Microbial nitrogen transformations in constructed wetlands treating contaminated groundwater

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by

Master of Science

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Mikrobielle Stickstoffumsetzungen in Pflanzenkläranlagen zur Behandlung von kontaminiertem Grundwasser

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Declaration

I hereby declare that this thesis has not been previously published or written by another person; neither has it been submitted nor accepted for any other academic award. It is the result of my original work carried out at Brandenburg University of Technology Cottbus, Germany, within the framework of the Ph.D. Programme "Environmental and Resource Management". All materials from other sources have been duly and adequately acknowledged.

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Summary

Groundwater is the main source for potable water and domestic use in numerous countries around the world. However, water quality can be affected by pollution, which influences the natural environment and human health. One of the widespread pollutants in water is ammonia which is toxic to fish and causes eutrophication of lakes and wetlands. Constructed wetlands are promising *in situ* water treatment methods thanks to enhanced microbial growth within the plants' rhizospheres, which creates an effective contaminant degradation zone. The ammonia in constructed wetlands can be removed either via total nitrification with further denitrification or partial nitrification coupled with anaerobic ammonium oxidation (anammox). However, so far, the role of anammox in constructed wetlands as well as its correlation with other nitrogen transformations remains unclear. The quantification of nitrogen turnover processes in constructed wetlands is difficult due to the complexity of the wetland systems.

Accordingly, the main aim of this research is to investigate pathways of ammonium removal in constructed wetlands treating contaminated groundwater. For this, several approaches were applied: a) physico-chemical parameters measurements; b) investigations of nitrogen stable isotope fractionation; c) stable isotope labelling approach; d) molecular biological methods. Also, seasonal and spatial variations in nitrogen transformations in several types of constructed wetlands (unplanted horizontal subsurface flow, planted horizontal subsurface flow, and floating plant root mat) were investigated.

The application of the stable isotope approach combined with common physico-chemical investigations enabled us to identify key factors influencing efficiency of nitrogen removal in constructed wetlands, which was the plant presence as substrate for attachment and growth of microorganisms. Ammonium removal efficiencies were not different between investigated seasons (spring, summer, and autumn), what could be explained by the fact that throughout all investigated seasons the air temperature remained high (above 10°C). While plant uptake accounted for significant part of ammonium removal during spring and summer in planted constructed wetlands, isotope fractionation patterns revealed that nitrification-denitrification were prevailing processes in planted constructed wetlands throughout the year, occurring in a linear way along the flow path, and not depending on depth in the root zone.

The research results also illustrated that in the planted horizontal subsurface flow constructed wetland, the functional genes of the nitrogen cycle were evenly distributed in a linear way along the flow path with prevalence at the superficial points. The same trend was observed for the nitrification and denitrification turnover rates using the isotope labeling techniques. Significant nitrate consumption under aerobic conditions diminishes nitrification rates and should therefore be taken into account when estimating nitrification turnover rates. This nitrate consumption was due to aerobic denitrification, the rate of which was comparable to that for anaerobic denitrification. Consequently, denitrification should not be considered as an exclusively anaerobic process. Phylogenetic analysis of hydrazine synthase (*hzsA*) gene clones indicated the presence of *Brocadia* and *Kuenenia* anammox species in the constructed wetland. Although anammox bacteria were detected by molecular methods, anammox

activity could not be measured and hence this process appears to be of low importance in nitrogen transformations in these freshwater ecosystems.

In conclusion, this research demonstrated that combination of physico-chemical measurements with stable isotope and molecular biological approaches is an effective tool for investigation of nitrogen transforming processes in constructed wetlands. Such information is not only valuable for understanding of the processes ongoing inside these wastewater treatment facilities but also necessary for further technological improvement of constructed wetlands.

Keywords: constructed wetlands; nitrogen; nitrification; denitrification; anammox.

Zusammenfassung

Grundwasser ist die wichtigste Quelle für Trink- und Brauchwasser in zahlreichen Ländern auf der ganzen Welt. Allerdings kann die Wasserqualität durch Verunreinigungen beeinträchtigt sein, die Umwelt und Gesundheit negativ beeinflussen. Ein weit verbreiteter Schadstoff im Wasser ist Ammonium, welches toxisch für Fische ist und die Eutrophierung von Seen und Feuchtgebieten bewirken kann. Pflanzenkläranlagen stellen ein effizientes *in situ* Wasserreinigungssverfahren dar. Das liegt insbesondere am beschleunigten mikrobiellen Wachstums innerhalb der Rhizosphäre der Pflanzen, die eine effektive Schadstoffabbauzone bildet. Das Ammonium kann in Pflanzenkläranlagen entweder über völlige Nitrifikation mit weiterer Denitrifikation oder teilweise Nitrifikation verbunden mit anaerober Ammoniumoxidation (Anammox) entfernt werden. Bislang ist die Rolle von Anammox in Pflanzenkläranlagen sowie deren Korrelation mit anderen Stickstoffumsetzungen noch nicht umfänglich geklärt. Die Quantifizierung der Stickstoffumsetzungsprozesse in Pflanzenkläranlagen ist eine Herausforderung aufgrund der biogeochemischen und hydrodynamischen Komplexität solcher Systeme.

Dementsprechend ist das Hauptziel dieser Arbeit, die Wege der Ammoniumentfernung in Pflanzenkläranlagen zur Behandlung von kontaminiertem Grundwasser zu untersuchen. Hierzu wurden verschiedene Ansätze angewendet: a) Messungen von physikalisch-chemischen Parametern; b) Untersuchungen der Fraktionierung von stabilen N-Isotopen im natürlichen Häufigkeitsbereich; c) Markierungsexperimente mit angereicherten stabilen Isotopensubstanzen; d) molekularbiologische Methoden. Außerdem wurden saisonale und räumliche Variationen der Stickstoffumsetzungen in verschiedenen Typen von Pflanzenkläranlagen (nicht bepflanzte horizontale Untergrundströmung, bepflanzte horizontale Untergrundströmung, und schwimmende Pflanzenwurzelmatte) untersucht.

Die Anwendung des stabilen Isotopen-Ansatzes verbunden mit physikalisch-chemischen Untersuchungen gibt uns die Möglichkeit, die wichtigsten Einflussfaktoren für die Effizienz der Stickstoffentfernung in Pflanzenkläranlagen zu identifizieren. Es konnte gezeigt werden, dass der Pflanzenbewuchs und die damit verbundene Wurzelzone der optimaler Lebensraum für die beim Schadstoffabbau aktiven Mikroorganismen war. Ammonium-Abbauraten zeigen keine saisonale Abhängigkeit (Frühling, Sommer, und Herbst), was der Tatsache geschuldet war, dass in allen untersuchten Jahreszeiten die Lufttemperatur über 10°C lag. Im Frühjahr und Sommer entfielen ein erheblicher Teil der Ammoniumentfernung auf die Nährstoffaufnahme durch die Pflanzen in den Pflanzenkläranlagen. Dennoch zeigten Isotopensignaturen, dass die Nitrifikation-Denitrifikation der vorherrschenden Prozess zur Ammoniumentfernung in Pflanzenkläranlagen über das gesamte Jahr ist, wobei ein linearer Zusammenhang zwischen Strömungsweg und Abbau zu beobachten ist. Kein Zusammenhang wurde zwischen Abbau und Tiefe der Wurzelzone gefunden.

Die Untersuchungen haben weiterhin gezeigt, dass in bepflanzten horizontalen unterirdischen Strömungspflanzenkläranlagen die funktionellen Gene des Stickstoffkreislaufs gleichmäßig entlang des Strömungspfades verteilt sind. Allerdings lässt sich diesbezüglich eine vertikale Zonierung erkennen mit dem bevorzugten Auftreten an oberflächennahen Messstellen. Der

gleiche Trend wurde für die Umsatzraten von Nitrifikation und Denitrifikation mit Hilfe der Isotopenmarkierungstechniken beobachtet. Signifikanter Nitratverbrauch unter aeroben Bedingungen vermindert Nitrifikationsraten und muss daher unbedingt bei der Schätzung von Nitrifikationsraten berücksichtigt werden. Dieser Nitratverbrauch stand im Zusammenhang mit aerober Denitrifikation, deren Rate vergleichbar war mit jener für die anaerobe Denitrifikation. Folglich sollte Denitrifikation nicht als ausschließlich anaerober Prozess betrachtet werden. Die phylogenetische Analyse von Hydrazin-Synthase (*hzsA*) Gen-Klonen zeigte die Anwesenheit von *Brocadia* und *Kuenenia* Anammox-Arten in der Pflanzenkläranlage. Obwohl Anammoxbakterien durch molekulare Methoden nachgewiesen wurden, konnte der eigentliche Anammoxprozess nicht gemessen werden. Das lässt den Schluss zu, dass Anammox von geringer Bedeutung für die Stickstoffumsetzung in diesen Süßwasser-Ökosystemen ist.

Insgesamt haben die Untersuchungen gezeigt, dass die Kombination von physikalisch-chemischen Messungen mit stabilen Isotopen und molekularbiologische Ansätzen ein effektives Werkzeug für die Untersuchung der Stickstoffumsetzungsprozesse in Pflanzenkläranlagen ist. Solche Informationen sind nicht nur für das Verständnis der Prozesse wertvoll, die in Abwasserbehandlungsanlagen ablaufen, sondern auch notwendig für die weiteren technologischen Verbesserungen von Pflanzenkläranlagen.

Schlagwörter: Pflanzenkläranlagen, Stickstoff, Nitrifikation, Denitrifikation, Anammox.

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List of Abbreviations and Acronyms

Anammox	Anaerobic Ammonium Oxidation
BOD	Biochemical Oxygen Demand
BOD_5	The Five-Day Biochemical Oxygen Demand
BTEX	Benzene, Toluene, Ethylbenzene, Xylene
COD	Chemical Oxygen Demand
CW	Constructed Wetland
DNRA	Dissimilatory Nitrate Reduction
EA	Elemental Analyzer
Eh	Redox potential
FPRM	Floating Plant Root Mat
GC-FID	Gas Chromatography-Flame Ionization Detector
GCMS	Gas Chromatography-Mass Spectrometry
HSSF	Horizontal Subsurface-Flow
IRMS	Isotope Ratio Mass Spectrometer
MTBE	Methyl *tert*-Butyl Ether
PRMF	Plant Root Mat Filter
qPCR	Quantitative Polymerase Chain Reaction
SF	Surface Flow
SSF	Subsurface Flow
TN	Total Nitrogen
TOC	Total Organic Carbon
TP	Total Phosphorus
TSS	Total Suspended Solids
VSSF	Vertical Subsurface-Flow

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1. Introduction

1.1 Impacts of nitrogen on the environment and importance of nitrogen removal on wastewater and groundwater treatment

From 1860 to 1960, sharply since 1960, there have been increased releases of nitrogen (N) into the environment as a result of increased fertilizer usage, fixation by crops, mineralization of animal manure, and atmospheric deposition, along with miscellaneous other sources (Galloway et al. 2003, Puckett 1995). In European countries approximately 18 % of fertilizer N ends up in wastewater in the form of ammonium (NH_4^+) or organic N (Mulder 2003). N contamination is of interest because of potential health effects in drinking water (Ward et al. 2005), nutrient enrichment of terrestrial (Galloway et al. 2003, Vitousek et al. 1997) and aquatic ecosystems (Rabalais 2002), and contributions to global warming (Galloway et al. 2003, Groffman et al. 2000). Anthropogenic N loading causes eutrophication which has substantial effects on ecosystem function and composition in estuaries. It can cause anoxia (no oxygen) or hypoxia (low oxygen) in stratified waters, and both anoxia and hypoxia appear to be becoming more prevalent in many estuaries and coastal seas (Harrison 1996).

Freshwater comprises 2.5 % of global water, and 29.9 % of freshwater is preserved as groundwater. One of the most common worldwide groundwater pollutants is nitrate (NO_3^-), which can cause detrimental effects on infant health. The World Health Organization recommends that drinking water should not contain more than 50 mg L^{-1} of NO_3^- (or 10 mg L^{-1} NO_3^--N), based on its potential to cause methaemoglobinaemia (WHO 2003). Epidemiological studies have linked exposure to NO_3^- at concentrations well below Environmental Protection Agency and World Health Organization standards to several cancers and negative birth outcomes (Ward et al. 2005). Especially in agricultural regions, dissolved NO_3^- is one of the most common contaminants that pose risk to diminish drinking water resources. In addition to contributing to impacts on water quality, the excess of NO_3^- adds uncertainty to estimates of nitrous oxide (N_2O) emissions, an important greenhouse gas (Groffman et al. 2000).

Another widespread pollutant in water is ammonia (NH_3), which is toxic to all vertebrates causing convulsions, coma and death (Randall & Tsui 2002). Under high pH NH_4^+ is turned into NH_3, which is one of the major toxic compounds as well as a critical long-term pollutant in marine environments (Ip et al. 2001), surface (Havens et al. 2001, Mangimbulude et al. 2012), and groundwater (Siljeg et al. 2010). NH_4^+ deposited to terrestrial and aquatic systems can be a further source of acidity, in that both biological uptake of NH_4^+ and nitrification produce hydrogen ions (Schindler et al. 1985). NH_3, due to its toxicity to fish and because it causes eutrophication of lakes and wetlands, is a serious environmental problem. The Council of the European Union set a recommended level of 0.05 mg L^{-1} and a maximum level of 0.5 mg L^{-1} of NH_4^+ (EEC 1998).

1.2 Constructed wetlands

Wetlands are land areas that are wet during a part or all of the year because of their location in the landscape, as they are frequently transitional between uplands (terrestrial systems) and continuously flooded (aquatic) systems (Kadlec & Wallace 2008). Constructed wetlands (CWs) are the wastewater treatment technology or system designed to employ ecological processes found in natural wetland ecosystems. According to Kadlec and Wallace (2008), modern CWs are man-made systems that have been constructed to emphasize specific characteristics of wetland ecosystems for improved treatment capacity. They are characterized by low capital costs, low operation and maintenance costs, and their perceived value for beautification and wildlife habitat improvement (Cole 1998). CWs are widely used in wastewater and groundwater treatment due to their low energy requirements and easy operation (Garcia et al. 2010). Wetlands, both constructed and natural, are promising in-situ water treatment method thank to enhanced microbial growth within the plants' rhizosphere, which creates an effective contaminant degradation zone (Kadlec & Wallace 2008). While microorganisms play the primary role in pollutant elimination, plants enhance the microbial activity to remove pollutants (Stottmeister et al. 2003). Due to the mosaic of aerobic and anaerobic zones within the root zone of the plants, contaminants can be removed by a variety of processes, aerobic as well as anaerobic.

Although originally introduced as a municipal wastewater treatment method, CWs have been successfully used to treat a variety of contaminated waters (Brix 1994a, Chen et al. 2006, Cooper 2009). Some of examples are:

- Domestic wastewater: even though applications are often limited due to the fact that in such waters N is often not controlled and therefore, NH_4^+ will be oxidized to NO_3^- and sent to groundwater in this form (Kadlec & Wallace 2008).
- Animal wastewater: mostly as primary treatment stage (Kadlec & Wallace 2008).
- Minewater treatment: however, designs remain to be further developed (Cooper 2009, Kadlec & Wallace 2008).
- Industrial wastewaters: very efficient in treating water that is high in biodegradable organic and N content (Chen et al. 2006, Kadlec & Wallace 2008).
- Leachate and remediation: groundwater is the main source for potable water and domestic use in numerous countries around the world, however, water quality can be affected by pollution, which influences the natural environment and human health, and alternatives to wetlands are extremely costly by comparison (Kadlec & Wallace 2008).
- Urban stormwater treatment (Kadlec & Wallace 2008).
- Agricultural wastewater (Cooper 2009, Vymazal 2009).

1.2.1 Types of constructed wetlands

CWs have basic classification based on the type of macrophytic growth, further classification is usually based on the water flow regime (Vymazal 2007). CWs can be designed in a variety of hydrologic modes. However, nowadays the two main types of CWs are distinguished as surface flow (SF) and subsurface flow (SSF) CWs (Kadlec & Wallace 2008) (Fig. 1.2-1). SF CWs are vegetated systems with open water surface and typically have water depths of less than 0.4 m. In SSF CWs, no free water is visible because the water flows through a porous medium planted with emergent water plants (helophytes). SSF CWs are further subdivided into horizontal flow (HSSF) and vertical flow (VSSF) systems depending on the direction of water flow through the porous soil (usually sand or gravel). Moreover, hybrid systems which combine different types of CWs are also used (Vymazal 2010). This chapter compares the different variants of CWs.

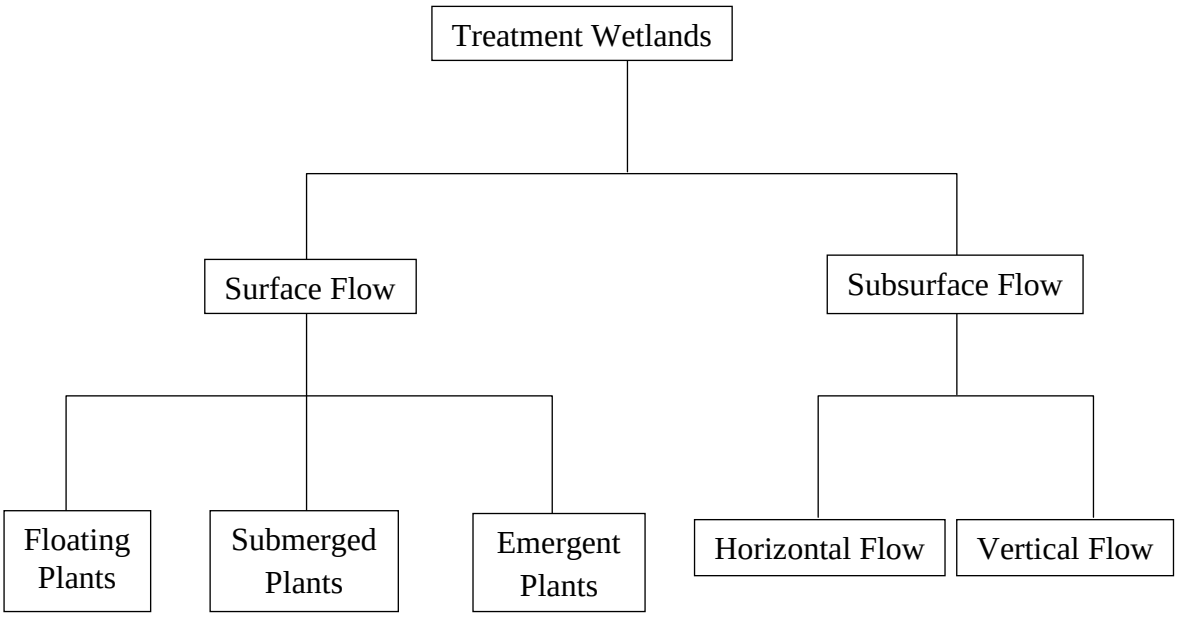

Fig. 1.2-1 Classification of constructed wetlands, source: Kadlec and Wallace (2008)

Surface flow constructed wetlands

A typical SF CW with emergent macrophytes is a shallow sealed basin or sequence of basins, containing 20 - 30 cm of rooting soil, with a water depth of 20 - 40 cm. Dense emergent vegetation covers a significant fraction of the surface, usually more than 50 %. Besides planted macrophytes, naturally occurring species may be present (Kadlec 1995). SF CWs have advantage of being closely mimic natural wetlands. CWs with SF are frequently used in North America (Kadlec & Wallace 2008) and Australia (Merz 2000).

Besides municipal wastewater, SF CWs with emergent vegetation have been used to treat various types of wastewaters. They are the most commonly used for advanced treatment of effluent from secondary or tertiary treatment processes. SF wetland systems offer low construction cost, but they generally have a lower contaminant removal efficiency compared

with SSF systems. There has been a recent attempt to develop an open-water zone, without vegetation, to improve the N removal efficiency, promote better inflow flux, and provide wildlife habitats (Jang et al. 2007).

SF CWs are efficient in removal of organics through microbial degradation and settling of colloidal particles. Suspended solids are effectively removed via settling and filtration through the dense vegetation. Attached and suspended microbial growth is responsible for removal of soluble biochemical oxygen demand (BOD). The major oxygen (O_2) source for these reactions is re-aeration at the water surface. N is removed primarily through nitrification/denitrification and NH_3 volatilization under higher pH values caused by algal photosynthesis. SF CWs also provide removal of phosphorus, but at relatively slow rates because of limited contact of water with soil particles which adsorb and/or precipitate phosphorus. Plant uptake represents only temporal storage because the nutrients are released to water after the plant decay (Vymazal 2010).

Horizontal subsurface flow constructed wetlands

HSSF wetlands are the most widely used in Europe. This type of CW was developed in the 1950s in Germany by Käthe Seidel who designed the HSSF CWs using coarse materials as the rooting medium (Seidel 1955). In the 1960s, Reinhold Kickuth suggested soil media with high clay content and called the system the "Root Zone Method" (Kickuth 1977). This first system consisted of a plastic-lined bed containing helophytes (emergent water plants) growing in soil. However, these soil-based systems, as a result of low hydraulic conductivity due to the use of silty soil suffered from surface overflow which prevented the wastewater from coming into contact with the plant roots. The problem of surface runoff was overcome by the use of more porous medium, such as gravel.

HSSF CWs are typically designed to treat primary effluent prior to either soil dispersal or surface water discharge. The design typically consisted of a rectangular bed planted with the common reed (*Phragmites australis*) and lined with an impermeable membrane. Mechanically pre-treated wastewater is fed in at the inlet and passes slowly through the filtration medium under the surface of the bed in a more or less horizontal path until it reaches the outlet zone where it is collected before discharge via level control arrangement at the outlet. Typical arrangement of HSSF CW has the depth of filtration bed usually 0.6-0.8 m in order to allow roots of wetland plants and namely *Phragmites* to penetrate the whole bed and ensure oxygenation of the whole bed through oxygen release from roots. Roots and rhizomes of reeds and all other wetland plants are hollow and contain air-filled channels that are connected to the atmosphere for the purpose of transporting oxygen to the root system. The majority of this oxygen is used by the roots and rhizomes themselves for respiration, but as the roots are not completely gas-tight, some oxygen is lost to the rhizosphere (Brix 1994a, Brix 1997). According to the working principle of HSSF CWs, the amount of oxygen released from roots and rhizomes should be sufficient to meet the demand for aerobic degradation of oxygen consuming substances in the wastewater as well as for nitrification of the NH_3. However, many studies have shown that the oxygen release from roots of different

macrophytes is far less than the amount needed for aerobic degradation of the oxygen consuming substances delivered with sewage and that anoxic and anaerobic decomposition play an important role in HSSF CWs (Brix 1990). As a result organic compounds are degraded aerobically as well as anaerobically by bacteria attached to plant underground organs (i.e. roots and rhizomes) and media surface and the removal of organics is generally very high in HSSF CWs (Vymazal 2005).

Due to the limited O_2 transfer inside the wetland, the removal of nutrients (especially N compounds) is limited, however, HSSF CWs remove the NO_3^- in the wastewater. Phosphorus is removed from wastewater in HSSF CWs primarily by ligand exchange reactions, where phosphate displaces water or hydroxyls from the surface of Fe and Al hydrous oxides. However, soil used for HSSF CWs (e.g. pea gravel, crushed stones) usually do not contain great quantities of Fe, Al or Ca and therefore, removal of phosphorus is generally low (Kadlec & Wallace 2008).

HSSF wetland systems are generally more expensive than SF wetlands, although maintenance costs remain low compared to alternatives. They are commonly used for secondary treatment for single-family homes or small cluster systems (Wallace & Knight 2006) or for small communities (Kadlec & Wallace 2008). However, there are many other applications to specialty wastewaters from industry. In general, HSSF wetlands have been utilized for smaller flow rates than SF wetlands, probably because of cost and space considerations. These systems are capable of operation under colder conditions than SF systems, because of the ability to insulate the top. A key operational consideration is the propensity for clogging of the media. HSSF wetlands do not provide the same opportunities for ancillary benefits that SF systems do. Unlike SF wetlands, because the water is not exposed during the treatment process, the risk associated with human or wildlife exposure to pathogenic organisms is minimized (Kadlec & Wallace 2008).

Vertical subsurface flow constructed wetlands

As HSSF wetlands have a limited capacity to oxidize NH_4^+ due to limited oxygen transfer, VSSF CWs were developed in Europe to provide higher levels of O_2 transfer, thus producing a nitrified effluent. They were originally introduced by Seidel (1965) to oxygenate anaerobic septic tank effluents. However, the VSSF CWs did not spread as quickly as HSSF CWs probably because of the higher operation and maintenance requirements due to the necessity to pump the wastewater intermittently on the wetland surface (Vymazal 2010). VSSF CWs consist of sand or gravel bed typically planted with wetland vegetation and water is equally distributed across the wetland surface. The water is fed in large batches and then the water percolates down through the medium. The new batch is fed only after all the water percolates and the bed is free of water. This enables diffusion of oxygen from the air into the bed. As a result, VSSF CWs are far more aerobic than HSSF CWs and provide suitable conditions for nitrification. On the other hand, VSSF CWs provide only partial denitrification (Vymazal 2010). VSSF CWs are very effective in removing organics and suspended solids as well as pathogens (EEC 1998). Removal of phosphorus is low unless media with high sorption

capacity are used (Vymazal 2009). Bed clogging might be a problem, particularly if the beds are operated without a resting period (Platzer & Mauch 1997). In order to achieve a good performance and at the same time to prevent clogging, it is important that the bed medium allows the passage of the wastewater through the bed before the next inflow arrives while at the same time holding the liquid back long enough to allow the contact with the bacteria growing on the media (Brix & Arias 2005).

As compared to HSSF CWs, VSSF CWs require less land (Brix & Arias 2005). Such CWs are able to clean contaminated waters with very high concentrations of contaminants even during cold winters (Brix et al. 2002). The ability of VSSF wetlands to oxidize NH_4^+ has resulted in their use in applications with higher NH_4^+ than municipal or domestic wastewater. Landfill leachates and food processing wastewaters can have NH_4^+ levels in the hundreds of milligrams per liter, and the key to reduction is the ability to nitrify. Successful VSSF wetlands therefore have formed part of the treatment process for those wastes (Kadlec 2003).

Floating plant root mat / non floating plant root mat filter

Recently, a new variant of CWs has been developed that engages helophytes, similar to those used in SF and SSF CWs. This is a floating root mat (FPRM), where the wetland plants are growing on the water surface or touching to the rooting proof bottom of the water body, and the root mat can function as a biofilter for the contaminated water. Generally, a floating root mat involves the growth of helophytes, usually rooted into the soil, but in this case converted into artificially macrophyte root mats floating in a pond or canal. These plants form a dense floating mat of roots and rhizomes, and by means of this a preferential hydraulic flow in the water zone between the root mat and the non-rooted bottom can be expected. The water is forced to flow through the root mat which operates as a filter when this root mat occupies the whole water body and touches the bottom of the pond or canal (Chen 2012).

Floating plant root mat (FPRM) and non-floating plant root mat filter (PRMF) are hybrids of helophytes-containing soil free ponds and conventional soil based CWs. Because of their specific structure, FPRM combines benefits from ponds and CWs, and is therefore used for the treatment of different types of wastewaters and removal of different pollutants such as suspended solids, nutrients, metals, and organic contaminants. The removal efficiencies are dependent on different factors such as the climatic conditions and the type of water. FPRM and PRMF are similar to ponds as they have an open water body, and are also similar to conventional soil based CWs as both of them use helophytes, but ponds are usually dominated by phytoplankton (Kadlec 2005). In the field of water treatment, FPRM was probably first used in eutrophicated lakes and rivers, for example, in Germany (Hoeger 1988). The development of a dense root mat by plant roots is important for the start-up of FPRM and PRMF. A great number of emergent water plant species like *Phragmites australis*, *Typha latifolia*, *Typha angustifolia*, *Juncus effusus* have the potential to grow as floating mats/islands, where most of them can form self-buoyant FPRM in nature or grow successfully on rafts with the potential to remove water contaminants (VanDuzer 2004).

The advantages of FPRM are direct uptake of nutrients from the water through the plant roots, shade preventing algal proliferation, easily coping with varying water levels, root network ensuring physical filtration and providing a large attachment surface for microorganisms and an ecological value/shelter for fauna. Disadvantages are seasonally dependent removal efficiencies and a relatively long start-up period. Unlike the facultative and aerated ponds, FPRM depend on the presence of macrophytes to achieve an enhanced removal of pollutants. In case of accumulation of too much sediment, the plant root mat can be easily shifted aside to allow its removal (Chen 2012).

1.2.2 Performance of various type of constructed wetlands for different contaminants

Suspended solids and biochemical oxygen demand

Removal of suspended solids and BOD are very high in all types of CWs (Table 1.2-1). While in SF and VSSF CWs the microbial degradation processes are mostly aerobic, in HSSF CWs anoxic and anaerobic processes prevail. The treatment efficiency is similar for SF and HF CWs, while for VSSF CWs the percentage efficiency is higher due to higher inflow concentrations. However, the outflow concentrations are comparable for all types of CWs (Table 1.2-1).

