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TEELE LIGI

Bacterial community structure and its genetic potential for nitrogen removal in the soils and sediments of a created riverine wetland complex

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II **Ligi, T**., Oopkaup, K., Truu, M., Preem, J.-K., Nõlvak, H., Mitsch, W.J., 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.

III **Ligi, T**., Truu, M., Oopkaup, K., Nõlvak, H., Mander, Ü., Mitsch, W.J., 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.

Author's contribution

Paper I: The author is responsible for the microbiological analysis (about 90%) and is partly responsible for the data analyses (about 50%) and for writing the manuscript (about 60%).

Paper II: The author performed most of the microbiological analysis (90%) and participated in the data analyses (30%) and in writing the manuscript (about 60%).

Paper III: The author performed most of the microbiological analysis (90%) and is partly responsible for the data analyses (60%) and for writing the manuscript (about 60%).

ABBREVIATIONS

amx%	Proportion of ANAMMOX bacteria specific 16S rRNA genes in the bacterial communities of soils and sediments according to the qPCR analysis
ANAMMOX	Anaerobic ammonium oxidation
ANOSIM	Analysis of similarities
Ca.	*Candidatus*
Cq	Quantification cycle (LinRegPCR program)
Ct	Threshold cycle
DNRA	Dissimilatory nitrate reduction to ammonium
ISD	Inverse Simpson's diversity
MENAP	Molecular Ecological Network Analyses Pipeline
MOM	Mississippi-Ohio-Missouri
n-damo	Nitrite-dependent anaerobic methane oxidation
ndamo%	Proportion of n-damo bacteria specific 16S rRNA genes in the bacterial communities of soils and sediments according to the qPCR analysis
nir%	Sum of *nirS* and *nirK* gene proportions in the bacterial communities of soils and sediments
nirK	Gene encoding copper-containing nitrite reductase
nirK%	*NirK* gene proportion in the bacterial communities of soils and sediments
nirK/nirS	Ratio of *nirK* and *nirS* gene proportions in the bacterial communities of soils and sediments
nirS	Gene encoding cythochrome cd1-containing nitrite reductase
nirS%	*NirS* gene proportion in the bacterial communities of soils and sediments
nosZ%	Sum of *nosZI* and *nosZII* gene proportions in the bacterial communities of soils and sediments
nosZ/nir	Ratio of *nosZ* and *nir* gene proportions in the bacterial communities of soils and sediments
nosZI	Clade I/typical nitrous oxide reductase encoding gene
nosZI%	*NosZI* gene proportion in the bacterial communities of soils and sediments
nosZI/nosZII	Ratio of *nosZI* and *nosZII* gene proportions in the bacterial communities of soils and sediments
nosZII	Clade II/atypical nitrous oxide reductase encoding gene
nosZII%	*NosZII* gene proportion in the bacterial communities of soils and sediments
Oo	Soil and sediment samples of permanently flooded areas of W wetlands and oxbow
ORWRP	Olentangy River Wetland Research Park
OTU	Operational taxonomic unit

Ox	Soil samples of oxbow
Ox_c	Soil samples of centre area of oxbow
Ox_{in}	Soil samples of inflow area of oxbow
Ox_{oo}	Soil samples of open area of oxbow
Ox_{out}	Soil samples of outflow area of oxbow
Ox_{trans}	Soil samples of transitional area of oxbow
PCoA	Principal coordinates analysis
pMEN	Phylogenetic molecular ecological network
qPCR	Quantitative polymerase chain reaction
Samx%	Proportion of ANAMMOX bacteria specific 16S rRNA gene sequences in the bacterial communities of soils and sediments according to the sequencing analysis
SF	Surface flow
Sndamo%	Proportion of n-damo bacteria specific 16S rRNA gene sequences in the bacterial communities of soils and sediments according to the sequencing analysis
SSF	Sub-surface flow
Trans	Samples of transitional areas of W wetlands and oxbow
TW	Treatment wetland
Up	Soil samples of upland areas
W upland	Soil samples of the upland area between W wetlands
W wetlands	Experimental freshwater TWs
W1	Vegetated freshwater TW
W2	Naturally colonised freshwater TW
W_c	Soil samples of centre areas of W wetlands
W_{in}	Soil samples of inflow areas of W wetlands
W_{om}	Mineral soil samples of permanently flooded areas of W wetlands
W_{oo}	Organic sediment samples of open areas of W wetlands (=W_{open})
W_{open}	Organic sediment samples of permanently flooded areas of W wetlands (=W_{oo})
W_{out}	Soil samples of outflow areas of W wetlands
W_{trans}	Soil samples of transitional areas of W wetlands

1. INTRODUCTION

Nitrogen is the main component of the Earth's atmosphere; however, only a limited amount of nitrogen is in a reactive form in nature. During the recent centuries, humans have interfered with the natural nitrogen cycle by using more chemically synthesised inorganic fertilisers, massively growing nitrogen-fixing plant species, and burning fossil fuels and biomass, thereby increasing the amount of nitrogen available to the biota. While anthropogenic nitrogen has produced benefits such as increased food quantity and quality, the loss of reactive nitrogen to air, land, and water from point and non-point sources has caused a cascade of environmental and health problems (Vitousek et al., 1997; Gruber and Galloway, 2008).

Most of the anthropogenic nitrogen released into the atmosphere and terrestrial ecosystems reaches streams, rivers, and lakes, and eventually ends up in estuaries and oceans (Hey et al., 2012), where, together with other nutrients, it causes algal blooms, reduced water quality, loss of habitat and natural resources, and severe hypoxia (Rabalais et al., 2009). An example of these drastic consequences is the 13,000 km^2 hypoxic zone ('dead zone') in the Gulf of Mexico caused by the use of synthetic nitrogen fertilisers, the extensive cultivation of nitrogen-fixing crops, such as soybean, the application of animal manure, and the release of municipal and industrial wastewaters in the Mississippi-Ohio-Missouri (MOM) Basin (Rabalais et al., 2002; Mitsch and Day, 2006; US Environmental Protection Agency). The landscape in this area has partly lost its ability to maintain a biogeochemical balance and buffering capacity, as 30 million ha of land has been drained in the MOM Basin during the 20^{th} century (Mitsch et al., 2001; Mitsch and Day, 2006).

In order to protect aquatic ecosystems from excessive nitrogen compounds, treatment wetlands (TWs) are proposed as an effective and low-cost solution to decrease nutrient concentrations in polluted waters by implementing a combination of physical, chemical, and biological processes. In addition to their ability to purify different types of wastewaters originating from point sources, TWs are effective systems against diffuse pollution, since they can be used to reduce nutrient concentrations already in the upstream water bodies and therefore protect greater rivers, lakes, and oceans from the negative consequences of nitrogen overload (Jordan et al., 2011; Vymazal, 2011; Hey et al., 2012). For that reason, the restoration and creation of 22,000 km^2 of wetlands in the MOM Basin has been suggested as a means to reduce the nutrient flow to the Gulf of Mexico (Mitsch et al., 2005; Mitsch and Day, 2006). Among different types of wetlands, Mitsch et al. (2005) have proposed the use of river diversion wetlands – wetlands on the adjacent floodplain or behind artificial levees that receive water by pumping or flood flow from the main channel of a river. In addition to their nutrient removal capacity, river diversion wetlands have several benefits, including restoring habitats, mitigating the effects of

flooding, protecting agricultural production, and increasing the quality of public health and local water (Mitsch et al., 2005; Mitsch and Day, 2006).

Microbial communities play a key role in TW biogeochemical cycles (Truu et al., 2009), and therefore it is crucial to understand how the composition and diversity of microbial communities are related to environmental parameters in the TWs, to use that knowledge in designing and operating TWs. The nitrogen cycle in TWs is extremely complex, comprising a wide variety of different biogeochemical processes; however, most of these merely transform nitrogen compounds from one form to another. Favourable conditions for processes with maximum nitrogen removal and minimum emission of the greenhouse gas nitrous oxide (N_2O) should be created in TWs. N_2O has 298 times higher global warming potential than that of carbon dioxide (IPCC, 2013), and it is assumed that it will have a prevailing impact on ozone layer destruction in the future (Portmann et al., 2012). Although several biological processes are known to be sources of N_2O emission (Butterbach-Bahl et al., 2013), only three bacterial-mediated pathways are known to remove nitrogen from TWs by producing gaseous nitrogen compounds without producing N_2O: the final step of denitrification, anaerobic ammonium oxidation (ANAMMOX), and nitrite-dependent anaerobic methane oxidation (n-damo) (Thamdrup, 2012). While denitrification has long been associated with nitrogen removal in TWs, ANAMMOX and n-damo are quite recently discovered pathways in the nitrogen cycle, and little information is available about those processes in TWs.

2. THE AIM OF THE STUDY

The main aim of the thesis was to characterise the bacterial community structure and its nitrogen removal potential in the soils and sediments of a created riverine wetland complex treating polluted river water.
The specific aims were:

- to characterise the structure of the bacterial community in relation to site-specific characteristics – soil chemical parameters, water regime in the wetlands, and soil type; and
- to evaluate the effect of site-specific factors – soil chemical parameters, water regime in the wetlands, and soil type – on the genetic potential of denitrification, anaerobic ammonium oxidation, and nitrite-dependent anaerobic methane oxidation in the bacterial communities of soils and sediments in the studied created riverine wetland complex.

3. LITERATURE REVIEW

3.1 Treatment wetlands

Treatment wetlands (TWs) are artificially created wetland systems designed to enhance and optimise certain physical and/or biogeochemical processes that occur in natural wetlands, with the primary purpose of removing contaminants from polluted waters (Fonder and Headley, 2013). They act as complex bio-reactors where different processes take place with the substrate, water, vegetation, and microorganisms (Lee et al., 2009). According to Fonder and Headley (2013), the common characteristics for all TWs are the presence of macrophytic vegetation, the existence of water-logged or saturated substrate conditions for at least part of the time, and the inflow of water with constituents that need to be removed.

Compared to conventional wastewater treatment systems such as the activated-sludge process, TWs have been considered as a green technology due to their minimal energy demand and lower installation, operation, and maintenance costs. In addition, TWs do not have very specific site requirements, they provide a habitat for wildlife, have high stability under changing environmental conditions, and provide opportunities for nutrient recycling and effluent reuse (Rousseau et al., 2008; Lee et al., 2009; Wu et al., 2014). Today constructed wetlands are acknowledged as a reliable wastewater treatment technology and represent a suitable solution for the treatment of different types of wastewater, as they have been successfully implemented for treating municipal, domestic, industrial, and agricultural wastewaters, urban and highway run-off, mine drainage, landfill leachate, etc. (Vymazal, 2011, 2014; Vymazal and Březinová, 2015).

Since the creation of the first full-scale TWs in the late 1960s (Vymazal, 2011), there has been remarkable development in this field, and numerous types of TWs have been constructed with different design and operational modes. According to Fonder and Headley (2013), TWs can be classified based on hydrology (water position, flow direction, saturation of media, and influent loading type) and vegetation characteristics (sessility, growth form). Most commonly, TWs are categorised according to the water surface position relative to the substrate or soil into surface flow (SF) and sub-surface flow (SSF) TWs. While in SSF TWs the wastewater flows through the vegetated bed of the substrate horizontally or vertically, in SF TWs the majority of wastewater flow occurs above a benthic substrate (Vymazal, 2007; Kadlec and Wallace, 2009; Fonder and Headley, 2013).

SF TWs usually consist of basins or channels, with soil or another suitable medium to support the vegetation (if present) and water at a relatively shallow depth flowing horizontally through the unit (Vymazal, 2014). They often resemble natural environments due to areas of open water and vegetation (emergent, submerged, and/or free-floating) either by design or as an

unavoidable consequence (Kadlec and Wallace, 2009). Plants have several physical benefits (filtering, velocity reduction, etc.), they conduct the uptake of nutrients and evapotranspiration, create microclimatic conditions, provide habitat for wildlife, give an aesthetic appearance to the system, can be used as bioindicators, etc. In addition, the rhizosphere provides a very important base for microorganisms (acting as an attachment surface, releasing gas and exudates, etc.) (Shelef et al., 2013). Compared to unvegetated lagoons, planted TWs have been reported to have higher treatment performance, and this is affected by the plant species used. The most commonly used macrophyte genera in SF TWs are *Typha*, *Scirpus*, *Phragmites*, *Juncus*, and *Eleocharis* (Vymazal, 2013a).

SF TWs are most commonly used to treat polluted waterbodies (Maniquiz et al., 2012; Ockenden et al., 2012; Dzakpasu et al., 2015), effluent from municipal wastewater treatment plants (Erler et al., 2011; Mulling et al., 2013; Mander et al., 2014), but also different industrial wastewaters from aquaculture, refineries, tanneries, breweries, explosive industries, etc. (reviewed by Vymazal, 2014). SF TWs are able to cope more successfully with higher inflow velocities and temporary changes in water levels than SSF TWs, and therefore they are used to purify urban, agricultural, and industrial storm waters (Beutel et al., 2009; Fonder and Headley, 2013; Lai, 2014). SF TWs can be used in different climates; however, ice formation can hydraulically preclude operation, and the rates of some removal processes are slower in cold water temperatures (Kadlec and Wallace, 2009). In addition, SF TWs are often combined with different types of SSF TWs (known as hybrid TWs) in order to implement the advantages of the different systems (Vymazal, 2013b).

3.2 Microbial community structure associated with pollutant removal in SF TWs

The composition of wastewater depends on its origin, but the most common substances in wastewater are different organic (proteins, detergents, solvents, grease, etc.) and inorganic (nutrients, acids, bases, etc.) compounds, micro-organisms (pathogenic and non-pathogenic bacteria, viruses, etc.), metals, etc. (Henze et al., 2001). The elimination of excess nutrients in wastewater is conducted in combination with different physical, chemical, and biological processes; however, microorganisms are known to play a fundamental role in the transformation and degradation of most pollutants in TWs (Faulwetter et al., 2009; Truu et al., 2009).

Microbially mediated processes in TWs are known to be affected by the type of substrate or filter material, hydraulic conditions, operational mode, the presence or absence of plants, wastewater properties (substrate and nutrient quality and availability), and environmental conditions during the operational period (Truu et al., 2009). However, there are still many uncertainties about the structure, spatial distribution, and activity of microbial communities in TWs. In

order to design TWs with maximal nutrient removal capability and minimal greenhouse gas emissions, a better understanding of the microbial communities and their relationships with environmental factors in these ecosystems is needed.

The interactions between different environmental factors determine the oxidation-reduction conditions prevailing in the wetland environment and consequently the forms of available electron acceptors, which in turn determine the potential for occurrence and spatial distribution of different microbially mediated processes. Microorganisms adapt to microhabitats that offer the most favourable conditions for microbial growth with respect to water and substrate availability, gas diffusion, and protection against predation. They live together in consortia with more or less sharp boundaries, interacting with each other and with other parts of the soil biota (Torsvik and Ovreas, 2002). Most of the microbes are attached to different surfaces such as soil, sediment, and filter material particles, but also to plant stems and leaves in TWs (Brix, 1994; Kaldlec and Wallace, 2009), indicating the importance of soils and sediments to system performance.

The microorganisms inhabiting TWs can be divided into two major groups based on their origin: indigenous microbes able to possess metabolic activity, survive and grow in a wetland system, and foreign microbes from wastewater (including pathogens) that are not able to survive or have any functional importance in a TW ecosystem (Truu et al., 2009). In addition, Mulling et al. (2014) showed that although the secondary treated wastewater contained a metabolically highly active and functionally diverse bacterial community from the wastewater treatment plant, the diversity and metabolic activity of the bacterial community decreased during residence in the pond and reed-bed TW system, and the effluent planktonic bacterial community resembled communities of physically similar natural ecosystems. Therefore, TWs are capable of reducing the input and impact of anthropogenic bacterial communities discharged by wastewater treatment plants into receiving surface waters.

In recent years, various research projects have been conducted to study the overall microbial community structure in SF TWs. High-throughput sequencing of 16S rRNA genes and metagenomic analyses have shown the dominance of the bacterial phylum *Proteobacteria*, while other prevalent phyla were *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Verrucomicrobia*, *Chloroflexi*, and *Firmicutes* in the soils of run-off, surface water, and municipal wastewater treating SF TW systems (Peralta et al., 2013; Bai et al., 2014; Arroyo et al., 2015). In addition to bacteria, different representatives of archaea and eukaryotes have been identified in SF TWs; however, their proportions in the microbial communities seem to be lower than those of bacteria. The metagenomic analysis of the water and rhizosphere soil of the SF TW purifying surface water showed the presence of fungi, algae, and protozoa in the soil and water samples (11.3% and 13.8% of all the sequences, respectively), but archaea-specific (phyla *Crenarchaeota* and *Euryarchaeota*) sequences were present only in the soil samples (4.9% of all the sequences) (Bai et al., 2014).

Phyla *Crenarchaeota* and *Euryarchaeota* specific archaea sequences were also detected in the soil samples of a SF TW treating polluted river water, where they constituted ~5–12% of all the sequences (Brooker et al., 2014).

TWs usually receive a considerable amount of carbon with the wastewater, which is rapidly utilised by heterotrophic bacteria in aerobic and anaerobic conditions using various electron acceptors. Simultaneously, the consumed carbon becomes available for different organisms again during the decomposition of the biomass (Kaldec and Wallace, 2009). In addition to carbon dioxide, which is the end product of different respiration processes, SF TWs are shown to be the source of an additional greenhouse gas, methane (Sha et al., 2011; Mander et al., 2014), produced by methanogenetic archaea in anaerobic conditions (Thauer et al., 2008). Therefore, it is also important to study microbial consortia associated with methane production (Brooker et al., 2014; He et al., 2015) and oxidation (Roy-Chowdhury et al., 2014; Arroyo et al., 2015), in order to design SF TWs with minimal methane emissions.

Different types of waste- and natural waters may also contain a substantial amount of sulphur compounds and their transformation in wetland systems is known to be closely related to carbon, nitrogen, and phosphorous cycles (Faulwetter et al., 2009; Pester et al., 2012; Wu et al., 2013). The highly important biotic sulphur transformation process in wetland systems is dissimilatory sulphate reduction into carbon dioxide and toxic sulphide in anaerobic conditions (Wu et al., 2013). The proportions of sulphate-reducing bacteria have been shown to be higher than the proportions of methanogens and nitrous oxide reductase gene possessing denitrifiers in the soil microbial communities of an SF TW treating river water (He et al., 2015).

In addition to macronutrients, TWs could also receive different pollutants such as pharmaceuticals, personal care products, pesticides, phthalates, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, etc. (Deblonde et al., 2011; Vymazal and Březinová, 2015), and their degradation pathways and impact on the natural and wetland systems' microbial communities are still not completely understood. Bai et al. (2014) detected sequences associated with the biodegradation pathways of sixteen different xenobiotic substances (nitrotoluene, benzoate, atrazine, etc.) in the water and rhizosphere soil samples of a SF TW purifying surface water. The removal of emerging organic contaminants (unregulated contaminants, which have long-term adverse effects on human health and ecosystems) in SF TWs has been associated with aerobic biodegradation combined with photodegradation (García-Rodríguez et al., 2014). It has also been suggested that antibiotics in wastewater affect microbial communities and promote the distribution of antibiotic resistance genes among microbial communities. Integrated TW systems (regulating pool and SF and SSF TWs) treating municipal wastewater have been shown to reduce the concentrations of antibiotics and antibiotic resistance genes in microbial communities with comparable efficiency to conventional treatment systems (Chen et al., 2015).

3.3 Nitrogen removal mechanisms in TW soils

The excess of nitrogen compounds in natural ecosystems is the most concerning problem, as inorganic nitrogen substances can be toxic to invertebrates and vertebrates, cause acidification of freshwater ecosystems, occurrence of toxic algae, eutrophication of aquatic ecosystems, and adverse effects on human health and the economy (Camargo and Alonso, 2006). Therefore, it is crucial to reduce the concentration of nitrogen compounds in the effluents of treatment systems to a level that does not cause problems in the environment.

The most common nitrogen forms in polluted waters are ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), nitrous oxide (N_2O), dissolved nitrogen gas (N_2) and nitrogen-containing organic compounds. In TWs, nitrogen is partitioned between the water, sediment, and biomass phases, and nitrogen compounds are transferred from one point/form to another through different physical, chemical, and biological processes. The prevalent physical transfer processes in TWs are particulate settling and resuspension, the diffusion of dissolved forms, plant translocation, litter fall, ammonia volatilization, and the sorption of soluble nitrogen on substrates (Kadlec and Wallace, 2009). In contrast to physical processes, where nitrogen compounds retain their molecular structure, in biological pathways the structure of nitrogen compounds is transformed. The biological nitrogen cycle is very complex, and several new processes have been discovered in recent years. The main known microbiological nitrogen pathways in TWs are ammonification, assimilation, nitrification, dissimilatory nitrate reduction to ammonium (DNRA), denitrification, and anaerobic ammonium oxidation (ANAMMOX) (Vymazal, 2007; Saeed and Sun, 2012). In most of the previously mentioned biological pathways, nitrogen compounds are transformed from one form to another and nitrogen is retained in the wetland system. Currently, only three pathways are known in which nitrogen compounds are converted into nitrogen gas and therefore removed from wetland systems: denitrification, ANAMMOX, and the relatively recently discovered nitrite-dependent anaerobic methane oxidation (n-damo) (Thamdrup, 2012).

