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its relationship with pollutant removal
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ORIGINAL PUBLICATIONS

This thesis consists of the following publications which are referred by their Roman numerals in the thesis.

I Truu, M, **Oopkaup, K**, Krustok, I, Kõiv-Vainik, M, Nõlvak, H, Truu, J (201X) Bacterial community activity and dynamics in the biofilm of an experimental hybrid wetland system treating greywater. Submitted.

II **Oopkaup, K**, Truu, M, Nõlvak, H, Ligi, T, Preem, JK, Mander, Ü, Truu, J (2016) Dynamics of Bacterial Community Abundance and Structure in Horizontal Subsurface Flow Wetland Mesocosms Treating Municipal Wastewater. *Water* 8: 457.

III Ligi, T, **Oopkaup, K**, Truu, M, Preem, JK, Nõlvak, H, Mitsch, JW, Mander, Ü, Truu, J (2014) Characterization of bacterial communities in soil and sediment of a created riverine wetland complex using high-throughput 16S rRNA amplicon sequencing. *Ecological Engineering* 72: 56–66.

IV Ligi, T, Truu, M, **Oopkaup, K**, Nõlvak, H, Mander, Ü, Mitsch, JW, Truu, J (2015) The genetic potential of N_2 emission via denitrification and ANAMMOX from the soils and sediments of a created riverine treatment wetland complex. *Ecological Engineering* 80: 181–190.

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Author's contribution
Author's contribution to the papers ('*' denotes a minor contribution, '**' denotes a moderate contribution, '***' denotes a major contribution).

	I	**II**	**III**	**IV**
Microbiological analysis		**		
Data analysis	***	***	**	**
Manuscript preparation	**	***	*	*

ABBREVIATIONS

ANAMMOX	anaerobic ammonium oxidation
BOD	biochemical oxygen demand
Ca.	*Candidatus*
COD	chemical oxygen demand
comammox	complete oxidation of ammonium to nitrate
DHA	dehydrogenase activity
DNRA	dissimilatory nitrate reduction to ammonium
DW	dry weight
GTW	greywater treatment wetland
HF	horizontal flow filter
HSSF	horizontal subsurface flow
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride
LECA	light-weight expanded clay aggregate
MC	mesocosm
MWTW	municipal wastewater treatment wetland
n-damo	nitrite-dependent anaerobic methane oxidation
NH_3-N	ammonia nitrogen
NH_4-N	ammonium nitrogen
nirK	gene encoding copper-containing nitrite reductase
nirS	gene encoding cythochrome cd1-containing nitrite reductase
NO_2-N	nitrite nitrogen
NO_3-N	nitrate nitrogen
N_2O	nitrous oxide
nosZ	nitrous oxide reductase encoding gene
OTU	operational taxonomic unit
PCR	polymerase chain reaction
PNA	potential nitrification activity
PO_4-P	phosphate phosphorus
qPCR	quantitative polymerase chain reaction
RE	removal efficiency
RTW	riverine treatment wetland
TN	total nitrogen
TOC	total organic carbon
TP	total phosphorus
TSS	total suspended solids
TW	treatment wetland

VF	vertical flow filter
VSSF	vertical subsurface flow
W_{oo}	organic sediment samples of the open areas of W wetlands of the riverine wetland complex
W_{om}	mineral soil samples of the open areas of W wetlands of the riverine wetland complex
W_{trans}	soil samples of transitional areas of W wetlands of the riverine wetland complex

1. INTRODUCTION

Wetlands have been recognized as natural resources throughout human history. These ecosystems function as a part of the landscape as well as have value through their functions which have proved to be useful to humans (Mitsch & Gossilink, 2000). One of these functions, the water purification capability of wetlands, is recognized as a beneficial feature in wastewater treatment (Scholz & Lee, 2005). Natural wetlands have been used as convenient wastewater discharge sites for centuries because of their capacity to treat low-quality polluted waters (Wentz, 1987). The growing knowledge about the functions and qualities of a natural wetland have caused a change in the perspective of wetlands since the 1950s and the use of natural wetlands for wastewater disposal begins to decrease (Vymazal, 2011a). Instead, the use of treatment wetlands is now preferred. Most of the biological pollutant removal processes are based on the exploitation of the concerted activity of microorganisms in different compartments of treatment wetland. Knowledge of the microbial community dynamics and structure and the relationship with the system properties and operation conditions is therefore crucial for the development and optimization of effective treatment wetlands.

1.1 Overview of treatment wetlands

Based on their main purpose, the artificially created wetlands, also called constructed wetlands, can be divided into subcategories: restored wetlands, created wetlands and treatment wetlands (Fonder & Headley, 2013). As the former two types are built for purposes other than water quality improvement, all the study sites described in this thesis categorize as treatment wetlands (TWs). These are the artificially created wetland systems designed to enhance and optimise certain physical and biogeochemical processes that occur in natural wetland ecosystems with a primary purpose to remove contaminants from polluted waters. They are often cost-effective alternatives or useful complements to the expensive wastewater treatment technologies such as trickling filters and activated sludge processes (Kadlec & Wallace, 2009). Based on their hydrologic mode, treatment wetlands can be divided into subtypes, where the three major types are free water surface (FWS), vertical subsurface flow (VSSF) and horizontal subsurface flow (HSSF) wetlands (Kadlec & Wallace, 2009).

   FWS or sometimes referred as surface flow wetlands are defined as typically shallow depth and vegetated aquatic systems with an exposed water surface similar to a natural marsh or swamp (Kadlec, 2009). In these systems, a wide variety of submerged, emergent and floating plants have been used, such as common reed (*Phragmites* spp.), cattail (*Typha* spp.), bulrush (*Scirpus* spp.) and sedge (*Carex* spp.) (Vymazal, 2013a). Macrophytes have several important properties related to the treatment process in FWS TWs that make them an

essential component of the wetland design. These properties include nutrient uptake and storage in the system as well as creating space for attachment and favourable microclimatic conditions for microorganisms by the root release of oxygen and exudates in the plant rhizosphere (Shelef et al., 2013; Truu et al., 2015). Plant uptake could be significant route for the nutrient removal, especially under low loading rates (Gottschall et al., 2007). It has been argued that the most important mechanism by which plants contribute to the TW treatment process is not in uptake of nutrients but rather in the physical effects of root structure combined with aeration (Tanner, 2001; Brisson & Chazarenc, 2009). Physical effects of the macrophytes' roots include filtering, flow velocity reduction, improved sedimentation, decreased resuspension, and even distribution of water as well as prevention of clogging (Stottmeister et al., 2003).

FWS TWs with emergent vegetation are able to deal with pulse flows and changing water levels and, therefore, can treat various types of wastewaters such as municipal wastewater (Andersson et al., 2005), agricultural drainage (Arroyo et al., 2010), landfill leachate (Ogata et al., 2015), metal ores mine drainage (Maine et al., 2017), etc. However, FWS TWs are most commonly used for the secondary or tertiary wastewater treatment process. Because of their design, FWS TWs are more susceptible to low temperatures and formation of ice can hydraulically preclude the winter operation; especially when the ice covers the open water and the oxygen-dependent treatment processes are reduced due to the decreased transfer of oxygen (Kadlec & Wallace, 2009).

Vertical subsurface flow TWs comprise a filter bed of graded gravel topped with sand or other porous filter materials which have high surface area for microbial biofilm formation (Vymazal, 2008). These TWs are fed intermittently with large batches, thus flooding the surface. Wastewater then percolates uniformly down or up through the substrate and is collected by a drainage network at the bottom. The filter drains completely, which allows air to refill the system (Brix & Arias, 2005). Therefore, the oxygen-dependent processes, such as mineralization of organic compounds, nitrification and nitrate assimilation are active in VSSF TWs (Saeed & Sun, 2017; Pelissari et al., 2018).

In horizontal subsurface flow TWs, the wastewater is fed by an inlet pipe and flows slowly through the porous medium under the surface of the filter to the outlet area where it is collected before leaving via a water level control structure (Vymazal, 1998). During the passage through the filter, the wastewater comes into contact with a network of aerobic, anoxic and anaerobic zones. Most of the environment is anoxic/anaerobic due to the permanent saturation of the filter (Vymazal, 2005). Consequently, this type of TW is suitable for anaerobic processes, like denitrification, anaerobic ammonium oxidation, nitrite-dependent anaerobic methane oxidation, methanogenesis, etc. (Vymazal & Kröpfelová, 2009; Coban et al., 2015). HSSF TWs are constructed with or without vegetation (Vymazal, 2011b). Filtration, bed insulation during the cold period, supply the growth of attached bacteria with substrate, oxygen release to the rhizosphere, nutrient uptake and storage as well as carbon sequestration are the

most important functions the macrophytes provide in these TWs (Vymazal, 2008).

In order to achieve a higher treatment effect especially in nitrogen removal, various types of constructed wetlands may be combined (Vymazal, 2005). Hybrid systems comprise most frequently vertical and horizontal flow systems arranged in a staged manner, but other types of hybrid systems have been designed as well for the treatment of wastewaters of different origin (Fonder & Headley, 2013; Vymazal, 2013b).

1.2 Microbially mediated removal of pollutants in treatment wetlands

While a variety of physicochemical pollutant removal mechanisms in TWs are well documented (Kadlec & Wallace, 2009), recognition that microbial activity play a major role in the nutrient cycling has steadily increased (Faulwetter et al., 2009; Truu et al., 2009). The properties of wetlands, such as substrate or filter material used, hydraulic conditions, presence or absence of plants in the system, and the characteristics of treated wastewater, but also environmental conditions during the operational period determine to a great extent the direction and rate of microbially mediated processes in TWs (Truu et al., 2009).

1.2.1 Nitrogen cycle

Nitrogen (N) compounds include a variety of inorganic and organic nitrogen forms that are essential for all living organisms. The variety of nitrogen compounds and their quantity in the treatment wetlands are dependent on the origin of wastewater entering the system. The most prevalent dissolved inorganic forms of nitrogen in wetlands are ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-). These nitrogen compounds, rather than particulate organic nitrogen, have the greatest impact on aquatic systems because they are easily available for uptake by microorganisms and plants (Lee et al., 2009). Gaseous nitrogen may exist in the form of dinitrogen (N_2), nitrous oxide (N_2O), nitric oxide (NO), nitrogen dioxide (NO_2), and ammonia (NH_3) in TW (Vymazal, 2007).

In the treatment wetlands, the nitrogen transformations may occur through several physicochemical processes, such as particulate settling and resuspension, the diffusion of the dissolved forms, plant translocation, ammonia volatilization and the sorption of soluble nitrogen on substrates (Kadlec & Wallace, 2009) as well as through the following biological pathways: ammonification, nitrification, denitrification, dissimilatory nitrate reduction to ammonium (DNRA), assimilation, anaerobic ammonium oxidation (ANAMMOX), and nitrogen fixation (Vymazal, 2007).

Ammonification (mineralization) is the process where organic N is biologically converted into ammonia. Organic pollutants containing nitrogen are

readily degraded in both aerobic and anaerobic zones of wetlands and during this process inorganic ammoniacal-nitrogen (NH_3-N) is released. Kinetically, ammonification proceeds more rapidly than nitrification. The rate of ammonification is fastest in the oxygenated zones of TW and it decreases in the oxygen deficient wetland zones where replacement of aerobic organisms by the facultative anaerobic and finally by the obligate anaerobic organisms takes place (Vymazal, 2007; Lee et al., 2009).