Table 1.2-1 Treatment efficiency (Eff, in %) of various types of CWs for organics and suspended solids. BOD_5 = the five-day biochemical oxygen demand, TSS = total suspended solids, inflow (In) and outflow (Out) concentrations in mg L^{-1}, n = number of CWs (from Vymazal (2010))

Type of CW	BOD_5				TSS			
	In	Out	Eff	n	In	Out	Eff	n
SF	161	42	74	50	185	43	77	52
HSSF	170	42	75	438	141	35	75	367
VSSF	274	28	90	125	163	18	89	98

Nutrients: nitrogen and phosphorus

Removal of nutrients in various types of CWs is presented in Table 1.2-2. Phosphorus retention is low in all types of CWs as compared to loads commonly occurring in wastewaters. In SSF CWs, the major removal mechanisms are adsorption and precipitation (Richardson 1985). However, materials which are commonly used for SSF CWs, such as washed gravel or crushed rock, provide very low capacity for sorption and precipitation (Vymazal 2007). Recently, manufactured filtration materials such as LECA (light weight clay aggregates) or by- and waste- products such as furnace steel slags have been tested in CWs (Vohla et al. 2005). The removal of phosphorus is very high with these substrates, but it is important to realize that sorption and precipitation are saturable processes and the sorption decreases over time.

Table 1.2-2 Treatment efficiency (Eff, in %) of various types of CWs for N and phosphorus. TP = total phosphorus, TN = total N, NH_4^+-N = ammonium-nitrogen, inflow (In) and outflow (Out) concentrations in mg L^{-1}, n = number of CWs (from Vymazal (2010))

Type of CW	TP				TN				NH_4^+-N			
	In	Out	Eff	n	In	Out	Eff	n	In	Out	Eff	n
SF	14.7	9.7	34	52	42.6	23.5	45	29	30	16	48	40
HSSF	9.6	4.8	50	272	63	36	43	208	36	22	39	305
VSSF	10.3	4.5	56	118	73	41	43	99	56	14.9	73	129

Removal of total N (Table 1.2-2) is also usually low due to low nitrification in HSSF CWs and low or zero denitrification in SF and VSSF CWs, respectively (Kadlec & Wallace 2008, Vymazal 2007). Volatilization may be a significant route for N removal in SF CWs. In VSSF CW, very high nitrification proceeds but, because of entirely aerobic conditions in the vertical bed, only limited denitrification takes place (Brix & Arias 2005). In order to achieve effective removal of total N, VSSF CWs could be combined with HSSF CWs which, in contrast, do not nitrify but provide suitable conditions for reduction of NO_3^- formed during nitrification in VSSF beds (Kadlec & Wallace 2008, Vymazal 2007, 2010). Plant uptake in all types of CWs is effective only when plants are harvested, but the amount sequestered in the aboveground biomass is usually very low and does not exceed 10 % of the inflow nutrient load (Vymazal 2009, 2010).

Sulfur

CWs generally are not as effective for removal of sulfur as for other contaminants. Although microbial routes provide for gaseous losses of hydrogen sulfide and dissimilatory sulfate reduction, these require the very low redox potentials usually found only in deeper wetland sediments. As a result, the median-observed concentration reduction is only 14 % for 32 CWs (Kadlec & Wallace 2008). Only some mine water CWs show more than 50 % reduction, and that may be attributed to the anaerobic mode of operation in some cases. HSSF CWs also sometimes satisfy this anoxic condition.

Pathogens

Pathogens are present in untreated domestic wastewaters as well as in runoff waters from animal sources. CWs, especially those that have long residence times (greater than about ten days) provide some disinfection. The extent of removal is strongly dependent on the hydraulic efficiency of the wetland. Empirical evidence is available that demonstrates that a significant die-off of indicator organisms and pathogenic species occurs in CWs. The most common indicator group is fecal coliforms, and in a review of 130 SSF CWs the median global removal was a 1.82 $\log_{10}$ reduction (Kadlec & Wallace 2008).

Organic chemicals

Organic chemicals pose somewhat more difficult set of problems because of their possible toxicity to plants and the limitations of aerobic and anaerobic degradation. This includes organics from very important generators: pesticides, petroleum and petrochemicals, food wastes, and remediation. Seeger et al. (2011) reported 99 % benzene removal in HSSF CWs, and 82 % methyl *tert*-butyl ether in FPRM. Other studies show generally good performance for hydrocarbons removal as well.

1.2.3 Removal mechanisms in constructed wetlands

The removal of contaminants from wastewater in biological treatment systems can be impacted by a number of physical, chemical and biological processes. Among the last ones, plants and microorganisms play the key role.

Role of plants

Plants influence contaminant removal processes in CWs mainly by the physical processes which they trigger. The wetland plants stabilize the bed surface, as their root systems prevent the formation of erosion channels, and provide the necessary conditions for physical filtration. Turnover of root mass also improves the soil hydraulic conductivity by the creation of macropores in a CWs soil, and this enhances interactions between roots and influent (Sim 2003). Furthermore, the plant roots release a wide variety of organic compounds, at rates up to 25 % of the total photosynthetically fixed carbon (C) (Sim 2003). This organic C is mainly used by denitrifying bacteria as a C source and thus stimulates the denitrification. Decaying plant material also provides a long-lasting, readily available C source for the microbial populations. Bacteria growth is stimulated by carbohydrates, amino acids, phenolics, aliphatic compounds, fatty acids, sterols, enzymes, vitamins, hormones and nucleosides released by secretion, diffusion or cell lysis (Jones et al. 2009).

Macrophytes release oxygen from roots into the rhizosphere and thus influence the biogeochemical cycles by changing the redox status in the CWs. These oxygen leakages change anaerobic environment to aerobic and therefore stimulate aerobic biodegradation pathways of contaminants, e.g. nitrification. The root oxygen release rates of *Phragmites* estimated by different techniques vary between 0.02 g m^{-2} day^{-1} (Brix 1990), 1-2 g m^{-2} day^{-1} (Gries et al. 1990), and 5-12 g m^{-2} day^{-1} (Armstrong & Armstrong 1990). Good nitrification potential of CWs planted with *Phragmites* is connected with its characteristics of roots growth. As *Phragmites* allocates 50 % of plant biomass to root and rhizome systems, increased root biomass allows for greater oxygen transport into the substrate, creating a more aerobic environment favoring nitrification reactions (Sim 2003). The oxygen fluxes depend on the species specific differences, seasonal variations as well as the oxygen demand of the surrounding medium.

Abundance and activity of bacteria attached to surfaces are higher than that of free-living bacteria (Hamilton 1987). Attached bacteria create microbial communities that are inserted in

polysaccharide/protein matrixes, e.g. biofilms, and the bacterial activity within these biofilms is synchronized by diffusion of nutrients into the biofilm and by internal processes within this layer (Atlas 1998). The development of microbial community is in dependence to surface characteristics where biofilms are growing (Hamilton 1987). There are several suitable surfaces for biofilm growth, such as carriers, macrophyte roots, litter etc. Due to a contact of these surfaces with flowing water, they are not less important than sediments for the various transformation processes (Bastviken et al. 2003). Therefore, another important role of plants in CWs is that they introduce surface area for attachment and growth of bacteria and protozoa. Microorganisms are growing on the submerged parts of leaves and stems as well as on the roots and rhizomes in the wetland soil (Brix 1997). Decomposing plant material enhances the biofilm growth by providing highly porous multiple layers of organic debris (litter/humus). These biofilms, both on above and belowground biomass as well as on dead macrophyte biomass, are responsible for the majority of microbial turnovers that occur in CWs.

Plants require nutrients for their growth and reproduction, which they uptake mainly by roots. Therefore, significant amounts of nutrients can be bound into the biomass of macrophytes. The uptake capacity of the wetland plants and accordingly the amount of nutrients which can be removed by harvesting is in the range between 30 to 150 kg P ha^{-1} $year^{-1}$ and 200 to 2500 kg N ha^{-1} $year^{-1}$ (according to the review of Brix (1994b)). These nutrients can be removed by the annual harvesting of the aboveground biomass (Brix 1997). However, considering substantial loadings into CWs with wastewater, the removal of nutrients by the harvesting is generally insignificant.

Recently, some unexplored functional mechanisms for plants in CWs were presented (Shelef et al. 2013). One of them salt phytoremediation, what implies use of halophytes to reduce salinity of the wastewater. Another newly described function of the plants is application of them as bioindicators in CWs. Bioindication is based on the assumption that plant performance can indicate the quality of the wastewater treatment. Accumulation of the highly reactive molecule hydrogen peroxide (H_2O_2) can be used as marker for the signaling of environmental stresses (Neill et al. 2002). Plants should be used as bioindicators of CW performance in the planning phase. Last but not the least, macrophytes provide aesthetic value for wastewater treatment systems and improved habitat for wildlife.

Physical and other processes

Among the main mechanisms responsible for contaminant removal in CWs, physico-chemical processes as sedimentation, adsorption and precipitation at the water-sediment, root-sediment and plant-water interfaces are of significant importance. As substrates may remove wastewater constituents by ion exchange/non-specific adsorption or specific adsorption/precipitation, a choice of substrate is crucial. Different substrates also may differ in their sorption capacities. Sorption may be reversible or irreversible due to mineralization of sorbed materials or to the formation of very strong chemical bonds (Kadlec & Wallace 2008). For example, the performance of various substrates for heavy metal removal was estimated

for CWs treating industrial wastewater (Scholz & Xu 2002). No statistically significant performance increases of lead and copper reduction were observed when using more expensive adsorption filter media. Besides, the good overall filtration performance not only for lead and copper, but also for BOD_5 and turbidity removal was reached during the set-up phase of 10 months. This implies that irreversible adsorption or precipitation onto the soil surface is a significant removal mechanism for pollutants (Chen 2012). Through sorption NH_3 can be stored for a short-term in CWs during drawdown periods until oxidized.

Sedimentation is crucial for removal of TSS from wastewater in CWs. Low water velocities, coupled with the presence of plant litter (in SF CWs) or sand/gravel media (in HSSF and VSSF CWs), promote settling and seizure of TSS. In SF CWs, the presence of dense wetland vegetation causes retaining of TSS by filtration. HSSF wetlands are very effective in trapping and retaining TSS from the wastewater, however, the accumulation of TSS material reduces the hydraulic conductivity of the wetland, often to a significant degree, and causes bed clogging (Kadlec & Wallace 2008).

Precipitation can refer to the reaction of phosphate ions with cations of metals such as Fe, Al, Ca, or Mg. For P removal, absorption and precipitation by wetland soils are generally considered more important than uptake by plants (Richardson & Craft 1993). However, wetland soils become soon saturated under any long-term increase in phosphorus loading (Kadlec & Wallace 2008). Sorption is important for phosphorus during the start-up period for a CW.

Role of microorganisms

Microorganisms but not the plants play the key role in the transformation of nutrients and organic contaminants in CWs. Pollutants in the wastewater can be removed by various pathways depending on the oxygen input by helophytes and the availability of other electron acceptors. For example, in HSSF CWs aerobic biodegradation occur only near roots and on the rhizoplane (the surface of the roots) (Faulwetter et al. 2009, Stottmeister et al. 2003). In the far proximity from roots, where zones are mainly free of oxygen, anaerobic processes such as denitrification, sulfate reduction and/or methanogenesis take place (Faulwetter et al. 2009, Stottmeister et al. 2003). Furthermore, microbially mediated reactions, such as organic matter decomposition and N transformations, are affected by temperature. N removal was found to be most efficient at temperatures above 15ºC (Akratos & Tsihrintzis 2007), or even at elevated temperatures up to 20-25ºC (Lee et al. 2009).

Microbial degradation is the main process in the removal of soluble/colloidal biodegradable organic matter in wastewater (Sim 2003). When dissolved organic matter is delivered by diffusion process into the biofilms attached on the plant roots and media, biodegradation can take place. For N, the main removal mechanism is microbial nitrification/denitrification, and incorporation into the plant biomass is only significant if plants are harvested and biomass is removed from the system. Phosphorus removal is provided through a complex of physical, chemical and microbiological processes, although adsorption and precipitation to the soil has

usually been considered to be the main removal process (Kadlec & Wallace 2008, Kröpfelová 2008); microbiota uptake and plant uptake may also play a role in the removal of phosphorus (Kröpfelová 2008).

1.3 Nitrogen transformation processes in constructed wetlands

N compounds can be affected by different processes such as microbial removal, N fixation, plant and microbial uptake, NH_3 volatilization, mineralization, and NO_3^- reduction to NH_4^+. The N cycle is characterized by complexity and the co-occurrence of many processes (Fig. 1.3-1). The main processes that can affect N removal in CWs are plant uptake and microbial assimilation or reduction to inert dinitrogen gas (N_2). NH_4^+ is the dominant form of N in sewage/effluent affected systems. Another source of NH_4^+ in CWs is ammonification. By hydrolysis and mineralization, organic N can be converted into NH_4^+. When NH_4^+ is formed or enters a CW with wastewater, it can be taken up by plants, immobilized into the organic material, or oxidized to NO_3^- by microorganisms. However, nitrification-denitrification processes play a dominant role in NH_4^+ removal in CWs (Coban et al. 2014). In case of nitrification, the produced NO_3^- can further undergo denitrification under anaerobic conditions, which is the stepwise reduction of NO_3^- via nitrite (NO_2^-) to N_2O and N_2 using organic C as an electron acceptor. Over the last decade there is also evidence that some microorganisms can reduce NH_4^+ directly to N_2 using NO_2^- or NO_3^- as an electron donor in a process known as anammox (anaerobic ammonium oxidation) (Lee et al. 2009). However, the quantification of these processes can be difficult due to a complexity of the wetland systems concerning flow dynamics, root exudation, substrates (gravel), and the mosaic of aerobic and anaerobic zones within the root zone of the plants (Kadlec 2000, Martin & Reddy 1997). For investigations of N cycle in CWs, a wide range of methods is applied, such as physico-chemical measurements, molecular biology techniques, isotope tracking using ^{15}N labels, and the $^{15/14}N$ natural abundance of various N compounds. However, none of these techniques is satisfactory as a result of high spatial and temporal variations in the N transformation processes (Groffman et al. 2006). Here the main N transformation processes as well as methods for investigations of N cycle will be discussed.

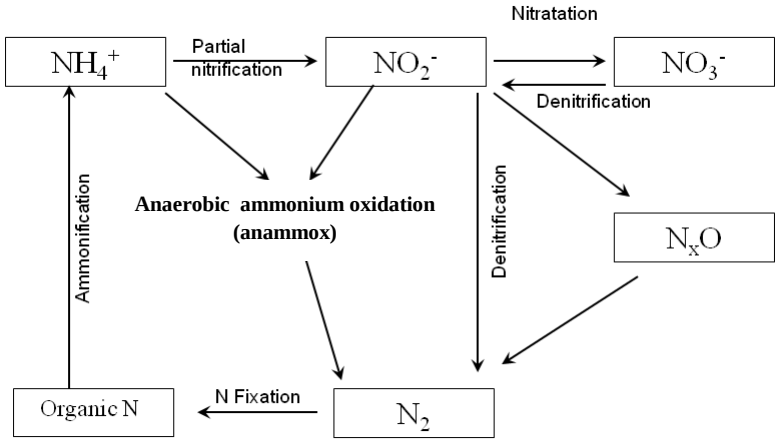

Fig. 1.3-1 Potential microbial N-transformation processes in CWs

1.3.1 Ammonification

Wastewaters contain varying amounts of organic N, depending upon the source. N in domestic sewage comprises about 60 % NH_4^+ and 40 % organic N (EPA 1993). Organic N is made up of a variety of compounds including amino acids, amino sugars, urea and uric acid, and purines and pyrimidines (Kadlec & Wallace 2008). Due to toxicity of NH_3 to living organisms, in mammals it is converted to a less toxic form by the addition of carbon dioxide (CO_2) (Kadlec & Wallace 2008). Urea (CN_2H_4O) is formed by mammals as a physiological mechanism to dispose of NH_3 and uric acid ($C_4N_4H_4O_3$) is produced by insects and birds for the same purpose. These organic forms of N are important in CW treatment because they are readily hydrolyzed, chemically or microbially, resulting in the release of NH_3 (Kadlec & Wallace 2008). Other organic forms of N, primarily as amino acids, which are the main components of proteins, typically make up from 1-7% of the dry weight of plants and animals. Also purines and pyrimidines are synthesized from amino acids to become the main building blocks of the nucleotides that make up DNA in living organisms.

Ammonification is the biological transformation of organic N from dead and decaying cells and tissues as well as direct excretion of urea to NH_4^+. This process occurs both aerobically and anaerobically, and heterotrophic microorganisms are considered to be involved (EPA 1993). Typical ammonification reactions are:

Urea breakdown

$CN_2H_4O + H_2O \Rightarrow 2\ NH_3 + CO_2$

Amino acid breakdown

$RCH(NH_2)COOH + H_2O \Rightarrow NH_3 + CO_2$ (Kadlec & Wallace 2008)

Kinetically, ammonification proceeds more rapidly than nitrification. The rates of ammonification are the highest in the oxygenated zone and decrease with the change from aerobic to anaerobic environment. A microgradient system of aerobic and anaerobic zones of CWs is highly suitable for this process, although ammonification occurs mostly in oxygen saturated areas. The rates are depending on temperature, pH, C/N ratio, available nutrients, and soil structure (Reddy & Patrick 1984). The optimal ammonification temperature is reported to be 40 - 60°C while optimal pH is between 6.5 and 8.5 (Vymazal 1995). A wide range of ammonification rates is reported in the literature, with values between 0.004 and 0.53 g N m^{-2} d^{-1} (Reddy & D'Angelo 1997, Tanner et al. 2002).

1.3.2 Nitrification

Nitrification is typically defined as the biological formation of NO_3^- or NO_2^- from compounds containing reduced N with oxygen as the terminal electron acceptor (DeGraaf et al. 1996). Nitrification has been typically associated with the chemoautotrophic bacteria, although it is now recognized that heterotrophic nitrification occurs and can be of significance (Paul &

Clark 1996). This is a two-step process, performed by two different groups of microorganisms (Paul & Clark 1996, Schmidt et al. 2003).

In the first step, nitritation, NH_4^+ is oxidized to NO_2^-:

$NH_4^+ + 1.5\ O_2 \Rightarrow NO_2^- + 2\ H^+ + H_2O$ (Vymazal 2007)

This reaction is performed by strictly chemolithotrophic (strictly aerobic) bacteria which obtain C largely from CO_2 or carbonates. While a wide range of ammonia oxidizing bacteria was found in soil, i.e. genera *Nitrosospira, Nitrosovibrio, Nitrosolobus, Nitrosococcus* and *Nitrosomonas*, in fresh waters only *Nitrosomonas europaea* was detected (Grant & Long 1981, Paul & Clark 1996, Schmidt 1982).

In the next nitrification step, nitratation, NO_2^- is oxidized to NO_3^-:

$NO_2^- + 0.5\ O_2 \Rightarrow NO_3^-$ (Vymazal 2007)

The second step in the process of nitrification is executed by facultative chemolitrotrophic bacteria which can also use organic compounds, in addition to NO_2^-, for the generation of energy for growth. In contrast with the ammonia oxidizing bacteria, only one species of nitrite oxidizing bacteria was found in the soil and freshwater, i.e. *Nitrobacter winogradskyi* (Grant & Long 1981).

The nitrification process is very oxygen demanding. If calculated based on the stoichiometric relationship, the theoretical O_2 consumption by the first reaction, nitritation, is about 3.43 g O_2 per gram of NH_3-N oxidized, and 1.14 by the second nitrification reaction, for a total of 4.57. Actual consumption is reportedly somewhat less, 4.3 g O_2 per gram of NH_3-N oxidized (Tchobanoglous et al. 1991). Moreover, yields produced by *Nitrosomonas* and *Nitrobacter* are 0.15 mg cells mg^{-1} NH_4^+-N oxidized and 0.02 mg cells mg^{-1} NO_2^--N oxidized, respectively. Therefore, the combined processes of cell synthesis create 0.17 g of dry weight biomass per gram of NH_4^+-N consumed (EPA 1993). In addition, alkalinity is needed, 7.07 g $CaCO_3$ per g of NH_4^+-N oxidized (Ahn 2006). Thus nitrification lowers the alkalinity and pH of the water. The doubling time of nitrifying bacteria is reported as 2-3 days (Gerardi 2006).

Nitrification is influenced by temperature, pH value, alkalinity of the water, inorganic C source, moisture, microbial population, and concentrations of NH_4^+-N and dissolved oxygen (Vymazal 1995). The optimum pH for nitrification is ranging between about 7.2 and 9.0 (Tchobanoglous et al. 1991). As most of CWs operate at pH about neutral, pH should have minor influence of nitrification in these systems. The optimum temperature for nitrification in pure cultures ranges from 25 to 35°C and in soils from 30 to 40°C (Vymazal 2007). The minimum temperatures for growth of *Nitrosomonas* and *Nitrobacter* according to Cooper (Cooper et al. 1996) are 5 and 4°C, respectively. Nitrification rates in CWs were reported to be in the range of 0.01-2.15 g N m^{-2} d^{-1} with the mean value of 0.048 g N m^{-2} d^{-1} (Reddy & D'Angelo 1997, Tanner et al. 2002).

1.3.3 Denitrification

Denitrification is most commonly defined as the process in which NO_3^- is converted into N_2 via intermediates NO_2^-, nitric oxide (NO), and N_2O (Paul & Clark 1996). Denitrification is carried out by a wide range of autotrophic and heterotrophic facultative anaerobic bacteria (such as *Paracoccus denitrificans, Pseudomonas, Micrococcus, Achromobacter* and *Bacillus*) that are able to use NO_3^- (and NO_2^-) as an electron acceptor under anoxic conditions. It can be presented as the following sequence of biochemical changes from NO_3^- to elemental gaseous N:

$NO_3^- \Rightarrow 2\ NO_2^- \Rightarrow 2\ NO \Rightarrow N_2O \Rightarrow N_2$ (Vymazal 2007)

Denitrification requires an external organic C source which is used as an electron donor in the respiratory chain. It can be, for example, glucose or methanol. However, in CWs the source of organic C is rather organic matter that is sometimes characterized by the Redfield ratio C:N:P = 106:16:1 (Schmid et al. 2007). In this case, the denitrification reaction can be written:

$84.8\ NO_3^- + (CH_2O)_{106}(NH_3)_{16}(H_3PO_4) \rightarrow 42.4\ N_2 + 106\ CO_2 + 16\ NH_3 + 148.8\ H_2O + H_3PO_4$

This reaction normally occurs in the presence of available organic substrate only under anoxic conditions (Eh = +350 to +100 mV). According to the equation above, the C requirement is 3.02 g organic matter per gram of NO_3^--N. Organic substances can be provided with inflowing water, through the decomposition of dead plant material or by living root exudates (Kadlec & Wallace 2008). The most labile form of organic C is the influent BOD, and this is preferable used by denitrifying bacteria to reduce oxidized forms of N. As can be seen from the equation, denitrification also produces alkalinity and therefore, increases the pH in CWs. The rate of denitrification is influenced by many factors, including NO_3^- concentration, microbial flora, type and quality of organic C source, hydroperiods, different plant species residues, the absence of O_2, redox potential, soil moisture, temperature, pH value, presence of denitrifying bacteria, soil type, water level, and the presence of overlying water (Ambus & Christensen 1993, Vymazal 1995, Willison et al. 1998).

Denitrification was shown to occur under the presence of O_2 as well (Mosier 1998, Robertson et al. 1995, Robertson & Kuenen 1984). One possible explanation for this can be that CWs have mosaics of aerobic and anaerobic zones in dependence of proximity from the rhizosphere. Such zonation is supported by stratification due to biofilm formation, where NH_3 oxidation takes place near the oxygenated microzone around a rootlet and denitrification can occur only microns away in the anaerobic bulk soil (Kadlec & Wallace 2008). In the SF CWs, water-sediment interface is characterized by the presence of oxygen gradient, and therefore, aerobic reactions can take place near the surface and anaerobic at bottom sediments (Reddy & Patrick 1984). However, a number of laboratory studies with batch cultures revealed an occurrence of denitrification under aerobic conditions, so called aerobic

denitrification (Robertson et al. 1995, Robertson & Kuenen 1984). In CWs, nitrification occurs in the plants' rhizosphere as it delivers oxygen and in the top layer of the water body where the aerobic denitrification could occur simultaneously. Gao et al. (2010) reported rather similar rates of denitrification in marine sediments under both oxic and anoxic conditions. However, there is a lack of studies in natural environments, verifying and quantifying rates of aerobic denitrification (Gao et al. 2010).

Other possible pathways of NO_3^- loss in CWs are microbial and plant uptake and dissimilatory NO_3^- reduction (DNRA). DNRA is a microbial process that transforms NO_3^- to NH_4^+ via formation of NO_2^- in anaerobic or low O_2 environments. DNRA was shown to be relevant under reduced conditions (Eh = -200 mV) (Buresh & Patrick 1981). However, nowadays there is growing evidence that DNRA can also take place with presence of O_2, especially under high C:N ratio of about 10 and low NO_3^- concentrations (Leahy & Colwell 1990) and it is stimulated by a presence of macrophytes (Stein & Arp 1998). Still, the rates of DNRA in CWs related to the total NO_3^- loss reported in literature are in a range between 2 and 9% (Kendall et al. 2007, Mariotti et al. 1988, Stein & Arp 1998). Immobilization (microbial and plant uptake) is another way of NO_3^- loss. Yet, NH_4^+ is a preferred N source for microorganisms and NO_3^- uptake by microorganisms is inhibited when both NH_4^+ and NO_3^- are present (Cresswell & Syrett 1979, Recous et al. 1990).

Alternatively, NO_3^- reduction can be coupled to iron or sulfur oxidation. The bacterium *Thiobacillus denitrificans* can reduce NO_3^- to N_2 while oxidizing elemental sulfur, or reduced sulfur compounds including sulfide (S_2^-), thiosulfate ($S_2O_3^{2-}$), and sulfite (SO_3^{2-}) (Ehhalt et al. 2001). Iron-driven denitrification occurs at relatively low temperatures and circumneutral pH (Augustin et al. 1998). So far, the potential importance of this process at an ecosystem-level and factors controlling it are poorly understood (Augustin et al. 1998). CWs may have many forms of sulfur in sediments as well as C compounds, and consequently both heterotrophic, C-driven, iron-driven, and sulfur-driven denitrification have been observed to occur simultaneously in CWs sediments (Augustin et al. 1998, Ehhalt et al. 2001).

1.3.4 Anaerobic ammonium oxidation - Anammox

A recently discovered microbial transformation pathway in the global N cycle is anammox, an anaerobic oxidation of NH_4^+ combined with NO_2^- reduction and N_2 as the end product (Strous & Jetten 2004):

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2\ H_2O$$

However, while anammox organisms have been found in many different natural environments, there is still lack of knowledge about the role of this reaction in CWs. Investigations are necessary to learn about competition between anaerobic NH_4^+ oxidizers and other groups of ammonia oxidizing bacteria in the ecology of various wetland systems (Zhu et al. 2010).

The anammox process is characterized by several unusual features. Firstly, the anammox bacteria are still non-cultivated in pure culture; therefore, so far their studies are based on tracer studies or in enrichment cultures. Secondly, they grow extremely slowly (doubling time from weeks to months). Thirdly, despite to the only recent discovery of them, nowadays anammox is considered to be a major factor in global N cycling. Ultimately, this process has a good potential for application in biotechnology (Strous & Jetten 2004).

Nowadays, there is clear evidence that the anammox process is responsible for 30-50 % of marine NH_4^+ oxidation. However, due to a lack of data, the overall importance of this process in freshwater ecosystems is still unknown (Strous & Jetten 2004). Tracer studies in laboratory incubation with ^{15}N-labeled NH_4^+ and unlabelled NO_2^- or NO_3^- are being widely applied for detection of anammox (Kuypers et al. 2003, Waki et al. 2010). By such method, anammox and denitrification rates are possible to be measured individually because of the differences in the isotope composition of the produced N_2 ($^{29}N_2$ for anammox, $^{30}N_2$ or $^{28}N_2$ for denitrification) (Strous & Jetten 2004).

The anammox process was enhanced in laboratory scale subsurface CWs by the inoculation with the effluent of a wastewater treatment plant (Paredes et al. 2007). By this approach, the N removal rates were increased, and consequently, inoculation with an active biomass is an efficient strategy for faster establishment of anammox process in CWs. Still, the anammox seemed as a minor process for N removal even with appropriate wastewater composition and limitation of oxygen supply and organic C. The explanation is the extremely slow rates of anammox bacteria, and thus, more time is needed to set up stable N removal via anammox process. Nevertheless, further research is necessary in pilot-scale CWs and in role of the rhizosphere and plant roots in total in such systems.

Furthermore, the anammox was studied in full-scale CWs in east of France (Dong & Sun 2007). Here, CWs were shown as suitable reactor for growth of anammox bacteria; good N turnover rates can be reached by co-existence of partial nitrification and anammox. Also, promising results of N removal through nitritation and anammox were obtained in the study with SF CWs (Tao & Wang 2009). Vegetation significantly increased NH_4^+ removal via anammox by providing anaerobic biofilm layers in unsaturated zones near plant roots. Apart from this, effects of pH and seasonal temperature variation (He et al. 2012) and influent N concentration (Tao et al. 2012) on partial nitrification/anammox were investigated.

