandeid kordi.

Alates 1940. aastast kuni tänaseni on antibakteriaalsete ainete tootmine ja tarbimine maailmas mitmekordistunud, sama aja jooksul on oluliselt suurenenud ka bakterite antibiootikumresistentsus, nii ohutute kui ka patogeensete bakterite hulgas. Tetratsükliini resistentsust määrava geeni esinemissagedus on mullabakterite hulgas ajavahemikul 1970–2008 kasvanud 15 korda, põhjuseks väetamine tetratsükliine sisaldava sõnniku või reoveesette kompostiga (Knapp *et al.*, 2010). Reoveesettes ja settekompostis esinevad bakterid on sageli antibiootikumresistentsed, kuna on elanud pikka aega antibiootikume sisaldavas keskkonnas

(Reinthaler *et al.*, 2003; Sahlström, *et al.*, 2009). Niisuguste bakterite sattumine keskkonda põhjustab ravimresistentsuse levikut. Mullabakterite antibiootikumresistentsus kujutab endast potentsiaalset ohtu inimeste ja loomade tervisele, sest resistentsust määravad geenid võivad transformeeruda ohututelt mullabakteritelt patogeensetele bakteritele horisontaalse geeniülekande teel (Davies, 1994).

Taimkatsed on näidanud, et antibakteriaalsed ained fluorokinoloonide, tetratsükliinide ja sulfoonamiidide rühmast akumuleeruvad mullast taimedesse (Migliore *et al.*, 1995; Brambilla *et al.*, 1996; Aruksaar *et al.*, 1998; Aruksaar *et al.*, 1999; Lillenberg *et al.*, 2003; Boxall *et al.*, 2006). Sel teel on võimalik ravimite sattumine mullast inimese toidulauale või loomasööda koostisesse. Erinevalt loomorganismidest, kus ravimite jäägid väljuvad ekskrementidega, taimedel väljutusmehhanism puudub. Seetõttu on võimalik ravimijääkide kontsentreerumine pika kasvuperioodi jooksul (Lillenberg, *et al.*, 2003). Tulemuseks võib olla kõrgem ravimijääkide sisaldus toidutaimedes, kui on lubatud loomsetes toitudes. Loomsele toormele kehtestatud MRL tuleneb ADI arvust (acceptable daily intake – päevane lubatud doos). ADI on päevas tarbida lubatud aine kogus inimese kehakaalu 1 kg kohta kogu eluaja jooksul, ilma tervist kahjustamata. Eristatakse toksikoloogilist ADI - aine ohutut doosi vältimaks otseseid kahjulikke kõrvaltoimeid organismile ja mikrobioloogilist ADI – aine ohutut doosi organismi normaalsele mikrofloorale, kusjuures $ADI_{tox} > ADI_{mic}$. Loomsele toormele kehtestatud MRL põhineb mikrobioloogilisel ADI arvul (EMA/EPMARs; EMEA/MRL/398/98). Toiduga saadav ravimikogus peab olema ohutu ka inimese organismis resideerivatele bakteritele.

Mõnede ravimite puhul on antud piirnormid algse ravimi ja tema metaboliitide summaarse sisalduse kohta: näiteks enrofloksatsiin (EMEA/MRL/820/02), mille peamiseks metaboliidiks loomorganismides on tsiprofloksatsiin (Mengozzi *et al.*, 1996; Küng *et al.*, 1993). Esimene neist on kasutusel ainult veterinaarmeditsiinis, teine ainult humaanmeditsiinis. Ka taimedes metaboliseerub omastatud enrofloksatsiin tsiprofloksatsiiniks.

10 mg/kg enrofloksatsiini sisaldusega mullas kasvanud salatis vedelikkromatograafilise HPLC meetodiga määramisel oli enrofloksatsiini ja tsiprofloksatsiini sisaldus vastavalt 300 μg/kg ja 70 μg/kg. Enrofloksatsiini ja tsiprofloksatsiini summaarseks sisalduseks

salatis saadi 370 μg/kg, mis ületab piimas ja lihas lubatud MRL 100 μg/kg (EMEA/MRL/820/02) üle kolme korra (Lillenberg *et al.*, 2003).

Kuna taimedele ei ole MRL kehtestatud, on võimalik taimse toidu ohutust hinnata lähtuvalt loomsele toormele kehtestatud piirnormist (EMEA/MRL/026/95; EMEA/MRL/820/02). Mõnede käesolevas töös uuritud ravimite puhul (norfloksatsiin ja ofloksatsiin) MRL loomse toorme jaoks puudub, sest need ravimid on kasutusel ainult humaanmeditsiinis. Norfloksatsiini ja ofloksatsiini sisaldust taimedes võib tinglikult võrrelda tsiprofloksatsiini ja enrofloksatsiini lubatud summaarse sisaldusega.

8.3. Uurimistöö eesmärgid

1. Uurida Eesti reoveesetet ning sette komposti mõningate keskkonnas kauem püsivate ja/või potentsiaalselt taimedesse akumuleeruvate antibakteriaalsete ainete: fluorokinoloonide, sulfoonamiidide ja tetratsükliinide leidumise suhtes.

2. Anda hinnang erinevatele komposti valmistamise tehnoloogiatele komposti ohutuks muutmise seisukohalt.

3. Uurida valitud ravimite akumuleerumist mullast toidutaimedesse.

4. Hinnata reoveesette komposti kui põllumajandusväetise ohutust, arvestades keskkonnakaitse ja toiduhügieeni nõudeid.

8.4. Materjal ja metoodika

8.4.1. Reoveesette ja komposti uuringud

Tallinna ja Tartu reoveesetet ja reoveesette komposti analüüsiti aasta jooksul. Tartus ja Tallinnas on reoveesette stabiliseerimise meetodid erinevad: Tartus toimub pressitud sette aunkompostimine: 25%-lise kuivainesisaldusega sete viiakse väljale aunadesse ja segatakse tugiainega (purustatud puukoor) vahekorras ~1/1. Reoainete bakteriaalse lagundamise tulemusena tõuseb aunas temperatuur kuni +71 °C. Aeroobsete bakterite elutegevuseks vajalike tingimuste tagamiseks segatakse aunasid mitu korda kuus. Tallinnas on kasutusel biopuhastis settinud toormuda anaeroobne stabiliseerimine – metaankääritamine

+37 °C juures. Anaeroobsete bakterite metabolismi tulemusena peaksid lagunema keemilised kontaminandid. Kääritatud sete, kuivainesisaldusega 28%, viiakse väljale aunadesse ja segatakse tugiainega (turvas) vahekorras 1/0,75. Aunade segamine toimub üks kord kuus.

Reoveesette proovid võeti enne segamist tugimaterjaliga. Mõlemas linnas võeti settest kolm proovi igal kuul aasta jooksul. Kompostiproovid võeti mõlemas linnas 2, 6 ja 12 kuud seisnud aunadest, kuus proovi auna erinevatest kohtadest. Kokku võeti 144 proovi, neist pooled Tartust, pooled Tallinnast. Ligikaudu 200 g reoveesetet või komposti koguti 500 ml mahuga klaaspurki, segati ja kaeti hermeetiliselt suletava kaanega. Enne analüüsimist hoiti proove temperatuuril +4 °C. Analüüsid teostati reeglina ühe nädala jooksul. Pikemaks säilitamiseks hoiti proove sügavkülmas –80 °C.

Töötati välja uus metoodika kolme antibiootikumide klassi – tetratsükliinide (TC), fluorokinoloonide (FQ) ja sulfoonamiidide (SA) määramiseks reoveesettes ja kompostis. Tsiprofloksatsiini (CIP), norfloksatsiini (NOR), ofloxatsiini (OFL), sulfadimetoksiini (SDM), sulfametoksasooli (SMX), tetratsükliini (TCL) ja doksütsükliini (DOX) ekstraheerimiseks kasutati PLE (pressurized liquid extraction) meetodit, ekstraktide puhastamiseks SPE (solid phase extraction) meetodit ja ekstraktid analüüsiti LC-MS (liquid chromatography-mass spectrometry) meetodil.

PLE. Ekstraktsioon viidi läbi kasutades ekstraheeriva solvendina 0,35% fosforhappe ja atsetonitriili segu 1:1, pH 2,5. Ekstraktsiooni aeg: 10 min, temperatuur: 100–110 °C, rõhk: 100–110 atm., kordus: 5 tsüklit.

SPE. Ekstrakti puhastamiseks kasutati kahte erinevat tüüpi ekstraktsioonipadruneid: SCX (*strong cation-exchange*) ja HLB (*hydrophilic-lipophilic balance*). Sulfoonamiidide määramisel andsid kõrgema saagise SCX padrunid, fluorokinoloonide ja tetratsükliinide puhul HLB padrunid. Kuigi sulfoonamiidide saagis HLB padrunite kasutamisel langes, jäi see siiski aktsepteeritavale tasemele. Seepärast kasutati edaspidi kõigi kolme antibiootikumide grupi üheaegseks määramiseks ainult HLB padruneid.

LC-MS. Antibiootikumide sisalduse määramiseks reoveesettes ja kompostis kasutati instrumenti Agilent Series 1100 LC- MSD Trap XCT. Meetodi määramispiirid olid HLB padrunite kasutamise korral CIP 1,8;

NOR 1,3; OFL 0,8; SMX 0,1; SDM 0,1; DOX 80 ja TCL 160 μg/kg. Standardhälbed olid vastavalt 0,18; 0,13; 0,08; 0,01; 0,01; 7,7 ja 15,7. Saagiste protsent varieerus olenevalt ainest ja ekstraktsioonipadruni tüübist. Materjalide ja metoodika detailne kirjeldus on avaldatud artiklis II (Lillenberg *et al.*, 2009).

8.4.2. Taimede kasvatamine, proovi ettevalmistamine ja uuringud

Taimi kasvatati kasvuhoones plastikpottides, kahes erinevas mullas: liiv-savi mullas pH_{KCl} 6,7; niiskusesisaldus 19,5% ja savi-liiv mullas pH_{KCl} 6,9; niiskusesisaldus 8% (Lisa 1). Antibiootikumid lisati mulda vesilahustena, nii et kõikide ainete lõppkontsentratsiooniks potis oli 10, 100, 500, 1000 μg/kg või 10 mg/kg mulla kuivkaalu kohta. Parema lahustuvuse saavutamiseks lahustati fluorokinoloonid eelnevalt 2 ml-s 0,1 mM ammooniumatsetaat/metanool puhverlahuses (75/25), pH 2,8 (kohandatud 0,1%-lise sipelghappega). Sulfoonamiidid lahustati eelnevalt 2 ml 0,3 M NaOH vesilahuses. Iga kontsentratsiooni jaoks võeti kolm paralleelpotti. Kontrolliks kasvatati taimi antibiootikumidevabas mullas, samuti kolmes paralleelpotis.

Katsetaimedeks olid lehtsalat *(Lactuca sativa L)*, porgand *(Daucus carota L),* kartul *(Solanum tuberosum L)* ja nisu *(Triticum vulgare L)*. Lehtsalati ja porgandi seemned osteti kauplusest, nisuseemned ja kartulid saadi EMÜ Põllumajandus- ja keskkonnainstituudi mullateaduse ja agrokeemia osakonnast. Mulla kogused pottides olid kartulil 5, nisul 3, porgandil 1,5 ja salatil 0,5 kg. Salati kasvuaeg viie antibiootikumi juuresolekul alates seemnete külvamisest oli 70 päeva, teistel taimedel 120 päeva (kartulil alates kartuli muldapanekust). Seejärel taimed koristati ja eraldati võsud juurtest. Mullaga kokkupuutunud taimeosad pesti hoolikalt jooksva vee all.

Söödavad osad kuivatati eraldi: salatil lehed, kartulil mugulad, porgandil peajuur ja nisul terad. Kartulid ja porgandid tükeldati enne kuivatamist. Kuivatamine toimus pimedas ruumis, et vältida fotokeemilisi reaktsioone, mis võiksid põhjustada fluorokinoloonide lagunemist (Hooper and Wolfson, 1991). Kuivanud taimed jahvatati peeneks purustusveskis. Täieliku kuivkaalu saavutamiseks hoiti taimset materjali termostaadis +45 °C juures 24 tundi. Enne analüüsimist hoiti taimede proove hermeetilistes plastikaatkottides sügavkülmas temperatuuril –80 °C.

Antibiootikumid ekstraheeriti taimsest materjalist LE *(liquid extraction)* meetodil. Ekstraktid puhastati SPE meetodil ja analüüsiti LC-MS meetodil.

LE. Antibiootikumid ekstraheeriti kuivatatud taimsest materjalist atsetonitriili ja äädikhappe seguga 1:1.

SPE. Ekstraktide puhastamiseks kasutati HLB padruneid.

LC-MS. Antibiootikumide sisalduse määramiseks kasutati instrumenti Agilent Series 1100 LC-MSD Trap XCT.

Ekstraheerimist on detailselt kirjeldatud artiklis V (Lillenberg *et al.*, 2010[2]). Antibiootikumide saagised varieerusid kõikide proovimaatriksite piirides 54-98%. Valideerimine teostati maatriksis, kus saavutati kõige madalam saagise protsent (porgandi juur liiv-savimullas: 54-78%), seega on valideerimise hinnang metoodikale antud konservatiivselt. Meetodi määramispiirid varieerusid sõltuvalt antibiootikumist: CIP 108,3; NOR 162,2; OFL 22,9; SDM 71,2 ja SMX 130,6 μg/kg. Standardhälbed olid vastavalt 2,7; 4,1; 0,6; 1,8 ja 3,3. Tulemused, mis jäid alla määramispiiri, näitavad küll antibiootikumide vähest sisaldust porgandis, kuid ei pruugi olla täpsed. Teistes taimedes mulla madalamatel kontsentratsioonidel saadud tulemuste usaldusväärsus on suurem. Antibiootikumide sisaldus määrati taimede söödavates osades: salati lehtedes, kartuli mugulates, porgandi juurtes ja nisu terades. Analüüsid tehti ka mõnedest mittesöödavatest taimeosadest: nisu ja porgandi lehtedest ning salati juurtest. Võrreldi antibiootikumide akumuleerumise ulatust taimede juurtesse ja lehtedesse ning samasse taimeosasse kahest erineva lõimisega mullast. Kokku tehti taimedest 154 analüüsi.

8.5. Tulemused ja arutelu

8.5.1. Antibiootikumide sisaldus reoveesettes ja kompostis

Uuritud reoveesetted sisaldasid fluorokinoloonide ja sulfoonamiidide jääke. Tetratsükliinide jääke setetest ei leitud. Sulfoonamiide leiti peaaegu kõigis setteproovides, kuid alla sõnnikule kehtestatud piirnormi 100 μg/kg. Fluorokinoloonide sisaldus tuvastati enamuses Tartu setteproovides 2-4 korda üle piirnormi, Tallinnas ainult mõnedes proovides, kuid

2-15 korda üle piirnormi. FQ kõrged sisaldused nii Tallinna kui Tartu reoveesetetes ilmnesid jaanuaris või veebruaris, Tartus ka augustis. Antibiootikumide keskmised sisaldused Tallinna ja Tartu reoveesetetes on esitatud joonistel (Figure 11, 12). Võetud proovide arvu juures (3 proovi 1 kord kuus) ei saa väita, et FQ sisalduse tõusud ja langused kuude lõikes oleks seaduspärasus. Antibiootikumide sisaldus reovee setetes varieerus mitte ainult kuude lõikes, vaid ka sama päeva proovides. Tetratsükliini reoveesettest ei leitud, kuid selle sisaldus tuvastati reovees. Doksütsükliini ei leitud ka reoveest. Tetratsükliini müüdud kogused on võrreldes fluorokinoloonidega väiksemad (Eesti ravimistatistika, 2002–2006; Eesti ravimistatistika, 2006–2008). Kuna TCL kasutatakse põhiliselt oftalmoloogias, satub see antibiootikum kanalisatsiooni harvemini.

Reoveesette kompostimisel antibiootikumid lõplikult ei lagunenud. Ravimijääkide kontsentratsioonid 2-, 6- ja 12-kuu vanustes kompostiaunades on esitatud tabelites (Table 9, 10). Kuus kuud seisnud kompostides leiti kõiki FQ jääke nii Tartus kui Tallinnas, SA jääke leiti ainult Tartus. FQ ja SMX jääke leiti Tartu kompostis veel 12 kuu pärast, Tallinnas olid need selleks ajaks lagunenud. SDM jääke 12 kuu vanusest kompostist ei leitud ei Tartus ega Tallinnas.

Antibiootikumide sisaldus kompostis oleneb nende sisaldusest settes, mis on nii kuude kui päevade lõikes ebaühtlane. Lisaks algmaterjalile oleneb antibiootikumi sisaldus kompostiproovis proovivõtu kohast, sest ka kompostiaunas, ehkki seda segatakse mitmeid kordi, ei ole antibiootikumide sisaldus ühtlane. Aasta keskmise fluorokinoloonide sisalduse arvutamine kompostis ei oma mõtet, sest kunagi ei segata kokku erinevate kuude reoveesettest valmistatud kompostiaunu. Põllule või haljasalale viiakse Tallinnas sama kuu settest valmistatud 6–8 kuud seisnud kompostiaun. Tartus viiakse samavana kompost nn. järelvalmimisauna, kus enam segamist ei toimu.

Fluorokinoloonid adsorbeeruvad tugevasti tahkete osakeste külge ega eemaldu sealt kergesti. See võimaldab seletada FQ erinevaid kontsentratsioone sama päeva reoveesettes ja sama kuu kompostiaunas, samuti nende kõrgemaid sisaldusi kuus kuud seisnud kompostis võrreldes kaks kuud seisnud kompostiga. Kompostiaunade keskmine FQ sisaldus on arvutatud kuue juhusliku proovi analüüsitulemuste järgi ja ei pruugi väljendada 100 tonnise auna tegelikku keskmist fluorokinoloonide

sisaldust. Kohati kõrge FQ sisaldusega ühe aasta vanune Tartu kompost võib põllule laotatuna põhjustada lokaalselt kõrgeid antibiootikumide sisaldusi mullas, mis ületavad mulla jaoks soovitatud piirnormi 10 μg/kg ja on kümneid kordi kõrgem teaduslikult põhjendatud piirnormist 1 μg/kg. Ühe aasta vanune Tallinna kompost sisaldas antibiootikumide jääke minimaalselt, millest võib järeldada, et Tallinnas kasutatav sette segamine turbaga on ravimijääkide eemaldamisel efektiivsem, kui kompostimine puukoorega Tartus. Küll aga sisaldas Tallinnas antibiootikumide jääke kuue kuu vanune kompost, mis loetakse juba valminuks ja turustamiskõlbulikuks (Figure 15).

8.5.2. Antibiootikumide akumuleerumine mullast taimedesse

Kõige suuremates kogustes akumuleerusid taimedesse SMX ja SDM. Loomsele toormele kehtestatud piirnorm lubab SA summaarseks sisalduseks 100 μg/kg. Katsetaimedes leiti mõnel juhul sellest palju kõrgemaid kontsentratsioone, kuid ainult 10, 1 ja 0,5 mg/kg antibiootikumide sisaldusega mullas kasvanud taimedes. Kõige madalam mulla ravimite sisaldus, millest taimed antibiootikume omastasid oli 0,01 mg/kg (Table 13, 14). Kõrgemad antibiootikumide sisaldused leiti taimede maa-alustes osades (Table 15, 16). Savi-liivmullast omastasid taimed antibiootikume reeglina rohkem kui liiv-savimullast (Figure 26, 27).

Antibiootikumide sisaldus mullas 10 mg/kg avaldas negatiivset mõju taimede kasvule ning osutus mõne taime puhul letaalseks. Hävinud nisu- ja salatitaimede lehtedes tuvastati SA summaarne sisaldus üle 900 μg/kg, salati juurtes üle 7000 μg/kg. Suuremates kogustes antibiootikume omastanud nisu ja salati lehtedes tekkis kloroos, taimed hakkasid närbuma 30. päeval ja eksperimendi lõpuks hävisid täielikult. Kloroosi teket on seostatud CIP akumuleerumisega lehtedes (Lillenberg, 2003). Fluorokinoloonidel on omadus adsorbeeruda mullaosakeste külge, seepärast on väga raske saavutada nende ühtlast kontsentratsiooni mullas. Nende akumuleerumine taimesse võib sõltuda ka juurte asukohast katsemullas. FQ ebaühtlane sisaldus mullas võib olla põhjuseks, miks mõnel juhul antibiootikumide kontsentratsioonid taimedes ei vähene korrellatsioonis kontsentratsioonide langusega mullas.

Antibiootikumide kergem omastamine savi-liivmullast on loogiline, sest desorptsioon toimub saviosakeste küljest raskemini (Nowara *et al.*, 1997). Taimede maa-alused osad, juured ja mugulad, on esimesed, kuhu antibiootikumid mullast jõuavad, seetõttu nende kõrgem sisaldus juurtes ja mugulates on ootuspärane. Kas lehtedessse jõuab uuritud antibiootikume tõepoolest vähem, kui maa-alustesse osadesse, või toimub lehtedes valguse mõjul FQ fotodegradatsioon (Hooper and Wolfson, 1991) ei ole antud töö põhjal võimalik öelda. Lehtedes toimuvate metabolismi protsesside tõttu on võimalik uuritud ainete muundumine nende metaboliitideks (Lillenberg *et al.,* 2003), mille sisaldust taimes ei uuritud, kuid mis võivad samuti omada antibakteriaalset toimet.

Teostatud uuring näitas, et fluorokinoloonid võivad akumuleeruda mullast toidutaimede söödavatesse osadesse, kui nende kontsentratsioon mullas on vähemalt 10 μg/kg. Kas selline akumuleerumine toimub ka reoveesette kompostiga väetatud mullast, vajab kontrollimist põllukatsetega.

8.5.3. Reoveesette komposti sobivus põllumajandusväetiseks

Antibiootikumide akumuleerumine toidutaimedesse võib osutuda ohtlikuks, sest taimse toiduga üliväikestes kogustes antibiootikumide omastamine põhjustab inimese organismis bakterite ravimresistentsuse kujunemise, mistõttu isegi siis, kui antibiootikumi sisaldus taimes jääb alla lihale ja piimale kehtestatud MRL, võib see olla ohtlik. Mitmesuguste antibiootikumide jääke sisaldavate toitude üheaegsel söömisel (näiteks liha, kartul, piim, lehtsalat, porgand) võib esineda ADI_{mic} ületamine.

Käesolevas uurimistöös akumuleerusid antibiootikumid mullast toidutaimedesse suuremas koguses, kui lubab MRL loomses toormes, kuid ainult mulla kõrgemate ravimi kontsentratsioonide juures: 0,5, 1 ja 10 mg/kg. Nii kõrged ravimite kontsentratsioonid võivad esineda mullas, kui väetamine toimub anaeroobselt töödeldud, kuid tugiainega täiendamata reoveesettega, milles FQ ja SA sisaldus on üle 1 mg/kg. Eestis nii kõrgeid ravimite kontsentratsioone tõenäoliselt mulda ei satu, sest väetamine toimub kompostiga, milles antibiootikumide sisaldused leiti olevat alla 100 μg/kg. Piirkontsentratsiooniks, mille juures antibiootikumide akumuleerumist mullast taimede söödavatesse osadesse veel täheldati, oli 10 μg/kg. Tartu ja Tallinna turustamiseks valmis kompostist leiti FQ rohkem kui 10 või ligi 10 μg/kg (CIP 9,

OFL 8 μg/kg). Need arvud ei ole konstantsed suurused, vaid muutuvad, sõltudes ravimite tarbimisest konkreetsel ajal, konkreetses linnas või asulas. Mulla ravimisisalduse piirnorm 10 μg/kg ei ole piisav vältimaks antibiootikumide akumuleerumist toidutaimede söödavatesse osadesse.

Ohu hindamisel tuleb lähtuda ravimijääkide sisalduse teaduslikult põhjendatud piirnormist mullas 1 μg/kg. Töös uuritud FQ sisaldused turustamiseks valmis kompostis olid sellest kümneid kordi suuremad (Tartus OFL 8, NOR 64, CIP 70 μg/kg; Tallinnas OFL 8, NOR 17, CIP 9 μg/kg). Sellise kompostiga väetamisel võivad mullas kujuneda lokaalselt küllalt kõrged, mulla mikroobidele letaalsed FQ kontsentratsioonid. SA kontsentratsioon turustamiseks valmis kompostis oli küll madal (Tartus SMX 2 μg/kg), kuid siiski kõrgem piirnormist, mistõttu väetamine niisuguse kompostiga võiks põhjustada ravimresistentsuse kujunemise mulla mikroobidel ja selle võimaliku ülekandumise patogeenidele. Ainult ravimisisalduse piirnormi 1 μg/kg rakendamisel kompostile võime eeldada, et mullas ei ületa ravimisisaldus bakterite antibiootikumresistentsust ennetavat kontsentratsiooni 0,01–0,1 μg/kg (Montforts, 2005).

8.6. Kokkuvõte ja järeldused

Töötati välja uus meetod sulfoonamiidide, fluorokinoloonide ja tetratsükliinide grupi antibiootikumide üheaegseks ekstraheerimiseks reoveesettest ja kompostist. Uuriti nende antibiootikumide sisaldust Tartu ja Tallinna reoveesettes ühe aasta jooksul, sisalduse muutust sette kompostimise käigus ja akumuleerumist mullast toidutaimedesse settes esinevate kontsentratsioonide juures. Võrreldi erinevate sette töötlemise tehnoloogiate efektiivsust antibiootikumide sisalduse vähendamisel. Töö tulemused on globaalse tähtsusega, sest andmed ravimisisalduste vähenemise kohta reoveesette töötlemise käigus, samuti ravimite akumuleerumise kohta mullast toidutaimedesse on uudsed kogu maailmas. Fluorokinoloonide sisaldus reoveesetetes oli kohati kõrge (CIP kuni 1520 μg/kg; NOR kuni 580 μg/kg; OFL kuni 157 μg/kg), kohati madal, varieerudes kuude ja isegi ühe päeva lõikes. Sulfoonamiidide sisaldus oli reoveesetetes alati madal. Tetratsükliine Eesti reoveesettest ega kompostist ei leitud, seepärast nende taimedesse akumuleerumist ei uuritud. Samas ei ole välistatud, et tetratsükliine võib sisalduda teiste riikide reoveesetetes, kui neid tarvitatakse suuremates kogustes ja sette

töötlemise tehnoloogiad erinevad meil kasutatavatest. Tetratsükliinide akumuleerumine mullast toidutaimedesse pakub teaduslikku huvi ja võiks olla edasiste uuringute teemaks.

Tallinna ja Tartu realiseerimisvalmis reoveesette kompost sisaldas antibiootikumide jääke kogustes, mis ületasid nii soovitusliku piirnormi mullale 10 μg/kg kui ka teaduslikult põhjendatud piirnormi 1 μg/kg. Niisugused antibiootikumide kogused kompostväetises võivad osutuda ohtlikuks mullaelustikule, rikkudes sealset bioloogilist tasakaalu, samuti inimestele ning loomadele, põhjustades antibiootikumresistentsuse sagenemist mullabakteritel ja selle võimalikku ülekandumist patogeenidele. Kõige rohkem sisaldas realiseerimisvalmis kompost CIP (70 μg/kg), seejärel NOR (64 μg/kg), OFL (8 μg/kg) ja SMX (2 μg/kg). SDM realiseerimisvalmis kompostist ei leitud.

Kui võrrelda Tallinna ja Tartu reoveesette töötlemise tehnoloogiaid ravimijääkide eemaldamise seisukohalt, on ilmne, et Tallinnas kasutatav anaeroobselt stabiliseeritud sette segamine turbaga on efektiivsem, kui pressitud sette aeroobne stabiliseerimine kompostimisel purustatud puukoorega Tartus. Tartu 12 kuud seisnud kompostist leiti märkimisväärses koguses antibiootikumide jääke, Tallinna kompostis olid need ravimid selleks ajaks praktiliselt lagunenud – sisaldus alla 1 μg/kg. Fluorokinoloonid adsorbeeruvad tugevasti tahkete osakeste külge ega eemaldu sealt kergesti. Adsorptsioon võimaldab seletada fluorokinoloonide erinevaid kontsentratsioone sama päeva reoveesette ja sama kuu kompostiauna proovides, samuti nende kõrgemaid sisaldusi kuus kuud seisnud kompostis võrreldes kaks kuud seisnud kompostiga.

Kõige madalama ravimisisaldusega mullast 10 μg/kg akumuleerusid taimede söödavatesse osadesse ainult OFL ja CIP. Kõrgematel ravimikontsentratsioonidel kasvanud taimedest leiti ka teisi antibiootikume: NOR ja SMX alates 100 μg/kg, SDM alates 500 μg/kg. Taimsele toidutoormele ravimijääkide MRL kehtestamise vajadus sõltub ravimite sisaldusest reoveesette kompostis (või sõnnikus). Reoveesette töötlemise täiustatud tehnoloogiad peaksid tagama ravimijääkide sisalduse vähenemise kompostväetises keskkonnale ohutu tasemeni – 1 μg/kg, mis välistaks ühtlasi ka nende akumuleerumise mullast taimedesse. Ravimijääkide akumuleerumine reoveesette kompostiga (või sõnnikuga) väetatud mullast toidutaimedesse vajab täiendavaid uuringuid, mis peaksid kaasama ka põllukatsed.

8.7. Ettepanekud

- Enne turustamist tuleks reoveesette komposti analüüsida ravimijääkide sisalduse suhtes.
- Ravimijääkide sisaldus kompostis ei tohiks ületada 1 μg/kg.
- Ravimijääkide sisalduse alandamiseks soovitatud tasemeni peaks komposti töötlemise ja hoiustamise aeg olema turbaga kompostimisel vähemalt aasta, puukoorega kompostimisel vähemalt kaks aastat.

REFERENCES

Adegoroye, A., Paterson, N., Li, X., Morgan, T., Herod, A.A., Dugwell, D.R., Kandiyoti, R. (2004). The characterization of tars produced during the gasification of sewage sludge in a spouted bed reactor. *Fuel*, **83**, 1949-1960.

Alder, A.C., McArdell, C.S., Golet, E.M., Ibric, S., Molnar, E., Nipales, N.S., Giger, W. (2001). Occurrence and fate of fluoroquinolone, macrolide, and sulfonamide antibiotics during wastewater treatment and in ambient waters in Switzerland. In: *Pharmaceuticals and Personal Care Products in the Environment: Scientific and Regulatory Issues*. Daughton, C.G., Jones-Lepp, T. (eds.). Symposium Series 791, American Chemical Society, Washington DC, pp. 56-69.

Al-Enezi, G., Hamada, M.F., Fawazi, N. (2001). Heavy metals contents of municipal waste water and sludge's in Kuwait. *In proceedings of the Fourth Middle East Conference on the Role of Environmental Awareness in Waste Management, Kuwait city, Kuwait, 10-12 Nov. 2001*, 299-307.

Alexy, R., Sommer, A., Lange, F.T., Kümmerer, K. (2006). Local use of antibiotics and their input and fate in a small sewage treatment plant – significance of balancing and analysis on a local scale vs. nationwide scale. *Acta Hydroch. Hydrob.*, **34 (6)**, 587-592.

Al-Muzaini, S.M., Al-Obied, T.E. (2010). Temporal changes on pollutants associated with sewage sludge. *J. Environ. Sci. Eng.*, **4 (9)**, 70-78.

Al-Muzaini, S., Samhan, O., Hamodan, M.F. (1991). Sewage related impact of Kuwait´s marine environment: a case study. *Wat. Sci. Tech.*, **23 (1-3)**, 181-189.

Al Sayed, M.H., Madany, I.M., Buali, A.R.M. (1995). Use of sewage sludge ash in asphaltic paving mixes in hot regions. *Construct. Build. Mater.*, **9 (1)**, 19-23.

Anderson, M. (2002). Encouraging prospects for recycling incinerated sewage sludge ash (ISSA) into clay-based building products. *J. Chem. Tech. Biotechnol.*, **77 (3)**, 352-360.

Angin, I., Yaganoglu, A.V. (2009). Application of sewage sludge as a soil physical and chemical amendment. *Ecoloji.*, **19 (73)**, 39-47.

Aruksaar, A., Püssa, T., Kuldkepp, P., Ivask, M. (1998). Effect of some antibacterial drugs on growth of barley and radish. *Transactions of EAAS*, **6**, 7-10.

Aruksaar, A., Püssa, T., Kuldkepp, P., Ivask, M. (1999). Influence of veterinary drugs to the plant growth and accumulation of drug residues in plants. *Transactions of EAAS*, **9**, 5-8.

Avisar, D., Primor, O., Gozlan, I., Mamane, H. (2010). Sorption of sulfonamides and tetracyclines to montmorillonite clay. *Water Air Soil Pollut.*, **209 (1- 4)**, 439-450.

Babel, S., Sae-Tang, J., Pecharaply, A. (2009). Anaerobic codigestion of sewage and brewery sludge for biogas production and land application. *Int. J. Environ. Sci. Tech.*, **6 (1)**, 131-140.

Banegas, V., Moreno, J.L., Moreno, J.I., Garcia, C., León, G., Hernández, T. (2007). Composting anaerobic and aerobic sewage sludges using two proportions of sawdust. *Waste Manage.*, **27**, 1317-1327.

Barber, W.P.F. (2009). Influence of anaerobic digestion on the carbon footprint of various sewage sludge treatment options. *Water* & *Env. J.*, **23**, 170-179.

Barbosa, J., Barron, D., Jimenez-Lozano, E. (1999). Electrophoretic behavior of quinolones in capillary electrophoresis. Effect of pH and evaluation of ionization constants. *J. Chromatogr. A*, **839**, 183-192.

Batt, A.L., Kim, S., Aga, D.S. (2007). Comparison of the occurrence of antibiotics in four full-scale wastewater treatment plants with varying designs and operations. *Chemosphere.*, **68 (3)**, 428-435.

Bean, D.C., Livermore, D.M., Hall, L.M. (2009). Plasmids imparting sulfonamide resistance in *Escherichia coli*: implications for persistence. *Antimicrob. Agents. Chemother.*, **53**, 1088-1093.

Benotti, M.J., Trenholm, R.A., Vanderford, B.J., Holady, J.C., Stanford, B.D., Snyder, S. (2009). Pharmaceuticals and endocrine disrupting compounds in U.S. drinking water. *Environ. Sci. Technol.*, **43 (3)**, 597-603.

Blackwell, P.A., Lützhøft, H-C.H., Ma, H.-P., Halling-Sørensen, B., Boxall, A.B.A., Kay, P. (2004).Ultrasonic extraction of veterinary antibiotics from soils and pig slurry with SPE clean-up and LC–UV and fluorescence detection. *Talanta*, **64**, 1058-1064.

Boyd, L.B., Atmar, R.L., Randall, G.L., Hamill, R.J., Steffen, D., Zechiedrich, L. (2008). Increased fluoroquinolone resistance with time in *Escherichia coli* from >17,000 patients at a large county hospital as a function of culture site, age, sex, and location. *BMC Infect. Dis.*, **8 (4)**, 1-7.

Boxall, A.B.A. *(*2008). Fate and transport of veterinary medicines in the soil environment. *In: Fate of Pharmaceuticals in the Environment and in Water Treatment Systems.* Aga, D.S. *(ed). CRC Press,* Boca Raton, FL, USA*, pp.* 123-138.

Boxall, A. B. A., Johnson, P., Smith, E. J., Sinclair C. J., Stutt, E., Levi, L.S. (2006). Uptake of veterinary medicines from soil into plants. *J. Agric. Food Chem.*, **54**, 2288-2297.

Boxall, A., Blackwell, P., Cavallo, R., Kay, P., Tolls, J. (2002). The sorption and transport of a sulphonamide antibiotic in soil systems. *Toxicol. Lett.*, **131**, 19-28.

Boxall, A., Kolpin, D., Halling-Sørensen, B., Tolls, J. (2003). Are veterinary medicines causing environmental risks? *Environ. Sci. Technol.*, **37 (15)**, 286A-294A.

Boxall, A., Fogg, L., Kay, P., Blackwell, P., Pemberton, E., Croxford, A. (2004). Veterinary medicines in the environment. *Rev. Environ. Contam. Toxicol.*, **180**, 1-91.

Brambilla, G., Casoria, P., Civitareale, C., Cozzolino, S., Gaudio, L., Migliore, L. (1996). Sulphadimetoxine as environmental tracer to evaluate phytotoxicity in crop. *In proceedings of Conference on Residues of Veterinary Drugs in Food, Veldhoven, Netherlands, 6-8 May 1996,* 292-295.

Bridle, T., Skrypski-Mantele, S. (2000). Assessment of sludge reuse options: a life cycle approach. *Water Sci. Tech.*, **41 (8)**, 131-135.

Burhenne, J., Ludwig, M., Spiteller, M. (1997). Photolytic degradation of fluoroquinolone carboxylic acids in aqueous solution. Part I: Primary photoproducts and half-lives. *Environ. Sci. Pollut. Res.*, **4**, 10-15.

Büyüksönmez, F., Sekeroglu, S. (2005). Presence of pharmaceuticals and personal care products (PPCP) in biosolids and their degradation during composting. *J. Res. Sci. Technol.*, **2**, 31-40.

Carballa, M., Omil, F., Lema, J.M. (2009). Influence of different pretreatments on anaerobically digested sludge characteristics: suitability for final disposal. *Water Air Soil Pollut.*, **199 (1-4)**, 311-321.

Carballa, M., Omil, F., Lema, J.M., Llompart, M., García-Jares, C., Rodríguez, I., Gómez, M, Ternes, T. (2004). Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. *Water Res.*, **38**, 2918-2926.

Carballa, M., Omil, F., Ternes, T., Lema, M.J. (2007). Fate of pharmaceutical and personal care products (PPCPs) during anaerobic digestion of sewage sludge. *Water Res.*, **41**, 2139-2150.

Carmosini, N., Lee, L.S. (2008). Sorption and degradation of selected pharmaceuticals in soil and manure. In: *Fate of Pharmaceuticals in the Environment and in Water Treatment Systems.* Aga, D.S. (ed). CRC Press, Boca Raton, FL, USA, pp. 139-165.

Cenni, R., Janisch, B., Spliethoff, H., Hein, K.R.G. (2001.) Legislative and environmental issues on the use of ash from coal and municipal sewage sludge co-firing as construction material. *Waste Manage.*, **21**, 17-31.

Choi, K.-J., Kim, S.G., Kim, C.-W., Kim, S.-H. (2007). Determination of antibiotic compounds in water by on-line SPE-LC/MSD. *Chemosphere*, **66**, 977-984.

Chopra, I., Roberts, M. (2001). Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.*, **65 (2)**, 232-260.

Christian, T., Schneider, R.J., Färber, H.A., Skutlarek, D., Meyer, M.T., Goldbach, H.E. (2003). Determination of antibiotic residues in manure, soil and surface waters. *Acta. Hydrochim. Hydrobiol.*, 2003, **31 (1)**, 36-44.

Collucci, M., Bork, H., Topp, E. (2001). Persistence of estrogenic hormones in agricultural soils (I. 17-beta estradiol and estrone). *J. Environ. Qual.*, **30**, 2070-2076.

Conerlya, O., Ohaniana, E.V. (2010). Pharmaceuticals in the environment: regulatory challenges ahead. *Hum. Ecol. Risk Assess.*, **16 (6)**, 1234-1241.

Council of the European Communities. (1991). Directive concerning urban wastewater treatment, 91/271/EEC. *Off. J. Eur. Communities*, **135**, 40-52.

Daughton, C., Ternes, T. (1999). Pharmaceuticals and personal care products in the environment: agents of subtle change? *Environ. Health Perspect.*, **107**, 907-937.

Davies, J. (1994). Inactivation of antibiotics and the dissemination of resistance genes. *Science*, **264 (5157)**, 375–382.

Davis, R.D. (1984). Crop uptake of metals (cadmium, lead, mercury, copper, nickel, zinc, and chromium) from sludge treated soil and its implication for soil fertility and for the human diet. In: *Processing and Use of Sewage Sludge.* L'Hermite, E., Ott, H. (eds.). Kluwer Acad. Publ., Dordrecht, Holland, pp. 349-357

Decree of Estonian Minister of the Environment No 78.(2004). State Gazette **I**, **9**, **52**.

Diaz-Cruz, M.S., Barceló D., (2005). LC-MS^2 trace analysis of antimicrobials in water, sediment and soil.*Trends Anal. Chem.*, **24**, 645-657.

Dolliver, H., Kumar, K., Gupta, S.C. (2007). Sulfamethazine uptake by plants from manureamended soil. *J. Environ. Qual.*, **36**, 1224-1230.

Dolliver, H., Gupta, S., Noll, S. (2008). Antibiotic degradation during manure composting. *J. Environ. Qual.*, **37**, 1245-1253.

Earll, M. (1999-2006). A guide to LogP and pKa measurements and their use. http://www.raell.demon.co.uk/chem/logp/logppka.htm#pka

Eesti ravimistatistika, 2002-2006. Ravimiamet. http://www.ravimiamet.ee/vvfiles/6/6eade06d6639a8a5bbe5048f91ee82f4.pdf

Eesti ravimistatistika, 2006-2008. Ravimiamet. http://www.ravimiamet.ee/vvfiles/1/Eesti%20ravimistatistika%202006-2008.pdf

EMEA/CVMP/055/96. (1996). Committee for Veterinary Medicinal Products. Note for guidance: environmental risk assessment for veterinary medicinal products other than GMO-containing and immunological products.

