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CHARACTERIZATION OF THE MICROBIAL POPULATION IN THE FILTER BODY OF SUBSURFACE VERTICAL FLOW CONSTRUCTED WETLANDS

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Preface

The presented study was carried out at the Institute for Sanitary Engineering and Water Pollution Control of the Department for Water, Atmosphere and Environment at the University of Natural Resources and Applied Life Sciences Vienna (BOKU). The work was part of the project "Characterization of microbial biocoenosis to optimise removal efficiency and design of subsurface flow constructed wetlands for wastewater treatment" funded by the Austrian Science Fund (FWF, project no.: P16212-B06), which started in May 2003 and ended May 2006.

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- Center for Anatomy and Cell Biology - Medical University of Vienna
- Clinical Institute of Hygiene and Medical Microbiology, Water Hygiene, Medical University Vienna
- Institute for Chemical Engineering, Working group Gene Technology, Vienna University of Technology
- Institute for Water Quality, Resources and Waste Management, Vienna University of Technology
- Department of Bioresources Working Group Environmental Molecular Analytics: Austrian Research Center, Seibersdorf
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Abstract

Vertical flow constructed wetlands (VFCWs) with intermittent loading, and sandy filter materials represent the latest generation of constructed wetlands and are able to fulfil the required effluent requirements. To obtain a more detailed knowledge of the microorganisms, mainly responsible for the purification performance of a VFCW, the distribution of the biomass, productivity and diversity of the microbial population, inhabiting the filter bodies of VFCWs, were determined within this study. Six of the eight investigated pilot-scale constructed wetlands (PSCWs) were planted with *Miscanthus sinensis giganteus*, whereas the other two were unplanted. For an evaluation of the results obtained from the PSCWs an outdoor full-scale constructed wetland (FSCW) planted with *Phragmites australis* was investigated additionally.

For the determination of the microbial biomass four different approaches (adenosine triphosphate (ATP) - content, fumigation – extraction for microbial carbon, substrate induced respiration, phospholipid fatty acids content), were applied to quantify the microbial biomass in seven different depths of the indoor and outdoor VFCWs. Additionally the bacterial biomass was determined within the different depth layers by a direct microscopic approach. These four methods used to determine the microbial biomass showed that >50 % of the total microbial biomass were located in the first cm and about 90 % of the microbial biomass were present in the first 10 cm of the filter body within the in- and outdoor VFCWs, due to the high nutrient content and the good oxygen supply. All four different methods used to estimate the microbial biomass gave similar results. Bacterial biomass accounted only for 17-54 % of the total microbial biomass (MB) within the first 10 cm of the filter bodies of the investigated VFCWs. Further investigations indicated that fungi play only a minor role within this system. The big discrepancy between microbial and bacterial biomass seems to be the result of an underestimation of the bacterial biomass due to methodical problems (e.g. incomplete displacement of cells from the filter material). The MB accounted for 3-13 % of the total organic carbon within the investigated VFCWs. During operation of the plant organic matter accumulated. However, this enrichment was only significant within the top 5 cm of the investigated VFCWs.

Easy biodegradable carbon is utilized by heterotrophic microorganisms and metabolized or assimilated for biomass growth (bacterial secondary production = BSP). For a localization of the microbial carbon degradation processes within the filter body of the investigated VFCWs, the BSP was determined in different depths, by measuring the incorporation rate radioactive labelled amino acid leucine. It was shown that the BSP decreased with increasing filter body depth, but not as strong as the number of bacteria. About 80 % of the BSP took place within the first 10 cm of the filter bodies of the VFCWs. The BSP was not fluctuating much within the same depth layer of the PSCWs, mainly due the hydraulic optimized sprinkler system which enabled an equal distribution of the wastewater across the one square meter surface. These findings emphasize the importance of an efficient hydraulic loading system to enable an equal utilization of the whole filter body.

The microbial and bacterial biomass and productivity in all depths of the filter body was not significantly different between the planted and unplanted systems. A comparison between the wastewater purification performances of planted and unplanted PSCWs also showed no significant difference. But it has to be noted critically that the plant growth on the indoor PSCWs was rather poor due to the indoor conditions and therefore it remains arguable if there are differences regarding the microbial community of planted and unplanted VFCWs.

Additionally a simple mass balance calculation of bacterial C - fluxes within VFCWs was performed. From this approach median doubling times of 33-35 h for the bacteria of the PSCWs have been calculated. This simple model for the carbon utilization within VFCWs revealed, that in the planted indoor PSCWs about 86% of the total carbon input of the system were utilized by bacteria for biomass growth and respiration (bacterial carbon demand), underlining the role of bacteria within the carbon removal of VFCWs.

Besides the microbial biomass and productivity distribution within the filter body of the VFCWs the characterization of the diversity of the bacterial community was another main focus within this study. For a general assessment of the diversity of bacteria inhabiting VFCWs, the molecular fingerprint technique “Terminal Restriction Fragment Length Polymorphism” (T-RFLP), was applied to assess potential vertical distribution patterns of bacterial diversity within the investigated VFCWs. No significant correlation between the depth-zone of the filter body and the bacterial diversity was observed, although few bacteria decreased or increased with depth. The results obtained from the outdoor and indoor VFCWs indicate, that constructed wetlands with similar design and operational mode are colonized by similar populations, showing only little variation in their composition over filter depth.

By construction of a clone library, based on the 16S rRNA, a brief inventory of the most dominant and metabolically active bacteria inhabiting the filter material, was done. The most abundant active bacteria belonged to the Actinobacteria and Betaproteobacteria as well as to unknown bacterial phylogenetic groups. Additionally Firmicutes, Planctomycetales, Chloroflexi and Bacteroidetes could have been detected within the investigated VFCWs.

Furthermore the diversity of ammonia oxidizing bacteria (AOB), which are performing the rate-limiting step of the nitrification process, was analyzed within the investigated FSCW. Therefore the molecular fingerprint technique “Denaturing Gradient Gel Electrophoresis” (DGGE) was applied, to display the diversity within the functional gene “ammonia monooxygenase”, present only in ammonia oxidizers. It was shown that AOB were present down to the bottom of the filter body (50 cm depth), which indicates, that ammonia oxidation takes place in all depth layers due to the aerobic conditions within the whole filter body. AOB were detected during the cold and warm season, showing that the presence of AOB species is not influenced by strong temperature changes, but no conclusions about their physiological status can be drawn from these results. The depth profiles showed no differences, with the only exception of a slightly higher diversity within the uppermost 0-5 cm of the filter body. The two dominant DNA-bands within in the DGGE-gel were present within all analyzed samples, and could have been affiliated to the AOB lineages *Nitrosomonas europaea / “Nitrosococcus mobilis*” and *Nitrosospira.* Whereas *Nitrosomonas* is a typical AOB present within most wastewater treatment plants and adapted to high ammonia concentrations, whereas *Nitrosospira* is more common in soil ecosystems. Although only very few *AOB* were detected in the wetland filter material, the investigated FSCW displayed a very efficient and stable nitrification performance around the whole year.

This study demonstrated that VFCWs with a filter body consisting of sandy filter material and intermittent loading with domestic wastewater provide optimal conditions for the development of an abundant, productive and diverse microbial community, which seem to be generally unaffected by the cultivated plants of the system.

Keywords

Ammonia oxidizing bacteria

Bacterial diversity

Bacterial secondary production

Microbial biomass

Vertical flow constructed wetlands

Kurzfassung

Vertikal durchströmte „bepflanzte Bodenfilter“ (BBF), auch Pflanzenkläranlagen genannt, mit intermittierender Beschickung und einem sandigen Filtermaterial repräsentieren die neueste Generation von BBF. Dieser Anlagen-Typus ist in der Lage, die in der österreichischen Abwasseremissionsverordnung für kommunales Abwasser von Kleinkläranlagen geforderten Ablaufgrenzwerte einzuhalten. Zur Optimierung des Systems BBF ist ein möglichst detailliertes Wissen über Mikroorganismen, die hauptverantwortlich für die Reinigungsleistung der Anlagen sind, notwendig. In dieser Studie wurde die Verteilung der mikrobiellen Biomasse sowie der bakteriellen Produktivität und Diversität innerhalb von vertikal durchströmten Pilotanlagen untersucht. Sechs der acht indoor Pilotanlagen waren mit *Miscanthus sinensis giganteus* bepflanzt, während die beiden anderen Anlagen unbepflanzt waren. Um die Übertragbarkeit der für die Pilotanlagen ermittelten Ergebnisse auf eine Anlage im realen Betrieb zu testen, wurde zusätzlich eine Freilandanlage mit gleichem Aufbau und identer Betriebsweise untersucht, die jedoch anstelle von *Miscanthus* mit *Phragmites australis* bepflanzt war.

Zur Bestimmung der mikrobiellen Biomasse in sieben unterschiedlichen Tiefen der Filterkörper der Pilot- sowie der Freilandanlage wurden vier unterschiedliche Methoden angewandt: die Bestimmung des Adenosin - Triphosphat - Gehalts, die Fumigation - Extraktion zur Ermittlung des mikrobiellen Kohlenstoffs, die substratinduzierte Respiration, sowie die Messung des Phospholipid - Fettsäuren - Gehalts. Weiters wurde auch die bakterielle Biomasse in den unterschiedlichen Tiefenschichten mittels einer direkten mikroskopischen Methode ermittelt.

Die vier Methoden zur Bestimmung der mikrobiellen Biomasse zeigten, dass in allen hier untersuchten BBF aufgrund der optimalen Nährstoff- und Sauerstoffversorgung mehr als 50 % der gesamten mikrobiellen Biomasse (MB) innerhalb des obersten Zentimeters des Filterkörpers lokalisiert waren; ca. 90 % der mikrobiellen Biomasse befanden sich innerhalb der obersten 10 cm Schicht. Die vier unterschiedlichen Methoden zu Abschätzung der MB lieferten ähnliche Ergebnisse. Der Anteil bakterieller Biomasse an der gesamten MB betrug 17-54 % innerhalb der obersten 10 cm Schicht der untersuchten Anlagen. Die Diskrepanz zwischen mikrobieller und bakterieller Biomasse kann nicht durch Pilz - Biomasse erklärt werden, da innerhalb dieser Studie gezeigt wurde, dass Pilze nur eine untergeordnete Rolle in diesem System spielen, vielmehr wurde dieser Unterschied vermutlich durch eine Unterschätzung der bakteriellen Biomasse (z.B. aufgrund unvollständiger Ablösung der Zellen vom Filtermaterial) verursacht. Der Anteil der MB am gesamten organischen Kohlenstoff betrug 3-13 %. Mit zunehmender Betriebsdauer der BBF kam es zu einer Akkumulation von organischem Material im Filterkörper, diese Anreicherung war jedoch nur in den obersten 5 cm der hier untersuchten Anlagen signifikant.

Vertikal durchströmte BBF sind sehr effiziente Systeme zur Entfernung von Kohlenstoff-Verbindungen aus Abwässern. Leicht abbaubarer Kohlenstoff wird von heterotrophe Mikroorganismen metabolisiert und zwecks Biomassewachstum assimiliert (bakterielle Sekundärproduktion = BSP). Durch die Bestimmung der Einbaurate der radioaktiv markierten Aminosäure Leucin konnte eine räumliche Verteilung der mikrobiellen Abbauprozesse innerhalb des Filterkörpers ermittelt werden. Es zeigte sich, dass die BSP mit zunehmender Filterkörpertiefe abnimmt; ca. 80 % der BSP fanden in den obersten 10 cm der im Rahmen dieser Studie untersuchen BBF statt. Daraus lässt sich schließen, dass auch der Großteil der mikrobiellen Abbauprozesse in dieser obersten Filterkörperschicht statt findet. Innerhalb einer Tiefenschicht schwankte die BSP kaum. Ein Grund dafür liegt in der gleichmäßigen Verteilung des Abwassers über die gesamte Filteroberfläche mittels eines effizienten Sprinkler-Systems. Diese Tatsache unterstreicht die Notwendigkeit eines optimierten Abwasser-Beschickungs-Systems, um die Ausnutzung des gesamten Filterköpervolumens zu ermöglichen.

Bezüglich der mikrobiellen und bakteriellen Biomasse sowie der Produktivität zeigte sich kein signifikanter Unterschied zwischen bepflanzten und unbepflanzten Anlagen. Ein Vergleich

der Reinigungsleistungen der bepflanzten und unbepflanzten Pilotanlagen zeigte ebenso keinen signifikanten Unterschied. Es muss jedoch angemerkt werden, dass *Miscanthus* aufgrund der schlechten Lichtverhältnisse nur sehr kümmerlich anwuchs, daher ist der Vergleich zwischen bepflanzten und unbepflanzten Pilotanlagen nur bedingt aussagekräftig.

Zusätzlich wurde eine einfache Massenbilanzierung der bakteriellen Kohlenstoffflüsse innerhalb des Systems BBF durchgeführt. Aus diesem Ansatz wurden Verdoppelungszeiten von 33-35 Stunden für die Bakterien der Pilotanlage berechnet. Dieses einfache Modell der bakteriellen Kohlenstoffflüsse zeigte, dass ungefähr 86% des Kohlenstoff-Eintrags in der Anlage von Bakterien zum Biomassewachstum und zur Respiration verbraucht wurden. Diese Zahlen unterstreichen die Bedeutung der Bakterien innerhalb des Kohlenstoffabbaus in BBF.

Neben der Ermittlung der Verteilung der mikrobiellen Biomasse und bakteriellen Produktivität innerhalb einer vertikal durchströmten Pflanzenkläranlage lag ein weiterer Schwerpunkt dieser Studie in der Charakterisierung der Diversität der Bakterien-gemeinschaften. Für eine generelle Beschreibung der bakteriellen Artenvielfalt der Bakterien innerhalb der Filterkörper der Pilot- sowie der Freilandanlage, wurde die molekularbiologische „Fingerprint-Technik“ “Terminal Restriction Fragment Length Polymorphism” (T-RFLP) angewandt. Im Rahmen der hier durchgeführten Untersuchungen konnte kein Unterschied in der Diversität in den einzelnen Tiefenschichten festgestellt werden, obwohl einige wenige Arten deutlich mit zunehmender Filterkörpertiefe zu- bzw. abnahmen. Es zeigte sich, dass BBF mit gleichem Aufbau und identer Betriebsweise auch von einer ähnlichen Bakteriengemeinschaft kolonisiert werden, die sich nur geringfügig in den einzelnen Tiefenschichten voneinander unterschieden.

Durch die Erstellung einer Klon-Bibliothek, basierend auf der 16S rRNA, konnte eine grobe Bestandsaufnahme der dominantesten metabolisch aktiven Bakterien der untersuchten BBF durchgeführt werden. Diese zeigte, dass Vertreter der Klasse Actinobacteria und Betaproteobacteria sowie bis jetzt noch nicht beschriebene phylogenetische Gruppen am häufigsten vertreten waren. Weiters konnten auch Bakterien der Klassen Firmicutes, Planctomycetales, Chloroflexi und Bacteroidetes in den untersuchten BBF nachgewiesen werden.

Zusätzlich wurde die Diversität von Ammoniak oxidierenden Bakterien (AOB) untersucht, die den geschwindigkeitsbestimmenden Schritt innerhalb des Nitrifikationsprozesses katalysieren, der wiederum eine zentrale Rolle innerhalb des Stickstoffabbaus in PKA spielt. Die molekularbiologische Fingerprint - Technik „Denaturierende Gradienten Gel Elektrophorese“ (DGGE) wurde angewendet um die Diversität des funktionellen Gens „Ammoniak Monooxygenase“, das ausschließlich in AOB vorkommt, wiederzugeben. AOB konnten bis in die untersten Schichten des Filterkörpers nachgewiesen werden. Daher ist anzunehmen, dass aufgrund der aeroben Verhältnisse im gesamten Filterkörper Nitrifikation stattfindet. Da AOB sowohl im Sommer als auch im Winter detektiert werden konnten, lässt sich daraus schließen, dass ihr Vorkommen nicht maßgeblich von Temperaturschwankungen beeinflusst wird. Allerdings sagt dieses Ergebnis nichts über die Stoffwechselaktivität der Bakterien aus. Die AOB-Gemeinschaft zeigte keine deutlichen Unterschiede zwischen den einzelnen Tiefenschichten, mit Ausnahme einer etwas höheren Diversität in den obersten 0 - 5 cm des Filterkörpers. Die zwei dominierenden DNA - Banden innerhalb des DGGE - Gels, die in jeder Tiefenschicht vorhanden waren konnten den AOB-Linien *Nitrosomonas europaea / “Nitrosococcus mobilis*” und *Nitrosospira.* zugeordnet werden. Während *Nitrosomonas* ein typischer AOB - Vertreter konventioneller Kläranlagen ist, kommt *Nitrosospira* eher in Bodenökosystemen vor. Obwohl nur sehr wenige verschiedene AOB innerhalb des Filtermaterials detektiert werden konnten, zeigte die Anlage eine stabile und effiziente Nitrifikationsleistung während der gesamten Untersuchungsperiode.

Diese Studie zeigt, dass vertikal durchströmte bepflanzte Bodenfilter mit intermittierender Beschickung und einem sandigen Filtermaterial optimale Bedingungen für die Entwicklung einer zahlreichen, diversen und produktiven mikrobiellen Lebensgemeinschaft bieten.

Schlagwörter

Ammoniak Oxidierer

Bakterielle Diversität

Bakterielle Sekundärproduktion

Mikrobielle Biomasse

Pflanzenkläranlagen

Vertikal durchströmte beflanzte Bodenfilter

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1 Introduction

1.1 General Background

The consumption of water by human activities produces polluted water, which is discharged back to the environment and may cause damage to human health and environment. Therefore the importance of preserving and maintaining the water quality is a major national and international task and responsibility. Improving global access to clean drinking water and safe sanitation is one of the least expensive and most effective means to improve public health and save lives.

In 2004, a total of 5.3 billion people had access to improved drinking water sources – up from 4.1 billion in 1990. Yet, due to the population growth, the number of people unserved has not changed significantly since then. About one sixth of the world's population - a total of 1.1 billion people - remain without access to improved drinking water. 2.6 billion people even do not have access to basic sanitation. Worldwide, about 1.7 million deaths a year - 90 % of which are children - are attributed to unsafe water, poor sanitation and hygiene, mainly through infectious diarrhoea (WHO / UNICEF report, 2004). With the increase of the human population and the higher degree of industrialisation also the amount of polluted water (wastewater) produced by residential areas and industry increased. At the beginning of the 20th century, a few cities and industries began to recognize that the discharge of wastewater directly into streams was detrimental, and this led to the construction of wastewater-treatment facilities (Moharikar et al., 2005).

In nature as well as in wastewater treatment plants (WWTPs) organisms are the key players in the purification process. In both natural and engineered systems the abilities of microorganisms to degrade diverse organic compounds are indispensable for the wastewater purification process. The successful exploitation of these features has been achieved for almost a century in biological wastewater treatment plants. These facilities are among the most important biotechnological applications, preventing the pollution of natural ecosystems and the spread of wastewater-born diseases such as cholera and typhoid fever (Daims et al., 2006a). Compared to chemical or physical technologies biological systems for wastewater treatment are more efficient, they have lower construction and operating costs, they are more robust and they do not need replacement once loaded (Moharikar et al., 2005).

Within wastewater treatment it can be distinguished between fixed film- or suspended growth systems. Within fixed-film treatment plants the biomass grows on a supporting media and the sewage passes over its surface. These systems include rotating biological contactors or diverse biofilters, like trickling filters, or soil / sand filters (e.g. constructed wetlands), whereas in suspended growth systems (like activated sludge tanks) the biomass is free floating and well mixed with the sewage. For a general comparison of different wastewater treatment systems it can be stated that, fixed-film systems (including constructed wetlands) are more able to cope with drastic changes in the amount of biological material and can provide higher removal rates for organic material and suspended solids than suspended growth systems (such as activated sludge). On the other hand suspended growth systems can be operated with a smaller area demand per person than fixed-film systems that treat the same amount of wastewater.

Today a wide range of treatment technologies for domestic wastewater (sewage) is available. Conventional wastewater treatment plants, based on the activated sludge process and a wide range of alternative techniques, including various forms of aquaculture systems (e.g. ponds and ditches) and wetland systems, are in use for wastewater treatment. Conventional wastewater treatment normally relies on large-scale plants including extensive wastewater collection systems. It is the preferred form of wastewater treatment in developed countries to a large extent because it is a well-known technology in which civil engineers

throughout the world are trained. It also minimizes the area required for treatment per person, which is an important consideration in urban areas where space is at a premium.

Alternatives to conventional wastewater treatment are often unknown and sometimes resisted by the engineering and public works communities. Their land requirements are larger than for conventional plants and therefore they are widely perceived as not applicable in urban areas where land is limited. Although the activated sludge process is still the most popular biological wastewater treatment technique, for both industrial and municipal wastewater, alternative technologies, like constructed wetlands, have become the favoured option for many wastewater treatment purposes.

Sustainability minimizes the short and long term environmental impacts of development activity through resource conservation, recycling, waste minimization, and the utilization of energy efficient and ecologically responsible materials and procedures (Campbell and Ogden, 1999). Sustainability challenges us also to reflect on wastewater treatment differently. The traditional wastewater management concept (urban wastewater collection system plus treatment of the wastewater in a central treatment plant) has been successfully applied over many decades in densely populated areas of industrialized countries (Wilderer and Schreff, 2000). Instead of focussing on end-of-pipe-treatment for emission prevention, attention shifts towards optimal resource utilization, favouring the development of decentralised systems (Balkema et al., 2002). Domestic wastewater can be treated close to where it is created or collected and transported via a sewer system to a municipal treatment plant. Instead of building large sewer systems on-site wastewater treatment systems are used especially in rural areas. Advantages of decentralized wastewater cleaning strategies are the following:

- low-cost due to the absence of extensive wastewater collection systems
- require less maintenance
- construction, repair and operation can be done by the local community or plot owner
- a large number of opportunities are granted for on-site water reuse and groundwater recharge
- failures of single units do not cause the collapse of the whole system

Constructed wetlands have often been integrated in decentralized wastewater treatment concepts and used as a beneficial option for on-site wastewater treatment. Given the pressing need for clean, disinfected water in developing countries this is an area where the application of the economical beneficial wastewater treatment system constructed wetlands can be play an important role within concepts to solve water supply and sanitation problems (e.g. Nelson et al., 2001).

1.2 Constructed wetlands (CWs) – a nature-oriented technology for wastewater treatment

Over the past 40 years, more and more interest has been expressed in the potential use of a variety of nature-oriented systems to help, to restore and to preserve the water quality. The potential for achieving improved water quality while creating valuable wildlife habitat has lead to a growing interest in the use of CWs for treating and recycling wastewater. Since the introduction of CWs for wastewater purification in the sixties (Seidel, 1966) this technology has been developed and become a well-accepted technique for treating different kinds of

wastewaters from point and nonpoint sources of water pollution. Wetlands rely on self-maintaining, self-regulating biological processes. A CW can be defined as “a designed and man-made complex of saturated substrates, emergent and submergent vegetation, animal life, and water, that simulates natural wetlands for human use and benefits” (Hammer, 1989). CWs provide an ecological and economical alternative to conventional WWTP. The knowledge accumulated over time about ways in which contaminants can be removed by simple natural combination of water plants and a filter material (soil or an artificial substrate), has led to the deliberate application of such systems in nature and ultimately to the creation of artificial systems with various states of naturalness (Stottmeister et al., 2003).

Constructed wetlands are engineered land-intensive biological treatment systems, which have been modelled on natural wetlands to take advantage of the processes, which decompose and retain nutrients in these systems. Therefore CWs are involving a macrophytic vegetation and a filter material. A complex microbial community, mainly associated to the filter material, developed in interaction with the wastewater, is primarily responsible for the purification performance of the system. A main difference between a constructed and a natural wetland is the much greater degree of control, thus allowing the establishment of experimental treatment facilities with well defined composition of substrate, vegetation and water regime (Brix, 1993). Depending on the wetland type the filter body can be supported by an adjacent drainage layer below the filter body, to collect the purified water and to release it e.g. into receiving waters. Also the constantly high nutrient load is in contrast to most natural wetlands. Especially in colder climates the application of wastewater with higher temperatures than the environment enables the operation also during winter with only minor reductions regarding the purification efficiency of the system.