3.3.1 Denitrification

Denitrification is the microbiologically mediated anoxic reduction of nitrate or nitrite to gaseous nitrous oxide or nitrogen gas. Generally, denitrification is the reverse reaction of nitrogen fixation, in the sense that it carries fixed nitrogen back into the atmosphere (Shoun et al., 2012), and it is considered to be the major mechanism of total nitrogen removal in TWs (Saeed and Sun, 2012).

For a long time, the ability to denitrify was associated only with bacteria; however, denitrifying species have also been discovered among archaea (Cabello et al., 2004; Graf et al., 2014) and fungi (Maeda et al., 2015). Bacteria capable of denitrification are widely distributed in the environment and exhibit high taxonomic diversity, as denitrifiers are spread among phylogenetically diverse

groups (Tiedje, 1988; Graf et al., 2014). A study comprising available microbial genomes including at least one denitrification-associated gene showed that 652 genomes could be classified into 18 phyla (Graf et al., 2014).

Most denitrifiers are facultative anaerobes using nitrogen oxides as the terminal electron acceptor and organic compounds as electron donors in oxygen limiting conditions to generate ATP. In addition to heterotrophs, some autotrophic denitrifiers have been identified that are able to use inorganic compounds such as sulphur (Beller et al., 2006), arsenic (Rhine et al., 2006), and hydrogen (Vasiliadou et al., 2006) as electron donors.

Environmental factors affecting denitrification are generally divided into proximal and distal regulators. While proximal factors affect denitrifying communities immediately, leading to instantaneous changes in denitrification rates (e.g. nitrate and oxygen concentrations, organic matter availability, and temperature), distal regulators control the composition and diversity of denitrifying communities for a longer term and on a greater scale (e.g. plant growth, management practices, soil texture, soil pH, and disturbance) (Wallenstein et al., 2006; Saggar et al., 2013).

A complete denitrification pathway consists of four respiratory processes, in which nitrate is reduced to nitrogen gas via intermediaries (nitrite, nitric oxide, and nitrous oxide) (Zumft, 1997). The first stage of denitrification, nitrate reduction to nitrite, is catalyzed by the membrane-bound nitrate reductase encoded by *nar* genes or by the periplasmic nitrate reductase encoded by *nap* genes. While the *nar* gene is expressed under anaerobic conditions, the regulation of the *nap* gene varies from one organism to another (Zumft, 1997; Philippot, 2002). Denitrifiers have been shown to possess either one or both types of nitrate reductase genes (Roussel-Delif et al., 2005). Studies incorporating different soils, sediments, and waters have shown that both types of nitrate reductase encoding genes are nearly equally represented in *Proteobacteria* communities (Bru et al., 2007). Nitrate reduction is a common process for denitrification and DNRA, therefore these genes are not used as molecular markers for detecting denitrification.

The reduction of nitrite to nitric oxide by nitrite reductase distinguishes denitrifiers from other nitrate-respiring bacteria. Two structurally different forms of nitrite reductase have been identified: the copper-containing nitrite reductase encoded by *nirK* gene and cythochrome cd1-containing nitrite reductase encoded by *nirS* gene (Hochstein and Tomlinson, 1988; Zumft, 1997). Most denitrifiers possess only one copy of either *nirS* or *nirK* genes per cell; however, some species have up to two *nirS* or four *nirK* gene copies per cell, and a few denitrifiers have both types of *nir* genes in their genomes (Graf et al., 2014). Although two types of nitrite reductases have the same function (Coyne et al., 1989), the *nir* gene type may differ within the same genus and even within the same species (Graf et al., 2014). There is no clear pattern in the distribution of *nirS*- and *nirK*-type denitrifiers in the environment, including wetland ecosystems, as different studies have shown different results (Hallin et

al., 2009; Yoshida et al., 2009; García-Lledó et al., 2011; Correa-Galote et al., 2013). According to the sequenced genomes available in the databases, the majority of denitrifying bacteria harbour the *nirK* gene (Graf et al., 2014).

It is still unknown why different classes of *nir* genes have evolved and what are the advantages of each group. There have been hypotheses on the possibilities of different evolutionary processes and alternative functions of *nir* genes (Jones et al., 2008). In addition, the maintenance of two different types of *nir* genes may be related to niche differentiation, as selective factors in habitats have a different effect on organisms with different *nir* gene types (Hallin et al., 2009; Jones and Hallin, 2010).

The result of nitrite reduction is toxic nitric oxide, and therefore it is crucial to reduce it rapidly to nitrous oxide. In denitrifiers, this process is mediated by membrane-bound nitric oxide reductase encoded by the *cnorB* or *qnorB* genes (Hendriks et al., 2000). In addition to denitrifiers, nitrous oxide reductase has been identified in different pathogenic strains, helping it to survive in oxygen-limited environments and at nitrosative stress (Philippot, 2005). Therefore, similarly to nitrate reduction, nitrite reductase encoding genes are not suitable representatives for evaluating the denitrification process. A study based on the available genomes showed that 35% and only 3.6% of organisms harbouring the *nirK* and *nirS* gene, respectively, lacked the *nor* gene (Graf et al., 2014).

A complete denitrification pathway ends with a nitrous oxide reduction to nitrogen gas catalysed by nitrous oxide reductase. While there are different sources of nitrous oxide in nature (denitrification, autotrophic and heterotrophic nitrification, nitrifier-denitrification within the same nitrifying microorganism, coupled nitrification-denitrification by distinct microorganisms, and DNRA) (Butterbach-Bahl et al., 2013), according to current knowledge, nitrous oxide reductase is the only known enzyme capable of reducing nitrous oxide (Thomson et al., 2012). Two different types of nitrous oxide reductase encoding genes have been identified: clades I and II, according to Jones et al. (2013), or typical and atypical, according to Sanford et al. (2012). Although the function of different types of *nosZ* gene encoded periplasmic enzymes is the same, the *nosZI* gene encoded nitrous oxide reductase transport via the Tat translocation pathway is energetically more costly than export mainly by the *nosZII* encoded enzyme via the Sec pathway (Lee et al., 2006; Jones et al., 2013). In general, the *nosZI* gene has been identified in the members of phylum *Proteobacteria* and archaea, while organisms with the *nosZII* gene belong to a broader range of taxa (Sanford et al., 2012; Jones et al., 2013).

Denitrification should be viewed as the modular assemblage of four partly independent processes, as the set of genes associated with denitrification may vary in different species, and not all denitrifiers possess all of the genes associated with this process (Zumft, 1997; Graf et al., 2014). For example, some denitrifiers do not have the *nosZ* gene and are emitters of nitrous oxide, while others have been shown to possess only the *nosZ* gene, while lacking other genes associated with denitrification (Jones et al., 2008; Sanford et al., 2012;

Graf et al., 2014), and have the ability to use nitrous oxide as a sole electron acceptor (Saggar et al., 2013). According to the analyses of 652 complete genomes harbouring denitrification genes, only one third of these organisms had a complete set of *nir*, *nor*, and *nosZ* genes (Graf et al., 2014).

3.3.2 Anaerobic ammonium oxidation

ANAMMOX process was discovered in the 1990s, and it forced researchers to re-evaluate the classical understanding of the nitrogen cycle, wherein ammonium could be transformed only aerobically by ammonium-oxidising bacteria (Thamdrup, 2012). ANAMMOX bacteria are chemolithoautotrophs that are able to oxidise ammonium coupled with nitrite reduction in the complete absence of oxygen, using carbon dioxide as the sole carbon source (Strous et al., 1999a). In addition, ANAMMOX bacteria have quite a versatile lifestyle, since they are known to use a variety of organic (e.g. propionate, acetate, and formate) and inorganic (e.g. ferrous iron) compounds as alternative electron donors, and iron and manganese oxides as alternative electron acceptors (Strous et al., 2006; van Niftrik and Jetten, 2012).

All known ANAMMOX organisms constitute a separate order *Brocadiales*, belonging to the phylum *Planctomycetes*. Currently, five different genera of ANAMMOX bacteria have been identified, and due to the impurity of cultures, by classical microbiological standards they have been given the status of *Candidatus* (*Ca*.): *Ca*. *Brocadia*, *Ca*. *Kuenenia*, *Ca*. *Anammoxoglobus*, *Ca*. *Jettenia*, and *Ca*. *Scalindua* (Jetten et al., 2010). One of the reasons for their complicated cultivation is their slow growth rate: they divide only once per week (single cells) or per two weeks (aggregated cells) under optimal conditions (van Niftric and Jetten, 2012). The optimal pH and temperature ranges for the ANAMMOX process are 6.7–8.3 and 20–40 °C, respectively (Strous et al., 1999b). ANAMMOX activity is negatively affected by high ammonium and nitrate concentrations; a 50% decrease in activity was detected in 55 mM and 45 mM concentrations, respectively. In addition, dissolved and organic compounds, nitrite, sulphide, phosphate, and acetate have been identified as ANAMMOX inhibitors (Strous et al., 1999b; Dapena-Mora et al., 2007).

The key processes of ANAMMOX catabolism are hypothesised to take place in a unique intracellular compartment called ‘anammoxosome’. This massive cell organelle is surrounded by a membrane which contains tightly packed atypical lipid molecules (‘ladderane’ lipids), creating a highly impermeable barrier to minimise the loss of ions and intermediates (Sinninghe Damsté et al., 2002). According to the analyses of *Ca. K. stuttgartiensis*, the metabolic pathway of the ANAMMOX process starts with the reduction of nitrite to nitric oxide by a cd1-type nitrite reductase followed by a hydrazine synthesis from nitric oxide and ammonium conducted by hydrazine synthase. Finally, highly toxic hydrazine is oxidised to nitrogen gas by a hydrazine dehydrogenase (Kartal et al., 2011). While *nirS*-type nitrite reductase has been

detected in the genome of *Ca. K. stuttgartiensis* (Strous et al., 2006), Hira et al. (2012) found a *nirK* gene encoded enzyme in the ANAMMOX bacterium strain KSU-1.

ANAMMOX bacteria were first discovered in experimental wastewater treatment systems (Mulder et al., 1995), but they have now been found in different ecosystems, including marine sediments (Li et al., 2010), ocean oxygen minimum zones (Kong et al., 2013), river water and sediments (Sonthiphand and Neufeld, 2013), wetland with high nitrogen input (Shen et al., 2015a), agricultural soils (Long et al., 2013; Shen et al., 2015b), and paddy soils (Wang et al., 2012).

3.3.3 Nitrite-dependent anaerobic methane oxidation

In addition to denitrification and ANAMMOX, nitrogen gas can be produced during the n-damo process, where 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 oxidise methane by methane monooxygenase via an intra-aerobic mechanism. Therefore, n-damo is a unique process which links carbon and nitrogen cycles. N-damo was first described in the enrichment cultures of anoxic freshwater canal sediments containing high amounts of nitrate (up to 1 mM) and saturated with methane (Raghoebarsing et al., 2006). Currently this process is associated with a bacterial group belonging to the candidate phylum NC10 with only one described species – *Ca. Methylomirabilis oxyfera* (Ettwig et al., 2010).

Ca. M. oxyfera is anaerobic Gram-negative mesophilic bacteria that possess a unique polygonal shape and lack intracytoplasmic membranes, which is a common feature among proteobacterial methanotrophs (Ettwig et al., 2010; Wu et al., 2012). They have a preference for nitrite over nitrate (Raghoebarsing et al., 2006) and similarly to ANAMMOX bacteria, a long doubling time (1–2 weeks) (Ettwig et al., 2010). In addition, the detection of genes encoding Calvin-Benson-Bassham cycle in *Ca. M. oxyfera* suggests autotrophic carbon dioxide fixation in those bacteria (Rasigraf et al., 2014). The sequencing of the complete genome of *Ca. M. oxyfera* revealed homologues of genes involved in denitrification and aerobic methane oxidation; however, known genes encoding enzymes producing nitrogen gas were missing. It has been hypothesised that nitrogen gas production in *Ca. M. oxyfera* might be dependent on a yet unknown 'nitric oxide dismutase' which mediates the conversion of two molecules of nitric oxide into oxygen and nitrogen gas (Ettwig et al., 2010).

To date, 16S rRNA sequences of the n-damo bacteria have been identified from paddy soil (Wang et al., 2012; Hu et al., 2014), natural and urban wetlands (Hu et al., 2014; Shen et al., 2015a), peatland (Zhu et al., 2012), sediments of a river (Shen et al., 2014) and a deep water lake (Deutzmann et al., 2014), etc., but data on the factors affecting the abundance of n-damo bacteria in natural ecosystems is very limited.

4. MATERIAL AND METHODS

4.1 Study site description

This dissertation is based on the study conducted in the experimental wetland complex located on the floodplain of the Olentangy River at the Wilma H. Schiermeier Olentangy River Wetland Research Park (ORWRP) in Columbus, Ohio, USA. The 21 ha facility includes two 1 ha kidney-shaped experimental freshwater wetlands (W wetlands) and a 3 ha river diversion wetland (oxbow), which differ mainly in terms of their water regime and vegetation type (Fig. 1).

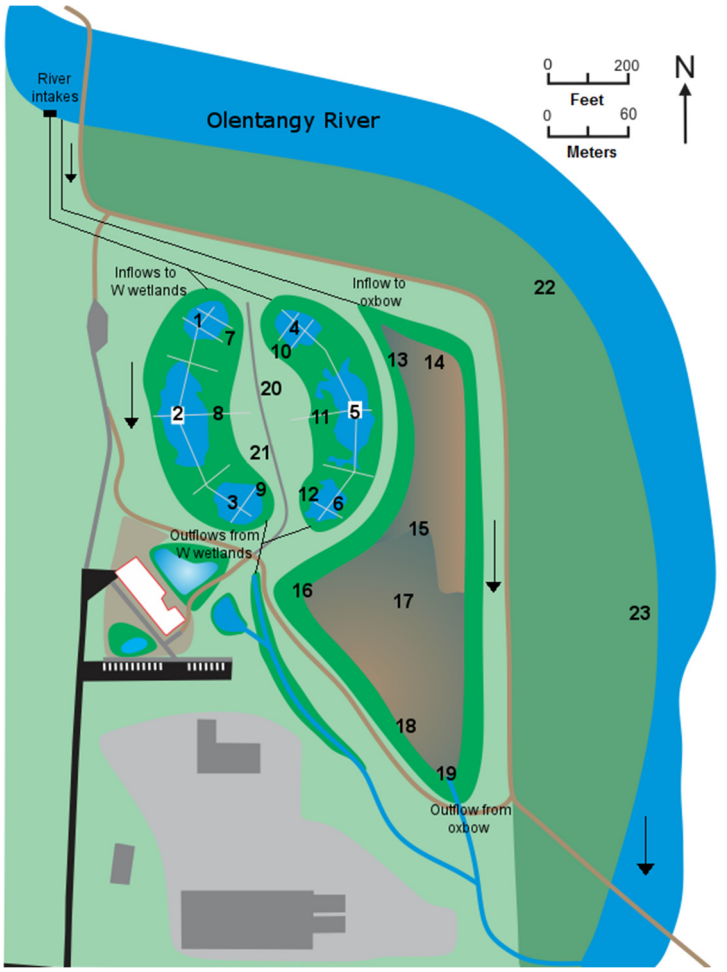

Figure 1. Scheme of the Olentangy River Wetland Research Park study area. The sampling sites are marked with numbers: 1–6 origin of the organic and mineral samples and 7–12 origin of the transitional areas samples of W wetlands (W1 and W2); 13–19 show sampling sites in oxbow and 20–23 in upland areas. The arrows indicate the direction of water flow in the TWs and in the river.

Since their creation in 1994, W wetlands have received equal amounts of water pumped from the Olentangy River (average flow 626–1552 m^3/day) based on a formula relating pumping rates to river stages to mimic the conditions naturally occurring in riverine wetlands (Mitsch et al., 2012). W wetlands were designed to have three distinct permanently flooded deepwater basins surrounded by shallower occasionally flooded marsh areas that were either planted (W1) or colonised naturally (W2) (Mitsch et al., 1998). The period from May to October is the warm and dry season (mean air temperature 21°C) during which the water level in the river is low, while during the cold and wet period from November to April (mean air temperature 4 °C) the W wetlands receive more frequent and greater quantities of water (Roy-Chowdhury et al., 2014).

In contrast to W wetlands, where water is pumped continuously throughout the year, the 3 ha oxbow only receives water through the check valve in cases when the water level in the river is higher than in the oxbow (typically 7–8 times/yr). In 1997, a year after its creation, the oxbow was planted with 6900 rootstocks representing 21 species (Fink and Mitsch, 2007).

A more detailed description of wetlands is presented in Papers I–III.

4.2 Sampling and grouping of soils and sediments

Twenty-nine soil and sediment samples from the permanent sampling sites of the study area were collected in March 2009. Twelve samples from the permanently and occasionally flooded areas of W wetlands, seven samples from the oxbow, and four samples from the upland areas were obtained from the 0–15 cm top soil layer. Additionally, six mineral soil samples from the open areas of W wetlands were collected below the organic layer, from a depth of 15–30 cm. The scheme of the study area with sampling sites is presented in Fig. 1.

The pH_{KCl} (pH) values and total C and N, NH_4-N, NO_3-N, P, Ca, K, and Mg content of the soil samples were determined by the Plant Biochemistry Laboratory of the Estonian University of Life Sciences using standard procedures (APHA, 1989).

In Paper I, the soil and sediment samples were grouped in order to compare the genetic potential of denitrification between the permanently and occasionally flooded areas in W wetlands (W_{open} and W_{trans}, respectively), different transitional areas (W_{trans} and Ox), and different wetland basins ($W_{wetlands}$ and Ox). In Papers II and III, the differences in the bacterial communities between the organic and mineral samples in the permanently flooded areas of W wetlands (W_{oo} and W_{om}) were compared in addition to the differences in water regime and transitional areas. The W_{oo} sampling group in Papers II and III is constituted from the same samples as sample group W_{open} in Paper I. Upland samples (Up) were used as a comparison group in the analyses. The samples of W wetlands and the oxbow were grouped according to the water flow direction

into the inflow, centre, and outflow areas of W wetlands (W_{in}, W_c, and W_{out}, respectively) and the oxbow (Ox_{in}, Ox_c, and Ox_{out}, respectively).

Additionally, oxbow samples were divided into two groups according to the water regime at the site: 1) Ox_{oo} – oxbow soil samples from sampling sites 14, 15, and 17 (Fig. 1) which are overflooded for a longer period (n = 3) and 2) Ox_{trans} – oxbow soil samples from sampling sites 13, 16, 18, and 19 (Fig. 1) which are overflooded for a shorter period (n = 4). In order to test the differences between the permanently and occasionally flooded areas in both type of wetland basins, the soil samples were grouped into two groups: 1) Oo – soil and sediment samples from W_{oo} and Ox_{oo} groups (n = 9) and 2) Trans – soil samples from W_{trans} and Ox_{trans} groups (n = 10).

4.3 Extraction of DNA and amplification and sequencing of bacterial 16S rRNA gene

Total community DNA extraction from the soil and sediment samples is described in Paper I.

The soil bacterial community was profiled using the Illumina HiSeq2000 sequencing combinatorial sequence-tagged PCR products amplified from the V6 region of the 16S rRNA gene. A detailed description of the PCR reactions, pooling of amplicons, DNA library preparation, and sequence analyses is given in Paper II.

In summary, the total initial number of sequences after assembling paired-end reads was 9,726,191, and the following quality control and alignment to the SILVA-compatible reference database provided 5,156,957 effective reads ranging from 52,503 to 319,859 within the samples. The taxonomic assignment of the obtained sequences was conducted using the Greengenes reference database (v 13_5) and Mothur (v 1.27). Sequences were clustered into operational taxonomic units (OTUs) with a 95% similarity level.

Sequences belonging to the phylum *Planctomycetes* were blasted against the ANAMMOX bacteria comprising order *Brocadiales* as described in Paper III. The results are presented as proportion of sequences belonging to the order *Brocadiales* from all the sequences in the sample (Samx%).

In order to detect the sequences belonging to *Ca. Methylomirabilis oxyfera*, the taxonomic assignment of the obtained sequences was conducted using the Greengenes reference database (v 13_8) and Mothur (v 1.35.1). The number of sequences of the *Ca. Methylomirabilis oxyfera* was determined by matching all of the sequences against full 16S rRNA genes of known *Ca. Methylomirabilis* genera sequences from the SILVA v 119 database (Pruesse et al., 2007). This was done using local alignment and identity over 90% in USEARCH v 7.0 (Edgar, 2010). The results are presented as the proportion of sequences belonging to the *Ca. Methylomirabilis* genera out of all of the sequences in the sample (Sndamo%).

4.4 QPCR conditions

The size of the bacterial community and its genetic potential for nitrogen removal through denitrification, ANAMMOX, and n-damo processes were determined in the study area by applying the quantitative polymerase chain reaction (qPCR). Two different primer pairs were used to assess the abundance of bacterial 16S rRNA genes in the soil and sediment samples amplifying the 156 bp long fragment from V4–V5 regions (Paper I) and exactly the same 111 bp long fragment as for sequencing analysis from V6 region (Paper II and III). The genetic potential of nitrite reduction in the bacterial community was evaluated by quantifying the *nirS* and *nirK* genes (Papers I–III) and N_2 emission by quantifying the nitrous oxide reductase encoding *nosZI* (Papers I–III) and *nosZII* (Paper III) genes and ANAMMOX (Paper III) and n-damo specific 16S rRNA genes.