EMA/EPMARs. European Medicines Agency – Maximum Residue Limits Reports. http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/landing/vet_mrl_search.jsp&murl=menus/medicines/medicines.jsp&mid=WC0b01ac058008d7ad

EMEA/MRL/026/95. (1995). Committee for Veterinary Medicinal Products. Sulphonamides (2). Summary report. http://www.ema.europa.eu/ema/index.jsp?curl=pages/includes/document/document_detail.jsp?webContentId=WC500015931&murl=menus/medicines/medicines.jsp&mid=WC0b01ac058009a3dc

EMEA/MRL/398/98 - FINAL. (1998). Committee for Veterinary Medicinal Products. Enrofloxacin (extension to sheep, rabbits and lactating cows). Summary report (3). http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2009/11/WC500014144.pdf

EMEA/MRL/820/02 - FINAL. (2002). Committee for Veterinary Medicinal Products. Enrofloxacin (extension to all food producing species). Summary report (5). http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2009/11/WC500014151.pdf

Endo, H., Nagayoshi,Y., Suzuki, K. (1997). Production of glass ceramics from sewage slugde. *Water Sci. Tech.*, **36 (11)**, 235-241.

EPA 530-R-99.009. (1999). Biosolids generation, use, and disposal in the United States. US Environmental Protection Agency. Municipal and Industrial Solid Waste Division, Office of Solid Waste.

EU Council Directive 86/278/EEC. (1986). On the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture.

Evangelou, V.P. (1998). Environmental soil and water chemistry: principles and applications. John Wiley and Sons, Inc., p. 352.

Farkas, M.H., Mojica, E.E., Patel, M., Aga, D.S., Berry, J.O. (2009). Development of a rapid biolistic assay to determine the changes in relative levels of intracellular calcium in leaves following tetracycline uptake by pinto bean plants. *Analyst*, **134(8)**, 1594-1600.

FEDESA - European Federation of Animal Health. (2001). Antibiotic use in farm animals does not threaten human health. FEDESA/ FEFANA Press, Brussels, Belgium, pp. 1-3.

Fent, K., Weston, A. A., Caminada, D. (2006). Ecotoxicology of human pharmaceuticals. *Aquat. Toxicol.*, **76 (2)**, 122-159.

Figueroa, R. A., Leonard, A., Mackay, A. A. (2004). Modeling tetracycline antibiotic sorption in clays. *Environ. Sci. Technol.*, **38 (2)**, 476-483.

Furczak, J., Joniec, J. (2007). Preliminary study of sludge effect on soil microbial activity of a podzolic soil under willow culture. *Int. Agrophys.*, **21**, 39-48.

Gao, J., Pedersen, J.A. (2005). Adsorption of sulfonamide antimicrobial agents to clay minerals. *Environ. Sci. Technol.*, **39**, 9509–9516.

García-Galán, M.J., Díaz-Cruz, M.S., Barceló, D. (2009). Combining chemical analysis and ecotoxicity to determine environmental exposure and to assess risk from sulfonamides. *Trends Anal. Chem.*, **28**, 804-819.

Garcia-Gil, J.C., Plkaza, C., Senesi, N., Brunetti, G., Polo, A. (2004). Effects of sewage sludge amendment on humic acids and microbiological properties of a semiarid Mediterranean soil. *Biol. Fert. Soils*, **39**, 320-328.

Gascó, G., Martínez-Iñigo, M.J., Lobo, M.C. (2004). Soil organic matter transformation after a sewage sludge application. *Electron. J. Environ. Agric. Food Chem.*, **3**, 716-722.

Gavalchin, J. and Katz, S.E.(1994). The persistence of fecal born antibiotics in soil. *J. AOAC Internat.*, **77**, 481-485.

Giergielewicz-Mozajska, H., Dabrowski, L., Namiesnik, J. (2001). Accelerated solvent extraction (ASE) in the analysis of environmental solid samples. *Crit. Rev. Anal. Chem.*, **31**, 149-165.

Giger, W., Alder, A.C., Golet, E.M., Kohler, H.-P.E., McArdell, C.S., Molnar, E., Siegrist, H., Suter M. J.-F. (2003). *Chimia*, **57**, 485-491.

Glassmeyer, S.T., Kolpin, D.W., Furlong, E.T., Focazio, M.J. (2008). Environmental presence and persistence of pharmaceuticals: an overview. In: *Fate of Pharmaceuticals in the Environment and in Water*

Treatment Systems. Aga, D. S. (ed.). CRC Press, Boca Raton, London, New York, pp. 3-51.

Global atlas of excreta, wastewater sludge, and biosolids management: moving forward the sustainable and welcome uses of a global resource. (2008). LeBlanc, R.J., Matthews, P., Richard, R.P. (ed.s). United Nations Human Settlements Programme (UN-HABITAT). Nairobi, Kenya. 609 p.

Golet, E.M., Alder, A.C., Giger, W. (2002[1]). Environmental exposure and risk assessment of fluoroquinolone antibacterial agents in wastewater and river water of the Glatt valley watershed, Switzerland. *Environ. Sci. Technol.*, **36 (17)**, 3645-3651.

Golet, E.M., Alder, A.C., Hartmann, A., Ternes, T.A., Giger, W. (2001). Trace determination of fluoroquinolone antibacterial agents in urban wastewater by solid-phase extraction and liquid chromatography with fluorescence detection. *Anal. Chem.*, **73 (15)**, 3632-3638.

Golet, E.M., Strehler, A., Alder, A.C., Giger, W. (2002). Determination of fluoroquinolone antibacterial agents in sewage sludge and sludge-treated soil using accelerated solvent extraction followed by solid-phase extraction. *Anal. Chem.*, **74**, 5455-5462.

Golet, E.M., Xifra, I., Siegrist, H., Alder, A.C., Giger, W. (2003). Environmental exposure assessment of fluoroquinolone antibacterial agents from sewage to soil. *Environ. Sci. Technol.*, **37 (15)**, 3243-3249.

Gros, M., Petrović, M., Barcelo, D. (2007). Wastewater treatment plants as a pathway for aquatic contamination by pharmaceuticals in the Ebro river Basin (Northeast Spain). *Environ. Toxicol. Chem.*, **26 (8)**, 1553-1562.

Gröning, J., Held, C., Garten, C., Claußnitzer, U., Kaschabek, S.R., Schlömann, M. (2007). Transformation of diclofenac by the indigenous microflora of river sediments and identification of a major intermediate. *Chemosphere*, **69**, 509-516.

Gu, C. and Karthikeyan, K.G. (2005). Sorption of the antimicrobial ciprofloxacin to aluminium and iron hydrous oxides. *Environ. Sci. Technol.*, **39**, 9166-9173.

Guibelin, E. (2002). Sustainability of thermal oxidation processes: strength for the new millennium. *Water Sci. Tech.*, **46 (10)**, 259-267.

GWRC – Global Water Research Coalition (2008). Development of an International Priority List of Pharmaceuticals Relevant for the Water Cycle. Prepared by: Kiwa Water Research, CIRSEE and TZW, London, p. 5.

Göbel, A., McArdell, C.S., Joss, A., Siegrist, H., Giger, W. (2007). Fate of sulfonamides, macrolides, and trimethoprim in different wastewater treatment technologies. *Sci. Total. Environ.*, **372**, 361-371.

Göbel, A., McArdell, C.S., Suter, M.J.F., Giger, W. (2004). Trace determination of macrolide and sulfonamide antimicrobials, a human sulfonamide metabolite, and trimethoprim in wastewater using liquid chromatography coupled to electrospray tandem mass spectrometry. *Anal. Chem.*, **76**, 4756-4764.

Göbel, A., Thomsen, A., McArdell, C.S., Alder, A.C., Giger, W., Theiβ, N., Löffler, D., Ternes, T.A. (2005[1]). Extraction and determination of sulfonamides, macrolides, and trimetoprim in sewage sludge. *J. Chromatogr. A*, **1085**, 179-189.

Göbel, A., Thomsen, A., McArdell, C.S., Joss, A., Giger, W. (2005). Occurrence and sorption behavior of sulfonamides, macrolides, and trimethoprim in activated sludge treatment. *Environ. Sci. Technol.*, **39**, 3981-3989.

Haiß, A., Kümmerer, K (2006). Biodegradability of the x-ray contrast compound diatrizoic acid, identification of aerobic degradation products and effects against sewage sludge micro-organisms. *Chemosphere*, **62**, 294-302.

Haller, M.Y., Müller, S.R., McArdell, C.S., Alder, A.C., Suter, M.J. (2002). Quantification of veterinary antibiotics (sulfonamides and trimethoprim) in animal manure by liquid chromatography– mass spectrometry. *J. Chromatogr. A*, **952**, 111-120

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P.F., Ingerslev, F., Holten Lützhøft, H.C., Jørgensen, S.E. (1998). Occurrence, fate and effects of pharmaceutical substances in the environment – a review. *Chemosphere*, **36 (2)**, 357-393.

Halling-Sørensen, B., Sengeløv, G., Tjørnelund, J. (2002). Toxicity of tetracyclines and tetracycline degradation products to environmentally relevant bacteria, including selected tetracycline resistant bacteria. *Arch. Environ. Contam. Toxicol.*, **42**, 263-271.

Hammerum, A.M., Sandvang, D., Andersen, S.R., Seyfarth, A.M., Porsbo, L.J., Frimodt-Moller, N., Heuer, O.E. (2006). Detection of sul1, sul2 and sul3 in sulphonamide resistant *Escherichia coli* isolates obtained from healthy humans, pork and pigs in Denmark. *Int. J. Food Microbiol.*, **106**, 235-237.

Hamscher, G., Sczesny, S., Höper, H., Nau, H. (2002). Determination of persistent tetracycline residues in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray

ionization tandem mass spectrometry. *Anal. Chem.*, **74 (7)**, 1509-1518.

Hara, K., Mino, T. (2008). Environmental assessment of sewage sludge recycling options and treatment processes in Tokyo. *Waste Manage.*, **28**, 2645-2652.

Harrison, E. Z., Oakes, S. R., Hysell, M., Hay, A. (2006). Review: Organic chemicals in sewage sludges. *Sci. Total Environ.*, **367**, 481 - 497.

Hartig, C., Storm, T., Jekel, M. (1999). Detection and identification of sulphonamide drugs in municipal waste water by liquid chromatography coupled with electrospray ionisation tandem mass spectrometry. *J. Chromatogr. A*, **854 (1-2)**, 163 -173.

Hartmann, A., Alder, A.C., Koller, T., Widmer, R.M. (1998). Identification of fluoroquinolone antibiotics as the main source of umuC genotoxicity in native hospital wastewater. *Environ. Toxicol. Chem.*, **17**, 377-382.

Heberer, T. (2002). Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. *Toxicol. Lett.*, **131 (1-2)**, 5-17.

Heberer, T., Stan, H.J. (1996). Vorkommen von polaren organischen Kontaminanten im Berliner Trinkwasser [Occurrence of polar organic contaminants in Berlin drinking water]. *Vom Wasser*, **86**, 19-31.

Heidler, J., Sapkota, A., Halden, R.U. (2006). Partitioning, persistence, and accumulation in digested sludge of the topical antiseptic triclocarban during wastewater treatment. *Environ. Sci. Technol.*, **40**, 3634-3639.

Herklotz, P. A., Gurung, P., Heuvel, B.V., Kinney, C. A. (2010). Uptake of human pharmaceuticals by plants grown under hydroponic conditions. *Sci. Total Environ.*, **78**, 1416- 1421.

Hermo, M.P., Barrón, D., Barbosa, J. (2005). Determination of residues of quinolones in pig muscle, comparative study of classical and microwave extraction techniques. *Anal. Chim. Acta*, **539**, 77-82.

Hirsch, R., Ternes, T., Haberer, K., Kratz, K. (1999). Occurrence of antibiotics in the aquatic environment. *Sci. Total Environ.*, **225**, 109-118.

Hirsch, R., Ternes, T.A., Haberer, K., Mehlich, A., Ballwanz, F., Kratz, K.-L. (1998). Determination of antibiotics in different water compartments via liquid chromatography -electrospray tandem mass spectrometry. *J. Chromatogr. A*, **815 (2)**, 213-223.

Holm, J.V., Rügge, K., Bjerg, P.L., Christensen, T.H. (1995). Occurrence and distribution of pharmaceutical organic compounds in the

groundwater downgradient of a landfill (Grindsted, Denmark). *Environ. Sci. Technol.*, **29**, 1415-1420.

Hong, J., Hong, J., Otaki, M., Jolliet, O. (2009). Environmental and economic life cycle assessment for sewage sludge treatment processes in Japan. *Waste Manage.*, **29**, 696-703.

Hooper, D.C., Wolfson, J.S. (1991). Fluoroquinolone antimicrobial agents. *N. Engl. J. Med.*, **324 (6)**, 384-394.

Hospido, A., Carballa, M., Moreira, M., Omil, F., Lema, J.M., Feijoo, G. (2010). Environmental assessment of anaerobically digested sludge reuse in agriculture: potential impacts of emerging micropollutants. *Water Res.*, **44**, 3225-3233.

Houdková, L., Boráň, J., Ucekaj, V., Elsäßer, T., Stehlík, P. (2008). Thermal processing of sewage sludge – II. *Appl. Therm. Eng.*, **28**, 2083-2088.

Ingerslev, F., Halling-Sørensen, B. (2000). Biodegradability properties of sulfonamides in activated sludge. *Environ. Toxicol. Chem.*, **19 (10)**, 2467-2473.

Iranpour, R., Cox, H.H.J., Kearney, R.J., Clark, J.H, Pincince, A.B., Daigger, G.T. (2004). Regulations for biosolids land application in US and European Union. *J. Residuals Sci. Technol.*, **1**, 209-222.

Jacobsen, A. M., Halling-Sørensen, B. (2006). Multi-component analysis of tetracyclines, sulfonamides and tylosin in swine manure by liquid chromatography–tandem mass spectrometry. *Anal. Bioanal. Chem.*, **384**, 1164-1174.

Jacobsen, A.M., Halling-Sørensen, B., Ingerslev, F., Hansen, S.H. J. (2004). Simultaneous extraction of tetracycline, macrolide and sulfonamide antibiotics from agricultural soils using pressurised liquid extraction, followed by solid-phase extraction and liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A*, **1038**, 157-170.

JEFCA. (2006). Joint Food and Agriculture Organization of the United Nations and World Health Organization Expert Committee on Food Additives. Summary of evaluations performed by the JEFCA (1956–2005) (1st through 65th meetings), (verified 2 Apr. 2007). FAO, Rome; WHO, Geneva, Switzerland. http://jecfa.ilsi.org/index.htm

Jin, Y.X., Chen, R.J., Sun, L.W., Qian, H.F., Liu, W.P., Fu, Z.W. (2009). Induction of estrogen-responsive gene transcription in the embryo, larval, juvenile and adult life stages of zebrafish as biomarkers of short-term exposure to endocrine disrupting chemicals. *Comp. Biochem. Physiol. C.,* **150 (3)**, 414-420.

Jjemba, P.K. (2002). The potential impact of veterinary and human therapeutic agents in manure and biosolids on plants grown on arable land: a review. *Agric. Ecosyst. Environ.*, **93**, 267-278.

Jones-Lepp, T.L., Stevens, R. (2007). Pharmaceuticals and personal care products in biosolids/sewage sludge: the interface between analytical chemistry and regulation. *Anal. Bioanal. Chem.*, **387 (4)**, 1173-1183.

Kaleta, A., Ferdig, M., Buchberger, W. (2006). Semiquantitative determination of residues of amphetamine in sewage sludge samples. *J. Sep. Sci.*, **29**, 1662-1666.

Kaonga, C.C., Kumwenda, J., Mapoma, H.T. (2010). Accumulation of lead, cadmium, manganese, copper and zinc by sludge worms; Tubifex tubifex in sewage sludge. *Int. J. Environ. Sci. Tech.*, **7**, 119-126.

Kah, M., Beulke, S., Brown, C.D. (2007). Factors influencing degradation of pesticides in soils. *J. Agric. Food*, **55**, 4487-4492.

Katzung, B.G. (2000). Basic and Clinical Pharmacology, 8th edition. Appleton and Lange, Norwalk, CT, USA. 1217 p.

Kay, P., Blackwell, P., Boxall, A. (2004). Fate and transport of veterinary antibiotics in drained clay soils. *Environ. Toxicol. Chem.*, **23**, 1136-1144.

Kim, S., Eichhorn, P., Jensen, J.N., Weber, A.S., Aga, D.S. (2005). Removal of antibiotics in wastewater: effect of hydraulic and solid retention times on the fate of tetracycline in the activated sludge process. *Environ. Sci. Technol.*, **39 (15)**, 5816-5823.

Kinney, C.A., Furlong, E.T., Kolpin, D.W., Burkhardt, M.R., Zaugg, S.D., Werner, S.L., Bossio, J.P., Benotti, M.J. (2008). Bioaccumulation of pharmaceuticals and other anthropogenic waste indicators in earthworms from agricultural soil amended with biosolid or swine manure. *Environ. Sci. Technol.*, **42**, 1863–1870.

Kinney, C.A., Furlong, E.T., Zaugg, S.D., Burkhardt, M.R., Werner, S.L., Cahill, J.D., Jorgensen, G.R. (2006). Survey of organic wastewater contaminants in biosolids destined for land application. *Environ. Sci. Technol.*, **40 (23), 72**07-7215.

Kipper, K., Herodes, K., Lillenberg, M., Nei, L., Haiba, E., Litvin, S.V. (2010). Plant uptake of some pharmaceuticals commonly present in sewage sludge compost. *In proceedings of 2nd International Conference on Chemical, Biological and Environmental Engineering, Cairo, Egypt, 2-4 November 2010,* 261-264.

Knapp, C.W., Dolfing, J., Ehlert, P. A. I., Graham, D.W. (2010). Evidence

of increasing antibiotic resistance gene abundances in archived soils since 1940. *Environ. Sci. Technol.*, **44 (2)**, 580-587.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton H. T. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. Streams, 1999-2000: a national reconnaissance. *Environ. Sci. Technol.*, **36 (6)**, 1202-1211.

Kreuzig R., Höltge S. (2005). Investigations on the fate of sulfadiazine in manured soil: laboratory experiments and test-plot studies. *Environ. Toxicol. Chem.*, **24**, 771-776.

Kulshrestha, P., Rossman, F.G., Jr., Aga, D.S. (2004). Investigating the molecular interactions of oxytetracycline in clay and organic matter: insights on factors affecting its mobility in soil. *Environ. Sci. Technol.*, **38 (15)**, 4097-4105.

Kumar, K., Gupta, S.C., Baidoo, S. K., Chander, Y., Rosen, C. J. (2005). Antibiotic uptake by plants from soil fertilized with animal manure. *J. Environ. Qual.*, **34**, 2082-2085.

Kümmerer, K. (2001). Drugs in the environment: emission of drugs, diagnostic aids and disinfectants into wastewater by hospitals in relation to other sources - a review. *Chemosphere*, **45**, **(6-7)**, 957-969.

Kümmerer, K. (2008). Pharmaceuticals in the environment – a brief summary. In: *Pharmaceuticals in the Environment. Sources, Fate, Effects and Risks. 3rd Edition*. Kümmerer, K. (ed.). Springer-Verlag, Berlin, Heidelberg, pp. 3-21

Kümmerer, K. (2009). The presence of pharmaceuticals in the environment due to human use – present knowledge and future challenges. *J. Environ. Manage.*, **90 (8)**, 2354-2366.

Kümmerer K. (2009[1]). Antibiotics in the aquatic environment – A review – Part I. *Chemosphere*, **75 (4)**, 417-434.

Kümmerer, K. (2010). Pharmaceuticals in the Environment. *Annu Rev. Environ. Reso*ur., **35**, 57-75.

Kümmerer, K., Henninger, A. (2003). Promoting resistance by the emission of antibiotics from hospitals and households into effluent. *Clin. Microbiol. Infect.*, **9**, 1203-1214.

Küng, K., Riond, J.L., Wanner, M. (1993). Pharmacokinetics of enrofloxacin and its metabolite ciprofloxacin after intravenous and oral administration of enrofloxacin in dogs. *J. Vet. Pharmacol. Ther.*, **16**, 462-468.

Lapen, D.R., Topp, E., Metcalfe, C.D., Li, H., Edwards, M., Gottschall, N., Bolton, P., Curnoe, W., Payne, M., Beck, A. (2008). Pharmaceutical

and personal care products in tile drainage following land application of municipal biosolids. *Sci. Total Environ.*, **399(1-3)**, 50-65.

Lazzari, L., Sperni, L. Bertin, P., Pavoni, B. (2000). Correlation between inorganic (heavy metals) and organic (PCBs and PAHs) micropollutant concentrations during sewage sludge composting processes. *Chemospher*e, **41 (3)**, 427-435.

Lee, C., Lee, Y., Schmidt, C., Yoon, J., von Gunten, U. (2007). Oxidation of N-nitrosodimethylamine (NDMA) with ozone and chlorine dioxide: kinetics and effect on NDMA formation potential. *Environ. Sci. Technol.*, **41**, 2056-2063.

Li, B., Zhang, T. (2010). Biodegradation and adsorption of antibiotics in the activated sludge process. *Environ. Sci. Technol.*, **44 (9)**, 3468-3473.

Lillenberg, M. (2003). Microbiological method for determination of fluoroquinolones in soil and food plants. Comparative analysis by HPLC. MSc thesis (food science), EPMÜ.

Lillenberg, M., Järvis, T. (2005). Toidutekkelised parasitaar- ja viirushaigused. OÜ Halo Kirjastus, Tartu, 91 p.

Lillenberg, M., Roasto, M., Püssa, T. (2003). Drug residues in environment. Estimation of fluoroquinolones in soil and food plants. *J. Agr. Sci.*, **14 (1)**, 13-26.

Lillenberg, M., Yurchenko, S., Kipper, K., Herodes, K., Pihl, V., Sepp, K., Lõhmus, R., Nei, L. (2009). Simultaneous determination of fluoroquinolones, sulfonamides and tetracyclines in sewage sludge by pressurized liquid extraction and liquid chromatography electrospray ionization-mass spectrometry. *J. Chromatogr. A*, **1216**, 5949-5954.

Lillenberg, M., Herodes, K., Kipper, K., Nei, L. (2010[2]). Plant uptake of some pharmaceuticals from fertilized soils. *In proceedings of ICEST, Bangkok, Thailand, 23-25 April,* 161-165.

Lillenberg, M., Litvin, S.V., Nei, L., Roasto, M., Sepp, K. (2010[3]). Enrofloxacin and ciprofloxacin uptake by plants from soil. *Agr. Research*, **8**, 807-814.

Lillenberg, M., Yurchenko, S., Kipper, K., Herodes, K., Pihl, V., Lõhmus, R., Ivask, M., Kuu, A., Kutti, S., Litvin, S.V., Nei, L. (2010[1]). Presence of fluoroquinolones and sulfonamides in urban sewage sludge and their degradation as a result of composting. *Int. J. Environ. Sci. Tech.*, **7 (2)**, 307-312.

Lindberg, R., Jarnheimer, P., Olsen, B., Johansson, M., Tysklind M. (2004). Determination of antibiotic substances in hospital sewage

water using solid phase extraction and liquid chromatography/mass spectrometry and group analogue internal standards. *Chemosphere*, **57**, 1479-1488.

Lindberg, R.H., Wennberg, P., Johansson, M.I., Tysklind, M., Andersson, B.A.V. (2005). Screening of human antibiotic substances and determination of weekly mass flows in five sewage treatment plants in Sweden. *Environ. Sci. Technol.*, **39 (10)**, 3421-3429.

Lindsey, M.E., Meyer, M., Thurman, E.M. (2001). Analysis of trace levels of sulfonamide and tetracycline antimicrobials in groundwater and surface water using solid-phase extraction and liquid chromatography/mass spectrometry. *Anal. Chem.*, **73 (19)**, 4640-4646.

Lizondo, M., Pons, M., Gallardo, M., Estelrich, J. (1997). Physicochemical properties of enrofloxacin. *J. Pharm. Biomed. A*nal., **15**, 1845-1849.

Liu, F., Ying, G.G., Zhou, Q.X. (2009). Terrestrial ecotoxicological effects of the antimicrobial agent triclosan. *Ecotoxicol. Environ. Saf.*, **72**, 86-92.

Loos, R., Gawlik, B.M., Locoro, G., Rimaviciute, E., Contini, S., Bidoglio, G. (2009). EU-wide survey of polar organic persistent pollutants in European river waters. *Environ. Pollut.*, **157 (2)**, 561-568.

Loraine, G.A., Pettigrove, M.E. (2006). Seasonal variations in concentrations of pharmaceuticals and personal care products in drinking water and reclaimed wastewater in southern California. *Environ. Sci. Technol.*, **40**, 5811-5816.

Lu, Y.J., Wu, X.W., Guo, J.F. (2009). Characteristics of municipal solid waste and sewage sludge co-composting. *Waste Manage.*, **29 (3)**, 1152-1157.

Lundin, M., Olofsson, M., Pettersson, G.J., Zetterlund, H. (2004). Environmental and economic assessment of sewage sludge handling options. *Resour. Conservat. Recycl.*, **41 (4)**, 255-278.

Lundström, E., Adolfsson-Erici, M., Alsberg, T., Björlenius, B., Eklund, B., Lavén, M. Breitholtz, M. (2010). Characterization of additional sewage treatment technologies: Ecotoxicological effects and levels of selected pharmaceuticals, hormones and endocrine disruptors. *Ecotoxicol. Environ. Saf.*, **73 (7)**, 1612-1619.

Mahzuz, H.M.A., Alam, R., Alam, N.M., Basak, R., Islam, S.M. (2009). Use of arsenic contaminated sludge in making ornamental bricks. *Int. J. Environ. Sci. Tech.*, **6 (2)**, 291-298.

Mantovi, P., Baldoni, G., Toderi, G. (2005). Reuse of liquid, dewatered, and composted sewage sludge on agricultural land: effects of long-

term application on soil and crop. *Water Res.*, **39**, 289-296.

Manya, J.J., Sanchez, J.L., Abrego, J., Gonzalo, A., Arauzo, J. (2006). Influence of gas residence time and air ratio on the air gasification of dried sewage sludge in a bubbling fluidized bed. *Fuel,* **85**, 2027-2033.

Marengo, J.R., Kok, R.A., O´Brien, G.K., Velagaletti, R.R., Stamm, J.L. (1997). Aerobic biodegradation of (C-14)-sarafloxacin hydrochloride in soil. *Environ. Toxicol. Chem*, **16 (3)**, 462-471.

Martinez, M., McDermott, P., Walker, R. (2006). Pharmacology of the fluoroquinolones: a perspective for the use in domestic animals. *Vet. Journal*, **172**, 10-28.

McKone, T.E., Maddalena, R.L. (2007). Plant uptake of organic pollutants from soil: bioconcentration estimates based on models and experiments. *Environ. Toxicol. Chem.*, **26 (12)**, 2494-2504.

Meyer, M. (2004). Use and environmental occurrence of veterinary pharmaceuticals in the United States. In: *Pharmaceuticals in the Environment: Sources, Fate, Effects and Risks*. Kümmerer, K. (ed.), Springer-Verlag, Heidelberg, Germany, pp. 155-163.

Mellon, M., Benbrook, C., Benbrook, K.L. (2001). Hogging it: Estimates of antimicrobial abuse in livestock. Union of Concerned Scientists, UCS Publications, Cambridge, MA, USA. 67 p.

Mendez-Arriaga, F., Gimenez, J., Esplugas, S., (2008). Photocatalytic degradation of non-steroidal antiinflammatory drugs with TiO_2 and simulated solar irradiation. *Water Res.*, **42 (3)**, 585-594.

Mengozzi, G., Intorre, L., Bertini, S. (1996). Pharmacokinetics of enrofloxacin and its metabolite ciprofloxacin after intravenous and intramuscular administrations in sheep. *Am. J. Vet. Res.*, **57 (7)**, 1040-1043.

Merck Manuals[1]. Online Medical Library. http://www.merckmanuals.com/professional/sec14/ch170/ch170f.html

Merck Manuals[2]. Online Medical Library. http://www.merckmanuals.com/professional/sec14/ch170/ch170n.html

Merck Manuals[3]. Online Medical Library. http://www.merckmanuals.com/professional/sec14/ch170/ch170n.html#sec14-ch170-ch170n-404f

Meremäe, K., Elias, P., Tamme, T., Kramarenko, T., Lillenberg, M., Karus, A., Hänninen, M.-L., Roasto; M. (2010). The occurrence of *Campylobacter spp.* in Estonian broiler chicken production in 2002-2007. *Food Control*, **21 (3)**, 272-275.

Metcalfe, C., Miao, X.-S., Koenig, B.G., Struger, J. (2003). Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. *Environ. Toxicol. Chem.*, **22**, 2881-2889.

Miao, X.-S., Yang, J.-J., Metcalfe, C. (2005). Carbamazepine and its metabolites in wastewater and in biosolids in a municipal wastewater treatment plant. *Environ. Sci. Technol.*, **39**, 7469-7475.

Migliore, L., Brambilla, G., Cozzolino, S., Gaudio, L. (1995). Effects on plants of sulphadimethoxine used in intensive farming *(Panicum miliaceum, Pisum sativum and Zea Mays). Agric. Ecosyst. Environ.*, **52**, 103-110.

Migliore, L., Brambilla, G., Casoria, P.,Civitareale, C., Cozzolino, S., Gaudio, L. (1996). Effect of sulphadimethoxine contamination on barley *(Hordeum distichum L., Poaceae, Liliopsida). Agricult. Ecosyst. Environm.*, **60**, 121-128.

Migliore, L., Cozzolino, S., Fiori, M. (2003). Phytotoxicity to and uptake of enrofloxacin in crop plants. *Chemosphere*, **52**, 1233–1244.

Monteiro, S. C., Boxall, A. B. A. (2009). Factors affecting the degradation of pharmaceuticals in agricultural soils. *Environ. Toxicol. Chem.*, **28 (12)**, 2546–2554.

Montforts, M.H.M.M. (2005). RIVM report 601500002/2005. The trigger values in the environmental risk assessment for (veterinary) medicines in the European Union: a critical appraisal. Performed by Expert Centre of Substances of the RIVM, Bilthoven, 21-37.

Monzó, J., Payá, J., Borrachero, M.V., Córcoles, A. (1996). Use of sewage sludge ash (SSA) -cement admixtures in mortars. *Cement Concr. Res.*, **26 (9)**, 1389-1398.

Monzó, J., Payá, J., Borrachero, M.V., Girbés, I. (2003). Reuse of sewage sludge ashes (SSA) in cement mixtures: the effect of SSA on the workability of cement mortars. *Waste Manage.*, **23**, 373-381.

Murakami, T., Suda, T., Mouri, S., Shigeta, J., Hirata, T., Fujimori, T., Huang, W. (2006). Cogasification of sewage sludge and plastic wastes: evaluation of experiment and application. *J. Jpn. Inst. Ene*rgy, **85**, 964-970.

Murakami, T., Suzuki, Y., Nagasawa, H., Yamamoto, T., Koseki, T., Hirose, H., Okamoto, S. (2009). Combustion characteristics of sewage sludge in an incineration plant for energy recovery. *Fuel Process.Technol.*, **90 (6)**, 778–783.

Murakami, T., Xu, G., Suda, T., Matsuzawa, Y., Tani, H., Fujimori, T.

(2007). Some process fundamentals of biomass gasification in dual fluidized bed. *Fuel*, **86**, 244 - 255.

Nakata, H., Kannan, K., Jones, P.D., Giesy, J.P. (2005). Determination of fluoroquinolone antibiotics in wastewater effluents by liquid chromatography-mass spectrometry and fluorescence detection. *Chemosphere*, **58**, 759-766.

National Waste Management Plan. (2002). Ministry of the Environment, Tallinn, pp. 48-49.

NEBRA - North East Biosolids and Residuals Association. (2007). A national biosolids regulation, quality, end use, and disposal survey – final report. http://www.nebiosolids.org/uploads/pdf/NtlBiosolidsReport-20July07.pdf.

Nei, L., Lillenberg, M. (2009[1]). Mackereth oxygen sensor: measurement uncertainty. *ECS Trans.*, **19 (22)**, 55-63.

Nei, L., Lillenberg, M. (2009[2]). Reoveesettest valmistatud komposti peab hoolega kontrollima. *Keskkonnatehnika*, **7**, 12-13.

Niessen, W.M.A. (1998). Analysis of antibiotics by liquid chromatography–mass spectrometry. *J. Chromatogr. A*, **812**, 53-75.

Nowara, A., Burhenne, J., Spiteller, M. (1997). Binding of fluoroquinolone carboxylic acid derivates to clay minerals. *J. Agric. Food, Chem.*, **45**, 1459-1463.

O´Connor, G.A., Granato, T.C., Basta, N.T. (2001). Bioavailability of Biosolids Molybdenum to Soybean Grain. *J. Environ. Qual.*, **30**, 1653-1658.

O´Connor, G.A. (1996). Organic compounds in sludge-amended soils and their potential for uptake by crop plants. *Sci.Total. Environ.*, **185**, 71-81.

Odegaard, H. (2003). Sludge minimization technologies - an overview. *Water Sci. Tech.*, **49 (10)**, 31-40.

Okuda, T., Yamashita, N., Tanaka, H., Matsukawa, H., Tanabe, K. (2009). Development of extraction method of pharmaceuticals and their occurrences found in Japanese wastewater treatment plants. *Environ. Int.*, 35, 815-820.

Oppel, J., Broil, G., Löffler, D., Römbke, J., Meller, M., Ternes, T. (2004). Leaching behaviour of pharmaceuticals in soil-testing-systems: a part of an environmental risk assessment for groundwater protection. *Sci. Total. Environ.*, **328**, 265-273.

O´Reilly, A., Smith, P. (1999). Development of methods for predicting the minimum concentrations of oxytetracycline capable of exerting a selection for resistance to this agent. *Aquaculture*, **180**, 1-11.

Palmada, J., March, R., Torroella, E., Espigol, C., Baleri, T. (2000). Determination of enrofloxacin and its active metabolite (ciprofloxacin) at the residue level in broiler muscle using HPLC with fluorescence detection. *References of Euroresidue IV. Veldhoven, 8-10 May, 2000,* 822-826.

Parolo, M.E., Savini, M.C., Vallés, J.M., Baschini, M.T., Avena, M.G. (2008). Tetracycline adsorption on montmorillonite: pH and ionic strength effects. *Appl. Clay Sci.*, **40 (1-4)**, 179-186.

Paterson, S., Mackay, D., Bacci, E., Calamari, D. (1991). Correlation of the equilibrium and kinetics of leaf-air exchange of hydrophobic organic chemicals. *Environ. Sci. Technol.*, **25 (5)**, 866-871

Paterson, S., Mackay, D., Gladman, A. (1991[1]). A fugacity model of chemical uptake by plants from soil and air. *Chemosphere*, **23**, 539-565.

Pérez, S., Eichhorn, P., Aga, D. S. (2005). Evaluating the biodegradability of sulfamethazine, sulfamethoxazole, sulfathiazole, and trimethoprim at different stages of sewage treatment. *Environ. Toxicol. Chem.*, **24 (6)**, 1361-1367.

Pet Education.com http://www.peteducation.com/article.cfm?c=0+1451&aid=1481

Picó, Y., Andreu, V. (2007). Fluoroquinolones in soil: risks and challenges. *Anal Bioanal Chem.*, **387**, 1287-1299.

Porubcan, L.S., Serna, C.J., White, J.L., Hem, S.L. (1978). Mechanism of adsorption of clindamycin and tetracycline by montmorillonite. *J. Pharm. Sci.*, **67**, 1081-1087.

Posyniak, A., Zmudzki, J., Mitrwska, K. (2005). Dispersive solid-phase extraction for the determination of sulfonamides in chicken muscle by liquid chromatography. *J. Chromatogr. A*, **1087**, 259-264.

Qiang, Z., Adams, C. (2004). "Potentiometric Determination of Acid Dissociation Constants (pKa) for Human and Veterinary Antibiotics," *Water Res.*, **38 (12)**, 2874-2890.

Qiting, J., Xiheng, Z. (1988). Combination process of anaerobic digestion and ozonization technology for treating wastewater from antibiotics production. *Water Treat.*, **3**, 285–291.

Radjenović, J., Petrović, M., Barceló, D. (2009.) Fate and distribution of pharmaceuticals in wastewater and sewage sludge of the conventional activated sludge (CAS) and advanced membrane bioreactor (MBR) treatment. *Water Res.*, **43 (3)**, 831-841.

Ravina, M., Campanella, L., Kiwi, J.(2002). Accelerated mineralization of the drug diclofenac via Fenton reactions in a concentric photo-

reactor, *Water Res.*, **36**, 3553-3560.

Redshaw, C. H., Wootton, V. G., Rowland, S. J. (2008). Uptake of the pharmaceutical fluoxetine hydrochloride from growth medium by *Brassicaceae*. *Phytochem.*, **69**, 2510- 2516.

Reiderer, M. (1990). Estimating partitioning and transport of organic chemicals in the foliage/atmosphere system: discussion of a fugacity based model. *Environ. Sci. Technol.*, **24**, 829-837.

Reinthaler, F.F., Posch, J., Feierl, G., Wüst, G., Haas, D., Ruckenbauer, G., Mascher, F., Marth, E.(2003). Antibiotic resistance of *E.coli* in sewage and sludge. *Water Res.*, **37 (8)**, 1685-1690.

Richardson, M.L., Bowron, J.M. (1985). The fate of pharmaceutical chemicals in the aquatic environment – a review. *J. Pharmacol.*, **37**, 1-12.

Riigi Teataja I. **9**, **52**. (2004). Keskkonnaministri määrus nr.78.

Roasto, M., Meremäe, K., Praakle-Amin, K., Hörman, A., Elias, T., Lillenberg, M., Elias, A., Kramarenko, T., Häkkinen, L., Põltsama, P., Mäesaar, M., Elias, P., Hänninen, M.-L. (2011). Termofiilsete kampülobakterite uuringud Eestis 2000-2010 (*Campylobacter spp.* in Estonian food chain in 2000-2010). *Agraarteadus (Journal of Agricultural Science)*, **22 (1)**, 31-39.

Roasto, M., Praakle-Amin, K., Meremäe, K., Tamme, T., Hörman, A., Lillenberg, M., Hänninen, M.-L.(2009). Food control and research on *Campylobacter spp. in Estonia. Archiv für Lebensmittelhygiene,* **60**, 109-115.

Rockefeller, A.A.(2002). Comment and controversies. Sewers, sewage treatment, sludge: damage without end. *New Solution*s, **12 (4)**, 341-346.

Rogers, H. R., Campbell, J. A., Crathorne, B., Dobbs, A. J. (1989). The occurrence of chlorobenzenes and permethrins in twelve UK sewage sludges. *Water Res.*, **23**, 913-921.

RxList[1]. The Internet Drug Index. http://www.rxlist.com/cipro-drug.htm

RxList[2]. The Internet Drug Index. http://www.rxlist.com/noroxin-drug.htm

RxList[3]. The Internet Drug Index. http://www.rxlist.com/floxin-drug.htm

RxList[4]. The Internet Drug Index. http://www.rxlist.com/oracea-drug.htm

Sacher, F., Lange, F.T.H., Brauch, H.J., Blankenhorn, I. (2001).

Pharmaceuticals in groundwaters. Analytical methods and results of a monitoring program in Baden-Wurttemberg, Germany. *J. Chromatogr. A*, **938**, 199-210.

Sahlström, L., Rehbinder, V., Albihn, A., Aspan, A., Bengtsson, B. (2009). Vancomycin resistant enterococci (VRE) in Swedish sewage sludge. Acta Vet. Scand., **51 (24)**, 1-9.

Sarmah, A.K., Meyer, M.T., Boxal, A.B. (2006). A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemospher*e, **65,** 725-759.

Sassman, S.A., Lee, L.S. (2005). Sorption of three tetracyclines by several soil: role of pH and cation exchange. *Environ. Sci. Technol.*, **39,** 7452-7459.

Schaumann, R., Rodloff, A.C. (2007). Activities of quinolones against obligately anaerobic bacteria. *Anti-Infective Agents Med. Chem.*, **6,** 49-56.

Schröder, H.F. (2002). Mass spectrometric monitoring of the degradation and elimination efficiency for hardly eliminable and hardly biodegradable polar compounds by membrane bioreactors. *Water Sci. Techn*ol, **46,** 57-64.

Schlüsener, M.P., Spiteller, M., Bester, K. (2003). Determination of antibiotics from soil by pressurized liquid extraction and liquid chromatography–tandem mass spectrometry. *J. Chromatogr.* A, **1003**, 21-28.

Schlüsener, M.P., Bester, K. (2006). Persistence of antibiotics such as macrolides, tiamulin and salinomycin in soil. *Environ. Pol*lut., **143**, 565-571.

Sczesny, S., Nau, H., Hamscher ,G. (2003). Residue analysis of tetracyclines and their metabolites in eggs and in the environment by HPLC coupled with a microbiological assay and tandem mass spectrometry. *J. Agric. Food Ch*em., **51,** 697-703.

Sirius Analytical Instruments. (2008). http://www.sirius-analytical.com/pKa

Smith, S.R. (2009). A critical review of the bioavailability and impacts of heavy metals in municipal solid waste composts compared to sewage sludge. *Environ. Int.*, **35 (1)**, 142-156.

Soda, S., Iwai, Y., Sei, K., Shimod, Y., Ike, M. (2010). Model analysis of energy consumption and greenhouse gas emissions of sewage sludge treatment systems with different processes and scales. *Water*

Sci.Tech., **61 (2)**, 365-373.

Spongberg, A.L., Witter, J.D. (2008). Pharmaceutical compounds in the wastewater process stream in Northwest Ohio. *Sci. Total. Environ.*, **397**, 148-157.