CWs can be used for different kinds of wastewater, including municipal wastewater, stormwater runoff, agricultural wastewater, coal mine drainage, landfill leachate and with some restrictions also for industrial polluted water. These systems can be applied for secondary treatment, responsible for degradation of the organic content of domestic or municipal wastewater, or for tertiary treatment as a polishing stage. By using CWs as a secondary wastewater treatment stage a primary treatment for the removal of solids is necessary. Brix and Arias (2005) recommend to pre-treat the sewage in a two- or three chamber sedimentation tank (septic tank) prior to loading on the CW. Constructed wetlands are also regarded as important elements in polishing conventionally treated wastewater for recreational and environmental applications (Ghermandi et al., 2007a).

CWs ranging in size from small-single family residential systems, to municipal facilities, up to a capacity for about 1,000 person equivalents (PE). The German guidelines distinguish between on-site constructed wetland systems ranging up to 50 PE and municipal systems up to a size of 1,000 PE (ATV, 1998). The limiting factors for the maximal size of a CW are the area demand and additionally the technical requirements (e.g. pump capacity) are increasing disproportional. Brix and Arias (2005) pointed out, that it is very important to make sure that the pump capacity is increased in proportion to the size of the systems in order to assure an even distribution of water across the entire filter bed surface.

Different filter material substrates have been applied as filter materials for CWs, sometimes natural soil is used, but generally a mixture of gravel and sand with almost no clay and silt content is recommended (non-binding filter material), to preserve the permeability of the material. Brix and Arias (2005) proposed a clay and silt content (particles less than 0.125 mm) of less than 0.5 % for vertical flow wetland to minimise the risk of clogging of the filter body. Furthermore they recommended a sand with a d_{10} between 0.25 and 1.2 mm, a d_{60} between 1 and 4 mm, and a uniformity coefficient ($U = d_{60}/d_{10}$) less than 3.5 mm. Stottmeister et al. (2003) suggested a relatively small range of effective grain size d_{10} from 0.06 to 0.1 mm for vertical flow CW and a of d_{10} >0.1 mm for horizontal flow systems. The k_f-value (a measure of the permeability of water to the filter material), resulting from the chosen grain size, should be around $(10^{-3}\text{-})10^{-4}$ m.s^{-1} (Vymazal et al., 1998).

CWs, also referred as planted (soil) filters, can be planted by many different plant species. The most common emergent macrophytes used for CWs are listed in table 1.1.

Table 1.1: Plant species common for constructed wetlands.

Scientific name	Common English name
Phragmites australis	common reed
Typha angustifolia	narrow-leaved cattail
Typha latifolia	broad-leaved cattail
Scirpus sp.	bulrushes
Juncus sp.	rushes
Carex sp.	sedges
Glyceria maxima	reed sweet grass
Cyperus sp.	flat sedge
Iris pseudacorus	yellow flag iris
Phalaris arundinacea	reed canarygrass
Schoenoplectus lacustris *	common club-rush
Caltha palustris *	marsh marigold
Alisma sp. *	water plantain
Acorus calamus *	sweet flag
Salix sp.*	willow
Alnus sp.*	alder
*Rumex sp.**	docks
*Rumex alpinus***	alpine dock

* used rather seldom

** used only for CWs on a higher sea level

1.2.1 Design diversity of constructed wetlands

From the beginning of the development of the CW technique for the purification of wastewater up to now a variety of different design types of CWs has been applied. A general division of constructed wetlands discriminates between surface flow systems with a free water surface (see figure 1.1) and subsurface wetlands, which can be subdivided again regarding their water flow within the filter body, into vertical flow and horizontal flow wetlands (see figure 1.2).

There are numerous different technological variants in terms of design, peripheral equipment, the used grain size and vegetation. A more detailed description of the different basic design types of constructed wetlands is given elsewhere (e.g. Vymazal et al., 1998). Anyway the precise technology chosen has an important influence on the contaminants biological degradation pathways and removal mechanisms.

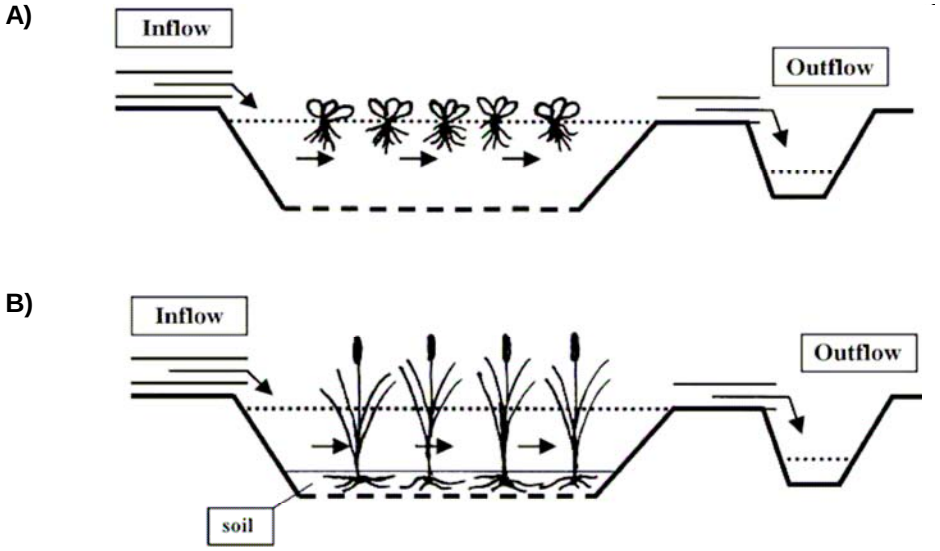


Figure 1.1: Surface flow constructed wetlands (SFCWs) with free floating plants (A) and with emergent macrophytes (B) (Vymazal, 2007).

Surface flow CWs (SFCWs) have the disadvantage to provide breeding areas for mosquito larvae and to produce more odour problems than subsurface flow constructed wetlands (SSFCWs). SSFCWs are generally more efficient regarding the removal of organic and inorganic nutrients, as well as of pathogens. Additionally they require less space, and are not subject to freezing. Disadvantages of SSFCWs include the risk of clogging of the filter media, due to excessive sludge accumulation. Whereas aerobic processes predominate in SSFCWs, anaerobic processes usually prevail in surface flow systems (Stottmeister et al., 2003).

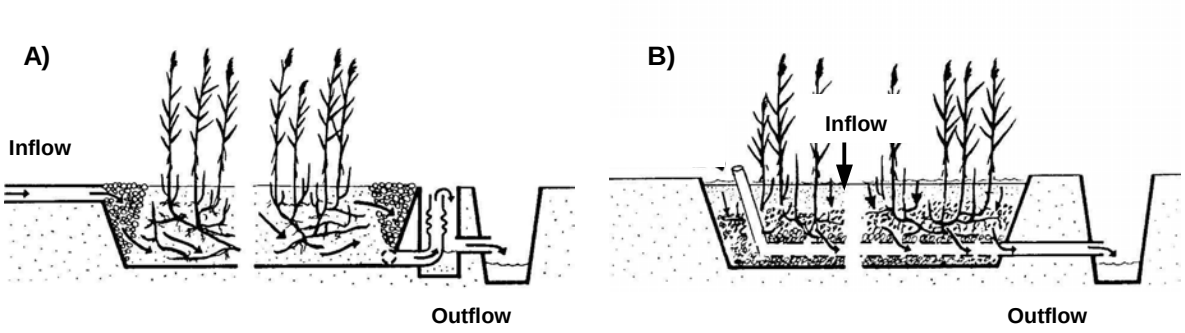


Figure 1.2: Subsurface flow constructed wetlands (SSFCWs) with horizontal flow (HFCW) (A) and with vertical flow (VFCW), (Brix, 1993, slightly modified).

In vertical flow systems the wastewater is distributed preferably spacious across the surface and passes trough filter body. The hydraulic loading normally is intermittent, allowing the filter pores to fill up with air between the loadings. In contrast horizontal filters are normally loaded continuously and the filter body is permanently water-saturated. Therefore aerobic and anaerobic processes occur within HFCWs, whereas in VFCWs nearly exclusively aerobic processes take place. Within horizontal subsurface flow systems surface runoff is evident in most of the systems due to low hydraulic conductivity of the filter material (Brix et al., 2007). HFCWs have the advantage of requiring less maintenance because the systems can be operated without pumps and siphons when exploiting natural hydraulic gradients and therefore needs less technical equipment than VFCW. But generally higher removal rates for different wastewater contaminants can be achieved by VFCW (see “treatment performances” below).

Various types of CWs can be combined in order to achieve higher removal efficiencies, especially for nitrogen (see “multistage hybrid systems” below). Additionally CWs can be combined with conventional treatment systems in order to exploit the specific advantages of the different systems (tertiary treatment).

Following many years of experience of operating CWs with different designs, recommendations, manuals and guidelines about the application of CWs have been compiled listing technical design criteria and operating parameters (US EPA, 2000; ATV, 1998; DWA, 2006; Brix and Arias, 2005; ÖNORM B 2505, 2008).

1.2.2 Treatment performances of constructed wetlands

It is almost impossible to directly compare the removal rates derived from different studies due to the great diversity and heterogeneity among constructed wetlands regarding design and operation, even if they are supposed to belong to the same design type. There are different parameters like the grain size and the chemical properties of the used filter material, the vegetation, the applied type of wastewater, the thickness of the filter body, the applied wastewater distribution system, the hydraulic loading rate, the intervals between the loadings, the hydraulic retention time within the system, the local climate and other factors, which are important for the removal efficiencies of the system. Thus it is difficult to compare results from various CWs, especially if they are derived from different studies. Therefore no absolute values for wastewater concentrations are compared within the following paragraph, which gives some examples of removal performances of different CW design types (excluding systems with floating and submerged plants).

Surface flow CWs (SFCWs) / subsurface flow CWs (SSFCWs):

Meutia (2001) compared surface and subsurface flow CWs situated in a tropical climate and showed that, the subsurface flow system is more suitable for treatment than the surface flow due to higher removal efficiencies. Also a comparison of subsurface flow wetlands regarding the removal capacity of heavy metals by Paredes et al. (2007a) demonstrated a significantly higher rate of zinc and chromium removal in comparison with surface flow systems. Reinoso et al. (2008) found higher removal rates for protozoan pathogens (*Cryptosporidium* and *Giardia*) and coliphages in a SSFCW wetland compared to a SFCW.

Vertical / horizontal flow CWs:

Different authors found higher removal rates for VFCWs, regarding the parameters BOD, COD, TSS, when comparing them with HFCWs (e.g. Puigagut et al., 2007). Whereas vertical flow constructed wetlands are very suitable for nitrification, by converting up to 99.9 % of the ammonia from the influent to nitrate (Langergraber et al., 2007b), horizontal flow systems are able to remove nitrogen from the system by converting the nitrogen into gaseous forms (denitrification; see chapter 1.3.1.4), but are limited in their ability to nitrify ammonia (Vymazal, 2007). For example Gasiunas et al. (2005) reported an average total nitrogen removal efficiency for a HFCW ranging from 37-44 %.

Rustige et al. (2003) found slightly higher phosphorus removal rates for horizontal systems , when comparing 62 vertical and horizontal flow CWs. Vymazal (2007) stated that, horizontal-flow systems have a higher potential for adsorption and precipitation of phosphors because the substrate is constantly flooded and there is not much fluctuation in redox potential in the bed. Vertical-flow systems, where wastewater is fed intermittently, may not be as effective because the oxygenation of the filter body may cause desorption and subsequent release of phosphorus. But generally the removal of phosphorus in vertical flow constructed wetland systems is rather low, typically 20–30 %. If higher removal rates are required, this can be

achieved e.g. by chemical precipitation of phosphorus with aluminium polychloride in the sedimentation tank, to meet requirements of 90 % phosphorus removal (Brix and Arias, 2005).

Matamoros et al. (2007) compared the two design types in terms of the removal efficiency of pharmaceuticals and personal care products (PPCPs) and demonstrated that the VFCW was more efficient for the removal of most of the investigated substances in comparison to a horizontal flow CW.

Alexandrino et al. (2004) investigated a VFCW and a HFCW by a PCR-based approach for the detection for the enteropathogenic bacteria *Campylobacter* and *Yersinia* and detected higher bacteria removal rates for the vertical flow system. In contrast to these results Ulrich et al. (2005) found no significant differences between horizontal and vertical filters regarding the removal of fecal indicator organisms and pathogenic microorganisms.

Multistage hybrid systems:

Various types of constructed wetlands may be combined in order to achieve higher treatment effect, especially for nitrogen. However, hybrid systems comprise most frequently VFCWs and HFCWs arranged in a staged manner. In combined systems, the advantages of HFCWs and VFCWs can be combined to complement each other. It is possible to produce an effluent low in BOD, which is fully nitrified and partly denitrified and hence has a much lower total-N concentrations. E.g. the combination of a VFCW, as the first stage provides suitable conditions (aerobic) for nitrification while a horizontal system as the second stage provides suitable conditions (anoxic / anaerobic) denitrification. (Vymazal, 2007). The effluent of the VFCW (= influent of the HFCW) can be mixed with its influent to provide organic carbon sources for the denitrification process (see chapter 1.3.1.4).

Planted / unplanted CWs:

Significant better performances regarding the removal efficiencies of organic and inorganic nutrients for planted CWs compared to unplanted systems have been reported by many authors:

De Feo (2007) reported that the vegetation played an essential role especially in the removal of nitrogen; he stated that for the wastewater with the highest loads the contribution of reeds in the removal of nitrogen was greater than that of biofiltration processes. The contribution of the vegetation was also detectable in the removal of orthophosphate. Caselles-Osorio and Garcia (2007) compared two SSFCWs; one planted and one were the aboveground biomass of common reed was cut. The CW with aboveground biomass exhibited more oxidized conditions and better removal efficiencies (on average 81 % for COD and 98 % for ammonium) than the wetland operated without aboveground biomass (73 % for COD and 72% for ammonium). Maine et al. (2007) demonstrated that macrophyte disappearance could be related to the overall toxicity of several environmental constrains as high pH and conductivity, metal concentrations, and sulphide presence. Villasenor et al. (2007) investigated four HFCWs planted with different plant species and one unplanted reference. All planted wetlands achieved higher removal rates for pollutants compared with the unplanted control wetland. Lin et al. (2007) showed that planted wetland cells exhibited significantly higher nitrate removal efficiencies (70-99 %) and soil denitrification rates (3.78-15 µg N_2O-N g^{-1} dry soil h^{-1}) than an unplanted covered wetland cell (0.1 µg N_2O-N g^{-1} h^{-1}). Also Calheiros et al. (2008) achieved significantly higher COD and BOD_5 removal rates for a horizontal subsurface flow constructed wetland planted with *Typha latifolia* treating tannery wastewater compared to an unplanted unit. In contrast to these results Lim et al. (2001) found no significant difference between planted and unplanted surface and subsurface flow wetlands regarding the BOD_5 removal.

It can be summarized that CWs can provide a suitable and efficient alternative to conventional wastewater treatment systems, able to fulfil the required stringent effluent standards (e.g. Paing and Voisin, 2005; Langergraber et al., 2007b; Brix et al., 2007). Generally for all CW types, especially for easily biodegradable organic compounds (BOD) and suspended solids (SS), high removal rates up to >98 % are possible; for fecal indicator bacteria rates >99 % can be achieved, if the systems are designed and operated properly (e.g Sleytr et al., 2007). Ammonia conversion and nitrogen removal by nitrification and denitrification is variable depending upon system design, retention time and oxygen supplies (Bastian and Hammer, 1993). The phosphorus removal rate also varies depending on the design type and especially on the used filter material, but generally it decreases rather rapidly with operation period of the plant, due to the depleted adsorption capacity of the filter substrate. Additionally CW are very efficient regarding the removal of heavy metals, pharmaceuticals and personal care products. Considering the different CW design types it can be concluded from various studies that subsurface flow CWs are more efficient than surface flow and vertical flow CW achieve higher removal rates than horizontal flow systems, except the removal of total nitrogen and phosphorus. Various studies demonstrated higher removal efficiencies for planted systems compared to unplanted systems. Highest removal rates can be achieved by multistage hybrid systems.

1.2.3 Advantages and disadvantages of constructed wetlands:

In the following a summary of the advantages and disadvantages of constructed wetlands, focused especially on the use of subsurface flow wetlands (SSFCW) is given (based on US EPA, 2000 with some modifications and additions):

Advantages:

- CWs provide an effective treatment in a passive manner and minimize mechanical equipment, energy, and skilled operator attention.
- They can be less expensive to construct and are usually less expensive to operate and maintain compared to mechanical treatment plants designed to produce the same effluent quality. The operation and the maintenance of CWs is not only cheaper it also requires less energy.
- Year-round operation for secondary treatment is possible in all climates, with some restrictions considering the purification performance in cold periods.
- Year-round operation for advanced or tertiary treatment is possible in warm to moderately temperature climates. A SSFCW provides more thermal protection than a free water surface wetland.
- CWs, in particular SSFCWs, are very effective and reliable for removal of organic compounds, suspended solids, pathogens, metals and some persistent organics in municipal wastewaters.
- The risk of contact by humans and animals with partially treated wastewater can be minimized. For a properly operated plant almost no disturbance of the inhabitants by bad odour occurs.
- Constructed wetlands provide a valuable wildlife habitat.
- High operational reliability due to the low technical requirements for the maintenance of the system.

Disadvantages:

- A CW requires a large land area compared to conventional mechanical treatment processes (e.g. according to the Austrian design standards (ÖNORM B 2505, 2008) the required surface area for subsurface vertical flow CW is 4 m^2 per person).
- Phosphorus, metals and some persistent organics removed in the system are bound in the wetland sediments and accumulate over time. To obtain a stable sufficient phosphorus removal rate over a longer operation period is not possible by using conventional filter materials.
- In cold climates the lower water temperatures reduce the rates of removal for BOD, NH_4 and NO_3. An increased detention time can compensate for these reduced rates but the increased wetland size in extremely cold climates may not be cost effective or technically possible.
- To achieve a significant removal of total nitrogen by nitrification and subsequent denitrification would require a filter body, which provides aerobic zones as well as anoxic zones with enough carbon sources. These conditions are not sufficient accomplishable within one wetland; therefore a combination of different wetland types or other methods for nitrogen removal is recommendable.
- Disturbances of the plant operation can occur due to a reduced permeability of the filter material (soil clogging) as a consequence of to high amounts of suspended solids in the influent. A reconstitution of the CW after such disturbances is very laborious.
- The long-term effectiveness of constructed wetlands is not well known. Wetland aging may be a problem, which may contribute to a decrease in contaminant removal rates over time.
- Temperature and fluctuations in flow affect wetland function and can cause a wetland to display inconsistent contaminant removal rates.

1.3 Microbial communities in conventional and nature-like wastewater treatment plants

Natural ecosystems (terrestrial or aquatic) are able to deal with a level of pollution up the specific self-purification capacity of the ecosystem. Basically microorganisms are responsible for the mineralization of pollutants. Systems built for wastewater treatment, regardless if they are based on conventional technologies (activated sludge and trickling filters) or nature-like techniques (like CWs), still depend on complex microbial communities. Even today in modern WWTPs these communities are not deliberately assembled from individual species with known function, because they remain the result of natural selection. But in conventional WWTPS there is the possibility to influence the selection of activated sludge community by modifying operational parameters like e.g. the oxygen content or the sludge age The art of constructing an efficient wastewater treatment facility is still to make the best use of natural processes by considering them in space and time. The group of microorganisms most directly involved in wastewater treatment are bacteria, which dominate all other microbial groups, both in number and biomass (Amann et al., 1998). The various types of microorganisms within wastewater treatment systems exist basically as biofilms within flocs in activated sludge, on different supporting materials (e.g. in trickling filters or CWs).

Prokaryotic microorganisms are most important within the wastewater treatment process due to their ability to supply energy for cell maintenance and growth through a plenitude of

metabolic pathways. The potential to utilize organic pollutants in the wastewater as carbon sources for metabolism varies enormously among microorganisms. The most rapid and complete degradation of pollutants is performed under aerobic conditions by catabolic pathways. Additionally anoxic processes like denitrification, Anammox (anaerobic ammonium oxidation), sulphate reduction or methanogenesis contribute to the degradation of pollutants.

1.3.1 Nitrogen transformation within wastewater treatment - an example for a nutrient degradation process catalyzed by bacteria

Municipal wastewater normally contains high concentrations of carbon-, nitrogen- and phosphorus-compounds. If wastewater is not treated sufficiently, and the nutrients are released into a receiving water, they are responsible for eutrophication of the environment. Municipal wastewater contains nitrogen mainly in form of organic nitrogen (proteins, amino acids, urea, nucleic acids) and ammonium (NH_4^+), the concentration of nitrate-nitrogen is very low (see e.g. table 4.2). Most of the urea is already degraded in the sewer systems, wherefore the nitrogen enters the WWTPs mainly in form of ammonium.

The nitrogen transformation comprises different reactions catalyzed by bacteria as summarized in figure 1.3 and discussed in the following chapter.

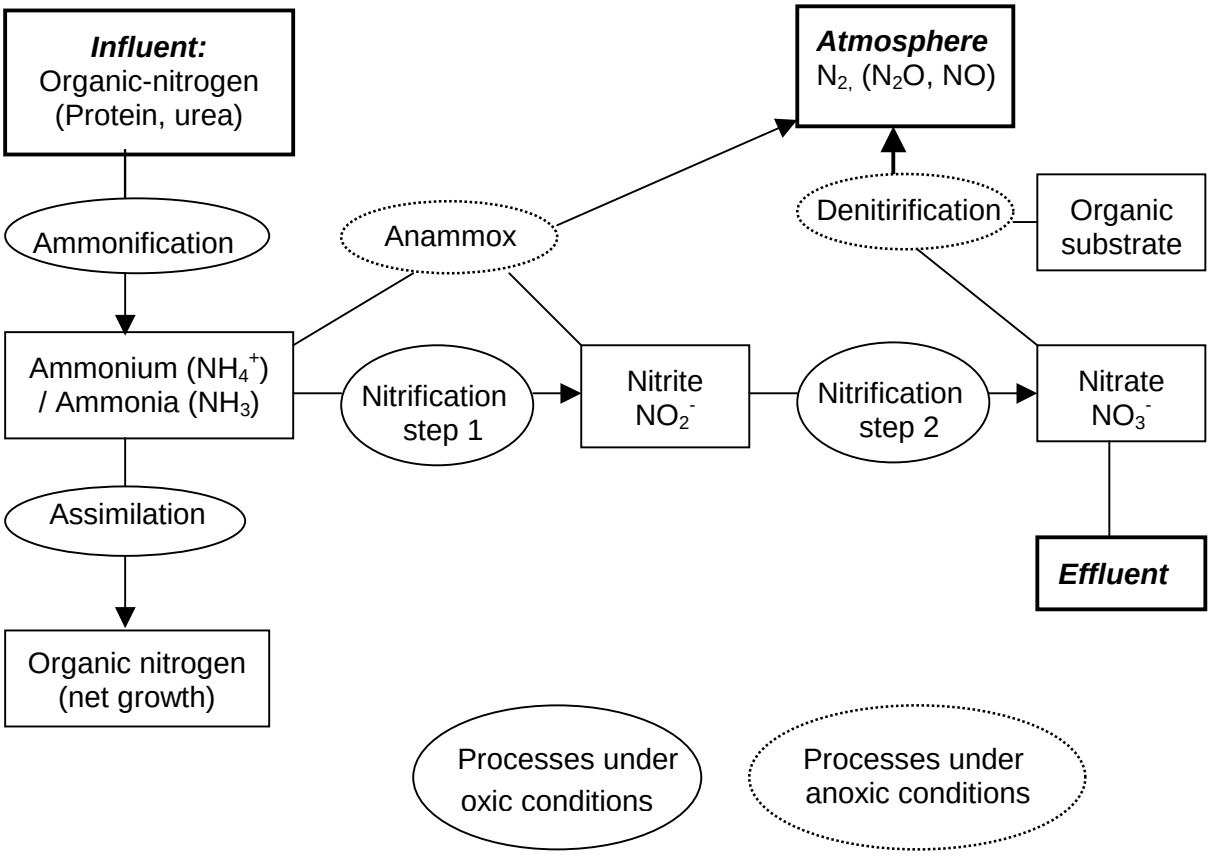


Figure 1.3: Decomposition of nitrogen-compounds during wastewater treatment.