Standard curves for two different target regions of 16S rRNA gene quantification were created in a similar manner; the details about the used primers, target gene amplification conditions, cloning procedure, and standard dilutions are described in Paper I and II.

All qPCR amplifications were performed with RotorGene® Q (QIAGEN, Ca, USA) in a 10 µl reaction mixture containing 5 µl Maxima SYBR Green Master Mix (Thermo Fisher Scientific Inc., MA, USA) and different specific concentrations of primers, 1 µl template DNA, and sterile distilled water. The detailed descriptions of the used qPCR reactions conditions, primer concentrations, and amplification programs for total bacterial and ANAMMOX specific 16S rRNA and functional gene quantification are described in Papers I–III.

The quantification of n-damo specific 16S rRNA genes was conducted using the similar qPCR reaction mixture containing 0.6 µM of forward (qP1F 5′-GGG CTT GAC ATC CCA CGA ACC TG-3′) and (qP2R 5′-CTC AGC GAC TTC GAG TAC AG-3′) reverse primers (Ettwig et al., 2009) under the following conditions: initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 61 °C for 30 s, and extension at 72 °C for 30 s and 80 °C for 15 s. The fluorescence signal was read after the second extension step. Immediately after qPCR amplification, melting curve analyses were performed by ramping up the temperature from 60 °C to 95 °C (0.35 °C/3 s) with continuous fluorescence recording.

4.5 QPCR data processing

In Papers I and II, a three-step quality control, which consists of visual inspection of amplification and melting curves (RotorGene Series Software v 2.0.2 (QIAGEN)), detection of irregular fluorescence reads (LinRegPCR program version 2012.3 by Ruijter et al., 2009), and dissimilar efficiencies, with outlier removal process was used to analyse the qPCR data of standard curves and environmental samples (Nõlvak et al., 2012). After quality control, target gene copy numbers were calculated from the standard curves and presented per gram of dry weight of sediment or soil (copies/g dw). Functional genes were normalised against bacterial 16S rRNA using amplification efficiencies and cycle threshold (Ct) values, as proposed by Ruijter et al. (2009).

In order to take into account the differences in amplification efficiencies of PCR products caused by the variability of soil and sediment properties, the following improvements were implemented into the calculations of qPCR data in Paper III compared to Papers I and II: 1) Ct values obtained by RotorGene® Q were replaced by the quantification cycle (Cq) values of remodelled amplification curves provided by the LinRegPCR program; 2) common threshold values for all gene amplicons were replaced by individual gene amplicon threshold values; and 3) the amplification efficiencies of samples and standard dilutions were considered in the calculations of gene copy numbers. The detailed description of the method used is described in Paper III.

In the current thesis, 16S rRNA gene abundance (16S rRNA, copies/g dw) and the proportions of nitrite reductases (*nirS*% and *nirK*%) and two clades of nitrous oxide reductases (*nosZI*% and *nosZII*%) encoding genes and ANAMMOX organisms specific 16S rRNA genes (amx%) in the bacterial communities presented in Paper III were used. The individual gene proportions were further used to calculate the proportion of total nitrite and nitrous oxide reductase genes (*nir*% and *nosZ*%, respectively) and their ratios (*nirK/nirS*, *nosZI/nosZII*, and *nosZ/nir*). The method described in Paper III was applied to normalise the n-damo specific 16S rRNA genes against the whole bacterial 16S rRNA gene count to represent the proportion of n-damo bacteria in bacterial communities (ndamo%).

4.6 Statistical analyses

Principal component analysis (PCA) of the soil chemical parameters was performed using the prcomp function from the stats package and visualised with the ggplot2 (Wickham, 2009) and ggbiplot (Vu, 2011) packages in the software R version 3.2.0 (R Development Core Team, 2015).

The detailed descriptions of the methods and programs used for Inverse Simpson's diversity (ISD), evenness, and richness indices, principal coordinates analysis (PCoA), analysis of similarities (ANOSIM), distance-based regression analysis, and Molecular Ecological Network Analyses Pipeline (MENAP) are

presented in Paper II. The modules presented in Paper II (Fig. 4) included five or more OTUs and are marked with letters, while modules comprising less than five OTUs were included in the current thesis and are marked with numbers.

Due to the non-normal distribution of the obtained gene parameter values and amplification efficiencies, all of the statistically significant differences in these parameters between studied groups were determined applying Kruskal-Wallis one-way analyses of variance via multiple comparisons of mean ranks. The correlations between different gene parameter values and between gene parameter values and soil chemical parameters were calculated with Spearman rank order correlations. Statistical analyses were performed using Statistica v 7 software (StatSoft, Inc., USA).

5. RESULTS AND DISCUSSION

5.1 Chemical properties of soils and sediments of the ORWRP wetland complex

The chemical composition of soils and sediments varied throughout the ORWRP study area. In general, pH values ranged between 5.44 and 7.16, referring to the slightly acidic or neutral conditions in the studied samples. 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 values of 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 showed a high variation in their P content (2.29–129.27 mg/kg), but the concentrations of Ca, K, and, Mg did not vary as much (1271–5597 mg/kg, 68–286 mg/kg, and 182–433 mg/kg, respectively).

The PCA based on the chemical parameters of soils and sediments (Fig. 2) showed that the first two principal components explain 72.5% of the total variance in the data set. PC1 was strongly correlated to the C/N ratio and to lesser extent to P, K, N, and Ca concentrations, while the values of Mg, pH, and C showed higher correlations with PC2. Of all the studied soil chemical parameters, the NO_3-N concentration had the smallest contribution to the distribution of samples on the PCA ordination plot.

Samples of W wetlands clustered together on the ordination plot according to their origin in the wetland basins, showing that the chemical conditions in the W_{oo}, W_{om}, and W_{trans} differed (Fig. 2 and Table 1). The effect of different hydrologic regimes on the soil physiochemical properties between permanently and occasionally flooded TWs was also demonstrated in the study conducted by Ahn and Peralta (2009). Similarly to W wetlands, the soil samples of the oxbow were variable in their chemical composition. The oxbow receives water on average 7–8 times per year, and this passes through the wetland within 9–12 days. In the eastern areas of the oxbow, water stays for a longer period, creating conditions more similar to the permanently flooded areas, while the conditions in the western edge of the basin more closely resemble the transitional areas of W wetlands. The Ox_{oo} samples were chemically more similar to the W_{om} than the W_{oo} samples, indicating that the chemical conditions in those areas are defined more by the initial soil type and less by the water regime at the site. Up samples originating from the area between the basins of W wetlands clustered in spatial proximity to W_{trans} samples, showing that certain similarities in the soil conditions before the creation of wetlands have been maintained, despite the differences in the water conditions at the site in the following years.

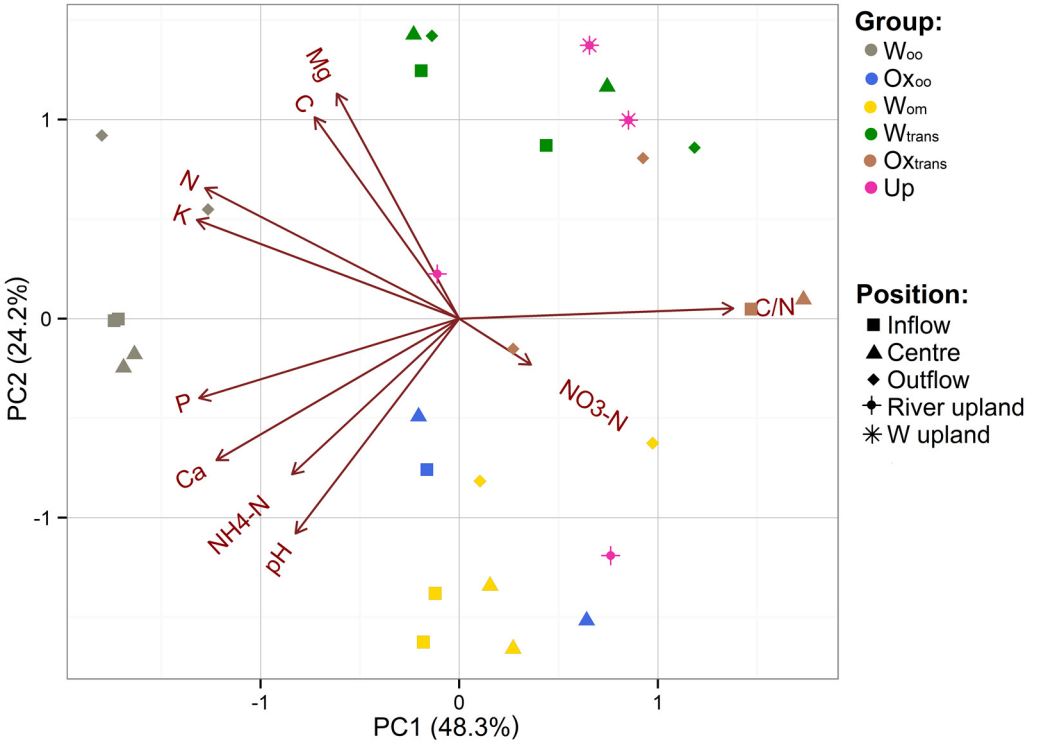

Figure 2. Ordination plot based on PCA demonstrating the grouping of soil and sediment samples according to their chemical composition. The value of a chemical parameter increases in the direction of the arrow, and the length of the arrow represents the strength of the correlation with ordination axes. Abbreviations of the soil groups: W_{oo} – organic sediment samples of open areas of W wetlands, Ox_{oo} – soil samples of open area of oxbow, W_{om} – mineral soil samples of open areas of W wetlands, W_{trans} – soil samples of transitional areas of W wetlands, Ox_{trans} – soil samples of transitional area of oxbow, Up – soil samples of upland areas, and W upland – soil samples of the upland area between W wetlands.

Table 1. Average values and standard deviations (in parentheses) of the chemical parameters of different soil groups. Abbreviations of the soil groups: W_{oo} – organic sediment samples of open areas of W wetlands, Ox_{oo} – soil samples of open area of oxbow, W_{om} – mineral soil samples of open areas of W wetlands, W_{trans} – soil samples of transitional areas of W wetlands, Ox_{trans} – soil samples of transitional area of oxbow, and Up – soil samples of upland areas.

Soil group	**Chemical parameter**									
	pH	**NH_4-N (mg/kg)**	**NO_3-N (mg/kg)**	**C (%)**	**N (%)**	**C/N**	**P (mg/kg)**	**Ca (mg/kg)**	**K (mg/kg)**	**Mg (mg/kg)**
W_{oo} (n=6)	6.81 (0.10)	60.9 (37.3)	0.41 (0.72)	2.92 (0.37)	0.36 (0.03)	8.0 (0.5)	92.2 (34.3)	5165 (279)	237 (29)	365 (41)
Ox_{oo} (n=3)	7.05 (0.10)	3.37 (0.33)	1.39 (1.13)	1.88 (0.44)	0.18 (0.06)	10.4 (0.94)	24.1 (7.9)	4332 (376)	118 (14)	287 (25)
W_{om} (n=6)	6.69 (0.36)	50.8 (25.2)	0.38 (0.41)	1.83 (0.13)	0.16 (0.01)	11.4 (0.5)	28.7 (21.8)	3736 (1035)	131 (17)	233 (32)
W_{trans} (n=6)	5.92 (0.20)	3.2 (3.0)	0.34 (0.45)	2.71 (0.45)	0.24 (0.05)	11.4 (0.9)	12.2 (8.6)	2203 (428)	160 (36)	373 (30)
Ox_{trans} (n=4)	6.16 (0.47)	3.20 (0.5)	0.81 (0.80)	1.96 (0.37)	0.16 (0.03)	12.7 (1.7)	6.7 (3.0)	1729 (610)	115 (53)	315 (60)
Up (n=4)	6.24 (0.77)	1.8 (2.3)	4.87 (4.11)	2.91 (0.60)	0.23 (0.04)	12.6 (0.4)	29.1 (22.6)	2356 (1066)	129 (29)	271 (67)

Several significant differences were detected in the measured soil chemical parameters between the studied soil groups, which are given in Papers I (Table 2) and II (Table 2). Half of the measured chemical variables showed the highest (C, N, P, Ca, and K) values in W_{oo} samples compared to other studied soil groups in W wetlands (Table 1 and Fig. 2), which may be related to the accumulation of sediments over the 15 years of operation. A study conducted ten years after the creation of the W wetlands demonstrated a higher sediment accumulation in the open water areas, while soil organic matter concentrations were greatest in the vegetated zones (Anderson and Mitsch, 2006). The higher content of inorganic C, P, and Ca in the permanently flooded areas of W wetlands have been related to the co-precipitation with $CaCO_3$, while macrophyte colonisation has been presumed to effectively prevent this process in transitional areas. In addition, while the sediments in the permanently flooded areas of W wetlands were found to be very homogenous (containing fine particulate matter), the sediment layer in the transitional areas was slightly more cohesive and heterogeneous, referring to their different origin (allochthonous or autochthonous input, respectively) (Anderson et al., 2005; Anderson and Mitsch, 2006). The accumulation of soil sediments is age-related in TWs, but it is not an entirely linear process, as soil development is considerably affected by a site-specific variability (i.e. site location, mixed wetland types, hydrologic connectivity, vegetation type, and other design features) (Wolf et al., 2011).

A study conducted at the same time (15 years after the creation) in W wetlands did not detect differences in nutrient removal efficiency between W1 and W2 (Mitsch et al., 2012). Mitsch et al. (2014) concluded that planting had a short-term (16 years or less) effect on plant diversity and perhaps a longer-term effect on very particular wetland functions (i.e. methane emission, accumulation of organic matter). The results of the current study showed that environmental conditions were similar in these wetland basins, and differences in the initial vegetation have not significantly affected soil chemical composition after fifteen years of operation, as the soil samples of two W wetlands were not statistically different according to the measured chemical characteristics, and no differences were detected in the ordination plot between the W1 and W2 samples (data not shown).

When soil chemical parameters of sampling sites were compared along the longitudinal gradient of basins of W wetlands and the oxbow, no significant differences were detected between the soil and sediment samples.

5.2 Composition of bacterial communities in the soils and sediments of the ORWRP wetland complex

The advent of massive parallel sequencing technologies has revolutionised microbial ecology studies, as it has made it possible to get closer to the true level of microbial taxonomic diversity (Kim et al., 2013). Amplicon-based metagenomics can provide broad coverage and an extensive redundancy of sequences for targeted genes, even at low abundances within a metagenome (Suenga, 2012), and this method has also become popular in SF TW studies (Peralta et al., 2013; Ansola et al., 2014; Brooker et al., 2014; Arroyo et al., 2015; He et al., 2015).

The bacterial community structure in the soils and sediments of the ORWRP was studied by applying a high-throughput 16S rRNA amplicon sequencing technique. The detailed results and discussion are presented in Paper II. In brief, the retrieved 16S rRNA gene sequences from all of the studied soil samples belonged to 56 different bacterial phyla (including candidate divisions). More than two-thirds of the sequences were classified into phyla *Proteobacteria*, *Acidobacteria*, *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia*, and *Chloroflexi*, indicating the relatively low percentage of organisms belonging to rare bacterial phyla in the studied soil bacterial communities in TWs. This finding is in accordance with several other studies in SF TWs (Peralta et al., 2013; Ansola et al., 2014; Brooker et al., 2014; Arroyo et al., 2015) and with the known pattern from general ecology studies stating that the majority of species are rare and a few species are abundant in the communities (Preston, 1948).

The water regime at the sampling site was one of the main factors affecting the microbial community structure in the study area, as permanently flooded W_{oo} and W_{om} samples differed from the occasionally flooded W_{trans} and Ox samples, while the Up soil microbial communities differed from the studied wetland communities. This trend was identified on different taxonomical levels (Fig. 2 and Supplementary Tables S1 and S2 in Paper II) and also on the ordination plot (Fig. 3a in Paper II), where differences between the transitional areas and permanently inundated areas were confirmed by the analysis of ANOSIM (Paper II). In addition, the bacterial communities in the occasionally flooded areas of W wetlands and the oxbow showed higher diversity and evenness (Table 3 in Paper II).

Similarly to the ordination of samples according to the chemical variables (Fig. 2), oxbow samples were rather widely distributed according to the bacterial community structure on the ordination plot (Fig. 3). Oxbow samples from the presumably more flooded sampling sites placed closer to the W_{om} and W_{oo} samples, while the rest of the oxbow samples were spread around the W_{trans} and Up groups. This confirms the suggestion that some parts of the oxbow were overflooded for longer periods and thus favoured by soil bacterial communities similar to the permanently flooded W wetland areas. Similarly to the transitional areas of W wetlands, bacterial communities of Ox_{trans} showed

greater diversity and evenness than Ox_{oo} (Table 2). In addition, bacterial communities of Ox_{oo} and Ox_{trans} differed in the proportions of certain bacterial phyla (e.g. *Proteobacteria*, *Acidobacteria*, and *Verrucomicrobia*) (Fig. 4), classes (e.g. *Gamma*- and *Alphaproteobacteria* and unclassified *Acidobacteria*) (Fig. 5), and orders (e.g. *Chromatiales*, *Rhizobiales*, and *Verrucomicrobiales*) (Fig. 6), which were also detected to be different between the occasionally and permanently flooded areas in W wetlands. This shows that bacterial community structure in the oxbow soils also depended on their position in the wetland. However, the differences between the Ox_{oo} and Ox_{trans} bacterial communities were less pronounced than between the respective areas in W wetlands, which may be due to the lower hydraulic load and younger age of the oxbow basin compared with W wetlands. Nor did the ANOSIM test detect any significant differences in the bacterial communities of Ox_{oo} and Ox_{trans} soil groups compared to the communities of W wetlands. When Ox_{trans} together with W_{trans} samples and Ox_{oo} together with W_{oo} samples were analysed as one group, respectively, the bacterial communities in the Trans samples were significantly different from the Oo samples and from Up ($p < 0.05$ in all cases). In addition, the bacterial communities in the Trans samples had greater diversity ($p < 0.001$), richness ($p < 0.05$), and evenness ($p < 0.001$) than those in the Oo samples. However, the Ox_{oo} soil group showed a certain resemblance also with the W_{trans} and Up samples by its proportions of some bacterial phyla (e.g. *Bacteroidetes*, *Actinobacteria*, and *Gemmatimonadetes*), classes (e.g. *Actionobacteria*, *Acidobacteria*, and *Gemmatimonadetes*) and orders (e.g. *Acidobacterales*, *Actinomycetales*, *Myxococcales*, and *Bacteroidales*). Similarly to the current study, Deng et al. (2014) detected higher proportions of *Myxococcales* in the hummock compared to hollow wetland soils using 16S rRNA pyrosequencing. The order *Myxococcales* mainly consists of aerobic bacteria that are able to degrade biological macromolecules and can survive unfavourable environmental conditions, such as limited nutrient supply, dryness, and cold and hot periods by forming myxospores (Dawid, 2000). The Up samples were more heterogenic in terms of their soil chemical composition than their bacterial community structure, suggesting that other factors than soil chemical composition impact the structure of the soil bacterial community in upland areas.

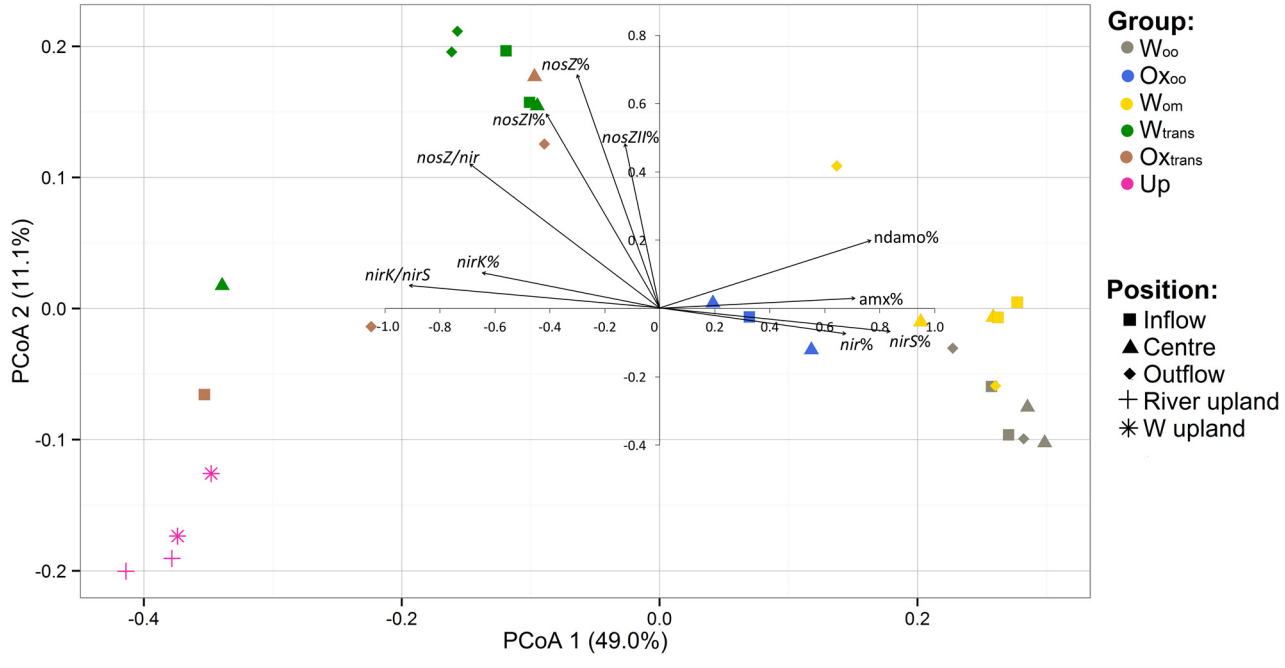

Figure 3. Ordination biplot of the bacterial community structure of soil and sediment samples and statistically significant correlations of the individual gene proportions in the bacterial communities with the first two PCoA axes according to the principal coordinate analysis based on the Bray-Curtis distance matrix. Abbreviations of the soil groups: W_{oo} – organic sediment samples of open areas of W wetlands, Ox_{oo} – soil samples of open area of oxbow, W_{om} – mineral soil samples of open areas of W wetlands, W_{trans} – soil samples of transitional areas of W wetlands, Ox_{trans} – soil samples of transitional area of oxbow, Up – soil samples of upland areas, and W upland – soil samples of the upland area between W wetlands.