The inorganic NH_4-N is mainly removed by nitrification-denitrification processes in TWs. Nitrification is usually defined as the biological oxidation of ammonium to nitrate with nitrite as an intermediate in the reaction sequence. Nitrification has been typically associated with the chemolithoautotrophic bacteria, although it is now recognized that heterotrophic nitrification can also occur (Zhang, Jinbo et al., 2015). The first step, the oxidation of ammonium to nitrite, is executed by strictly aerobic chemolithotrophic bacteria or archaea. The second step in the process of nitrification, the oxidation of nitrite to nitrate, is performed by facultative chemolithotrophic bacteria which can also use organic compounds, in addition to nitrite, for the generation of energy for growth (Vymazal, 2007). These two reactions are catalysed by physiologically distinct clades of microorganisms. Recently, a novel clade of nitrite-oxidising bacterial genus *Nitrospira* were found that are able to perform complete oxidation of ammonium to nitrate (comammox) (Daims et al., 2015; van Kessel et al., 2015). These bacterial clades have been found from many different ecosystems (e.g. engineered treatment systems, rice paddy soil, forest soil), but their ecological background and relevance in the whole nitrogen cycle is still unclear (Daims & Wagner, 2018; Lawson & Lücker, 2018; Wang et al., 2018).

Denitrification is defined as a three or four step nitrogen reduction process in which nitrate is converted into dinitrogen or nitrous oxide via intermediates nitrite and nitric oxide (Zumft, 1997). Despite denitrifiers ability to utilize nitrate as electron acceptors, oxygen is more preferred if present in bulk water (Saeed & Sun, 2012). Each of the reduction steps is catalysed by the respective enzymes, i.e. periplasmic (Nap) or membrane-bound (Nar) nitrate reductases, nitrite reductases (CuNir and cd1Nir), nitric oxide reductases (cNor,qNor and qCuANor) and nitrous oxide reductases (Nos) encoded by *nap* and *nar*, *nirK* and *nirS*, *nor* and *nosZ* genes, respectively. However, denitrification is a modular pathway where an organism may not always possess the full set of enzymes and thus, perform only a subset of the steps within the pathway (Zumft, 1997). Approximately one-third of the bacterial genomes that possess the *nir* genes encoding the nitrite reductases catalysing the dissimilatory reduction of NO_2 to NO, are currently known to lack *nosZ*, that makes them producers of a greenhouse gas N_2O (Jones et al., 2008). On the other hand, several studies have found that the *nosZ* gene is present in a large range of archaeal and bacterial phyla, some of which include taxa commonly found in high abundance in different ecosystems (Zumft & Kroneck, 2006; Jones et al., 2011; Graf et al., 2014). The *nosZ* gene can be used as a target for the different populations of the denitrifying bacteria capable of nitrous oxide reduction (Jones et al., 2013). A

number of studies have used both *nir* genes and *nosZ* gene as molecular markers to study denitrification in various environments, including TWs (Chon et al., 2011; García-Lledó et al., 2011; Song et al., 2011; Ji et al., 2012; Wang, XJ. et al., 2012; Correa-Galeote et al., 2013; Fu et al., 2017; Li et al., 2019). While heterotrophic denitrification process has been associated with TWs, autotrophic denitrifiers have been identified that are able to use inorganic compounds (Bezbaruah & Zhang, 2003; Ahn, 2006; Zhai et al., 2016; Xu et al., 2017). In the treatment wetlands, it is supposed that microsites with steep oxygen gradients can be established which allow a sequential occurrence of nitrification and denitrification in very close proximity. Sufficient amount of organic carbon is needed as an electron donor and a carbon source for the nitrate reducing denitrifying organisms (van Oostrom & Russell, 1994; Sliekers et al., 2002).

Oxidation of ammonium to N_2 (ANAMMOX) can occur under anaerobic conditions in TWs. The nitrite is an electron acceptor and carbon dioxide is the sole carbon source in this process (Thamdrup & Dalsgaard, 2002). The anammox bacteria, identified thus far belong to the order *Brocadiales* of the phylum *Planctomycetes* and are autotrophic organisms (Jetten et al., 2001). Therefore, stimulating the growth of anammox bacteria in a wastewater treatment system reduces the need for an organic carbon source, which is required in the conventional denitrification process. ANAMMOX activity was found positively correlated with the denitrification process in inland river sediments (Zhou et al., 2014). These findings suggest that microbial symbiosis and co-metabolism commonly exist in natural ecosystems. ANAMMOX is more common in bioreactors, wastewater plants and landfills and has minor importance in most soils (Hu et al., 2011).

Newly discovered nitrite-dependent anaerobic methane oxidation (n-damo) is a process where in addition to the denitrification and ANAMMOX, nitrogen gas can be produced (Raghoebarsing et al., 2006). During this process nitrite is reduced to nitric oxide and the latter is hypothesised to be dismutated into nitrogen gas and oxygen. Finally, the produced internal oxygen is used as an electron acceptor to oxidize methane. This process is associated with a bacterial group belonging to the candidate phylum NC10 with only one described species '*Candidatus* (*Ca.*) Methylomirabilis oxyfera' (Ettwig et al., 2010). Recently a novel archaeal lineage was found, named '*Ca.* Methanoperedens nitroreducens', capable of n-damo process (Haroon et al., 2013). '*Ca*. M. oxyfera' have been identified in natural wetlands (Hu et al., 2014; Chen et al., 2015), anaerobic sludge blanket (Ma et al., 2017) and TWs (Yang et al., 2017; Xu, Y. et al., 2018), while '*Ca*. M. nitroreducens' was found in freshwater marsh sediments (Shen et al., 2017).

Similarly to the assimilatory nitrate reduction, also dissimilatory nitrate reduction to ammonium (DNRA) results in ammonium production, but the former process is regulated by oxygen and is unaffected by ammonium (Tiedje, 1988). Therefore, this dissimilatory pathway fits for the anaerobic environments. As a result, nitrogen is more easily available for plant and microbial uptake as well as less prone to losses via leaching or in form of gaseous

compounds. During the respiratory ammonification, small amounts of N_2O may be released as a by-product (Stremińska et al., 2012). The N_2O is produced when NO in the cell is detoxified. Some DNRA bacteria can also link N_2O reduction to N_2 with energy conservation (Hallin et al., 2018). Nitrate consumption by DNRA process has been found high in FWS TW (Jahangir et al., 2017) and created freshwater wetland (Scott et al., 2008).

1.2.2 Carbon cycle

Organic substrates in domestic wastewater are highly differentiated. Simple and complex carbon compounds flow into the wastewater treatment plant in dissolved or suspended form. Based on their sensibility to biodegradation, these compounds can be divided into readily biodegradable compounds (carbohydrates, proteins and fats) and slowly biodegradable compounds (inert compounds). In the treatment process, they are transformed into simple compounds, oxidized, assimilated or not decomposed (Kadlec & Wallace, 2009; Makowska & Spychała, 2014). Sufficient quantity and diversity of the fractions of carbon compounds are essential for carrying out a variety of removal processes, e.g. organic carbon is needed for heterotrophic and inorganic carbon for autotrophic microorganisms (Truu et al., 2009). Within TWs, the organic matter is usually degraded by aerobic respiration (mineralization) or anaerobic processes such as fermentation and methanogenesis (Reddy & D'Angelo, 1997; Scholz & Lee, 2005), however, other processes such as photochemical oxidation, sedimentation, volatilization, and sorption have an impact on the faith of organic matter as well (Kayranli et al., 2010). Anaerobic degradation of organic matter is less efficient than decomposition occurring under aerobic conditions (Scholz & Lee, 2005). Degradable carbon compounds are rapidly utilized in carbon cycle of TWs. Many carbon removal pathways lead to the microbial production of carbon dioxide (CO_2) and methane (CH_4), which are both important greenhouse gases. As their water dissolution capability is limited, active transfer of carbon to and from the atmosphere in TWs is present. It has been shown that the temporal and spatial pattern of the emission of methane and CO_2 is very variable within TWs (Mander et al., 2005; Mander et al., 2014). Methane flux in wetlands is a function of the activity of methanogenic archaea and methanotrophic bacteria (Johansson et al., 2004; López et al., 2018). It has been shown that influent wastewater C/N ratio has a strong effect on CO_2 and methane emissions and that the optimum C/N ratio, at which TWs can achieve a low level of greenhouse gases flux is 5:1 (Yan et al., 2012; Maucieri et al., 2017). In addition to the methane oxidation, the methanotrophic bacteria present in the TWs can enhance the biodegradation of xenobiotic pollutants due to their methane monooxygenase enzymes that simultaneously with the methane oxidation also co-metabolically oxidize many organic pollutants (Chang & Alvarez-Cohen, 1996).

1.2.3 Phosphorus cycle

Phosphorus (P) is an essential element for all life forms, although even slightly elevated concentrations of phosphorus can lead to an eutrophication in various types of aquatic ecosystems (Correll, 1998). In TWs, phosphorus occurs in a form of phosphate in organic and inorganic compounds (Vymazal, 2007). Free orthophosphate is the only form of phosphorus believed to be utilized directly by algae and macrophytes and thus represents a major link between organic and inorganic phosphorus cycling in wetlands. Another group of inorganic phosphorus compounds are linearly condensed and cyclic polyphosphates (Vymazal, 1995). Organic P forms can be divided into two groups: 1) easily decomposable P (nucleic acids, phospholipids or sugar phosphates); and 2) slowly decomposable organic P (inositol phosphates or phytin) (Dunne & Reddy, 2005). TWs provide an environment for the interconversion of all forms of phosphorus. Soluble reactive phosphorus is taken up by plants and converted to tissue phosphorus or may be sorbed to the particles of wetland soils and sediments.

Phosphorus transformations in TWs are following: peat/soil accretion, adsorption/desorption, precipitation/dissolution, plant/microbial uptake, fragmentation and leaching, mineralization and burial (Kadlec & Wallace, 2009). However, sorption as well as storage in biomass are saturable processes that have a finite capacity and therefore cannot contribute to the long-term sustainable P removal (Dunne & Reddy, 2005). Microbial uptake of P is very fast, but the magnitude (amount of stored P) is very low. Soil microorganisms participate in the solubilization of soil P. Measurements of the precise amounts of soil P solubilized by soil organisms is complicated by the concomitant mineralization of organic P in TWs (Vymazal, 2007).

In subsurface flow constructed wetlands, phosphorus is mainly sorbed by or precipitated on filter material (Vohla et al., 2011). TWs have shown their ability to remove large amounts of P from wastewater using special filter materials with an enhanced P sorption capacity (Ádám et al., 2006). Materials with high pH and Ca and Mg rich expanded soil aggregates or hydrated oil shale ash (an industrial by-product) have shown high (even up to 95%) phosphorus removal efficiency (Vohla et al., 2005; Kaasik et al., 2008; Kasak et al., 2011; Karabelnik et al., 2012). Even if a medium with high P binding capacity has been selected, it may be saturated after a few years (Arias et al., 2001). Over 80% of the phosphorus removal from wastewater is shown to be a result of P accumulation in the filter material via adsorption and only 20% is accumulated into wetland plants and immobilized by microbes in TWs (Mander et al., 2003).

1.3 Microbial community structure in treatment wetlands

Despite the fact that many pollutants are removed from wastewater through chemico-physical or plant mediated processes such as volatilization, sedimentation, sorption, photodegradation, plant uptake, transpiration flux, accretion and

chemical procedures (Kadlec & Wallace, 2009), it is also well known that microbiologically mediated reactions play key role in the treatment process in TWs (Faulwetter et al., 2009; Truu et al., 2009; Weber, 2016). Due to the rapid growth rates and quick response to the changes, microbial populations can also serve as the most sensitive and rapid bioindicators for evaluating the effect of various pollutants in environment (Urakawa & Bernhard, 2017). There are still many uncertainties about the structure, spatial distribution and activity of microbial communities in TWs. Therefore, in the engineered systems, it is vital to discern the factors that affect these microbial community attributes. That knowledge can be utilized for improving the design and operation of TWs, so that a balance between a stable and active microbial community and an effectively performing system could be achieved.