The release of ammonium by the discharge of wastewater treatment systems into receiving waters causes local oxygen depletion and free ammonia is toxic to fish and other aquatic organisms therefore a reduction within WWTPs is necessary to prevent eutrophication in aquatic environments. Thus also for small wastewater treatment plants (less than 500 PE) a maximum effluent concentration for ammonia-nitrogen of 10 mg L^{-1} is required to fulfil the stringent Austrian effluent standards (1. AEVkA, 1996). But no standard for total nitrogen of the discharge of small plants is given with in this norm, which implicates that denitrification is not essentially within plants of this size.

1.3.1.1 Ammonification

The first step within the nitrogen-decomposition is the microbial hybridization of organic-nitrogen into ammonia (NH_3) or ammonium (NH^+_4) by ammonification. Free ammonia and ammonium represent two forms of reduced inorganic nitrogen which exist in equilibrium depending upon the pH and temperature of the waters in which they are found. Above a pH of 8 the nitrogen occurs mainly as ammonia, whereas below 8 the equilibrium shifts towards the appearance of ammonium.

Ammonification mainly occurs under oxidized conditions and is carried out by virtually all microorganisms that are involved in the decay of dead organic matter. Ammonification rates are dependent on temperature, pH, C/N ratio, available nutrients and within in soils on conditions like texture and structure. The optimal ammonification temperature is 40-60℃ and the optimal pH ranges from 6.5 to 8.5 (Vymazal, 2007). Urea ($CO(NH_2)_2$), uric acid, and organic nitrogen of faeces are all substrates for microbial ammonification. The microorganisms derive metabolically useful energy from the oxidation of organic-nitrogen to ammonium. In addition, much of the ammonium is assimilated and used as a nutrient for metabolic purposes. However, if microorganisms produce ammonium in quantities that exceed their own requirements, as it is usually the case, the surplus is excreted into the ambient environment and is available for use as a nutrient by plants, or as a substrate for further microbial processes like nitrification or Anammox.

1.3.1.2 Nitrification

Nitrification is usually defined as the biological oxidation of ammonium to nitrate with nitrite as an intermediate in the reaction sequence. Chemoautotrophic nitrifying bacteria derive energy from the oxidation of ammonia or nitrite and use carbon dioxide as carbon source.

Nitrification is influenced by temperature, pH value, alkalinity, inorganic C source, moisture, microbial population structure, and concentration of ammonium-N and dissolved oxygen. The optimum temperature for nitrification in pure cultures ranges from 25 to 35℃ and in soils from 30 to 40℃. The pH range for growth of pure cu ltures of ammonia oxidizers is 5.8 to 8.5, and the pH range for growth of nitrite oxidizers is 6.5 to 8.5 (Princic et al., 1998). Approximately 4.3 mg O_2 per mg ammoniacal nitrogen oxidized to nitrate nitrogen is needed (Vymazal, 2007).

The nitrification process is divided into two steps, the ammonia oxidation and the nitrite oxidation as show in figure 1.4.

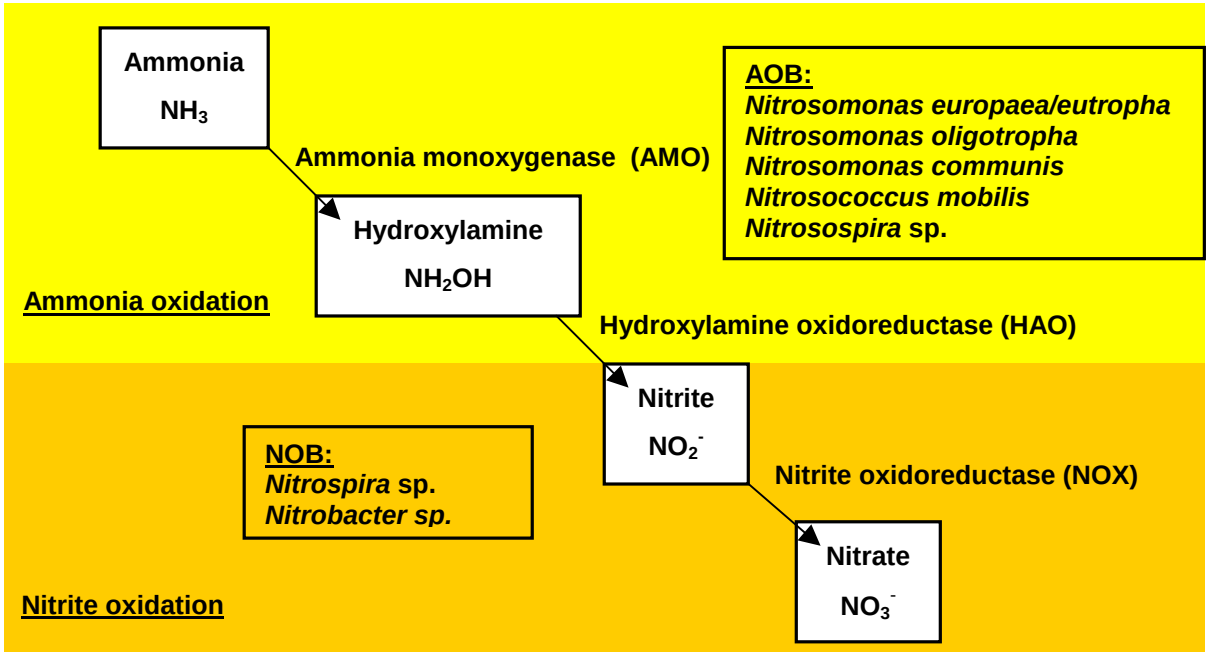


Figure 1.4: Aerobic nitrification performed by ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB).

Aerobic ammonia oxidation:

Most likely ammonia (NH_3) and not ammonium (NH_4^+) is the substrate for this oxidation process. The conversion of ammonia to nitrite via hydroxylamine, performed by ammonia oxidizing bacteria (AOB), is the first step of nitrification:

$$NH_4 + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O \quad [\Delta G^\circ - 275 \text{ kJ mol}^{-1}]$$

AOB are generally considered as aerobic chemolithoautotrophs, but recently organic compounds have been described that can serve them as carbon sources. Some ammonia oxidizers can obtain their energy for growth from both aerobic or anaerobic ammonia oxidation (see chapter 1.3.1.3). But under anoxic conditions the ammonia oxidation activity is relatively low. The reducing equivalents are transferred to the terminal electron acceptors O_2 (under oxic conditions: dissolved oxygen > 0,8 mg L^{-1}). Ammonia oxidation is initiated by the enzyme ammonia monooxygenase (AMO), that oxides ammonia to hydroxylamine. Oxygen and dinitrogen tetroxide are the most likely electron acceptors for this enzyme. The hydroxylamine resulting from ammonia oxidation is further oxidized to nitrite by the hydroxylamine oxidoreductase (HAO). The specific aerobic ammonia oxidation activity is stimulated by NO_2. (Schmidt et al., 2003).

Diversity of aerobic ammonia oxidizing bacteria (AOB):

Cultivation-dependent analyses of environmental AOB diversity is time consuming and tedious due to the slow growth rates of these bacteria. Traditionally, AOB were classified by cell morphology into the five different genera: *Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosovibrio* and *Nitrosolobus* (Bothe et al., 2000). On the basis of 16S rRNA sequence homology, *Nitrosospira, Nitrosovibrio* and *Nitrosolobus* were proposed to be combined into one common genus *Nitrosospira*. With the exception of *Nitrosococcus*, all genera represent closely related organisms of the β-subclass of Proteobacteria. The genus *Nitrosococcus* is phylogenetically not homogeneous (Teske et al., 1994). *N. mobilis* is a β-subclass organism,

whereas other *Nitrosococcus* species (consists only of marine AOB species) belong to the γ-class. The genus *Nitrosomonas* (including *Nitrosococcus mobilis*) is affiliated to the β-class (Purkhold et al., 2003; Siripong and Rittmann, 2007).

In soils the typical AOB species are *Nitrosomonas europaea*, *Nitrospira briensis*, *Nitrosococcus nitrosus*, *Nitrosococcus oceanus*, *Nitrosolobus multiformi.* Horz et al. (2004) stated that an increased nitrogen deposition significantly altered the structure of a soil ammonia-oxidizing community; resulting in a shift towards the dominance of bacteria most closely related to *Nitrosospira* sp.

But the capability of oxidizing ammonia is not only restricted to the domain bacteria. Könneke et al. (2005) report the first time the isolation of a marine Crenarchaeota that grows chemolithoautotrophically by aerobically oxidizing ammonia to nitrite. In the following many other authors confirmed the occurrence of ammonia-oxidizing archaea (AOA) in different aquatic ecosystems like marine sediments and water columns (e.g. Francis et al., 2005), in a thermal spring (Weidler et al., 2007), or in a subterranean estuary (Santoro et al., 2008). But also in diverse terrestrial habitats AOA have been detected (e.g. Adair and Schwartz, 2008). Leininger et al. (2006) could even demonstrate that AOA were predominating among ammonia-oxidizing prokaryotes in soils. Additionally AOA have been also detected in wastewater treatment plant bioreactors (Park et al., 2006). The possibility that some archaea carry an *amoA* gene was first demonstrated by Schleper et al. (2005) and later confirmed by Francis et al. (2005); in both studies primers specifically targeting the archaeal *amoA* gene were applied to document the distribution and diversity of uncultivated archaea in marine and terrestrial ecosystems. Lam et al. (2007) could prove the active expression of putative (*amoA*) genes of marine group I Crenarchaeota detected in the Black Sea water column, whereas Treusch et al. (2005) demonstrated the expression of two proteins in mesophilic Crenarchaeota from grassland soils related to subunits of the ammonia monooxygenase.

Not many data about archaea in CWs are available. Truu et al. (2005) found a very high diversity of archeae in a horizontal flow CW and Nicomrat et al. (2006b) detected archaea in a constructed wetland treating acid coal mine drainage. These findings indicate that archaea might play an important role within the microbial population of CWs. The possible occurrence of AOA in constructed wetlands has not been investigated up to now (also not within this study) and remains an interesting topic for further research.

Nitrite oxidation:

The second step of nitrification is the oxidation of nitrite to nitrate.

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^-\ [\Delta G^\circ - 74\ kJ\ mol^{-1}]$$

The reaction is catalyzed by the membrane-bound nitrite oxidoreductase (NOX) of nitrite oxidizing bacteria (NOB). NOB are facultative chemolithoautotrophic organisms, some of them are capable of heterotrophic growth under oxic as well as anoxic conditions but heterotrophic growth is significantly slower than lithoautotrophic growth. NOB are much more sensitive to oxygen limitation than AOB. It has been reported that hydroxylamine, ammonia and nitrogen oxide can inhibit NOB (Schmidt et al., 2003).

Diversity of NOB:

NOB are more phylogenetically diverse than AOB. All known NOB belong to a different phylogenetic group: genus *Nitrobacter* belongs to the Alphaproteobacteria, genus *Nitrococcus* to the Deltaproteobacteria, genus *Nitrospina* to the Betaproteobacteria, and the genus *Nitrospira* belongs to the separate phylum *Nitrospirae* (Teske et al., 1994). In soils the

species *Nitrobacter winogradsky*, *Nitrospina gracilis*, and *Nitrococcus mobilis* are the typical NOB (Chen et al., 2003).

Diversity of nitrifiers within wastewater treatment systems:

Nitrifying bacteria are extremely difficult to cultivate. Nitrification failures within municipal WWTPs frequently occur, since nitrifiers are inhibited by several environmental and engineering factors, including low temperature, extreme pH, low dissolved oxygen concentration, and a wide variety of chemical inhibitors. Additionally they are very slow growing, and this makes nitrifiers susceptible to washout of conventional WWTPs (Wagner et al., 2002; Siripong and Rittman, 2007).

Nitrosomonas europaea traditionally have been considered as the main ammonia oxidizing bacteria within WWTP. Although species of *N. europaea* abundant in many WWTPs, the total AOB diversity can be much higher as demonstrated by comparative sequence analyses of almost 200 *amoA* gene fragments revealing that a wide variety of different betaproteobacterial ammonia oxidizers occurs in nitrifying WWTPs (Wagner and Loy, 2002). It has been observed that different AOB species are dominating in different treatment plants. In addition to *N. europaea*, *Nitrosomonas eutropha, Nitrosococcus mobilis*, members of the *Nitrosomonas marina* cluster, and another phylogenetic lineage, for which no cultured representative exists, were found in different WWTPs (Wagner and Loy, 2002). But it has been demonstrated that also *Nitrosospira* can be the dominating AOB species within a full-scale activated sludge plant (Coskuner and Curtis, 2002), whereas Wagner and Loy stated (2002), that ammonia oxidizers of the genus *Nitrosospira* are not important in nitrifying WWTPs, in contrast to other ecosystems. *Nitrosomonas* seems to preponderate in engineered, high-ammonia systems, whereas in low-ammonia environments a predominance of *Nitrosospira* has been found (Sundberg et al. 2007). The analyses of the AOB community of seven different WWTP by Siripong and Rittmann (2007) revealed that *Nitrosomonas europaea / eutropha*, *Nitrosomonas oligotropha*, *Nitrosomonas communis*, but also *Nitrosospira* lineages were present in all WWTP with only slightly variations due to seasonal temperature changes. The authors hypothized that the coexistence of various AOB is an evidence of functional redundancy, a feature that may help to maintain the stability of the system for nitrification. Additionally also *Nitrosococcus mobilis* was identified as the key AOB in some wastewater treatment systems (Juretschko et al., 1998); but this species occurs only under higher salt concentrations.

Nitrobacter species traditionally have been considered as the main nitrite oxidizing bacteria within WWTPs. Coskuner and Curtis (2002) demonstrated that *Nitrospira* can be present along with *Nitrobacter*. *Nitrospira* are often the dominant NOB in activated-sludge systems (Juretschko et al., 1998; Harms et al., 2003; Daims et al., 2006b). These findings are of great interest for plant operators, due to the fact that e.g. *Nitrobacter* and *Nitrospira* respond differently to shifts in the growth conditions (that occur infrequently in WWTPs), due to their different ecophysiological requirements (Daims et al., 2006a). *Nitrospira*-like nitrite oxidizers are probably *K*-strategists (with high substrate affinities and low maximum activity or growth rate) for oxygen and nitrite and thus can outcompete *Nitrobacter* under substrate-limiting conditions (Wagner and Loy, 2002). Coexistence of different nitrifiers, who perform the same task allow communities to maintain physiological capabilities when conditions change. Thus, a high level of nitrifier diversity is thought to confer performance stability (Siripong and Rittman, 2007). The dominance of different species is caused by the presence of different selection pressures in wastewater treatment facilities; like influent characteristics, environmental conditions and design and operation of the system. Yuan and Blackall (2002) pointed out, that optimising the microbial community structure properly should be an explicit aim for the design and operation of a treatment plant.

For constructed wetlands similar AOB species have been detected. Gorra et al. (2007), Abd El Haleem et al. (2000) and Sundberg et al. (2007) detected only sequences related to the *Nitrosospira* lineage in different designed CWs. Ibekwe et al. (2003) and Tietz et al. (2007a;

see chapter 4.3) could demonstrate that *Nitrosomonas europaea* / "*Nitrosococcus mobilis*" was present together with *Nitrosospira* within subsurface horizontal flow CWs used to treat dairy washwater. *Nitrosospira* seems to play a more pronounced role in CW than in conventional WWTP, this is reasonable due to the fact that *Nitrosospira sp.* are the dominating AOB species within most soils (Horz et al., 2004; Mahmood et al., 2006). The diversity of NOB within CWs has not been investigated up to now.

1.3.1.3 Anaerobic ammonia oxidation (anammox)

In the anaerobic ammonium oxidation process, ammonia is oxidized with nitrite as primary electron acceptor under strictly anoxic conditions:

$$NH_4^+ + NO_2^- \rightarrow N_2^- + 2H_2O \quad [\Delta G° - 357 \text{ kJ mol}^{-1}]$$

Microorganisms capable of anammox involve basically two categories. *Nitrosomonas eutropha*, a classic aerobic ammonium oxidizer, belongs to one group which oxidizes ammonium with NO_2 as electron acceptor under anoxic conditions (Zhang et al., 2008). The so-called anammox bacteria can be found in a group of deep-branching Planctomycetes (see table 1.2).

Table 1.2: Anammox bacteria discovered up to date (Zhang et al., 2008).

Genus	Species	Source
Brocadia	*Candidatus Brocadia anammoxidans*	Wastewater
	Candidatus Brocadia fulgida	Wastewater
Kuenia	*Candidatus Kuenenia stuttgartiensis*	Wastewater
Scalindua	*Candidatus Scalindua brodae*	Wastewater
	Candidatus Scalindua wagneri	Wastewater
	Candidatus Scalindua sorokinii	Wastewater, seawater
Others	*Candidatus Jettenia asiatica*	Not reported
	Candidatus Anammoxoglobus propionicus	Synthetic water

These anammox bacteria use a complex reaction mechanism involving hydrazine as an intermediate. The reactions are assumed to be carried out in a unique prokaryotic organelle, the anammoxosome. This organelle is surrounded by ladderane lipids, which make the organelle nearly impermeable to hydrazine and protons. The anammox bacteria have been detected in many marine and freshwater ecosystems and were estimated to contribute up to 50 % of oceanic nitrogen loss (Op den Camp et al., 2006). For the species *Brocadia anammoxidans* and *Kuenenia stuttgartiensis* a temperature of 37℃ and a pH of 8 have been reported by Schmidt et al. (2003) as optimal conditions for their growth.

The direct integration of anammox into wastewater treatment requires a preceding partial nitrification step from ammonia to nitrite, with simultaneous inhibition of NOB, which yields enough ammonium and nitrite as substrates for the anammox reaction. This provides resource-efficient nitrogen removal without aeration or the addition of extra organic carbon. Because aeration is required only for the initial partial oxidation of ammonia to nitrite, the total costs are lower than those of the traditional nitrification-denitrification pathway (Daims et al., 2006b). Although this technology has been tested within conventional full-scale treatment plants (cited in Zhu et al., 2008 and Zhang et al., 2008), as well as in pilot-scale CWs (Paredes et al., 2007b) and even full-scale CWs (Dong and Sun, 2007) the mechanisms

remain still rather unclear. Additionally the establishment of anammox organisms in full-scale plants is difficult due to their very slow growth rates (see also table 1.3 “N-removal”).

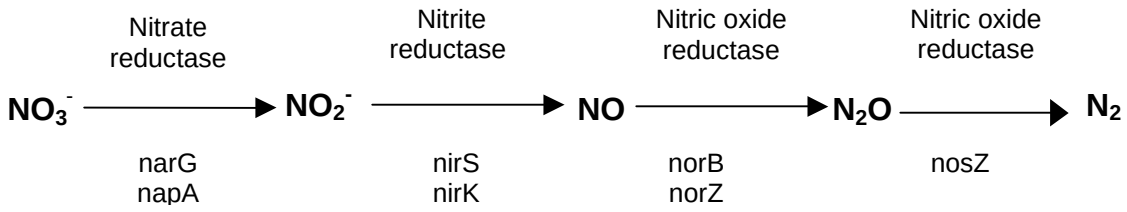
But nevertheless the anammox process is a promising tool to conventional N-removal mechanisms (involving nitrification and denitrification) within different kinds of wastewater treatment plants.

1.3.1.4 Denitrification

Denitrification is the reduction of nitrate to nitrogen gas, via nitrite and nitric and nitrous oxides.

$$6(CH_2O) + 4NO_3^- \rightarrow 6CO_2 + 2N_2 + 6H_2O$$

This process is performed by various chemoorganotrophic, lithoautotrophic and phototrophic bacteria, especially under oxygen-reduced or anoxic conditions. Most denitrifying bacteria are facultative anaerobic heterotrophs. Denitrification can be described as a kind of anoxic respiration. Electrons originated from e.g. organic matter, reduced sulphur compounds, or molecular hydrogen are transferred to oxidized nitrogen compounds instead of oxygen, in order to build up a proton motive force usable for ATP regeneration (Schmidt et al., 2003). The following enzymes are involved in this reduction process:



Dinitrogen is the main end product of denitrification, while nitrogenous gases like nitric oxide and nitrous oxide are occurring as intermediates and are released in more or less low concentration depending on the concentration of dissolved oxygen and the expression of denitrification enzymes (Schmidt et al., 2003).

Environmental factors known to influence denitrification rate include the absence of O_2, redox potential, soil moisture, temperature, pH value, presence of denitrifiers, organic matter, and nitrate. The optimal pH ranges between 6 and 8. Low oxygen concentrations, favour the production of nitrite from ammonia over the production of nitrate. The nitrite can then be denitrified to nitrous oxide and / or dinitrogen without being converted to nitrate. This process has been termed “partial nitrification-denitrification” (Vymazal, 2007).

The ability to denitrify is distributed widely within both bacteria and archaea. Approximately 50% of the cultivated phyla possess denitrifiers (Prosser, 2007). Members of the genera *Alcaligenes, Pseudomonas, Methylobacterium, Bacillus, Paracoccus and Hyphomicrobium* could have been isolated as part of the denitrifying microbial community from WWTPs (Wagner et al., 2002). Due to the great diversity of denitrifiers molecular studies have focused on functional genes for denitrifiers (*nirK, nirS, nosZ*), by applying more than one primer set to detect all denitrifiers. The broad phylogenetic range of denitrifiers makes it difficult to link community structure to environmental conditions. By combining FISH with MAR (microautoradiography) denitrifiers have been identified in activated-sludge (Wagner and Loy, 2002).

In the ideal wastewater treatment process, bacteria denitrify by using carbon compounds from the influent wastewater as electron donors. As a means to improve control strategies for nitrogen removal, external carbon compounds (e.g. methanol) can be added to enhance denitrification rates. In anoxic zones of CWs organic substrates (as carbon source for denitrifiers) are often not available, because they have been already decomposed.

Denitrification is the final step within the classical pathway of biological nitrogen removal in wastewater treatment plants, and is also important because of the emission of N_2O contributing to the greenhouse effect.

1.3.2 Comparison of conventional activated sludge wastewater treatment plants (AS-WWTPs) and subsurface vertical flow constructed wetlands (VFCWs) with a focus on factors relevant for the development of microorganisms

Table 1.3: Differences between conventional activated sludge wastewater treatment plants (AS-WWTP) and subsurface vertical flow constructed wetlands (VFCW) as habitats for microorganisms, considering the chemical, physical and biological conditions and processes within the two systems.

