Promoters: Prof. dr. ir. Diederik Rousseau
Department of Industrial Biological Sciences
Laboratory of Industrial Water and Ecotechnology
Prof. dr. ir. Gijs Du Laing
Department of Applied Analytical and Physical Chemistry
Laboratory of Analytical Chemistry and Applied Ecochemistry
Dean: Prof. dr. ir. Marc Van Meirvenne
Rector: Prof. dr. Anne De Paepe

Assessing and improving the removal of contaminants of emerging concern in intensified constructed wetlands

Hannele Auvinen

Thesis submitted in fulfillment of the requirements for the degree of
Doctor (PhD) in Applied Biological Sciences

Dutch translation of the title: Evalueren en verbeteren van de verwijdering van opkomende micropolluenten in geïntensifieerde rietvelden

Cover illustration: Detail of an intensified full-scale constructed wetland at Huize Walden, Malle, Belgium (by Hannele Auvinen)

Printed by: University Press, Zelzate, Belgium

Please refer to this thesis as follows:
Auvinen, H. (2017). Assessing and improving the removal of contaminants of emerging concern in intensified constructed wetlands. PhD thesis, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium, pp. 150.

ISBN: 978-90-5989-985-8

Preface

Four years ago the thought of writing this preface seemed something very distant and even impossible to imagine. I am in gratitude to many people for supporting me and for sharing their knowledge with me. All that help has gotten me until the end of this PhD.

First of all, I would like to thank my promoters Prof. Diederik Rousseau and Prof. Gijs Du Laing for giving me the opportunity to do my research independently and for the guidance during these years. I am also very grateful to the members of the examination committee, Prof. Mieke Uyttendaele, Prof. Stijn Van Hulle, Dr. Ralf Kaegi and Prof. Peter Goethals for their encouragement and valuable comments.

In the daily work at the office and the laboratory I met many people who had an important role in this thesis. Thank you Ann, Ellen, Joachim and Roseline for your help in the laboratory with the analyses. Also thank you Katharina, Volker and Wilhelm for the good co-operation. My sincere thanks go also to the master thesis students, Bryan, Iva, Laura, Laurens, Lize, Pieter-Jan and Viviana, for the dedication you showed to our work. I would also like to thank the colleagues in the offices in Kortrijk and at the Coupure - Bjorge, Caroline, Dave, Frederik, Han, Isabel, Ivona, Joël, Lies, Mark, Michael, Natalia, Veerle, Wim, Yannick and Yoann - for the nice talks and for all your help with the experiments and lessons.

I also got a lot of support from the participants of the European COST action ES1205 (Engineered nanomaterials from wastewater treatment & stormwater to rivers) regarding my research on the nanoparticles. I would especially like to thank Lars and Lena, Ralf, Karen, Geert and Kela. Also, thank you Vincent for the good and fun co-operation on our presentations.

Special thanks go to Dion for helping me with the pilot, for sharing his knowledge and especially for being available for any questions at any time.

I would also like to thank the personnel at A.Z. Groeninge and Huize Walden, especially Jo Dendauw, Joyce De Cokere and the technical personnel, for the co-operation and their help during the experiments. I am also thankful to Ann-Sofie Vandendriessche and Filip Dewaele from Aquafin for giving the opportunity to set up my experiment on Aquafin site.

Last but not least, my warmest thanks go to my families in Finland and Belgium for their support and for being the listening ear during these years. Wouter, in the end we could say it was a bit of a team work finishing this thesis. While I did the writing, you organized the catering, the child care, checked my statistics, spelling and so much more. I am really honored to have you by my side and I do not think there is a challenge we would not tackle. Nea ja Max, teitä haluaisin kiittää kaikkein eniten. Te muistutatte aina minua siitä mikä elämässä oikeasti on tärkeää.

Hannele
Hämeenlinna, 9th April 2017

Contents

List of abbreviations

ACWD	Average well color development
API	Active pharmaceutical ingredient
ATL	Atenolol
BPEI	Branched polyethyleneimine
BSP	Bisoprolol
BOD_5	Biological oxygen demand
CBZ	Carbamazepine
CEC	Contaminant of emerging concern
CEC	Cation exchange capacity
CLLP	Community-level physiological profiling
COD	Chemical oxygen demand
CSUP	Carbon source utilization pattern
CW	Constructed wetland
DCF	Diclofenac
DF	Darf field
DGGE	Denaturing gradient-gel electrophoresis
DLS	Dynamic light scattering
DZP	Diazepam
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
EC50	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
EDX	Energy-dispersive X-ray spectroscopy
ENM	Engineered metallic nanomaterial
EPS	Extracellular polymeric substance
EU	European Union
FWS	Free water-surface
GA	Gum arabic
GBP	Gabapentin
HAADF	High-angle annular dark field
HRT	Hydraulic retention time
HSSF	Horizontal sub-surface flow
HQ	Hazard quotient

ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry
I.E.	Inhabitant equivalent
IS	Internal standard
LC-MSMS	Liquid chromatography tandem mass spectrometry
LOD	Limit of detection
LOEC	Lowest observed effective concentration
MEC	Measured environmental concentration
MFM	Metformin
MTP	Metoprolol
MWCO	Molecular weight cut-off
n.a.	Not available
NOEC	No-effect concentration
NOM	Natural organic matter
NP	Nanoparticle
OM	Organic matter
PC	Principal component
PCA	Principal component analysis
PCR	Polymerase chain reaction
PCS	Photon correlation spectroscopy
PEC	Predicted environmental concentration
PNEC	Predicted no-effctive concentration
PVA	Polyvinyl alcohol
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
SBR	Sequencing batch reactor
SF	Surface flow
SDS	Sodium dodecyl sulfate
SD	Standard deviation
SMX	Sulfamethoxazole
SPE	Solid phase extraction
STL	Sotalol
SRHA	Suwannee river humic acid
SS	Suspended solids
SSF	Sub-surface flow
STEM	Scanning transmission electron microscopy
TEC	Trace element composition
TEM	Transmission electron microscopy
TMD	Tramadol
TOC	Total organic carbon
TSS	Total suspended solids
TST	Telmisartan
UF	Ultra filtration
VMM	Vlaamse milieumaatschappij

VSSF	Vertical sub-surface flow
VST	Valsartan
WWTP	Wastewater treatment plant

Chapter 1

Scope and aim of this thesis

Wastewater contains a wide variety of micropollutants, such as pharmaceuticals, pesticides, nanomaterials, fire retardants and plasticizers, in addition to the common contaminants, i.e. organic matter, suspended solids and nutrients. The development of sensitive analytical instrumentation in the last 20 years has increased our awareness about the presence of these micropollutants in the environment, their transformations and fate and also, about their alarming and often unforeseen environmental effects (Noguera-Oviedo, Aga, 2016). These "new" pollutants are measured in environmental samples mostly at very low concentrations (ng/L - μg/L) and are collectively named contaminants of emerging concern (CECs). In this work, focus is laid on pharmaceuticals and metallic engineered nanomaterials (ENMs). Pharmaceuticals were chosen for this study because of their widespread presence in domestic and hospital wastewater, the two types of urban wastewater investigated in this work. Among ENMs, silver nanoparticles (Ag-NPs) were targeted due to their various applications in household products. Also, due to the toxicity of pharmaceuticals and Ag-NPs in the environment, it is important to study their removal during wastewater treatment.

The Water Framework Directive (2000/60/EC and its amendments) is the corner stone of European water policy. It aims to prevent the deterioration of the quality of surface waters, to protect them and, where necessary, to improve the quality (European Parliament, 2000). In 2001, the first list of so called priority substances, i.e. substances which pose a significant risk to or via the aquatic environment, was established and environmental quality standards in surface waters were set for these substances (European Parliament, 2001). The list includes e.g. heavy metals, industrial chemicals, herbicides and pesticides. The priority substances list has been updated at several occasions and in 2013, an additional watch list was established (European Parliament, 2013) and updated in 2015 (European Commission, 2015a). Although no regulations are yet applied to the substances on the watch list, more information about the presence of these substances in European surface waters is gathered. Several pharmaceutical compounds (diclofenac, three hormones and three antibiotics) are on this list.

Research has shown that wastewater treatment plants are an important point source of CECs into the environment because common wastewater treatment systems are not designed to remove these contaminants (Carballa et al., 2004). Advanced oxidation techniques, such as ozonation, are often brought forward as solutions to mitigate the discharge of pharmaceuticals (Audenaert et al., 2014). However, due to their high start-up and maintenance costs and requirement for technical personnel for operation, they cannot be considered as an option for small-scale treatment systems and remote locations. Metallic ENMs are nowadays widely used because of their specific properties arising from their small particle size and are even incorporated in many general household products (Brar et al., 2010). The increasing production and use of these materials increases the risk of them being released into the environment. The removal of metallic ENMs during wastewater treatment does not follow the same principles as that of pharmaceuticals because metal contamination can only be relocated. Pharmaceuticals are organic compounds and their biodegradation is at least theoretically possible. The biodegradation of different pharmaceuticals is not equally efficient because of their various chemical structures. The structure may be non-biodegradable as is for many compounds comprising polycyclic structures and halogens. The degradation pathways of certain structures can also be incomplete and hence, result in the production of non-biodegradable transformation products. Both pharmaceuticals and ENMs can undergo further chemical and physical removal processes in CWs, such as attachment of these compounds onto surfaces. Plants can also play a role in retaining these compounds.

Constructed wetlands (CWs) are man-made ecosystems in which natural processes take place to treat wastewater. CWs are mainly used as small-scale decentralized treatment systems at single households, small communities or industries which are remotely located. They can also be utilized as tertiary treatment when placed after e.g. activated sludge treatment or rotating biological contactor. In Flanders, CWs are applied as a (partial) treatment system at approximately only 10% of Flemish wastewater treatment systems owned by Aquafin NV. In other European countries (perhaps due to the less dense population) CWs are more common. For example, in France, most wastewater treatment systems (>85%) have the treatment capacity of less than 5000 I.E and currently some 20% of these systems are CWs (Millot, 2017). In the future, the refurbishment of existing ponds and sand filters can increase the proportion of CWs to 50% of all treatments plants smaller than 5000 I.E. The importance of CWs as individual treatment systems is rising also in Flanders, because European Union member states need to organize wastewater treatment of small communities and plan implementation of individual treatment systems for single households to meet the environmental quality standards stated in the EU Water Framework Directive (2000/60/EC) (Auvinen et al., 2016a).

CWs are an attractive option in remote locations in comparison to conventional wastewater treatment systems because they can be less costly to build and maintain and because of their green appearance they are better integrated with the rural landscape. Their maintenance does not require special technical skills, although lack of proper maintenance can cause malfunctions such as clogging. Perhaps the most important challenge of CWs is that they have a large footprint and CWs are therefore rarely suitable for urban locations. CWs come in various configurations which are categorized based on two main design parameters: hydrology and flow path. As a consequence, the pollutant removal mechanisms as well as the ambient conditions, such as dissolved oxygen concentration, inside the CW affecting the pollutant removal efficiency can be different depending on the type of CW (Vymazal, 2011).

In terms of CECs, it is important to study the removal processes in CWs for several reasons: Firstly, CWs are a common wastewater treatment system in Europe (Vymazal, 2002; Rousseau et al., 2004; Puigagut et al., 2007) and their use is rising. Secondly, the CWs which are located in remote areas discharge their effluent in small water ways where low or even negligible dilution occurs. In the rural areas these streams can have high biodiversity which is put at risk by the pollutant emissions from the wastewater treatment system. Thirdly, when CWs are located near the wastewater source, as is usual, the sewer system is short and low dilution is expected to occur (unless combined sewer systems are used) possibly inducing high concentrations of pollutants in the influent. Finally, according to literature further information is needed on factors affecting pharmaceutical removal. Furthermore, little is known about the processes ENMs undergo in CWs and their removal efficiency in these systems.

The specific research questions are selected to address these concerns. What is the fate of Ag-NPs in CWs? Do CWs function as sinks for these particles similarly to activated sludge treatment systems and which transformations do they undergo during treatment? Is it possible that the aging of the CW or active aeration increases the release of ENMs from CWs? A lot of research has already been conducted on the removal of pharmaceuticals in CWs. From literature it appears that redox potential and dissolved oxygen concentration have an effect on the pharmaceutical removal efficiency (e.g. Zhang et al. (2012b)) but as the effect of active aeration, i.e. intensification, is inadequately studied, it is the main research line of the second part of this thesis. Can the removal of selected pharmaceuticals be improved by applying active aeration and/or by lengthening the hydraulic retention time? How does an intensified CW perform when the influent has a high concentration of pharmaceuticals? What is the ecological effect of the pharmaceutical discharge from a full-scale intensified CW?

This thesis begins with a literature overview on possible transformation and removal processes of the selected CECs in CWs (*Chapter 2*). Chapter 2 also discusses the possible release routes of ENMs from CWs and the potential toxicity of metallic ENMs on CW microbial communities. Chapter 2 further reviews scientific literature on the removal efficiency of selected pharmaceuticals in different types of CWs and evaluates the risks involved in the discharge of pharmaceuticals via wastewater effluent.

The main objective of the research chapters is to contribute to the understanding of the fate of CECs in constructed wetlands. Two types of CECs, pharmaceuticals and Ag-NPs, were chosen as the target components in this thesis because of their known toxic properties and ubiquitous presence in domestic and hospital wastewater. Figure 1.1 illustrates the interplay between the research chapters.

Different types of setups are used to comprehensively understand their fate and behavior in intensified CWs. The fate of Ag-NPs is studied using microcosms simulating CWs. It is necessary to apply such small-scale setups to be able to investigate the effect of different parameters on the fate of Ag-NPs (*Chapter 3*). Aeration and increasing organic matter, are chosen as the target parameters. Intensified (i.e. aerated) CWs are the newest development in wetland technology. They offer more efficient treatment than conventional CWs and require only about one third of the land area needed for a conventional system. The aeration brings along extra costs but these costs are remarkably lower (∼50%) than by an activated sludge treatment. During the lifetime of a CW, which is some 20 years, organic and inorganic matter builds up in the substrate bed. Earlier research has shown that ENMs are efficiently attached onto surfaces and especially to organic matter. It is thus investigated how the increasing of organic matter (leaf litter), simulating aging, affects the Ag-NP fate.

ENMs can undergo different processes inside a constructed wetland. In *Chapter 4* some of these processes are studied in detail to fill up the knowledge gained in the microcosm experiments. Hence in this chapter, the effect of different types of substrates on the Ag-NP removal from water phase and the role of wetland plants (typical CW helophyte *Phragmites australis*) are investigated.

The known toxicity of Ag-NPs and their affinity for organic matter were the incentives for the investigations in *Chapter 5*. In this chapter the impact of Ag-NPs on microorganisms habiting the microcosm biofilm is investigated and compared to the effects caused by Ag ions.

In *Chapters 6 and 7*, the removal of pharmaceuticals in pilot- and full-scale CWs is studied. A pilot-scale CW was chosen for the first investigations because the operation of such a system can easily be adjusted and it can easily be transported which then again allows comparing between different types of wastewater. The removal of various types of pharmaceuticals is already widely studied in different types of CWs. However, little is known about the performance of actively aerated (intensified) CWs. Therefore, a pilot-scale CW was built to investigate the suitability of such a system to treat the pharmaceutical load in domestic and hospital wastewater (*Chapter 6*). It is likely that hydraulic retention time (HRT) also plays an important role in defining the removal efficiency of pharmaceuticals. This is because pharmaceuticals often have complex chemical structures and their biodegradation can be increased by longer contact time between the organism and pollutant. The interplay of aeration and HRT in improving the removal efficiency of a certain pharmaceutical is studied in batch experiments (*Chapter 7*).

CWs often discharge their effluent in small water courses and hereby possibly pose a risk to the local biodiversity. The removal efficiency of pharmaceuticals in a full-scale CW is investigated in *Chapter 7* and the risk posed by effluent discharge from this CW into an effluent-dominated stream is analyzed based on hazard quotients.

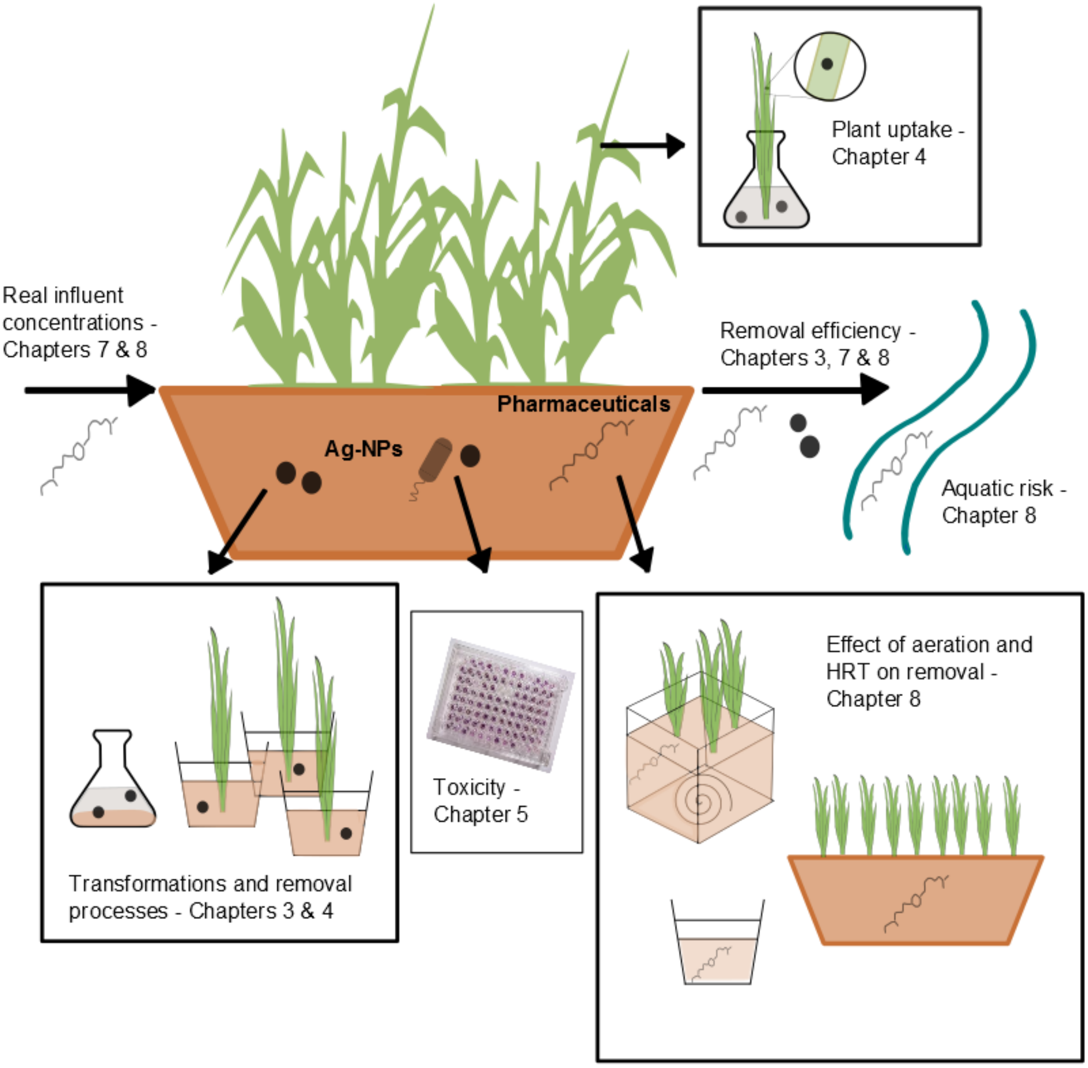


Figure 1.1: Schematic representation of the interplay between the research chapters. The fate of two different types of CECs is investigated, and the combining factor for these experiments is constucted wetlands.

Chapter 2

Assessing the transformations and removal of metallic nanoparticles and pharmaceuticals in constructed wetlands - a review

This chapter is partly based on **Auvinen H.**, Gagnon V., Rousseau D.P.L., Du Laing G. 2017. Fate of metallic nanoparticles in constructed wetlands: prospection and future research perspectives. Reviews in Environmental Science and Bio/Technology (in press).

2.1 Introduction

A typical constructed wetland (CW) for wastewater treatment is a sub-surface flow CW, where water flows either vertically or horizontally through a gravel or sand bed. CWs are planted to increase the microbial activity in the bed, to increase nutrient removal and for aesthetic reasons. CWs are commonly used as decentralized wastewater treatment systems in rural areas because, on one hand, of their robust treatment and low maintenance requirement and, on the other hand, because of their large land area requirement, which restricts their use in urban areas. In addition to domestic wastewater, also industrial wastewater, stormwater and landfill leachate are treated in CWs (Kadlec, Wallace, 2009). When used for the treatment of domestic wastewater, CWs are most often used as a secondary treatment for settled wastewater but in some cases also as tertiary treatment after activated sludge treatment or rotating biological contactor. In France, CWs are often designed to treat domestic wastewater without primary settling (Molle et al., 2005). This creates a sludge layer on top of the wetland which is disposed of about every 10 years. The chemical content of this sludge may restrict its use as soil amendment. Both types of CECs, engineered nanomaterials (ENMs) and pharmaceuticals are likely to occur in the wastewater that is treated in CWs.

ENMs can be discharged to the wastewater via the production, usage and disposal phases (Figure 2.1). For example, silver nanoparticles (Ag-NPs) and titanium dioxide nanoparticles (TiO_2-NPs) are used in common household products. As they are used in textiles, plastics and cosmetics, they can be released from the product during washing and end up in wastewater (e.g. Benn, Westerhoff (2008)). Also, ENMs can be released from (weathered) materials, e.g. from painted facades during rainfall and then be transported with runoff (Kaegi et al., 2008). A large amount of ENM containing materials are disposed of in landfills (Keller, Lazareva, 2013) and leaching could occur (Gottschalk, Nowack, 2011).

Pharmaceuticals and more precisely their active pharmaceutical ingredients (APIs) are a group of compounds with very different functional and chemical properties (Kümmerer, 2010). Many APIs can be partially metabolized by the (human) body resulting in the excretion of both parent compound and its metabolite(s). These compounds, named collectively pharmaceutical residues, reach the wastewater treatment plant where they can undergo further abiotic and biotic transformations.

In this chapter, we provide an overview of transformation and removal processes of ENMs and pharmaceuticals. Based on literature data we discuss the probable fate of ENMs in CWs and evaluate the removal efficiency of the selected pharmaceuticals in CWs. We also discuss the likelihood of ENMs being released from CWs, assess the potential toxicity of ENMs on the wastewater treatment processes in CWs and analyze the environmental impact posed by the discharged pharmaceuticals.

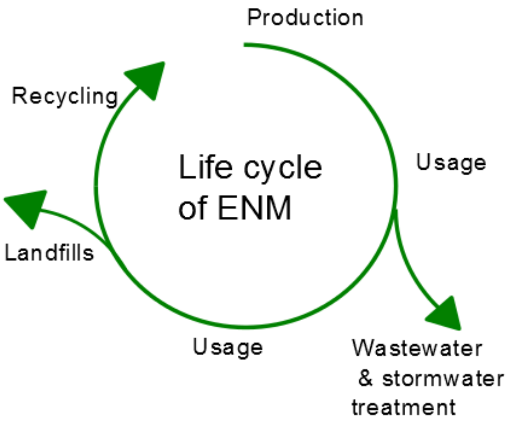


Figure 2.1: Possible release of ENM to the environment during their life cycle

2.2 Predicted environmental concentrations of engineered nanomaterials

At the moment, little is known about the environmental concentrations of ENMs because current analytical methodologies do not allow distinguishing between naturally occurring nanoscale materials and ENMs (Von der Kammer et al., 2012). However, several calculations of predicted environmental concentrations (PEC) based on a probabilistic material flow analysis from a life-cycle perspective of ENM containing products have been performed. Blaser et al. (2008) estimated that the concentration of Ag-NPs in wastewater influent ranges between 2 to 18 μg/L which is somewhat higher than measured for nanosized Ag in real wastewater influent (<1.5 μg/L; Li et al. (2013)). TiO_2-NPs and ZnO-NPs are frequently used in sun screens and can be released from the skin in the shower. Considering TiO_2-NPs in a typical sun screen (containing ~5% TiO_2 w/v), it can be calculated that approximately 0.45 mg Ti (0.05 g/L * 15 mL * 48 mol/(48+2*16 mol) = 0.45 mg Ti) is washed off from the skin in the shower after using some 15 mL of sun screen on a sunny day. The concentration of Ti in the grey water can be estimated to ~10 μg/L.

Li et al. (2013) showed that the concentration of the nanosized Ag decrease considerably during wastewater treatment, to <12 ng/L. This is in agreement with modeling studies: Sun et al. (2014) predicted concentrations of 0.06 - 16 ng/L, 13 - 110 μg/L and 0.05 - 0.29 μg/L for Ag-NPs, TiO_2-

NPs and zinc oxide NPs (ZnO-NPs) in wastewater effluent, respectively. The concentrations further decrease upon discharge to surface water due to dilution. The concentrations of Ag-NPs, TiO_2-NPs and ZnO-NPs in European surface water were estimated to be very low, on average 0.66 ng/L, 0.53 μg/L and 0.09 μg/L, respectively (Sun et al., 2014). Although Markus et al. (2013) predicted that the contribution of Ag-NPs, TiO_2-NPs and ZnO-NPs in the current total load of these metals in the rivers Rhine and Meuse is very low, the toxic effects of these particles in small streams and especially very locally in the sediments at the discharge point need to be considered carefully. Furthermore, the accuracy of such model estimates has not yet been verified. The models are based on estimations and approximations on e.g. production volumes and concentrations leaching from a product during its life cycle, which bring along (currently still) large uncertainties.

2.3 Possible transformation processes affecting the fate of engineered nanomaterials in constructed wetlands

When ENMs are released to an aquatic medium they can undergo physical, chemical and biological transformations which affect their fate and behavior in the environment. These transformations include primarily processes such as dissolution, aggregation, sedimentation, sorption onto particulate matter and other solid surfaces and interaction with dissolved ions. The transformation processes are dependent on both the properties of the ENM (e.g. type, coating, size) and the prevailing environmental conditions (e.g. natural organic matter and dissolved oxygen). In the following sections, the main transformation processes of different ENMs reported in earlier literature are reviewed and the environmental factors affecting these processes are discussed in relation to their possible fate in CWs.

2.3.1 Aggregation and sedimentation

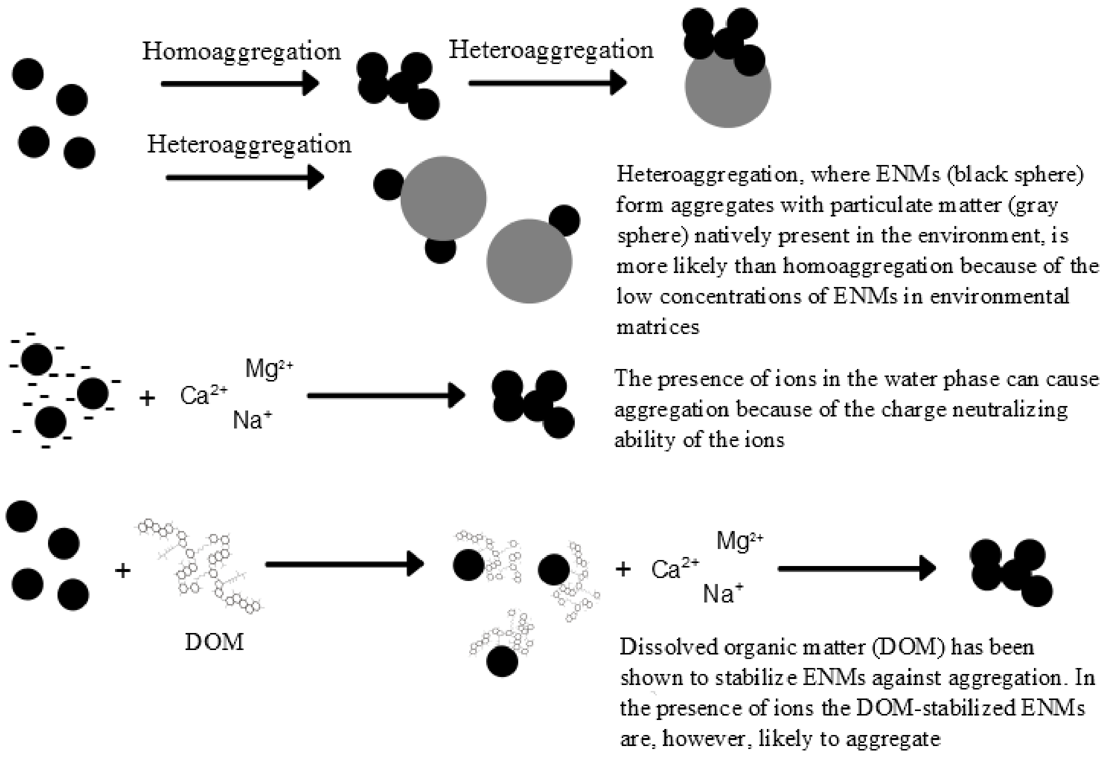
Aggregation and subsequent sedimentation of ENMs decrease their mobility and bioavailability by restricting their distribution in the aquatic environment. Also, the surface area of the particles decreases due to aggregation which in turn decreases their reactivity, affecting the release of ions for instance (see further Section 2.3.2).

Nanosized dispersed particles are not likely to settle. They move about in the dispersion by Brownian motion and along the streaming water. When this movement brings particle surfaces in contact, thermodynamic interactions allow aggregation to occur (Zhang, 2014). ENMs can undergo two forms of aggregation in the environment, homo- and heteroaggregation. Whereas in homoaggregation particles of the same type collide and form aggregates, in heteroaggregation ENM aggregates with another kind of particulate or colloidal matter (Quik et al., 2014). When ENMs are scarcely present, as in most environmental media, the likelihood of homoaggregation is low (Hotze et al., 2010). In wastewater heterogenic particulate matter is ubiquitous and hence, aggregates formed with this matter and ENMs are much more likely to be formed than aggregates composed of only ENMs.

Because the properties of ENMs are related to their small size, the ENMs are mostly coated to enhance their dispersion stability, i.e. to prevent aggregation (Hotze et al., 2010). The mechanism of stabilization, electrostatic or steric, can have an impact on the behavior of the ENM in the environment (Christian et al., 2008). Electrostatically stabilized ENMs are kept in dispersion through the repulsion of similar surface charges and are thus more susceptible to changes in ionic concentrations in the media than sterically stabilized particles having an isolating polymer coating to keep them in dispersion. Several studies have investigated the effect of ionic strength on the aggregation behavior of different types of ENMs. Huynh, Chen (2011) and Li et al. (2010) studied the effects of NaCl, $MgCl_2$ and $CaCl_2$ on Ag-NPs with different coatings. The common conclusion that can be drawn from these studies is that the type of ion is more determinant of the aggregation than the mechanism of stabilization. For example, complete aggregation of citrate-coated (i.e. electrostatic stabilization) or polyvinylpyrrolidone (PVP)-coated (i.e. steric stabilization) Ag-NPs is achieved by >10 mM of monovalent ions, while only >1 mM of divalent ions is needed for complete aggregation. Divalent ions, such as e.g. Ca^{2+}, have a stronger charge neutralizing ability than monovalent ions, e.g. Na^+ (El Badawy et al., 2012). Also TiO_2-NPs, cerium dioxide NPs (CeO_2-NPs), copper oxide nanoparticles (CuO-NPs), copper nanoparticles (Cu-NPs) and ZnO-NPs have been observed to undergo aggregation and sedimentation at elevated ionic strength (Zhang et al., 2009; Zhou, Keller, 2010; Gallego-Urrea et al., 2014). Although the natural concentrations of the ions are somewhat lower (in the range of 0.5 - 5 mM; Verbanck et al. (1989)) than in the studies on synthetic waters, a cumulative effect can be expected in real freshwater and wastewater samples.

Similarly to coating, natural dissolved organic matter (DOM) which attaches on the surface of the ENMs has a stabilizing effect on the ENMs due to which the ENMs are more persistent to aggregation (Gallego-Urrea et al., 2014). DOM has been shown to cause disaggregation and subsequent decrease in Ag-NP size distribution at concentrations 5 - 20 mg/L total organic carbon (TOC) as Suwannee river humic acid (SRHA) and up to 15 mg Ag/L (Kanel et al., 2015; Fabrega et al., 2009; Metreveli et al., 2015). Similarly, the presence of DOM has been shown to have a stabilizing effect on TiO_2-NPs (Zhang et al., 2009), CeO_2-NPs (Van Hoecke et al., 2011), CuO-NPs (Ben-Moshe et al., 2010) ZnO-NPs (Zhang et al., 2009), iron trioxide NPs (Fe_2O_3) (Ben-Moshe et al., 2010; Zhang et al., 2009); Zhang et al. 2009), gold NPs (Au-NPs) (Stankus et al., 2011) and nickel oxide NPs (NiO-NPs) (Zhang et al., 2009). However, there are also contradictory results showing no significant impact of SRHA (2.5 - 10 mg TOC/L) on the aggregation state of carbonate-coated Ag-NPs or disaggregation of pre-agglomerated Ag-NPs (Piccapietra et al., 2012).

The dissolved fraction of the total natural organic carbon is some $20 - 30\%$ in wastewater influent but in effluent nearly 100% (Dignac et al., 2000). Studies on dissolved organic carbon (DOC) removal in activated sludge treatment plants have reported concentrations of $10 - 20$ mg DOC/L in wastewater effluent (Katsoyiannis, Samara, 2007; Dignac et al., 2000) proving the relevance of the above mentioned studies in the context of wastewater treatment. In CWs similar removal of the particulate fraction is expected due to efficient removal of total suspended solids (TSS) (Vymazal, 2009) but the composition and concentration of DOM may differ from that in an activated sludge treatment system. A study of Barber et al. (2001) on tertiary surface-flow CWs treating domestic wastewater, pulp mill and dairy effluent showed that CWs receiving treated wastewater can even produce an increase in DOC. The autochthonous DOM in CWs originates primarily from microbial activity (Quanrud et al., 2004), plant debris (Pinney et al., 2000) and plant root exudates (Glatzel et al., 2003). This indicates that the DOM discharged from CWs differs probably greatly from that of activated sludge treatment systems and may have a different impact on the stability of ENMs. However, in wastewater the ionic strength, especially the presence of Ca^{2+}, is probably more determinant of aggregation than DOM. Metreveli et al. (2015) showed that above a relatively low concentration of Ca^{2+} ($\sim$20 mg/L; moderately soft water) Ag-NPs aggregated until sedimentation independently of the presence or absence of SRHA.



Heteroaggregation, where ENMs (black sphere) form aggregates with particulate matter (gray sphere) natively present in the environment, is more likely than homoaggregation because of the low concentrations of ENMs in environmental matrices

The presence of ions in the water phase can cause aggregation because of the charge neutralizing ability of the ions

Dissolved organic matter (DOM) has been shown to stabilize ENMs against aggregation. In the presence of ions the DOM-stabilized ENMs are, however, likely to aggregate

Figure 2.2: The aggregation process of ENMs in environmental samples.

2.3.2 Dissolution

When an ENM is dissolved, metal ions are released from its surface. The dissolution process of ENMs has environmental relevance from the risk analysis perspective because ions are often more toxic than ENMs (Levard et al., 2013a; Sotiriou, Pratsinis, 2010; Li et al., 2011; Moore et al., 2016). However, dissolution can possibly reduce the mobility as Ag^+ and Ce^{2+} appear to be more efficiently bound to soil/sediment than their nanoparticle counterparts (Cornelis et al., 2012; Van Koetsem et al., 2015).

Odzak et al. (2014) compared the dissolution tendencies of four different types of ENMs in artificial aqueous media chemically similar to environmental waters, and found clear differences in the dissolution rates of the ENMs. While citrate, gelatin, PVP and chitosan coated Ag-NPs were very poorly soluble (only some %), ZnO-NPs were very rapidly largely dissolved. The dissolution of CuO-NPs was found to be incomplete and faster than that of carbon-coated Cu-NPs but both dissolved to a smaller extent than ZnO-NPs. Quik et al. (2014) studied the environmental transformations of CeO_2-NPs in different surface waters and observed only minor dissolution (<0.4%).

The dissolution rate is also related to particle size; small ENMs being dissolved faster than larger ones due to their greater surface-to-volume ratio and subsequent increased reactivity (Zhang et al., 2011b). As a consequence, the dissolution rate of aggregated ENMs is reduced (Li, Lenhart, 2012).

Odzak et al. (2014) observed that the dissolution tendency is to some extent also dependent on the coating of the ENM. The presence of NOM in the water also inhibits the dissolution of ENMs and this is a dose dependent manner (Liu, Hurt, 2010). The inhibition is likely caused by NOM attaching onto the surface of the ENM which results in steric stabilization analogue to a coating (Delay et al., 2011).

Ions dissolved in the water can affect the dissolution rate of ENMs. The reaction with sulfide, i.e. sulfidation, can either decrease or increase the dissolution process (Levard et al., 2011; Ma et al., 2013, 2014) (see Section 2.3.3). ZnO-NPs can react with phosphates to form precipitates which have lower solubility than the ZnO-NPs themselves (Rathnayake et al., 2014). The reaction between Ag-NPs and chloride ions can decrease the release of Ag^+ by formation of a solid AgCl precipitate on the outer layer of the ENM (Li et al., 2010) if the molar Cl/Ag ratio is ≤ 535) (Levard et al., 2013b). However, at environmentally relevant (low) concentrations of Ag-NPs (see Section 2.2) in wastewater the Cl/Ag molar ratio is much higher (in the range of 10^5 if 1 μg Ag/L and 100 mg Cl/L is assumed) and this promotes the formation of readily soluble Ag-Cl complexes instead (Levard et al., 2013b).

The dissolution tendency of some ENMs depends also on the solution pH. For example, the dissolution of Ag-NPs is promoted in acidic pH (Peretyazhko et al., 2014). However, the pH in domestic wastewater is between 7 and 8 (Henze, Comeau, 2008) and therefore, the effect of pH on dissolution of ENMs is not further considered for CWs treating domestic wastewater. Ag-NPs are more easily dissolved if there is oxygen present in the environment because oxygen causes the Ag-NPs to oxidize to silver oxides (Ag_2O) which easily dissolve upon contact with water (Levard et al., 2012). In CWs the dissolved oxygen concentration depends on the type (vertical sub-surface (VSSF) or horizontal sub-surface flow (HSSF)) and possible application of active aeration. Higher dissolved oxygen concentrations are usually measured in VSSF CWs than in HSSF CWs because the batch feeding mode applied in VF CWs allows the pores to be filled with air between feeding events.

2.3.3 Sulfidation

The reaction of ENMs with sulfides is interesting because sulfidation of Ag-NPs has been shown to decrease their toxicity (Reinsch et al., 2012; Levard et al., 2013a). When sulfide concentration is low, e.g. in surface water, sulfidation of Ag-NPs occurs via oxidative dissolution and subsequent precipitation (Figure 2.3; Liu et al. (2011)). First, Ag^+ is formed via oxidative dissolution in the presence of dissolved oxygen, after which these ions precipitate with sulfides to form silver sulfide (Ag_2S). For the reaction to occur, sulfide can be either free or in the form of metal sulfides (e.g. CuS, ZnS) (Thalmann et al., 2014). When sulfide concentration is high, e.g. within HSSF CWs, sulfidation of Ag-NPs occurs via a direct particle-fluid reaction where the Ag phase is converted to Ag_2S phase (Figure 2.3; Liu et al. (2011)).

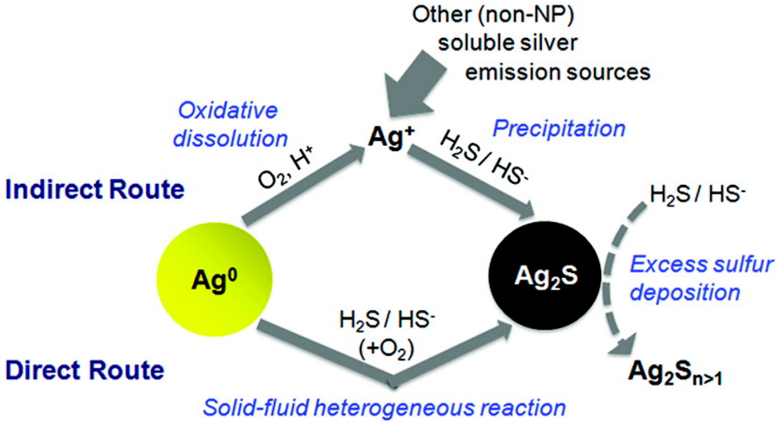


Figure 2.3: Sulfidation pathways of Ag-NPs. Reprinted with permission from Liu et al. (2011): Kinetics and mechanisms of nanosilver oxysulfidation. Environmental Science and Technology 45 (17), 7345-7353. Copyright 2017 American Chemical Society.

Ma et al. (2013) studied the sulfidation mechanism of ZnO-NPs in the laboratory. While a solid Ag_2S phase is formed during the sulfidation of Ag-NPs, the ZnO core becomes covered by nanocrystalline zinc sulfide (ZnS). The ZnS shell does not quench the leaching of Zn^{2+} as efficiently as the Ag_2S phase (Levard et al., 2011). In contrast to Ag- and ZnO-NPs, the sulfides formed of CuO-NPs are more prone to dissolution than the original NP (Ma et al., 2014). The main reason is proposed to be the formation of poorly ordered easily soluble Cu_xS_y phases instead of poorly soluble CuS. The reaction occurs through both dissolution and reaction with sulfide as well as a direct solid-fluid sulfidation.

Sulfidation has been identified as one of the primary transformation mechanisms of Ag-NPs in the sewer systems (Kaegi et al., 2013) as well as in pilot- and full-scale activated sludge treatment systems (Kaegi et al., 2013; Kent et al., 2014) and in terrestrial and freshwater wetland mesocosms (Moore et al., 2016; Lowry et al., 2012a). Lombi et al. (2013) showed that Ag-NPs were sulfidized also in laboratory-scale anaerobic digestors fed with real sludge and wastewater and that the reaction occurred irrespective of surface functionalities or core composition (Ag or AgCl) of the NP. Lowry et al. (2012a) noticed however that sulfidation in the freshwater wetland mesocosms was slower than expected based on laboratory studies and that the sulfidation had occurred only partially after 18 months and leaching of Ag^+ was still possible. In sewers and during primary treatment sulfidation is expected to be rather fast. Kaegi et al. (2011) reported nearly complete sulfidation after 2 h during wastewater treatment and Brunetti et al. (2015) showed that 60 - 80% of the spiked Ag-NPs underwent sulfidation in the sewer system. Once fully sulfidized, Ag_2S is very stable as it does not oxidize during prolonged aeration (Choi et al., 2009) and remains stable in wastewater sludge over more than 50 years (Donner et al., 2015).

ZnO-NPs are also prone to sulfidation in environmental matrices although they may not be equally persistent as sulfidized Ag-NPs. In short-term, the sulfidation of ZnO-NPs seems to be efficient as Brunetti et al. (2015) showed that the spiked ZnO-NPs nearly completely sulfidized in a full-scale sewer experiment. Lombi et al. (2012) studied the fate of ZnO-NPs during anaerobic digestion of sewage sludge and in conditions simulating the post-processing of digested sludge in composting/stockpiling. First, the ZnO-NPs underwent sulfidation which represented 67 - 96% of the total Zn. The Zn speciation was affected by the long (two months) incubation simulating composting/stockpiling and during this phase, ZnS was oxidized and Zn became associated with iron precipitates and complexed by phosphates.

The sulfide concentration of the wastewater (in sewer $\sim$4 mg S/L; Kaegi et al. (2013)) is expected to increase in CWs due to microbial sulfate reduction, where sulfate-reducing bacteria catalyze the production of sulfide from sulfate in the absence of dissolved oxygen (Wu et al., 2013). If we assume the sulfate removal efficiency to be 30% (based to laboratory-scale experiments of Wiessner et al. (2005)) and an average sulfate concentration of $\sim$30 mg S/L for domestic wastewater influent (Yoda et al., 1987), an increase of $\sim$9 mg S/L can be expected in CWs. Bearing in mind the low predicted environmental concentrations of ENMs (see section 2.2), the S:Ag and S:ZnO molar ratio's would be in the range of 10^4. Since the molar ratios necessary for the sulfidation of Ag-NPs and ZnO-NPs is much lower (in the range of 0.5 - 1; Levard et al. (2011)), (partial) sulfidation of these ENMs in CWs seems unavoidable.

2.3.4 Attachment to solids

In general, adsorption of molecules onto surfaces can result from three types of interactions, physical, chemical and electrostatic interactions (Voice, Weber, 1983). In the environment, these interactions are usually involved in the sorption process simultaneously at varying degrees because of the heterogeneous chemical structure of natural (suspended) solids. In case of ENMs, their surface properties, such as surface charge and hydrophobicity/hydrophilicity, are likely to affect the adsorption efficiency (Lin et al., 2010). Hence, the coating of the ENM plays an important role in defining its sorption tendency. Song et al. (2011) investigated the effects of organic coatings of different hydrophobicity on Ag-NP adsorption onto chemically heterogeneous collector surfaces. The study indicated that ENM coatings with higher hydrophobicity (PVP and gum arabic (GA)) had greater attachment to hydrophobic surfaces than particles with a less hydrophobic coating (citrate). However, it is crucial to note that the surface properties of ENMs are affected by molecules present in the environmental matrix and hence, to predict the adsorption tendency of the ENMs based on their pristine structure is not possible. In the environment ENMs are subjected to interactions with ions (e.g. sulfides, phosphates) and macromolecules (e.g. humic acids, proteins) which are likely to result in modifications in the (surface) structure and charge of the ENMs and hence, affect their fate in the environment (Lowry et al., 2012b; Lin et al., 2010).

ENMs are, in general, shown to efficiently attach to soil and sediment, especially to the organic fraction present (Cleveland et al., 2012; Coutris et al., 2012), although Ag-NPs and CeO_2-NPs have been shown to be less prone to adsorption than their ions, indicating that these ENMs have a higher mobility than their ions (Van Koetsem et al., 2015; Cornelis et al., 2012). In wastewater treatment, the organic matter functions as a sink for the ENMs (Table 2.1). The results in Table 2.1 showing that ENMs partition to great extent to organic matter, support the hypothesis that organic matter, when suspended, is likely to be a major transport medium for ENMs. Thus, enhancing the removal of TSS during wastewater treatment is likely to result in improved removal of ENMs (Kaegi et al., 2013).

The removal of TSS is mostly very efficient in CWs. The long-term monitoring of 10 Czech HSSF CWs treating domestic wastewater showed that the CWs reliably decreased the TSS concentration to mostly under 20 mg/L independent of the influent concentration (maximum reported concentration 1457 mg/L) (Vymazal, 2011). During the life time of the CW, solids are building up within the substrate, especially at the inlet of the CW. Although the fraction of organic matter in these solids can vary greatly (5 – 82%; Caselles-Osorio et al. (2007)) since it is dependent on the type of wastewater and loading rate, these solids can form a sink for ENMs. The sludge layer on top of the French type VSSF CWs can function similarly reducing the amount of ENMs reaching the actual CW substrate. The surface of the substrate is habited by micro-organisms which form a biofilm in all SSF CWs. Extracellular polymeric substance (EPS) present in the biofilm efficiently adsorbs ENMs (Puay et al., 2015). In mature CWs plant roots form a large surface area which likely serves as a binding site for ENMs (Jacob et al., 2013), possibly (at least partly) due to biofilm formation on top of the root surface. In addition, metal retention in the roots can improve if iron plaque is formed on the roots (Jiang et al., 2009). This iron precipitate is caused by oxidation of ferrous iron in anoxic conditions (Jiang et al., 2009), e.g. upon exposure to wastewater from metallurgical industry or landfill leachate.

Table 2.1: Retention of ENMs during wastewater treatment. The experimental influent concentrations were between 0.3 - 10 mg/L.

Type of ENM	**Process description**	**Coating**	**Retention (%)**	**Observations**	**Reference**
Ag	Pilot-scale WWTP consisting of non-aerated tank and settler average sludge age of 14 days	Polyethylene fatty acid ester	85	Based on mass balance suggests 5% of Ag left the WWTP with the effluent	Kaegi et al. (2011)
	Batch-tests on activated sludge; contact time 24 h	Citrate, PVP	99		Kaegi et al. (2013)
	Batch-tests on activated sludge; contact time 3 h	Carboxyl	39		Kiser et al. (2010)
		None	97		Kiser et al. (2010)
	Simulated WWTP with primary settler, and SBR	Citrate	100	>90% of Ag-NPs remained in the water stream after primary clarification; complete removal after aeration and secondary clarification	Hou et al. (2012)
TiO_2	Batch-tests on activated sludge; contact time 3 h	None	23		Kiser et al. (2010)
CeO_2	Model WWTP with aeration tank and settler; sludge age not reported	None/surfactant	>94		Limbach et al. (2008)
Cu	Batch-tests on activated sludge (filtrates); contact time 20 h	n.a.	95	Mainly removed by aggregation and settling rather than biosorption	Ganesh et al. (2010)
ZnO	Simulated WWTP with primary settler and SBR.	None	100	Simulated primary settler removed 70% ZnO-NPs. Further removal in SBR.	Hou et al. (2012)
Au	Batch-tests on activated sludge; contact time 24 h	Citrate, PVP	99		Kaegi et al. (2013)

2.3.5 Plant uptake

Some plant species are known to be able to take up metals and when the metals are principally accumulated in the aboveground biomass at high concentrations, one speaks of hyperaccumulators (for review see Krämer (2010)). There is no one species which can accumulate different metals at equal amounts (Weiss et al., 2006). Generally, only free metal ions, soluble metal complexes and metal ions occupying exchangeable sites or attached on inorganic soil constituents are bioavailable for plants (Salt et al., 1995). Metals existing in the environment bound to organics, as precipitates or in the silicate mineral structure (indigenously present) are not bioavailable for plants. Also ENMs have been shown to be bioavailable to plants to some extent. Their bioavailability to plants is restricted by their size as particles up to only about 5 nm in diameter can traverse an undamaged cell wall efficiently (Dietz, Herth, 2011). Also, the surface charge and hence, the coating, can affect the uptake efficiency and transport within the plant. Zhu et al. (2012), who studied the fate of Au-NPs with different surface functionalization, observed that the Au-NPs with a negatively charged coating were the least efficiently attached onto the root surface prior to uptake but more efficiently transported to leaves once inside the plant, compared to positively charged Au-NPs.

Van Koetsem et al. (2016b) studied the uptake of citrate-coated Ag-NPs in a hydroponic culture of *Elodea canadensis* and observed a dose-dependent reduction of 20 – 57% in the total Ag concentration in the growth medium within 72 h resulting in the accumulation of 25 – 110 mg Ag/kg in plant biomass. The uptake of Ag^+ was approximately 4 and 8 times higher than the uptake of Ag-NPs at the highest initial Ag doses tested (0.5 – 1 mg/L) (no significant difference at initial doses 0.05 – 0.25 mg/L). Lowry et al. (2012a) investigated the fate of PVP-coated Ag-NPs in a freshwater emergent wetland and found that only a few percent of the spiked Ag ended up in plant tissue of *Juncus effuses, Carex lurida, Panicum virgatum* and *Lobelia cardinalis.* Furthermore, the majority of the accumulated Ag was found in the roots. Low translocation of Ag-NPs to aboveground biomass has also been observed in other rooted plants, such as *Vigna unguiculata* and *Triticum aestivum* in terrestrial environments (Wang et al., 2015). In the same study of Wang et al. (2015), they observed that Ag_2S was taken up by the plant roots to a somewhat larger extent than Ag-NPs. Also CuO- and ZnO-NPs are both mainly accumulated in the root zone of wetland plants *Phragmites australis* and *Schoenoplectus tabernaemontani* (Zhang et al., 2015a, 2014b).

Jacob et al. (2013) showed that high phosphorus concentration can limit the uptake of TiO_2-NPs in *Elodea Canadensis.* Van Koetsem et al. (2016b) found no link between the uptake of Ag-NPs and phosphorus concentration but reported correlation between the uptake efficiency and conductivity and specific ion content. This likely evidences that the ENM uptake efficiency is dependent of occurring environmental transformations which are determined by the ambient conditions. In CWs, adsorption to organic matter will restrict plant uptake of ENMs as metal species bound to organics are unavailable to plants. Also, aggregation (occurring e.g. due to high ionic strength (conductivity)) for example can restrict plant uptake efficiency by increasing the particle size of ENMs.

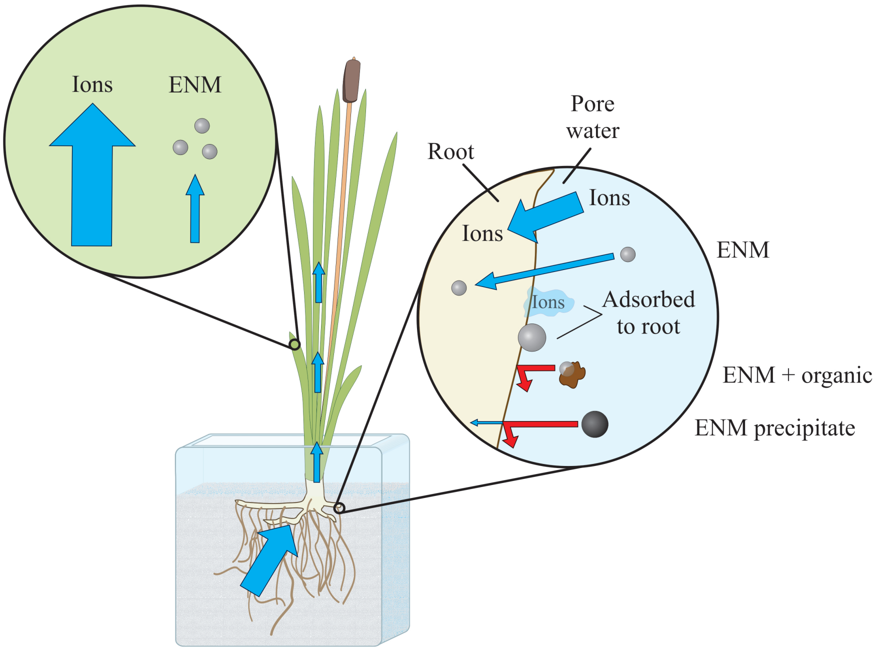


Figure 2.4: Schematic illustration of the uptake of ENMs and metallic ions by plants. The translocation efficiency of the different species is indicated with the thickness of the arrows. The uptake and translocation of ENMs is restricted by their size and hence, it is likely that larger particles are less efficiently taken up by the plant and translocated within the plant than smaller ones.