The microbial community inhabiting TWs can be divided into two major groups based on their origin: autochthonous microbes (present in TW) able to possess metabolic activity, survive and grow in a wetland system, and allochthonous microbes (wastewater introduced, including pathogens) most of which are not able to survive or have any functional importance in a TW ecosystem (Truu et al., 2009). Part of the allochthonous microbes could be included into the wetlands permanent microbial community and the role of allochtonous microbes could be more significant in the early stages of the microbial community succession in TW.

According to Knelman and Nemergut (2014), combinations of four processes affect microbial community assembly: dispersal and diversification add new taxa to communities while selection and drift affect their relative abundances. Microbial community adapts to microhabitats present in the TW filter material and live together in consortia with more or less sharp boundaries that interact with each other and with other parts of the filter biota (Torsvik & Øvreås, 2002). The set of biotic interactions is thought to be a key factor controlling microbial community structure and diversity in different habitats (Nemergut et al., 2013). The physical structure of the TWs filters and the varying composition of wastewater could cause spatial isolation within microbial communities. High spatial isolation has been shown to yield high microbial diversity, whereas lower spatial isolation showed much lower diversity and was dominated by a few microorganisms (Tiedje et al., 2001). The study of the role of temporal scale in the assembly of microbial communities in wastewater treatment systems has gained importance during recent years (Vuono et al., 2015; Jiang et al., 2018). However, the collective effect of community assembly processes (e.g., dispersal, drift, and selection) on microbial process in wastewater treatment systems are still poorly understood, and they constitute a key knowledge gap in process-based modelling of biogeochemical cycles in such engineered systems (Xia et al., 2018).

Newly established TWs require stabilisation period until stable treatment efficiency is achieved as well as stable microbial community is formed. Several studies have shown that the length of this period could be between 75–100 days (Pell & Nyberg, 1989; Ragusa et al., 2004; Weber & Legge, 2011; Ramond et

al., 2012), however longer periods (up to 700 days) have demonstrated as well (Song et al., 2011; Samsó & García, 2013). Still, the dynamics of microbial community in TWs during the start-up period is not clearly understood and molecular studies utilising high-throughput sequencing to provide thorough information about microbial community structure in this period are lacking.

Many authors have studied different aspects of microbial communities in TWs and their relationship with treatment processes, such as community diversity (Sleytr et al., 2009; Ramond et al., 2012; Peralta et al., 2013; Ansola et al., 2014; Arroyo et al., 2015; Ibekwe et al., 2016; Zhang et al., 2016), spatial distribution (Krasnits et al., 2009; Bouali et al., 2014; Cao et al., 2017), activity (Iasur-Kruh et al., 2010; Yi et al., 2016) and functional properties (Button et al., 2015; Fan et al., 2016; Hu et al., 2016; Lv, T. et al., 2017). So far, a majority of the research about microbial communities in TWs has been based on older molecular techniques and recent methods, for instance amplicon sequencing and shotgun metagenomics, have started to emerge (Weber, 2016; Sánchez, 2017).

Due to the distinctive hydraulic and environmental conditions, microbial community activity and assembly in FWS, VSSF and HSSF TWs take place in spatially heterogenous environment. Nurk et al. (2005) have shown higher metabolic diversity and activity in the hydraulically saturated filter bed compared to the dry filter in HSSF TW. In the case of a VSSF TW treating municipal wastewater, more than 50% of the microbial biomass and bacterial activity was concentrated in the first upper centimeters and about 95% in the first 10 cm of the sandy substrate filter as was shown in an indoor pilot scale study (Tietz et al., 2007).

Recent studies about microbial community profiling in TWs have shown the dominance of the bacterial classes *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* together with the phyla *Bacteroidetes*, *Actinobacteria* and *Acidobacteria* in vertical flow TWs (Adrados et al., 2014; Ibekwe et al., 2016; Rodríguez-Martínez et al., 2016); classes *Betaproteobacteria* and *Gammaproteobacteria* from phylum *Proteobacteria* as well as the phyla *Bacteroidetes*, *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Firmicutes* in horizontal flow TWs (Adrados et al., 2014; Zhong et al., 2014; Yi et al., 2016); and classes of *Proteobacteria* such as *Alphaproteobacteria*, *Betaproteobacteria*, and *Deltaproteobacteria* with other prevalent phyla *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Verrucomicrobia*, *Chloroflexi*, and *Firmicutes* in FWS TW systems (Peralta et al., 2013; Ansola et al., 2014; Bai et al., 2014; Arroyo et al., 2015).

As can be seen from the results above, analysis of microbial community structure based on higher taxonomic level profiling does not present clear distinction between different TW types. New advanced molecular techniques, like the high-throughput sequencing, have enabled to get exceeding number of microbial sequences from soils and TWs filter materials, allowing to estimate more precisely the diversity of microbial communities (Escobar-Zepeda et al., 2015; Sánchez, 2017). Coupling the clustering sequences by the similarity into operational taxonomic units (OTUs) with the various statistical approaches can

be applied to study the microbial community alpha and beta diversity in TWs (Bouali et al., 2014; Zhong et al., 2014; Guan et al., 2015; Babatunde et al., 2016; Long et al., 2016; Zheng et al., 2018). The amplicon-based sequencing approach could be complemented with the high-throughput meta-approaches based on the shotgun principle, which consists of sequencing biomolecules like DNA or proteins. Rapidly advancing techniques, such as metagenomics (the study of all genes in a microbial community), and metaproteomics (the study of proteins from a microbial community), could enable cultivation-independent analysis of a whole microbial community in TWs. The information obtained using combination of different omic approaches could provide a better understanding of the relative roles of deterministic and stochastic factors in microbial community assembly in TWs and allow for defining the predictive models of microbial communities in TWs.

2. THE AIM OF THE STUDY

The main aim of this thesis is to characterize the bacterial community structure and diversity as well as relate the findings to the pollutant removal potential in different types of treatment wetlands.

The specific objectives were:

- to assess the temporal dynamics of bacterial community activity, abundance and succession in the biofilm of two newly established experimental TW systems treating domestic greywater and municipal wastewater;
- to study the bacterial community structure in soils and sediments of a free water surface TW system treating polluted river water in an intensive agricultural area;
- to evaluate the nitrogen removal potential of the bacterial community in the studied TWs;
- to estimate the relationship between bacterial community structure and treatment efficiency of TWs treating domestic greywater and municipal wastewater;
- to analyse the effect of different wastewater and TW types on bacterial community structure and nitrogen removal potential in the system.

3. MATERIAL AND METHODS

Two studies in experimental wastewater treatment wetlands (Publications I and II) and two studies in a created riverine treatment wetland complex (Publications III and IV) were used in the current thesis. All studies focus on bacterial community structure and abundance in the treatment wetlands and relate the microbial community parameters to the carbon and nitrogen removal potential.

3.1 Overview of the case study sites

3.1.1 Experimental greywater treatment hybrid wetland system (Publication I)

The study was carried out in an experimental hybrid greywater treatment wetland system (GTW) where the efficiency of filter materials in greywater treatment was examined. The whole system treated greywater (water from showers, hand basins, kitchen and laundry) from a household of a five-member family for 10 months (from October 2009 to August 2010). The study system comprised of a septic tank (2 m^3) followed by a collective well and four subsystems each consisting of three parallel VSSF filters (0.02 m^3, 20 cm layer of filter material in each) after which was a hydraulically saturated HSSF filter (0.05 m^3, 60 cm layer of filter material). Light expanded clay aggregates (LECA, Filtralite® and FiltraliteP®) with varied properties and crushed and screened hydrated oil shale ash sediment were used as filter materials. Different subsystems contained different filter media combinations. The experiment was divided into three operational periods, based on the greywater re-circulation and loading rate applied to the system. During the first operational period (from October 2009 to January 2010), the greywater (32.5 l/d) passed both filters before it was recirculated (300% rate, 60 cycles/d). Whereas the water circulation through alkaline horizontal filter (average pH 8.9) kept pH level high also in the vertical filters, the flow regime was changed so that the greywater was recirculated only within the vertical filters (second period). Total hydraulic loading rate was raised to 80 l/d on the 120th day of the experiment (third period). A detailed description of the system, filter materials and operational characteristics is given in Karabelnik et al. (2012), Kasak et al. (2011) and Publication I.

3.1.2 Horizontal subsurface flow wetland mesocosms (Publication II)

A 150-day municipal wastewater treatment study was conducted in horizontal subsurface flow filters of an experimental unplanted hybrid treatment wetland (MWTW HF). The mesocosms (MC) were fed with raw domestic wastewater combined with effluents from the dairy and meat industries, pumped from an

inlet of the activated sludge treatment plant. Wastewater was pumped into a septic tank (2 m^3) followed by six parallel vertical subsurface flow MCs (total area 6 m^2) and was subsequently collected into a distribution well. The pre-treated wastewater was divided equally between 21 parallel horizontal sub-surface flow (HSSF) MC cells (length: 1.5 m, width: 0.2 m, depth: 0.6 m). The three HSSF MCs used in this study were filled with LECA, with a particle size of 2–4 mm, forming the wetland media and providing a high surface area environment for microbial biofilm attachment. The retention time and hydraulic loading rate of the HSSF MCs were kept stable during the trial period at 1.2 day and ≤20 mm/d, respectively. The MCs were covered with 5 cm thick insulation slabs during winter to avoid freezing of the wastewater due to low air tem-peratures. Detailed description and scheme of the study system is provided by Nurk et al. (2009), Zaytsev et al. (2011) and Publication II.

3.1.3 Created riverine treatment wetland complex (Publication III and IV)

The studied experimental wetland complex (RTW) is located on the floodplain of the Olentangy River at the Olentangy River Wetland Research Park in Columbus, Ohio, USA. The 21 ha facility is composed of two 1 ha in size kidney-shaped experimental freshwater wetlands (W wetlands) and a 3 ha river diversion oxbow (Ox), which differ mainly in water regime and vegetation type (Mitsch et al., 1998). The W wetlands have received equal amounts of water pumped from the Olentangy River (average flow 626–1552 m^3/d). The water retention time in the W wetlands is about 3–4 days and eventually the water is released into the Olentangy River. W wetlands were designed to have three distinct permanently flooded deepwater basins (water level 30–60 cm) surrounded by shallower occasionally flooded marsh areas (water level 0–30 cm) that were either planted (W1) or colonised naturally (W2). In contrast to the W wetlands, where the water is pumped throughout the year, the Ox is gravity-fed from the Olentangy River. A detailed description of the wetlands and their operational characteristics is given in Publication III.

3.2 Soil, filter media and water sampling

In greywater purification experiment (Publication I), samples for microbiological analyses were taken in two weeks interval over five-month period and additionally on day 225 and 290. Composite filter material samples (about 30 g) comprised of 5–6 sub-samples were taken from 0–5 cm layer of each VF and 10–15 cm layer of the HF. Water samples from the collective well (inflow to VFs) and outflows of both type of filters were also collected at each sampling time. Electric conductivity, O$_2$ concentration, pH and water temperature were measured on site. Biological oxygen demand (BOD$_7$), chemical oxygen demand

(COD), total organic carbon (TOC), total suspended solids (TSS), NH_4-N, NO_2-N, NO_3-N, total nitrogen (TN), PO_4-P, total phosphorous (TP), SO_4^{2-}, Ca, and Mg contents were determined from the wastewater samples in laboratory using standard procedures.