	AS-WWTP	VFCW
Oxygen content / supply	Within aeration tanks the oxygen supply can be provided by surface stirring, by submersed pumps or stirrers. These aeration systems provide more or less constant aerobic conditions within the whole tank. For an efficient carbon degradation an oxygen content of 0.5-1.0 mg L^{-1} is necessary and for nitrification an oxygen concentration of about 2.0 mg L^{-1} is required. In the non-aerated phase anoxic conditions (absence of oxygen) occur, suitable for denitrification.	VFCWs with an intermittent loading mode and a sandy filter material require no energy to provide aerobic areas up to the deepest zones of the filter body (assuming that the plant is operated under non-saturated conditions and that no disturbances like e.g. soil clogging occurs). In the VFCW investigated within this study (grain size of the filter material: 0.06-4 mm) the content of dissolved oxygen varied between 2.5 and 9.4 mg L^{-1} due to the filter body pores, which are filled up with air between the hydraulic loadings (Tietz et al. 2007b, see chapter 4.1).
Water content	Aquatic system (99.7-99.8 % water content and about 0.2-0.3.% suspended solids)	VFCWs are terrestrial systems with regular floodings. The water content depends on the grain size of the filter material particles. For the sandy substrates of the CWs investigated within this study the water content varied between 20 and 90 % of water saturation (Tietz et al. 2007b, see chapter 4.1).
Surface available for biofilm development	Activated sludge flocs in wastewater treatment plants are biofilms of a special kind, lacking a	In biological filters bacteria attach themselves to the filter media and a biofilm arises on the surfaces of

	carrier material (Manser and Siegrist, 2006). Bacteria produce extrapolymeric substances (EPS) to stick together and to form the typical activated sludge flocs. Sludge flocs are passively distributed with the water flow in the aeration tank.	the filter grains. A large available surface area for biofilm growth is one of the most essential properties of filter media used in biofilm processes. The available surface area is dependent on the grain size and density. Bacterial abundance normally is inversely related to sediment grain size, because smaller particles have a higher surface area per unit mass (Battin et al., 2001). But the surface area available for biofilm growth is low compared to natural soils if a sandy filter material is used due to missing clay and silt fraction. (Tietz et al., 2007b; see chapter 4.1).
Microbial diversity	Rather low (see chapter 1.3.3)	High (see chapter 1.3.3)
Heterogeneity of the habitat / possibilities for the development of microniches	AS-WWTPs provide a more homogeneous environment relative to most natural ecosystems (Daims et al., 2006a). Despite the mechanical disturbances during the aeration phase different microniches following physico-chemical gradients exist within one activated sludge floc (with a typical diameter of 0.1–2 mm). It is assumed that the available substrates and the oxygen in the boundary layer between floc and aqueous phase and in the floc interior are only transferred by diffusion. This gives rise to pronounced concentration gradients in the activated sludge flocs. In contrast, nitrite is only produced inside the floc and is therefore scarcely influenced by diffusion (Manser and Siegrist, 2006). *Nitrospira*-like bacteria with preference for different nitrite concentrations, were found to coexist in the same nitrifying biofilm, where they inhabit microniches along nitrite concentration gradients (Maixner et al., 2006). AS-flocs are complex nitrifying biofilms were AOB and NOB are arranged according to their physicochemical re-quirements. Heterotrophic bacteria were found to occupy the outer part of the biofilm while AOB were distributed in the inner part of the biofilm (Aoi, 2002).	Like in AS-flocs filter material particles provide different microniches on their surface, which are mainly defined by physicochemical concentration gradients of oxygen, pH and / or nutrients. Soil-like ecosystems have a strong heterogeneity and a variety of different microniches. Microniches develop especially within soil aggregates or in the rhizosphere. The rhizosphere provides a big variety of distinct microniches depending on oxygen leakage (especially at the root tips) and nutrient enrichment by rhizodeposition (see rhizosphere effect). The diversity of microniches available for colonization in soil means that there will be hot spots for microbial activity. E.g. anoxic microniches within filter material aggregates enable that denitrification, sulphur reduction and other anaerobic processes can take place locally restricted also in well-aerated systems.

Continuity / fluctuations	Due to the circulation of the sludge-wastewater mixture during the aeration phase the activated sludge flocs are exposed to a mechanical load, which breaks bigger aggregates and prevents the formation of bigger more complex structures. Additionally smaller flocs do not settle adequately and are washed out with the treated water and therefore cannot persist within the system. Because of the shifts between aeration and non-aeration phase physicochemical parameters like oxygen, pH, temperature, ammonia and nitrate concentrations fluctuate permanently.	Stable conditions within the filter body enable the development of a more complex microbial community. Especially for organisms with slower growth rates, like nitrifiers or anammox-microorganisms a continuous less disturbed environment enables a permanent development. The oxygen-, water- and nutrient-content of the filter material changes significantly during hydraulic loading especially in the uppermost layer of the filter material, but with increasing depth the physicochemical conditions are remaining more constantly.
Biofilm / free bacteria	Most of the bacteria, especially those that are physiological active, are associated within biofilms forming the typical activated sludge flocs. Within AS-flocs closer interactions of different physio-logical types of microorganisms, e.g., of ammonia and nitrite oxidizers are possible, and bacteria are better protected from protozoan grazing or harmful substances (Schramm et al., 1999).	Most of the bacteria are associated with biofilm growth on the filter material (e.g. Silyn-Roberts and Lewis, 2003; Larsen and Greenway, 2004). Additionally plant roots serve as carriers for attached microbial growth (Münch et al., 2007) (see rhizosphere-effect).
Biomass retention time	The sludge retention time must be high enough (approximately seven days) to ensure that also slow growing organisms (e.g. nitrifiers) can establish.	Microorganisms are attached to the surface of the filter medium and therefore remain within the system.
Hydraulic retention time	Detention time of the wastewater within the aeration tank: about 4 - 14 hours	Depending on the grain size of the used filter material, the thickness of the filter body and the content of suspended solids of the wastewater. The hydraulic retention time of the wastewater within system with a sandy filter material varies normally between 0.5 and 4 days.
Rhizosphere-effect	Non (no plants)	Plant rhizosphere enhances microbial density and activity by the release of oxygen and exudates (organic acids, sugars and vitamins) and dead organic root matter, which may serve as substrates for microorganisms (Atkinson and Watson, 2000).

		Münch et al. (2005) found an enhancement of the bacterial density up to a distance of 50 mm from *Phragmites australis* roots within a laboratory batch reactor. Münch et al. (2007) stated that the rhizoplane biofilm of *Glyceria maxima* (from a hydroponic system for nitrogen removal) is remarkably thin and related to the age and morphology of the roots. Additionally the extent of the rhizosphere effect shows plant specific differences (e.g. Gagnon et al., 2007). But on the other hand Larsen and Greenway (2004) and Tietz et al. (2007b; see chapter 4.1) found no significant difference between the amount of microbial biomass in the bulk-soil within planted and unplanted CWs. But owning to the relatively low amount of carbon released by plants in comparison to the wastewater content; it can be assumed that rhizodeposition is only significant in VFCWs with very low carbon loads like e.g. mine drainage (Stottmeister et al., 2003).
Disturbances of the plant operation due to specific microbial growth	Excessive growth of filamentous bacteria causes bulking and foaming of the activated sludge, (by extensive growth of e.g. *Microthrix parvicella* or *Nocardia limicola*) and therefore impede the separation of sludge from the treated wastewater.	After a continuing organic overload of the CW followed by a clogging of the system the plant can be reconstituted by taking the system out of operation for a period of several weeks and allow to dry out. If this does not help, it might be necessary to remove and replace the upper 10 cm of the filter sand (Brix and Arias, 2005). The organic overload produces an layer consisting mainly of suspended solids from the wastewater and a surplus of biomass, whose degradation leads to a depletion of oxygen. The restoration measures are very time and labour-intensive.
Possibilities to investigate and monitor microbial communities within the system	Microscopic analyses and culture-based investigations of AS have a long tradition and are rather easily to perform, although their force of expression is limited (e.g. Amann et al., 1998). Culture independent methods are much more labour-,	Due to the more constant conditions within the microbial community within a filter body of a VFCW (compared to a AS-tank), because microorganisms are fixed to the filter material and remain in a stable equilibrium as

	time-intensive and more expensive and therefore the classical methods are still useful for a monitoring of the system. Due to the rather homogenous matrix of AS (compared with CWs) the way of homogenising a sample is not so complicated and also quantitative analyses are possible (e.g. Shannon et al., 2007), although the disaggregation of AS-flocs requires special protocols (e.g. Ziglio et al., 2002). Additionally it has to be considered that a domestic sewage is a very complex matrix, which can contain substances, that hamper and inhibit analyses of the microbial community. Additionally already kits for DNA extraction from wastewater are available, as well as kits for FISH analyses of specific AS-organisms (http://www.vermicon.de/).	"biofilm-inhabitants", a routine monitoring of the microbial community is not necessary. Additionally the possibilities to change parameters relevant for the microbial population are very limited and therefore the investigation of the microorganisms is only relevant for research purpose. Due to the complex soil-like matrix of a CW filter body investigations of microbial communities are rather challenging. The first problem is that a complete desorption of the biofilm bacteria from the filter substrate is impossible. Secondly fine soil and wastewater particles accumulating within the filter body can inhibit PCR-based analyses. And thirdly due to the inhomogeneity of the filter material (coarse grain size) the results of parallel analyses can vary enormously.
Possibilities to control / vary physicochemical parameters	Wastewater treatment plants with a complex configuration require a higher level of process control. Purification processes can be controlled by changing the oxygen content, the hydraulic retention time of the aeration tank and the primary settlement tank, by regulating the recycle sludge flow rate, or by dosing precipitants (e.g. aluminium- or iron-chloride for p-removal) or nutrients (e.g. methanol for denitrification).	The physicochemical parameters, which can be influenced during plant operation of CW, are the oxygen and water content. Water and oxygen content of the filter body are related to the hydraulic loading mode, depending on the amount of wastewater per load and the time intervals between the loadings. If anoxic zones are required the plant has to be operated in a water saturated mode (e.g. by adjusting the height of the outlet of the system). An aeration of the system is not possible without constructional measures, like aeration tubes within the filter material, but they are not used routinely for VFCWs.
N-removal (see also chapter 1.3.1)	The main processes of N-elimination within wastewater treatment, ammonification, nitrification, denitrification and/or anammox, can occur within in the same AS-WWTP. Properly designed AS-WWTP with conventional nitrification-denitrifi-cation can obtain total N-removal	The processes that are relevant for nitrogen decomposition in VFCWs are ammonification and especially nitrification. Denitrification, the major removal mechanism for N-removal, normally barely occurs in VFCWs due to the lack of anoxic

	rates up to 95% (Schmidt et al., 2003). Members of the phylum *Planctomycetes* responsible for anammox have been detected in large amounts in WWTP with nitrogen removal and anoxic niches (e.g. Schmidt et al., 2003).	zones with adequate organic matter concentrations. Other N-removal pathways like plant uptake, ammonia adsorption (due to the missing clay fraction of the filter material) and volatilisation are considered to play only minor roles within VFCWs (Vymazal, 2007). The relevance of the anammox process, which is autotrophic and therefore requires no organic matter, within VFCWs is still relatively unknown. Paredes et al. (2007b) found very low numbers of anammox organisms within a planted fixed-bed reactor. They inoculated the reactor with anammox biomass and achieved a 4-5fold increase of the nitrogen removal rate. Dong and Sun (2007) detected ANNAMOX bacteria in the aerobic-anoxic interface of a VFCW constructed with an aeration pipe in the middle of the filter body.
P-removal	Due to the capacity of specific bacteria to accumulate polyphosphate intracellularly phosphorus is fixed in bacteria and can be removed from the wastewater by settlement of the activated sludge within the settling tank.	The mechanisms for P-removal in CWs include sorption on the filter body material, storage in biomass and the formation and accretion of new sediments. The first two processes, however are saturable, meaning they have a finite capacity and therefore, cannot contribute to long-term sustainable P removal (Kadlec et al., 2000). The phosphorus accumulated by bacteria gets mineralized and the phosphorus is released again after decay of the cells. Therefore nearly the entire phosphorus removal capacity of CW is caused by soil adsorption. Phosphorus removal by P-uptake by plant roots and subsequent harvesting plays only a minor role (Vymazal, 2007).

Pathogen removal	The efficiency of conventional wastewater treatment processes in removing pathogenic micro-organisms has been investigated in several studies (e.g. Lucena et al., 2004). The removal efficiency of pathogenic and indicator micro-organisms in wastewater treatment plants vary according to the treatment process type, retention time, other biological flora present in activated sludge, O_2-concentration, pH, temperature and the efficiency in removing suspended solids. Typical a properly working biological–chemical treatment process may achieve 90–99% microbial reductions (Koivunen et al., 2003). Up to 5.7 logs removal of Coliforms and 5.5 logs of coliphages were observed in the conventional treatment process with advanced tertiary treatment (Zhang and Farahbakhsh, 2007). The bacterial removal within AS-WWTP is a result of the adsorption of bacteria onto the activated sludge floc, removal due through predation by protozoa and metazoans, cell lysis caused by other bacteria or phages and natural die-off.	Generally the removal efficiency is considered to be a result of a combination of chemical and physical factors, including mechanical filtration and sedimentation as well as biological mechanisms, like antimicrobial activity of root exudates, predation by nematodes and protists, activity of lytic bacteria or viruses, retention in biofilms and natural die-off (Vacca et al., 2005). For a VFCW high removal rates for fecal indicator organisms could have been achieved. E.g. *Escherichia coli* was reduced of 4.35 log units, total Coliforms of 4.31 log units, and Enterococci of 4.80 log units, respectively (Sleytr et al., 2007, see annex V). The pathogen removal efficiency in CWs is primary influenced by the used filter material, the hydraulic loading rate and the resulting hydraulic residence time. The influence of plants on the pathogen removal is still in discussion. Whereas Vacca et al. (2005) as well as Sleytr et al. (2007; see annex V) found no correlation between the removal efficiency of enteric bacteria and the presence of plants, Wand et al. (2007) observed higher removal efficiencies for a planted sand filter in comparison to an unplanted filter.
	The performance of wastewater treatment in the elimination of microbes is usually evaluated with bacterial indicators. However, there is some concern about the suitability of these indicators for evaluating the removal of other pathogens such as viruses and protozoa, leading to a general consensus on the need for alternative indicators (Lucena et al., 2004).	
Possibilities to remove biomass (and nutrients fixed within) from the system	Excess sludge (containing water, dead and living microbial biomass and diverse organic material) from primary and secondary treatment is settled and removed for further treatment. These solids are normally processed in three steps: digestion (to remove organic matter and stabilize the sludge), dewatering (decreases the volume), and disposal (e.g. incineration or land application) (Moharikar et al., 2005).	The microbial biomass developed within the filter body can not be removed. Plant biomass can be removed from the system by harvesting. But the nutrients within the macrophytes are translocated from the shoots through the roots prior to autumn senescence, and therefore they can not be taken out of the system by harvesting at

	the end of the vegetation period. Therefore the removal of nitrogen and phosphorus via harvesting of aboveground plant biomass is low but it can be substantial for lightly loaded systems (about 100-200 gN m^{-2} yr^{-1} and 10-20 gP m^{-2} yr^{-1} can be removed) (Vymazal, 2007). But plants are normally not harvested from CWs in cold climates because of their isolating effect during winter.

1.3.3 Importance of bacterial diversity, activity and biomass within wastewater treatment systems

Microbial communities within wastewater treatment systems (conventional as well as extensive treatment technologies) have been regarded as “black boxes” for a long time. Especially with the development of culture-independent techniques, like PCR-based techniques (clone libraries and molecular fingerprint techniques), or by the application of fluorescent in situ hybridization (FISH) more detailed insights into the microbial community compositions of conventional WWTP have been achieved within the last fifteen years (e.g. Amann et al., 1998; Wagner et al., 2002; Daims et al., 2006a). Since the early nineties, a great variety of analytical and investigative methods have been developed to analyse species composition, spatial structure (or architecture) and functional properties of microbial aggregates such as activated sludge flocs and biofilms. Simultaneously, novel methods became available which allow advanced mathematical modelling and numerical simulation of biological, chemical and physical processes in bioaggregates and bioreactors. Especially the FISH-technique enables an *in situ* monitoring of microbial biofilm communities. FISH is highly effective for detecting specific bacteria and analyzing the spatial organization of a complex microbial community, due to the possibility of detecting specific bacterial cells at a single-cell level by *in situ* hybridization using phylogenetic markers (16S-rRNA-targeting oligonucleotide probes) labeled with a fluorescent compound (Wagner et al., 1993). In future these techniques could provide a rapid, direct and specific detection method for routinely monitoring of the microbial communities of AS-WWTP to detect harmful microorganisms within the treatment unit and in the in- and outflow. When FISH analyses are performed with complex environmental samples, difficulties related to the presence of microbial cell aggregates and nonuniform background fluorescence is often encountered (Wagner et al., 1993). Especially in samples from soil-like systems with a high degree of organic matter and a high content of fine material the background fluorescence hampers these analyses. FISH analyses were performed within CW systems. Nicomrat et al. (2006a) performed FISH counts within constructed wetlands receiving acid mine drainage and reported of problems with low fluorescence intensities. These can be caused by low rDNA content in dormant and / or inactive microorganisms and thick cell walls that resist the probe’s ability to penetrate through the cell wall.

PCR-based fingerprint techniques (e.g. Denaturing Gradient Gel Electrophoresis = DGGE, Terminal Restriction Fragment Length Polymorphism = T-RFLP, Single Strand Conformation Polymorphism = SSCP), as well as the clone library approach are important tools to elucidate the bacterial diversity within wastewater treatment systems (see also chapter 3.3.3). Fingerprint techniques, which allow a high sample throughput, are very useful tools to compare microbial communities within different wastewater treatment facilities, or to detect population shifts under different operation conditions, or to monitor seasonal variations of the

microbial population. To obtain a general fingerprint of the investigated community the diversity of the 16S rDNA can be analyzed. To get an indication of the active members of the community it is advisable to target the 16S rRNA. Like other PCR-based techniques these methods can be also restricted on the investigation of the diversity of a group of bacteria with a specific physiological potential by screening the diversity of a functional gene instead of 16S rDNA (see also chapter 3.3.3.2).

In some instances it may be necessary to obtain a complete inventory of the prokaryotic microorganisms in a wastewater treatment plant. In this case the only possibility is to create a clone library based on 16S rDNA (or rRNA to identify physiological active organisms) of the microbial community (see also chapter 3.3.3.3).

Different culture independent analyses revealed a diverse microbial community within AS-WWTP: Wagner et al. (2002) reported that within five lab scale and three full-scale AS-WWTP thirteen bacterial divisions, of the thirty-six currently identified bacterial domains were detected, indicating considerable microbial diversity in WWTPs. Betaproteobacteria were the most frequently retrieved phylogenetic group. Apart from Proteobacteria, molecular isolates affiliated to Bacteroidetes, Chloroflexi and Planctomycetales were retrieved in significant numbers. Similar results were obtained by e.g. Kapley et al. (2007), who investigated the bacterial diversity of a biological wastewater treatment facility that receives wastewater from different industries. The phyla Proteobacteria, Actinobacteria Firmicutes, Planctomycetales and Bacteroidetes were identified. The total diversity was composed of members of five known phyla, represented by thirty-seven genera, with the Proteobacteria constituting the most abundant phylum detected. However, a very large fraction of the diversity represented a hitherto unidentified bacterial population.

Apart from the excessively studied activated sludge bacterial communities during the last seven years, also microbial populations of different types of constructed wetlands created for the treatment of various types of wastewater have been investigated by different culture-independent molecular techniques (Silyn-Roberts and Lewis, 2001, 2003; Walsh et al., 2002; Baptista et al., 2003, 2008; Ibekwe et al., 2003, 2007; Hallberg and Johnson, 2005; Truu et al. 2005; Yan et al., 2005; Ahn et al., 2007; Ishida et al., 2006; Nicomrat et al., 2006a, 2006b, 2008; De Journett et al., 2007; Gorra et al., 2007; Jin and Kelly, 2007; Sawaittayothin and Polprasert, 2007). Excluding the studies which applied special wastewater types, like acid mine drainage, to the investigated CWs it can be summarized that the bacterial communities of CWs are rather diverse like in conventional wastewater treatment systems and also the community compositions are comparable. Ibekwe et al. (2007) revealed that Gamma-, Beta- and Deltaproteobacteria were the dominant groups within a free water surface flow constructed wetland. According to these results Walsh et al. (2002) demonstrated that Proteobacteria were the dominating group in this system. Baptista et al. (2008) compared the bacterial diversity within planted and unplanted horizontal subsurface-flow constructed wetlands and found no significant difference within the community structure of the two systems.

Furthermore special attention is paid on how to relate the composition of the community of microbes present in wastewater treatment systems to the *in situ* function of individual populations and how such information might be used to manage and control these systems better. The functional diversity of a microbial community has been defined as the occurrence and distribution of physiological and metabolic traits among members of the investigated community (Torsvik and Ovreas, 2007). For functions of an ecosystem, like organic matter decomposition and element cycling quantitative and qualitative (community composition) analyses of the microbial consortium can be regarded as an integral part. It is however an open question whether changes in community composition within functional groups will influence processes within the ecosystem. This is due to the functional redundancy present in many environmental communities. E.g. the coexistence of various nitrifiers in different WWTPs is an evidence of functional redundancy, a feature that may help maintain the stability of the system for nitrification (Siripong and Rittman, 2007).

Connections between the phylogenetic affiliation and the function of bacteria are possible when analyzing functional genes like *amoA* (AOB) (see chapter 3.3.3.2), *nirK, nirS, nosZ* (denitrifiers) (see chapter 1.3.1.4), *pmoA* (methane oxidizers), *mcrA* / *mrtA* (methanogenic archaea) or *dsz* (desulfurizing bacteria).

To identify the most important species (key players) within a specific functional group (e.g. AOB) is important because it allows to draw conclusions about the specific different ecophysiological requirements of these species. But these characteristics are mostly derived from pure cultures of these organisms. The first problem arising from this approach is that most bacteria identified within a wastewater treatment facility are not culturable. About 5 to 15% of the bacteria found within AS-WWTP are culturable (Wagner et al., 1993). The second problem is that the behaviour in pure cultures does not necessarily reflect the requirements within natural communities. But to gain more information about the physiological potential of the identified bacteria is only possible within pure cultures, even in the age of modern molecular biology.

Important aspects and implications of quantitative measurements of microbial biomass and activity for AS-WWTP and CW systems are summarized in table 1.4.

Table 1.4: Relevance of the assessment of diverse parameters related to microbial biomass and activity within for AS-WWTP and CW systems.

	AS-WWTP	**CW systems**
In *situ* measurements of population structures	Possible by FISH + CLSM (confocal laser scanning microscopy)	Very difficult to determine due to the solid matrix of the filter material.
Spatial distribution patterns of microbial biomass	Not relevant	Important also for evaluation of the design criteria of the system.
Spatial distribution of bacterial activity / productivity / viability	The formation of active bacteria within an activated sludge floc helps to understand the and requirements and interactions of bacteria. *In situ* measurement are possible e.g. by microsensors.	Helps to understand the requirements of bacteria and is also important for evaluation of design criteria of the system.
Quantitative determination of total biomass	Can be useful to establish the optimal excess sludge rate.	Comparison of differently configured systems (e.g. using diverse filter substrata, planted / unplanted).

Classical methods for the determination of the active biomass within AS-WWTP rely mainly on respirometric analyses by measuring e.g. the oxygen uptake rate (OUR) (e.g. Cronje et al., 2002). For the investigation of biomass in CWs additional methods developed for analyses of soil microbial communities are employed (see table 3.2).

Besides the many papers investigating the microbial biomass, activity, productivity and viability within AS-WWTP some papers dealing with the measurement of microbial activity, productivity and / or growth rates within CWs have been found (Baptista et al., 2003; Tao et al., 2007, Gagnon et al., 2007). Other papers investigated the spatial distribution of the microbial biomass within the filter body of CWs (Larsen and Greenway, 2004; Nguyen, 2000; Silvan et al., 2003; Nurk et al., 2005) and the biofilm development, respectively (Ragusa et al., 2004; Flood et al., 1999; Silyn-Roberts and Lewis, 2003). The investigations on the

spatial distribution of the microbial biomass within the filter body of CWs showed a strong negative correlation with increasing depth and an reduction of biomass with increasing distance from the influent for horizontal systems, respectively. Whether the vegetation of the CW has a significant influence on the microbial biomass of the bulk-soil or not, seems to be controversial; whereas Gagnon et al. (2007) demonstrated that microbial density and activity were significantly higher within in a CW planted with *Phalaris arundinacea* compared to an unplanted system, Larsen and Greenway (2004) found no significant difference between a planted and an unplanted CW. If comparing quantitative results from different studies it has to be considered, that the design, the mode of operation and the composition of the applied wastewater can vary extremely among different CWs, and therefore it is not possible to compare directly the results obtained from theses studies.