2.4 Possible toxic effects of ENMs on the wastewater treatment process in constructed wetlands

The toxicity of ENMs is undoubtedly related to the toxicity of the metal it is composed of. There are thus ENMs which are composed of an element ubiquitous in nature (e.g. Ti), ENMs which contain elements essential for cell structures (e.g. Cu and Zn) and ENMs composed of metals toxic at low concentrations (e.g. Ag). It is thus logical that the toxic concentrations of the different ENMs vary greatly and that low concentrations of certain ENMs could even have positive effects on e.g. bacterial growth due to supplementation of essential elements. Several researchers have compared the toxicities of different types of ENMs. Luo et al. (2014) compared the toxicities of Au- and Ag-NPs on ammonia-oxidizing bacteria at concentrations 0.05 – 2 mg/L, and found no adverse effects of Au-NPs on the bacterial community. Instead, Ag-NPs decreased the bacterial abundance and diversity resulting in 19 and 46% reduction in the ammoxidation activity at concentrations 0.05 and 2 mg Ag/L, respectively. Shah et al. (2014) observed adverse effects on the richness of a mixed soil microbial community when exposed to Ag- and TiO_2-NPs at a concentration of 0.0625 mg NPs/kg soil but ZnO- and Cu-NPs did not affect the bacterial community structure. Ma et al. (2015), who studied the effect of Ag (ion and NP), zero-valent Fe-NPs, TiO_2-NPs and CeO_2-NPs on the nitrification function and bacterial community structure, observed decreased nitrification efficiency and shifts in community structure only at prolonged exposure to high concentrations (20 mg/L) of Ag-species. In the study of Moore et al. (2016), the impacts on sediment microbial consortia exposed to pulse Ag- or Cu-NPs were rather short-term and evidenced of environmental resilience.

The actual mechanism causing the toxicity is not entirely clear. Although many studies show that the release of free ions from the particles is the main cause of toxicity, there are also studies showing that the toxic effects cannot be explained only by the amount of ions leaching from the ENMs and hence, there are likely to be particle specific effects as well (Yuan et al., 2013).

Hence, the properties of an ENM can influence its toxicity. For example, El Badawy et al. (2011) showed that the toxicity of Ag-NP on *Bacillus sp.* is dependent on the surface charge of the ENM because the chances for cell-particle interactions increase with decreasing magnitude of the negative zeta-potential. As a consequence, the most negatively charged Ag-NPs (uncoated and citrate-coated) exhibited complete growth inhibition at 75 and 600 μg/L, the less negative PVP-coated Ag-NPs at 13 μg/L and the positively charged branched polyethyleneimine (BPEI)-coated Ag-NPs at 3 μg/L. Also the size of the ENM could be significant in defining its toxicity. Choi, Hu (2008) found that the fraction of Ag-NPs less than 5 nm in the suspension correlated with the inhibition of nitrifying bacteria. However, Levard et al. (2013b), who studied the transformations of Ag-NPs and their toxicity to higher organisms, found little correlation between the size of the aggregates and the observed toxicity and suggested that the reduction in Ag^+ due to AgCl formation had the dominant toxicity decreasing effect.

When ENMs undergo transformations in the environment, it is likely that their toxicity is altered. Sulfidation has been observed to be an important mechanism at decreasing the toxicity of Ag-NPs to bacteria (Reinsch et al., 2012). Moreover, Choi et al. (2009) showed that sulfide had the most pronounced effect on relieving the toxicity of Ag-NPs among the tested ligands (SO_4^{2-}, Cl^-, PO_4^{3-}, EDTA). In this study, the inhibition of nitrification was reduced by 80% in the presence of 0.5 mg/L sulfide and 1 mg/L Ag (as Ag-NPs). In addition, other environmental factors such as dissolved oxygen concentration and ionic strength, can affect the dissolution/aggregation behavior of ENMs and thereby, affect their toxicity on organisms (see Sections 2.3.1 and 2.3.2).

It should be noted that comparing the toxicities of ENMs and their transformation products is not straightforward since their toxicities can depend on the target organism among other factors. Choi et al. (2008) observed that AgCl colloids and Ag-NPs were less toxic to *Escherichia coli* than Ag^+ but nitrifying bacteria were especially susceptible to Ag-NPs, more than to Ag^+ and AgCl colloids: 1 mg Ag/L as Ag-NPs inhibited the microbial respiration by 86% while in the case of Ag^+ and AgCl colloids the inhibitory effect was only approximately 45%. In a similar experiment, Choi, Hu (2008) observed that the presence of 0.14 mg Ag/L of polyvinyl alcohol (PVA) coated Ag-NPs, 0.27 mg Ag/L of Ag^+ and 0.25 mg Ag/L of AgCl colloids inhibited the growth of nitrifiers by 50%.

There are some studies where the effects of ENMs on actual wastewater treatment processes have been investigated. Alito, Gunsch (2014) studied COD and ammonium removal efficiencies in laboratory-scale sequencing batch reactors (SBRs) which were spiked with citrate- and GA-coated Ag-NPs (0.2 mg/L). Although the treatment efficiency was recovered within 24 h, the COD and ammonium removal efficiencies were initially affected by 20 − 30% and by 1 − 15%, respectively. When the effect of CeO_2-NPs on the nitrogen removal process in an SBR was studied by Hou et al. (2015), they observed no significant effect on total nitrogen removal at 1 mg/L but at 10 and 50 mg/L the total nitrogen removal efficiency reduced by approximately 30%. The resilience of the bacterial communities in sludge is probably related to the protection by the EPS matrix which accounts for the floc formation in activated sludge (Henriques, Love, 2007). The adsorption of ENMs by EPS can decrease the diffusive transportation through the EPS (Choi et al., 2010) and thus, protect the bacteria from the ENMs.

In CWs, the cells habiting the biofilm are better protected against toxins than planktonic cells due to EPS (Choi et al., 2010), similarly to sludge flocs. A study on wastewater dwelling bacteria showing that the community profile of a biofilm culture did not change after exposure to 200 mg/L of Ag-NPs, while nearly all planktonic bacteria died at 1 mg/L illustrates the protective effects of EPS (Sheng, Liu, 2011). However, the biofilm bacteria can still experience stress due to the presence of ENMs (Hou et al., 2015). Under stress, bacteria are unable to protect themselves from intracellular reactive oxygen species which can damage cellular structures when accumulating in the cell (Choi, Hu, 2008). The concentrations of ENMs in the biofilm increases during the lifetime of a CW. Lesage et al. (2007) studied the accumulation of Zn and other bulk metals in a HSSF CW and measured concentrations reaching 1400 mg Zn/kg dry matter in the wetland substrate after 6 years of operation. During the sampling they measured an average Zn concentration of 120 μg/L in in the influent. As this is more than 100 times higher than the concentration predicted for ZnO-NPs in domestic wastewater (1 μg/L; calculated with a conservative 70% removal efficiency (Markus et al., 2013) from a modeled value for wastewater effluent 0.29 μg/L (Sun et al., 2014)), the likelihood of NP induced toxicity seems irrelevant at current production volumes.

2.5 Possible release of engineered nanomaterials from constructed wetlands

Effluent, harvested plant material and removed sludge (French type CW) can be regarded as potential routes for ENMs to be released from CWs. Also, in cases where the CW is restored due to e.g. clogging, by removing the gravel, washing it and returning it back to the CW, ENM containing wastewater can be produced.

Due to the affinity of ENMs for organic matter as discussed earlier (see section 2.3.4) and the efficient removal of suspended solids in CWs (e.g. Vymazal (2009)) the concentrations of ENMs released from CWs are during normal operation likely to be low and comparable to activated sludge treatment systems. The results from a study investigating the release of Ag-NPs from CW microcosms support this theory (See Chapter 3). Over the years the CW bed becomes more filled with SS and plant roots and this can result in topographic and vegetative heterogeneities creating faster and slower flow paths through the wetland substrate (Dierberg et al., 2005). Fast flow of influent through the CW (i.e. short-circuiting) may decrease the removal efficiency of ENMs similarly to other contaminants. Also, active aeration may cause mixing of a fraction of influent with effluent especially in systems with a small length-to-width ratio. In addition, sudden changes in the influent flow rate or aeration may cause detachment of biofilm and subsequent increased discharge of ENMs embedded in the biofilm.

In conventional CWs sludge is not needed to be removed during operation. In French type VF CWs, where unsettled wastewater is treated, the sludge layer is occasionally removed, and in this case, if the sludge is applied to (agricultural) land, contamination of the soil by ENMs may occur (Deng et al., 2014). The mass of the sludge deposit corresponds to approximately 40% of the SS introduced with the wastewater (Molle et al., 2005) and due to the mineralization of organic matter over the years, the metal concentration in the sludge deposit layer is bound to increase. Molle (2003) measured concentrations of e.g. Cu and Zn in a sludge deposit collected after 11 years of operation and stored since collection for 5 years. The concentrations found (196 mg Cu/kg dry matter and 221 mg Zn/kg dry matter) would not have restricted the application of the sludge on agricultural land (European Commission, 1986). However, the European sewage sludge directive does not directly concern ENMs in sludge. Additional toxicity and bioavailability data should be gathered to allow the establishment of environmental quality standards although it should be taken into account ha

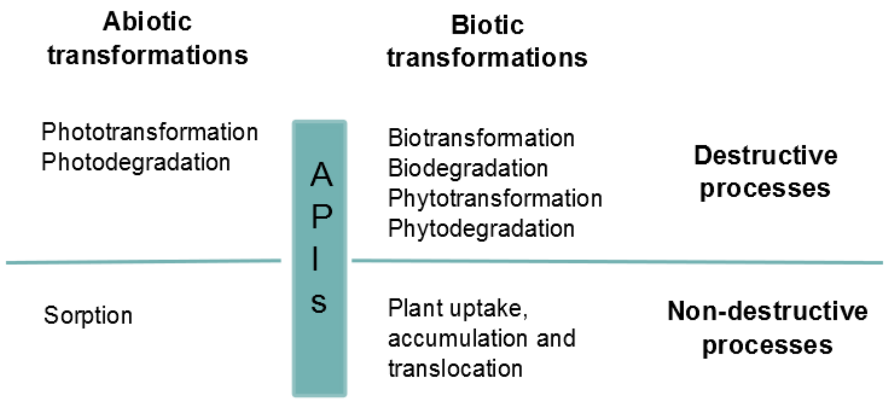


Figure 2.5: Possible transformation processes of pharmaceutical active ingredients (APIs) in CWs

measuring the concentration of ENMs in sludge can currently not be required because of the lack of reliable techniques to detect ENMs in environmental samples (Ganzleben et al., 2011).

Plants which are harvested at regular intervals from the CW may also contain ENMs which can be released upon composting. Vymazal et al. (2010) studied the accumulation of metals in the aboveground tissue of *Phalaris arundinacea* growing in a HSSF CW treating domestic wastewater. The values for annual accumulated mass in the aboveground tissue per given area (so called standing stock) were for e.g. Zn and Cu 28.5 g $Zn/m^2/a$ and 8.8 g $Cu/m^2/a$. If we assume similar uptake efficiency for ZnO-NPs as for Zn^{2+} and estimate the ZnO-NP:Zn ratio in domestic wastewater to be 0.008 (120 μg Zn/L (Lesage et al., 2007) and 1 μg ZnO-NPs/L; the latter calculated with a conservative 70% removal efficiency (Markus et al., 2013) from a modeled value for wastewater effluent 0.29 μg/L (Sun et al., 2014)), the standing stock for ZnO-NP in *P. arundinacea* would be 0.2 g $Zn/m^2/a$. In comparison to the bulk metal counterpart, the mass of ENMs is thus predicted to be low and the risk posed by the ENM containing plant material from CWs seems insignificant.

2.6 Removal mechanisms of pharmaceuticals in constructed wetlands

CWs are complex systems where the removal of pharmaceutical residues can occur by several mechanisms (Garcia-Rodríguez et al., 2014)(Figure 2.5). Biotransformation or -degradation is the main process leading to partial or complete transformation of these compounds. Other mechanisms capable of transforming pharmaceutical compounds are phototransformation or -degradation, where transformation occurs by exposure to sunlight, and phytotransformation or -degradation, where plants metabolize the pharmaceutical compound. In sorption and plant uptake the chemical structure of the contaminant is not altered and thus, these processes only relocate the contamination. The relative importance of a particular removal mechanism depends on the chemical structure and properties of the pharmaceutical being treated, the wetland type (e.g. surface flow (SF), sub-surface flow (SSF)), design (e.g. type of plants and substrate) and operational parameters (e.g. hydraulic retention time (HRT), aeration) as well as environmental conditions at the treatment site (e.g. temperature) (Imfeld et al., 2009).

2.6.1 Biotransformation and -degradation

Microorganisms (mainly bacteria in CWs) can transform many pharmaceuticals and their metabolites (Kümmerer, 2010). It is likely that the extent of biodegradation depends on the chemical structure of the contaminant and recalcitrance can be ascribed to the presence of certain structures in the molecule such as the chlorine atom (Kimura et al., 2005). Because of the complex structure of these compounds, their biodegradation is often incomplete (biotransformation). The transformation products, which are products of microbial metabolism and thus the result of incomplete degradation, have different properties than the parent compound, and this affects their toxicity and persistence in the environment (Trautwein, Kümmerer, 2011). Also, the prevailing environmental conditions play an important role in defining the transformation rate.

It seems that the redox potential in the environment affects the degradation of pharmaceuticals through promoting aerobic/anoxic/anaerobic degradation by bacteria. Therefore, while e.g. ibuprofen is better degraded in the presence of oxygen (i.e. high redox potential) (Ávila et al., 2013), carbamazepine is shown to be better degraded in anoxic/anaerobic conditions (i.e. low redox potential) (Matamoros et al., 2005). This highlights the importance of applying both oxidative and reductive processes in CW systems to reach efficient removal of pollutants requiring differing redox conditions for degradation.

2.6.2 Photolytic transformation and degradation

Photolytic transformation/degradation can occur directly or indirectly: pharmaceutical compounds which contain e.g. aromatic rings or heteroatoms can either absorb solar radiation and undergo a chemical reaction leading to their transformation (direct reaction) or be transformed by photosensitized strong oxidant species such as hydroxyl radicals generated from humic acids (indirect reaction) (Boreen et al., 2003; Zepp et al., 1981). Photolytic transformation is only likely to occur in SF CWs where the water is exposed to sunlight. Even then the coverage of plants can decrease the efficiency of photolysis (Matamoros et al., 2012). Examples of pharmaceuticals which have been shown to be prone to photolytic transformation are diclofenac, ibuprofen, sulfamethoxazole, sulfapyridine, triclosan and naproxen (Anderson et al., 2013; Matamoros et al., 2012; Zhang et al., 2013). In some cases the human metabolism/microbial transformation of the pharmaceutical can result in altered photodegradability, as is shown for tramadol which is less susceptible to photolysis than its O-demethylated transformation product (Rühmland et al., 2015).

2.6.3 Plant uptake and phytotransformation and -degradation

Translocation of pharmaceuticals to plant tissue occurs via diffusion (Dietz, Schnoor, 2001) and it is mainly dependent on the lipophilicity of the contaminant (Trapp, Karlson, 2001). Moderately lipophilic compounds (log Kow 0.5 – 3) are taken up best while highly lipophilic compounds are sorbed by the roots or the soil and very polar compounds are poorly transported through biomembranes. It seems, however, that the uptake efficiency is also dependent on the type of plant. Zhang et al. (2013) reported that *Scirpus validus* was able to remove 28 – 62% of clofibric acid from the water phase when grown hydroponically and similarly, Dordio et al. (2009b) found a removal of clofibric acid of more than 50% with *Typha spp.* In contrast to these studies, the concentration of clofibric acid did not decrease in an experimental system with duckweed (Reinhold et al., 2010).

In the plant tissue the pharmaceutical compound can be subjected to metabolic processes, i.e. phytotransformation and possibly phytodegradation. Plant enzymes degrade the contaminant to a stable intermediate which is stored in the plant, preferably compartmentized in the vacuole and apoplast where it can do least harm to the plant (Pilon-Smits, 2005). Before storage the pollutant is usually also conjugated with glutathione or glucose to further increase the tolerance to plant tissues. There are only few studies looking into the transformation products of pharmaceuticals in plant tissue because of the low concentrations of the transformation products and complexity of analysis in solid matrices. Li et al. (2016) studied the transformation of ibuprofen by *T. angustifolia* in a CW mesocosm and observed that ibuprofen in the plant tissue was transformed via carboxylation and hydroxylation and postulated that the uneven distribution in the plant tissues could be caused by the compartmentation used by the plant to dispose of harmful substances.

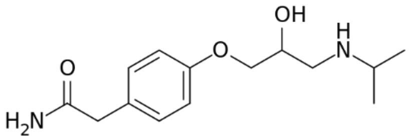
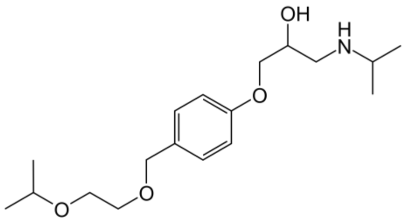
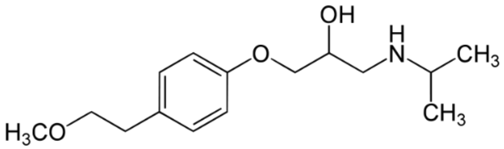
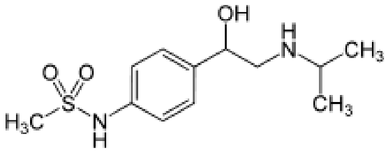
2.6.4 Sorption

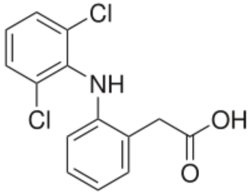
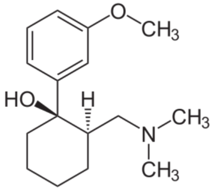
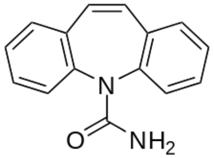
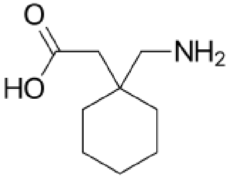
In CWs the suspended solids originating from the wastewater influent and accumulated within the wetland bed, as well as the substrate self, the plant roots, and the biofilm covering the substrate and the roots, can adsorb pharmaceuticals. The presence of many functional groups in pharmaceuticals implies that several sorption mechanisms can be involved in the sorption process (Tolls, 2001). Hence, in addition to hydrophobic partitioning (estimated by water-octanol partitioning coefficient, log K_{ow}), also cation exchange, cation bridging, surface complexes and hydrogen bonding can play a role in the sorption of pharmaceuticals on surfaces. This means that not only the properties of the sorbate are important but also the characteristics of the wetland substrate (e.g. pH, organic matter composition and reactivity) in defining the adsorption rate. One of the common pharmaceuticals that is widely believed to be prone to sorption is carbamazepine, (at least partly) due to its hydrophobicity (Matamoros et al., 2005). Among the studied pharmaceuticals the sartans, telmisartan and valsartan, have the highest log K_{ow} (8.42 and 4.00, respectively) implicating potential sorption onto solids. Several studies have investigated the sorption of pharmaceuticals to organic solids, especially activated sludge (Table 2.2). In general, it can be concluded that for compounds having a sorption coefficient (K_d) below 300 L/kg SS, the effect of sorption (to sewage sludge) on the overall removal of the pharmaceuticals is irrelevant (Joss et al., 2005).

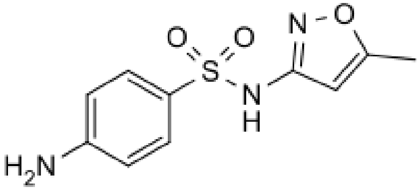
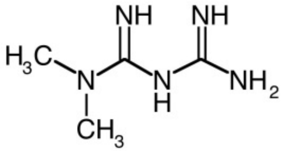
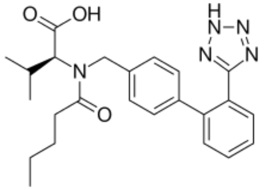
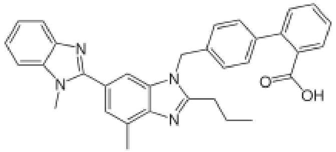
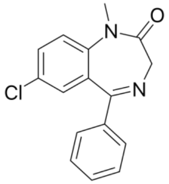
2.7 Description of the pharmaceuticals studied in this work

A selection of pharmaceuticals was made based on the following criteria: a) to have a wide spectrum of pharmaceuticals with different properties (i.e. biodegradability) and b) to target pharmaceuticals with high (expected) occurrence in the wastewater to be treated. In addition, diclofenac was selected for the study because its presence in surface waters is of increased interest due to the assigned European wide monitoring program for diclofenac (European Parliament, 2013). The target pharmaceuticals (atenolol, bisoprolol, carbamazepine, diazepam, diclofenac, gabapentin, metformin, metoprolol, sotalol, sulfamethoxazole, telmisartan, tramadol and valsartan) belong to seven therapeutic classes (Table 2.2).

Table 2.2: Properties of the selected pharmaceuticals

Therapeutic class	**Pharmaceutical**	**Acronym**	**Molecular structure**	**Log K_{ow}[1]**	**K_d (L/kg SS)**	**Reference**
Beta-blockers	Atenolol	ATL		0.16	64 38 1-8	Radjenović et al. (2007) Maurer et al. (2007) Scheurer et al. (2010)
	Bisoprolol	BSP		1.87	n.a.	
	Metoprolol	MTP		1.88	1 10-90	Maurer et al. (2007) Scheurer et al. (2010)
	Sotalol	STL		0.24	37 10-60	Maurer et al. (2007) Scheurer et al. (2010)

Therapeutic class	**Pharmaceutical**	**Acronym**	**Molecular structure**	**Log K$_{ow}$^{1}**	**K$_d$ (L/kg SS)**	**Reference**
Analgesic	Diclofenac	DCF		4.51	118	Radjenović et al. (2007)
					1.58	Ternes et al. (2004a)
					16	Joss et al. (2006)
					151	Hyland et al. (2012)
	Tramadol	TMD		3.01	47	Wick et al. (2009)
Anticonvulsant	Carbamazepine	CBZ		2.46	135	Radjenović et al. (2007)
					17	Ternes et al. (2004a)
					89	Hyland et al. (2012)
					25.5	Wick et al. (2009)
					10	Jones et al. (2005)
	Gabapentin	GBP		-1.1	n.a.	

Therapeutic class	Pharmaceutical	Acronym	Molecular structure	Log K_{ow}[1]	K_d (L/kg SS)	Reference
Antibiotic	Sulfamethoxazole	SMX		0.89	77 160-500 269 10	Radjenović et al. (2007) Joss et al. (2006) Hyland et al. (2012) Jones et al. (2005)
Anti-diabetic	Metformin	MFM		-2.64	3	Jones et al. (2005)
Angiotensin	Valsartan	VST		4.0	n.a.	
	Telmisartan	TST		8.42	n.a.	
Anxiolytic	Diazepam	DZP		2.82	20 138 53	Ternes et al. (2004a) Hyland et al. (2012) Wick et al. (2009)

[1] Obtained from: SRC PhysProp Database:http://esc.srcinc.com/fatepointer/search.asp

2.8 Removal efficiency of pharmaceuticals in constructed wetlands

The occurrence of pharmaceuticals in wastewater influents and effluents of CWs has been extensively studied. The removal is a result of simultaneously occurring removal mechanisms (Section 2.6), although some mechanisms may be more common in some CW configurations than others (e.g. phototransformation or oxic/anoxic/anaerobic biodegradation). Table 2.3 gives an overview of the removal efficiencies of the selected pharmaceuticals in several pilot- and full-scale CWs.

Atenolol, one of the most widely used beta blockers, has been shown to be removed quite efficiently in SF, HSSF and VSSF CW systems (Table 2.3). A much less pronounced reduction in the concentration was observed for BSP, MTP and STL which is in agreement with studies on activated sludge showing that BSP, MTP and STL are less prone to biodegradation than ATL (Gabet-Giraud et al., 2010; Wick et al., 2009). It has been postulated that the removal of ATL would be more efficient in the presence of oxic conditions (Burke et al., 2014) but in a recent study it was shown that the removal efficiency was actually more efficient at low redox conditions (<-100 mV; 99%) than at high redox conditions (>100 mV; 88%) (Rühmland et al., 2015). However, this result can also be affected by seasonality (Matamoros, Salvadó, 2012); warm temperatures of summer resulting in more active biotransformation (99% removal) than in the winter time (88% removal). STL has been categorized among the most recalcitrant pharmaceutical compounds, together with DCF and CMZ (Oulton et al., 2010). Therefore, the negative removal efficiencies observed in the SF CW by Breitholtz et al. (2012) are likely to be caused mainly by the combination of poor removal efficiency and variation in the influent concentrations.

The removal of DCF has been extensively monitored in various types of CWs (Table 2.3). Its removal efficiency spans the whole range from 0 to 100% and the actual trigger to its efficient removal is uncertain. Although DCF is reported to be photolabile (Andreozzi et al., 2003) its removal reached only maximum 40% in a SF CW while higher removal was reached in SSF systems. It has been proposed that both oxic and anoxic/anaerobic conditions are needed for removing DCF (Ávila et al., 2014b) and this can explain why the removal is improved in hybrid systems. Ávila et al. (2015) obtained a removal of 85% for DCF in a CW system combining VSSF, HSSF and SF CWs in series.

There are only few studies on CWs reporting the removal of TMD. Based on these studies, it seems that SSF systems are more efficient than SF CWs at decreasing the concentration of TMD (Table 2.3). However, when Rühmland et al. (2015) studied the removal of TMD and its metabolites/transformation products (O-desmethyl-TMD, N-desmethyl-TMD and N,O-didesmethyl-TMD), they found that the total concentration of these compounds did not decrease significantly, implying that TMD is transformed rather than mineralized during HSSF treatment. The transformation of TMD was found to be more efficient during summer than in the winter.

The removal of CBZ has been extensively studied because of its recalcitrance and subsequent poor removal in all types of CWs (Table 2.3). CBZ is very poorly biodegradable due to its complex aromatic structure (Table 2.2). Instead, because of its moderately hydrophobic nature, it can be removed from the water phase to some extent by sorption (Conkle et al., 2012) and plant uptake (Zhang et al., 2013).

GBP is similarly to CBZ used in the treatment of epilepsy but its removal in CWs is much less studied (Table 2.3). Studies on activated sludge treatment have shown very efficient (99.5%) removal of GBP (Yu et al., 2006) while maximum 88% of GBP was removed in a secondary HSSF CW (Chen et al., 2016); difference possibly caused by the presence/absence of dissolved oxygen.

The removal efficiency of SMX, a common antibiotic, varies greatly within and between different studies without any apparent trend (Table 2.3). One reason for this is expected to be the possible retransformation of its acetylated human metabolite back to the active parent compound during wastewater treatment (Göbel et al., 2005). When the concentration of both compounds are measured in the influent and effluent samples, removal efficiencies up to 70 – 87% were obtained (Hijosa-Valsero et al., 2011; Rühmland et al., 2015). In contrast to other similar studies only targeting SMX, high average removal efficiency was obtained for SMX in a SF CW (Conkle et al., 2008).

MFM is one of the most prescribed medications worldwide and therefore detected in high concentrations in wastewater influent (Oosterhuis et al., 2013). Its fate in CWs is, however, not yet studied. In activated sludge treatment MFM is rapidly aerobically transformed to guanylurea which is persistent in the environment (Scheurer et al., 2012; Trautwein, Kümmerer, 2011). Similarly to MFM, there is no explicit data on the transformations of VST and TST in CWs. In activated sludge treatment the concentration of VST has been reported to decrease by approximately 80% (Kasprzyk-Hordern et al., 2009).

Many studies report very low (below detection limit) concentrations of DZP in wastewater influent (Carballa et al., 2004; Reif et al., 2011; Jelic et al., 2011) and therefore, there is only limited data available on its removal. Jelic et al. (2011) concluded though that sorption to activated sludge contributes significantly to the removal of DZP.

In general, from the results discussed here it is clear that estimating the removal efficiency of a certain pharmaceutical compound in a CW is very difficult. The variability in the removal efficiencies is likely not only affected by the type of CW, but also its operational parameters, such as HRT, among other factors. There are only a few studies published investigating the effect of HRT on pharmaceutical removal in CWs, and further research is certainly advisable. Zhang et al. (2012a, 2015b) studied the effect of HRT on the removal of selected pharmaceuticals, among which DCF and CBZ, in SSF CWs. They reported that an increase of 1 – 12% in the removal efficiency in the planted systems was achieved when the HRT of the CWs was increased from 2 d to 4 d. Also, Ávila et al. (2014a) and Matamoros et al. (2007) found some correlation between removal efficiency and HRT.

In the previous, the removal of pharmaceutical residues has only been discussed in traditional CW systems. Many different types of CWs have been designed (for a review see: Wu et al. (2015)) to improve their performance and to intensify the treatment to obtain a smaller foot print; a well-known dilemma of CWs. As oxygen transfer within the CW is one of the crucial problems limiting its treatment performance (e.g. organic matter removal, nitrification) and making it necessary to apply long HRT, research has been done on e.g. adapted feeding mode and active aeration to improve the oxygen transfer in the wetland. Currently, the effect of intensified systems on the pharmaceutical removal efficiency is only scarcely studied.

Ávila et al. (2013) studied the effect of batch feeding on the removal efficiency of DCF in a hybrid HSSF CWs and observed an improvement of approximately 20% by using batch feeding strategy instead of continuous feeding (Table 2.3: control line). They assumed that enhanced removal was caused by the improved oxygenation of the CW bed between the feeding events. Similar study was conducted by Zhang et al. (2012b) on single stage HSSF CW. In this study the removal of DCF was significantly improved by batch feeding in comparison to continuous feeding but no difference was observed for CBZ.

Active aeration results in more efficient performance which allows higher organic loading of the CW and results subsequently in a smaller land area requirement, making CWs a possible treatment option for locations where a conventional CW would not fit. To date there is only one study looking into the effects of active aeration on pharmaceutical removal efficiency. Ávila et al. (2014b) compared unsaturated VSSF CWs with different substrates (gravel and sand) to actively aerated VSSF CW with saturated gravel bed. They found that the overall performance of the aerated CW was better than that of the unsaturated gravel-bed CW, but that the best results (also pharmaceutical removal) were reached by the unsaturated sand-bed CW. However, in this study only two pharmaceuticals were targeted (diclofenac, ibuprofen). Therefore, further research on various pharmaceuticals and aeration regimes is necessary.

Table 2.3: Removal efficiencies of selected pharmaceuticals in CWs

	Influent (ng/L)	Effluent (ng/L)	Removal (%)	Type of CW	Reference
ATL	1442	284	80	I:Full-scale SF	1
	137	31	77	III:Full-scale HSSF	2
	734	383	48	III:Full-scale HSSF	3
	1100-2000	530-1000	29-54	III:Full-scale SF	6
	20-210	-	88-99	III:Pilot-scale VSSF	10
	400-1100	-	58-99	II:Full-scale HSSF	11
BSP	97-160	76-100	22-38	III:Full-scale SF	6
MTP	211	25	88	I:Full-scale SF	1
	182	162	11	III:Full-scale HSSF	3
	600-1500	620-1100	-3-33	III:Full-scale SF	6
STL	174	148	15	I:Full-scale SF	1
	323	306	5	III:Full-scale HSSF	3
	520-1000	550-1200	-21-23	III:Full-scale SF	6
DCF	131	51	53	III:Full-scale HSSF	2
	284	271	5	III:Full-scale HSSF	3
	3200	3	>99	H:Pilot-scale HSSF	4
	2800	1400 (control line)	50	H:Pilot-scale HSSF	5
	380-670	290-390	24-40	III:Full-scale SF	6
	670-2730	<230-600	>46-86	H:Full-scale SF:HSSF	7
	820	-	73	II:Pilot-scale VSSF	9
	2200	-	20-35	III:Pilot-scale VSSF	10
	1100	-	17-95	II:Full-scale HSSF	11
TMD	410-740	460-600	-12-31	III:Full-scale SF	6
	930-1200	-	16-80	III:Pilot-scale VSSF	10
	700-800	-	54-85	II:Full-scale HSSF	11
CBZ	173	154	11	III:Full-scale HSSF	2
	372	387	-4	III:Full-scale HSSF	3
	290-1000	340-850	-17-15	III:Full-scale SF	6
	2060	-	20-26	II:Pilot-scale VSSF	9
	2200-4970	-	<0-13	III:Pilot-scale VSSF	10
GBP	20-200	-	-53-88	II:Full-scale HSSF	11
SMX	4090	918	78	I:Full-scale SF	1
	61	64	-7	III:Full-scale HSSF	2
	214	180	16	III:Full-scaleSHQSF	3
	49-140	85-140	-104-15	III:Full-scale SF	6
	620[a]	83-250[a]	59-97[a]	II:Pilot-scale SF/HSSF	8
	320-490[a]	-	-38-70[a]	III:Pilot-scale VSSF	10
MFM			n.a.		
VST			n.a.		
TST			n.a.		
DZP			n.a.		

[a] Sum of SMX and its metabolite N-acetyl-SMX

[1]Conkle et al. (2008); [2]Lee et al. (2011); [3]Verlicchi et al. (2013); [4]Ávila et al. (2010); [5]Ávila et al. (2013); [6]Breitholtz et al. (2012); [7]Hijosa-Valsero et al. (2010a); [8]Hijosa-Valsero et al. (2011); [9]Matamoros et al. (2007); [10]Rühmland et al. (2015); [11]Chen et al. (2016)

2.9 Environmental impact of pharmaceuticals in surface waters

2.9.1 Possible adverse effects in the ecosystem

Pharmaceuticals are designed to have specific pharmacological and physiological functions and they can have significant (cumulative) effect on the metabolism of non-target organisms and the ecosystem as a whole (Bendz et al., 2005). Because the environmental concentrations of pharmaceuticals are very low, acute toxicity to aquatic organisms is mostly not observed and hence, the adverse effects are caused by chronic exposure (Péry et al., 2008). One remarkable example of such effects is the feminization of fish in rivers receiving wastewater effluent. The role of steroid estrogens is implicit in the development of intersex conditions, altered mating behavior, skewed sex ratios and reduced reproduction (Gross-Sorokin et al., 2006).

The concerns related to antibiotics in the environment are caused by the potent formation of antibiotic resistant bacteria and subsequent human health threat (Boxall et al., 2003). Moreover, antibiotics can select for resistant bacteria even at lower concentrations than the levels considered safe according to accepted environmental quality standards (Berendonk et al., 2015). However, it is not confirmed whether the discharge of antibiotics into the environment is the major source of resistant bacteria in the environment or is it rather the transfer of already resistant bacteria present in wastewater (Kümmerer, 2009).

Pharmaceuticals which are poorly biodegradable and hydrophobic tend to adsorb onto soil and sediment. In particular, river biofilms have been shown to accumulate pharmaceuticals (Aubertheau et al., 2016). River biofilms represent a major primary production source and hence, the contamination of these biofilms may cause bioaccumulation and biomagnification problems. However, the risk for humans to be exposed to pharmaceuticals via food has been estimated low (Schulman et al., 2002; McEneff et al., 2014).

2.9.2 Effective concentrations vs. measured environmental concentrations

In this section, the data on toxicity of the selected pharmaceuticals is reviewed. Table 2.4 summarizes observed effects of the selected pharmaceuticals on different species. The extent of the toxic effects is dependent on the non-target species (i.e. species not intentionally receiving treatment) and the type and concentration of the pharmaceutical in question (Crane et al., 2006). In wastewater effluent the concentration of single pharmaceuticals varies from some ng/L to tens of μg/L (Verlicchi, Zambello, 2014) and the toxic concentrations listed in Table 2.4 are clearly higher than this. The table 2.4 illustrates examples of toxicity tests conducted in the laboratory using typical end points such as mortality and decreased growth. Although these experiments were defined as chronic (to distinguish them from even shorter acute tests of some hours) the exposure periods applied were still rather short. The effects can also be much more subtle (e.g. on genetic level).

However, in wastewater effluent the pharmaceutical residues appear as a mixture which can even have a higher toxicity than what is assumed based on the effects of the single substances (Cleuvers, 2003). Studies have shown that the initial estimation of environmental risk posed by a multicomponent mixture can be based on the toxicity data of the single components and their predicted environmental concentration (PEC) / predicted non-effect concentration (PNEC) ratio (Backhaus, Faust, 2012). Only if environmental risk is increased based on this initial estimation, more elaborate analyses on the mixture effects (either antagonistic or synergistic) are required.

CWs discharge their effluents often in small streams where limited dilution takes place. In the effluent dominated streams the concentration of pharmaceuticals can be high and therefore, it is of the essence to assess the effect of the discharged pharmaceutical residues on aquatic organisms. Furthermore, little is known about the long-term effects of low concentrations of pharmaceuticals mixtures on aquatic organisms.

It is difficult to define the concentration of pharmaceuticals that can be regarded as safe in the environment in a straightforward manner. The exact effects in aquatic systems upon chronic exposure are currently not yet known. Also, a high variability in toxic effects caused by single and different combinations of pharmaceuticals can be expected as discussed earlier. The European Joint Research Center has conducted experiments on European rivers and proposed 100 ng/L as a warning limit for most investigated pharmaceutical substances (Loos et al., 2009). However, this limit is not based on toxicity testing, but it rather reflects a "normal" level of pharmaceutical pollution in European rivers. Approximately $\sim$90% of all measured concentrations were below this level. In the future, it is probably advisable to set limitations for pharmaceutical discharge based on the sensitivity of waterway.

Table 2.4: Toxicity data on the effects of the selected pharmaceuticals on aquatic organisms

	Species	**Organism**	**Exposure period (d)**	**LOEC**[1] (mg/L)	**NOEC**[2] (mg/L)	**EC50**[3] (mg/L)	**Adverse effect observed on**	**Reference**
ATL	*H.vulgaris*	Invertebrate	17	>10	-	-	-	Pascoe et al. (2003)
	P.promelas	Fish embryo	28	3.2-10	≥ 10	-	-	Winter et al. (2008)
	P.promelas	Fish	21	1-10	3.2->10	-	Size	Winter et al. (2008)
	L.minor	Plant	7	-	-	>320	-	Cleuvers (2003)
	D.subspicatus	Algae	3	-	-	620	Growth	Cleuvers (2003)
BSP				n.a.				
MTP	*L.minor*	Plant	7	-	-	>320	-	Cleuvers (2003)
	D.subspicatus	Algae	3	-	-	7.3	-	Cleuvers (2003)
STL				n.a.				
DCF	*D.rerio*	Fish embryo	10	4	8	-	Mortality	Ferrari et al. (2003)
	P.subcapitata	Algae	4	10	20	-	Growth	Ferrari et al. (2003)
	B.calyciforus	Rotifer	2	12.5	25	-	Reproduction	Ferrari et al. (2003)
	C.dubia	Invertebrate	7	1	2	-	Reproduction	Ferrari et al. (2003)
	L.minor	Plant	7	-	-	7.5	Growth	Cleuvers (2003)
	D.subspicatus	Algae	3	-	-	72	Growth	Cleuvers (2003)
TMD	*P.putida*	Bacterium	0.67	>1000	-	-	-	Bergheim et al. (2012)

	Species	Organism	Exposure period (d)	LOEC[1] (mg/L)	NOEC[2] (mg/L)	EC50[3] (mg/L)	Adverse effect observed on	Reference
CBZ	*D.rerio*	Fish embryo	10	25	50	-	Mortality	Ferrari et al. (2003)
	P.subcapitata	Algae	4	>100	>100	-	-	Ferrari et al. (2003)
	B.calyciforus	Rotifer	2	0.38	0.75	-	Reproduction	Ferrari et al. (2003)
	C.dubia	Invertebrate	7	0.025	0.1	-	Reproduction	Ferrari et al. (2003)
	L.minor	Plant	7	-	-	25.5	Growth	Cleuvers (2003)
	D.subspicatus	Algae	3	-	-	74	Growth	Cleuvers (2003)
GBP				n.a.				
SMX	*P.subcapitata*	Algae	4	0.09	-	-	-	Ferrari et al. (2004)
	C.meneghiniana	Diatom	4	1.25	-	-	-	Ferrari et al. (2004)
	C;leopolensis	Blue-green algae	4	0.006	-	-	-	Ferrari et al. (2004)
	B.calyciforus	Rotifer	2	25	-	-	-	Ferrari et al. (2004)
	C.dubia	Crustacean	7	0.25	-	-	-	Ferrari et al. (2004)
	D.rerio	Fish embryo	10	>8	-	-	-	Ferrari et al. (2004)
MFM	*L.minor*	Plant	7	-	-	110	Growth	Cleuvers (2003)
	D.subspicatus	Algae	3	-	-	>320	-	Cleuvers (2003)
VST	*D.subspicatus*	Algae	3	-	-	72	Growth	Bayer et al. (2014)
TST				n.a.				
DZP	*H.vulgaris*	Invertebrate	17	-	0.01	-	Polyp regeneration	Pascoe et al. (2003)

[1] NOEC: The highest concentration used in a toxicity test that does not cause a toxic effect that is statistically significantly different from the control.

[2] LOEC: The lowest tested concentration which produced a statistically significant difference from the control.

[3] EC50: Concentration at which 50 % of the organisms were observed to be affected.

2.10 Conclusions

ENMs are present in our everyday household products. The increasing production volumes (Robichaud et al., 2009) make it important to study the fate of ENMs in wastewater treatment systems to reliably estimate the environmental risk they pose.

During the usage of an ENM containing product, ENMs can be released to the washing water (Benn, Westerhoff, 2008; Mitrano et al., 2014) and end up in wastewater. In the sewer system, ENMs are likely to bind with organic matter and some types may sulfidize but little losses to the sewer biofilm are expected (Kaegi et al., 2013; Brunetti et al., 2015). The current estimates on the ENM concentration in wastewater reaching the treatment plant vary from some μg/L to hundreds of μg/L depending on the type of ENM. Although these values are low and unlikely to cause toxicity to microbial communities in CWs, caution is warranted due to the expected increase in the production of ENMs in the future.

The ENMs are expected to further transform in CWs. The type and extent of these transformations depend on the type of the ENM and the prevailing environmental conditions in the CW. Some differences in the fate of ENMs in HSSF CWs versus VSSF CWs can thus be expected due to e.g. different dissolved oxygen conditions. In general, important transformations that are likely to occur in CWs are sulfidation, sorption onto organic matter and other solids and heteroaggregation with suspended particles. Plant uptake is likely to have a minor role in the removal of ENM from the water phase, although adsorption of ENMs onto the root surfaces can be substantial. Hence, wetland plants can play an important role in ENM immobilization in the rhizosphere. Although sulfidation in CWs seems unavoidable, it is impossible to say to what extent it occurs. This may be important for the bioavailability of the metal species as ions are better taken up by plants than ENMs or it may impact the toxicity caused on the biofilm. The biofilm is not predicted to be adversely affected by the ENMs in the water phase but in long-term the accumulating ENMs may become toxic to the micro-organisms. The concentrations of ENMs released from a CW with effluent, harvested plant material and (in some special cases) sludge are expected to be low due to the efficient retention of ENMs within the substrate.

Pharmaceuticals are chemically complex compounds which even at very low concentration can have adverse and unforeseen effects in the ecosystem. They are used in large quantities throughout the world and the consumption is expected to increase due to the aging of the population (UN 2015).

While modern wastewater treatment systems efficiently remove organic carbon and nutrients, the influent stream contains also a lot of different trace substances, such as pharmaceuticals, for which these treatment systems are not designed. Hence, the elimination of pharmaceuticals at the wastewater treatment plant is insufficient and these compounds are detected in wastewater effluent, receiving waters and even ground water and drinking water. Constructed wetlands (CWs) are mainly used as small-scale wastewater treatment systems in remote areas. The emissions from a small-scale treatment system may seem insignificant in comparison to large treatment plants but actually the contrary is true. CWs often discharge their effluent in water ways which have a rich biodiversity or in small streams where possibly only little dilution occurs. It is thus likely that the emissions from the CWs do have an impact on the ecosystem in these streams.

CWs can contribute to the removal of pharmaceuticals via several simultaneously occurring processes. The combination and extent of the processes involved in the removal of the specific pharmaceutical depend on the characteristics of the pharmaceutical and on the prevailing environmental conditions in the CW. The multitude of possible removal mechanisms in a CW is considered an asset in comparison to other wastewater treatment systems.

A lot of research on pharmaceutical removal in CWs has already been conducted (for a review see e.g Verlicchi, Zambello (2014)). Although studies have indicated that improved oxygenation of the CW can aid the removal of some compounds (Zhang et al., 2012b), no detailed investigations on the effect of active aeration have yet been performed. Since active aeration enhances the removal of organics, the CW can be designed with a smaller footprint. This compact design would be beneficiary for locations where conventional (i.e. larger) CWs do not fit, e.g. hospitals or other urban locations. Although counteractive to aeration in terms of decreasing the footprint, another possible way to improve pharmaceutical removal in CWs is to increase the HRT. The effect of HRT has not yet been studied extensively but indications have been found that e.g. clofibric acid, ketoprofen and salisylic acid removal correlates with the HRT (Zhang et al., 2015b). In short, the discharge from the CW can affect the biodiversity of the receiving stream and therefore, it is necessary to explore the possibilities of adapting the operation of the CW to improve the effluent water quality.

Chapter 3

Fate of citrate-coated silver nanoparticles in constructed wetlands - a microcosm study

This chapter is based on **Auvinen H.**, Kaegi R., Rousseau D.P.L., Du Laing G. 2017. Fate of citrate-coated silver nanoparticles in constructed wetlands - a microcosm study. Water, Air and Soil Pollution 228:97.

3.1 Introduction

Silver nanoparticles (Ag-NPs) are used in common household products, such as textiles, biocidal sprays, food packaging material and toys, because of their antimicrobial properties (Vance et al., 2015). During washing, Ag-NPs can be released from textiles (Benn, Westerhoff, 2008; Mitrano et al., 2014) and will thus be transported to the wastewater treatment plant through the municipal sewer system. Even though the concentration of Ag-NPs in the raw wastewater is currently low (Li et al., 2013), the predicted increase in their production and use may lead to elevated amounts of Ag-NPs released into the wastewater in the future (Peralta-Videa et al., 2011). The specific use of Ag-NPs in household products in combination with the toxicity of Ag (Doiron et al., 2012; Ratte, 1999) and the resulting environmental risk (Colvin, 2004), have led to several scientific publications studying the removal of Ag-NPs from wastewater streams (Kaegi et al., 2013; Lombi et al., 2013).

These studies revealed that sulfidation of Ag-NPs and attachment of Ag-NPs to sludge biomass are the most important processes mitigating the toxicity of Ag-NPs (Reinsch et al., 2012) and removing them from the wastewater stream through sedimentation in a secondary clarifier. According to Kaegi et al. (2011), sulfidation of Ag-NPs is a fast process and at current Ag concentrations measured in wastewater, is neither limited by the sulfide availability in the wastewater nor by the hydraulic retention time in wastewater systems. Due to the efficient removal of Ag-NPs during the wastewater treatment (sulfidized) Ag-NPs will be accumulated in the sewage sludge. Kim et al. (2010) identified individual nanosized silver sulfide particles in the sludge of a full-scale wastewater treatment plant. In a microcosm study simulating emergent freshwater wetland, spiked Ag-NPs quickly settled to the bottom and transformed into Ag_2S (Lowry et al., 2012a), further demonstrating the importance of the sulfidation of Ag-NPs.

The removal of engineered nanoparticles (NPs) in constructed wetlands (CWs) has received little attention to date. CWs are often applied for domestic wastewater treatment, usually in remote areas and small communities, and can also be used to treat landfill leachate among others types of wastewater (Kadlec, Wallace, 2009). Different compositions of the influent water, different designs, operation principles and varying ages of the CWs affect the conditions within the CWs, for example, oxygen content, redox conditions, retention time, and accumulation of organic matter within the CWs. Aeration of CWs enhances their treatment efficiency (Fan et al., 2013; Nivala et al., 2007; Zhang et al., 2010b), but aeration may result in the release of Ag^+ due to the oxidative dissolution of Ag-NPs (Liu et al., 2011). Ag^+ may be of even greater environmental concern than Ag in its particulate form (Behra et al., 2013). Furthermore, the conditions within CWs can change dynamically over the typical lifetime of CWs (20 – 30 years) owing to changes on a time scale of minutes (e.g. accepting stormwater overflows) to years (e.g. clogging, vegetation development). Physicochemical conditions of the surrounding media influence the transformations of NPs, and thus define the persistence, reactivity, bioavailability and toxicity of (transformed) NPs in the environment (Lowry et al., 2012b). To assess the risk associated with an increasing use of (Ag)-NPs in consumer products and to better understand the environmental distribution of (Ag-)NPs, it is therefore of interest to study the removal of (Ag-)NPs in CWs.

Therefore, we investigated the retention of Ag-NPs in microcosms simulating CWs treating domestic wastewater and studied the distribution and transformation of Ag-NPs (citrate-coated) within the CW microcosms. Although only one type of Ag-NP was studied, earlier research indicated that coating (citrate or PVP) does not affect the fate of Ag-NPs during wastewater treatment (Kaegi et al., 2013). We derived the removal efficiency of the Ag-NPs by comparing the Ag concentration measured in samples collected from the influent and from the effluent of the CWs. Ag concentrations of the digested biofilm and plant material were used to establish the distribution of Ag within the microcosms and the transformation (sulfidation) of the Ag-NPs in the biofilm was evaluated by detailed electron microscopy analyses. By aerating and adding organic matter (OM) to the microcosms we assessed whether these factors influence the amount of Ag discharged from the microcosms.

3.2 Materials and methods

3.2.1 Experimental design

Microcosms simulating sub-surface flow CWs were monitored during this experiment. The experiment lasted for 25 weeks including an initial adaptation period during which the plants were allowed to grow and biofilm to develop before the Ag-NP dosing was initiated. The microcosms were sampled once a week for 18 weeks. To investigate the effect of aeration and OM on the performance of the CWs, different microcosms were set up as shown in Table 3.1. Aeration is sometimes applied in CWs to stimulate the removal of organic carbon (e.g. Fan et al. (2013)), but could also foster the oxidation of Ag-NPs which may affect their fate in the microcosms. During the lifetime of CWs, there is build-up of OM and reed leaves were added to simulate the accumulation of OM in an aging CW.

The microcosms were built in polypropylene containers by filling them with 2 L of washed gravel (⌀ 6/8 mm; porosity 36.5%±1.3%; Kranendonk NV, The Netherlands). A similar setup has been used earlier to assess the effects of ENMs on the microbial community in CW microcosms (Button et al., 2016). A schematic illustration of the setup can be found in section 5.2.1 (Button et al., 2016). The chemical composition and the cation exchange capacity and pH of the gravel used is given in section 4.3.2 Table 4.1 (Auvinen et al., 2016b). The depth of the gravel layer was approximately 15 cm.

Table 3.1: Setup of the different treatments. The microcosms were constructed in triplicate for each treatment.

Microcosm	Aeration applied	OM added	Ag-NP added
Air	X	-	X
OM	-	X	X
Positive control	-	-	X
Negative control	-	-	-

The microcosms were planted with common reed (*Phragmites australis*), obtained from a local garden center. Little amounts of potting soil was still present between the fine roots upon planting. The setups were fitted with a 32-mm-diameter perforated central sampling tube and a small outlet spout made of silicone tubing with a plastic stopper for draining. The aeration was provided in the AIR microcosms with an aquarium air stone and pump (Hozelock 320). The reed leaves added in the OM microcosms were placed in a rain water tank for one month before the beginning of the experiment where the leaves partially degraded. Then, the leaves were chopped in small pieces and 100 g was mixed thoroughly with the gravel.

In total, the influent water of the CWs was spiked 18 times with Ag-NPs (50 μg Ag/week/microcosm). The concentration occurring in the microcosms (100 μg/L) was higher than expected to occur in real domestic wastewater (Blaser et al., 2008) to be able to detect residual Ag via microscopy and measure it in plant material where concentrations were predicted to remain low. Synthetic domestic wastewater (OECD, 2001) was used as influent (490 mL per microcosm). The Ag-NPs were added in the influent to guarantee their even distribution in the microcosm. The spiking of the influent was done individually per microcosm and the synthetic wastewater was added to the microcosm immediately after spiking the wastewater with Ag-NPs to minimize the holding time and possible subsequent transformations of Ag-NPs. In the study of Button et al. (2016) where also synthetic wastewater and same Ag-NPs were used, no sulfidation nor chlorination was observed directly after adding the Ag-NPs to the wastewater solution.

The microcosms were operated in batch mode with weekly draining of the effluent equating to a hydraulic retention time (HRT) of 7 days. The microcosms were drained top-down via the outlet spout so that the water was completely replaced by freshly prepared influent. The water was sampled after draining the complete volume and mixing it well. This procedure differs from the one explained in chapter 5 to account for the issues observed there. The evaporating water was replaced twice a week with tap water.