State Agency of Medicines[1]." Estonian Statistics on Medicines 2002-2006. http://www.ravimiamet.ee/vvfiles/3/Eesti%20ravimistatistika%202002-2006.pdf

State Agency of Medicines[2]. Estonian Statistics on Medicines 2006-2008.

http://www.ravimiamet.ee/vvfiles/1/Eesti%20ravimistatistika%202006-2008.pdf

State Agency of Medicines[3]. Veterinary products authorised in Estonia. http://193.40.10.165/register/register.php?keel=eng&inim_vet=vet

Steger-Hartmann, T., Kümmerer, K., Hartmann, A. (1997). Biological degradation of cyclophosphamide and its occurrence in sewage water. *Ecotox. Environ. Safety*, **36 (2)**, 174 -179.

Stevens, J. L., Northcott, G. L., Stern, G. A., Tomy, G.T., Jones, K.C. (2003). PAHs, PCBs, PCNs, organochlorine pesticides, synthetic musks, and polychlorinated n-alkanes in UK sewage sludge: survey results and implications. *Environ. Sci. Technol.*, **37**, 462-467.

Stoob, K., Singer, H.P., Stettler, S., Hartmann, N., Mueller, S.R., Stamm, C.H. (2006). Exhaustive extraction of sulfonamide antibiotics from aged agricultural soils using pressurized liquid extraction. *J. Choromatogr. A*, **1128**, 1-9.

Stuer-Lauridsen, F., Birkved, M., Hansen, L.P., Holten Lützhøft, H.C., Halling-Sørensen, B. (2000). Environmental risk assessment of human pharmaceuticals in Denmark after normal therapeutic use. *Chemosphere*, **40 (7)**, 783-793.

Stumpf, M., Ternes, T. A., Wilken, R.-D., Rodrigues, S.V., Baumann, W. (1999). Polar drug residues in sewage and natural waters in the state of Rio de Janeiro, Brazil: *Sci. Total Environ.*, **225**, 135-141.

Suzuki, S., Tanaka, M., Kaneko, T. (1997). Glass-ceramic from sewage sludge ash. *J. Mater. Sci.*, **32**, 1775-1779.

Shenkera, M., Harusha, D., Ben-Aria, J., Chefetz, B. (2011). Uptake of carbamazepine by cucumber plants – a case study related to irrigation with reclaimed wastewater. *Chemosphere*, **82 (6)**, 905-910.

Zhang, C., Wang, Y. (2010). Determination and correlation of 1-octanol/water partition coefficients for six quinolones from 293.15 K to

323.15 K. *Chem. Res. Chinese Universities*, **26 (4)**, 636-639.

Zhang, C., Wang, Y., Wang, F. (2007). Determination and temperature dependence of n-octanol/water partition coefficients for seven sulfonamides from (298.15 to 333.15) K. *Bull. Korean Chem. Soc.*, **28 (7)**, 1183-1186.

Zwiener, C., Glauner, T., Frimmel, F.H. (2000). Biodegradation of pharmaceutical residues investigated by SPE-GC/ITD-MS and on-line derivatization. *J. High Res. Chromatog.*, **23 (7-8)**, 474-478.

Zühlke, S., Dünnbier, U., Heberer, T. (2004). Detection and identification of phenazone-type drugs and their microbial metabolites in ground- and drinking water applying solid-phase extraction and gas chromatography with mass spectrometric detection. *J. Chromatogr. A*, **1050**, 201-209.

Taruya, T., Okuno, N., Kanaya, K. (2002). Reuse of sewage sludge as raw material of Portland cement in Japan. *Water Sci. Tech.*, **46 (10)**, 255-258.

Tay, J.-H., Show, K.-Y. (1991). Clay blended sludge as lightweight aggregate concrete materials. *J. Environ. Eng. Div. – ASCE*, **117**, 834-844.

Thiele-Bruhn, S. (2003). Pharmaceutical antibiotic compounds in soils – a rewiew. *J. Plant. Nutr. Soil Sci.*, **166**, 145-167.

Thiele-Bruhn, S., Beck, I-C. (2005). Effects of sulfonamide and tetracycline antibiotics on soil microbial activity and microbial biomass. *Chemosphere*, **59 (4)**, 457-465.

Thiele-Bruhn, S., Seibicke, T., Schulten, H.-R., Leinweber, P. (2004). Sorption of sulfonamide pharmaceutical antibiotics on whole soils and particle-size fractions. *J. Environ. Qual.*, **33**, 1331-1342.

Thomsen, A., Göbel, A.., Mc.Ardell, C.S., Alder, A.C., Giger, W. (2003). Determination of sulfonamide and macrolide antibiotics in sewage sludge using accelerated solvent extraction and LC-MS/MS. *Presentation of project results on Poseidon Symposium, 4-5 Nov 2003, Braunschweig, Germany.*

Ternes, T.A. (1998). Occurrence of drugs in German sewage treatment plants and rivers. *Water Res.*, **32 (11)**, 3245-3260.

Ternes, T.A., Andersen, H., Gilberg, D., Bonerz, M. (2002). Determination of estrogens in sludge and sediments by liquid extraction and GC/MS/MS. *Anal. Chem.*, **74**, 3498-3504.

Ternes, T.A., Bonerz, M., Herrmann, N., Löffler, D., Keller, E., Bago Lacida, B., Alder, A.C. (2005). Determination of pharmaceuticals,

iodinated contrast media and musk fragrances in sludge by LC/tandem MS and GC/MS. *J. Chromatogr. A*, **1067**, 213-223.

Ternes, T.A., Stüber, J., Hermann, N., McDowell, D., Ried, A., Kampmann, M., Teiser, B. (2003). Ozonation: a tool for removal of pharmaceuticals, contrast media and musk fragrances from wastewater? *Water Res.*, **37**, 1976-1962.

Tolls, J. (2001). Sorption of veterinary pharmaceuticals in soils: a review. *Environ. Sci. Technol.*, **35**, 3397-3406.

Topp, E., Hendel, J., Lapen, D., Chapman R. (2008[1]). Fate of the non-steroidal anti-inflammatory drug naproxen in agricultural soil receiving liquid municipal biosolids. *Environ. Toxicol. Chem.*, **27**, 2005-2010.

Topp, E., Hendel, J.G., Lu, Z., Chapman, R. (2006). Biodegradation of caffeine in agricultural soils. *Can. J. Soil. Sci.*, **86**, 533-544.

Topp, E., Monteiro, S.C., Beck, A., Coelho, B.B., Boxall, A.B.A., Duenk, P.W., Kleywegt, S., Lapen, D.R., Payne, M., Sabourin, L., Li, H., Metcalfe, C.D. (2008). Runoff of pharmaceuticals and personal care products following application of biosolids to an agricultural field. *Sci. Total. Environ.*, **396**, 52-59.

Toussaint, B., Bordin, G., Janosi, A., Rodriguez, A.R. (2002). Validation of a liquid chromatography-tandem mass spectrometry method for the simultaneous quantification of 11 (fluoro)quinolone antibiotics in swine kidney. *J. Chromatogr. A*, **976**, 195-206.

Toussaint, B., Chedin, M., Bordin, G., Rodriguez, A.R. (2005). Determination of (fluoro)quinolone antibiotic residues in pig kidney using liquid chromatography-tandem mass spectrometry. I. Laboratory-validated method. *J. Chromatogr. A*, **1088**, 32-39.

Trapp, S., Matthies, M., Scheunert, I., Topp, E. (1990). Modeling the bioconcentration of organic chemicals in plants. *Environ. Sci. Technol.*, **24**, 1246 -1252.

Trautwein, C., Metzger, J., Kümmerer, K. (2008). Aerobic biodegradability of the calcium channel antagonist verapamil and identification of the dead-end metabolite 2-(3,4-dimethoxyphenyl)-2isopropyl-5-(methylamino) pentane nitrile. *Chemosphere*, **72**, 442-450.

Tremier, A., de Guardia, A., Massiani, C., Paul, E., Martel, J. L. (2005). A respirometric method for characterising the organic composition and biodegradation kinetics and the temperature influence on the biodegradation kinetics, for a mixture of sludge and bulking agent to be co-composted. *Bioresour. Technol.*, **96**, 169-180.

Tsadilas, C.D., Matsi, T., Barbayiannis, N., Dimoyiannis, D. (1995). Influence of sewage sludge application on soil properties and on the distribution and availability of heavy metal fractions. *Soil Sci. Plant Anal.*, **26**, 2603-2619.

Tshervjakova, V., Terezova, A. (1986). Pharmacology and Dispencary. Valgus, Tallinn, Estonia, pp. 98-112 (in Estonian).

Turiel, E., Martín-Esterban A., Tadeo, J.L. (2006). Multiresidue analysis of quinolones and fluoroquinolones in soil by ultrasonic-assisted extraction in small columns and HPLC-UV. *Anal. Chim. Acta*, **562**, 30-35.

USGS (2002). US Geological Survey. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams. USGS Fact Sheet FS-027-02. http://toxics.usgs.gov/pubs/FS-027-02/pdf/FS-027-02.pdf

Walters, E., McClellan, K., Halden, R.U. (2010). Occurrence and loss over three years of 72 pharmaceuticals and personal care products from biosolids-soil mixtures in outdoor mesocosms. *Water Res.*, **44 (20)**, 6011-6020.

Webber, M. D., Lesage, S. (1989). Organic contaminants in Canadian municipal sludges. *Waste Manag. Res.*, **7**, 913-921.

WHO. (2003). World Health Organization. GEMS/Food regional diets: Regional per capita consumption of raw and semi-processed agricultural commodities. Global Environment Monitoring System/ Food Contamination Monitoring and Assessment Programme (verified 2 Apr. 2007). Geneva, Switzerland. http://www.who.int/foodsafety/chem/gems_regional_diet.pdf

Wise, R. (2002). Antimicrobial resistance: priorities for action. *J. Antimicrob. Chemoth.*, **49**, 585-586.

Worden, R.M., Grethlein, A.J., Jain, M.K., Datta, R. (1991). Production of butanol and ethanol from synthesis gas via fermentation. *Fuel*, **70**, 615-619.

Wu, C., Spongberg, A.L., Witter, J.D., Fang, M., Czajkovski, K.P. (2010). Uptake pharmaceutical and personal care products by soybean plants from soils applied with biosolids and irrigated with contaminated water. Environ. *Sci. Technol.*, **44**, 6157-6161.

Xia, K., Bhandari, A., Das, K., Pillar, G. (2005). Occurrence and fate of pharmaceuticals and personal care products (PPCPs) in biosolids. *J. Environ. Qual.*, **34**, 91-104.

Yan, S., Carlson, K. (2003). Evolution of antibiotic occurrence in a river through pristine urban and agricultural landscapes. *Water Res.*, **37**, 4645-4656.

Pamba.ru™ . Справочник лекарств. http://www.pamba.ru/therapeutic/sulfanilamides/long/sulfadimethoxine/

ACKNOWLEDGEMENTS

I would like to express my greatest thanks to my supervisor Professor Lembit Nei for his marvellous optimism and everlasting energy. His valuable advice and encouragement have greatly contributed to the realization of this work.

I am also very grateful to my supervisor Professor Kalev Sepp, who always did his best for supporting our experiments as well as my international PhD courses and presentations at conferences.

This work was done in active co-operation with chemists and physicists from the University of Tartu. Great thanks to our team members: Dr Viljar Pihl, Associate Professor Koit Herodes, PhD student Karin Kipper, Associate Professor Sergey Yurchenko and Dr Rünno Lõhmus. My warm thanks to Ms Sandra V. Litvin for making corrections to our manuscripts.

I am greatly thankful to Mr Mati Perker, Mr Harri Terase, Mr Urmas Tiivoja and Mr Hillar Toomiste, the managers of the Tallinn and Tartu Wastewater Treatment Plants. Without their kind assistance this work would have been impossible to accomplish.

My sincere thanks to the colleagues from the Department of Soil Science and Agrochemistry: Professor Emeritus Paul Kuldkepp, Associate Professor Alar Astover, Mr Tõnu Tõnutare and Mr Avo Toomsoo for their advice and help in performing the plant experiments.

I would like to take the opportunity to express my sincere thanks to my friendly colleagues from the Department of Food Hygiene – PhD student Dea Anton, Associate Professor Kadrin Meremäe, Associate Professor Emeritus Alida Kiis and PhD student Piret Raudsepp for their help and advice, whenever I needed them.

My very special thanks to the head of the Department of Food Hygiene, Associate Professor Mati Roasto for promoting the perfect working milieu and supporting me from the times of performing my MSc work

until completing this PhD thesis; to my colleague Dr Terje Elias, who encouraged me to start with PhD studies.

My warm thanks to my family for the patience and understanding my frequent absence from home, while this work took the time that would have otherwise belonged to them. A special thanks to my son Hendrik for the help with the plant experiments.

This study was funded by the Estonian Environmental Investment Centre, Estonian Science Foundation (grants No 6658 and No 7127), Estonian research target project SF 0180058s07 and the Estonian Nanotechnology Competence Centre.

I

ORIGINAL PUBLICATIONS

Lillenberg, M., Roasto, M., Püssa, T. (2003).
DRUG RESIDUES IN ENVIRONMENT.
ESTIMATION OF FLUOROQUINOLONES IN SOIL AND
FOOD PLANTS.
Journal of Agricultural Science, 14 (1), 13-26.

RAVIMIJÄÄGID KESKKONNAS. FLUOROKINOLOONIDE MÄÄRAMINE MULLAS JA TOIDUTAIMEDES

M. Lillenberg, M. Roasto, T. Püssa

ABSTRACT. Drug Residues in Environment. Estimation of Fluoroquinolones in Soil and Food Plants. *All drugs approved by the authorities have undergone pharmacokinetic and animal toxicological studies. But there is still lack of knowledge about their fate and effects on the environment. After excretion, these drugs and their metabolites can contaminate the environment. Fluoroquinolones are broad spectrum synthetic antibiotics used both in human and veterinary medicine. There exists a couple of structurally very closely related fluoroquinolones – enrofloxacin and ciprofloxacin. The last one is the main metabolite of the first. Enrofloxacin with its metabolites enters the manure and further the soil. It has been shown that both fluoroquinolones are strongly adsorbed to soil and resistant to physical, chemical and microbiological attacks. The fluoroquinolones can be uptaken by plants growing in soil fertilised with manure and in such way finally reach human as well as animal food. Very small amounts of broad spectrum antibiotics in everyday food may generate the strains of resistant microorganisms in human and animal organism. There is scarce data available concerning the actual fate and effect of the drug residues in the environment and in food plants. Methods of quantitative assay of these compounds are needed.*

The aim of the research was to study the uptake and accumulation of fluoroquinolones by plants cultivated in soil amended with drugs. For that purpose microbiological agar-diffusion method for estimation of content of antibiotics residues in plants and soil was put up. In the role of testorganism Bacillus subtilis *was used. The results were controlled by chromatography (HPLC) after 10 months.*

Keywords: *contamination, environment, enrofloxacin, ciprofloxacin, residues, manure, soil, food plants, microbiological agar-diffusion method,* Bacillus subtilis, *HPLC method.*

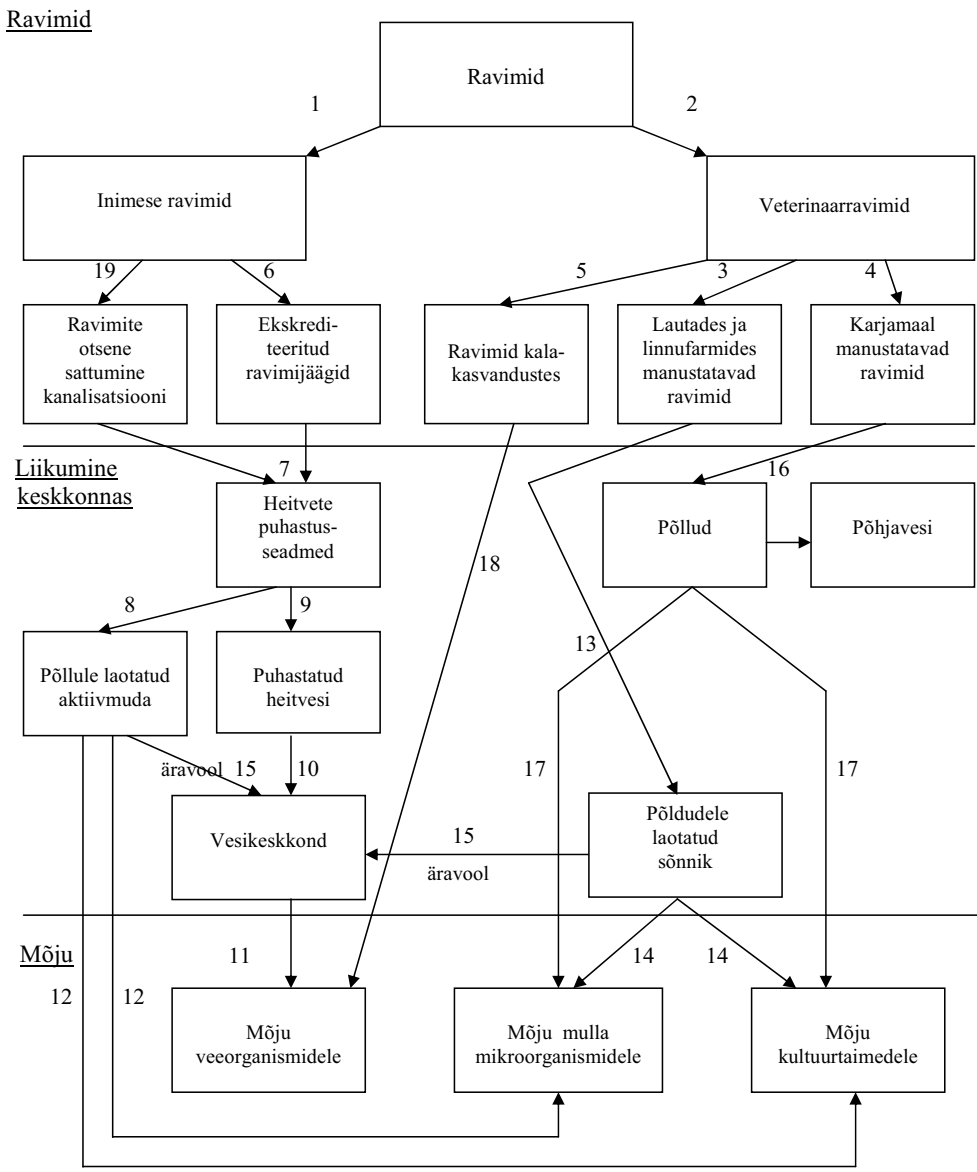
Sissejuhatus

Tänapäeval on kõik riigi tervishoiuameti poolt lubatud ravimid läbinud põhjalikud farmakokineetilised ja loomtoksikoloogilised uuringud. Seni teatakse aga vähe ravimite edasisest saatusest pärast eksrediteerimist. Keskkonda sattunult võivad ravimid ja nende metaboliidid mõjuda kahjulikult vee ja mulla elustikule.

Fluorokinoloonid on laia toimespektriga sünteetilised ravimid. Et vältida ristuva resistentsuse teket, ei kasutata üldjuhul loomade ja inimeste raviks üht ja sama antibiootikumi. Ent on olemas kaks väga lähedase struktuuriga fluorokinolooni – enrofloksatsiin ja tsiprofloksatsiin, millest esimene leiab kasutamist veterinaar- ja teine humaanmeditsiinis. Ainevahetuse lõpp-produktidena jõuavad nii enrofloksatsiin ja selle põhimetaboliit tsiprofloksatsiin ekskrementidesse. Põllumajandusloomade sõnnikut kasutatakse laialdaselt orgaanilise väetisena, sealhulgas toidutaimede kasvatamisel. On alust arvata, et teiste ravimijääkide kõrval sisaldab sõnnik ka fluorokinoloonide jääke. Koos mullavees lahustunud mineraalainetega võivad taimed omastada ka vees lahustunud fluorokinoloone. Sel teel on võimalik ravimijääkide sattumine nii loomasööta kui ka inimese toidulauale. Nende pidev väikestes kogustes omastamine võiks põhjustada patogeensete mikroobide ravimresistentsust, esile kutsuda allergiaid ja kahjustada maksa. Loomne toidutoore allub rangele riiklikule kontrollile – ravimijääke kas ei tohi seal üldse olla või siis on kehtestatud nende lubatud sisalduse maksimumpiir (*MRL – maximum residue limit*). Antibiootikumide võimalikku akumuleerumist taimedesse on seni vähe uuritud. Et pole teada, millistes kogustes on taimed võimelised omastama antibiootikume, puuduvad taimse toidutoorme kohta ka vastavad piirnormid.

Käesoleva töö eesmärgiks oli uurida fluorokinoloonide akumuleerumist mullast taimedesse ja näidata nende antibakteriaalse toime säilimist seal. Selleks töötati välja mikrobioloogiline agar-difusioon meetod fluorokinoloonide määramiseks mullas ja taimedes. Testorganismina kasutati bakterit *Bacillus subtilis*. Enrofloksatsiini säilimist salatis kontrolliti 10 kuu pärast kromatograafiliselt HPLC-meetodiga.

Võtmesõnad: saastamine, keskkond, enrofloksatsiin, tsiprofloksatsiin, ravimijäägid, orgaaniline väetis, muld, toidutaimed, mikrobioloogiline agar-difusioon meetod, *Bacillus subtilis,* HPLC-meetod.



Joonis 1. Ravimite võimalikud liikumisteed keskkonnas
Figure 1. *Routes of Medical Substances in the Environment* (Halling-Sørensen *et al.*, 1998)

Kirjanduse ülevaade

Ravimijääkide liikumine keskkonnas

Kuni viimase ajani ei pööratud ravimijääkide saatusele keskkonnas erilist tähelepanu. Põhjuseks, miks ravimid võivad keskkonda sattudes aga kahjulikeks osutuda, on asjaolu, et nad on loodud eesmärgiga mõjutada bioloogilisi objekte. Neil on piisav lipofiilsus, mis võimaldab läbida biomembraane, ja stabiilsus, mis hoiab ära nende inaktiveerumise enne raviefekti tekitamist. Nii on ravimitel vajalikud omadused, et akumuleeruda organismides ja kutsuda esile muutusi vee ja pinnase ökosüsteemides. Mõne enam kasutatava ravimi aastane tarbimine võib olla küllalt suur (Halling-Sørensen *et al*., 1998). Näiteks Taanis on hinnatud antibiootikumide aastaseks summaarseks tarbimismääraks ligikaudu 34 tonni (Stuer-Lauridsen *et al*., 2000).

Joonisel 1 on näidatud mitmesuguste ravimite võimalikud liikumisteed keskkonnas. Ravimid võib liigitada üldiselt kaheks: ühed, mida kasutatakse inimese ravimisel (1) ja teised, mida kasutatakse loomade puhul (2). Veterinaarravimeid kasutatakse lautades ja linnufarmides kas kasvustimulaatoritena söötade koostises või teraapias (3). Kariloomi ravitakse ka otse karjamaal (4). Kalakasvandustes antakse ravimeid sööda koostises, mida puistatakse vette (5). Inimese või looma organismist väljuvad ravimid uriini ja väljaheidetega kas muundumata kujul või metaboliitidena. Inimese ekskrementidega jõuavad ravimijäägid kanalisatsioonivõrgustikku (6) ja sealt edasi heitvete puhastusseadmesse (7).

Brasiilias tehtud uurimistöö näitab, et erinevate ravimite puhul varieerub puhastusseadet muutumatuna läbinud ainehulk 12%-st kuni 90%-ni (Stumpt *et al*., 1999). Siit järeldub, et puhastusseadmed ei kõrvalda ravimijääke täielikult. Märkimisväärne kogus neist jõuab pinnavette ja võib sealt sattuda ka joogivette. Ravimijääkide võimalik saatus võib pärast puhastusseadmesse sisenemist olla põhimõtteliselt üks järgmistest:

1) aine on kergesti degradeeritav ning juba puhastusseadmes toimub tema kiire ja täielik lagunemine süsinikdioksiidiks ja veeks;

2) aine on lipofiilne ja raskesti degradeeritav ning ta akumuleerub muutumatuna puhastusseadme aktiivmudasse (8);

3) aine metaboliseeritakse küll hüdrofiilsemaks, kuid ta ei lagune, vaid läbib puhastusseadme (9) ja jõuab vesikeskkonda (10).

Kui jõkke jõudnud metaboliit on bioloogiliselt aktiivne, võib see mõjutada veeorganisme (11). Aktiivmuda, mille koostises on ka lipofiilsed ravimijäägid, laotatakse põldudele väetiseks (12). Sel viisil satuvad ravimid pinnasesse, kus võivad mõjutada mikroorganisme ja akumuleeruda kultuurtaimedesse. Loomakasvatuses kasutatavad kasvustimulaatorid ja ravimid jõuavad kas muutumatutena või metaboliitidena sõnnikusse (13). Viimane laotatakse väetisena põldudele. Nii jõuavad ka veterinaarravimid pinnasesse, kus võivad mõjutada mullaorganisme ja kultuurtaimi. Aktiivmuda või sõnnikuga põldudele sattunud hüdrofiilsete omadustega ravimijäägid võivad vihmasadude tagajärjel vee äravooluga jällegi pinnavette sattuda (15). Karjamaadel loomadele manustatavad ravimid läbivad looma organismi ja väljuvad ekskrementidega (16). Sel viisil tekivad pinnases lokaalselt eriti tugevad ravimijääkide kontsentratsioonid, mis avaldavad tugevat mõju mullaorganismidele ja taimedele (17). Pinnasesse jõudnud ravimid ja nende metaboliidid kas mineraliseeritakse mullaorganismide poolt või jõuavad muutumatutena põhjavette. Kalakasvandustes kasutatavad ravimid puistatakse kalatoidu koostises otse veekogudesse. Suur hulk sellisest ravitoidust jääb kalade poolt puutumata, langeb veekogu põhja ja võib mõjutada setetes ning bentoses elavaid organisme (18). Teadmata kogus humaanmeditsiinis kasutatavate ravimite ülejääkidest arvatakse sattuvat otse kanalisatsiooni (19).

Ravimijääkide säilimine keskkonnas

Raviaine säilimisaeg keskkonnas sõltub tema molekuli ehitusest. Mulla mikroorganismid lagundavad ravimid kas nende orgaanilisteks metaboliitideks või süsihappegaasiks ja veeks. Antibiootikumide all mõistetakse üldiselt mikroobide, seente, loomsete ja taimsete organismide poolt produtseeritavaid aineid, millel on võime pidurdada teiste mikroorganismide elutegevust või nad surmata. Antibiootikumide tootmise võime on organismidel välja kujunenud pikaajalise evolutsiooni tulemusena ja see kujutab endast tähtsat tegurit olelusvõitluses (Tshervjakova, Terezova, 1986). Samas on evolutsiooni tulemusena looduses välja kujunenud ka biodegradatsiooni rajad looduslike antibiootikumide mineraliseerumiseks. Tänapäeval toodetakse ja kasutatakse aga laialdaselt ka sünteetilisi ja poolsünteetilisi antibakteriaalseid aineid, mis on loodusele "võõrad" ja raskesti lagundatavad. Seetõttu püsivad ka näiteks fluorokinoloonid keskkonnas kaua. On tõestatud fluorokinoloonide tugev seostumine sõnniku ja mullaga, mis võib olla nende aeglase degradeerumise täiendavaks põhjuseks (Marengo *et al*., 1997). Enro- ja tsiprofloksatsiini sisaldust sõnnikus ega sõnnikuga väetatud mullas ei ole uuritud. Teatakse, et enrofloksatsiini elimineerimine

loomorganismist toimub 80% ulatuses läbi neerude ja 20% ulatuses sapi kaudu (Crumplin, 1986). Fluorokinoloonide kontsentratsioon uriinis võib ületada 100–300 korda nende kontsentratsiooni seerumis (Montay *et al*., 1984). On alust arvata, et vedela sõnnikuga väetatud põllumajanduslik maa võib saastuda fluorokinoloonidega (Nowara *et al*., 1997).

On püütud arvutuslikult ennustada ravimijääkide kontsentratsioone sõnnikuga väetatud mullas. Sealjuures on arvestatud metaboliitide teket, ekskretsiooni teid ja kiirust, sõnniku kogumise, hoidmise ja põllule laotamise korraldust jne. Kõik need mõjurid ei oma ennustamisel võrdset tähtsust. Lautades peetavate loomade puhul, kui sõnnikus segunevad uriin ja roe ning sõnnikut hoitakse enne põllule laotamist hoidlates, on ekskretsiooni teed ja kiirus väiksema tähtsusega. Karjamaal ravitud loomade ekskrementidega jõuavad ravimijäägid aga otse mulda. Sel juhul on nimetatud faktorid ravimijääkide kontsentratsiooni ennustamisel olulise tähtsusega. Põhiline osa ravimijääkidest jõuab mulda siiski lautadest saadud sõnnikuga. Ennustused põhinevad Taani Keskkonnakaitse Agentuuri andmetel. Sigade ja veiste sõnnik laotatakse põldudele vastavalt 150 kg N / ha ja 265 kg N / ha aastas. Arvutati, kui suur võiks ravimijääkide kontsentratsioon olla 10 cm sügavusel mullas. Väikseim ennustatav kontsentratsioon seasõnnikuga väetamisel saadi hormoonide (oksütoksiin ja vasopressiin) puhul: 0,01–0,05 µg kg mulla kohta, suurim kontsentratsioon aga ravi eesmärgil kasutatavate antibiootikumide puhul: 1–9 µg/g. Sealhulgas arvutati enrofloksatsiini võimalikuks kontsentratsiooniks mullas 3,81 µg/g. Kasvustimulaatorite puhul saadi selliseks kontsentratsiooniks 0,2–1,3 µg/g ning seedetegevust ja ainevahetust reguleerivate ravimite puhul 0,04–5,7 µg/g.

Lehmasõnniku suurema lämmastikusisalduse tõttu olid arvutuslikud ravimijääkide kontsentratsioonid mullas lehmasõnnikuga väetamisel mõnevõrra suuremad kui seasõnniku puhul. Kesknärvisüsteemi ravimite jääke arvutati lehmasõnnikuga väetatud mullas vahemikus 0,09 µg /g (ksülosiin) kuni 28 µg /g (metamisoolnaatrium).

Näitena selle kohta, kuidas neid kontsentratsioone saada, toome enrofloksatsiini sisalduse leidmise seasõnnikuga väetatud mullas (150 kg N / ha aastas):

Looma keskmine eluskaal	100	kg
Enrofloksatsiini annustamine	10	mg/kg
Päevane doos	1	g
Sõnnikutoodang looma kohta päevas	4,8	kg
N-sisaldus päevases sõnnikus	0,03	kg
Vajalik põllupind saadud N kogusele	1,75	m^2
N kontsentratsioon antud põllupinnal	0,57	g/m^2
Enrofloksatsiini kontsentratsioon	3,81	µg/g

Kõik need arvutused on tehtud lähtudes halvimast võimalikust situatsioonist (ekskrementidega eraldub maksimaalne kogus ravimijääke) (Halling-Sørensen *et al*., 2002).

Mõistetavalt on need ennustused ligikaudsed ja vajavad kinnitamist katsetega.

Ravimijäägid taimedes

Ravimijääkide võimalikku liikumist mullast taimedesse on seni vähe uuritud. Kirjanduses on andmed sulfadimetoksiini akumuleerumisest otra. Sulfadimetoksiini sisaldus odra juurtes ja lehtedes määrati kromatograafilise (HPLC) meetodiga. Juurtes oli sulfadimetoksiini ligikaudu 4 korda rohkem kui lehtedes-vartes, vastavalt 79 ja 18 µg/g (sulfadimetoksiini sisaldus kasvumullas 100 µg/g). On tõstatatud küsimus MRL (maksimaalse lubatud sisalduse) kehtestamise vajalikkusest veterinaarravimite jääkidele taimedes (Brambilla *et al.,* 1996).

Käesolevas töös käsitletud enrofloksatsiini ja tsiprofloksatsiini summaarne MRL lihas on 100 µg/kg (Riigi…, 2000). Taimse toidutoorme korral on MRL kehtestatud vaid pestitsiidijääkidele.

Eestis on kvalitatiivse mikrobioloogilise agar-difusioonmeetodiga püütud määrata tsiprofloksatsiini ja sulfadimetoksiini akumuleerumist aedoas, redises ja suvinisus (Aruksaar *et al*., 1999). Tsiprofloksatsiin andis inhibitsioonitsooni ainult redises (pH 8, tsoonide läbimõõdud kasvumulla kontsentratsioonidel 50 ja 100 µg/g vastavalt 12 ja 13 mm). Kasutatud meetod ei näidanud sulfadimetoksiini akumuleerumist üheski taimes. Ometi on kirjanduses näidatud sulfadimetoksiini akumuleerumist rea taimede maapealsetes osades (Brambilla *et al*., 1996; Migliore *et al*., 1995). Et negatiivse tulemuse andis ka sulfadimetoksiiniga segatud muldade mikrobioloogiline analüüs, teevad autorid järelduse, et toodud tulemuste alusel ei saa teha otsust ravimijääkide sisalduse kohta taimedes (Aruksaar *et al*., 1999).

Siit tuleneski vajadus töötada välja spetsiaalselt taimedele ja mullale kohandatud mikrobioloogiline meetod ravimijääkide määramiseks.

Materjalid ja meetodid

Taimekasvatus

Taimede kasvatamine toimus selleks kohandatud ruumis, mida õhutati ja valgustati päevavalguslampidega, imiteerimaks looduslikke tingimusi. Eksperimendiks valiti 3 taime: lehtsalat (*Lactuca sativa*), oder (*Hordeum vulgare L.*) ning harilik kurk (*Cucumis sativus L.*). Taimede seemned külvati plastikpottidesse, milles oli 1 kg väetisevaba ja fluorokinoloonide lahusega segatud mulda. Kaalutud mullakogused segati eelnevalt fluorokinoloonide lahustega plastikaatkotis. 200 ml destilleeritud vees lahustati vajalik kogus enro- või tsiprofloksatsiini ja lahus lisati mullakogustele nii, et antibiootikumi lõppkontsentratsiooniks potis saadi 10, 50, 200 või 500 mg/kg. Iga kontsentratsiooni jaoks võeti kolm paralleelpotti. Kontrolliks kasvatati iga taimeliiki fluorokinoloonidevabas mullas 5 paralleelpotis.

Taimi kasteti läbi potis oleva plastiktoru, mis avanes poti põhjas. Selline kastmine oli vajalik, et vältida fluorokinoloonide uhtmist ülemistest mullakihtidest alumistesse ja säilitada potis võimalikult ühtlane kontsentratsioon. Eksperiment kestis 28 päeva, salati puhul tsiprofloksatsiiniga mullas 42 päeva. Seejärel taimed koristati, eraldati juured, kuivatati ja jahvatati peeneks. Mullaproovid võeti kolme paralleelpoti ülemistest kihtidest ja segati. Kontrollmuldi ja kontrolltaimi kasutati kontrolliks ja spaikimisteks (fluorokinoloonide lisamine etteantud hulga ekstraheerimissegusse), et saada kalibreerimisgraafikuid arvutusteks. Fluorokinoloonidega segatud muldi ja taimi analüüsiti mikrobioloogilisel agar-difusioonmeetodil.

Mikrobioloogiline agar-difusioonmeetod fluorokinoloonide määramiseks mullas ja taimedes. Kromatograafiline (HPLC) meetod

Testkultuurid. *B. subtilis* BGA või ATCC 6633 eosesuspensioon.

Lahused. Trimetoprimi (TMP) vesilahus 100 µg/ml. Tsiprofloksatsiini ja enrofloksatsiini vesilahused spaikimiseks: kontsentratsioon 0,5, 1, 2, 5, 10, 20, 40, 80 µg/ml.

Testsööde. Testagar pH-8 (Merck) valmistatakse vastavalt tootja õpetusele ja autoklaavitakse. Kui söötme temperatuur on langenud 48 kraadini, lisatakse söötmele TMP vesilahust 100 µl 100 ml söötme kohta.

Inokulum. Söötmele lisatakse *B. subtilis'e* spoorisuspensiooni 1 ml 100 ml söötme kohta.

Testsöötme kogus. 6 ml söödet mõõdetakse steriilse pipetiga plastikust Petri tassidesse Ø 90 mm.

Spaikimine mulda. 2 g antibiootikumivaba õhkkuiva kontrollmulda segatakse 10 ml fluorokinolooni lahusega 50ml-lises korgiga plastiktuubis. Fluorokinoloonide lahused tehakse kontsentratsioonidega 0,5; 1; 2; 5; 10; 20; 40 ja 80 µg/ml, et saada spaikmuldades kontsentratsioonid vastavalt 2,5; 5; 10; 25; 50; 100; 200; 400 µg 1 g õhkkuiva mulla kohta. Plastiktuube hoitakse 5 tunni jooksul toatemperatuuril pöörleval vertikaalsel segajal. Seejärel tsentrifuugitakse 30 min 4000 p/min. Supernatant eemaldatakse dekanteerimise teel ja sade kuivatatakse Petri tassidel üle öö. Mullad autoklaavitakse 1 at juures 30 min.

Spaikimine taimedesse. 50 mg kuivatatud ja peeneks jahvatatud antibiootikumivaba taimset materjali segatakse 2 ml-stes korgiga plastiktuubides 1 ml fluorokinolooni lahusega. Spaikimislahused valmistatakse kontsentratsioonidega 0,5; 1; 2; 5, 10 µg/ml. Plastiktuube hoitakse toatemperatuuril pöörleval vertikaalsegajal 3 tundi ja seejärel tsentrifuugitakse 10 min 4000 p/min. Supernatant eemaldatakse Pasteuri pipetiga 1 ml Eppendorfi topsidesse. Sade kuivatatakse õhu käes Petri tassil üle öö.

Proovi võtmine. Analüüsitav taimne materjal kuivatatakse ja peenestatakse. Taimset materjali kaalutakse prooviks 2,5 mg. Autoklaavitud mulda kaalutakse prooviks 5 mg.

Analüüsi käik. Petri tassides tardunud inokuleeritud testsöötmele asetatakse pintsettidega steriilne põhjata metallsilinder Ø 6 mm. Proov puistatakse silindrisse. Silinder eemaldatakse. Iga proov analüüsitakse paralleelselt kahel Petri tassil. Samal testsöötmel analüüsitakse kahe paralleelina ka kontrollproov (0) ja spaigitud proovid. Vedelike (kalibreerimislahused, spaikimise supernatandid) analüüsiks asetatakse testsöötmele steriilsete pintsettidega immutamata paberkettake Ø 6 mm. Steriilse pipetiga mõõdetakse 13,6 µl analüüsitavat vedelikku, millega immutatakse kettake.

Selline kogus on vajalik, et kettake märguks maksimaalselt ning samas ei valguks vedelik üle ketta ääre söötmesse. Petri tassid proovidega asetatakse antibiootikumi difundeerumiseks geeli 22 tunniks külmkappi +4…+6 °C juurde. Seejärel inkubeeritakse proove termostaadis temperatuuril 37 °C 18 tundi. Bakterikasvu inhibitsioonitsooni diameetrid mõõdetakse ning arvutatakse paralleelproovide keskmine väärtus.

Kalibreerimine muldadele. Arvestades, et kogu spaigitud antibiootikum seostub mullaga (adsorptsioon ligi 100%), konstrueeritakse kalibreerimisgraafik teljestikus lg kalibreerimismuldade kontsentratsioonidest (x-telg) ja kalibreerimismuldade inhibitsioonitsoonide diameetrid (y-telg).

Kalibreerimine taimedele. Et taimsesse materjali ei seostu kogu spaigitud antibiootikum, tehakse antud juhul kalibreerimisgraafik seostumise protsenti arvestades. Viimane arvutatakse spaikimiste supernatantide diameetrite järgi graafikult teljestikus lg spaikimislahuste kontsentratsioonist (x) ja inhibitsioonitsooni diameeter (y). Edasi arvutatakse taimega seostumise astme (%) järgi tegelik antibiootikumi kontsentratsioon spaigitud taimedes. Et leida antibiootikumi kontsentratsiooni proovis, konstrueeritakse graafik teljestikus lg antibiootikumi kontsentratsioon spaiktaimes (x) ja spaiktaimede inhibitsioonitsooni diameeter (y).

Arvutused. Antibiootikumi kontsentratsioon proovis arvutatakse kalibreerimisgraafiku võrrandi järgi y=ax+b, kus a ja b on konstandid, x on lg antibiootikumi kontsentratsioonist proovis, y on proovi inhibitsioonitsooni diameeter. Antibiootikumi kontsentratsioon proovis = antilog x.

HPLC-meetod. Flurokinoloonide kromatograafiliseks määramiseks taimedes võeti aluseks L. Migliore poolt (Migliore, 2001) mõneti modifitseeritud kirjanduslik meetod (Palmada *et al*., 2000).

Tulemused ja arutelu

Fluorokinoloonid mullas

Fluorokinoloonide sisalduse määramiseks katsemuldades tehti esmalt kindlaks nende seondumisaste mullaosakeste külge. Selleks tehti spaikimislahused kontsentratsioonidega 10, 20, 40, 80, 120 µg/ml ja viidi antibiootikumivabasse kontrollmulda. Mõõdeti muldade supernatantide ja spaikimislahuste inhibitsioonitsoonide diameetrid. Kalibreerimisgraafiku järgi arvutati enrofloksatsiini ja tsiprofloksatsiini kontsentratsioon supernatantides ja viimase alusel nende seondumise aste (%) mullaga. Kui spaikimismulla supernatant bakterikasvu inhibitsiooni ei andnud, siis arvestati, et kogu spaigitud antibiootikum on seondunud kontrollmullaga.