Significant advances have been made in the microbiological and biochemical areas of activated sludge. These advances have been driven by the development of new analytical techniques, that allow microorganisms to be studied in situ in the activated sludge environment. However, there has been little cross-linking and overlap between the engineering and technology and microbiology and biochemistry paradigms. (Cronje et al., 2002). For mathematical simulations describing the biological, chemical and physical degradation processes, and thereby enabling a prediction of the performance of activated sludge WWTPs (e.g. Henze et al., 2000), the growth and decay rate, and the value of active biomass play a fundamental role. The current design and simulation models include active biomass as a fundamental parameter, discriminating between heterotrophic and autotrophic biomass. However these parameters remain mostly purely hypothetical because they are not quantitatively measured. Some studies tried to experimentally quantify these microbial parameters for the investigated treatment system, instead of relying on theoretical values (Cronje et al., 2002; Ismail et al., 2007). Accurate estimates of microbial population concentrations and the direct, *in situ* determination of kinetic parameters are necessary for the calibration and validation of existing models of biological nutrient removal within WWTP.

These simulation models established for AS-WWTP to describe the transport and the reactions of the main constituents of wastewater have been adapted to different kinds of wastewater treatment systems, e.g. to subsurface CWs (Langergraber, 2008). The numerical model HYDRUS-2D / CW2D (Langergraber and Simunek, 2005) is able to describe the biochemical elimination and transformation processes for organic matter, nitrogen and phosphorus based on the mathematical formulation introduced by Henze et al. (2000) for activated sludge systems. Furthermore they can be used to evaluate and improve existing design criteria of CWs (Langergraber et al., 2007c; see annex IV). For a critical review of the mathematical modelling of structure and function of microbial communities within WWTP see Wilderer et al. (2002).

It has been excepted by scientists from all disciplines, involved in the investigation of wastewater treatment processes, that an enhanced knowledge of the structure, the function, the spatial distribution and depending on the climate seasonal fluctuations of the bacterial population are necessary for further plant optimization. But there are still concerns whether it is necessary to know about all the details on the micro-scale when designing and operating large-scale treatment plants. These questions are discussed and partly answered in two very interesting articles by Wilderer et al. (2002) and Daims et al. (2006a). Wilderer et al. (2002) stated that it is far from being accepted that the increased level of analytical resolution in time and space satisfies the needs of wastewater engineers. But certainly shedding light on the black box of microbial systems is important for any further development in biotechnology. Particular for the optimization of operation and design of wastewater treatment systems at a practical level, a more scientific rather than empirical (trial and error) approach could be highly valuable in many cases. However it is most important to bridge the gap between science and engineering in the field of wastewater technology, by increasing the awareness and valuation of each other's knowledge and expertise. The next challenge in the field microbial ecology is to elucidate the mechanisms and requirements of decomposition activities and to characterise the specific physiological requirements and the *in situ* location

of the species involved within these processes and furthermore to apply these findings to improve the process performance and stability of diverse wastewater treatment systems.

One of the most interesting aspects in the research field of wastewater treatment is the multidisciplinary nature of the topic. There is a great need to promote interdisciplinary research involving engineers, microbiologist, chemists, ecologist, aquatic biologists, and others. A lot of basic research has been done within each of this research subjects, but for future investigations it will be more and more of great interest to combine all these findings for further improvements and optimizations of the wastewater treatment systems, considering design criteria and the way of operating the system.

2 OBJECTIVES AND AIMS

For an optimization of the system constructed wetland regarding design criteria as well as the operation mode it is a fundamental need to understand the basic biological, chemical and physical processes taking place in the filter body. Microorganisms, which are responsible for the turnover of organic carbon and the transformation of nitrogen compounds in a CW, have been often regarded as a “black box” due to complexity and diversity of the community. By designing a CW it is important to create an environment, which is optimal for the processes responsible for the retention and degradation of wastewater compounds.

Vertical flow constructed wetlands (VFCWs) with intermittent loading which use a sandy substrate as filter material represent the latest generation of subsurface flow constructed wetlands and are state of the art for this technology for treatment of domestic wastewater (e.g. Langergraber and Haberl, 2001). To obtain a deeper understanding of the quantity, diversity, and activity of the microorganisms inhabiting the filter body of a VFCW the biomass, productivity and community composition of the microbial population within the filter body of vertical flow constructed wetlands were investigated.

The project was carried out in three parts:

The first part comprised preliminary experiments to optimize the sampling procedure and to adapt methods originally derived from soil microbiology for determination of microbial biomass and productivity within the filter bodies of indoor pilot-scale VFCWs:

- Several methods based on different measuring principles are applied to quantify the microbial biomass in the filter body of constructed wetlands and a method for estimating the bacterial productivity in sediments is adopted. To implement these methods to constructed wetlands it is necessary to adapt them to the special characteristics of the artificial filter substrate (e.g. low water content, in homogeneity due to the relatively coarse filter material, inhibiting substance from the wastewater).

The second part of the project includes the application of the methods developed within part I to assess the spatial distribution of microbial and bacterial biomass and bacterial production within the filter bodies of indoor and outdoor VFCWs:

- Investigation of the microbial and bacterial biomass in the filter body by the application of different methods to reveal potential biomass distribution patterns within planted and unplanted indoor and a planted outdoor VFCW.
- Estimation of the bacterial carbon utilization by measuring the bacterial production within planted and unplanted indoor and a planted outdoor VFCW.

The third part of this project focuses on the diversity of bacteria inhabiting the filter body of VFCWs:

- By applying culture-independent methods a brief inventory of the most dominant and metabolically active bacteria inhabiting the filter material should be done to assess potential vertical distribution patterns of bacterial diversity within planted and unplanted indoor and a planted outdoor VFCW.

- By using a molecular fingerprint technique the most important ammonia oxidizing bacteria (which are performing the rate-limiting step of the nitrification process) in an outdoor VFCW should be determined to obtain a more detailed understanding of nitrogen degrading process.

The scientific outcome of part II and III is presented within three published peer reviewed papers (see chapters 4.1-4.3) and one paper submitted for publication (see chapter 5). A summary and conclusion of the four papers is given within chapter 6.

3 Material and methods

3.1 Treatment systems

3.1.1 Pilot scale system

Eight subsurface vertical flow pilot-scale constructed wetlands (PSCWs), with a surface area of 1 m^2, each have been constructed in the technical laboratory hall of the Institute of "Sanitary Engineering and Water Pollution Control". The 50 cm deep filter bodies consist of sandy substrate with a grain size of 0.06-4 mm (d_{10}=0.2 mm, d_{60} = 0.8 mm). An intermediate layer of 10 cm thickness with a gravel size of 4-8mm prevents fine particles to be washed out into the 15cm thick drainage layer made of coarse gravel with a diameter of 16-32 mm (see figure 3.1).

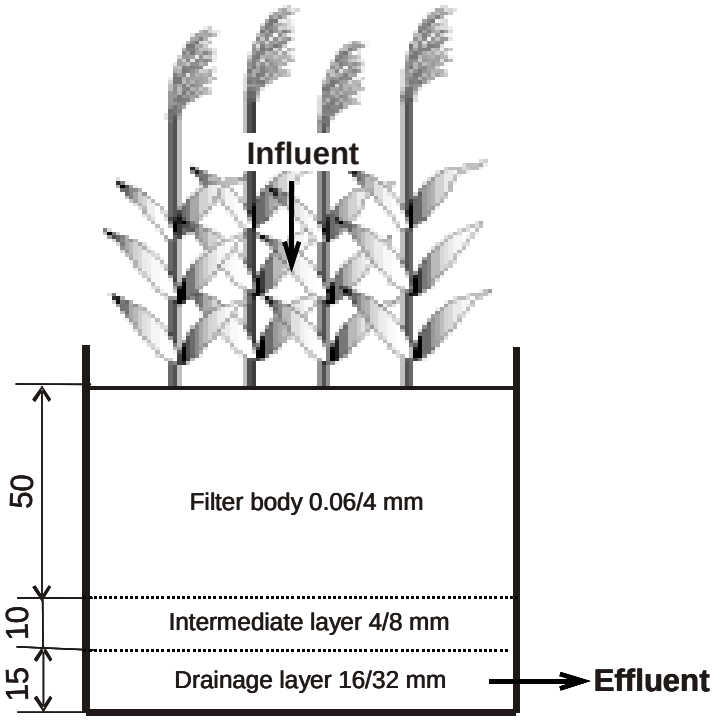


Figure 3.1: Schematic illustration of a pilot-scale constructed wetland (PSCW).

Fifteen litres of municipal wastewater, which were pre-treated in a settling tank, were spread every six hours on the surface of the filter body using a sprinkler system that was constructed to ensure a homogenous distribution even for coarser sandy substrate (see figure 3.2). This hydraulic loading rate of 60 L m^{-2} d^{-1} results in an organic load of approximately 20 g COD m^{-2} d^{-1}, corresponding to a specific surface area of 4 m² per person equivalent COD (PE_{COD}) (when considering a specific pollutant load for mechanically pre-treated wastewater of 80 g COD per day).

Figure 3.2: Wastewater influent system of the PSCW.

Six PSCWs were planted with *Miscanthus sinensis giganteus* (china reed) growing under a 1000 W mercury-vapour lamp and an additional UV-lamp specific for plant growth whereas the other two were unplanted without lamps above the filter surface to prevent extensive algae growing (for an overview see figure 3.3). All eight systems were operated non water-saturated (free drainage) and without clogging problems during the whole operation period. The PSCWs were operated automatically using a LabView® program.

Figure 3.3: Overview of the eight indoor pilot-scale constructed wetlands (PSCWs).

3.1.2 Full scale system

To evaluate the results derived from the indoor pilot scale plants an experimental full-scale outdoor constructed wetland (FSCW), located in Ernsthofen (Lower Austria), was investigated (for an overview and a schematic illustration of the CW see figure 3.4 and 3.5, respectively). The plant consists of three parallel-operated subsurface vertical flow beds planted with a surface area of about 20 m^2 each (table 3.1). The wastewater was mechanically pre-treated by a three-chamber septic tank. The plant was constructed according to the Austrian design standards (ÖNORM B 2505, 2008). Design and operation criteria are equivalent to the indoor plant; also the same filter material was used with the difference that all beds were planted with *Phragmites australis* (common reed). The wastewater was distributed via a network of four parallel pipes (diameter 5 cm; distance between the pipes 1 m) with holes every 0.75 m (with a diameter of 8 mm). The filter beds were loaded four times a day with municipal wastewater, which was collected by means of three tile drains after the filter body passage. The hydraulic loading rates varied between 20 and 40 g COD m^{-2} d^{-1} for the three beds. For this study samples were taken exclusively from bed two, which was loaded with 43 l m^{-2} d^{1}; which is equivalent to an organic load of 27 g COD m^{-2} d^{-1}, corresponding to a specific surface area of 3 m^2 per person.

Table 3.1: Layout of the experimental FSCW in Ernsthofen (Langergraber, 2007).

Parameter	Unit	Filter bed 1	Filter bed 2	Filter bed 3
Surface area	m^2	17.8	18.5	18.2
Specific surface area	m^2 PE_{COD}^{-1}	4	3	2
Organic loading (design)	g COD m^{-2} d^{-1}	20	27	40
Organic loading (measured)	g COD m^{-2} d^{-1}	17.5±0.5	23.4±0.7	35.1±1.5
Daily hydraulic loading	L	573	795	1177

Figure 3.4: Overview of the full-scale vertical flow constructed wetland (FSCW) in Ernsthofen.

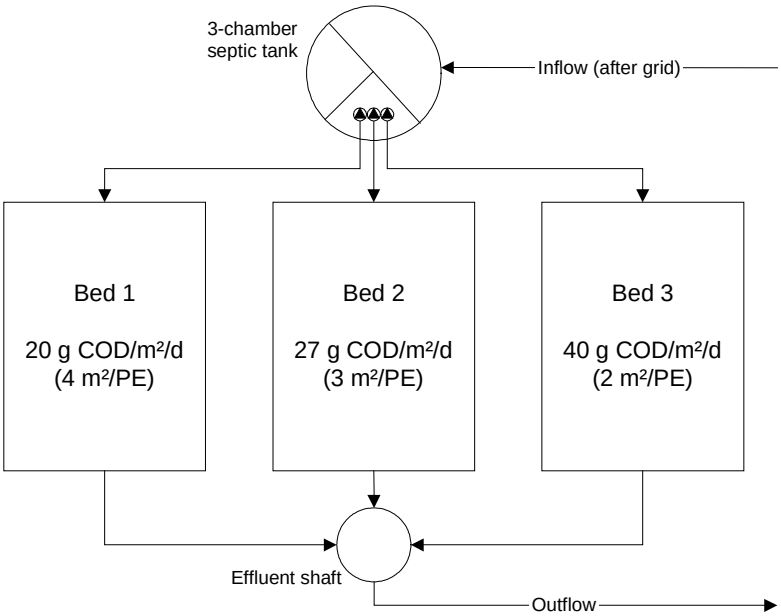


Figure 3.5: Schematic layout of the experimental full-scale plant in Ernsthofen (Langergraber et al., 2007a).

3.2 Sampling

3.2.1 Filter body samples (also referred as “soil samples”)

Samples were taken from seven different depths of the filter body: 0-1 cm, 1-5 cm, 5-10 cm, 10-20 cm, 20-30 cm, 30-40 cm, 40-50 cm. Samples from a depth of 0-10 cm were taken by a syringe cut at the top (inner diameter: 2.5 cm; see figure 3.6). Samples from 10-50 cm were removed by a drill corer (inner diameter: 10 cm) carefully separately for each depth layer to avoid a mixing of the different depths.

Figure 3.6: Syringe cut at the top to take samples from depths of 0-10 cm of the CW filter body.

Sampling for preliminary analyses

First samples for preliminary pre-investigations were taken from the eight indoor plants (PSCW) beginning three months after operation start of the plants, ending fifteen months later. Several sampling campaigns with samples taken from two different depths of the filter body (5-10 and 30-40 cm) were performed to develop, adapt and optimize the protocols of the applied methods mainly originated from microbial soil ecology. The samples were taken from different distances from the center of the filter surface to avoid biases from potentially non-uniform distributed wastewater. These samples were processed immediately in the lab.

Sampling of horizontal and vertical profiles

To check possible variations within one depth layer depending on the distance from the center of filter body samples from the PSCW were taken 0-10, 10-20, 20-30, 30-40 and 40-50 cm away from the center in a depth of 0-1 cm and 40-50 cm, respectively.

For vertical profiles samples were taken from 0-1, 1-5, 5-10, 10-20, 20-30, 30-40 and 40-50 cm depth from the eight different PSCWs, as well as from the full-scale plant. The depth profiles were taken in a distance of 20 cm from the center of the filter body and in 20 cm distance from the wastewater pipes for the outdoor CW, respectively.
Filter body samples were taken two years after operation start of the PSCW over a period of two years. For the full-scale plant the sampling period lasted for on year beginning two years after plant operation start.

For a more detailed description of these sampling campaigns and storage of the samples see Tietz at al. (2007b) chapter 4.1.2.2; Tietz et al. (2008) chapter 4.2.2.2.; Tietz et al. (2007a) chapter 4.3.2.2 and chapter 5.1.2.2.

Rhizosphere samples (also referred as “rhizosphere-soil”)

The filter body material attached to the roots and rhizomes (see figure 3.7) were carefully scraped by a palette-knife to remove the attached material.

Figure 3.7: Roots and rhizomes from *Miscanthus sinensis giganteus* with adherent filter body material.

3.2.2 Wastewater samples

Chemical and bacteriological analyses

Samples from the influent and the eight effluents of the PSCWs were taken on a monthly basis for routine chemical and bacteriological analyses over a period of two years (see also Tietz et al., 2007b, chapter 4.1.2.3 and Tietz et al., 2008, chapter 4.2.2.3). Only the COD (chemical oxygen demand) of the influent was measured twice a month. For the outdoor plant water samples have been collected by the local plant staff on a weekly basis over a period of 20 months and have been analyzed in the plant laboratory by cuvette tests for suspended solids (SS), organic matter (COD and BOD_5), nitrogen (NH_4–N, NO_2–N, and NO_3–N) and phosphorus (PO_4–P). The plant lab data have been checked every two months by analyzing parallel samples in the university lab. In addition the following parameters have been analyzed monthly in the lab of the Institute of Sanitary Engineering and Water Pollution Control: Total organic carbon (TOC), organic and total nitrogen (N_{org} and TN, respectively), and total phosphorus (TP). The routinely measured parameters were monitored weekly over a period of about one year, except TOC (total organic carbon), which was measured monthly.

3.3 Significance of the applied methods to characterise the microbial community in the filter body of the constructed wetlands

In the following chapter the relevance, the principles and the limitations of the methods chosen for the investigations of the objectives of this study are discussed.

3.3.1 Methods to determine the microbial and bacterial biomass

Microbial biomass has been defined as the part of organic matter in soil that constitutes living microorganisms smaller than 5-10 μm^3 (Alef and Nannipieri, 1995). Soil microbial parameters were probably the first indicators of soil quality used. Therefore, the quantitative description of microbial communities in soil has become a topic of profound interest, but remains one of the most difficult tasks facing microbial ecology (Zelles, 1999). Microbial biomass acts both as a nutrient reservoir and a catalytic force in decomposition and is crucial for understanding nutrient fluxes within and between ecosystems (Smith and Paul, 1990). Microbial biomass can also be a sensitive indicator of environmental toxicity attributable pesticides, metals, and other anthropogenic pollutants (Robertson et al.,1999).

The microbial population of a soil habitat is a very complex and diverse community, with different biochemical characteristics, associated with soil particles and organized in different microcosms. The microbial soil community comprises nematodes, protozoa, fungi and bacteria. Due to this diversity and complexity it is not possible to capture the complete microbial compartment by the application of one single method. Therefore many methods focusing on different aspects of the microbial soil community have been developed to function as indicators for microbial soil biomass.

To quantify the microbial biomass one has to distinguish between direct methods and indirect methods. Direct methods are trying to assess the total biomass, or the sum of all cells, or to measure the quantity of specific compounds existing in every eukaryotic and prokaryotic cell (microbial biomarkers). Indirect methods are focusing on the measurement of specific physiological activities of the microbial community and therefore they are more often used as indicators for microbial activity than as a measure for microbial biomass. Table 3.2 gives a

summary of the most commonly used methods to estimate the quantity of microbial biomass in soils and sediments including their relevance and limitations.

Table 3.2: Methods to quantify total microbial and bacterial biomass in soils and sediments.

Direct methods	Relevance / limitations	Method described by (e.g.)
Fumigation-Extraction (FE)	Comprises total microbial biomass (MB); can distinguish between C-, N- and P-biomass; includes also dead biomass	Sparling and West (1988)
Fumigation-Incubation (FI)	Similar to FE but laborious procedure; no longer in common use	Jenkinson and Powlson (1976)
Adenosine triphosphate (ATP) - content / Adenylate energy charge	Correlates with MB; the adenylate energy charge provides information about the activity of the community	Bai et al. (1989)
Nucleic acid (DNA, RNA) quantification	RNA / DNA ratio correlates with the metabolic state of the community; not very reliable due to adsorption of DNA on detritus and clay; not very often in use	Dell'Anno et al. (1998)
Phospholipid Fatty Acids (PLFAs) analysis	Total sum of PLFAs correlates with MB; specific PLFAs are biomarkers for gram-positive, -negative bacteria; *actinomycetes*, specific physiological (e.g. nitrite-oxidizers, sulphate-reducers, methanotrophs) and phylogenetic bacterial groups (e.g. *Bacteroides / Flavobacterium*, *Sphingomonas*), fungi, protozoa, and for eukaryotes	Frostegård et al. (1991) Zelles (1999)
Cultivation-based methods (e.g. Plate count, MPN-methods)	Number of cultivable bacteria (e.g. heterotrophic bacteria or by using selective media for detection of specific groups) and fungi can be quantified; not very meaningful due to the low proportion of culturable organisms	Craig et al. (2002) Rowe et al. (1977)
Microscopic direct counts of fluorescently labeled cells	Often applied for bacteria; can be used to quantify specific bacterial groups by applying FISH-probes (fluorescent in situ hybridization: application of specific oligo-nucleotides linked to a fluorescent dye)	Weinbauer et al. (1998) Kepner and Pratt (1994) Bertraux et al. (2007)
Immunofluorescent staining assays	By staining organisms with fluorescently labeled antibodies a quantification of target organisms (species specific) is possible; however there are unspecific binding-reactions of antibodies to organic matter of the soil, thus this method is not often in use in modern literature	Schmidt (1973)

Indirect methods	Relevance / limitations	Method described by
Substrate induced respiration (SIR)	Comprises the metabolically active biomass; easy to perform method which gives a crude estimation about the TMB	Anderson and Domsch (1978)
Enzyme-activities (e.g.: dehydrogenase, arginin-deaminase, phosphatase, glucosidase, protease, DMSO-reduction, hydrolysis of FDA)	Limited correlation with TMB; indicator for bacterial or microbial activity; mostly enzyme activities are measured after the addition of substrate and therefore these results have to be interpreted carefully as they express a potential activity and not the original soil conditions	Gong (1997) Nannipieri et al. (1983) Alef and Kleiner (1986) Sparling and Searle (1993) Schnürer and Rosswall, (1982)

Within this study four different methods, common in soil microbiology, were chosen to determine the microbial biomass within the filter bodies of the investigated CWs (see chapters 3.3.1.1-3.3.1.4). Additionally one method to measure the bacterial biomass was applied (see chapter 3.3.1.5). These four methods are based on different principles; a combination of these techniques allows a more accurate quantification of microbial biomass.

3.3.1.1 Substrate induced respiration (SIR)

The measurement of soil respiration is a simple and well known method to assess biological activity. Respiration as a universal process is not only restricted to microorganisms but is also carried out by other organisms inhabiting soil habitats. Respiration can be measured by the uptake rate of oxygen or by the amount of released carbon dioxide. SIR is defined as the soil respiration measured in the presence of an added easily degradable substrate (in most cases glucose is used) in contrast to the basal respiration (without addition of substrate). For an accurate determination of the SIR-rate the saturating amount of glucose response has to be ascertained for every sample type within preliminary experiments. The measurement of the SIR rate starts after a short adaptation period after the glucose addition, which the community needs for uptake and utilization of the nutrient. The enhanced rate of respiration stays reasonable stable for up to 6-8 hours, afterwards a further increase of the respiration rate occurs attributed to cell division and population growth. Therefore the maximal rate of respiration during the “plateau” phase following substrate addition can be considered as activity of the initial population.

The respiration depends on the physiological state of the cells and is influenced by different abiotical factors like soil moisture, temperature, the availability of nutrients and soil structure. But the SIR-method appears to give a reliable estimate of microbial-C over a wide range of soils and pH conditions (Sparling and West, 1988). The SIR-respiration rates can be converted into microbial via a formula given by Anderson and Domsch (1978) who calibrated the SIR-technique with the fumigation-incubation method and hence related the SIR response to soil microbial biomass. This is a rather simple method but the application of the conversion factor from the literature originated from different kinds of soils has to be considered with reservations.

The SIR was measured for this study by using the “Isermeyer method” for determining the CO_2 evolution according to Anderson and Domsch (1978) (see figure 3.8 for the experimental set-up; the applied protocol is described within Tietz et al. (2007b; chapter 4.1.2.9).

Figure 3.8: Experimental set-up for the measurement of the "Substrate Induced Respiration" of the CW filter body samples.

3.3.1.2 Chloroform fumigation-extraction (C-FE) for measuring microbial biomass-carbon and -nitrogen

Chloroform fumigation of soil kills and lyses microbial cells; thus the cell material is no longer attached to the soil matrix and can be extracted from soil and the total organic carbon (TOC) of the soil extract is measured. To reveal the proportion of TOC derived from the biomass an unfumigated sample is treated in the same way and TOC is determined in the extract. The difference of TOC-fumigated minus TOC-unfumigated (C-flush) should represents the organic carbon originated from microbial biomass, which became extractable by the fumigation-treatment. The soil biota represents 1-3 % of the soil carbon and 3-5 % of soil nitrogen (Alef and Nannipieri, 1995).