The ^{15}N tracer experiment and molecular biology techniques were applied for an estimation of the presence and activity of anammox bacteria in two parallel lab-scale VSSF CWs (Zhu et al. 2011). Moreover, one of the systems was inoculated with activated sludge. Thus, an enhancement of anammox biodiversity, activity, and abundance was obtained and evaluated. The coexistence of ammonia oxidizing bacteria and archaea, and anammox has been firstly reported in CWs ecosystems as well. Therefore, this is the first try to accomplish anammox process and increase anammox activity in CWs.

1.4 Nitrogen isotope fractionation in constructed wetlands

Natural abundances of the rare stable isotope of N, ^{15}N, are now being used widely in research on N cycling in organisms and ecosystems. Variations in $\delta^{15}N$ among samples reflect N isotope fractionations and mixture processes. The fractionation occurs because more energy is needed to break or form chemical bonds involving ^{15}N than ^{14}N. Consequently, ^{14}N-containing molecules react faster (e.g. with an enzyme) than those containing ^{15}N (Robinson 2001). N_2 gas in the atmosphere presents most of N in the biosphere, and this enormous pool is well mixed with an isotope composition that is set to $\delta^{15}N = 0$ ‰ by convention. There is a wide range reported for N isotope values for NH_4^+ and NO_3^- in precipitation from about -20 ‰ to 10 ‰, and some of the more negative values are related to soil and anthropogenic emissions in highly industrialized areas (Fry 2006). Thus, the $\delta^{15}N$ values may be used to trace human pollutant plumes and identify the sources and fates of N that human activities are currently adding to many ecosystems.

Another important aspect is the utilization of process-related stable isotope fractionation effects in many parts of the N-cycle. Natural samples are mixtures of N compounds from a variety of sources (Hauck 1973), and therefore, the qualitative and quantitative interpretation of N isotope signatures can be challenging, if taking into account not only denitrification, but other processes such as nitrification and plant assimilation. This quantification, using stable isotope ratios in concert with concentration data, is always based on isotopic enrichment factors. The N isotope fractionation in a system at any point in time reflects the balance among denitrification (increased $\delta^{15}NO_3^-$ with loss of NO_3^-, ε = negative), nitrification (decreased $\delta^{15}NO_3^-$ with increased NO_3^-, ε = positive, or if conversion of NH_4^+ to NO_3^- is complete $\varepsilon = 0$ ‰) and assimilation (no change in $\delta^{15}NO_3^-$ with loss of NO_3^-, $\varepsilon = 0$ ‰) (Lund et al. 1999).

Reported isotope enrichment factors for denitrification are based on field data as well as laboratory studies (e.g. (Böttcher et al. 1990, Knoller et al. 2011, Lehmann et al. 2003, Mengis et al. 1999)). For several decades, the dual isotope approach investigating both nitrogen and oxygen isotopic composition of NO_3^- has been successfully used to delineate NO_3^- sources, to reveal NO_3^- transport pathways, and to identify N transformation processes such as nitrification or denitrification in aquifers (e.g. (Aravena & Robertson 1998, Bohlke & Denver 1995, Einsiedl & Mayer 2006, Osenbruck et al. 2006)), lakes (e.g. (Bozau et al. 2006, Knoeller & Strauch 1999, Lehmann et al. 2003)), marine environments (e.g. (Brandes et al. 1998, Casciotti & McIlvin 2007, Voss et al. 2001)), and wetlands (e.g. (Itoh et al. 2011, Sidle & Goodrich 2003)).

Stable N isotope ratios were measured for estimating denitrification in a large CWs in southern California (Lund et al. 1999). ^{15}N enrichment factors were calculated, and they were significantly different from those found before in laboratory and groundwater studies where denitrification contributed to NO_3^- losses. The possible explanation can be an extensive macrophyte growth in these CWs, which was absent in other systems. Also, a quantitative assessment of the contribution of denitrification to the overall NO_3^- losses can be made only

by making some assumptions to the impacts of plant assimilation and nitrification on $\delta^{15}N$ in wetland systems. Therefore, future research in evaluation of ^{15}N enrichment for plant assimilation and nitrification in correlation with the denitrification for the overall N transformation in wetlands is necessary.

Recently, a new attempt to assess denitrification by using stable N isotope fractionation in small CWs was performed (Sovik & Morkved 2008). Based on the method offered by Lund et al. (Lund et al. 1999), the denitrification and assimilation by plants rates were estimated. ^{15}N enrichment factors were similar to the former study, smaller than in laboratory experiments but comparable to what has previously been found in groundwater and large wetlands studies. However, use of the method to quantify the contribution of denitrification to the N removal is still questionable due to a lot of assumptions which are a base for the method. Furthermore, there has been found in other investigations of the same working group, that the method of measuring the natural abundance of $\delta^{15}N\text{-}NO_3^-$ is not suitable for estimating denitrification contribution to NO_3^- removal in CWs treating wastewater, when NH_4^+ and organic N concentration in inlet are high and the system is partially aerobic (Sovik & Morkved 2007).

A conceptual model for the quantification of both nitrification and denitrification within any one site of the wetland was first developed by Erler and Eyre (Erler & Eyre 2010). For this aim, the natural abundance and stable isotope amendments were combined. Also, the net isotope effect (η) of nitrification was quantified; however, η was dependent on the rate constants that control denitrification, and consequently, further estimations of denitrification are non-reliable. Therefore, the method is only valid for the relationship between η and the actual rate of denitrification. Investigations are needed to show the proportions between η and the rate of denitrification, then, more detailed natural abundance measurements can be used to compare denitrification rates within the CW.

An isotope mass balance approach was applied to small CWs in Central Switzerland (Reinhardt et al. 2006). The natural isotopic composition of NO_3^- as well as NH_4^+, biomass, and sediment particular N were examined in the wetlands. N isotope fractionations of such biological processes, as nitrification, mineralization and denitrification were assessed and quantified, and factors which potentially influence these values were examined. Also, conclusions were made on how isotopic signatures can be used for designing management strategies for wetlands of particular interest. However, the information was limited as samples were taken only from inflow and outflow.

Advances in stable isotope methods allow getting accurate measurements at very low concentrations. Measurements of the $^{15/14}N$ natural abundance of both NO_3^- and NH_4^+ should be used to identify spatial variations in N transformations at a fine spatial resolution throughout the CWs with different substrates. Another special interest in N isotope investigations in CWs can be comparing pathways of N removal in different types of CWs, and investigating seasonal variations in N transformation processes in CWs, especially when getting NH_4^+ rich inflowing wastewater.

1.5 Aim of the work

The objective of this work was to improve the basic knowledge about nitrogen transformation processes in constructed wetlands. Therefore, experiments for investigating the nitrogen removal in pilot-scale constructed wetlands have been performed. The thesis will contribute to filling the lacks and will address and seek answers to the following topics:

- Better understanding of the dynamics of nitrogen cycle processes, and particularly the spatial distribution of nitrogen species in constructed wetlands and the seasonal variations.
- Investigations of nitrogen transformation processes by stable isotope fractionation for different types of constructed wetlands.
- Getting enrichment factors for each individual nitrogen transformation process in constructed wetlands.
- Exploring the process of anammox and its interaction with other processes of nitrogen cycle in horizontal subsurface-flow constructed wetlands.
- Clarifying the role of anammox in nitrogen removal in horizontal subsurface-flow constructed wetlands.
- Measuring bacterial abundance and activity of main processes of nitrogen cycle at different locations in constructed wetlands.
- Understanding of the key factors influencing the efficiency of nitrogen removal in constructed wetlands.

2. Materials and methods

2.1 The Leuna Megasite and constructed wetland design

The CWs in the pilot-scale experimental study site were built as a part of CoTra (Compartment Transfer) project in June 2007 at the Leuna Megasite near Leipzig, Germany (Fig. 2.1-1). As Leuna has been a location of chemical industry since the beginning of the last century, a range of contaminants has been spilled into groundwater due to accidental spills, improper handling, and damages due to heavy bombing during World War II. Consequently, the contamination is complex, and the main pollutants are petroleum hydrocarbons (BTEX), methyl *tert*-butyl ether (MTBE), and NH_4^+ (Martienssen et al. 2006). The main goal of the project was to develop a near-natural groundwater remediation technique for contaminated megasites by transferring of contaminated groundwater from anaerobic environments to mosaic aerobic/anaerobic environments, which enhances biodegradation/transformation processes. This can be realized using CWs.

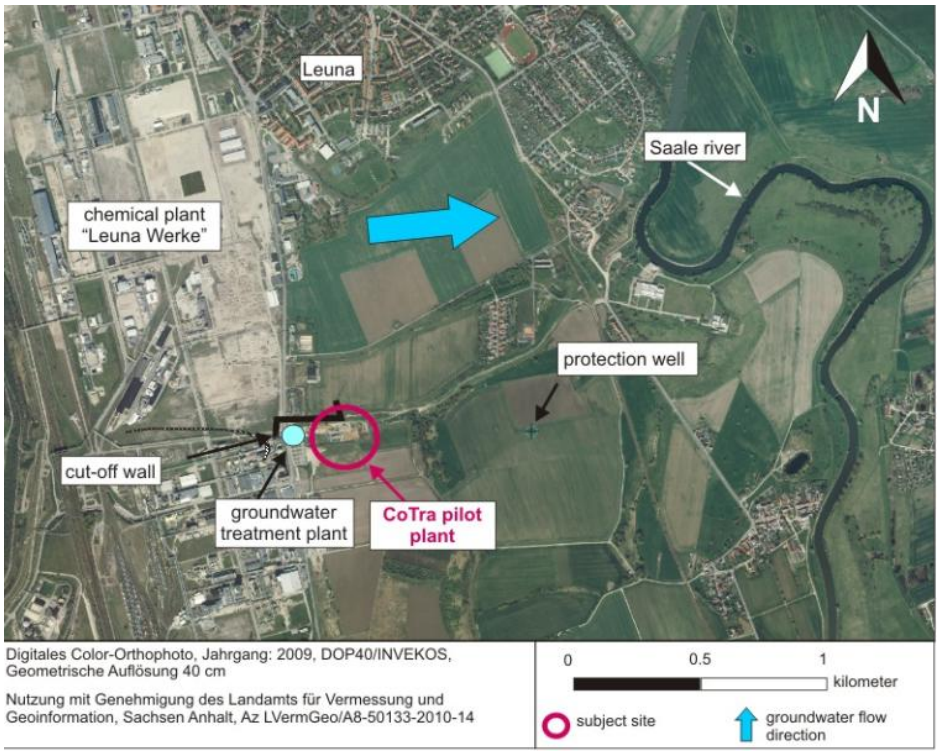

Fig. 2.1-1 The Leuna Megasite

It enabled the investigation of different near-natural groundwater treatment wetland systems, e. g. HSSF CW, VSSF CW, aerobic trench systems, tidal flow CW and FPRM systems, with the focus on optimizing and understanding the removal processes of the main contaminants BTEX, MTBE and NH_4^+ (Fig. 2.1-2). The experimental plant was equipped with the ability of online measurement for water parameters, and the in- and outflow volumes were recorded using a flow meter. Regular sampling of the organic contaminants and inorganic compounds was provided by staff and delivered the basic concentration data for the in- and outflow. Additionally, sampling campaigns within the systems along the flow path were also

undertaken. A local weather station measured rainfall, ambient temperature and solar radiation.

There were three experimental CWs taken into consideration for this study. The operation conditions of the systems were as follows:

S11 - FPRM, established in April 2008 with water level of 15 cm, after September 2009 water level at 30 cm;

S15 - unplanted HSSF CW, established in June 2007; water level at 40 cm;

S16 - planted HSSF CW, established in June 2007; water level at 40 cm.

Fig. 2.1-2 The pilot scale plant in Leuna (courtesy of M. Kaestner)

All the CWs were placed in containers with the dimension of 5 m × 1.1 m × 0.6 m. The HSSF CWs were filled up with gravel (grain size 2-3.2 mm) up to a height of 50 cm, and the water level was set to 40 cm, resulting in a vadose zone of 10 cm. The FPRM was filled with plant root mats, with plants only supported by the densely woven root bed (no gravel). Both planted CWs (S11 and S16) were planted with common reed (*Phragmites australis*). The contaminated groundwater was pumped from a 15 m depth well at an inflow rate of 6 L h^{-1} in each wetland. The theoretical hydraulic retention time (assuming no water loss) was therefore 6.88 days for the gravel-based CWs. Main characteristics of inflow water during the period of investigations are shown in Table 2.1-1.

Table 2.1-1 Average (n = 37) concentration of organic and inorganic contaminants of the groundwater used as the wetland's influent in Leuna (2012-2013)

Contaminant	**Mean concentration [mg L^{-1}]**	**Standard deviation**
Benzene	4.1	4.0
Methyl-*tert*-butyl ether (MTBE)	0.4	0.4
NH_4^+-N	23.4	5.0
NO_2^--N	0.2	0.2
NO_3^--N	b.d.l.	-
Total organic carbon (TOC)	17.5	7.0
Chemical oxygen demand (COD)	45.0	22.0
The five-day biological oxygen demand (BOD_5)	21.0	14.0
b.d.l. - below detection limit		

2.2 Sampling procedure

Inflow and outflow water samples as well as pore water samples were collected every two weeks from July 2012 through June 2013, with exception of winter season (November 2012 – March 2013), making a total of 14 sampling days. Water from the filter was pumped using a peristaltic pump (REGLO digital, Ismatec) from several points distributed along the flow path (1 m, 2.5 m, 4 m) and the depth of the filter (0.2 m, 0.3 m, 0.4 m for HSSF CW; 0.3 m for FPRM). The temperature and redox potential were measured on-site using a flow-through cell equipped with a redox electrode ($Pt/Ag^+/AgCl/Cl^-$; Sentix ORP, WTW, Germany). pH was measured in situ using a SenTix41 electrode with pH 537 Microprocessor (WTW, Weilheim, Germany). The samples for NH_4^+, NO_2^-, and NO_3^- concentrations were transferred into 25 mL brown glass bottles and measured in the field laboratory. An additional 50 ml of sample were collected for NO_3^- isotope analysis, and 0.5-2 L of sample (dependent on NH_4^+ concentration) for NH_4^+ isotope analysis was acidified to pH 2 using 98 % sulfuric acid. All samples were stored at 4^oC until analysis. Brief overview of the measured parameters is shown in Table 2.2-1.

Table 2.2-1 Overview of measured parameters in the CWs

Method type	Parameter	Measurement System/Method
Physico-chemical parameters	Eh	Using Pt-Ag^+/AgCl/Cl^--electrodes
	pH	Using pH 537 microprocessor and SenTix 41 pH electrod
	Ammonia-N Nitrate-N Nitrite-N	Ion chromatography, with UV and conductivity detectors
	CH_4 N_2O MTBE Benzene	Gas chromatography
N isotope fractionation	Natural abundances of $^{15/14}N$ in NH_4^+ Natural abundances of $^{15/14}N$ in NO_3^-	Isotope ratio mass spectrometry
N tracer experiments	N_2 production rates from N transformations	Mass spectrometry
Molecular biology	Abundance of specific bacteria Determination of species of bacteria	Q-PCR with specific primers Cloning and sequencing

2.3 Analytical methods

Concentrations of N-compounds

Analysis of inorganic ions (NH_4^+, NO_2^-, NO_3^-) was conducted using a photometer (Spectroquant® Nova 60, Merck) and the Merck quick tests (number 1.00683.0001 for NH_4^+, 1.09713.0001 for NO_3^-, and 1.14776. for NO_2^-).

Natural abundances of $^{15/14}N$ in NH_4^+ and NO_3^-

NH_4^+ was liberated from aqueous solutions (samples) as NH_3 under basic pH and re-dissolved in a standard solution of sulfuric acid. The preparation of NH_4^+ followed the basic principle of a Kjeldahl distillation (Saez-Plaza et al. 2013). The resulting NH_4^+ sulfate was homogenized and weighed into tin capsules that were combusted in an elemental analyzer (EA) Vario ISOTOPE Cube (Elementar Analysensysteme GmbH, Hanau) connected to an isotope ratio mass spectrometer (IRMS) Isoprime 100 (Isoprime Ltd, Cheadle Hulm). For nitrification incubations, NH_4^+ isotopic composition was measured by oxidizing NH_4^+ to NO_2^- using a bromate solution, and then reacting with acetic acid buffered sodium azide to create N_2O (Zhang et al. 2007). The $^{15/14}N$ of the resultant N_2O was measured on an IRMS Delta V plus (Thermo Electron GmbH, Bremen) with a Gasbench II (Thermo Electron GmbH, Bremen). The $^{15/14}N$ is expressed as delta (δ)-notation in per mil (‰) relative to the

international referenced material AIR. Each sample batch was run with the international reference materials for calibration USGS25 ($\delta^{15}N$: -30.4‰), and USGS26 ($\delta^{15}N$: +53.7‰) (Adamsen & Reeder 1983), plus an internal standard ($\delta^{15}N$: 0‰). For both methods the analytical precision was ±0.4‰.

The NO_3^- isotope signature was determined using the denitrifier method (McIlvin & Casciotti 2011) and measured on a IRMS Delta V plus (Thermo Electron GmbH, Bremen) with a Gasbench II (Thermo Electron GmbH, Bremen). That method allowed for a measurement of $\delta^{15}N$ and $\delta^{18}O$ by measuring N_2O produced by controlled reduction of sample NO_3^- by the bacterial strain *Pseudomonas chlororaphis* (ATCC #13985) in 12 ml Exetainer vials. Analytical precision for $\delta^{15}N$ was ±0.4 ‰ and ±1.6 ‰ for $\delta^{18}O$. For calibration of the N and O isotope measurement, the references NO_3^- IAEA-N3 ($\delta^{15}N$: +4.7 ‰ AIR; $\delta^{18}O$: +25.6 ‰ VSMOW), USGS32 ($\delta^{15}N$: +180 ‰ AIR; $\delta^{18}O$: +25.7 ‰ VSMOW), and USGS 35 ($\delta^{15}N$: +2.7 ‰ AIR; $\delta^{18}O$: +57.5 ‰ VSMOW) were used. (McIlvin & Casciotti 2011)

Organic compounds

The samples for benzene and MBTE determination were analysed by means of a headspace GC with a flame ionization detector (FID) (Agilent 6890 GC). The size of the (Agilent DB-MTBE) column was 30 m × 0.45× mm × 2.55 µm, and the following procedures were performed: 35°C (6 min), 4°C/min to 120°C, 20°C/min to 280°C (5 min). N was used as the carrier gas. Prior to the analysis, the samples were equilibrated at 80°C for 30 minutes.

Nitrous oxide and methane

CH_4 and N_2O analyses of water samples were carried out by a modified GC system consisting of a static headspace sampler (DANI HSS 86.50) coupled to a GC-14B (Shimadzu). Samples are automatically heated up to 60°C and shaken rigorously for 7 min prior to GC analysis. Then an aliquot of a headspace gas sample is transferred to a sample loop (3 ml) by pressurising the sample vial with nitrogen. To prevent entering of halogens into the GC an ascarite trap was installed between vial and the sample loop. The sample loop is then switched into the carrier gas stream of the GC. The carrier gas (N_2) has a mean flow rate of 35 ml min^{-1} at a total column pressure of 200 kPa. Gas separation is achieved by a 1/8”column (4 m) packed with HayeSep Q 80/100 mesh (Supelco, Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The GC column consists of a pre-column (1 m) and a separation column (3 m). A back flush regime is installed in between to prevent water and high-boiling components from entering the separation column. Oven temperature is set to 35°C. The GC is equipped with FID for measuring CH_4 and an electron capture detector (ECD) for N_2O analysis. For N_2O detection Argon/Methan (90 %/10 %) is additional supplied as make-up gas to improve ECD sensitivity and stability. A standard gas calibration was carried out prior to gas sample analyses by using four different standard gases with five replicates (Linde AG, Pullach, Germany) (CH_4: 0.33, 0.97, 5.02, 409.8 ppm; N_2O: 0.79, 5.03, 17, 123.6). For each GC run standard gas with highest concentration was additionally analysed (five replicates) to perform daily correction.

2.4 Water balance and contaminated mass loads calculations

The loss of water due to evapotranspiration has to be considered for the calculation of the contaminant loads in the water. The water balance depends primarily on evapotranspiration, which is affected by ambient temperature, wind conditions, air humidity and the physiological activity of the plants. These environmental factors, depending on the season and daily weather conditions, may cause a dilution or accumulation of the substances in the pore water of CWs.

The percentage of water loss (V_{wl} (Δt)) during a defined period (sampling day) were calculated as per Eq. 1. The difference between the influent water volume, precipitation and the effluent water volume results in water loss from the system:

$$V_{wl}(\Delta t) = \frac{V_{in}(\Delta t)+V_{rain}(\Delta t)-V_{out}(\Delta t)}{V_{in}(\Delta t)+V_{rain}(\Delta t)} \times 100 \qquad (1)$$

where V_{in} (Δt), V_{out} (Δt) and V_{rain} (Δt) are the inflow volume, outflow volume and volume of rainfall registered during time period Δt, respectively. Considering the significant water losses, the concentrations of N compounds do not reflect the actual N mass fluxes. Therefore, daily loads along the flow path from inflow to outflow were calculated and correlated with N dynamics along the flow path. The contaminant mass loads were calculated on the basis of water volume flow rates and contaminant concentrations. The decrease of contaminant mass load along the flow path was calculated assuming that the water loss was linear over distance, which means the water flow rate at each calculated distance was recalculated by the linear water loss (Eq. 2).

$$M_{ij} = C_{ij} \times \left[V_{in} - \frac{(V_{in}-V_{out})}{L} \times L_i\right] \qquad (2)$$

Here, M_{ij} is the contaminant mass flux in mg d^{-1} at distance *i* m and depth *j* cm; C_{ij} is the contaminant concentration in mg L^{-1} at distance *i* m and depth *j* cm; V_{in} is the inflow rate in L d^{-1}; V_{out} is the outflow volume in L d^{-1}; L_i is the distance from the inflow at point *i* m; *L* is the length of the CW in m.

2.5 Microbial community characterization and molecular biology tests

DNA extraction and quantitative polymerase chain reaction assay

Samples of gravel and roots in HSSF CW were taken from 9 sampling points: according to the distance from the flow path, 1 m, 2.5 m, and 4 m, and according to depth at 0.2 m, 0.3 m, and 0.4 m. DNA was isolated from 0.82±0.06 g of mixed sample of gravel and roots using the Fast DNA® SPIN Kit for Soil and the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA). The quality of DNA was checked with PCR targeting 16S rRNA gene and the products were run on the 1.0 % agarose gel 20 min at 120 mV (Fig. 2.5-1).

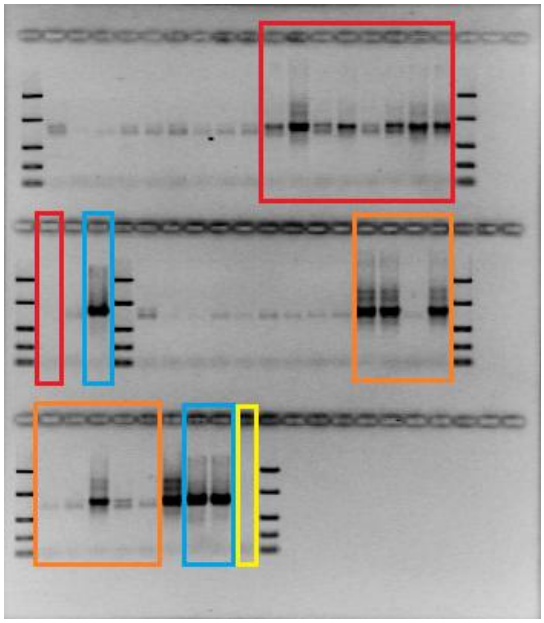

Fig. 2.5-1 The agarose gel with products of the PCR targeting 16S rRNA gene, where original samples are labeled in red; sample dilutions (1:10) are labeled in orange; positive controls are labeled in blue; negative control is labeled in yellow

The copy numbers of nitrogen transformation gens were determined by quantitative PCR. qPCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems) and published primer sets for *hzsA* (Harhangi et al. 2012), *nirS*, *nirK*, *nosZ*, *amoA* and 16S rRNA genes (for sequences, coding genes and annealing temperature see Table 2.2-3). A dilution series of cloned 16S rRNA, *amoA*, *nirS*, *nirK*, *nosZ* and *hzsA* gene fragments (pGEM-T easy; Promega, Madison, WI, USA) were used to prepare DNA standards with known quantities of target DNA (insert source see Table 2.5-1).

Reactions were carried out using KAPA™ SYBR® FAST qPCR MasterMix. PCR efficiency for the different assays ranged between 84 and 98 %. Diluting of DNA extracts was performed in order to avoid inhibition of PCR by inhibitors contained in the gravel material.

Table 2.5-1 Primers and standard information of performed qPCRs

Gene	Primer	Sequence (5´-3´)	Annealing temp. (ºC)	Reference	Standard clone
16S rRNA gene	*Nad F*	TCCTACGGGA GGCAGCAGT	57	(Nadkarni et al. 2002)	*Pseudomonas putida*
	Nad R	GGACTACCAG GGTATCTAAT CCTGTT			
hydrazine synthase	*hzsA 1597F*	WTYGGKTATC ARTATGTAG	55	(Harhangi et al. 2012)	*Kuenenia stuttgartiensis*
	hzsA 1857R	AAABGGYGAA TCATARTGGC			
anammox 16S rRNA gene	*amx381F*	TTCGCAATGC CCGAAAGG	59	(Schmid et al. 2003)	*Kuenenia stuttgartiensis*
	amx550R	GAACAACGCT TGCCGCCTCT			
a subunit of ammonium mono-oxigenase, *amoA*	*amoA 1F*	GGGGTTTCTA CTGGTGGT	57	(Rotthauwe et al. 1997)	*Nitrosomonas europaea*
	amoA 2R	CCCCTCKGSA AAGCCTTCTT C			
dissimilatory nitrite reductase, *nirS*	*nirS cd3AF*	GTSAACGTSA AGGARACSGG	57	(Throback et al. 2004)	*Pseudomonas stutzeri*
	nirS R3cd	GASTTCGGRT GSGTCTTGA			
dissimilatory nitrite reductase, *nirK*	*nirK 1F*	GGMATGGTKC CSTGGCA	45	(Braker et al. 1998)	*Blastobacter denitrificans*
	nirK 5R	GCCTCGATCA GRTTRTGGTT			
Nitrous oxide reductase, *nosZ*	*nosZ 2F*	CGCRACGGCA ASAAGGTSMS SGT	65	(Henry et al. 2006)	*Paracoccus denitrificans*
	nosZ 2R	CAKRTGCAKS GCRTGGCAGA A			

Cloning, sequencing, and phylogenetic analysis of anammox

The qPCR products targeting the *hzsA* gene were purified using the MinElute PCR Purification Kit (Qiagen, Chatsworth, CA), ligated and cloned using the pGEM-T Easy Vector System according the manufactures protocol (Promega, Madison, WI, USA). Sixty-eight colonies were picked up, amplified with M13 primers (M13-F GTTTTCCCAGTCACGAC, M13-R CAGGAAACAGCTATGAC) and sequencing was performed at the Macrogen sequencing facility (Macrogen Inc., Amsterdam, Netherlands). Sequences were aligned using BioEdit Sequence Alignment Editor program (version BioEdit v7.2.5; North Carolina State University, Raleigh, NC). Phylogenetic analysis was carried out using the Mega 6.0 (Tamura et al. 2013).

2.6 Laboratory experiments for enrichment factor determination

Denitrification

The site-specific enrichment factor for denitrification was measured by placing 40 g of homogenized samples of gravel and roots from different depths and distances into serum bottles and filling with 0.12 L of inflow water. The experiment was running in three replicates. Also, 15 ml of 3-(N-morpholino) propanesulfonic acid (MOPS) buffer was added to the final concentration of 50 mM. Then bottles were flushed with N_2 gas and sealed. Samples were preincubated for 24 hours at 20°C in the dark with shaking in order to remove residual oxygen. Subsequently, organic C (equimolar mixture of lactate, acetate, benzoate, and ethanol) and NO_3^- were added to each bottle to bring them up to a final concentration of 7 mg NO_3^--N L^{-1}. Furthermore, 20 ml of sample was taken at 1, 2, and 3.5 h after substrate addition with a syringe, filter with 0.25 mm and stored at +4°C until analysis. Sampling points were chosen based on preliminary experiment, where 90 % of NO_3^- was removed after 3.5 hours.

Nitrification

The site-specific enrichment factor for nitrification was determined in an analogous way. 100 g of homogenized samples of gravel and roots from planted HSSF CW were placed in 500 ml Schott bottles with 0.3 L of inflow water. The experiment was running in three replicates. Samples were covered by screw caps to allow air access and incubated at 20°C in the dark with shaking. Subsequently, NH_4^+ was added to the final concentration of nearly 150 mg NH_4^+-N L^{-1}. 0.025-0.1 L of sample dependent on NH_4^+ concentration was taken with a syringe immediately after substrate addition, and then again after 44, 75, and 100 hours. Concentration of NH_4^+ was measured immediately, and for isotope measurements samples were acidified to pH 2 by 98 % sulfuric acid and stored at +4 °C until analysis.