3.2.2 Nanoparticle suspension

Citrate-coated Ag-NPs were obtained from PlasmaChem GmbH (Berlin, Germany) as a colloidal suspension (pH 6 – 8). The suspension was stored in the dark at 4 – 8°C. The concentration of the stock dispersion was measured prior to use by inductively coupled plasma-mass spectrometry (ICP-MS) as described later in section 3.2.3. The dispersion was digested prior to analysis as described later in section 3.2.3 for influent and effluent samples. The hydrodynamic diameter (Z-average) of the particles in the stock solution was determined by using a Photon Correlation Spectrometer (PCS; 100M Malvern Instruments Ltd). All measurements were performed in triplicate at 25°C using a helium-neon laser (633 nm) and a scattering angle of 150°. The average particle size was determined based on intensity and number. The particles in the stock suspension were additionally investigated by a scanning transmission electron microscope (STEM) and the particle size distribution was extracted from STEM images using the ParticleSizer software (Wagner, 2016).

3.2.3 Total silver analysis

Digestion of the samples

Influent and effluent water samples were digested in the microwave oven (Mars 5 and 6) after adding 4 mL of concentrated HNO_3 and 1 mL of H_2O_2 to 10 mL of sample. The digestion program consisted of the following steps: 10 min at 55°C; 10 min at 75°C; 40 min at 100°C. The performance of the digestion method was investigated as a preliminary study prior to another research project studying the fate of Ag-NPs in natural water samples previously conducted in our lab (Van Koetsem et al., 2015, 2016a).

At the end of the experiment, total Ag content in the different compartments of the microcosms was also determined. The gravel was mixed carefully after removing the plants and sub-samples of 100 g were treated with 100 mL of potassium-free phosphate buffer (10 mM Na_2HPO_4, 8.5 g/L NaCl, pH 7.4) to detach the biofilm and the organic matter from the gravel (Button et al., 2016). The sample was shaken (orbital shaker) in the buffer solution for 2 h at moderate speed (200 rpm), after which the buffer was directly decanted. A sub-sample of 10 mL was digested following the protocol described above and the Ag concentration was measured to assess the fraction of Ag associated with the biofilm and the organic matter. To the remaining gravel sample 20 mL of 5% HNO_3 was added to allow the determination of Ag firmly attached to the gravel. After shaking the sample for 1 h at moderate speed (200 rpm), the acidic solution was directly decanted and 10 mL of sample was digested as described above. These sequential extractions were conducted in duplicate for each microcosm.

To determine the amount of Ag attached to the biofilm on the microcosm walls, 100 mL of 5% HNO_3 and 5 mL H_2O_2 were added to the empty container after the experiment and shaken vigorously. 10 mL of sample were digested and analyzed for total Ag as described above.

The plant samples were divided in two parts: aboveground tissue (i.e. stems and leaves) and belowground tissue. Due to the very fine structure of the roots, these samples may still have contained small amounts of potting soil. Both types of plant samples were dried in an oven overnight (40°C), cut into pieces and ground to a fine powder with mortar and pestle. A sub-sample of 1 g of plant material was digested as described in Du Laing et al. (2003). Minor amounts of particulate matter were still observed in the digest. This material was left to settle, and only the supernatant was used for the analysis of total Ag.

Analysis of Ag

Digested samples (<15 mL) were first diluted to 25 mL with Milli-Q water and then diluted (1:10) with an acidified (1% HNO_3) internal standard (10 μg Ga/L and 10 μg Rh/L) solution. Thereafter, the total silver concentration was measured using ICP-MS (PerkinElmer Sciex Elan DRC-e). The instrumental parameters and the calibration ranges are given in Appendix A Table A1. External calibration standards were used for ICP-MS analyses, and recalibrations were performed every 20 samples. Blank samples and reference standards were included at the beginning and the end of each intra-analysis batch of 20 samples for quality control purposes. The detection limit varied during the experiment between 0.02 and 0.12 μg Ag/L. The detection limit was defined as the sum of the average Ag concentration measured in blank samples and 3 times standard deviation of these samples.

3.2.4 Total organic carbon, total suspended solids, dissolved oxygen and pH

Thirteen weeks after the beginning of the Ag-NP spiking, total organic carbon (TOC) and total suspended solids (TSS) were determined on effluent samples weekly for five weeks. TOC was measured in non-filtered samples using a TOC-analyzer (TOC-V_{CPN}, Shimadzu). The detection limit of these measurements varied between 1.2 and 3.9 mg/L. The detection limit was defined as the sum of the average TOC concentration measured in blank samples and 3 times standard deviation of these samples. TSS was determined gravimetrically after filtering 100 mL of effluent through a paper filter (Macherey Nagel 640m). The total Ag concentration in the filtrate was determined twice (analysis according to sections 3.2.3 and 3.2.3).

The dissolved oxygen (DO) content in the microcosms was measured twice during the experiment (week 4 and 7) by using a portable DO meter (HI9142, Hanna Instruments). The DO was measured in situ 3 - 4 days after spiking of Ag-NPs by using the perforated sampling tube inside the microcosm. The pH was measured twice during the experiment (week 4 and 7) from the influent and drained effluents by using a bench-top pH meter (520A, Orion Research Inc.).

3.2.5 Scanning transmission electron microscopy and energy-dispersive X-ray analysis (STEM-EDX)

Samples from the stock suspension and from the biofilm extracts were analyzed using a scanning transmission electron microscope (STEM HD 2700Cs, Hitachi), operated at an acceleration voltage of 200 kV. Individual Ag-NPs, or their transformation products (Ag_2S) were localized using a high-angle annular dark-field (HAADF) detector. Elemental analysis of selected NPs was performed with an energy-dispersive X-ray (EDX) analysis system (EDAX) attached to the microscope.

A few drops of the stock suspension were pipetted on a holey carbon TEM grid (Plano, GmbH, Germany) and subsequently drawn through the grid by using a paper towel. After particle deposition, the grid was washed with a drop of demineralized water. Two preparation protocols were applied to deposit biofilm samples on TEM grids. The first protocol is very simple and can be applied in any laboratory, however, it requires high particle concentrations due to the low amount of sample that is eventually retained on the TEM grid. The second protocol is more sophisticated and requires a dedicated infrastructure. However, suspension with much lower particle concentrations can successfully be prepared for STEM analyses, as the centrifugation procedure concentrates the particles from the suspension on the TEM grid.

In the first protocol, a few drops of the aqueous biofilm extracts obtained after the extraction with phosphate buffer were drawn through a TEM grid (Plano GmbH, Germany) with a paper towel. In the second protocol, the liquid samples (approximately 200 mL, duplicate samples from each microcosm were combined) were centrifuged (5 min at 700 x g) and the solid fraction was freeze dried and stored at -18°C. The samples were later thawed under a gentle argon flow, packed in moist absorbing clay and sent for further processing. The samples were ground to a fine powder with mortar and pestle. The powder was added to a 2.0 mL Eppendorf tube and filled with 1.6 mL of ultrahigh quality water (NANOpure, Diamond, Barnstead, Thermo Scientific). The dispersion was vortexed for 1 min and then split into 2 Eppendorf tubes. The two tubes were sonicated with a Hielscher UP200St Vial tweeter for 2 minutes (amplitude 75%, cycle 50%). Temperature was monitored during sonication in a separate tube filled with distilled water and the temperature did not rise above 45°C. The dispersions were diluted to reach final concentrations of $\sim$0.3 mg sample/mL and 0.07 mg sample/mL. Two different concentrations were used to evaluate which one is better suited for the STEM analysis. These suspensions were centrifuged (1 h at 25,000 x g) on Formvar – Carbon coated TEM grids (Quantifoil Micro Tools GmbH, Germany). The described treatment was necessary to evenly distribute the NPs on the TEM grid. Due to the grinding and sonication process, no conclusions can be drawn about the association between the (transformed) Ag-NPs and other colloids/particles in the original sample but the speciation of Ag-NPs should not change during sample preparation.

3.2.6 Data analysis

Statistical analyses were performed by using SPSS Statistics 22 software. Firstly, the data were examined for normal distribution using the Shapiro-Wilk's test. Because the normality criterion of all samples was not met, a non-parametric test (Mann-Whitney U) was performed. This test was done pairwise to determine whether the Ag mass in the effluent and in the different compartments of the microcosm differed significantly between the treatments. The level of significance was set at $p=0.05$.

3.3 Results

3.3.1 Characterization of Ag-NPs

The total Ag concentration of the stock Ag-NP suspension determined by ICP-MS was 97±3 mg/L. Results from PCS measurements showed a number-weighted average particle diameter of 10 nm (11.1±0.2 nm). However, the intensity-weighted average particle diameter was close to 100 nm (90.9±7.9 nm) indicating that also larger particles (most probably Ag-NP aggregates) were present in the stock suspension. The particle size distribution extracted from STEM images (approximately 150 particles) revealed an average diameter of approximately 13 nm (Figure 3.1) confirming that intensity-weighted particle size distributions were biased by the presence of aggregates.

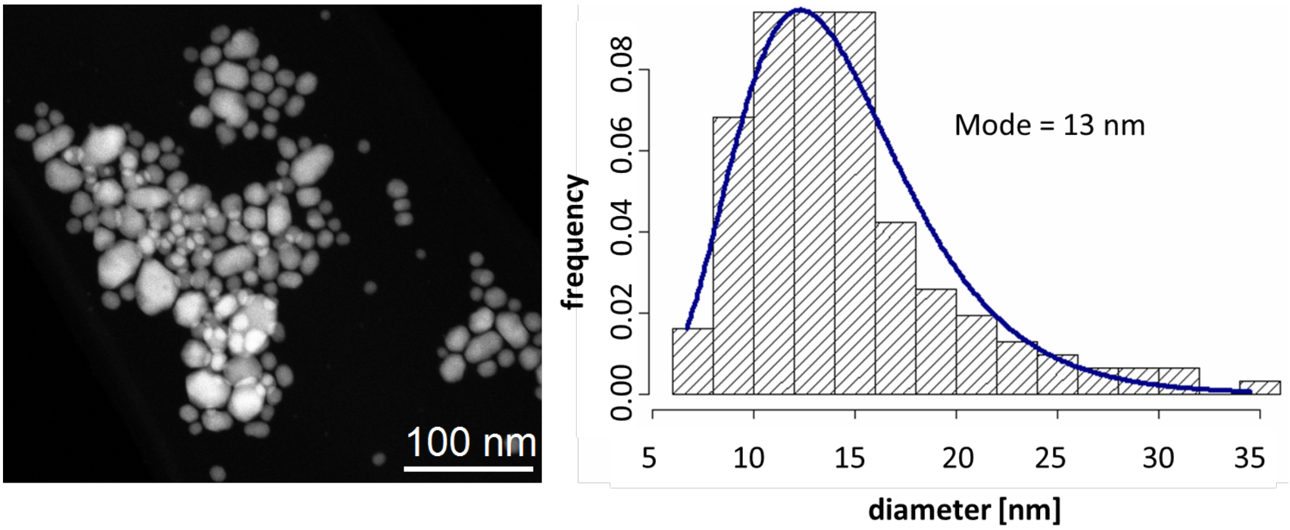


Figure 3.1: Left: STEM image of the Ag-NP from the stock dispersion. Right: particle size distribution extracted from STEM images. The distribution was fitted to a log-normal distribution.

3.3.2 Dissolved oxygen (DO) and pH

The influent had a pH of 8.0 whereas the pH of the effluents was slightly lower (7.4 – 7.7). Due to the similarity of the effluent pH in all treatments, the pH is not considered to have caused differences in the fate of Ag-NPs between the different treatments. In the aerated treatments, the DO concentration was 3.2±0.1 mg/L. Without aeration, the DO concentration was below detection limit (detection limit reported by the manufacturer 0.1 mg/L).

3.3.3 Total Ag mass and removal of the Ag-NPs in CWs

After the initial acclimatization phase, 50 μg of Ag-NPs was spiked weekly during 18 weeks resulting in a total nominal mass of 900 μg of Ag-NPs added to the microcosms (except for the negative control). The Ag concentration in the influent water was measured in 15 out of 18 spiking events (due to technical difficulties) and resulted in an average mass of 52±5 μg of Ag applied per spiking event. For the mass balance calculations we assumed that an average mass of 52 μg Ag was dosed in the microcosms in three spiking events where the influent Ag concentration could not be measured. The total Ag concentration in the effluent (measured at 15 events) and the effluent volume were used to calculate the mass of total Ag leaving the microcosms. The total Ag mass released from the microcosms with the effluent was lowest for the positive control and for the aerated microcosms, 90.5±56.8 μg and 94.4±17.8 μg, respectively (Table 3.2), and there was no significant difference between these treatments ($p>0.05$). The highest total mass of Ag was measured in the effluent of the microcosms with added OM, 190±21.3 μg, which differed significantly from the other two set-ups ($p<0.05$). For the three events where no Ag concentrations were available, we used the average Ag concentrations in the effluent of the individual experiments to complete the mass balance calculations. The Ag fractions retained in the wetland microcosms, calculated from the difference between integrated influent and effluent Ag masses, were 0.8 and 0.9. The lowest removal (80%) was obtained for the OM treatment and in the other two treatments (positive control and air) 90% of the Ag was removed. The Ag concentrations measured in all samples from the negative controls were below the detection limit.

3.3.4 Distribution of Ag within the microcosms

The largest percentage of Ag that was recovered within the microcosms, was found in the biofilm extracts (Table 3.2). Independent of the treatment, approximately 95% of the Ag recovered was associated with the substrate (sub-samples biofilm and gravel). The large standard deviation associated with the Ag mass in the gravel of the OM treatment is caused by one of the six sub-samples, where unusually high Ag concentrations were obtained (Table 3.2). A few % of Ag was lost to the biofilm that grew on the walls of the microcosms and less than 1% was found in the plant roots. In the leaves and stems of the plants, the Ag concentrations remained below the detection limits.

Table 3.2: The mass balance and distribution of total Ag within the microcosms. The total mass (μg Ag) is calculated for water and solid samples based on the measured concentration in the given medium and the volume or mass of the given medium.

	Discharge and overall removal			**[2]Ditribution within the microcosm compartments**										
Setup	**Influent**	**Effluent**	**[1]Removal efficiency**	**Biofilm**		**Gravel**		**Plant roots**		**Plant shoots**		**Microcosm walls**		**[3]Total recovery**
	μg Ag	μg Ag	%	μg Ag	%	μg Ag	%	μg Ag	%	μg Ag	%	μg Ag	%	%
Negative control	<LOD	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-	-
Positive control	937±6	91±57	90	265±31	77	61±24	18	3.2±1.9	0.9	<LOD	-	17±6	4.9	49
OM	922±10	190±21	79	29±16	65	104±104	30	2.8±1.1	0.8	<LOD	-	16±1	4.5	61
Air	931±7	94±18	90	248±25	81	44±7	15	1.2±0.5	0.4	<LOD	-	11±9	3.7	44

[1] Calculated from the difference between Ag masses measured in the influent and in the effluent.

[2] Normalized by the sum of Ag mass recovered in the different microcosm compartments. The percentage represents the proportion of Ag mass recovered in a given compartment to the sum of Ag mass recovered in all microcosm compartments.

[3] The percentage represents the proportion of Ag mass recovered in (effluent and all microcosm compartments) to the sum of Ag mass spiked (influent).

LOD:Limit of detection

3.3.5 Correlation of TSS/TOC and total Ag content

TSS and TOC were measured weekly between weeks 13 – 17 in the effluent samples. TSS concentrations in the effluents of the positive control and the aerated treatments ranged from 3.7 to 178 mg/L, with the lowest values systematically measured in one of the three replicates of the positive control. The high TSS concentration in the effluents of the OM setups (121 – 320 mg/L) demonstrates the effect of OM addition within the substrate. The solids in the effluents of the positive control and the aerated treatments originate from biofilm, degrading plant roots and potting soil that was still present between the roots upon planting. Similarly to the TSS values, the TOC concentrations in the effluents of the positive control, aerated and OM setups ranged from 4.7 to 97.9 mg/L, TOC being highest in the OM setups. A more detailed overview of the TSS and TOC concentrations in the different treatments can be found in Table A2 in Appendix A.

The total Ag concentration correlated well with the TSS concentration in the effluent (R_2=0.81, Figure 3.2). Also Ag and TOC concentrations were positively correlated, but the correlation was considerably less pronounced (R_2=0.59) compared to the correlation observed between Ag and TSS. The Ag concentration in the filtrate from the TSS analysis was analyzed twice during the experiment. The total Ag concentration in these filtrates was below the detection limit in all samples (data not shown).

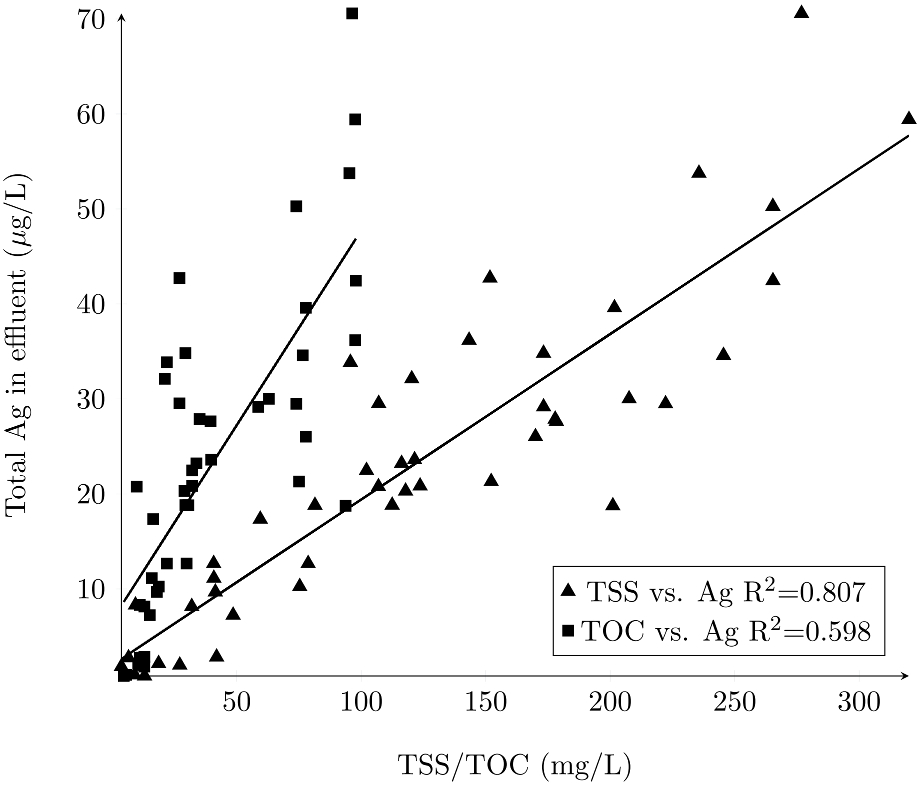


Figure 3.2: Correlation between TSS or TOC and Ag concentration in microcosm effluents (n=45).

3.3.6 Morphology and elemental composition of Ag-NP in the biofilm

The detected Ag-NPs were of comparable sizes as the pristine particles in the stock suspension (Figure 3.3). EDX analyses of individual particles revealed that Ag was always associated with sulfur (S), suggesting that Ag-NPs transformed into Ag_2S during the treatment. To a first approximation, the intensity ratio between S(Kα) and Ag(Lα) should reflect the atomic ratio of the transformed Ag-

NPs. However, due to variable contributions of S from the background (organic matter) to the signal intensity, a quantitative evaluation of the signal intensities was not performed. Because of the presence of organic matter in the samples the recording of high resolution phase contrast images and phase identification based on lattice spacings was not possible. Thus, due to the small size of the particles and in combination with considerable amounts of S in the biofilms, the presence of minor amounts of metallic Ag (partially sulfidized Ag-NP) cannot be excluded.

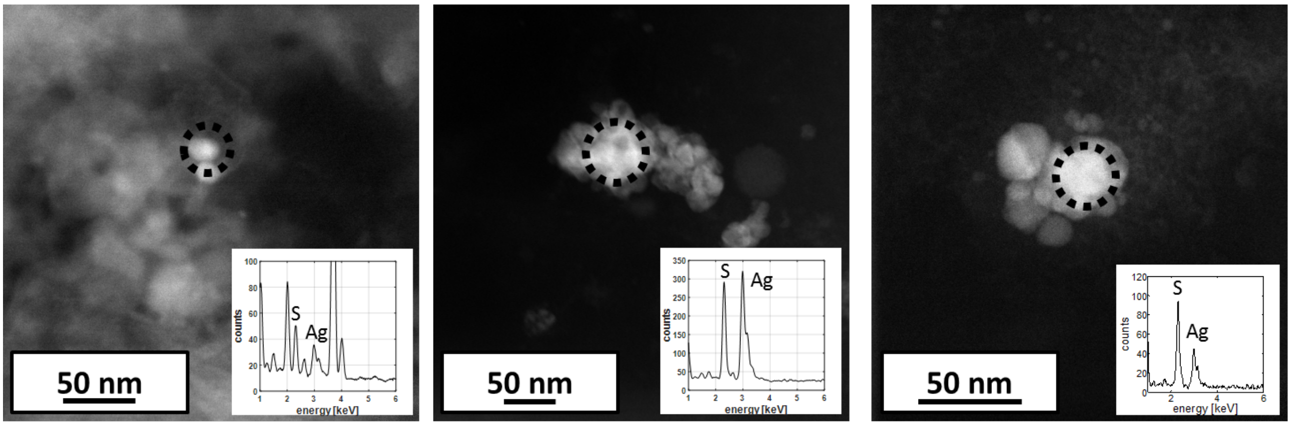


Figure 3.3: STEM images (high angular annular dark field) of (transformed) Ag-NPs detected in the biofilm of the different microcosms (left: positive control; middle: OM; right: air). Insets show the EDX spectra of the particles marked with the dashed circle. The varying Ag – S ratios result from the variable background contribution of S which is present in the biofilm.

3.4 Discussion

Our results revealed an efficient retention of Ag-NPs in the CWs and indicated that most of the retained Ag-NPs were attached to/incorporated in the biofilm. However, in total only between 40% and 60% of the total Ag was recovered. The results from the Ag measurements in the influent were in agreement with the nominal mass applied in the microcosms. In the effluent samples, no residual materials were observed in the digested samples. We thus assume that results from the influent and effluent measurements were very robust. However, it is not clear whether the sequential extraction procedure quantitatively extracted the Ag from the gravel. Also, after the acid treatment of the emptied microcosms, remaining biofilm material was observed on the walls. In addition, the digestion protocol did not completely digest the plant material as residual particulate matter was observed in the digest. Thus, we assume that the poor mass balance closure for Ag resulted from the non-quantitative recovery of Ag from the different compartments within the microcosm, and thus did not affect the results of the Ag removal efficiencies calculated for the CWs from measured influent and effluent Ag concentrations. In similar experiments in the future it is important to apply additional extraction steps.

The good correlation between the TSS and the Ag content in the effluent observed in all experiments further suggests that Ag-NPs were dominantly attached to the particles/biomass leaving the CWs which is in good agreement with earlier laboratory-, pilot- and full-scale studies (e.g. Kaegi et al. (2011); Kim et al. (2010); Ma et al. (2014)). In these studies it was concluded that the Ag-NPs mainly accumulate in sludge and are therefore efficiently removed from the water phase. This hypothesis is supported by the significantly higher Ag fraction that passed the CWs in the OM treatment and the correspondingly lower fraction found in the biofilm compared to the other treatments

(Table 3.2). The addition of organic matter probably provided additional surfaces for the attachment of Ag-NPs but also resulted in a higher fraction of Ag that passed the CWs as more organic matter also left the CWs. However, the Ag fractions extracted from the microcosm walls at the end of the experiments were comparable for all three experiments (11 – 17 μg Ag) and may indicate that the addition of OM did not substantially affect the biofilm, but mainly provided additional surfaces for Ag-NP attachment. Furthermore, the total recovery of Ag was highest in the OM experiment, which is in line with our hypothesis that the poor Ag mass closure is related to the incomplete extraction/digestion of Ag from the compartments within the CWs. The higher Ag fraction passing though the CWs in the OM experiment therefore resulted in reduced amounts of Ag remaining in the CWs which in turn improved the mass closure of Ag. In similar experiments in the future it is advisable to apply more extraction cycles to improve the mass closure. However, in the batch experiments described in the following chapter (Chapter 4) also show that the Ag-NP removal from the water phase is improved in the presence of biofilm and that only minor amounts of Ag-NP are taken up by reed, hence confirming the conclusions made here.

In general, the high retention efficiencies of 80% – 90% of Ag-NPs in combination with the fact that Ag-NPs are dominantly attached to organic matter are consistent with high annual mean removal efficiencies (80±15%) of TSS reported from 17 CWs (Vymazal, 2009). The results indicate that the retention of solids in the wetland becomes essential in limiting the release of Ag (or particulate bound pollutants in general), which again is in line with the absence of Ag in filtered effluents samples. The low concentrations detected in the filtrates could be caused by adsorption of free Ag-NPs and Ag^+ on the paper filter. However, Van Koetsem et al. (2016a) has studied the recovery of these citrate-coated Ag-NPs and Ag^+ during filtration through the same paper filters as used in this study and they concluded that approximately 60% and 95% of Ag-NPs and Ag^+ were recovered in the filtrates, respectively. Hence, it is logical to assume that the low concentrations observed in the filtrates of this study indicate only negligible amounts of free Ag^+, and even Ag-NPs, in the samples.

Aeration did not result in an increased concentration of Ag in the water phase although aeration could have caused Ag^+ formation through oxidative dissolution of Ag-NPs (Liu et al., 2011). This can be well explained by the sulfidation of the Ag-NPs, which was also observed by STEM-EDX in samples from the aerated microcosms. Sulfidation results in dramatically reduced release of Ag^+ (Levard et al., 2011). As the DO in the aerated microcosms remained rather low (3.2 mg/L), it may well be possible that anoxic zones were present within the matrix or the biofilm where sulfate reducing bacteria may have developed.

In this study, we used citrate-coated Ag-NPs and another coating might stabilize Ag-NPs more efficiently. However, also polyvinylipyrrolidone (PVP) which sterically stabilizes the particles did not influence the removal efficiencies during activated sludge treatment (Kaegi et al., 2013). Thus, we assume that our results are also applicable for Ag-NPs with other types of coatings.

3.5 Conclusions

Ag-NPs were very efficiently removed (80 - 90%) from synthetic wastewater in microcosms simulating CWs. The largest fraction of Ag-NPs was attached to or incorporated in the biofilm developed on the gravel bed of the CWs. The fraction of Ag-NPs that passed the CWs, was bound to solids present in the effluent. Thus, an increasing retention of TSS in CWs would lead to a proportional decrease in Ag being discharged from the CWs. Results from STEM-EDX analyses suggested that Ag-NPs were dominantly sulfidized, even in the aerated treatments. Anoxic/anaerobic zones within the biofilm most likely favored the growth of sulfate reducing bacteria resulting in the sulfidation of the Ag-NPs. Aeration did not affect the retention efficiency of total Ag in the microcosms and the distribution of total Ag in the aerated microcosms was similar to that of the positive control. The addition of OM provided additional surfaces for the attachment of Ag-NPs and resulted in a slightly reduced retention of Ag-NPs in the microcosms due to increased discharge of TSS with the effluent. Although this study describes laboratory-scale setups many conclusions and predictions on the fate of ENMs in full-scale CWs can be made. The biofilm is likely to function as the main sink for the Ag-NPs due to its high affinity for Ag-NPs. The biofilm thickness and the percentage of substrate covered by biofilm will increase with operation time and are thus higher in full-scale CWs than the studied microcosms, hence indicating larger biomass being able to accumulate Ag-NPs in full-scale CWs than in the microcosms studied. Also, plant roots, occupying a large volume in full-scale CWs, offer an important attachment site for biofilm and hence for Ag-NPs. As Ag-NPs are mainly accumulating in biofilm sudden high flow of influent, large fluctuations in aeration force or the presence of toxic compounds in the influent could induce the detachment of biofilm and hence, cause temporary release of (transformed) Ag-NPs from the CW. As the CW ages and more organic matter is accumulating within the CW bed, clogging could occur and lead to short-circuiting and possibly increased discharge of Ag-NPs. In general, the results obtained in this study implicate that the biofilm in CWs will act as a sink for Ag-NPs, similarly to activated sludge, and the release of (transformed) Ag-NPs is during normal operation primarily determined by the discharge of TSS.

Chapter 4

Substrate- and plant-mediated removal of citrate-coated silver nanoparticles in constructed wetlands

This chapter is based on **Auvinen H.**, Vásquez Sepúlveda V., Rousseau D.P.L., Du Laing G. 2016. Substrate- and plant-mediated removal of citrate-coated silver nanoparticles in constructed wetlands. Environmental Science and Pollution Research 23(21), 21920-21926.

4.1 Introduction

Silver nanoparticles (Ag-NPs) are used in a wide range of household products, such as high-performance clothing, food packaging and wound bandages (Vance et al., 2015), because of the bactericidal properties of Ag (Doiron et al., 2012; Ratte, 1999). Ag-NPs can be released from these products during usage (Benn, Westerhoff, 2008; Mitrano et al., 2014), and will therefore be transported to the wastewater treatment plant which acts as a barrier controlling the release of NPs to the environment. The potential adverse effects on aquatic organisms have raised concerns about the discharge of (Ag-)NPs with wastewater effluent (Fabrega et al., 2011; Klaine et al., 2008) and thus, understanding the fate and behavior of (Ag-)NPs in different environmental and biological systems can help improve the risk assessment (Lowry et al., 2012b).

Although constructed wetlands (CWs) are used to treat wastewater that potentially contains NPs, such as municipal wastewater and landfill leachate (Kadlec, Wallace, 2009; Keller, Lazareva, 2013), the fate of NPs in CWs has received little attention to date. In activated sludge treatment, Ag-NPs are likely to precipitate, mainly as sulfides (Ag_2S), and attach to sludge biomass, and these processes result in efficient removal of Ag-NPs in the secondary clarifier (Kaegi et al., 2011; Kim et al., 2010; Lombi et al., 2013). The formation of Ag_2S from polyvinylpyrrolidone (PVP)-coated Ag-NPs has been reported also in a fresh water emergent wetland (Lowry et al., 2012a). These solids were found nearby the location where they were dosed indicating rapid formation and low mobility of these precipitates.

In addition to sulfidation and attachment to biosolids, aggregation, plant uptake, and adsorption on the surfaces of the substrate and plant roots can play a role in the retention of (Ag-)NPs in CWs. Sub-surface flow CWs, where the treated water flows through the wetland substrate, have a high contact surface area and long hydraulic retention time which can improve the attachment of NPs in these systems. The surface area is (mainly) created by the (biofilm covered) substrate and plant roots. The biofilm excretes extracellular polymeric substances (EPS) which are responsible for the adhesion of bacterial communities onto surfaces (Tsuneda et al., 2003). Its high adhesion capacity makes EPS also efficient at binding toxicants, such as NPs (Sheng, Liu, 2011). Research on phototrophic biofilm cultures in rotating angular bioreactors simulating natural aquatic environment showed, however, that Ag-NPs are not necessarily immobilized by EPS but can move through the EPS structure resulting in widespread effects in the biofilm ecosystem (González et al., 2015). The specific conditions (e.g. pH, dissolved organic carbon) within the biofilm define the properties of the Ag-NPs and subsequently the exposure of the biofilm organisms (Kroll et al., 2014). The tendency of NPs to adsorb onto plant material is related to the coating of the NP (Sharif et al., 2013). NPs coated with non-polar substances are more likely to bind onto plant roots than NPs with polar coating because of their hydrophobic character.

In this study, we investigated the distribution of Ag after supplying citrate-coated Ag-NPs to the water phase in batch experiments representing partial processes occurring in a CW. By using citrate-coated Ag-NPs, we focused on studying silver species that are presumed to have high mobility in the sewer system, enabling them to reach the CW. Although in reality Ag-NPs are prone to transformations in the sewer system (Brunetti et al., 2015; Kaegi et al., 2013), the resulting less mobile silver sulfides are assumed to be rapidly removed from the aqueous phase in the sewer, the settling tank prior to the CW or the first part of the CW.

In the first experiment, the removal of citrate-coated Ag-NPs from the water phase was studied after bringing the NPs in contact with wetland substrates in synthetic wastewater. The effect of different (biofilm covered) solid substrates, sand, gravel and zeolite, as well as the effect of synthetic wastewater on the removal efficiency was investigated. In the second experiment, the distribution of Ag-NPs was studied in a hydroponic culture of *Phragmites australis*, a common helophyte in CWs. Separate experiments were conducted to be able to distinguish between removal induced by substrate- and plant-mediated processes.

4.2 Materials and Methods

4.2.1 Batch experiments

Constructed wetland substrates and biofilm

The effect of wetland substrates and biofilm on the removal of Ag-NPs from the water phase was studied in a 24-h batch experiment. Sand (∅ 1/4-1/2 mm), gravel (∅ 8-16 mm) and zeolite (Clinoptilolite, ∅ 5-10 mm) were selected to represent the solid substrates of a wetland system. The matrices were purchased from local hardware stores and distributors (Hubo, Zottegem, Belgium; Kranendonk N.V., Waalwijk, The Netherlands; Benopet®, Zingem, Belgium). They were washed with tap water prior to use, in order to remove any fine particles present, and dried in the oven at 105°C overnight. The gravel samples containing biofilm were obtained from a laboratory-scale horizontal sub-surface flow wetland, originally inoculated with activated sludge and fed with synthetic wastewater prepared according to (Weber, Legge, 2011).

The substrates were introduced in Erlenmeyer flasks (300 mL) and 100 mL of spiked synthetic wastewater (Weber et al., 2011) was added in each flask. The dry weight of substrate was 30 g except for biofilm samples which had a wet weight of 30 g (29.5±0.1 g dry weight). Additionally, the concentration of Ag in the control samples containing spiked synthetic wastewater and spiked ultrapure water was monitored. Citrate-coated Ag-NPs were spiked at initial concentration of 100 μg/L. The experiment was conducted in triplicate. The Erlenmeyers were shaken in an orbital shaker at room temperature for 24 h at 100 rpm. The samples for the analysis of total Ag were taken before shaking as well as directly after shaking was stopped. The total Ag remaining in the empty Erlenmeyer was also determined.

Hydroponic culture of *Phragmites australis*

To analyze the Ag uptake capacity of *P. australis* and to determine the distribution of Ag in the plant tissues, a hydroponic culture of *P. australis* was set up. Three strong looking, green reed plants, named active plants, and three rhizomes without green leaves, named passive plants, were selected for this experiment. The roots of the active and passive plants differed in structure, the passive plants having thinner roots than the active plants. As wetland plants can oxidize the root zone by transporting oxygen to the rhizome from the shoots and thereby cause oxidation of e.g. metals (Allen et al., 2002), the overall removal of Ag from water phase can be dependent on the activity of the plant.

The plants were obtained from a laboratory-scale sub-surface flow CW fed with synthetic wastewater (Weber, Legge, 2011) without being exposed to Ag-NPs. During the batch experiment, the plants were cultivated for 4 weeks under controlled temperature and light conditions (room temperature; light cycle 16 h + 8 h) in synthetic wastewater (190 mL) containing 100 μg Ag/L added as citrate-coated Ag-NPs. Also, the stability of the Ag concentration in two unplanted controls containing spiked synthetic wastewater was monitored and the Ag content in an unexposed plant was analyzed. The spiked synthetic wastewater was replaced once a week. At the end of each week, the culture water was decanted, its volume was recorded for mass balance calculations and a sample was taken for the total Ag analysis. A fresh synthetic wastewater was prepared and a sample for total Ag analysis was taken before adding the water to the plant culture. At the end of the experiment the total Ag content associated with the plant tissues and the glass recipient was determined.

4.2.2 Characterization of the nanoparticle stock suspension

An aqueous suspension of Ag-NPs (as given by the supplier: pH 6-8; z-potential -50 ± 5 mV) stabilized with citrate was purchased from PlasmaChem GmbH (Berlin, Germany). The size distribution was determined with a Photon Correlation Spectrometer (PCS; 100M Malvern Instruments Ltd, UK). All measurements were performed in triplicate, at 25°C using a helium-neon laser (633 nm) and scattering angle of 150°. The average particle size was determined based on both intensity and number. The concentration of the silver suspension was verified by ICP-MS analysis prior to use in the experiment. The Ag-NPs used in this experiment were the same ones as used in the experiments described in the previous chapter (Chapter 3). A STEM image of the particles is shown there. Certified reference material was not use as it is not available for Ag-NPs. However, the recovery of spiked Ag was checked and it was above 95%.

4.2.3 Characterization of the substrates

The pure substrates were characterized for pH (520A, Orion Research Inc.), conductivity (LF537, WTW), organic matter content (loss at ignition), cation exchange capacity (CEC) and trace element composition (TEC) according to Van Ranst et al. (1999). TEC was determined by ICP-OES (Vista-

MPX CCD Simultaneous ICP-OES, Varian, Agilent Technologies, Santa Clara, CA, USA) after *aqua regia* open vessel microwave digestion.

4.2.4 Total Ag analysis

Digestion of the samples

The water samples (5 mL) were digested with 2 mL of HNO_3 (Chem-Lab, Zedelgem, Belgium) by open vessel microwave digestion (MARS 5, CEM Corp., Matthews, NC, USA). The digestion program consisted of the following steps: 10 min at 55°C; 10 min at 75°C; 40 min at 100°C. The optimization of the digestion method was done earlier (Van Koetsem, 2015).

At the end of the hydroponic culture experiment, the plants were divided in three parts: roots and old and new aboveground tissues. Leaves that were present at the start of the experiment were defined as old aboveground tissue. In the roots, both the amount of Ag adsorbed and Ag taken up was determined. For the determination of the mass of Ag adsorbed to the roots, two extraction protocols were applied. Firstly, the roots were submerged in 50 mL of Milli-Q water and sonicated for 10 min. Secondly, the roots were submerged in 50 mL of 1% HNO_3 and sonicated for 5 min. Both aqueous samples were digested as described above. The mass of recovered Ag was measured after both steps and the sum accounted for the total mass adsorbed by plant biomass.

For the determination of the mass taken up by the plant, roots and old and new aboveground tissues were cut in small pieces and dried for 24 hours at 40°C. After this, the samples were ground to smaller size with mortar and pestle. The solid samples (approximately 0.3 g) were acid digested with 5 mL 65% HNO_3 and 1 mL H_2O_2 overnight at room temperature, after which they were digested by open vessel microwave digestion as described above.

To determine the adsorption to the Erlenmeyer glass, 100 mL 5% HNO_3 (Chem-Lab, Zedelgem, Belgium) and 1 ml H_2O_2 (Chem-Lab, Zedelgem, Belgium) were added to the empty Erlenmeyer flask and shaken for 24 hours in an orbital shaker (200 rpm). The aqueous samples were digested as described above.

ICP-MS

Digested samples (<10 mL) were first diluted to 10 mL with Milli-Q water and then diluted (1:10) with an acidified (1% HNO_3) internal standard solution (10 μg Rh/L and 10 μg Ga/L (Chem-Lab, Zedelgem, Belgium) in 2% HNO_3). All samples were analyzed for total silver content by means of inductively coupled plasma-mass spectrometry (ICP-MS) (Elan DRC-e, PerkinElmer, Inc., Waltham, MA, USA). External calibration standards (see Appendix A Table A1) for the applied calibration range) were used for ICP-MS analyses, and recalibrations were performed every 20 samples. Blank samples and reference standards were included at the beginning and the end of each intra-analysis batch of 20 samples for quality control purposes. The detection limit of the ICP-MS was 0.016 μg/L.

4.2.5 Data analysis

The data were first controlled for normality by using the Shapiro-Wilk's test and for homogeneity of variances by using the Levene's test. Thereafter, the data were examined for significant differences by using One-way ANOVA or Welch test in combination with Tukey Post-Hoc test or the Games-Howell test. All statistical analyses were performed with the software SPSS Statistics 22.

4.3 Results

4.3.1 Characterization of the nanoparticle stock suspension

According to the intensity based analysis, the average particle size of the Ag-NP stock suspension was 90.7±7.9 nm and according to the volume based analysis, 11.1±0.2 nm. Although large particles were present only at a low relative percentage, their presence resulted in high scattering intensity. The concentration of the stock suspension was 97.03±3.13 mg Ag/L.

4.3.2 Characterization of the solid matrices

The different matrices (sand, gravel and zeolite) were characterized in order to determine how their physicochemical properties could affect the fate of nanoparticles in wetland systems containing these matrices. The sand samples had the highest conductivity, whereas the zeolite samples differed from the others by higher loss at ignition and cation exchange capacity (CEC) (Table 4.1).

Table 4.1: Physicochemical properties of substrates sand, gravel and zeolite

	Sand	**Gravel**	**Zeolite**
pH-H_2O	7.9 ± 0.1	8.2 ± 0.1	7.9 ± 0.1
Conductivity (μS/cm)	1280 ± 67	30.8 ± 4.6	34.3 ± 3.2
CEC (meq/100 g)	2.3 ± 0.5	1.4 ± 0.3	10.0 ± 0.8

In addition, the concentrations of major and trace elements and silver were analyzed for the different substrates (Appendix B Table B1). Zeolite contained clearly a higher concentration of K, Al, Pb and Zn than sand and gravel. On the other hand, a higher concentration of Ca was found in gravel. The Ag concentration in sand and gravel was below the detection limit of the method, but the Ag concentration of zeolite was 0.36±0.04 μg/g.

4.3.3 Effect of type of substrate and presence of biofilm on the removal of Ag grom the water phase

Figure 4.1 shows the percentage Ag removed from the water phase by the different treatments. The Ag-NPs were not completely stable in solution and a minor fraction (~10%) of the initial concentration was lost during 24 h in ultrapure water (C-MQ) and synthetic wastewater (C-WW). Sand induced the best removal efficiency with 85% in comparison to 55% with zeolite and 20% with gravel. The presence of biofilm on the gravel (Biofilm) increased the removal efficiency by gravel significantly ($p<0.05$) to 67%. The mass of biofilm covering the gravel, measured via loss on ignition, was 1.7 mg/g gravel.

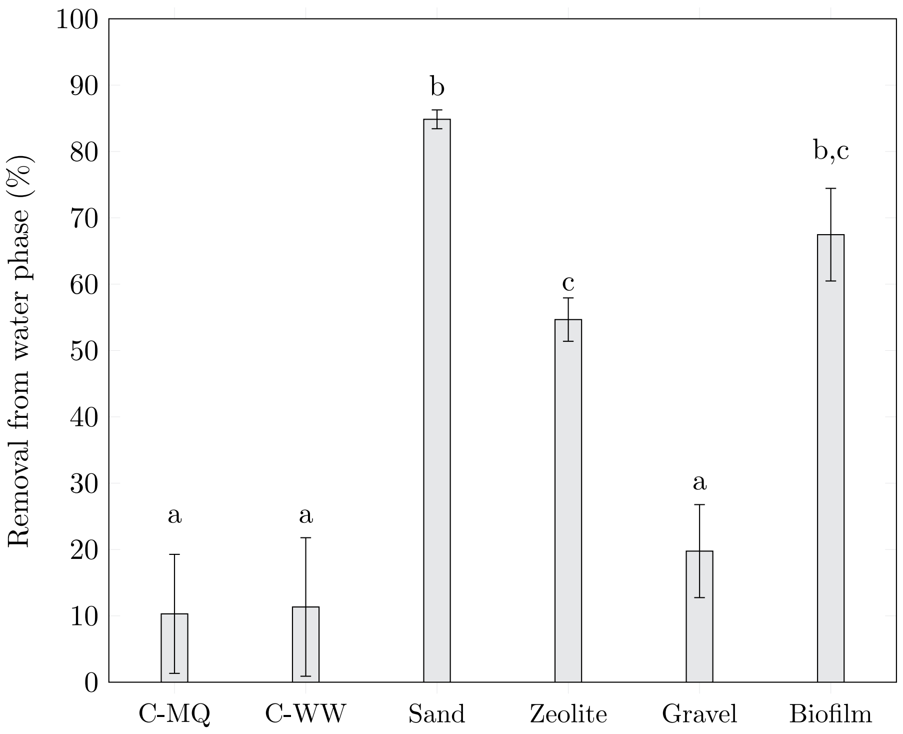


Figure 4.1: The removal efficiency of Ag from the water phase by solid substrates in a batch experiment of 24 h (average±SD, n=3). Significant differences are marked with differing letters a, b and c.

4.3.4 Removal of Ag-NPs in a hydroponic culture of *Phragmites australis*

A mass balance was established to identify the distribution of Ag in a hydroponic culture of *P. australis*. During the 4-week experiment, the majority of the total Ag recovered was found in the water phase (Table 4.2). Attachment and uptake by the roots were determined as the most important plant-mediated processes (13 - 41%) while only small amounts of Ag were measured in the aboveground tissues (0 - 1%). Attachment to the glass recipient was only substantial in the control samples whereas only a few % of the recovered Ag was found attached on the glass surface in the planted systems. The Ag content in the unexposed control plant was below the detection limit. The weight of the roots after the experiment was similar for both active and passive plants (11.4±3.2 g dry weight and 11.9±5.3 g dry weight, respectively).

Figure 4.2 shows the concentration of Ag found in the water phase after each week. At week 3 the concentration of Ag in the water phase started to increase in case of the active plants and at week 4 in case of the passive plants. It is necessary to note, however, that the so called passive plants started to grow leaves as well, making it difficult to differentiate activity levels between the plant types. The unplanted controls behaved very differently in comparison to each other: in control 1 there was more adsorption in the glass recipient than in control 2 (Table 4.2) and therefore, the Ag concentration of the water phase increased more rapidly in control 2 than the control 1 (Figure 4.2).

Table 4.2: Mass balance of Ag after the 4-week plant experiment (average ± SD, n=3). In the case of old and new leaves composite samples of the three replicates were analysed because of the low mass of the material and hence, no SD can be shown. No significant differences observed between the two types of plants. Asterisk (∗) marks loss of sample.

		Control 1		**Control 2**		**Active plant**		**Passive plant**	
		µg Ag	%	µg Ag	%	µg Ag	%	µg Ag	%
IN	Total	67.3	100	67.0	100	73.1±6.4	100	67.2±0.9	100
OUT	Water phase	36.2	54	33.2*	50	43.0±11.2	58	28.1±10.3	42
	Attachment onto the root surface	-		-		13.7±1.6	19	27.6±10.3	41
	Uptake by the roots	-		-		13.8±8.2	19	8.7±6.6	13
	Translocation to the old leaves	-		-		0.9	1	-	-
	Translocation to the new leaves	-		-		0.01	0	0.1	0.1
	Attachment onto the glass recipient	30.0	45	11.1	17	2.4±0.5	3	3.1±0.9	5
	Total	66.2	98	44.3	66	73.1±11.8	100	67.6±11.4	101

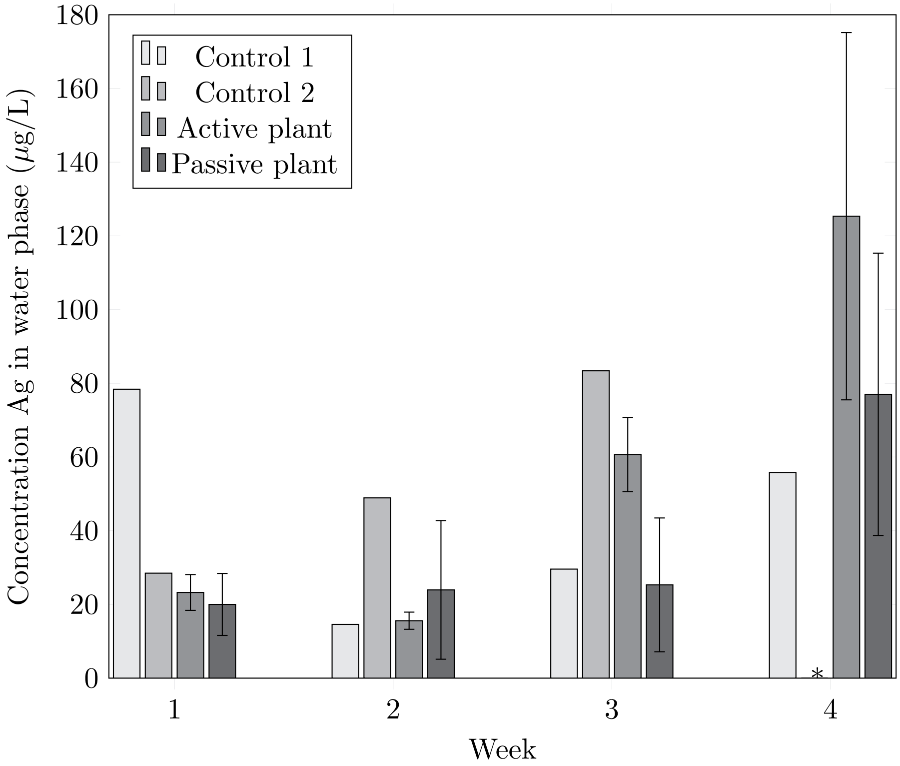


Figure 4.2: Total Ag concentration found in the water phase during the experiment (average ± SD). No significant differences observed between the different types of plants.

4.4 Discussion

The results from the batch experiment with different wetland substrates showed efficient removal of Ag-NPs from the water phase in 24 h, although the Ag-NPs were added to the system in a mobile, citrate-coated form. More than 50% of the initial concentration of Ag was removed from the water phase in the presence of sand and zeolite, in comparison to the removal of ~20% with gravel present. The removal by sand and zeolite was significantly different from the control samples where a decrease in Ag concentration was also observed (~10%). The process of aggregation and sedimentation is the likely explanation for the decrease in concentration in the control sample as attachment onto the walls of the Erlenmeyer was found to be negligible in the time frame of 24 h (data not shown). Indication of the formation of aggregates was also seen in the PCS measurement where the intensity based analysis gave a larger particle size than the number based analysis. Additional heteroaggregation between the Ag-NPs and small particulate matter detached from the substrates has probably occurred in the batch experiment (Velzeboer et al., 2014) due to the incomplete removal during washing or shear forces during shaking (especially zeolite). The higher removal efficiencies of sand and zeolite are also in agreement with the higher CEC and porosity of zeolite (Huang et al., 2000), and smaller particle size of sand in comparison to gravel, properties improving adsorption capacity. In general, the attachment of nanoparticles on surfaces is governed by both the surface area and interactions between the particle and the surface, such as hydrophobic and electrostatic interactions (Chapter 2). Dissolution of the Ag-NPs can also have taken place before or simultaneously with aggregation/adsorption (Zhang et al., 2011a). Also, the organic content of the substrates can be linked to the removal of Ag from the water phase (Colman et al., 2012). In the current study, zeolite presented a high loss on ignition, indicating a higher organic matter content than sand and gravel, and this can have improved its Ag removal efficiency. This is also supported by the fact that biofilm-coated gravel was more efficient at removing Ag(-NPs) from the water phase than clean gravel (67% vs. 20%). It is likely that the removal by sand and zeolite increases in the presence of a biofilm. This also confirms the conclusion made in the previous chapter (Chapter 3) that biofilm has a major impact on retaining Ag-NPs. Also, Sheng, Liu (2011) found that the Ag concentration in the Ag-NP suspension decreased rapidly when incubated in the presence of biofilm. In case of urban wastewater systems, Ag-NPs have been found to be efficiently retained by suspended organic matter, but less so by biofilms present in sewer systems (Kaegi et al., 2013). However, as CWs operate at a long retention time, the concentration of Ag-NPs adsorbed to the biofilm can increase due to a long contact time between water and biofilm. Microorganisms habiting the CW biofilm can possibly be adversely affected by the Ag-NP sequestering in the biofilm. Hence, in the following chapter (Chapter 5) the effects of Ag-NPs and Ag ions on CW biofilm are investigated.

In the hydroponic culture experiment, the control samples behaved very differently when compared to each other: Control 2 exhibited a lower attachment onto glass than control 1 (17% vs. 45%, respectively). Subsequently, the percentage of Ag recovered in the water phase of control 2 was 50% at week 3 while 50% was only reached after 4 weeks in control 1. This is possibly due to one Erlenmeyer being older than the other and thus having a rougher surface which can enhance NP attachment on the surface. Although this caused the control samples not being reproducible, we find that it is still relevant to discuss the distribution in the planted cultures, because this data is only in a minor way affected by the attachment of Ag on the glass surface (3 and 5% of Ag recovered from glass surface; Table 4.2).

The Ag associated with the plant biomass was mainly attached on the root surface (19 - 41%

of total Ag added) and to a smaller extent taken up by the roots (13 – 19% of total Ag added). Translocation to the aboveground tissues accounted only for 1% of the total Ag added to the system and more Ag was found in the old tissue than in the new tissue. These findings are in agreement with results reported earlier in literature. Lowry et al. (2012a) investigated the translocation of Ag from Ag-NPs in different wetland plants in a long-term simulated freshwater wetland experiment of 18 months, and they found that only 0.2 – 3% of the total Ag added to the system was found in plant tissue, majority of which in the roots. Jacob et al. (2013) studied the accumulation of Ti from TiO_2-NPs in a wetland species *Rumex crispus* in which the translocation of Ti was less than 1% of the total plant associated Ti after hydroponic culturing of 18 days. As was concluded in the review of Miralles et al. (2012) the translocation of metallic nanoparticles is dependent on the exposure time and the type of NP with small NPs being more easily transported than large ones. Plants in full-scale constructed wetlands provide surface also for biofilm attachment (Kadlec, Wallace, 2009), and can thus possibly sequester larger amounts of (Ag-)NPs because of the high adsorption capacity of biofilm than assumed based on a short-term study where biofilm is not fully developed. When the amount of Ag present in the water phase was studied after each week, a rising trend was observed in all samples. This can be caused by several reasons. Firstly, dissolution from the aggregated particles can have occurred (Zhang et al., 2011a). Secondly, it is possible that the attachment became less efficient in the course of the experiment due to the reducing amount of binding sites on the root(/glass) surfaces. Finally, the Ag-NPs attached on the root(/glass) surfaces can have dissolved into Ag^+ (Ma et al., 2012) or detached. Further research would be necessary to identify the Ag release mechanism. Differences, although not statistically significant, were observed in Ag concentration in the water phase of the active and passive plants. It can be speculated that longer root zone oxidation by the active plants resulted in the oxidation of attached Ag and thus higher Ag concentration in the water phase. Also, the thinner roots of the passive plants, which were observed when starting up the experiment, can have improved the adsorption capacity of the passive plants in comparison to the active plants.