Table 2. Average values and standard deviations (in parentheses) of the Inverse Simpson's Diversity (ISD), richness (number of OTUs at 95% similarity), and evenness indices of the bacterial communities of the studied soil groups. Abbreviations of the soil groups: W_{oo} – organic sediment samples of open areas of W wetlands, Ox_{oo} – soil samples of open area of oxbow, W_{om} – mineral soil samples of open areas of W wetlands, W_{trans} – soil samples of transitional areas of W wetlands, Ox_{trans} – soil samples of transitional area of oxbow, and Up – soil samples of upland areas.

Soil group	ISD	Richness	Evenness
W_{oo} (n=6)	20.8 (5.5)	945 (84)	0.68 (0.02)
Ox_{oo} (n=3)	42.6 (9.0)	949 (19)	0.74 (0.02)
W_{om} (n=6)	25.8 (3.7)	954 (41)	0.69 (0.02)
W_{trans} (n=6)	114.5 (8.4)	1000 (37)	0.80 (0.01)
Ox_{trans} (n=4)	106.7 (29.0)	1013 (77)	0.79 (0.02)
Up (n=4)	110.6 (19.3)	868 (50)	0.80 (0.01)

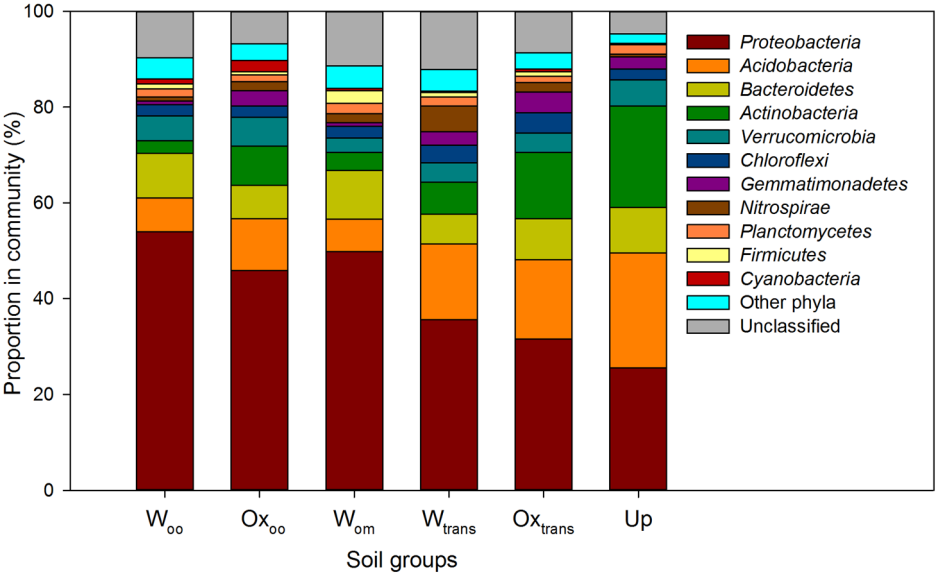

Figure 4. Average proportions of bacterial phyla in the studied soil groups. Only phyla representing more than 2% at least in one soil group are presented. Other phyla are summarised and indicated as other phyla in the figure. Abbreviations of the soil groups: W_{oo} – organic sediment samples of open areas of W wetlands, Ox_{oo} – soil samples of open area of oxbow, W_{om} – mineral soil samples of open areas of W wetlands, W_{trans} – soil samples of transitional areas of W wetlands, Ox_{trans} – soil samples of transitional area of oxbow, and Up – soil samples of upland areas.

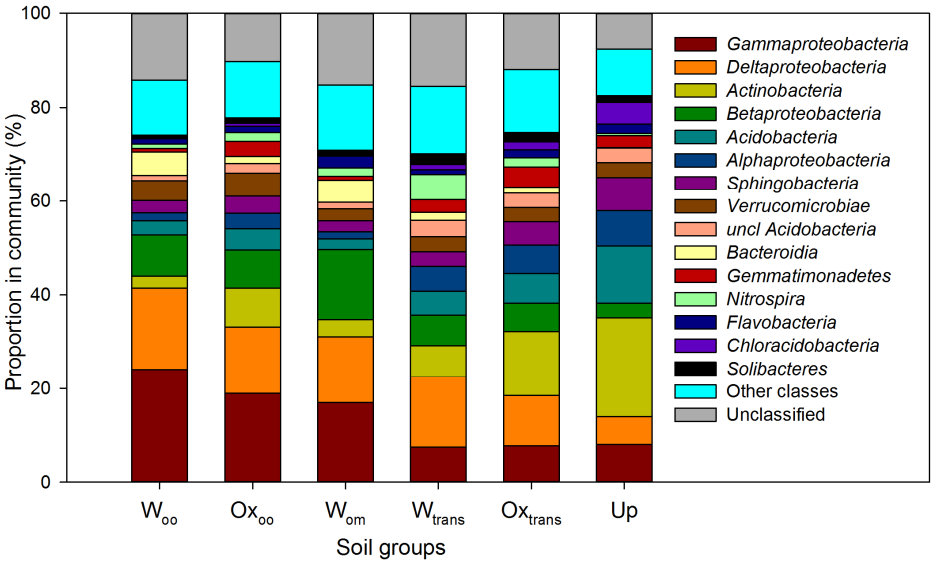

Figure 5. Average proportions of bacterial classes in the studied soil groups. Only classes representing more than 2% at least in one soil group are presented. Other classes are summarised and indicated as other classes in the figure. Abbreviations of the soil groups: W_{oo} – organic sediment samples of open areas of W wetlands, Ox_{oo} – soil samples of open area of oxbow, W_{om} – mineral soil samples of open areas of W wetlands, W_{trans} – soil samples of transitional areas of W wetlands, Ox_{trans} – soil samples of transitional area of oxbow, and Up – soil samples of upland areas.

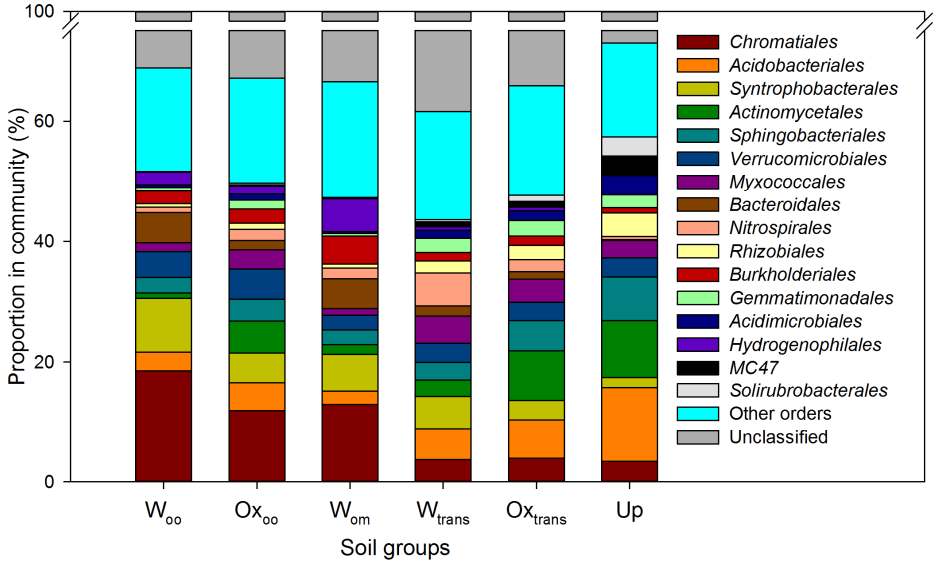

Figure 6. Average proportions of bacterial orders in the studied soil groups. Only orders representing more than 2.5% at least in one soil group are presented. Other orders are summarised and indicated as other orders in the figure. Abbreviations of the soil groups: W_{oo} – organic sediment samples of open areas of W wetlands, Ox_{oo} – soil samples of open area of oxbow, W_{om} – mineral soil samples of open areas of W wetlands, W_{trans} – soil samples of transitional areas of W wetlands, Ox_{trans} – soil samples of transitional area of oxbow, and Up – soil samples of upland areas.

Although the sulphur content in the studied soil samples was not determined, different sulphur-related processes seem to be important in the soils and sediments of the studied wetlands, as a relatively high proportion of taxa comprising purple sulphur bacteria (e.g. order *Chromatiales*), sulphur oxidizers (e.g. genera *Sulfuricurvum* and *Thiobacillus*), and sulphate reducers (e.g. families *Desulfobacteraceae* and *Desulfobulbaceae* and genera *Desulfobacca* and GOUTA19) were detected from the soil groups of W wetlands and Ox_{oo} (Fig. 6 and Supplementary Tables S1 and S2). According to the known sulphate reducers taxa (reviewed by Muyzer and Pester, 2008), the average proportions of sulphate-reducing bacteria in the bacterial communities of W_{oo}, W_{om}, and W_{trans} were 6.56 ±1.18%, 5.94 ±2.42%, and 6.78 ±2.56%, respectively, while lower values were detected in the Ox_{oo} (4.52 ±1.19%), Ox_{trans} (3.48 ±2.1%), and Up (1.87 ±0.39%) samples. The same sulphate-reducing bacterial taxa have also been determined previously in different wetland soils (reviewed by Pester et al., 2012). He et al. (2015) showed that the proportions of sulphate-reducing bacteria specific *dsrAB* genes ranged from 7 to 17% in soils of restored wetland flooded with river water. Differences between the occasionally and permanently flooded areas were also detected in the proportions of families comprising sulphur reducers, as *Desulfobacteraceae* showed higher proportions in W_{oo}, while *Syntrophobacteraceae* was more common in W_{trans} samples (Supplementary Table S1). This is in accordance with the results of a study in which sequences belonging to *Desulfobacteraceae* were detected mainly in lagoon environments, while *Syntrophobacteraceae* were detected mainly in vegetated sediments of natural wetlands and TWs (Arroyo et al., 2015).

According to the molecular ecological network analyses (Fig. 7 and Supplementary Table S3), the bacterial communities of W wetlands' soil groups formed four and those of Ox formed five modules (only modules including five or more OTUs are presented in Fig. 4 in Paper II). The networks of the W_{trans} and Ox groups comprised more OTUs and had more significant relationships between OTUs within the same sample group compared to W_{oo} and W_{om}, referring to the more complicated ecological interactions within the bacterial communities of occasionally flooded areas compared to permanently flooded areas. The more comprehensive network in Ox compared to the soil groups of W wetlands may be related to the more variable environmental conditions in the oxbow sampling sites, as samples were collected from all over the wetland basin, while sampling areas among the soil groups of the W wetlands had more similar conditions. The generation of smaller networks comprising less than five samples was not feasible with MENAP, and therefore the comparison of the molecular ecological networks of Ox_{oo} and Ox_{trans} was not possible in this study.

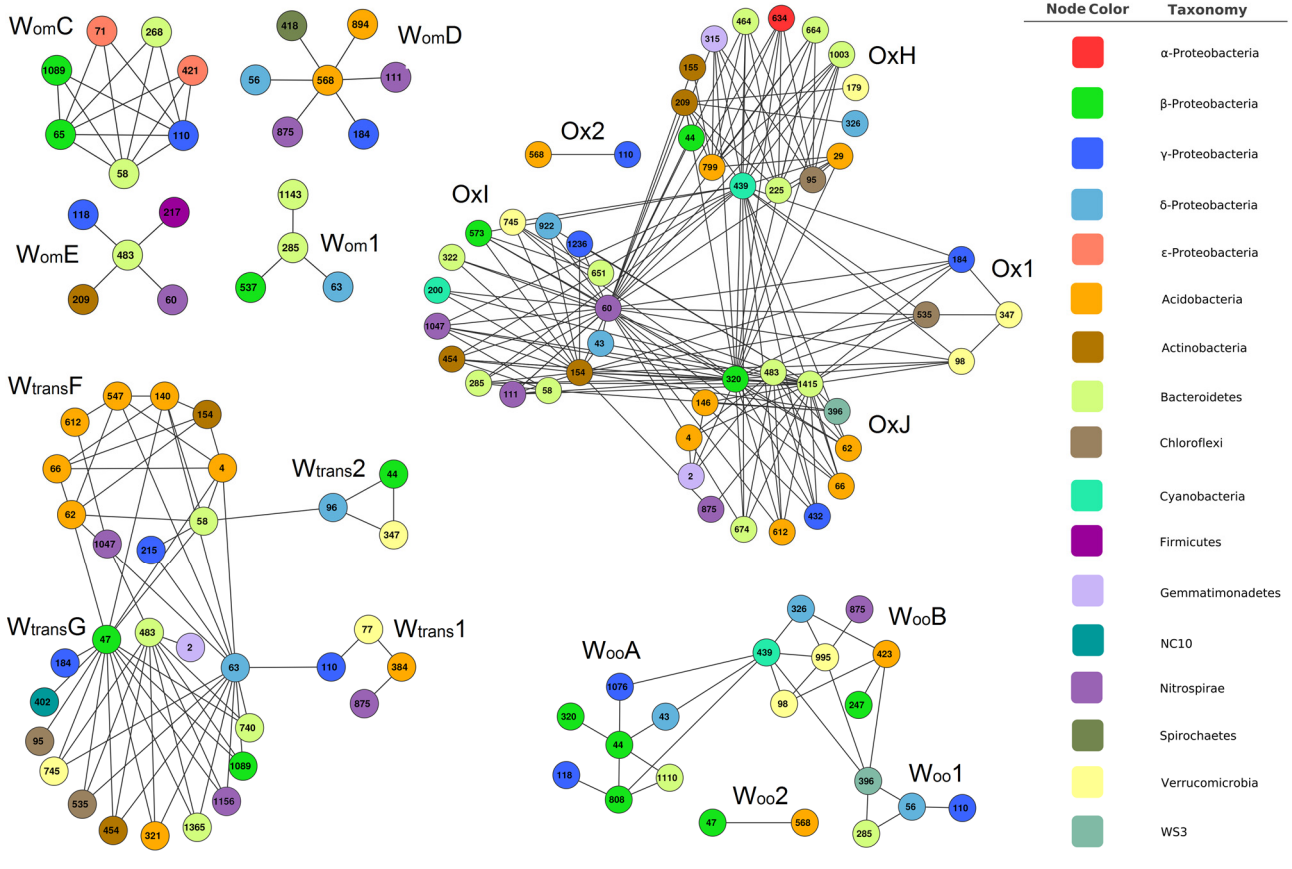

Figure 7. Molecular ecological networks with module structures by the simulated annealing approach in organic sediment samples of open areas of W wetlands (modules $W_{oo}A$, $W_{oo}B$, $W_{oo}1$, and $W_{oo}2$), mineral soil samples of open areas of W wetlands (modules $W_{om}C$, $W_{om}D$, $W_{om}E$, and $W_{om}1$), soil samples of transitional areas of W wetlands (modules $W_{trans}F$, $W_{trans}G$, $W_{trans}1$, and $W_{trans}2$), and soil samples of oxbow (modules OxH, OxI, OxJ, Ox1, and Ox2). Nodes (OTUs) are coloured by representative phyla. Relationships (correlation threshold 0.85) between the OTUs within the sample group are presented with lines.

The majority of OTUs forming the modules in different soil and sediment groups belonged to different taxa, indicating that despite the similarities between the two types of constantly and occasionally flooded areas, the interactions between the bacterial phylotypes in those soil groups can be different (Fig. 7 and Supplementary Table S3). Nevertheless, all of the obtained networks of the soil groups contained two common OTUs belonging to the order *Chromatiales* and the genus *Nitrospira*. In addition, W_{oo} and W_{om} shared four OTUs (belonging to the orders *Syntrophobacterales*, *Methylococcales*, and *Sphingobacteriales* and the class *Chloracidobacteria*), while W_{oo} and W_{trans} shared only two OTUs (belonging to the class *Betaproteobacteria* and the genus *Thiobacillus*). 18 common OTUs were detected in the ecological networks of Ox and W_{trans} (Supplementary Table S3). The higher number of common OTUs involved in the networks of occasionally inundated soil groups compared to the soil groups of permanently flooded areas suggests that although the fluctuating water regime has created conditions for more complex and diverse bacterial communities, some microbial groups involved in the interactions are similar. For example, six of ten OTUs of the module $W_{trans}F$ belonged to the phylum *Acidobacteria* (three belonged to the class *Chloracidobacteria*) and four of them were also present in OxJ. In addition, modules OxJ and $W_{trans}G$ shared one OTU belonging to the order *Sphingobacteriales*, which showed several relationships within both modules with other members, including OTUs from the orders *Acidobacteriales* and *Sphingobacteriales*. The three largest modules of the Ox soil group also contained several OTUs belonging to the order *Sphingobacteriales*, which were significantly related to each other as well as OTUs from the order *Acidobacteriales*.

The effect of the hydrologic regime on microbial community structure and diversity has also been demonstrated in a length heterogeneity PCR-based study in TWs (Ahn and Peralta, 2009), as discussed in Paper II. In addition, Ansola et al. (2014) studied bacterial communities in natural wetlands and TWs using pyrosequencing of the bacterial 16S rRNA gene, and found that the distribution of samples adhered to a gradient from flooded (lagoon) to dry-wet areas (zones with *Typha latifolia* and *Salix atrocinerea*). The results of the current study suggest that the fluctuating water level created a wide spectrum of diverse environmental conditions, suitable for more different bacterial groups, resulting in more complex communities and excluding the rise of single dominating bacterial groups, while the more stable conditions in permanently flooded areas represented favourable environments for a few specific bacterial groups. This was in agreement with the notion that competitive interactions are key determinants of community structure and diversity (Tiedje et al., 2001; Treves, 2003). Soils subjected to considerable seasonal fluctuations in environmental conditions, such as nutrient availability, water content, temperature, or higher heterogeneity of resources, have high spatial isolation, and the competition between community members is nearly absent, creating high microbial diversity, whereas soil communities with low spatial isolation allow competitive interactions, have

much lower diversity and are dominated by a few members. In addition, a study evaluating the effects of fluctuating oxygen regimes on wet tropical forest soils proposed that soil bacteria are adapting to fluctuating redox regimes and generally possess physiological tolerance mechanisms which allow them to endure unfavourable redox periods (Pett-Ridge and Firestone, 2005).

The transitional areas of W wetlands and the oxbow are covered with emergent plants, while in the permanently flooded zones the vegetation is almost absent or moderate, consisting mainly of submerged aquatic vegetation and macroalgae (Mitsch et al., 2012; Mitsch et al., 2014). Plant communities can have an impact on the formation of different soil bacterial communities in permanently and occasionally flooded areas of the ORWRP. Other studies have shown an effect of vegetation on soil microbial community structure in SF TWs, and the effect was dependent on the plant species (Ruiz-Rueda et al., 2009; Arroyo et al., 2013; Arroyo, 2015) as well as plant/plant interactions (Bisseger et al., 2014).

Statistical analyses did not detect significant differences between the bacterial communities in the organic sediments and mineral soils of permanently flooded areas of W wetlands (Fig 2., Fig. 3., and Table 3 in Paper II). The environmental conditions were not similar in the W_{oo} and W_{om} samples, as differences were detected in these groups' chemical parameters (Table 1 in Paper II). Similarly, He et al. (2015) concluded that bacterial community composition was more influenced by site position in the wetland than the depth or season in the restored wetlands. In this study, both W_{om} and W_{oo} ecological networks comprised a similar number of OTUs, which did not show a high number of correlations. Although only six common OTUs were detected between the networks of W_{oo} and W_{om} (Fig. 7 and Supplementary Table S3), these networks contained several OTUs belonging to the same order (e.g. W_{om}C and W_{oo}A contained OTUs from the orders *Sphingobacteriales*, *Burkholderiales*, and *Chromatiales*), indicating that connected bacterial groups in W_{oo} and W_{om} belong to similar taxa, and differences appear on the lower taxonomic level.