It should be noted that these site-specific enrichment factors were measured using only substrate from the HSSF CW because only this system had consistent N removal throughout

the year, and therefore, the enrichment factors were the most representative for the microbial N transformation processes occurring in CWs.

2.7 Measuring N-transformation rate with ^{15}N isotopic tracing method

Anammox and denitrification

For anammox and denitrification potential turnover rates determination, the same sampling schema as for molecular biology investigations was used. However, samples were taken only from 3 points: 1 m distance from inflow and 0.2 m depth, 2.5 m distance from inflow and 0.3 m depth, and 4 m distance from inflow and 0.4 m depth. The presence, activity, and potential of anammox and denitrifying bacteria were measured as described by Risgaard-Petersen et al. (2004). Homogenized samples of gravel and roots of known weight and density (16.28±1.27 g, 1.98±0.14 g cm^{-3}) were transferred to the 22 mL glass vials together with inflow water, purged by N_2, and sealed. The experiment was running in three replicates. The slurries were then pre-incubated for 24 h to remove NO_x^- in sediment and incubation media. Subsequently, 100 µl of N_2-purged stock solution of each isotopic mixture, i.e. (1) $^{15}NH_4^+$ (^{15}N at.%: 98), (2) $^{15}NO_3^-$ (^{15}N at.%: 98), and (3) $^{15}NH_4^+$ (^{15}N at.%: 98) + $^{14}NO_3^-$ was added resulting in a final concentration of about 100 µM N. Incubations in the dark and at 20 ºC were stopped at intervals 0 h, 2 h, 4 h, and 6 h by adding 200 µl of a 7 M $ZnCl_2$ solution. The experiment was repeated but only set $^{14}NH_4^+$ 1 mM + $^{15}NO_3^-$ 200 µM was used with longer incubation time of 12 h, 24 h, and 48 h.

The analyses of ^{15}N-N_2 abundance in the samples were conducted using a GCMS-QP2010Plus (Shimadzu). The system is equipped with a ShinCarbon ST column (100/120 mesh, 1.33 m × 1 mm ID, Restek) connected downstream via an automatic 6-port-valve (Valco Instruments Co. Inc.) to a Molsieve 5A column (10 m × 0.53 mm ID, 0.50 µm film, Agilent) and helium as the carrier gas. Gas samples were applied via a sample loop attached to a second automatic 6-port-valve (Valco Instruments Co. Inc.), which is assembled between the injector outlet and ShinCarbon ST inlet. The concentration of N_2 was calculated based on a calibration function gained from standard gas calibration (N_2 2530 ppm, loop size: 0.01, 0.5, and 2 mL, n=5 for each loop size). For N_2 analysis a gas aliquot of 500 µL was withdrawn from the headspace of a sample vial by gas-tight syringe and flushed through a 100 µL sample loop (attached to the 6-port-valve). Afterwards, the sample loop was immediately switched into the GCMS carrier gas stream and masses $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ were determined. The system set-up for N_2 analysis was as follows: 40°C oven temperature, 200 °C I/F and ion source temperature, total flow 14 mL min^{-1}, column flow 8 mL min^{-1}.

The rate of anammox and denitrification were calculated from the production of $^{29}N_2$ in the samples amended with $^{15}NH_4^+$ + $^{14}NO_3^-$ and $^{30}N_2$ in the samples amended with $^{15}NO_3^-$ respectively using the equations of Spott and Stange (2011). Given that the HSSF CW had maximum N removal in summer, the incubation temperature for the calculation of potential turnover rates under simulated *in situ* conditions in this system was chosen based on summer average air temperature (+20°C) (Coban et al. 2014).

Potential ammonia oxidation rates

For NH_3 oxidation potential turnover rates determination, pool enrichment/dilution method was used according to Wessel and Tietema (1992). The sampling points were chosen analogous with denitrification and anammox incubations. Homogenized samples of gravel and roots from HSSF CW of known weight and density (178.55±16.78 g, 1.98±0.14 g cm^{-3}) were placed in 500 mL Schott bottles with 0.1 L of inflow water. The experiment was running in three replicates. Samples were closes with air access and incubated at +20°C in the dark with shaking. Subsequently, two sets of incubations were set up. To the first set 5 mL 10 % of $^{15}NH_4^+$ with 98 at.% was added to the final concentration of 177.38 mM NH_4^+ L^{-1}; to the second 1 mL 10 % of $^{15}NO_3^-$ with 98 at.% was added resulting in the final concentration of 7.08 mM NO_3^-.

Between 6 - 8 mL of sample was taken with a syringe immediately after substrate addition, and then again after 3, 12, and 24 h. Samples were filtered via 0.2 mm and stored in +4°C until analysis. In all incubations atom% and concentration of $^{15}NO_3^-$ were measured. For measurements, the SPINMAS technique was used which is a direct coupling of a SPIN (sample preparation unit for inorganic N) to a common quadropole Mass Spectrometer (GAM 400, InProcess Instruments GmbH, Bremen, Germany) (Stange et al. 2007).

In the ammonia oxidation rates experiment, the net nitrification rates in $^{15}NH_4^+$ amendments were determined using the isotope mixing equation (e.g., (Spott et al. 2006)) and the $^{15}NO_3^-$ amendments were used to determine the gross nitrification rate and the NO_3^- consumption rates by the pool dilution approach using the equations (3) and (4) respectively from Wessel and Tietema (1992):

$$p = \frac{ln\frac{f_t - k}{f_0 - k}}{ln\frac{W_t}{W_0}} \times \frac{W_0 - W_t}{t} \quad (3)$$

$$c = \left[1 + \frac{ln\frac{f_t - k}{f_0 - k}}{ln\frac{W_t}{W_0}}\right] \times \frac{W_0 - W_t}{t} \quad (4)$$

where p - gross production rate of the enriched pool,

c - gross consumption rate of the enriched pool,

f - ^{15}N abundance of the enriched pool,

k - naturally-present ^{15}N abundance,

W - amount of ^{14}N plus ^{15}N in the enriched pool,

t - time,

and 0 – initial state.

2.8 Data analysis

Mean values of two samples were taken for organic concentrations at one sampling point. Differences between treatments and over time and distance and depth were determined using analysis of variance (ANOVA). The statistical analysis was performed by SPSS Statistics 21 software, and the differences were regarded as significant at $p < 0.05$. Error bars represent ±1 standard deviation (SD).

2.9 List of equipment and chemicals

Equipment used in this work:

- a redox electrode ($Pt/Ag^+/AgCl/Cl^-$; Sentix ORP, WTW, Germany)
- a SenTix41 electrode with pH 537 Microprocessor (WTW, Weilheim, Germany)
- a photometer (Spectroquant® Nova 60, Merck)
- an elemental analyzer (EA) Vario ISOTOPE Cube (Elementar Analysensysteme GmbH, Hanau)
- an isotope ratio mass spectrometer (IRMS) Isoprime 100 (Isoprime Ltd, Cheadle Hulm)
- an IRMS Delta V plus (Thermo Electron GmbH, Bremen) with a Gasbench II (Thermo Electron GmbH, Bremen)
- a headspace GC with a flame ionization detector (FID) (Agilent 6890 GC) the (Agilent DB-MTBE)
- a modified GC system consisting of a static headspace sampler (DANI HSS 86.50) coupled to a GC-14B (Shimadzu)
- a 1/8"column (4 m) packed with HayeSep Q 80/100 mesh (Supelco, Sigma-Aldrich Chemie GmbH, Steinheim, Germany)
- the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA)
- the Macrogen sequencing facility (Macrogen Inc., Amsterdam, Netherlands)
- a GCMS-QP2010Plus (Shimadzu). The system is equipped with a ShinCarbon ST column (100/120 mesh, 1.33 m × 1 mm ID, Restek) connected downstream via an automatic 6-port-valve (Valco Instruments Co. Inc.) to a Molsieve 5A column (10 m × 0.53 mm ID, 0.50 µm film, Agilent)
- a second automatic 6-port-valve (Valco Instruments Co. Inc.)
- the SPINMAS technique which is a direct coupling of a SPIN (sample preparation unit for inorganic N) to a common quadropole Mass Spectrometer (GAM 400, InProcess Instruments GmbH, Bremen, Germany)

Chemicals used in this work:

- 98 % sulfuric acid
- the Merck quick tests (number 1.00683.0001 for NH_4^+, 1.09713.0001 for NO_3^- and 1.14776. for NO_2^-)
- bromate solution
- acetic acid

- sodium azide
- the Fast DNA® SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA)
- 1.0 % agarose
- the MinElute PCR Purification Kit (Qiagen, Chatsworth, CA)
- the pGEM-T Easy Vector System (Promega, Madison, WI, USA)
- 3-(N-morpholino) propanesulfonic acid (MOPS)
- sodium lactate
- sodium acetate
- sodium benzoate
- ethanol
- sodium nitrate
- ammonium chloride
- sodium nitrate (^{15}N at.%: 98)
- ammonium chloride (^{15}N at.%: 98)
- zinc chloride

3. Results and discussion

3.1 Comparison of HSSF CWs and FPRM for the removal of ammonium

This chapter gives the results of treatment performance of three wetland systems in Leuna in 2012-2013: the five-year old FPRM (S11) with a water level of 30 cm, the unplanted HSSF CW (S15) and the planted HSSF CW (S16).

3.1.1 Treatment performance

The efficiency of NH_4^+ removal for the investigated systems is shown in the Fig. 3.1-1. The results show that during summer and spring, the planted HSSF CW exhibited a statistically significant better performance than the unplanted HSSF CW for NH_4^+ removal; however, there was no significant difference between these systems during autumn. The FPRM showed a statistically significant better removal in comparison with the unplanted HSSF CW only in spring. Furthermore, the planted HSSF CW demonstrated better performance than the FPRM in summer season. The significant *p* values are presented in Table 3.1-1.

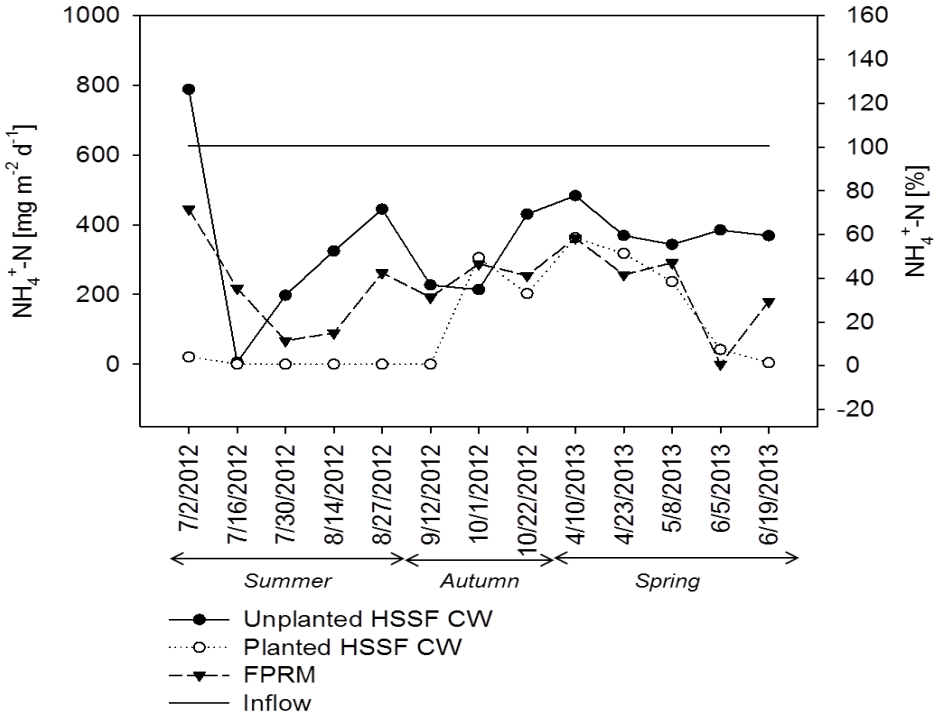

Fig. 3.1-1 NH_4^+-N loads in the inflow and outflow of the different constructed wetland types: unplanted HSSF, planted HSSF and FPRM. Mean inflow load is shown as a horizontal solid line

Table 3.1-1 ANOVA test for NH_4^+ outflow loads comparing the unplanted HSSF CW, the FPRM, and the planted HSSF CW in summer, autumn and spring seasons (n = 13)

	Unplanted HSSF-CW − FPRM	**Unplanted HSSF-CW − Planted HSSF-CW**	**FPRM−Planted HSSF-CW**
Summer	.384	**.029**	**.014**
Autumn	.582	.347	.467
Spring	**.032**	**.032**	.798

The importance of plants as substrate for microorganisms in our wetlands is clearly illustrated by the fact that the unplanted HSSF CW had statistically significantly lower NH_4^+-N removal in summer and spring period in comparison with the planted HSSF CW and in spring in comparison with the FPRM. Plant roots and rhizomes introduce surface area for attachment and successful growth of microorganisms. Moreover, by release of oxygen into the subsurface they enhance nitrification, and by release of organic carbon they enhance denitrification (Brix 1997). According to the presence of differences between hydroponic FPRM and gravel-based planted HSSF CW only in summer, presence of gravel as substrate for attachment and growth of microorganisms plays a minor role in the N removal in planted CWs.

Unplanted HSSF CW removed 53.6±32.2 % of inflowing NH_4^+-N in summer (n = 5), 55.4±20.5 % in autumn (n = 3), and 41.7±6.2 % in spring (n = 5). The planted HSSF CW had the following NH_4^+ removal efficiencies: 99.7±0.6 % in summer, 77.1±20.1 % in autumn, and 65.0±32.3 % in spring. FPRM achieved 73.2±8.4 % in summer, 63.5±2.6 % in autumn, and 61.6±28.2 % in spring. However, there were no statistically significant differences in removal efficiencies between seasons for planted HSSF CW ($F(2,8) = 2.061$, $p = .190$) as well as for FPRM ($F(2,8) = 0.459$, $p = .647$) and unplanted HSSF CW ($F(2,8) = 0.389$, $p = .690$). The lack of seasonal differences in removal efficiencies can be explained by that most of microbial N transformations occur at temperatures above 15°C, and throughout all these seasons the temperature was mainly higher than 15°C or slightly lower during autumn (Fig. 3.1-3) (Kuschk et al. 2003).

These degradation rates are significantly higher than those reported by Seeger et al. (2011) during summer season for the same systems (54±17% for planted HSSF CW and 41±7% for FPRM). However, this can be justified by the fact that NH_4^+-N concentration decreased from average 45 mg L^{-1} to 23 mg L^{-1} since 2009 when Seeger collected samples. This can reflect the increased removal efficiency with time, as the rate of removal remained constant at 23 mg NH_4^+-N L^{-1} over the entire investigation period of these systems. Vymazal (2007) reports average 42 % removal efficiency of HSSF CWs with average removed loadings 250 g m^{-2} yr^{-1}, which are similar to our removed loads (229 g m^{-2} yr^{-1}) for the planted HSSF SW in summer.

The spatial distribution of NH_4^+ loads was determined as a function of the distance from the inflow and the depth of the CWs (Fig. 3.1-2). The significant p values between depths, distances and treatments are given in Table 3.1-2. No significant differences were observed in NH_4^+ loads between the depths in all the systems except at 4 m distance from inflow in planted HSSF CW, indicating that the same processes were occurring at 0.2 and 0.3 m depths in this system. This can be explained by the fact that these depths had much higher root density in comparison with 40 cm as the main root growth of *Phragmites australis* was only recorded in the top soil zone down to a depth of 20-30 cm (Börnert 1990).

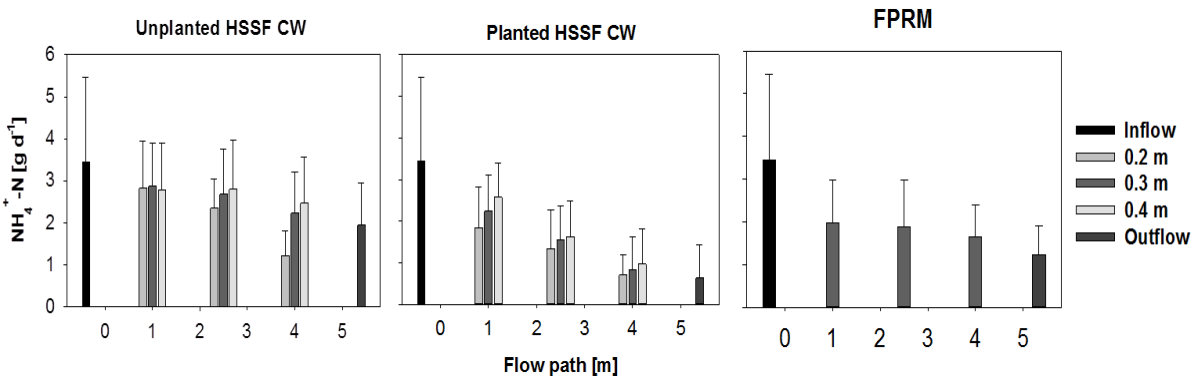

Fig. 3.1-2 Mean NH_4^+-N loads in the inflow, outflow and along the flow path and at three depths of the different constructed wetland types: unplanted HSSF, planted HSSF and FPRM (n = 13)

Table 3.1-2 ANOVA test for differences in NH4+ loads between depths, distances and three treatment systems (A: Unplanted HSSF CW; B: Planted HSSF CW; C: FPRM) in the unplanted HSSF CW, FPRM and the planted HSSF CW (n = 13)

	Depths		**Distances**[a]			**Treatments**		
	A	B	A	B	C	A-B	A-C	B-C
1 m	.976	.123	.153	**.004**	**.026**	**.010**	**.015**	.399
2.5 m	.499	.671	.359	**.001**	.844	**.000**	**.033**	.200
4 m	**.003**	.654	**.008**	**.000**	.509	**.000**	.309	**.001**
Outflow	-	-	.936	.395	.148	**.001**	**.045**	**.049**

a: Comparison of the concentration difference between 1 m with inflow, 2.5 m with 1 m, 4 m with 2.5m, and outflow with 4 m.

Considering the change in pollutant load along the flow distance from the inlet, a significant decrease in NH_4^+ loads was obtained in the unplanted HSSF CW between distances of 2.5 and 4 m, in the planted HSSF CW at distances up to 4 meter and only up to 1 meter in the FPRM (Figure 3.1-2, Table 3.1-2). It illustrates that most of NH_4^+ in the planted HSSW CW is removed up to 4 m distance. However, no significant removal after 1 m in the FPRM is evidence that this system was not as efficient for NH_4^+ removal as the planted HSSW CW. As for the unplanted HSSF CW, lack of statistically significant variations between distances demonstrates that this system has insignificant NH_4^+ removal except for 4 m distance from inlet. For the three treatment systems, significantly lower NH_4^+ loads were achieved in the planted HSSF CW compared to the unplanted HSSF CW along the flow path, at 1 m, 2.5 m, and outlet in the FPRM compared to the unplanted HSSF CW, and at 4 m and outlet in the FPRM compared to the planted HSSF-CW (Table 3.1-2).

Using the plant uptake rates reported by Molle et al. (2008) for *P. australis* during growth (0.2 to 0.67 g N m^{-2} d^{-1}), the percentage of N removed by plants in the HSSF CW was estimated to be from 32 to 100 % of the total N removal during summer season (0.63 g N m^{-2} d^{-1}), and from 80 to 100 % of the total N removal in spring (0.25 g N m^{-2} d^{-1}). Plant uptake was calculated to be responsible for between 43 and 100 % (summer) and between 54 and 100 % during spring of N removal in FPRM.

According to the differences between N removal efficiencies and estimated plant uptake, there are other processes responsible for N removal, which can be nitrification with further denitrification. For verifying this, stable isotope investigations were applied, what will be discussed later.

Confirming the finding of previous works (Lee et al. 2009, Vymazal 2007), plant presence was the key factor influencing N removal efficiency in these CWs. Based on the knowledge that the systems measured here are broadly representative of HSSF CWs and FPRM, it is then possible to use the high-resolution isotope data to untangle when and where, and to what extent different processes are driving N removal rates.

3.1.2 Physico-chemical parameters

Throughout the whole investigation period, pH in the systems varied within the range between 6.8 and 8.8, which is close to the optimum pH for nitrifying bacteria (Vymazal 2007). Daily air average temperatures are shown in Fig. 3.1-3, with average seasonal temperature 18.0°C in summer, 12.7°C in autumn, and 16.3°C in spring. As most of microbial N transformations occur at temperatures above 15^{o}C, temperature is an important factor influencing N removal transformation processes (Kuschk et al. 2003). Throughout all investigated seasons the air temperature was above 10°C (Figure 3.1-3), and therefore, the treatment efficiency of all studied CWs remained high.

As shown in Fig. 3.1-3, the water loss calculated from inflow and outflow streams varied between 14 % in spring and 99 % in summer for the planted HSSF CW and 9 % and 34 % respectively for the FPRM. The unplanted HSSF CW showed steady water loss 14±5 % throughout all the seasons. The FPRM as hydroponic system had free water surface and much lower plant density (observed visually). Considering this and statistically significantly higher water loss by planted HSSF CW in comparison with unplanted HSSF CW and FPRM ($F(2,39) = 25.148$, $p = .000$), loss of water in wetlands was mainly via transpiration by plants, not via evaporation from surface. Water loss is also evidence of plant activity, as clearly shown by lack of variation in the unplanted treatment. Thus, given that NH_4^+ is the preferred N source for plants (Kadlec & Wallace 2008, Wiessner et al. 2013), the maximum water loss in summer and spring would also mean that the plants are receiving relatively more NH_4^+ in these months than during autumn months.

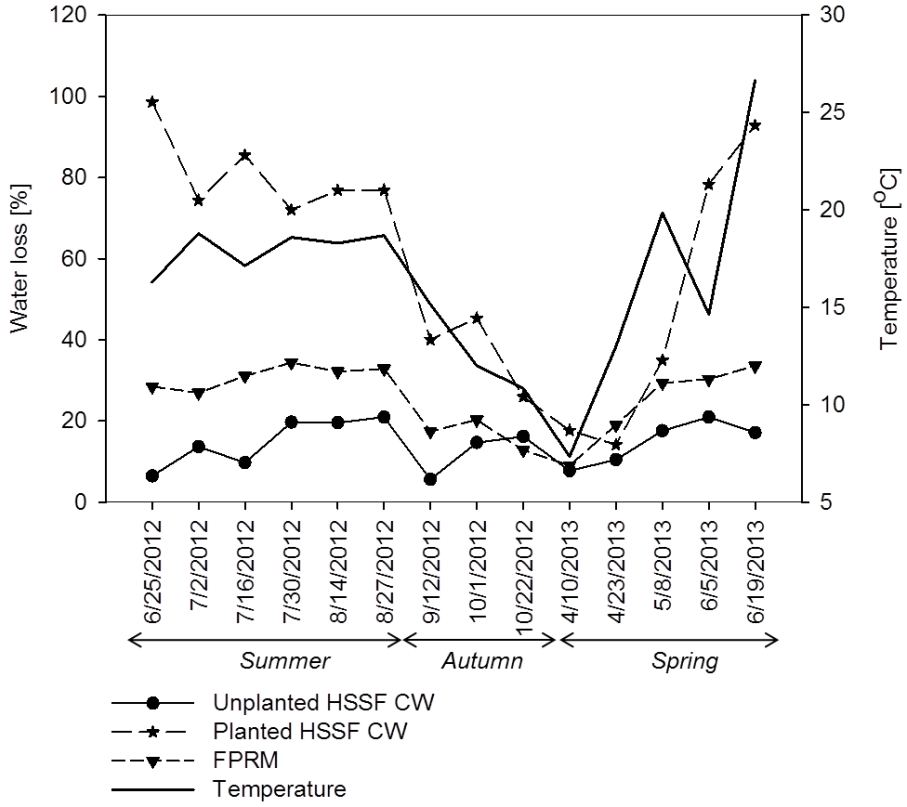

Fig. 3.1-3 Air temperature and water loss (Eq. 1) in pilot-scale CWs throughout the sampling period

Redox potential, which results are presented in Fig. 3.1-4, varied dependent on system, distance from inflow, and depth. There was a statistically significant difference in redox values between different wetland types ($F(2,348) = 97.799$, $p = .000$). The unplanted HSSF CW was characterized by stable redox conditions at ~24 mV. The most negative redox values were observed in the planted HSSF CW, and these decreased statistically significantly with distance from inflow ($F(4,138) = 26.046$, $p = .000$) and depth ($F(3,139) = 3.974$, $p = .009$) (from -71 to -119 mV). While some amount of oxygen entered the systems with the inflow, plant roots remained main oxygen source along the flow path. Root exudates (Brix 1997) and oxygen release by *P. australis* which can range from 0.02 to 12 g m^{-2} d^{-1} (Armstrong & Armstrong 1990, Brix 1990, Gries et al. 1990) both enhance microbial activity substantially. Therefore, low redox is product of the increased microbial turnover in the planted systems. Redox values in the FPRM were more positive than in the planted HSSF CW, between -66 and 0 mV, as this is a hydroponic system with free water surface. The redox potential data obtained show that all three systems were limited by oxygen. Chen (2012) showed that the oxygen concentration was less than 0.5 mg L^{-1} in May and less than 0.15 mg L^{-1} in July in the same systems. NH_4^+ and root exudates were probably competing with benzene and MTBE for electron acceptors (in particular oxygen). Thus, it can be assumed that the treatment performance of these three pilot-scale CWs could be limited by an insufficient oxygen input by the helophyte roots and the surface diffusion into their pore water.

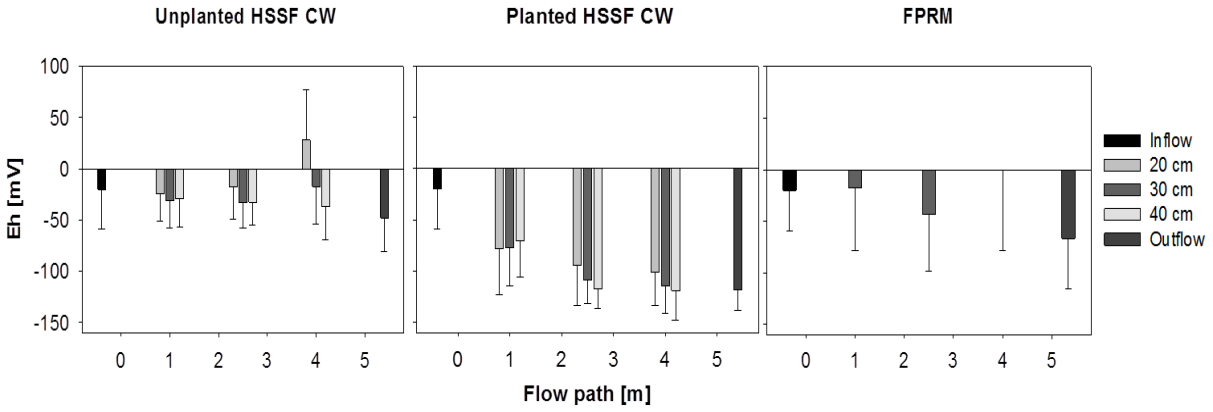

Fig. 3.1-4 Redox potential as a mean for all seasons in unplanted and planted HSSF CWs and FPRM (*n* = 13)

3.1.3 Other compounds

Organic contaminants

The spatial distribution of benzene and MTBE was determined as a function of the distance from the inflow and the depth of the CWs during the summer and autumn months (Fig. 3.1-5, 3.1-6). Considering the pollutants' loads changes along the flow distance from the inlet, a significant decrease in benzene was obtained in the planted HSSF CW and the FPRM ($F(4,10) = 8.344$, $p = .003$). There was no significant decrease in MTBE loads with the distance from the inlet for any system ($F(4,10) = 1.756$, $p = .214$). There was significant difference between depths in any system neither for benzene ($F(2,15) = .246$, $p = .785$) nor for MTBE ($F(2,15) = 1.401$, $p = .277$). The planted HSSF-CW showed statistically significant higher benzene ($F(1,18) = 15.612$, $p = .001$) and MTBE ($F(1,18) = 8.196$, $p = .010$) removal along the flow path than the unplanted HSSF-CW. Reasons for these observations might be that the plants enhance the microbial activity and phytovolatilization (Reiche et al. 2010). For all the distances in the FPRM the loads of benzene following the inlet were below 0.08 g d^{-1} and this system had statistically significant higher benzene removal in comparison with planted and unplanted HSSF CWs ($F(1,22) = 8.249$, $p = .009$). The same pattern was observed for MTBE ($F(1,22) = 8.283$, $p = .009$). In general, the results indicate that in the FPRM, more favorable conditions for benzene and MTBE removal occurred compared to both HSSF CWs along the flow path. Anaerobic degradation of petroleum hydrocarbons by microorganisms has been shown in some studies to occur only at negligible rates (Leahy & Colwell 1990). Given that the FPRM had free water surface and more aerobic conditions which was illustrated by the higher redox values (Fig. 3.1-4), it is not surprising then that this system had the highest benzene and MTBE removal.