4.5 Conclusions

The main goal of this study was to assess the effect of wetland substrates, biofilm and plant biomass on the removal of citrate-coated Ag-NPs from synthetic wastewater. It was shown that sand and zeolite induced efficient removal of Ag from solution, while the presence of gravel reduced the Ag concentration measured in the water phase only by ∼20%, thus only ∼10% more than in the control sample. The removal of Ag from the water phase can be partly explained by aggregation-sedimentation. The removal by sand and zeolite is presumably further improved by their properties, high porosity and CEC for zeolite and small particle size for sand, which increase their adsorption capacity. Biofilm on the gravel increased the Ag removal efficiency of gravel significantly indicating of important mechanism of Ag sequestering in CWs. Further experiments should be conducted to verify adsorption as the main removal process and to elucidate the long-term capability of these substrates to bind Ag-NPs (desorption).

In the presence of plant biomass the largest fraction of the recovered Ag was found in the water phase during the 4-week experiment (0.42 and 0.58) followed by the fraction of Ag attached on the root surface (0.19 and 0.41). Almost all Ag associated to the plant material (minimum 97%) was attached to and taken up by the roots rendering translocation to aboveground tissues negligible. Starting from week 3 - 4, the Ag concentration in the water phase began to increase, most likely evidencing desorption/dissolution behavior and/or saturation of surfaces by Ag. Based on these findings, Ag-NPs are likely to be removed from the water phase when being in contact with wetland substrates, and the removal is further increased by the presence of biofilm. It was further observed that *P. australis* has a minor role in removing Ag-NPs from the water phase, as desorption/dissolution of the plant-associated Ag can occur and translocation to aboveground tissues is very low.

Chapter 5

Susceptibility of constructed wetland microbial communities to silver nanoparticles: a microcosm study

This chapter is based on Button M., **Auvinen H.**, Van Koetsem F., Hosseinkhani B., Rousseau D., Weber K.P. and Du Laing G. 2016. Susceptibility of constructed wetland microbial communities to silver nanoparticles: a microcosm study. Ecological Engineering, 97, 476-485.

5.1 Introduction

Silver nanoparticles (Ag-NPs) are increasingly integrated into textiles, food packaging and many household items as an antimicrobial agent (Vance et al., 2015). The magnitude of production and breadth of applications are growing thereby increasing the likelihood of Ag release to the environment. Both ionic Ag and Ag-NPs are potentially toxic (Morones et al., 2005), and recent studies have shown that Ag can leach from Ag-NP containing consumer products into the wastewater system (Benn, Westerhoff, 2008). According to Geranio et al. (2009) some garments can release up to 45% of the total Ag embedded in the textile after a single wash. This released Ag enters wastewater treatment plants and is predicted to then be transferred in unknown amounts to the natural environment (Blaser et al., 2008) giving rise to the risk of exposure and accumulation in biota inhabiting aquatic ecosytems (Cleveland et al., 2012).

The investigation of Ag-NP toxicity on wetland systems can be completed within the context of natural wetlands, or wetlands built for water pollution control (referred to here as constructed wetlands). Existing studies have focused on the behavior of Ag-NPs in natural wetlands. Lowry et al. (2012a) found that a large percentage of silver dosed to natural wetland mesocosms was transformed into sulfidised (Ag_2S) silver with rapid transfer of silver from the water column into the sediment. Relatively small amounts of Ag were accumulated in different plant species whilst large body burdens were observed in mosquito fish and chironomids (Lowry et al., 2012a). Colman et al. (2014) investigated ecosystem-level effects of Ag-NPs in natural wetland mesocosms reporting that exposure led to a series of effects including leaf senescence, declines in phytoplankton biomass, negative impacts to water chemistry, and an overall increase in methane release. Colman et al. (2014) used admittedly high exposure concentrations (2.5 mg/L) more in line with laboratory toxicological studies than likely environmental concentrations, but demonstrated the unique challenges and complexities of such studies, and the ecosystem-level impacts that silver can have. Colman et al. (2014) also observed a relatively clear convergence regarding the effects of different Ag treatments with ionic silver and Ag-NPs showing similar results, likely due to the rapid transformation of silver once within the mesocosm systems as demonstrated by Lowry et al. (2012a).

Although detailed Ag-NP studies have been completed with natural wetland mesocosm systems, no studies have yet been completed on wetlands designed for water pollution control. Constructed wetlands (CWs) rely on microbiological processes for water treatment (Faulwetter et al., 2009). For processes such as organic degradation, nitrification, or denitrification microbial communities housed within biofilm surrounding the CW bed media (usually gravel) play the dominant mechanistic role. With their increasing use as a low-cost, low-maintenance and effective mode of wastewater treatment the constructed wetland represents a system likely to receive Ag released from consumer products. In particular the large numbers of small-scale constructed wetland systems now employed primarily in European countries but also increasingly in North America for the treatment of greywater from individual residences.

Estimated environmental concentrations for Ag-NPs based on a combination of modeled and analytical results range from 10^{-4} to 10^{2} μg/kg or μg/L with the highest concentrations occurring in sediments and wastewater treatment plant effluents (Gottschalk et al., 2013). No estimates on current or expected Ag-NP loadings to CWs have been made in the literature. CWs receiving greywater from a single home represent the system type most at risk for potentially high Ag-NP concentrations. Geranio et al. (2009) found X-STATICTM fabric to release approximately 314 μg Ag/g fabric, and AgKilBactTM fabric to release approximately 377 μg Ag/g fabric after a single wash. Assuming a relatively small high efficiency washer (approximately 50 L of water) processing 20 sports shirts (approximately 2 kg total weight), upward of 15 mg/L could be estimated as a worst case scenario leaving a washing machine and therefore entering CWs. That estimate is based on clothing containing 100% X-STATICTM or AgKilBactTM. This type of silver treated fibre is commonly incorporated into fabric in the range of 5 - 20%. Assuming a 10% final concentration in clothing the washer effluent (influent to a single home CW) could reasonably contain 1.5 mg Ag/L. This estimate would of course become even lower when averaged over an entire days water release from a single home and when considered that the leaching decreases at each washing.

Understanding the effect of Ag-NPs on microbial communities from constructed wetlands is important for the continued operation of wetlands for water pollution control in the face of growing Ag-NP inlet loadings. If Ag-NPs have an exceptionally toxic effect, constructed wetlands which are already in use and otherwise operating efficiently for water treatment may not be viable for future wastewater applications, or could require augmentation to ensure continued operation. Several studies have looked at effects in non-CW microbial communities with differing results. Low levels of Ag-NPs (0.14 mg/kg) were shown to cause reduced enzyme activity in the microbial communities of contaminated soils (Colman et al., 2013), whilst concentrations up to 1.0 mg/L reportedly had no effect on the genetic diversity of microbial communities in estuarine sediments (Bradford et al. 2009). In contrast, Doiron et al. (2012) reported that exposure concentrations as low as 5 μg/L of polymer-coated Ag-NPs resulted in decreased bacterial abundance in marine microcosms. Similarly, Fabrega et al. (2009) saw a decrease in the growth of marine biofilm in the presence of Ag-NPs.

Genetic fingerprinting methods are frequently used to study microbial communities in environmental samples (Boon et al., 2002). In denaturing gradient gel electrophoresis (DGGE), the number and relative abundance of the dominant ribotypes in a sample can be determined based on the number and intensity of the bands. Community-level physiological profiling (CLPP) can be used to assess the catabolic function of microbial communities based on substrate utilisation (Weber, Legge, 2010a). CLPP is a rapid and sensitive method to measure differences in catabolic activity, richness, diversity, and has been successfully applied to the assessment of microbial assemblages in constructed wetlands (Weber, Legge, 2010a; Weber et al., 2011; Zhang et al., 2010a). In addition, the first known *ex-situ* method of microbial community functional (based on the catabolic capabilities) toxicity testing was recently developed and described using the concepts underlying the CLPP method and applied to constructed wetland samples exposed to Au-NPs (Weber et al., 2014).

This study set out to evaluate the impacts of different types of Ag-NPs (citrate and polyvinylpyrrolidone coated) in addition to ionic Ag on constructed wetland microbial communities associated with both the biofilm and interstitial waters. Changes in microbial community function and structure were monitored in wetland microcosms over a period of 28 days following exposure to 100 μg/L Ag-NPs. The distribution and toxicity of each type of Ag-NP and potential for development of microbial community resistance was further assessed via *ex-situ* community based functional toxicity testing over a wider exposure range.

5.2 Materials and methods

5.2.1 Experimental design

CW microcosms (Figure 5.1) were built in triplicate for each type of Ag-NP (citrate, and polyvinylpyrrolidone (PVP) coated), ionic Ag, and an unexposed control, using 5 L polypropylene containers giving a total of 12 experimental systems. The microcosms were filled with 2 kg of coarse granitic gravel (⌀ 10 - 15 mm) and inoculated by homogeneously mixing 0.5 kg of similar biofilm-containing gravel obtained from the surface of a local horizontal subsurface flow constructed wetland, treating tertiary domestic wastewater (De Pinte, Belgium). The microcosms were fitted with a 32-mm-diameter perforated central sampling well and a small outlet spout made of silicone tubing with a plastic stopper for draining. The microcosms were fed with 0.5 L of a simulated wastewater (pH 7.4) based on that described by Weber et al. (2011), consisting of 1 g/L molasses, 0.049 g/L urea, 0.0185 g/L $\mathrm{NH}_4 H_2PO_4$, (Merck, Darmstadt, Germany). After filling the water level was just below the surface of the gravel. The microcosms were operated in batch mode with weekly draining equating to a hydraulic retention time (HRT) of 7 days. Solids were retained in the microcosms for the duration of the experiment. The microcosms were allowed to acclimatize for 19 days (longest time permitted due to project constraints) before the simulated wastewater was spiked to a final Ag concentration of 100 μg/L. The exposure concentration is higher than some predicted environmental concentrations e.g. Blaser et al. (2008), but falls within a reasonable worst-case scenario for CWs (as described earlier). The chosen concentration also provides a balance between environmental relevance, analytical feasibility and the aim of investigating the development of Ag resistance in the exposed microbial community over the relatively short term of the experiment. After the acclimatization period, the microcosms were operated for 28 days.

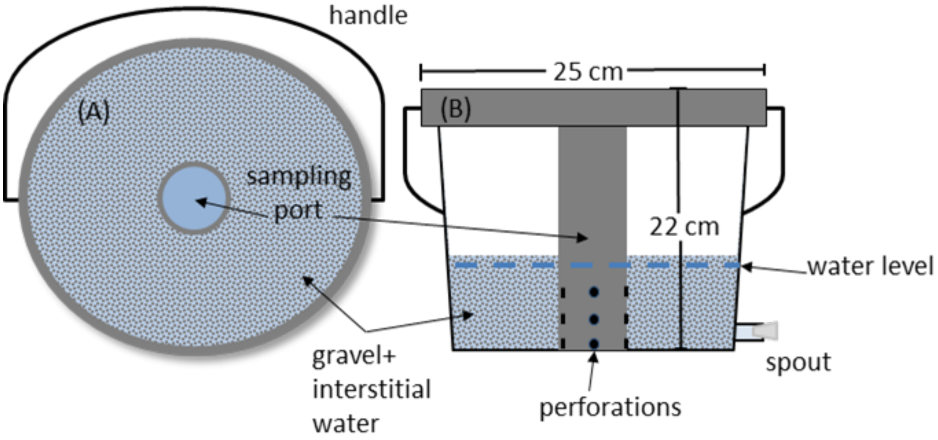


Figure 5.1: Diagram of a constructed wetland microcosms (A) aerial view and (B) cross-sectional view. Diagram is not to scale.

5.2.2 Nanoparticle stock solutions

Citrate Ag-NPs with a manufacturer stated average particle size of 10 nm and at a concentration of 100 mg/L were obtained from PlasmaChem GmbH (Berlin, Germany) as a colloidal dispersion. PVP Ag-NPs in powder form (20 - 30 nm, SkySpring Nanomaterials, Inc., Houston, TX, USA) were dispersed using Milli-Q water (EMD Millipore Corp., Billerica, MA, USA) followed by 15 minutes in an ultrasonic bath (Sonorex Super RK103H, Bandelin electronic GmbH, Berlin, Germany) to give a final concentration of 100 mg/L. The ionic Ag standard solution (Plasma HIQU, 1000 ± 2 μg Ag/mL in 2 – 5% HNO_3) was purchased from ChemLab NV (Zedelgem, Belgium). All Ag-NP suspensions were stored in the dark at 4 - 8°C. The concentrations of the Ag stock dispersions were verified by ICP-MS analysis (Section 5.2.3) prior to their use in spiking the simulated wastewater.

5.2.3 Nanoparticle characterization

Characterisation of the PVP and citrate Ag-NPs used in this study was performed on representative stock solutions and the nanoparticle spiked simulated wastewater. Scanning Transmission Electron Microscope images (STEM) (Quanta FEG 250, FEI, Oregon, USA) were collected to qualitatively assess the particle morphology and provide size distribution data. The elemental identity of the imaged particles was confirmed using energy dispersive X-ray spectroscopy (EDX) (EDAX, USA). A representative EDX spectra is included as supplementary information (Appendix C Figure C3). A droplet of each sample was placed onto a carbon/formvar coated copper TEM grid and allowed to dry. Images were collected under high vacuum at an accelerating voltage of 30 Kv and aspot size of 1 nm using a dark field (DF) STEM detector. The nanoparticle size distribution was measured for single particles or particle agglomerations in a representative image. Particle diameter was derived from the area measured using the analyze particles function in the free software program ImageJ. The mean particle diameter, based on number distribution, and the zeta potential was determined using a Dynamic light scattering instrument (DLS) (Brookhaven 90 Plus/Zeta, Brookhaven Instruments Corp., New York, US). All sample measurements were performed in triplicate at 22°C using a 659 nm laser positioned at a measuring angle of 90°. The results of this characterization are shown in Figure 5.2. Total Ag content in the different Ag-NPs stock solutions was quantified using inductively coupled plasma-optical emission spectrometry (ICP-OES) (Vista-MPX CCD Simultaneous ICP-OES, Varian, Agilent Technologies, Santa Clara, CA, USA). Sample aliquots of 2.5 mL were combined with 3.0 mL 65% HNO+3 and subjected to open vessel microwave digestion (MARS, CEM Corp., Matthews, NC, USA) at 100°C and 600 W for 1 h. The concentration of all stock solutions was verified before spiking the wastewater to be fed into the wetland microcosms.

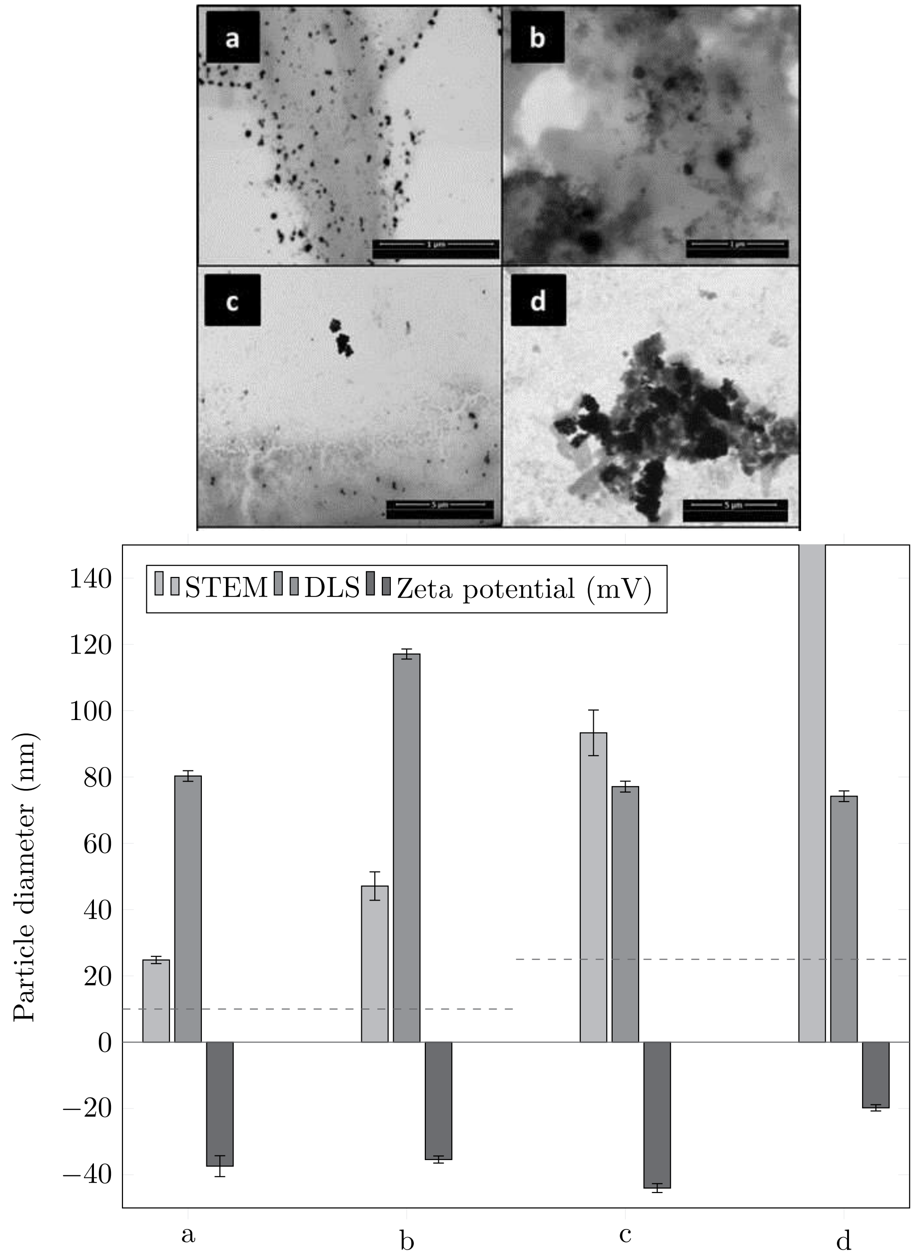


Figure 5.2: STEM images for citrate coated Ag-NPs in the original stock solution (a) and in the simulated wastewater (b), PVP coated Ag-NPs in the original stock solution (c) and in the simulated wastewater (d). Bar chart shows the particle diameter calculated using STEM images and DLS (average±standard error). The horizontal dashed line indicates the particle size quoted by the supplier. Particle diameter for PVP Ag-NPs in simualted wastewater according to STEM was 615±72 nm.

The percentage of ionic Ag in Ag-NP stock solutions (defined here as <10 kDa molecular weight cut-off (MWCO)) was determined using centrifugal ultrafiltration (UF) devices (Amicon Ultra-4, EMD Millipore Corp., Billerica, MA, USA; MWCO of 10kDa). For this, 4.0 mL was pipetted from the stocks/samples into UF devices and subjected to centrifugation (Megafuge 1.0, Heraeus, Hanau, Germany) at 3735 g for 15 min. Afterwards, the filtrates were diluted ten times with internal standard solution (IS) (10 μg Rh/L (Chem-Lab, Zedelgem, Belgium) in 2% HNO_3), and analysed directly for total Ag content by means of inductively coupled plasma-mass spectrometry (ICP-MS) (Elan DRC-e, PerkinElmer, Inc., Waltham, MA, USA). Both citrate and PVP coated Ag-NPs were >99% particulate. External calibration standards were used for ICP-MS analyses, and recalibrations were performed every 20 samples. Blank samples and reference standards were included at the beginning and the end of each intra-analysis batch of 20 samples for quality control purposes. All measurements were performed in triplicate unless stated otherwise. The detection limit of the ICP-MS was 0.016 μg Ag/L.

5.2.4 Water chemistry measurements

Water quality was characterized weekly at the end of the 7 day HRT in the sampling well for pH (Model 520A pH meter, Orion Research Inc., Boston, MA, USA), dissolved oxygen (DO) (Portable DO meter, HI 9143, Hanna Instruments, Woonsocket, RI, USA), and total organic carbon (TOC). TOC in the wastewater was measured using a TOC-V_{cpn} analyser (Shimadzu, Kyoto, Japan) within 3 days of sample collection during which time the samples were stored in a refrigerator at 4°C.

5.2.5 Microbial community analysis

Community-level physiological profiling

Community-level physiological profiling (CLPP) was used to study impacts to microbial community function in the microcosms. The profiles were created by gathering substrate utilization data from 96 well (31 carbon sources and 1 blank in triplicate) Biolog® Ecoplates (Biolog, Hayward, CA, USA). Interstitial waters were collected after 28 days from the sampling well of each microcosm using a long glass 50 mL pipette (following a 3 times well volume flush) into 50 mL autoclaved glass bottles. Biofilm samples were collected after 28 days by shaking 75 g of gravel from each microcosm in 750 mL phosphate buffer saline (10 mM Na_2HPO_4 (Chem-Lab, Zedelgem, Belgium), 8.5 g/L NaCl (Chem-Lab, Zedelgem, Belgium), pH 7.4) for 3 h (approximately 200 rpm) and in the dark according to Weber, Legge (2010b). The 75 g of gravel was comprised of 25 g from replicated system giving a composite sample. Each well of the Biolog® Ecoplate was inoculated with 100 μL of sample using an 8 channel pipette. Direct inoculation without pre-treatment of the samples was possible as both the interstitial water and biofilm extracts were sufficiently clear. The plates were incubated in the dark at room temperature and were read using an absorbance microplate reader at 590 nm (Infinite® 200PRO, Tecan Group Ltd., Männedorf, Switzerland and PowerWaveX340, BioTek Instruments, Inc., Winooski, VT, USA) at 0, 14, 24, 43, 48, 65, 72 and 115 h post inoculation. Data from the time point of 65 h was selected for further analysis based on the suggestions of (Weber, Legge, 2010a).

DNA extraction and purification

Total DNA was extracted from the interstitial water and biofilm samples (previously detached from gravel using the above protocol for prepping CLPP solutions) by filtering 50 mL of each through a 0.22 μm membrane filter (Millipore, Bedford, MA, USA) using a sterile vacuum filtration system and the filters stored at -20°C. For DNA extraction 200 mg of glass beads and 1000 μL of lysis buffer (100 mM Tris/EDTA/NaCl, 1% PVP40, 2% SDS, pH 7) (Sigma Aldrich/ Chem-Lab, Belgium) were added to the tube containing the rolled filter paper. The tube was then shaken vigorously twice with a FastPrep® automated homogenizer (MP Biomedicals, Santa Ana, CA, USA) for 30 s at 1600 rpm and then centrifuged for 5 min at maximum speed (16220 rpm). Thereafter, the supernatant was mixed with 500 μL of phenol:chlorophorm:isoamilic alcohol (Sigma Aldrich, Belgium), after which it was centrifuged for 1 min at maximum speed (16220 rpm). Then, the supernatant was mixed with 700 μL of chloroform (Sigma Aldrich, Belgium) and centrifuged for 1 min at maximum speed (16220 rpm). 45 μL of 3 M sodium acetate and 500 μL of ice-cold isopropyl alcohol (both Sigma Aldrich, Belgium) were then added to the supernatant. The tubes were mixed and kept at -20°C at least for 1 h, after which they were centrifuged for 30 min at 4°C at maximum speed (16220 rpm). Finally, the pellet was left to dry and thereafter resuspended in 50 μL of Tris-EDTA buffer (Sigma Aldrich, Belgium). The extracted DNA samples were purified using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The concentration of DNA in the samples was measured with a NanoDrop spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA) and the quality of the DNA was verified using agarose gel electrophoresis. The samples were stored at -20°C until further processing.

PCR amplification

The V3 region of 16S rRNA genes from the microbial community was amplified by PCR using Universal primers (338F-ACTCCTACGGGAGGCAGCAG with a GC-clamp and 518R-ATTACCGCGGCTGCTGG) based on the protocol described by Øvreås et al. (1997). The master mix contained the following components: 0.2 μM of each primer, 200 μM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 10x Taq Reaction Buffer ($MgCl_2$-free), 1.25 U/50 μL of Taq DNA Polymerase, 400 ng/μL 31 of bovine serum albumin, and DNase and RNase free filter sterilised water (Thermo Scientific, Waltham, MA, USA). 1 μL of extracted DNA was added to 24 μL of mastermix. The PCR was carried out in a thermal cycler T100^{TM} (Bio-Rad, Hercules, CA, USA) as follows: an initial denaturation step for 5 min at 94°C; then 30 cycles of 1 min at 95°C, 1 min annealing at 53°C, and 2 min DNA synthesis at 72°C; concluding with a final elongation step of 10 min at 72°C.

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was used to assess the structure of the microbial community in the wetland microcosms. Triplicated samples were pooled and run as a single sample. DGGE was performed for PCR products using an INGENY phor-U 2x2 (Ingeny International BV, The Netherlands) according to the manufacturer's instructions. The PCR products were loaded on a polyacrylamide gel (8% wt/vol) (acrylamide/bisacrylamide solution (37.5:1), Bio-Rad, Hercules, CA, USA) in 1xTAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4). The gradient ranged from 45% to 60%. The 100% denaturant contained 7 M urea (Bio-Rad, Hercules, CA, USA) and 40% (v/v) formamide (Bio-Rad, Hercules, CA, USA).

Samples were run for 18 h at 100 V and 60°C. After the run, the gel was stained with SYBR Green solution (50 μL/L in 1x TAE-buffer; Applied Biosystems, Grand Island, NY, USA) for 20 min, after which the gel was imaged on a UV trans illumination table (OptiGo, Isogen Life Science, De Meern, The Netherlands). The images were imported into an analysis software (GelCompar II, Applied Maths, Belgium) for determination of the number and intensity of bands. See Appendix C Figure C2 for DGGE gel image.

Ex-situ dose-response testing of Ag-NPs

Ex-situ experiments were conducted to test the effects of Ag-NPs on the catabolic capabilities of biofilm microbial communities within the wetland microcosms and adaptation of the community to Ag toxicity. Biofilm microbial communities were detached from the gravel of microcosms that had previously been exposed to 100 μg/L for 28 days and from the control with no Ag exposure as described above and detailed in Weber et al. (2014). An aliquot (19.6 mL) microbial community solution was placed in a sterile 50 mL C-tube and 0.4 mL of Ag-NP stock solution added to give final exposure concentrations of 0.5, 1, 2, 5 mg Ag/L. The sample was thoroughly mixed by 30 second hand shaking and then each well of a Biolog® Ecoplate was inoculated with 100 μL. The plate was then incubated for 5 days and absorbance readings were collected as described above.

Data analysis

CLPP data were analyzed according to Weber, Legge (2010a) based on average well color development (AWCD), richness and diversity. Richness is a measure of the number of different carbon sources utilised by a microbial population, and is calculated here as the number of wells with a corrected absorbance greater than 0.25 AU. Diversity is expressed here in terms of the Shannon index applied to the utilization of all 31 individual substrates contained on the Biolog® Ecoplate. Carbon source utilization patterns (CSUPs) from CLPPs and banding patterns from DGGE were subjected to principal component analysis (PCA). Normality of the PCA data was assessed based on the data distribution as given in Weber et al. (2007). The kurtosis an skewness of each variable and their standard errors were determined and the corresponding z values were calculated based on these values. Z values smaller than 1.96 for both parameters were defined as an indication of normally distributed data. Homoscedasticity was assessed based on variance ratio (i.e. highest variance / lowest variance) according to Weber et al. (2007). Variance ratio <1.5 was defined as an indication of homogeneous distribution of variances. If necessary, the normality and homoscedasticity was improved by using data transforms (Taylor power law transform and logaritmic transform) according to Weber et al. (2007). The mean CSUP of the triplicated treatments was used in PCA analysis. The principal components (PCs) were extracted from the covariance matrix of the data. The PCs of the CLPP data are based on a linear combination of different carbon sources and those of the DGGE data are based on different bacterial genes. The impact of these variables on defining the PCs is illustrated in the loading plots in Appendix C Figure C1. The statistical significance of differences ($p<0.05$) in results was assessed using a factorial ANOVA followed by a post-hoc Tukey HSD for differences between each treatment and group and a one way ANOVA followed by a 2-sided Dunnett's test comparing each dose concentration against the control. Differences are not significant in the absence of a reported p-value. Statistical analyses were performed in STATISTICA version 12, StatSoft® and XLSTAT 2013, Addinsoft©.

5.3 Results and discussion

5.3.1 Water quality

Water quality within the CW microcosms was comparable for all treatments and did not fluctuate significantly over time. Over the course of the 28 day exposure period water pH in all systems was in the neutral range (7.3 – 8.2), TOC was 2.4 – 4.5 mg/L (measured in the effluent) and DO was typically low but showed an increase from less than 1 mg/L after 7 days of operation to between 2 and 5 mg/L for the remaining duration of the experiment. There was no indication of an impact of Ag exposure on water quality based on these parameters (see Appendix C for Table C1).

5.3.2 Nanoparticle characterization

Characterisation results for the investigated Ag-NPs are shown in Figure 5.2 (Section 5.2.3). The mean particle diameter measured in the stock solutions using DLS were 80.3 and 77.1 nm for citrate and PVP coated Ag-NPs respectively. Analysis of STEM images differed with values of 24.8 and 93.3 nm. These values are higher than the manufacturer stated sizes and suggest agglomeration had occurred to some extent in the stock solutions prior to addition to the simulated wastewater. Agglomeration was also evident in the simulated wastewater by both DLS and STEM imaging, and was most evident for PVP coated Ag-NPs in the simulated wastewater (Figure 5.2d). This was also reflected in the zeta potential of the stock and wastewater solution whereby PVP coated Ag-NPs in the wastewater solution showed a zeta potential of lower magnitude (-20 mV) and therefore higher propensity for agglomeration. Citrate coated Ag-NPs in both the stock and wastewater solutions (and the PVP stock) had comparatively high negative Zeta potential suggesting greater particle stability. From these results and despite the relative stability suggested based on zeta potential results it is clear that agglomeration of particles was occurring to some extent prior to addition to the wetland microcosms. EDX spectra (Appendix C Figure C3) show no significant Ag sulfidation or chlorination to occur directly after adding Ag to the original simulated wastewater solution, however this does not discount it from happening while in the microcosms over longer time periods. Agglomeration is an important parameter to monitor as the decreased surface area and increased mass of particle agglomerates will alter behavior including rates of dissolution and partitioning behavior.

The mass of Ag-NPs added to the systems in the spiked simulated wastewater was reduced by approximately 98% in the water column after 7 days (the maximum period between batch loading of wastewater) for all systems. This amounted to the removal of 98 ppb, leaving a concentration of 2 ppb. All of this remaining silver was verified to be Ag-NPs (via 10 kDa MWCO filters) with no ionic silver detected (even in the system dosed with ionic silver). Due to these low concentrations characterization of the remaining particles via DLS or TEM was not possible.

The distribution of Ag in the microcosms was determined at the end of the 28 day exposure period (Figure 5.3) and the majority was found to reside in the biofilm with levels approximately tenfold higher than in the interstitial water. This distribution was comparable for all treatments with the exception of citrate coated Ag-NPs where the mass was approximately half the amount in the biofilm compared to other Ag types after 28 days of operation. This was not explained by concomitant higher levels in the interstitial water and may be due to increased loss during the weekly drain and fill cycle, or due to partitioning into the accumulated organic matter and sediment rather than the biofilm. The overall recovery of Ag based on the sum of biofilm and interstitial water Ag was approximately 55%. In a study by Weber et al. (2014), evidence was given for the microbiological degradation of the citrate coatings surrounding Au-NPs. Although not accounted for in this study it is possible that degradation of the citrate coatings occurred here as well. This could affect partitioning behavior through increased formation of Ag_2S compounds leading to deposition in the sediment and lower availability to the biofilm dwelling microbes. Minimal ionic Ag was detected in the interstitial water of any of the microcosms including those dosed with ionic Ag, which again suggests rapid particle formation/association. Rapid decreases in the concentration of Ag from the interstitial water (water column) have been reported elsewhere (Colman et al., 2014; Lowry et al., 2012a). In a comprehensive study by Colman et al.

(2014) using natural wetland mesocosms, Ag concentrations in the interstitial water fell by up to 67% within 24 hours of dosing. The decrease was attributed to release of large quantities of chloride ions (Cl^-) from submerged and floating macrophytes which led to the formation of AgCl(s). In the absence of plants in the present study the observed low levels of Ag in the interstitial water could be partially due to some AgCl(s) formation via small amounts of added Cl^- in the simulated wastewater feed, however more likely due to the formation of Ag_2S(s), via larger amounts of added sulphur in the simulated wastewater feed, which is known to occur readily under low oxygen conditions or through reaction with organic matter (Levard et al., 2011, 2012). This would explain the lack of ionic Ag after 28 days exposure with the low levels of DO (1 - 3 mg/L) observed here. As stated the entire mass of Ag added to the systems is not accounted for in Figure 5.3. As this study was focused on the microbial community the biofilm was sampled in such a way that mainly gravel-adhered biofilm was collected and did not include significant amounts of accumulated solids from the sediment layer at the base of the microcosms. It is suspected that unaccounted Ag had settled into the sediment layer or been removed during the drain-fill cycle. Lowry et al. (2012a) investigated the long-term fate of Ag-NPs in a simulated natural wetland reporting that Ag-NPs added to the water column rapidly settled into aquatic sediments and that a large fraction of dosed Ag was sulfidized to Ag_2S(s). Water chemistry data collected over the same period was similar in all systems throughout the period, further suggesting that the type of Ag-NP or ionic silver dosing did not affect the systems in different ways (Appendix C Table C1).

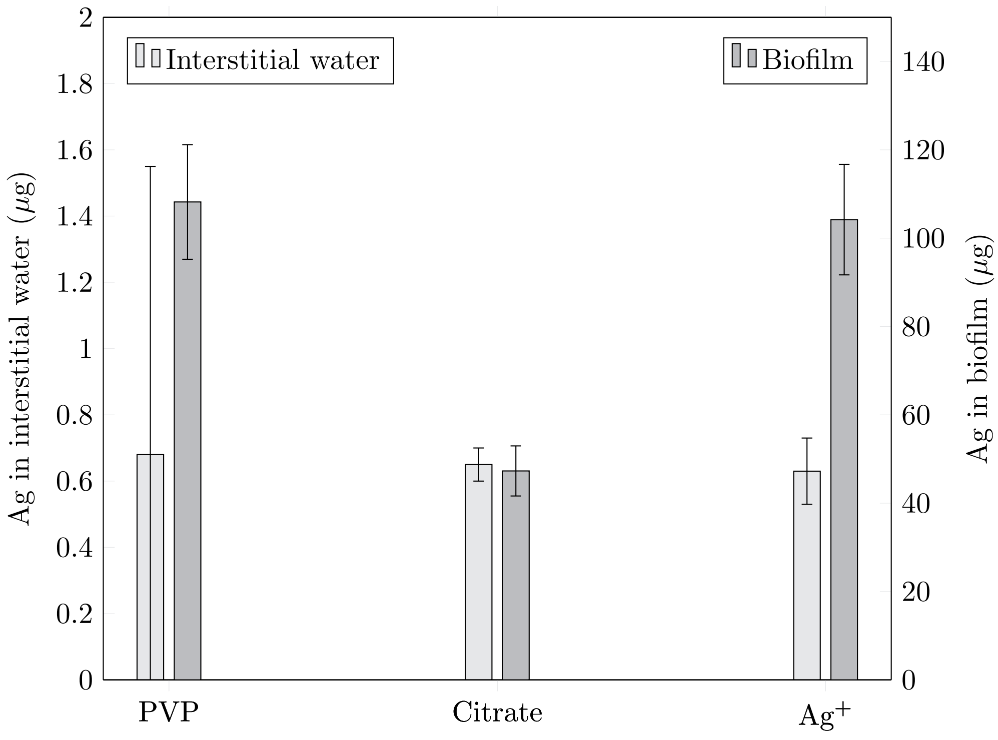


Figure 5.3: The total mass of Ag measured in the interstitial water and biofilm of the experimental microcosms after 28 days of operation. Values were determined using total Ag data derived from microwave assisted acid digested samples with ICP-MS analysis. Error bars represent 1 standard deviation (n=3). No Ag was detected in the control microcosms.

5.3.3 Microcosm microbial community impacts

The richness and diversity of the microbial community in terms of both function and structure was examined at the end of the 28 day exposure period (Figure 5.4A-D). Functional diversity and richness, Figure 5.4A and 5.4C respectively, was higher for the biofilm microbial community in comparison to the interstitial water for all treatments. PVP Ag-NPs appeared to have a slightly positive impact on the interstitial water community function with richness and diversity being slightly higher than the control, whereas all other exposures resulted in functional richness below that of the control. Structural diversity and richness, Figure 5.4B and 5.4D respectively, was higher in the interstitial water compared to the biofilm. The dose of 100 μg/L used in this study was selected to provide some degree of relevance to concentrations that might be encountered in constructed wetlands. Overall, no distinct impacts either negative or positive were seen in the functional or structural richness or diversity of the CW microcosm microbial communities.

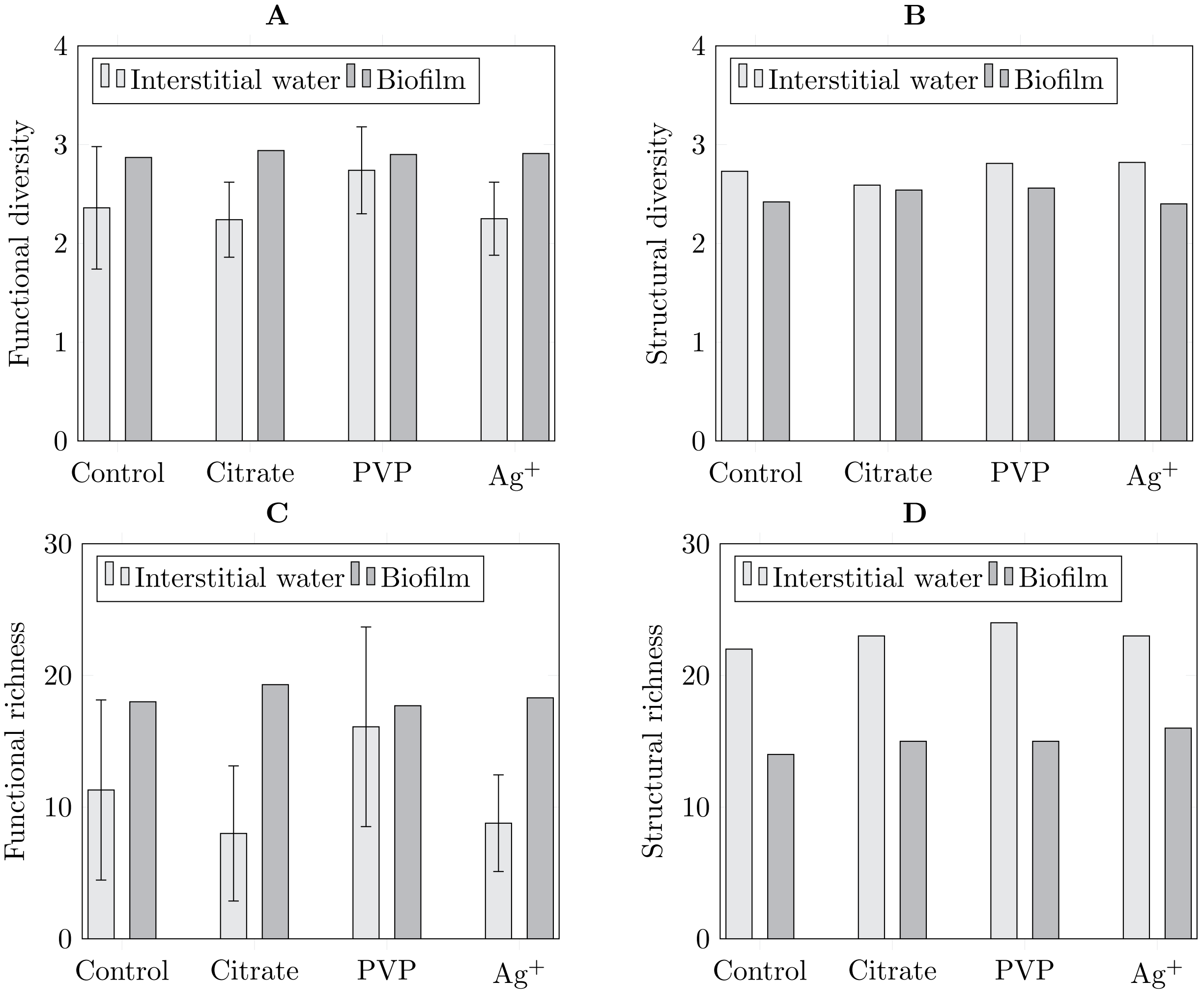


Figure 5.4: Functional and structural diversity and richness of microbial communities in wetland microcosms after 28 days exposure to 100 μg/L of Ag-NPs or ionic Ag in simulated wastewater. Diversity is calculated using the Shannon index. Richness is calculated as the number of identifiable bands in a DGGE sample or as the number of carbon sources utilized in CLPP. Error bars for interstitial water samples are based on 1 standard deviation (n=3). Biofilm samples were combined and run as a single sample.

Silver nanoparticle toxicity effects have been recorded for microbial communities at concentrations lower than that used here (100 μg/L). For example, Kroll et al. (2016) found significant effects to stream microbial communities (periphyton) after exposure to 20 μg/L PVP coated Ag-NPs or ionic silver. Specifically they found a decrease in diversity and population density. Additionally, when examining the biofilm (extracellular polymeric substances contained in the biofilm) the ratio of polysaccharides to proteins changed (assumed from C/N ratio), and the biofilm mass was reduced by about 50%. Although the results found here do not report any significant effects, they are consistent with other studies looking at microbial communities and biofilms associated with water treatment (as opposed to environmental microbial communities such as periphyton). Zhang et al. (2014a) investigated the exposure of membrane bioreactor biofilm, treating synthetic wastewater, to 60 days of Ag-NPs at a concentration of 100 μg/L. They found no significant impact to the microbial communities or water treatment performance as the Ag-NPs (or ionic silver) were adsorbed or precipitated quickly in the system. Zhang et al. (2014a) also found a developed silver resistance in the biofilms, as measured through the abundance of the silE gene (silver resistance gene).

Some of the resilience of biofilms can be attributed to the ability for the extracellular polymeric substance (EPS) matrix to act as a barrier, limiting the contact of silver (ionic or particle form) with the microbial communities themselves. Joshi et al. (2012) showed the production of EPS by engineered *E. coli* provided protection from Ag-NPs most likely due to an enhanced aggregation effect in the EPS matrix. Joshi et al. (2012) also showed the addition of an artificial EPS analogue (xanthan) to provide a similar protection. Kang et al. (2014) also showed that the EPS produced by *E. coli* in suspension hindered the intracellular penetration of ionic silver, and that during the penetration of ionic silver through the biofilm matrix ionic silver was reduced into nanoparticles (10 - 30 nm in diameter) and immobilized in the EPS matrix before they could reach the cell. It is likely that the general resilience of the CW biofilm microbial communities recorded here is in part due to the EPS matrix acting as a barrier. With respect to the interstitial communities it is possible that the interstitial microbial communities were also able to produce an EPS exudate, as shown with suspended *E. coli* by Kang et al. (2014), however the lack of significant effects are also likely due to the rapid removal of the silver from the water column.

It is also noted that there is significant potential for the ongoing accumulation of Ag within the aquatic sediment and, although not evaluated here, could lead to eventual re-release of Ag in significant concentrations to the interstitial water and biofilm. Two recent studies more representative of a sudden mobilisation event of Ag-NPs from aquatic sediment investigated the impacts of elevated levels (2 mg/L) of Ag and found negative, cascading, ecosystem level impacts to water chemistry and plants in natural wetland mesocosms (Colman et al., 2014) and significantly increased mortality in *Fundulus heteroclitus* larvae and embryo in microcosms (Bone et al., 2015). However, in the latter study, Bone et al. (2015) found that the results of microcosm exposures were not replicable at the meso-scale and attributed the disparity in part to decreased levels of UV light in laboratory microcosm experiment.

Principal component analysis (PCA) was used to further investigate impacts to the microbial community function and structure after 28 days of exposure. Analysis of the carbon source utilization patterns (CSUPs) and DGGE banding patterns revealed that the functional potential of the biofilm microbial community differed from that of the interstitial water, but within the biofilm there were no differences observed between treatments and the control (Figure 5.5A). In the case of interstitial water samples, the microbial community treated with ionic Ag had developed differently from the other treatments and the control. According to the PCA analysis using DGGE data, the interstitial water and biofilm samples are populated by different microbial communities and again less variation was observed in the biofilm communities, with the ionic Ag exposed interstitial waters developing differently than the other interstitial communities (Figure 5.5B) suggesting a slight impact for this type of Ag exposure when compared to Ag-NPs. Although no statistical analysis could be completed for the structural data this subtle effect is discussed here as it is not surprising for ionic silver to have an effect of some magnitude.

Differing toxicities for ionic Ag and Ag-NPs have been described but the reported data are not consistent. Kwok et al. (2012) demonstrated that both citrate and PVP Ag-NPs were less toxic to early life stage *O. latipes* than gum arabic (GA) Ag-NPs, but also noted that all NPs were three to ten times less toxic than ionic Ag ($AgNO_3$). Conversely Bone et al. (2015) found that Ag-NPs were more toxic than ionic Ag to *F. heteroclitus* embryos in mesocosm exposures. The microcosm microbial community impacts noted here, however, are more pertinent to subtle changes in function and structure rather than blatantly negative effects. Microbial community analysis as employed can be advantageous when trying to elucidate more subtle shifts in ecosystem function for which the potential ramifications are less clear. Microbial community function is known to vary depending on location within a constructed wetland. Interstitial water communities typically demonstrate a lower functional richness and different physiological profile compared to gravel or rhizospheric associated microbial communities (Weber, Legge, 2013). The observations of lower functional diversity and richness in interstitial water communities compared to the biofilm (Figure 5.4A and 5.4C) and different carbon source utilisation patterns for each community (Figure 5.5A) conform to this existing understanding of microbial functionality in constructed wetlands. It is interesting then to observe the opposite trend for community structure as determined by DGGE (Figure 5.4B and 5.4D) with higher structural richness and diversity in the interstitial water compared to the biofilm. Biofilm community structural profiles were also distinct from interstitial water communities based on the occurrence and relative intensity of DGGE bands (Figure 5.5B). A possible explanation for this is that the biofilm develops competitively to maximize function and may therefore be composed of a smaller number of species that are better suited to the specific experimental conditions, such as exposure to Ag, leading to higher function but with reduced species richness. With this in mind a more in depth analysis of the biofilm communities was completed with a specific goal to investigate a potential developed resistance to silver.

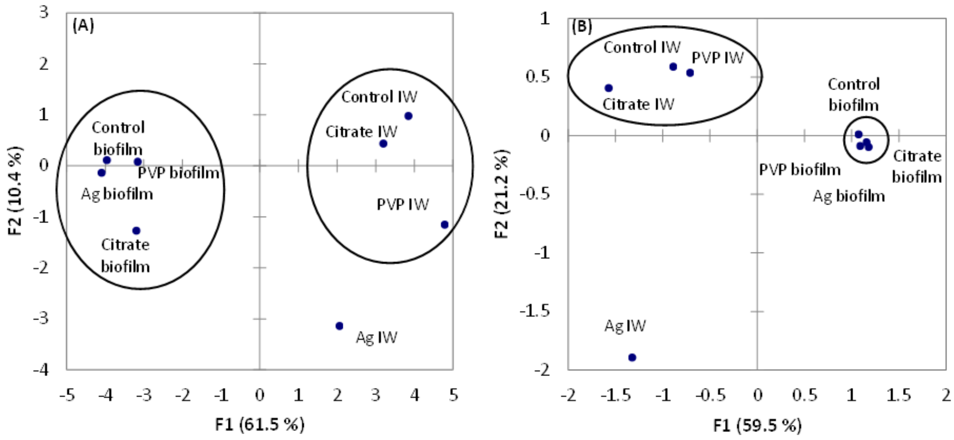


Figure 5.5: Principal component analysis of A) community structure based on the DGGE banding patterns and B) community function based on CSUPs of the Biolog® Ecoplates after 28 days exposure in laboratory microcosms. IW = interstitial water, Ag = ionic Ag, citrate = citrate coated Ag-NPs, PVP = PVP coated Ag-NPs.

5.3.4 Microbial community Ag resistance

The effect of Ag-NPs and ionic Ag on the development of community resistance was assessed *ex-situ* via the quantification of catabolic capabilities after 28 days exposure to Ag (previously exposed), and a control group with no exposure (denoted as Ag naïve). A significant ($p<0.05$) decrease in the catabolic capability with increasing dose was observed for all Ag types except for PVP Ag-NP exposed communities with prior exposure (Figure 5.6). The minimum community level catabolic effect concentration (MCLCEC), as described by Weber et al. (2014) varied between treatments being lowest with ionic Ag (0.5 mg/L). The MCLCEC for citrate Ag-NPs was 1 mg/L and for the communities treated with PVP Ag-NPs, an MCLCEC was only observed in the Ag naïve community at a concentration of 5 mg/L. The exposure to higher concentrations of ionic Ag ceased the catabolic activity of the microbial community completely (at 1 mg/L), which was not observed for PVP and citrate NPs, and hence these results correlate well with earlier research showing that ionic Ag is more toxic to microorganisms than nano-Ag (Chapter 2). Previous exposure to NPs or ionic Ag appeared in some cases to increase resistance to Ag toxicity with significant differences ($p<0.05$) in effect between the previously exposed and naïve communities at 5 mg/L for PVP Ag-NPs and 0.5 mg/L for ionic Ag. These results are consistent when analyzing raw data (as shown) or normalizing to the original no-dose response. P-values for comparisons between naïve and previously exposed communities for ionic silver at 0.5 mg/L are 0.005 and 0.015 for non-normalized and normalized data respectively. P-values for comparisons between naïve and previously exposed communities for PVP Ag-NPs at 5 mg/L are 0.011 and 0.013 for non-normalized and normalized data respectively. Data has been displayed here as non-normalized to better assist in future study comparisons between different authors. These concentrations are higher than any predicted environmental concentrations for surface waters or sediments (Gottschalk et al., 2013), however are within the realm of possibility for CWs. These results highlight the possibility for variability in the development of Ag resistance depending on the mode of exposure. Current understanding of microbial resistance to Ag is based mainly on clinical studies with exposure to ionic Ag (Silver et al., 2006). A better understanding of Ag resistance in the environment will be possible when the mode of toxicity is clearly understood, which is not yet the case (Choi et al., 2010). It has been suggested that the mode of toxicity for Ag-NPs is similar to that of Ag ions (Sintubin et al., 2011) acting through the inhibition of essential enzyme activity. The *ex-situ* dose response experiments performed in this study (Figure 5.6) showed significant differences in the toxicity trends and resistance development for each type of Ag-NP and ionic Ag.

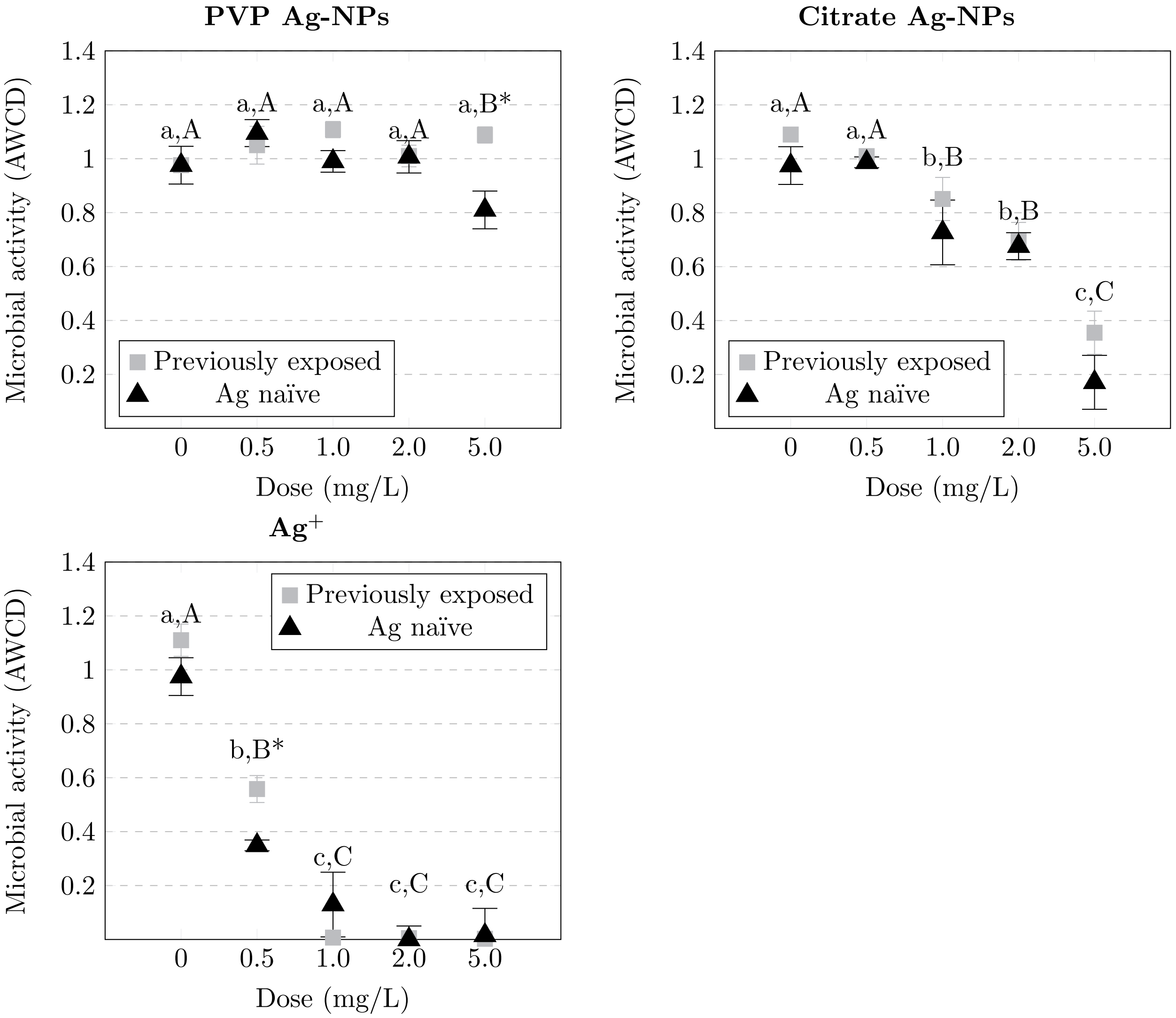


Figure 5.6: Dose-response curves for the effect of different types of Ag-NPs on biofilm microbial communities. Exposures were carried out *ex-situ* using Biolog® Ecoplates. Previously exposed refers to communities having undergone 28 days exposure to 100 μg/L Ag. Naïve refers to the control communities maintained under identical conditions but without previous exposure to Ag. Error bars are 1 standard deviation (n=3). Asterisk ($*$)= significant difference ($p<0.05$) between previously exposed and naïve groups.