Enrofloksatsiini spaikimislahuste kontsentratsioonidel 20, 40, 80, 120 µg/ml oli enrofloksatsiini seondumine mullaga vastavalt 99,6; 99,5; 99,5; 99,4%. Kontsentratsioonil 10 µg/ml enrofloksatsiini spaikimislahuse supernatant inhibitsioonitsooni ei andnud ja seondumisastmeks loeti siin 100%. Tulemus on kooskõlas kirjanduse andmetega, kus enrofloksatsiini adsorptsioon mullale pH 5,9 juures oli 99,5%. Meie katses kasutati mulda, mille pH oli 6,0.

Tsiprofloksatsiini puhul oli mullaga seondumise aste spaikimislahuste kontsentratsioonidel 10, 20, 40, 80 ja 120 µg/ml vastavalt 100; 100; 99,9; 99,6 ja 99,5%.

Mida nõrgem oli fluorokinoloonide spaikimislahuste kontsentratsioon, seda suurem oli nende mullaga seondumise aste. Üldiselt võib öelda, et seondumisaste lähenes 100 protsendile kõigil kontsentratsioonidel. Seetõttu võeti eksperimentaalmuldade fluorokinoloonide sisalduse arvutamisel aluseks spaikmuldade kalibreerimisgraafik, arvestades, et kogu spaigitud antibiootikum oli ka mullaga seostunud.

Metoodika väljatöötamisel arvutati ka agar-difusioonmeetodi katseviga. Selleks analüüsiti tsiprofloksatsiiniga mulda sama testsöötmega täidetud 10 erineval Petri tassil. Katseviga oli seda väiksem, mida väiksem oli mulla antibiootikumisisaldus. Mulla tsiprofloksatsiini algkontsentratsioonide 500, 200, 50 ja 10 µg/g korral oli suurim hälve mõõdetud inhibitsioonitsooni diameetrite vahel vastavalt ±2; 2; 1,5 ja 1 mm. Sellest lähtudes ei saa mikrobioloogilise agar-difusioonmeetodiga määratud fluorokinoloonide sisaldused olla väga täpsed. Kontsentratsiooni täpsustamiseks on vajalik kromatograafiline analüüs.

Mullaproovid võeti taimekasvatuseksperimendi 2., 14. ja 28. päeval. Nagu selgub tabelitest 1 ja 2, fluorokinoloonide kontsentratsioon mullas katse jooksul oluliselt ei muutu. Võrreldes enrofloksatsiini sisaldusi katsemuldades 2. ja 28. päeval, selgub, et enamasti see katse lõpuks veidi tõuseb, mõnel juhul jääb samaks, kuid kontsentratsioonide erinevus jääb üldjuhul katsevea piiresse (tabel 1). Kolme päeva proovide keskmine määratud antibiootikumisisaldus võib olla suurem kui kasvumulla nominaalkontsentratsioon, ent ka see vahe jääb katsevea piiresse (tabel 3 ja 4). Tsiprofloksatsiini sisaldus katsemuldades eksperimendi lõpuks väheneb (tabel 2). Lõpp-järeldusi teha oleks aga ennatlik, sest tsiprofloksatsiini sisalduse vahe eksperimendi alguses ja lõpus võetud

proovides jääb jällegi katsevea piiridesse. Võrreldes katsemuldade algkontsentratsioone eksperimendi 3 päeva keskmiste kontsentratsioonidega, võib öelda, et mulda lisatud fluorokinoloonide antimikroobne aktiivsus püsis suhteliselt muutumatuna 28 päeva jooksul (tabel 3).

Kirjanduse andmetel metaboliseerub enrofloksatsiin osaliselt tsiprofloksatsiiniks nii looma organismis (Mengozzi *et al*., 1996) kui ka mullas (Nekrassova, 2001). Oletatakse, et tsiprofloksatsiini juuresolekul enrofloksatsiini antimikroobne aktiivsus võimendub – ilmneb sünergism (Mengozzi *et al*., 1996). Et seda mikrobioloogilise agar-difusioonmeetodiga näidata, oleks ilmselt vajalik pikem katseaeg. Küll aga kinnitavad meie eksperimendi tulemused kirjanduses mainitud fluorokinoloonide stabiilsust mullas (Golet *et al*., 2002).

Tabel 1. Enrofloksatsiini sisaldus mullas eksperimendi 2., 14. ja 28. päeval
Table 1. *Concentration of Enrofloxacin in Soil on the 2., 14. and 28. Day of the Experiment*

Muld *Soil*	Algkonts. *Initial conc*., µg/g	i diameeter / *Inhib. zone diam*. (mm)			Enrofloksatsiini konts. / *Enrofloxacin conc*. (µg/g)		
		2. päev *2nd day*	14. päev *14th day*	28. päev *28th day*	2. päev *2nd day*	14. päev *14th day*	28. päev *28th day*
Oder *Barley*	500	41,5	41,5	42	487	487	520
	200	34,5	35	35	193	206	206
	50	24	25	25,5	48	55	59
	10	12,5	14,25	14,5	11	13	14
Salat *Lettuce*	500	41	42	42,5	455	520	555
	200	35	37	37	206	268	268
	50	25,25	26,25	26	57	65	63
	10	11,5	12	12	9	10	10
Kurk *Cucumber*	500	41,75	42	41,75	503	520	503
	200	36	35,5	36	235	220	235
	50	23,5	25	24,5	45	55	52
	10	12	12	12,5	10	10	11

* määratud enrofloksatsiini kontsentratsioonid on ümardatud täisarvuni / *conc. of estimated enrofloxacin is rounded*
* spaikimine ja kalibreerimine tabelis 3 / *spiking and calibration in table 3*

Tabel 2. Tsiprofloksatsiini sisaldus mullas eksperimendi 2., 14. ja 28. päeval
Table 2. *Concentration of Ciprofloxacin in Soil on the 2., 14. and 28. Day of the Experiment*

Muld Soil	Algkonts. *Initial conc*., µg/g	i diameeter / *Inhib.zone diam*. (mm)			Tsiprofloksatsiini konts. / *Ciprofloxacin conc*. (µg/g)		
		2. päev *2nd day*	14. päev *14th day*	28. päev *28th day*	2. päev *2nd day*	14. päev *14th day*	28. päev *28th day*
Oder Barley	500	37	36	35,75	567	494	477
	200	31,5	31	30,5	266	248	231
	50	21	21	20	62	62	54
	10	8	7,5	7,5	10	10	10
Salat Lettuce	500	36,75	36,5	36,5	548	529	529
	200	32	31,5	31	284	266	248
	50	21,75	21	20,5	69	62	58
	10	8,5	8,25	8	11	11	10
Kurk Cucumber	500	37,75	37	36,5	629	567	529
	200	31	31	30,5	248	248	231
	50	21	20	19	62	54	47
	10	8,5	8	8	11	10	10

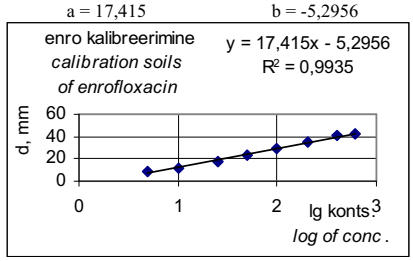
*määratud tsiprofloksatsiini kontsentratsioonid on ümardatud täisarvuni /*conc.of estimated ciprofloxacin is rounded*
* spaikimine ja kalibreerimine tabelis 3 / *spiking and calibration in table 3*

Tabel 3a. Enro spaikmullad
Table 3a. *Calibration soils of enrofloxacin*

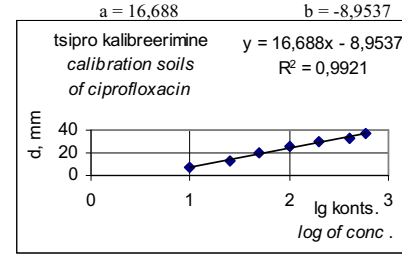
Konts. / Conc., µkg/g	lg konts. log of conc.	i tsoon / i zone, mm
5	0,699	8,5
10	1	12
25	1,398	17,25
50	1,699	23,5
100	2	29,25
200	2,301	35,25
400	2,602	41
600	2,778	43

Tabel 3b. Tsipro spaikmullad
Table 3b. *Calibration soils of ciprofloxacin*

Konts. / Conc., µkg/g	lg konts. log of conc.	i tsoon / i zone, mm
5	0,699	
10	1	7,5
25	1,398	13,25
50	1,699	20
100	2	26
200	2,301	30
400	2,602	33,5
600	2,778	37



Joonis 2a. Enro kalibreerimisgraafik
Figure 2a. *Calibration curve of enrofloxacin*



Joonis 2b. Tsipro kalibreerimisgraafik
Figure 2b. *Calibration curve of ciprofloxacin*

Tabel 4. Fluorokinoloonide sisaldus mullas. Kolmel päeval võetud proovide keskmine
Table 4. *Content of fluoroquinolones in soil. Average of three measurements*

Muld *Soil*	Algkonts. *Initial conc.,* µkg/g	Enrofloksatsiin / Enrofloxacin			Tsiprofloksatsiin / Ciprofloxacin		
		i d, mm *d i, mm*	lg konts. *log of conc.*	konts. / conc., µkg/g	i d, mm *d i, mm*	lg konts. *log of conc.*	konts. / conc., µkg/g
Oder	500	41,7	2,698570	500	36,25	2,708754	511
Barley	200	34,8	2,302360	201	31	2,394157	248
	50	24,8	1,728142	53	20,7	1,776947	60
	10	13,75	1,093631	12	7,7	0,997944	10
Salat	500	41,8	2,704312	506	36,6	2,729727	537
Lettuce	200	36	2,371266	235	31,5	2,424119	266
	50	25,8	1,785564	61	21,1	1,800916	63
	10	11,8	0,981659	10	8,25	1,030902	11
Kurk	500	41,8	2,704312	506	37,1	2,759689	575
Cucumber	200	35,8	2,359781	229	30,8	2,382172	241
	50	24,3	1,699431	50	20	1,735001	54
	10	12,2	1,004628	10	8,2	1,027906	11

* i d / d i - inhibitsioonitsooni diameeter / *diameter of inhibition zone*
* määratud kontsentratsioonid on ümardatud täisarvuni / *conc. of estimated fluoroquinolones are rounded*

Fluorokinoloonid taimedes

Fluorokinoloonide sisalduse leidmiseks katsetaimedes arvestati nagu mulla puhulgi antibiootikumi seondumise astet spaikimisel. Spaikimislahused kontsentratsioonidega 0,5; 1; 2; 5 ja 10 µg/ml segati kuivatatud taimse materjaliga metoodikas kirjeldatud viisil. Fluorokinoloonide seondumisaste taimse materjaliga arvutati samuti nagu nende seondusmisaste mullaga. Fluorokinoloonide seondumisaste (%) taimega olenes taime liigist ja spaikimislahuse kontsentratsioonist. Nagu mulla puhulgi oli fluorokinolooni seondumisaste seda kõrgem, mida madalam oli spaikimislahuse kontsentratsioon. Enrofloksatsiini seondumisaste salatiga oli olenevalt spaikimislahuse kontsentratsioonist 64–100%, odraga 28–100%, kurgiga 23–100%. Tsiprofloksatsiini seondumisaste salatiga oli

analoogselt 90–100%, odraga 64–100% ja kurgiga 70–100%. Taimega seondumise astme järgi arvutati fluorokinolooni tegelik kontsentratsioon spaiktaimes ja selle järgi koostati kalibreerimisgraafik.

Eksperimendi tulemustest nähtub, et fluorokinoloonid tõepoolest tõusevad koos mullaveega taimedesse ja akumuleeruvad seal, säilitades oma antimikroobse aktiivsuse. Sama sisalduse korral mullas oli enrofloksatsiini sisaldus katsetaimedes üldjuhul suurem kui tsiprofloksatsiini sisaldus. Taimedest oli fluorokinoloonide sisaldus suurim salatis ja väikseim kurgis (tabel 5). Kõrge tsiprofloksatsiini kontsentratsioon salati puhul võib olla tingitud sellest, et viimast kasvatati tsiprofloksatsiiniga segatud mullas 14 päeva kauem kui teisi taimi. Fluorokinoloonide nominaalkontsentratsioonil 10 µg/g kasvanud taimedest ei andnud inhibitsioonitsooni ükski, välja arvatud salat, mida oli kasvatatud tsiprofloksatsiini juuresolekul kauem. Oder ja kurk andsid inhibitsioonitsooni ainult kõige kõrgemal nominaalkontsentratsioonil mullas. Märkimisväärne on see, et salatis, mis oli kasvanud mulla tsiprofloksatsiini nominaalkontsentratsioonil 10 µg/g, määrati nominaalsest üle 4 korra suurem tsiprofloksatsiini sisaldus. Inhibitsioonitsooni diameeter oli küll väikseim, mida on võimalik mõõta – 7 mm (analüüsitav materjal söötmel 6 mm) –, kuid spaiksalat ei andnud kontsentratsioonidel 10 ja 20 µg/g üldse bakterikasvu inhibitsiooni, seega on tulemus usaldatav. Tulemus näitab, et pikema kasvuaja puhul võib antibiootikum taimes ka kontsentreeruda.

Tabel 5. Fluorokinoloonide sisaldus taimedes
Table 5. *Concentration of Fluoroquinolones in Plants*

Taim / *Plant*	Algkonts. mullas *Initial conc. in soil,* µg/g	Enrofloksatsiin / *Enrofloxacin*		Tsiprofloksatsiin / *Ciprofloxacin*	
		i d, mm	konts. / *conc.,* µg/g	i d, mm	konts. / *conc.,* µg/g
Oder / B*arley*	500	20	20	11,5	13
	200	15	14	–	–
	50	7	8	–	–
	10	–	–	–	–
Salat / L*ettuce*	500	25	76	16	223
	200	18	27	14,25	163
	50	9	7	8,5	58
	10	–	–	7	44
Kurk / *Cucumber*	500	21	36	13	40
	200	16	22	–	–
	50	7	9	–	–
	10	–	–	–	–

*i d – inhibitsioonitsooni diameeter mm / *diameter of inhibition zone*

Kromatograafiline võrdlusanalüüs HPLC-meetodil

Et kontrollida akumuleerunud fluorokinoloonide säilumist katsetaimedes, tehti 10 kuud pärast taimede kogumist kromatograafiline võrdlusanalüüs. Selleks valiti salat, mis oli kasvanud enrofloksatsiini juuresolekul. Kromatograafiline meetod võimaldab välja selgitada, kas enrofloksatsiin taimes on osaliselt metaboliseerunud tsiprofloksatsiiniks, samuti määrata nende kahe fluorokinolooni summaarset sisaldust taimes. Kuivatatud taimset materjali säilitati kinnistes plastiktuubides pimedas ruumis toatemperatuuril 10 kuud.

Analüüsiks võeti mulla kõikidel enrofloksatsiini nominaalkontsentratsioonidel kasvanud salatitaimed. Kromatogrammid näitasid, et enrofloksatsiin oli säilinud kõikides proovides ja osaliselt metaboliseerunud tsiprofloksatsiiniks. Enro- ja tsiprofloksatsiini summaarne sisaldus salatis HPLC-meetodi järgi oli algkontsentratsioonidel 500, 200, 50 ja 10 µg/g vastavalt 12,7; 3; 1 ja 0,37 µg/g (tabel 6). Saadud tulemusi võrreldi mikrobioloogilise meetodiga määratud enrofloksatsiini sisaldustega salatis. Mikrobioloogiline meetod saab näidata ainult antibakteriaalsete ainete summaarset sisaldust uuritavas materjalis. Kromatograafia näitas, et enrofloksatsiini juuresolekul kasvanud salat sisaldas ka tsiprofloksatsiini. Kas nimetatud metaboliit oli tekkinud taimes või juba eelnevalt mullas, on ilma täiendavate uuringuteta raske öelda. Mikrobioloogilise meetodiga määratud enrofloksatsiini sisaldus salatis oli mitu korda suurem, kui kromatograafilise meetodiga saadud kontsentratsioonid. Suure tõenäoliselt fluorokinoloonid taimses materjalis 10 kuu jooksul osaliselt lagunevad.

Mikrobioloogilise meetodiga vahetult pärast eksperimendi lõppu määratud suured fluorokinoloonide sisaldused võivad viidata ka teiste antimikroobselt aktiivsete metaboliitide tekkele. Ka kirjanduses on andmeid, et

loomorganismis võib enrofloksatsiinist peale tsiprofloksatsiini tekkida ka seni tundmata metaboliite (Küng *et al*., 1993). Viimased kas lagunevad hiljem või on siis kromatograafiliselt määratavad teistel lainepikkustel.

Tabel 6. Fluorokinoloonide sisaldus salatis HPLC-meetodi järgi
Table 6. *Concentration of Fluoroquinolones in* Lettuce *by HPLC Method*

Enrofloksatsiini algkonts. mullas, µg/g *initial conc. of enrofloxacin in soil,* µg/g	Enrofloksatsiini konts., µg/g *enrofloxacin conc.*, µg/g	Tsiprofloksatsiini konts., µg/g *ciprofloxacin conc.*, µg/g	Enro + tsipro konts., µg/g *conc.enrofloxacin + ciprofoxacin,* µg/g	Tekkinud tsipro, % *ciprofloxacin formed,* %
500	10,8	1,9	12,7	15
200	2,4	0,6	3	20
50	0,8	0,2	1	20
10	0,3	0,07	0,37	19

Kromatograafiline meetod näitab fluorokinoloonide sisaldust salatis ka väikseimal mulla enrofloksatsiini algkontsentratsioonil. Kontsentratsioon 0,37 µg /g jääb alla mikrobioloogilise meetodi määramispiiri. Seetõttu sobib fluorokinoloonide väga väikeste sisalduste määramiseks ainult kromatograafiline meetod. Mikrobioloogilise meetodi eelis on see, et ta on lihtsam, odavam ja näitab ära enrofloksatsiini ning tema tuntud ja tundmatute metaboliitide summarse antimikroobse aktiivsuse.

Järeldused

Eksperimendi tulemused näitavad, et pikema kasvuaja jooksul võib salatisse akumuleeruda küllalt palju ühel või teisel viisil mulda sattunud antibiootikumi. Võimalik, et ka teistes taimedes oleks fluorokinoloonide sisaldus pikema kasvuaja järel suurem. Kui toetuda kirjanduse andmetele (Halling-Sørensen *et al*., 2002), võiks enrofloksatsiini ja tema metaboliitide summaarne sisaldus sõnnikuga väetatud mullas olla halvimal juhul 3,8 µg/g. Mikrobioloogilise meetodiga määrati mulla tsiprofloksatsiini kontsentratsioonil 10 µg/g kasvanud salatis tsiprofloksatsiini sisalduseks 44 µg/g. See on taimes üle 4 korra suurem, kui oli kasvumullas. Et enrofloksatsiini sisaldus oli taimedes üldiselt suurem, võib arvata, et salati pikema kasvuaja jooksul enrofloksatsiiniga mullas see isegi ületaks tsiprofloksatsiini sisalduse. Lihtne arvutus näitab, et kui mulla tsiprofloksatsiini kontsentratsioonil 10 µg/g jõudis taimesse 42 päeva jooksul fluorokinolooni 44 µg/g, siis mulla kontsentratsioonil 3,8 µg/g võiks taimesse jõuda sama kontsentreerumisastme korral ligikaudu 15 µg/g. Selline kontsentratsioon peaks olema määratav ka lihtsa mikrobioloogilise agar-difusioonmeetodiga.

Loomses toidutoormes on enro- ja tsiprofloksatsiini summaarse sisalduse lubatud piirnorm MRL 100 µg/kg ehk 0,1 µg/g. 15 µg/g taimse materjali kohta ületaks selle väärtuse mitmekordselt. Ka 10 kuu pärast kromatograafilise meetodiga määratud fluorokinoloonide summaarne sisaldus 0,37 µg/g on üle piirnormi. Veelgi pikema kasvuaja puhul võiks fluorokinolooni taimes olla veelgi suuremas koguses. Itaalias on tõstatatud küsimus MRLi vajadusest veterinaarravimite jääkidele taimedes (Brambilla *et al*., 1996). Võib-olla tasuks selle üle mõelda ka Eestis? Oleks vaja uurida ravimijääkide sisaldust sõnnikuga väetatud mullas ja pikema kasvuperioodiga taimedes. Ravimijääke võib olla rohkem juurviljades ja köögiviljades, mille söödav osa on mullas (Brambilla *et al*., 1996). Inimesele ohtlik võiks olla ravimijääkide sisaldus kartulis, sest kartuli kasvuaeg on pikk, söödav osa on mullas ja kartulit süüakse Eestis keskmiselt rohkem kui teisi toidutaimi. Igapäevatoiduga väikestes kogustes pidevalt omastatavad fluorokinoloonid võiksid põhjustada resistentsete bakteritüvede tekkimist inimorganismis.

Kokkuvõte

Käesolevas töö uuriti fluorokinoloonide säilumist mullas ja nende akumuleerumist toidutaimedesse. Eksperimendiks valiti 3 taime: lehtsalat, oder ja harilik kurk, mida kasvatati laboratooriumis enro- või tsiprofloksatsiiniga segatud mullas nominaalkontsentratsioonidel 500, 200, 50 ja 10 µg/g. Fluorokinoloonide sisalduse määramiseks mullas ja taimedes töötati välja mikrobioloogiline agar-difusioonmeetod. Testorganismiks oli *Bacillus subtilis.* Eksperiment kestis 28 päeva, salati puhul tsiprofloksatsiiniga mullas 42 päeva. Põhitulemused on järgmised.

- Mullas püsis fluorokinoloonide kontsentratsioon eksperimendi jooksul muutumatuna.

- Enrofloksatsiini sisaldus tuvastati kõigis mulla enrofloksatsiini kontsentratsioonidel 500, 200 ja 50 µg/g kasvanud taimedes. Enrofloksatsiini algkontsentratsioonil 10 µg/g kasvanud taimedes mikrobioloogiline meetod fluorokinolooni sisaldumist ei näidanud.
- Tsiprofloksatsiini sisaldumine tuvastati odras ja kurgis ainult mulla algkontsentratsioonil 500 µg/g.
- Kauem kasvatatud salatis leiti tsiprofloksatsiini kõikidel mulla algkontsentratsioonidel. Mulla nominaalkontsentratsioonil 10 µg/g oli tsiprofloksatsiini sisaldus suurem kui kasvumullas – 44 µg/g. Seega võivad fluorokinoloonid pikema kasvuaja jooksul taimes kontsentreeruda.
- Kromatograafilise HPLC-meetodiga kontrolliti 10 kuu pärast fluorokinoloonide sisaldust salatis, mis oli kasvanud enrofloksatsiiniga segatud mullas. Osaliselt oli enrofloksatsiin metaboliseerunud tsiprofloksatsiiniks. Fluorokinoloonide summaarne sisaldus salatis oli väiksem kui vahetult pärast eksperimendi lõppu mikrobioloogilise meetodiga määratud sisaldus. Järeldatakse, et 10 kuu jooksul taimesse akumuleerunud fluorokinoloonid osaliselt lagunevad. Kromatograafiline meetod näitas enrofloksatsiini ja tsiprofloksatsiini sisaldust salatis ka kõige madalamal mulla algkontsentratsioonil 10 µg/g, kus mikrobioloogiline meetod ei näidanud: summaarne fluorokinoloonide sisaldus salatis 0,37 µg/g oli suurem kui fluorokinoloonide lubatud maksimaalne sisaldus loomses toidutoormes – 0,1 µg/g.
- Arutluse alla tuleks võtta ravimijääkide piirnormi vajalikkus toidutaimedes.
- Väljatöötatud mikrobioloogiline agar-difusioonmeetod fluorokinoloonide määramiseks mullas ja taimedes sobib kohandamiseks ka teistele ravimijääkidele.

Kirjandus

Aruksaar, A., Püssa, T., Kuldkepp, P., Ivask, M. Antibakteriaalsete veterinaarravimite mõju taimede kasvule ning ravimijääkide akumuleerumine taimedes. – Akadeemilise Põllumajanduse Seltsi Toimetised, nr 9, lk 5–8, 1999.

Brambilla, G., Casoria, P., Civitareale, C., Cozzolino, S., Gaudio, L., Migliore, L. Sulphadimetoxine as Environmental Tracer to Evaluate Phytotoxity in Crop. – Conference on Residues of Veterinary Drugs in Food, Proceedings, p. 292–295, Veldhoven, 6–8 May, 1996.

Crumplin, G. C. The Mechanism of Action of Quinolones, in: Quinolones – Their Future in Clinical Practice. – Int. Congr. Symp. Ser – R. Soc. Med., No. 104, p. 1–15, 1986.

Golet, E. M., Strehler, A., Alder, A. C., Giger, W. Determination of Fluoroquinolone Antibacterial Agents in Sewage Sludge and Sludge-Treated Soil Using Accelerated Solvent Extraction Followed by Solid-Phase Extraction. – Anal. Chem., No. 74, p. 5455–5462, 2002.

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P. F., Ingerslev, F., Holten Lützhoft, H. C., Jørgensen, S. E. Occurrence, Fate and Effects of Pharmaceuticals in the Environment. – Chemosphere, No. 36 (2), p. 357–393, 1998.

Halling-Sørensen, B., Nors Nielsen, S., Jensen, J. Environmental Assessment of Veterinary Medicinal Products in Denmark. – Environmental Project, No. 659, p. 18–34, 2002.

Küng, K., Riond, J. L., Wanner, M. Pharmacokinetics of Enrofloxacin and its Metabolite Ciprofloxacin after Intravenous and Oral Administration of Enrofloxacin in Dogs. – J. Vet. Pharmacol. Ther., No. 16, p. 462–468, 1993.

Marengo, J. R., Kok, R. A., O´Brien, G. K., Velagaletti, R. R., Stamm, J. L. Aerobic Degradation of 14C-Sarafloxacin Hydrochloride in Soil. – Environm. Toxicol. and Chemistry, No. 16 (3), p. 462–471, 1997.

Mengozzi, G., Intorre, L., Bertini, S. Pharmacokinetics of Enrofloxacin and its Metabolite Ciprofloxacin After Intravenous and Intramuscular Administrations in Sheep. – Am. J. Vet. Res., No. 57 (7), p. 1040–1043, 1996.

Migliore, L., Brambilla, G., Cozzolino, S., Gaudio, L. Effects on Plants of Sulphadimethoxine Used in Intensive Farming *(Panicum miliaceum, Pisum sativum* and *Zea mays)* – Agricult. Ecosyst. Environm., No. 52., p. 103–110, 1995.

Migliore, L. Method for Enrofloxacin Determination in Plants (modification from Palmada *et al*., 2000). – Via della Ricerca Scientifica, Roma, 2001.

Montay, G., Goueffon, Y., Roquet, F. Adsorption, Distribution, Metabolic Fate and Elimination of Pefloxacin Mesylate in Mice, Rats, Dogs, Monkeys and Humans. – Antimicrob. Agents and Chemother, No. 25, p. 463–472, 1984.

Nekrassova, O. Chromatographic Methods for Determination of Some Antibiotics from Soil. – MSc Thesis, Environmental Protection Institute of EAU, Tartu, 2001.

Nowara, A., Burhenne, J., Spiteller, M. Binding of Fluoroquinolone Carboxylic Acid Derivates to Clay Minerals. – J. Agric. Food Chem., No. 45, p. 1459–1463, 1997.

Palmada, J., March, R., Torroella, E., Espigol, C., Baleri, T. Determination of Enrofloxacin and its Active Metabolite (Ciprofloxacin) at the Residue Level in Broiler Muscle Using HPLC with Fluorescence Detection. – References of Euroresidue IV, p. 822–826, Veldhoven, 8–10 May, 2000.

Riigi Teataja I, 6, 38. Toidus lubatud saasteainete loetelu ja piirnormide toidugruppide kaupa kehtestamine. – VVm nr 14, lisa 3, 2000.

Stuer-Lauridsen, F., Birkved, M., Hansen, L. P., Holten Lützhoff H-C., Halling-Sørensen, B. Environmental Risk Assessment of Human Pharmaceuticals in Denmark After Normal Therapeutic Use. – Chemosphere, No. 40, p. 783–793, 2000.

Stumpf, M., Ternes, T. A., Wilken, R-D., Rodrigues, S. V., Baumann, W. Polar Drug Residues in Sevage and Natural Waters in the State of Rio de Janeiro, Brazil. – Sci. Tot. Environ., No. 225, p. 135–141, 1999.

Tshervjakova, V. Terezova, A. Farmakoloogia ja retseptuur. – Tallinn, "Valgus" 1986, lk. 98–112.

Drug Residues in Environment. Estimation of Fluoroquinolones in Soil and Food Plants

M. Lillenberg, M. Roasto, T. Püssa

Summary

Drugs as an essential part of modern human and veterinary medicine are absorbed, distributed, metabolised and finally excreted from organism. After excretion drugs and their metabolites can contaminate the environment. Residues of pharmaceuticals used in human medicine occur in water upon passing sewage treatment plants. Veterinary drugs can enter the environment directly by slurry and manure used as fertilizers. Medical substances used on fish farms will be exposed directly to the receiving waters (Halling – Sørensen *et al*., 2002). Very little is still known about the actual fate of drugs in the environment. The medical substances have many of the necessary properties to bioaccumulate and provoke effects both in the aquatic and terrestrial ecosystems. Veterinary drugs can be uptaken by plants growing in soil fertilised with manure. In such way drug residues can finally reach human as well as animal food.

Fluoroquinolones are broad spectrum synthetic antibiotics used both in human and veterinary medicine. In order to restrict the development of cross resistance the fluoroquinolones used in human medicine are not generally used in veterinary practice and *vice versa.* There still exists a couple of very closely related fluoroquinolones – enrofloxacin and ciprofloxacin. The first one is widely used in veterinary medicine and the second one is licensed for human medicine. Ciprofloxacin is the main metabolite of enrofloxacin in animal organism After excretion enrofloxacin and its metabolites occure with manure in soil. It has been shown that both fluoroquinolones are strongly adsorbed to soil and stable for a long time. Fluoroquinolones may be able to move with soil water into food plants and accumulate there. These tiny amounts of broad spectrum antibiotics in everyday food may generate the strains of resistant microorganisms in human and animal organism. Bacterial resistance in turn is the well known painful problem in chemotheraphy. Today nothing is known about accumulation of fluoroquinolones in plants, therefore method of quantitative assay of these compounds was needed.

Content of two fluoroquinolones – enrofloxacin and ciprofloxacin – was studied in soil and in three food plants: barley *(Hordeum vulgare),* lettuce *(Lactuca sativa)* and cucumber *(Cucumis sativus)* cultivated in this soil. The plants were grown in plastic pots, filled with soil amended with either enrofloxacin or ciprofloxacin (initial concentrations of both 500, 200, 50 or 10 μg/g soil). Experiment lasted from seeding to harvesting 28 days, only in case of lettuce in presence of ciprofloxacin longer – 42 days.

Microbiological agar-diffusion method was elaborated for estimation of the concentration of fluoroquinolones both in soil and plants. In the role of test organism the fluoroquinolone sensitive bacterium *Bacillus subtilis* was used.

Microbiological Agar-Diffusion Method for Estimation of Fluoroquinolones in Soil and Plants

Testbacterium. *B. subtilis* BGA or ATCC 6633 spore suspension.

Solutions. Water solution of trimethoprim 100 μg/ml (TMP), water solutions of enrofloxacin and ciprofloxacin 0.5; 1; 2; 5; 10; 20; 40 and 80 μg/ml.

Test medium. Test agar pH 8 from Merck, prepare according to instructions of producer and sterilise in autoclave 50 min under 1 bar. After decreasing the temperature of test agar to 48 °C, add the solution of TMP 100 μl/100ml.

Inoculum. Add spore suspension of *B. subtilis* 1ml/100ml.

Test amount. Fill Petri dishis Ø 90 mm with 6 ml of inoculated test agar.

Spiking of soil. In order to prepare calibration soils, mix 2 g of air-dry drug-free plant growth soil with 10 ml of fluoroquinolone solution in 50 ml plastic centrifuge tubes by end-over-end method during 5 hours at room temperature. Centrifuge tubes at 4000 rpm during 30 min. Remove supernatants by decantation. Dry sediments on plastic Petri dishes at room temperature overnight. Solutions of fluoroquinolones with concentrations of 0.5; 1; 2; 5; 10; 20; 40 and 80 µg/ml will give the calibration soils with concentrations of 2.5; 5; 10; 25; 50; 100; 200 and 400 µg/g respectively. Sterilise soils in autoclave 30 min. under 1 bar.

Spiking of plants. In order to prepare the calibration plants, mix 50 mg of dried and ground leaves and stems of 3 different plants grown in fluoroquinolone-free soil with 1 ml of following antibiotic solutions in water: 0.5; 1; 2; 5 and 10 µg/ml in 2ml plastic tubes. Rotate tubes end-over-end during 3 hours at room temperature and centrifuge 4000 rpm 10 min. Remove supernatants by a plastic Pasteur pipette into 1 ml Eppendorf tubes. Dry sediments on plastic Petri dishes at room temperature overnight.

Samples. 2,5 mg ground dry plant material or 5 mg autoclaved soil.

Microbial inhibition test. Inoculated culture medium is ready for test after gelifying during 30 min. on Petri dish. Put the stainless steel cylinder Ø 6 mm on the gel. Pour 2.5 mg plant material or 5 mg soil on the gel through cylinder. Remove cylinder. In case of solutions of antibiotics or spiking supernatants dip 13.6 µl of the solution on 6 mm blank paper disk. Test on the same gel control samples and spiked samples. Test all samples in two parallels. Keep Petri dishes with samples in refrigerator at 4–6 °C during 22 hours for pre-diffusion and thereafter incubate during 18 hours at 37 °C. The inhibition circle of microbial growth will appear. Measure the diameter of the inhibition circle. Take the average of two parallel samples.

Calibration soils. The adsorption rate of fluoroquinolones to soil is close to 100%. Use measured avarage diameters of spiked soils for constructing the calibration curves in axes: log from antibiotic concentrations (x) and diameter of inhibition circles (y) of spiked soils.

Calibration plants. The adsorption rate of fluoroquinolones to plants is less than 100%. It depends on plant and concentration of spiking solution. Find out the rate of adsorption. Analyse spiking solutions and supernatants by microbial inhibition test. Construct the calibration curve in axes: log from concentrations (x) and diameter of inhibition circles (y) of spiking solutions. The concentration of antibiotic in supernatant shows the adsorption rate. If supernatant gives the inhibition circle, find the concentration of antibiotic in supernatant using the calibration curve. Calculate the % of adsorption of antibiotic to plant material and concentration of antibiotic in spiked plants. If the supernatant does´nt give the inhibition circle, all the added antibiotic is adsorbed by plant material – the adsorption rate is 100%. Construct the calibration curve in axis: log from calculated concentration of antibiotic in spiked plants (x) and diameter of inhibition circle of spiked plants. To find out the concentration of antibiotic in plant samples use last calibration curve, different for every plant.

Calculations. Calculate the concentration of antibiotic in soil and plant samples using equation of calibration curve $y = ax+b$, where a and b are constants, x = log from concentration of antibiotic in sample and y = diameter of inhibition circle of sample. Concentration of antibiotic in sample = 10^{x}.

HPLC method. For estimation of fluoroquinolones in plants by HPLC use method of J. Palmada (Palmada *et al*., 2000), modified by L. Migliore (Migliore, 2001).

Results and conclusion

The content of fluoroquinolones in soil didn´t change remarkably during the experiment time. The difference of concentrations was in limits of error (in concentrations 500, 200, 50 and 10 µg/g error in diameter of inhibition circles was ± 2; 2; 1.5 and 1 mm, respectively) (tables 1, 2). The averages of drug concentrations of three soil samples taken on the 2., 14. and 28. day of experiment were close to nominal drug concentrations in soil (table 3, 4).

Making use of agar-diffusion method the accumulation of fluoroquinolones in all plants grown at highest drug nominal concentration (500 μg/g) was approved. Enrofloxacin was discovered in all plants grown at drug nominal concentrations 500, 200 and 50 μg/g). At the lowest nominal concentration (10 μg/g) the presence of enrofloxacin in plants was not detected. Ciprofloxacin in barley and cucumber was discovered only in plants grown at drug nominal concentration 500 μg/g. In general, enrofloxacin accumulated in plants better than ciprofloxacin. In lettuce, cultivated during 42 days, ciprofloxacin was found at all nominal drug concentrations. The level of ciprofloxacin in plant (44 μg/g) was considerably higher, than nominal concentration of ciprofloxacin in soil (10 μg/g). It means that ciprofloxacin is accumulated and concentrated by lettuce (table 5).

The presence of enrofloxacin in lettuce leaves kept in dried state during 10 months was studied making use of chromatographic (HPLC) method. The chromatograms showed that beside enrofloxacin lettuce contained also ciprofloxacin (table 6). The agar-diffusion method applied directly after finishing of plant growing experiment revealed higher contents of enrofloxacin than the sum of contents of enro and ciprofloxacin determined by HPLC method.

It can be concluded, that during time both enro- and ciprofloxacin undergo partial degradation in the plant material. It is supposed, that beside ciprofloxacin other antibacterially active metabolites of enrofloxacin are formed in lettuce. After 10 months, the sum of enro- and ciprofloxacin in lettuce, cultivated in soil with nominal concentration of enrofloxacin 10 μg/g, was 0.37 μg/g by HPLC method. It exceeded the MRL 0,1 μg/g (100 μg/kg), fixed for the content of fluoroquinolones in food of animal origin. A discussion concerning necessity fixing of MRL values for antibiotic residues in food plants is included.

Elaborated microbiological agar-diffusion method for estimation of the concentration of fluoroquinolones in soil and plants can be modified for other antibiotic residues.

II

Lillenberg, M., Yurchenko, S., Kipper, K., Herodes, K., Pihl, V., Sepp, K., Lõhmus, R., Nei, L. (2009). SIMULTANEOUS DETERMINATION OF FLUOROQUINOLONES, SULFONAMIDES AND TETRACYCLINES IN SEWAGE SLUDGE BY PRESSURIZED LIQUID EXTRACTION AND LIQUID CHROMATOGRAPHY ELECTROSPRAY IONIZATION-MASS SPECTROMETRY. *Journal of Chromatography A*, 1216, 5949-5954.

Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.

This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/copyright

Author's personal copy

Journal of Chromatography A, 1216 (2009) 5949–5954

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Simultaneous determination of fluoroquinolones, sulfonamides and tetracyclines in sewage sludge by pressurized liquid extraction and liquid chromatography electrospray ionization-mass spectrometry

Merike Lillenberg[a], Sergei Yurchenko[b], Karin Kipper[b], Koit Herodes[b], Viljar Pihl[b], Kalev Sepp[c], Rünno Lõhmus[d], Lembit Nei[e,*]

[a] Department of Food Science and Hygiene, Estonian University of Life Sciences, Kreutzwaldi 58A, 51014 Tartu, Estonia
[b] Institute of Chemistry, University of Tartu, Jakobi 2, 51014 Tartu, Estonia
[c] Department of Landscape Management and Nature Conservation, Estonian University of Life Sciences, Kreutzwaldi 5, 51014 Tartu, Estonia
[d] Institute of Physics, University of Tartu, Riia 142, 51014 Tartu, Estonia
[e] Department of Environmental Protection, Tartu College of Tallinn University of Technology, Puiestee 78, 51008 Tartu, Estonia

ARTICLE INFO

Article history:
Received 6 March 2009
Received in revised form 8 June 2009
Accepted 10 June 2009
Available online 13 June 2009

Keywords:
Sewage sludge
Fluoroquinolones
Tetracyclines
Sulfonamides
PLE
SPE
HPLC–ESI-MS

ABSTRACT

A new scheme for the quantitative determination of traces of fluoroquinolones (FQs), tetracyclines (TCs) and sulfonamides (SAs) in sewage sludge was developed. The compounds were simultaneously extracted from sewage sludge by pressurized liquid extraction (PLE). A novel and effective method for PLE was developed. Solid-phase extraction was used for cleaning up the extracts. Identification and quantification of the compounds was done using high-performance liquid chromatography with electrospray ionization mass spectrometry in selected reaction monitoring mode. The best recovery of FQs and TCs was obtained by using hydrophilic–lipophilic balance cartridges, recoveries ranged 59% for norfloxacin to 82% for ofloxacin and 95% for doxycycline; for SAs strong cation-exchange cartridges were more efficient, recoveries were 96% for sulfamethoxazole and 43% for sulfadimethoxine. Limit of quantification ranged from 0.1 ng/g for SAs to 160 ng/g for tetracycline. Method precision for TCs was 5.06% and 1.12%, and for SAs 0.43% and 2.01%. FQs precision ranged from 0.77% to 1.89%.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

The amount of sewage sludge increases rapidly all over the world [1]. Although by its nature a hazardous waste, sewage sludge may be used as a fertilizer, if after relevant treatment it is safe for soil, surface and ground water, plants, people and animals [2]. Before utilization the sludge should be analyzed for heavy metals, *E. coli* and helmints' eggs. If the contents of named pollutants do not exceed the trigger values, the sewage sludge is considered to be safe as an ingredient of a fertilizer [3]. Recent research has shown that in addition to these pollutants treated sewage sludge always contains traces of several pharmaceuticals, including antibiotics [4–6]. Although their concentrations are much lower than the levels of traditionally known organic pollutants the potential long-term effects of these compounds to humans, plants and animals cannot be ignored. No trigger values exist for drug residues in sewage sludge neither in Estonia [3] nor in the European Union [7]. The most closely related act is the EU directive EMEA/CVMP/055 establishing trigger values for drug residues in manure [8]. The content of drug residues should not exceed 100 μg/kg in manure and 10 μg/kg in the soil fertilized with manure.