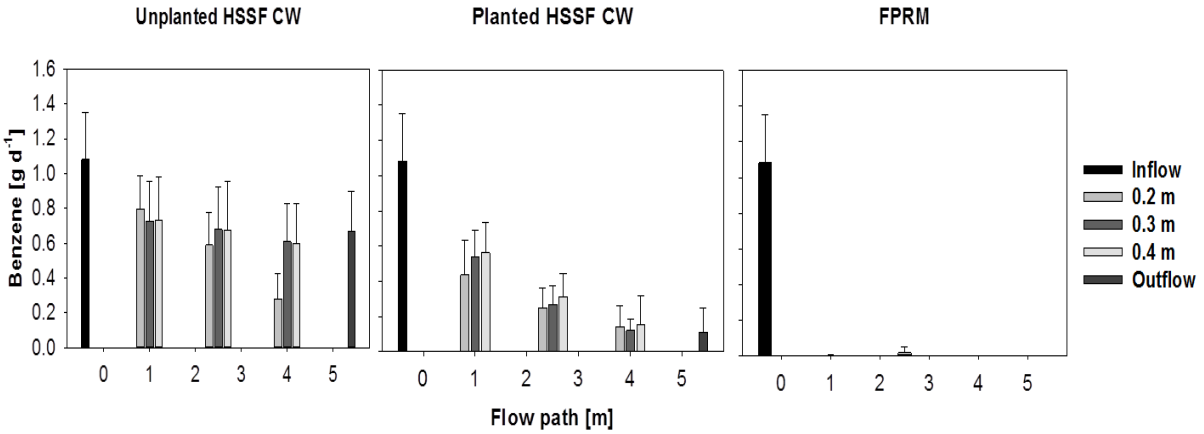

Fig. 3.1-5 Mean benzene loads in the inflow, outflow and along the flow path and at three depths of the different CW types: unplanted HSSF, planted HSSF, and FPRM (n = 6). For the FPRM the loads of benzene following the inlet were below 0.08 g d^{-1}

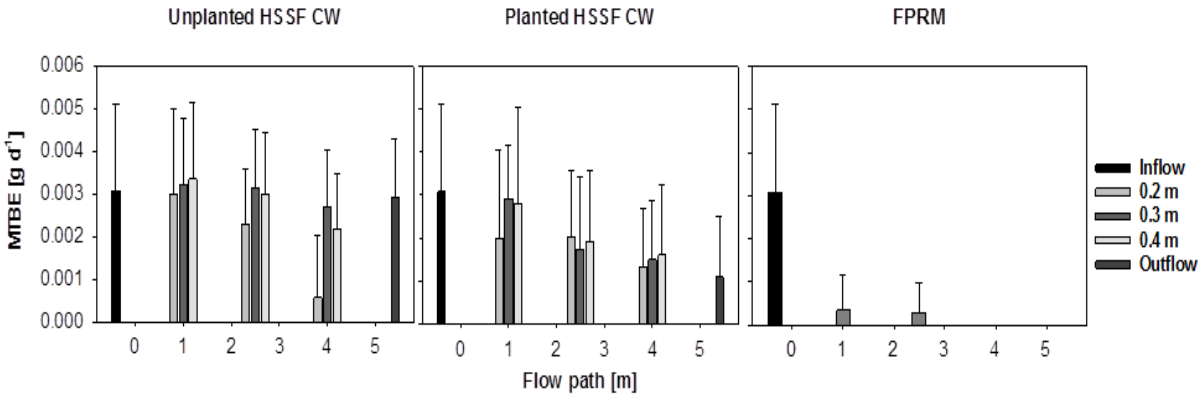

Fig. 3.1-6 Mean MTBE loads in the inflow, outflow and along the flow path and at three depths of the different CW types: unplanted HSSF, planted HSSF, and FPRM (n = 6)

Other nitrogen compounds

Both NO_2^- and NO_3^- were present along the flow path in all CWs. The spatial distribution of NO_2^- and NO_3^- loads was determined as a function of the distance from the inflow and the depth of the CWs. There were no changes in NO_2^- loads along the flow distance from the inlet ($F(3,20) = .550$, $p = .654$) and with depth ($F(2,18) = .200$, $p = .821$). Also, there was no difference between the treatments ($F(2,21) = .536$, $p = .593$). This leads us to the conclusion that NO_2^- was present in all CWs as intermediate of nitrification and/or denitrification processes. NO_2^- is toxic to the ammonia oxidizing bacteria (Stein & Arp 1998), and that is why no significant NO_2^- accumulation occurs as it is immediately oxidized or reduced after the formation. Presence of NO_2^- with no accumulation in our systems is evidence of nitritation process, i.e. oxidation of NH_4^+ to NO_2^-, and further either nitratation, i.e. oxidation of NO_2^- to NO_3^-, or denitrification, i.e. reduction of NO_2^- to N_2O or N_2.

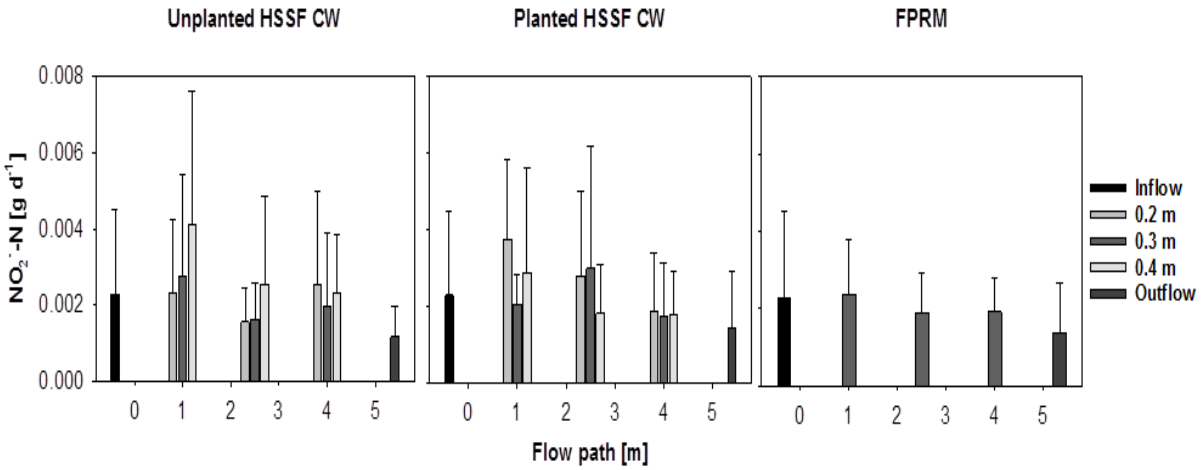

Fig. 3.1-7 Mean NO_2^- loads in the inflow, outflow and along the flow path and at three depths of the different CW types: unplanted HSSF, planted HSSF, and FPRM (n = 12)

As for NO_3^- loads, in any CW no accumulation or removal of NO_3^- was observed. There were no changes along the flow distance from the inlet ($F(3,20) = .122$, $p = .946$) and with depth ($F(2,18) = .469$, $p = .633$). As inflowing water contained only NH_4^+, all NO_3^- was formed via the nitrification process. However, it was not accumulated at any points in any system, what is evidence for simultaneous occurrence of nitrification and denitrification at every sampling point (Akratos & Tsihrintzis 2007). Since the planted HSSF CW had the highest NH_4^+ removal, this system also had the highest denitrification rates and therefore, the lowest apparent NO_3^- loads. The unplanted HSSF CW had higher NO_3^- loads ($F(1,18) = 8.395$, $p = .010$) along the flow path than the planted HSSF CW. This might be due to enhanced denitrification via plant root exudates as C source, which was absent in case of the unplanted HSSF CW. Also the FPRM had higher NO_3^- loads ($F(1,12) = 16.495$, $p = .002$) along the flow path than the planted HSSF CW. The FPRM had higher redox values in comparison with the planted HSSF CW, and therefore, was less favorable for the occurrence of the denitrification process, which requires anoxic conditions (Lee et al. 2009). There was no statistically significant difference between NO_3^- loads in the unplanted HSSF CW and the FPRM.

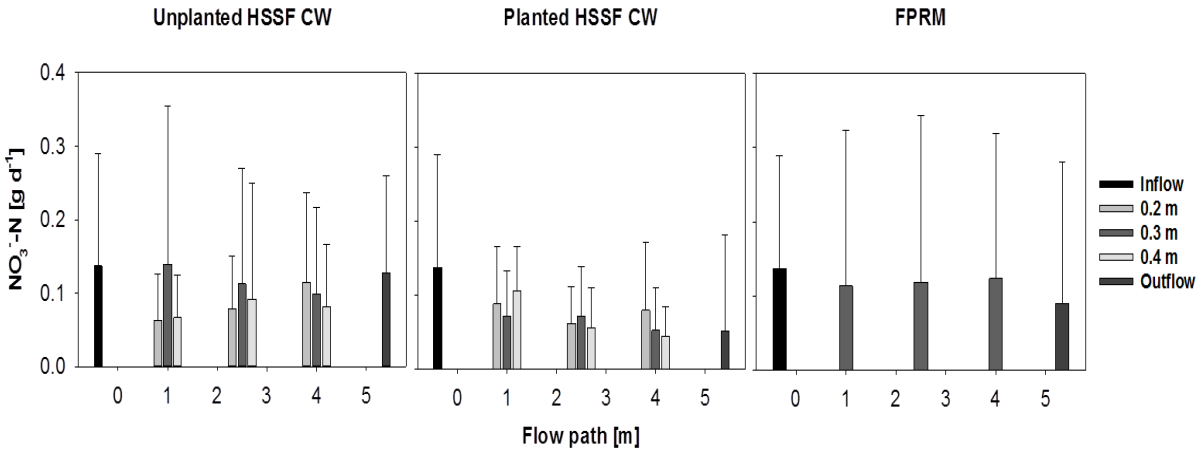

Fig. 3.1-8 Mean NO_3^- loads in the inflow, outflow and along the flow path and at three depths of the different CW types: unplanted HSSF, planted HSSF, and FPRM (n = 12)

Dissolved gases

Natural as well as constructed wetlands can be sources of various greenhouse gases such as nitrous oxide (N_2O) and methane (CH_4) (Teiter & Mander 2005). N_2O is increasing in the atmosphere at a rate of about 0.3 % year^{-1} (Mosier 1998). It has an atmospheric lifetime of about 120 years, a global warming potential of 296 relative to CO_2 over a 100 year time horizon, and is responsible for about 6 % of anticipated warming (Ehhalt et al. 2001). CH_4 is another greenhouse gas increasing in the atmosphere at the rate of about 0.8 % year^{-1} (Mosier 1998). CH_4 in the atmosphere has a lifetime of 8.4 years. On a 100 year time horizon, CH_4 has a global warming potential of 23 relative to CO_2, and is responsible for about 20 % of anticipated warming (Ehhalt et al. 2001). Both, denitrification which produce N_2O and CH_4 formation depend on the oxygen status of the soil or sediment. Regarding this, the spatial and temporal variability of fluxes of both N_2O (Ambus & Christensen 1993, Augustin et al. 1998) and CH_4 (Saarnio et al. 1997, Willison et al. 1998) is extremely high. In addition to the oxygen status, denitrification rates in soils are influenced by C availability, NO_3^- availability, temperature, and pH (Erich et al. 1984). Biological CH_4 oxidation is an important environmental process preventing the release into the atmosphere of the excessive CH_4 produced in anoxic soils and sediments. Natural wetlands release about 20 % (110 Tg) of the total annual global CH_4 emissions (Galchenko et al. 1989). In contrast to soil and natural wetlands, far fewer studies have been carried out on N_2O and CH_4 fluxes from CWs for wastewater treatment (Teiter & Mander 2005).

Dissolved N_2O and CH_4 were measured in the CWs along the flow path at three distances from the inlet and three depths as well as in inflow and outflow. There were significant seasonal variations in CH_4 concentrations ($F(2,72) = 118.678$, $p = .000$) (Fig.3.1-9). The lowest CH_4 emissions were observed in summer. Similar results were obtained by Saarnio et al. (1997) in an oligotrophic pine fen. This can be explained by the enhanced CH_4 oxidation potential due to higher temperatures in summer (Fig. 3.1-3). The decrease in temperature in autumn increased the CH_4 production potential via reducing of *in situ* decomposition processes, which possibly utilized unused substrates in the CWs (Saarnio et al. 1997) and therefore, increase CH_4 emissions. As for spring season, such high CH_4 emissions could be that the substrates in the CWs were actively converted to CH_4 from the autumn through the winter under the snow cover and emitted during the early spring thaw of soil (Saarnio et al. 1997).

As for the difference between the treatments, the unplanted HSSF CW showed higher CH_4 concentrations than other systems in summer ($F(2,21) = 18.532$, $p = .000$) and in spring ($F(2,21) = 10.789$, $p = .001$). However, in autumn only the FPRM had significantly lower concentrations than other systems ($F(2,21) = 25.472$, $p = .000$). This is evidence of CH_4 transport by vascular plants in planted CWs which lower the CH_4 concentrations. Saarnio et al. (1997) reported increase in CH_4 concentrations with depth which probably reflects a decrease in CH_4 transport by vascular plants and a decrease in substrate availability for methanogenesis. However, in the CWs the concentration of CH_4 did not change with depth ($F(2,51) = .583$, $p = .562$). Furthermore, the CH_4 concentrations did not change with the flow

path ($F(4,70) = .367$, $p = .831$) except for the planted HSSF CW in summer ($F(3,6) = 25.066$, $p = .001$). Augustin et al. (1998) reported complex interactions between the management systems, weather, and soil type, which influence CH_4 emission rates, and in the investigated systems CH_4 concentrations seem to reflect the complex mosaic structure of aerobic and anaerobic zones in CWs.

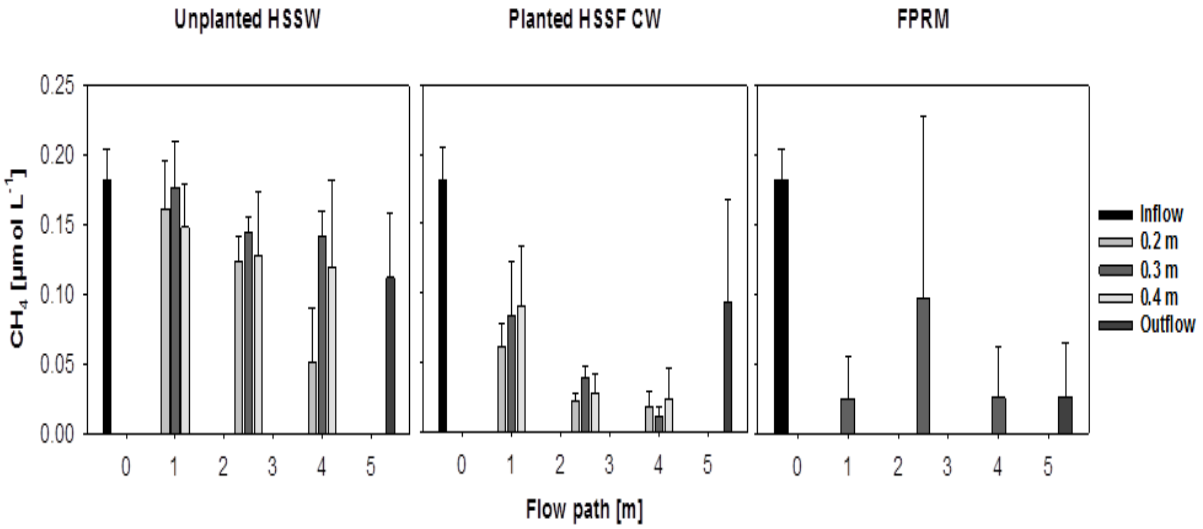

Fig. 3.1-9 Mean CH_4 concentrations for the summer season in the inflow, outflow and along the flow path and at three depths of the different CW types: unplanted HSSF, planted HSSF, and FPRM (n = 5)

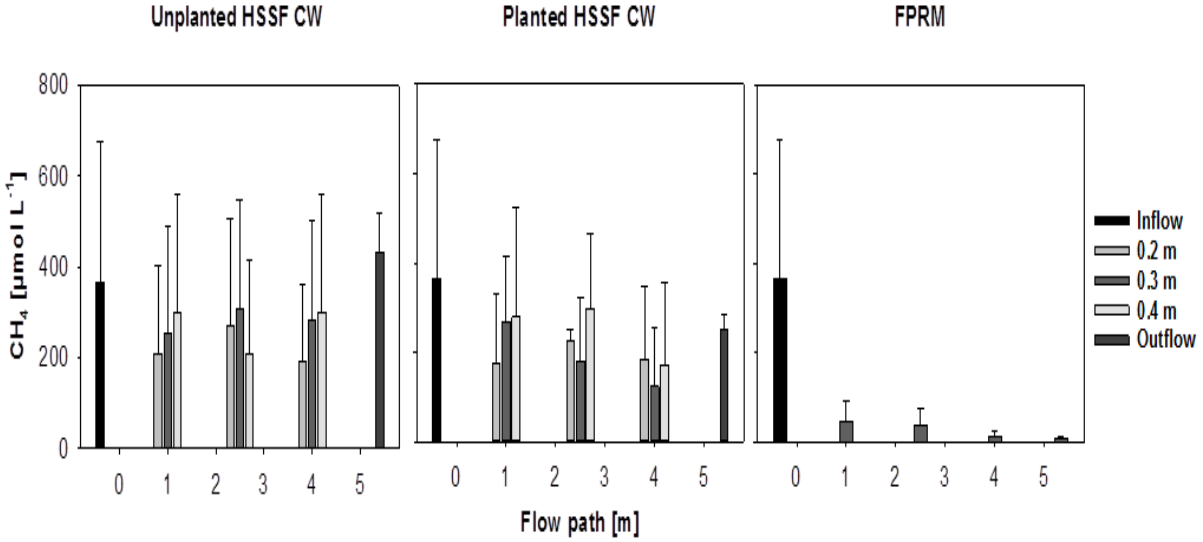

Fig. 3.1-10 Mean CH_4 concentrations for the autumn season in the inflow, outflow and along the flow path and at three depths of the different CW types: unplanted HSSF, planted HSSF, and FPRM (n = 3)

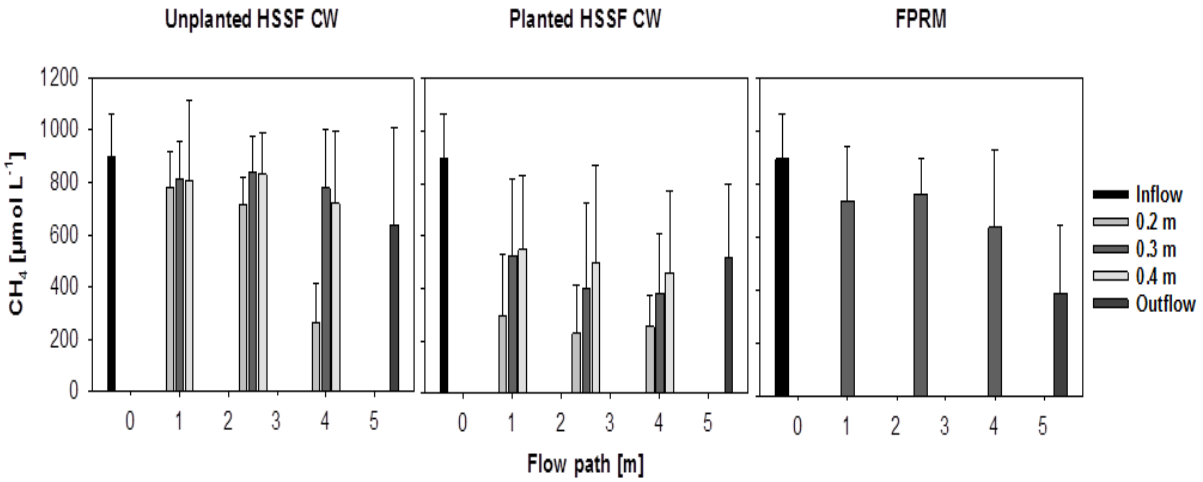

Fig. 3.1-11 Mean CH_4 concentrations for the spring season in the inflow, outflow and along the flow path and at three depths of the different CW types: unplanted HSSF, planted HSSF, and FPRM (n = 5)

As for N_2O concentrations, there were statistically significant variations ($F(2,72) = 105.740$, $p = .000$) between the investigated seasons. The highest N_2O concentrations were observed in spring and the lowest in summer. Such peak of N_2O emissions in spring can be explained by the spring thaw of soil (Christensen & Tiedje 1990). The spring emissions may be caused by denitrification and the C source mediating the increased N_2O production may be microorganisms killed by freezing or detritus that becomes available by the freezing/thawing process. Therefore, the organic matter availability and temperature were the main factors influencing the N_2O emissions. However, low N_2O concentrations in summer did not correlate with most of studies (Huang et al. 2013, Teiter & Mander 2005), although other studies reported the highest emissions during the autumn (Sovik & Klove 2007) or spring (Allen et al. 2007).

There was no statistically significant difference between the treatments in summer ($F(2,21) = 3.319$, $p = .056$). However, in autumn the FPRM had higher N_2O concentrations in comparison with other systems ($F(2,21) = 10.584$, $p = .001$) and in spring, the planted HSSF CW had lower N_2O concentrations in comparison with other systems ($F(2,21) = 18.926$, $p = .000$). Role of plants in N_2O emissions remains unclear (Huang et al. 2013). Wetland plants can increase N_2O emission by active transport through their culms (Picek et al. 2007). This may be one of pathways to contribute to the total gas emissions in CWs' ecosystems (Cheng et al. 2007). Oxygen released from roots of macrophytes is assumed to stimulate nitrification and also may influence N_2O production (Inamori et al. 2008). However, results vary widely in the literature in a way that fluxes are higher in some cases (Ström et al. 2007) and lower (Wild et al. 2001) in planted CWs compared with unplanted systems. For example, Picek et al. (2007) argued that the presence of plants might increase emissions of such gases as CO_2 and CH_4 as a source of available C for microorganisms in CWs, and this is consistent with the measured N_2O concentration results for the FPRM in autumn. Contrariwise, less N_2O was shown to be from aerated CWs planted with *P. australis* and *T. angustifolia* than those from unplanted (Maltais-Landry et al. 2009), what is similar to the results obtained in spring.

There were no statistically significant changes in N_2O concentrations with depth ($F(2,51) = .032$, $p = .968$). The N_2O concentrations did not change with the flow path ($F(4,70) = .367$, $p = .831$). Since N_2O emissions are controlled by oxygen, the absence of spatial variations in N_2O concentrations in our CWs is another evidence of complex mosaic structure of aerobic and anaerobic zones in CWs.

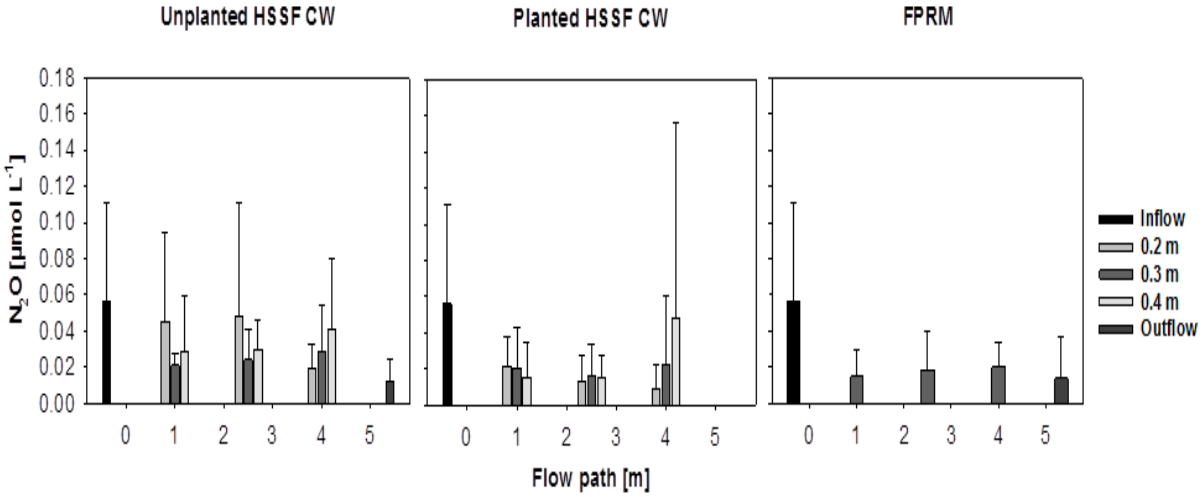

Fig. 3.1-12 Mean N_2O concentrations for the summer season in the inflow, outflow and along the flow path and at three depths of the different CW types: unplanted HSSF, planted HSSF, and FPRM (n = 5)

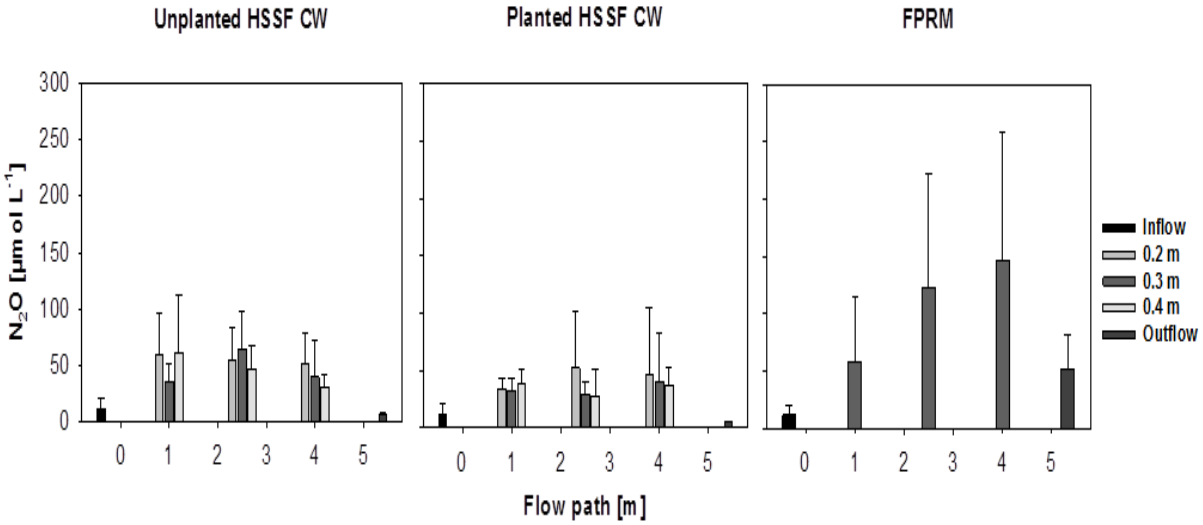

Fig. 3.1-13 Mean N_2O concentrations for the autumn season in the inflow, outflow and along the flow path and at three depths of the different CW types: unplanted HSSF, planted HSSF, and FPRM (n = 3)

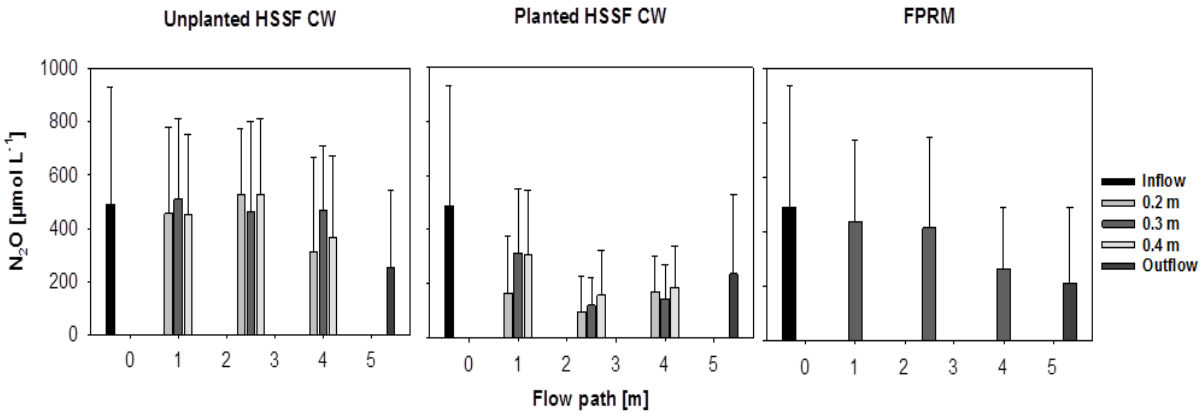

Fig. 3.1-14 Mean N_2O concentrations for the spring season in the inflow, outflow and along the flow path and at three depths of the different CW types: unplanted HSSF, planted HSSF, and FPRM (n = 5)

3.1.4 Conclusions from the ammonium removal assessment

The planted horizontal subsurface flow constructed wetland showed better treatment efficiency than unplanted horizontal subsurface flow constructed wetland for ammonium removal in summer and spring on a pilot-scale. This illustrates the key role of plants for contaminant removal as substrate for microorganisms in constructed wetlands. High removal efficiency of ammonium by the planted horizontal subsurface flow constructed wetland illustrates that this system can be efficiently used for the ammonium treatment, however, only under loads up to 250 g m^{-2} yr^{-1}. The floating plant root mat indicated lower ammonium treatment efficiency than the planted horizontal subsurface flow constructed wetland in summer, and this illustrates that presence of gravel as substrate for attachment and growth of microorganisms also plays a role, even though only minor, in the nitrogen removal in planted constructed wetlands. This confirms that floating plant root mat as a variant of constructed wetlands with an open water body, and therefore, higher oxygen concentrations could be a cost-competitive variant and an alternative for the treatment of the contaminants with aerobic degradation pathways. Examples of such pollutants can be benzene and MTBE, as this system showed the best treatment efficiency for these contaminants.