The mechanisms by which biofilm-bound microbial communities resistance to Ag toxicity may increase over time conceivably involves a combination of increasing numbers of persistent cells (Keren et al., 2004), gene expression responses (Lenz et al., 2008), efflux systems (Silver, Phung, 1996) or horizontal gene transfer (Gunawan et al., 2013). Although not yet mentioned in the literature for CW microbial communities, one of the simplest and more easily understood mechanisms of resistance may be that biofilm dwelling microbes are inherently provided physical protection by extracellular polymeric substances as shown in conventional wastewater treatment systems (Sheng, Liu, 2011). Resistance may therefore be due at least in part to a microbial community's ability to build and maintain viable biofilms. Many factors will influence this process including development time prior to exposure and environmental factors such as shear stress, oxygenation and nutrient availability. Along the lines of Blanck et al. (1988), this study shows there may be some potential for creating pollution induced tolerance in CW microbial communities. The period of exposure in this experiment was relatively short and therefore, longer-term studies to elucidate the potential for the development of Ag resistance and its physiological basis in wetland biofilms would be useful. Hence, a long-term study would give further insight into whether the observed effects are becoming more severe or whether the system is able to recover its function despite increasing concentrations of Ag. The length of such a study should be minimum 1 year to take the maturation process of the wetland biofilm into account. Additional experiments using sulfidised Ag-NPs would also be informative. As observed by Lowry et al. (2012a), Ag-NP transformation can occur fairly rapidly. Better understanding sulfidisation kinetics in the relatively short distribution systems preceding CWs would also be helpful to fully understand what particle types require priority investigation moving forward.

5.4 Conclusions

This is the first study to look at the effects of different Ag-NPs on both the function and structure of microbial communities in constructed wetlands. Low doses of Ag did not exert significant toxic effects in the short term whether ionic or in nanoparticle form but did lead to subtle changes in both functional and structural microbial community profiles. Higher doses of Ag-NPs (>500 μg/L) significantly reduced microbial community function in *ex-situ* tests in the case of citrate Ag-NPs and ionic Ag. PVP coated Ag-NPs were shown to have limited toxicity in this study. Some evidence of the development of resistance to toxicity was observed in previously exposed microbial communities for all types of Ag (particulate and ionic). Longer term and larger scale studies to elucidate the potential effects of Ag accumulation in constructed wetlands are needed to answer important remaining questions on the potential environmental impacts of continued accumulation and eventual release of Ag over time.

Chapter 6

Treatment of domestic and hospital wastewater in an aerated sub-surface flow constructed wetland

This chapter is based on **Auvinen H.,** Havran I., Hubau L., Vanseveren L., Gebhardt W., Linnemann V., Van Oirschot D., Du Laing G., Rousseau D. 2016. Removal of pharmaceuticals by a pilot aerated sub-surface flow constructed wetland treating municipal and hospital wastewater. Ecological Engineering 100: 157-164.

6.1 Introduction

Several studies have shown that effluent from urban wastewater treatment is an important source of organic micropollutants such as pharmaceutical residues in surface waters (Michael et al., 2013). In Flanders, some rivers were investigated for the occurrence of selected pharmaceuticals by VMM (the Flemish Environment Agency) in the context of a European project FATE EUMORE. A third (34%) of these samples contained a higher concentration of pharmaceuticals than the warning level (mostly 100 ng/L) (European Commission, 2015b; Loos et al., 2009). Recently, also Vergeynst et al. (2014, 2015) detected quite a number of pharmaceuticals in Flemish surface waters and wastewater effluents.

Pharmaceuticals are partly metabolized but not completely degraded by the human body and therefore, the unaltered parent compounds and their metabolites are excreted (Ternes et al., 2004b). At the wastewater treatment plant these compounds can undergo biological degradation in which their molecular structure can be further transformed but they are rarely completely mineralized (e.g. Trautwein, Kümmerer (2011)). Constructed wetlands (CWs), which are mainly used as decentralized wastewater treatment and as treatment systems for small communities, can contribute efficiently to decreasing pharmaceutical load to the environment. The ecosystem of the small water courses where CWs discharge (Rousseau et al., 2004) can easily be compromised if dilution in the receiving water is low. Therefore, it is important to study the performance and improve the CW technology towards more efficient pharmaceutical removal.

Extensive investigations on the effects of different types of wetland configurations, plants and treatment strategies on the behavior of different pharmaceuticals have already been performed, for example, in Spain (Ávila et al. (2015, 2014a, 2013, 2010), Matamoros, Salvadó (2012); Matamoros, Bayona (2006), Reyes-Contreras et al. (2012), Hijosa-Valsero et al. (2016, 2010a, 2011)) and Germany

(Ávila et al. (2014b); Carranza-Diaz et al. (2014)). However, further research on different types of CWs is needed to gain insight into the factors affecting the degradation of pharmaceuticals.

The aim of this study was to assess the performance of an aerated pilot-scale sub-surface flow (SSF) CW treating municipal and hospital wastewater. In the first part of the experiment, the pilot CW was fed with settled municipal wastewater of a small community. The effects of active aeration on its pharmaceutical removal efficiency as well as the robustness of its performance during increased hydraulic loading were studied. In the second part of the experiment, the pilot CW was relocated to a hospital site and its treatment performance was assessed during continuous and intermittent aeration. Hospital wastewater has typically a higher concentration of pharmaceutical residues than municipal wastewater. Separate treatment of this water would be advisable for several reasons: firstly, to reduce the load of pharmaceuticals on the centralized wastewater treatment plant (WWTP) and secondly, to prevent discharge of pharmaceutical residues to the environment due to combined sewer overflows and sewer leakages. Also, suppressing the problem with dilution is not a proper solution for pharmaceuticals because the adverse effects that they cause can already occur at very low concentrations (Chapter 2). This is also against the principles of cleaner production.

6.2 Materials and methods

6.2.1 Experimental setup and location

A transportable pilot-scale subsurface flow constructed wetland was built using a plastic container with an approximate total volume of 1 m^3. The cubic tank was filled with an 80 cm layer of coarse Rhine gravel (∅ 8-16 mm, approximate porosity 40%; Kranendonk N.V.) and evenly planted with mature *Phragmites australis* plants obtained from laboratory-scale CWs treating WWTP effluent. The plants were allowed to re-root and biofilm to develop for 8 weeks before the sampling was started. During this adaptation period the wetland was fed with wastewater (hydraulic retention time (HRT) 1 d) and aerated continuously.

Influent was evenly distributed on the CW surface over the horizontal length of the tank on one side. On the opposite side, effluent was collected at the bottom of the tank in a perforated inversed T-shaped pipe connected to an elbow which discharged the effluent at a level approximately 5 cm below the gravel surface. As a consequence, the water flow direction was mainly vertical, although the applied aeration was expected to cause mixing. Two perforated PVC tubes were positioned in the middle parallel to each other to allow the measurement of on-site parameters (temperature, pH, dissolved oxygen) inside the wetland. Aeration of the wetland was provided with a fish pond air pump (Secoh, JDK-20; design capacity 23 L/min) connected to a perforated aeration tube that was placed on the bottom of the tank. At setting the flow rate and defining the HRT, a nominal organic loading rate of 80 g $O_2/d/m^2$ (BOD_5) was used. The value was chosen based on earlier experiments on actively aerated CWs (D. Van Oirschot, personal communication, 12.12.2014 and 5.8.2016). An online database was consulted for the BOD_5 data that has been measured earlier regularly at the site (VMM, 2016).

In March 2015, the pilot CW was placed at the wastewater treatment plant of Aalbeke (Kortrijk, Belgium) which treats combined sewer municipal wastewater from a small community (design capacity 450 I.E.). This treatment plant is the property of Aquafin N.V. (www.aquafin.be), the company responsible for municipal wastewater treatment in Flanders. Wastewater for the pilot CW was taken batch-wise (10 times per day) from the existing primary settler by using an immersion pump

(POW67915, PowerPlus). The operational parameters of the pilot CW can be found in Table 6.1. After the measurement campaigns, the pilot CW was kept operational (regular feeding and continuous aeration) until the transfer to the hospital site.

In October 2015, the setup was transported to the Campus Kennedylaan of the hospital AZ Groeninge (Kortrijk, Belgium). Raw wastewater was taken directly from the hospital sewer (approximately 300 L/d) by means of a macerator pump (DRK 10-1K, Duijvelaar Pompen) and delivered to a 1 m^3 settling tank. The settling time was 7 hours and the accumulated sludge was removed every 2 - 3 days by manually opening a bottom valve. The settled sewage was pumped to the pilot CW batch-wise (5 times per day) by using a peristaltic pump (530S, Watson-Marlow). The intake of the pump was attached to a piece of Styrofoam to assure that only the supernatant was pumped into the experimental wetland. The operational parameters of the pilot CW can be found in Table 6.1.

In both locations, the aeration was controlled by a timer. In Aalbeke, three measurement campaigns were conducted: In the first (AER-HRT1) and second campaign (AER-HRT0.5), the aeration was continuously on. The second campaign differed from the first one by a lower HRT (0.5 d and 1 d, respectively). The third campaign (N-AER), with no aeration, was started after an adaptation period of one week and an HRT of 1 d was utilized. It lasted for two weeks: N-AER1 indicating the first week of the campaign and N-AER2 the second. In AZ Groeninge, there were two measurement campaigns: In the first one (AER100), the aeration was continuously on, and in the second one (AER50), aeration time was reduced to 50 %. The sampling for AER50 was begun after 2 weeks of adaptation.

Table 6.1: Operational parameters during the measurement campaigns

	Measurement campaign	**Nominal HRT(d)**	**Q (L/d)**	**Aeration (on/off;h)**	**Duration (weeks)**
Aalbeke - municipal wastewater	AER-HRT1	1	360±18	24/0	1
	AER-HRT0.5	0.5	680±15	24/0	1
	N-AER1	1	350±17	0/24	1
	N-AER2	1	310±35	0/24	1
AZ Groeninge - hospital wastewater	AER100	2	200	24/0	2
	AER50	2	200	4/4	2

6.2.2 Sampling

In Aalbeke, influent and effluent samples were taken simultaneously, but in AZ Groeninge, the effluent sample was taken 2 days (duration of the nominal HRT) after the influent sample, to be able to take the changing composition of the influent into account. Approximately 80 mL of influent and effluent samples were collected during each feeding/discharge event to obtain a mixed sample from that day by using a peristaltic pump (Sci-Q323, Watson Marlow). The sampling was automated to occur from an overflow beaker at programmed time points. In Aalbeke, the samples were taken every 2.5 hours during 24 h (total of 10 feeding/discharge events per day). In AZ Groeninge, there were 5 feeding/discharge events, the difference caused by a long settling time necessary. The mixed sample was preserved in a cool box protected from sun light until the next morning when the sample bottle was replaced.

In general, the selection of sampling strategy is crucial for valid results. Ávila et al. (2010) presented a strategy based on continuous injection of known concentrations of target pharmaceuticals with which reliable results on the pharmaceutical removal efficiency could be achieved. In the current study, injection of pharmaceuticals could not be applied because of the high concentration of the target compounds in the influent water. In Aalbeke, the pharmaceutical concentrations in the influent were more stable than in AZ Groeninge and the changes were merely gradual rather than sharp. For this reason, simultaneous sampling of influent and effluent was chosen as the sampling strategy. In AZ Groeninge however, much larger daily variations in the concentration of the target components were observed. Although the applied aeration is likely to cause mixing and thus some stabilization of the concentration fluctuations, we believe that sampling based on the (nominal) HRT is a representative choice in case the stability of the influent concentrations is uncertain.

6.2.3 Analysis methods

Physico-chemical measurements

Temperature, pH and dissolved oxygen (DO) were monitored in the wetland on site. The measurements were done by using a multimeter (HQ40d, Hach).

Chemical oxygen demand (COD) was measured spectrophotometrically (DR2800, Hach) using test kits according to the manufacturer's instructions (LC1500, Hach). The ammonium nitrogen content (Nessler method) was determined using standard methods (Greenberg et al., 1992). Nitrate concentration was measured in the Aalbeke samples by using an ion chromatograph (761, Metrohm), and in the AZ Groeninge samples by using test kits according to manufacturer's instructions (LCK340, Hach).

Pharmaceuticals

A number of pharmaceuticals was selected for the study. In Aalbeke, the selection of the pharmaceuticals was done on the basis of a preliminary investigation of two grab samples taken on consecutive days. In AZ Groeninge, the pharmaceuticals were selected based on water quality measurements by the Flemish Environment Agency in 2014 (VMM 2016) and drug delivery data obtained from the hospital pharmacy. In Aalbeke, the studied pharmaceuticals were carbamazepine (CBZ), diclofenac (DCF), metformin (MFM), sotalol (STL), tramadol (TMD) and valsartan (VST). In AZ Groeninge, the studied pharmaceuticals were atenolol (ATL), bisoprolol (BSP), carbamazepine, diclofenac, gabapentin (GBP) and sulfamethoxazole (SMX).

Directly after sampling, samples were filtered twice (1 μm and 0.45 μm; Whatman GL/B), and preserved frozen until analysis. Commercially available solid phase extraction (SPE) cartridges filled with Oasis HLB material from Waters (Milford, MA, USA) were used for concentration of the target analytes. The cartridges were conditioned in a first step with 5 mL methanol and in a second step with 5 mL ultra-pure water. Before SPE concentration the samples were filtered by means of 0.45 μm membrane filters. The extracted analytes were desorbed from the SPE material by rinsing the cartridges with three portions of 2 mL methanol forced by gravity. The cartridge eluates were evaporated to dryness using a gentle stream of nitrogen at 60°C in a Turbovap system (Biotage). The dry residues were reconstituted in 1 mL methanol–water (1:1v/v) and 10 μL were injected into the LC–MSMS system.

LC-separations were carried out with a Hypersil Gold aQ column (RP-C18, 5 μm, spherical; 125 x 2.1 mm I.D.) from Thermo Fisher Scientific (Runcorn, UK) Gradient elution by means of (A) methanol in combination with (B) Milli-Q-purified water was applied both containing 2 mM ammonium acetate and 0.1% acetic acid. The gradient was programmed as follows: Starting with

20% A / 80% B the concentration was increased linearly to 90% A / 10% B within 12 min. Up to 22 min the composition was kept constant. The eluent composition was back to the starting conditions after 23 min and kept until the end of the analytical run (30 min). The overall flow rate was 0.2 mL/min.

For quantification the LC-MSMS mass spectrometer was used in selected reaction monitoring (SRM) mode (Thermo Fisher Scientific LTQ Orbitrap). The collision energy was optimised on the most abundant product-ion (Gebhardt, Schröder, 2007; Schröder et al., 2010) (Table 6.2).

Table 6.2: Mass to charge ratios of the target components

Precursor ion (m/z)	**Product ion (m/z)**	**Component**	
130.10	71.20	MFM	
237.14	194.26	CBZ	
247.10	204.10	CBZ-D10	Internal standard
264.18	58.00	TRD	
273.17	133.20	STL	
296.10	214.18	DCF	
300.00	217.10	DCF-D4	Internal standard
436.19	207.12	VST	
267.17	145.20	ATL	
326.23	116.10	BSP	
172.13	154.20	GBP	
254.06	156.10	SMX	

6.2.4 Data analysis

Statistical analyses were performed with the SPSS Statistics 22 software. First, the data on pharmaceutical removal efficiencies were examined for normal distribution by using the Shapiro-Wilk test and homogeneity of variances by using the Levene's test. If both assumptions were met, one-way ANOVA was performed together with Tukey Post-Hoc test to define the significance of the differences. In case of normal data but non-homogeneous variances the Welch test together with Games-Howell Post-Hoc test was performed. Non-normal data sets were further analyzed by using a non-parametric test (Mann-Whitney U Test) to define the significance of the differences. The significance level was set at $p=0.05$.

6.3 Results and discussion

6.3.1 Common water quality parameters

Table 6.3 outlines the water quality parameters measured during the experimental period. In Aalbeke, the removal of COD and ammonium nitrogen was efficient during the aerated measurement campaigns AER-HRT1 and AER-HRT0.5, but hampered due to oxygen limitation during N-AER. Nitrate concentrations of 14.8 - 25.1 mg N/L were measured in the effluents during AER-HRT1 and AER-HRT0.5 due to high DO and subsequent low denitrification efficiency. During the non-aerated campaign N-AER, nitrification efficiency decreased significantly ($p<0.05$). Similar results were obtained in AZ Groeninge. COD removal efficiency was decreased there due to intermittent aeration (50%). However, in spite of the reduction in COD removal, still good nitrification was achieved. This could be explained by the altered aeration regime: The measured COD is partly particulate and its detachment of the wetland bed can be influenced by aeration. It is also possible that the altered aeration regime affected the redox potential within the wetland bed and therefore also the biotransformations of organic matter and ammonium. All in all, as nitrification-denitrification is assumed to be the major pathway of total nitrogen removal in CWs (Saeed, Sun, 2012), its inhibition could be defined as a major bottle-neck of the treatment efficiency. Nitrate removal is important in Flanders because the whole territory is classified as a nitrate sensitive area by the European Commission (European Commission, 1991).

Table 6.3: Common water quality parameters (average values). Standard deviation based on: n(AER-HRT1, AER-HRT0.5, N-AER1, N-AER2)=5; n(AER100, AER50)=2)

	Aalbeke								**AZ Groeninge**			
	AER-HRT1		**AER-HRT0.5**		**N-AER1**		**N-AER2**		**AER100**		**AER50**	
	In	Out	In	Out	In	Out	In	Out	In	Out	In	Out
T* (°C)		15		16		17		22		12		7
SD		2		2		2		2		3		3
pH*		7.2		7.0		7.1		7.5		7.3		8.7
SD		0.2		0.3		0.5		0.1		0.1		1.1
DO* (mg/L)		8.1		8.3		0.5		0.3		8.9		8.7
SD		1.6		1.8		0.2		0.1		0.3		1.1
COD (mg/L)	168	26	222	29	173	100	263	154	667	113	168	26
SD	67	5	7	5	76	30	41	18	237	57	67	5
NH_4^+ (mg N/L)	24	<2	33	6	22	24	41	42	42	<2	24	<2
SD	9	-	2	12	12	10	11	1	9	0.1	9	-
NO_3^- (mg N/L)	<5	21	<5	15	<5	<5	<5	<5	<5	125	<5	21
SD	-	5	-	9	-	-	-	50	-	1	-	5

$*$ Measured inside the wetland

6.3.2 Occurrence of pharmaceuticals in wastewater

Table 6.4 shows the average pharmaceutical concentrations measured in the settled influent. In Aalbeke, the average influent pharmaceutical concentrations varied from 0.27 μg/L for CBZ to 2.83 μg/L for VST. In AZ Groeninge, the lowest measured average concentration (0.60 μg/L) was for SMX and the highest (8.34 μg/L) for ATL. The average concentrations of CBZ and DCF, the two pharmaceutical compounds analyzed on both sites, were higher at AZ Groeninge than in Aalbeke (the average concentrations were factor 5.4 higher for CBZ and factor 3.5 higher for DCF). The results on influent concentrations obtained here are in agreement with similar studies on treatment of municipal and hospital wastewater (Vergeynst et al., 2015; Van Nuijs et al., 2010; Verlicchi et al., 2012). The concentrations of ATL, DCF and GBP are also in the same range as earlier measured by VMM in a screening study at AZ Groeninge (VMM, 2016) but the concentrations for CBZ obtained here were higher than reported earlier (0.05 – 2.8 μg/L). There is no earlier monitoring data available for SMX and BSP.

Table 6.4: Average concentration for target pharmaceuticals in influent and effluent (ng/L). Standard deviation (SD) based on: n(AER-HRT1, AERHRT0.5, N-AER1)=4; n(N-AER2, AER100, AER50)=5

	Aalbeke								**AZ Groeninge**			
	AER-HRT1		**AER-HRT0.5**		**N-AER1**		**N-AER2**		**AER100**		**AER50**	
	In	Out	In	Out	In	Out	In	Out	In	Out	In	Out
ATL									8340	450	6030	170
SD									4020	380	7760	130
BSP									2420	350	1940	520
SD									1270	110	960	230
CBZ	320	250	270	300	340	310	1250	960	5200	4570	1730	2370
SD	110	150	90	90	30	30	150	280	1160	690	720	1590
DCF	490	260	670	550	1210	770	2000	1870	5220	3340	2380	1420
SD	360	220	220	200	420	400	400	340	1800	580	630	1030
GBP									2330	1190	3660	1660
SD									1490	860	2420	860
MFM	910	<10	1210	<10	1280	790	3000	210				
SD	180	-	600	-	320	190	250	280				
STL	540	320	880	680	1190	860	2600	2080				
SD	120	180	450	290	430	340	640	320				
SMX									60	30	210	50
SD									80	30	390	30
TMD	610	600	630	740	440	410	620	740				
SD	260	350	220	220	240	290	150	290				
VST	2270	<10	2830	<10	1550	1040	2700	2590				
SD	1400	-	980	-	670	420	420	390				

6.3.3 Removal efficiency of pharmaceuticals

In Aalbeke, the selected pharmaceuticals can be roughly categorized in two groups based on their removal efficiency: one group was defined as readily degradable compounds (MFM, VST) which obtained nearly complete removal; the second group was defined as recalcitrant compounds (CBZ, DCF, STL, TMD) which had low removal efficiencies (Figure 6.1 and Table 6.4).

The removal efficiencies of CBZ, DCF and STL were low ($< 50\%$) during the whole experiment and the removal efficiency of TMD was mostly negative. Also previously low (and even negative) removal efficiencies have been reported for TMD. Breitholtz et al. (2012) studied the removal of pharmaceuticals in full-scale surface flow CWs and the obtained removal efficiencies ranged between -12 and 26 %. Low removal efficiency together with anomalies in the sampling caused by daily concentration fluctuations of the influent and the concentrating effect of evapotranspiration can explain the negative removal efficiencies of TMD obtained in the current study. Hijosa-Valsero et al. (2010b) studied mesocosm-scale wetlands with various treatment designs, and found also low CBZ and DCF removal in planted sub-surface flow CW ($< 40\%$). Also, Matamoros, Bayona (2006) reported in their experiment on horizontal SSF CW low removal efficiency of DCF ($<50\%$). Similarly to CBZ and DCF, STL has been classified among the most resistant to biodegradation (Oulton et al., 2010). Research on activated sludge systems shows a reduction of less than 30% for STL (Jelic et al., 2011; Gabet-Giraud et al., 2010; Oulton et al., 2010). Conkle et al. (2008) reported a removal efficiency of only 20 % in a surface flow CW with a hydraulic retention time of 27 d.

At AZ Groeninge, ATL and BSP were moderately degraded (>75% and >50% respectively; Figure 6.2, Table 6.3). The values reported in literature for ATL and BSP removal are generally lower than the values found in the current study. ATL has been shown to degrade in activated sludge treatment by 14 - 40% and BSP by 20 - 50% (Rosal et al., 2010; Wick et al., 2009) with an exception of higher removal for ATL (94%) (Vieno et al., 2006). Variable removal efficiencies have been reported for ATL in CWs as primary treatment (80%), secondary treatment (66%) and tertiary treatment (27 - 53%) (Breitholtz et al., 2012; Conkle et al., 2008; Verlicchi et al., 2013).

Variable and mainly poor removal was observed for CBZ, DCF, GBP and SMX. In contrast to the findings in the literature and for unclear reasons, the average removal of GBP was only 33 - 37%. GBP has been reported to be readily biodegradable during activated sludge treatment (Kasprzyk-Hordern et al., 2009; Yu et al., 2006) and removal efficiencies up to 88% were found in a full-scale horizontal sub-surface flow CW (Chen et al., 2016). Variable results on the removal of SMX have been reported earlier in literature. SMX was removed in a full-scale tertiary CW for maximum 12% but also negative removal efficiencies were reported (Breitholtz et al., 2012). In pilot-scale tertiary horizontal SSF CWs the removal efficiency was 16% (Verlicchi et al., 2013). On the contrary, Hijosa-Valsero et al. (2011) reported that a secondary horizontal SSF CW was able to remove 87% of SMX and its primary metabolite N-acetyl-SMX. The variability in the results may be caused by the retransformation of this metabolite to the parent compound during wastewater treatment (Göbel et al., 2005). Also, the influent concentration variations and the sampling strategy according to nominal HRT are likely to play a role in the current study. Furthermore, the detection of low concentrations in influent could be impaired due to strong matrix effects (Ibánez et al., 2013).

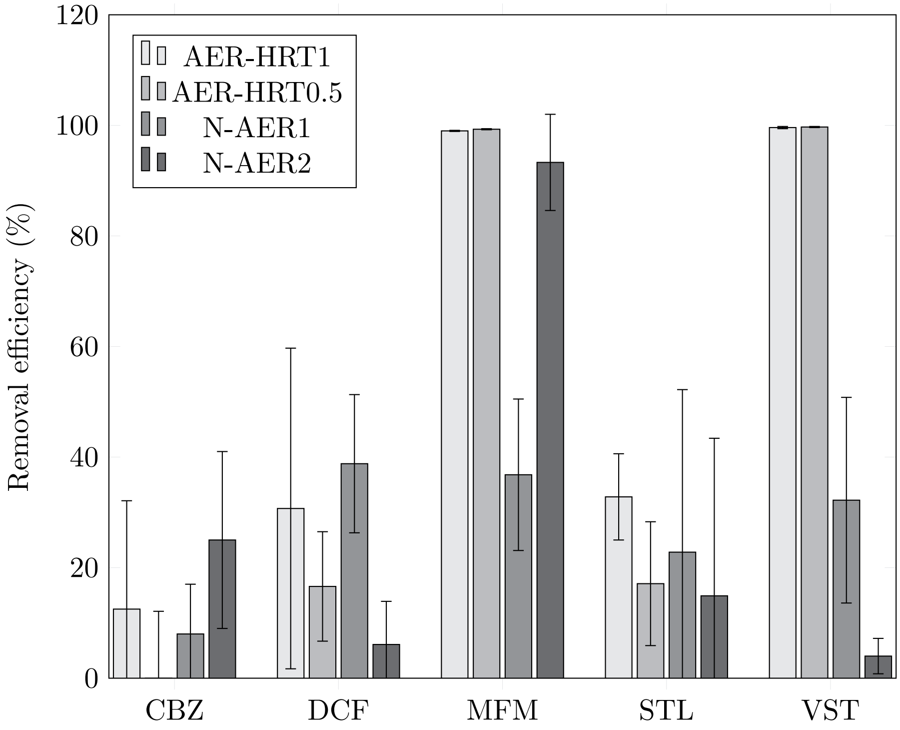


Figure 6.1: The removal efficiency of the selected pharmaceuticals in domestic wastewater (Aalbeke) (Average ± SD, n=4 for AER-HRT1, AER-HRT0.5 and N-AER1; n=5 for N-AER2). Only negative removal efficiencies were obtained for TMD.

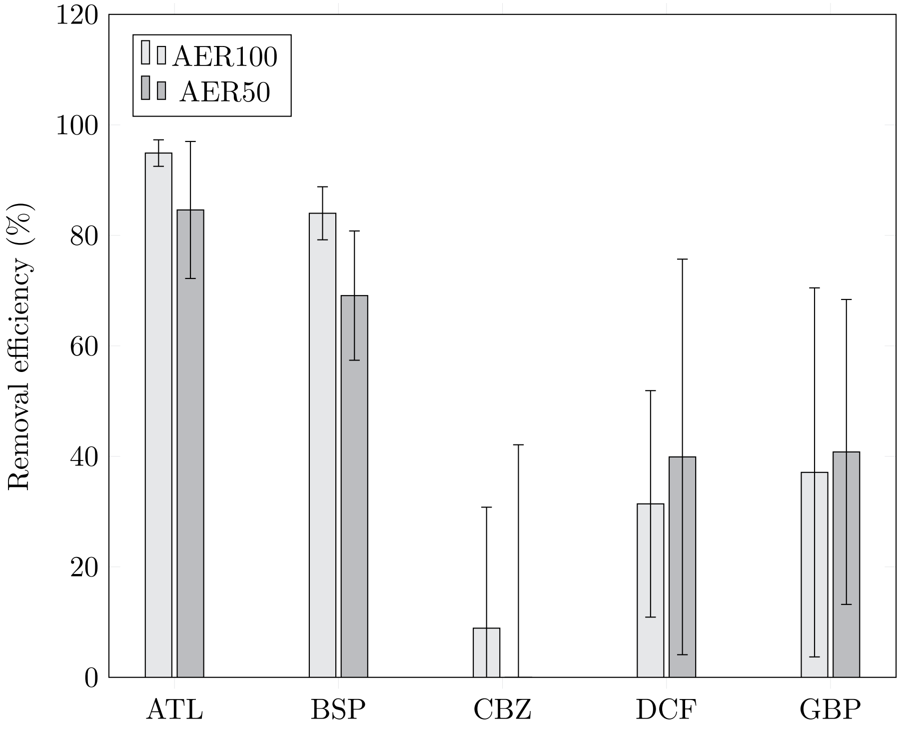


Figure 6.2: The removal efficiency of the selected pharmaceuticals in hospital wastewater (AZ Groeninge) (Average values ± SD, n=5). Only negative removal efficiencies were obtained for SMX.

6.3.4 Effect of aeration on the removal efficiency of pharmaceuticals

Although aeration is widely used in CWs to improve the degradation of organic matter and nitrification (Liu et al., 2016; Wu et al., 2014), its effect on the removal of pharmaceuticals is still inadequately studied. Earlier studies have indicated that pharmaceutical removal efficiency can be greatly affected by the dissolved oxygen concentration in the environment (Conkle et al., 2012). In CWs, the oxygen status of the filter bed depends on the type of the CW and, in general, unsaturated vertical sub-surface flow (VSSF) systems have a higher redox potential than saturated horizontal sub-surface flow (HSSF) CWs. Ávila et al. (2014a) who studied pharmaceutical removal in hybrid CWs concluded that the VSSF stage contributed more to the removal than the HSSF stage. Zhang et al. (2012b) tested the influence of batch and continuous feeding of horizontal sub-surface flow CW on the removal of several pharmaceutical compounds. They found significant improvement in the removal of diclofenac, ibuprofen, salicylic acid and ketoprofen with batch mode which suggests the enhancement of biodegradation processes by drain and fill cycles. Similarly, Ávila et al. (2013) found that the occurrence of high redox potentials within the CW due to batch feeding improved the removal of diclofenac and ibuprofen significantly. Carbamazepine, naproxen, clofibric acid and paracetamol removal was not improved due to batch feeding in these experiments implicating that their biodegradation is not dependent on the availability of oxygen or that the alteration in the feeding mode did not result in a change in redox conditions large enough to improve/decrease removal efficiency of these compounds. In a study of Ávila et al. (2014b) active aeration, i.e. more drastic impact on the redox conditions than achieved by feeding mode alteration, improved the removal of ibuprofen and triclosan in saturated gravel bed vertical flow (VF) CWs in comparison to free-draining gravel bed VF CWs. In contrast to the previously mentioned studies, diclofenac was removed better in the less oxidized wetlands with lower redox potentials, possibly because the removal of diclofenac is likely to occur via a combination of anoxic and aerobic microbial metabolic pathways (Ávila et al., 2014b).

In the current study, continuous aeration improved the removal efficiency of MFM and VST significantly ($p<0.05$) and increased hydraulic loading did not significantly decrease the removal efficiency of these compounds ($p>0.05$). MFM was completely removed during the aerobic measurement campaigns and it was also, interestingly, removed even during the N-AER phase, with a sharp rise in removal efficiency during the last week of sampling (Figure 6.1, Table 6.4). This might imply that MFM is prone to anoxic/anaerobic degradation as well and that anoxic/anaerobic microorganisms are also capable of transforming this compound after an adaptation period (2 - 3 weeks). Mrozik, Stefaska (2014) obtained similar results when studying the biodegradation of MFM in aerobic and anaerobic soils. The degradation efficiency of MFM was lower in anaerobic conditions but had a rising trend during the 60 days of observation. VST was removed completely in aerobic conditions but the removal decreased significantly when aeration was stopped. The earlier reported results on superior removal during aerobic wastewater treatment (∼80% in activated sludge, (Kasprzyk-Hordern et al., 2009) in comparison to nearly no removal in the sewer system (Jelic et al., 2015) further demonstrates the importance of aerobic conditions at enhancing VST removal.

The removal efficiencies of any of the tested pharmaceuticals were not significantly affected by the decrease in aeration time from continuous to 50% ($p>0.05$). However, the removal efficiencies of ATL and BSP, >75% and >50%, respectively, were better than earlier reported for secondary CWs (see 6.3.3), and this can be caused by aeration used in the current study. This theory is supported by the fact that ATL has earlier been reported to biodegrade both under aerobic and anoxic conditions but the degradation was faster when oxygen was present (Pomiès et al., 2015). Also, the removal of

BSP was less efficient in anaerobic conditions than aerobic conditions when studied in the laboratory with WWTP sludge (Lahti, Oikari, 2011).

6.4 Conclusions

High concentrations of pharmaceuticals from different therapeutic classes were measured in settled municipal wastewater and hospital effluent. Aeration was necessary in both cases to meet the discharge standards set for COD at the HRT applied (0.5 – 2 d). The results demonstrated that the single stage SSF CW was insufficient in preventing the discharge of pharmaceuticals. Nonetheless, the removal of certain pharmaceuticals could be improved by using active aeration. MFM and VST were significantly better removed when the system was aerated. Although, MFM was also removed nearly completely after a lag-phase without aeration, indicating the adaptation of the microbial community within the CW. Also, comparison with literature values suggested that aeration had a positive impact on the removal of ATL and BSP. Decreasing the hydraulic retention time (1 d to 0.5 d) or a reduction of aeration time to 50% did not significantly affect the removal efficiency of the targeted pharmaceuticals indicating robust performance of the system.

The pharmaceutical removal efficiency could possibly be improved by applying a longer HRT since it has been shown to enhance the removal of some pharmaceuticals (Zhang et al., 2012a). Alternatively, complementary treatment steps, e.g. hybrid CWs, could also improve the pharmaceutical removal efficiency due to the variability in the degradation processes (Ávila et al., 2014a). To fully explore the capacity of aerated CWs, the effect of these modifications on the pharmaceutical removal efficiency should be examined. In addition, the majority of studies on pharmaceutical removal in CWs is focused on the parent compounds in spite of research showing that they are mostly only partly degraded (Joss et al., 2006). Although some of the microbial metabolites are even more recalcitrant than the parent compound, many of them are likely to be biodegradable (Quintana et al., 2005). It should thus be investigated whether aeration can promote the mineralization of these metabolites and thereby contribute to lowered pharmaceutical load and toxicity in the water ways.

Chapter 7

Pharmaceutical removal in an aerated full-scale constructed wetland and assessment of its aquatic risk

This chapter is based on **Auvinen H.,** Gebhardt W., Linnemann V., Du Laing G., Rousseau D. 2017. Water Science and Technology (submitted).

7.1 Introduction

Many pharmaceuticals show such persistence to biodegradation that their presence in surface waters is used as an indicator of wastewater contamination (Vystavna et al., 2013). The environmental concentrations of pharmaceuticals are usually very low, in the range of ng/L but some commonly used substances which are poorly removed during wastewater treatment can occur at μg/L levels (Ashton et al., 2004; Lindqvist et al., 2005; Loos et al., 2009). It is likely that the highest concentrations of pharmaceuticals are detected in small streams where limited dilution occurs.

The chronic effects that pharmaceutical residues can pose in the environment are difficult to identify and quantify. Therefore, the data on the ecotoxicity of pharmaceuticals is derived from experiments in the laboratory and only a small part of this data is targeting effects after chronic exposure, i.e. long-term exposure at low concentration (Quinn et al., 2008). An initial estimate of the aquatic risk of the pharmaceuticals can be calculated via hazard quotients (HQs). The HQs compare the measured environmental concentration (MEC) and the predicted no-effect concentration (PNEC) for a specific organism observed in the laboratory experiments (Santos et al. 2007). If the ratio MEC / PNEC is higher or equal to 1, the particular pharmaceutical can have adverse ecological effects (Gros et al., 2010).

Constructed wetland (CWs) are mostly used at rural and remote locations as wastewater treatment systems for single households and small communities. They discharge in small rivers and water courses which often have high biodiversity (Matamoros et al., 2016) making them susceptible to anthropogenic pollution. The configurations vary from surface flow systems to sub-surface flow systems and hybrids where several (different types of) CWs are applied in the treatment train. The configuration, the operation and the ambient environmental conditions within the CW are likely to affect the pharmaceutical removal efficiency. Several studies on pharmaceutical removal efficiencies in different types of CWs have already been performed (for a review see Verlicchi, Zambello (2014)) but there is still need to explore factors that could improve the removal efficiency. For example, dissolved oxygen content is likely to play an important role in the removal of pharmaceuticals. Improved removal efficiency of e.g. diclofenac, ibuprofen and ketoprofen has been observed during discontinuous feeding which replenishes the oxygen in the substrate pores (Ávila et al., 2013; Zhang et al., 2012b). Ávila et al. (2014b) studied the effect of active aeration on pharmaceutical removal in sub-surface flow CWs, and concluded that the actively aerated saturated CW performed similarly to the typical unsaturated CW. However, their research included only a limited number of pharmaceutical substances and therefore, further research is needed to conclusively define the effect of active aeration on different types of pharmaceuticals.

The main objective of this study is to evaluate the removal efficiency in a full-scale constructed wetland treating wastewater from a healthcare facility and analyze the ecological impact of the effluent discharge in an effluent-dominated stream. In addition, the effect of active aeration and hydraulic retention time (HRT) on the removal efficiency are studied in a separate batch experiment.

7.2 Materials and methods

7.2.1 Full-scale constructed wetland

The full-scale CW investigated in this study was built in 2015 and it is located at a health care facility in the Province of Antwerp in Belgium. The CW comprises of a vertical sub-surface flow (VSSF) part followed by a horizontal sub-surface flow (HSSF) part having a total surface area of 240 m^2 (40 x 6 m) and a depth of 110 cm. The CW bed contains porous light expanded clay aggregate (LECA; ∅ 8/16, Argex) granules. The HSSF part is partly filled with tobermorite (calcium silicate hydrate mineral) to increase the phosphorous removal. The CW is planted with *Phragmites australis* and *Iris pseudacorus*. Aeration is provided in the CW with the Forced Bed Aeration technology (FBA®, Rietland). The aeration time is controlled automatically based on the flow rate of the incoming wastewater (4 h/d per 10 m^3/d) and the capacity of the air pumps is 150 m^3/h. In this way the electricity costs of aeration can be minimized. In general, the energy requirements of an aerated sub-surface flow CW have been estimated to be nearly 50% lower than those of activated sludge treatment when applied in small-scale (0.49 $kWh/m^3/d$ vs. 0.88 $kWh/m^3/d$) (Austin, Nivala, 2009). The design capacity of the system is 340 I.E. but at the time of sampling the complete capacity of the system was not in use and therefore, the HRT of the system was long, approximately 10 d (design HRT 3 - 4 d). The CW receives wastewater from a septic tank at intervals and flow rate dependent on water consumption. The effluent flow rate varied during the sampling period from 6 m^3d^{-1} to 16 m^3d^{-1} based on 5 daily measurements during 5 consecutive days. The CW discharges effluent in a small forest creek where dilution occurs only by rainfall. The creek runs in a sandy ground and is shaded by the forest trees. The water depth in the creek was 0 - 10 cm (partly dry) and its flow rate low (partly stagnant).

During the sampling period the weather was dry and the average temperature was ~10°C. Grab samples were taken from a reservoir tank where influent is collected after the septic tank and from an effluent collection well at the end of the CW from where the effluent is directly discharged into the creek. One influent and one effluent mixed sample were obtained per day and one such sample was based on 5 grab samples taken every 2 – 3 hours during day time. The sampling campaign lasted for 5 days. In addition, two grab samples were taken from the creek on the third sampling day (at noon) at distances 50 m and 100 m from the effluent discharge point.

7.2.2 Batch experiment

A microcosm scale batch experiment was set up in order to investigate in more detail the role of HRT and active aeration on the removal efficiency of selected pharmaceuticals. The substrate (1.3 L per setup) was put in a plastic container (∅ 20 cm, h ∼5 cm) where influent (0.5 L) was added. The substrate (LECA) and influent were fetched from the full-scale CW and stored at 4°C until the start of the batch experiment (2 days). Four treatments were applied in the microcosms (Table 7.1). Aeration was applied by means of one aquarium air pump (Hozelock 320) and air stones. The experiment was conducted inside at constant temperature (20°C) and the setups were covered to prevent light penetration. Effluent samples were obtained by draining the whole liquid volume from the microcosms.

Table 7.1: Treatments during batch experiment

Treatment	Applied HRT (d)	Aeration applied
HRT2-AIR	2	Yes
HRT6-AIR	6	Yes
HRT2-NO-AIR	2	No
HRT6-NO-AIR	6	No

7.2.3 Analysis methods

Common water quality parameters

Dissolved oxygen (DO) and pH were measured once in the full-scale CW using a multimeter HQ40d (Hach). The measurements were conducted in influent, at 3 locations along the length of the CW and in effluent. The mixed influent and effluent samples obtained during the sampling campaign of the full-scale CW were analyzed for chemical oxygen demand (COD), ammonium (NH_4^+) and nitrate (NO_3^-) by using kits according to manufacturer's instructions (LCI500, LCK305, LCK340; Hach, Belgium). The influent and effluent samples from the batch experiment were analyzed for DO and pH (HQ40d, Hach).

Analysis of pharmaceuticals

Twelve pharmaceuticals from 6 different therapeutic classes were targeted in this study. The selected pharmaceuticals were atenolol (ATL), bisoprolol (BSP), carbamazepine (CBZ), diazepam (DZP), diclofenac (DCF), gabapentin (GBP), metformin (MFM), metoprolol (MTP), sotalol (STL), telmisartan (TST), tramadol (TMD) and valsartan (VST). The analysis of the target pharmaceuticals was done using an LS-MSMS system (Thermo Fisher Scientific LTQ Orbitrap) after purification and concentration of the samples using solid phase extraction (SPE). SPE was done using commercially available SPE cartridges filled with Oasis HLB material from Waters (Milford, MA, USA). The analytical procedure is described in Chapter 6.

7.2.4 Data analysis

Statistical analyses on the pharmaceutical removal efficiencies were performed by using the SPSS Statistics 24 software. Since the data had partly non-normal distribution as observed by using the Shapiro-Wilk test, the data sets were further analyzed by using a non-parametric test (Kruskal-Wallis

H test) with Bonferroni post hoc test to define the significance of the differences. Spearman's rank order correlations was run to determine the correlation between removal efficiency and DO concentration. The significance level was set at p=0.05.

7.2.5 Aquatic risk assessment

The hazard quotients (HQs) were calculated based on the measured environmental concentration (MEC) and predicted no-effect concentration (PNEC) according the following equation:

$$HQ = \frac{MEC}{PNEC}$$

The PNEC was estimated based on chronic toxicity data using an assessment factor of 1000 applied to the lowest EC50 value reported (Vestel et al., 2016) or NOEC values with an assessment factor of 10 (Jin et al., 2012). The variation in the HQs was calculated based on the lowest and the highest MEC in the effluent/creek.

The preliminary risk assessment based on HQs was done using small water organisms and plant species (*Brachionus calyciflorus, Lemna minor, Desmodesmus subspicatus, Ceriodaphnia dubia* and *Daphnia magna*) as model organisms. The PNEC-values calculated based on literature data are shown in Table 7.2.

Table 7.2: PNEC values obtained based on literature data

Type	Species	PNEC (μg/L)	Factor applied	Exposure	Reference
Algae	*D. subspicatus*	74	1000	3 d	Cleuvers (2003)
Rotifer	*B. calyciflorus*	38	10	48 h	Ferrari et al. (2003)
Crustacean	*C.dubia*	2.5	10	7 d	Ferrari et al. (2003)
Crustacean	*D.magna*	76.3	1000	n.a.	Ginebreda et al. (2010)
Plant	*L.minor*	25.5	1000	7 d	Cleuvers (2003)

7.3 Results

7.3.1 Water quality based on conventional parameters during full-scale treatment

The full-scale CW performance was monitored during the sampling campaign for COD, NH_4^+ and NO_3^- and on-site measurements pH and DO were measured once (Table 7.3). The high pH in the effluent water is likely to be caused by the tobermorite mineral in the substrate. Due to the oxic conditions in the CW (10.6±0.1 mg/L), the COD and NH_4^+-N removal were high (98% and >98%, respectively). The denitrification efficiency was limited, likely due to the aeration applied, and hence, approximately 50% of NH_4^+-N in the influent was discharged as NO_3^--N.

7.3.2 Dissolved oxygen concentration and pH during the batch experiment

The pH did not change markedly during the batch experiment (Table 7.4). The DO was much higher in the effluent of the aerated microcosms (7.7±1.0 mg/L) than in the microcosms without aeration (0.9±0.5 mg/L).

Table 7.3: Conventional water quality parameters during full-scale treatment. Average values ± standard deviation (n=5; except for pH and DO in CW n=3).

	In	CW	Out	Removal (%)
pH	7.5	7.7±0.5	8.6	
DO (mg/L)	0.7	10.6±0.1	11.3	
COD (mg/L)	486±128		11±1	98
NH_4^+ (mg N/L)	68±7		<2	>98
NO_3^- (mg N/L)	<5		33±3	

Table 7.4: DO and pH during the batch experiment. Average values ± standard deviation (n=3)

	pH	DO
Influent	7.5	3.6
HRT2-AIR	6.5±0.0	7.9±0.6
HRT6-AIR	6.4±0.2	7.5±1.1
HRT2-NO-AIR	7.0±0.0	1.2±0.2
HRT6-NO-AIR	7.0±0.1	0.6±0.5

7.3.3 Occurrence of pharmaceuticals in the influent

The selected pharmaceuticals occurred in the influent at varying levels (Figure 7.1, Table 7.5). The lowest average concentration (40±20 ng/L) was measured for DZP and the highest one (50.66±32.74 μg/L) for MFM. TST and VST were not detected in any of the samples. The concentrations fluctuated also greatly from day to day (standard deviation near average concentration) due to daily variations in water consumption for e.g. bathing. In general, the influent pharmaceutical concentrations are so high that they are comparable to concentrations occurring in hospital effluent (Auvinen et al., 2017).

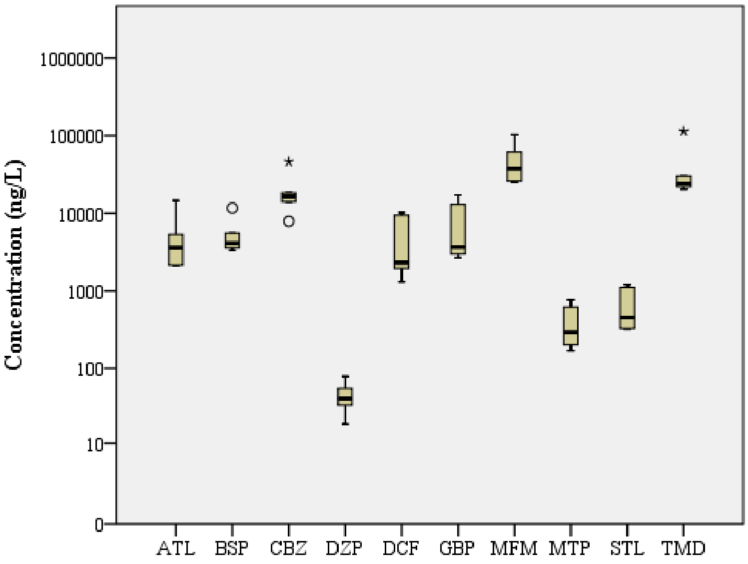


Figure 7.1: Box plots on the influent pharmaceutical concentrations of the full-scale CW (n=5). Note the logarithmic scale. The tick marks $*$ and $\circ$ mark the outliers.

7.3.4 Removal of selected pharmaceuticals during full-scale treatment

Very efficient removal of the selected pharmaceuticals was achieved during the full-scale treatment (in general >90%) (Table 7.5). Although MFM and TMD were present in the influent at the highest concentrations, their efficient removal in the CW lowered their concentrations in the effluent to ≤30 ng/L. The highest average concentration observed in the effluent was for CBZ (1280±300 ng/L). The average concentrations of ATL, BSP and DCF were below 100 ng/L and the average concentrations of DZP, GBP, MTP and STL were below the detection limit (10 ng/L). In the creek only CBZ and TMD were detected in the two grab samples (1380±520 ng/L and 60±20 ng/L, respectively). On the day when the creek water was sampled, only CBZ and TMD were detected in the effluent.

Table 7.5: Removal of selected pharmaceuticals during full-scale treatment

	Influent (ng/L)	**Effluent (ng/L)**	**Removal efficiency (%)**
ATL	5570±5220	90±20	98
BSP	5670±3480	10±10	~100
CBZ	20580±14800	1280±300	94
DZP	40±20	<10	78
DCF	5040±4370	50±90	99
GBP	7910±6740	<10	~100
MFM	50660±32740	<10	~100
MTP	410±270	<10	98
STL	680±440	30±10	99
TMD	42180±40320	<10	~100

7.3.5 Effect of active aeration and hydraulic retention time on the removal efficiency

Figure 7.2 illustrates the relations between removal efficiency, aeration and HRT. Aeration improved the removal of GBP significantly. The removal of MFM and STL was improved significantly with aeration at HRT 2 d but at HRT 6 d the removal was statistically equally efficient with or without aeration. The removal efficiencies of GBP, MFM (at HRT 2 d) and STL (at HRT 2 d) correlated also well with the DO concentration in the effluent (r_s=0.8, $p<0.05$). The concentration of TST was below the detection limit (10 ng/L) in all effluent samples. The variable removal efficiencies observed for VST are likely to be caused by the low influent concentrations (20±10 ng/L) and subsequent difficulties in quantification.

The removal efficiency of CBZ was improved with increasing HRT. The longer HRT improved the removal of DCF only during aeration and oppositely, the longer HRT enhanced the removal of TMD when aeration was not applied.

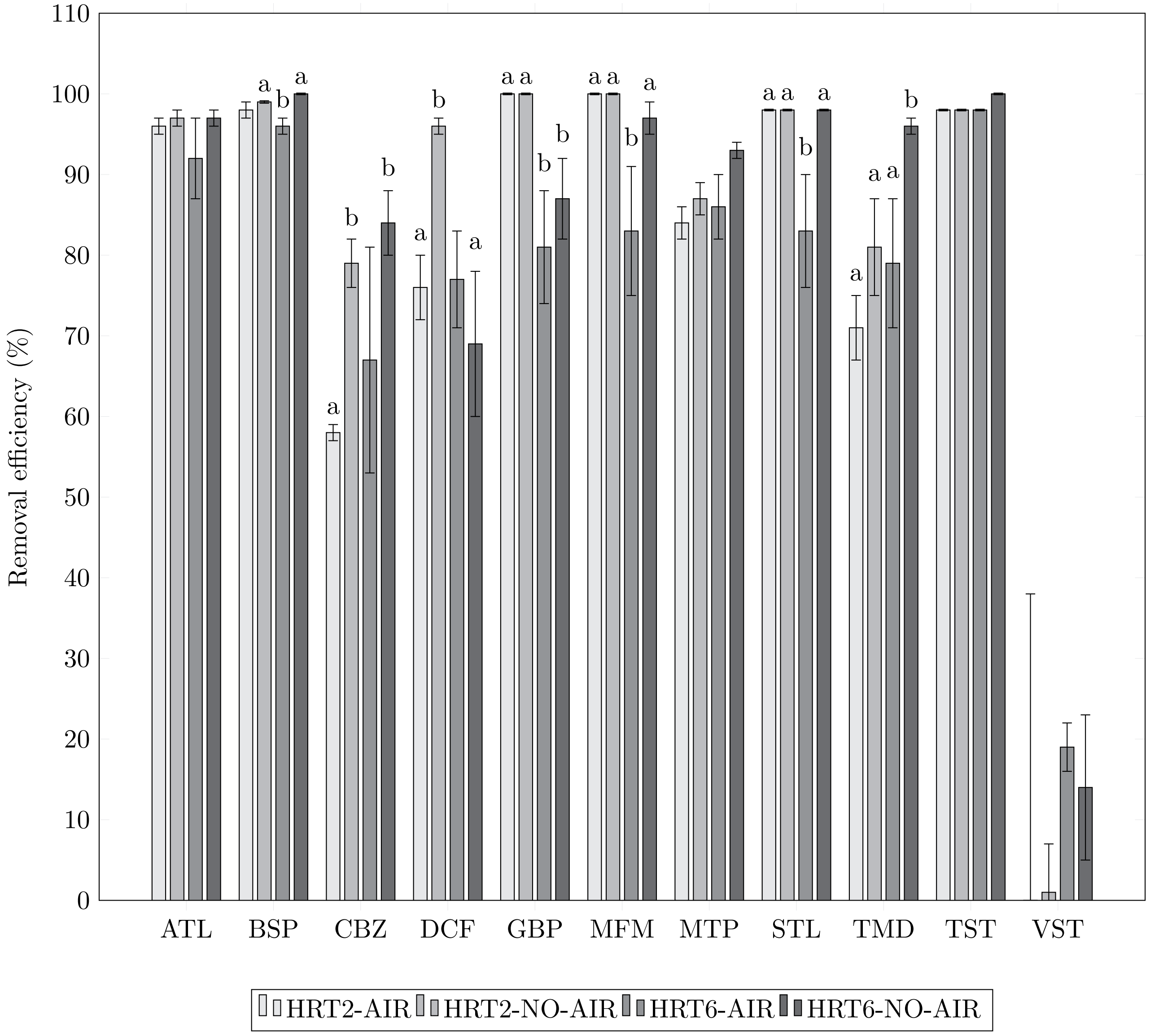


Figure 7.2: Dependency of the removal efficiencies of the selected pharmaceuticals on aeration and HRT (average ± standard deviation; n=3, except for HRT2-NO-AIR n=2 due to loss of sample). Statistically significant differences are marked with differing letters a and b.