The sewage sludge from Estonian wastewater treatment plants has never been analyzed for pharmaceuticals and as a rule the relevant levels are unknown. The selection of drugs was made by considering their stability in soil [4,9] and/or their potential accumulation into plants [10–13]. It has been shown that tetracyclines (TCs) and fluoroquinolones (FQs) can bind strongly to solid particles and this phenomenon might be an additional reason for their slow degradation [14–17]. Sulfamethoxazole is relatively stable in sewage sludge [18] and sulfonamides (SAs) are found to be present there [5]. Therefore, these antibiotics may contaminate agricultural fields, disturb natural balance and accumulate in crops and vegetables [11–13]. Even very small amounts of antibiotics in everyday food may generate the strains of resistant bacteria in human and animal bodies, provoke allergy and affect the liver [13].

Analytical tools have been developed for different media, e.g. feed products [19–22], environmental water samples [23–26], soil [4,13,15,27–29] and manure [15,30], while only few methods

* Corresponding author.
E-mail address: lembit.nei@ttu.ee (L. Nei).

0021-9673/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2009.06.029

Author's personal copy

have been designed for extraction of antibacterial agents from sewage sludge [4,31]. The existing methods [15] cannot be used for simultaneous extraction of FQs, SAs and TCs from sewage sludge.

Several extraction techniques have been applied for the determination of antibiotics from solid phase, such as ultrasonic-assisted extraction (USE) [6,32,33], microwave-assisted extraction (MAE) [34,35], pressurized liquid extraction (PLE) and accelerated solvent extraction (ASE) [4,15]. For extracts clean-up liquid–liquid extraction (LLE) [30,36] and solid-phase extraction (SPE) [4,21,23,25] were used. ASE or PLE have clear advantages over other methods such as higher precision, smaller amounts of extraction solvent and reduced sample preparation time [37]. Sludge extraction is usually followed by pre-concentration and clean-up of the PLE extracts using SPE with different cartridges [4,28,31].

Most of the relevant analytical methods reported are based on liquid chromatography–mass spectrometry (LC–MS) [38,39], LC–tandem MS (LC–MS/MS) [19,40] and HPLC-UV [24,35]. Optimum conditions with regard to extraction solvent and number of extraction cycles were established. Identification and quantification of the pharmaceuticals were carried out by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) using electrospray ionization (ESI).

The aim of this work was to develop a specific extraction method for the quantification of FQs, SAs and TCs in sewage sludge using the same extraction process.

2. Experimental

2.1. Chemicals and materials

Antibiotics were purchased from Riedel-de-Haën (Seelze, Germany)—three fluoroquinolones: ciprofloxacin (CIP, purity 99.8%), norfloxacin (NOR, purity 99.9%) and ofloxacin (OFL, purity 99.3%); two tetracyclines: tetracycline hydrochloride (TCL, purity 97.3%) and doxycycline hyclate (DOX, purity 99.5%); two sulfonamides: sulfadimethoxine (SDM, purity 99.4%) and sulfamethoxazole (SMX, purity 99.9%). Strong cation-exchange (SCX) cartridges (Strata SCX (55 μm, 70 Å) 500 mg/6 mL) were supplied by Phenomenex (Torrance, CA, USA); Hydrophilic–lipophilic balanced (HLB) cartridges (Oasis HLB (60 μm), 500 mg/6 mL) by Waters (Milford, MA, USA). Acetonitrile and methanol were obtained from J.T. Baker (Deventer, The Netherlands), phosphoric acid from Lachema (Brno, Czech Republic), citric acid monohydrate from Fisher Scientific (Pittsburgh, PA, USA), formic acid from Riedel-de-Haën, ammonium acetate from Fluka (Buchs, Germany). All solvents were of reagent grade or higher quality.

2.2. Sample collection and storage

The samples were taken from anaerobically digested sludge (before mixing with peat) in Tallinn and from untreated sludge (before composting with tree bark) in Tartu. Approximately 200 g of sludge (content of dry matter was 28% in Tallinn and 25% in Tartu) was placed into a 500 mL glass jar and mixed thoroughly. The jar was covered hermetically with a lid. Before analyzing the samples were stored in refrigerator at temperature +4 °C in the dark. The samples were analyzed as soon as possible, typically within a week. Alternatively they were stored in polypropylene vials frozen at temperature −80 °C.

2.3. Pressurized liquid extraction (PLE)

PLE was performed using an in-house designed system schematically depicted in Fig. 1. The extractor was designed using ultra high vacuum components. For surviving high pressure the stainless steel chamber cylinder wall thickness was 10 mm and for sealing flanges copper gaskets were used. Volume of the pressure chamber was 55 mL. Standard HPLC valves and stainless steel tubing were used.

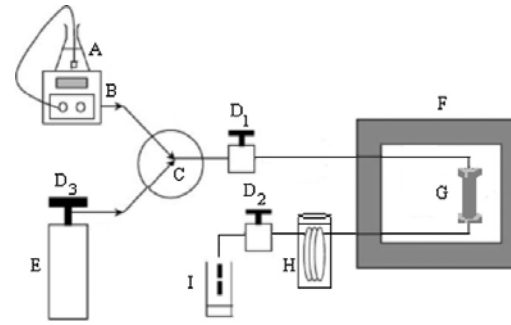


Fig. 1. Pressurized liquid extraction (PLE) system: A, extraction solvent; B, HPLC pump; C, three-way switching valve; D1 and D2, static valves; D3, the valve of argon gas; E, argon tank; F, oven; G, extraction cell; H, cooling coil; I, extract collection vial.

9 ± 1 g (wet weight, ww) of sewage sludge sample was mixed 1:1 with sand, and 9 ± 1 g of sludge/sand blend was packed into cellulose filter and placed into the extraction cell mounted in an oven. Extraction was performed with 0.35% phosphoric acid and acetonitrile mixture (1:1, v/v) adjusted to pH 2.50 with 0.01 M citric acid monohydrate. For one extraction cycle approximately 30 mL of solvent was pumped into the extraction cell with static valve D1 open. The system was pressurized with argon using valve D3; subsequently the cell was heated. The operating conditions were as follows: temperature in the range 100–110 °C with a 30 min heat-up time, pressure in the range 100–110 atm (10,130–11,143 kPa), static extraction 10 min, 5 cycles and solvent flush volume 60%. The extracted analytes were purged from the sample cell using pressurized argon for 40 s. The solvent used for flushing of the extraction cell was collected with static valve D2 open after the first cycle of extraction. Subsequent cycles of extraction were carried out using the same operating conditions. The extract cooling was accomplished by stainless steel tubing in cold water. The total volume of the extract collected was in the range of 150–160 mL.

2.4. Solid-phase extraction

The extracts collected by PLE were cleaned up by SPE. Antibiotics as CIP, NOR, OFL, TCL, DOX, SDM and SMX were extracted using SCX and HLB cartridges. Two different cartridges were tested with the aim of securing the best possible recoveries. For SPE procedure the vacuum manifold, supplied by Agilent Technologies, was used. For extraction with SCX cartridges the cartridges were preconditioned with 6 mL of methanol and 6 mL of buffer solution (1 mM ammonium acetate and 0.1% formic acid, pH 2.8). A portion (80 mL) of sludge PLE extract was diluted to 500 mL with H_2O (pH adjusted to 2.0) and then percolated through the cartridge at a flow rate ~1.5 mL/min using the vacuum manifold. After extraction, the compounds were eluted from cartridges using 20 mL of 20% ammonia water solution in 40% methanol. For extraction with HLB cartridges the cartridges were preconditioned with 20 mL of methanol and 10 mL of Milli-Q water. Dilution of PLE extract was preformed as for SCX cartridges. Flow rate of sample loading was ~6 mL/min. After extraction, the compounds were eluted from cartridges using 12 mL of methanol. The SPE extracts were concentrated on polypropylene vials in N_2 stream. Polypropylene vials were used to avoid sorption to glass walls and samples were not evaporated to complete dry-

Author's personal copy

Table 1
m/z and base peaks of precursor and product ions used in the LC–MS analysis of antibiotics.

Compound	Parent ion	*m/z*	fragment ion 1	fragment ion 2	fragment ion 3
			m/z		
SDM	$[M+H]^+$	311	156	108	245
SMX	$[M+H]^+$	254	108 $[H_2NPhO]^+$	92 $[H_2NPhH]^+$	156 $[H_2NPhSO_2]^+$
TCL	$[M+H]^+$	445	410 $[M-H_2O-NH_3+H]^+$	427 $[M-H_2O+H]^+$	325
DOX	$[M+H]^+$	445	428 $[M-NH_3+H]^+$		
CIP	$[M+H]^+$	332	288 $[M-CO_2+H]^+$	314 $[M-H_2O+H]^+$	294 $[M-H_2O+H-HF]^+$
NOR	$[M+H]^+$	320	276 $[M-CO_2+H]^+$	233	302 $[M-H_2O+H]^+$
OFL	$[M+H]^+$	362	318 $[M-CO_2+H]^+$	344 $[M-H_2O+H]^+$	

ness. Residues were dissolved in 1 mL of 1:1 solution of methanol with buffer solution (1 mM ammonium acetate and 0.1% formic acid, pH 2.8).

2.5. LC–MS

The SPE extracts were analyzed by LC–MS (Agilent Series 1100 LC-MSD Trap XCT (Santa-Clara, CA, USA)) equipped with a binary pump, a degasser, an auto-sampler and a column thermostate. Antibiotics were chromatographed using a Phenomenex Synergi Hydro-RP column (250 mm × 4.6 mm, 4 μm) equipped with a Phenomenex SecurityGuard cartridge AQ 4 mm × 2 mm. Electrospray interface (ESI) was used in positive ion mode for ionization. Selected reaction monitoring was used, total intensity of fragments presented in Table 2 was used for quantification. For instrument control and data analysis software: Agilent ChemStation for LC Rev. A. 10.02; MSD Trap Control version 5.2 and Data Analysis for LC-MSD Trap 3.2. were used. Gradient elution with methanol and ammonium buffer solution (1 mM ammonium acetate and 0.1% formic acid, pH 2.8) was used. The linear gradient with a flow rate 0.4 mL/min started at 35% methanol for 20 min and was raised to 80% within 20 min, after that methanol concentration was lowered to 35% in 5 min. Column temperature was set to 30 °C and the injection volume was 5 μL.

Under these chromatographic conditions the separation of seven antibiotic drugs was successfully performed. Antibiotics were detected using electrospray ionization in the positive ion mode which ensured a better sensitivity. The maximum number of observed fragmentation reactions was four at any time. Ions detected are shown in Table 1. Stock solutions of 1 mg/mL in the appropriate solvent were prepared. Stock solution for SDM was 0.5 mg/mL due to its poor solubility. The working standard contained 7 antibiotics at 0.1 mg/mL. Solutions were stored at −20 °C.

For calibration antibiotic solutions were prepared in eluent (35% methanol). The calibration graphs with peak area versus concentration were composed.

In order to take into account possible matrix effects standard addition experiments were carried out at least once for each batch of samples. Recovery was calculated from standard addition experiment data. The results presented are corrected with recoveries.

3. Results and discussion

3.1. PLE

The developed method is based on the combination of PLE, SPE, LC–MS and its subsequent application to perform screening of sewage sludge for a total of 7 structurally and chemically diverse antibiotics. The variables optimized were the extraction solvent and pH, time, temperature, pressure and the number of extraction cycles. Methanol and acetonitrile aqueous solutions showed lower extraction efficiencies for most of the studied antibiotics. The mixture of 0.35% phosphoric acid and acetonitrile (1:1, v/v) with 0.01 M citric acid monohydrate was finally chosen as an extraction solvent. During PLE optimization it was found that 10 min extraction times were insufficient, and with times >15 min the recoveries for TCs decreased. The decrease in recovery was also observed at temperatures exceeding 120 °C, resulting either from thermal degradation or from a loss in method selectivity due to the more efficient extraction of interfering matrix components. The effect of pressure on extraction efficiency of antibiotics was studied in the range from 70 to 110 atm (7091–11,143 kPa), and no significant impact on the extraction efficiency was followed in this range. The next variable studied was the number of extraction cycles. Five consecutive simple extractions were applied to the same sample with the identical extraction conditions. The recoveries were considered negligible (lower than 2%) during the fourth cycle. The fifth cycle showed no antibiotics. In the current study, five extraction cycles were applied when treating the samples.

Spiking and recovery experiments with quartz sand were preformed during the method development. Recoveries for TCL, DOX, NOR, CIP and SMX ranged from 55 to 100%.

During PLE method development a serious carryover was observed, especially after the extraction of spiked samples. Repetitive cleaning with different solvents appeared to be of low efficiency. For cleaning the PLE vessel an original simple solution is proposed: a small volume of ethanol was burnt in the extraction

Table 2
Validation data of 7 antibiotics in sewage sludge ($n=5$) using SPE with HLB cartridge.

Compound	Unfortified sludge sample (original)		Fortified sludge sample (spiked)			Rec[b], %	LOQ, ng/g
	Concentration, ng/g	Standard deviation	Concentration, ng/g	Standard deviation	RSD[a], %		
TCL	n.d.[c]	-	310.66	15.72	5.06	27	160
DOX	n.d.	-	682.37	7.67	1.12	95	80
NOR	2.59	0.13	42.58	0.80	1.89	58	1.3
CIP	6.55	0.18	50.65	0.39	0.77	61	1.8
OFL	0.54	0.08	12.34	0.11	0.92	84	0.8
SMX	0.11	0.01	1.40	0.01	0.43	91	0.1
SDM	n.d.	-	0.45	0.01	2.01	52	0.1

[a] Relative standard deviation.
[b] Average recovery of antibiotics.
[c] Not detected.

Author's personal copy

vessel and subsequently the vessel was rinsed with the extraction solvent.

3.2. SPE

For the PLE extracts clean-up procedure two types of SPE cartridges – SCX and HLB – were used. To optimize the SPE procedure, SCX cartridges with various sorbents based on silica (supplied by Phenomenex) and polymer (supplied by Alltech) were applied. From different types of SCX cartridges the most reliable results were obtained when using Phenomenex SCX modified silica cartridges due to the better isolation of antibiotics from the sample. Different solvents, such as 1:4 NH_4OH/water in methanol or in acetonitrile were tested for eluting antibiotics from the cartridges. The comparison of the tested solvents showed that the higher content of organic modifier resulted in higher recoveries. Best results with SCX cartridges were obtained when using 1:4:1 NH_4OH/water/methanol.

The pH of the PLE extracts was adjusted to pH 2.0 before SPE analysis using conc. HCl. Higher pH values resulted in lower recoveries. Studied antibiotics have rather wide pK_a range, and the pH of the solvent should be lower than pK_a of the analyzed antibiotic (the lowest pK_a value was in the case of DOX: $pK_a = 2.8$). Good repeatability was achieved using SCX.

To increase the accuracy of the method, the HLB cartridges were used due to their ability to retain both hydrophilic and hydrophobic compounds [25]. The sample pH was adjusted to 2.0 and methanol was used for the elution. Comparison of the two cartridges (Table 2 and Fig. 2) showed that HLB cartridges were more efficient and reliable: the standard deviation of the results ranged from 0.011 to 0.175 and the recoveries ranged from 26% to 95% (overall recovery from standard addition experiments). As an exception, SMX had better recovery and smaller standard deviation with SCX cartridges.

3.3. Method validation

For calibration standards appropriate dilutions from working standard in the concentration range 0.5–500 ng/mL ($n = 7$) for NOR, CIP, OFL, 0.1–500 ng/mL ($n = 8$) for SMX, SDM and 10–5000 ng/mL ($n = 6$) for TCL and DOX were made. The calibration graphs showed excellent linearity in the studied concentration ranges ($r^2 \geq 0.9994$). An unweighted linear regression analysis of representative calibration curves resulted in a slope 966,229.6 (OFL), 163,082.8 (NOR), 652,880.7 (CIP), 399,829.1 (SDM), 74,338.5 (SMX), 414,634.6 (DOX), 11,211.1 (TCL). The intercepts of the calibration curves were not statistically significant at 95% confidence level.

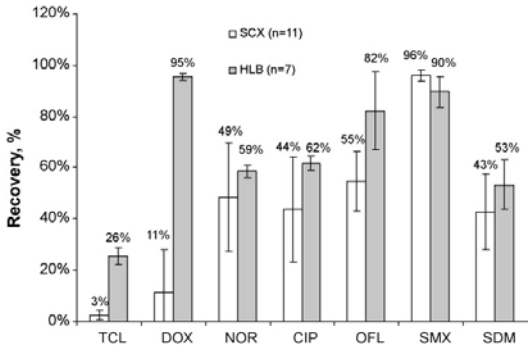


Fig. 2. Recovery percentage for antibiotics from sewage sludge by using SCX and HLB, error bars for 2 standard deviation. TCL—tetracycline, DOX—doxycycline, CIP—ciprofloxacin, NOR—norfloxacin, OFL—ofloxacin, SMX—sulfamethoxazole, SDM—sulfadimethoxine.

The described method was validated for the simultaneous determination of 7 antibiotics belonging to three different antibiotic groups – FQs, SAs and TCs – from sewage sludge. The average recovery rates for the studied antibiotics by using SCX cartridges were: SMX—96% (standard deviation, SD = 1.0%), SDM—43% (SD = 7.4%), OFL—55% (SD = 5.9%), NOR—49% (SD = 10.6%), CIP—44% (SD = 10.2%), DOX—11% (SD = 8.4%), TCL—3% (SD = 0.9%). By using HLB cartridges the average recovery rates were: SMX—90% (SD = 3.0%), SDM—53% (SD = 4.8%), OFL—82% (SD = 7.6%), NOR—59% (SD = 1.3%), CIP—62% (SD = 1.4%), DOX—95% (SD = 0.6%), TCL—26% (SD = 1.7%) (Fig. 2). There was no significant difference between the recoveries in the case of two sludges from two Estonian cities. An excellent recovery for SMX was obtained when using SCX cartridges. However, SCX cartridges could be utilized only for cleaning up the extracts of 5 antibiotics: 3 FQs and 2 SAs, whereas the recovery of TCs was too low. Much better recovery and repeatability for all the antibiotics were obtained when using the HLB cartridges but the recovery of SMX was smaller than in case of SCX. Therefore, SPE by using HLB cartridges is more efficient and can be applied for determination of 6 antibiotics from sewage sludge: CIP, NOR, OFL, SDM, SMX and DOX. Determination of TCL needs to be improved.

Limit of quantifications (LOQ) were estimated (as 10 times the standard deviation) from five replicate analyses of unspiked and spiked sludge samples using HLB cartridges. For the antibiotics present in the sludge, standard deviation of the determinations was used; for the rest of pharmaceuticals results obtained from spiked samples were used. Method precision was estimated as relative standard deviation from five replicate analyzes. The validation data is presented in Table 2.

3.4. Application to Tallinn sewage sludge

Different samples of sewage sludge were analyzed. The sewage sludge from sewage treatment plants of the two Estonian largest cities, Tallinn and Tartu, was used. The sludge treatment technology in Tallinn is anaerobic stabilization—methane fermentation and mixing with peat. The highest contents of antibiotics were found in Tallinn: CIP 425.5 μg/kg and NOR 162.3 μg/kg (ww). Maximum content of CIP exceeds the trigger value for manure (100 μg/kg) over four times. Contents of OFL (37.6 μg/kg), SDM (20.5 μg/kg) and SMX (6.1 μg/kg) were lower. The average contents of antibiotics were: CIP 32.8 μg/kg, NOR 20.8 μg/kg, OFL 4.0 μg/kg SDM 2.0 μg/kg and SMX 1.0 μg/kg (ww). In other studies the antibiotic content in sludge was described as dry weight (dw). Comparing with other studies the highest CIP content in Tallinn sludge 1.52 mg/kg (dw) was comparable with its content in Gothenburg (1.4–3.4 mg/kg, dw) and Floda (0.5–0.9 mg/kg, dw) [6]. The highest content of OFL in Tallinn sludge 0.13 mg/kg (dw) was comparable with its content in Gothenburg (0.0–0.1 mg/kg, dw) [6]. The highest content of NOR in Tallinn sludge 0.58 mg/kg (dw) was comparable with its content in Floda (0.1–0.4 mg/kg, dw) [6].

Typical chromatograms for OFL, CIP, NOR and SMX for Tallinn sewage sludge after PLE and HLB are shown in Fig. 3.

3.5. Application to Tartu sewage sludge

In Tartu sewage sludge is treated by aerobic stabilization—composting with tree bark. The content of SAs was quite low in both cities, under 10 μg/kg, but at least one of them was present in every sludge sample. The contents of SMX ranged from 0.0 to 3.9 μg/kg and SDM 0.0 to 22.2 μg/kg (ww). In Tartu the highest concentrations of antibiotics were: NOR—109.8 μg/kg and CIP—110.6 μg/kg (ww). OFL was present in every sludge sample from Tartu and the highest concentration was 39.2 μg/kg (ww). The average contents of antibiotics were: CIP—35.5 μg/kg, NOR—25.0 μg/kg, OFL—10.9 μg/kg, SDM—1.9 μg/kg and SMX—0.9 μg/kg (ww). Aver-

Author's personal copy

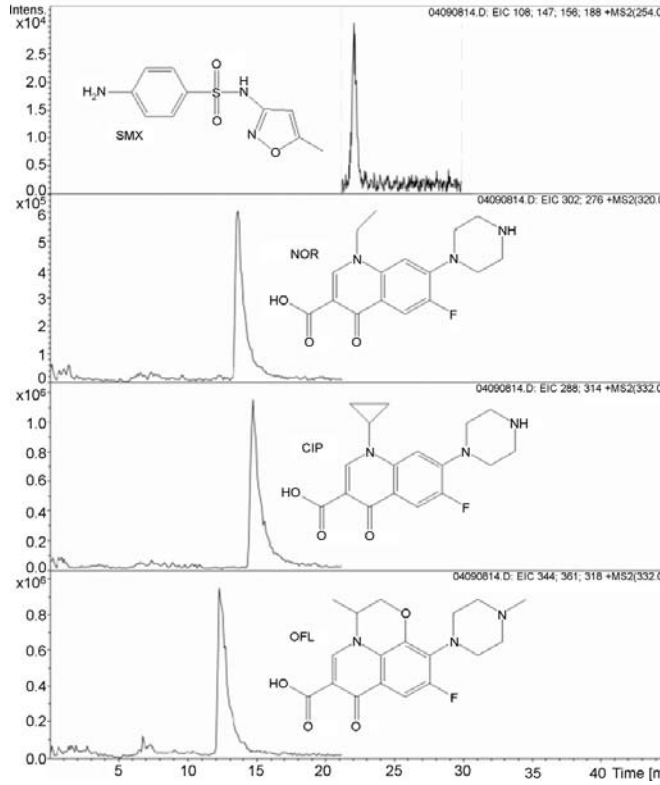


Fig. 3. Chromatograms, Tartu sewage sludge, June 2008: OFL—10.26 ng/g; CIP—58.59 ng/g; NOR—58.55 ng/g; SMX—1.45 ng/g.

age contents of FQs were slightly higher than in the case of Tallinn sludge.

4. Conclusion

A novel reliable method for simultaneous determination of fluoroquinolones, sulfonamides and tetracyclines in sewage sludge was developed. The compounds were extracted from sewage sludge by PLE. The solution of 0.35% phosphoric acid and acetonitrile (1:1, v/v) with 0.01 M citric acid monohydrate was used as an extraction solvent. Extracts were cleaned up by SPE using two different cartridges, SCX and HLB, from which HLB was more efficient and can be applied for determination of 6 antibiotics from sewage sludge. The results showed that antibiotics are present in Estonian sewage sludge and their content may exceed the relevant trigger values for manure.

Acknowledgements

This work was funded by Estonian Environmental Investment Centre, Estonian Science Foundation (Grant No. 6658 and 7127), Estonian research target project SF 0180058s07 and Estonian Nanotechnology Competence Centre. We thank Mr Mati Perker, Mr Harri Terase and Mr Urmas Tiivoja, the managers of Tallinn and Tartu Wastewater Treatment Plants, for their kind assistance. We also thank Ms Sandra V. Litvin for making corrections to the manuscript.

References

[1] G. Carbonell, J. Pro, N. Gomez, M.M. Babin, C. Fernandez, E. Alonso, J.V. Tarazona, Ecotoxicol. Environ. Saf. 72 (2009) 1309.
[2] A.J. Moffat, A.T. Armstrong, J. Ockleston, Biomass Bioenergy 20 (2001) 161.
[3] E.M. Golet, A. Strehler, A.C. Alder, W. Giger, Anal. Chem. 74 (2002) 5455.
[4] A. Thomsen, A. Göbel, C.S. Mc.Ardell, A.C. Alder, W. Giger, Poseidon Symposium, Braunschweig, 2003.
[5] R.H. Lindberg, P. Wennberg, M. Johansson, M. Tysklind, B.A.V. Andersson, Environ. Sci. Technol. 39 (2005) 3421.
[6] Decree of Minister of the Environment No.78, State Gazette I, 9, 52, 2004.
[7] EUR-Lex–31986L0278-EN; Council Directive 86/278/EEC of 12 June 1986.
[8] EMEA/CVMP/055/96, The European Agency for the Evaluation of medicinal Products (EMEA), Veterinary Medicines Evaluation Unit, London, 1996.
[9] S. Sczesny, H. Nau, G. Hamscher, J. Agric. Food Chem. 29 (2003) 697.
[10] L. Migliore, G. Brambilla, S. Cozzolino, L. Gaudio, Agric. Acosyst. Environ. 52 (1995) 103.
[11] G. Brambilla, P. Casoria, C. Civitareale, S. Cozzolino, L. Gaudio, L. Migliore, Conference on Residues of Veterinary Drugs in Food, Veldhoven, 1996, Proceedings, p. 292.
[12] A. Aruksaar, T. Püssa, P. Kuldkepp, M. Ivask, Trans. Estonian Acad. Agric. Soc. 9 (1999) 5.
[13] M. Lillenberg, M. Roasto, T. Püssa, J. Agric. Sci. 14 (2003) 13.
[14] J.R. Marengo, R.A. Kok, G.K. OïBrien, R.R. Velagaletti, J.L. Stamm, Environ. Toxicol. Chem. 16 (1997) 462.
[15] T. Christian, R.J. Schneider, H.A. Färber, D. Skutlarek, M.T. Meyer, H.E. Goldbach, Acta Hydrochem. Hydrobiol. 31 (2003) 36.
[16] N. Carmosini, L.S. Lee, D.S. Aga (Eds.), Sorption and Degradation of Selected Pharmaceuticals in Soil and Manure, CRC Press, Boca Raton, London, New York, 2008, p. 139.
[17] S. Thiele-Bruhn, M.O. Aust, Arch. Environ. Contam. Toxicol. 47 (2004) 31.
[18] M.L. Richardson, J.M. Bowron, J. Pharm. Pharmacol. 37 (1985) 1.
[19] B. Toussaint, G. Bordin, A. Janosi, A.R. Rodriguez, J. Chromatogr. A 976 (2002) 195.
[20] B. Toussaint, M. Chedin, G. Bordin, A.R. Rodriguez, J. Chromatogr. A 1088 (2005) 32.

Author's personal copy

[21] A. Posyniak, J. Zmudzki, K. Mitrwska, J. Chromatogr. A 1087 (2005) 259.
[22] M.P. Hermo, D. Barrón, J. Barbosa, J. Chromatogr. A 1104 (2006) 132.
[23] R. Lindberg, P. Jarnheimer, B. Olsen, M. Johansson, M. Tysklind, Chemosphere 57 (2004) 1479.
[24] H. Nakata, K. Kannan, P.D. Jones, J.P. Giesy, Chemosphere 58 (2005) 759.
[25] M.E. Lindsey, M. Meyer, E.M. Thurman, Anal. Chem. 73 (2001) 4640.
[26] K.-J. Choi, S.G. Kim, C.-W. Kim, S.-H. Kim, Chemosphere 66 (2007) 977.
[27] M.P. Schlüsener, M. Spiteller, K. Bester, J. Chromatogr. A 1003 (2003) 21.
[28] A.M. Jacobsen, B. Halling-Sørensen, F. Ingerslev, S.H. Hansen, J. Chromatogr. A 1038 (2004) 157.
[29] K. Stoob, H.P. Singer, S. Stettler, N. Hartmann, S.R. Mueller, C.H. Stamm, J. Chromatogr. A 1128 (2006) 1.
[30] A.M. Jacobsen, B. Halling-Sørensen, Anal. Bioanal. Chem. 384 (2006) 1164.
[31] A. Göbel, A. Thomsen, C.S. McArdell, A.C. Alder, W. Giger, N. Theis, D. Löffler, T. Ternes, J. Chromatogr. A 1085 (2005) 179.
[32] P.A. Blackwell, H-C.H. Lützhøft, H.-P. Ma, B. Halling-Sørensen, A.B.A. Boxall, P. Kay, Talanta 64 (2004) 1058.
[33] E. Turiel, A. Martín-Esterban, J.L. Tadeo, Anal. Chim. Acta 562 (2006) 30.
[34] S. Morales-Moñoz, J.L. Lique-García, M.D. Lique de Castro, J. Chromatogr. A 1059 (2004) 25.
[35] M.P. Hermo, D. Barrón, J. Barbosa, Anal. Chim. Acta 539 (2005) 77.
[36] M.Y. Haller, S.R. Müller, C.S. McArdell, A.C. Alder, M.J. Suter, J. Chromatogr. A 952 (2002) 111.
[37] H. Giergielewicz-Mozajska, L. Dabrowski, J. Namiesnik, Crit. Rev. Anal. Chem. 31 (2001) 149.
[38] W.M.A. Niessen, J. Chromatogr. A 812 (1998) 53.
[39] M.S. Díaz-Cruz, D. Barceló, Trends Anal. Chem. 24 (2005) 645.
[40] T.A. Ternes, M. Bonerz, N. Herrmann, D. Löffler, E. Keller, B. Bago Lacida, A.C. Alder, J. Chromatogr. A 1067 (2005) 213.

III

Nei, L., **Lillenberg, M**.(2009).
MACKERETH OXYGEN SENSOR:
MEASUREMENT UNCERTAINTY.
The Electrochemical Society Transactions, 19(22), 55-63.

Mackereth Oxygen Sensor: Measurement Uncertainty

L. Nei[a] and M. Lillenberg[b]

[a] Department of Environmental Protection, Tartu College, Tallinn University of Technology, Puiestee 78, 51008 Tartu, Estonia
[b] Department of Food Science and Hygiene, Estonian University of Life Sciences, Kreutzwaldi 58A, 51014 Tartu, Estonia

The measurement of oxygen concentrations in natural and waste water is a major requirement of environmental monitoring. Since 1953 amperometric membrane-covered sensors are commonly used for dissolved oxygen measurements. The device consists of a two-electrode electrochemical cell with a thin gas-permeable membrane separating the electrodes and the electrolyte solution from the analyzed media. The reliability of dissolved oxygen analysis has been extensively discussed in the relevant literature. There is a remarkable disagreement between the values of measurement uncertainty of dissolved oxygen measurement reported by different authors. We draw out the main uncertainty sources and show the potential measures for increasing the reliability of continuous electrochemical oxygen measurements, carried out in environmental monitoring and waste water treatment.

Introduction

Electrochemical oxygen sensors have found wide application since the introduction of the Clark sensor over 50 years ago. Many modifications to the original design of this device have been proposed [1-8] since the publication of the relevant patent [9], but the essential basis of the sensor remains unchanged in that a miniature noble metal electrode, typically platinum or gold, is covered with a thin layer of electrolyte solution retained by a thin gas-permeable membrane. The necessary indicator (sensing) electrode potential is applied from an external source. Despite of the wide usage, several disadvantages and limitations to the Clark sensor are known. Of these, the instability of the output current is the most significant, leading to frequent and elaborate pre-calibrations in the laboratory [10]. This is at best undesirable in environmental monitoring and sewage treatment efficiency control, when continuous measurements are carried out. Clark sensors are successfully exploited in clinical work, where small sample amounts are essential. The uncertainty of oxygen measurement with a Clark sensor significantly depends on the accuracy and frequency of the calibration procedure.

In 1962 a different oxygen sensor design, more suitable for environmental monitoring, was proposed by Mackereth [11, 12]. The relatively large electrodes – tubular silver cathode and lead anode – were ″isolated″ from the external medium by a polyethylene tubular membrane. The indicator electrode potential of this galvanic sensor was ″created″ internally. The stability of the oxygen reduction current over periods of at least many months of continuous operation was declared [12]. Following the lines of this design, several modifications of the Mackereth sensor were developed for field conditions, where the stability of the operating characteristics and the long-term measurement reliability are of great importance [13-17]. The measurement uncertainty in

case of this sensor type strongly depends on the drifts of a number of sensor characteristics.

Measurement uncertainty estimates based on various preconditions and models have been presented in the scientific literature [18-23]. The measurement uncertainty has been declared to be somewhat between 1% and 4%. By all means, this is valid only under certain and very favorable conditions. Practitioners know well that at low oxygen concentrations the measurement uncertainty may reach up to several tens of percents. Recently ″model-based measurement uncertainty estimation in amperometric dissolved oxygen concentration measurement″ was proposed [24]. In this excellent paper, several important uncertainty sources in amperometric dissolved oxygen measurement are explored and uncertainty estimation procedure based on a ″detailed measurement model″ is presented. Still, the work is mainly based on theoretical considerations and some of the factors resulting from the design and the electrochemical reactions have not been considered in the relevant model. The aim of this work was to complement the model presented in [24] and to show some possible ways of reducing uncertainty in oxygen measurements.

Theory

A Mackereth-type oxygen sensor (see figure 1) consists of a perforated Perspex body, a tubular indicator electrode from Ag [11], Cu/Ni [25] or Cr/Ni alloy [24], an anode formed from Cd or Pb strips, and a tubular polyethylene membrane separating the electrodes and the electrolyte solution from the analyzed media. In the current study we applied the following electrode materials and electrolyte solutions: Cu-30%Ni alloy – indicator electrode; Cd-strips – anode; 27% KOH or a solution containing 15% KOH, 39% C_2H_5OH and 46% H_2O – electrolyte solution.

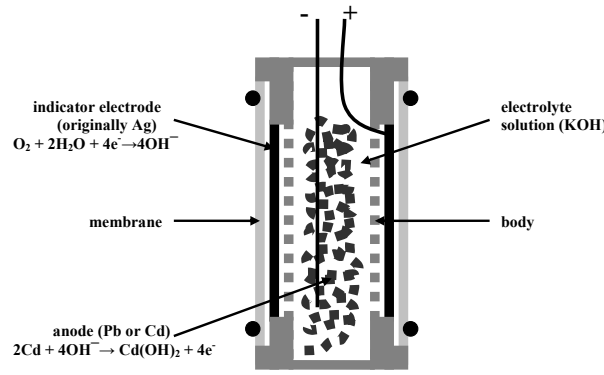


Figure 1. Schematic drawing of a conventional galvanic Mackereth oxygen sensor.

Oxygen diffuses through the membrane. The potential of the indicator (working) electrode is controlled at a value providing the diffusion-limited electrochemical reduction process of di-oxygen. The sensor current is proportional to the oxygen concentration in the analyzed media:

$$I_d = nFA \frac{1}{\frac{l_m}{P_m} + \frac{l_e}{P_e}} Po_2 \quad [1]$$

where n = 4: the number of electrons involved in the cathode reaction, F is Faraday's constant; A is the indicator electrode area (*ca* 10 cm^2 in our experiments), l_m is the thickness of the membrane (we commonly use 50 μm polyethylene), l_e is the thickness of the electrolyte layer between the indicator electrode and the membrane, P_m and P_e are the permeabilities of oxygen in the membrane and electrolyte layer, and Po_2 is the partial pressure of oxygen in gaseous media ($Po_2 = k_H\ c_{O2}$, where k_H is the Henry constant and c_{O2} the concentration of dissolved oxygen in liquid).

Sources of uncertainty

Uncertainty sources of dissolved oxygen measurement are associated with (1) calibration procedure and (2) drift of the parameters of the oxygen analyzer. According to [24] oxygen measurement uncertainty originates from (1) calibration and (2) measurement. Uncertainty resulting from calibration procedure has been analyzed in detail in [20] and [24]. In this paper we mainly concentrate on the most significant uncertainty sources related to oxygen concentration measurements with a Mackereth sensor and show some possible ways of reducing them. For convenience, the uncertainty components originating from calibration are united here into a single quantity.

Calibration

Dissolved oxygen analyzers do not measure the oxygen concentration directly. Therefore, the device must be standardized to a reference condition that compares an electrical current to a known dissolved oxygen concentration [26]. The calibration of dissolved oxygen sensor is usually performed in air-saturated water at certain temperatures. The major uncertainty sources in calibration are the limited accuracy of temperature measurement, temperature instability and uncertainty of dissolved oxygen concentration in the calibration solution [24]. There is also a mismatch of temperature between the sensor and the calibration medium, but this is negligible if compared to the other factors related to temperature. In [20] it has been shown that both the imperfect saturation of the air with water vapor and the unstable CO_2 content of the indoor air may contribute to the accuracy of calibration. It is easy to exclude these factors experimentally via passing the air used for preparing aqueous solutions with known oxygen concentrations through KOH solution and water. Numerous tables of oxygen solubility in distilled water have been published. As a rule, the differences between the oxygen concentration values in these tables do not exceed 0.05 mg dm^{-3}. Careful studies have shown that uncertainty $u(I_{cal})$ originating from the calibration procedure carried out using air-saturated water is within 1.5% [20].

Dissolved oxygen measurement

According to [20], the following sources of dissolved oxygen measurement uncertainty can be listed: (1) background current (or its drift); (2) the effective drift of the diffusion layer thickness; (3) changes in properties of the membrane (slight deformation, aging,

etc). In addition to these uncertainty sources, the following factors are considered in the current paper: (4) measurement response time; (5) change in the activity of indicator electrode surface.

Background current. In environmental monitoring and sewage aeration control the measured oxygen concentrations are often far below saturation. The presence of the traces of pharmaceuticals and other organic pollutants in natural and waste water is a phenomenon with unpredictable consequences [27]. The efficiency of the degradation of organic pollutants present in sewage and sewage sludge remarkably depends on dissolved oxygen concentration [28, 29]. The activated sludge process used in sewage treatment needs the dissolved oxygen concentrations to be settled within a narrow interval, normally 0.5 to 2 mg dm^{-3}. Typically in oxygen-free samples a background current is present in a sensor due to the reduction of impurities, but mainly di-oxygen, diffusing to the cathode from the inner electrolyte solution and from the plastic body of the sensor [15]. At dissolved oxygen concentrations below 5 mg dm^{-3}, the background current of the membrane-covered oxygen sensor becomes the primary uncertainty source. The background current of the Mackereth sensor measured in oxygen-free medium is usually below 0.5% of the corresponding current values obtained from air-saturated samples. During calibrations in air-saturated water or during the storage in air between the measurements the sensor gets ″soaked″ with oxygen. Depending on the design of the sensor it takes more or less time in oxygen-free surrounding before all oxygen has been ″consumed″ by the indicator electrode or ″rinsed″ off the sensor. Due to this phenomenon, it seems to be essential that the magnitude of the background current of an oxygen sensor sufficiently depends on oxygen concentration in the surrounding environment. The value of the background current also depends on the sample temperature and the time during which the sensor is kept at ″high″ or ″low″ oxygen concentrations.

With the aim of quantifying the background current under different measuring conditions, a galvanic Mackereth sensor with an additional ″inner″ cathode was developed (see figure 2). Both the indicator electrode (sensing electrode or cathode) and the supporting cathode were made from the same material (Cu-Ni alloy) and were approximately of the same size (their ″external″ surface areas were *ca* 10 cm^2). The current measured in the circuit supporting cathode - anode allowed to estimate the magnitude of the background current in a conventional Mackereth sensor used for measurements in samples with different oxygen concentrations. When the sensor was held in an oxygen-free sample for at least 2 hours, this ″background″ current was less than 0.2% of the current in the circuit indicator electrode - anode measured in air-saturated sample at the same temperature (20 ^{0}C). In air-saturated water and in oxygen-saturated water these ″background″ currents increased respectively to 1.6% and to 5.3%. It takes at least 12 hours after the ″pure″ oxygen experiment to restore the 0.2% background current level under oxygen-free conditions. As the background current is not persistent, it is difficult to eliminate the relevant uncertainty by the means of calibration. Uncertainty $u(I_0)$ resulting from the (drift of) the background current of the conventional galvanic sensor used under ″typical″ conditions (stored in air, the range of the measured oxygen concentrations is from zero to 10 mg dm^{-3}) is as minimum 0.16 mg dm^{-3}. The sensor with two cathodes used for estimating the residual current of the conventional sensor is able to ″consume″ more efficiently the oxygen present in the electrolyte solution reservoir and in the insulating plastic body of the sensor.

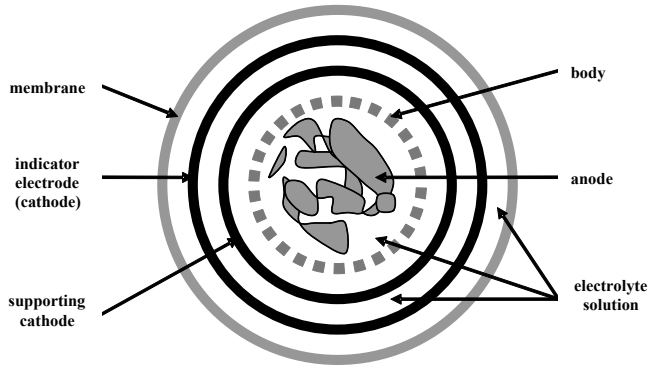


Figure 2. A schematic drawing of an oxygen sensor with supporting cathode (top view).

Ways of reducing the residual current include using a cathode of a large area, a small exposure area of the electrolyte reservoir, and a minimum plastic material in contact with this reservoir [28]. Even if the background current is significant, provided that it is constant, low oxygen concentrations can still be measured by using an imposed opposing current in the measuring circuit to compensate for the residual. Unfortunately, the background current of the oxygen sensor is not constant.