This method was first published by Jenkinson and Powlson (1976). The criticism or concern, that chloroform dissolves more than just biomass, by rendering other, non-living fractions of the soil organic matter extractible, was again rejected by Jenkinson et al. (2004). But on the other hand they had to admit that this possibility could not be entirely excluded, although they showed that the quantity of non-biomass C thus solubilised was tiny compared to that from killed biomass.

But the most serious criticism about this method is the application of the conversion factors (k_{EC}) for the transformation from C-flush (= difference of TOC-fumigated minus TOC-unfumigated) into biomass-C proposed e.g. by Sparling and West (1988) and for the conversion from N-flush into N-biomass (Brookes et al., 1985). Several authors (e.g. Sparling and West, 1988) measured the k_{EC}-factor by adding a soluble ^{14}C-labeled substrate such as glucose to the soil; incubated for 1-2 days and measured the extractable TOC after fumigation. The k_{EC}-factor is calculated by the following equation:

$$k_{EC} = (\text{glucose-}^{14}C \text{ added}) - (CO_2\,^{14}C \text{ respired}) \;/\; (^{14}C \text{ in extracts of fumigated sample})$$

The problem of this approach lies in the assumption that all of the insoluble ^{14}C is present as live microbial biomass, with non-stabilized in non-living-structures such as insoluble polysaccharides, making the k_{EC} values thus obtained to small (Jenkinson et al., 2004). Therefore the application of these conversion factors can lead to an underestimation of the biomass values obtained from the C-FE-method. Joergensen (1996) assessed a range k_{EC}-values varying from 0.23-0.84 on the basis of 153 soils by indirect calibration using the

fumigation-incubation method. Some other authors calibrated the C-FE-results with other methods for determination of microbial biomass, for example with the SIR-method (e.g. Sparling et al., 1990) but they yielded k_{EC} values similar to those of Joergensen (1996). Therefore some authors (e.g. Friedel et al., 2002) suggested not to convert the C-flush into biomass-C. Jenkinson et al. (2004) argued that there are similarities between the biomass in different kinds of soils and in their opinion this legitimates the application of these factors for different types of soils. But we have to keep in mind that all these published values were derived from different kinds of soils and therefore their applicability to other soil-like sample types like sediments or filter materials of CWs has to be still questioned. In this study we converted the C- and N-flushes by k_{EC}-factor and k_{EN}-factor respectively (published by Wu et al., 1990 and Brookes et al., 1985, respectively), because we wanted to compare three different microbial biomass-methods and therefore we had to convert the results into the same unit.

The short exposure time to chloroform vapor (24h) proposed by Jenkinson and Powlson (1976) was criticized by Davidson et al. (1989). Haubensak et al. (2002) evaluated the fumigation time necessary for achieving the largest and most consistent C and N releases and recommended that effect of fumigation period and water content should be assessed for different sample types. But they also concluded that that across contrasting soil types in general half of the observed soils required only a fumigation time of 24 hours. Therefore a chloroform incubation time of 1 d can be considered as a reasonable compromise between a maximal achievable flush and an expectable biomass assay time (Jenkinson et al., 2004).

Another limitation of the method is that in dry samples (water content < than 30 % of the maximal water holding capacity) microorganisms are apparently less effected by they fumigant (Sparling and West, 1989).

A further cause of troubles is the use of unpurified chloroform. This can cause enormous flushes of CO_2, usually accompanied by the disappearance of inorganic N (Jenkinson et al., 2004). But this can be easily avoided by taking care to use only chloroform without any additives (e.g. ethanol is often used as stabilizer).

An advantage of this method is that it allows determining biomass-C and –N within the same extract (extraction-medium is a 0.5 M K_2SO_4-solution). It is also possible to determine the amount of ninhydrin-reactive nitrogen (detects nitrogen bonded in amino acids) within the same extract. The ninhydrin-N is a reliable and sensitive indicator for the amount of soil biomass and has a strong linear relationship to biomass-C and –N determined by the C-FE-method (Joergensen and Brookes, 1990). An extraction of the fumigated and non-fumigated samples with a $NaHCO_3$-solution enables the determination of the P-flush of the analyzed samples (Brookes et al., 1982), which can be converted to biomass-P with the same restrictions as for C- and N-biomass.

A description of the protocol applied within this study to measure the biomass-carbon and –nitrogen by the C-FE-method is given by Tietz et al. (2007b; chapter 4.1.2.7).

3.3.1.3 Adenosine triphosphate (ATP) – content

ATP is an important energy compound in the metabolism of all living microorganisms and is absent in dead cells and in other non-living forms of soil organic matter. It has only a transient extracellular existence in soil; essentially, all measured soil ATP comes from living organisms (Alef and Nannipieri, 1995). Therefore the ATP content has been used as an indicator for living (active) microbial biomass in different soils (e.g. Dyckmans et al., 2003), sediments (e.g. Nakamura and Takaya, 2003) and constructed wetlands (Tao et al., 2007). Nannipieri et al. (1990) stated that ATP, which is measured immediately after sampling, reflects rather microbial activity than microbial biomass. By measuring the adenylates ADP (adenosine diphosphate) and AMP (adenosine monophosphate) in addition to ATP the

adenylate energy charge (AEC) can be calculated, defined as ([ATP] + 0,5 [ADP]) / ([ATP] + [ADP] + [AMP]). The AEC can be used as an indicator for the physiological and metabolic status of microorganisms (e.g. Brookes et al., 1983).

However caution must be taken by using the ATP-content as a measure for microbial biomass due to the following reasons (according to Alef and Nannipieri, 1995):

- ATP concentrations in the cells are not constant and dependent on the physiological state.
- A serious obstacle to interpreting and comparing ATP levels in different soils results from various methods used to extract ATP from soil (e.g. Martens, 2001). The completeness of the extraction cannot be checked practically. A complete desorption of ATP from soils with a high clay content could not be achieved.
- ATP measurements reflect more the status of microbial activity than the biomass size.

After appropriate extraction of the adenylates (ATP, ADP and AMP) from soils they can be measured either with the fire-fly luciferin-luciferase system (Brookes et al., 1987) or by the ion-aired reverse-phase high performance liquid chromatograph approach (Bai et al., 1989), which was applied within this study (for an example of a chromatogram showing the three adenylates measured within this study see annex I).

The determination of the ATP-content was applied as on out of four different methods to assess the microbial biomass of the filter bodies of the investigated CWs. ADP and AMP were not analyzed, because preliminary experiments showed that their extraction efficiencies, tested by three consecutive loops of repeated extractions of the same sample, was rather low (within the first extraction round) and varied with the filter body depth of the analyzed samples. In contrast to these results almost the total extractable amount of ATP was detected within the first extraction, independent of the sampling depth.

Different correlations between the ATP-content and the biomass-C, obtained from parallel measurements of different methods for the determination of microbial biomass-C (e.g. fumigation-extraction or substrate induced respiration) and ATP-analysis, are available (e.g. Contin et al., 2000; Tsai et al., 1997). Within this study the ATP-results have been converted into biomass by the relationship given by Dyckmans et al. (2003). A description of the protocol applied to measure the ATP-content is given by Tietz et al. (2007b; chapter 4.1.2.8).

3.3.1.4 Phospholipid Fatty Acid (PLFA) analysis

Viable microorganisms have an intact membrane, which contains fatty acids as components of its phospholipids (PLs). The fatty acids are responsible for the fluidity, integrity, permeability, and the activities of membrane-bound enzymes. Analysis of PLFA environmental community profiles is an effective tool for monitoring the microbial response to their environment. PLFA profiles simultaneously contain general information about the phylogenetic identity and the physiological constitution of the community. Fatty acids from these lipids (PLFAs) are particularly suitable to characterizing microbial communities for the following reasons:

- decompose within day after cell death (not found in storage products)
- are found in all living organisms with the exception of archaebacteria*

- differ between phylogenetic subgroups and different physiological types of the microbial population
- provide indications of the nutritional status of the organisms (turnover / adaptability)
- give information about the stress the cells are exposed to
- indicators for viable microbial biomass

* Archaeal membranes contain ether-linked phospholipids (PLEL) instead of the ester-linked PL of bacteria and eukaryotes and they are not detected within PLFA assays. Only Gattinger et al. (2002) found significant amounts of PLFAs proportions (up to 35%) within eight monocultures of Euryarcheota. But no other evidence of the appearance of PLFAs in archaea could have been found in the literature.

The PLs are extracted with organic solvents from the cell membranes and walls of the microorganisms. The lipids are fractionated into neutral lipids, glycolipids and phospholipids on silicic acid containing columns. The fatty acids of the PL are converted into their corresponding FAMES (fatty acid methyl esters) via esterification. The FAMES are separated and quantified with a gas chromatograph and the identification of the different esters can be conducted with a mass spectroscopy (for an example of a chromatogram showing the FAMES measured within the filter body of the CW see annex II).

Environmental samples show a wide range of PLFAs (between 30 and 50 different compounds (Boschker and Middelburg, 2002). Lipid profiles can provide insight into microbial community structure because of a relative abundance of certain PLFAs, which differ considerably among specific groups of microorganisms (Zelles, 1999). A lot of research has been done to characterize the PLFAs composition of many taxonomic and physiologic groups of microorganisms. A large database derived mostly from information on isolated pure cultures of microorganisms is already available (e.g. Lechevalier, 1989). Some PLFAs are the dominating PLFA within on group whereas others appear in different groups of microorganisms or they are even ubiquitary. Therefore community PLFAs patterns have to be interpreted very carefully and we have to keep in mind that we cannot obtain a list of the taxa occurring in an environmental sample from these analyses.

PLFA patterns are applied to reveal shifts within the PLFAs composition and can be used to detect changes within the microbial community caused by changes within their environment. Cells adapt their PLFA compositions in order to modify membrane fluidity and permeability as a consequence of changing environmental conditions (Petersen and Klug, 1994). Therefore PLFA-analyses are suitable for detecting rapid changes in living populations (Zelles, 1999). The influence of contaminations on the microbial population by heavy metals (e.g. Rajapaksha et al., 2004), fumigants applied to agricultural soils to control pathogens (Ibekwe et al., 2001), osmotic stress (Mukhopadhyay et al., 2006), or polycyclic aromatic hydrocarbons (Slater et al., 2008) have been assessed by changes in the PLFA composition. The impact of other disturbances on microorganisms, like soil sieving (Petersen and Klug, 1994), or changes in temperature, nutrient or water content (Wilkinson et al., 2002), or changes through soil depth profiles (Fierer et al., 2003) was also estimated via the PLFA-approach.

PLFA analyses have been also used assess microbial biomass of environmental samples (e.g. Petersen et al., 1991; Baath et al., 1992; Zelles et al., 1997; Bailey et al., 2002; Baath and Anderson, 2003; Leckie et al., 2004). PLFAs make up a relatively constant proportion of biomass of naturally occurring communities under undisturbed conditions (Lechevalier, 1989). Additionally many authors found significant correlations between the microbial biomass of environmental samples measured by different methods and their content of total PLFAs (e.g. Frostegård et al., 1991; Zelles et al., 1994; Leckie et al., 2004; Bailey et al., 2002).

For several studies PLFA-analyses were additionally used to discriminate between bacterial and fungal biomass in soil (Frostegård and Baath, 1996; Baath and Anderson, 2003; Bailey et al., 2002).

A description of the protocol applied in this study to determine the PLFAs within the filter body samples is given by Tietz et al. (2008; chapter 4.2.2.6).

3.3.1.5 Microscopic direct counts and measurement of bacterial cell volume

Quantification of bacterial numbers by microscopic direct counts:

Quantification of total bacterial numbers is a basic and essential task in several areas of microbiology, including public health, biotechnology, pharmaceutical industries, food and water, and natural environments (Lebaron et al., 1998).

Total counts in soils are usually determined using fluorescent dyes, such as DAPI or SYBR green, due to fluorescence enhancement if they are bound to nucleic acids (Klauth et al. 2004). The most common dyes are DAPI, Acridine Orange (AO) and the blue-excitable green fluorescent dyes (SYBR green I, II, gold; SYTO; YO-PRO; TOTO; YO-YO, TO-PRO or Pico Green). AO, which has been used especially in the beginning of the development of the microscopic direct count method, was replaced by DAPI, whereas nowadays the green fluorescent SYBR dyes are the bacterial stains of choice, due to their higher affinity to nucleic acids (Weinbauer et al., 1998) and their higher fluorescent yield compared with AO and DAPI (Nybroe et al., 2007). These dyes bind to DNA (dsDNA and ssDNA) as well as to RNA with different preferences. For instance, the quantum yield of SYTO-16 for DNA is much higher than those of SYTO-13 and SYTO-11, which have higher binding affinities for RNA (Lebaron et al., 1998). This staining technique enables the enumeration of bacteria as well as of viruses (e.g. Patel et al., 2007).

Typically, soil and sediment bacteria are stained in solution with fluorescent dyes, collected onto membrane filters, and enumerated by using epifluorescence microscopy. In contrast to most dyes the SYTO dyes penetrate intact live cells, while others are generally applied to samples after fixation (e.g. by formaldehyde). By epifluorescent direct count techniques all bacteria, whether they are viable or not, with intact nucleic acid bind to the fluorescent dyes and produce a visible signal. The detection of the stained cells can be obtained by epifluorescence microscopy, by laser scanning microscopes, by solid state cytometry or by flow cytometry. Due to the fact that the direct count method includes also dead cells this technique overestimates the actual number of bacteria in contrast to traditional culture dependent techniques, which underestimates the number of viable bacterial cells (see chapter 3.3.3). The “LIVE / DEAD” staining procedure (Boulos et al., 1999) allows discriminating between viable (referred as bacteria with an intact membrane) and non-viable cells (with damaged cell membrane) by staining the cells simultaneously with probidium iodide and SYTO9. The green fluorescent SYTO9 is able to enter all cells and is used for assessing total cell counts, whereas red fluorescing PI enters only cells with damaged cytoplasmic membranes. The emission properties of the stain mixture bound to DNA change due to the displacement of one stain by the other and quenching by fluorescence resonance energy transfer (Stocks, 2004).

Epifluorescence microscopic direct count is a high-throughput method (about 30 min are necessary for the preparation of one sample) with a relatively high sensitivity. Of course it does not permit differentiation of bacterial cells on the basis of taxonomy and it cannot be used to estimate the microbial biomass without knowledge of the average cell volume within the analyzed sample.

In the following the major drawbacks / limitations of the microscopic direct count method are summarized:

- incomplete desorption of bacteria from soil / sediment particles
- unspecific staining occurs frequently in soils and sediments and can hamper the enumeration of bacteria
- particle-masking effects within soil / sediment aggregates
- high background fluorescence
- fading of the fluorescence signal during light-exposure
- overestimation of bacterial abundance due to the fact that also dead cells are included

The combination of the dye SYBR Green with aluminium oxide filters, which has been applied within this study, has the advantage to reduce sufficiently the background fluorescence and to enable a good discrimination between sediment particle (appear orange under blue excitation) and bacterial cells (appear green under blue excitation) (see figure 3.9). The problem of particle-masking effects due to the formation of aggregates, which consist of inorganic (mineral particles) and organic material (e.g. detritus; extracellular polysaccharides and living biomass), can be reduced by chemical disintegration (e.g. by sodium pyrophosphate) and mechanically destruction (e.g. by ultrasonication). The fading of the fluorescence signal under light-excitation can be retarded by mounting the stained filter with anti-fading solutions. The most frequently recommended additives for mounting media are p-phenylenediamine (PPD), n-propyl gallate (NPG), 1,4-diazobicyclo[2,2,2]-octane (DABCO) (Ono et al., 2001) and ascorbic acid (Weinbauer et al., 1998). Ready to use antifading kits are also commercially available.

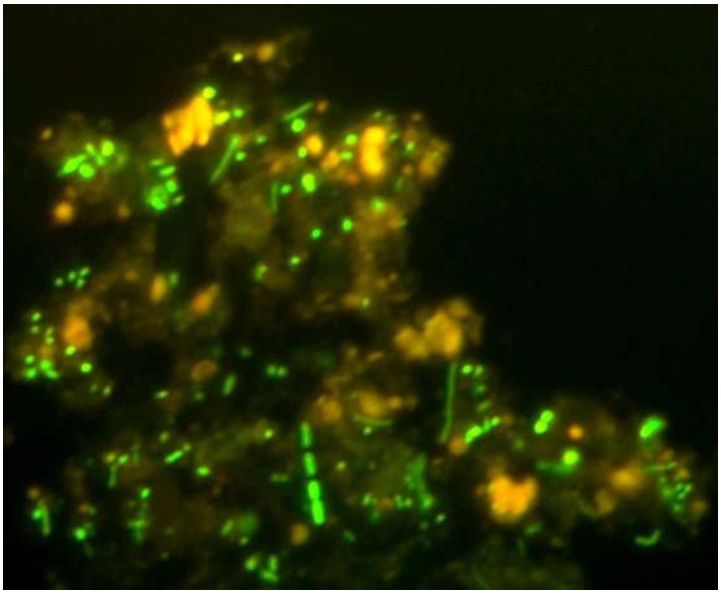


Figure 3.9: Bacteria / soil aggregate from the CW filter material stained with SYBR Green I (1600x magnification; before disaggregation treatment by ultrasonication).

Measurement of cell volume:

To quantify the bacterial biomass carbon via the determination of bacterial cell numbers it is indispensable to measure the average volume of the cells in the investigated habitat. By measuring the length and width of the bacterial cells via the application of automated image analysis systems, it is possible to calculate the biovolume of the cells by using the equation given by Battin et al. (2001) (see Tietz et al. 2007b, chapter 4.1.2.9). This equation is applied under the assumption that the approximate shapes of the bacteria were either spheres or cylinders terminating in hemispheres (Gasol et al., 1995). To obtain representative values for the biovolume it is recommended to count between 400 (Felip et al., 2007) and 1000 cells (Pernthaler et al., 1997). Fluorescent staining protocols may influence the apparent size of bacterial cells.

Based on these measurements the bacterial carbon content per cell can be estimated by applying an allometric volume-to-carbon conversion factor e.g. published by Norland (1993) (see Tietz et al. 2007b, chapter 4.1.2.9). By multiplying the bacterial carbon content per cell with the number of bacterial cells the bacterial biomass carbon per sample can be determined.

Pernthaler et al. (1997) reported that cells brightly stained, in this case by probes conjugated with the indocarbocyanine fluorescent dye Cy3 bacteria, usually appeared to be larger than their corresponding DAPI images. Also the dye SYBR Green I, shows a very bright and intensive fluorescence (see figure 3.9) and therefore can lead to overestimate the width and length of the measured cells.

A description of the protocol applied within this study to determine the bacterial abundance and to measure the bacterial cell volume is given by Tietz et al. (2007b; chapters 4.1.2.9 and Tietz et al., 2008 4.2.2.4).

3.3.2 Measurement of the bacterial productivity (bacterial secondary production =BSP)

Bacterial secondary production is defined by the transformation of organic matter into heterotrophic biomass during a distinct time period. BSP is theoretical unconstrained by the inputs of organic carbon to the system from primary production or other sources (Cole, 2000). A sizeable fraction of organic matter degraded and assimilated by microbes is normally not respired or mineralized for cellular growth. This suggests that microbial secondary production constitutes a significant route of carbon and nutrient fluxes in ecosystems (Buesing and Gessner, 2006).

The most common approach for the in situ measurement of the BSP is based on the incorporation of radioactive labeled molecules into the bacterial biomass during a distinct time period. The two most common methods are the measurement of the incorporation rate of radioactive labeled amino acid leucine into the protein pool (Simon and Azam, 1989) and the determination of the incorporation rate of the radioactive labeled nucleotide thymidine into the bacterial DNA (Fuhrman and Azam, 1980). Whereas the leucine-approach is a good indicator for the biomass increase the thymidine - approach correlates with the DNA synthesis rate and therefore also with the cell division rate. Due to the fact that bacterial cells incorporate ten times more leucine than thymidine the leucine - method is also one order of magnitude more sensitive than the thymidine - method (Simon and Azam, 1989). Big discrepancies between the results of the leucine- and the thymidine– approach can be possible, due to unbalanced growth of the bacterial population (Kirschner and Velimirov, 1999). Unbalanced growth leads also to a decoupling of BSP and bacterial abundance. Under unfavorable environmental conditions the bacterial protein synthesis appears to increase more rapidly than cell duplication; thus specific growth rates of protein synthesis reflects more accurately the variability of the available organic substrate (Pulido-Villena and Reche, 2003).

By multiplying the incorporation rates with theoretical or empirical conversion factors the biomass-carbon production (in [g C g sample^{-1} h^{-1}]) can be calculated. Additionally other issues, like the intracellular isotope dilution, substrate saturation, linearity of the incorporation rate over incubation time, extraction efficiency, restriction of macromolecule labeling to bacteria (specificity) and nonspecific incorporation of radio labeled leucine into molecules other than proteins, or adsorption of radiolabel molecules to the sample matrix have to be considered to obtain reliable bacterial production estimates. The specific substrate saturation, the linearity over incubation time, and the extraction efficiency have to be determined for each specific sample type. Due to the fact that the experiments are performed under substrate saturated conditions it can be assumed that no intracellular isotope dilution occurs (Tietz et al., 2008; see chapter 4.2.2.5). The permissibility of applying conversion factors from the literature, originated from completely different ecosystems (planktonic marine bacteria, freshwater sediments, or soils) to transform the incorporation rates of the CW samples into biomass-C production, are discussed in Tietz et al. (2008, chapter 4.2.4.1).

Buesing and Gessner (2003) examined the specificity of leucine incorporation into bacterial proteins by measuring the incorporation rates in the presence of prokaryotic and eukaryotic inhibitors and demonstrated that no significant leucine incorporation into eukaryotes occurred during short-term incubations, but on the other hand they pointed out that significant incorporation by fungi could not be ruled out. Fischer and Pusch (1999) stated that an increased uptake at higher leucine concentrations (>50 µM) could be partly attributed to eukaryotes. Therefore also the fungal biomass was estimated indirectly within this study as an indicator for the fungal productivity, which was not assessed within this study due to complexity of the method (incorporation of ^{14}C-acetate into ergosterol; e.g. Verma et al., 2003).

A description of the incorporation of radioactive labeled leucine applied within this study to measure the BSP within the filter body of the constructed wetlands is given by Tietz et al. (2007b, chapter 4.1.2.9 and Tietz et al. 2008, chapter 4.2.2.5).

3.3.3 Methods to assess the bacterial community structure and diversity

Investigations of microbial composition and diversity in natural and anthropogenically impacted or created habitats are important in the characterization of such habitats, since microbes are key players in many environmental processes (Nogales et al., 2001). Analyses of bacterial abundance by fluorescence microscopy revealed that 1g of soil (Torsvik et al., 1990) might contain more than 10^{10} bacteria. By applying conventional culture-based methods only 0.1 to 1 % of the total bacteria could have been detected in pristine forest, up to 10% in environments like arable soils (Torsvik et al., 1998), and up to 15 % within very eutrophic environments like wastewater (Wagner et al., 1993). Furthermore, because relatively nutrient-rich media are generally used for cultivation, copiotrophic bacteria rather than the bacteria dominant in the natural community may be selected (Moyer et al., 1994). Applying molecular based cultivation-independent methods enables also the detection of bacteria, which have not been cultured in the laboratory yet. Since the late eighties particularly sequence analyses of 16S ribosomal RNA genes (16S rDNA) have proven to be powerful tools for investigating the microbial diversity in environmental samples originated from various habitats (e.g. Cheng and Foght, 2007; Martinez et al., 2006; Labbe et al., 2007; Lozupone and Knight, 2007; Hovda et al., 2007). Several characteristics of the 16S rRNA (rRNA) gene, such as its essential function, ubiquity, and evolutionary properties, have allowed it to become the most commonly used molecular marker in microbial ecology (Case et al., 2007). Beneath the highly conserved regions the 16S rRNA also includes hypervariable regions. Therefore sequencing of the 16S rRNA genes enables diversity analyses of the total bacterial community (e.g. Muyzer et al., 1993), as well as diversity investigations for distinct functional groups (e.g. Purkhold et al., 2003) down to investigations on species level (e.g. Kuppeveld et al., 1992).