The bacterial community structure of the inflow, centre, and outflow areas of W wetlands and the oxbow were compared and, similarly to measured chemical parameters, no statistically significant differences were detected between the studied diversity parameters in soils and sediments of these wetland areas, indicating that the overall microbial community structure in the TWs is not strongly affected by water quality at the site.

5.3 Chemical factors affecting the composition of bacterial communities in the soils and sediments of the ORWRP wetland complex

The distance-based regression analysis showed that over half of the variability in bacterial community structure can be explained by soil chemical parameters (NH_4-N 33.4%; Ca 10.4%; NO_3-N 6.0%; pH 4.3%; C 4.1%). Bacterial community structure was also related to the origin of the sample (soil and wetland type and water regime), since the use of sample location in the analysis as a co-variable decreased the explanatory capability by 18.3%, showing the relationship between the chemical parameter values and sampling position (Paper II).

The high explanatory effect of different nitrogen compounds refers to the relationship between different nitrogen fractions and bacterial community structure in the soils and sediments of the study area. A study conducted in natural and treatment wetlands found a correlation between bacterial community structure and water nitrogen content (Arroyo et al., 2015). Carbon content, type, and availability are known to be important factors affecting the diversity of different soil microbial communities (Eilers et al., 2010; Lagomarsino et al., 2012), but usually only a small proportion of the total carbon in soils is readily available for bacteria. This study also found that the total carbon pool had an effect on the structure of the bacterial community in the soils and sediments of ORWRP. Arroyo et al. (2015) showed that bacterial community structure was related to the soil organic matter in SF TWs and natural wetlands in Spain, and the studies conducted in SF TWs and natural wetlands in northern Virginia demonstrated the relationship between bacterial community structure and soil C/N values, which is an indicator of the quality of the organic matter of soil (Ahn and Peralta, 2009; Peralta et al., 2013).

Different studies have shown the relationship between bacterial community structure and pH in soil environments, as discussed in Paper II. In the current study, the soil pH value explained a relatively small proportion of the variability in the bacterial communities compared to other measured soil parameters. Several other studies have also not found significant correlations between bacterial community structure and pH in the soils of TWs (Ansola et al., 2014; Arroyo et al., 2015).

Several statistically significant relationships were detected, when the relationships between obtained structure and chemical parameters of pMEN modules were analysed (Table 5 in Paper II). In addition, module $W_{om}1$ was correlated positively with K values ($r = 0.92$; $p < 0.01$), Ox2 with P and Ca concentrations ($r = 0.80$; $p < 0.05$ and $r = 0.80$; $p < 0.05$, respectively) and Ox1 with NH_4-N ($r = 0.78$; $p < 0.05$) values in the soil. In two cases, modules within the same soil group were correlated with the same chemical parameter, although the relationships' directions were different since Ox2 was correlated positively and OxI negatively with P, and $W_{om}1$ was correlated positively and $W_{om}C$ negatively with K content in the soils. In addition, the modules $W_{trans}F$ and OxI,

which showed positive and negative relationships with pH values, respectively, contained three common OTUs (belonging to the orders *Flavobacteriales*, *Acidimicrobiales*, and *Nitrospirales*) (Fig. 7 and Supplementary Table S3). The relationships between bacterium and environment may be related to the organisms' relationships within the consortium. This may be the reason why such variable results have been shown between pH and soil bacterial communities, as pH may have a different influence on the subcommunities within the whole community.

5.4 Bacterial abundance and genetic potential for nitrogen removal in the soils and sediments of the ORWRP wetland complex

The abundance of bacteria in the soils and sediments of the wetland complex was evaluated using two different bacterial 16S rRNA primer sets and calculation methodologies.

According to the bacteria-specific 16S rRNA primers (785FL/919R) and calculation methods used in Paper I, the abundance of the 16S rRNA gene in all the soil and sediment samples of the wetland complex ranged from $3.60 * 10^8$ to $1.17 * 10^{10}$ copies/g dw (Supplementary Table S4). In order to assure the uniformity of the results, the abundance of 16S rRNA gene copy numbers in the soils and sediments of the TW complex in Paper II was determined using the same primer set (L-V6/R-V6) as was used for sequencing the 16S rDNA. The 16S rRNA gene abundances quantified with the L-V6/R-V6 primer set varied from $9.37 * 10^8$ to $1.50 * 10^{10}$ copies/g dw (Paper II) and were statistically higher ($p < 0.01$) than the results obtained with the primer set 785FL/919R. However, the coverage of 785FL/919R and L-V6/R-V6 primer sets was found to be quite similar according to the SILVA ribosomal RNA database (79.5% and 81.7%, respectively). The differences in the gene copy numbers may be related to the statistically ($p < 0.001$) lower amplification efficiencies of the primers 785FL/919R (1.779 ± 0.087) compared to primers L-V6/R-V6 (1.898 ± 0.082). When the *nirS*, *nirK*, and, *nosZI* genes were normalised against the 16S rRNA gene data obtained using different 16S rRNA primer pairs, the proportions of denitrification-related functional genes were not statistically different (Papers I and II).

In Paper III, the same L-V6/R-V6 primer set was used; however, the calculation methodology of 16S rRNA gene abundance and functional gene proportions in the bacterial community was improved, as described in section 4.5 of this study. The abundance of the bacterial 16S rRNA gene, according to the L-V6/R-V6 primers, ranged from $1.04 * 10^9$ to $3.03 * 10^{10}$ copies/g dw across the study area (Paper III). The improvement of the calculation methodology did not statistically affect the results of the 16S rRNA gene abundance, but significantly higher values of *nirS*%, *nirK*%, and *nosZI*% were

obtained ($p < 0.001$ in all cases) compared to the results in Paper II. The importance of the calculation methodology on the gene quantification results from environmental samples was also shown by Nõlvak et al. (2012).

The abundances of 16S rRNA gene values were significantly higher in W_{oo} compared to W_{trans} ($p < 0.05$) and W_{om} ($p < 0.01$), respectively, and in Oo compared to Trans ($p < 0.01$) (Table 3). This indicates that there are more suitable conditions for bacteria in the organic rich sediments of permanently flooded TW areas. 16S rRNA abundance was positively related to N, C, K, and Mg concentrations in the soil, while a negative correlation was detected with the soil C/N value (Table 4 in Paper III).

5.4.1 Nitrite reduction potential in the soils and sediments of the ORWRP wetland complex

Nitrite reductase is considered to be the key enzyme of denitrification, since it catalyses the reduction of soluble nitrite into gaseous nitric oxide. Two different types of *nir* genes have been widely used as molecular markers to study denitrification in various environments, including SF TWs (Henry et al., 2004; Graham et al., 2010; García-Lledó et al., 2011; Wakelin et al., 2011; Correa-Galote et al., 2013; Jones et al., 2014; Veraart et al., 2014).

The presence of *nirS* and *nirK* genes was detected in all of the studied samples, referring to the existence of genetic potential for nitrite reduction throughout the study area (Table 3). While *nirK*% ranged from 6.74 to 17.04%, *nirS*% showed greater variations between samples, ranging from 2.55 to 40.18% in the bacterial communities of the studied samples (Paper III), referring to the fact that a relatively high proportion of bacteria possess *nir* genes in the studied bacterial communities. Other TW studies have detected similar or somewhat lower *nirS* and *nirK* gene proportions (García-Lledó et al., 2011; Correa-Galote et al., 2013). In addition, Jones et al. (2014) have shown that *nirS* and *nirK* gene proportions in the soil communities ranged between 3–21% and 4–26%, respectively, indicating that bacteria possessing *nir* genes may also constitute a remarkable proportion of bacterial communities in grassland and agricultural soils. The comparison of the results based on the quantification of different genes between the studies is complicated due to the differences in applied qPCR methodologies, as discussed in Paper I. In addition, one must consider that although most known denitrifiers possess one copy of either *nirS* or *nirK* genes per cell, some species have up to two *nirS* or four *nirK* gene copies per cell, and few organisms have both types of *nir* genes in their genomes (Graf et al., 2014).

Table 3. Average values and standard deviations (in parentheses) of gene parameter values in the studied soil groups. Abbreviations of the soil groups: W_{oo} – organic sediment samples of open areas of W wetlands, Ox_{oo} – soil samples of open area of oxbow, W_{om} – mineral soil samples of open areas of W wetlands, W_{trans} – soil samples of transitional areas of W wetlands, Ox_{trans} – soil samples of transitional area of oxbow, and Up – soil samples of upland areas.

Soil group	**Target gene abundance (copies/g dw)**	**Proportion of genes in the bacterial community (%)**										
	16S rRNA	***nirS*%**	***nirK*%**	***nir*%**	***nirK/ nirS***	***nosZI %***	***nosZII %***	***nosZ*%**	***nosZI/ nosZII***	***nosZ/ nir***	**amx%**	**ndamo%**
W_{oo} (n=6)	$2.41 * 10^{10}$ ($4.10 * 10^{9}$)	32.9 (5.0)	10.3 (3.0)	43.2 (6.7)	0.31 (0.08)	1.02 (0.25)	1.69 (0.60)	2.71 (0.82)	0.63 (0.13)	0.06 (0.01)	0.0055 (0.0055)	0.0123 (0.0042)
Ox_{oo} (n=3)	$1.74 * 10^{10}$ ($1.12 * 10^{10}$)	28.0 (2.2)	10.9 (2.1)	38.9 (4.6)	0.39 (0.06)	1.34 (0.47)	1.56 (0.34)	2.90 (0.74)	0.86 (0.25)	0.07 (0.01)	0.0004 (0.0003)	0.0105 (0.0036)
W_{om} (n=6)	$3.79 * 10^{9}$ ($2.93 * 10^{9}$)	23.0 (4.4)	7.7 (0.6)	30.7 (4.8)	0.34 (0.05)	1.87 (1.33)	1.00 (0.26)	2.87 (1.26)	2.09 (1.80)	0.10 (0.06)	0.0025 (0.0023)	0.0087 (0.0048)
W_{trans} (n=6)	$1.18 * 10^{10}$ ($7.27 * 10^{9}$)	15.9 (5.3)	13.5 (2.2)	29.4 (6.0)	0.93 (0.34)	2.47 (1.18)	2.39 (0.38)	4.86 (1.09)	1.08 (0.61)	0.17 (0.06)	0.0145 (0.0340)	0.0072 (0.0033)
Ox_{trans} (n=4)	$6.97 * 10^{9}$ ($3.65 * 10^{9}$)	14.0 (2.1)	13.6 (2.2)	27.6 (3.7)	0.98 (0.15)	2.52 (0.53)	2.02 (0.52)	4.54 (0.23)	1.40 (0.80)	0.17 (0.02)	0.0003 (0.0003)	0.0020 (0.0016)
Up (n=4)	$7.46 * 10^{9}$ ($3.40 * 10^{9}$)	4.3 (2.9)	12.5 (3.2)	16.8 (6.0)	3.31 (0.99)	1.33 (0.36)	0.86 (0.09)	2.19 (0.57)	1.63 (0.26)	0.13 (0.03)	0.0001 (0.0002)	0.0001 (0.0001)

NirS% showed higher values in the W_{oo} samples than in W_{trans} ($p < 0.01$), while differences were not detected in the respective values of *nirK*%. When the permanently and occasionally flooded oxbow soil samples were added to the respective soil groups of W wetlands, the differences in *nirS*% between areas with different water regimes became more significant ($p < 0.001$), and higher *nirK*% were detected in Trans soils compared to Oo group ($p < 0.05$). The preference of conditions prevailing in permanently flooded areas by *nirS*-type denitrifiers was also confirmed by the lower values of *nirK/nirS* in W_{oo} and Oo compared to W_{trans} and Trans groups, respectively ($p < 0.01$ and $p < 0.001$). When all the soil groups were compared, the highest *nirK/nirS* was detected in the Up samples. *NirS*% and *nirK*% also showed correlations with bacterial community structure, being significantly correlated with the first axis of PCoA ($r = 0.83$, $p < 0.001$ and $r = –0.66$, $p < 0.001$, respectively); however, while *nirS*% were related to community structure in permanently flooded areas, *nirK*% showed relationships with the communities of upland and transitional areas (Fig. 3). In addition, only *nirS*% were related to the consortia of bacterial OTUs (Table 5 in Paper II).

The results of this study indicate a certain niche preference between the two types of nitrite reductase possessing bacteria (Papers I and II). *NirK*-gene possessing bacteria may be more tolerant for fluctuating water regime, while bacteria with *nirS* gene seem to prefer the conditions prevailing in more permanently flooded areas. In addition, different environmental factors affected differently these two types of *nir* genes when all the samples were analysed together (Table 3 in Paper III) or separately in the studied soil groups (Supplementary Table S5; Table 4 in Paper I), indicating that the effect of chemical parameters on bacteria possessing *nir* genes may also be different in different ecosystems. For example, *nirK*% were related positively to soil NO_3-N concentration only in the W_{trans} soil group, whereas no relationship was detected between those parameters in the W_{oo} soil group (Supplementary Table S5; Table 4 in Paper I). A laboratory experiment conducted with the soils from W wetlands showed that potential denitrification rates in the transitional area samples of W wetlands were positively influenced by nitrate addition, while this effect was not detected in the cases of permanently flooded areas (Song et al., 2014). An extended discussion of the niche preference of two *nir* genes is presented in Papers I and II.

The overall nitrite reduction potential in the bacterial communities was assessed using the sum of the two types of *nir* gene proportions. The values of *nir*% ranged between 11.6 and 50.9%, and were higher in W_{oo} compared to W_{trans} ($p < 0.01$), and in Oo compared to the Trans samples ($p < 0.001$). Bacterial communities in the permanently flooded areas have a greater proportion of bacteria with a potential for nitrite reduction, and their abundance was also higher compared to areas with a fluctuating water regime (Paper I). This is also in accordance with the results from a study conducted in the W wetlands of the same study area, indicating a higher potential denitrification activity in open water areas (3.78 g N/m^2 yr) compared to marsh areas (2.77 g N/m^2 yr)

measured from 2004 through 2009 (Song et al., 2014), and other studies conducted in different wetland systems indicating higher denitrification activity in the permanently flooded areas (Burgin and Groffman, 2012; Peralta et al., 2013).

In addition to water regime in the wetland basin, nitrite reduction potential is also affected by soil type, since higher values of *nirK*%, *nirS*%, and *nir*% were detected in the bacterial communities of organic sediments compared to the mineral soils below them ($p < 0.05$). Although the bacterial communities of these two substrates did not differ significantly in terms of the structure of their bacterial community, environmental conditions for bacteria in general and denitrifiers may be more preferable in the upper sediment layer, which is probably related to the chemical composition of organic and mineral soil layers (Fig. 2 and Table 1). Higher values of *nirS* and *nirK* genes were detected in the top sediment layer compared to the underlying layer in two Danish agricultural streams (Veraart et al., 2014).

5.4.2 Nitrous oxide reduction potential in the soils and sediments of the ORWRP wetland complex

Nitrous oxide reductase encoding *nosZI* and *nosZII* genes were detected in all of the studied soil and sediment samples, indicating the presence of genetic potential for nitrous oxide reduction in soils all over the TW complex study area (Table 3).

NosZI% and the recently discovered *nosZII*% were quite equally represented in the bacterial communities of the TW complex soils ranging between 0.56–4.59% and 0.48–3.01%, respectively (Paper III). Since the second clade of the *nosZ* gene was discovered recently, a limited amount of information is available about this type of nitrous oxide reductase gene in wetlands, and especially in TWs. Lower values of *nosZI* and *nosZII* gene proportions were found in the sediments of SF TW purifying the effluent from wastewater treatment plant (~0.4% and ~0.2%, respectively) (Jones et al., 2013), while *nosZII* genes were almost undetectable in the sediments of natural and restored wetlands (Hathaway et al., 2015). Similar representation of *nosZ* gene clades was detected also in sediments of boreal lakes with high nitrate content (0.9–4.7% and 1.3–5.2% for clade I and II, respectively) (Saarenheimo et al., 2015).

Both *nosZI*% and *nosZII*% and their sum, which ranged between 1.47–6.69%, were higher in the occasionally flooded than in the permanently inundated areas of W wetlands ($p < 0.01$, $p < 0.05$, and $p < 0.05$, respectively) in the ORWRP study area. When proportions of different genes encoding nitrous oxide reductase and their sum were analysed in Oo and Trans groups, differences in *nosZI*% and *nosZ*% became even greater between permanently and occasionally flooded areas ($p < 0.01$ in both cases) and higher values of *nosZI*/*nosZII* were detected in Trans group ($p < 0.05$). The higher genetic potential for N_2O reduction of transitional area bacterial communities was a somewhat surprising result, as nitrous oxide reductase is assumed to be the most oxygen sensitive enzyme in the denitrification pathway (discussed in Paper I).

When the organic sediments in the permanently flooded areas were compared with mineral soils below, higher *nosZII*% were detected in the bacterial communities of organic sediments ($p < 0.05$), while no differences in *nosZI*% or *nosZ*% were identified. This shows that although the bacterial communities in the upper sediment layer had higher genetic potential for nitrite reduction, the nitrous oxide reduction potential in the community was not affected by the soil type, and both soil layers contributed similarly to N_2O reduction in TWs. Thus, further TW studies should also consider including deeper soil layers in the analysis.

The study found a relationship between *nosZ* gene proportions and bacterial community structure, but in contrast to *nir* genes, *nosZI*% and *nosZII*% were related to the PCoA axis 2 ($r = 0.57$; $p < 0.01$ and $r = 0.48$; $p < 0.01$, respectively), and only *nosZI*% were correlated with PCoA axis 1 ($r = –0.43$; $p < 0.05$) (Fig. 3). In addition, only *nosZI*% showed correlation with the bacterial consortium within the soil groups (Table 5 in Paper II).

NosZI% and *nosZII*% were differently correlated with measured soil chemical variables (Table 3 in Paper III and Supplementary Table S5), indicating that similarly to *nir* genes, organisms possessing different types of nitrous oxide reductase genes also have different requirements for environmental conditions, as discussed in Paper III. This suggestion is also in agreement with the conclusions of a study conducted in agricultural fields by Jones et al. (2014).

The proportions of nitrite reductase encoding genes were higher than those of nitrous oxide reductase genes in all of the studied soil samples, which is in accordance with the results based on the available genomes of denitrifiers, showing that not all denitrifiers possessing the *nir* gene have the *nosZ* gene (Jones et al., 2008; Sanford et al., 2012; Graf et al., 2014). When the bacterial communities of permanently and occasionally flooded areas were compared using the ratio of nitrous oxide and nitrite reductase encoding gene proportions, the higher *nosZ/nir* values were detected in the bacterial communities of the W_{trans} and Trans areas compared to the W_{oo} ($p < 0.01$) and Oo areas ($p < 0.001$), respectively. Although the soils of the ORWRP were sources of N_2O, the lower genetic potential for N_2O emission via denitrification was found in the microbial communities of occasionally flooded areas. The gas measurements in the ORWRP study area have shown the highest N_2O emission in the occasionally (0.191 g N/m^2 yr) and the lowest in the permanently (0.061 g N/m^2 yr) flooded areas (Hernandez and Mitsch, 2006), constituting only a small fraction of the denitrification rates measured with acetylene blocking method (Mitsch et al., 2012). N_2O is also known to be produced in several other microbially mediated pathways, as pointed out in section 3.3.1, and therefore it is difficult to determine the exact sources of N_2O emission.

5.4.3 ANAMMOX potential in the soils and sediments of the ORWRP wetland complex

The presence of ANAMMOX specific bacteria in the soils and sediments was revealed through the analysis of the obtained 16S rRNA sequences and was also confirmed by the qPCR results (Paper III). ANAMMOX specific gene proportions varied in the studied bacterial communities, ranging from 0.00005% to 0.084%, and representatives from genera *Ca. Scalindua*, *Ca. Brocadia*, and *Ca. Kuenenia* were detected in the ORWRP study area samples. Wheras bacteria from genera *Ca. Brocadia* and *Ca. Kuenenia* have been found in different terrestrial and freshwater ecosystems, the representatives of genus *Ca. Scalindua* have mainly been related to marine ecosystems (detailed discussion in Paper III).

While the values of different *nir* and *nosZ* genes were not affected by distance from the inlet, the bacterial communities of W_{in} significantly ($p < 0.01$) differed from W_{out} by the higher values of amx%, indicating that the inflow areas of W wetlands provided more suitable environmental conditions for ANAMMOX bacteria. Especially high amx% were detected in the upper soil layer of the W1 wetland (0.014% in site 1 and 0.084% in site 7; Fig. 1), which refer to the occurrence of "hotspots" of ANAMMOX bacteria in the soils of TWs. Differences were also detected in the diversity of ANAMMOX bacteria; the proportions of *Ca. Brocadia* decreased and *Ca. Scalindua* increased in the direction from inflow to outflow in W wetlands (Fig. 8). In the oxbow, differences in the longitudinal gradient were not detected in amx%, while the microbial communities in the Ox_{in} were, analogically to W_{in}, dominated by the representatives of *Ca. Brocadia*. No statistically significant differences were detected between different soil types or areas with different water regimes in amx% (Table 3); however, when Trans and Oo samples were analysed together, amx% were statistically higher ($p < 0.05$) in the Oo samples (according to the geometric mean). In addition, amx% were positively correlated with PCoA axis 1 ($r = 0.70$; $p < 0.001$) on the ordination biplot of bacterial community structure (Fig. 3), referring to the importance of the wetland water regime at the site to these organisms. The ANAMMOX bacterial community structure was also affected by water regime in W wetlands; *Ca. Brocadia* was the dominant genera in W_{oo} samples, while *Ca. Scalindua* was the most abundantly represented ANAMMOX genera in the bacterial communities of W_{trans}.