The sampling during the five-month period of the experimental hybrid constructed wetland system treating municipal wastewater was conducted on days 26, 45, 64, 94, and 150 (Publication II). At each sampling event, five subsamples of wetland media were collected from a depth of 25–35 cm with a soil corer at even distances along the longitudinal transect of the MC. The subsamples were mixed to form a composite sample for each MC. Influent and effluent wastewater samples were taken on day 26. The following indicator parameter values were determined from wastewater samples: TN, NH_4-N, NO_2-N, NO_3-N, TOC, and BOD_7.

Twenty-nine soil and sediment samples from the permanent gas sampling sites of the SFW TW complex treating polluted river water were collected in March 2009 (Publication III and IV). Six samples from the permanently (W_{oo} – organic sediment samples) and six samples from occasionally flooded areas (W_{trans} – transitional areas) of W wetlands, seven samples from the oxbow (Ox), and four samples from the upland areas (Up – 2 samples between W1 and W2 and 2 samples near the Olentangy River) were obtained from the 0–15 cm top soil layer. Additionally, six mineral soil samples were collected from 15–30 cm layer of the open areas of W wetlands (W_{om} – mineral soil samples). In the context of this thesis, only W wetlands flooded and mineral soil samples were considered. The pH values and total carbon (TC), TN, NH_4-N, NO_3-N, P, Ca, K, and Mg content of the soil samples were determined as the background data.

3.3 Microbiological analyses of soil and filter media

3.3.1 Microbial activity measurements (Publication I)

Dehydrogenase activity (DHA) and potential nitrification activity (PNA) measurements were performed on each sampling day in order to evaluate the development of a microbial biomass on the filter material particles and its nitrification potential according to the protocol of von Mersi (1996) and Kandeler (1996), respectively. Microbial activity was analyzed by dehydrogenase enzyme activity via the reduction of the compound 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to a red-colored formazan (INTF) over 2 hours incubation period and analyzed colorimetrically. 1mM $(NH_4)_2SO_4$ solution was used as a substrate for nitrification activity measurements and the samples were incubated in a water bath shaker at 25°C for 5 hours. The dehydrogenase and potential nitrification activity were calculated on dry filter material (dry weight – dw) bases and expressed as µg INTF/g dw 2h and µg N/g dw 5h, respectively.

Based on the results of water quality and activity measurements, the filter media samples of the most stable subsystem (higher activity, less clogging and sufficient treatment efficiency) were chosen for the further molecular analysis of microbial community.

3.3.2 DNA extraction, quantitative PCR and preparation of PCR products for sequencing

Total bacterial DNA was extracted from 0.25 g of crushed VF filter material (Publication I) and 0.25–0.5 g of soil (Publication III) using PowerSoil DNA Isolation Kit and 10 g of HF filter material (Publications I and II) using PowerMax Soil Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) following the protocols of the producer. An exception was the homogenisation step for PowerSoil DNA Isolation Kit protocol that was carried out at 5,000 rpm for 20 s using Precellys® 24 (Bertin Technologies, Montigny-le-Bretonneux, France). The quantity and quality of DNA extracts were determined spectrophoto-metrically using Infinite M200 (Tecan AG, Grödig, Austria). The extracted DNA was stored at –20 °C until further analyses.

In the case of all experiments, bacterial abundance was estimated by quantifying bacterial specific 16S rRNA gene fragment using L-V6 and R-V6 primers (Gloor et al., 2010) amplifying V6 hypervariable region of this gene. For estimating the potential of denitrification and the proportion of nitrous oxide reducers in microbial community of filter media, *nirK* and *nirS*, *and nosZ* clade I and clade II, respectively were used as the marker genes (Publications II, III and IV). In order to evaluate potential of ANAMMOX process in the RTW, ANAMMOX-specific 16S rRNA gene fragment was targeted (Publication IV). All the quantifications were performed on quantitative PCR (qPCR) system RotorGene® Q (QIAGEN, Foster City, CA, USA). The calculation of the target gene copy numbers were performed as described in Nõlvak et al. (2016) and the results are presented per gram of dry filter material weight (copies/g dw). In order to evaluate the proportion of the functional groups in bacterial com-munity, the respective target genes were normalized against 16S rRNA gene considering individual amplification efficiencies and cycle threshold values as was proposed by Ruijter et al. (2009). The results were presented as percentages of detected gene copies in the total bacterial community. The detailed descriptions of the used qPCR reactions conditions, primer concentrations, and amplification programs can be found from the Publications II–IV.

For the analysis of bacterial community structure, the same 16S rRNA gene fragment as was applied in qPCR analysis was used. All PCR reactions were performed in a 20 µl reaction mixture using a Phusion Hot Start High Fidelity Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) according to the instructions of the manufacturer. Preparation of the 2x100bp paired-end DNA library was performed using the NEXTflex® PCR-Free DNA Sequencing Kit (BIOO Scientific Corp., Austin, TX, USA) and the sequencing was performed

using the Illumina® HiSeq 2000 system (Illumina Inc., San Diego, CA, USA). The detailed description of the preparation of amplicons for sequencing is given in the Publications I–III.

3.3.3 Sequencing data processing and statistical analyses

The paired-end reads were assembled into composite reads with SHERA (Rodrigue et al., 2010, Publications II and III) or Pear v 0.9.6 (Zhang et al., 2013, Publication I), using default settings. The assembled reads were processed using Mothur (Schloss et al., 2009, different versions). For data denoising, sequences were discarded if the average sequencing quality score dropped below 25 over 25-bp sliding window, were shorter than 70 bp, had any ambiguous bases, and had longer than 6 homopolymers. Chimeras were detected with uchime algorithm in *de novo* mode (Edgar, 2010). Silva v 119 (Pruesse et al., 2007) and Greengenes v 13_5 (McDonald et al., 2012) were used as reference databases for taxonomic assignment in Publication I and Publications II–IV, respectively. 60% confidence in bootstrap values was used for classifying sequences. All reads that were unable to taxonomically assign were removed from the following analysis. For ANAMMOX-specific bacterial community analyses, all sequences taxonomically assigned to the phylum *Planctomycetes* were used in a blast search against the order *Brocadiales* extracted from the Greengenes v 13_5 database using local alignment and identity over 90% in Usearch v 7.0 (Edgar, 2010). As the number of ANAMMOX-specific bacterial sequences have increased and become more accurate in the databases, this process was repeated for this thesis using reference sequences from Silva database v 132.

Denoised sequences were clustered into operational taxonomic units (OTU) using Swarm v 2.1.5 (Mahé et al., 2015, Publication I) or CROP v 1.33 (Hao et al., 2011, Publications II and III). In Publication I OTU count numbers were standardized before statistical tests with metagenomeSeq v 1.15.3 (Paulson et al., 2013) cumulative-sum scaling method in R v 3.3.0 (R Core Team, 2017). Mothur was used to calculate the alpha diversity indices and Bray-Curtis dissimilarity based distances between samples. The beta diversity (BD$_{Total}$) in Publication I, calculated as the total variation of the bacterial communities, was obtained by computing the sum-of-squares of the relative abundance data for the OTUs and the bacterial genera (Legendre & De Cáceres, 2013) in R with adespatial package v 0.1. The total sum-of-squares of the community composition data was partitioned into the additive components – the species contributions to beta diversity (SCBD) and the local contributions of individual sampling units to beta diversity (LCBD). The temporal variation in the composition of the bacterial communities was decomposed into replacement and richness difference indices (Legendre & Gauthier, 2014).

Distance-based regression analysis was applied using the DISTLM programme with forward selection procedure to identify influent wastewater chemical characteristics that explain significant amounts of variation in bacterial

community structure (McArdle & Anderson, 2001). Statistically significant microbial relationships between OTUs were calculated using SparCC with 10000 permutations (Friedman & Alm, 2012, Publication I) or The Molecular Ecological Network Analyses Pipeline (Deng et al., 2012, Publications II–III). Spearman's rank correlation coefficient values were calculated with Statistica v 7.1 (StatSoft, Tulsa, USA).

4. RESULTS AND DISCUSSION

4.1 Wastewater characteristics and treatment wetland systems performance

The pH was around neutral during the study period in the water entering to the GTW system but it increased significantly (by 1.2 and 0.6 units, p<0.001 in both cases) during the passage through both VFs and HF (Table 1, Publication I). The oxygen concentration was low in the initial greywater and as was expected, the concentration increased almost an order of magnitude in VFs and decreased again in HF. Similarly to the other studies dealing with greywater purification (Eriksson et al., 2002; Ghunmi et al., 2011), this study found that the organic matter content was high in the inflow of VFs and as indicated by COD/BOD_7, about half of this was easily degradable fraction. Around 60% of the TN was removed by the whole system, while the removal efficiency of ammonia was around 20% in the system. About half of the TN was removed in VFs and the removal efficiency increased with time (r=0.48, p<0.05) in these filters. Nearly 10% of the nitrogen was removed in HF and the removal efficiency did not change significantly during the experimental period in this filter. Most of the phosphorus was in soluble form in the raw greywater and its removal efficiency was at similar ranges in both types of filters (around 30%). Statistical analysis did not detect significant differences in the treatment efficiencies related to the changes in the system operational parameters during the experiment.

In the HFs of MWTW, a slight increase in wastewater pH during the experiment was detected similarly to GTW (Publication II). An increase in pH of the treated water during the passage through the filters was detected at the beginning of the experiment but the difference between influent and effluent decreased by the experimental time. Although the concentrations of TN and ammonia were considerably higher (about three times) in the influent of HFs of MWTW compared to the studied greywater, the removal efficiencies of TN, NH_4-N and TOC (all around 20%) in this system were similar to the respective removal efficiencies of the HF of GTW. Significant relationships were found between the MWTW system operation time and the removal efficiencies of NH_4-N (r=0.73, p<0.01) and organic matter (BOD_7 RE r=0.65, p<0.05 and TOC RE r=0.52, p<0.05).

In the soils and sediments of RTW, the pH was slightly acidic or neutral (from 5.4 to 7.2; Publication III). In general, W_{oo} had higher soluble P, NH_4-N, K, and Ca contents, the lowest C/N ratios, and the most variable NO_3-N content compared to the other studied soil groups. W_{om} had the lowest C, N, and Mg contents and W_{trans} was distinguished by the lowest soluble P concentrations and lowest pH. The most variable soil chemical parameters were the concentrations of NH_4-N and NO_3-N (<0.05–104.4 mg/kg and <0.17–9.88 mg/kg, respectively), while the contents of total C and N and their ratio varied in a smaller range

(1.39–3.80%, 0.12–0.42%, and 7.41–14.17, respectively). In addition, the studied soil and sediment samples differed by their P contents (2.3–129.3 mg/kg). However, the soil samples of the two W wetlands were not statistically different by their chemical characteristics indicating that the initial vegetation had not significantly affected soil chemical composition after fifteen years of operation. In addition, when soil chemical parameters of sampling sites were compared along the longitudinal gradient of basins of W wetlands, no significant differences were detected between the soil and sediment samples. Mitsch et al. (2012) monitored the conditions and treatment efficiency of the wetland complex during 15 years from the creation of W wetlands and did not detect difference in the nutrient removal efficiency between these two W wetlands after 15 years. The study also showed that the wetlands had been sinks for 26%–60% of nitrogen by mass (and 22%–29% by concentration of NO_3-N) for the last 5 years of the 15-year study period and the denitrification accounted for only 2%–10% of the nitrogen removed by these wetlands. Another study, conducted within the same period in these RTW W wetlands found that temporal variation in denitrification activity primarily depended on temperature and nitrate concentration in soil (Song et al., 2012). Additionally, significantly lower denitrification rates were recorded in the shallow marshes compared to the open water inflow and outflow areas and deep marsh areas by this study.