The redox potential data obtained show that all three systems were limited by oxygen and spatial variations in dependence on root depth indicated that plant roots remained main oxygen source along the flow path. Even though temperature is considered to be an important factor influencing nitrogen removal processes, there was lack of seasonal variations for ammonium treatment efficiency in our constructed wetlands, what could be explained by the fact that throughout all investigated seasons the air temperature remained high (above 10°C). Water loss was evidence of plant activity, and the maximum water loss in summer and spring showed that the plants are receiving relatively more ammonium in these months than during autumn months. Presence of nitrite and nitrate at all points in all investigated constructed wetlands with no accumulation was evidence for simultaneous occurrence of nitrification and denitrification at every sampling point. In all constructed wetlands, nitrous oxide and methane concentrations seemed to reflect the complex mosaic structure of aerobic and anaerobic zones.

3.2 Seasonal and spatial variations in stable nitrogen isotope fractionation in HSSF CWs and FPRM

3.2.1 Ammonium

During nitrification under closed system conditions, the concentration of NH_4^+-N concentration can be related to its δ^{15}N-NH_4^+ values as per a Rayleigh equation (Eq. 5)

$$\delta\,(\mathrm{NH_4^+ - N}) = \delta_0\,(\mathrm{NH_4^+ - N}) + \varepsilon \ln f \qquad (5)$$

where ε is the enrichment factor for N during nitrification and f is the fraction of the original NH_4^+-N pool remaining at a given time/distance (expressed as C (NH_4^+-N)/C_0 (NH_4^+-N)). The enrichment factor for the planted HSSF CW measured in the potential NH_4^+ oxidation laboratory experiment was -7.8 ‰ (Fig. 3.2-1). This is somewhat higher than the range of enrichment factors reported for ammonia oxidizing bacteria, between -14.2 and -38.2 ‰ (Casciotti et al. 2003). However, the increase in δ^{15}N-NH_4^+ was observed only between first two time points, 0 and 44 hours. Taking into consideration only these points, the enrichment factor would be -44.6±2.6 ‰, which is slightly lower than the range reported before. We hypothesize that such relatively low enrichment factors reflect the high loading of substrate (150 mg NH_4^+-N L^{-1}) (Singleton et al. 2007). After this, at the samplings 75 and 100 hours, the δ^{15}N-NH_4^+ dropped down by 4-5 ‰, indicating that nitrification was no longer the only process controlling changes in NH_4^+ concentration (Eq. 5). This can be explained by simultaneously occurring mineralization and nitrification, because these incubations included both plant material and sediments, which could be easily mineralized under aerobic conditions.

Under aerobic conditions, mineralization is characterized by fast rates (Vymazal 2007) and associated with an epsilon of 0 ‰ (Lund et al. 2000). The δ^{15}N reported for organic N sources in wetlands were shown to be between 0.7 and 5.5 ‰ (Bernot et al. 2009, Reinhardt et al. 2006). Thus, while mineralization influences all time points, it becomes a greater influence as the rates of nitrification diminish with the concentration of inorganic N, according to Michaelis–Menten kinetics. The initial enrichment between time 0 and 44 hours was driven mostly by nitrification, but later it was dampened by ongoing influx by somewhat lighter mineralized N.

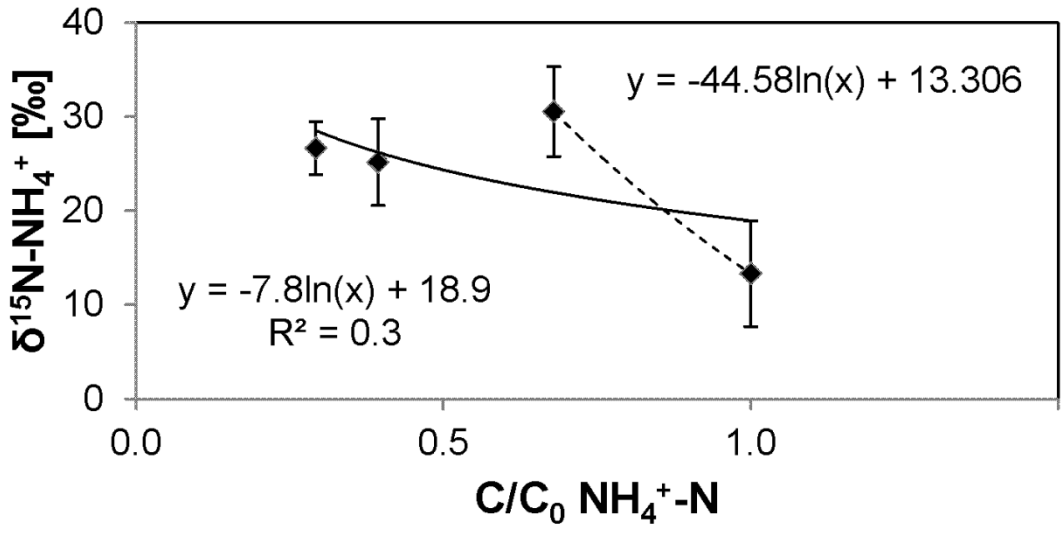

Fig. 3.2-1 Rayleigh plots of changes in NH_4^+ isotopic composition versus concentration measured in laboratory experiment for determination of potential enrichment factor (ε) for nitrification. The solid line refers to all points and reflects the enrichment factor for nitrification and mineralization occurring simultaneously. The dashed line shows the site-specific ε value for nitrification based on the first two points, when nitrification was the dominant process controlling NH_4^+ in the system

Based on the field measurements, loads were calculated by combining concentration and water loss, and *f* in the Eq. (5) was modified and expressed as L (NH_4^+-N)/L_0 (NH_4^+-N). Applying Eq. (5) to field data yields an 'apparent' enrichment factor ε, because closed system conditions are rarely achieved under field conditions (Knoeller et al. 2006). Even though field measurements might not be precise for quantitative evaluation of nitrification, they are still useful for distinguishing nitrification from plant uptake and/or dilution.

The $\delta^{15}N\text{-}NH_4^+$ values and loads in the CWs are presented in Table 3.1-1. NH_4^+-N loads were statistically significantly changing over depth ($F(2,231) = 3.956$, $p = .020$). Furthermore, there were no statistically significant differences in $\delta^{15}N\text{-}NH_4^+$ values between depths ($F(3,188) = 0.774$, $p = .510$), indicating that the same processes were occurring at 0.2, 0.3, and 0.4 m depth. Also, the $\delta^{15}N\text{-}NH_4^+$ in planted HSSF CW and FPRM increased over distance from inflow while NH_4^+-N loads and Eh decreased (Fig. 3.1-1, 3.1-4). This indicates that NH_4^+ oxidation occurred linearly with the flow of contaminated water from inflow to outflow.

Table 3.2-1 Mean $\delta^{15}N\text{-}NH_4^+$ and loads of NH_4^+-N of the pore water of experimental pilot-scale along flow path from inflow (0 m) to outflow (5 m)

Distance from inflow [m]	Depth [m]	Unplanted HSSF CW		Planted HSSF CW		FPRM	
		$\delta^{15}N\text{-}NH_4^+$ [‰ v.AIR]	Loads [g NH_4^+-N d^{-1}]	$\delta^{15}N\text{-}NH_4^+$ [‰ v.AIR]	Loads [g NH_4^+-N d^{-1}]	$\delta^{15}N\text{-}NH_4^+$ [‰ v.AIR]	Loads [g NH_4^+-N d^{-1}]
Summer							
0		9.64	6.01	9.64	6.01	9.64	6.01
1	**0.2**	8.84	4.22	9.68	3.15		
	0.3	10.29	4.08	7.83	3.15	14.75	2.61
	0.4	9.87	4.19	8.26	2.98		
2.5	**0.2**	10.06	3.06	12.78	1.86		
	0.3	9.84	3.92	11.37	1.60	16.42	2.71
	0.4	9.37	4.10	13.48	1.79		
4	**0.2**	9.46	1.03	19.59	0.30		
	0.3	9.91	3.30	20.15	0.37	17.80	1.84
	0.4	9.22	3.89	19.85	0.43		
5		9.90	3.06	2.28	0.06	18.79	1.47
Autumn							
0		9.24	4.02	9.24	4.02	9.24	4.02
1	**0.2**	9.24	2.79	13.86	2.13		
	0.3	9.29	3.09	10.95	2.43	13.47	1.89
	0.4	9.31	2.65	10.49	2.69		
2.5	**0.2**	9.28	2.58	15.34	2.46		
	0.3	9.36	2.59	13.83	2.50	14.91	1.64
	0.4	9.32	2.87	13.74	2.67		
4	**0.2**	10.14	1.60	20.70	1.43		
	0.3	9.53	2.04	18.46	1.98	16.71	1.60
	0.4	9.52	2.24	18.02	1.90		
5		9.80	1.77	24.21	1.40	17.76	1.49
Spring							
0		7.55	3.21	7.55	3.21	7.55	3.21
1	**0.2**	7.32	2.50	12.68	1.37		
	0.3	7.45	2.64	10.12	2.29	7.21	2.78
	0.4	7.13	2.53	8.61	3.05		
2.5	**0.2**	7.55	2.24	13.00	1.09		
	0.3	7.79	2.41	11.82	1.83	7.71	2.56
	0.4	7.68	2.61	10.29	2.01		
4	**0.2**	8.58	1.35	14.01	0.96		
	0.3	7.82	2.38	14.79	1.32	8.28	2.14
	0.4	8.06	2.11	12.55	1.67		
5		8.10	3.21	13.63	1.18	10.22	1.20

The apparent enrichment factor for planted HSSF CW was lower in autumn (-14.1 ‰) than in summer (-4.2 ‰) and spring (-5.5 ‰) (Fig. 3.2-2). The apparent enrichment factors for FPRM were very close to planted HSSF CW, somewhat lower in autumn (-8.1 ‰), and higher in summer (-6.1 ‰) and spring (-3.3 ‰). As the laboratory experiment has shown, the influence of mineralization may also explain high enrichment factors for all seasons in planted HSSF CWs (mean ε = -7.9 ‰). In the control (unplanted) system there was no enrichment of $\delta^{15}N\text{-}NH_4^+$ along the flow path despite the observed decrease in NH_4^+ loads. This can be explained by microbial assimilation (ε = 0 ‰) (Lund et al. 1999), as a high C/N ratio of 2.5:1 was observed in the system, and calculated assimilation rates based on TOC (data not shown) imply N loads decrease through incorporation into the microbial biomass. The microbial assimilation could assign part of N removal in planted CWs as well as plant uptake (32 to 100 % in planted HSSF CW). However, the increase in $\delta^{15}N\text{-}NH_4^+$ can be explained only by nitrification because this is the process which causes isotope fractionation (Casciotti et al. 2003). Anammox was found to play a minor role in CWs which will be shown further in this work and has been shown in other studies (Castine et al. 2012, Zhu et al. 2011) and the loss of NH_4^+ through free ammonia volatilization is insignificant at pH<7.5 (Vymazal 2007). Therefore, nitrification is occurring throughout all seasons in both planted HSSF CW and FPRM, and plant uptake must not account for 100 % of the measured NH_4^+ removal. This illustrates the key role of plants for enhancing microbial processes as plant roots will provide sufficient substrate for attachment of microorganisms and their growth.

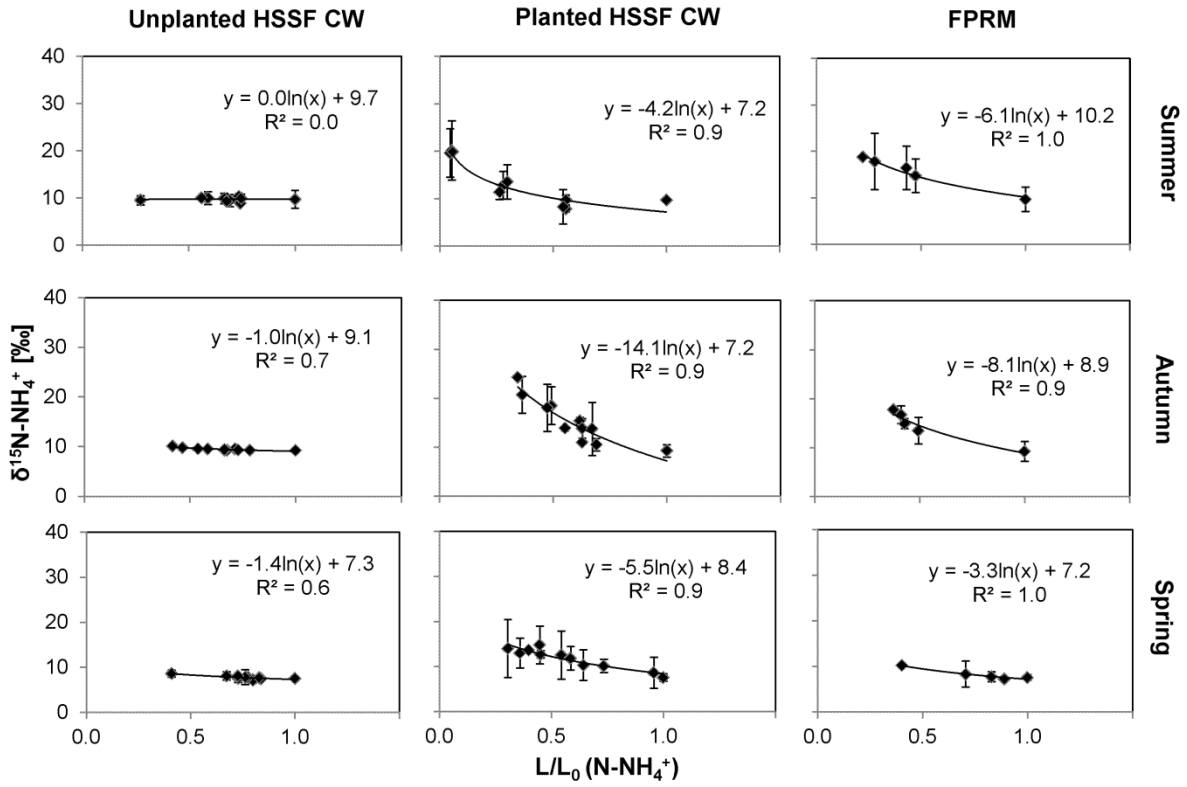

Fig. 3.2-2 Rayleigh plots for unplanted and planted HSSF CWs and FPRM showing relationships between $\delta^{15}N\text{-}NH_4^+$ and L/L_0 of $N\text{-}NH_4^+$ for summer (n = 2), autumn (n = 2), and spring (n = 3)

For the visualization of the seasonal variations in the planted HSSF CW, a method of geostatistical interpolation (kriging) was applied (Fig. 3.2-3). By this, the trends of $\delta^{15}N\text{-}NH_4^+$ distribution across the planted HSSF CW in dependence on a season can be illustrated. In all seasons $\delta^{15}N\text{-}NH_4^+$ was equally distributed along the flow path with somewhat prevalence of lighter $\delta^{15}N\text{-}NH_4^+$ in the 0.4 m depth. Furthermore, the $\delta^{15}N\text{-}NH_4^+$ increased with the flow path as bacteria has uptaken lighter isotope (^{14}N) and residual NH_4^+ became more enriched with the heavy isotope (^{15}N). This is another evidence of microbially mediated process, in this case nitrification. In summer, this trend was the most clear to observe, as in this season the planted HSSF CW had the best removal efficiency of NH_4^+, i.e. the highest amount of $^{14}NH_4^+$ was taken up by bacteria and therefore, the highest amount of $^{15}NH_4^+$ was remaining in the residual pore water. However, the even distribution of $\delta^{15}N\text{-}NH_4^+$ throughout all seasons illustrates that the seasonal variations in biodegradation mechanisms of NH_4^+ was absent and the nitrification was the key process influencing the NH_4^+ removal not only in summer but also during the autumn and spring seasons.

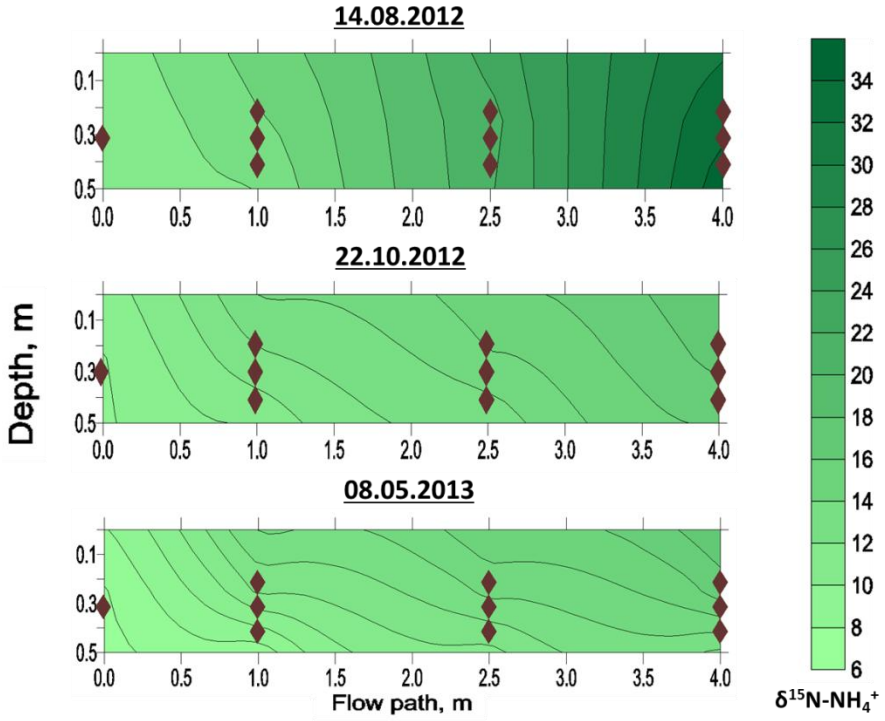

Fig. 3.2-3 Visualization of seasonal variations of N-isotope fractionation in planted HSSF CW: Kriging. The color scale for $\delta^{15}N\text{-}NH_4^+$ is shown on the right. The sampling points are marked in brown

There are several possible explanations for the higher apparent enrichment factor for nitrification in autumn. The first is negligible plant uptake during this season, what makes nitrification, mineralization, and microbial assimilation the responsible processes for NH_4^+ removal. When plant uptake is absent, nitrifying bacteria will increase their turnover rates because the competition for the substrate will be absent. Furthermore, as our laboratory experiment has shown, mineralization is less prominent under higher nitrification rates. And as the results of the unplanted HSSF CW showed, the microbial assimilation could account only for a small part of N removal. Second possible reason could be lower mineralization

rates under lower temperatures (Vymazal 2007). The enrichment factor will increase as nitrification will mask influence of mineralization even more (Fig. 3.2-1). In any case, nitrification is the prevailing process in planted CWs throughout the year, not depending on depth in the root zone, especially in autumn when temperatures are lower and plant uptake is negligible.

3.2.2 Nitrate

During denitrification, the relationship between NO_3^--N and δ^{15}N-NO_3^- values can be expressed using a Rayleigh equation (Eq. 6) in an analogous way with NH_4^+-N (Eq. (5)),

$$\delta\,(\mathrm{NO_3^-} - \mathrm{N}) = \delta_0\,(\mathrm{NO_3^-} - \mathrm{N}) + \varepsilon \ln f \qquad (6)$$

where *f* indicates the change in NO_3^- loads (L (NO_3^--N)/L_0 (NO_3^--N)) and ε is the enrichment factor for denitrification. Using Eq. (6), the site-specific enrichment factor for denitrification from the laboratory experiment was calculated to be -16.9±4.4 ‰ (Fig. 3.2-4). This is comparable with reported laboratory enrichment factors for denitrification (Granger et al. 2008, Kritee et al. 2012, Spoelstra et al. 2010). The anaerobic conditions and absence of NH_4^+ accumulation (data not shown) make it reasonable to assume that denitrification was the only process affecting NO_3^- in these incubations (i.e., nitrification and mineralization were absent). This enrichment factor experiment also revealed a high denitrification potential in the wetland substrates: 37 % NO_3^- was reduced within one hour.

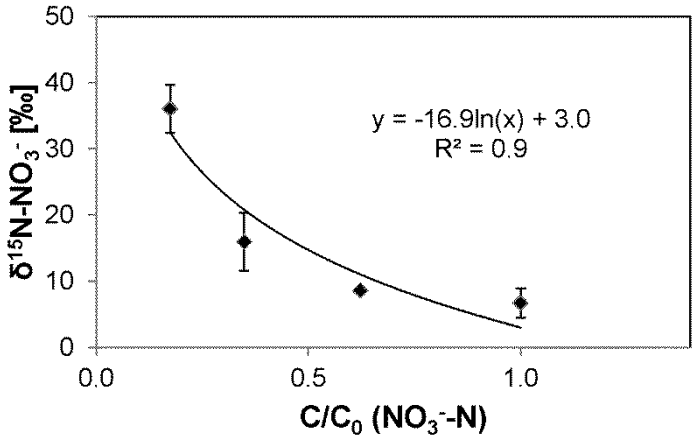

Fig. 3.2-4 Rayleigh plots of changes in NO_3^- isotopic composition versus concentration measured in laboratory experiment for determination of potential enrichment factor (ε) for denitrification

However, as in the field study the NO_3^- concentrations in the inflow were below detection limits, it was impossible to assign a clear 'L_0' value for NO_3^-, as all measured NO_3^- was produced by nitrification within the planted CWs (as supported by the δ^{15}N-NH_4^+ data). Indeed, despite clear potential for denitrification and negative range of redox values to support the process (Fig. 3.1-4), there was no relationship between δ^{15}N-NO_3^- and loads of N-NO_3^- in any of the CWs (Fig. 3.2-5). Neither the loads of NO_3^- nor the enrichment of δ^{15}N had any relationship with distance. However, as no accumulation of NO_3^- was observed and only plant uptake could not explain all N removal (32 to 100 %), it is reasonable to conclude

that simultaneous nitrification – denitrification masked the NO_3^- isotope signature, rather than a complete absence of denitrification (Akratos & Tsihrintzis 2007, Wankel et al. 2009). Although previous studies in wetlands do report apparent enrichment factors for the denitrification (Fukuhara et al. 2007, Lund et al. 2000, Reinhardt et al. 2006), the expression of this enrichment factor in the environment must depend on the relative rates of nitrification (producing 'light' NO_3^-) and denitrification (producing 'heavy' NO_3^-). So it is then predictable that in the CWs measured here, where the dominant N source was NH_4^+, nitrification would be a dominant enough process to mask any denitrification isotope effects. Accordingly, the lack of significant apparent enrichment factors for denitrification in these CWs indicates that the stable isotope approach is unsuitable for the estimation of denitrification in the systems obtaining NH_4^+ rich inflow water.

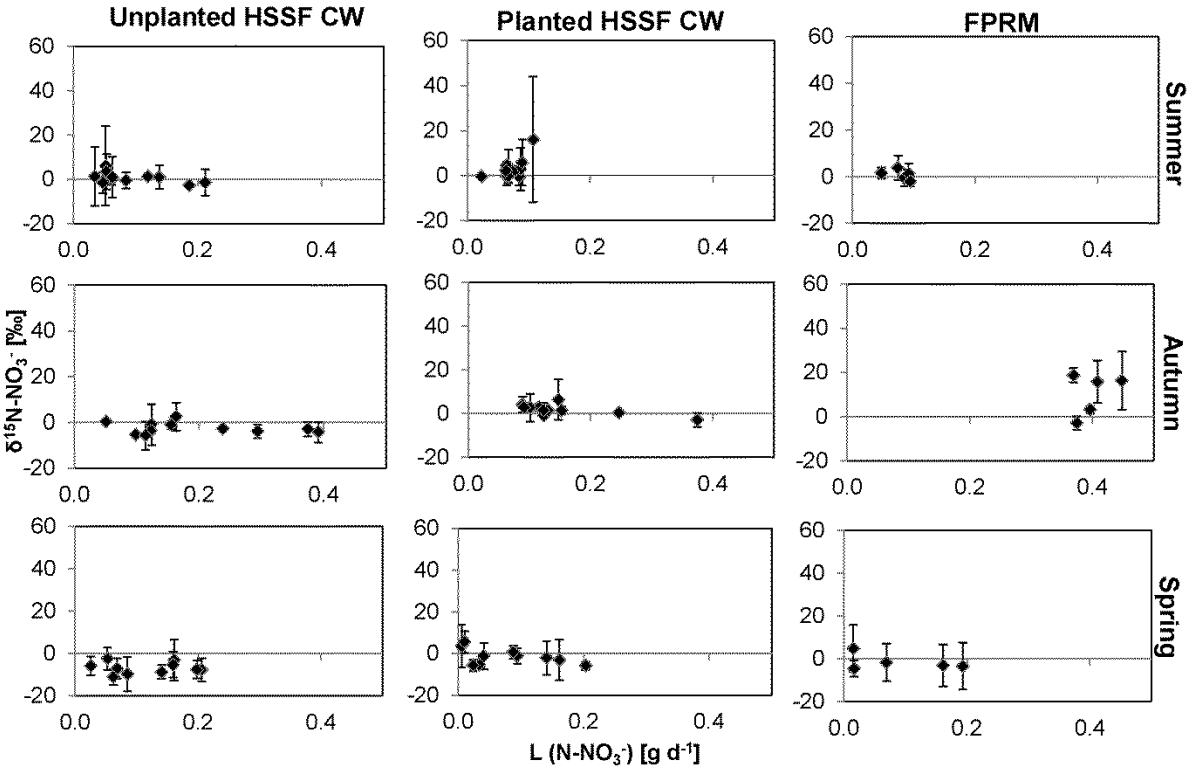

Fig. 3.2-5 Rayleigh plots for unplanted and planted HSSF CWs and FPRM showing relationships between δ^{15}N-NO_3^- and L of N-NO_3^- for summer (n = 3), autumn (n = 2), and spring (n = 3)

The stable isotope techniques, e.g. ratios of $^{15/14}$N and $^{18/16}$O isotopes, help identify NO_3^- sources (Kendall et al. 2007). The stable nitrogen isotope signature of nitrate (δ^{15}N-NO_3^-) is also an effective indicator of denitrification in groundwater, due to the isotopic fractionation that occurs during denitrification (Mariotti et al. 1988). Denitrification can be assessed through the characteristic enrichment shift of N and O isotopes in NO_3^- in soil and groundwater (Böttcher et al. 1990, Fukada et al. 2004). The δ^{18}O in NO_3^- was measured in order to estimate whether the dual isotope analysis of NO_3^- can support the occurrence of denitrification. However, in our systems the δ^{18}O data were not representative and the dual isotope plot did not show any increase in δ^{18}O and δ^{15}N (Fig. 3.2-6). This might be due to organic contamination of our samples, as they contained benzene and MTBE and this can

intervene into N_2O production by denitrifying bacteria in the denitrifier method used for the samples preparation. Still, unfortunately, the reason for high fluctuations in $\delta^{18}O\text{-}NO_3^-$ is still unknown and remains to be discovered.

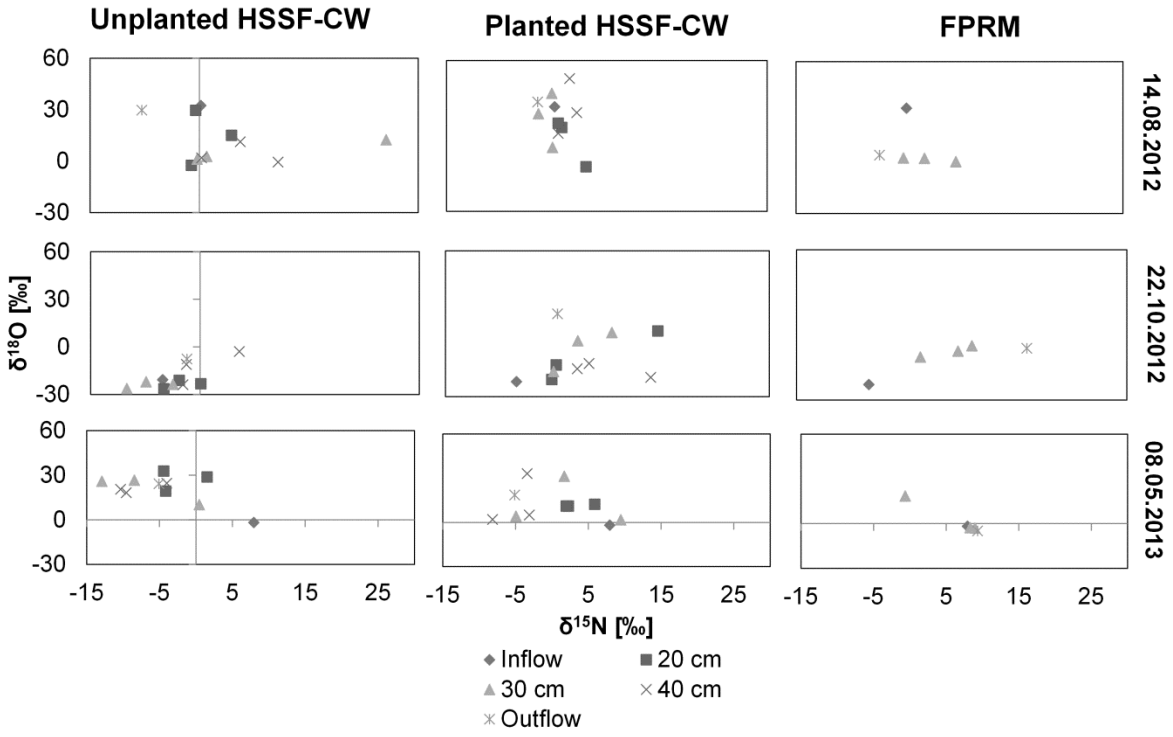

Fig. 3.2-6 Dual NO_3^- isotope plot for unplanted HSSF CW, planted HSSF CW, and FPRM showing relationships between $\delta^{15}N\text{-}NO_3^-$ and $\delta^{18}O\text{-}NO_3^-$ for summer (14.08.2012), autumn (22.10.2012), and spring (8.05.2013). Mean values of samples for every season are not shown due to high variations between the samples

3.2.3 Conclusion from natural abundance isotope investigations

The significant enrichment of ^{15}N in ammonium was observed to be clearly correlated to the decrease of ammonium loads along the flow path through our constructed wetlands. This strongly indicates the occurrence of nitrification. Lack of ^{15}N enrichment in nitrate implies that other processes such as nitrification and mineralization are superimposed on denitrification. The application of the stable isotope approach combined with common physico-chemical investigations enabled us to identify key factors influencing efficiency of nitrogen removal in constructed wetlands, which was the plant presence as substrate for attachment and growth of microorganisms. Isotope fractionation patterns revealed that nitrification-denitrification were prevailing processes in planted constructed wetlands throughout the year, occurring in a linear way along the flow path, and not depending on depth in the root zone. Therefore, planted constructed wetlands could be a good solution for the treatment of ammonium contaminated groundwater.