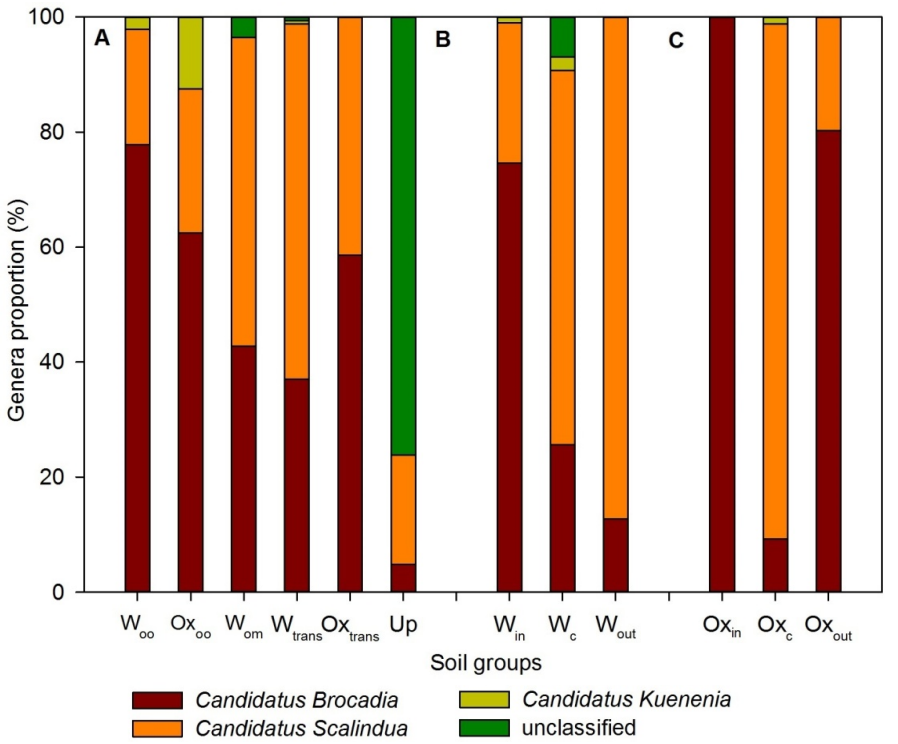

Figure 8. Genera proportions of the order *Brocadiales* in the studied soil groups. Abbreviations of the soil groups: (1) W_{oo} – organic sediment samples of open areas of W wetlands, Ox_{oo} – soil samples of open area of oxbow, W_{om} – mineral soil samples of open areas of W wetlands, W_{trans} – soil samples of transitional areas of W wetlands, Ox_{trans} – soil samples of transitional area of oxbow, and Up – soil samples of upland areas (A); (2) W_{in} – soil samples of inflow areas of W wetlands, W_c – soil samples of centre areas of W wetlands, and W_{out} – soil samples of outflow areas of W wetlands (B); (3) Ox_{in} – soil samples of inflow area of oxbow, Ox_c – soil samples of centre area of oxbow, and Ox_{out} – soil samples of outflow area of oxbow (C).

ANAMMOX bacteria were also detected in the upland areas, but their proportions were lower than in W_{oo} soil samples ($p < 0.05$). In addition, a majority of the sequences of order *Brocadiales* remained unclassified in these areas. This may indicate that ANAMMOX bacteria are also able to survive in aerobic areas, but their abundance is low and the diversity of ANAMMOX bacteria in those areas is different compared to wetland soil groups. Recently, ANAMMOX bacteria specific hydrazine synthase genes (~10^5 copies/g) were also detected in the aerobic zones of a wastewater treatment plant (dissolved oxygen > 2 mg/L) (Wang et al., 2015). Shen et al. (2015b) detected ANAMMOX bacteria specific sequences in different vegetable field soils and showed the presence of variable *Brocadiales* genera, including *Ca. Brocadia*. Most of the ANAMMOX studies

have been conducted in different profoundly anoxic environments, and databases contain sequences mostly from those ecosystems, but still unknown types of ANAMMOX bacteria may inhabit areas where the favourable oxic-anoxic boundary may be only a few millimetres thick (Zhu et al., 2010).

The ANAMMOX bacterial proportions in the community were correlated positively with pH and NH_4-N, P, Ca, and K content, and negatively with C/N values in the studied soils and sediments (Table 3 in Paper III). The positive relationship between ANAMMOX specific 16S rRNA gene abundance and ammonium but not nitrate concentrations was also evident in agricultural soils (Shen et al., 2015b), while no correlations were found between *hszB* gene abundance or proportions in the bacterial communities and pH, ammonium, nitrate-nitrite nitrogen or total nitrogen values in the paddy field soils (Wang et al., 2012).

According to the sequencing analysis, ANAMMOX specific 16S rRNA gene proportions varied between 0–0.17% across the studied soil bacterial communities. In contrast to qPCR analysis, which detected ANAMMOX bacteria specific genes from all of the studied samples, the sequencing analyses did not find order *Brocadiales* specific sequences from sites 5, 13, and 21 (Fig. 1). No significant differences were detected between different soil types in Samx%; however, W_{trans} and Trans samples showed higher Samx% compared to W_{oo} and Oo samples, respectively ($p < 0.05$ in both cases) (Supplementary Table S6). Samx% were not correlated with none of the measured soil chemical parameters. These results demonstrate that although the presence of ANAMMOX bacteria specific 16S rRNA sequences can be detected by both methods, the results are not fully compatible.

The differences between the results of sequencing and qPCR analyses may be related to the differences in the coverage of the used ANAMMOX specific and universal 16S rRNA primers or limitations of reference databases (Lim, 2011). In addition, different ANAMMOX functional gene (hydrazine synthase encoding *hszA* and *hszB* genes) specific primers were tested in the current study in order to quantify the proportion of ANAMMOX bacteria (Harhangi et al., 2012; Wang et al., 2012), but they were discarded due to the appearance of non-specific PCR products.

5.4.4 The evidence of novel nitrogen transformation processes in the soils and sediments of the ORWRP wetland complex

In addition to denitrification and ANAMMOX, evidence of autotrophic denitrification and the relatively recently discovered n-damo processes was detected in the ORWRP study area.

N-damo specific 16S rRNA gene proportions varied in the studied bacterial communities, ranging from 0 to 0.019% according to the qPCR results. The highest ndamo% were identified in the organic sediment samples of the W2 wetland (sites 5 and 6; Fig. 1), while in one of the Up area samples between W

wetlands no n-damo specific sequences were detected (site 21; Fig. 1). Statistical analysis did not detect differences in ndamo% between different soil types, water regimes, or along the vertical gradient of W wetlands or the oxbow (Table 3). However, the ndamo% was lower in the Up soils than in the W_{oo} ($p < 0.05$). When the Oo and Trans soils were compared, the ndamo% was higher in the permanently flooded areas compared to the occasionally flooded areas ($p < 0.01$). In addition, ndamo% was also positively related to PCoA axis 1 ($r = 0.76$; $p < 0.001$) (Fig. 3), pointing to a relationship with water regime in the studied wetlands. In contrast to amx%, statistically significant relationships were detected between the obtained pMEN modules structure and ndamo%; modules $W_{trans}1$ and $W_{trans}2$ were correlated positively ($r = 0.87$; $p < 0.05$ and $r = 0.91$; $p < 0.05$, respectively) and module $W_{oo}2$ negatively ($r = –0.82$, $p < 0.05$) with ndamo%. Furthermore, one OTU of module $W_{trans}G$ belonged to phylum NC10 (Fig. 7 and Supplementary Table S3). The results of this study indicate that the proportions of n-damo bacteria in the studied soil communities seem to be affected by water regime, as conditions created by the more permanent water table are more favourable than those in occasionally inundated or drier areas.

In this study, the proportion of n-damo specific bacteria in the community was relatively equally represented in organic and mineral soil layers. N-damo specific bacteria are shown to have higher abundance in deeper sediments. The highest *Ca. M. oxyfera* specific 16S rRNA gene abundance in natural and urban wetlands was detected at the depth of 50–60 cm (Hu et al., 2014), while in a minerotrophic peatland the maximum abundance values were found at the depth of 80–85 cm (Zhu et al., 2012). Considering these findings, the 0–15 cm or 15–30 cm soil layers analysed in this study may not comprise the maximum abundance of n-damo bacteria. However, the proportions of *Ca. M. oxyfera* in the bacterial communities of permanently flooded areas in the ORWRP study area were comparable with values reported in different wetland soils at a depth of 20–30 cm (Hu et al., 2014) and river sediments at a depth of 0–3 cm (Shen et al., 2014).

Several significant relationships were also detected between ndamo% and chemical parameters. Ndamo% was positively correlated with Ca ($r = 0.67$; $p < 0.001$), NH_4-N ($r = 0.58$; $p < 0.001$), P ($r = 0.48$; $p < 0.01$), pH ($r = 0.46$; $p < 0.05$), and K ($r = 0.40$; $p < 0.05$), while negative correlations were detected between ndamo% and C/N ($r = –0.67$; $p < 0.001$) and NO_3-N ($r = –0.44$; $p < 0.05$) values in the soil. A study conducted in paddy soil also found a positive correlation between n-damo gene abundance and NH_4-N values; however, no relationships were detected between n-damo proportions in the bacterial community and chemical parameters (Wang et al., 2012).

According to the sequencing analysis, 0.07% of all the obtained 16S rRNA sequences belonged to *Ca. M. oxyfera*. The number of *Ca. M. oxyfera* specific sequences ranged from 1 to 490 sequences per sample and Sndamo% varied between 0.0007 and 0.34%, showing higher values in W_{om} compared to Up

($p < 0.01$) and W_{oo} ($p < 0.05$) (Supplementary Table S6). Brooker et al. (2014) also detected more 16S rRNA sequences belonging to the phylum NC10 from the deeper layers in the permanently flooded inflow areas of the same W1 wetland (0–15 cm versus 15–30 cm). However, except for the correlation between Sndamo% and C values ($r = –0.44$; $p < 0.05$), similarly to ndamo%, Sndamo% was correlated with NH_4-N ($r = 0.51$; $p < 0.01$), Ca ($r = 0.45$; $p < 0.05$), and C/N ($r = –0.51$; $p < 0.01$) values in the soil, and with PCoA axis 1 ($r = 0.71$; $p < 0.001$), indicating a certain similarity between the results obtained by sequencing and qPCR methods of n-damo specific genes compared to ANAMMOX.

Although the presence of n-damo specific 16S rRNA genes was detected using qPCR and sequencing methods, the n-damo proportions obtained with two different methods did not coincide completely. *Ca. M. oxyfera pmoA* gene specific primers were also tested in order to quantify the n-damo bacteria in the current study (Luesken et al., 2011), but similarly to the tested ANAMMOX functional gene specific primers, they were unsuitable due to the presence of non-specific PCR products.

In the current study, ndamo% were correlated with amx% ($r = 0.63$; $p < 0.001$). In addition, amx% and ndamo% values were positively correlated with *nirS*% (Supplementary Table S3 in Paper III and $r = 0.86$; $p < 0.001$, respectively). The proportions of ANAMMOX, n-damo, and *nirS* genes were higher in the Oo than Trans samples, and correlated similarly with chemical parameters, indicating a certain similar preference for environmental conditions of different *nirS* gene possessing bacteria. In addition to denitrifiers, *nirS* genes have been detected from the genomes of ANAMMOX (discussed in Paper III) and also from n-damo bacteria (Ettwig et al., 2010). Recent studies have revealed the co-occurrence of ANAMMOX and n-damo organisms in paddy soils (Wang et al., 2012) and in an urban wetland with high nitrogen input (Shen et al., 2015a). However, while in paddy soils both bacterial groups showed the highest values in deeper layers (30–60 cm), niche segregation was detected in the urban wetland, as ANAMMOX bacteria were more abundant in the shallow (0–10 cm and 20–30 cm), and n-damo in the deeper (50–60 cm and 90–100 cm) soil layers.

According to the sequencing analyses, one of the greatly represented genera in the soil samples of TWs was *Thiobacillus* (Supplementary Table S2), which is known to contain species capable of sulphur-based autotrophic denitrification (Robertson and Kuenen, 2006). While heterotrophic denitrification is a very effective process for nitrogen removal in cases when a sufficient amount of an organic carbon source is present in the environment, autotrophic denitrification does not require carbon in organic form, and its efficiency is lower than the heterotrophic process. For example, facultatively anaerobic *T. denitrificans* and *T. thiophilus* are chemolithoautotrophs that are able to reduce nitrogen oxides by using elemental or reduced sulphur compounds as electron donors (Batchelor and Lawrence, 1978; Kellermann and Griebler, 2009). *T. denitrificans* possesses

all the necessary genes for complete denitrification (Shao et al., 2010). Genus *Thiobacillus* was shown to exist more abundantly in the bacterial communities of arctic wetland sediments compared to lake sediments (Stoeva et al., 2014). *T. denitrificans* sequences were also detected in the sediments of a restored wetland that was permanently flooded with river water (He et al., 2015). In addition to the genus *Thiobacillus*, sequences belonging to other sulphur-based autotrophic denitrifiers containing genera *Paracoccus* and *Sulfurimonas* were detected from the samples of the current study area, but their proportions constituted less than 0.04% of the determined sequences in each sample. Therefore, in addition to heterotrophic denitrification, autotrophic denitrifiers may also have a role in the nitrogen cycle in the ORWRP study area, but further studies are needed to confirm this hypothesis.

5.4.5 Nitrogen removal pathways in the soils and sediments of the ORWRP wetland complex

Over the 15 years of operation, the NO_3-N removal efficiency in W wetlands decreased from 35% to 25%; however, for the last six years (2005–2010), nitrogen retention was steady or even slowly improving (average retention of NO_3-N was 26.4 g N/m^2 yr and total N was 38.8 g N/m^2 yr) (Mitsch et al., 2012; Mitsch et al., 2014). No obvious trends in denitrification rates over the period 2004–2009 were detected, and the average denitrification potential measured by the acetylene blocking method was only 3.2 g N/m^2 yr (Song et al., 2014; Mitsch et al., 2014). An additional mass-balance study conducted in 2008–2009 in the same W wetlands concluded that the annual nitrogen loss through denitrification was 2.7 g N/m^2 yr, accounting for only 3.5% of the difference between inflow and outflow (Batson et al., 2012). Other processes, such as sedimentation and groundwater seepage, accounted for over 50% of nitrogen removal, but the fate of a significant part of the nitrogen retention is nonetheless unknown (Batson et al., 2012; Mitsch et al., 2012; Mitsch et al., 2014).

The putative network of nitrogen transformation processes related to nitrate removal in W wetlands is presented in Fig. 3 in Paper III. Although n-damo and sulphur-based autotrophic denitrification processes were marked as hypothetic pathways in Fig. 3 (Paper III), later analyses showed evidence of the presence of those processes in the soils and sediments of W wetlands.

The results of this study show that a significant proportion of bacteria in the microbial communities of soils and sediments of TWs possess denitrification-related genes, and the genetic potential for denitrification is high. In addition, although the dominating nitrogen removal pathway in all of the studied soil bacterial communities seems to be denitrification, ANAMMOX and n-damo specific gene sequences were detected in almost all of the samples, indicating that those processes can contribute to nitrogen removal in the W wetlands and also in the oxbow, but their contribution is quite low compared to denitrification in the upper layer of soils and sediments. Taking into account the findings of

recent studies (Jones et al., 2013; Wei et al., 2015), the available primer sets used for quantifying and sequencing the marker genes of the denitrification, ANAMMOX, and n-damo processes may underestimate their real proportions, and the design of specific primers is still challenging.

It is assumed that similarly to ANAMMOX, the n-damo process is not prevalent in freshwater sediments and water-logged soils with high organic matter input due to the competition with denitrifiers for nitrite. In environments where ammonium is not limited and organic matter concentration is low, ANAMMOX bacteria are assumed to outcompete n-damo bacteria in the competition for nitrite (Thamdrup, 2012). In this study, we detected denitrification, ANAMMOX, and n-damo specific genes in almost all of the samples, which refer to a very broad range of environmental conditions in the soils and sediments of the ORWRP study area. Facultatively anaerobic denitrifiers and anaerobic ANAMMOX and n-damo bacteria were detected within the 0–15 cm soil and sediment layers. A study conducted in the same study area in 2008–2010 showed that the conditions in the soils of W wetlands (0–8 cm, 8–16 cm) were anaerobic throughout the year according to the redox potential measurements (Roy-Chowdhury et al., 2014). However, redox potential can change considerably in space and/or in time in soils (Vorenhout et al., 2004; Thomas et al., 2009), and suitable niches can be created for very different bacteria. Therefore, in order to determine the more precise spatial distribution of those nitrogen gas producing processes in wetland soils, more samples should be collected along a depth gradient with narrow thickness.

The bacterial community analysis revealed the presence of different bacterial families and genera related to sulphur cycling, including sulphate reduction (Supplementary Tables S1 and S2). The presence of sulphur-reducing bacteria was also shown in an earlier microcosm study using the sediments and water from the W1 wetland, showing a rapid depletion of water sulphate concentration in the mixture of open and transitional area sediments (0–15 cm), while a considerably lower decline was detected in the case of deeper sediments (15–30 cm) (Brooker et al., 2014). In addition, the results from redox potential measurements conducted by Roy-Chowdhury et al. (2014) indicate that the electron acceptors for other lower energy yield processes, such as sulphate reduction and methanogenesis, may be prevalent already in the upper layers of the W wetland soils.

Sulphate reducers are known to use a wide range of alternative electron acceptors and donors, and inhabit very variable environments including different wetlands (Muyzer and Stams, 2008; Pester et al., 2012). For example, a study conducted by He et al. (2015) showed that the proportions of sulphate reducers specific *dsrAB* genes were 2–2.5 times higher compared to the *nosZ* genes in the soil microbial communities of a restored wetland flooded with river water. Sulphides can have a stimulatory or inhibitory effect on denitrification, since some autotrophic denitrifiers are able to use sulphide as an electron donor, while the inhibitory effect of sulphide on nitrous oxide reductase may lead to

higher N_2O emission (Sørensen et al., 1980; Bowels et al., 2012). Since ANAMMOX bacteria are also known to be inhibited even by very low sulphide concentrations (Dapena-Mora et al., 2007), both denitrification and ANAMMOX processes could possibly be affected by the sulphur cycle in the soils of the ORWRP. However, a recent study by Russ et al. (2014) demonstrated cooperation between ANAMMOX bacteria and sulphide-dependent autotrophic denitrifiers on the laboratory scale, which may indicate that ANAMMOX bacteria could also exist in environments where sulphur cycling is active and actual sulphide concentrations remain below the μM range. Concurrently, the results of the current study support the idea that more information is needed about sulphur-associated metabolism in TWs to assess its potential impact on nitrogen, carbon etc. transformation processes, in order to construct TWs with high nitrogen removal efficiency.

6. CONCLUSIONS

The first TWs were constructed more than fifty years ago and they have been used all over the world to reduce excessive nitrogen concentration in different types of wastewaters. Despite the advancements made in the design and operation of TWs over that time, there is still a paucity of information about the microbial communities and nitrogen removal processes in those systems. Based on a microbiological analysis of the soils and sediments of a wetland complex treating polluted river water, the assessment of TWs' bacterial community structure and its nitrogen removal potential was performed in this study. The results and data analysis presented in this thesis allow to make the following conclusions:

Proteobacteria, *Acidobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Verrucomicrobia* were the dominant bacterial phyla in all the soils and sediments of the TWs. Water regime was an important factor determining the structure of bacterial communities in the soils and sediments within the TW basins as the bacterial communities of permanently flooded areas differed from the occasionally flooded TWs' zones. The soil bacterial communities in areas with a fluctuating water regime were more diverse, and phylogenetic groups were more evenly represented. The differences in bacterial community structure between the permanently and occasionally flooded areas in the oxbow were less pronounced compared to the artificially flooded TWs. The ecological networks within bacterial communities in the occasionally flooded soils were more complex than in the case of communities of the organic rich or mineral soils in the permanently flooded areas. The bacterial communities of the soils and sediments of the TWs were not affected by soil type or distance from the inlet.

The results of the study revealed a potential for several nitrogen removal processes in the soils and sediments of the TW complex. The genetic potential of the denitrification of the TW bacterial communities was dependent on the site's hydrological conditions. Nitrite reduction potential was highest in the bacterial communities of TWs' permanently flooded zones. The proportions of bacteria with *nirS* gene exceeded the proportions of bacteria possessing *nirK* gene in the bacterial communities of permanently flooded soils and sediments of TWs, while the upland soil communities were dominated by *nirK*-type nitrite reducing bacteria. Bacteria with different *nir* genes had a certain niche preference in the soils of TWs. *nirK* gene possessing bacteria were more tolerant of drier or fluctuating water regimes, while bacteria with *nirS* genes favoured conditions characteristic of more permanently flooded areas. The nitrite reduction potential of bacterial communities of the TW was affected by soil type; higher potential for this processes was detected in the organic rich sediments of W wetlands.