4.2 The dynamics of bacterial community abundance and activity in the treatment wetland systems

In GTW VFs, the bacterial abundance evaluated by the 16S rRNA gene abundance increased rapidly after the start of the system operation and reached its maximum (1.3×10^{10} copies/g dw) by the day 85 (Publication I). After a three-month operational period, in response to the first change in the system operation, the bacterial abundance rapidly dropped about one order of magnitude and the 16S rRNA gene abundances stayed at the level below 2×10^9 copies/g dw for the rest of the experiment. DHA reached its maximum level (540 µg INTF/g dw/2h) after the second change in the system operation. There were no rapid changes in DHA value and the activity remained above the value of 270 µg INTF/g dw/2h up to the end of the experiment in these filters. Statistical analysis detected a significant negative relationship (p<0.05) between DHA and 16S rRNA gene abundance in VFs of this system. Both these observed parameters were significantly related to the inflow organic matter quality (COD/BOD_7) and TP content but the relationships were opposite.

In the HF of this system, both the bacterial abundance and DHA stayed substantially below of those measured in VFs. The 16S rRNA gene abundance varied between 2.5×10^8 and 1.8×10^9 copies/g dw and the maximum was measured on day 106 in this filter. Rapid and simultaneous decreases of both bacterial abundances and activity were recorded after the second change in the

system operation and although the 16S rRNA gene abundance remained below 5×10^8 copies/g dw after that drop, the DHA value increased up to 240 INTF/g dw/2h by the day 225. No statistically significant relationships between DHA, 16S rRNA gene abundance, inflow chemical parameters and pollutant removal efficiencies in HF were found.

In the influent of HFs of MWTW, the bacterial 16S rRNA gene abundance was between 5.6×10^5 and 8.1×10^7 copies/ml during the first 94 days of the system operation, after that a decrease in this gene abundance to 1.0×10^5 copies/ml was recorded (Publication II). In contrast, a steady increase in the bacterial abundances of the developing biofilm on the filter material particles was detectable. The 16S rRNA gene abundance increased during the first 94 days of the system operation and then stabilised. The replicates of MCs differed only slightly by their bacterial 16S rRNA gene abundances during the study period. Statistical analyses revealed that the bacterial community abundance in HSSF filters was affected by the temperature in MCs as well as by influent nitrate concentration.

After the 15 years of operation, the 16S rRNA gene abundance was between 3.79×10^9 and 2.41×10^{10} copies/g dw in the soils and sediments of W wetlands of the Olentangy RTW (Publications III and IV). Bacterial abundance was significantly higher ($p<0.05$) in permanently flooded organic sediments compared to the mineral soils and sediments of the occasionally flooded areas. The bacterial abundance was positively correlated to N, C, K, and Mg concentrations in soil, while a negative correlation was detected with soil C/N value.

Table 1. Averages and standard deviations (in parentheses) of the wastewater characteristics and removal efficiencies (RE) of different pollutants in the greywater treatment wetland (GTW), horizontal subsurface flow filters of the municipal wastewater treating wetland (MWTW HF) and W wetlands of riverine treatment wetland complex (RTW); W_{oo} – organic sediment samples of open areas; W_{om} – mineral soil samples of open areas; W_{trans} – soil samples of transitional areas). *TC and TN values are presented in percentages for RTW soils and sediments.

Parameter	GTW				MWTW HF			RTW		
	Influent (n=9)	VF effluent (n=45)	HF effluent (n=15)	RE (%)	Influent (n=5)	Effluent (n=5)	RE (%)	W_{oo} (n=6)	W_{om} (n=6)	W_{trans} (n=6)
pH	6.8 (±0.3)	8.0 (±0.3)	8.6 (±0.3)	–	7.6 (±0.3)	8.3 (±0.3)	–	6.8 (±0.1)	6.7 (±0.4)	5.9 (±0.2)
O$_2$ (mg/l)	0.4 (±0.6)	3.5 (±0.7)	2.6 (±0.5)	–			–	–	–	–
TSS (mg/l)	135 (±25)	16 (±7)	11 (±6)	92 (±3)	–	–	–	–	–	–
TC (mg/l)	203 (±34)	117 (±29)	123 (±28)	39 (±10)	–	–	–	2.92* (±0.37)	1.83* (±0.13)	2.71* (±0.45)
TOC (mg/l)	155 (±29)	40 (±11)	34 (±10)	79 (±4)	19 (±6.1)	14 (±7.0)	26 (±32)	–	–	–
COD/BOD	1.8 (±0.3)	3.8 (±2.0)	4.3 (±2.5)	–	–	–	–	–	–	–
TN (mg/l)	13 (±26)	6.0 (±1.2)	5.4 (±1.3)	57 (±10)	25 (±5)	18 (±4)	24 (±17)	0.36* (±0.03)	0.16* (±0.01)	0.24* (±0.05)
NH$_4$-N (mg/l)	3.5 (±2.1)	2.3 (±1.6)	2.8 (±1.6)	–	9.4 (±5.1)	8.0 (±4.2)	16 (±12)	60.9 (±37.3)	50.8 (±25.2)	3.2 (±3.0)
NO$_3$-N (mg/l)	0.02 (±0.01)	0.07 (±0.13)	0.02 (±0.01)	–	12 (±1.6)	8 (±3)	33 (±24)	0.41 (±0.72)	0.38 (±0.41)	0.34 (±0.45)
TP (mg/l)	7.2 (±3.6)	5.3 (±3.5)	4.3 (±2.2)	34 (±32)	–	–	–	92.2 (±34.3)	28.7 (±21.8)	12.2 (±8.6)
PO$_4$-P (mg/l)	5.4 (±3.2)	4.6 (±3.1)	3.7 (±2.0)	–	–	–	–	–	–	–

4.3 The dynamics of bacterial community structure in treatment wetlands

The bacterial community structure in the soils and sediments of the RTW and filter material of the GTW and MWTW was studied by applying a high-throughput 16S rRNA amplicon sequencing technique. Taxonomical profiling of the 16S rRNA sequences revealed a large number of phyla from all studied sites (from 36 to 56 different bacterial phyla). Moving window analysis based on Bray-Curtis dissimilarity of the relative abundances of phyla revealed that the temporal changes in the bacterial community structure during last three sampling occasions were minor in all GTW filters as well as in HF of the MWTW (data not shown). This allowed to combine the relative abundances on phylum level and present the average values together with the W wetland samples of RTW (Figure 1).
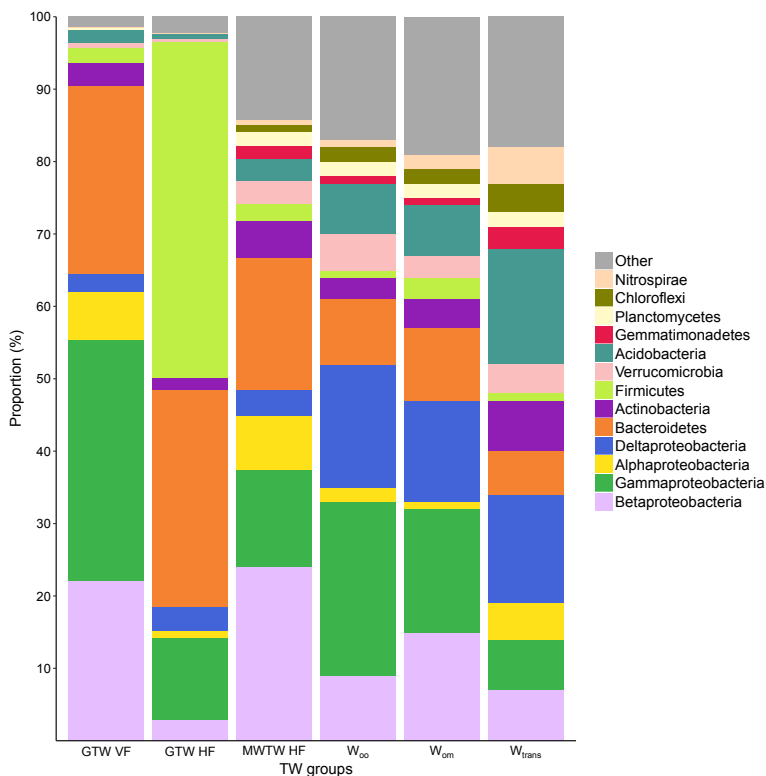
Figure 1. Average proportions of bacterial phyla in the studied TW groups. In the case of *Proteobacteria*, four most abundant classes are shown. Only taxa representing more than 1% at least in one TW group are presented. Abbreviations of the TW groups: GTW VF – vertical flow filter of the greywater treatment wetland, GTW HF – horizontal flow filter of the greywater treatment wetland, MWTW HF – horizontal subsurface flow filters of the municipal wastewater treating wetland, W_{oo} – organic sediment samples of the open areas of W wetlands of the riverine wetland complex (RWT), W_{om} – mineral soil samples of the open areas of W wetlands of the RWT, W_{trans} – soil samples of transitional areas of W wetlands of the RWT.

The structure of the bacterial community of the developing bacterial biofilm differed from the communities detected on the initial filter materials used in VFs and HF of GTW. The bacterial community of the VF units was dominated by classes of *Beta-* and *Gammaproteobacteria* and phylum *Bacteroidetes*. Similar dominant bacterial groups have been shown also in other studies investigating greywater treatment systems (Dalahmeh et al., 2014; Rodríguez-Martínez et al., 2016). The proportion of *Proteobacteria* was significantly higher in VFs than in HF of this system where the members of phylum *Firmicutes* gained significant importance whereas the proportion of *Bacteroidetes* remained at the same level during the performance of the filter. On the genus level, 30 and 24 dominant bacterial genera (≥1% at least in one sample) were identified during the experimental period in VFs and HF of the GTW, respectively. Only seven of them such as *Tolumonas*, *Acinetobacter*, *Xanthomonas*, *Xenophilus*, *Polaro-monas*, *Pseudomonas* and *Legionella* were represented in both types of filters. Substantial changes in the proportions of several genera such as *Tolumonas*, *Acinetobacter*, *Xanthomonas*, *Thiotrix*, *Arenimonas*, and *Dechloromonas* were detected after the first change in the system operation on day 106 in VFs.

A similar tendency was observed in the HF of MWTW, where the structure of the developing bacterial community differed from the initial community on LECA and from the community entering into the MCs with wastewater during the experimental period. Contrary to the results obtained from the HF of GTW, *Proteobacteria* was the most abundant phylum in the inflow, initial filter material and in all these filters during the experimental period accounting up to 57% of the total number of sequences. This group was followed by *Bacteroidetes* in the filter material biofilm, *Actinobacteria* on raw LECA and *Firmicutes* in the influent. *Proteobacteria* differed also by the composition and proportions of classes in those system components with *Betaproteobacteria* being the most abundant in the filter material and *Gammaproteobacteria* on initial LECA and in the influent. The taxonomic composition of the bacterial community in the HF of MWTW was similar to those found in the hybrid systems of TWs treating municipal wastewater (Arroyo et al., 2010; Iasur-Kruh et al., 2010) and in the activated sludge samples of wastewater treatment plants (Wang, X. et al., 2012).