7.3.6 Aquatic risk assessment

The HQs were calculated only for CBZ due to the very low concentrations of other pharmaceuticals (<100 ng/L) present in the effluent. The HQs, which were based on minimum and maximum concentrations detected in effluent and the creek, ranged from 0.01 to 0.7; not indicating possible toxicity to the target organisms by CBZ discharge.

7.4 Discussion

In contrast to earlier experiments on beta-blockers (ATL, BSP, MTP, STL) in activated sludge systems (Wick et al., 2009), excellent removal of these compounds was obtained in the current study. Also in earlier studies on CWs, lower removal efficiency of ATL, BSP and STL has been noted, MTP and STL being the more recalcitrant types (11 – 80%; Conkle et al. (2008). Dordio et al. (2009a) studied the removal of ATL in unplanted microcosms filled with LECA granules and concluded that the efficient removal obtained (82%) over 4 days was primarily caused by adsorption of ATL to the LECA granules because new material (no biofilm) was used in the study. It is thus possible that the combination of biodegradation and adsorption onto the LECA granules enabled the excellent removal efficiency observed in the current study. The full-scale CW is only recently (in late 2015) taken into operation and this has possibly an effect on the adsorption capacity of the LECA (not saturated, biofilm not fully developed). The removal efficiency of STL further depended on aeration and correlated positively with the DO concentration of the effluent. The removal efficiency of STL was however not dependent on the aeration when HRT of 6 d was used instead of 2 d. Anoxic biotransformations are in general slower than oxic ones and hence, a longer HRT is needed to obtain the same treatment efficiency.

GBP has earlier been reported to be quite efficiently removed in CWs (88%; Chen et al. (2016)). Based on the batch experiment, GBP is readily biodegradable in oxic conditions and it can be removed even at a short HRT. In a previous study, where hospital wastewater was treated in an aerated pilot-scale sub-surface flow CW, GBP was only removed by 33 -37% (Auvinen et al., 2017). It is possible that the high organic loading applied in the aforementioned study restricted the removal of GBP.

Although many studies on CWs report low removal efficiency for DCF (<50%; e.g. Matamoros, Bayona (2006)), excellent removal of pharmaceuticals has earlier been observed especially in hybrid systems. Ávila et al. (2010), who studied the removal of DCF in a CW system comprising of two horizontal sub-surface flow CWs in series, showed that DCF was removed by >97% at a similar hydraulic loading rate (0.028 m/d) as applied in the current study. Similarly, a removal efficiency of 89% of DCF was observed in a hybrid CW where vertical sub-surface flow CW is followed by a horizontal sub-surface flow CW and a surface flow CW (Ávila et al., 2015). The reason for the better removal in hybrid systems can be related to the presence of both anoxic and aerobic conditions occurring in these types of CWs, which are likely to be essential for the degradation process of DCF (Ávila et al., 2014b). Based on the batch experiment it seems that HRT also plays a role in the removal efficiency of DCF in aerobic conditions. It is possible that the oxic pathway necessary for the degradation is limiting the removal in the microcosms without aeration and hence, the removal is not improved even at longer HRT.

MFM was shown to be efficiently removed in the pilot-scale CW (6). In that study, MFM was removed promptly during aeration but a lag-phase occurred when aeration was not used. Similar behavior was observed in the current study, where the removal efficiency in the non-aerated microcosms was improved with increasing HRT.

Poor removal of TMD has been reported in earlier literature. Auvinen et al. (2017) observed negative removal efficiencies for TMD in a pilot-scale sub-surface CW. Breitholtz et al. (2012) studied full-scale free-surface flow CWs and observed removal efficiencies ranging from negative values to 26%. They explained the low removal to be partly caused by the sub-zero temperatures and subsequent slow biotransformations. Although Auvinen et al. (2017) did not find a correlation between aeration and removal efficiency for TMD in their study, it is possible that the applied HRT (1 d) was too short to obtain significant removal with or without aeration. In the current study, the increase in HRT (from 2 d to 6 d) increased the removal of TMD when aeration was not applied indicating that the anoxic pathway is preferred but adequate HRT is necessary.

CBZ is generally considered as a recalcitrant component and therefore, its efficient removal in the current study is somewhat surprising. The applied aeration did not decrease its removal significantly, although some studies indicate better removal at low redox conditions (Matamoros et al., 2005). CBZ has also been observed to be removed by adsorption to LECA (Dordio et al., 2009c) similarly to ATL (Dordio et al., 2009a). The fact that the removal of CBZ was improved by increasing HRT can be linked to the improved adsorption efficiency and/or be due to better biodegradation during longer contact time. It is also possible that the observed unusually high effluent pH of the full-scale CW affected the adsorption behavior. In earlier experiments pH has been shown to affect the dissociation of the pharmaceutical and its subsequent attachment to soil/sediment by ion exchange (Lorphensri et al., 2006).

Because the CW discharges into a small forest creek where little to no dilution occurs, it was important to assess the effect of pharmaceuticals on the ecotoxicity in this creek. Due to the efficient removal of all targeted pharmaceuticals the initial estimation of the aquatic risk in the forest creek is insignificant for the model organisms. Final conclusions on the risk should only be drawn after further investigations where more pharmaceuticals are targeted and where the degradation products of the pharmaceuticals are taken into account. Also, the differences in water consumption and aeration regime during day and night may have an effect on the discharge and hence, an effect on the potential toxicity of the effluent.

7.5 Conclusions

The full-scale CW produced a high quality effluent in terms of COD, NH_4^+ and the targeted pharmaceuticals. The removal efficiency of all targeted pharmaceuticals was >90%; higher than generally seen in CWs. The excellent removal is expected to be caused by the hybrid design of the CW where oxic and anoxic zones are both present, long HRT (10 d) and the presence of LECA which has been shown adsorb (at least) ATL and CBZ efficiently.

Aeration was shown to increase the removal of only a few pharmaceuticals, namely GBP, MFM and STL. The removal of MFM and STL was equally efficient with and without aeration when the longer HRT (6 d) was applied. TMD was better removed when aeration was not applied at long HRT. DCF showed opposite behavior and its removal improved with increasing HRT as aeration was applied. Due to the overall efficient removal of the targeted pharmaceuticals, the aquatic risk was considered low in a preliminary assessment.

Further research should aim at validating the results obtained during the batch experiment. This could be done when the full-scale CW is in full operation and its HRT is decreased to the design HRT of 3 – 4 d. The adsorption on LECA could decrease with increasing biofilm growth during longer operation time and cause a decrease in the removal efficiencies (Dordio et al., 2007). However, because of the large specific surface area of the porous LECA, the area occupied by biofilm is larger than in conventional CWs filled with gravel, possibly enhancing the treatment. In future research attention should also be paid to the discharge of pharmaceutical degradation products, such as quanylurea (from MFM) which could be present at high concentrations in the effluent (Scheurer et al., 2012). Although the water quality of the effluent based on common parameters meets the requirements, the removal of NO_3^- could possibly be improved by adjusting the aeration regime of the horizontal stage of the CW. The discharge of NO_3^- is an important issue in Flanders which is categorized as a nitrate sensitive area by the European Union (European Commission, 1991). The impact of adjusting the aeration regime on the pharmaceutical removal efficiency should be studied further. Although a decrease in aeration time (100% to 50%) did not decrease the pharmaceutical removal efficiency significantly in the pilot experiment (Chapter 6), the aeration needs to be sufficient for the conversion of organic matter and nitrification and similarly, there is likely to be a threshold for the pharmaceuticals.

Chapter 8

Synthesis

8.1 General discussion

Wastewater is an important source of micropollutants, such as pharmaceuticals and metallic engineered nanomaterials, in surface waters. Although these contaminants are likely to be present at very low concentrations in surface water, the possible adverse effects on aquatic organisms (Gross-Sorokin et al., 2006; Blinova et al., 2010) and the potential risk to human exposure via drinking water (Nödler et al., 2013) and the food chain (Ebbs et al., 2016) are the main reasons why research on improving the removal of these contaminants from wastewater is important. In constructed wetlands, the removal is based on simultaneously occurring biological, chemical and physical processes, which were explained in detail in *Chapter 2*, and the multitude of which can be an asset at obtaining efficient removal of contaminants with different characteristics. Despite the large number of papers on pharmaceutical removal in CWs, further knowledge on the factors affecting, and hence possibly improving, the removal of pharmaceuticals from wastewater is necessary. Therefore in this work, focus was laid on intensified, i.e. aerated, CWs where dissolved oxygen concentration higher than usual may increase the efficiency of pharmaceutical removal as indicated by e.g. Zhang et al. (2012b). In case of ENMs, earlier research on their fate in CWs is inexistent. The widespread and increasing use of ENMs and the utilization of CWs to treat many potentially ENM containing wastewater streams were the incentive to study their fate in CWs.

The aim of this thesis was to assess the removal of pharmaceuticals and (Ag-)nanoparticles and identify factors improving their removal in constructed wetlands. In this section, the fate and behavior of these contaminants is discussed and their potential environmental risk is analyzed based on the data obtained during the course of this PhD research.

8.1.1 Engineered nanomaterials

Transformations in constructed wetlands

Metallic ENMs differ from their bulk metal counterparts by their large specific surface area (area-to-volume ratio) and surface functionalization due to which these ENMs have often unique properties and reactivities (*Chapter 2*). Their physico-chemical characteristics, such as chemical composition, size and surface functionalization, affect their fate, bioavailability and toxicity in the environment (Levard et al., 2012). Also environmental factors, such as dissolved oxygen concentration and presence of NOM, can affect the fate of ENMs in the environment by altering the ENM speciation and morphology (*Chapter 3*). The transformed ENMs may present different behavior in the environment compared to the untransformed ENMs.

During activated sludge treatment, ENMs are shown to be efficiently retained in sludge flocs comprised largely of organic matter (Kaegi et al., 2011). In CWs where similar flocs are not present, Ag-NPs were shown to be mainly attached to the biofilm, which covers the substrate and plant roots, and to degrading plant debris (*Chapter 3, Chapter 5*). The importance of biofilm for the Ag-NPs removal was further demonstrated in *Chapter 4*, where it was shown that removal of Ag-NPs from the water phase did occur in the presence of CW substrates and was increased significantly in the presence of biofilm (investigated for gravel). It was further concluded that the properties of the substrate (e.g. cation exchange capacity and porosity) also likely to play a role in defining the removal from the water phase. It is most likely that the organic matter present in the influent and which accumulates especially at the inlet of CWs functions as an additional sink for ENMs (*Chapter 2*). Similar behavior is expected in cases where raw wastewater is applied on the surface of the CWs (the so called French type CWs) where a sludge deposit is formed on top of the CW bed (*Chapter 2*).

During wastewater treatment sulfides are formed due to microbial sulfate reduction in anoxic conditions. Several types of ENMs have shown to undergo sulfidation, i.e. the transformation to sulfide minerals during activated sludge treatment (Kaegi et al., 2011). The environmental implications of sulfidation depend on the type of ENM. For example, sulfidized CuO-NPs are more prone to dissolution than the original NPs whereas Ag_2S is very stable (Ma et al., 2014). Detailed STEM-EDX analysis showed that the Ag-NPs in the CW microcosms were sulfidized and that aeration could not prevent the sulfidation (*Chapter 3*). Sulfidation of Ag-NPs prevents the release of Ag^+ (dissolution) because Ag_2S has a very low solubility (Choi et al., 2009). Dissolution of (transformed) Ag-NPs could affect their bioavailability and toxicity since small ions are more bioavailable than large ENMs and they are in general considered more toxic than ENMs (*Chapter 2, Chapter 5*). In the microcosm experiments no ionic Ag (based on ultrafiltration experiments) was detected in the effluent samples suggesting that the dissolution of Ag-NPs was low (possibly due to efficient sulfidation) or that the dissolved ions were efficiently retained in the solids in the microcosm (*Chapter 5*).

Retention and possible release from constructed wetlands

Efficient removal of Ag-NPs was achieved during the microcosm experiment where only 10 – 20% of the spiked Ag-NPs (measured as total Ag) were measured in the effluent. This indicates that the CW microcosms functioned as sinks for Ag-NPs similarly to activated sludge treatment systems (*Chapter 3*). In *Chapter 5* it was shown that the removal was independent of the type of coating (PVP, citrate) and also Ag^+ showed similar removal efficiency as the Ag-NPs. This shows that in a complex environment, such as wastewater, the coating of the ENM does not play an important role in defining its fate. This is probably because the ions in the wastewater alter the surface properties of the ENM (*Chapter 2*) and result in a more uniform behavior of the ENMs in the CW. Therefore, although only Ag-NPs were investigated in this thesis, it is likely that other types of ENMs will undergo aggregation and attach to the organic matter in the CW. Their tendency to transform (e.g. sulfidize) or dissolve is, however, more defined by the chemical structure of the ENM in question. For example, while the sulfidation of Ag-NPs restricts their dissolution, the opposite is true for CuO-NPs as discussed in *Chapter 2*.

The concentration of the discharged Ag correlated significantly with the concentration of TSS present in effluent (R^2=0.8, $p<0.05$) suggesting that Ag was primarily bound to the solids in the effluent (*Chapter 3*). Similarly, TSS has been shown to be a major transport medium for Ag-NPs in activated sludge treatment (Kaegi et al., 2013). In general, TSS removal is efficient in CWs but (temporary) malfunctions and operational defects (e.g. clogging, overland flow, sudden high flow rate, drying out, influent toxicity) could increase TSS discharge due to biofilm detachment or influent short-circuiting (*Chapter 2*). Solids accumulation and subsequent clogging is one of the major problems with SSF CWs (Nivala, Rousseau, 2009). It occurs quite often in CWs and may be difficult to observe and remedy without extensive interventions. Active aeration has been shown to decrease the solids accumulation within the substrate but to increase biofilm growth (Chazarenc et al., 2009). Further investigations on its effect on clogging are still needed. If a part of the biofilm detaches from the substrate, it is likely to be retained and metabolized within the substrate pores unless it is washed out by peak flow (Rousseau et al., 2008; Hua et al., 2013). Hence, CWs seem quite resilient towards perturbations. Usage of sand and other small-grain substrate can improve TSS removal, but its effect on clogging needs to be taken into account. Polishing treatments, such as sand or membrane filtration, are unlikely to be cost-efficient measures to reduce TSS discharge from CWs, and hence, a better way to ensure efficient TSS removal in a CW is to make sure the CW is maintained properly. Proper maintenance includes cleaning out grids and inlet zone of debris and harvesting plant material at yearly (or bi-annual) basis. These measures will help prevent clogging of the CW bed. It is also necessary to monitor the system for any signs of clogging and take action when this occurs. Automatic monitoring of flooding for instance would allow to take immediate action. Proper hydrodynamic design of CWs (e.g. width-to-length ratio) is essential at preventing short-circuiting, although also uncontrollable factors such as vegetation may have a major impact on channeling within the substrate (Persson, Wittgren, 2003).

Metal accumulation in plant material is possible but the uptake of ENMs by plants is restricted by the large size of ENMs in comparison to ions (*Chapter 2*). During the microcosm experiment, only minor amounts of Ag (< 1% of the total mass recovered within the microcosm compartments) were found attached or taken up by the roots of *P. australis* during 25 weeks of operation (*Chapter 3*). The 4-week hydroponic culture experiment on *P. australis* showed that some uptake is possible, especially by the roots, but translocation to the leaves is insignificant (*Chapter 4*). The comparison of the results from these two experiments suggests that the attachment of Ag-NPs on biofilm/organic matter is more likely than attachment to/uptake by the roots. As a consequence, the levels of Ag-NPs in the harvested plant biomass are expected to be very low and not to cause any restrictions on the application of the plant biomass e.g. as soil amendment after composting.

Although sludge is not produced in CWs, solids are separated in a primary settling tank producing primary sludge. ENMs are likely to partition to the primary sludge efficiently due to their high affinity for organic material (*Chapters 2-5*). In addition, the removal of ENMs during primary treatment will decrease the concentration of ENMs in the CW influent. Only in case of the French type CWs sludge is produced during the normal treatment process (i.e. no clogging) and the sludge deposit on top of the CW needs to be disposed of occasionally (Molle et al., 2005). When the sludge is applied on agricultural land, concerns are raised on food safety due to metal leaching and subsequent accumulation in crops (Ebbs et al., 2016). Although the sludge being a primary sink for ENMs, the current ENM concentrations in the influent wastewater are estimated to be low and hence, the concentrations of ENMs in sludge are likely to be insignificant in comparison to bulk metals (*Chapter 2*). However, some caution is warranted since production volumes of ENMs have a rising trend. Also, proper handling of the wastewater produced upon refurbishment of CWs (Cooper et al., 2006) is necessary since this is likely to contain high concentrations of metals, among which (transformed)

ENMs (*Chapter 2*).

Possible adverse effects on the microorganisms in constructed wetlands

ENMs are potentially toxic (Choi, Hu, 2008) and can hence cause toxicity to microorganisms in CWs possibly ultimately affecting the performance of the CW. The water phase is unlikely (at current production volumes) to contain such high concentrations of ENMs which would harm the ecosystem in the CW (*Chapter 2, Chapter 5*). However, the accumulating ENMs could in long-term possibly lead to toxic concentrations within the substrate. In the microcosm experiment described in *Chapter 5*, the microcosms were exposed to an increasing concentration of Ag-NPs (up to 80 μg/kg substrate) during the monitoring period of one month. During this time no significant alterations in the metabolic capacity or the microbial community structure were observed which could have indicated possible toxicity. Although the exposure concentration used in this experiment is very low in comparison to bulk metal concentrations observed within CW substrate after years of operation (>100 mg/kg) (*Chapter 2*), the experiment gives an indication of the possible ENM-specific toxicity.

8.1.2 Pharmaceuticals

Removal efficiency in constructed wetlands

The use of CWs to treat domestic wastewater is increasing due to the stringent regulations to safeguard surface waters in the European Union (*Chapter 2*). This is also noticeable in Belgium where CWs are nowadays built as treatments systems for single households and small communities in the rural areas (Auvinen et al., 2016a).

A large number of studies on pharmaceutical removal in CWs has been published in the recent decade. In general, the removal efficiencies have been found very variable and dependent of the pharmaceutical in question (*Chapter 2*). This was also observed during the pilot-scale studies on domestic and hospital wastewater (*Chapter 6*). On one hand, carbamazepine and tramadol were shown to be the most recalcitrant compounds of the pharmaceuticals studied with a removal efficiency varying from negative to 20%. On the other hand, some components such as atenolol and metformin, were removed nearly completely. The overall removal efficiencies of the studied compounds were higher during the full-scale experiment where, despite the high influent concentrations, a removal of >90% was achieved (*Chapter 7*). The unexpected improved removal was expected to be caused by a combination of factors (hybrid design, long HRT and LECA as substrate). The main conclusion of the pilot-scale studies was that the pilot CW was insufficient in preventing the discharge of pharmaceuticals, and as the pilot system was operated at a quite high organic loading rate, it was hypothesized that the removal could be improved by increasing the HRT.

Pharmaceuticals were observed to be present in high concentrations in the hospital (*Chapter 6*) and in the wastewater of the health care facility (*Chapter 7*). For more enhanced removal, it is a common recommendation to treat such wastewaters near the source (Ternes et al., 2004b; Pauwels, Verstraete, 2006). The experiment on the full-scale CW showed that efficient removal of pharmaceuticals is possible to obtain. In cases where the hospital is located in a more urban environment where place is limited, it would be necessary to apply higher influent loading rates. The pilot-scale study showed that robust removal of COD and NH_4^+ could be obtained even at high loading rate (on average 150 g COD/m^2/d and 10 g NH_4^+/m^2/d) but high concentrations of pharmaceuticals were still present in the effluent (*Chapter 6*). With the expected future regulations on pharmaceutical concentrations in surface waters and wastewater (currently implementation of the watch list; European Parliament (2013)), it would be necessary to enhance the removal of pharmaceuticals during wastewater treatment, possibly using complimentary treatment steps, such as ozonation (De Buyck, 2015).

Effect of design on the removal efficiency of pharmaceuticals

Earlier research has shown that the vertical flow part in a hybrid CW comprising vertical and horizontal CWs has the largest effect on pharmaceutical removal (Ávila et al., 2014a). The higher efficiency of VSSF CWs was claimed to be caused by higher dissolved oxygen concentrations due to the unsaturation of the substrate bed in this type of CWs. Hence we assessed the application of active aeration. The removal of metformin, valsartan and gabapentin was clearly dependent on aeration (*Chapter 6, Chapter 7*). However, degradation of metformin appeared also to be possible when aeration was not applied but an adaptation of the microbial metabolism was then necessary (*Chapter 6, Chapter 7*). The removal of bisoprolol, sotalol, diclofenac and tramadol seemed to depend on aeration as well as HRT applied (*Chapter 7*). In case of bisoprolol, sotalol and diclofenac aeration in combination with long HRT (6 d) induced the best removal efficiency observed during the batch experiment. For tramadol aeration decreased its removal efficiency. Removal of carbamazepine was independent of aeration but increased with increasing HRT (*Chapter 7*), which likely explains (at least partly) the very poor removal efficiencies obtained during the pilot-scale experiments (*Chapter 6*).

These examples show that the presence of both oxic and anoxic conditions in CWs can aid in improving the removal efficiency because some pharmaceuticals are better removed in oxic and some in anoxic conditions. Hence, these results support the selection of hybrid systems when improved pharmaceutical removal is targeted. It is possible that equally efficient pharmaceutical removal could be achieved in CW hybrid systems comprising VSSF and HSSF CWs as in partly intensified CWs, as was suggested in a study of Ávila et al. (2014b). However, to maintain the oxygen transfer capability in the VSSF CW, rigorous maintenance is necessary. Currently, the application of hybrid CW systems is quite modest in Belgium (Flanders): less than 10% of the 406 CWs in the complete database on Flemish CWs reported in Auvinen et al. (2016a) were hybrid systems. It was further reported that only 5 out of 35 Aquafin operated CW treatment systems were hybrid CWs; a very low number in comparison to the 22 systems comprising submerged aerated filter/rotating biological contactors and a tertiary CW.

In addition to HRT and dissolved oxygen concentration, this study suggested that the excellent removal efficiencies obtained during the full-scale experiment were caused by efficient adsorption of the pharmaceuticals on the LECA substrate (*Chapter 7*). Due to the short duration of the study, it is not possible to determine whether the adsorption remains equally efficient and hence, suggestions on the application of LECA as a means of pharmaceutical adsorbent cannot be made.

Aquatic risks due to pharmaceutical discharge

Since CWs are often located in rural areas and discharge in small water ways, assessing the risk that the pharmaceutical discharge poses in these water ways for aquatic ecosystem health is essential (*Chapter 2*). Currently in Flanders the norms for discharge from wastewater treatment plants are based on the size of the treatment plant instead of the vulnerability (factors such as dilution and ecological diversity) of the receiving stream. In The Netherlands and United States of America more specific regulation is applied on wastewater treatment plants to ensure the carrying capacity of the receiving streams is not exceeded (Environmental Protection Agency (EPA), 1992; Rousseau et al., 2003). Although these norms apply currently only for nutrients, possible future regulatory standards will be addressing pharmaceuticals as well. In Switzerland works are ongoing to equip all large and medium sized wastewater treatment plants and some of the smaller treatment plants (>8000 I.E.) with an advanced treatment step (ozonation/activated carbon) to enhance pharmaceutical removal and hence to safequard the surface waters (Audenaert et al., 2014).

Due to the difficulties of assessing environmental toxicity *in situ*, laboratory tests are most often conducted to estimate the risks (Quinn et al., 2008). Safety factors are applied to the results to account also for organisms which are possibly more susceptible than the target organism (Vestel et al., 2016). In this manner a preliminary assessment of the aquatic risk can be performed quite reliably.

The efficient removal of pharmaceuticals decreased the aquatic risk below the toxicity threshold on the selected target organisms during full-scale experiment (*Chapter 7*). In this study, only carbamazepine was discharged in potentially toxic concentrations. Because of the high concentrations of the targeted pharmaceuticals, it is however advisable to investigate the discharge of a larger selection of pharmaceuticals to more reliably assess the toxicity. Sometimes the combination of pharmaceuticals is also more toxic than expected based on the toxicities of the single compounds (Cleuvers, 2003). If we for example consider the effluent concentrations from the pilot-scale CW treating hospital wastewater, the effluent would appear toxic based on the preliminary assessment with hazard quotients (*Chapter 6*). Furthermore, a thorough ecotoxicological assessment needs to take the effects of transformation products also into account (Olvera-Vargas et al., 2016).

8.2 General conclusions

8.2.1 Engineered nanomaterials

- (Ag-)NPs are likely to be efficiently removed in CWs. Biofilm surrounding the substrate and plant roots as well as organic matter retained within the substrate are the main sinks for (Ag-)NPs. Ag-NPs undergo further transformations, such as sulfidation, which restricts their dissolution and subsequently decreases their toxicity.

- Active aeration, when resulting in low concentrations of dissolved oxygen, did not prevent sulfidation of the Ag-NPs and did not increase the discharge of total Ag. Aerated CWs are usually equipped with a zone where aeration is not applied or where it is regulated to allow the development of anoxic zones for denitrification. In these zones, microbial sulfate reduction can also take place and induce sulfidation of Ag-NPs.

- Organic matter build-up during the aging of the CWs is likely to offer additional adsorption sites for (Ag-)NPs and hence, result in more efficient retention of (Ag-)NPs if the organic matter is retained within the CW substrate.

- The release of (transformed) (Ag-)NPs from CWs with effluent is likely to be low. The discharged (Ag-)NPs are attached to solids present in the effluent and hence, controlling the TSS removal increases the retention of (Ag-)NPs. Regular maintenance, such as clearing the inlet of debris and harvesting of plants, and monitoring of CWs is essential at preventing the discharge of TSS.

- Plant uptake of Ag-NPs has a low impact on the removal efficiency, although adsorption to the roots can occur to some extent. With the low translocation, possible release of Ag from harvested and composted plant material is insignificant and is not likely to restrict the applications of the composted plant material.

- No significant toxicity (based on metabolic capacity or the microbial community structure) on the biofilm microbial community was observed succeeding an exposure of 80 μg Ag/kg substrate. However, some caution is warranted due to the predicted rise in future ENM production volumes.

8.2.2 Pharmaceuticals

- The removal efficiency of pharmaceuticals was highly dependent on the type of pharmaceuticals as were the factors affecting the removal efficiency. Aeration was shown to improve the removal of gabapentin, metformin and valsartan significantly. The removal of metformin could also occur without aeration after microbial adaptation. The removal efficiency of carbamazepine correlated significantly with HRT. For some components both factors, (no) aeration and increased HRT, together caused significantly improved removal. The removal of sotalol and diclofenac was significantly improved with aeration at long HRT but in case of tramadol, no aeration induced better removal. Also, efficient removal of the pharmaceuticals during full-scale treatment was likely to be partly caused by the LECA substrate presenting high adsorption capacity for some pharmaceuticals.

- Based on these results, active aeration may not be necessary for efficient pharmaceutical removal, although the presence of both oxic and anoxic conditions is likely to enhance the overall removal. Hence, the application of hybrid CWs is recommended when enhanced pharmaceutical removal is targeted. However, active aeration may provide a solution for (urban) locations, such as hospitals, where space is critical because the intensification of the treatment induces a decrease in the foot-print.

- In case of high pharmaceutical concentrations, such as hospital wastewater, separate treatment of this wastewater is recommended. Due to the space constraints CWs may not be suitable as a sole technology to treat these wastewaters. In such cases aeration is needed to intensify the treatment although complimentary treatment (e.g. ozonation) is likely to be required.

- In the preliminary toxicity assessment, the effluent of the full-scale CW was not expected to cause toxicity in the aquatic organisms targeted.

8.3 Perspectives

8.3.1 Engineered nanomaterials

- Based on the conclusion that the release of ENMs can primarily be controlled by the release of TSS, it is possible that the factors causing the release of TSS also induce the discharge of (transformed) ENMs. Further research should thus address (temporary) situations where the TSS concentration of the effluent increases. Such situations could be short-circuiting caused by clogging, detachment of biofilm due to increased toxicity of the influent or sudden peaks in influent flow. Also, when active aeration is applied in CWs with a low length-to-width ratio, influent could escape due to mixing by aeration.

- This study investigated the toxicity of Ag-NPs on CW microcosm biofilm. However, the exposure time in this experiment was rather short and hence, further research is needed to illustrate the effects of long-term exposure to low concentrations. Also, more realistic scenarios where both bulk metals and ENMs (combined and separately) are applied, are advisable to identify the contribution of the different metal species on the possible toxicity.

8.3.2 Pharmaceuticals

- The results obtained during the batch experiment on aeration and HRT should be validated in full-scale experiments. The planned increase in the hydraulic loading of the investigated CW would offer a possibility for the validation experiments.

- The overall efficient removal of the selected pharmaceuticals was assumed to be partly due to the presence of LECA substrate as it was in earlier literature shown to adsorb pharmaceuticals efficiently. The long-term effect of its adsorption capacity should however be investigated since the biofilm growth on the surface of the LECA granules could hinder the adsorption process.

- Based on the overall high influent concentrations of the selected pharmaceuticals in the influent of the full-scale CW, it is likely that other non-targeted pharmaceuticals were also present. Final conclusions on the effluent toxicity can thus be drawn only after a more elaborate screening of the effluent.

- Despite the large amount of publications on pharmaceutical removal in CWs, only a small part addresses transformation products. Literature shows that pharmaceuticals are mostly not completely mineralized and that the transformation products may be even more toxic and/or persistent in the environment as the mother component. Hence, further research is needed to obtain extensive data on the fate of these transformation products in CWs. Furthermore, no data yet exists on the effects of aeration on the removal of these transformation products.

- Efficient removal of pharmaceuticals can be obtained during normal operation in a CW as shown in the full-scale study (Chapter 7). It should still be investigated how treatment instabilities, such as fluctuations in the influent composition, loading and flow rate, affect pharmaceutical removal.

Bibliography

Alito Christina L., Gunsch Claudia K. Assessing the effects of silver nanoparticles on biological nutrient removal in bench-scale activated sludge sequencing batch reactors // Environmental Science & Technology. 2014. 48. 970–976.

Allen Winthrop C, Hook Paul B, Biederman Joel a, Stein Otto R. Wetlands and Aquatic Processes Temperature and Wetland Plant Species Effects on Wastewater Treatment and Root Zone Oxidation // Journal of Environmental Quality. 2002. 31. 1010–1016.

Anderson Julie C, Carlson Jules C, Low Jennifer E, Challis Jonathan K, Wong Charles S, Knapp Charles W, Hanson Mark L. Performance of a constructed wetland in Grand Marais, Manitoba, Canada: Removal of nutrients, pharmaceuticals, and antibiotic resistance genes from municipal wastewater. // Chemistry Central Journal. 2013. 7, 1. 54–69.

Andreozzi Roberto, Marotta Raffaele, Paxéus Nicklas. Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment // Chemosphere. 2003. 50, 10. 1319–1330.

Ashton Danielle, Hilton Martin, Thomas Kevin V. Investigating the environmental transport of human pharmaceuticals to streams in the United Kingdom // Science of the Total Environment. 2004. 333, 1-3. 167–184.

Aubertheau Elodie, Stalder Thibault, Mondamert Leslie, Ploy Marie-Cécile, Dagot Christophe, Labanowski Jérôme. Impact of wastewater treatment plant discharge on the contamination of river biofilms by pharmaceuticals and antibiotic resistance // Science of the Total Environment. 2016. 579. 1387–1398.

Audenaert Wim T.M., Chys Michael, Auvinen Hannele, Dumoulin Ann, Rousseau Diederik, Van Hulle Stijn W.H. (Future) Regulation of Trace Organic Compounds in WWTP Effluents as a Driver for Advanced Wastewater Treatment // Ozone News. 2014. 42, 6. 17–23.

Austin David, Nivala Jaime. Energy requirements for nitrification and biological nitrogen removal in engineered wetlands // Ecological Engineering. 2009. 35, 2. 184–192.

Auvinen Hannele, Du Laing Gijs, Meers Erik, Rousseau Diederik . Constructed Wetlands Treating Municipal and Agricultural Wastewater – An Overview for Flanders, Belgium // In: Vymazal J. (Ed.). 2016a. Natural and constructed wetlands. Springer international publishing.

Auvinen Hannele, Havran Iva, Hubau Laurens, Vanseveren Lize, Gebhardt Wilhelm, Linnemann Volker, Van Oirschot Dion, Du Laing Gijs, Rousseau Diederik P.L. Removal of pharmaceuticals by a pilot aerated sub-surface flow constructed wetland treating municipal and hospital wastewater // Ecological Engineering. 2017. 100. 157–164.

Auvinen Hannele, Vásquez Sepùlveda Viviana, Rousseau Diederik, Du Laing Gijs. Substrate- and plant-mediated removal of citrate-coated silver nanoparticles in constructed wetlands // Environmental Science and Pollution Research. 2016b. 23, 21. 21920–21926.

Ávila Cristina, Bayona Josep M., Martín Isabel, Salas Juan José, García Joan. Emerging organic contaminant removal in a full-scale hybrid constructed wetland system for wastewater treatment and reuse // Ecological Engineering. 2015. 80. 108–116.

Ávila Cristina, Matamoros Víctor, Reyes-Contreras Carolina, Piña Benjamí, Casado Marta, Mita Luigi, Rivetti Claudia, Barata Carlos, García Joan, Bayona Josep Maria. Attenuation of emerging organic contaminants in a hybrid constructed wetland system under different hydraulic loading rates and their associated toxicological effects in wastewater // Science of the Total Environment. 2014a. 470-471. 1272–1280.

Ávila Cristina, Nivala Jaime, Olsson Linda, Kassa Kinfe, Headley Tom, Mueller Roland A., Bayona Josep Maria, Garci ́a Joan. Emerging organic contaminants in vertical subsurface flow constructed wetlands: Influence of media size, loading frequency and use of active aeration // Science of the Total Environment. 2014b. 494-495. 211–217.

Ávila Cristina, Pedescoll Anna, Matamoros Víctor, Bayona Josep María, García Joan. Capacity of a horizontal subsurface flow constructed wetland system for the removal of emerging pollutants: An injection experiment // Chemosphere. 2010. 81, 9. 1137–1142.

Ávila Cristina, Reyes Carolina, Bayona Josep María, García Joan. Emerging organic contaminant removal depending on primary treatment and operational strategy in horizontal subsurface flow constructed wetlands: Influence of redox // Water Research. 2013. 47, 1. 315–325.

Backhaus Thomas, Faust Michael. Predictive Environmental Risk Assessment of Chemical Mixtures : a conceptual framework // Environmental Science & Technology. 2012. 46. 2564–2573.

Barber Larry B., Leenheer Jerry A., Noyes Ted I., Stiles Eric A. Nature and transformation of dissolved organic matter in treatment wetlands // Environmental Science and Technology. 2001. 35, 24. 4805–4816.

Bayer Anne, Asner Robert, Schüssler Walter, Kopf Willi, Weiß Klaus, Sengl Manfred, Letzel Marion. Behavior of sartans (antihypertensive drugs) in wastewater treatment plants, their occurrence and risk for the aquatic environment // Environmental Science and Pollution Research. 2014. 21, 18. 10830–10839.

Behra Renata, Sigg Laura, Clift Martin J D, Herzog Fabian, Minghetti Matteo, Johnston Blair, Petri-Fink Alke, Rothen-Rutishauser Barbara. Bioavailability of silver nanoparticles and ions: from a chemical and biochemical perspective. // Journal of the Royal Society, Interface. 2013. 10, 87. 20130396.

Ben-Moshe Tal, Dror Ishai, Berkowitz Brian. Transport of metal oxide nanoparticles in saturated porous media // Chemosphere. 2010. 81, 3. 387–393.

Bendz David, Paxéus Nicklas a., Ginn Timothy R., Loge Frank J. Occurrence and fate of pharmaceutically active compounds in the environment, a case study: Höje River in Sweden // Journal of Hazardous Materials. 2005. 122, 3. 195–204.

Benn Troy M., Westerhoff Paul. Nanoparticle silver released into water from commercially available sock fabrics // Environmental Science and Technology. 2008. 42, 11. 4133–4139.

Berendonk Thomas U, Manaia Célia M, Merlin Christophe, Kassinos Despo Fatta, Cytryn Eddie, Walsh Fiona, Bürgmann Helmut, Huovinen Pentti, Stefani Stefania, Schwartz Thomas, Kisand Veljo, Baquero Fernando, Martinez José Luis. Tackling antibiotic resistance: the environmental framework // Nature Reviews Microbiology. 2015. 13. 310–317.

Bergheim Marlies, Gieré Reto, Kümmerer Klaus. Biodegradability and ecotoxicitiy of tramadol, ranitidine, and their photoderivatives in the aquatic environment // Environmental Science and Pollution Research. 2012. 19, 1. 72–85.

Blanck Hans, Wängberg Sven-Ake, Molander Sverker. Pollution-Induced Community Tolerance—A New Ecotoxicological Tool // In: Cairns, J., Pratt, J.P. (Eds.). 1988. Functional testing of aquatic biota for estimating hazards of chemicals. ASTM.

Blaser Sabine A., Scheringer Martin, MacLeod Matthew, Hungerbühler Konrad. Estimation of cumulative aquatic exposure and risk due to silver: Contribution of nano-functionalized plastics and textiles // Science of the Total Environment. 2008. 390, 2-3. 396–409.

Blinova Irina, Ivask Angela, Heinlaan Margit, Mortimer Monica, Kahru Anne. Ecotoxicity of nanoparticles of CuO and ZnO in natural water // Environmental Pollution. 2010. 158, 1. 41–47.

Bone Audrey J., Matson Cole W., Colman Benjamin P., Yang Xinyu, Meyer Joel N., Di Giulio Richard T. Silver nanoparticle toxicity to Atlantic killifish (Fundulus heteroclitus) and Caenorhabditis elegans: A comparison of mesocosm, microcosm, and conventional laboratory studies // Environmental Toxicology and Chemistry. 2015. 34, 2. 275–282.

Boon Nico, De Windt Wim, Verstraete Willy, Top Eva M. Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from di¡erent wastewater treatment plants // FEMS Microbiology Ecology. 2002. 39. 101–112.

Boreen Anne L., Arnold William A., McNeill Kristopher. Photodegradation of pharmaceuticals in the aquatic environment: A review // Aquatic Sciences. 2003. 65, 4. 320–341.

Boxall Alistair B. A., Kolpin Dana W., Halling-Sørensen Bent, Tolls Johannes. Are veterinary medicines causing environmental risks? // Environmental Science & Technology. 2003. 37, 15. 286–294.

Brar Satinder K., Verma Mausam, Tyagi R. D., Surampalli R. Y. Engineered nanoparticles in wastewater and wastewater sludge - Evidence and impacts // Waste Management. 2010. 30, 3. 504–520.

Breitholtz Magnus, Näslund Maria, Stråe Daniel, Borg Hans, Grabic Roman, Fick Jerker. An evaluation of free water surface wetlands as tertiary sewage water treatment of micro-pollutants // Ecotoxicology and Environmental Safety. 2012. 78. 63–71.

Brunetti Gianluca, Donner Erica, Laera Giuseppe, Sekine Ryo, Scheckel Kirk G, Khaksar Maryam, Vasilev Krasimir, De Mastro Giuseppe, Lombi Enzo. Fate of zinc and silver engineered nanoparticles in sewerage networks. // Water research. 2015. 77. 72–84.

Burke Victoria, Richter Doreen, Hass Ulrike, Duennbier Uwe, Greskowiak Janek, Massmann Gudrun. Redox-dependent removal of 27 organic trace pollutants: Compilation of results from tank aeration experiments // Environmental Earth Sciences. 2014. 71, 8. 3685–3695.

Button Mark, Auvinen Hannele, Van Koetsem Frederik, Hosseinkhani Baharak, Rousseau Diederik P.L., Weber Kela P., Du Laing Gijs. Susceptibility of constructed wetland microbial communities to silver nanoparticles : A microcosm study // Ecological Engineering. 2016. 97. 476–485.

Carballa Marta, Omil Francisco, Lema Juan M., Llompart María, García-Jares Carmen, Rodríguez Isaac, Gómez Mariano, Ternes Thomas. Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant // Water Research. 2004. 38, 12. 2918–2926.

Carranza-Diaz Otoniel, Schultze-Nobre Luciana, Moeder Monika, Nivala Jaime, Kuschk Peter, Koeser Heinz. Removal of selected organic micropollutants in planted and unplanted pilot-scale horizontal flow constructed wetlands under conditions of high organic load // Ecological Engineering. 2014. 71. 234–245.

Caselles-Osorio Aracelly, Puigagut Jaume, Segú Emma, Vaello Núria, Granés Francesc, García David, García Joan. Solids accumulation in six full-scale subsurface flow constructed wetlands // Water Research. 2007. 41, 6. 1388–1398.

Chazarenc Florian, Gagnon Vincent, Comeau Yves, Brisson Jacques. Effect of plant and artificial aeration on solids accumulation and biological activities in constructed wetlands // Ecological Engineering. 2009. 35, 6. 1005–1010.

Chen Yi, Vymazal Jan, Brezinová Tereza, Kozeluh Milan, Kule Lumír, Huang Jingang, Chen Zhongbing. Occurrence, removal and environmental risk assessment of pharmaceuticals and personal care products in rural wastewater treatment wetlands // Science of the Total Environment. 2016. 566-567. 1660–1669.

Choi Okkyoung, Clevenger Thomas E., Deng Baolin, Surampalli Rao Y., Ross Louis, Hu Zhiqiang. Role of sulfide and ligand strength in controlling nanosilver toxicity // Water Research. 2009. 43, 7. 1879–1886.

Choi Okkyoung, Deng Kathy Kanjun, Kim Nam Jung, Ross Louis, Surampalli Rao Y., Hu Zhiqiang. The inhibitory effects of silver nanoparticles, silver ions, and silver chloride colloids on microbial growth // Water Research. 2008. 42, 12. 3066–3074.

Choi Okkyoung, Hu Zhiqiang. Size dependent and reactive oxygen species related nanosilver toxicity to nitrifying bacteria. // Environmental Science & Technology. 2008. 42, 12. 4583–4588.

Choi Okkyoung, Yu Chang Ping, Esteban Fernández G., Hu Zhiqiang. Interactions of nanosilver with Escherichia coli cells in planktonic and biofilm cultures // Water Research. 2010. 44, 20. 6095–6103.

Christian P., Von Der Kammer F., Baalousha M., Hofmann Th. Nanoparticles: Structure, properties, preparation and behaviour in environmental media // Ecotoxicology. 2008. 17, 5. 326–343.

Cleuvers Michael. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects // Toxicology Letters. 2003. 142. 185–194.

Cleveland Danielle, Long Stephen E., Pennington Paul L., Cooper Emily, Fulton Michael H., Scott Geoffrey I., Brewer Timothy, Davis Jeff, Petersen Elijah J., Wood Laura. Pilot estuarine mesocosm study on the environmental fate of Silver nanomaterials leached from consumer products // Science of the Total Environment. 2012. 421-422. 267–272.

Colman Benjamin, Wang Si Yi, Auffan Melanie, Wiesner Mark R., Bernhardt Emily S. Antimicrobial effects of commercial silver nanoparticles are attenuated in natural streamwater and sediment // Ecotoxicology. 2012. 21, 7. 1867–1877.

Colman Benjamin P., Arnaout Christina L., Anciaux Sarah, Gunsch Claudia K., Hochella Michael F., Kim Bojeong, Lowry Gregory V., McGill Bonnie M., Reinsch Brian C., Richardson Curtis J., Unrine Jason M., Wright Justin P., Yin Liyan, Bernhardt Emily S. Low Concentrations of Silver Nanoparticles in Biosolids Cause Adverse Ecosystem Responses under Realistic Field Scenario // PLoS ONE. 2013. 8, 2.

Colman Benjamin P., Espinasse Benjamin, Richardson Curtis J., Matson Cole W., Lowry Gregory V., Hunt Dana E., Wiesner Mark R., Bernhardt Emily S. Emerging contaminant or an old toxin in disguise? Silver nanoparticle impacts on ecosystems // Environmental Science and Technology. 2014. 48, 9. 5229–5236.

Colvin Vicki L. The potential environmental impact of engineered nanomaterials // Nature Biotechnology. 2004. 22, 6. 760–760.

Conkle Jeremy L., Gan Jay, Anderson Michael A. Degradation and sorption of commonly detected PPCPs in wetland sediments under aerobic and anaerobic conditions // Journal of Soils and Sediments. 2012. 12, 7. 1164–1173.

Conkle Jeremy L., White John R., Metcalfe Chris D. Reduction of pharmaceutically active compounds by a lagoon wetland wastewater treatment system in Southeast Louisiana // Chemosphere. 2008. 73, 11. 1741–1748.

Cooper David, Griffin Paul, Cooper Paul. Factors affecting the longevity of subsurface horizontal flow systems operating as tertiary treatment for sewage effluent // Proceedings of the 10th International Conference on Wetland Systems for Water Pollution Control, 23–29 September 2006; Ministerio de Ambiente, do Ordenamento do Territori e do Desenvolvimento Regional (MAOTDR) and IWA: Lisbon, Portugal. 2006. 621–629.

Cornelis Geert, Doolette Casey, Thomas Madeleine, McLaughlin Mike J., Kirby Jason K., Beak Douglas G., Chittleborough David. Retention and dissolution of engineered silver nanoparticles in natural soils // Soil Science Society of America Journal. 2012. 76. 891.

Coutris Claire, Joner Erik Jautris, Oughton Deborah Helen. Aging and soil organic matter content affect the fate of silver nanoparticles in soil // Science of the Total Environment. 2012. 420. 327–333.

Crane Mark, Watts Chris, Boucard Tatiana. Chronic aquatic environmental risks from exposure to human pharmaceuticals - review // Science of the Total Environment. 2006. 367. 23–41.

De Buyck Pieter-Jan. Ozonisatie en rietvelden voor de verwijdering van farmaceutica uit afvalwater // MSc thesis, Ghent University, Belgium. 2015. pp. 104.

Delay Markus, Dolt Tamara, Woellhaf Annette, Sembritzki Reinhard, Frimmel Fritz H. Interactions and stability of silver nanoparticles in the aqueous phase: Influence of natural organic matter (NOM) and ionic strength // Journal of Chromatography A. 2011. 1218, 27. 4206–4212.

Deng Ying-qing, White Jason C., Xing Bao-shan. Interactions between engineered nanomaterials and agricultural crops: implications for food safety // Journal of Zhejiang University SCIENCE A. 2014. 15, 8. 552–572.

Dierberg Forrest E., Juston John J., DeBusk Thomas A., Pietro Kathy, Gu Binhe. Relationship between hydraulic efficiency and phosphorus removal in a submerged aquatic vegetation-dominated treatment wetland // Ecological Engineering. 2005. 25, 1. 9–23.

Dietz Annette C., Schnoor Jerald L. Advances in phytoremediation // Environmental Health Perspectives. 2001. 109, Suppl. 1. 163–168.

Dietz Karl Josef, Herth Simone. Plant nanotoxicology // Trends in Plant Science. 2011. 16, 11. 582–589.

Dignac Marie F., Ginestet Philippe, Rybacki Danielle, Bruchet Auguste, Urbain Vincent, Scribe Pierre. Fate of wastewater organic pollution during activated sludge treatment: Nature of residual organic matter // Water Research. 2000. 34, 17. 4185–4194.

Doiron Kim, Pelletier Émilien, Lemarchand Karine. Impact of polymer-coated silver nanoparticles on marine microbial communities: A microcosm study // Aquatic Toxicology. 2012. 124-125. 22–27.

Donner Erica, Scheckel Kirk, Sekine Ryo, Popelka-Filcoff Rachel S., Bennett John W., Brunetti Gianluca, Naidu Ravi, McGrath Steve P., Lombi Enzo. Non-labile silver species in biosolids remain stable throughout 50 years of weathering and ageing // Environmental Pollution. 2015. 205. 78–86.

Dordio Ana, Pinto José, Barrocas Dias Cristina, Pinto Ana Paula, Palace Carvalho Alfredo J., Teixeira Dora Martins. Atenolol removal in microcosm constructed wetlands // International Journal of Environmental Analytical Chemistry. 2009a. 89, 8-12. 835–848.

Dordio Ana V., Duarte Cátia, Barreiros Margarida, Carvalho A. J Palace, Pinto A. P., Costa Cristina Teixeira da. Toxicity and removal efficiency of pharmaceutical metabolite clofibric acid by Typha spp. - Potential use for phytoremediation? // Bioresource Technology. 2009b. 100, 3. 1156–1161.

Dordio Ana V., Estêvão Candeias A. J., Pinto A. P., Teixeira da Costa C., Palace Carvalho A. J. Preliminary media screening for application in the removal of clofibric acid, carbamazepine and ibuprofen by SSF-constructed wetlands // Ecological Engineering. 2009c. 35, 2. 290–302.

Dordio Ana V., Teimão José, Ramalho Idália, Carvalho a. J Palace, Candeias a. J Estêvão. Selection of a support matrix for the removal of some phenoxyacetic compounds in constructed wetlands systems // Science of the Total Environment. 2007. 380, 1-3. 237–246.

Du Laing Gijs, Tack F. M G, Verloo Marc G. Performance of selected destruction methods for the determination of heavy metals in reed plants (Phragmites australis) // Analytica Chimica Acta. 2003. 497, 1-2. 191–198.

Ebbs Stephen D, Bradfield Scott J, Kumar Pawan, White Jason C, Musante Craig, Ma Xingmao. Accumulation of zinc, copper, or cerium in carrot (Daucus carota) exposed to metal oxide nanoparticles and metal ions // Environmental Science: Nano. 2016. 3. 114–126.

El Badawy Amro M., Scheckel Kirk G, Suidan Makram, Tolaymat Thabet. The impact of stabilization mechanism on the aggregation kinetics of silver nanoparticles // The Science of the Total Environment. 2012. 429. 325–331.

El Badawy Amro M., Silva Rendahandi G., Morris Brian, Scheckel Kirk G., Suidan Makram T., Tolaymat Thabet M. Surface charge-dependent toxicity of silver nanoparticles // Environmental Science and Technology. 2011. 45, 1. 283–287.

Environmental Protection Agency (EPA) . Program Overview: Total Maximum Daily Loads (TMDL) // Available in: https://www.epa.gov/tmdl/program-overview-total-maximum-daily-loads-tmdl. 1992.

European Commission . Council Directive of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture // Official Journal of the European Communities. 1986. 4, 7. 6–12.

European Commission . Council Directive concerning the protection of waters against pollution caused by nitrates from agricultural sources // Official Journal of the European Communities. 1991. L 375, 31.12.1991. 1–8.

European Commission . Commission implementing decision 2015/495 of 20 March 2015 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council // Official Journal of the European Union. 2015a. L78. 40–42.

European Commission Joint Research Centre. FATE map // Available in: http://fate-gis.jrc.ec.europa.eu/geohub/MapViewer.aspx?id=3. 2015b.

European Parliament . Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy // Official Journal of the European Communities. 2000. L 269, September 2000. 1–15.

European Parliament . Decision No. 2455/2001/EC of the European Parliament and of the Council establishing the list of priority substances in the field of water policy and amending Directive 2000/60/EC. // Official Journal of the European Union L 331. 2001. 2001, October. 1–5.

European Parliament . Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy // Official Journal of the European Union. 2013. 2013, July. L 226/1–L 226/17.

Fabrega Julia, Fawcett Shona R, Renshaw Joanna C, Lead Jamie R. Silver nanoparticle impact on bacterial growth: Effect of pH, concentration, and organic matter // Environmental Science & Technology. 2009. 43, 19. 7285–7290.

Fabrega Julia, Luoma Samuel N., Tyler Charles R., Galloway Tamara S., Lead Jamie R. Silver nanoparticles: Behaviour and effects in the aquatic environment // Environment International. 2011. 37, 2. 517–531.