The laboratory experiments for the potential ammonium oxidation enrichment factor determination helped us to illustrate occurrence of nitrification with the mean enrichment factor of -7.8 ‰, what was well comparable with the field enrichment factor (-7.9 ‰).

Furthermore, it showed the simultaneously occurring mineralization and nitrification. The laboratory experiments for the potential denitrification enrichment factor determination demonstrated high denitrification rates with the mean enrichment factor of -16.9 ‰. As the field data did not give us any representative values for the nitrogen isotope in nitrate due to overlapping of nitrification with denitrification, the data from the laboratory experiment were the key evidence of denitrification occurrence in the planted constructed wetlands. Therefore, additional approaches for stable isotope investigations such as laboratory experiments beside field measurements should be considered when attempting to quantify nitrification and especially denitrification in constructed wetlands obtaining ammonium rich inflow water.

3.3 Investigations on anammox, nitrification and denitrification in the HSSF CW

In this chapter, the results of qPCR with primers for functional genes of the N-cycle in samples of gravel and roots from HSSF CW are presented. Furthermore, the results of isotope labelling experiments for the activity of anammox, denitrification, and nitrification in the CW will be discussed. Furthermore, the anammox biodiversity in the HSSF CW is shown.

3.3.1 Overview of N-transforming bacterial abundance in the HSSF CW

For qPCRs, set of primers was applied for total bacteria and N-transforming bacteria in order to obtain an overview about quantitative ratios between different bacteria of the N-cycle and their spatial distribution (Fig. 3.3-1). For total bacteria, a universal probe and primer set which specifically detects 16S rRNA genes of the domain Bacteria were used (Nadkarni et al. 2002). Throughout the flow path, the highest bacterial number of 6.21×10^8 copy number g^{-1} sample was found at 4 m distance from the inlet, 0.2 m depth, and the lowest of 6.78×10^7 copy number g^{-1} sample at 4 m from inflow, 0.4 m depth. This correlates well with the highest root density of *P. australis* at 0.2-0.3 m depths (Börnert 1990).

For denitrifying bacteria, several functional gene based primers were used. The main reason for using only the functional gene based primers is that denitrifying bacteria are phylogenetically diverse. They belong to all major phylogenetic groups except for the *Enterobacteriaceae*, obligate anaerobes, and gram-positive bacteria other than *Bacillus* spp. (Zumft 1992). For this reason denitrifying bacteria can be defined rather as a physiological than phylogenetic group. Nitrite reductase is the key enzyme in the dissimilatory denitrification process. The reduction of NO_2^- to NO can be catalyzed by the products of two different nitrite reductase genes: one product contains copper (the product of *nirK*), and the other contains cytochrome cd_1 (the product of *nirS*). The two genes seem to occur equally exclusively in a given strain, but both types have been also found in different strains of the same species (Coyne et al. 1989). While *nirS* is more widely distributed, *nirK* is found in only 30 % of the denitrifiers studied (Braker et al. 1998). However, *nirK* is found in a wider range of physiological groups (Coyne et al. 1989). Since denitrifiers are not defined by close phylogenetic relationship, an approach involving 16S rRNA molecules is not suitable for general detection of this physiological group in the environment and therefore, only functional-based primers can be used.

In the planted HSSF CW, both *nirS* and *nirK* genes were very abundant. The *nirK* gene occurred with a minimum at 2.5 m from the inlet, 0.2 m depth, and maximum at 4 m from the inlet, 0.4 m depth. Interestingly, the trend for *nirS* gene distribution was vice versa, the minimum of *nirS* was found at 4 m from inflow, 0.4 m depth, and the maximum at 2.5 m from inflow, 0.2 m depth. The apparent predominance of either *nirK* or *nirS* at different locations may reflect differences in denitrifier enzyme properties in CWs where this process is strongly pronounced (Priemé et al. 2002). Different sampling points in the planted HSSF CW are different with respect to several parameters, e.g., oxygen status, organic carbon content, and pH, which may have profound influence on the composition of the denitrifying

communities. Priemé et al. (2002) has shown that the majority of *nirK* clones did not branch in the phylogenetic tree with any known denitrifying bacteria, indicating that the investigated soils had unique denitrifiers not known among cultivated denitrifiers. High *nirK* abundance due to high biodiversity can also be the case in our HSSF CW. Previous studies indicated that diverse denitrifying bacterial communities are present in environments such as CWs (Ruiz-Rueda et al. 2009, Sundberg et al. 2007).

Nitrous oxide reduction is the final step in the denitrification pathway. The *nosZ* gene which is encoding enzyme catalyzing this step is largely unique to denitrifying bacteria, although a few non-denitrifier species capable of reducing nitrous oxide have been identified (Zumft 1992). In our samples, *nosZ* gene had the lowest ratio to total bacterial number, 0.23 %, at 2.5 m from inflow, 0.4 m depth, and the highest ratio of 1.92 % at 1 m from inflow, 0.2 m depth. The relative abundance of this denitrifying gene was in good agreement with previous CW studies where the ratio of *nosZ* to total bacteria was reported to range from 0 % to 3.0 % (Chon et al. 2011).

Ammonia-oxidizing organisms including ammonia oxidizing archaea and ammonia-oxidizing bacteria are essential in nitrification processes that oxidize NH_4^+ to NO_2^- and NO_3^-, the key and often rate limiting step in N removal. Both ammonia oxidizing archaea and bacteria have the same functional *amoA* gene encoding the α-subunit of ammonia monooxygenase which catalyzes the first step in ammonia oxidation (Nicol & Schleper 2006). In our samples, *amoA* gene had the lowest ratio to total bacterial number, 0.06 %, at 2.5 m from inflow, 0.4 m depth, and the highest ratio of 0.36 % at 4 m from inflow, 0.2 m depth. Given that these bacteria require oxygen for their metabolism, it is not surprising then that they were mostly located at the top points where the highest root density of *P. australis* was and therefore, the highest oxygen concentrations.

In order to quantify anammox abundance and ratio to total bacteria, at first step primers for the anammox bacterial 16S rRNA gene (amx) were applied. Anammox 16S rRNA gene had the lowest ratio to total bacterial number, 10.00 %, at 4 m from inflow, 0.4 m depth, and the highest ratio of 26.83 % at 4 m from inflow, 0.2 m depth. However, the use of the 16S rRNA gene to study biodiversity has the disadvantage that it is not related to the physiology of the target organism and that such anammox primers do not capture all diversity (Schmid et al. 2007). On the other hand, anammox 16S rRNA gene primers may target not only the anammox group but other *Planctomycetes* as well (Humbert et al. 2010) and by this, significantly overestimate the number of anammox bacteria. The use of the 16S rRNA gene to study biodiversity has the disadvantage that it is not related to the physiology of the target organism and that such primers do not capture all diversity. Therefore, the use of functional gene markers provides a good alternative (Harhangi et al. 2012). The hydrazine synthase of anammox, which forms an N-N bond from NO and NH_4^+ and is encoded by the *hzsC*, *hzsB*, *hzsA* genes, is not present in other genomes sequenced so far and might thus be a very good biomarker for anammox bacteria (Harhangi et al. 2012). This novel and unique functional *hzsA* biomarker for anammox bacteria was used to our samples and obtained similar spatial distribution of anammox ratios to the total bacteria: the lowest of 0.05 % at 4 m from inflow,

0.4 m depth, and the highest of 0.29 % at 1 m from inflow, 0.2 m depth. Still, the *hzsA* gene copy number was two orders of magnitude lower than that one of anammox 16S rRNA gene.

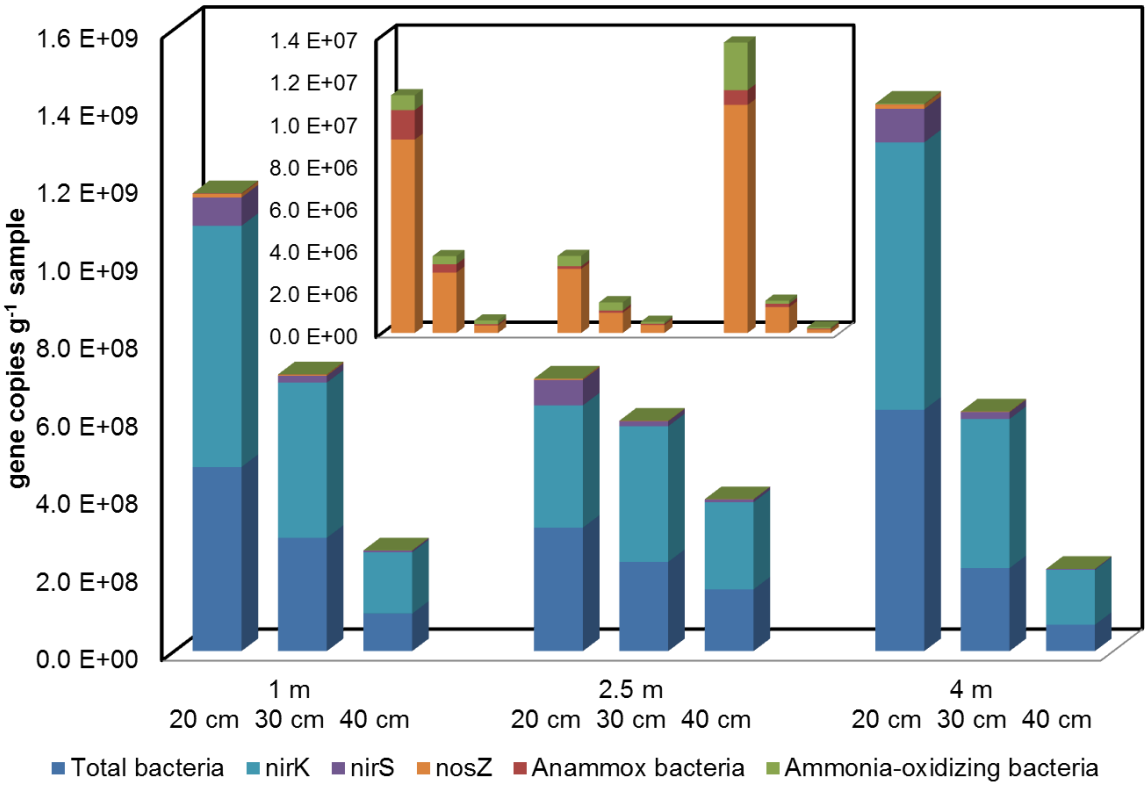

Fig. 3.3-1 Overview of quantitative ratios between different bacteria of N-cycle and their spatial distribution

3.3.2 Anammox

Potential anammox activity

To determine potential activity for anammox and denitrification, the isotope labeling experiment was performed with three sets of incubations ($^{15}NH_4^+$, $^{15}NO_3^-$, and $^{15}NH_4^+$ + $^{14}NO_3^-$) and subsequent additional set $^{14}NH_4^+$ + $^{15}NO_3^-$. $^{15}NH_4^+$ set was used as control for anoxic conditions created, $^{15}NO_3^-$ set was used for denitrification, and $^{15}NH_4^+$ + $^{14}NO_3^-$ as well as $^{14}NH_4^+$ + $^{15}NO_3^-$ were used for the denitrification and anammox turnover rates determination. The results showed in case of the incubations amended with $^{15}NH_4^+$ no accumulation of $^{29}N_2$ or $^{30}N_2$, indicating that all ambient O_2 and $^{14}NO_x^-$ had been consumed during the 24 h preincubations. When both $^{15}NH_4^+$ and $^{14}NO_3^-$ were added, accumulation of neither $^{29}N_2$ nor $^{30}N_2$ could be observed (i.e. no ^{15}N enrichment in sampled N_2 could be detected) in any incubation. This implies that $^{15}NH_4^+$ was not utilized for N_2 production and hence, hints at an absence of the anammox process. However, when applying only $^{15}NO_3^-$ or $^{14}NH_4^+$ + $^{15}NO_3^-$ with higher concentrations and longer incubation time, labeled N_2 was produced. This indicates utilization of labeled NO_3^- for N_2 production and hence, clearly hints at a denitrification activity. In fact, when using the equations of Spott and Stange (2011) for

calculating the fraction of anammox N_2 (i.e. hybrid N_2) in those treatments the resulting contributions were ≤0.08 % on the total N_2 mixture, which is just below the detection limit of 0.1 %. Moreover, observed accumulation of $^{29}N_2$ within sampled N_2 was extremely weak (0.78 % to 0.84 %), which also indicates the absence or insignificance of the anammox process in the HSSF CW (Fig. 3.3-2).

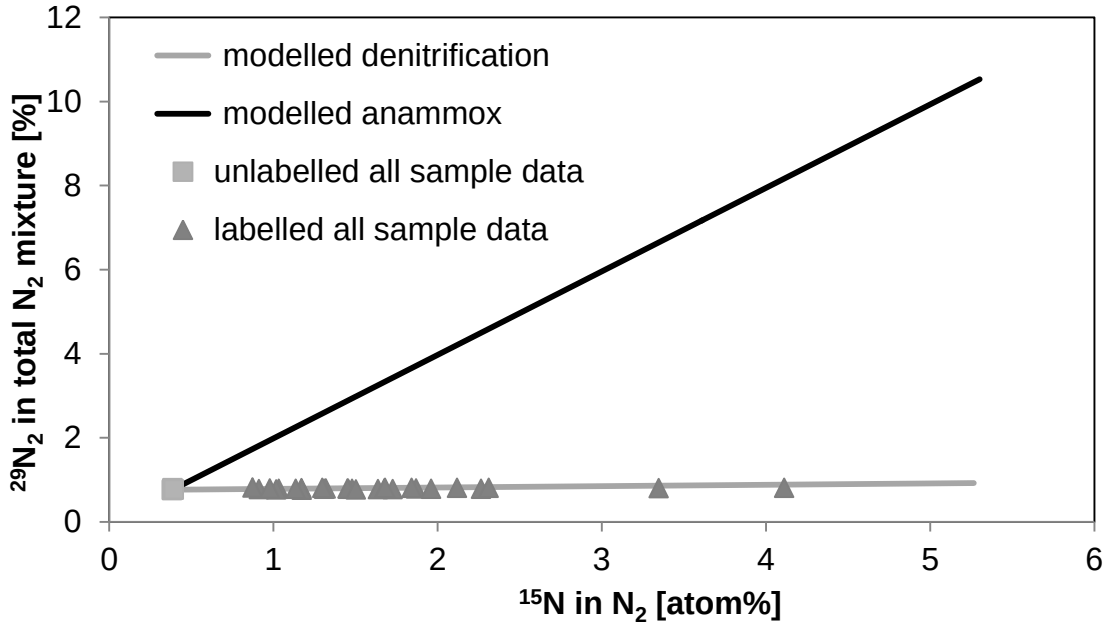

Fig. 3.3-2 Comparison of measured $^{29}N_2$ data with modelled $^{29}N_2$ data when assuming an increasing contribution of either only denitrification N_2 or only anammox N_2 to N_2 atmosphere with natural ^{15}N abundance in HSSF CW incubations with $^{14}NH_4^+$ + $^{15}NO_3^-$ (98 at.%). It can be seen that a strong accumulation of $^{29}N_2$ which could only result from the anammox process was absent over incubation time

Absence of $^{29}N_2$ and $^{30}N_2$ accumulation in the control incubations with only $^{15}NH_4^+$ indicated that NH_4^+ could not be oxidized and therefore, anoxic conditions for anammox were created (all ambient O_2 had been consumed during the 24 h preincubations). In the incubations conducted in the presence of $^{15}NH_4^+$ + $^{14}NO_3^-$, absolutely no ^{15}N enrichment could be detected in N_2. This obviously hints at the absence of relevant anammox activity. Similar results were found in the incubations with $^{15}NO_3^-$. Here, ^{15}N enrichment in N_2 was clearly detectable, but no significant accumulation of $^{29}N_2$ (Fig. 3.3-2) but only of $^{30}N_2$ occurred, which also suggest absence of anammox activity, even though up to 1.39×10^6 copy number g^{-1} sample was detected. Bale et al. (2014) detected anammox activity with already 4.2×10^5-1.4×10^6 copies *hzsA* gene g^{-1} in marine environment. The possible obstruction for the anammox bacteria could be using of NO_3^- as substrate instead of NO_2^-. However, given that anammox bacteria perform dissimilatory NO_3^- reduction to NO_2^- (Kartal et al. 2007), not only NO_2^- but also NO_3^- should be utilizable in combination with NH_4^+ for anammox. On the one hand, the combination of unlabelled NH_4^+ and labelled NO_3^- can produce $^{14}N^{15}N$ (i.e. $^{29}N_2$) via classical anammox pathway as well as $^{15}N^{15}N$ (i.e. $^{30}N_2$) via dissimilatory NO_3^- reduction to NH_4^+ and a further anammox process (Kartal et al. 2007). In the last case, anammox and denitrification would be indistinguishable. On the other hand, the experiment of Kartal et al.

(2007) illustrated that when using a labelled pool of NO_3^- and unlabelled pool of NH_4^+, 90 % of the $^{15}NO_3^-$ was converted to $^{14}N^{15}N$ (i.e. $^{29}N_2$) and only minor amount to $^{15}N^{15}N$ (i.e. $^{30}N_2$). This proves in principle that focusing on $^{29}N_2$ evolution should allow the detection of the anammox process (Kartal et al. 2007). Instead of anammox, however, in all $^{15}NO_3^-$ treatments high denitrifying activity was detected. One could therefore assume that denitrifiers should produce NO_2^- which could be then subsequently used by the anammox process. Lam et al. (2009) reported that anammox bacteria obtain at least 67 % of their NO_2^- from NO_3^- reduction, and less than 33 % from aerobic ammonia oxidation. However, the absence of any detectable anammox activity (the detection limit 0.1 %) the suggests that such kind of co-existence of anammox and denitrification did not occur in the CW.

Analysis of operating parameters in our HSSF CW and sufficient operating time of six years to establish the microbial community indicated the presence of all suitable conditions for anammox process (Zhu et al. 2010). Taking into account low redox values also proven by the presence of CH_4 and N_2O (Fig.3.1-9 - Fig.3.1-14), an oxic/anoxic interface was successfully present as a result of plants' rhizosphere activity. With high organic carbon of this inflow of contaminated groundwater and the carbon from the rhizodeposition products, NO_2^- produced by ammonia oxidizing bacteria may be predominantly used for denitrification instead of anammox. Low concentrations of contaminants and low C/N ratio below 2 stimulates anammox (Reiche et al. 2010), and in our study a C/N ratio of about 2 was relatively high, which would create advantageous conditions for growth of denitrifying bacteria. Therefore, anammox process might play a role in CWs, however in the systems where they will not be outcompeted by denitrifying bacteria, i.e. under low C/N ratio (He et al. 2012).

Advantage of treating contaminated waters by anammox in CWs would be mitigating of N_2O emissions as unlike denitrification anammox does not produce N_2O as byproduct (Van de Graaf et al. 1997). However, Erler et al. (2008) investigated that even when anammox contributed up to 30 % to N removal, high N_2O emissions were detected. Alternatively, CWs were considered to be a minor source of N_2O emissions and it was suggested that if all global domestic wastewater were treated by wetlands the share of the trace gas emission budget would still be less than 1 % (Teiter & Mander 2005). This means that absence of anammox process in CWs for waste- and groundwater treatment should not produce a negative impact on the global warming.

Anammox bacterial abundance

The abundance of anammox genes in biofilms attached on gravel and roots from HSSF CW was quantified with qPCR using *hzsA* primers, the phylogenetic marker for anammox bacteria (Harhangi et al. 2012). The results are shown in Fig. 3.3-3. The anammox bacteria were detected at all points in the CW with the highest abundance at the depth of 0.2 m, 1.39×10^6 copy number g^{-1} sample, and the lowest at 0.4 m depth, 3.69×10^4 copy number g^{-1} sample ($F(2,24) = 7.157$, $p = .004$). The abundance of the *hzsA* gene was changing with the distance from the inflow along the flow path at each depth ($p < 0.05$).

At the next step, anammox 16S rRNA gene primers were used. The results are shown in Fig. 3.3-4. The anammox bacteria were detected at all points in the CW with the highest abundance at the depth of 0.2 m, 1.67×10^8 copy number g^{-1} sample, and the lowest at 0.4 m depth, 6.78×10^6 copy number g^{-1} sample ($F(2,33) = 10.271$, $p = .000$). The anammox 16S rRNA gene was changing with the distance from the inflow along the flow path only at 0.2 m depth ($F(2,5) = 21.469$, $p = .004$).

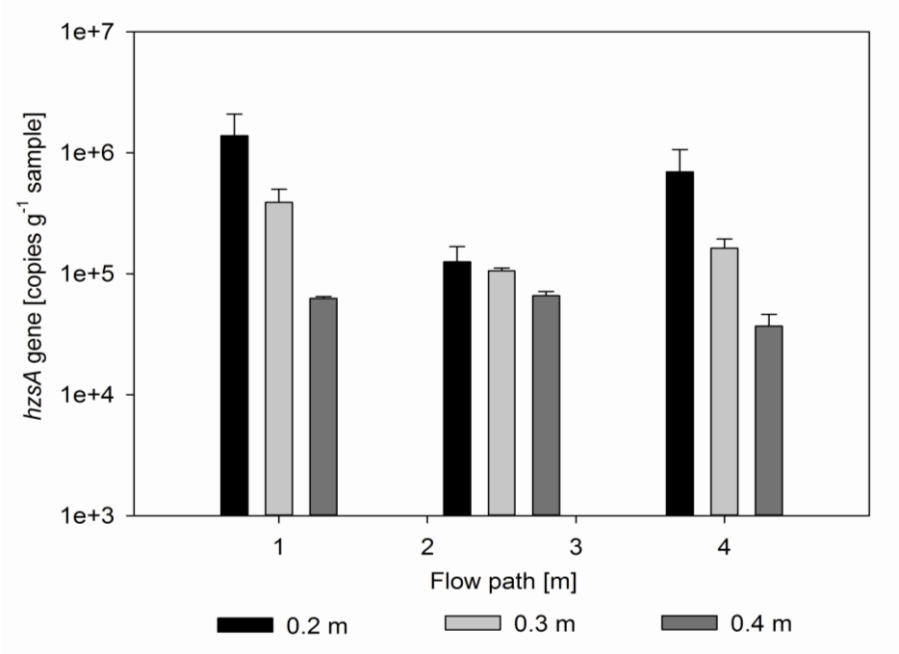

Fig. 3.3-3 The spatial variations of anammox bacterial abundance targeting on *hzsA* gene in samples of gravel and roots in the HSSF CW

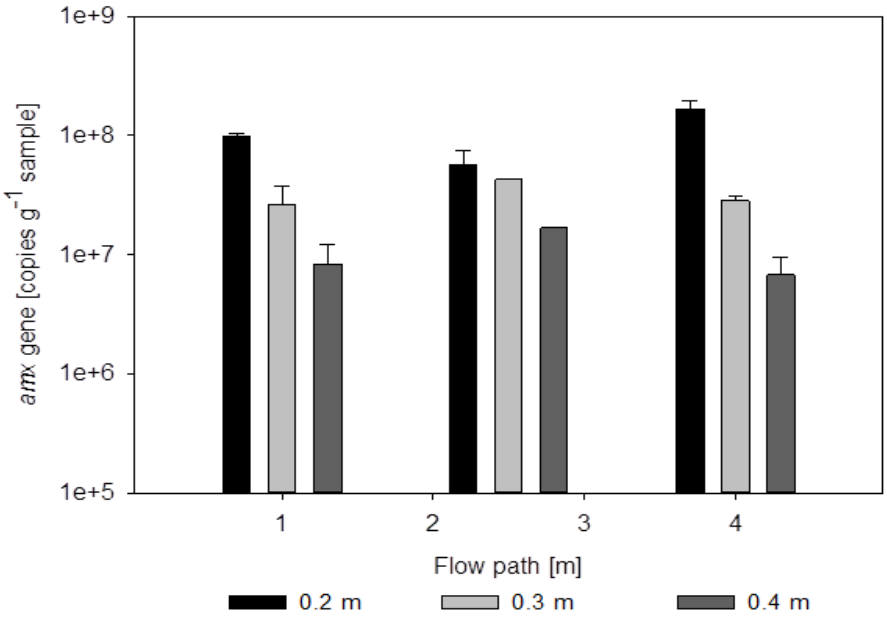

Fig. 3.3-4 The spatial variations of anammox bacterial abundance targeting on anammox 16S rRNA gene in the samples of gravel and roots in the HSSF CW

The hydrazine synthase gene was reported as a unique phylogenetic biomarker for anammox bacteria and is found only as one copy per cell (Harhangi et al. 2012). Therefore, this primer set is better than traditional *amx* primers as *amx* primers may target not only anammox group but other *Planctomycetes* as well (Humbert et al. 2010). Using traditional *amx* primers for the same samples gave us two orders of magnitude higher copy numbers of anammox bacteria (Fig. 3.3-4), and this results are similar to Bale et al. (2014). However, even using a highly specific target for anammox does not allow for correlating the abundance of the bacteria with their activity (Wang et al. 2012). So far, only one study in CWs detected active anammox process without stimulation and/or enhancement (Erler et al. 2008). While all main groups of bacteria of N cycle can be present in the system, some of them might be inactive and contributing to the total N turnover insignificantly (Philippot & Hallin 2005). The presence of O_2 and high concentrations of organic C make the freshwater ecosystems unsuitable for the occurrence of the anammox process.

Biodiversity of anammox bacteria

Positive results of qPCR targeting the *hzsA* gene made it possible to obtain the sequence information of a *hzsA* clone library from selected sampling points. The presence of anammox bacteria and diversity of the anammox community were studied by analysis of the *hzsA* gene clone library sequences in the gravel and root samples from HSSF CW. Positive qPCR results were obtained with all the sampling points. After cloning and sequence analysis, 68 clones appeared to represent *hzsA* genes. These clones were obtained from 3 locations inside the CW: sampling point (SP) 1 represents 1 m distance from inflow and 0.2 m depth, SP2 stand for 1 m distance from inflow and 0.3 m depth, and SP3 corresponds to 4 m distance from inflow and 0.2 m depth.

Three species of anammox bacteria, “Ca. Brocadia fulgida”, “Ca. Brocadia anammoxidans”, and “Ca. Kuenenia stuttgartiensis” were detected in the HSSF CW. Phylogenetic analysis of the *hzsA* genes (Fig. 3.3-5) showed that most of sequences in both SP1 and SP3 were related to “Ca. Brocadia fulgida”. One *hzsA* clone sequences from the SP1 and three *hzsA* clone sequences from the SP3 were closely related to the sequence of “Ca. Brocadia anammoxidans”, while two other SP1 clones with two SP2 clones clustered together with “Ca. Kuenenia stuttgartiensis”. No Jettenia asiatica- or Scalindua sp.- like *hzsA* sequence was obtained. While “Ca. Brocadia fulgida” and “Ca. Kuenenia stuttgartiensis” could be found at locations SP1 and SP3 (i.e. 1 and 4 m distance from the inflow), “Ca.Kuenenia” was detected at SP1 and SP2 (i.e. 1 m distance from the inflow). This reflects the presence of the various microniches in CWs suitable for different anammox species (Zhu et al. 2010). Other studies also showed high biodiversity of anammox in terrestrial ecosystems (Humbert et al. 2010). Any “Ca. Scalindua”- like *hzsA* sequence was found, which is not unexpected, since “Ca. Scalindua” species were shown to be the dominant anammox bacteria in marine ecosystems (Schmid et al. 2007). “Ca. Jettenia” was absent in the obtained sequences.