In the FWS RTW, *Proteobacteria* was the most dominant phylum in all studied soil groups and opposite to GTW and MWTW systems, the class *Delta-proteobacteria* was prevalent. The latter was followed by *Bacteroidetes* and *Acidobacteria* in all soil types of the permanently flooded areas, and *Acido-bacteria* and *Actinobacteria* in the soils of the transitional areas of W wetlands. The phylum *Nitrospirae* was detected in all studied TWs, but in the transitional areas of RTW the proportion of this bacteria was remarkably higher than in the other studied TW units (Figure 1). The genus *Nitrospira* from this phylum contain also some species that have been recently shown to have a unique metabolic pathway – on contrary to the other *Nitrospira* species, they perform a complete nitrification pathway summoned as comammox (Daims et al., 2015; van Kessel et al., 2015). The proportion of the representatives of this phylum

was around 1% also in the HF of MWTW. Similar proportions of this bacterial group were found in floating TWs purifying wastewater from the secondary effluent (Gao et al., 2017) and FWS TW treating swine wastewater (Jia et al., 2017).

The OTU-based analysis indicated differences in the diversity of bacterial community between the studied TW systems. While bacterial community diversity index values were similar in VFs of GTW and the mineral soils and organic sediments of open areas in RTW, in HF of GTW the diversity was about 25% lower and in HFs of MWTW and transitional areas of W wetlands 4 and 5 times higher, respectively than in the studied VFs and permanently flooded areas of the W wetlands. The community diversity increased in MWTW and GTW systems during the first three months that followed by almost stable state up to the end of the experiment. It should be kept in mind that the bacterial diversity index values could be affected by the OTU clustering method and the comparison of these values between different studies must be taken carefully. The results of the principal coordinate analysis, based on the Bray–Curtis dissimilarity, show that the bacterial community differences between the sampling events were substantially greater than the community differences between the replicate mesocosms in the cases of GTW and MWTW systems. Similar bacterial community successional patterns were detected for the MWTW MCs and GTW systems with larger changes in the community structure during the first months of operation. Later, the temporal variation in community structure decreased. This stabilisation period during TW start-up phase has been demonstrated also by other authors (Ragusa et al., 2004; Weber & Legge, 2011; Ramond et al., 2012). In the case of RTW, ordination analysis revealed that the samples of W wetlands open areas (W_{oo} and W_{om}) separated from the samples of transitional areas. Multivariate analysis of similarities (ANOSIM) indicated that the bacterial communities of W_{oo} were not different from the W_{om} communities, but both diverged from the W_{trans} community.

The results of the analysis of beta-diversity partitioning show that the temporal changes in the bacterial community structure in VFs and HF of GTW, as well as in HF of MWTW were dominated by species replacement, which accounted for about 90% in VFs of GTW and 70% in HFs of both systems (Figure 2, Publication I). While the contribution of replacement was constant over the study period, the contribution of richness difference was higher during the first sampling. These findings suggest that the temporal pattern in the relative importance of different assembly mechanisms during the succession of biofilm bacterial communities are dependent on the filter type. The results indicate that the deterministic and stochastic processes simultaneously contributed to the succession of the bacterial communities in the biofilms but the relative importance and temporal dynamics of these different processes over time were variable.
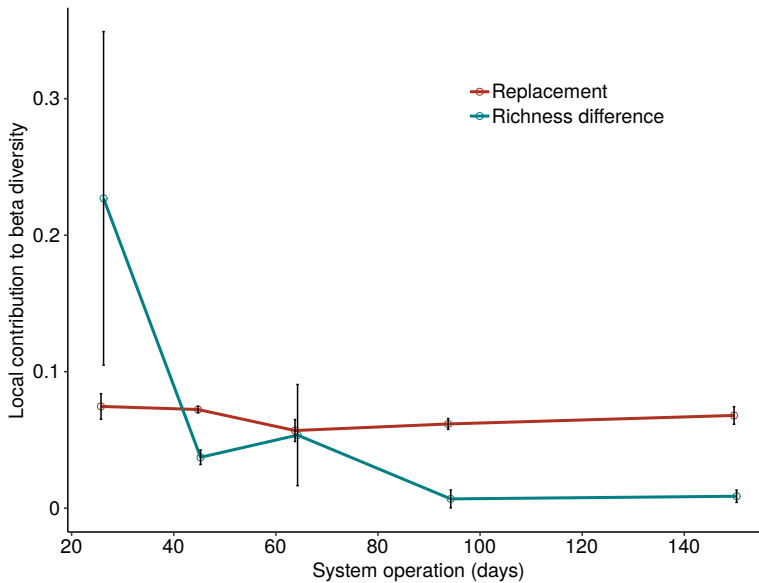
Figure 2. Local contributions to the beta diversity components (replacement and richness difference) during the experimental period in the horizontal subsurface flow filters of the municipal wastewater treating wetland. Vertical lines indicate standard deviations (n=3).

Co-inertia analysis was used to reveal the similarity in community succession in GTW VF and HF. Strong congruence between the temporal dynamics of bacterial community structure in VF and HF on the genus level was revealed (RV=0.84, p<0.001). The similarity in the temporal dynamics of bacterial communities in the two studied filter units was not due to similar pattern in the temporal variation of the relative abundance values of the same bacterial genera.

The co-occurrence analysis revealed several strongly related bacterial consortia (modules) in the bacterial communities of the studied treatment systems. The number of modules and correlations between them differed significantly between the systems. A correlation network with the smallest number of modules was detected in W wetlands of RTW, where OTUs from the W_{om} clustered into three modules, and OTUs from the W_{oo} and W_{trans} soil groups clustered into two modules. In the VFs of GTW, the obtained network composed of three large modules with numerous (50–79) members, while in HF, the bacterial network composed of 19 modules with 1–18 OTUs in each module. In contrast, five modules with an average clustering coefficient of 0.230 were revealed in the HFs of MWTW.

4.3.1 Factors affecting bacterial community structure
in the studied treatment wetlands

According to the distance-based regression analysis, the system operational time was significantly related (p<0.001) with the bacterial community structure in the HF of MWTW MCs. Influent chemical characteristics such as contents of nitrate, total nitrogen and TOC as well as the pH had a cumulative effect that explained 77% of the variation in temporal dynamics of bacterial community structure. Similarly, the temporal dynamics of bacterial community structure in the HF of GTW was explained by inflow pH and the contents of NH_4-N and TOC (66% cumulatively, p<0.01). In the VFs of GTW, the results of the regression analysis showed that the inflow pH and concentrations of TN, NH_4-N, TOC, COD/BOD, and TP significantly explained variations (70% cumulatively, p<0.01) in bacterial community structure. Several statistically significant relationships between bacterial community composition and soil chemical parameters were found in soils and sediments of the W wetlands of RTW. When all samples were incorporated into the regression analysis, then the analysis revealed that the microbial community structure was affected by NH_4-N, Ca, NO_3-N, and C concentrations and pH value in the studied medium (soil or sediment). A cumulative effect of chemical variables explained 58% of the total variation in bacterial community structure in these TWs.

4.3.2 Relationship between pollutant removal efficiency and
bacterial community structure in different types of TWs

Numerous statistically significant relationships between bacterial community structure and treatment efficiencies of the experimental systems (GTW and HF of MWTW) were found (Publicaton I and II). In the HF of MWTW, strong positive correlations between the bacterial community diversity and the removal efficiencies of ammonia (r=0.82), BOD_7 (r=0.65), TOC (r=0.58) and nitrate (r=0.52) (p<0.05) were found. In addition, certain bacterial consortia, as identified by the co-occurrence analysis, were related to the treatment efficiencies of MWTW and GTW systems. Several mutually related bacterial groups had significant positive correlations with the removal efficiencies of NH_4-N, NO_3-N, and BOD_7 in MWTW. In VFs of GTW, the removal efficiencies of TN, NH_4-N, and TP were related to the certain bacterial consortia, but in HF of this system only relationship with TP removal revealed.

The application of the random forest approach revealed representatives from 20 bacterial genera that were linked to the treatment efficiency of the VFs of GTW. From these genera, *Acidovorax* and *Terrimonas* were related to both TOC and TN removal, and *Rheinheimeria* to TOC and TP removal. Furthermore, relationships between the genera *Tolumonas*, *Blastocatella* and *Pedobacter* and the removal of TN and NH_4-N from VFs were found. *Tolumonas* has been found in bacterial communities of different types of bioreactors treating

municipal wastewater (Świątczak & Cydzik-Kwiatkowska, 2018; Xu, Z. et al., 2018) as well as from constructed wetlands (Wang et al., 2016). The genus *Blastocatella* has been linked to ammonia removal in aerobic wastewater treatment systems (Ouyang et al., 2017) and nitrate removal in bioreactors (Peng et al., 2018). Genus *Dokdonella* is abundant in heterotrophic ammonia-oxidizing microbial communities in bioreactors with efficient nitrification activity (Fitzgerald et al., 2015) and the genus *Brevundimonas* harbours strains with aerobic denitrification capability (Lv, P. et al., 2017).

The removal of organic compounds from greywater was related to six genera and five of them such as *Arenimonas, Myroides, Acinetobacter, Terrimonas,* and *Rheinheimera* were important in the bacterial community succession according to the results of the beta-diversity partitioning analysis. Bacteria from the genus *Rheinheimera* has been found from biofilms of aerobic wastewater treatment systems and the members of this genus can readily degrade organic matter (Naz et al., 2016; Naz et al., 2018). *Rheinheimera* together with *Arenimonas* are abundant in the VF biofilms (Xu et al., 2016).

This positive relationship between bacterial community diversity and filter treatment efficiency in the VFs of GTW suggests that an increase in the number of bacterial taxa is accompanied by the increase in the number of different metabolic processes in the VF biofilm. In the HF, the analysis did not detect any significant relationships between the community diversity and pollutants removal efficiencies.

4.4 Nitrogen removal potential in the studied treatment wetlands

4.4.1 Nitrification potential

Results of the PNA measurements in the greywater treating system indicated very low nitrification potential during the first operational period (78 days) in GTW VFs (Publication I). The activity increased during the second half of the study period. Ye and Li (2009) also found that the maximum nitrification activity was established during a two-month period in a newly constructed hybrid TW treating domestic wastewater. The phylogenetic affiliation of the bacterial 16S rRNA gene sequences obtained from the VSSF filters of this system resulted in four bacterial genera, namely *Nitrosomonas*, *Nitrosospira*, *Nitrospira*, and *Nitrobacter*, known to be associated with the nitrification process. Similar bacterial groups were detected also in HF of MWTW and RTW soils and sediments (Table 2, Figure 3). *Nitrosomonas* was the most abundant ammonia-oxidizing and *Nitrospira* nitrite-oxidizing bacterial genus. However, their abundance remained low and this could explain the low nitrification activity in GTW during the study period. In the HF of MWTW, similarly to VF of GTW, the proportions of *Nitrosomonas* and *Nitrospira* increased over the operational period (Figure 3).

Table 2. Average proportions and standard deviations (in parentheses, n=6) of nitrifying bacterial genera in the studied soil groups of riverine treatment wetland complex (RTW). W_{oo} – organic sediments of open areas of W wetlands; W_{om} – mineral soils of open areas of W wetlands; W_{trans} – transitional area soils of W wetlands.