Isolation of rRNA genes for phylogenetic analyses may be less selective and provide a more representative view of a microbial community structure than classical techniques, but such an approach has other potential limitations. Biases can occur during sample collection, cell lysis, nucleic acid extraction, PCR amplification, separation of amplified DNA, application of nucleic probes and data analyses (Wintzingerode et al., 1997). In the following a summary of the potential biases and pitfalls which can occur by applying molecular diversity analyses, especially to soil and sediment samples, is given:

- First losses and so caused biases within the investigation of the community composition can occur during the sample collection and further storage of the samples (especially when RNA is analyzed). Sessitsch et al. (2002) recommended lyophilisation, storage at -20℃ as well as storage in glycerol stocks at -80 ℃ as appropriate for the storage of soils and subsequent RNA isolation.
- Differences in the efficiencies of nucleic acid extraction are caused by difficulties in cell lysis (e.g. for spores and other structures with resistant cell walls) and the ability of different bacterial groups to adhere more or less strongly to the sample matrix (e.g. soil particles). Moreover, DNA liberated from easily lysed organisms can become adsorbed to soil colloids leading to an underestimation of the amount of DNA. Thus the extracted fraction corresponds to about only 25 to 35% of the total bacteria present in the soil. Due to cell structure of gram-negative bacteria (thinner cell wall compared to gram positive bacteria) there is a risk that the extraction procedure will favour the lysis of gram-negative bacteria (Frostegård et al., 1999).
- The preferential amplification of some sequence types relative to others by PCR leads to an unrepresentative composition of the community DNA. For example rDNAs of extremely thermophilic archaebacteria often cannot be amplified by the usual PCR methods (Reysenbach et al., 1992).
- Inhibition of PCR can be caused by the sample matrix (e.g. humic acids). A dilution of the samples can lead to disregard of sequences with a lower abundance. Therefore a nucleic acid purification step prior to PCR can be useful (More et al., 1994), or the addition of BSA (bovine serum albumin) to the PCR (Kreader et al., 1996).

But nevertheless PCR-based culture independent molecular approaches are the most promising methods for the investigation of the structural and functional diversity of environmental bacterial communities. Beside very labour-intensive constructions of clone libraries the development and application of molecular fingerprint techniques to analyze PCR amplicons from complex environments represents an important breakthrough in this field. In the following three molecular methods which have been employed within this study to characterize the bacterial diversity of CWs, are described. The T-RFLP approach was employed for a general assessment of the bacterial diversities within different CW samples, whereas the DGGE technique was applied to determine the diversity within an important functional bacteria group. Additionally the clone library method has been applied to provide a brief inventory of the most dominant active species within the analyzed systems.

3.3.3.1 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

For this community profiling method the separation of PCR products amplified by universal primers for the conserved regions of the 16S rRNA gene, present in all bacteria, was done. It is a high-throughput and reproducible method, that allows the semi-quantitative analysis of the diversity of a particular gene in a community. The primer set used for the PCR contains one fluorescently labeled primer at the 5`-terminus (it is also possible to label the second primer with a different fluorescent dye). The 16S rDNA PCR-products are digested by one or

more frequently cutting enzymes and the resulting fragments are separated via capillary electrophoresis. Due to sequence variations the length of the fluorescently labeled terminal restriction site should be different for each species. A laser reader detects the labeled fragments (T-RFs) and generates a profile based on the size of the fragments. The digital output of the electrophoresis provides information on the size of the product in base pairs; the fluorescence intensity of the peaks expresses the relative abundance of the species within the community (evenness or structure) and the number of peaks is a measure of the richness. The addition of a DNA size standard marked with a distinct fluorescent dye to the digested fragments allows the assessment of the relative size of each T-RF. (For examples of T-RFLP profiles obtained within this study see annex III).

In the following the limitations and drawbacks of this method are summarized:

- One peak can comprise numerous T-RFs differing only in a few base pairs; extremely few T-RFs are truly specific for a single species. An increased specificity can result from analysis of digests with multiple enzymes (Dunbar et al., 2001).
- Impossibility of retrieving suitable phylogenetic information from the generated T-RFs, since fragments are difficult to isolate and are normally too short to be sequenced properly and would if sequencing would be possible, not yield enough sequence information for phylogenetic analysis (Oros-Sichler et al., 2007).
- Populations comprising between 0.1 and 1 % of a bacterial community can be detected in T-RF profiles (Dunbar et al., 2000).
- For highly complex (soil) bacterial communities, the method has been shown to be ineffective in assessing relative phylotype richness and structure (Dunbar et al., 2001).
- An incomplete digestion by the restriction enzyme may, as a result of the generation of partially digested fragments, lead to an overestimation of the overall diversity within a community (Osborn et al., 2000).

T-RFLP analysis is a powerful comparative molecular fingerprinting technique, which is widely used to describe the microbial community structure within different kinds of ecosystems to assess potential temporal and spatial variations, caused by natural and / or engineered interventions. A specific identification of distinct peaks within a complex T-RFLP profile is not directly accomplishable. But in combination with the construction of a clone library and by performing T-RFLP analysis of each clone the phylogenetic information of specific peaks can be revealed and thus identification is possible (e.g. Hackl et al., 2004). Another simpler approach, but with higher error rate is the prediction of theoretical fragment lengths based on the sequences derived from the clone library (manuscript, chapter 5).

Due to the complex matrix of results derived from T-RFLP profiles from different bacterial communities statistical tools like cluster analysis (e.g. Hartmann and Widmer, 2006), principal component analysis and discriminant analysis (Parka, 2006) are very useful for the interpretation of the results.

Within this study the T-RFLP method was applied to detected possible differences between the investigated outdoor and indoor system and between “bulk soil” and “rhizosphere soil”, respectively. Additionally the in- and outflows of the systems where analyzed for a qualitative assessment of the bacterial reduction efficiency of the investigated wastewater treatment systems.

The protocol for analysis of the T-RFLP profiles of the filter body samples, as well as for the in- and outflows samples of the CWs investigated within this study are described in chapter 5.1.2.3.

3.3.3.2 Denaturing Gradient Gel Electrophoresis (DGGE) analysis of the ammonia oxidizing community

DGGE is a profiling technique, which is based on the melting behaviour of DNA fragments of the same length depending on their G+C-content and their nucleotide sequence composition within a chemical gradient of increasing denaturing strength. One of the primers used to amplify the target sequence contains a “GC clamp” (GC-rich sequence attached to the 5`end), which prevents the complete separation of the double stranded DNA during its movement through the denaturing gradient of the gel. The melting radically reduces the mobility of the nucleic acid in the gel. As a consequence of the different sequences of the DNA-fragments they are separated within the gel. Each band of the so originated banding pattern corresponds to a single species; therefore the number of bands can serve as a measure of richness of ribotypes. A clear advantage of the method is that it can be combined with additional analyses. Thus excision of selected bands, followed by sequencing can lead to the phylogenetic affiliation of the ribotypes that make up a band in the gel (e.g. Oros-Sichler et al., 2007).

Due to the complexity of the microbial communities of constructed wetlands, especially in the upper layer, detected with general primers for eubacteria (Truu et al., 2005), more restricted approaches with primers specific for functional genes have been applied within this study. The group of ammonia-oxidizing bacteria (AOB) (see chapter 1.3.1.2) have a coherent phylogeny and defined nutritional requirements and are of profound practical importance in natural and engineered environments (Coskuner et al., 2005).

The enzyme ammonia monooxygenase (AMO) is catalyzing the first step of the oxidation of ammonia to hydroxylamine (see figure 1.4). For a cultivation independent way to assess the diversity within the AOB specific or semi specific PCR primers for the amplification of 16S rDNA (e.g. Voytek and Ward, 1995) or the ammonia monooxygenase structural gene *amoA* (e.g. Rotthauwe et al., 1997) are applied. Rotthauwe et al. (1997) demonstrated that sequence analyses of the *amoA* gene represent a very powerful molecular tool for analyzing indigenous ammonia-oxidizing communities due to its specificity, its fine-scale resolution of closely related populations, and the fact that a functional trait rather than a phylogenetic trait is detected. Rotthauwe et al. (1997) also showed that the topology of an *amoA*-based phylogenetic tree is in good agreement with the topology of the corresponding 16S rDNA tree. Additionally Juretschko et al. (1998) displayed that nucleotide sequence similarities of closely related ammonia oxidizers were significantly lower for the *amoA* genes than for the corresponding 16S rRNA within the activated sludge originated from a nitrification-denitrification basin of an industrial wastewater treatment plant. This indicates a better phylogenetic resolution of the *amoA* approach compared to 16S rDNA analyses to discriminate between different species within an AOB population, but on the other hand the *amoA* sequence contains less phylogenetic information due to its shorter length (the complete 16S-rDNA comprises 1509 bp, whereas the amoA sequence constitutes 453 bp).

Although PCR-DGGE represents a very suitable tool for the investigation of the diversity of microbial communities in soils, some drawbacks are limiting the applicability and operational range of the method:

- Only organisms that occur in a population above a given threshold of about 0.1-1 % of the total target number can be detected (Muyzer et al., 1993).
- Quantification of the targets by measuring the intensity of bands is inherently difficult as a result of the qualitative nature of the (competitive) PCR.
- Co-Migration of different sequences: One band can be the result of different DNA fragments originated from different organisms (Gafan and Spratt, 2005)

- The method reaches its limits in its ability to present the diversity in very complex microbial communities (e.g. soils) by using the 16S rRNA gene as a marker (e.g. Burr et al., 2006).
- The excision of bands (for further analyses) that are very close to each other may be difficult.
- The size of the amplified DNA fragments is limited to about 500 bp, which severely hampers subsequent probe hybridization (e.g. for FISH) and phylogenetic analyses (Wilderer et al., 2002)

A description of the DGGE protocol applied to assess the community structure of the AOB within the filter body of the CWs investigated within this study is given by Tietz et al. (2007a, chapter 4.3.2.5).

3.3.3.3 Construction of a clone library based on the 16S rRNA

Cloning and sequencing of PCR amplified functional genes from environmental samples are powerful tools for investigating the ecology and role of microorganisms within diverse ecosystems (e.g. Wakelin et al., 2008; Niu et al., 2006; Collins et al., 2006). To make an inventory of the species composition the clone library technique is the method of choice, although its high time- and work-expenses. The targeted gene (e.g. the 16S rRNA gene or functional genes) within the extracted community DNA or RNA is amplified with specific primers and ligated into a plasmid (vector). The plasmids are transformed into competent *E. coli* cells and amplified by the proliferation of *E. coli* (see figure 3.10). Afterwards the target sequences within the vectors are again amplified by PCR followed by sequencing for identification of the clones.

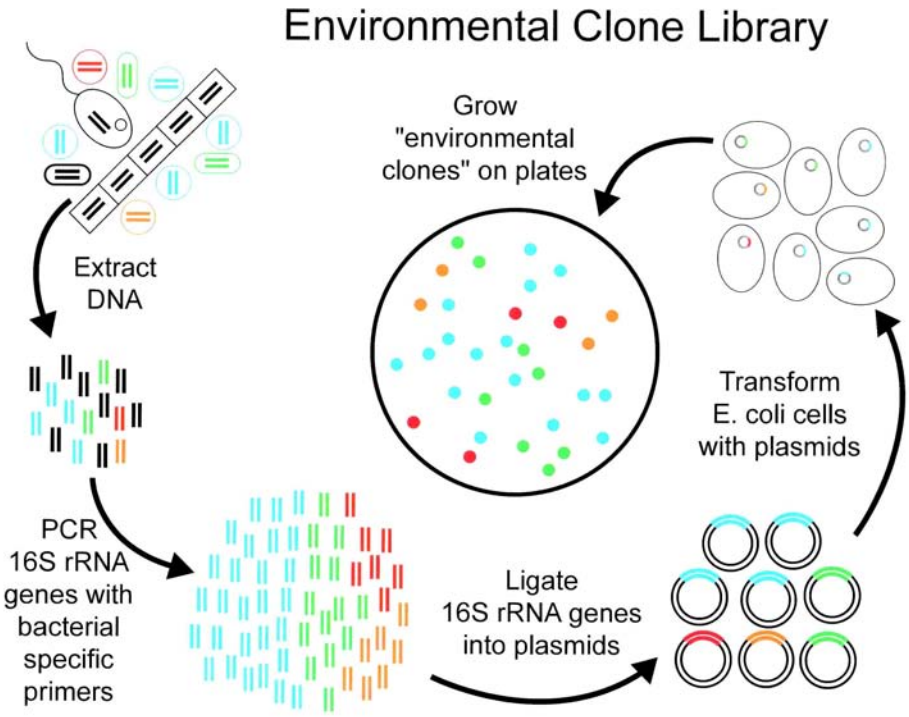


Figure 3.10: Schematic illustration of the construction of a clone library from an environmental sample (UMCES, 05/2008).

Since metabolically active cells usually contain higher numbers of ribosomes than inactive cells, a 16S rRNA library generated from total extracted RNA may be considered to reflect predominantly the diversity of the metabolically active members of the community (Nogales et al., 2001). The identification of the most abundant metabolically active bacteria within the investigated system is one of the first steps in developing optimal in situ bioremediation strategies by providing ideal conditions for the organisms. A second step should be to find out which function these bacteria play in the wastewater degradation process. But assigning physiologies and functions to the hosts of the 16S rRNA gene sequences is complicated in many cases by the lack of characterized closer relatives and by the diversity of phenotypes among closer relatives in some groups (Janssen, 2006). A detailed investigation of the physiological potentials of the bacteria inhabiting the habitat, their requirements, and their exact local appearance and spatial distribution within the system could help to optimize the community composition favouring the organisms which are efficient in decomposing wastewater compounds.

By sequence analysis of 16S rRNA genes the most dominant species inhabiting the system are identified resulting in a more detailed description of the community structure. Analyses of 16S rDNA libraries can reveal the existence of yet undescribed and uncultivated bacteria, which may play important roles in pollutant degradation or other community processes, which are necessary to provide sustainable conditions for the system. But it is important to realize that libraries of PCR-amplified 16S rRNA and 16S rRNA genes may not represent a complete or accurate picture of the bacterial community (Janssen, 2006). Additionally to biases occurring during nucleic acid extraction and PCR (see chapter 3.3.3) the varying number of copies of 16S rRNA or 16S rRNA genes per cells and a possible selection of certain DNA during cloning steps may cause biases in the contribution of the various bacterial groups to the library. Furthermore clone libraries with less than 400 cloned sequences and libraries consisting predominately of sequences less than 300 nucleotides give an incomplete picture of the community composition (Janssen, 2006).

The protocol from the construction of the clone library to the sequencing of the 16S rRNA genes as performed within this study is described in chapters 5.1.2.5-5.1.2.7.

3.3.3.4 Comparison of the DGGE-, the T-RFLP- and the clone library-technique

Burr et al. (2006) promoted a combination of DGGE and 16S rDNA cloning to assess the bacterial diversity, which worked successfully for two moderately complex environmental bacterial communities but was limited in its ability to present the diversity in a soil microbial community, due to its complexity. Also a little reduced resolution of the DGGE method was obvious, by detection of slightly fewer bands on DGGE gels than peaks on T-RFLP traces. All molecular fingerprint techniques reach their limits by displaying the diversity of very complex environmental samples due to a too low resolution. By analyzing distinct functional groups of the community by analyzing functional marker genes (e.g. *amoA* instead of the 16S rRNA gene) this problem can be avoided.

The choice which technique will be applied often is influenced in fact by the expertise and equipment available in the laboratory. Hence, in spite of the fact that the two fingerprint techniques are based on different principles they result in comparable results. For example Nunan et al. (2005) reported that DGGE and T-RFLP analyses led to similar general conclusions. Smalla et al. (2007) applied the DGGE, T-RFLP and SSCP approach to analyze the community diversity of four replicate soil samples, evaluated the results by cluster analysis and concluded that all three methods led to similar results. The construction of a clone library is the only method applied within this study, which provides information about the species composition of the investigated community; but with the big disadvantage that this method is very labour - intensive and therefore the comparison of numerous bacterial communities is very difficult.

Within this study a combination of three different molecular methods was used to investigate the community structure and diversity in this system (Tietz et al., 2007a, see chapter 4.3 and chapter 5 manuscript). The applications of „Denaturing Gradient Gel Electrophoresis“ (DGGE) and „Terminal Restriction Fragment Length Polymorphism“ (T-RFLP) allow the fingerprinting of a community by analyzing the polymorphism of a certain gene. By recovering specific bands from the DGGE – gel this technique enables also further analyses like sequencing (Tietz et al. 2007a). By constructing a clone library based on the 16S rRNA with additional sequencing of the clones a more detailed description of the community structure can be obtained by identifying the most dominant active species inhabiting the system (see chapter 5 manuscript).

4 Publications

4.1 Publication I: Characterization of microbial biocoenosis in vertical subsurface flow constructed wetlands

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Authors: Alexandra Tietz, Alexander Kirschner, Günter Langergraber, Kirsten Sleytr, Raimund Haberl

Abstract

In this study a quantitative description of the microbial biocoenosis in subsurface vertical flow constructed wetlands fed with municipal wastewater was carried out. Three different methods (substrate induced respiration, ATP measurement and fumigation-extraction) were applied to measure the microbial biomass at different depths of planted and unplanted systems. Additionally, bacterial biomass was determined by epifluorescence microscopy and productivity was measured via ^{14}C leucine incorporation into bacterial biomass. All methods showed that >50 % of microbial biomass could be found in the first cm and about 95 % in the first 10 cm of the filter layer. Bacterial biomass in the first 10 cm of the filter body accounted only for 15-23 % of the total microbial biomass within this depth layers. Whether fungi or methodical uncertainties are mainly responsible for the difference between microbial and bacterial biomass remains to be examined. A comparison between the purification performance of planted and unplanted pilot-scale subsurface vertical flow constructed wetlands (PSCWs) showed no significant difference. The microbial biomass in all depths of the filter body was also not different in planted and unplanted systems. Compared with data from soils the microbial biomass in the PSCWs was high, although the specific surface area of the used sandy filter material available for biofilm growth was lower, especially in the beginning of the set-up of the PSCWs, due to missing clay and silt fraction.

Keywords

Bacterial production, microbial biomass, subsurface vertical flow constructed wetlands

4.1.1 Introduction

Wastewater purification in constructed wetlands is a result of the interactions between plants, soil and microorganisms. Until now, constructed wetlands have often been seen as a "black box" and were investigated only with regard to their removal efficiencies for nutrients and hygienically relevant bacteria (e.g. Vacca et al., 2005). Only a few publications attempted to obtain a more detailed description of the microbial biocoenosis within the filter body of constructed wetlands. Some of these papers focused on microbial activity and productivity (e.g. Baptista et al., 2003); others investigated the microbial biomass and biofilm development (e.g. Larsen and Greenway, 2004), and only a very small number of papers have been found that investigated the microbial diversity within constructed wetlands by describing the bacterial community composition (e.g. Truu et al., 2005). Thus, there is a significant lack of information concerning the microbial community, their biomass, productivity

and community diversity in constructed wetlands; but this information is highly needed for a better understanding of the degradation processes in these systems.

In natural wetlands it is established knowledge that the major part of the degradation and transformation processes regarding organic compounds is carried out by bacteria and fungi. Bacteria are dominating these processes in the wet sediment and the water body itself, both under oxic and anoxic conditions (Kirschner and Velimirov, 1999). In soil, and on plant litter in streams, and on dead shoots of marsh plants, on the other hand, the biomass of fungi often exceeds bacterial biomass (Bailey et al., 2002a; Buesing and Gessner, 2006). However, the contribution of bacteria and fungi to degradation of organic matter in natural wetlands is still in debate (Buesing and Gessner, 2006) and there is no consistent knowledge on the situation in constructed wetlands.

In addition, it is a long time discussed topic (e.g. Brix, 1997) whether plants do significantly contribute to the organic matter and nutrient removal in constructed wetlands. It is well known that roots stimulate the microorganisms by the so called “rhizosphere effect” (Stottmeister et al., 2003) but, to our knowledge, there are only few studies available investigating the microbial degradation processes and biocoenosis in planted compared to unplanted wetlands.

The aim of the present study was to get a detailed description of the microbial and bacterial biomass and bacterial productivity in planted and unplanted subsurface vertical flow constructed wetlands. Several methods based on different measuring principles were applied to quantify the microbial biomass within the filter body of constructed wetlands and a method for estimating bacterial productivity in sediments (Buesing and Gessner, 2003) was adopted. In addition, the results shall provide a basis for a future determination of stoichiometric and kinetic factors of CW2D, a model developed to simulate degradation and transformation processes in subsurface flow constructed wetlands (Langergraber and Simunek, 2005).

4.1.2 Material and Methods

4.1.2.1 Description of the investigated constructed wetland (CW) systems

The investigations were carried out at eight indoor pilot-scale subsurface vertical flow constructed wetlands (PSCWs) with a surface area of 1 m^2 each. The 50 cm main filter layer consists of a sandy substrate with a grain size of 0.06-4 mm with an adjacent drainage layer. The PSCW receives four times a day 15 litres of mechanically settled municipal wastewater per day and square meter which results in an organic load of 20 g COD $m^{-2}d^{-1}$ (i.e. a specific surface area of 4 m² per person). Six PSCWs are planted with *Miscanthus sinensis giganteus* whereas the other two are unplanted. Tracer experiments (with potassium chloride) revealed a hydraulic retention time of the systems of 2.5-3 days.

One year after operation start, the aboveground density of the plants was observed for 12 months by monthly counting of the plant stems, and amounted between 40 and 130 stems per m^2. The root density was not analyzed during these investigations. On the surface of the unplanted filters algae growth was observed covering between 5 and 25 % of the surface area. For sampling these regions were excluded. For illumination of each PSCW a 1000 Watt mercury-vapour lamp and an additional UV-lamp specific for plant growth (Osram, Vienna, Austria) were installed.

4.1.2.2 Sampling

Samples were taken from different depths of the filter layer of the two years old planted and unplanted PSCWs. Samples from deeper layers of the filter body were taken by a drill corer (inner diameter: 10 cm), whereas samples from the three upper strata were removed by a syringe cut at the top (inner diameter: 2.5 cm). Filter body samples were taken from depths of 0-1, 1-5, 5-10, 10-20, 20-30, 30-40 and 40-50 cm.

Three sampling campaigns with a complete depth profile were performed for planted and unplanted PSCWs during May to November 2005. Five replicate samples for each of the three top layers (0–10 cm) were collected with a syringe and combined to a composite sample. For the four deepest layers (from 10 to 50 cm) the samples originated from one big core. On three additional occasions, samples were taken from the three uppermost layers of planted and unplanted PSCWs because preliminary results have revealed that the main microbial activity is located within the top 10 cm. The sampling locations were chosen randomly and carefully marked to avoid a second sampling at the same point. The samples were analyzed immediately for bacterial production and stored at 4 °C for analysis of microbial biomass and TOC for maximally 10 days. Samples for microscopic direct counts were preserved in formaldehyde (4 % final conc. in phosphate buffered saline) and stored at 4 °C (max. for four weeks) until further analyses w ere conducted. Representative samples for cell volume estimation were stored no longer than one week.