In oxygen-free samples the background currents in the circuits indicator electrode – anode and supporting cathode – anode of the sensor schematically depicted in figure 2, become equal in 5 minutes. As a result of opposing these two currents the total ″zero″ current did not exceed 0.01% from the (resulting) sensor current in air-saturated water, allowing the extension of dissolved oxygen measurement range to less than 10 μg dm^{-3}.

Drift of the diffusion layer thickness. According to equation 1, the drift of the diffusion layer thickness has an impact on the sensor current. The main cause of this drift is the instability of the thickness of the electrolyte layer between the membrane and the indicator electrode, taking place due to temperature changes, membrane aging and electrochemical reactions changing the volume of the electrolyte solution in the sensor. It is extremely difficult to measure the effective distance of the membrane from the indicator electrode. We assume that the change in the electrolyte layer thickness does not exceed 2 μm in one day and 5 μm during one year period provided that the sensor is handled in good manner. Experiments involving the output current measurements of the sensors with different l_e values allowed to calculate (using equation 1) the values of P_m and P_e. In the case of polyethylene membrane and 27% KOH electrolyte solution at 25 ^{0}C $P_m \cong P_e$. According to this, the 2μm electrolyte layer thickness drift results in the 2% change of the sensor current.

One of the ways to decrease this uncertainty component $u(\Delta l_e)$ is to find an electrolyte solution with higher permeability. This was secured by using an electrolyte solution prepared from H_2O, KOH, and C_2H_5OH. The solution containing 15% KOH and 39% C_2H_5OH proved to be appropriate. Higher ethanol concentrations caused a sufficient increase in the background current of the sensor. This electrolyte solution allowed the

elimination of the impact of electrolyte layer thickness change on measurement uncertainty.

Changes in the properties of the membrane. The unique characteristic of the Clark or Mackereth sensor is the membrane serving both to protect the sensing electrode and to give a reproducible electrochemical device. Although in principle a variety of membranes could be used for oxygen sensors, in practice the majority of them are supplied with polyethylene or fluorinated plastics [30]. In general, they have permeability that does not change noticeably over time. The P_m values are influenced by changes in properties of the membrane: deformation, aging, etc. During six months the average drift of the dissolved oxygen concentration value measured at 5 ^{0}C under saturation conditions was found to be equal to 1.2% [24]. This instability involves all the uncertainty factors listed above, and it remains unclear how in [24] the uncertainty $u(\Delta P_m)$ corresponding to the ″pure″ change of the membrane properties was determined. Implicit experiments (illustrated in figure 3) prove that this parameter in the case of our common Mackereth-type sensor does not exceed 1%. During the first month of operation the oxygen reduction current decreased ca 6% (mainly due to the partial blocking of the indicator electrode surface). After that the sensor showed an excellent stability for at least one year (see figure 3): the variation of the current did not exceed 2% in this period.

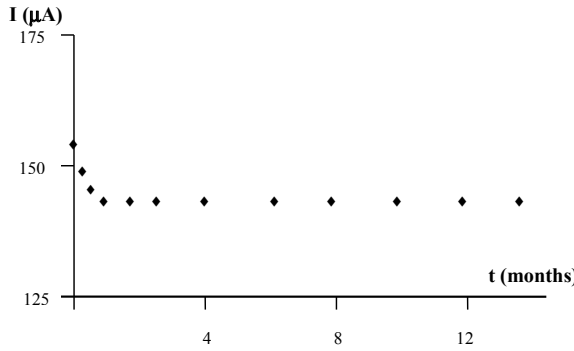


Figure 3. Output current I of a common Mackereth sensor during continuous operation, t is time.

Change in the activity of indicator electrode surface. During the first month of operation the current decrease of 6% is followed (see figure 3) due to the partial indicator electrode surface deactivation. Voltammetric measurements showed that the electrode surface is (presumably partly) covered with $Cd(OH)_2$. Afterwards, the sensor exhibits an excellent stability [15]. The only possible way of excluding the uncertainty component $u(\Delta A)$ arising from the deactivation of the indicator electrode is to use the sensor from the second month after mounting.

Response time. It is absolutely wrong to exclude the uncertainty $u(I_{trans})$ associated with the response time from the total uncertainty budget. The transient current for a sensor on going from de-aerated media into air-saturated water and back is in a few per cent of the steady-state value in a couple of minutes. The 95% response time of the sensor shown in figure 1 was 40-60 s. After 5 minutes the current reached the 2% zone of the steady-state

current and after 15 minutes the 1% zone. On the basis of this we should take into account the uncertainty component originating from the response time. Most likely in every-day work this value may reach up to 2% from the sensor current in air-saturated media. Using the sensor, schematically presented in figure 2, with a supporting cathode, and opposing the currents present in the circuits cathode – anode and supporting cathode – anode this source of uncertainty becomes negligible. For the both sensor types the current transients corresponding to a step change in oxygen concentration from air-saturation to zero level are shown in figure 4.

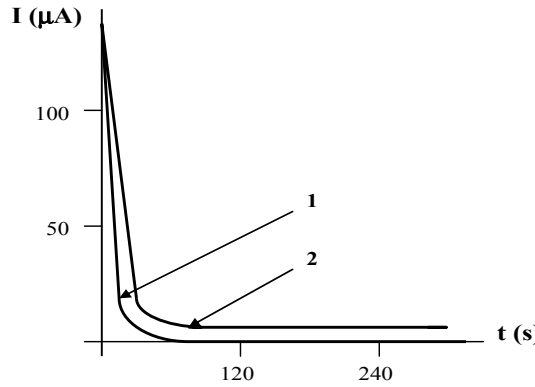


Figure 4. Current transient for a step change in oxygen concentration from air-saturation to zero level at 25 ^{0}C, I is the output current, t is time; 1 – sensor with the supporting cathode; 2 – conventional Mackereth sensor.

Other possible sources of uncertainty. There are still several other randomly occurring instability sources of the sensor: leakage of the electrolyte solution, unstable reference potential, localized electrolyte concentration changes and gas bubbles in the electrolyte solution [15]. The impact of these factors on the dissolved oxygen measurement uncertainty is mostly below 2% provided the sensor is in good condition. Alternatively, this component of uncertainty may exceed even 10% or more.

Once all the anode material has been consumed, the sensor no longer detects oxygen and fails [31]. This failure occurs rapidly and with little or no warning. The sensors presented in this paper last between 1.5 and 3 years, but they may fail within a couple of hours. It is of utmost importance to estimate the working condition of the oxygen sensor before, after, and presumably during oxygen concentration measurements. There are various intelligent ways of doing this [31].

Dissolved oxygen measurement uncertainty budgets

The contributions of different uncertainty sources to the dissolved oxygen measurement uncertainty are presented in Table 1 where: $u(I_{cal})$ originates from the calibration procedure, $u(I_0)$ results from the (drift of) the background current, $u(\Delta l_e)$ corresponds to the drift of the diffusion layer thickness, $u(\Delta P_m)$ - to the change of the membrane

properties, u(ΔA) arises from the deactivation of the indicator electrode (decrease of the active surface area) and $u(I_{trans})$ is associated with the response time of the sensor.

TABLE 1. Dissolved oxygen measurement uncertainty budgets.

Sensor	***Conventional*** [b]	***Modified*** [c]	***Conventional*** [b]	***Modified*** [c]
Oxygen content in water sample	*10 mg dm^{-3}*	*10 mg dm^{-3}*	*1 mg dm^{-3}*	*1 mg dm^{-3}*
$u(I_{cal})$, %	1.5	1.5	1.5	1.5
$u(I_0)$, %	1.6	0.01	5	0.1
$u(\Delta I_e)$, %	2	0.1	2	0.1
$u(\Delta P_m)$, %	1.2	1.2	1.2	1.2
u(ΔA), %	2	2	2	2
$u(I_{trans})$, %	2	0	2	0
$U(Co_2)$, % [a]	4.3	2.8	6.4	2.8
$U(Co_2)$, mg dm^{-3}	0.43	0.28	0.06	0.03

[a] $U(Co_2) = (\Sigma u_i^2)^{1/2}$; [b] conventional sensor – figure 1; [c] modified sensor – figure 2

According to the results presented in Table 1 it becomes evident, that no single uncertainty source is heavily dominating in dissolved oxygen measurement with both sensor types at oxygen concentrations around 10 mg dm^{-3}. If, instead of 10 mg O_2 dm^{-3} the measurements are carried out at 1 mg O_2 dm^{-3}, the picture changes: the impact of the uncertainty resulting from the background current increases remarkably. At lower oxygen concentrations the impact of the background current becomes predominating, when the conventional oxygen sensor is used. The level of uncertainty becomes too high (over 6%) when measuring dissolved oxygen concentrations below 1 mg dm^{-3} with a conventional Mackereth sensor, schematically depicted in figure 1, due to the higher contribution of the background current. The modified oxygen sensor (figure 2) with supporting inner cathode (which eliminates the background current) is free of this remarkable uncertainty component.

Conclusion

No single uncertainty source is heavily dominating in dissolved oxygen measurement at oxygen concentrations around 10 mg dm^{-3}. If, instead of 10 mg O_2 dm^{-3}, the measurement is carried out at 1 mg O_2 dm^{-3}, the picture changes: at lower oxygen concentrations the impact of the background current increases remarkably and becomes predominating, when a conventional oxygen sensor is used. The utilization of the modified sensor with supporting cathode and modified electrolyte solution leads to the significant drop of the impacts of background current and diffusion layer drift on the uncertainty of dissolved oxygen measurement. This is especially valuable in sewage treatment efficiency control, when oxygen concentrations are clearly below saturation.

Acknowledgments

The authors thank Ms Sandra V. Litvin for making corrections to this manuscript. L. N. thanks the Estonian Science Foundation for funding his research.

References

1. R. Neville, *US Patent 3 071 530* (1963).
2. D. E. Carritt and J. W. Kanwisher, *Anal. Chem.*, **31,** 5 (1959).
3. D. P. Lucero, *Anal. Chem.*, **41,** 613 (1969).
4. I. Bergman, *US Patent 1 200 595* (1970).
5. H. Suzuki, A. Suguma and N. Kojima, *Sensors and Actuators B*, **10,** 91 (1993).
6. H. Suzuki, A. Suguma, N. Kojima, F. Takei and K. Ikegami, *Biosensors Bioelectron*, **6,** 395 (1991).
7. W. Schelter, W. Gumbrecht, B. Montag, U. Sykora and W. Erhardt, *Sensors and Actuarors B*, **6,** 91 (1992).
8. T. G. Anjos and C. E. W. Hahn, *Sensors and Actuators B*, **135**, 224 (2008).
9. L. C. Clark, *US Patent 2 913 386* (1959).
10. R. G. Compton and R. J. Northing, *J. Chem. Soc. Faraday Trans.*, **86,** 1077 (1990).
11. F. J. H. Mackereth, *US Patent 3 322 662* (1967).
12. F. J. H. Mackereth, *J. Sci. Instrum.*, **41,** 38 (1964).
13. K. Bergmann, R. Marvet, V. Past, J. Raudsepp, T. Tenno and T. Jaetma, *SU Inventor's Cert. 604 403* (1977).
14. A. Koorits, L. Nei, V. Past, E. Reinot and T. Tenno, *SU Inventor's Cert. 1 408 992* (1987).
15. L. Nei and R. G. Compton, *Sensors and Actuators B*, **30,** 83 (1996).
16. L. Nei, *Fresenius J. Anal. Chem*., **367**, 436 (2000).
17. L. Nei, *ECS Trans.*, **2,** 33 (2007).
18. S. C. Chang, J. R. Stetter and C. S. Cha, *Talanta*, **40,** 461 (1993).
19. A. Mashirin, A. Koorits and T. Tenno, *Acta et Comment. Univ. Tartuensis*, **743,** 77 (1986).
20. K. Hellat, A. Mashirin, L. Nei and T. Tenno, *Acta et Comment. Univ. Tartuensis*, **757,** 184 (1986).
21. F. H. Dawson and P. Henville, *J. Phys. E: Sci. Instrum.*, **18,** 526 (1985)
22. H. Nösel, *Messtecnik*, **1,** 15 (1973).
23. L. Jalukse, I. Leito, A. Mashirin and T. Tenno, *Accred. Qual. Assur.*, **9,** 340 (2004).
24. L. Jalukse and I. Leito, *Meas. Sci. Technol.*, **18,** 1 (2007).
25. L. Nei, *Electroanalysis*, **7,** 471 (1995).
26. J. H. Hargraeves and C. H. Trucker, *SRAC Publication,* **4601** (2002).
27. C. G. Daughton and T. A. Ternes, *Environ. Health Persp.*, **107,** 907 (1999).
28. M. Carbella, F. Omil, J. M. Lema, M. Llompart, C. Carcía-Jares, I. Rodríguez, M. Gómez and T. Ternes, *Water Res.*, **38,** 2918 (2004).
29. V. Matamoros, M. Hijosa and J. M. Bayona, *Chemosphere*, **75,** 200 (2009).
30. M. L. Hitchman, *Measurement of Dissolved Oxygen*, pp. 71-73 and 88, Wiley Interscience, New York (1978).
31. P. R. Warburton, R. S. Sawtelle, A. Watsin and A. Q. Wang, *Sensors and Actuators B*, **72,** 197 (2001).

IV

Lillenberg, M., Yurchenko, S., Kipper, K., Herodes, K., Pihl, V., Lõhmus, R., Ivask, M., Kuu, A., Kutti, S., Litvin, S.V., Nei, L. (2010). PRESENCE OF FLUOROQUINOLONES AND SULFONAMIDES IN URBAN SEWAGE SLUDGE AND THEIR DEGRADATION AS A RESULT OF COMPOSTING. *International Journal of Environmental Science and Technology,* 7 (2), 307-312.

Int. J. Environ. Sci. Tech., 7 (2), 307-312, Spring 2010
ISSN: 1735-1472
© IRSEN, CEERS, IAU

Presence of fluoroquinolones and sulfonamides in urban sewage sludge and their degradation as a result of composting

*[1]M. Lillenberg; [2]S. Yurchenko; [2]K. Kipper; [2]K. Herodes; [2]V. Pihl; [3]R. Lõhmus; [4]M. Ivask; [4]A. Kuu; [4]S. Kutti; [4]S. V. Litvin; [4]*L. Nei*

[1]*Department of Food Science and Hygiene, Estonian University of Life Sciences, Kreutzwaldi 58A, 51014 Tartu, Estonia*

[2]*Institute of Chemistry, University of Tartu, Jakobi 2, 51014 Tartu, Estonia*

[3]*Institute of Physics, University of Tartu, Riia 142, 51014 Tartu, Estonia*

[4]*Department of Environmental Protection, Tartu College of Tallinn University of Technology, Puiestee 78, 51008 Tartu, Estonia*

Received 5 October 2008; revised 5 February 2010; accepted 25 February 2010; avaiable online 1 March 2010

ABSTRACT: The concentrations of some widely used pharmaceuticals, namely fluoroquinolones (ciprofloxacin $C_{17}H_{18}FN_3O_3$, norfloxacin $C_{16}H_{18}FN_3O_3$ and ofloxacin $C_{18}H_{20}FN_3O_4$) and sulfonamides (sulfadimethoxine $C_{12}H_{14}N_4O_4S$ and sulfamethoxazole $C_{10}H_{11}N_3O_3S$) were determined in urban sewage sludge utilized for making compost. The levels of degradation of these pharmaceuticals resulting from sludge treatment were assessed. The concentrations of the studied pharmaceuticals sufficiently varied both in sewage sludge and in compost and due to this phenomenon the possible danger resulting from the presence of pharmaceuticals in sewage sludge, used for composting, can not be ignored. The concentrations of the studied pharmaceuticals were lower in compost, if compared to the relevant concentrations in sewage sludge. The highest pharmaceutical concentration in sewage sludge - 426 µg/kg - was detected in the case of ciprofloxacin. The highest concentrations present in compost were 22 µg/kg of norfloxacin and 20 µg/kg of ciprofloxacin. Results show that before using the sewage sludge for making compost or before using the compost a fertilizer for food plants, they should be carefully tested against the content of commonly used pharmaceuticals.

Keywords: *Ciprofloxacin; Norfloxacin; Ofloxacin; Pharmaceuticals; Soil pollution; Sulfadimethoxine; Sulfamethoxazole*

INTRODUCTION

Due to rapid increase in urban population the amount of sewage sludge has increased dramatically in the past 20 y. Environmental pollution caused by sewage sludge has become a serious social problem, which hinders urban development. It is of utmost importance to find ways of effective re-usage of this waste and reduce its impact on the environment (Lu *et al.*, 2009). Sewage sludge can be used to generate energy which could be saved on the fossil fuels conventionally used as a source of energy (Babel *et al.*, 2009). Incineration is costly and contributes to air pollution and landfill space is becoming scare (Mahzuz *et al.*, 2009). The use of sewage sludge in agriculture is one of the major causes of environmental pollution (Nouri *et al.*, 2008). Sewage sludge is the residue from the treatment of domestic and industrial wastewater. It contains useful organic matter and nutrients for plants (Kaonga *et al.*, 2010). The contents of nitrogen, phosphorus and organic matter are up to 10 times higher in sewage sludge and its compost, if compared to common Estonian agricultural soils. Still, its usage as a fertilizer is limited due to a large number of toxic pollutants found in this matter. As the amount of sewage sludge is growing rapidly all over the world, the problems involving sewage sludge safety, treatment and usage are a global concern. Composting or aerobic biological treatment of organic wastes is an ancestral way to reduce wastes and to reuse organic matter. Among the range of existing organic wastes, sewage sludge composting enables the production of a quality product that may be used as a soil conditioner or as an organic fertilizer (Tremier *et al.*, 2005; Suthar and Sing, 2008) since its organic matter content can vary from 50 % to 70 % of the total solids content (Banegas *et al.*, 2007). The quality of compost depends on its

*Corresponding Author Email: *lembit.nei@ttu.ee*
Tel.: +372 5345 8322; Fax: +372 620 4801

environmental compatibility and the correspondence to market necessities. These features involve the absence of toxic inorganic and organic compounds, which may enter the food chain through the plant uptake (Lazzari *et al.*, 2000). Pharmaceuticals are often present in sewage sludge. Their quantities rise from year to year. In spite of the fact that very low drug levels in the environment can have undesirable ecological and health effects, until now the problems related to the presence of pharmaceuticals in sewage sludge and its compost have received little attention (Carballa *et al.*, 2007). Over the past decade, the scientific community has become increasingly interested in the impact of pharmaceutical contaminants on the environment and human health. A large proportion of pharmaceuticals is introduced into the environment via sewage treatment plants. A number of papers present the studies involving the occurrence and removal of pharmaceutical compounds in sewage treatment systems (Jones *et al.*, 2007; Matamoros *et al.*, 2009; Radjenović *et al.*, 2009). The presence and possible accumulation of these substances in sewage sludge are acknowledged. Current national and international regulations governing the application of sewage sludge ignore the presence and fate of pharmaceuticals. It has been shown that the degradation of pharmaceuticals may take place during bio-solid composting (Buyuksonmez and Sekeroglu, 2005). Potentials of phototrophic bacteria in treating pharmaceutical wastewater are shown in Madukasi *et al.*, (2010). No systematic work concerning biodegradation of pharmaceuticals during sewage sludge composting has been published. Remarkable amounts of pharmaceuticals enter the terrestrial environment by fertilizing with sewage sludge compost or manure (Halling-Sørensen *et al.*, 1998). No trigger values exist for their residues in sewage compost in the European Union. The content of drug residues should not exceed 100 µg/kg in manure and 10 µg/kg in soil (EMEA/CVMP/055/96, 1996). Little information is available on degradation of pharmaceuticals in the environment. It has been shown that antibacterial drugs can bind strongly to solid particles, which could be an additional reason for their slow degradation (Marengo *et al.*, 1997; Carmosini and Lee, 2008). Their residues with long persistence may be harmful for aquatic and terrestrial organisms; they may accumulate in food plants and finally reach human or animal food (Lillenberg *et al.*, 2003). Plants unlike animals have no excretion. As a result concentrations of drug residues in food plants may exceed the maximum residue limits (MRL) for meat and milk. Antibacterial drugs consumed in very small amounts with everyday food may generate the strains of resistant bacteria in human and animal organism, provoke allergy and affect the liver (Lillenberg *et al.*, 2003). The sewage sludge and sewage sludge compost from sewage treatment plants of the two Estonian largest cities – Tallinn and Tartu – were analyzed. Also, three random sewage samples from Tartu were analyzed with the aim of estimating the level of the residues of the studied pharmaceuticals. The selection of pharmaceuticals was made considering their stability in soil during 6-12 months period (Golet *et al.*, 2002a) and their potential ability to accumulate into plants (Migliore *et al.*, 1995; Migliore *et al.*, 1996; Lillenberg *et al.*, 2003). These pharmaceuticals include fluoroquinolones and sulfonamides: ciprofloxacin (CIP), norfloxacin (NOR), ofloxacin (OFL), sulfadimethoxine (SDM) and sulfamethoxazole (SMX). Fluoroquinolones and sulfonamides represent the most commonly used families of antibiotics (Pérez *et al.*, 2005; Picó and Andreu, 2007). CIP is the most widely prescribed fluoroquinolone in the world, followed by OFL. That was the reason why we carried out some experiments with the aim of studying the uptake of CIP by lettuce. NOR, an oral broad-spectrum antibacterial agent is very common in Europe (Picó and Andreu, 2007). SDM is indicated for the treatment of a wide range of respiratory, genitourinary tract, enteric, and soft tissue infections. SMX is an anti-bacterial sulfonamide that inhibits the synthesis of dihydrofolic acid, a compound that bacteria must be able to make to survive. As one of the most consumed sulfonamides in human medicine, this pharmaceutical has been reported most frequently and is usually considered ecologically harmful (García-Galán *et al.*, 2009). In the present study SDM and SMX were chosen as target antibiotics because of their widespread use (Isidori *et al.*, 2005; De Liguoro *et al.*, 2007). The current study was completed in 2007 and 2008 in Tartu, Estonia.

MATERIALS AND METHODS

The sewage sludge in Tartu is treated by composting – mixing with tree bark (volume ratio 2:3). The methane fermentation and mixing with peat (volume ratio 4:3) are used in Tallinn. The samples were taken in 2007-2008 from anaerobically digested sludge (before mixing with peat) in Tallinn and from untreated sludge (before composting) in Tartu, three samples in a month from both cities during a year. The compost samples were taken from stacks with different ages: 2, 6 and 12 months, 6 samples from different parts of stack in Tallinn and Tartu. Three stacks in both cities were piled up two

months before sampling. Two stacks in each city were made six months before taking samples; and one stack in Tartu and one in Tallinn had been stored for twelve months. The total amount of samples was 144, half of them were obtained from Tartu and half from Tallinn. Approximately 200 g of sludge (content of dry matter 28 % in Tallinn and 25 % in Tartu) or compost was placed into 500 mL glass jar, mixed thoroughly and covered hermetically with lid. Before analyses, the samples were stored in refrigerator at temperature + 4 °C in the dark. Lettuce was grown in plastic pots (Fig. 1), filled with soil amended with CIP. The initial concentrations of CIP in soil were: 500, 200, 50 and 10 mg/kg. Lettuce was harvested on the 42nd day after seeding. The concentration of CIP was determined using the agar diffusion methodology described in Lillenberg *et al.* (2003). Dried, grounded and weighted plant material was placed on an agar medium inoculated with antibiotic-sensitive bacterium *Bacillus subtilis*. The pharmaceutical diffused from plant material to the agar medium and inhibited the bacterial growth around the studied matter. The measured diameter of the inhibition circle allowed to determine the concentrations of the studied pharmaceutical in plant samples. A simple calibration procedure, using spiked plant matter, was used for generating calibration plots. The uncertainty of the drug concentration measurement was ± 10%. This methodology was chosen due to its availability, inexpensiveness and rapidness, if compared to the routinely used chromatographic work. Pharmaceuticals used for standardization were purchased from Riedel-de-Haën. Purity: CIP - 99.8 %, NOR - 99.9 %, OFL - 99.3 %, SDM - 99.4 %, SMX - 99.9 %. Acetonitrile and methanol were obtained from J.T.Baker, phosphoric acid from Lachema, citric acid monohydrate from Fisher Scientific, formic acid from Riedel-de-Haën, ammonium acetate from Fluka Chemie AG. All solvents were of reagent grade or higher quality. The following equipment was used for analysis: in-house built pressurized liquid extraction (PLE) system, hydrophilic-lipophilic balanced (HLB) solid phase extraction (SPE) cartridges (Oasis HLB (60 µm), 500 mg/6 mL) by Waters (Milford, MA, USA) and Agilent Series 1100 LC-MSD Trap XCT (Santa-Clara, CA, USA) with an analytical column Phenomenex Synergi Hydro-RP (250 mm x 4.6 mm, 4 um). Pharmaceuticals were extracted by PLE method, the extracts cleaned up by SPE method (Lillenberg *et al.*, 2009). The SPE step was necessary for removal of other organic contaminants that suppressed ionization and thus disabled the detection of minor amounts of substance. HPLC-MS/MS (liquid chromatography coupled with tandem mass spectrometry) was used for analysis (Lillenberg *et al.*, 2009). Relative standard deviation (RSD) of the determinations was within 2 %.

Fig. 1: Lettuce, 40th day after seeding

RESULTS AND DISCUSSION

In most of the studied sewage sludge and compost samples the concentrations of pharmaceuticals were clearly extremely low, but (surprisingly) in some sludge samples the content of pharmaceuticals still exceeded 100 µg/kg, and in some sewage compost samples the drug concentrations were above 10 µg/kg. The relevant concentration ranges of the detected pharmaceuticals for the whole set of studied sewage sludge and compost samples are presented in Table 1. Remarkable concentration variations can be followed due to the heterogeneity of the studied matter. Our results concerning the content of the studied residues of pharmaceuticals in Tartu sewage and some data concerning the occurrence of these compounds in U.S. streams have been included for comparison into Table 1. Interestingly CIP and OFL were not present in the randomly taken sewage samples in Tartu. In spite of this in some sludge and compost samples their presence was obvious, reaching to 111 µg/kg in the case of CIP and 39 µg/kg in the case of OFL. The large variability of the concentrations of pharmaceuticals in sewage and sewage sludge was apparent. As a result of this the concentration of pharmaceuticals varies noticeably within the same compost stack. An example of this phenomenon is presented in Table 2. The results presented in Table 2 show that the content of fluoroquinolones differs up to 1.8 times within the same stack. The determined

concentrations of sulfonamides were much lower, if compared to the concentrations of fluoroquinolones. Surprisingly, the concentration of SDM was under the detection limit (0.01 µg/kg). For another Tartu compost stack the SDM concentrations were in the range 0.1 - 0.4 µg/kg. The concentrations of the studied pharmaceuticals - fluoroquinolones and sulfonamides - sufficiently varied both in sewage sludge and in compost. Therefore the possible danger resulting from the content of pharmaceuticals in sewage sludge can not be ignored. The concentrations of the studied pharmaceuticals in compost were up to one order of magnitude lower, if compared to the relevant concentrations in sewage sludge. Partly this effect is caused by adding peat or tree bark to sewage sludge. Still, the main reason of the decrease in pharmaceutical concentrations during composting is the applied sludge treatment technology. The safest way to exclude exposing plants to pharmaceuticals in to ensure that these substances are adequately degraded before sewage sludge compost is applied onto arable land. The decomposition of pharmaceuticals was faster in the case of Tallinn composting technology. Interestingly, SDM was present in most sludge and 2 month stored compost samples, although this antimicrobial was not marketed in the years 2007 and 2008 in Estonia. It is possible that 3 old supplies were put to use or small amounts of this chemical were imported from other countries. It is known that fluoroquinolones are eliminated in conventional sewage treatment by 90 % with sorption to sewage sludge being the main process responsible (Golet *et al.*, 2002b, 2003). For sulfonamides, sorption to sewage sludge has been found to be of minor importance (Göbel *et al.*, 2005, 2007). As the highest concentrations were detected for CIP, the results of the experiments carried out with the aim of studying the uptake of CIP by lettuce (and described in detail in Lillenberg *et al.*, 2003) are shown in a generalized form in Table 3. In the case of relatively low CIP concentrations in soil (less than 50 mg/kg) a remarkable accumulation of this pharmaceutical by lettuce was followed. This clearly shows that before using the sewage sludge compost as a fertilizer for crops it should be tested against the residues of commonly used pharmaceuticals, and the presence and breakdown of a variety of other compounds of concern needs to be studied under realistic conditions, leading to field-scale trials. Further investigations on the on the mobility and bioavailability, persistence, degradation pathways and kinetics of pharmaceuticals present in sewage, sewage sludge, compost, soils and plants are needed. Other bulking agents, as for example sawdust (Banegas *et al.*, 2007) can be considered for use with sewage sludge.

Table 1: Pharmaceuticals in sewage sludge and compost

		Concentrations of pharmaceuticals, µg/kg				
Place	Sample	NOR	CIP	OFL	SMX	SDM
Tartu	sewage*	0.07	0.00	0.00	0.42	0.01
	sludge	0.0-110	2.6-111	0.5-39	0.0-2.8	0.0-7.9
	compost	0.0-22	0.0-20	0.0-3.2	0.0-0.9	0.0-4.2
Tallinn	sludge	0.0-162	0.0-426	0.0-38	0.0-6.0	0.0-20
	compost	0.0-5.4	0.0-7.1	0.0-0.5	0.0-0.3	0.0-0.2
USA	streams**	0.12	0.49	0.03[1]	0.15	0.06

*3 sewage samples were taken randomly: average concentrations of pharmaceuticals

**139 stream sites in on areas considered susceptible to contamination from wastewater, median concentrations are presented (Kolpin *et al.*, 2002; except[1] - detected in Seine River: Tamtam *et al.*, 2008)

Table 2: The concentrations of pharmaceuticals in compost samples obtained from different parts of the same compost stack in Tartu

	Concentrations of pharmaceuticals (µg/kg)				
Sample No.	NOR	CIP	OFL	SMX	SDM
1	12.0	13.1	2.8	0.6	0.0
2	11.1	11.0	2.6	0.2	0.0
3	12.0	12.7	2.4	0.5	0.0
4	7.7	9.1	1.8	0.4	0.0
5	10.8	10.6	2.3	0.0	0.0
6	6.6	7.7	1.6	0.2	0.0

Table 3: Ciprofloxacin uptake by lettuce

Initial CIP concentration in soil, mg/kg	500	200	50	10
CIP in lettuce, 42nd day after seeding, mg/kg	223	163	58	44

CONCLUSION

This study showed that the concentrations of the studied fluoroquinolones (ciprofloxacin, norfloxacin and ofloxacin) and sulfonamides (sulfadimethoxine and sulfamethoxazole) - sufficiently varied both in sewage sludge and in compost. The concentrations of pharmaceutical residues in compost were significantly lower, if compared to the relevant concentrations in sewage sludge. The degradation of pharmaceutical residues was more efficient in Tallinn probably due to anaerobical sludge digestion (compost was made by mixing the treated sewage sludge with peat), if compared to the results obtained in Tartu (raw sewage sludge was mixed with tree bark). It is concluded that before using the sewage sludge compost as a fertilizer it should be carefully tested against the content of different pharmaceuticals. The content of pharmaceuticals in the compost made from sewage sludge may easily lead to the elevated concentrations in food plants, if the compost is used as a fertilizer. This work shows that studies of the sewage sludge used for making compost and the development of novel sewage sludge treatment technologies are needed with the aim of solving environmental problems related to sewage sludge exploitation.

ACKNOWLEDGEMENTS

This work was funded by Estonian Environmental Investment Centre, Estonian Science Foundation (different grants) and Estonian research target project SF 0180058s07.

REFERENCES

Babel, S.; Sae-Tang, J.; Pecharaply, A., (2009). Anaerobic co-digestion of sewage and brewery sludge for biogas production and land application. Int. J. Environ. Sci. Tech., 6 (1), 131-140 **(10 pages)**.

Banegas, V.; Moreno, J. L.; Moreno, J. I.; García, C.; León, G.; Hernández, T., (2007). Composting anaerobic and aerobic sewage sludges using two proportions of sawdust. Waste Manage., 27 (10), 1317-1327 **(11 pages)**.

Buyuksonmez, F.; Sekeroglu, S., (2005). Presence of pharmaceuticals and personal care products (PPCPs) in biosolids and their degradation during composting. J. Resour. Sci. Tech., 2 (1), 31-40 **(10 pages)**.

Carballa, M.; Omil, F.; Ternes, T.; Lema, M. J., (2007). Fate of pharmaceutical and personal care products (PPCPs) during anaerobic digestion of sewage sludge. Water Resour., 41(10), 2139-2150 **(12 pages)**.

Carmosini, N.; Lee, L. S., (2008). Sorption and degradation of selected pharmaceuticals in soil and manure, in: Aga, D. S. (Ed.), Fate of pharmaceuticals in the environment and in water treatment systems. CRC Press, Boca Raton, London, New York.

De Liguoro, M.; Poltronieri, C.; Capolongo, F; Montesissa, C., (2007). Use of sulfadimethoxine in intensive calf farming: Evaluation of transfer to stable manure and soil. Chemosphere, 68 (4), 671-676 **(6 pages)**.

EMEA/CVMP/055/96, (1996). Commitee for Veterinary Medicinal Poducts. Note for guidance: environmental risk assessment for veterinary medicinal products other than GMO-containing and immunological products.

García-Galán, M. J.; Díaz-Cruz, M. S.; Barceló, D., (2009). Combining chemical analysis and ecotoxicity to determine environmental exposure and to assess risk from sulfonamides. Trends Anal. Chem., 28 (6), 804-819 **(16 pages)**.

Göbel, A.; McArdell, C. S.; Joss, A.; Siegrist, H.; Giger, W. (2007). Fate of sulfonamides, macrolides, and trimethoprim in different wastewater treatment technologies. Sci. Total Environ. 372 (2-3), 361-371 **(11 pages)**.

Göbel, A.; Thomsen, A.; McArdell, C. S.; Joss, A.; Giger, W., (2005). Occurrence and Sorption Behavior of Sulfonamides, Macrolides, and Trimethoprim in Activated Sludge Treatment. Swiss Federal Institute for Environmental Science and Technology (EAWAG), CH-8600 Dbendorf, Switzerland. Environ. Sci. Tech., 39 (11), 3981-3989 **(9 pages)**.

Golet, E. M.; Alder, A. C.; Giger, W., (2002a). Environmental exposure and risk assessment of fluoroquinolone antibacterial agents in wastewater and river water of the Glatt valley watershed, Switzerland. Environ. Sci. Tech., 36 (17), 3645-3651 **(7 pages)**.

Golet, E. M.; Strehler, A.; Alder, A. C.; Giger, W., (2002b). Determination of fluoroquinolone antibacterial agents in sewage sludge and sludge-treated soil using accelerated solvent extraction followed by solid-phase extraction. Anal. Chem., 74 (21) 5455-5462 **(8 pages)**.

Golet, E. M.; Xifra, I.; Siegrist, H.; Alder, A. C.; Giger, W., (2003). Environmental exposure assessment of fluoroquinolone antibacterial agents from sewage to soil. Environ. Sci. Tech., 37 (15), 3243-3249 **(7 pages)**.

Halling-Sørensen, B.; Nielsen, S. N.; Lanzky, P. F.; Ingerslev, F.; Lützhøft, H. C. H.; Jørgensen, S. E., (1998). Occurrence, fate and effects of pharmaceutical substances in the environment - A review. Chemosphere, 36 (2), 357-394 **(38 pages)**.

Isidori, M.; Lavorgna, M.; Nardelli, A., (2005). Toxic and genotoxic evaluation of six antibiotics on non-target organisms. Sci. Total Environ., 346 (1-3), 87-98 **(12 pages)**.

Jones O. A. H.; Voulvoulis N.; Lester J. N., (2007). The occurrence and removal of selected pharmaceutical compounds in a sewage treatment works utilising activated sludge treatment. Environ. Pollut., 145 (3), 738-744 **(7 pages)**.

Kaonga, C. C.; Kumwenda, J.; Mapoma, H. T., (2010). Accumulation of lead, cadmium, manganese, copper and zinc by sludge worms; *Tubifex tubifex* in sewage sludge. Int. J. Environ. Sci. Tech., 7 (1), 119-126 **(8 pages)**.

Kolpin, D. W.; Furlong, E. T.; Meyer, M. T.; Thurman, E. M.; Zaugg, S. D.; Barber, L. B.; Buxton H. T., (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. Environ. Sci. Tech., 36 (6), 1202-1211 **(10 pages)**.

Lazzari, L.; Sperni, L.; Bertin, P.; Pavoni, B., (2000). Correlation between inorganic (heavy metals) and organic (PCBs and PAHs) micropollutant concentrations during sewage sludge composting processes. Chemosphere, 41 (3), 427-435 **(9 pages)**.

Lillenberg, M.; Roasto, M.; Püssa, T., (2003). Drug residues in environment. Estimation of fluoroquinolones in soil and food plants. J. Agr. Sci., 14 (1), 13-26 **(14 pages)**.

Lillenberg, M.; Yurchenko, S.; Kipper, K.; Herodes, K.; Pihl, V.; Sepp, K.; Lõhmus, R; Nei, L., (2009). Simultaneous determination of fluoroquinolones, sulfonamides and tetracyclines in sewage sludge by pressurized liquid chromatography and electrospray ionization-mass spectrometry. J. Chromatographia. A, 1216 (32), 5949-5954 **(6 pages)**.

Lu, Y. J.; Wu, X. W.; Guo, J. F., (2009). Characteristics of municipal solid waste and sewage sludge co-composting. Waste Manage., 29 (3), 1152-1157 **(6 pages)**.

Madukasi, E. I.; Dai, X.; He, C.; Zhou, J., (2010). Potentials of phototrophic bacteria in treating pharmaceutical wastewater. Int. J. Environ. Sci. Tech., 7 (1), 165-174 **(10 pages)**.

Mahzuz, H. M. A.; Alam, R.; Alam, N. M.; Basak, R.; Islam, S. M., (2009). Use of arsenic contaminated sludge in making ornamental bricks. Int. J. Environ. Sci. Tech., 6 (2), 291-298 **(8 pages)**.

Marengo, J. R.; Kok, R. A.; O´Brien, G. K.; Velagaletti, R. R.; Stamm, J. L., (1997). Aerobic biodegradation of (C-14)-sarafloxacin hydrochloride in soil. Environ. Toxicol. Chem., 16 (3), 462-471 **(10 pages)**.

Matamoros V.; Hijosa M.; Bayona J. M., (2009). Assessment of the pharmaceutical active compounds removal in wastewater treatment systems at enantiomeric level. Ibuprofen and naproxen. Chemosphere, 75 (2), 200-205 **(6 pages)**.

Migliore, L.; Brambilla, G.; Casoria, P.; Civitareale, C.; Cozzolino, S.; Gaudio, L., (1996). Effect of sulphadimethoxine contamination on barley (*Hordeum distichum L, Poaceae, Liliopsida*). Agricult. Ecosyst. Environm., 60 (2-3), 121-128 **(8 pages)**.

Migliore, L.; Brambilla, G.; Cozzolino S.; Gaudio, L., (1995). Effect on plants of sulfadimethoxine used in intensive farming (*panicum-miliaceum, pisum-sativum and zea-mays*). Agricult. Ecosyst. Environ., 52 (2-3), 103-110 **(8 pages)**.

Nouri, J.; Mahvi, A. H.; Jahed, G. R.; Babaei, A. A., (2008). Regional distribution pattern of groundwater heavy metals resulting from agricultural activities. Environ. Geo., 55 (6), 1337-1343 **(7 pages)**.

Pérez, S.; Eichhorn, P.; Aga D. S., (2005). Evaluating the biodegradability of sulfamethazine, sulfamethoxazole, sulfathiazole and trimethoprim at different stages of sewage treatment. Environ. Toxicol. Chem., 24 (6), 1361-1367 **(7 pages)**.

Picó, Y.; Andreu, V., (2007). Fluoroquinolones in soil - risks and challanges. Anal. Bioanal. Chem., 387, 1287-1299 **(13 pages)**.

Radjenovic J.; Petrović M.; Barceló D., (2009). Fate and distribution of pharmaceuticals in wastewater and sewage sludge of the conventional activated sludge (CAS) and advanced membrane bioreactor (MBR) treatment. Water Res., 43 (3), 831-841 **(11 pages)**.

Suthar, S.; Singh, S., (2008). Vermicomposting of domestic waste by using two epigeic earthworms (Perionyx excavatus and Perionyx sansibaricus). Int. J. Environ. Sci. Tech., 5 (1), 99-106 **(8 pages)**.

Tamtam, F.; Mercier, F.; Le Bot, B.; Eurin, J., Dinh, Q. T.; Clément, M.; Chevreuil, M., (2008). Occurrence and fate of antibiotics in the Seine River in various hydrological conditions. Sci. Total Environ., 393 (1), 84-95 **(12 pages)**.

Tremier, A.; de Guardia, A.; Massiani, C.; Paul, E.; Martel, J. L., (2005). A respirometric method for characterising the organic composition and biodegradation kinetics and the temperature influence on the biodegradation kinetics, for a mixture of sludge and bulking agent to be co-composted. Bioresour. Tech., 96 (2), 169-180 **(12 pages)**.