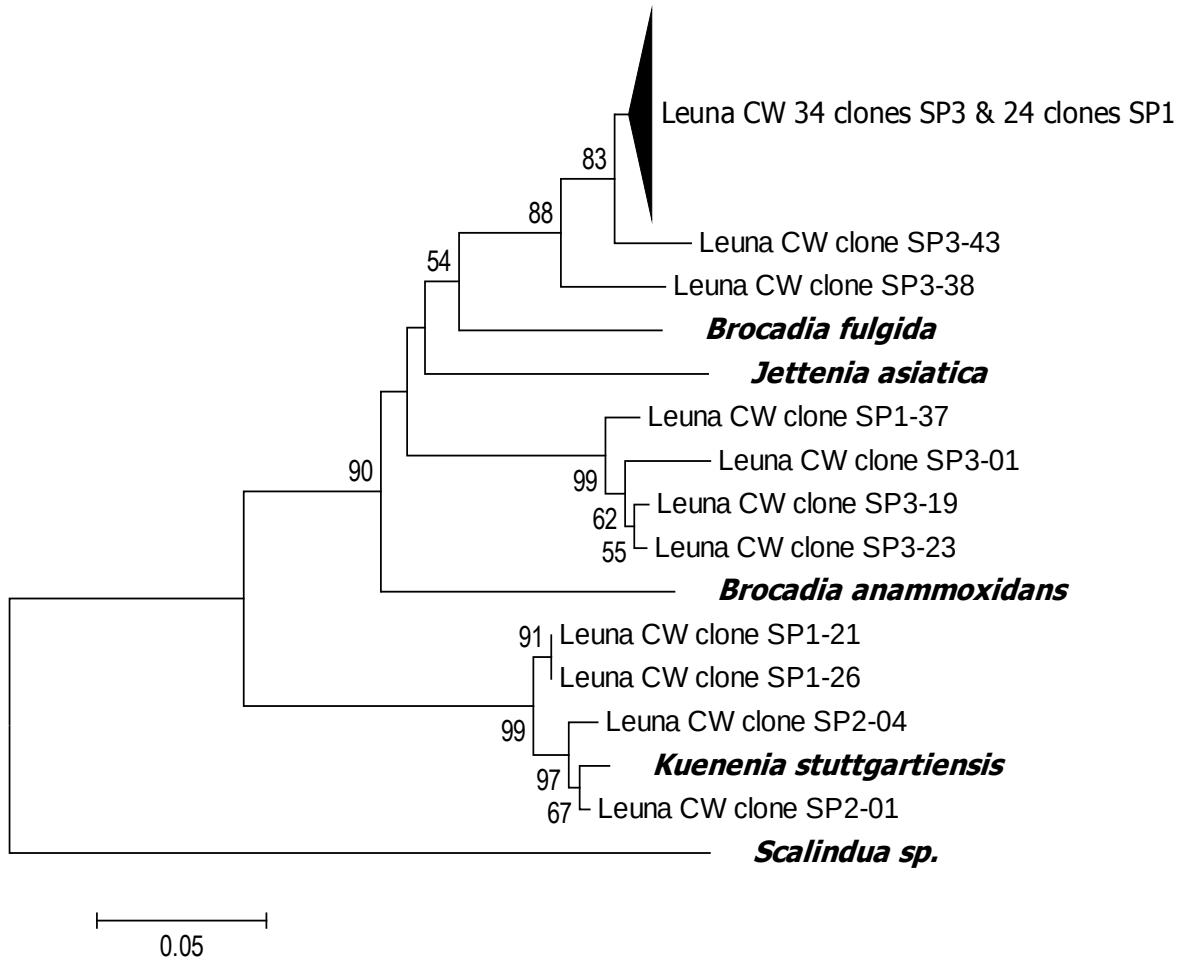

Fig. 3.3-5 Phylogenetic analysis of *hzsA* gene sequences from samples of gravel and roots in the HSSF CW (68 in total). The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.05544029 is shown. Bootstrap values of ≥50 (500 replicates) are shown at the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. The bar represents 5% sequence divergence. All positions containing gaps and missing data were eliminated. There were a total of 258 positions in the final dataset

3.3.3 Denitrification

Denitrifying bacterial abundance

Abundance of denitrifying genes was measured with qPCR targeting *nirS* and *nirK*, which are key enzymes responsible for NO_2^- reduction as the central step of the denitrification process. The results showed high copy number of denitrifying genes, between 1.43×10^8 and $7.74 \times 10^8 g^{-1}$ sample (Fig. 3.3-6). Furthermore, denitrifying bacteria were most abundant at 0.2 m and least abundant at the depth of 0.4 m ($F(2,32) = 12.278$, $p = .000$). The *nirS and nirK* genes were equally abundant with the distance from the inflow along the flow path at each depth ($p > 0.05$).

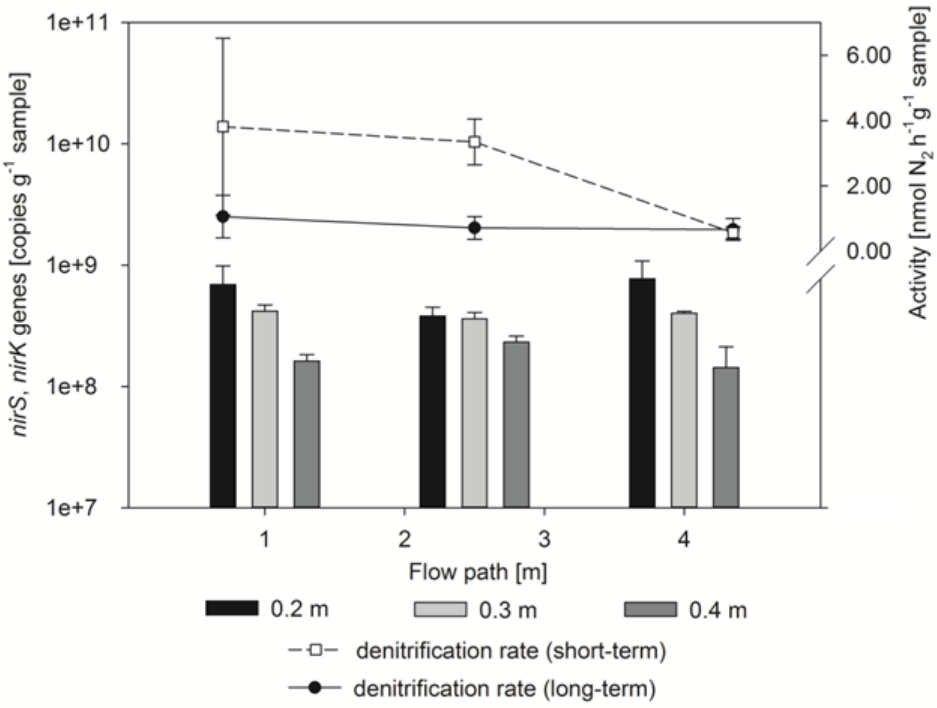

Fig. 3.3-6 The spatial variations of denitrification bacterial abundance targeting the *nirS* and *nirK* genes and the denitrification rates in short-term and long-term experimental set-up in samples of gravel and roots in the HSSF CW

Also, abundance of N_2O reducing denitrifying bacteria was measured with qPCR targeting the *nosZ* gene which encodes for the enzyme responsible for N_2O reduction. The results showed much lower copy number of *nosZ* gene than *nirS* with *nirK* genes, between 1.68×10^5 and 1.07×10^7 g^{-1} sample (Fig. 3.3-7). Furthermore, there was a strong dependency of this gene's distribution with depth ($F(2,33) = 12.012$, $p = .000$). The *nosZ* gene was most abundant at 0.2 m and least abundant at the depth of 0.4 m. The abundance of the *nosZ* gene was changing with the distance from the inflow along the flow path at each depth ($p < 0.05$).

The functional genes of denitrifying bacteria were abundant along the flow path at all depths. The highest number of denitrifying bacteria was shown at 0.2 m and the lowest at the 0.4 m depth, and this trend was consistent with the calculated turnover rates for denitrification. The denitrifying genes, *nirS* and *nirK*, were evenly distributed along the flow path.

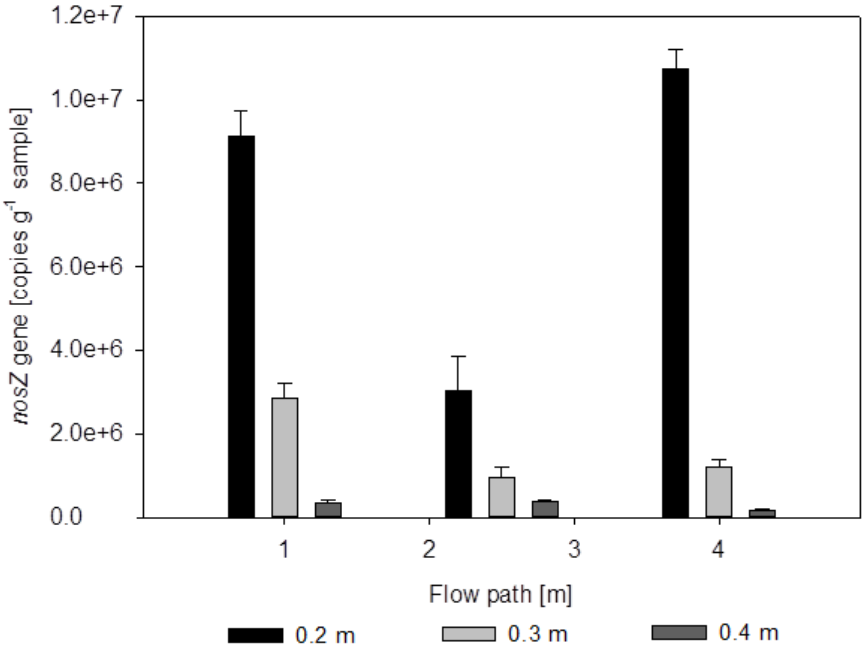

Fig. 3.3-7 The spatial variations of denitrification bacterial abundance targeting on *nosZ* gene in samples of gravel and roots in the HSSF CW

Denitrifying bacterial activity

At the next step, the potential denitrification rate was calculated with isotope labeling technique using the approach of Spott and Stange (2011). In three short-term sets of incubations ($^{15}NH_4^+$, $^{15}NO_3^-$, and $^{15}NH_4^+ + {}^{14}NO_3^-$) and the following long-term incubation with $^{14}NH_4^+ + {}^{15}NO_3^-$, only in the incubation sets with $^{15}NO_3^-$ labeled N_2 was produced, what made it possible to calculate denitrification rates. The results are presented in Table 3.3-1 and Fig. 3.3-6. Interestingly, there was a significant difference between rates calculated from the short-term and long-term incubations ($F(1,50) = 17.157$, $p = .000$). In case of short-term incubation, the maximum denitrification rates were detected at the 0.2 m depth and the minimum at the 0.4 m ($F(2,22) = 6.714$, $p = .005$), which showed the similar trend with the distribution of the denitrifying genes. However, in case of long-term experimental set-up there was no difference between depths ($F(2,24) = 1.181$, $p = .324$) and the rates were lower.

Table 3.3-1 Calculated turnover rates for N-turnover processes at different points in the HSSF CW

Distance from inflow [m]	**Depth [m]**	**Net nitri.[a] nmol NO_3^- h^{-1} g^{-1} sample**	**Gross nitri.[a] nmol NO_3^- h^{-1} g^{-1} sample**	**Nitrate consumption nmol NO_3^- h^{-1} g^{-1} sample**	**Anaerobic denitri.[b] (short-term) nmol N_2 h^{-1} g^{-1} sample**	**Anaerobic denitri.[b] (long-term) nmol N_2 h^{-1} g^{-1} sample**
1	**0.2**	1.60±0.87	7.09±0.33	109.31±89.03	3.82±2.71	1.06±0.65
2.5	**0.3**	2.12±0.23	6.84±3.72	164.23±200.9	3.35±0.70	0.72±0.35
4	**0.4**	1.26±0.10	1.33±0.29	7.34±12.19	0.57±0.19	0.67±0.33

a:nitrification, b:denitrification

As for denitrification potential turnover rates, the activity was depending on depth only in short-term incubations. Besides, the turnover rates in long-term denitrification incubations were much lower than in short-term ones. The possible reason for this could be a decrease of turnover rates over time in a batch experiment with the decrease of substrate concentration, according to Michaelis–Menten kinetics. This would also smooth out the difference between different sampling points. Therefore, only short-term incubations (up to 6 hours) for denitrification rate measurements should be used in further experiments.

As the inflowing water contained MTBE and benzene, these carbon sources were available throughout the flow path in the CW. Only low concentration of MTBE (0.4±0.4 mg L^{-1}) was present in inflow, therefore, benzene together with root excudates remained the main sources of organic carbon in our CW. The concentrations of the MTBE and benzene dissolved in pore water were measured and loads were calculated in order to determine where this C source could play an important role. The highest loads of MTBE and benzene were detected at 0.2 m depth and the lowest at 0.4 m depth (Fig. 3.1-5). However, this differences were significant neither for MTBE ($F(2,6) = .230$, $p = .801$) nor for benzene ($F(2,6) = .093$, $p = .912$).

In case of denitrification activity, the highest rates were also observed at 0.2 m depth and the lowest at 0.4 m. Given that such organic C source as benzene was present throughout the whole flow path (Fig. 3.1-5) and as the root density decreased with depth, we can assume that the root exudates play the main role in organic carbon delivery for the denitrification process. Another conclusion follows that denitrification is not inhibited by the presence of oxygen, what will be discussed further.

3.3.4 Nitrification

Ammonia oxidizing bacterial abundance

Abundance of ammonia oxidizing genes was estimated with qPCR targeting the α subunit of ammonium monooxygenase (*amoA*), which is the key enzyme in the aerobic ammonia oxidation process. The measured copy number was similar to the copy number of the *hzsA* gene but lower than the copy number of denitrifying genes, between 6.77×10^4 and $2.24 \times$

10^6 g^{-1} sample (Fig. 3.3-8). As with the anammox and denitrifying genes copy number, ammonia oxidizing gene copies show that these bacteria were most abundant at 0.2 m and least abundant at the depth of 0.4 m ($F(2,33) = 12.968$, $p = .000$). The abundance of the *amoA* gene was changing with the distance from the inflow along the flow path at each depth ($p < 0.05$).

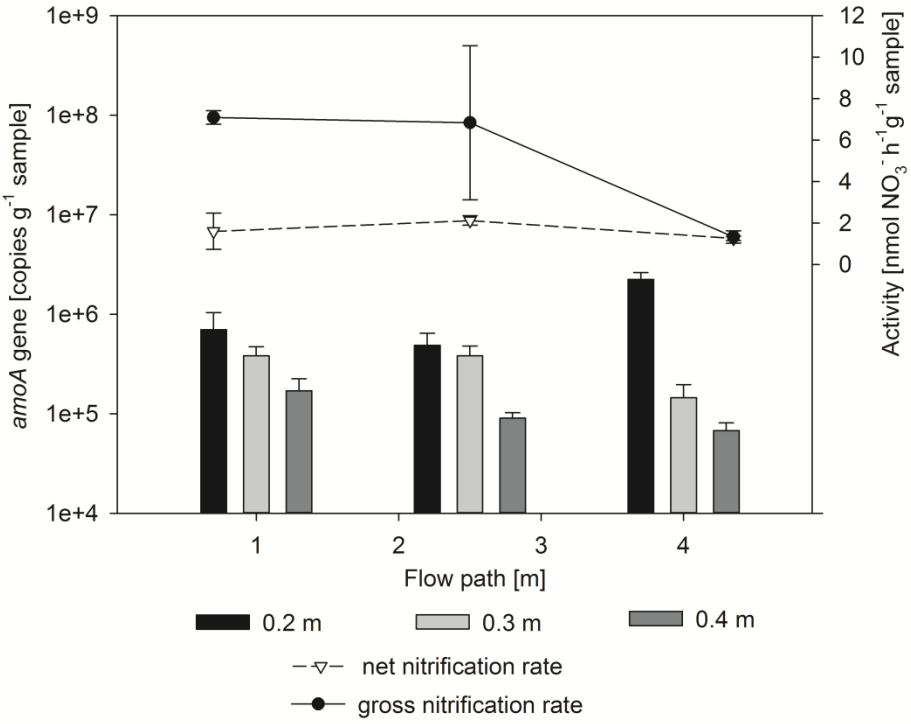

Fig. 3.3-8 The spatial variations of ammonia oxidizing bacterial abundance quantified via the *amoA* gene and the nitrification rates calculated using the mixing equation (net) and using the equation (1) (gross) in samples of gravel and roots in the HSSF CW

The functional genes of nitrifying bacteria were abundant along the flow path at all depths. This is well supported by the even distribution of $\delta^{15}N\text{-}NH_4^+$ along the flow path in the same system (Coban et al. 2014). The highest number of all investigated groups of bacteria was shown at 0.2 m and the lowest at the 0.4 m depth, and this trend was consistent with the calculated turnover rates for nitrification.

Ammonia oxidizing bacterial activity

Also, the net nitrification rates in $^{15}NH_4^+$ amendments were determined using the isotope mixing equation and the $^{15}NO_3^-$ amendments were used to determine the gross nitrification rate and the NO_3^- consumption rates using the equations (1) and (2) respectively. The results are presented in Table 3.3-1 and Fig. 3.3-8. The gross nitrification rates were significantly higher than the net nitrification rates ($F(1,49) = 15.599$, $p = .000$). The significant NO_3^- consumption rates were observed which were not depending on the location ($F(2,23) = .578$, $p = .569$). Likewise the distribution of the ammonia oxidizing genes, the gross nitrification rates were depending on the location of the sample, i.e. depth and distance along the flow path ($F(2,23) = 5.428$, $p = .012$) (Fig. 3.3-8).

The nitrification rates were calculated using isotope mixing equation for $^{15}NH_4^+$ amendments and using the equations (1) from Wessel and Tietema (1992) for $^{15}NO_3^-$ amendments. In the last case, NH_4^+ was not additionally added or primarily present in incubations and therefore, it derived from the mineralization of the organic material. Thus, in these incubations nitrification rate is equal to mineralization. Calculation of nitrification rates based on isotope mixing equation and $^{15}NH_4^+$ amendments does not consider a possible NO_3^- consumption. Nitrification estimation method from Wessel and Tietema (1992) produces the result as 'gross nitrification' and thus, account a possible NO_3^- consumption, unlike the isotope mixing equation. At depths 0.2 m and 0.3 m, where high NO_3^- consumption were detected, the results of nitrification rates using those two calculation methods were quite different. Likewise, at 0.4 m depth, where NO_3^- consumption was not significant, there was also no difference between net nitrification and gross nitrification rates. However, at this depth the nitrification rates were also much lower. It can be concluded that under high nitrification rate high NO_3^- consumption can also take place and should be account for the calculations of the nitrification rates. Therefore, the approach of Wessel and Tietema (1992) should be used for the nitrification turnover rate measurements.

The nitrification rates were depending on the location what can be explained by the enhancement of the microbial activity in the zones with the higher root density, i.e. the top sampling points. Furthermore, the highest rates of nitrification were detected at the 0.2 and 0.3 m depth where ammonia oxidizing bacteria were also the most abundant. This could imply the importance of plants' rhizosphere for the oxygen delivery as no oxygen is permitting into the system via surface in the HSSF CW (Vymazal & Kröpfelová 2008).

3.3.5 Aerobic denitrification

There could be several possible ways for the detected NO_3^- consumption under aerobic conditions in $^{15}NO_3^-$ amendments in the incubation experiment (Table 3.3-1). Dissimilatory nitrate reduction to ammonium (DNRA) is a microbial process that transforms NO_3^- to NH_4^+ via formation of NO_2^- in anaerobic or low O_2 environments. DNRA was shown to be relevant under reduced conditions (Eh = -200 mV) (Buresh & Patrick 1981). However, nowadays there is growing evidence that DNRA can also take place with presence of O_2, especially under high C:N ratio of about 10 and low NO_3^- concentrations (Leahy & Colwell 1990) and it is stimulated by presence of macrophytes (Stein & Arp 1998). Still, the rates of DNRA in CWs related to the total NO_3^- consumption reported in literature are in a range between 2 and 9 % (Kendall et al. 2007, Mariotti et al. 1988, Stein & Arp 1998) and therefore could not be of high importance in our incubations considering significant NO_3^- consumption. Immobilization (microbial uptake) could be another way of NO_3^- consumption. However, NH_4^+ is a preferred N source for microorganisms and NO_3^- uptake by microorganisms is inhibited when both NH_4^+ and NO_3^- are present (Cresswell & Syrett 1979, Recous et al. 1990). Taking into account the occurrence of nitrification in these incubations, NH_4^+ was present and therefore, NO_3^- uptake should be negligible. Denitrification was assumed to be an exclusively anaerobic process which occur only with absence of oxygen (Bryan 1981). However, in the laboratory studies evidence was found for denitrification to occur under

aerobic conditions as well (Robertson et al. 1995) and to be persistent at high O_2 levels (Lloyd et al. 1987). Aerobic denitrification or co-respiration implies the simultaneous use of both O_2 and NO_3^- as oxidizing agents and can be performed by various genera of microorganisms (Robertson & Kuenen 1984). Gao et al. (2010) concluded that O_2 dynamics did not strongly affect N consumption by denitrification in the presence of abundant NO_x^-, but rather denitrification coexisted with O_2 respiration. Therefore, the NO_3^-consumption rates under aerobic conditions in our incubations could be assigned to aerobic denitrification.

Reports about aerobic denitrification rates in natural environments are missing except for one study in marine sediments (Gao et al. 2010). This is the first report of aerobic denitrification rates in the freshwater ecosystems. At each depth examined in the incubations, the potential denitrification rate under aerobic conditions was much higher than measured under anaerobic conditions. However, given that the aerobic denitrification (NO_3^- consumption) is expressed in nmol NO_3^- h^{-1} g^{-1} sample and the anaerobic denitrification in nmol N_2 h^{-1} g^{-1} sample and that 2 mol of NO_3^- transform to 1 mol of N_2, rates of aerobic denitrification could be overestimated. The previous findings in the sea sediments reported quite similar rates of denitrification under aerobic and anaerobic conditions (Gao et al. 2010). The maximum denitrification rate was not observed in the deepest depth interval with the lowest redox potential, but rather in the surface 0.2 m depth. It is well related to the nitrification rates and implies simultaneous occurrence of nitrification and denitrification, as it has been shown by the stable isotope approach in Coban et al. (2014). Our CW was obtaining NH_4^+ rich contaminated groundwater, and therefore, it would be consistent to assume that denitrifying bacteria should be located in a close proximity to nitrifying bacteria in order to obtain NO_3^- from them. Biofilm formation is advantageous for this as it creates an oxic-anoxic interface and therefore, provides processes stratification (Fukada et al. 2004).

3.3.6 Conclusions from the molecular-biological and isotope labeling studies

A number of quantitative polymerase chain reactions were conducted in order to investigate the ratios between total bacteria and nitrogen transforming bacteria, and the spatial distribution of various bacteria of the nitrogen cycle. The investigated bacteria of the nitrogen cycle were present along the flow path at all distances. Most bacteria had the highest abundance at 0.2 m depth and the lowest at 0.4 m depth in the investigated horizontal subsurface flow constructed wetland. However, there were different trends in dependence on distance along the flow path. The apparent predominance of different genes at different locations may reflect differences in the specific enzyme properties. Different sampling points in the planted horizontal subsurface flow constructed wetland are different with respect to several parameters, e.g., oxygen status, organic carbon content, and pH, which may have profound influence on the composition of the microbial communities.

Although anammox bacteria were detected by molecular-biological techniques and their abundance was comparable with other anammox studies where activity of these bacteria was detected, in our horizontal subsurface flow constructed wetland anammox activity could not be detected and therefore, this process appeared to be of low importance in nitrogen transformations in the studied ecosystem of constructed wetland. The anammox process

might play a role in some constructed wetlands, *i.e.* in those systems where they will not be outcompeted by denitrifying bacteria such as at low carbon-to-nitrogen ratio. However, absence of anammox process in constructed wetlands for waste- and groundwater treatment should not produce a negative impact on the global warming. Nevertheless, high biodiversity of anammox species was found in our system: "Ca. Brocadia fulgida", "Ca. Brocadia anammoxidans", and "Ca. Kuenenia stuttgartiensis" were detected.

The nitrification and denitrification turnover rates were the highest close to the air-constructed wetland interface as well as the functional genes of these processes. Denitrification turnover rates were depending on incubation time. The turnover rates decrease over time in a batch experiment with the decrease of substrate concentration. Therefore, only short-term incubations for denitrification rate measurements should be used in further experiments. Nitrification rates were calculated with and without taking into account the loss of nitrate, and in the last case the rates were much smaller. This is because significant nitrate consumption was detected, even under aerobic conditions, what should be taken into account when estimating nitrification rates. This nitrate consumption was apparently due to aerobic denitrification. Therefore, in constructed wetlands denitrification should not be considered as an exclusively anaerobic process.

4. Concluding remarks

The aim of this study was to investigate the nitrogen cycle in constructed wetlands. For this, three different types of constructed wetlands were chosen to provide treatment comparison from ammonium and organic compounds polluted groundwater. Samples were taken from different locations on the constructed wetlands to provide spatial variations, and measurements were done throughout the year except for the winter season to investigate the seasonal variations. Several comprehensive approaches were applied: physico-chemical measurements, stable isotope fractionation and isotope labeling approaches, and molecular-biological investigations.

It was shown that the planted horizontal subsurface-flow constructed wetland had the highest treatment performance for ammonium. The floating plant root mat indicated lower ammonium treatment efficiency than the planted horizontal subsurface flow constructed wetland only in summer, and this illustrates that presence of gravel as substrate for attachment and growth of microorganisms also plays a role, even though only minor, in the nitrogen removal in planted constructed wetlands.

There was no significant seasonal variation in ammonium removal. High removal efficiency of ammonium by the planted horizontal subsurface flow constructed wetland illustrated that this system can be efficiently used for the ammonium treatment under moderate air temperature of above 10 °C, *i.e.* throughout the year except for the winter season. However, absolute ammonium removal can be reached only under reasonable loads, what is average 250 g ammonium-nitrogen m^{-2} yr^{-1}.

The floating plant root mat as a variant of constructed wetlands without planting media could be a cost-competitive variant and an alternative for the treatment of distinctively contaminated waters, such as for the contaminants benzene and methyl-tert-butyl-ether, as this system is showed the best treatment efficiency for these contaminants. Nevertheless, further process optimization is especially necessary in this case to fulfill German national regulations for discharging into natural receiving streams.

The investigations of the nitrogen stable isotope fractionation revealed a clear correlation of the enrichment of heavy isotope of nitrogen in ammonium to the decrease of ammonium loads along the flow path through the constructed wetlands. This strongly indicated the occurrence of nitrification. The co-occurrence of three processes, nitrification, denitrification, and mineralization was proven by the laboratory experiments for the determination of potential process-specific enrichment factors.

Furthermore, the application of stable isotope fractionation approach combined with common physico-chemical investigations enabled us to identify key factor influencing efficiency of nitrogen removal in constructed wetlands, which was also the plant presence as substrate for attachment and growth of microorganisms. Isotope fractionation patterns revealed that nitrification-denitrification were prevailing processes in planted constructed wetlands

throughout the year, occurring in a linear way along the flow path, and not depending on depth in the root zone.

The anammox process was detected neither by stable isotope fractionation approach nor by isotope labelling incubations. However, anammox bacteria were identified by molecular-biological techniques and the information about their diversity in the horizontal subsurface flow constructed wetland obtained. Still, absence of anammox activity implies that this process is of low importance in nitrogen transformations in the studied ecosystem of constructed wetland. The anammox process might be relevant in other types of constructed wetlands, for example which have low carbon-to-nitrogen ratio. However, absence of anammox process in constructed wetlands for waste- and groundwater treatment should not produce a negative impact on the global warming and therefore, there is no further need of trying to enhance this process in constructed wetlands.

Unlike the stable isotope fractionation, the results of laboratory experiments for potential turnover rates for nitrification and denitrification revealed that these processes mostly occur at 0.2-0.3 m depths, which is the root depth of *Phragmites australis*. The incubation time for denitrification turnover rate experiment was tested, and application of only short-term incubations was proposed.

Furthermore, it was shown that significant nitrate consumption occurs under the aerobic conditions as well. Therefore, when accounting nitrification rates, only gross nitrification rates with considering nitrate losses should be used.

The nitrate consumption under aerobic conditions could be assigned only to aerobic denitrification. The rates of the aerobic denitrification were significant in comparison with anaerobic denitrification. This is the first report of aerobic denitrification rates in freshwater ecosystem. Obviously, in constructed wetlands denitrification should not be considered as an exclusively anaerobic process, probably due to processes stratification via biofilm formation.

Future research should be addressed to applied and basic topics. In applied research guidelines like effect of different plant species, in basic research, nitrogen transformation processes such as dissimilatory nitrate reduction and interactions of nitrogen cycle with other elemental cycles such as sulphur are examples of still open questions.

By improving knowledge in these above mentioned aspects it will be in future possible to broaden the application fields of constructed wetlands and give better advices to engineers in which special cases constructed wetlands can be an advantageous treatment option in comparison to the numerous other available technologies.

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