Fan Jinlin, Zhang Bo, Zhang Jian, Ngo Huu Hao, Guo Wenshan, Liu Feifei, Guo Yeye, Wu Haiming. Intermittent aeration strategy to enhance organics and nitrogen removal in subsurface flow constructed wetlands // Bioresource Technology. 2013. 141. 117–122.

Faulwetter Jennifer L., Gagnon Vincent, Sundberg Carina, Chazarenc Florent, Burr Mark D., Brisson Jacques, Camper Anne K., Stein Otto R. Microbial processes influencing performance of treatment wetlands: A review // Ecological Engineering. 2009. 35, 6. 987–1004.

Ferrari Benoît, Paxéus Nicklas, Lo Giudice Roberto, Pollio Antonino, Garric Jeanne. Ecotoxicological impact of pharmaceuticals found in treated wastewaters : study of carbamazepine, clofibric acid, and diclofenac // Ecotoxicology and Environmental Safety. 2003. 55. 359–370.

Ferrari Benoît Benoit, Mons Raphael, Vollet Bernard, Fraysse Benoît Benoit, Paxeus Nicklas, Vollat Bernard, Fraysse Benoît Benoit, Paxéus Nicklas, Lo Giudice Roberto, Pollio Antonino, Garric Jeanne, Iudice R Oberto L O G, Ollio A Ntonino P, Arric J Eanne G. Environmental risk assessment of six human pharmaceuticals: are the current environmental risk assessment procedures sufficient for the protection of the aquatic environment? // Environmental toxicology and chemistry. 2004. 23, 5. 1344–54.

Gabet-Giraud V., Miège C., Choubert J. M., Ruel S. Martin, Coquery M. Occurrence and removal of estrogens and beta blockers by various processes in wastewater treatment plants // Science of the Total Environment. 2010. 408, 19. 4257–4269.

Gallego-Urrea Julián A, Perez Holmberg Jenny, Hassellöv Martin. Influence of different types of natural organic matter on titania nanoparticle stability: effects of counter ion concentration and pH // Environmental Science: Nano. 2014. 1, 2. 181.

Ganesh Rajagopalan, Smeraldi Josh, Hosseini Turaj, Khatib Leila, Olson Betty H, Rosso Diego. Evaluation of nano copper removal and toxicity in municipal wastewaters // Environmental Science & Technology. 2010. 44. 7808–7813.

Ganzleben Catherine, Pelsy Florent, Hansen Steffen, Corden Caspar, Grebot Ben, Sobey Martine. Review of environmental legislation for the regulatory control of nanomaterials // Retrieved from the European Union website: http://ec.europa.eu/environment/chemicals/nanotech/pdf/review_legislation.pdf. 2011.

Garcia-Rodríguez Aida, Matamoros Víctor, Fontàs Clàudia, Salvaó Victòria. The ability of biologically based wastewater treatment systems to remove emerging organic contaminants–a review // Environmental science and pollution research international. 2014. 21, 20. 11708–11728.

Gebhardt Wilhelm, Schröder Horst. Liquid chromatography-(tandem) mass spectrometry for the follow-up of the elimination of persistent pharmaceuticals during wastewater treatment applying biological wastewater treatment and advanced oxidation // Journal of Chromatography A. 2007. 1160, 1-2. 34–43.

Geranio L., Heuberger M., Nowack B. The behavior of silver nanotextiles during washing // Environmental Science and Technology. 2009. 43, 21. 8113–8118.

Ginebreda Antoni, Munoz Isabel, Alda Miren López de, Brix Rikke, López-Doval Julio, Barceló Damià. Environmental risk assessment of pharmaceuticals in rivers: Relationships between hazard indexes and aquatic macroinvertebrate diversity indexes in the Llobregat River (NE Spain) // Environment International. 2010. 36, 2. 153–162.

Glatzel Stephan, Kalbitz Karsten, Dalva Mike, Moore Tim. Dissolved organic matter properties and their relationship to carbon dioxide efflux from restored peat bogs // Geoderma. 2003. 113, 3-4. 397–411.

Göbel Anke, Thomsen Angela, McArdell Christa S., Joss Adriano, Giger Walter. Occurrence and sorption behavior of sulfonamides, macrolides, and trimethoprim in activated sludge treatment // Environmental Science and Technology. 2005. 39, 11. 3981–3989.

González Aridane G., Mombo Stéphane, Leflaive Joséphine, Lamy Alexandre, Pokrovsky Oleg S., Rols Jean Luc. Silver nanoparticles impact phototrophic biofilm communities to a considerably higher degree than ionic silver // Environmental Science and Pollution Research. 2015. 22, 11. 8412–8424.

Gottschalk Fadri, Nowack Bernd. The release of engineered nanomaterials to the environment. // Journal of Environmental Monitoring. 2011. 13, 5. 1145–1155.

Gottschalk Fadri, Sun Tianyin, Nowack Bernd. Environmental concentrations of engineered nanomaterials: Review of modeling and analytical studies // Environmental Pollution. 2013. 181. 287–300.

Greenberg Arnold.E., Clesceri Lenore.S., Eaton Andrew.D. Standard methods for the examination fo water and wastewater. Washington, DC: American Public Health Association, 1992.

Gros Meritxell, Petrović Mira, Ginebreda Antoni, Barceló Damià. Removal of pharmaceuticals during wastewater treatment and environmental risk assessment using hazard indexes // Environment International. 2010. 36, 1. 15–26.

Gross-Sorokin Melanie Y, Roast Stephen D, Brighty Geoffrey C. Assessment of Feminization of Male Fish in English Rivers by the Environment Agency of England and Wales // Environmental Health Perspectives. 2006. 114, April. 147–151.

Gunawan Cindy, Teoh Wey Yang, Marquis Christopher P., Amal Rose. Induced adaptation of Bacillus sp. to antimicrobial nanosilver // Small. 2013. 9, 21. 3554–3560.

Henriques Inês D S, Love Nancy G. The role of extracellular polymeric substances in the toxicity response of activated sludge bacteria to chemical toxins // Water Research. 2007. 41, 18. 4177–4185.

Henze Mogens, Comeau Yves. Wastewater Characterization // In Henze M., van Loosdrecht M.C.M. (Eds.): Biological Wastewater Treatment: Principles Modelling and Design. 2008. IWA Publishing.

Hijosa-Valsero María, Fink Guido, Schlüsener Michael P., Sidrach-Cardona Ricardo, Martín-Villacorta Javier, Ternes Thomas, Bécares Eloy. Removal of antibiotics from urban wastewater by constructed wetland optimization // Chemosphere. 2011. 83, 5. 713–719.

Hijosa-Valsero María, Matamoros Víctor, Martín-Villacorta Javier, Bécares Eloy, Bayona Josep M. Assessment of full-scale natural systems for the removal of PPCPs from wastewater in small communities // Water Research. 2010a. 44, 5. 1429–1439.

Hijosa-Valsero María, Matamoros Víctor, Sidrach-Cardona Ricardo, Martín-Villacorta Javier, Bécares Eloy, Bayona Josep M. Comprehensive assessment of the design configuration of constructed wetlands for the removal of pharmaceuticals and personal care products from urban wastewaters // Water Research. 2010b. 44, 12. 3669–3678.

Hijosa-Valsero María, Reyes-Contreras Carolina, Domínguez Carmen, Bécares Eloy, Bayona Josep M. Behaviour of pharmaceuticals and personal care products in constructed wetland compartments: Influent, effluent, pore water, substrate and plant roots // Chemosphere. 2016. 145. 508–517.

Hotze Ernest M, Phenrat Tanapon, Lowry Gregory V, Mellon Carnegie. Nanoparticle aggregation : challenges to understanding transport and reactivity in the environment // Journal of Environmental Quality. 2010. 39. 1909–1924.

Hou Jun, You Guoxiang, Xu Yi, Wang Chao, Wang Peifang, Miao Lingzhan, Ao Yanhui, Li Yi, Lv Bowen. Effects of CeO2 nanoparticles on biological nitrogen removal in a sequencing batch biofilm reactor and mechanism of toxicity. // Bioresource technology. 2015. 191. 73–8.

Hou Linlin, Li Kaiyang, Ding Yuanzhao, Li Yan, Chen Jian, Wu Xiaolei, Li Xiqing. Removal of silver nanoparticles in simulated wastewater treatment processes and its impact on COD and NH4 reduction // Chemosphere. 2012. 87, 3. 248–252.

Hua Guofen F., Li Long, Zhao Yaquin Q., Zhu Wei, Shen Juaqin Q. An integrated model of substrate clogging in vertical flow constructed wetlands // Journal of Environmental Management. 2013. 119. 67–75.

Huang Limin, Wang Zhengbao, Sun Jinyu, Miao Lei, Li Quanzhi, Yan Yushan, Zhao Dongyuan. Fabrication of ordered porous structures by self-assembly of zeolite nanocrystals // Journal of the American Chemical Society. 2000. 122, 14. 3530–3531.

Huynh Khanh A., Chen Kai Loon. Aggregation kinetics of citrate and polyvinylpyrrolidone coated silver nanoparticles in monovalent and divalent electrolyte solutions // Environmental Science and Technology. 2011. 45, 13. 5564–5571.

Hyland Katherine C., Dickenson Eric R V, Drewes Jörg E., Higgins Christopher P. Sorption of ionized and neutral emerging trace organic compounds onto activated sludge from different wastewater treatment configurations // Water Research. 2012. 46, 6. 1958–1968.

Ibánez Maria, Gracia-Lor Emma, Bijlsma Lubertus, Morales Emilio, Pastor Laura, Hernández Felix. Removal of emerging contaminants in sewage water subjected to advanced oxidation with ozone // Journal of Hazardous Materials. 2013. 260. 389–398.

Imfeld Gwenaël, Braeckevelt Mareike, Kuschk Peter, Richnow Hans H. Monitoring and assessing processes of organic chemicals removal in constructed wetlands // Chemosphere. 2009. 74, 3. 349–362.

Jacob Donna L., Borchardt Joshua D., Navaratnam Leelaruban, Otte Marinus L., Bezbaruah Achintya N. Uptake and translocation of Ti from nanoparticles in crops and wetland plants // International Journal of Phytoremediation. 2013. 15, 2. 142–153.

Jelic Aleksandra, Gros Meritxell, Ginebreda Antoni, Cespedes-Sánchez Raquel, Ventura Francesc, Petrovic Mira, Barcelò Damiá. Occurrence, partition and removal of pharmaceuticals in sewage water and sludge during wastewater treatment // Water Research. 2011. 45, 3. 1165–1176.

Jelic Aleksandra, Rodriguez-Mozaz Sara, Barceló Damia, Gutierrez Oriol. Impact of in-sewer transformation on 43 pharmaceuticals in a pressurized sewer under anaerobic conditions // Water Research. 2015. 68. 98–108.

Jiang Fu Ying, Chen Xin, Luo An Cheng. Iron plaque formation on wetland plants and its influence on phosphorus, calcium and metal uptake // Aquatic Ecology. 2009. 43, 4. 879–890.

Jin Xiaowei, Zha Jinmiao, Xu Yiping, Giesy John P., Richardson Kristine L., Wang Zijian. Derivation of predicted no effect concentrations (PNEC) for 2,4,6-trichlorophenol based on Chinese resident species // Chemosphere. 2012. 86, 1. 17–23.

Jones O. A.H., Voulvoulis N., Lester J. N. Human Pharmaceuticals in Wastewater Treatment Processes // Critical Reviews in Environmental Science and Technology. 2005. 35, 4. 401–427.

Joshi Nimisha, Ngwenya Bryne T., French Christopher E. Enhanced resistance to nanoparticle toxicity is conferred by overproduction of extracellular polymeric substances // Journal of Hazardous Materials. 2012. 241-242. 363–370.

Joss Adriano, Keller Elvira, Alder Alfredo C., Göbel Anke, McArdell Christa S., Ternes Thomas, Siegrist Hansruedi. Removal of pharmaceuticals and fragrances in biological wastewater treatment // Water Research. 2005. 39, 14. 3139–3152.

Joss Adriano, Zabczynski Sebastian, Göbel Anke, Hoffmann Burkhard, Löffler Dirk, McArdell Christa S., Ternes Thomas a., Thomsen Angela, Siegrist Hansruedi. Biological degradation of pharmaceuticals in municipal wastewater treatment: Proposing a classification scheme // Water Research. 2006. 40, 8. 1686–1696.

Kadlec Robert H., Wallace Scott D. Treatment Wetlands. Boca Raton: CRC Press, 2009. 2nd ed.

Kaegi R., Ulrich A., Sinnet B., Vonbank R., Wichser A., Zuleeg S., Simmler H., Brunner S., Vonmont H., Burkhardt M., Boller M. Synthetic TiO2 nanoparticle emission from exterior facades into the aquatic environment // Environmental Pollution. 2008. 156, 2. 233–239.

Kaegi Ralf, Voegelin Andreas, Ort Christoph, Sinnet Brian, Thalmann Basilius, Krismer Jasmin, Hagendorfer Harald, Elumelu Maline, Mueller Elisabeth. Fate and transformation of silver nanoparticles in urban wastewater systems // Water Research. 2013. 47, 12. 3866–3877.

Kaegi Ralf, Voegelin Andreas, Sinnet Brian, Zuleeg Steffen, Hagendorfer Harald, Burkhardt Michael, Siegrist Hansruedi. Behavior of metallic silver nanoparticles in a pilot wastewater treatment plant // Environmental Science and Technology. 2011. 45, 9. 3902–3908.

Kanel Sushil R., Flory Jason, Meyerhoefer Allie, Fraley Jessica L., Sizemore Ioana E., Goltz Mark N. Influence of natural organic matter on fate and transport of silver nanoparticles in saturated porous media: laboratory experiments and modeling // Journal of Nanoparticle Research. 2015. 17, 3.

Kang Fuxing, Alvarez Pedro J J, Zhu Dongqiang. Microbial Extracellular Polymeric Substances Reduce Ag+ to Silver Nanoparticles and Antagonize Bactericidal Activity Microbial Extracellular Polymeric Substances Reduce Ag + to Silver Nanoparticles and // Environmental Science & Technology. 2014. 48. 316–322.

Kasprzyk-Hordern Barbara, Dinsdale Richard M., Guwy Alan J. The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters // Water Research. 2009. 43, 2. 363–380.

Katsoyiannis Athanasios, Samara Constantini. The fate of dissolved organic carbon (DOC) in the wastewater treatment process and its importance in the removal of wastewater contaminants. // Environmental Science and Pollution Research International. 2007. 14, 5. 284–292.

Keller Arturo A., Lazareva Anastasiya. Predicted releases of engineered nanomaterials: from global to regional to local // Environmental Science & Technology Letters. 2013. 1, 1. 65–70.

Kent Ronald D., Oser Joel G., Vikesland Peter J. Controlled evaluation of silver nanoparticle sulfidation in a full-scale wastewater treatment plant // Environmental Science and Technology. 2014. 48, 15. 8564–8572.

Keren Iris, Kaldalu Niilo, Spoering Amy, Wang Yipeng, Lewis Kim. Persister cells and tolerance to antimicrobials // FEMS Microbiology Letters. 2004. 230, 1. 13–18.

Kim Bojeong, Park Chee Sung, Murayama Mitsuhiro, Hochella Michael F. Discovery and characterization of silver sulfide nanoparticles in final sewage sludge products // Environmental Science and Technology. 2010. 44, 19. 7509–7514.

Kimura Katsuki, Hara Hiroe, Watanabe Yoshimasa. Removal of pharmaceutical compounds by submerged membrane bioreactors (MBRs) // Desalination. 2005. 178. 135–140.

Kiser Mehlika A., Ryu Hodon, Jang Hyunyoung, Hristovski Kiril, Westerhoff Paul. Biosorption of nanoparticles to heterotrophic wastewater biomass // Water Research. 2010. 44, 14. 4105–4114.

Klaine Stephen J, Alvarez Pedro J J, Batley Graeme E, Fernandes Teresa F, Handy Richard D, Lyon Delina Y, Mahendra Shaily, McLaughlin Michael J, Lead Jamie R. Nanomaterials in the environment: behavior, fate, bioavailability, and effects. // Environmental toxicology and chemistry / SETAC. 2008. 27, 9. 1825–1851.

Krämer Ute. Metal hyperaccumulation in plants. // Annual Review of Plant Biology. 2010. 61. 517–34.

Kroll Alexandra, Behra Renata, Kaegi Ralf, Sigg Laura. Extracellular polymeric substances (EPS) of freshwater biofilms stabilize and modify CeO2 and Ag nanoparticles // PLoS ONE. 2014. 9, 10.

Kroll Alexandra, Matzke Marianne, Rybicki Marcus, Obert-Rauser Patrick, Burkart Corinna, Jurkschat Kerstin, Verweij Rudo, Sgier Linn, Jungmann Dirk, Backhaus Thomas, Svendsen Claus. Mixed messages from benthic microbial communities exposed to nanoparticulate and ionic silver: 3D structure picks up nano-specific effects, while EPS and traditional endpoints indicate a concentration-dependent impact of silver ions // Environmental Science and Pollution Research. 2016. 23, 5. 4218–4234.

Kümmerer Klaus. Antibiotics in the aquatic environment - A review - Part I // Chemosphere. 2009. 75, 4. 417–434.

Kümmerer Klaus. Pharmaceuticals in the environment // Annual Review of Environment and Resources. 2010. 35. 57–75.

Kwok Kevin W H, Auffan Melanie, Badireddy Appala R., Nelson Clay M., Wiesner Mark R., Chilkoti Ashutosh, Liu Jie, Marinakos Stella M., Hinton David E. Uptake of silver nanoparticles and toxicity to early life stages of Japanese medaka (Oryzias latipes): Effect of coating materials // Aquatic Toxicology. 2012. 120-121. 59–66.

Lahti Marja, Oikari Aimo. Microbial transformation of pharmaceuticals naproxen, bisoprolol, and diclofenac in aerobic and anaerobic environments // Archives of Environmental Contamination and Toxicology. 2011. 61, 2. 202–210.

Lee Sungyun, Kang Su Il, Lim Jae Lim, Huh Yu Jeong, Kim Kap Soon, Cho Jaeweon. Evaluating controllability of pharmaceuticals and metabolites in biologically engineered processes, using corresponding octanol-water distribution coefficient // Ecological Engineering. 2011. 37, 10. 1595–1600.

Lenz Ailyn P., Williamson Kerry S., Pitts Betsey, Stewart Philip S., Franklin Michael J. Localized gene expression in Pseudomonas aeruginosa biofilms // Applied and Environmental Microbiology. 2008. 74, 14. 4463–4471.

Lesage E., Rousseau D. P L, Meers E., Tack F. M G, De Pauw N. Accumulation of metals in a horizontal subsurface flow constructed wetland treating domestic wastewater in Flanders, Belgium // Science of the Total Environment. 2007. 380, 1-3. 102–115.

Levard Clément, Hotze E. Matt, Lowry Gregory V., Brown Gordon E. Environmental transformations of silver nanoparticles: Impact on stability and toxicity // Environmental Science and Technology. 2012. 46, 13. 6900–6914.

Levard Clément, Hotze Ernest M., Colman Benjamin P., Dale Amy L., Truong Lisa, Yang X. Y., Bone Audrey J., Brown Gordon E., Tanguay Robert L., Di Giulio Richard T., Bernhardt Emily S., Meyer Joel N., Wiesner Mark R., Lowry Gregory V. Sulfidation of silver nanoparticles: Natural antidote to their toxicity // Environmental Science and Technology. 2013a. 47, 23. 13440–13448.

Levard Clément, Mitra Sumit, Yang Tiffany, Jew Adam D., Badireddy Appala Raju, Lowry Gregory V., Brown Gordon E. Effect of chloride on the dissolution rate of silver nanoparticles and toxicity to E. coli // Environmental Science and Technology. 2013b. 47, 11. 5738–5745.

Levard Clément, Reinsch Brian C., Michel F. Marc, Oumahi Camella, Lowry Gregory V., Brown Gordon E. Sulfidation processes of PVP-coated silver nanoparticles in aqueous solution: Impact on dissolution rate // Environmental Science and Technology. 2011. 45, 12. 5260–5266.

Li Lingxiangyu, Hartmann Georg, Schuster Michael. Quantification of nanoscale silver particles removal and release from municipal wastewater treatment plants in Germany // Environmental Science & Technology. 2013. 47. 7317–7323.

Li Mei, Zhu Lizhong, Lin Daohui. Toxicity of ZnO nanoparticles to Escherichia coli: Mechanism and the influence of medium components // Environmental Science and Technology. 2011. 45, 5. 1977–1983.

Li Xuan, Lenhart John J. Aggregation and dissolution of silver nanoparticles in natural surface water // Environmental Science and Technology. 2012. 46, 10. 5378–5386.

Li Xuan, Lenhart John J., Walker Harold W. Dissolution-accompanied aggregation kinetics of silver nanoparticles // Langmuir. 2010. 26, 22. 16690–16698.

Li Yifei, Zhang Jiefeng, Zhu Guibing, Liu Yu, Wu Bing, Ng Wun Jern, Appan Adhityan, Tan Soon Keat. Phytoextraction, phytotransformation and rhizodegradation of ibuprofen associated with Typha angustifolia in a horizontal subsurface flow constructed wetland // Water Research. 2016. 102. 294–304.

Limbach L.K., Bereiter R., Galli R., Stark W.J. Removal of oxide nanoparticles in a model waste water treatment plant // Environmental Science and Technology. 2008. 42. 5828–5833.

Lin Daohui, Tian Xiaoli, Wu Fengchang, Xing Baoshan. Fate and transport of engineered nanomaterials in the environment. // Journal of Environmental Quality. 2010. 39, 6. 1896–1908.

Lindqvist Niina, Tuhkanen Tuula, Kronberg Leif. Occurrence of acidic pharmaceuticals in raw and treated sewages and in receiving waters // Water Research. 2005. 39, 11. 2219–2228.

Liu Huaqing, Hu Zhen, Zhang Jian, Ngo Huu Hao, Guo Wenshan, Liang Shuang, Fan Jinlin, Lu Shaoyong, Wu Haiming. Optimizations on supply and distribution of dissolved oxygen in constructed wetlands: A review // Bioresource Technology. 2016. 214. 797–805.

Liu Jingyu, Hurt Robert H. Ion release kinetics and particle persistence in aqueous nano silver colloids // Environmental Science and Technology. 2010. 44, 6. 2169–2175.

Liu Jingyu, Pennell Kelly G., Hurt Robert H. Kinetics and mechanisms of nanosilver oxysulfidation // Environmental Science and Technology. 2011. 45, 17. 7345–7353.

Lombi Enzo, Donner Erica, Taheri Shima, Tavakkoli Ehsan, Jämting Asa K., McClure Stuart, Naidu Ravi, Miller Bradley W., Scheckel Kirk G., Vasilev Krasimir. Transformation of four silver/silver chloride nanoparticles during anaerobic treatment of wastewater and post-processing of sewage sludge // Environmental Pollution. 2013. 176. 193–197.

Lombi Enzo, Donner Erica, Tavakkoli Ehsan, Turney Terence W., Naidu Ravi, Miller Bradley W., Scheckel Kirk G. Fate of zinc oxide nanoparticles during anaerobic digestion of wastewater and post-treatment processing of sewage sludge // Environmental Science and Technology. 2012. 46, 16. 9089–9096.

Loos Robert, Gawlik Bernd Manfred, Locoro Giovanni, Rimaviciute Erika, Contini Serafino, Bidoglio Giovanni. EU-wide survey of polar organic persistent pollutants in European river waters // Environmental Pollution. 2009. 157, 2. 561–568.

Lorphensri Oranuj, Intravijit Jittipong, Sabatini David A., Kibbey T. C G, Osathaphan Khemarath, Saiwan Chintana. Sorption of acetaminophen, 17a-ethynyl estradiol, nalidixic acid, and norfloxacin to silica, alumina, and a hydrophobic medium // Water Research. 2006. 40, 7. 1481–1491.

Lowry Gregory V., Espinasse Benjamin P., Badireddy Appala Raju, Richardson Curtis J., Reinsch Brian C., Bryant Lee D., Bone Audrey J., Deonarine Amrika, Chae Soryong, Therezien Mathieu, Colman Benjamin P., Hsu-Kim Heileen, Bernhardt Emily S., Matson Cole W., Wiesner Mark R. Long-term transformation and fate of manufactured Ag nanoparticles in a simulated large scale freshwater emergent wetland // Environmental Science and Technology. 2012a. 46, 13. 7027–7036.

Lowry Gregory V., Gregory Kelvin B., Apte Simon C., Lead Jamie R. Transformations of nanomaterials in the environment // Environmental Science & Technology. 2012b. 46, 13. 6893–6899.

Luo Zhuanxi, Chen Zheng, Qiu Zhaozheng, Li Yancai, Laing Gijs Du, Liu Aifen, Yan Changzhou. Gold and silver nanoparticle effects on ammonia-oxidizing bacteria cultures under ammoxidation // Chemosphere. 2014. 120. 737–742.

Ma Rui, Levard Clément, Marinakos Stella M., Cheng Yingwen, Liu Jie, Michel F. Marc, Brown Gordon E., Lowry Gregory V. Size-controlled dissolution of organic-coated silver nanoparticles // Environmental Science and Technology. 2012. 46, 2. 752–759.

Ma Rui, Levard Clément, Michel F. Marc, Brown Gordon E., Lowry Gregory V. Sulfidation mechanism for Zinc oxide nanoparticles and the effect of sulfidation on their solubility // Environmental Science Technology. 2013. 47. 2527–2534.

Ma Rui, Stegemeier John, Levard Clement, Dale James, Noack Clinton W, Yang Tittany, Brown Gordon, Lowry Gregory. Sulfidation of copper oxide nanoparticles and properties of resulting copper sulfide // Environmental Science: Nano. 2014. 1. 347–357.

Ma Yanjun, Metch Jacob W., Vejerano Eric P., Miller Ian J., Leon Elena C., Marr Linsey C., Vikesland Peter J., Pruden Amy. Microbial community response of nitrifying sequencing batch reactors to silver, zero-valent iron, titanium dioxide and cerium dioxide nanomaterials // Water Research. 2015. 68. 87–97.

Markus Arjen A., Parsons John R., Roex Eewin W M, Kenter G. C M, Laane Remi W P M. Predicting the contribution of nanoparticles (Zn, Ti, Ag) to the annual metal load in the Dutch reaches of the Rhine and Meuse // Science of the Total Environment. 2013. 456-457. 154–160.

Matamoros Víctor, Bayona Josep M. Elimination of pharmaceuticals and personal care products in subsurface flow constructed wetlands. // Environmental Science & Technology. 2006. 40, 18. 5811–5816.

Matamoros Víctor, Nguyen Loc Xuan, Arias Carlos a., Salvadó Victòria, Brix Hans. Evaluation of aquatic plants for removing polar microcontaminants: A microcosm experiment // Chemosphere. 2012. 88, 10. 1257–1264.

Matamoros Víctor, Puigagut Jaume, García Joan, Bayona Josep M. Behavior of selected priority organic pollutants in horizontal subsurface flow constructed wetlands: A preliminary screening // Chemosphere. 2007. 69, 9. 1374–1380.

Matamoros Víctor, Rodríguez Yolanda, Albaigés Joan. A comparative assessment of intensive and extensive wastewater treatment technologies for removing emerging contaminants in small communities // Water Research. 2016. 88. 777–785.

Matamoros Víctor, Salvadó Victòria. Evaluation of the seasonal performance of a water reclamation pond-constructed wetland system for removing emerging contaminants // Chemosphere. 2012. 86, 2. 111–117.

Matamoros Viítor, García Joan, Bayona Josep M. Behavior of selected pharmaceuticals in subsurface flow constructed wetlands: A pilot-scale study // Environmental Science and Technology. 2005. 39, 14. 5449–5454.

Maurer Max, Escher Beata I., Richle Philip, Schaffner Christian, Alder Alfredo C. Elimination of beta-blockers in sewage treatment plants // Water Research. 2007. 41, 7. 1614–1622.

McEneff Gillian, Barron Leon, Kelleher Brian, Paull Brett, Quinn Brian. A year-long study of the spatial occurrence and relative distribution of pharmaceutical residues in sewage effluent, receiving marine waters and marine bivalves // Science of the Total Environment. 2014. 476-477. 317–326.

Metreveli George, Philippe Allan, Schaumann Gabriele E. Disaggregation of silver nanoparticle homoaggregates in a river water matrix // Science of the Total Environment. 2015. 535. 35–44.

Michael Irene, Rizzo Luigi, McArdell Christa S., Manaia Célia M., Merlin Christophe, Schwartz Thomas, Dagot Christophe, Fatta-Kassinos Despo. Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: A review // Water Research. 2013. 47, 3. 957–995.

Millot Yoann. Use of alternative matrix materials and configurations for optimizing nitrogen removal in constructed wetlands // PhD thesis, INSA Lyon & Ghent University, France. 2017. pp. 211.

Miralles Pola, Church Tamara L., Harris Andrew T. Toxicity, uptake, and translocation of engineered nanomaterials in vascular plants // Environmental Science and Technology. 2012. 46, 17. 9224–9239.

Mitrano Denise M., Rimmele Elisa, Wichser Adrian, Erni Rolf, Height Murray, Nowack Bernd. Presence of nanoparticles in wash water from conventional silver and nano-silver textiles // ACS Nano. 2014. 8, 7. 7208–7219.

Molle Pascal. Filtres plantes de roseaux: limites hydrauliques et rétention du phosphore // PhD thesis, IRSTEA, France. 2003. pp. 280.

Molle Pascal, Liénard A., Boutin Catherine, Merlin G., Iwema A. How to treat raw sewage with constructed wetlands: an overview of the French systems // Water Science and Technology. 2005. 51, 9. 11–21.

Moore Joe D., Stegemeier John P., Bibby Kyle, Marinakos Stella M., Lowry Gregory V., Gregory Kelvin B. Impacts of pristine and transformed Ag and Cu engineered nanomaterials on surficial sediment microbial communities appear short-lived // Environmental Science and Technology. 2016. 50, 5. 2641–2651.

Morones Jose Ruben, Elechiguerra Jose Luis, Camacho Alejandra, Holt Katherine, Kouri Juan B, Ramírez Jose Tapia, Yacaman Miguel Jose. The bactericidal effect of silver nanoparticles. // Nanotechnology. 2005. 16, 10. 2346–2353.

Mrozik Wojciech, Stefaska Justyna. Adsorption and biodegradation of antidiabetic pharmaceuticals in soils // Chemosphere. 2014. 95. 281–288.

Nivala Jaime, Hoos M. B., Cross C., Wallace Scott, Parkin Gene. Treatment of landfill leachate using an aerated, horizontal subsurface-flow constructed wetland // Science of the Total Environment. 2007. 380, 1-3. 19–27.

Nivala Jaime, Rousseau Diederik P L. Reversing clogging in subsurface-flow constructed wetlands by hydrogen peroxide treatment: Two case studies // Water Science and Technology. 2009. 59, 10. 2037–2046.

Nödler Karsten, Hillebrand Olav, Idzik Krzysztof, Strathmann Martin, Schiperski Ferry, Zirlewagen Johannes, Licha Tobias. Occurrence and fate of the angiotensin II receptor antagonist transformation product valsartan acid in the water cycle - A comparative study with selected β-blockers and the persistent anthropogenic wastewater indicators carbamazepine and acesulfame // Water Research. 2013. 47, 17. 6650–6659.

Noguera-Oviedo Katia, Aga Diana S. Lessons learned from more than two decades of research on emerging contaminants in the environment // Journal of Hazardous Materials. 2016. 316. 242–251.

OECD . OECD guidelines for the testing of chemicals - 303A Activated sludge units // Available in: http://www.oecd.org/env/ehs/testing/34898616.pdf. 2001.

Odzak Niksa, Kistler David, Behra Renata, Sigg Laura. Dissolution of metal and metal oxide nanoparticles in aqueous media // Environmental Pollution. 2014. 191. 132–138.

Olvera-Vargas Hugo, Leroy Sébastien, Rivard Michael, Oturan Nihal, Oturan Mehmet, Buisson Didier. Microbial biotransformation of furosemide for environmental risk assessment: identification of metabolites and toxicological evaluation // Environmental Science and Pollution Research. 2016. 23, 22. 22691–22700.

Oosterhuis Mathijs, Sacher Frank, Laak Thomas L. ter. Prediction of concentration levels of metformin and other high consumption pharmaceuticals in wastewater and regional surface water based on sales data // Science of the Total Environment. 2013. 442. 380–388.

Oulton Rebekah L, Kohn Tamar, Cwiertny David M. Pharmaceuticals and personal care products in effluent matrices: A survey of transformation and removal during wastewater treatment and implications for wastewater management. // Journal of environmental monitoring : JEM. 2010. 12, 11. 1956–1978.

Øvreås L, Forney L, Daae F L, Torsvik V. Distribution of bacteroplankton in meromictic lake Sælenvannet, as deteremined by denaturing gradient gel electrophorresis of PCR-amplified gene fragments coding for 16S rRNA // Applied and Environmental Microbiology. 1997. 63, 9. 3367–3373.

Pascoe David, Karntanut Wanchamai, Müller Karsten T. Do pharmaceuticals affect freshwater invertebrates ? A study with the cnidarian Hydra vulgaris // Chemosphere. 2003. 51. 521–528.

Pauwels B., Verstraete Willy. The treatment of hospital wastewater: An appraisal // Journal of Water and Health. 2006. 4, 4. 405–416.

Peralta-Videa Jose R., Zhao Lijuan, Lopez-Moreno Martha L., Rosa Guadalupe de la, Hong Jie, Gardea-Torresdey Jorge L. Nanomaterials and the environment: A review for the biennium 2008–2010 // Journal of Hazardous Materials. 2011. 186, 1. 1–15.

Peretyazhko Tanya S, Zhang Qingbo, Colvin Vicki L. Size-Controlled Dissolution of Silver Nanoparticles at Neutral and Acidic pH Conditions: Kinetics and Size Changes // Environmental Science & Technology. 2014. 48, 20. 11954–11961.

Persson Jesper, Wittgren Hans B. How hydrological and hydraulic conditions affect performance of ponds // Ecological Engineering. 2003. 21, 4-5. 259–269.

Péry A M, Gust M, Vollat B, Mons R, Ramil M, Fink G, Ternes T, Garric J. Fluoxetine effects assessment on the life cycle of aquatic invertebrates // Chemosphere. 2008. 73, 3. 300–304.

Piccapietra Flavio, Sigg Laura, Behra Renata. Colloidal stability of carbonate-coated silver nanoparticles in synthetic and natural freshwater // Environmental Science and Technology. 2012. 46, 2. 818–825.

Pilon-Smits Elizabeth. Phytoremediation // Annual Review of Plant Biology. 2005. 56, 1. 15–39.

Pinney Michael L., Westerhoff Paul K., Baker Larry. Transformations in dissolved organic carbon through constructed wetlands // Water Research. 2000. 34, 6. 1897–1911.

Pomiès M., Choubert J. M., Wisniewski C., Miège C., Budzinski H., Coquery M. Lab-scale experimental strategy for determining micropollutant partition coefficient and biodegradation constants in activated sludge // Environmental Science and Pollution Research. 2015. 22, 6. 4383–4395.

Puay Ni-Qing, Qiu Guanglei, Ting Yen-Peng. Effect of Zinc oxide nanoparticles on biological wastewater treatment in a sequencing batch reactor // Journal of Cleaner Production. 2015. 88. 139–145.

Puigagut Jaume, Villaseñor José, Salas Juan José, Bécares Eloy, García Joan. Subsurface-flow constructed wetlands in Spain for the sanitation of small communities: A comparative study // Ecological Engineering. 2007. 30, 4. 312–319.

Quanrud David M., Karpiscak Martin M., Lansey Kevin E., Arnold Robert G. Transformation of effluent organic matter during subsurface wetland treatment in the Sonoran Desert // Chemosphere. 2004. 54, 6. 777–788.

Quik Joris T K, Velzeboer Ilona, Wouterse Marja J., Koelmans A. A., Meent Dik van de. Heteroaggregation and sedimentation rates for nanomaterials in natural waters // Water Research. 2014. 48, 1. 269–279.

Quinn Brian, Gagné François, Blaise Christian. An investigation into the acute and chronic toxicity of eleven pharmaceuticals (and their solvents) found in wastewater effluent on the cnidarian, Hydra attenuata // Science of the Total Environment. 2008. 389, 2-3. 306–314.

Quintana José Benito, Weiss Stefan, Reemtsma Thorsten. Pathways and metabolites of microbial degradation of selected acidic pharmaceutical and their occurrence in municipal wastewater treated by a membrane bioreactor // Water Research. 2005. 39, 12. 2654–2664.

Radjenović Jelena, Petrović Mira, Barceló Damià, Petrović Mira. Advanced mass spectrometric methods applied to the study of fate and removal of pharmaceuticals in wastewater treatment // Trends in Analytical Chemistry. 2007. 26, 11. 1132–1144.

Rathnayake Sewwandi, Unrine Jason M., Judy Jonathan, Miller Anne Frances, Rao William, Bertsch Paul M. Multitechnique investigation of the pH dependence of phosphate induced transformations of ZnO nanoparticles // Environmental Science and Technology. 2014. 48, 9. 4757–4764.

Ratte Hans Toni. Bioaccumulation and toxicity of silver compounds: A review // Environmental Toxicology and Chemistry. 1999. 18, 1. 89–108.

Reif R., Besancon A., Le Corre K., Jefferson B., Lema J. M., Omil F. Comparison of PPCPs removal on a parallel-operated MBR and AS system and evaluation of effluent post-treatment on vertical flow reed beds // Water Science and Technology. 2011. 63, 10. 2411–2417.

Reinhold Dawn, Vishwanathan Saritha, Park Jung Jae, Oh David, Michael Saunders F. Assessment of plant-driven removal of emerging organic pollutants by duckweed // Chemosphere. 2010. 80, 7. 687–692.

Reinsch Brian. C., Levard Clément, Li Z., Ma Ri., Wise A., Gregory K. B., Brown G. E., Lowry Gregory V. Sulfidation of silver nanoparticles decreases Escherichia coli growth inhibition // Environmental Science and Technology. 2012. 46, 13. 6992–7000.

Reyes-Contreras Carolina, Hijosa-Valsero María, Sidrach-Cardona Ricardo, Bayona Josep M., Bécares Eloy. Temporal evolution in PPCP removal from urban wastewater by constructed wetlands of different configuration: A medium-term study // Chemosphere. 2012. 88, 2. 161–167.

Robichaud Christine Ogilvie, Uyar Ali Emre, Darby Michael R, Zucker Lynne G, Wiesner Mark R. Estimates of Upper Bounds and Trends in Nano-TiO2Production As a Basis for Exposure Assessment // Environmental Science & Technology. 2009. 43, 12. 4227–4233.

Rosal Roberto, Rodríguez Antonio, Perdigón-Melón José Antonio, Petre Alice, García-Calvo Eloy, Gómez María José, Agüera Ana, Fernández-Alba Amadeo R. Occurrence of emerging pollutants in urban wastewater and their removal through biological treatment followed by ozonation // Water Research. 2010. 44, 2. 578–588.

Rousseau Diederik P L, Vanrolleghem Peter a., De Pauw Niels. Constructed wetlands in Flanders: A performance analysis // Ecological Engineering. 2004. 23, 3. 151–163.

Rousseau Diederik P.L., De Pauw Niels, Geenens Dieter, Griffin P., Vanrolleghem Peter A. Do constructed wetlands contribute to a better river water quality? // The Proceedings of Second World Wide Workshop for Young Environmental Scientists, 14-17 May 2003, Vitry-sur-Seine, France. 2003. 61–71.

Rousseau Diederik P.L., Lesage Els, Story Anke, Vanrolleghem Peter A., De Pauw Niels. Constructed wetlands for water reclamation // Desalination. 2008. 218, 1-3. 181–189.

Rühmland Sabine, Wick Arne, Ternes Thomas A., Barjenbruch Matthias. Fate of pharmaceuticals in a subsurface flow constructed wetland and two ponds // Ecological Engineering. 2015. 80. 125–139.

Saeed Tanveer, Sun Guangzhi. A review on nitrogen and organics removal mechanisms in subsurface flow constructed wetlands: Dependency on environmental parameters, operating conditions and supporting media // Journal of Environmental Management. 2012. 112. 429–448.

Salt D E, Blaylock M, Kumar N P, Dushenkov V, Ensley B D, Chet I, Raskin I. Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants // Bio/technology. 1995. 13, 5. 468–474.

Scheurer Marco, Michel Amandine, Brauch Heinz Jürgen, Ruck Wolfgang, Sacher Frank. Occurrence and fate of the antidiabetic drug metformin and its metabolite guanylurea in the environment and during drinking water treatment // Water Research. 2012. 46, 15. 4790–4802.

Scheurer Marco, Ramil Maria, Metcalfe Chris D., Groh Stefanie, Ternes Thomas A. The challenge of analyzing beta-blocker drugs in sludge and wastewater // Analytical and Bioanalytical Chemistry. 2010. 396, 2. 845–856.

Schröder Horst, Gebhardt Wilhelm, Thevis Mario. Anabolic, doping, and lifestyle drugs, and selected metabolites in wastewater-detection, quantification, and behaviour monitored by high-resolution MS and MS n before and after sewage treatment // Analytical and Bioanalytical Chemistry. 2010. 398, 3. 1207–1229.

Schulman Lisa J, Sargent Edward V, Naumann Bruce D, Faria Ellen C, Dolan David G, Wargo John P. A Human Health Risk Assessment of Pharmaceuticals in the Aquatic Environment. Human and aquatic risk assessment. // Human and Ecological Risk Assessment: an International Journal. 2002. 8, 4. 657–680.

Shah Vishal, Jones Jamilee, Dickman Jenifer, Greenman Steven. Response of soil bacterial community to metal nanoparticles in biosolids // Journal of Hazardous Materials. 2014. 274. 399–403.

Sharif Fariya, Westerhoff Paul, Herckes Pierre. Sorption of trace organics and engineered nanomaterials onto wetland plant material // Environmental Science: Processes & Impacts. 2013. 15, 1. 267.

Sheng Zhiya, Liu Yang. Effects of silver nanoparticles on wastewater biofilms // Water Research. 2011. 45, 18. 6039–6050.

Silver Simon, Phung Le T. Bacterial heavy metal resistance: new surprises // Annual Reviews in Microbiology. 1996. 50, 1. 753–789.

Silver Simon, Phung Le T., Silver Gregg. Silver as biocides in burn and wound dressings and bacterial resistance to silver compounds // Journal of Industrial Microbiology and Biotechnology. 2006. 33, 7. 627–634.

Sintubin Liesje, De Gusseme Bart, Van Der Meeren Paul, Pycke Benny F G, Verstraete Willy, Boon Nico. The antibacterial activity of biogenic silver and its mode of action // Applied Microbiology and Biotechnology. 2011. 91, 1. 153–162.

Song Jee Eun, Phenrat Tanapon, Marinakos Stella, Xiao Yao, Liu Jie, Wiesner Mark R, Tilton Robert D, Lowry Gregory V. Hydrophobic Interactions Increase Attachment of Gum Arabic- and PVP-Coated Ag Nanoparticles to Hydrophobic Surfaces // Environmental Science & Technology. 2011. 45. 5988–5995.

Sotiriou Georgios A., Pratsinis Sotiris E. Antibacterial activity of nanosilver ions and particles // Environmental Science and Technology. 2010. 44, 14. 5649–5654.

Stankus Dylan P., Lohse Samuel E., Hutchison James E., Nason Jeffrey a. Interactions between natural organic matter and gold nanoparticles stabilized with different organic capping agents // Environmental Science and Technology. 2011. 45, 8. 3238–3244.

Sun Tian Yin, Gottschalk Fadri, Hungerbühler Konrad, Nowack Bernd. Comprehensive probabilistic modelling of environmental emissions of engineered nanomaterials // Environmental Pollution. 2014. 185. 69–76.

Ternes Thomas A., Herrmann Nadine, Bonerz Matthias, Knacker Thomas, Siegrist Hansruedi, Joss Adriano. A rapid method to measure the solid-water distribution coefficient (Kd) for pharmaceuticals and musk fragrances in sewage sludge // Water Research. 2004a. 38, 19. 4075–4084.

Ternes Thomas A., Joss Adriano, Siegrist Hansruedi. Scrutinizing Pharmaceuticals and Personal Care Products in Wastewater Treatment // Environmental Science and Technology. 2004b. 38, 20. 392–399.

Thalmann Basilius, Voegelin Andreas, Sinnet Brian, Morgenroth Eberhard, Kaegi Ralf. Sulfidation kinetics of silver nanoparticles reacted with metal sulfides // Environmental Science and Technology. 2014. 48, 9. 4885–4892.

Tolls Johannes. Sorption of veterinary pharmaceuticals in soils: A review // Environmental Science and Technology. 2001. 35, 17. 3397–3406.

Trapp Stefan, Karlson Ulrich. Aspects of phytoremediation of organic pollutants // Journal of Soils and Sediments. 2001. 1, 1. 37–43.

Trautwein Christoph, Kümmerer Klaus. Incomplete aerobic degradation of the antidiabetic drug Metformin and identification of the bacterial dead-end transformation product Guanylurea // Chemosphere. 2011. 85, 5. 765–773.

Tsuneda Satoshi, Aikawa Hirotoshi, Hayashi Hiroshi, Yuasa Atsushi, Hirata Akira. Extracellular polymeric substances responsible for bacterial adhesion onto solid surface // FEMS Microbiology Letters. 2003. 223, 2. 287–292.

VMM . Geoloket // Available in: http://geoloket.vmm.be. 2016.

Van Hoecke Karen, De Schamphelaere Karel a C, Van Der Meeren Paul, Smagghe Guy, Janssen Colin R. Aggregation and ecotoxicity of CeO2 nanoparticles in synthetic and natural waters with variable pH, organic matter concentration and ionic strength // Environmental Pollution. 2011. 159, 4. 970–976.

Van Koetsem Frederik. Idenfication of factors affecting the physicochemical behaviour and fate of engineered metallic nanoparticles released into aquatic environments // PhD thesis, Ghent University, Belgium. 2015. pp. 320.

Van Koetsem Frederik, Geremew Tewodros Tilahun, Wallaert Elien, Verbeken Kim, Van der Meeren Paul, Du Laing Gijs. Fate of engineered nanomaterials in surface water: Factors affecting interactions of Ag and CeO2 nanoparticles with (re)suspended sediments // Ecological Engineering. 2015. 80. 140–150.

Van Koetsem Frederik, Verstraete Simon, Wallaert Elien, Verbeken Kim, Van Der Meeren Paul, Rinklebe Jörg, Du Laing Gijs. Use of filtration techniques to study environmental fate of engineered metallic nanoparticles: Factors affecting filter performance // Journal of Hazardous Materials. 2016a. 322. 105–117.

Van Koetsem Frederik, Xiao Yi, Luo Zhuanxi, Du Laing Gijs. Impact of water composition on association of Ag and CeO2 nanoparticles with aquatic macrophyte Elodea canadensis // Environmental Science and Pollution Research. 2016b. 23, 6. 5277–5287.

Van Nuijs Alexander L.N., Tarcomnicu Isabela, Simons Ward, Bervoets Lieven, Blust Ronny, Jorens Philippe G, Neels Hugo, Covaci Adrian. Optimization and validation of a hydrophilic interaction liquid chromatography-tandem mass spectrometry method for the determination of 13 top-prescribed pharmaceuticals in influent wastewater // Analytical and Bioanalytical Chemistryhemistry. 2010. 398, 5. 2211–2222.

Van Ranst E., Verloo M., Demeyer A., Pauwels J.M. Manual for the soil chemistry and fertility laboratory: analytical methods for soil and plants equipment, and management of consumables. Ghent: Ghent University, 1999. 243 pp.

Vance Marina E., Kuiken Todd, Vejerano Eric P., McGinnis Sean P., Hochella Michael F., Hull David Rejeski. Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory // Beilstein Journal of Nanotechnology. 2015. 6, 1. 1769–1780.

Velzeboer Ilona, Quik Joris T.K., Meent Dik van de, Koelmans Albert A. Rapid settling of nanoparticles due to heteroaggregation with suspended sediment // Environmental Toxicology and Chemistry. 2014. 33, 8. 1766–1773.

Verbanck Michel, Vanderborght Jean-pierre, Wollast Roland. Major ion content of urban wastewater: assessment of per capita loading // Research Journal of the Water Pollution Control Federation. 1989. 61, 11/12. 1722–1728.

Vergeynst Leendert, Haeck Ashley, De Wispelaere Patrick, Van Langenhove Herman, Demeestere Kristof. Multi-residue analysis of pharmaceuticals in wastewater by liquid chromatography-magnetic sector mass spectrometry: Method quality assessment and application in a Belgian case study // Chemosphere. 2015. 119. S2–S8.

Vergeynst Leendert, Van Langenhove Herman, Joos Pieter, Demeestere Kristof. Suspect screening and target quantification of multi-class pharmaceuticals in surface water based on large-volume injection liquid chromatography and time-of-flight mass spectrometry // Analytical and Bioanalytical Chemistry. 2014. 406, 11. 2533–2547.

Verlicchi P., Al Aukidy M., Galletti A., Petrovic M., Barceló D. Hospital effluent: Investigation of the concentrations and distribution of pharmaceuticals and environmental risk assessment // Science of the Total Environment. 2012. 430. 109–118.

Verlicchi P., Galletti A., Petrovic M., Barceló D., Al Aukidy M., Zambello E. Removal of selected pharmaceuticals from domestic wastewater in an activated sludge system followed by a horizontal subsurface flow bed - Analysis of their respective contributions // Science of the Total Environment. 2013. 454-455. 411–425.

Verlicchi Paola, Zambello Elena. How efficient are constructed wetlands in removing pharmaceuticals from untreated and treated urban wastewaters? A review // Science of the Total Environment. 2014. 470-471. 1281–1306.

Vestel Jessica, Caldwell Daniel J., Constantine Lisa, D'Aco Vincent J., Davidson Todd, Dolan David G., Millard Steven P., Murray-Smith Richard, Parke Neil J., Ryan Jim J., Straub Jürg Oliver, Wilson Peter. Use of acute and chronic ecotoxicity data in environmental risk assessment of pharmaceuticals // Environmental Toxicology and Chemistry. 2016. 35, 5. 1201–1212.

Vieno Niina M., Tuhkanen Tuula, Kronberg Leif. Analysis of neutral and basic pharmaceuticals in sewage treatment plants and in recipient rivers using solid phase extraction and liquid chromatography-tandem mass spectrometry detection // Journal of Chromatography A. 2006. 1134, 1-2. 101–111.

Voice Thomas C., Weber Walter J. Sorption of hydrophobic compounds by sediments, soils and suspended solids-I. Theory and background // Water Research. 1983. 17, 10. 1433–1441.

Von der Kammer Frank, Ferguson P. Lee, Holden Patricia A., Masion Armand, Rogers Kim R., Klaine Stephen J., Koelmans Albert A., Horne Nina, Unrine Jason M. Analysis of engineered nanomaterials in complex matrices (environment and biota): General considerations and conceptual case studies // Environmental Toxicology and Chemistry. 2012. 31, 1. 32–49.

Vymazal Jan. The use of sub-surface constructed wetlands for wastewater treatment in the Czech Republic: 10 years experience // Ecological Engineering. 2002. 18, 5. 633–646.

Vymazal Jan. The use constructed wetlands with horizontal sub-surface flow for various types of wastewater // Ecological Engineering. 2009. 35, 1. 1–17.

Vymazal Jan. Long-term performance of constructed wetlands with horizontal sub-surface flow: Ten case studies from the Czech Republic // Ecological Engineering. 2011. 37, 1. 54–63.

Vymazal Jan, Kröpfelová Lenka, Švehla Jaroslav, Štíchová Jana. Can multiple harvest of aboveground biomass enhance removal of trace elements in constructed wetlands receiving municipal sewage? // Ecological Engineering. 2010. 36, 7. 939–945.

Vystavna Y., Le Coustumer P., Huneau F. Monitoring of trace metals and pharmaceuticals as anthropogenic and socio-economic indicators of urban and industrial impact on surface waters // Environmental Monitoring and Assessment. 2013. 185, 4. 3581–3601.

Wagner Thorsten. ij-particlesizer: ParticleSizer 1.0.3 Zenodo. 2016.

Wang Peng, Menzies Neal W, Lombi Enzo, Sekine Ryo, Blamey F Pax C, Hernandez-Soriano Maria C, Cheng Miaomiao, Kappen Peter, Peijnenburg Willie J G M, Tang Caixian, Kopittke Peter M. Silver sulfide nanoparticles (Ag2S-NPs) are taken up by plants and are phytotoxic // Nanotoxicology. 2015. 5390, September. 1–9.

Weber Kela P., Grove Jason a., Gehder Matthias, Anderson William a., Legge Raymond L. Data transformations in the analysis of community-level substrate utilization data from microplates // Journal of Microbiological Methods. 2007. 69, 3. 461–469.

Weber Kela P., Legge Raymond L. Community-level physiological profiling // In: Cummings, S.P. (Ed.). 2010a. Bioremediation. Humana Press.

Weber Kela P., Legge Raymond L. Method for the detachment of culturable bacteria from wetland gravel // Journal of Microbiological Methods. 2010b. 80, 3. 242–250.

Weber Kela P., Legge Raymond L. Dynamics in the bacterial community-level physiological profiles and hydrological characteristics of constructed wetland mesocosms during start-up // Ecological Engineering. 2011. 37, 5. 666–677.

Weber Kela P., Legge Raymond L. Comparison of the catabolic activity and catabolic profiles of rhizospheric, gravel-associated and interstitial microbial communities in treatment wetlands // Water Science and Technology. 2013. 67, 4. 886–893.

Weber Kela P., Mitzel Michael R., Slawson Robin M., Legge Raymond L. Effect of ciprofloxacin on microbiological development in wetland mesocosms // Water Research. 2011. 45, 10. 3185–3196.