The nitrous oxide reduction potential of the bacterial community was also affected by water regime. The proportions of different *nosZ* gene possessing bacteria were higher in the bacterial communities of the occasionally flooded

areas. Bacteria with the *nosZ* clade I or II gene were quite equally represented in the bacterial communities of the TW complex soils. Bacteria with different *nosZ* gene clades had different requirements for soil chemical conditions. Similarly to the nitrite reduction potential, the potential of nitrous oxide reduction was not affected by distance from the inlet.

The proportions of genes encoding nitrite and nitrous oxide reductases in the bacterial communities were affected differently by soil chemical parameters.

The genetic potential for the production of the greenhouse gas N_2O via partial denitrification was detected in all of the studied areas of the ORWRP wetland complex, but it was lower in the case of the bacterial communities of occasionally flooded areas than in permanently flooded areas.

In addition to denitrification, the potential for ANAMMOX and recently discovered n-damo processes was detected in the soils and sediments of the TW complex. The proportions of bacteria conducting these two processes were also affected by water regime at the site; the most suitable conditions for ANAMMOX and n-damo bacteria were in the permanently flooded areas. The ANAMMOX and n-damo specific bacteria had similar requirements for the soil chemical composition in the TW complex. Representatives of the ANAMMOX bacterial genera *Ca. Brocadia*, *Ca. Kuenenia*, and *Ca. Scalindua*, out of which the latter has previously been associated mainly with marine environments, were identified from the soils and sediments of the wetland complex.

Based on the results of this study, it can be concluded that the bacterial communities in the soils and sediments of TWs have the genetic potential for several nitrogen removal processes; nevertheless, denitrification is the main process performing this function in the soils and sediments of the studied TWs. The creation of TW areas with a fluctuating water regime could help to decrease the negative impact of N_2O emission from partial denitrification from the TWs soils.

In addition, nitrogen cycling is closely linked to other nutrient cycles in the soils and sediments of TWs, and these relationships should be further studied and considered when designing efficiently functioning TWs.

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SUMMARY IN ESTONIAN

Vabaveelise tehismärgalakompleksi bakterikoosluse struktuur ja selle lämmastikuärastuse geneetiline potentsiaal

Viimaste sajandite jooksul on sünteetiliste lämmastikväetiste kasutamise, õhulämmastikku siduvate taimede massilise kasvatamise ning fossiilsete kütuste ja biomassi põletamise tulemusena kordades suurenenud bioloogiliselt kättesaadava lämmastiku hulk eri ökosüsteemides, mis omakorda on põhjustanud ulatuslikke keskkonnaprobleeme. Enamik inimtegevuse tagajärjel keskkonda sattunud lämmastikust jõuab varem või hiljem magevee- ja rannikuökosüsteemidesse, kus see põhjustab koos teiste toitainetega vetikate õitsenguid, vee kvaliteedi langust, elukeskkondade ja looduslike ressursside kadumist ning surnud tsoonide tekkimist.

Üks maailma ulatuslikumaid surnud tsoone on 13 000 km^2 suurune hüpoksiline ala Mehhiko lahes, mille on põhjustanud USA kesklääne piirkonnast Mississippi, Ohio ja Missouri (MOM) jõgede sissekantud liigsed toitained. Peamised lämmastikuallikad MOMi jõgedes on lämmastikväetiste kasutamine, liblikõieliste taimede, nagu sojauba, kasvatamine, loomakasvatusest pärinev sõnnik ning munitsipaal- ja tööstuslikud reoveed. Lisaks on nimetatud piirkonnas 20. sajandi jooksul kuivendatud ligi 30 miljonit hektarit maad, mistõttu on kadunud puhvertsoonid jõgede ülemjooksude ja suudmeveekogude vahel.

Veeökosüsteeme on võimalik liigsete toitainete eest kaitsta tehismärgalade rajamisega. Tehismärgalad on konstrueeritud lagundama või siduma eri tüüpi saasteaineid, rakendades selleks looduslikele märgaladele iseloomulikke bioloogilisi, keemilisi ja füüsikalisi protsesse. Lisaks punktreostusallikast pärinevate reovete käitlemisele on tehismärgalad leidnud rakendamist ka hajureostuse tagajärjel saastunud veekogude puhastamisel, et vähendada bioloogiliselt kättesaadava lämmastiku jõudmist suurematesse veeökosüsteemidesse. Eespool mainitud põhjustel on hakatud MOMi piirkonda kavandama ulatuslikku, ligi 22 000 km^2 suurust tehismärgalade võrgustikku, mille tulemusena loodetakse suurendada hapniku kontsentratsiooni Mehhiko lahes, vähendada laialdaste üleujutuste ohtu, parandada piirkonna üldist veekvaliteeti ning luua uusi elukeskkondi. Märgalade konstrueerimise, funktsioneerimise ja taastamise uurimise eesmärgil on Olentangy jõe paremkaldale (USA, Ohio, Columbus) rajatud Olentangy River Wetland Research Parki (ORWRP) nimeline uurimiskeskus, mille juurde kuulub ka põllumajandusliku tegevuse tagajärjel reostunud jõevett puhastav tehismärgalade kompleks.

Mikroobikooslustel on tehismärgalade lämmastikuringes võtmeroll ning seetõttu on väga oluline mõista mikroobikoosluste ja keskkonnaparameetrite vahelisi seoseid kõnealustes ökosüsteemides. Tehismärgalades tuleks luua sobilikud tingimused eelkõige sellistele bioloogilistele protsessidele, mis tagaks maksimaalse lämmastikuärastuse ning millega kaasneks võimalikult väike

dilämmastikoksiidi (N_2O) emissioon. N_2O on süsihappegaasist 298 korda ohtlikum kasvuhoonegaas ja seda peetakse üheks peamiseks osoonikihi hõrenemise põhjustajaks tulevikus. Kuigi looduses on väga mitmeid N_2O allikaid, muundavad vaid vähesed protsessid lämmastikühendid keskkonnale ohutuks molekulaarseks lämmastikuks (N_2) ilma N_2O tekketa. Sellised protsessid on teadaolevalt denitrifikatsiooni viimane etapp, anaeroobne ammooniumi oksüdeerimine (ANAMMOX) ja hiljuti tuvastatud nitritist sõltuv metaani anaeroobne oksüdeerimine (n-damo).

Käesoleva töö eesmärk oli analüüsida ORWRP läbivooluliste tehismärgalade kompleksi muldade ja setete bakterikoosluse struktuuri, hinnata selle lämmastikuärastuse geneetilist potentsiaali ning analüüsida nende näitajate seoseid keskkonnaparameetritega (keemilised parameetrid, veerežiim ja pinnasetüüp).

ORWRP tehismärgalade kompleksi kuuluvad kaks 1994. aastal rajatud 1 ha suurust vabaveelist tehismärgala ja 1996. aastal rajatud 3 ha suurune soot. Kui vabaveelistesse tehismärgaladesse toimub vee pumpamine Olentangy jõest ööpäev ringi vastavalt jõe voolumustrile (keskmiselt 626–1552 m^3 päevas), siis sooti siseneb vesi vaid juhul, kui jõe veetase on kõrgem märgala tasapinnast (7–8 korda aastas). Kunstlikult üleujutatud tehismärgaladesse on rajatud kolm püsivalt üleujutatud ala, mis on ümbritsetud ajutiselt üleujutatud taimestatud aladega. Läänepoolsesse tehismärgalasse istutati 13 erinevat kohaliku piirkonna magevee märgaladele iseloomulikku taimeliiki, kuid idapoolse tehismärgala taimestik on kujunenud looduslikult.

Proovialalt koguti 29 sette- ja mullaproovi 2009. aasta märtsis. Pinnase bakterikoosluse taksonoomilise profiili määramiseks kasutati 16S rRNA V6 hüpervarieeruva piirkonna geenifragmentide sekveneerimist Illumina® HiSeq 2000 platvormil. Bakteriaalse 16S rRNA geeni arvukuste ning denitrifikatsiooni, ANAMMOXi ja n-damo protsesside spetsiifiliste geenikoopiate osakaalude hindamiseks mikroobikoosluses rakendati kvantitatiivset PCR meetodit.

Töö tulemused näitasid, et bakterite arvukust setetes ja muldades mõjutasid nii veerežiim kui ka mullatüüp. Bakterikooslus oli kõige arvukam püsivalt üleujutatud alade orgaanikarikkas settekihis.

Tehismärgalade setete ja mulla bakterikooslused erinesid kaldavalli kooslustest. Kõige arvukamalt oli tehismärgalade pinnase bakterikooslustes esindatud hõimkond *Proteobacteria*, millele järgnesid *Acidobacteria*, *Bacteroidetes*, *Actinobacteria* ja *Verrucomicrobia* hõimkondade esindajad. Kaldavalli bakterikooslustes domineerisid peaaegu võrdselt hõimkondade *Proteobacteria*, *Actinobacteria* ja *Acidobacteria* esindajad.

Veerežiim oli oluline faktor bakterikoosluste struktuuri kujunemisel. Tehismärgalade püsivalt üleujutatud alade setete bakterikoosluste taksonoomiline struktuur erines ajutiselt üleujutatud alade omast. Lisaks olid ajutiselt üleujutatud alade bakterikooslused mitmekesisemad ja ühtlasema liikide jaotusega kui püsivalt üleujutatud alade omad. Arvatavasti loob kõikuv veetase laialdasema keskkonnatingimuste spektri, mis soodustab keerukamate koosluste kujunemist

ja takistab üksikute dominantsete rühmade teket. Soodi püsivalt ja ajutiselt üleujutatud alade muldade bakterikooslused olid sarnasemad kui kunstlikult üleujutatud tehismärgalade vastavate alade kooslused, mis võib olla seotud soodi väiksema hüdraulilise koormuse ja lühema opereerimisajaga.

Veerežiim mõjutas ka suhteid sette- ja mullaproovide bakterikoosluste sees. Ajutiselt üleujutatud tehismärgalade mulla bakterikooslustes ilmnes arvukamalt bakterite vahelisi seoseid kui püsivalt üleujutatud orgaanikarikaste setete ja mineraalsete muldade bakterikooslustes.

Tehismärgalade pinnase bakterikoosluse struktuuri ei mõjutanud pinnase tüüp ega kaugus tehismärgala sissevoolust.

Enam kui pool märgalade mikroobikoosluse struktuuris esinevast varieeruvusest oli seotud pinnaseproovide keemilise koostisega (NH_4-N, Ca, NO_3-N, pH ja C sisaldusega), mis omakorda oli tugevalt mõjutatud teistest keskkonnaparameetritest (pinnase tüüp, veerežiim).

Kõikides uuritud tehismärgalade ja kaldavallide muldades esines denitrifikatsiooni läbiviivaid ensüüme kodeerivaid geene. Suurim nitriti redutseerimise potentsiaal esines püsivalt üleujutatud alade setete bakterikooslustes. Pidevalt üleujutatud alade tingimused olid soodsamad *nirS* geeni omavatele bakteritele, samal ajal kui *nirK* geeni kandvad bakterid olid arvukamalt esindatud kuivematel ja fluktueeriva veetasemega aladel. Sarnaselt kogu bakterikooslusega oli ka nitriti redutseerimise potentsiaali omavate bakterite osakaal kõrgem ülemises, orgaanikarikkas settekihis kui selle all asetsevas mineraalses kihis.

Uuringu tulemused näitasid, et ohtliku kasvuhoonegaasi dilämmastikoksiidi redutseerimise potentsiaal erines tehismärgalakompleksi erinevate piirkondade setete ja muldade bakterikooslustes. Sarnaselt nitriti redutseerimise potentsiaaliga oli ka N_2O redutseerimise potentsiaal mõjutatud asukoha veerežiimist ja kõrgemad *nosZ* geeniga bakterite osakaalud esinesid ajutiselt üleujutatud alade bakterikooslustes. *NosZ* klaad I või II kuuluva geeniga bakterid olid suhteliselt võrdselt esindatud tehismärgalade pinnase mikroobikooslustes. Kuigi nii *nosZI* kui *nosZII* geeniga mikroorganismide osakaalud olid kõrgemad ajutiselt üleujutatud alade muldade bakterikooslustes, olid erinevat tüüpi *nosZ* geeni omavate bakterite osakaalud koosluses mõjutatud erinevalt keskkonna keemiliste parameetrite poolt.

Nitriti redutseerimise potentsiaal ületas dilämmastikoksiidi redutseerimise potentsiaali kõikides tehismärgalade kompleksi sette ja mulla bakterikooslustes, mis viitab denitrifikatsioonist pärineva N_2O emissioonile tehismärgalades. Kõige väiksem denitrifikatsiooniga seotud N_2O emissiooni geneetiline potentsiaal esines ajutiselt üleujutatud muldade bakterikooslustes. Nitriti ja dilämmastikoksiidi reduktaasi kodeerivate geenide osakaalud bakterikooslustes olid mõjutatud erinevalt mulla keemiliste parameetrite poolt.

Perekondade *Thiobacillus*, *Paracoccus* ja *Sulfurimonas* esindajate tuvastamine tehismärgalade setete ja muldade bakterikooslustes osutab sellele, et lisaks heterotroofsetele denitrifitseerijatele võib tehismärgalade pinnases esineda ka autotroofseid denitrifitseerijaid.

Tehismärgalade mulla ja sette bakterikooslustest tuvastati ka ANAMMOXi ja n-damo protsesse läbiviivaid baktereid ning nende osakaalud varieerusid tehismärgalade kompleksi muldade bakterikooslustes. Sarnaselt denitrifitseerijatega olid mõlema bakterigrupi arvukused koosluses mõjutatud veerežiimi poolt. Sobilikumad tingimused ANAMMOXi ja n-damo bakteritele olid püsivalt üleujutatud alade muldade bakterikooslustes (võrreldes üleminekualadega), kuid kõige ebasoodsamad tingimused neid protsesse läbiviivatele bakteritele esinesid aga kaldavallide bakterikooslustes. Lisaks viitasid ANAMMOXi ja n-damo spetsiifiliste bakterite sarnastele keskkonnatingimuste eelistustele ka seosed samade keemiliste parameetritega.

ANAMMOXi bakterite koosluse struktuur oli mitmekesine: viiest teadaolevast ANAMMOXi perekonnast tuvastati tehismärgalade kompleksi pinnasest *Ca. Brocadia*, *Ca. Scalindua* ja *Ca. Kuenenia* perekondadesse kuuluvaid baktereid.

Kuigi tehismärgalade setetes ja muldades on olemas potentsiaal erinevateks lämmastikuärastuse protsessideks, näitavad käesoleva töö tulemused et denitrifikatsioon on peamine seda funktsiooni läbiviiv protsess uuritud märgalade kompleksi pinnase bakterikooslustes. Töö tulemused lubavad järeldada, et ulatuslikumate muutuva veerežiimiga alade loomine võiks vähendada osalisest denitrifikatsioonist tulenevat N_2O emissiooni tehimärgalade setetest ja muldadest.

Lisaks näitavad käesoleva uurimuse tulemused, et lämmastiku eemaldamine tehismärgalade setetes ja muldades toimub väga erinevate protsesside vahendusel ning on tihedalt seotud teiste aineringetega, mis vajavad põhjalikumat uurimist, et rakendada neid teadmisi efektiivsemalt opereerivate tehismärgalade rajamisel.

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APPENDIX

Supplementary Table S1

Average values and standard deviations (in parentheses) of the proportions of the fifteen most abundant families in the studied soil groups. Abbreviations of the soil groups: W_{oo} – organic sediment samples of open areas of W wetlands, Ox_{oo} – soil samples of open area of oxbow, W_{om} – mineral soil samples of open areas of W wetlands, W_{trans} – soil samples of transitional areas of W wetlands, Ox_{trans} – soil samples of transitional area of oxbow, and Up – soil samples of upland areas.

Family	W_{oo} (n=6)	Family	Ox_{oo} (n=3)	Family	W_{om} (n=6)	Family	W_{trans} (n=6)	Family	Ox_{trans} (n=4)	Family	Up (n=4)
Desulfobacteraceae	3.23 (0.63)	*Nocardioidaceae*	3.13 (2.23)	*Hydrogenophilaceae*	5.43 (3.66)	*Syntrophobacteraceae*	3.47 (0.95)	*Nocardioidaceae*	3.95 (3.61)	*Sinobacteraceae*	2.51 (1.15)
Hydrogenophilaceae	2.13 (0.94)	*Syntrophobacteraceae*	2.18 (0.45)	*Comamonadaceae*	3.29 (3.56)	*Thermodesulfo*[a]	2.25 (0.91)	*Solibacteraceae*	1.98 (0.60)	*Hyphomicrobiaceae*	2.47 (0.80)
Syntrophobacteraceae	1.92 (0.26)	*Hydrogenophilaceae*	1.40 (0.32)	*Flavobacteriaceae*	2.43 (1.75)	FW	2.22 (1.52)	*Syntrophobacteraceae*	1.90 (0.52)	*Flavobacteriaceae*	1.86 (1.10)
Rhodocyclaceae	1.37 (0.73)	*Verrucomicrobiaceae*	1.34 (1.01)	*Desulfobacteraceae*	2.25 (0.81)	*Solibacteraceae*	2.22 (0.87)	*Flavobacteriaceae*	1.50 (0.61)	*Syntrophobacteraceae*	1.80 (0.34)
Chromatiaceae	1.13 (0.22)	*Solibacteraceae*	1.15 (0.19)	*Syntrophobacteraceae*	1.35 (0.46)	*Haliangiaceae*	1.30 (0.39)	*Gemmatimonadaceae*	1.21 (0.71)	*Nocardioidaceae*	1.77 (1.13)
Flavobacteriaceae	1.02 (0.55)	*Flavobacteriaceae*	1.10 (0.43)	*Solibacteraceae*	1.14 (0.40)	*Rhodospirillaceae*	1.13 (0.32)	*Sphingomonadaceae*	1.17 (0.27)	*Xanthomonadaceae*	1.65 (0.56)
Syntrophaceae	0.96 (0.17)	*Xanthomonadaceae*	1.07 (0.58)	*Thermodesulfo*[a]	1.08 (0.39)	*Sinobacteraceae*	1.13 (0.41)	*Hyphomicrobiaceae*	1.14 (0.39)	*Solibacteraceae*	1.35 (0.76)
Desulfobulbaceae	0.80 (0.09)	*Desulfobacteraceae*	1.03 (0.23)	*Helicobacteraceae*	0.98 (1.15)	*Hyphomicrobiaceae*	1.08 (0.37)	*Haliangiaceae*	1.06 (0.29)	*Spartobacteriaceae*	1.32 (0.66)
Verrucomicrobiaceae	0.74 (0.16)	*Flexibacteraceae*	0.89 (0.11)	*Syntrophaceae*	0.91 (0.20)	*Flavobacteriaceae*	0.96 (0.26)	*Sinobacteraceae*	0.99 (0.64)	*Micromonosporaceae*	1.30 (0.44)
Ignavibacteriaceae	0.70 (0.08)	*Burkholderiaceae*	0.83 (0.33)	*Chromatiaceae*	0.89 (0.60)	*Nitrospiraceae*	0.92 (0.48)	*Flexibacteraceae*	0.97 (0.43)	*Flexibacteraceae*	1.26 (0.59)
Solibacteraceae	0.64 (0.03)	*Haliangiaceae*	0.75 (0.18)	*Spirochaetaceae*	0.87 (0.40)	*Geobacteraceae*	0.83 (0.28)	*Xanthomonadaceae*	0.86 (0.38)	*Rhodospirillaceae*	1.06 (0.23)
Myxococcaceae	0.62 (0.23)	*Sphingomonadaceae*	0.72 (0.17)	*Rhodocyclaceae*	0.77 (0.50)	PRR-10	0.66 (0.27)	FW	0.86 (0.70)	*Microbacteriaceae*	1.05 (0.50)
Burkholderiaceae	0.59 (0.32)	*Thermodesulfo*[a]	0.70 (0.24)	*Nocardioidaceae*	0.75 (1.30)	*Syntrophaceae*	0.63 (0.25)	*Rhodospirillaceae*	0.85 (0.39)	*Sphingomonadaceae*	1.00 (0.53)
Thermodesulfo[a]	0.54 (0.16)	FW	0.69 (0.51)	*Desulfobulbaceae*	0.71 (0.26)	*Gemmatimonadaceae*	0.62 (0.48)	*Thermodesulfo*[a]	0.70 (0.72)	*Mycobacteriaceae*	0.85 (0.13)
Flexibacteraceae	0.52 (0.11)	*Myxococcaceae*	0.67 (0.39)	FW	0.66 (0.34)	*Desulfobacteraceae*	0.60 (0.43)	*Micromonosporaceae*	0.67 (0.46)	*Haliangiaceae*	0.79 (0.44)

[a] – *Thermodesulfovibrionaceae*

Supplementary Table S2

Average values and standard deviations (in parentheses) of the proportions of the fifteen most abundant genera in the studied soil groups. Abbreviations of the soil groups: W_{oo} – organic sediment samples of open areas of W wetlands, Ox_{oo} – soil samples of open area of oxbow, W_{om} – mineral soil samples of open areas of W wetlands, W_{trans} – soil samples of transitional areas of W wetlands, Ox_{trans} – soil samples of transitional area of oxbow, and Up – soil samples of upland areas.