Genera	RTW soil groups		
	W_{oo}	W_{om}	W_{trans}
Ammonia-oxidizing bacteria			
Nitrosomonas	0.023% (±0.029)	0.014% (±0.012)	0.008% (±0.009)
Nitrosospira	0.001% (±0.001)	0.001% (±0.002)	0.002% (±0.003)
Nitrite-oxidizing bacteria			
Nitrobacter	0.002% (±0.002)	0.001% (±0.001)	0.002% (±0.001)
Nitrospira	0.205% (±0.052)	1.433% (±1.069)	0.929% (±0.484)
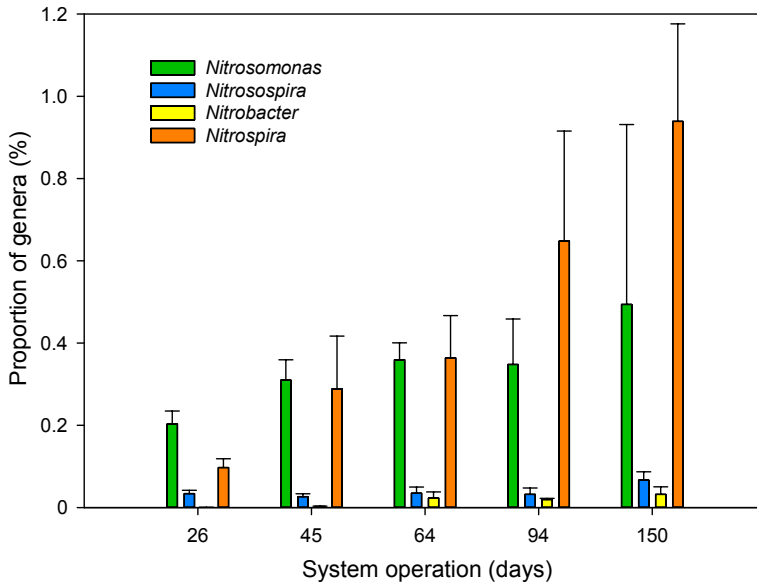
Figure 3. Dynamics of the proportions of nitrifying bacterial genera (with standard deviations, n=3) in the bacterial community of horizontal subsurface flow filters of the municipal wastewater treating wetland system.

Whereas the proportions of those genera were over two times higher in the former indicating possibility of nitrification potential also in the HF of MWTW. *Nitrosomonas* and *Nitrospira* are shown to dominate in ammonia and nitrite oxidation, respectively also in conventional wastewater treatment plants (Yao & Peng, 2017) and other VF wetlands (Pelissari et al., 2017). Genus *Nitrosomonas* species can contribute substantially to nitrogen removal under low oxygen (0.5–3%) conditions through process called nitrifier-denitrification (Zhu et al., 2013).

Nitrospira supply urease-negative ammonia oxidizers with ammonia and receive nitrite produced by ammonia oxidation in return, leading to a reciprocal feeding interaction of nitrifiers. In addition, *Nitrospira* couple formate oxidation with nitrate reduction in case of anoxia (Koch et al., 2015). Although, both PNA and the proportion of the sequences belonging to the nitrifiers genera were scarce in HF of GTW system of current study, these two dominating groups of nitrifiers were also found in considerable amounts in HSSF filters of a hybrid TW system receiving municipal sewage (Zhai et al., 2016).

Unlike this study, Li, B. et al. (2018) found that *Nitrosospira* together with *Nitrosomonas* were prevalent in FWS TW. Fan et al. (2016) suggested that the ammonia-oxidizing archaea were more critical than AOB for the ammonia removal from river water in FWS TWs.

4.4.2 Denitrification potential

The quantification of the targeted denitrification genes showed that in MWTW HF, the proportions of all *nir* genes increased about 2.3 times (up to 3.74%) during the first 64 days of the system performance in microbial community but then followed a decrease that ended with 0.98% of these genes at the end of the experiment (Publication II). This result is in agreement with the previous studies that evaluated the proportion of denitrifiers in municipal wastewater-fed FWS TWs (Chon et al., 2011). Unlike the aforementioned results, the proportion of *nirK* gene ranged from 7.67 to 13.51% and *nirS* gene from 15.93 to 32.93% in the microbial communities of the studied RTW samples, which refers to the fact that a relatively high proportion of bacteria possess *nir* genes in the studied bacterial communities (Publication III and IV). The *nir* genes proportions obtained in this study and shown in different TWs by other authors stay within similar range to the proportions in bacterial communities of grassland and agricultural soils (Jones et al., 2014). The dynamics of the abundances of both *nir* genes in MWTW HF samples followed the same pattern as the 16S rRNA gene abundance during the experimental period. A remarkable increase in the abundances of both these genes was recorded within the first 26 days of the system operation, followed by a slight increase of *nirK* gene up to day 150 and a stabilisation of *nirS* gene after day 94. In RTW, the proportions of *nirS* showed higher values in the organic sediments of the permanently flooded areas than in transitional area sediments ($p<0.01$), while differences were not detected in the respective values of *nirK* proportions. On average, the proportion of *nirS* exceeded *nirK* by 6 times in MWTW and 3 to 4 times in RTW. Similar results have been demonstrated by the other authors as well (Kandeler et al., 2006; Li et al., 2019). Although it was assumed that *nirS* and *nirK* cannot co-occur in the same bacterial cell (Jones et al., 2008), recent discoveries have demonstrated otherwise (Graf et al., 2014). Therefore, it is difficult to distinguish *nirS*-type and *nirK*-type bacterial communities.

The absolute abundances of both *nir* genes had positive relationships with the influent nitrate concentration and removal efficiencies of ammonia, TOC and BOD_7 ($p<0.05$ in all cases) in MWTW. This finding is consistent with that of García-Lledó et al. (2011), who found similar results in free water surface CW. However, in the Olentagy RTW only ammonia concentration in water was significantly related ($p<0.05$) to *nir* genes proportions, but not nitrate concentration. In MWTW HFs, the proportions of *nir* genes were significantly related to the influent nitrogen, ammonia, nitrite and TOC concentrations ($p<0.05$). Overall, the proportion of *nirS* gene had more statistically significant relationships with the MWTW water characteristics compared to *nirK* gene. In RTW, water regime was the main driver for *nir* genes possessing microbial communities, as *nirS*-type denitrifiers were prevalent in permanently flooded areas and higher values of *nirK*/*nirS* were found in transitional areas. In addition to the water regime in the wetland basin, nitrite reduction potential was also affected by soil type, since higher proportion values of *nirK*, *nirS*, and *nir* genes were detected in the bacterial communities of organic sediments compared to the mineral soils beneath them ($p<0.05$). Azziz et al. (2017) also showed in a mesocosm experiment studying denitrification in paddy soil that the community structure of *nirS*-type denitrifiers was more related to the analysed factors than the *nirK*-type organisms and soil type was the main factor that influenced this community structure. In addition, Hou et al. (2018) observed in a long-term fertilization study that the *nirS*-type denitrifier community was more sensitive to the rhizosphere effect than the *nirK*-type community.

The proportions of *nosZ* genes from clades I and II appeared to be similar (from 1.0 to 2.5%) across the RTW soil groups (Publication IV). The bacterial communities of W_{trans} possessed significantly more *nosZ* genes compared to W_{oo} ($p<0.05$). From 1.3 to 2.4 times bigger proportions of organisms possessing one or the other clade of *nosZ* were found in W_{trans} samples ($p<0.05$ in all cases). The proportion of *nosZ*II was significantly higher in organic sediments of open areas of W wetlands than in the mineral soils beneath them ($p<0.05$). The ratio of the two *nosZ* gene clades (*nosZ*I/*nosZ*II) varied large extent (from 0.51 to 5.46) in the wetland complex area. The proportion of *nosZ* genes in bacterial community was negatively correlated to pH value ($p<0.05$) and no significant relationship with nitrogen or nitrogen compounds were found in this study site. Similar results were obtained also by Correa-Galote et al. (2013) in a FWS TW. Graf et al. (2014) found that the *nosZ* genes had a significantly higher frequency of co-occurrence with *nirS* than with *nirK*. The results suggest that *nirS*-type denitrifiers possess more often genetic tools for performing the complete denitrification pathway and thus contribute less to N_2O emissions than *nirK*-type denitrifiers under favourable environmental conditions.

4.4.3 ANAMMOX potential

Anaerobic ammonium oxidation (ANAMMOX) is one of the two main processes part of the nitrogen removal pathways producing dinitrogen gas (Thamdrup & Dalsgaard, 2002). The presence of ANAMMOX specific bacteria in the RTW soil groups was examined through the analysis of the obtained 16S rRNA sequences and was also confirmed by the qPCR results (Publication IV). Although the sequencing analysis of the ANAMMOX specific 16S rRNA gene proportions varied between 0–0.17% across the studied soil bacterial communities in RTW, the proportions of these organisms was 0.00005% to 0.084% in bacterial community according to the qPCR analysis in the soils and sediments of this studied site. These proportions were up to six orders of magnitude lower than the proportions of *nosZ* genes in this site. Significant relationships between relative abundance of anammox bacteria and pH value, P and C/N content were detected ($p<0.05$). However, statistical analyses did not detect a significant difference in the values of anammox bacterial proportions between wetland areas with different water regimes as well as between different soil types. Although the initial affiliation of the ANAMMOX sequences showed a dominance of '*Ca*. Brocadia' (Publication IV), the most resent repeated screening of the 16S rRNA gene sequences using bacterial genera known to be associated with the ANAMMOX process revealed that '*Ca*. Jettenia' was the most prevalent genus in all the studied soil groups (Table 3). This discrepancy in results shows that the reference databases have improved since the first analysis and enable more precise affiliation of the sequences. The second dominant genus was '*Ca*. Scalindua' and that was confirmed by both these analyses. Over half of the retrieved sequences were affiliated to '*Ca*. Scalindua' in a study of anammox bacteria in estuarine and coastal wetlands (Jiang et al., 2017). Li, P. et al. (2018) also found '*Ca*. Scalindua' to be present in mangrove wetland sediments while, in addition to the latterly mentioned genus, also '*Ca*. Kuenenia' was found to be one of the prevalent genera in FWS TW (Waki et al., 2015), on the contrary to the Olentangy RTW, where the minor proportions of '*Ca*. Kuenenia' was found.

The potential for the ANAMMOX process was found also in the HF of MWTW where 1.14% out of all the obtained sequences across the samples were identified as representatives of the phylum *Planctomycetes* and 0.34% of these sequences belonged to the order *Brocadiales*. Similarly to RTW, '*Ca*. Jettenia' was the most prevalent anammox bacterial genus in these filters. Still the abundance of anammox bacteria remained low in this type of TW. The finding is in concordance with a study by Coban et al. (2015), who also found low numbers of ANAMMOX bacteria but detected no anammox activity in a benzene and ammonia rich groundwater-fed HSSF TW. Small proportions of anammox bacterial genera were also found in GTW (Table 3). This minor ANAMMOX genetic potential in greywater treatment systems could be explained by the low concentrations of N compounds in greywater and due to the sensitivity of the ANAMMOX process to environmental parameters (Gross et al., 2015).

Table 3. Average proportion of anaerobic ammonium-oxidizing bacterial genera in the studied treatment wetlands. Abbreviations of the TW groups: GTW VF – vertical flow filter of the greywater treatment wetland, GTW HF – horizontal flow filter of the greywater treatment wetland, MWTW HF – horizontal subsurface flow filters of the municipal wastewater treating wetland, W_{oo} – organic sediment samples of the open areas of W wetlands of the riverine wetland complex (RWT), W_{om} – mineral soil samples of the open areas of W wetlands of the RWT, W_{trans} – soil samples of transitional areas of W wetlands of the RWT. nd – not detected.