Weber Kela P., Petersen Elijah J., Bissegger Sonja, Koch Iris, Zhang Jun, Reimer Kenneth J., Rehmann Lars, Slawson Robin M., Legge Raymond L., O'Carroll Denis M. Effect of gold nanoparticles and ciprofloxacin on microbial catabolism: A community-based approach // Environmental Toxicology and Chemistry. 2014. 33, 1. 44–51.

Weiss Jeff, Hondzo Miki, Biesboer David, Semmens Michael. Laboratory study of heavy metal phytoremediation by three wetland macrophytes. // International Journal of Phytoremediation. 2006. 8, 3. 245–259.

Wick Arne, Fink Guido, Joss Adriano, Siegrist Hansruedi, Ternes Thomas A. Fate of beta blockers and psycho-active drugs in conventional wastewater treatment // Water Research. 2009. 43, 4. 1060–1074.

Wiessner Arndt, Kappelmeyer Uwe, Kuschk Peter, Kästner Matthias. Sulphate reduction and the removal of carbon and ammonia in a laboratory-scale constructed wetland // Water Research. 2005. 39, 19. 4643–4650.

Winter Matthew J, Lillicrap Adam D, Caunter John E, Schaffner Christian, Alder Alfredo C, Ramil Maria, Ternes Thomas A, Giltrow Emma, Sumpter John P, Hutchinson Thomas H. Defining the chronic impacts of atenolol on embryo-larval development and reproduction in the fathead minnow (Pimephales promelas) // Aquatic Toxicology. 2008. 86. 361–369.

Wu Haiming, Zhang Jian, Ngo Huu Hao, Guo Wenshan, Hu Zhen, Liang Shuang, Fan Jinlin, Liu Hai. A review on the sustainability of constructed wetlands for wastewater treatment: Design and operation // Bioresource Technology. 2015. 175. 594–601.

Wu Shubiao, Kuschk Peter, Brix Hans, Vymazal Jan, Dong Renjie. Development of constructed wetlands inperformance intensifications for wastewater treatment: A nitrogen and organic matter targeted review // Water Research. 2014. 57. 40–45.

Wu Shubiao, Kuschk Peter, Wiessner Arndt, Müller Jochen, Saad Rania A B, Dong Renjie. Sulphur transformations in constructed wetlands for wastewater treatment: A review // Ecological Engineering. 2013. 52. 278–289.

Yoda Motoyuki, Miyaji Yusho, Kitagawa Mikio. Long Term Competition Between Sulfate Reducing and Methane Producing Bacteria for Acetate in Anaerobic Biofilm // Water Research. 1987. 21, 12. 1547–1556.

Yu Jim T., Bouwer Edward J., Coelhan Mehmet. Occurrence and biodegradability studies of selected pharmaceuticals and personal care products in sewage effluent // Agricultural Water Management. 2006. 86, 1-2. 72–80.

Yuan Zhihua, Li Jiangwei, Cui Li, Xu Bin, Zhang Hongwu, Yu Chang Ping. Interaction of silver nanoparticles with pure nitrifying bacteria // Chemosphere. 2013. 90, 4. 1404–1411.

Zepp Richard G., Baughman George L., Schlotshauer Patricia F. Comparison of photochemical behavior of various humic substances in water: I. Sunlight induced reactions of aquatic pollutants photosensitized by humic substances // Chemosphere. 1981. 10. 109–117.

Zhang Chiqian, Liang Zhihua, Hu Zhiqiang. Bacterial response to a continuous long-term exposure of silver nanoparticles at sub-ppm silver concentrations in a membrane bioreactor activated sludge system // Water Research. 2014a. 50. 350–358.

Zhang Chong Bang, Wang Jiang, Liu Wen Li, Zhu Si Xi, Ge Han Liang, Chang Scott X., Chang Jie, Ge Ying. Effects of plant diversity on microbial biomass and community metabolic profiles in a full-scale constructed wetland // Ecological Engineering. 2010a. 36, 1. 62–68.

Zhang Dong Qing, Gersberg Richard M., Hua Tao, Zhu Junfei, Tuan Nguyen Anh, Tan Soon Keat. Pharmaceutical removal in tropical subsurface flow constructed wetlands at varying hydraulic loading rates // Chemosphere. 2012a. 87, 3. 273–277.

Zhang Dong Qing, Gersberg Richard M., Zhu Junfei, Hua Tao, Jinadasa K. B S N, Tan Soon Keat. Batch versus continuous feeding strategies for pharmaceutical removal by subsurface flow constructed wetland // Environmental Pollution. 2012b. 167. 124–131.

Zhang Dong Qing, Hua Tao, Gersberg Richard M., Zhu Junfei, Ng Wun Jern, Tan Soon Keat. Carbamazepine and naproxen: Fate in wetland mesocosms planted with Scirpus validus // Chemosphere. 2013. 91, 1. 14–21.

Zhang Dongqing, Hua Tao, Xiao Fei, Chen Chunping, Gersberg Richard M., Liu Yu, Ng Wun Jern, Tan Soon Keat. Uptake and accumulation of CuO nanoparticles and CdS/ZnS quantum dot nanoparticles by Schoenoplectus tabernaemontani in hydroponic mesocosms // Ecological Engineering. 2014b. 70. 114–123.

Zhang Dongqing, Hua Tao, Xiao Fei, Chen Chunping, Gersberg Richard M., Liu Yu, Stuckey David, Ng Wun Jern, Tan Soon Keat. Phytotoxicity and bioaccumulation of ZnO nanoparticles in Schoenoplectus tabernaemontani // Chemosphere. 2015a. 120. 211–219.

Zhang Dongqing, Ni Wangdong, Gersberg Richard M., Ng Wun Jern, Tan Soon Keat. Performance Characterization of Pharmaceutical Removal by Horizontal Subsurface Flow Constructed Wetlands Using Multivariate Analysis // Clean - Soil, Air, Water. 2015b. 43, 8. 1181–1189.

Zhang Lie Yu, Zhang Lan, Liu Yong Ding, Shen Yin Wu, Liu Hao, Xiong Ying. Effect of limited artificial aeration on constructed wetland treatment of domestic wastewater // Desalination. 2010b. 250, 3. 915–920.

Zhang Wen. Nanoparticle Aggregation: Principles and Modeling // In: Capco, D.G., Chen, Y. (Eds.). 2014. Nanomaterial. Springer Science+Business Media.

Zhang Wen, Yao Ying, Li Kungang, Huang Ying, Chen Yongsheng. Influence of dissolved oxygen on aggregation kinetics of citrate-coated silver nanoparticles // Environmental Pollution. 2011a. 159, 12. 3757–3762.

Zhang Wen, Yao Ying, Sullivan Nicole, Chen Yongsheng. Modeling the primary size effects of citrate-coated silver nanoparticles on their ion release kinetics // Environmental Science and Technology. 2011b. 45, 10. 4422–4428.

Zhang Yang, Chen Yongsheng, Westerhoff Paul, Crittenden John. Impact of natural organic matter and divalent cations on the stability of aqueous nanoparticles // Water Research. 2009. 43, 17. 4249–4257.

Zhou Dongxu, Keller Arturo A. Role of morphology in the aggregation kinetics of ZnO nanoparticles // Water Research. 2010. 44, 9. 2948–2956.

Zhu Zheng-Jiang, Wang Huanhua, Yan Bo, Zheng Hao, Jiang Ying, Miranda Oscar R, Rotello Vincent M, Xing Baoshan, Vachet Richard W. Effect of surface charge on the uptake and distribution of gold nanoparticles in four plant species. // Environmental Science & Technology. 2012. 46, 22. 12391–8.

Summary

Contaminants of emerging concern (CECs), such as pharmaceuticals and metallic engineered nanomaterials (ENMs), can pose a risk to the environment even at very low concentrations (ng/L - μg/L). The human body metabolizes pharmaceuticals usually only partly which results in the excretion of both the parent compound and its metabolite(s). ENMs are applied in many common household products, such as textiles, plastics and cosmetics, and are released from these products during washing. ENMs have extraordinary properties arising from their small size and these properties differentiate ENMs from larger-sized bulk metals. Wastewater treatment plants are thus potential point sources of CECs into the environment if efficient removal of these contaminants cannot be achieved. Although the presence of CECs in surface waters is currently not yet regulated, considerable efforts are made to gain more insight into the presence of pharmaceuticals in surface waters in the European Union.

Constructed wetlands (CWs) are mostly applied as decentralized wastewater treatment systems in the rural areas. In general, CW treatment consists of a primary settling of coarse matter and a subsequent CW or CWs for secondary treatment. The CW is an engineered artificial wetland in which wastewater is treated by naturally occurring chemical, physical and biological processes. CWs can be filled with gravel or coarse sand to increase the microbial activities and mostly they are planted with typical wetland vegetation such as common reed or cattail to improve nutrient removal and to give them a green appearance. CWs which are located in remote areas discharge their effluent often in small water ways where low or even negligible dilution occurs. The potential risk on these streams arising from the pollutant emissions from the wastewater treatment system is the primary incentive for this PhD research. The main objective of this PhD research was hence to contribute to the understanding of the fate of (silver) nanoparticles (Ag-NPs) and pharmaceuticals in constructed wetlands by assessing factors affecting and possibly improving their removal in CWs.

Currently little is known about the processes ENMs undergo in CWs and their removal efficiency in these systems. *Chapter 2* thus aims to predict the possible transformation processes ENMs undergo in CWs based on literature data in related fields, such as activated sludge treatment. The potential release of ENMs from CWs with effluent water, harvested plant material and incorporated in sludge and the possible toxicity in CWs are also discussed. This chapter also reviews the main pharmaceutical removal mechanisms in CWs and discussed factors affecting the removal. Also, a comprehensive review on pharmaceutical removal efficiencies in pilot- and full-scale CWs is presented and finally, the aquatic risks posed by pharmaceuticals are discussed.

Laboratory-scale microcosm experiments simulating CWs treating domestic wastewater were set up to experimentally analyze the retention of Ag-NPs in CWs (*Chapter 3*). This experiment also addressed the effect of aging (accumulation of organic matter) and aeration on the fate of Ag-NPs in the microcosms. The transformations of the Ag-NPs in the microcosms were studied with detailed microscopy studies (STEM-EDX). The results showed that the Ag-NPs were very efficiently removed

(80 - 90%) from the wastewater and that the biofilm developed on the gravel bed was the main sink for the Ag-NPs in the microcosms. The fraction of Ag-NPs that passed the CWs was bound to solids present in the effluent, suggesting that additional measures to decrease the discharge of suspended solids with effluent will increase the removal efficiency of (Ag-)NPs. Plants had a minor role in the retention of Ag-NPs in the microcosms. Results from STEM-EDX analyses suggested that Ag-NPs were dominantly sulfidized, and low dissolved oxygen concentrations in the microcosms did not prevent sulfate reduction. Aeration did also not affect the retention efficiency of total Ag in the microcosms and the distribution of total Ag in the aerated microcosms was similar to that of the positive control. The aging (i.e. addition of organic matter) resulted in a slightly reduced retention of Ag-NPs in the microcosms due to increased discharge of suspended solids with the effluent.

In *Chapter 4*, the effects of plants and different wetland substrates on the removal of Ag-NPs from the water phase was examined in more detail. Different substrates are applied in CWs and they may have differing influence on the fate of ENMs in CWs. Also, many plants species have been shown to accumulate metals and hence, it is logical to assume that they could play a role in the retention of ENMs in CWs as well. The batch experiments showed that the removal of Ag-NPs from the water phase was dependent on the type of substrate and whether it was covered with biofilm or not. The results (gravel¡zeolite¡sand) indicated that the particle size (sand) and cation exchange capacity (zeolite) could increase the removal efficiency. Biofilm covered gravel increased the removal of Ag-NPs from the water phase to 67% in comparison to 20% by clean gravel, illustrating the importance of organic matter at retention of (Ag-)NPs. Ag uptake by plants was low during the 4-week experiment in a hydroponic culture. Ag-NPs remained mainly in the water phase and were adsorbed in/taken up by the roots but translocation to the shoots was negligible.

Since the Ag-NPs are mainly retained in the biofilm within the CW substrate, toxicity may arise. Ag has been shown to be toxic to many organisms even at low concentrations. In *Chapter 5*, it was investigated whether the biofilm community function (metabolism) or the structure (species) are affected by the exposure to Ag-NPs. In this experiment also ionic Ag was incorporated to compare the toxic effects. Ionic Ag is usually reported to be more toxic than Ag-NPs but ionic Ag can also be released from Ag-NPs. No alterations in the community function nor structure were observed during one month of exposure to an increasing Ag concentration (at most 80 μg/kg) and this was proposed to be caused by the protective effects of the biofilm structure.

Many earlier studies have indicated the potential correlation between oxic conditions and pharmaceutical removal and hence, the effect of intensified, i.e. aerated, CWs was chosen as the main topic of the second part of this PhD research. In *Chapter 6*, the performance of an aerated pilot-scale CW treating domestic and hospital wastewater was assessed. The pilot CW was able to produce high-quality effluent even at a high organic loading rate but its pharmaceutical removal efficiency was inadequate. The removal of certain pharmaceuticals, such as metformin and valsartan, was shown to be improved during aeration. To be able to treat hospital wastewater in CWs, further optimization of e.g. the hydraulic retention time (HRT) is necessary and the introduction of complimentary treatment needs to be analyzed.

In *Chapter 7*, the removal of pharmaceuticals was studied in a full-scale aerated CW. In addition, the effect of aeration and HRT on the removal efficiency was assessed in a batch experiment. It was considered important to evaluate the aquatic risk posed by the emissions from the full-scale CW because it discharged its effluent in a small forest creek. Excellent overall removal of pharmaceuticals was observed in the full-scale CW and therefore, the aquatic risk it poses on the forest creek was estimated insignificant. The removal efficiency is likely to be caused by a combination of factors, such as hybrid design, low loading and presence of LECA as substrate. The batch experiment revealed that the removal of certain pharmaceuticals can be improved by active aeration and/or increased HRT.

In *Chapter 8*, the main results obtained during this PhD research are compiled and discussed in the view of the latest research. This chapter also contains an overview of the main conclusions and suggestions are given for future research based on the experience acquired during this research. In short, silver nanoparticles were shown to be efficiently removed in CWs by attaching to organic matter. They were shown to undergo further transformations which restricts their dissolution and subsequently decreases their toxicity. Intensification, i.e. active aeration, of the CW when resulting in low concentrations of dissolved oxygen, did not increase the discharge of silver from the CW. Proper maintenance and monitoring of the CWs were recommended as the most suitable methods to control the discharge of nanomaterials from CWs. The removal of the selected pharmaceuticals was observed to be highly dependent on the type of pharmaceutical. Based on the experiments on pharmaceutical removal, active aeration may not be necessary for efficient removal, although the presence of both oxic and anoxic conditions is likely to enhance the overall removal. Hence, the application of hybrid CWs is recommended when enhanced pharmaceutical removal is targeted. However, active aeration may provide a solution for (urban) locations, such as hospitals, where space is critical because the intensification of the treatment induces a decrease in the foot-print.

Samenvatting

Afvalwater bevat naast de typische organische stoffen en nutriënten ook andere verontreinigingen. Deze andere vormen van verontreiniging, zoals geneesmiddelen en metallische nanopartikels (Engels: engineered nanomaterials (ENMs)), worden collectief opkomende micropolluenten (Engels: contaminants of emerging concern (CECs)) genoemd. Ze zijn zelfs in zeer lage concentraties (ng/L - μg/l) een mogelijk risico voor het milieu. Het menselijk lichaam verwerkt geneesmiddelen slechts gedeeltelijk waardoor zowel het oorspronkelijk geneesmiddel als eventuele nevenproducten afgescheiden worden. ENMs komen voor in de meest diverse huishoudproducten, zoals textiel, plastiek en cosmetica. Tijdens het wassen kunnen de ENMs tevens vrijkomen. De ENMs hebben, door hun kleine grootte, buitengewone eigenschappen die afwijken van deze van bulkmetalen.

Waterzuiveringsinstallaties kunnen dus mogelijks een bron van CECs in het milieu zijn als de verwijdering van CECs niet efficiënt is. Hoewel er tot op vandaag nog geen wetgeving is met betrekking tot CECs in het oppervlaktewater, vinden er wel reeds grote inspanningen plaats in de Europese Unie om meer inzicht te verkrijgen in de aanwezigheid en concentraties van geneesmiddelen in oppervlaktewateren.

Rietvelden (Engels: constructed wetlands) zijn waterzuiveringsinstallaties die meestal decentraal aangelegd worden in afgelegen gebieden omdat in deze regio's het aanleggen van riolering niet kostenefficiënt is. In de meeste rietveldsystemen vinden twee processen plaats. Eerst wordt de grove materie verwijderd door bezinking waarna het water naar een rietveld (of rietvelden in serie) gestuurd wordt voor secondaire behandeling. Een rietveld is een artificieel moeras waar afvalwater behandeld wordt door natuurlijk voorkomende chemische, fysische en biologische processen. In bepaalde gevallen worden rietvelden gevuld met grind of grof zand om de microbiële activiteit te verhogen. Ze zijn meestal beplant met typische helofyten zoals riet en lisdodde om de verwijdering van nutriënten te verbeteren en het geheel een groene uitstraling te geven. Rietvelden lozen hun effluent vaak in kleine beken en rivieren. De mogelijke risico's die het lozen van geneesmiddelen en ENMs in zulke waterlopen met zich meebrengen, zijn de belangrijkste motivatie voor dit onderzoek geweest. Het hoofddoel van deze thesis is het gedrag van zilver ENMs en geneesmiddelen in rietvelden beter te begrijpen door de invloed van verschillende factoren, die de verwijdering mogelijks kunnen verbeteren, te bestuderen.

Momenteel is er weinig gekend over de processen die ENMs ondergaan in rietvelden en hoe efficiënt de verwijdering van ENMs is. In *hoofdstuk 2* worden de mogelijke transformatieprocessen in detail besproken op basis van een literatuurstudie in gerelateerde domeinen zoals actief slib waterzuivering. De mogelijke lozing van ENMs uit rietvelden (door middel van het effluent, het geoogste plantenmateriaal of het slib) en de mogelijke toxiciteit van ENMs op micro-organismen in het rietveld worden ook besproken. In dit hoofdstuk worden ook de voornaamste mechanismen en omgevingsfactoren besproken die de verwijdering van geneesmiddelen beïnvloeden. De potentiële risico's op het aquatische milieu door het lozen van geneesmiddelen worden ook overlopen.

Hoofdstuk 3 beschrijft een labo experiment waar het gedrag van zilver nanopartikels in rietvelden werd gesimuleerd met microkosmossen. In dit experiment werd ook onderzocht of veroudering van het rietveld (accumulatie van organisch materiaal) en beluchting de verwijdering van de ENMs kunnen beïnvloeden. De transformaties van de ENMs in de microkosmos werden bestudeerd door middel van microscopie (STEM-EDX). De resultaten toonden aan dat de ENMs zeer efficiënt verwijderd werden uit het afvalwater (80 - 90%) en dat het grootste deel van de ENMs zich vasthechten aan de biofilm. De ENMs die werden geloosd met het effluent waren evenwel geadsorbeerd aan vaste deeltjes. Dit doet vermoeden dat maatregelen die vaste deeltjes in het effluent verminderen, de verwijderingsefficiëntie van zilver ENMs in het rietveld ook kunnen verhogen. Planten hadden slechts een klein effect op de verwijdering van de ENMs in de microkosmos. De microscopieresultaten toonden aan dat de ENMs gesulfideerd waren en dat de toegepaste aeratie de sulfaatreductie niet kon voorkomen. Beluchting had ook geen effect op de verwijderingsefficiëntie of de distributie van de ENMs in de microskosmos. Veroudering (het toevoegen van organisch materiaal) resulteerde in een lichte verlaging van de vewijderingsefficiëntie van de ENMs door een daling in de verwijdering van zwevende deeltjes.

Hoofdstuk 4 bestudeert de effecten van planten en verschillende substraatmaterialen op de verwijdering van zilver ENMs uit de waterfase. Er worden verschillende substraten gebruikt in rietvelden die mogelijks een verschillend effect hebben op de verwijdering van ENMs. Veel plantensoorten kunnen metalen accumuleren en dus is het logisch om te veronderstellen dat ze ook een rol kunnen spelen in het vasthouden van ENMs. Het batch experiment toonde aan dat de verwijdering van de ENMs uit de waterfase afhankelijk was van het type substraat en de aanwezigheid van een biofilm. De resultaten (grind ¡ zeoliet ¡ zand) wezen aan dat een kleine partikelgrootte (zand) en een hoge kationuitwisselingscapaciteit (zeoliet) de verwijderingsefficiëntie kunnen verhogen. Grind dat bedekt was met een biofilm kon 67% van de ENMs van de waterfase verwijderen in vergelijking met 20% met grind zonder biofilm. Dit toont weer het belang van organische materie aan op de retentie van ENMs. Ag opname door de planten was laag tijdens het experiment van 4 weken in een hydrocultuur. De ENMs bleven voornamelijk in de waterfase en werden geadsorbeerd/opgenomen in de wortels van de planten. Transport naar de bladeren was verwaarloosbaar.

Omdat het merendeel van de zilver ENMs in de biofilm van het rietveld blijft, kunnen ze toxisch worden voor de micro-organismen. Het is eerder aangetoond dat zilver zelfs in lage concentraties toxisch kan zijn. In *hoofdstuk 5* werd onderzocht of de metabolische capaciteiten en diversiteit aan soorten in de biofilm beïnvloed worden door blootstelling aan zilver ENMs. In dit experiment werden de effecten van ENMs vergeleken met ionisch Ag die volgens verschillende studies giftiger is dan zilver nanopartikels. Zilver ENMs kunnen ook wel zilver ionen vrijgeven. De studie toonde geen veranderingen aan in het metabolisme of soortenrijkdom die veroorzaakt zou zijn door de blootstelling aan verhoogde Ag concentraties (maximaal 80 μg/kg). Vermoedelijk komt dit door de beschermende werking van de biofilm.

Eerdere studies wijzen op een mogelijke positieve correlatie tussen aerobe omstandigheden en de verwijdering van geneesmiddelen. Daarom werd de studie van een geïntensifieerd rietveld, waar actieve aeratie is toegepast, in dit deel van het onderzoek besproken. In *hoofdstuk 6* werd de verwijdering van geselecteerde geneesmiddelen onderzocht in een belucht piloot-schaal rietveld die huishoudelijk en ziekenhuis afvalwater zuiverde. Het gezuiverde water had een goede kwaliteit op basis van organische stoffen en ammonium, zelfs tijdens hoge organische belasting. Maar de verwijderingsefficiëntie van de geneesmiddelen was onvoldoende. Er werd wel aangetoond dat aeratie een positief effect had op de verwijdering van sommige farmaceutica zoals metformin en valsartan. Om afvalwater van een ziekenhuis goed te kunnen behandelen met een rietveld is er verdere optimalisatie nodig. Dit kan door optimalisatie van de hydraulische retentietijd en/of met de toevoeging van complementaire behandelingen.

Hoofdstuk 7 behandelt de verwijdering van geneesmiddelen in een full-scale belucht rietveld. Het effect van aeratie en HRT op de verwijderingsefficiëntie werd daarnaast bestudeerd in een batch experiment. Het behandelde water van het full-scale rietveld kwam terecht in een kleine beek waardoor het belangrijk werd geacht om het aquatische risico door de lozing van geneesmiddelen te bestuderen. Uitstekende verwijdering van geneesmiddelen werd vastgesteld in het full-scale rietveld en daarom werd het aquatisch risico in de beek als laag beschouwd. De hoge verwijderingsefficiëntie is waarschijnlijk te danken aan een combinatie van factoren zoals designfactoren, de lage belasting en het gebruik van geëxpandeerde kleikorrels als substraat. Het batch experiment toonde aan dat verwijderingsefficiëntie van bepaalde geneesmiddelen verhoogd kan worden door actieve aeratie en/of door een verhoogde HRT.

Hoofdstuk 8 vat de belangrijkste resultaten van dit onderzoek samen en bespreekt ze in het licht van de meest recente literatuur. Dit hoofdstuk lijst ook de belangrijkste conclusies op en geeft aanbevelingen voor verder onderzoek. Er werd aangetoond dat zilver nanopartikels efficient wervijderd worden door de adhesie aan organisch materiaal zoals biofilm. Deze nanopartikels transformeren ook waardoor hun oplosbaarheid en toxiciteit verminderen. Beluchting van het rietveld (aangetoond met redelijk lage zuurstof concentraties) veroorzaakte geen toename in de lozing van zilver van het rietveld. Regelmatig onderhoud en opvolging van het rietveld werden genoemd als de beste maatregelen om de lozing van nanopartikels te controleren. De verwijdering van de geselecteerde geneesmiddelen varieerde sterk afhankelijk van de type geneesmiddel. Gebaseerd op de experimenten is beluchting mogelijks niet noodzakelijk om efficiënte verwijdering van geneesmiddelen te behalen, hoewel de aanwezigheid van zowel oxische als anoxische zones de algemene verwijderingsefficiëntie wel kan verbeteren. Daarom is de toepassing van hybride CWs aanbevolen als verbeterde verwijdering van geneesmiddelen het doel is. Beluchte rietvelden kunnen wel een oplossing bieden voor stedelijke locaties zoals ziekenhuizen waar er plaats te kort is, omdat een belucht systeem maar een derde van de oppervlakte van een gewoon rietveld nodig heeft.

Tiivistelmä

Jätevesissä on tunnettujen epäpuhtauksien - orgaanisen aineksen ja ravinteiden – lisäksi myös monia muita saastuttavia aineita. Niitä kutsutaan yhteisnimityksellä ”contaminants of emerging concern” (CECs) koska, vaikka ne esiintyvät jätevedessä vain hyvin pieninä pitoisuuksina (ng/L-μg/L), niiden epäillään aiheuttavan haittaa ympäristölle. Tämä väitöskirja keskittyy lääkeaineisiin ja metallisiin nanopartikkeleihin. Ihmiselimistö hajottaa ja muuntaa lääkeaineita vain osittain ja siksi jätevedestä löydetään sekä lähtöainetta että sen metaboliatuotteita. Metallisia nanopartikkeleita käytetään tavallisissa kodin tavaroissa, kuten muovissa, tekstiileissä ja kauneudenhoitotuotteissa, joista partikkelit voivat pesun yhteydessä vapautua. Metallisten nanopartikkelien suosio perustuu niiden erityislaatuisiin ominaisuuksiin, jotka johtuvat niiden pienestä koosta ja, jotka erottavat ne tavallisista metalleista. Lääkejäämiä ja metallisia nanopartikkeleja voi siis päästä ympäristöön pistekuormana jätevedenpuhdistamoilta mikäli näitä epäpuhtauksia ei voida poistaa riittävän tehokkaasti. Nykyinen vesilainsäädäntö ei koske CEC-epäpuhtauksia, mutta mittavia seurantakampanjoita on järjestetty koko EU:n alueella, jotta enemmän tietoa lääkejäämien esiintyvyydestä ja pitoisuuksista pintavesissä saataisiin.

Keinotekoisia kosteikkoja (engl. constructed wetlands) käytetään Keski- ja Etelä-Euroopassa yleisesti hajautetussa jätevedenpuhdistuksessa, koska viemäröinnin rakentaminen haja-asutusalueelle ei ole kustannustehokasta. Kosteikkokäsittelyssä jätevesi puhdistetaan ensin karkeasta aineksesta laskeuttamalla, minkä jälkeen vesi johdetaan varsinaiseen kosteikkoon tai niiden sarjaan. Kosteikossa jätevesi puhdistuu luonnollisesti tapahtuvien kemiallisten, fysikaalisten ja biologisten prosessien seurauksena. Kosteikoita on eri tapaisia, ja valinta tehdään käsiteltävän veden laatuun, tarvittavaan puhdistustehoon, puhdistusyksikön sijaintiin ym. tekijöihin perustuen. Kosteikko voi siten olla täytetty kiviaineksella biologisen aktiivisuuden lisäämiseksi ja se on useimmiten istutettu ravinteiden poiston parantamiseksi ja vihreän yleisilmeen luomiseksi. Puhdistettu jätevesi johdetaan jokiin tai ojiin, joiden vedenlaatuun ja ekologiaan jätevedet saattavat vaikuttaa. Näin on erityisesti purkuvesistössä joissa on vähäinen virtaus ja sen seurauksena puhdistetun jäteveden huono sekoittuminen virtaan. Lääkeaineiden ja metallisten nanopartikkelien mahdolliset haittavaikutukset tälläisissä tyypillisissä purkuvesistöissä ovat kannustin tälle tutkimukselle. Tämän tutkimustyön pääasiallisena tarkoituksena olikin kasvattaa tietämystä lääkeaineiden ja metallisten nanopartikkelien poistamisesta jätevedestä tutkimalla tekijöitä, jotka niiden mahdollisesti edesauttavat niiden poistamista kosteikkokäsittelyssä.

Nykyään tiedetään hyvin vähän siitä mitä metallisille nanopartikkeleille tapahtuu kosteikkokäsittelyn aikana. *Kappaleessa 2* on käyty yksityiskohtaisesti läpi miten niiden ennustetaan käyttäytyvän kosteikkokäsittelyssä perustuen tietoon muilta vastaavilta aloilta. Lisäksi kappaleessa käsitellään riskiä että metallisia nanopartikkeleita vapautuisi kosteikosta veden, lietteen tai kasvimateriaalin mukana ja arvioidaan niiden mahdollisesti aiheuttamaa haittaa kosteikon toiminnalle. Tässä kappaleessa käsitellään myös lääkeaineiden poistamista jätevedestä vertailemalla viimeaikaisia tutkimustuloksia

eri kosteikoista ja käsittelemällä tekijöitä, jotka mahdollisesti vaikuttavat lääkeaineiden poistamistehokkuuteen kosteikoissa. Lisäksi kappaleessa arvioidaan lääkeaineiden aiheuttamaan ympäristöriskiä.

Kappaleessa 3 kuvaillaan laboratoriokokeita, joissa hopea nanopartikkelien käyttäytymistä keinotekoisessa kosteikossa simuloitiin mikrokosmoksilla. Lisäksi kokeessa tutkittiin kuinka kosteikon ikääntyminen (orgaaninen aines lisääntyy) ja ilmastus vaikuttavat hopea-nanopartikkelien pidättymiseen kosteikkoon. Mikroskopialla tutkittiin nanopartikkelien muodonmuutoksia, sillä niillä voi olla vaikutusta partikkelien toksisuuteen. Mikrokosmokset pidättivät hopea-nanopartikkeleita erittäin tehokkaasti (80 – 90%) ja suurin osa partikkeleista kiinnittyi biofilmiin ja orgaaniseen ainekseen. Veden mukana poistunut hopea oli myös kiinnittynyt kiinteään ainekseen, minkä seurauksena voitiin todeta että lisätoimenpiteet, jotka vähentävät kiintoaineksen määrää puhdistetussa vedessä, vähentävät myös (hopea) nanopartikkelien päästöjä. Kasveilla oli vähäinen vaikutus hopea-nanopartikkeleiden pidättymiseen. Mikroskopialla voitiin selvittää, että hopea- nanopartikkelit olivat reagoineet sulfidin kanssa muodostaen vaikealiukoista hopeasulfidia. Hopeasulfidin muodostuminen on toivottavaa, koska hopeasulfidin on todettu olevan eliöille vähemmän haitallista kuin hopea-nanopartikkelien. Ilmastus ei vaikuttanut hopea-nanopartikkelien pidättymiseen eikä reaktioon sulfidien kanssa. Orgaanisen aineksen kerääntyminen kosteikon ikääntyessä edesauttaa hopea-nanopartikkelien pidättymistä mikäli orgaaninen aines ei pääse poistumaan kosteikosta.

Kappaleessa 4 erilaisten kosteikon täyttömateriaalien ja kasvien vaikutusta hopea-nanopartikkelien pidättyvyyteen tutkittiin tarkemmin. Panoskokeilla selvitettiin onko täyttömateriaalin valinnalla väliä nanopartikkelien poistamisessa jätevedestä. Useat kasvilajit kykenevät ottamaan metalleja vedestä ja varastoimaan niitä rakenteisiinsa. Niiden vaikutuksesta nanopartikkeleihin ei kuitenkaan ole vielä paljon tietoa. Tässä kokeessa hopea-nanopartikkelien poistaminen vedestä oli tehokkainta hiekan ja zeoliitin läsnäollessa mutta vähäisempää sorassa. Mikäli sora oli päällystetty biofilmillä, sen tehokkuus poistaa nanopartikkeleita vedestä kasvoi huomattavasti (67% vs. 20%), todistaen orgaanisen aineksen tärkeyden nanopartikkelien pidättymisessä. Tutkimuksessa käytetty kosteikoissa tyypillinen kasvilaji, järviruoko, varastoi hopea- nanopartikkeita lähinnä juuristoonsa.

Koska hopea-nanopartikkelit varastoituvat lähinnä biofilmiin, on mahdollista, että metallipitoisuudet nousevat myrkyllisiksi kosteikon mikrobeille. *Kappaleessa 5* tutkittiin vaikuttaako kasvava hopea-nanopartikkelialtistus kosteikkomikrokosmosten mikrobiyhteisön metaboliaan tai rakenteeseen (altistus max 80 μg/kg). Tutkimuksessa huomioitiin sekä nanopartikkelit että hopeaionit, joita nanopartikkeleista voi liueta ja joiden uskotaan aiheuttavan hopea-nanopartikkelien toksisuuden. Kuukauden tutkimusjakson aikana negatiivisia vaikutuksia ei huomattu vertailussa kontrollikosteikkoon. Biofilmin uskotaan suojelevan kosteikon mikrobiyhteisöä epäpuhtausten haittavaikutuksilta.

Useat aikaisemmat tutkimustulokset ovat näyttäneet, että lääkeaineiden poistuminen on yhteydessä liuenneen hapen esiintyvyyteen kosteikossa, ja siksi ilmastetut kosteikot on otettu tämän tutkimuksen teemaksi. *Kappaleessa 6* käsitellään pilot-kosteikon kykyä poistaa lääkeaineita kotitalouksien ja sairaalan jätevedestä. Vaikka puhdistetun veden laatu olikin hyvä mitattuna orgaanisen aineksen ja ammoniakin pitoisuuksilla, lääkeaineiden poistaminen ei ollut riittävän tehokasta. Lääkeaineiden puhdistusteho vaihteli suuresti lääkeaineesta riippuen. Ilmastus paransi tiettyjen lääkeaineiden poistamista (metformiini ja valsartaani). Jotta kosteikkokäsittelyä voitaisiin soveltaa sairaalan jätevesien puhdistukseen, kosteikon toimintaa täytyy optimoida esimerkiksi säätämällä hydraulista viipymisaikaa, ja lisäksi tarvitaan mahdollisesti täydentäviä puhdistusprosesseja korkean orgaanisen ja lääkeainekuormituksen takia.

Kappaleessa 7 käsitellään täyden mittakaavan kosteikon toimintaa ja arvioidaan sen lääkeaineiden päästöjen vaikutusta purkuvesistössä, joka oli pieni metsäoja. Lisäksi kappaleessa käsitellään laboratoriokeiden tuloksia ilmastuksen ja hydraulisen viipymäajan vaikutuksista lääkeaineiden poistotehokkuuteen. Kosteikossa saavutettiin erittäin hyvä lääkeaineiden poistotehokkuus (>90%), ja sen vuoksi myös sen ekologinen riski ojan ekologialle arvioitiin alustavasti vähäiseksi. Erinomaisen tehokkuuden epäillään johtuneen monista tekijöistä, kuten kosteikon tyypistä, pitkästä viipymäajasta ja tehokkaasti adsorboivasta täyttöaineesta. Panoskokeen perusteella hydaulista viipymäaikaa pidentämällä ja ilmastuksella voidaan vaikuttaa joidenkin lääkeaineiden poistamistehokkuuteen. Yleisesti ottaen nämä tulokset tukevat sellaisten kosteikoiden valintaa lääkeaineiden poistamiseksi, joissa aerobiset ja anoksiset olosuhteet ovat läsnä. Näin on usein ns. hybridikosteikoissa, joissa jätevesi käsitellään useissa eri tyyppisissä kosteikoissa peräjälkeen tai osittain ilmastetuissa kosteikoissa.

Kappeessa 8 on koottu yhteen tämän tutkimuken tärkeimmät tulokset. Niiden perusteella on myös annettu tutkimusaiheet, jotka tulevaisuudessa tarvitsevat huomiota. Hopean nanopartikkeleiden osoitettiin kiinnittyvän tehokkaasti orgaanisen ainekseen kosteikossa ja läpikäyvän muutoksia, jotka rajoittavat niiden liukenemista ja toksisuutta. Ilmastuksella ei ollut vaikutusta hopean poistumistehokkuuteen vesifaasista. Asianmukainen kosteikkojen kunnossapito ja seuranta ovat suositeltavat toimet nanomateriaalien purkuvesisitöihin pääsyn rajoittamiseksi. Lääkeaineiden poistamistehokkuuksissa huomatttiin suuria eroja eri lääkeaineiden välillä. Tulosten perusteella kosteikon ilmastus ei mahdollisesti ole välttämätöntä hyvän poisamistehokkuuden saavuttamiseksi, mutta sekä hapellisten että hapettomien olosuhteiden läsnäolo todennäköisesti parantaa keskimääräistä puhdistustehoa, koska jotkin lääkeaineet poistuvat paremmin hapellisissa kuin hapettomissa olsouhteissa ja toisinpäin. Näin ollen on suositeltavaa käyttää hybridi kostikkoja, mikäli tavoitellaan parannettua lääkeaineiden poistamistehoa. Ilmastus voi tarjota ratkaisun tiheäänrakennetuilla alueilla, koska ilmastuksen avulla kosteikon tarvitsema pinta-ala pienenee noin kolmasosaan.

Curriculum vitae

Personal information

Name	Hannele Auvinen
Address	Kuusisenmutka 7 13210 Hämeenlinna Finland
Telephone	+358 44 239 06 51
E-mail	Hannele.Auvinen@UGent.be Hannele.Auvinen@gmail.com
Date of birth	4.5.1984
Place of birth	Helsinki, Finland
Nationality	Finnish

Education

2013–2017	Doctor (PhD) in Applied Biological Sciences Ghent University,Faculty of Bioscience Engineering, Department of Industrial Biological Sciences, Laboratory of Industrial Water an Ecotechnology Department of Applied Analytical and Physical Chemistry, Laboratory of Analytical Chemistry and Ecochemistry,
Topic:	"Assessing and improving the removal of contaminants of emerging concern in intensified constructed wetlands"
2003-2008	Master of Science (MSc) in Environmental Bioechnology Tampere University of Technology (TUT), Tampere, Finland
Topic:	"Performance and microbial community profile of a sulfidogenic fluidized-bed bioreactor treating acid mine drainage at low temperature"

Professional activities during PhD research

Tutoring

Hubau L. (2016). Verwijderen van farmaceutica uit ziekenhuisafvalwater met behulp van een pilootschaal rietveld. Master thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Industrial Biological Sciences, Ghent University, Ghent.

Vanseveren L. (2016). Pilootschaal rietveld voor verwijdering farmaceutica uit afvalwater. Master thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Industrial Biological Sciences, Ghent University, Ghent.

Havran I. (2015). Impact of aeration on the performance of wastewater treatment wetlands – a pilot scale study. Master thesis submitted in partial fulfillment of the requirements for the degree of International Master of Science in Environmental Technology and Engineering, Ghent University, Ghent.

De Buyck P.-J. (2015). Ozonisatie en rietvelden voor de verwijdering van farmaceutica uit afvalwater. Master thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Industrial Biological Sciences, Ghent University, Ghent.

Espinoza García L.V. (2015). Biogeochemical fate of silver nanoparticles in laboratory-scale constructed wetlands. Master thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Environmental Sanitation, Ghent University, Ghent.

Vásquez Sepùlveda V. (2014). Fate of silver nanoparticles in treatment wetlands. Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Environmental Sanitation, Ghent University, Ghent, Belgium.

Lefevere B. (2014). De verwijdering van geneesmiddelen met rietvelden. Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Bioscience Engineering, Ghent University, Ghent, Belgium.

De Buyck P.-J. (2014). Verwijdering van micropolluenten uit afvalwater: uitdieping op ozonisatie en behandeling in rietvelden. Thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Science in Bioscience Engineering, Ghent University, Ghent, Belgium.

Lefevere B. (2013). De verwijdering van organische micropolluenten in rietvelden. Thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Industrial engineering, Hogeschool West-Vlaanderen, Kortrijk, Belgium.

Evaluation of theses

De Dobbelaere B.,Detand T., Dhont C., Ponnet N. (2016). Aanwezigheid en transport van technologiekritische elementen in het aquatische en terrestrische milieu. Project report submitted in partial fulfillment of the requirements for the degree of Bachelor of Science in Bioscience Engineering, Ghent University, Ghent, Belgium.

Jan De Coninck. (2015). Speciatie-analyse en -modellering voor de recuperatie van palladium uit afvalwater van de spiegelindustrie. Thesis submitted in partial fulfillment of the requirements forthe degree of Master of Science in Bioscience Engineering, Ghent University, Ghent, Belgium.

Educational activities

Organization and supervision of laboratory courses

Microbiële ecologie during academic years 2013-2014 and 2014-2015.

Macro-ecologie en Ecotechniek during academic years 2013-2014 and 2015-2016.

Publications

Published articles

Auvinen H., Gagnon V., Rousseau D., Du Laing G. 2017. Fate of metallic nanoparticles in constructed wetlands: prospection and future research perspectives. Reviews in Environmental Science and Bio/Technology (in press).

Auvinen H., Kaegi R., Rousseau D., Du Laing G. 2017. Fate of silver nanoparticles in constructed wetlands – a microcosm study. Water, Air and Soil Pollution 228:97.

Auvinen H., Havran I., Hubau L., Vanseveren L., Gebhardt W., Linnemann V., Du Laing G., Rousseau D. 2017. Removal of pharmaceuticals by a pilot aerated sub-surface flow constructed wetland treating municipal and hospital wastewater. Ecological Engineering 100: 157-164.

Auvinen H., Vásquez Sepúlveda V., Rousseau D., Du Laing, G. 2016. Substrate- and plant-mediated removal of citrate-coated silver nanoparticles in constructed wetlands. Environmental Science and Pollution Research. 23(21): 21920-21926.

Button M., *Auvinen H.*, Van Koetsem F., Hosseinkhani B., Rousseau D., Weber K., Du Laing G. 2016. Susceptibility of constructed wetland microbial communities to silver nanoparticles: A microcosm study. Ecological Engineering 97: 476-485.

Audenaert W., Chys M., *Auvinen H.*, Dumoulin A., Rousseau D., Van Hulle S. 2014. (Future) Regulation of Trace Organic Compounds in WWTP Effluents as a Driver for Advanced Wastewater Treatment. Ozone News 42 (6):17-23.

Book chapter

Auvinen H., Du Laing G., Meers E., Rousseau D. 2016. Constructed Wetlands Treating Municipal and Agricultural Wastewater – An Overview for Flanders, Belgium. In: Vymazal J. (ed.) Natural and constructed wetlands.Springer International Publishing. p. 179-208.

Oral presentations in conferences and workshops

COST ES1205, Final conference Transfer of engineered nanomaterials from wastewater treatment & stormwater to rivers. Aveiro, Portugal, 7-8 February 2017.

WETPOL 6th International Symposium on Wetland Pollutant Dynamics and Control. York, UK, 13-18 September 2015.

IWA 14th International Conference on Wetland Systems for Water Pollution Control, Shanghai, China, 12-16 October 2014.

COST ES1205 Workshop Transfer of engineered nanomaterials from wastewater treatment & stormwater to rivers. Pula, Croatia, 2-4 June 2014.

Other activities

Acted as a Belgian delegate at the meetings of the Management Committee of COST Action ES1205, Pula, Croatia (2-4.6.2014) and Tallinn, Estonia (7-8.2015).

Short Term Scientific Mission (STSM) at Federal Institute of Hydrology, Koblenz, Germany, 1-5 September 2014. Funding by: COST Action ES1205.

Appendix A

Table A1: Instrumental parameters and calibration conditions of ICP-MS

Instrumental parameter	**Setting**
Nebulizer gas flow	0.75 L/min
Auxiliary gas flow	1.2 L/min
Plasma gas flow	15 L/min
Lens voltage	7.3 V
Plasma RF power	1300 W
Sweeps/reading	30
Dwell time/AMU	75 ms
Integration time	2250 ms
DRC mode	0.4 mL CH_4 /min
RPq	0.7
Calibration range	0, 0.2, 0.5, 1, 2, 4, 8, 20 μg Ag/L

Table A2: Aveage (±SD, minimum (Min) and maximum (Max) values for TOC/TSS (mg/L) for microcosm effluents, n=15)

	Positive control		**OM**		**Air**	
	TOC (mg/L)	TSS (mg/L)	TOC (mg/L)	TSS (mg/L)	TOC (mg/L)	TSS (mg/L)
Average	19±11	62±54	80±17	213±56	22±8	92±51
Min	5	4	40	121	11	19
Max	40	179	98	320	35	178

Appendix B

Table B1: Trace element composition of the studied substrates (average ± SD, n=3; LOD=limit of detection

	Sand	**Gravel**	**Zeolite**
Al (mg/g)	0.47±0.03	0.38±0.22	27.2±2.2
Ca (mg/g)	26.4±5.8	371±4	19.5±1.0
Fe (mg/g)	2.01±0.19	1.31±0.40	3.35±0.38
K (mg/g)	0.18±0.01	0.14±0.09	16.4±1.3
Mg (mg/g)	0.51±0.03	2.62±0.48	4.45±0.11
Na (mg/g)	1.23±0.10	0.12±0.009	1.44±0.06
Ag (μg/g)	<LOD	<LOD	0.36±0.04
Cd (μg/g)	<LOD	<LOD	<LOD
Cr (μg/g)	<LOD	<LOD	<LOD
Cu (μg/g)	<LOD	0.91±0.38	1.63±0.17
Mn (μg/g)	39.6±0.6	85.8±23.2	77.1±29.2
Ni (μg/g)	1.26±0.19	2.34±0.30	2.50±1.16
Pb (μg/g)	2.18±0.63	0.82±0.82	25.9±2.1
Zn (μg/g)	5.26±0.27	6.60±2.92	26.3±3.4

Appendix C

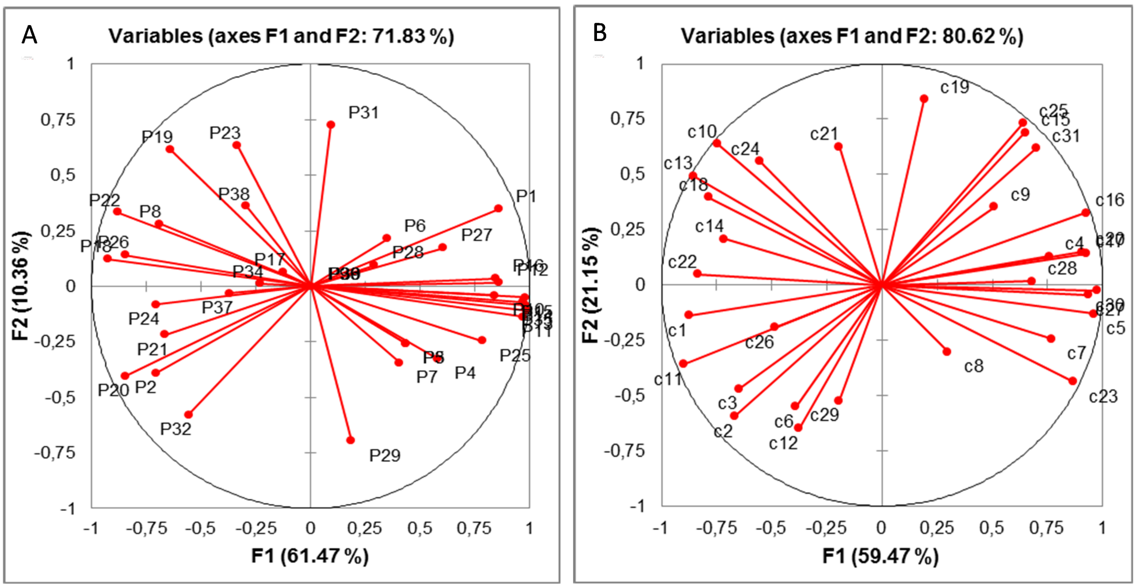


Figure C1: Loading plots for A) DGGE-based PCA and B) CLPP-based PCA

Table C1: Water quality measurements over the 28 day exposure period. A = control, B = citrate coated Ag-NPs, C = PVP coated Ag-NPs, D = ionic Ag. SD = 1 standard deviation of the mean (n=3)

	Day 7				**Day 14**				**Day 21**				**Day 28**			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
pH	7.5	7.4	7.3	7.4	8.2	7.9	7.8	8.0	7.7	7.8	7.8	7.8	7.7	7.7	7.7	7.7
SD	0.1	0.0	0.1	0.1	0.1	0.1	0.0	0.0	0.1	0.1	0.0	0.1	0.1	0.0	0.0	0.1
DO (mg/L)	0.1	0.4	0.1	0.3	4.5	2.9	4.0	3.6	5.1	4.4	4.1	2.9	2.2	2.6	1.1	1.8
SD	0.1	0.4	0.2	0.1	0.6	0.5	1.0	0.6	0.3	0.4	0.9	1.1	1.4	1.1	0.7	2.6
TOC (mg/L)	4.5	3.2	3.8	3.4	3.2	3.7	3.5	3.3	3.0	3.8	2.8	3.0	2.9	3.5	2.4	2.8
SD	1.9	0.2	0.6	0.3	0.1	0.4	0.2	0.3	0.3	0.7	0.2	0.2	0.6	1.9	0.1	0.6

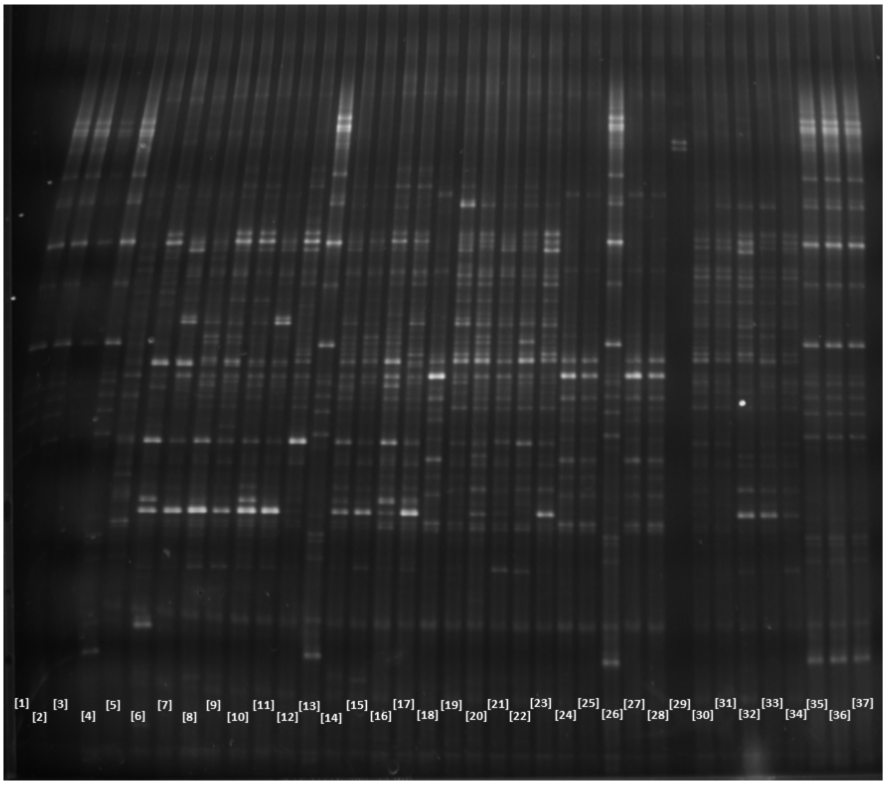


Figure C2: DGGE gel image including all samples. Lane number with sample description shown in table below image, W indicates the week of sampling. Samples are interstitial waters unless stated as biofilm. 5:Biofilm W0; 6:Control W0; 7:Control W1; 8:Citrate W1; 9:PVp W1; 10:Ag$^+$ W1; 12:Control W2; 14:Citrate W2; 15:PVP W2; 16:Ag$^+$ W2; 19:Control W4; 21:Citrate W4; 22:Ag$^+$ W4; 23:PVP W4; 24:PVP biofilm W4; 25:Citrate biofilm W4; 27:Control biofilm W4; 28:Ag$^+$ biofilm W4; 30:Control W3; 31:Ag$^+$ W3; 32:PVP W3; 34:Citrate W3

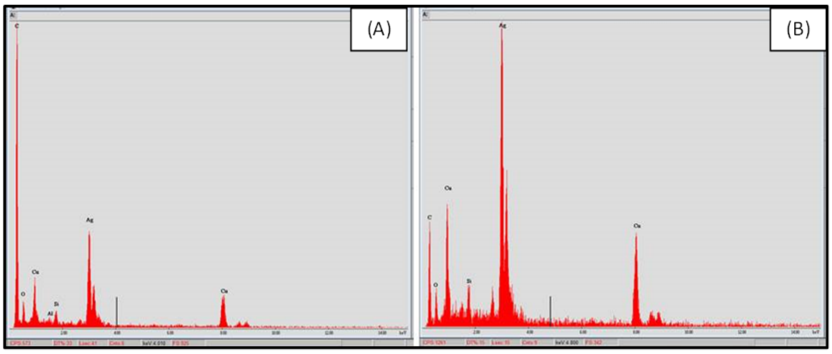


Figure C3: EDX spectra corresponding to Ag-NPs in the STEM images shown in Figure 5.2. For (A) PVP coated, and (B) Citrate coated Ag-NPs in the simulated wastewater solution.