AUTHOR (S) BIOSKETCHES

Lillenberg, M., M.Sc., Lecturer, Institute of Food Science and Hygiene; Ph.D. Student, Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Tartu, Estonia. Email: *merike.lillenberg@emu.ee*

Yurchenko, S., Ph.D., Research Fellow, Institute of Chemistry, University of Tartu, Estonia. Email: *sergei.yurchenko@mail.ee*

Kipper, K., M.Sc., Ph.D. Student, Institute of Chemistry, University of Tartu, Estonia. Email: *karin.kipper@gmail.com*

Herodes, K., Ph.D., Associate Professor, Institute of Chemistry, University of Tartu, Estonia. Email: *koit.herodes@ut.ee*

Pihl, V., Ph.D., Research Fellow, Institute of Chemistry, University of Tartu, Estonia. Email: *viljar.pihl@ut.ee*

Lõhmus, R., Ph.D., Senior Research Fellow, Institute of Physics, University of Tartu, Estonia. Email: *runno.lohmus@ut.ee*

Ivask, M., Professor, Department of Environmental Protection, Tartu College, Tallinn University of Technology, Tartu, Estonia. Email: *mari.ivask@ttu.ee*

Kuu, A., Ph.D., Lecturer, Department of Environmental Protection, Tartu College, Tallinn University of Technology, Tartu, Estonia. Email: *annely.kuu@ttu.ee*

Kutti, S., M.Sc., Department of Environmental Protection, Tartu College, Tallinn University of Technology, Tartu, Estonia. Email: *sanderkutti@gmail.com*

Litvin, S. V., B.Sc., M.Sc. Student, Tartu College, Tallinn University of Technology, Tartu, Estonia. Email: *sandlitv@yahoo.com*

Nei, L., Director and Professor, Tartu College, Tallinn University of Technology, Tartu, Estonia. Email: *lembit.nei@ttu.ee*

How to cite this article: (Harvard style)

Lillenberg, M.; Yurchenko, S.; Kipper, K.; Herodes, K.; Pihl, V.; Lõhmus, R.; Ivask, M.; Kuu, A.; Kutti, S.; Litvin, S. V.; Nei, L., (2010). Presence of fluoroquinolones and sulfonamides in urban sewage sludge and their degradation as a result of composting. Int. J. Environ. Sci. Tech., 7 (2), 307-312.

Lillenberg, M., Herodes, K., Kipper, K., Nei, L. (2010).
PLANT UPTAKE OF SOME PHARMACEUTICALS
FROM FERTILIZED SOILS.
Proceedings of 2010 International Conference on Environmental Science and Technology, Bangkok, Thailand, 23-25 April, 161-165.

ISBN 978-1-84626-xxx-x
Proceedings of 2009 International Conference on Environmental Science and Technology
Bangkok, Thailand, 23-25 April, 2010, pp. xxx-xxx

Plant Uptake of some Pharmaceuticals from Fertilized Soils

Merike Lillenberg [1], Koit Herodes [2], Karin Kipper [2] and Lembit Nei [3 +]

[1] Department of Food Science and Hygiene, Estonian University of Life Sciences, Kreutzwaldi 58A, 51014 Tartu, Estonia

[2] Institute of Chemistry, University of Tartu, Jakobi 2, 51014 Tartu, Estonia

[3] Department of Environmental Protection, Tartu College of Tallinn University of Technology, Puiestee 78, 51008 Tartu, Estonia

Abstract. Land application of sewage sludge can be a source of the contamination of food plants by pharmaceutical products. In this study the uptake of ciprofloxacin, ofloxacin, norfloxacin, sulfadimethoxine and sulfamethoxazole from soil into lettuce was demonstrated. In spite of this phenomenon the concentrations of the studied pharmaceuticals were clearly low in the plant samples, if compared to their concentrations in soil.

Keywords: pharmaceuticals, sewage sludge, plant uptake, compost

1. Introduction

Remarkable amounts of pharmaceuticals can enter the terrestrial environment by fertilizing with sewage sludge compost or manure [1]. Medical substances have many of the necessary properties to bio-accumulate and provoke changes in ecosystems. Most of the relevant studies are dealing with elevated drug concentrations. The accumulation of pharmaceuticals into plants depends on many factors and mainly on their soil concentrations [2-5]. No trigger values have been established for drug residues in sewage compost in the European Union [6]. Trigger values have been established for drug residues in manure [7]. These should not exceed 100 μg/kg in manure and 10 μg/kg in soil fertilized with manure. According to [8], these figures should be remarkably lower. Plants unlike animals have no excretion. Therefore, drug residues may accumulate into plants [5]. No limits have been set for drugs in plant products at present. Some of the studies claim that the uptake of some drugs from soil into plants is negligible [4, 9], but in [10] the importance of the relevant research is pointed out. It is considered to be very useful to learn the degradation of pharmaceuticals when diverse sewage sludge treatment technologies are applied.

Sulfonamides have been detected from raw sewage sludge [11] and from treated sewage [12]. Sulfamethoxazole is not biodegradable in sewage treatment tanks [13]. Elevated concentrations of fluoroquinolones have been found from treated sewage and from soil fertilized with sewage sludge [14]. Tetracyclines do not decompose in soil during a 6 months period [15]. The slow degradation is explained with their strong adsorption to solid particles [16-18]. Different ways of sewage sludge treatment including composting and methane fermentation are known. Although the usage of such compost as a fertilizer widely takes place in East Europe, the presence of drug residues in this matter has been ignored. The possible degradation of drug residues as a result of sludge composting still needs to be studied.

Humans may be exposed to residues of drugs in the environment by a number of routes including the consumption of crops that have accumulated substances from fertilized soils [4]. Experiments at elevated

+ Corresponding author. Tel.: + 372 53458322; fax: +372 6204801.
E-mail address: lembit.nei@ttu.ee.

concentrations of pharmaceuticals in soil have shown that they are able to accumulate in plants [19]. There is a need to determine the significance of uptake into plants from soil under "real" conditions as a potential route for pharmaceuticals. Therefore, the current study was conducted to investigate the potential for some widely used pharmaceuticals - fluoroquinolones, tetracyclines and sulfonamides – to be taken up by food plants (namely lettuce in this study) from soil fertilized with sewage sludge or its compost.

2. Experimental

2.1. Chemicals and equipment

Pharmaceuticals were purchased from Riedel-de-Haën (Seelze, Germany) - three fluoroquinolones: ciprofloxacin (CIP, purity 99.8%), norfloxacin (NOR, purity 99.9%) and ofloxacin (OFL, purity 99.3%); two sulfonamides: sulfadimethoxine (SDM, purity 99.4%) and sulfamethoxazole (SMX, purity 99.9%). Strong cation exchange (SCX) cartridges (Strata SCX (55 μm, 70 Å) 500 mg/6mL) were supplied by Phenomenex (Torrance, CA, USA); Hydrophilic-lipophilic balanced (HLB) cartridges (Oasis HLB (60 μm), 500 mg/6 mL) by Waters (Milford, MA, USA). Acetonitrile and methanol were obtained from J. T. Baker (Deventer, The Netherlands), phosphoric acid from Lachema (Brno, Czech Republic), citric acid monohydrate from Fisher Scientific (Pittsburgh, PA, USA), formic acid from Riedel-de-Haën, ammonium acetate from Fluka (Buchs, Germany). All solvents were of reagent grade or higher quality.

2.2. Liquid extraction

250 mg of dried lettuce (roots or leaves) was extracted with 10 mL of 1:1 (v/v) mixture of acetonitrile and 1% acetic acid, then homogenized with laboratory homogenizer DIAX 900 (Heidolph Instruments, Germany) 25 000 rpm, sonicated (5'), vortexed (1') and centrifuged at 8000 rpm. The supernatant was then separated and dried by nitrogen stream. Approximately 15 mL of 1 % acetic acid was added to the 1 mL of evaporation residue.

2.3. Solid-phase extraction

The extract collected by liquid extraction was cleaned up by solid phase extraction (SPE). Antibiotics - CIP, NOR, OFL, SDM and SMX - were extracted using HLB cartridges. For SPE procedure the vacuum manifold, supplied by Agilent Technologies, was used. HLB cartridges were preconditioned with 20 mL of methanol and 10 mL of Milli-Q water. The sample was loaded at a rate 6 mL/min. After extraction, the compounds were eluted from cartridges using 12 mL of methanol. The SPE extracts were concentrated in polypropylene vials in N_2 stream [22]. Residue was dissolved in 1 mL of 10 % methanol with buffer solution (5 mM 1,1,1,3,3,3-hexafluoro-2-propanol, pH adjusted to 9.0 with NH_4OH).

2.4. LC-MS

The SPE extracts were analyzed by LC–MS (Agilent Series 1100 LC-MSD Trap XCT (Santa-Clara, CA, USA)) equipped with a binary pump, a degasser, an auto-sampler and a column thermostat. Antibiotics were chromatographed using a Waters XBridge C18 column (150 mm × 3 mm, 3.5 μm) equipped with a Waters Guard Cartridge 4.6 mm × 20 mm. Electrospray interface (ESI) was used in positive ion mode for ionization. Selected reaction monitoring was used. Full MS^2 were recorded and the following transitions were applied for quantification: OFL m/z 362→ 261, 318, NOR m/z 320→302, 276, CIP m/z 332→288, 314, SMX m/z 254→108, 188, SDM m/z 311→108, 156, 218, 245. For instrument control and data analysis software: Agilent ChemStation for LC Rev. A. 10.02; MSD Trap Control version 5.2 and Data Analysis for LC-MSD Trap 3.2. were used. Gradient elution was carried out with methanol and HFIP buffer solution (5 mM 1,1,1,3,3,3-hexafluoro-2-propanol, pH adjusted to 9.0 with NH_4OH). The linear gradient started at 10% methanol and was raised to 100% within 50 min, after that methanol concentration was 100% for 5 min then lowered to 10% in 5 min and kept in 10% for 5 min. The eluent flow rate was 0.3 mL/min, the column temperature was set to 30 °C and the injection volume was 10 μL.

2.5. Lettuce samples

Aqueous solutions of the studied pharmaceuticals were mixed with soil. The final concentration of each pharmaceutical was 10 mg/kg (dry weight). With the aim of assuring better dissolution of the studied

pharmaceuticals fluoroquinolones were dissolved in 2 ml of 0.1 mM ammonium acetate buffer solution with pH=2.8 and sulfonamides were dissolved in 2 ml of 0.3 M NaOH. The seeds of lettuce *(Lactuca sativa)* were sowed into the pots. Lettuce was cultivated in the presence of the five pharmaceuticals during 70 days from sowing. Then the lettuce was collected, dried and milled. The roots were separated from the leaves. The milled lettuce (both the leaves and the roots) was hold in hermetical plastic bags at –80 °C. Before analysis the samples were dried at 45 °C. The novel methodology for the simultaneous determination of the studied pharmaceuticals is described in detail in [22].

3. Results and Discussion

A pilot study of sewage sludge from two Estonian largest cities, Tartu and Tallinn, was performed in 2008. The selection of pharmaceuticals was made considering their stability in soil and potential accumulation into plants. These pharmaceuticals included fluoroquinolones and sulfonamides. The concentrations of NOR, OFL and CIP; SDM and SMX were determined in sewage sludge. In all samples the residues of fluoroquinolones and sulfonamides were present. The highest contents of fluoroquinolones were: 110 µg/kg (NOR), 39 µg/kg (OFL) and 111 µg/kg (CIP) in Tartu; 162 µg/kg (NOR), 38 µg/kg (OFL) and 426 µg/kg (CIP) in Tallinn. All these concentrations exceed the trigger value for manure. The contents of sulfonamides remained below the trigger value for manure. The highest concentrations in Tartu were 8 µg/kg (SDM) and 3 µg/kg (SMX); in Tallinn 21 µg/kg (SDM) and 6 µg/kg (SMX). For sewage sludge compost the relevant concentrations were somewhat 3-30 times lower.

Recoveries for the detected pharmaceuticals varied from 86 to 97%. Typical chromatograms for SMX, SDM, NOR; CIP and OFL, obtained in the case of lettuce root sample, are shown in Fig. 1.

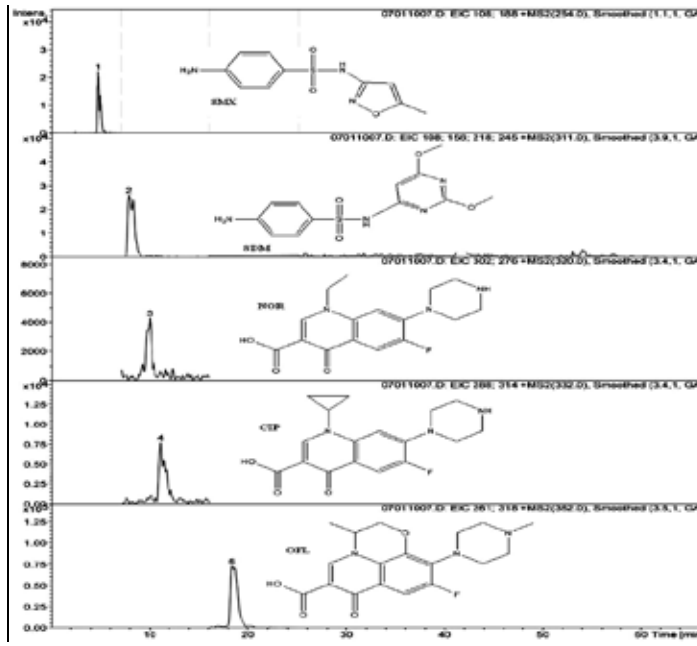


Fig. 1: Chromatograms for SMX, SDM, NOR, CIP and OFL in lettuce root samples.

None of the studied pharmaceuticals was detected in the control samples of lettuce leaves or roots. The results showing the uptake of the studied pharmaceuticals into lettuce roots and leaves from soil (with the 10 mg/kg concentrations of each studied pharmaceutical), are presented in table 1.

Table 1. Pharmaceuticals in different parts of lettuce

	Concentrations of pharmaceuticals, µg/kg				
Sample	CIP	NOR	OFL	SMD	SMX
roots	560	500	1180	3700	3650
leaves	220	n.d.	140	n.d.	n.d.

n.d. – not detected

Although all the studied pharmaceuticals were present in Tallinn and Tartu sewage sludge, raw sludge is not used for fertilizing soils. In Tartu sewage sludge is treated by composting – mixing with tree bark (volume ratio 2:3). The methane fermentation and mixing with peat (volume ratio 4:3) are used in Tallinn. As a result the levels of the residues of pharmaceuticals are subsequently diminishing. In spite of the fact that no limits have been set for pharmaceutical residues in plant products, some maximum residue values have been established for milk, meat and other animal-based products. For example, the total concentration of sulfonamides and the total concentration of ciprofloxacin and enrofloxacin in animal-based food products should not exceed 100 µg/kg (both) [23]. The results presented in table 1 have been received at relatively high concentrations (10 mg/kg) of pharmaceuticals in soil, but for CIP and OFL the possibility exists that even under "mild" conditions their concentrations in lettuce leaves may approach to undesirable levels.

A number of pharmaceuticals have been shown to be taken up by plants from soils [4]. Predictions of the potential exposure of the studied pharmaceuticals following common exploitation pathways indicate that the intake of these chemicals by humans via plant-derived food is low and the effects on human health, as in the case of a variety of veterinary medicines [4], are unlikely.

4. Conclusion

In the current study, uptake of ciprofloxacin, norfloxacin, ofloxacin, sulfadimethoxine and sulfamethoxazole was demonstrated in lettuce. The uptake of fluoroquinolones and sulfonamides by plants like lettuce does not seem to be a major human health risk, as the detected levels of the studied pharmaceuticals were relatively low, if compared to their soil concentrations. Further studies are needed to determine the uptake of different types of pharmaceuticals and other organic pollutants by various crop plants.

5. Acknowledgements

This work was funded by Estonian Environmental Investment Centre, Estonian Science Foundation (grant no 7127) and Estonian research target project SF 0180058s07.

6. References

[1] B. Halling-Sørensen, S. Nors Nielsen, P. F. Lanzky, F. Ingerslev, H. C. Holten Lützhøft, and S. E. Jørgensen Occurrence, fate and effects of pharmaceutical substances in the environment – a review. *Chemosphere*. 1998, **36** (2): 357-393.

[2] L. Migliore, G. Brambilla, S. Cozzolino, and L. Gaudio. Effects on plants of sulphadimethoxine used in intensive farming *(Panicum miliaceum, Pisum sativum and Zea Mays). Agric. Ecosyst. Environ.* 1995, **52** (2-3): 103-110.

[3] G. Brambilla, P. Casoria, C. Civitareale, S. Cozzolino, L. Gaudio, and L. Migliore. Sulphadimetoxine as environmental tracer to evaluate phytotoxity in crop. *Proc. of Conference on Residues of Veterinary Drugs in Food.* Veldhoven: 1996, pp. 292-295.

[4] A. B. A. Boxall, P. Johnson, E. J. Smith, C. J. Sinclair, E. Stutt, and L. S. Levi. Uptake of veterinary medicines from soil into plants. *J. Agric. Food Chem*. 2006, **54** (6): 2288-2297.

[5] H. Dolliver, K. Kumar, S. C. Gupta. Sulfamethazine uptake by plants from manure-amended soil. *J. Environ. Qual*. 2007, **36** (4): 1224-1230.

[6] EUR-Lex – 31986L0278-EN; *Council Directive* 86/278/EEC of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture.

[7] EMEA/CVMP/055/96. Environmental risk assessment for veterinary medicinal products other than GMO - containing and immunological products. The European Agency for the Evaluation of Medicinal Products, 1997.

[8] M. H. M. M. Montforts, The trigger values in the environmental risk assessment for (veterinary) medicines in the European Union: a critical appraisal. RIVM report 601500002/2005, p. 37.

[9] S. Thiele Bruhn. Pharmaceutical antibiotic compounds in soils – a review. *J. Plant. Nutr. Soil Sci*. 2003, **166** (4), 145-167.

[10] P. K. Jjemba. The potential impact of veterinary and human therapeutic agents in manure and biosolids on plants grown on arable land: a review. *Agric. Ecosyst. Environ*. 2002, **93** (1-3): 267-278.

[11] A. Thomsen, A. Göbel, C.S. Mc.Ardell, A.C. Alder, and W. Giger. Determination of sulfonamide and macrolide antibiotics in sewage sludge using accelerated solvent extraction and LC-MS/MS. Presentation of project results on Poseidon Symposium, Braunschweig, 2003.

[12] R. H. Lindberg, P. Wennberg, M. I. Johansson, M. Tysklind, and B. A. V. Andersson. Screening of human antibiotic substances and determination of weekly mass flows in five sewage treatment plants in Sweden. *Environ. Sci. and Tech.* 2005, **39** (10): 3421-3429.

[13] M. L. Richardson, and J. M. Bowron. The fate of pharmaceutical chemicals in the aquatic environment – a review. *J. Pharmacol*. 1985, **37** (1): 1-12.

[14] E. M. Golet, A. Strehler, A. C. Alder, and W. Giger, Determination of fluoroquinolone antibacterial agents in sewage sludge and sludge-treated soil using accelerated solvent extraction followed by solid-phase extraction. *Anal. Chem*. 2002, **74** (21): 5455-5482.

[15] S. Sczesny, H. Nau, and G. Hamscher. Residue analysis of tetracyclines and their metabolites in eggs and in the environment by HPLC coupled with a microbiological assay and tandem mass spectrometry. *J. Agric. Food Chem*., 2003, **51** (3): 697-703.

[16] J. R. Marengo, R. A. Kok, G. K. O´Brien, R. R. Velagaletti, and J. L. Stamm. Aerobic degradation of c-sarafloxacin hydrochloride in soil. *Environm. Toxicol. and Chem*. 1997, 16 (3): 462-471.

[17] N. Carmosini, and L. S. Lee. Sorption and Degradation of Selected Pharmaceuticals in Soil and Manure. In: D. S. Aga (ed.). *Fate of Pharmaceuticals in the Environment and in Water Treatment Systems*. CRC Press, 2008, pp. 179-195.

[18] T. Christian, R. J. Schneider, H. A. Färber, D. Skutlarek, M. T. Meyer, and H. E. Goldbach. Determination of antibiotic residues in manure, soil and surface waters. *Acta. Hydrochim. Hydrobiol*. 2003, **31** (1): 36-44.

[19] M. Lillenberg, M. Roasto, and T. Püssa. Drug residues in environment. Estimation of fluoroquinolones in soil and food plants. *J. Agric. Sci.* 2003, **14** (1): 13-26.

[20] M. P. Hermo, D. Barron, and J. Barbosa. Determination of residues of quinolones in pig muscle: comparative study of classical and microwave extraction techniques. *Anal. Chim. Acta*. 2005, **539** (1-2): 77-82.

[21] K. Ganzler, I. Szinai, and A. Salgo. Effective sample preparation method for extracting biologically active compounds from different matrices by a microwave technique. *J. Chromatogr*. 1990, **520**: 257-62.

[22] M. Lillenberg, S. Yurchenko, K. Kipper, K. Herodes, V. Pihl, K. Sepp, R. Lõhmus, and L. Nei. Simultaneous determination of fluoroquinolones, sulfonamides and tetracyclines in sewage sludge by pressurized liquid extraction and liquid chromatography electrospray ionization-mass spectrometry. *J. Chromatogr. A*. 2009, **1216** (32): 5949-5954.

[23] Maximum levels of certain contaminants in foodstuffs. Decree of the Government of Estonia. *Riigi Teataja (State Gazette)*. 2000, **6** (38): https://www.riigiteataja.ee/ert/act.jsp?id=613934

VI

Kipper, K., Herodes, K., **Lillenberg, M**., Nei, L.,
Haiba, E.; Litvin, S.V. (2010).
PLANT UPTAKE OF SOME PHARMACEUTICALS
COMMONLY PRESENT IN SEWAGE SLUDGE COMPOST.
Proceedings of 2nd International Conference on Chemical,
Biological and Environmental Engineering,
Cairo, Egypt, 2-4 November, 2010, 261-264

Plant Uptake of some Pharmaceuticals Commonly Present in Sewage Sludge Compost

Karin Kipper and Koit Herodes
Institute of Chemistry
University of Tartu
Jakobi 2, 51014 Tartu, Estonia

Merike Lillenberg
Department of Food Science and Hygiene
Estonian University of Life Sciences
Kreutzwaldi 58A, 51014 Tartu, Estonia

Lembit Nei*, Egge Haiba and Sandra V Litvin
Tartu College
Department of Environmental Protection
Tallinn University of Technology
Puiestee 78, 51008 Tartu, Estonia
*corresponding author, e-mail: lembit.nei@ttu.ee

***Abstract*—The application of sewage sludge compost as a fertilizer can be a source of the contamination of food plants by pharmaceutical products. In this study the uptake of ciprofloxacin, ofloxacin, norfloxacin, sulfadimethoxine and sulfamethoxazole from soil into potato was demonstrated. The concentrations of the studied pharmaceuticals were of considerable magnitude in the plant samples, if compared to their concentrations in soil.**

Keywords - sewage sludge; pharmaceutical; plant uptake; compost

I. INTRODUCTION

Sewage sludge is the residue from the treatment of domestic and industrial wastewater. It contains organic matter and nutrients that are useful for plants [1]. The contents of nitrogen, phosphorus and organic matter are up to 10 times higher in sewage sludge and its compost, if compared to common agricultural soils [2]. Still, its usage as a fertilizer is restricted due to a large number of toxic pollutants found in this matter. Composting or aerobic biological treatment of organic wastes is an ancestral way to reduce wastes and to reuse organic matter. Among the range of existing organic wastes, sewage sludge composting enables the production of quality compost that may be used as a soil conditioner or as an organic fertilizer [3], since its organic matter content can vary from 50% to 70% of the total solids content [4]. Compost is a relatively stable material similar to humus. It is fine textured and has low moisture. The quality of compost depends on its environmental compatibility and the correspondence with market necessities. These features involve the absence of toxic inorganic and organic substances that may enter the food chain through the plant uptake [5].

Among these, pharmaceuticals are frequently present in sewage sludge. Their quantities rise from year to year. In spite of the fact that very low drug levels in the environment can have undesirable ecological and health effects, until now the problems related to the presence of pharmaceuticals in sewage sludge and its compost have received little attention [6]. Over the past decade, the scientific community has become increasingly interested in the impacts of pharmaceutical contaminants on the environment and human health. A large proportion of pharmaceuticals are introduced into the environment via sewage treatment plants. The presence and possible accumulation of these substances in sewage sludge are known, but little information is available on biodegradation of these pollutants. Current national and international regulations governing the application of sewage sludge ignore the presence and fate of pharmaceuticals. It has been shown that the degradation of pharmaceuticals may take place during bio-solid composting [7]. It is considered to be very useful to study the degradation of pharmaceuticals if diverse sewage sludge treatment technologies are to be applied. Still, no systematic work concerning biodegradation of pharmaceuticals during sewage sludge composting has been published. The utilization of untreated raw sewage sludge in agriculture is prohibited.

Medical substances have many of the necessary properties to bio-accumulate and provoke changes in ecosystems. There exist no trigger values for drug residues in sewage compost in the European Union. Plants unlike animals have no excretion. Therefore, drug residues may accumulate into plants. As a result, concentrations of drug residues in food plants may exceed the maximum residue levels (MRL) for meat and milk. No limits have been set for drug residues in plant products at present time. Various treatment technologies including composting and methane fermentation are known. Although the usage of such compost as a fertilizer widely takes place in Eastern Europe, the presence of drug residues in this matter has been ignored.

978-1-4244-8747-9/10/ $ 26.00 © 2010 IEEE

The possible degradation of drug residues as a result of sludge composting needs to be studied.

Humans may be exposed to residues of drugs in the environment by a number of routes including the consumption of crops that have accumulated substances from fertilized soils [8]. Experiments at elevated concentrations of pharmaceuticals in soil have shown that they are able to accumulate in plants [9]. The methodology for the simultaneous determination of the studied pharmaceuticals from sewage sludge is described in detail in [10]. There is a need to determine the significance of uptake into plants from soil under "real" conditions as a potential route for pharmaceuticals. Therefore, the current study was conducted to investigate the potential for some widely used pharmaceuticals – fluoroquinolones and sulfonamides (ciprofloxacin $C_{17}H_{18}FN_3O_3$, norfloxacin $C_{16}H_{18}FN_3O_3$ and ofloxacin $C_{18}H_{20}FN_3O_4$, sulfadimethoxine $C_{12}H_{14}N_4O_4S$ and sulfamethoxazole $C_{10}H_{11}N_3O_3S$) – to be taken up by food plants (namely potatoes in this study) from soil fertilized with sewage sludge or its compost.

II. Experimental

A. Chemicals and Equipment

Pharmaceuticals were purchased from Riedel-de-Haën (Seelze, Germany) - three fluoroquinolones: ciprofloxacin (CIP, purity 99.8%), norfloxacin (NOR, purity 99.9%) and ofloxacin (OFL, purity 99.3%); two sulfonamides: sulfadimethoxine (SDM, purity 99.4%) and sulfamethoxazole (SMX, purity 99.9%). Hydrophilic-lipophilic balanced (HLB) cartridges (Oasis HLB (60 μm), 500 mg/6 mL) by Waters (Milford, MA, USA). Acetonitrile and methanol were obtained from J. T. Baker (Deventer, The Netherlands), acetic acid and ammonia from Riedel-de-Haën, 1,1,1,3,3,3-hexafluoro-2-propanol from Sigma (St. Louis, MO, USA). All solvents were of reagent grade or higher quality.

B. Liquid Extraction

250 mg of dried potato sample was extracted with 10 mL of 1:1 (v/v) mixture of acetonitrile and 1% acetic acid, then homogenized with laboratory homogenizer DIAX 900 (Heidolph Instruments, Germany) 25 000 rpm, sonicated (5'), vortexed (1') and centrifuged at 8000 rpm. The supernatant was then separated and dried by nitrogen stream. Approximately 15 mL of 1 % acetic acid was added to the 1 mL of evaporation residue.

C. Solid-Phase Extraction

The extract collected by liquid extraction was cleaned up by solid phase extraction (SPE). Antibiotics - CIP, NOR, OFL, SDM and SMX - were extracted using HLB cartridges. For SPE procedure the vacuum manifold, supplied by Agilent Technologies, was used. HLB cartridges were preconditioned with 20 mL of methanol and 10 mL of Milli-Q water. The sample was loaded at a rate of 6 mL/min. After extraction, the compounds were eluted from cartridges using 12 mL of methanol. The SPE extracts were concentrated in polypropylene vials in N_2 stream. Residue was dissolved in 1 mL of 10 % methanol with buffer solution (5 mM 1,1,1,3,3,3-hexafluoro-2-propanol, pH adjusted to 9.0 with NH_4OH).

D. Liquid Chromatography - Mass Spectrometry

The SPE extracts were analyzed by liquid chromatography–mass spectrometry (LC–MS) (Agilent Series 1100 LC-MSD Trap XCT (Santa-Clara, CA, USA)) equipped with a binary pump, a degasser, an auto-sampler and a column thermostat. Antibiotics were chromatographed using a Waters XBridge C18 column (150 mm × 3 mm, 3.5 μm) equipped with a Waters Guard Cartridge 4.6 mm × 20 mm. Electrospray interface (ESI) was used in positive ion mode for ionization. Selected reaction monitoring was used. Full MS^2 were recorded and the following transitions were applied for quantification: OFL m/z 362→ 261, 318, NOR m/z 320→302, 276, CIP m/z 332→288, 314, SMX m/z 254→108, 188, SDM m/z 311→108, 156, 218, 245. For instrument control and data analysis software: Agilent ChemStation for LC Rev. A. 10.02; MSD Trap Control version 5.2 and Data Analysis for LC-MSD Trap 3.2 were used. Gradient elution was carried out with methanol and hexafluoroisopropanol (HFIP) buffer solution (5 mM 1,1,1,3,3,3-hexafluoro-2-propanol, pH adjusted to 9.0 with NH_4OH). The linear gradient started at 10% methanol and was raised to 100% within 50 min, after that methanol concentration was 100% for 5 min, then lowered to 10% in 5 min and kept in 10% for 5 min. The eluent flow rate was 0.3 mL/min, the column temperature was set to 30 °C and the injection volume was 10 μL.

E. Plant Samples

Aqueous solutions of the studied pharmaceuticals were mixed with soil. The final concentration of each pharmaceutical was 10 mg/kg and 1 mg/kg (dry weight). To assure better dissolution of the studied pharmaceuticals fluoroquinolones were dissolved in 2 ml of 0.1 mM ammonium acetate buffer solution with pH=2.8 and sulfonamides were dissolved in 2 ml of 0.3 M NaOH. The potatoes were planted into the pots and cultivated in the presence of the five pharmaceuticals during 120 days from planting. Then the potatoes were collected, washed, dried and milled. The milled potatoes were hold in hermetical plastic bags at –80 °C. The samples were dried at 45 °C before analysis.

III. Results and Discussion

The selection of pharmaceuticals was made according to their stability in soil and their potential accumulation into plants. Typical chromatograms for SMX, SDM, NOR, CIP and OFL obtained in the case of potato sample are shown in Fig. 1. Recoveries for the detected pharmaceuticals varied from 80 to 94%. The average concentration values showing the accumulation of these pharmaceuticals into potatoes are presented in table 1. The variation of the concentrations detected from three parallel samples was within ±35%, which is fully acceptable in the case of biological objects. The upper numbers in each row show the concentrations of pharmaceuticals in potatoes cultivated in loamy soil, the

concentrations presented below these correspond to potato samples obtained from plants cultivated in sandy soil.

TABLE I. CONCENTRATIONS OF PHARMACEUTICALS IN POTATO SAMPLES

Pharmaceuticals	**In Potato Samples, µg/kg**				
In Soil, µg/kg	***CIP***	***NOR***	***OFL***	***SMD***	***SMX***
10,000	140 80	230 100	150 100	1,500 170	3,900 390
1,000	n.d. n.d.	n.d. n.d	33 36	37 40	n.d. n.d.

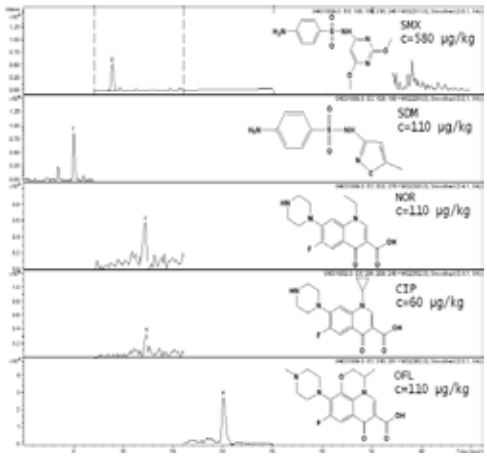


Figure 1. Chromatograms for SMX, SDM, NOR, CIP and OFL in potato samples.

Sulfonamides are among the most commonly used antibiotics in veterinary medicine and to a lesser extent in human medicine [11]. They are both fairly water-soluble and polar [12]. The low adsorption of sulfonamides on soil particles is known [13] and due to this phenomenon they are "free" to migrate into plants. An opposite behavior is characteristic to fluoroquinolones. It has been shown that more than 90% of applied ciprofloxacin and ofloxacin is adsorbed on different soils [13]. For this reason no significant migration of fluoroquinolones from soil into plants takes place. The amounts of fluoroquinolones going into potato do not depend much on soil type (table 1). In loamy soil the molecules of sulfonamides attach to clay particles [11], reducing their uptake by plants.

Figure 2. Potatoes, 30th day after planting. Pots on the left – the concentrations of SMX, SDM, NOR, CIP and OFL (each) in sandy soil were 10,000 µg/kg; pots on the right – the concentrations of the pharmaceuticals were 1,000 µg/kg (each).

Figure 2 clearly shows that the growth rate of a potato considerably depends on the concentration of pharmaceuticals in the soil. In this experiment all five pharmaceuticals were cumulatively present in soil. On the left the pots were filled with soil having the 10,000 µg/kg concentrations of each pharmaceutical. In pots on the right the relevant concentrations were 10 times lower (1,000 µg/kg).

Soils and sediments are the ultimate sinks for different pollutants [14]. Recently the uptake of ciprofloxacin, norfloxacin, ofloxacin, sulfadimethoxine and sulfamethoxazole was demonstrated in lettuce [15]. The results were obtained at relatively high concentrations (10 mg/kg) of pharmaceuticals in soil. The uptake of fluoroquinolones and sulfonamides by plants like lettuce did not seem to be a major human health risk, as the detected levels of the studied pharmaceuticals in lettuce leaves were relatively low if compared to their soil concentrations. Still in the case of CIP and OFL the possibility exists that even under "mild" conditions their concentrations in lettuce leaves may reach undesirable levels. The concentrations of sulfonamides in lettuce roots were of the same magnitude if compared to the eatable part of potato (3,700 µg/kg in the case of both SMD and SMX). Once released into the environment, sulfonamides become very mobile, and as a consequence they may affect many non-target organisms. As there are usually several pollutants of the same family or the same type that coexist in soil, synergic or antagonistic effects should be considered [16].

The CIP, NOR and OFL concentrations in lettuce roots – 560, 500 and 1,200 µg/kg, respectively [15] – were lower than the concentrations of sulfonamides, but they still exceeded the concentrations that were detected from potato samples. Therefore, further studies are needed to determine the uptake of different pharmaceuticals and other organic pollutants from different types of soils by various crop plants.

IV. CONCLUSIONS

Only a relatively small number of investigations have been published on the mobility and bioavailability of pharmaceuticals. In the current study uptake of ciprofloxacin, norfloxacin, ofloxacin, sulfadimethoxine and sulfamethoxazole was demonstrated in the case of potato. The uptake of fluoroquinolones and especially sulfonamides by plants like potato might pose health risk, as the detected levels of the studied pharmaceuticals were of considerable magnitude, if compared to their soil concentrations. The low adsorption of sulfonamides on soil may cause contamination of food plants. Further studies should be conducted to determine the uptake of different types of pharmaceuticals and other organic pollutants by various crop plants, and sorption kinetics at the soil-root interface has to be studied.

ACKNOWLEDGMENT

M.L. thanks Mr. Avo Toomsoo and Mr. Tõnu Tõnutare for fruitful discussions, Dr. Alar Astover and Mr. Hendrik Peterson for their assistance in carrying out experiments. This work was funded by Estonian Environmental Investment Centre, Estonian Science Foundation (grant no 7127) and Estonian research target project SF 0180058s07.

REFERENCES

[1] C. C. Kaonga, J. Kumwenda, and H. T. Mapoma, "Accumulation of lead, cadmium, manganese, copper and zinc by sludge worms; Tubifex tubifex in sewage sludge", Int. J. Environ. Sci. Tech., vol. 7, Winter 2010, pp. 119-126.

[2] M. Lillenberg, S. Yurchenko, K. Kipper, K. Herodes, V. Pihl, R. Lõhmus, M. Ivask, A. Kuu, S. Kutti, S. V. Litvin, and L. Nei, **"**Presence of fluoroquinolones and sulfonamides in urban sewage sludge and their degradation as a result of composting", Int. J. Environ. Sci. Tech., vol. 7, Spring 2010, pp. 307-312.

[3] A. Tremier, A. de Guardia, C. Massiani, E. Paul, and J. L. Martel, "A respirometric method for characterising the organic composition and biodegradation kinetics and the temperature influence on the biodegradation kinetics, for a mixture of sludge and bulking agent to be co-composted", Bioresour. Technol., vol. 96, Jan. 2005, pp. 169-180, doi: 10.1016/j.biortech.2004.05.005.

[4] V. Banegas, J. L. Moreno, J. I. Moreno, C. García, G. León, and T. Hernández, "Composting anaerobic and aerobic sewage sludges using two proportions of sawdust", Waste Management, vol. 27, 2007, pp. 1317-1327, doi: 10.1016/j.wasman.2006.09.008.

[5] L. Lazzari, L. Sperni, P. Bertin, and B., Pavoni, "Correlation between inorganic (heavy metals) and organic (PCBs and PAHs) micropollutant concentrations during sewage sludge composting processes", Chemosphere, vol. 41, Aug. 2000, pp. 427-435, doi: 10.1016/S0045-6535(99)00289-1.

[6] M. Carballa, F. Omil, T. Ternes, and M. J. Lema, "Fate of pharmaceutical and personal care products (PPCPs) during anaerobic digestion of sewage sludge", Water Res., vol. 41, May 2007, pp. 2139-2150, doi: 10.1016/j.watres.2007.02.012

[7] F. Buyuksonmez, and S. Sekeroglu, "Presence of pharmaceuticals and personal care products (PPCPs) in biosolids and their degradation during composting", J. Res. Sci. Technol., vol. 2, Jan. 2005, pp. 31-40.

[8] H. Dolliver, K. Kumar, and S. C. Gupta, "Sulfamethazine uptake by plants from manure-amended soil", J. Environ. Qual., vol. 36, June 2007, pp. 1224-1230, doi: 10.2134/jeq2006.0266.

[9] M. Lillenberg, M. Roasto, and T. Püssa, "Drug residues in environment. Estimation of fluoroquinolones in soil and food plants", J. Agric. Sci., vol. 14, 2003, pp. 13-26.

[10] M. Lillenberg, S. Yurchenko, K. Kipper, K. Herodes, V. Pihl, K. Sepp, R. Lõhmus, and L. Nei, "Simultaneous determination of fluoroquinolones, sulfonamides and tetracyclines in sewage sludge by pressurized liquid extraction and liquid chromatography electrospray ionization-mass spectrometry", J. Chromatogr. A., vol. 1216, Aug. 2009, pp. 5949-5954, doi: 10.1016/j.chroma.2009.06.029.

[11] S. Thiele-Bruhn, "Pharmaceutical antibiotic compounds in soils – a review", J. Plant Nutr. Soil Sci., vol. 166, Apr. 2003, pp. 145-167, doi: 10.1002/jpln.200390023.

[12] S. Thiele-Bruhn, T. Seibicke, H.-R. Schulten, and P. Leinweber, "Sorption of sulfonamide pharmaceutical antibiotics on whole soils and particle-size fractions", J. Environ. Qual., vol. 33, 2004, pp. 1331-1342.

[13] J. Beausse, "Selected drugs in solid matrices: a review of environmental determination, occurrence and properties of principal substances", Trends in Anal. Chem., vol. 23, Nov.-Dec. 2004, pp. 753-761, doi: 10.1016/j.trac.2004.08.005.

[14] L. Nei, J. Kruusma, M. Ivask, and A. Kuu, "Novel approaches to bioindication of heavy metals in soils contaminated by oil shale wastes", Oil Shale, vol. 26, Sept. 2009, pp. 424-431, doi: 10.3176/oil.2009.3.07.

[15] M. Lillenberg, K. Herodes, K. Kipper, and L. Nei, "Plant Uptake of some Pharmaceuticals from Fertilized Soils", Proceedings of the 2010 International Conference on Environmental Science and Technology, Apr. 2010, pp. 161-165.

[16] M. J.García-Galán, M. S. Díaz-Cruz, and D.Barceló, "Combining chemical analysis and ecotoxicity to determine environmental exposure and to assess risk from sulfonamides", Trends Anal. Chem., vol. 28, June 2009, pp. 804-819, doi: 10.1016/j.trac.2009.04.006.

VII

Lillenberg, M., Litvin, S.V., Nei, L., Roasto, M., Sepp, K. (2010). ENROFLOXACIN AND CIPROFLOXACIN UPTAKE BY PLANTS FROM SOIL.
Agronomy Research, 8, 807-814.