Genera	Wetland type					
	GTW VF	**GTW HF**	**MWTW HF**	W_{oo}	W_{om}	W_{trans}
'*Ca.* Brocadia'	0.001%	0.002%	0.001%	0.005%	0.003%	0.011%
'*Ca.* Scalindua'	0.002%	0.003%	0.002%	0.076%	0.036%	0.160%
'*Ca.* Jettenia'	0.023%	0.024%	0.335%	0.420%	0.220%	0.640%
'*Ca.* Kuenenia'	<0.001%	<0.001%	nd	nd	<0.001%	<0.001%

5. CONCLUSIONS

TWs have been used over fifty years for purification of different types of wastewater. This process is mostly related to microbiologically mediated reactions, however, there are still many uncertainties about the structure, spatial distribution and activity of microbial communities in TWs. Knowledge of the microbial community dynamics and structure and the relationship with the system properties and operation conditions is therefore crucial for the development and optimization of effective TWs. Based on a combination of different microbiological analysis approaches, the assessment of bacterial community abundance, activity, structure and diversity, as well as their relation to the pollutant removal potential in different types of TWs, was performed in this study. The results presented in this thesis allow to make the following conclusions:

The origin of wastewater, treatment wetland type and the treatment system hydrologic mode affected the bacterial community composition of the treatment system. Vertical flow filters of GTW were dominated by classes of *Gamma*- and *Betaproteobacteria*, and phylum *Bacteroidetes*, while in the horizontal flow filter *Firmicutes* was the prevalent phylum, followed by *Bacteroidetes*. The differences between the two filter types were also detected on the genus level. Only seven out of thirty prevailing genera were common to both filter types. MWTW horizontal sub-surface flow filters were dominated by different classes of *Proteobacteria*, but like in GTW, phylum *Bacteroidetes* was also prevalent. Soils and sediments of RTW stood out from the rest of the studied treatment wetlands, as the proportions of *Deltaproteobacteria*, *Acidobacteria*, and *Nitrospirae* were noticeably greater.

In the GTW and HF of MWTW, highest bacterial abundance was reached by the end of the three-month working period. While the bacterial community abundance stabilised and remained high for the rest of the study period in the HF of MWTW, a sudden decrease was detected in the bacterial abundance of VF and HF of GTW affected by the changes in the system operational parameters.

The assessment of the diversity of bacterial communities among the studied TW systems showed congruent results. The community diversity increased during the start-up period in MWTW and GTW systems over three-month, followed by the almost stable state up to the end of the experiment. In addition, comparable bacterial community successional pattern was detected in the MWTW and GTW filter units with greater changes in community structure during the first operation months of the system. In RTW, the bacterial community structure of wetlands' permanently flooded areas was distinct from the soil and sediments of the transitional areas.

The beta-diversity partitioning results showed that the temporal change in the biofilm bacterial community structure of TW filters was dominated by species replacement. While the contribution of species replacement was constant over the time, the contribution of richness difference was higher during the

system start-up period. The results indicate that deterministic and stochastic processes simultaneously contributed to the succession of the bacterial communities in the TW biofilms but the relative importance of these different processes over time were variable. In addition to endogenous factors, the bacterial community in TWs' biofilms was shaped by influent chemical parameters and was characterised by overdispersed phylogeny.

The diversity of the GTW and MWTW bacterial community and relative abundance of certain bacterial genera was positively linked to system treatment efficiency. Bacterial co-occurrence analysis revealed that organic matter and nitrogen removal in the studied TWs was performed by several small subsets of tightly interconnected bacterial species.

The results from this study suggest that the nitrogen removal occurs via the following pathways in the studied TWs: (i) in vertical flow filters and FWS TW via coupled autotrophic and heterotrophic nitrification and denitrification while the contribution of the aerobic denitrification is temporally variable; (ii) in horizontal flow filters via heterotrophic nitrification coupled with aerobic and anaerobic denitrification, and nitrifier-denitrification; (iii) in limited amount via ANAMMOX process with the highest potential for this process in soils and sediments of the RTW complex.

Based on the results of this thesis, it can be concluded that TWs should be designed and operated in a way that supports the development of diverse microbial communities with a high abundance of metabolically diverse nitrifying and denitrifying microbes.

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SUMMARY IN ESTONIAN

Tehismärgalade mikroobikoosluse struktuur ja selle seos süsteemi puhastusefektiivsusega

Märgalad on ökosüsteemid, mille erinevaid funktsioone on inimkond osanud enda kasuks tarbida juba sajandeid. Üks nendest kasulikest omadustest on märgalade võime eemaldada saastunud veest erinevaid reoaineid. Selliste ökosüsteemide kasutamine veepuhastuseks on toimunud viimase poole sajandi jooksul ning on nüüdseks kujunenud üheks arvestatavaks alternatiiviks veepuhastuses. Selline tehnoloogia põhineb looduslike isepuhastusprotsesside kasutamisel kunstlikult loodud ökosüsteemides, kus oskuslikult valitud pinnase, veerežiimi ja taimestiku koosmõjul kujundatakse keskkond, mis soodustab mikroorganismide arengut ning tagab vajalikud puhastusprotsessid. Sellist tüüpi veepuhastuseks loodud süsteeme nimetatakse tehismärgaladeks. Tehismärgalade erinevad omadused, sealhulgas eri tüüpi filtermaterjalid, tööparameetrid ning samuti taimestiku olemasolu või puudumine, mõjutavad mikroobikoosluste struktuuri, ruumilist jaotust ja aktiivsust tehiskeskkondades. Kuna mikroobsed protsessid on tehismärgalades reovee puhastusel olulise tähtsusega, aitavad mikroobiökoloogia alased uuringud laiendada teadmisi mikroobikoosluste kujunemisest ja seostest tehismärgalade tööparameetritega, võimaldades seeläbi luua stabiilse ja metaboolselt aktiivse mikroobikoosluse jaoks sobiliku keskkonna ning selle kaudu tõsta erinevate reoainete puhastamise efektiivsust tehismärgalades.

Käesoleva doktoritöö eesmärgiks oli uurida mikroobikoosluse arvukust ja struktuuri eri tüüpi tehismärgalades. Lisaks sellele vaadeldi, kuidas mikroobikoosluse parameetrid on seotud tehismärgalade puhastusefektiivsusega ja millised keskkonnafaktorid mõjutavad mikroobikoosluse stabiliseerumist uuritavates süsteemides. Uurimisobjektideks olid kolm eri tüüpi tehismärgala, millest kaks olid vastrajatud hübriidsed tehismärgalad ja üks üle 15 aasta kasutuses olnud vabaveeline jõevett puhastav tehismärgalade kompleks. Vastrajatud süsteemidena uuriti eksperimentaalse hallvett puhastava tehismärgala (HTM) vertikaal- ja horisontaalvoolulisi filtreid kümne kuu vältel ja munitsipaalreovett puhastava tehismärgala horisontaalvoolulist filtrit (MTMHF) viie kuu vältel. Vabaveeliste tehismärgalade (JTM) kompleks on rajatud põllumajandusliku hajureostuse eemaldamiseks USA Ohio osariigis asuvast Olentangy jõest. Probleemiks on eelkõige lämmastikuühendid, mille allikateks on peamiselt olmereovesi, lämmastikväetiste kasutamine taimekasvatuses, loomakasvatusest pärinev sõnnik ning selliste liblikõieliste taimede, nagu sojauba, kasvatamine.

Saadud tulemused näitasid, et reovee päritolu ja koostis, tehismärgalade tüüp ja töörežiim mõjutavad bakterikoosluse kujunemist ja struktuuri tehismärgalas. HTM-i vertikaalvooluliste filtrite materjalidel kujunenud biokiles olid enamlevinud bakterirühmadeks *Gamma-* ja *Betaproteobacteria* klassid ja hõimkond *Bacteroidetes*. Horisontaalvoolulise filtri materjalil oli märgata *Firmicutes* hõimkonna olulist kasvu süsteemi tööperioodi vältel. Erinevused kahe filtersüsteemi vahel tulid selgelt esile ka bakteriperekondade tasemel. Kolmekümnest

enamlevinud perekonnast ainult seitse olid kahes filtersüsteemis sarnased. Erinevalt HTM-i horisontaalfiltritest olid MTMHF-i materjali mikroobikoosluses domineerivad hõimkondade *Proteobacteria* ja *Bacteroidetes* esindajad. JTM-i sette- ja mullaproovides olid teistest uuritud tehismärgaladest erinevalt arvukamad *Deltaproteobacteria*, *Acidobacteria* ja *Nitrospirae* bakterirühmade esindajad.

Mikroobikoosluse struktuuri kujunemisel oli näha sarnast ajalist suktsessiooni HTM-i ja MTMHF-i vahel. Mikroobikoosluse mitmekesisus tõusis märgatavalt mõlema süsteemi töörežiimi esimese kolme kuu jooksul ja seejärel hakkas stabiliseeruma, sealhulgas olid algsed muutused mikroobikoosluse kujunemisel suurema varieeruvusega kui pärast stabiliseerumist. Statistilise analüüsi tulemused näitasid, et veerežiim oli oluline faktor bakterikoosluste struktuuri kujunemisel JTM-i mullas ja setes. Tehismärgalade püsivalt üleujutatud alade setete bakterikoosluste liigiline koosseis erines ajutiselt üleujutatud alade omast. Lisaks olid ajutiselt üleujutatud alade bakterikooslused mitmekesisemad ja ühtlasema liikide jaotusega kui püsivalt üleujutatud alade kooslused. Muutuv veetase võib luua suurema keskkonnatingimuste spektri, mis soodustab mitmekesisema koosluse kujunemist ja pärsib üksikute dominantsete mikroobirühmade teket.

Töö tulemused näitasid, et nii HTM-i ja MTMHF-i mikroobikoosluse mitmekesisus kui ka teatud bakteriperekondade osakaalud olid seotud nende süsteemide puhastusefektiivsustega. Võrgustiku analüüsi tulemused kinnitasid mitmete koosesinevate bakterirühmade mõju nii lämmastiku kui orgaaniliste ühendite eemaldamisele reoveest. Enamik tehismärgalade mikroobikoosluse struktuuris esinenud varieeruvusest oli mõjutatud reovee keemilistest näitajatest ja puhastussüsteemi ajalisest muutusest. JTM puhul mõjutasid koosluse liigilist koosseisu sellised olulised keskkonnaparameetrid nagu pinnase tüüp ja veerežiim.

Uuritavates tehismärgalades olid peamisteks lämmastikuärastuse protsessideks: (i) vertikaalvoolulistes filtersüsteemides ja vabaveelises JTM-s autotroofne ja heterotroofne nitrifikatsioon ning denitrifikatsioon; (ii) horisontaalvoolulistes filtersüsteemides aeroobse ja anaeroobse denitrifikatsiooni esinemine koos heterotroofse nitrifikatsiooniga ning autotroofsete nitrifitseerijate vahendatud denitrifikatsioon; (iii) vähesel määral anaeroobne ammooniumi oksüdeerimine, eelkõige vabaveelises JTM-s.

Käesoleva doktoritöö tulemused lubavad järeldada, et erinevate reoainete parimaks eemaldamiseks tuleks tehismärgalasid konstrueerida ja opereerida meetodil, mis loob sobiva keskkonna mitmekesise ja suure metaboolse aktiivsusega mikroobikoosluse kujunemiseks.

ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to my supervisors Prof. Jaak Truu and Dr. Marika Truu, for their guidance and support during my PhD studies. My special recognition belongs to Prof. Ülo Mander and the cool gang from 'Room 134' with whom I had the fortune to work together – Mikk, Teele, Kuno, Kärt, Kertu and Jens for their advice and all the good laugh. You kept the spirits up even during the hard times. I would also like to thank all my co-authors and colleagues from the Institute of Ecology and Earth Science.

My deepest appreciation belongs to my very dear grandparents, who never stopped believing in me and supported me on this journey. Last but not least, I am truly grateful to my family and friends for always being there for me. This could not have been possible without all of you!

PUBLICATIONS

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DISSERTATIONES TECHNOLOGIAE CIRCUMIECTORUM UNIVERSITATIS TARTUENSIS

1. **Sille Teiter.** Emission rates of N_2O, N_2, CH_4 and CO_2 in riparian grey alder forests and subsurface flow constructed wetlands. Tartu, 2005, 134 p.
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