Genus	**W_{oo}** (n=6)	**Genus**	**Ox_{oo}** (n=3)	**Genus**	**W_{om}** (n=6)	**Genus**	**W_{trans}** (n=6)	**Genus**	**Ox_{trans}** (n=4)	**Genus**	**Up** (n=4)
Desulfobacterium	2.87 (0.61)	*Thiobacillus*	1.20 (0.26)	*Thiobacillus*	5.23 (3.65)	*Ca. Solibacter*	2.22 (0.87)	*Ca. Solibacter*	1.98 (0.60)	*Flavobacterium*	1.72 (1.04)
Thiobacillus	1.94 (0.93)	*Ca. Solibacter*	1.15 (0.19)	*Polaromonas*	2.67 (2.95)	4-29	1.91 (1.23)	*Flavobacterium*	1.39 (0.57)	*Ca. Solibacter*	1.35 (0.76)
Ca. Solibacter	0.64 (0.03)	*Luteolibacter*	0.91 (0.65)	*Flavobacterium*	1.98 (1.61)	GOUTA19	1.06 (0.70)	*Kaistobacter*	0.88 (0.23)	*Rhodoplanes*	1.06 (0.14)
Anaeromyxobacter	0.62 (0.23)	*Flavobacterium*	0.89 (0.32)	*Desulfobacterium*	1.90 (0.74)	*Nitrospira*	0.92 (0.48)	*Cytophaga*	0.83 (0.42)	*Cytophaga*	0.98 (0.56)
Cupriavidus	0.58 (0.32)	*Desulfobacterium*	0.83 (0.18)	*Ca. Solibacter*	1.14 (0.40)	*Flavobacterium*	0.79 (0.25)	4-29	0.73 (0.54)	*Mycobacterium*	0.85 (0.13)
Flavobacterium	0.53 (0.34)	*Cupriavidus*	0.81 (0.32)	*Sulfuricurvum*	0.98 (1.15)	*Geobacter*	0.78 (0.24)	*Thiobacillus*	0.56 (0.80)	*Pedomicrobium*	0.80 (0.49)
Luteolibacter	0.49 (0.20)	*Cytophaga*	0.80 (0.12)	TM3	0.73 (0.44)	*Rhodoplanes*	0.55 (0.19)	*Gemmatimonas*	0.54 (0.29)	*Kaistobacter*	0.77 (0.47)
Cytophaga	0.48 (0.14)	4-29	0.69 (0.51)	4-29	0.65 (0.34)	*Thiobacillus*	0.53 (0.31)	*Rhodoplanes*	0.54 (0.25)	*Chthoniobacter*	0.57 (0.27)
Geobacter	0.31 (0.10)	*Anaeromyxobacter*	0.66 (0.39)	GOUTA19	0.53 (0.34)	*Cytophaga*	0.45 (0.21)	*Geobacter*	0.51 (0.39)	*Nitrospira*	0.48 (0.20)
Desulfobacca	0.29 (0.04)	*Kaistobacter*	0.59 (0.12)	*Desulfosporosinus*	0.43 (0.46)	*Desulfobacterium*	0.45 (0.35)	*Nitrospira*	0.38 (0.14)	MC18	0.46 (0.33)
Thiocapsa	0.29 (0.07)	*Geobacter*	0.47 (0.31)	*Algoriphagus*	0.41 (0.35)	*Desulfobacca*	0.41 (0.17)	GOUTA19	0.37 (0.38)	*Pseudomonas*	0.38 (0.23)
TM3	0.28 (0.13)	*Nitrospira*	0.46 (0.23)	*Cupriavidus*	0.36 (0.17)	*Anaeromyxobacter*	0.35 (0.26)	TM3	0.33 (0.48)	*Ca. Entotheonella*	0.37 (0.21)
Algoriphagus	0.24 (0.15)	GOUTA19	0.35 (0.23)	*Luteolibacter*	0.35 (0.31)	TM3	0.29 (0.18)	*Desulfobacterium*	0.30 (0.36)	*Bradyrhizobium*	0.23 (0.11)
GOUTA19	0.23 (0.09)	TM3	0.24 (0.17)	*Cytophaga*	0.28 (0.15)	*Gemmatimonas*	0.27 (0.14)	*Chthoniobacter*	0.25 (0.15)	*Streptomyces*	0.22 (0.04)
Nitrospira	0.20 (0.05)	*Desulfobacca*	0.24 (0.11)	*Clostridium*	0.27 (0.18)	*Kaistobacter*	0.21 (0.10)	*Luteolibacter*	0.25 (0.19)	*Kribbella*	0.22 (0.19)

Supplementary Table S3
Taxonomic classification of OTUs comprised in the molecular ecological network of various studied soil groups. Abbreviations of the soil groups: W_{oo} – organic sediment samples of open areas of W wetlands, W_{om} – mineral soil samples of open areas of W wetlands, W_{trans} – soil samples of transitional areas of W wetlands, and Ox – soil samples of oxbow.

Soil group	Module	OTU	Phylum	Class	Order	Family	Genus
W_{oo} (n=6)	W_{oo}A	43	*Proteobacteria*	*Deltaproteobacteria*	*Myxococcales*	*Myxococcaceae*	*Anaeromyxobacter*
		44	*Proteobacteria*	*Betaproteobacteria*	unclassified	unclassified	unclassified
		118	*Proteobacteria*	*Gammaproteobacteria*	*Methylococcales*	unclassified	unclassified
		320	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	*Cupriavidus*
		808	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	unclassified	unclassified
		1076	*Proteobacteria*	*Gammaproteobacteria*	*Chromatiales*	unclassified	unclassified
		1110	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	unclassified	unclassified
	W_{oo}B	98	*Verrucomicrobia*	*Verrucomicrobiae*	*Verrucomicrobiales*	*Verrucomicrobia*[a]	unclassified
		247	*Proteobacteria*	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	unclassified
		326	*Proteobacteria*	*Deltaproteobacteria*	*Desulfuromonadales*	unclassified	unclassified
		423	*Acidobacteria*	*Chloracidobacteria*	unclassified	unclassified	unclassified
		439	*Cyanobacteria*	*Chloroplast*	*Stramenopiles*	unclassified	unclassified
		875	*Nitrospirae*	*Nitrospira*	*Nitrospirales*	*Nitrospiraceae*	*Nitrospira*
		995	*Verrucomicrobia*	unclassified	unclassified	unclassified	unclassified
	W_{oo}1	56	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*	unclassified	unclassified
		110	*Proteobacteria*	*Gammaproteobacteria*	*Chromatiales*	unclassified	unclassified
		285	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	unclassified	unclassified
		396	WS3	PRR-12	Sediment-1	PRR-10	unclassified
	W_{oo}2	47	*Proteobacteria*	*Betaproteobacteria*	*Hydrogenophilales*	*Hydrogenophilaceae*	*Thiobacillus*
		568	*Acidobacteria*	*Chloracidobacteria*	unclassified	unclassified	unclassified
W_{om} (n=6)	W_{om}C	58	*Bacteroidetes*	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Flavobacterium*
		65	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Polaromonas*
		71	*Proteobacteria*	*Epsilonproteobacteria*	*Campylobacterales*	*Helicobacteraceae*	*Sulfuricurvum*

Soil group	Module	OTU	Phylum	Class	Order	Family	Genus
		110	*Proteobacteria*	*Gammaproteobacteria*	*Chromatiales*	unclassified	unclassified
		268	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	*Cyclobacteriaceae*	*Algoriphagus*
		421	*Proteobacteria*	*Epsilonproteobacteria*	*Campylobacterales*	*Helicobacteraceae*	*Sulfuricurvum*
		1089	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	unclassified
	$W_{om}D$	56	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*	unclassified	unclassified
		111	*Nitrospirae*	*Nitrospira*	*Nitrospirales*	*Thermodesulfo*[b]	GOUTA19
		184	*Proteobacteria*	*Gammaproteobacteria*	unclassified	unclassified	unclassified
		418	*Spirochaetes*	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	TM3
		568	*Acidobacteria*	*Chloracidobacteria*	unclassified	unclassified	unclassified
		875	*Nitrospirae*	*Nitrospira*	*Nitrospirales*	*Nitrospiraceae*	*Nitrospira*
		894	*Acidobacteria*	unclassified	unclassified	unclassified	unclassified
	$W_{om}E$	60	*Nitrospirae*	*Nitrospira*	*Nitrospirales*	FW	4-29
		118	*Proteobacteria*	*Gammaproteobacteria*	*Methylococcales*	unclassified	unclassified
		209	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Nocardioidaceae*	unclassified
		217	*Firmicutes*	*Bacilli*	*Bacillales*	unclassified	unclassified
		483	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	unclassified	unclassified
	$W_{om}1$	63	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*	unclassified	unclassified
		285	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	unclassified	unclassified
		537	*Proteobacteria*	*Betaproteobacteria*	unclassified	unclassified	unclassified
		1143	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	unclassified	unclassified
W_{trans} (n=6)	$W_{trans}F$	4	*Acidobacteria*	*Acidobacteria*	*Acidobacteriales*	unclassified	unclassified
		58	*Bacteroidetes*	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Flavobacterium*
		62	*Acidobacteria*	*Chloracidobacteria*	unclassified	unclassified	unclassified
		66	*Acidobacteria*	*Acidobacteria*	*Acidobacteriales*	unclassified	unclassified
		140	*Acidobacteria*	*Chloracidobacteria*	unclassified	unclassified	unclassified
		154	*Actinobacteria*	*Actinobacteria*	*Acidimicrobiales*	unclassified	unclassified
		215	*Proteobacteria*	*Gammaproteobacteria*	*Chromatiales*	*Sinobacteraceae*	unclassified

Soil group	Module	OTU	Phylum	Class	Order	Family	Genus
		547	*Acidobacteria*	*Chloracidobacteria*	unclassified	unclassified	unclassified
		612	*Acidobacteria*	iii1-8	32-20	unclassified	unclassified
		1047	*Nitrospirae*	*Nitrospira*	*Nitrospirales*	FW	4-29
	$W_{trans}G$	2	*Gemmatimonadetes*	unclassified	unclassified	unclassified	unclassified
		47	*Proteobacteria*	*Betaproteobacteria*	*Hydrogenophilales*	*Hydrogenophilaceae*	*Thiobacillus*
		63	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*	unclassified	unclassified
		95	*Chloroflexi*	SOGA31	unclassified	unclassified	unclassified
		184	*Proteobacteria*	*Gammaproteobacteria*	unclassified	unclassified	unclassified
		321	*Acidobacteria*	*Acidobacteria*	*Acidobacteriales*	unclassified	unclassified
		402	NC10	unclassified	unclassified	unclassified	unclassified
		454	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	unclassified	unclassified
		483	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	unclassified	unclassified
		535	*Chloroflexi*	SOGA31	unclassified	unclassified	unclassified
		740	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	unclassified	unclassified
		745	*Verrucomicrobia*	*Verrucomicrobiae*	*Verrucomicrobiales*	unclassified	unclassified
		1089	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	unclassified
		1156	*Nitrospirae*	*Nitrospira*	*Nitrospirales*	FW	unclassified
		1365	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	unclassified	unclassified
	$W_{trans}1$	77	*Verrucomicrobia*	unclassified	unclassified	unclassified	unclassified
		110	*Proteobacteria*	*Gammaproteobacteria*	*Chromatiales*	unclassified	unclassified
		384	*Acidobacteria*	RB25	unclassified	unclassified	unclassified
		875	*Nitrospirae*	*Nitrospira*	*Nitrospirales*	*Nitrospiraceae*	*Nitrospira*
	$W_{trans}2$	44	*Proteobacteria*	*Betaproteobacteria*	unclassified	unclassified	unclassified
		96	*Proteobacteria*	*Deltaproteobacteria*	*Myxococcales*	unclassified	unclassified
		347	*Verrucomicrobia*	*Verrucomicrobiae*	*Verrucomicrobiales*	unclassified	unclassified
Ox (n=7)	OxH	29	*Acidobacteria*	*Acidobacteria*	*Acidobacteriales*	unclassified	unclassified
		44	*Proteobacteria*	*Betaproteobacteria*	unclassified	unclassified	unclassified

Soil group	Module	OTU	Phylum	Class	Order	Family	Genus
		95	*Chloroflexi*	SOGA31	unclassified	unclassified	unclassified
		155	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	unclassified	unclassified
		179	*Verrucomicrobia*	*Verrucomicrobiae*	*Verrucomicrobiales*	*Verrucomicrobiaceae*	*Luteolibacter*
		209	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Nocardioidaceae*	unclassified
		225	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	unclassified	unclassified
		315	*Gemmatimonadetes*	*Gemmatimonadetes*	unclassified	unclassified	unclassified
		326	*Proteobacteria*	*Deltaproteobacteria*	*Desulfuromonadales*	unclassified	unclassified
		439	*Cyanobacteria*	*Chloroplast*	*Stramenopiles*	unclassified	unclassified
		464	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	unclassified	unclassified
		634	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Kaistobacter*
		664	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	unclassified	unclassified
		799	*Acidobacteria*	*Acidobacteria*	*Acidobacteriales*	unclassified	unclassified
		1003	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	*Flexibacteraceae*	*Cytophaga*
	OxI	43	*Proteobacteria*	*Deltaproteobacteria*	*Myxococcales*	*Myxococcaceae*	*Anaeromyxobacter*
		58	*Bacteroidetes*	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Flavobacterium*
		60	*Nitrospirae*	*Nitrospira*	*Nitrospirales*	FW	4-29
		111	*Nitrospirae*	*Nitrospira*	*Nitrospirales*	*Thermodesulfo*[b]	GOUTA19
		154	*Actinobacteria*	*Actinobacteria*	*Acidimicrobiales*	unclassified	unclassified
		200	*Cyanobacteria*	*Oscillatoriophycideae*	unclassified	unclassified	unclassified
		285	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	unclassified	unclassified
		322	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	unclassified	unclassified
		454	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	unclassified	unclassified
		573	*Proteobacteria*	*Betaproteobacteria*	unclassified	unclassified	unclassified
		651	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	unclassified	unclassified
		745	*Verrucomicrobia*	*Verrucomicrobiae*	*Verrucomicrobiales*	unclassified	unclassified
		922	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophobacteraceae*	unclassified
		1047	*Nitrospirae*	*Nitrospira*	*Nitrospirales*	FW	4-29

Soil group	Module	OTU	Phylum	Class	Order	Family	Genus
		1236	*Proteobacteria*	*Gammaproteobacteria*	unclassified	unclassified	unclassified
	OxJ	2	*Gemmatimonadetes*	unclassified	unclassified	unclassified	unclassified
		4	*Acidobacteria*	*Acidobacteria*	*Acidobacteriales*	unclassified	unclassified
		62	*Acidobacteria*	*Chloracidobacteria*	unclassified	unclassified	unclassified
		66	*Acidobacteria*	*Acidobacteria*	*Acidobacteriales*	unclassified	unclassified
		146	*Acidobacteria*	*Acidobacteria*	*Acidobacteriales*	unclassified	unclassified
		320	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	*Cupriavidus*
		396	WS3	PRR-12	Sediment-1	PRR-10	unclassified
		432	*Proteobacteria*	*Gammaproteobacteria*	*Legionellales*	*Coxiellaceae*	unclassified
		483	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	unclassified	unclassified
		612	*Acidobacteria*	iii1-8	32-20	unclassified	unclassified
		674	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	unclassified	unclassified
		875	*Nitrospirae*	*Nitrospira*	*Nitrospirales*	*Nitrospiraceae*	*Nitrospira*
		1415	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	unclassified	unclassified
	Ox1	98	*Verrucomicrobia*	*Verrucomicrobiae*	*Verrucomicrobiales*	*Verrucomicrobia*[a]	unclassified
		184	*Proteobacteria*	*Gammaproteobacteria*	unclassified	unclassified	unclassified
		347	*Verrucomicrobia*	*Verrucomicrobiae*	*Verrucomicrobiales*	unclassified	unclassified
		535	*Chloroflexi*	SOGA31	unclassified	unclassified	unclassified
	Ox2	110	*Proteobacteria*	*Gammaproteobacteria*	*Chromatiales*	unclassified	unclassified
		568	*Acidobacteria*	*Chloracidobacteria*	unclassified	unclassified	unclassified

[a] – *Verrucomicrobiasubdivision3*
[b] – *Thermodesulfovibrionaceae*

Supplementary Table S4
Average values and standard deviations (in parentheses) of bacterial 16S rRNA gene abundances in the studied soil groups according to the two different 16S rRNA gene-specific primer pairs. The abundance of 16S rRNA genes are calculated using the method described in Papers I (785FL/919R) and II (L-V6/R-V6). Abbreviations of the soil groups: W_{oo} – organic sediment samples of open areas of W wetlands, Ox_{oo} – soil samples of open area of oxbow, W_{om} – mineral soil samples of open areas of W wetlands, W_{trans} – soil samples of transitional areas of W wetlands, Ox_{trans} – soil samples of transitional area of oxbow, and Up – soil samples of upland areas.

Soil group	**16S rRNA (copies/g dw)**	
	785FL/919R	**L-V6/R-V6**
W_{oo} (n=6)	$7.41 * 10^9$ ($3.41 * 10^9$)	$1.18 * 10^{10}$ ($3.07 * 10^9$)
Ox_{oo} (n=3)	$4.51 * 10^9$ ($1.47 * 10^9$)	$8.69 * 10^9$ ($2.22 * 10^9$)
W_{om} (n=6)	$1.15 * 10^9$ ($8.88 * 10^8$)	$3.26 * 10^9$ ($2.23 * 10^9$)
W_{trans} (n=6)	$4.44 * 10^9$ ($2.02 * 10^9$)	$7.91 * 10^9$ ($3.86 * 10^9$)
Ox_{trans} (n=4)	$2.61 * 10^9$ ($9.24 * 10^8$)	$5.81 * 10^9$ ($2.31 * 10^9$)
Up (n=4)	$1.68 * 10^9$ ($1.25 * 10^9$)	$4.64 * 10^9$ ($3.10 * 10^9$)

Supplementary Table S5

Statistically significant correlations between gene parameter values and soil chemical characteristics in different soil groups according to the Spearman rank order correlations analysis. Abbreviations of the soil groups: W_{oo} – organic sediment samples of open areas of W wetlands, W_{om} – mineral soil samples of open areas of W wetlands; W_{trans} – soil samples of transitional areas of W wetlands, and Ox – soil samples of oxbow.

Soil group	Gene parameter value	Chemical variable									
		pH	NH_4-N (mg/kg)	NO_3-N (mg/kg)	C (%)	N (%)	C/N	P (mg/kg)	Ca (mg/kg)	K (mg/kg)	Mg (mg/kg)
W_{oo} (n=6)	16S rRNA	–0.89*				0.89*					
	nirK/nirS		–0.89*				0.94**				
	nosZI%	–0.89*			0.83*	0.89*				0.83*	
	nosZII%		–0.83*								
	nosZI/nosZII							0.89*			
	nosZ/nir				0.83*					0.83*	
	amx%							0.89*			
	ndamo%							0.86*			
W_{om} (n=6)	*nirK*%								0.94***	0.83*	0.83*
	amx%	0.83*									
W_{trans} (n=6)	16S rRNA										0.83*
	nirK%			0.89*							
	nirK/nirS										–0.83*
	nosZ%										–0.83*
	nosZ/nir										–0.83*
Ox (n=7)	*nirS*%	0.89**					–0.79*	0.86*	0.79*		
	nir%	0.86*						0.79*			
	nirK/nirS	–0.79*						–0.92**			
	nosZI%	–0.93**							–0.93**		
	nosZ%						0.89**	–0.93**	–0.96***		
	nosZI/nosZII	–0.79*		–0.79*							
	nosZ/nir	–0.86*					0.89**	0.93**	–0.86*		

* – $p < 0.05$
** – $p < 0.01$
*** – $p < 0.001$

Supplementary Table S6
Average proportions and standard deviations (in parentheses) of ANAMMOX and n-damo specific 16S rRNA sequences in the bacterial communities of the studied soil groups. Abbreviations of the soil groups: W_{oo} – organic sediment samples of open areas of W wetlands, Ox_{oo} – soil samples of open area of oxbow, W_{om} – mineral soil samples of open areas of W wetlands, W_{trans} – soil samples of transitional areas of W wetlands, Ox_{trans} – soil samples of transitional area of oxbow, and Up – soil samples of upland areas.

Soil group	Samx%	Sndamo%
W_{oo} (n = 6)	0.0042 (0.0034)	0.1020 (0.0473)
Ox_{oo} (n = 3)	0.0029 (0.0018)	0.0762 (0.0137)
W_{om} (n = 6)	0.0038 (0.0022)	0.1898 (0.0791)
W_{trans} (n = 6)	0.0394 (0.0319)	0.0718 (0.0060)
Ox_{trans} (n = 4)	0.0547 (0.0795)	0.0595 (0.0448)
Up (n = 4)	0.0016 (0.0025)	0.0013 (0.0006)

PUBLICATIONS

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Other publications:

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