Agronomy Research **8** (1), 807–814, 2010

Enrofloxacin and Ciprofloxacin Uptake by Plants from Soil

M. Lillenberg[1], S. V. Litvin[2], L. Nei[2], M.Roasto[1] and K. Sepp[3]

[1] Department of Food Science and Hygiene, Estonian University of Life Sciences, Kreutzwaldi 58A, 51014 Tartu, Estonia
[2] Department of Environmental Protection, Tartu College of Tallinn University of Technology, Puiestee 78, 51008 Tartu, Estonia, e-mail: lembit.nei@ttu.ee
[3] Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Kreutzwaldi 64, 51014 Tartu, Estonia

Abstract. Very small amounts of pharmaceuticals present in everyday food may generate strains of resistant microorganisms in human and animal organisms. This study involves the uptake and accumulation of some widely used fluoroquinolones – enrofloxacin and ciprofloxacin – by plants cultivated in soil augmented with drugs using the microbiological agar diffusion method. *Bacillus subtilis* was used as the test bacterium. The three plants chosen for the experiment were lettuce *(Lactuca sativa),* common barley *(Hordeum vulgare L.)* and cucumber *(Cucumis sativus L.),* which were cultivated in a laboratory in soils mixed with enro- or ciprofloxacin at nominal concentrations of 500, 200, 50 and 10 μg/g. The concentrations of fluoroquinolones remained unchanged in the soil during the experiment. The presence of enrofloxacin was detected in all plants grown at enrofloxacin concentrations of 500, 200 and 50 μg/g. The presence of ciprofloxacin was only detected in barley and cucumber grown in soil with a base concentration of 500 μg/g. In lettuce, which had a longer vegetation period, the presence of ciprofloxacin was detected at all concentrations. The content of ciprofloxacin in the lettuce was 44 μg/g at a soil concentration of 10 μg/g: fluoroquinolones accumulate in a plant during the vegetation period.

Key words: enrofloxacin, ciprofloxacin, pharmaceutical residue, plant uptake, microbiological agar-diffusion method, *Bacillus subtilis*

INTRODUCTION

By the end of the 20th century, growing concerns had emerged about the occurrence and fate of pharmaceuticals in the environment, and these concerns have continued to grow steadily ever since (Martínez-Carballo et al., 2009). The reason why pharmaceuticals may become harmful in the environment is that they are designed to affect biological objects. They have lipophilicity, which enables them to permeate bio-membranes, and stability, which prevents their inactivation before the therapeutic effect. Therefore, drugs have the properties they need to accumulate in organisms and cause changes in water and soil ecosystems. The annual consumption of widely used drugs can be quite extensive (Halling-Sørensen et al., 1998). In Denmark, the total annual use of antibiotics is about 34 tonnes (Stuer-Lauridsen et al., 2000).

Generally, drugs can be divided into two groups: drugs used in human medicine

and drugs used in veterinary medicine. Veterinary medicines are used in livestock barns and bird farms as growth stimulators. Livestock are also treated directly on pastures. On fish farms, drugs are introduced into water as a component of feed. Medicines used by humans or animals are excreted in an unchanged form or as metabolites. Drug residue from human excrement reaches the sewage system and finally ends up in sewage treatment plants. An unknown amount of human medicines enter the sewage system as raw sewage.

Depending on the drug, 12–90% of them pass through sewage treatment plants unaltered (Stumpf et al., 1999). It can be concluded that treatment facilities do not remove drug residue completely. A considerable amount of drugs enter surface water and can end up in drinking water. The fate of drug residue after entering a treatment facility may be one of the following: (*1*) the substance is easily degradable and decomposes fast and fully into CO_2 and water in the facility; (*2*) the substance is lipophilic and does not degrade easily, instead accumulating unaltered in the activated sludge of the facility; or (*3*) the substance is metabolised into a more hydrophilic matter but does not degrade at all, instead passing through the treatment plant and entering the aqueous environment.

Sewage sludge containing drug residue is used as a fertiliser in fields (Lillenberg et al., 2009; Radjenović et al., 2009; Lapen et al., 2008). Drugs reach the soil in this way, where they can affect microorganisms and accumulate in plants. Growth stimulators and medicines used in animal breeding reach manure either unaltered or as metabolites, finally entering the fields. On pastures, drugs go through cattle and are excreted. In this manner, extremely high concentrations of drug residue are concentrated locally in the soil, and essentially have a strong impact on soil organisms and plants. Drugs and their metabolites which have reached the soil are either mineralised by soil organisms or enter groundwater unaltered (Halling-Sørensen et al., 1998).

The lifetime of drugs in the environment depends on the structure of their molecules. The microorganisms of soil decompose drugs into either organic metabolites or carbon dioxide and water. The ability to produce antibiotics is a long-term evolutionary process and represents an important factor in the struggle for existence (Tshervjakova & Terezova, 1986). At the same time, the pathways of biodegradation have evolved in nature to mineralise natural antibiotics.

Synthetic and semi-synthetic antibacterial substances are currently in wide use. They are ‘strangers’ to nature and hard to degrade. Fluoroquinolones belong to a group of drugs that remain in the environment for a long period of time. The strong adsorption of fluoroquinolones to manure and soil can be one reason for their slow degradation (Marengo et al., 1997). The presence of enro- or ciprofloxacin in manure or soil fertilised with manure has not been studied. It is known that the elimination of enrofloxacin from animal organisms occurs through the kidneys (80%) (Crumplin, 1986). The concentration of fluoroquinolones in urine can exceed its concentration in serum up to 100–300 times (Montay et al., 1984). Enrofloxacin metabolises in animal organisms partly into ciprofloxacin (Mengozzi et al., 1996). It can be concluded that arable land fertilised with liquid manure may be contaminated with fluoroquinolones (Nowara et al., 1997). The antimicrobial ability of enrofloxacin becomes stronger when ciprofloxacin is present due to synergism (Mengozzi et al., 1996).

Attempts have been made to predict the concentrations of drug residues using

calculations (predicted environmental concentration – PEC). The formation of metabolites, methods of excretion, the collection and preservation of manure and the way it is spread in the field, etc. are taken into account. When animals are treated on pastures, drug residue in their excrement goes directly into the soil. In this case, the factors concerning drug residue concentration that are taken into account when making predictions are very importance. Major amounts of drug residue enter the soil with manure from animal barns.

The possible mobility of drug residue from soil to plants has been studied relatively little, although there is data on the accumulation of sulfadimetoxin in barley (Brambilla et al., 1996). The content of sulfadimetoxin was approximately four times higher in the roots than in the leaves and stems: 79 and 18 μg/g respectively (the content of sulfadimetoxin in the soil being 100 μg/g). The study concluded that a MRL (maximum residue limit) on veterinary medicine residue in plants should be imposed (Brambilla et al., 1996).

Fluoroquinolones are synthetic, wide-range drugs. Generally, the same drugs are not used on animals and humans so as to avoid the development of cross-resistance. Although the two fluoroquinolones – enrofloxacin and ciprofloxacin – are very close in structure, the former is used in veterinary medicine and the latter in human medicine. As final products of metabolism, both enrofloxacin and its metabolite ciprofloxacin end up in excrement (Boxall et al., 2006). Livestock manure is commonly used as organic fertiliser. One of its uses is on the fields where food plants are grown. The manure includes the residue of fluoroquinolones in addition to other drug residue. Plants can also intake fluoroquinolones along with minerals. The intake of drugs in small amounts can lead to drug resistance in pathogenic microbes and cause allergies and liver damage. Raw materials of animal origin are subject to strict state controls. There are no limits concerning raw materials of plant origin. The total MRL of enrofloxacin and ciprofloxacin in meat is 100 μg/kg (State Herald, 2000). In the case of raw materials of plant origin, MRL is set only for pesticide residue.

The aims of this work are to evaluate the uptake and accumulation of fluoroquinolones – enrofloxacin and ciprofloxacin – from soil into plants using a multiple concentration test and to determine their stability in soil.

MATERIALS AND METHODS

The following materials and chemicals were used: *B. subtilis* BGA spore suspension from Merck; test agar pH 8 from Merck; trimethoprim (TMP) from Sigma; ciprofloxacin and enrofloxacin from Bayer; Petri dishes Ø 90 mm; bottomless metal cylinders Ø 6 mm; blank paper disk Ø 6 mm from Becton-Dickinson & Co; and soil free of fertilisers, pH 6.0.

The plants were grown in a room specially prepared for this purpose, aired and lighted with daylight lamps. Three plants were chosen for the experiment: lettuce *(Lactuca sativa),* common barley *(Hordeum vulgare L.)* and cucumber *(Cucumis sativus L.)*. The seeds were planted in plastic pots with 1 kg of fertiliser-free soil and soil mixed with fluoroquinolone solution. The weighed soil was first mixed with fluoroquinolone solution. The necessary amount of enro- or ciprofloxacin was dissolved in 200 ml of distilled water and added to the soil. The total concentration of

the antibiotic in the pots was 10, 50, 200 or 500 mg/kg. Three parallel pots were prepared for each level of concentration. Each plant species was grown in fluoroquinolone-free soil for test purposes. The irrigation of the plants took place through a plastic tube at the bottom of the pot. This type of irrigation was necessary to avoid flushing fluoroquinolones from the upper to the lower levels and to maintain as even a concentration as possible in the pot. The experiment lasted for 28 days (42 days in the case of the lettuce). The plants were then harvested and their roots removed, dried and ground. Soil samples were taken on the 2nd, 14th and 28th days of the experiment. The soil was sterilised in an autoclave for 30 minutes under 1 bar of pressure.

In order to estimate the level of fluoroquinolones in the test soils, their adhesion level to soil particles was determined. Spiking solutions with concentrations of 0.5, 1, 2, 5, 10, 20, 40 and 80 μg/ml were prepared and mixed with test soil free of antibiotics. In order to prepare the soil samples for calibration, 2 g of air-dry drug-free soil was mixed with 10 ml of fluoroquinolone solution in 50 ml plastic centrifuge tubes using the end-over-end method over 5 hours at room temperature. The tubes were centrifuged at 4000 rpm for 30 minutes. Supernatants were removed by decantation. Sediment was dried on plastic Petri dishes at room temperature overnight. Solutions of fluoroquinolones with concentrations of 0.5, 1, 2, 5, 10, 20, 40 and 80 μg/ml were used to prepare calibration soils with concentrations of 2.5, 5, 10, 25, 50, 100, 200 and 400 μg/g respectively.

To determine the content of fluoroquinolones in the plants, their adsorption to plant material *in vitro* was estimated (Lillenberg et al., 2003). In order to prepare the calibration plants, 50 mg of dried and ground leaves and stems of three different plants grown in fluoroquinolone-free soil were mixed with 1 ml of the following antibiotic solutions in water: 0.5, 1, 2, 5 and 10 μg/ml in 2 ml Eppendorf tubes. The tubes were rotated end-over-end over 3 hours at room temperature and centrifuged at 4000 rpm for 10 minutes. Supernatants were removed using a plastic Pasteur pipette into 1 ml tubes. Sediment was dried on plastic Petri dishes at room temperature overnight.

The soil, plants, spiking solutions and supernatants were analysed using the agar diffusion method (Lillenberg et al., 2003). Three parallel determinations were performed for each sample. The measurement uncertainty of the drug concentrations was within ±10%. The test agar was sterilised in an autoclave for 50 min under 1 bar of pressure. After decreasing the temperature of the test agar to 48°C, 100 μl of aqueous solution of TMP (conc.100 μg/ml) and 1 ml *B. subtilis* spore suspension were added to 100 ml of the medium. Petri dishes were filled with 6 ml of inoculated test agar and after a period of 30 min a Ø 6 mm stainless steel cylinder was placed on the gel. 2.5 mg of dried and ground plant material or 5 mg of autoclaved soil was poured on the gel through the cylinder, after which the cylinder was removed. In the case of antibiotic solutions or spiking supernatants, a 6 mm blank paper disk was placed on the gel and 13.6 μl of the solution was dipped on the disk. The experimental plant and soil samples and spiked samples were tested on the same gel. Petri dishes with samples on the gel were kept in a refrigerator at 4–6 °C for 22 hours for pre-diffusion, and thereafter incubated for 18 hours at 37 °C. An inhibition circle of microbial growth appeared on the gel around the sample. Its diameter was measured and the average of parallel samples calculated. The average diameters of the spiked soils were used to construct the calibration curves in axes: log from antibiotic concentrations (x) and diameter of

inhibition circles (y) of spiked soils.

The adsorption level of fluoroquinolones to the plant or soil material was determined: the spiking solutions and supernatants were analysed using the microbial inhibition test. The calibration curve was constructed in axes: log from concentrations (x) and diameter of inhibition circles (y) of spiking solutions. The concentration of the antibiotic in the supernatant showed the adsorption rate. When the supernatant produced an inhibition circle, the concentration of antibiotics in the supernatant was determined by the calibration curve. The level of adsorption of the antibiotic to plant or soil material and concentration of the antibiotic in spiked plants or soils were calculated. When the supernatant did not produce an inhibition circle, all of the added antibiotic was adsorbed by the plant or soil – an adsorption rate of 100%. The calibration curves were constructed in axis: log from calculated concentration of antibiotic in spiked material (x) and diameter of inhibition circle of spiked material.

The antibiotic concentrations in the soil and plant samples were calculated using the following equation: $y = ax + b$, where a and b are constants, x = log from concentration of antibiotic in sample and y = diameter of inhibition circle of sample.

RESULTS AND DISCUSSION

Fluoroquinolones in soil

The adsorption rate of enrofloxacin for soil at concentrations of 10, 20, 40 and 80 μg/ml was 100, 99.6, 99.5 and 99.5% respectively. These results accord with those previously published (Nowara et al., 1997), where the adsorption of enrofloxacin in soil with a pH of 5.9 was 99.5%. In the case of ciprofloxacin, the level of adsorption to soil at concentrations of 10, 20, 40 and 80 μg/ml was 100, 100, 99.9 and 99.6% respectively. The lower the level of concentration of the fluoroquinolone spiking solution, the higher the adsorption rate with soil. Generally, it can be concluded that the adsorption rate was close to 100% at all concentrations.

Table 1 and 2 show that the concentration of fluoroquinolones did not change during the experiment; this was also shown in earlier publications (Golet et al., 2002). When comparing enrofloxacin content in test soils on days 2 and 28, it can be said that no sufficient changes in the relevant concentrations could be seen (Table 1). The content of ciprofloxacin in the sample soils remained almost unchanged (Table 2).

Fluoroquinolones in plants

The level of fluoroquinolone adsorption depended on plant species and spiking solution concentration. As the results in the soil showed, the lower the level of concentration of the fluoroquinolone spiking solution, the higher its adsorption rate. The adsorption rate of enrofloxacin in the lettuce was 64–100%, barley 28–100% and cucumber 23–100%. The adsorption rate of ciprofloxacin in the lettuce was 90–100%, barley 64–100% and cucumber 70–100%. Thereafter, the mobility of enrofloxacin and ciprofloxacin from soil to plants was studied. The results of the experiment showed that fluoroquinolones reach plants and accumulate there while retaining their antimicrobial activity. At the same initial concentration in soil, the content of

Table 1. Concentration of enrofloxacin in soil on 2nd, 14th and 28th days of experiment.

Plant	*Enrofloxacin* (μg/g)			
	initial	2nd day	14th day	28th day
Barley	500	487	487	520
	200	193	206	206
	50	48	55	59
	10	11	13	14
Lettuce	500	455	520	555
	200	206	268	268
	50	57	65	63
	10	9	10	10
Cucumber	500	503	520	503
	200	235	220	235
	50	45	55	52
	10	10	10	11

Table 2. Concentration of ciprofloxacin in soil on 2nd, 14th and 28th days of experiment.

Plant	*Ciprofloxacin* (μg/g)			
	initial	2nd day	14th day	28th day
Barley	500	567	494	477
	200	266	248	231
	50	62	62	54
	10	10	10	10
Lettuce	500	548	529	529
	200	284	266	248
	50	69	62	58
	10	11	11	10
Cucumber	500	629	567	529
	200	248	248	231
	50	62	54	47
	10	11	10	10

enrofloxacin was higher in the test plants than the content of ciprofloxacin. The accumulation of fluoroquinolones was dependant on plant species: its content was highest in lettuce and lowest in cucumber. The high rate of ciprofloxacin in lettuce was due to its vegetation period being 14 days longer compared to the growing period of the other plants in soil mixed with ciprofloxacin (Table 3). It is remarkable that for the lettuce grown in soil with a nominal concentration of ciprofloxacin of 10 μg/g, the content of ciprofloxacin was more than four times higher than predicted. The results showed that when the vegetation period is longer, antibiotics accumulate in the plant.

The results of the experiment showed that during the vegetation period a remarkable amount of ciprofloxacin can accumulate in lettuce. The total amount of enrofloxacin and its metabolites PEC in soil fertilised with manure could be up to 3.8

µg/g in the worst case (Halling-Sørensen et al., 2002). In our experiment with ciprofloxacin concentration of 10 µg/g in the soil, the ciprofloxacin content of the lettuce was 44 µg/g – more than four times higher in the plant than in the soil.

Table 3. Concentration of fluoroquinolones in plants.

Plant	*Enrofloxacin* or *ciprofloxacin* concentration in soil, µg/g	*Enrofloxacin* concentration in plant, µg/g	*Ciprofloxacin* concentration in plant, µg/g
Barley	500	20	13
	200	14	n.d.
	50	8	n.d.
	10	n.d.	n.d.
Lettuce	500	76	223 *
	200	27	163 *
	50	7	58 *
	10	n.d.	44 *
Cucumber	500	36	40
	200	22	n.d.
	50	9	n.d.
	10	n.d.	n.d.

*the lettuce was grown for 42 days in soil containing ciprofloxacin and for 28 days in soil containing enrofloxacin

CONCLUSIONS

Enrofloxacin and its metabolite ciprofloxacin as terrestrial contaminants must be monitored due to their wide presence in the environment, their chemical resistance and their ability to migrate from soil to crop. Enrofloxacin and ciprofloxacin uptake from soil by food plants was evident in our experiments. The significance of the route involving the uptake of the studied medicines from the soil by plants in terms of risk to human health was confirmed by our experiments. Further studies concerning the plant uptake of a wide spectrum of commonly used pharmaceuticals from soils fertilised with sewage sludge or its compost are needed with the aim of ensuring food safety.

ACKNOWLEDGMENTS. This work was partly funded by the Estonian Environmental Investment Centre. The authors thank Ms. Karin Muoni for translating the majority of this paper from Estonian into English.

REFERENCES

Boxall, A.B.A., Johnson, P., Smith, E.J., Sinclair, C.J., Stutt, E. & Levy, L.S. 2006. Uptake of veterinary medicines from soils into plants. *J. Agric. Food Chem.* **54**(6), 2288–2297.

Brambilla, G., Casoria, P., Civitareale, C., Cozzolino, S., Gaudio, L. & Migliore, L. 1996. Sulphadimetoxine as an environmental tracer in evaluating phytotoxicity in crops. In Haagsma N. & Ruiter A. (eds): *EuroResidue III. Third conference on residue of veterinary drugs in food (FECS no.123) Veldoven (The Netherlands), 6–8 May 1996.* Springer Berlin/Heidelberg, Utrecht, pp. 292–295.

Crumplin, G.C. 1986. The mechanism of action of quinolones and in quinolones and their future in clinical practice. *Int. Congr. Symp. Ser. R. Soc. Med.* **104**, 1–15.

Golet, E.M., Strehler, A., Alder, A.C. & Giger, W. 2002. Determination of fluoroquinolone antibacterial agents in sewage sludge and sludge-treated soil using accelerated solvent extraction followed by solid-phase extraction. *Anal. Chem.* **74**(21), 5455–5462.

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P.F., Ingerslev, F., Holten Lützhoft, H. C. & Jørgensen, S.E. 1998. Occurrence, fate and effects of pharmaceuticals in the environment. *Chemosphere* **36**(2), 357–393.

Halling-Sørensen, B., Nors Nielsen, S. & Jensen, J. 2002. *Environmental Assessment of Veterinary Medicinal Products in Denmark. Environmental Project, No. 659.* Danish Environmental Protection Agency, 97 pp.

Lapen, D.R., Topp, E., Metcalfe, C.D., Li, H., Edwards, M., Gottschall, N., Bolton, P., Curnoe, W., Payne, M. & Beck, A. 2008. Pharmaceutical and personal care products in tile drainage following land application of municipal biosolids. *Sci. Total Environ.* **399**(1–3), 50–65.

Lillenberg, M., Roasto, M., Püssa, T. 2003. Drug residue in the environment. Estimation of fluoroquinolones in soil and food plants. *J. Agricult. Science* **14**(1), 13–26.

Lillenberg, M., Yurchenko, S., Kipper, K., Herodes, K., Pihl, V., Sepp, K., Lõhmus, R. & Nei, L. 2009. Simultaneous determination of fluoroquinolones, sulfonamides and tetracyclines in sewage sludge by pressurised liquid extraction and liquid chromatography electrospray ionization-mass spectrometry. *J. Chromatogr. A* **1216**(32), 5949–5954.

Marengo, J.R., Kok, R.A., O´Brien, G.K., Velagaletti, R.R. & Stamm, J.L. 1997. Aerobic degradation of 14C-sarafloxacin hydrochloride in soil. *Environm. Toxicol. and Chemistry* **16**(3), 462–471.

Martínez-Carballo, E., González-Barreiro, C., Scharf., S. & Gans, O. 2007. Environmental monitoring study of selected veterinary antibiotics in animal manure and soils in Austria. *Environ. Pollut.* **147**(2), 570–579.

Mengozzi, G., Intorre, L. & Bertini, S. 1996. Pharmacokinetics of enrofloxacin and its metabolite ciprofloxacin after intravenous and intramuscular administration in sheep. *Am. J. Vet. Res.* **57**(7), 1040–1043.

Montay, G., Goueffon, Y. & Roquet, F. 1984. Adsorption, distribution, metabolic fate and elimination of pefloxacin mesylate in mice, rats, dogs, monkeys and humans. *Antimicrob. Agents and Chemother.* **25**, 463–472.

Nowara, A., Burhenne, J. & Spiteller, M. 1997. Binding of fluoroquinolone carboxylic acid derivates to clay minerals. *J. Agric. Food Chem.* **45**(4), 1459–1463.

Radjenović, J., Petrović, M. & Barceló, D. 2009., Fate and distribution of pharmaceuticals in waste water and sewage sludge of conventional activated sludge (CAS) and advanced membrane bioreactor (MBR) treatment. *Water Res.* **43**(3), 831–841.

Riigi Teataja I, 6, 38. Toidus lubatud saasteainete loetelu ja piirnormide toidugruppide kaupa kehtestamine /List of contaminants allowed in food and establishment of maximum levels by food group/. – VVm nr. 14, Lisa 3, 2000. (State Herald I, 2000, *in Estonian*) .

Stuer-Lauridsen, F., Birkved, M., Hansen, L.P., Holten Lützhoff H-C. & Halling-Sørensen, B. 2000. Environmental risk assessment of human pharmaceuticals in Denmark after normal therapeutic use. *Chemosphere* **40**(7), 783–793.

Stumpf, M., Ternes, T.A., Wilken, R-D., Rodrigues, S.V. & Baumann, W. 1999. Polar drug residue in sewage and natural waters in the State of Rio de Janeiro, Brazil. *Sci. Tot. Environ.* **225**(1–2), 135–141.

Tshervjakova, V. & Terezova, A. 1986. *Pharmacology and Dispensary.* Valgus, Tallinn, pp. 98–112 (in Estonian).

CURRICULUM VITAE

First name: Merike
Surname: Lillenberg
Date of birth: 02.01.1954
Employment: Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Sciences, Department of Food Hygiene, Kreutzwaldi 58 A, Tartu, 51014;
Telephone: +372 731 3432
E-mail: merike.lillenberg@emu.ee
Position: Lecturer

Education: University of Tartu, Biology 1983
Scientific degree:
MSc in Food Science
Institution and year of issuing degree:
Estonian Agricultural University, Tartu, 2003.
Foreign language: English, Russian

Career
Institution and position held:

2009 – …	lecturer, Department of Food Hygiene, Estonian University of Life Sciences.
2006–2008	extraordinary researcher, Department of Food Science and Hygiene, Estonian University of Life Sciences.
2004–2011	PhD student, Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences.
2003–2004	microbiologist, Department of Food Hygiene and Control, Estonian Agricultural University.
2000–2008	instructor, Environmental Protection Institute, Estonian Agricultural University.
2000–2001	analyst and quality policy consultant, Laboratory of Food Hygiene, Estonian Agricultural University.
1999–2000	microbiologist, Environmental Protection Institute, Estonian Agricultural University.

1991–1992	teacher of biology and chemistry at Laeva Secondary School.
1988–1991	researcher and instructor, Department of Biology, University of Tartu.
1984–1987	researcher, Department of Physiology, Estonian Agricultural Academy.

Scientific-organisational administrative activities:

2008– …	Member of Society of Environmental Toxicology and Chemistry (SETAC)
2005– …	Member of MSc (Food Science) commission, Department of Food Science, Estonian University of Life Sciences.

Directions of research: Environmental science and food hygiene.

Participation in research projects:

2009–2010	Environmental Investment Centre (EIC) project no 48: "Drug residues in sewage sludge compost and their accumulation from soil into crop plants via fertilization", principal executor.
2005–2008	Base financing project: „Biological and Chemical Hazards in Food Production Chain and Possibilities of Minimizing", principal executor.
2002–2004	Grant project no 4979 „Contamination of the Foodstuff with Thermophilic Campylobacteria, Severity of the Problem and Possibilities of Prevention", principal executor.
2007–2008	Environmental Investment Centre (EIC) project no 44: „Biodegradation of drug residues during sewage sludge composting", principal executor.
2005–2008	European Social Foundation project no 1.0101 - 0240 "Tippspetsialisti rakendamine toiduhügieeni alase õppe- ja teadustöö kvaliteedi tõstmiseks Eesti Põllumajandusülikoolis", executor.

Training activities:

VL.0644 General Food Hygiene – 9.0 ECTS, part: Food born parasitosis and virosis;

VL.0397 Cell and Molecular Biology – 2.0 ECTS;
VL.0256 Animal Biology – 3.0 ECTS;
PK.0229 General Biology – 3.0 ECTS, part:
Laboratory works (2000-2008).

n-service training:

Summer School in Molecular Biology:
„Chromosomes, chromatin and epigenetics.“
Palmse Mõis, Lääne-Virumaa, Estonia,
26 June - 2 July 2007 – 4 ECTS;

Summer School „Teaching in University“,
Arossa villa, Võrumaa, Estonia,
16 -18 Aug. 2006 – 3 ECTS;

PCR workshop.
AS SurgiTech, Tartu, Estonia,
27 Jan., 2004.

Awards: Scholarship of Town Council Fund of EMU, 2008.

ELULOOKIRJELDUS

Eesnimi: Merike
Perekonnanimi: Lillenberg
Sünniaeg: 02.01.1954
Töökoht: Eesti Maaülikool, Kreutzwaldi 1, 51014,
Veterinaarmeditsiini ja loomakasvatuse instituut,
Toiduhügieeni osakond
Telefon: +372 731 3432
E-post: merike.lillenberg@emu.ee
Ametikoht: Lektor

Haridustee:

2004–2011	Eesti Maaülikool, Põllumajandus- ja keskkonna-instituut, doktoriõpe
2002–2003	Eesti Maaülikool, Loomaarstiteaduskond, magistriõpe
1978–1983	Tartu Riiklik Ülikool, Bioloogia-geograafia teaduskond, bioloogia

Teenistuskäik:

2009–…	Eesti Maaülikool, Veterinaarmeditsiini ja loomakasvatuse instituut, Toiduhügieeni osakond, loomabioloogia lektor
2006–2008	Eesti Maaülikool, Veterinaarmeditsiini ja loomakasvatuse instituut, erakorraline teadur
2003–2004	Eesti Maaülikool, veterinaarmeditsiini ja loomakasvatuse instituut, toiduhügieeni ja -kontrolli osakond, mikrobioloog
2000–2008	Eesti Maaülikool, Põllumajandus- ja keskkonnainstituut, üldbioloogia praktikumi juhendaja
2000–2002	Eesti Põllumajandusülikool, Loomaarstiteaduskond, toiduhügieeni laboratoorium, analüütik, kvaliteedi konsultant

1999–2000	Eesti Põllumajandusülikool, Keskkonnakaitse Instituut, Keskkonnakeemia ja -ökoloogia labor, mikrobioloog
1991–1992	Laeva Põhikool, bioloogia-keemia õpetaja
1988–1991	Tartu Riiklik Ülikool, bioloogia osakond, geneetika ja tsütoloogia kat., nooremteadur, geneetika praktikumi juhendaja
1984–1987	Eesti Põllumajanduse Akadeemia, Veterinaaria tk. füsioloogia kateeder, nooremteadur

Teadus- või akadeemiline kraad: MSc (toiduteadus)
Kraadi välja andnud asutus, aasta: Eesti Põllumajandusülikool, 2003

Keelteoskus: Inglise keel, vene keel

Teadusorganisatsiooniline ja –administratiivne tegevus:

2008–…	Rahvusvahelise Keskkonnatoksikoloogia ja -keemia ühingu (SETAC) liige
2005–…	Eesti Maaülikool, Veterinaarmeditsiini ja loomakasvatuse instituut, toiduteaduse kutsemagistrikraadi kaitsmiskomisjoni liige

Teadustöö põhisuunad: toiduhügieen, keskkonnahügieen

Osalemine uurimisprojektides:

2009–2010	KIK projekt nr. 48 „Reoveesette kompostväetises sisalduvate ravimijääkide akumuleerumine mullast toidutaimedesse“, põhitäitja
2007–2008	KIK projekt nr. 44 „Ravimijääkide biodegradatsioon reoveesette kompostimisel“, põhitäitja
2005–2008	Baasfinantseerimise projekt „Bioloogilised ja keemilised ohud toidu tootmise ahelas ja nende minimeerimise võimalused“, põhitäitja
2005–2008	ESF projekt, meede 1.1. „Tippspetsialisti rakendamine toiduhügieeni alase õppe- ja teadustöö kvaliteedi tõstmiseks Eesti Põllumajandusülikoolis“, täitja

2002–2004 ETF grandiprojekt „Toiduainete saastatus termofiilsete kampülobakteritega, probleemi tõsidus ja tõrje võimalused“, põhitäitja

Erialane enesetäiendamine:

Molekulaarbioloogia suveülikool „Chromosomes, chromatin and epigenetics.“
Palmse Mõis, Lääne-Virumaa,
26. juuni–2. juuli 2007 – 4 EAP;

Suveülikool „Õpetamine kõrgkoolis“,
Arossa villa, Võrumaa,
16.–18. August 2006 – 3 EAP;

PCR koolitus.
AS SurgiTech, Tartu,
27. jaanuar, 2004.

Tunnustused: EMÜ raefondi stipendium, 2008.

LIST OF PUBLICATIONS

1.1 Articles indexed by Thomson Reuters Web of Science

Lillenberg, M., Yurchenko, S., Kipper, K., Herodes, K., Pihl, V., Lõhmus, R., Ivask, M., Kuu, A., Kutti, S., Litvin, S.V., Nei, L. (2010). Presence of fluoroquinolones and sulfonamides in urban sewage sludge and their degradation as a result of composting. *International Journal of Environmental Science and Technology*, **7 (2)**, 307–312.

Meremäe, K., Elias, P., Tamme, T., Kramarenko, T., **Lillenberg, M**., Karus, A., Hänninen, M.-L., Roasto; M. (2010). The occurrence of *Campylobacter spp.* in Estonian broiler chicken production in 2002-2007. *Food Control*, **21 (3)**, 272–275.

Lillenberg, M., Yurchenko, S., Kipper, K., Herodes, K., Pihl, V., Sepp, K., Lõhmus, R., Nei, L. (2009). Simultaneous determination of fluoroquinolones, sulfonamides and tetracyclines in sewage sludge by pressurized liquid extraction and liquid chromatography electrospray ionization-mass spectrometry. *Journal of Chromatography A*, **1216**, 5949–5954.

Roasto, M., Praakle-Amin, K., Meremäe, K., Tamme, T., Hörman, A., **Lillenberg, M.**, Hänninen, M.-L. (2009). Food control and research on *Campylobacter spp.* in Estonia. *Archiv für Lebensmittelhygiene,* **60,** 109–115.

1.2 Peer-reviewed articles in other international research journals with an ISSN code and international editorial board

Lillenberg, M., Litvin, S.V., Nei, L., Roasto, M., Sepp, K. (2010). Enrofloxacin and ciprofloxacin uptake by plants from soil. *Agronomy Research*, **8**, 807–814.

Nei, L., **Lillenberg, M**. (2009). Mackereth oxygen sensor: measurement uncertainty. *The Electrochemical Society Transactions*, **19 (22)**, 55–63.

Roasto, M., Meremäe, K., Praakle-Amin, K., Hörman, A., Elias, T., **Lillenberg, M.**, Elias, A., Kramarenko, T., Häkkinen, L., Põltsama,

P., Mäesaar, M., Elias, P., Hänninen, M.-L. (2011). Termofiilsete kampülobakterite uuringud Eestis 2000–2010 (*Campylobacter spp.* in Estonian food chain in 2000–2010). *Agraarteadus (Journal of Agricultural Science)*, **22 (1)**, 31–39.

1.3 Articles in Estonian and other peer-reviewed research journals with a local editorial board

Lillenberg, M., Roasto, M., Püssa, T. (2003). Ravimijäägid keskkonnas. Fluorokinoloonide määramine mullas ja toidutaimedes. (Drug residues in environment. Estimation of fluoroquinolones in soil and food plants). *Agraarteadus* (*Journal of Agricultural Science)*, **14 (1)**, 13–26.

3.1 Articles in collections indexed by the Thomson Reuters ISI proceedings

Kipper, K., Herodes, K., **Lillenberg, M.**, Nei, L., Haiba, E., Litvin, S.V. (2010). Plant uptake of some pharmaceuticals commonly present in sewage sludge compost. *Proceedings of 2nd International Conference on Chemical*, *Biological and Environmental Engineering*, *Cairo*, *Egypt*, *2–4 November*, *2010*, 261–264.

Lillenberg, M., Herodes, K., Kipper, K., Nei, L. (2010). Plant uptake of some pharmaceuticals from fertilized soils. *Proceedings of 2010 International Conference on Environmental Science and Technology*, *Bangkok*, *Thailand*, *23–25 April*, 161–165.

3.4 Articles/presentations published in conference proceedings not listed in Section 3.1

Nei, L., **Lillenberg, M.**, Haiba, E., Kipper, K., Herodes, K. (2010). Sewage sludge – a nutrient-rich fertilizer or hazardous waste. In: *Innovative Approaches and Sustainable Technology: Kohtla-Järve*, *27–28 April 2010*, 17–21.

Roasto, M., Püssa, T., Kiis, A., Tamme, T., **Lillenberg, M**. (2003). The research activities of the Department of Food Hygiene of Veterinary Faculty of Estonian Agricultural University (EAU) during the years 2000–2003. *Veterinaarmeditsiin*, 57–63.

5.2 Published meeting abstracts, not indexed by Thomson Reuters Web of Science

Lillenberg, M., Herodes, K., Kipper, K., Nei, L. (2010). Fluoroquinolones and sulfonamides in sewage sludge and its compost. In: *Abstract book: SETAC Europe 20th Annual Meeting, Seville*, *Spain*, *23–27 May 2010*, 192–193.

Kipper, K. **Lillenberg, M**., Yurchenko, S., Herodes, K., Pihl, V., Sepp, K., Lõhmus, R., Nei, L. (2009). Determination of antibiotic residues in sewage sludge by pressurized liquid extraction and LC-ESI-MS. In: *Book of Abstracts: 5th Conference by Nordic Separation Science Society, Tallinn*, *Estonia, 26–29 Aug. 2009*, 102.

Lillenberg, M., Yurchenko, S., Kipper, K., Herodes, K., Nei, L.(2008). Degradation of antibiotic residues during sewage sludge composting. In: *Abstract Book: SETAC North America 29th Annual Meeting*, *Tampa*, *Florida*, *USA*, *16–20 Nov. 2008*, 197.

Lillenberg, M., Herodes, K, Yurchenko, S., Kipper, K., Sepp, K., Nei, L. (2008). Antibiotic residues in some fertilizers. In: *Abstract Book: SETAC Europe 18th Annual Meeting, Warsaw*, *Poland*, *25–29 May 2008*, 79–80.

6.2 Text books

Lillenberg, M., Järvis, T. (2005). Toidutekkelised parasitaar- ja viirushaigused. Text book for students of Institute of Veterinary Medicine and Animal Sciences. Tartu, Halo Kirjastus. 91 p.

6.3 Popular science articles

Nei, L., **Lillenberg, M**. (2009). Reoveesettest valmistatud komposti peab hoolega uurima. *Keskkonnatehnika*, **7**, 12–13.

APPROBATION

International and regional conferences

Poster presentations

Sevilla, Spain 2010. SETAC Europe 20th Annual Meeting 23–27 May 2010. Presentation: Fluoroquinolones and sulfonamides in sewage sludge and its compost.

Tampa, Florida, USA 2008. SETAC North America 29th Annual Meeting 16–20 Nov. 2008. Presentation: Degradation of antibiotic residues during sewage sludge composting.

Warsaw, Poland 2008. SETAC Europe 18th Annual Meeting 25–29 May 2008. Presentation: Antibiotic residues in some fertilizers.

Oral presentations

Tartu, Estonia 2003. Conference of Veterinary Medicine 20–22 Nov. 2003. Presentation: Investigation of transfer of veterinary drug residues in agroecosystems. Estimation of fluoroquinolones in soil and food plants.

ANNEXES

ANNEX 1. Analyses of the soils for plant experiments.

Content of antimicrobials (AM) in soil µg/kg (dw)	Sample No.	pH $_{KCl}$	C%	N%	C/N	P_AL mg/kg	K_AL mg/kg	>0.063	<0.002	0.002-0.063	text_class
Analyses of the loamy soil											
AM free soil (control)	1	6.6	2.5	0.2	11.9	232	273	53.8	9.6	36.6	loamy
AM free soil (control)	2	6.5	2.6	0.2	10.6	254	314	52.1	18.6	29.3	loamy
10	3	6.6									
10	4	6.8									
100	5	6.7									
100	6	6.7									
500	7	6.7									
500	8	6.5									
1000	9	6.7									
1000	10	6.5									
10 mg/kg	11	6.8									
10 mg/kg	12	6.8									
Average in soil containing AM		6.7									
Analyses of the sandy soil											
AM free soil (control)	1	6.9	2.5	0.2	11.9	232	273	81.8	6	12.2	loamy sand
AM free soil (control)	2	6.9	2.6	0.2	10.6	254	314	81.5	5.6	12.9	loamy sand
10	3	7									
100	4	6.9									
500	5	6.9									
1000	6	6.9									
10 mg/kg	7	6.9									
Average in soil containing AM		6.9									

ANNEX 2. Analyses for pH, content of plant nutrients and heavy metals in sewage sludge compost ready for utilization from Tartu STP.

Compound	Mean content in the compost stacks mg/kg (dm)*			Calculated average content in compost mg/kg (dm)	Trigger values for heavy metals mg/kg (dm)
	stack No.1	stack No.2	stack No.3		
Dry matter	254000	328000	327000	303000	
Nitrogen (N)	31800	20500	18880	27727	
Phosphorus (P)	22000	24000	17000	21000	
Calcium (Ca)	60000	69000	57000	62000	
Magnesium (Mg)	8400	9900	8600	8967	
Potassium (K)	3700	4400	3300	3800	
Cadmium (Cd)	0.8	0.9	1.2	1.0	20
Chromium (Cr)	120	140	120	127	1000
Mercury (Hg)	0.9	0.7	0.6	0.7	16
Nickel (Ni)	31	28	32	30	300
Lead (Pb)	42	40	35	39	750
Zinc (Zn)	520	610	600	577	2500
Organic matter	66%	47%	57%	57%	
$pH_{\text{water extract}}$	7.6	8.2	7.5	7.8	

* Data from Tartu Waterworks. The three compost stacks were sampled. 50 samples from each compost stack were mixed and one analysis from the mixture was made. The analyzes were performed in the laboratory of Tartu Environmental Research Ltd.

VIIS VIIMAST KAITSMIST

VAHUR PÕDER

COMPATIBILITY OF ENERGY CONSUMPTION WITH THE CAPACITY OF WIND GENERATORS.
ENERGIA TARBIMISE SOBIVUS TUULEGENERAATORITE VÕIMSUSEGA.
Prof. **Andres Annuk**
21. juuni 2011

MARIT KOMENDANT

ANTENNAL CONTACT CHEMORECEPTION IN GROUND BEETLES (COLEOPTERA: CARABIDAE). JOOKSIKLASTE (COLEOPTERA: CARABIDAE) ANTENNAALNE KEMORETSEPTSIOON.
Vanemteadur **Enno Merivee**; Professor **Anne Luik**
6. mai 2011

MARIS HORDO

APPLICATION OF DENDROCLIMATOLOGICAL METHODS FOR FOREST GROWTH MODELLING.
DENDROKLIMATOLOOGILISTE MEETODITE KASUTAMINE PUISTU KASVUKÄIGU MODELLEERIMISEL.
Prof. **Andres Kiviste**, dr. **Helena Henttonen**, dr. **Samuli Helama**
27. aprill 2011

TATJANA KUZNETSOVA

PLANTATIONS OF NATIVE AND INTRODUCED TREE SPECIES IN THE RECLAMATION OF OIL SHALE POST-MINING AREAS.
KODUMAISTE JA VÕÕRLIIGILISTE PUISTUTE KASV PÕLEVKIVIKARJÄÄRIDE TASANDATUD PUISTANGUTEL.
Juhtivteadur **Malle Mandre**, vanemteadur **Henn Pärn**
25. jaanuar 2011

MIHKEL KIVISTE

CONDITION AND RESIDUAL BEARING CAPACITY OF EXISTING REINFORCED CONCRETE STRUCTURES.
OLEMASOLEVATE RAUDBETOONITARINDITE SEISUND JA JÄÄKKANDEVÕIME.
Prof. **Jaan Miljan**
21. jaanuar 2011

ISBN 978-9949-484-02-7

roheline trükis

Trükitud taastoodetud paberile looduslike trükivärvidega © Ecoprint