4.1.2.3 Chemical and microbial analyses of wastewater samples

Influent and effluent samples were taken monthly for analysis of organic matter (BOD_5, COD, TOC), total suspended solids (TSS), ammonia- (NH_4-N), nitrate-nitrogen (NO_3-N) and total phosphorus (TP) according to the German standard methods (German Standard Methods for the Examination of Water, Wastewater and Sludge, 1993). Furthermore, heterotrophic plate counts (HPC), *Escherichia coli (E. coli)*, total Coliforms (TC) and Enterococci (EC) were determined. The number of heterotrophic bacteria was determined by the pour plate method with yeast extract agar (ISO 6222, 1999). TC and *E. coli* were enumerated by the membrane filtration method by using Chromocult agar (Alonso et al., 1998). For Enterococci determination the membrane filters were incubated on Slanetz and Bartley agar (ISO 7899-2, 2000). All culture media were purchased from Merck (Darmstadt, Germany). For the determination of total bacterial cells in wastewater, 40 ml of water samples were preserved immediately with 0.2 µm filtered formaldehyde (5 % final concentration).

4.1.2.4 Chemical and physical measurements in the PSCW filter layer

Total organic carbon (TOC) and total organic nitrogen (TON) content of the substrate samples were measured by combustion at 900 °C with the C/N-analyzer Vario Max (Elementar; Hanau, Germany). Water content was measured online in situ by a soil moisture profiling probe (EnviroSCAN; Sentek, Stepney, Australia). The pH-value was measured with a pH-sensor after a 24-hour extraction of 10 g of filter body material with 25 ml of 0.01 M $CaCl_2$. Dissolved oxygen (DO) and temperature were measured online and in situ using a multi-channel instrument with 4 sensors (EC-sensor-OM-E200302 3600; Orbisphere; Vesenaz-Geneva, Switzerland).

4.1.2.5 Bacterial abundance and biomass determined by microscopic direct counts and measurement of cell volume

The filter material-cell aggregates of the fixed samples were chemically disintegrated by a 0.1 M sodium pyrophosphate solution and mechanically destroyed by an ultra sonication bath (Branson 5510; 135 W, 42 KHZ; 45 min). The samples were diluted with particle-free (< 0.2 µm) filtered water according to their bacterial concentrations and stained with the fluorescent dye SYBR Green I (10000 x dilution in DMSO; Molecular Probes; Leiden, NL). After incubation for ten minutes they were filtered on a 0.2 µm aluminium oxide filter (Anodisc; Whatman; Middlesex, UK) following the protocol of Weinbauer et al. (1998). The cells were enumerated by using an epifluorescence microscope (Zeiss Axioplan; Göttingen, Germany) with an excitation wavelength of 450-490 nm and an emission of 520 nm. From two filters per sample 30 randomly chosen microscopic fields with 10-20 bacteria were counted. Additionally, the length (l; [µm]) and width (w; [µm]) of 600 cells were determined for representative samples by the use of an AxioCam MRc digital camera with the corresponding AxioVision software (Zeiss; Göttingen, Germany). The volume of the cells (V; [μm^3]) was calculated from the equation given by Battin et al. (2001):

$$V = (w^2 \times \pi / 4) \times (l - w) + (\pi \times w^3 / 6)$$

By applying the allometric model describing the relationship between cell volume and cell carbon content from Norland (1993) we calculated the bacterial biomass C (C; [fgC]):

$$C = 120 \times V^{0.72}$$

Total bacterial cell counts in the preserved water samples were determined by the use of the fluorescent nucleic acid dye DAPI according to the protocol of Porter and Feig (1980).

4.1.2.6 Bacterial secondary production

For the estimation of bacterial secondary production the incorporation rate of ^{14}C-leucine was measured according to a modified protocol after Buesing and Gessner (2003). Preliminary tests showed that a leucine concentration of 40 µM (ARC 656A, ARC; St. Louis, MO; specific activity 325 mCi $mmol^{-1}$; diluted 32 x with non-radioactive leucine) was necessary to reach substrate-saturated conditions during incubation. Briefly, four replicate samples, consisting of 1 g fresh weight sediment, and one blank were incubated at in situ temperature for 1 hour. The incubation was stopped with trichloracetic acid (5 % final conc.) followed by several washing steps to remove leucine not incorporated in the protein fraction. This resulted in blank values always less than 0.1 % of the sample values. After an alkaline extraction of the proteins in NaOH, EDTA and SDS in a boiling water bath for 1 h and incubation over night at room temperature, an aliquot was measured for radioactivity with a liquid scintillation counter (Canberra Packard SC 1900TR). Leucine incorporation rates were converted to carbon production using the theoretical conversion factor of 1.55 kg C mol $leucine^{-1}$ recommended by Simon and Azam (1989) and an empirically derived conversion factor of 0.104 kg C mol $leucine^{-1}$ (Michel and Bloem, 1993). In both cases it was assumed that no intracellular isotope dilution occurred.

4.1.2.7 Fumigation-extraction for biomasses C and -N

As an alternative approach for the determination of microbial biomass C the fumigation-extraction method was performed after a modified protocol of the method described by Sparling and West (1988). 25 g of the filter body samples were fumigated with ethanol-free chloroform for 24 hours in a desiccator. After removal of the fumigant by evacuation of the desiccator the organic C was extracted from three fumigated and from two non-fumigated samples with 100 ml 0.5 M K_2SO_4 for 30 min. The extracts were filtered through 0.45 µm nitrocellulose filters (Millipore) and analyzed for total organic carbon by a Dohrmann Phoenix 8000 UV Persulfate TOC Analyzer. The filter body microbial biomass C was calculated by division of the difference between the TOC concentrations of fumigated and non-fumigated filter body extracts by a k_{EC}-value of 0.45 (Wu et al., 1990). For biomass N the extracts were analyzed for total Kjeldahl nitrogen (TKN). The differences between the TKN concentrations of fumigated and non-fumigated samples were divided by a k_{EN}-value of 0.54 (Brookes et al., 1985).

4.1.2.8 ATP measurements

ATP, ADP and AMP were measured after an extraction of the adenylates from the filter body samples with DMSO. A phosphate buffer (10 mM Na_3PO_4 with EDTA; pH = 12) was added for further dispersion. After a 1:10 dilution in a 0.5 M KH_2PO_4 buffer the extracts were filtered through 0.45 µm nitrocellulose filters (Millipore). During one hour incubation at 85 °C the adenine nucleotides reacted with chloracetaldehyde to form their corresponding etheno-derivatives, which were quantified by a high performance liquid chromatograph (HP 1090 HPLC, Agilent chromatograph) equipped with a fluorescent detector (Agilent 1100 Series FLD) according to the protocol given by Bai et al. (1989).

Preliminary experiments showed that the extraction efficiency for AMP and ADP was not as good as for ATP and due to these results in the following only ATP was determined. The ATP values were converted into biomass C after the relationship given by Dyckmans et al. (2003):

$$1 \text{ nmol ATP} = 135 \text{ µg microbial biomass C}$$

4.1.2.9 Substrate induced respiration

Biomass C was also measured by substrate induced respiration (SIR) according to Anderson and Domsch (1978) using the Isermeyer method for determining the CO_2 evolution (Isermeyer, 1952). Preliminary tests showed that 2 mg glucose per g filter material were necessary to reach a maximal initial respiration rate. 50 g of filter material (2 replicates) were put into nylon socks after an one-hour pre-incubation after the addition of a mixture of glucose and sea-sand (1:5). The socks were fixed to the edge of 250 ml glass bottles containing 20 ml of 0.1 M NaOH. After incubation for 4 h the CO_2 trapped in the NaOH was determined by titration with 0.1 M HCl. The results were converted into biomass C based on the relationship between CO_2 production and microbial biomass given by Anderson and Domsch (1978):

$$1 \text{ µg microbial biomass C g soil}^{-1} = (\text{µl } CO_2 \text{ g soil}^{-1} \text{ h}^{-1}) \times 40.04 + 0.37$$

4.1.2.10 Data analysis

All data were related to g of DW (dry weight) soil (filter material). Spearman-Rank correlation was performed to test for the relationship between the investigated variables. Potential differences between planted and unplanted PSCWs were tested by the non-parametric Mann-Whitney U-test. The different depths of the filter body were compared by the non-parametric Kruskal-Wallis test. Statistical significance was assumed at a probability level of $p < 0.05$. All statistical analyses were made with the software package SPSS 11.0 for Mac (SPSS Inc.; Chicago, Illinois, USA).

4.1.3 Results

4.1.3.1 Chemical and physical characteristics of the PSCW filter layer

The temperature was rather constant over the year due to the stable indoor conditions (table 4.1). Between the hydraulic loadings, oxygen diffuses into the filter material and provides optimal conditions for oxygen consuming processes like nitrification down to the deepest layers. TOC accumulates especially in the 0-1 cm layer due to the high amount of biomass in this layer and because of the deposition of suspended solids from wastewater.

Table 4.1: Maximum, minimum and mean values of chemical and physical characteristics of the filter layer material of the indoor pilot-scale subsurface vertical flow constructed wetlands (PSCWs).

Method	Max.	Min.	Mean value
pH (-)	7.52	5.97	6.69
Temperature (℃)	25.8	17.9	20.9
DO (mg L^{-1})	9.4	2.5	8.5 (between loadings) 4.1 (during loading)
TOC (mg g DW $soil^{-1}$)	20.0	0.2	10.1 (in 0-1cm depth) 2.7 (in 40-50cm depth)
TON (mg g DW $soil^{-1}$)	2.4	<0.01	1.2 (in 0-1cm depth) 0.1 (in 40-50cm depth)
C/N ratio	87	3.6	15
Water content (% of water sat.)	100	20	80 - 90 (in 0-1cm depth during loading) 20 - 30 (in 0-1cm depth between loadings) 55 - 65 (in 40-50 cm depth)

4.1.3.2 Wastewater analysis

Results of the chemical and microbial wastewater analyses are shown in table 4.2. The removal efficiencies are high as typically observed for vertical flow constructed wetlands (e.g. Weedon, 2003) and did not vary much over time except for phosphorus removal. The removal rate for TP decreased from 47 % in the first year to 30 % in the second year and to 0 % in the third year due to the diminished adsorption capacity of the filter material. There were no significant differences in the removal efficiency of planted and unplanted systems ($p > 0.05$, $n = 14$-100) for all investigated variables.

Table 4.2: Medians and minimum and maximum values (in brackets) of the chemical and bacteriological wastewater characteristics measured in the in- and effluent of planted PSCWs and calculated removal efficiencies.

Methods	Influent		Effluent		Removal
Chemical	(mg L^{-1})	n	(mg L^{-1})	n	(%)
COD	415 (141–1270)	46	< 20*	24	> 95
BOD_5	133 (41 – 320)	24	< 3*	24	> 98
TSS	100 (58 – 430)	24	1.7 (1.0 – 15)	95	98
TOC	144 (51 – 272)	24	4.8 (2.8 – 8.1)	100	97
NH_4-N	43 (19 – 63)	24	< 0.03*	100	99
NO_3-N	< 0.1*	24	43.4 (16 – 70.2)	94	-
TP	6.6 (3.2 – 8.1)	24	3.7 (0.5 – 7.6)	93	0 - 47
Bacteriological	log CFU ml^{-1}	n	log CFU ml^{-1}	n	log removal
HPC	6.0 (5.7 – 6.5)	22	3.2 (2.2 – 4.1)	22	2.8
E. coli	6.0 (5.3 – 6.9)	22	2.5 (0.6 – 4.2)	22	3.5
Total Coliforms	6.8 (5.6 – 7.2)	22	3.1 (1.0 – 4.5)	22	4.3
Enterococci	6.1 (5.6 – 7.0)	22	2.2 (0.3 – 3.9)	22	4.8
Total bacterial counts	log cells ml^{-1}	n	log cells ml^{-1}	n	log removal
MDC	8.0 (7.9 – 8.2)	12	6.0 (5.8 – 6.2)	12	2.0

* limit of detection
HPC = Heterotrophic plate count
MDC = Microscopic direct counts

4.1.3.3 Bacterial biomass and production in the filter

The measurements of the width and length of 600 cells resulted in an average cell volume of 0.13 μm^3 corresponding to an average bacterial carbon content of 28 fg C per cell. As shown in figure 4.1 the bacterial biomass decreased very rapidly with the depth of the filter body and reached its lowest values in 30-40 cm. The bacterial biomass C accounted for 2.5 % of TOC in the top layer and for 0.5 % in deeper layers.

The bacterial production rates showed a weaker decrease within the first 10 cm than bacterial biomass and a more pronounced reduction within the 10-20 cm depth layer. Below 20 cm the values remained rather constant. Leucine incorporation rates were transformed into bacterial carbon with two different conversion factors (figure 4.1), which resulted in values with a large range reaching from 0.18 µg to 52 µg C g DW $soil^{-1}$ h^{-1}. The values for bacterial biomass and production were significantly different between the different depths ($p < 0.05$, n = 6-13).

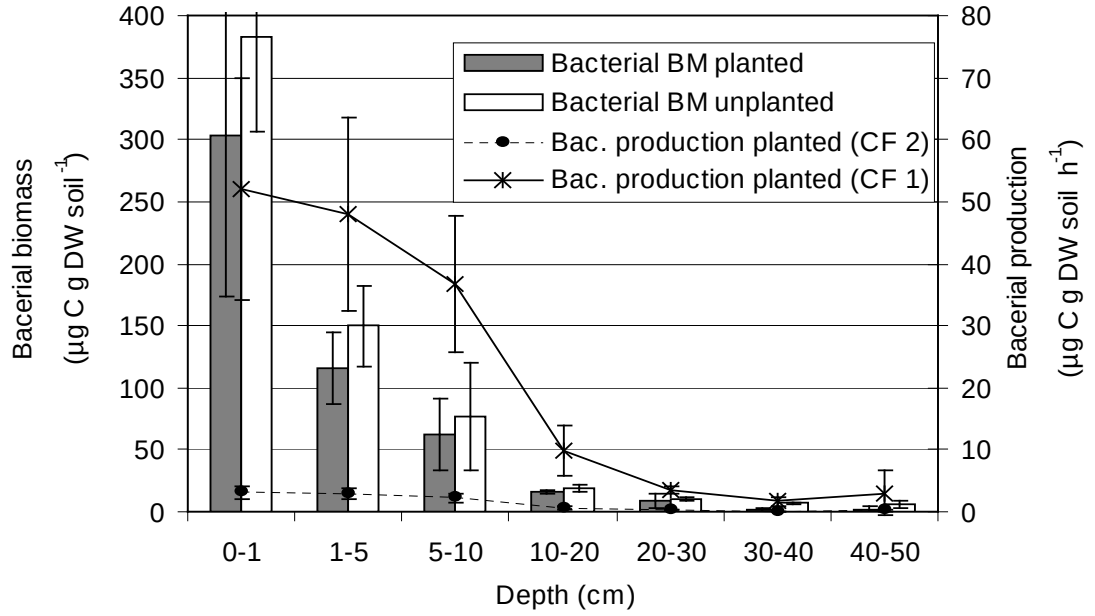

Figure 4.1: Comparison of bacterial biomass determined by microscopic direct counts and volume measurements in planted and unplanted PSCWs and bacterial secondary production rates measured by incorporation of ^{14}C-labeled leucine in planted PSCWs calculated with a maximal (CF 1; Simon and Azam, 1989) and minimal conversion factor (CF 2; Michel and Bloem (1993). (Means ± standard deviations of six replications for 0-10 cm and of three replications for 10-50 cm).

4.1.3.4 Microbial biomass in the filter body of the PSCWs

The three different methods (ATP, SIR and fumigation-extraction) used to quantify the microbial biomass resulted in values of similar range.

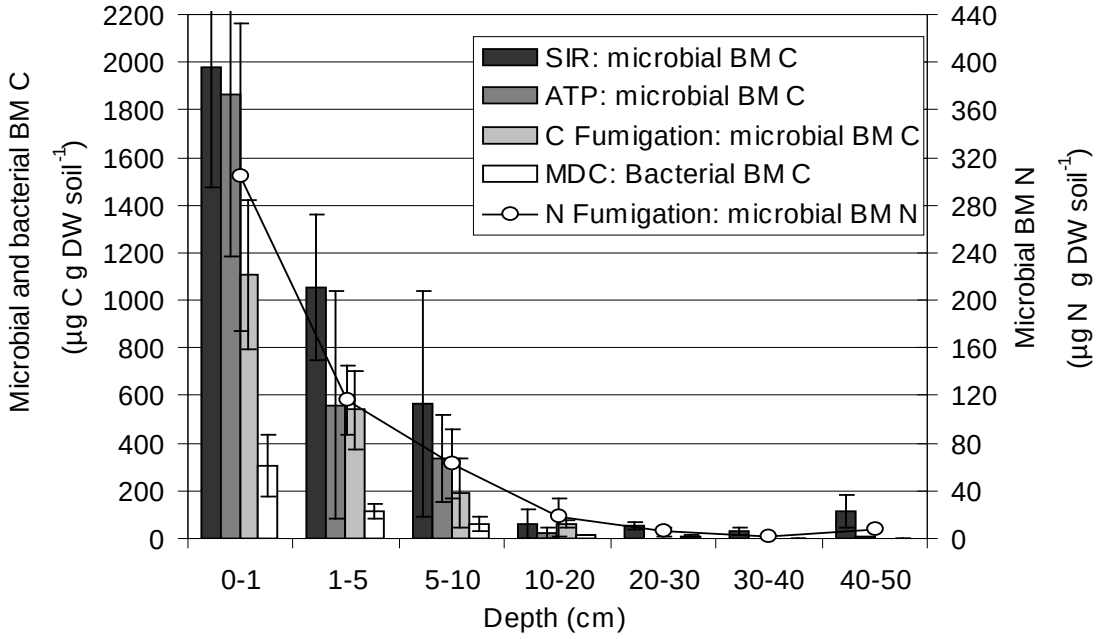

Figure 4.2: Comparison of different methods used to characterise the microbial biomass C, -N and the bacterial biomass C determined via microscopic direct counts (MDC) and volume measurements of the cells in planted PSCWs. (Means ± standard deviations of six replications for 0-10 cm and of three replications for 10-50 cm).

The average range of the contribution of bacterial biomass C to microbial biomass C was between 15 and 23 % in the top 10 cm of the filter body and increased in deeper layers (table 4.3). The proportion of microbial biomass C on TOC varied between 4.8 in deeper layers and 15.4 % in the top 5 cm layer. Microbial biomass N decreased from 300 µg g DW soil^{-1} in the top layer to 18 µg g DW soil^{-1} at 20 cm depth. Below this depth, microbial biomass N was mostly below detection limit. The average of the calculated C/N ratio was 5.3. Significant correlation coefficients ($r > 0.84$; $p < 0.001$, $n = 26\text{-}51$) were found between the different methods to describe biomass and activity of the microbial community (table 4.4). The microbial biomass at different depths was significantly different ($p < 0.05$, $n = 15\text{-}40$).

Table 4.3: Distribution of total microbial biomass (MB) referred to 1 g of DW soil of planted and unplanted PSCWs (calculated from the mean values of all three methods used to characterise the MB) and contribution of bacterial biomass (BB) to microbial biomass over the depth.

Depth	Total microbial biomass		Contribution of BB to MB	
	Planted	Unplanted	Planted	Unplanted
(cm)	(%)	(%)	(%)	(%)
0-1	53.5	59.9	19.7	17.8
1-5	27.2	24.0	14.8	21.3
5-10	15.0	11.0	14.5	22.6
10-20	2.2	2.5	25.3	26.2
20-30	0.7	1.3	45.0	26.4
30-40	0.4	0.4	15.2	58.5
40-50	1.0	0.9	7.4	18.0

4.1.3.5 Comparison of the total microbial and bacterial biomass in planted and unplanted PSCWs

A comparison between the total microbial biomass in planted and unplanted PSCW showed no statistically significant difference in all depth layers for all applied methods ($p > 0.05$, $n = 146$). Also for bacterial biomass and production no significant differences between planted and unplanted systems were detected. The slightly higher mean value for bacterial biomass within the first 10 cm of the unplanted PSCW apparent in figure 4.3 was also not significantly different ($p > 0.05$, $n = 6$).

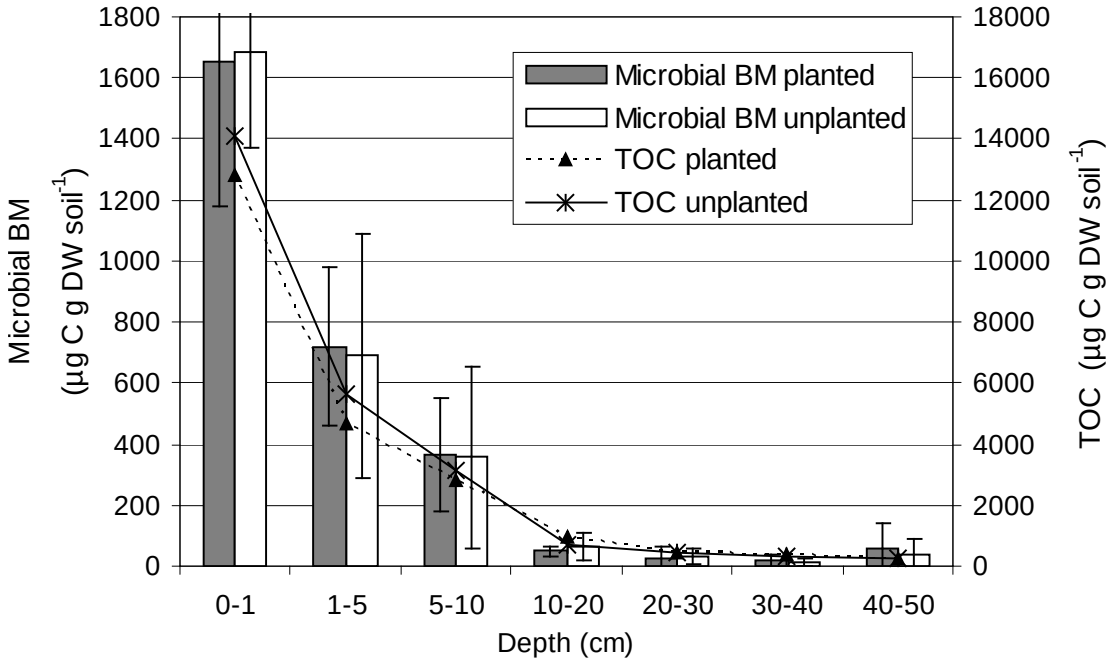

Figure 4.3: TOC and microbial biomass C in different depths of planted and unplanted PSCWs. (Means ± standard deviations of six replications from microbial biomass determinations by fumigation extraction, SIR and ATP for 0-10 cm and of three replications for 10-50 cm).

4.1.4 Discussion

4.1.4.1 Comparison of different methods used to characterise the microbial biomass

Results from the different methods applied to quantify the microbial and bacterial biomass and production showed similar patterns over depth with high biomass values / activities in the upper 10 cm and decreasing values with depth (figure 4.1-4.3). High correlation coefficients were observed between all methods used to characterise the microbial biomass (table 4.4). The good correlation between TOC and the other methods shows the good indicator function for microbial biomass of this easy to measure method.

Table 4.4: Spearman rank correlation coefficients of the different methods used to characterise the microbial biomass and productivity (FE-C/-N = fumigation extraction, MDC = microscopic direct counts, SIR = substrate induced respiration, LEU = leucine incorporation, TOC = total organic carbon). All correlations were highly significant at a probability level of p < 0.001. (In the brackets the number of measurements used for the correlation is given.)

Method	FE-C	FE-N	MDC	LEU	SIR	TOC
ATP	0.94 (33)	0.97 (30)	0.95 (39)	0.92 (39)	0.92 (39)	0.91 (32)
FE-C		0.94 (39)	0.94 (33)	0.96 (33)	0.89 (33)	0.88 (26)
FE-N			0.96 (30)	0.88 (30)	0.86 (30)	0.95 (26)
MDC				0.87 (51)	0.90 (39)	0.91 (33)
LEU					0.88 (39)	0.84 (32)
SIR						0.87 (35)

We observed a big discrepancy between microbial and bacterial biomass, indicating that fungal biomass may contribute to a large extent to the total microbial biomass in the PSCWs.

An overestimation of microbial biomass due to not suitable conversion factors for the transformation of the results from the three different methods applied to measure the biomass into microbial carbon can be an alternative explanation for the big discrepancy between microbial and bacterial biomass. In addition, an underestimation of the bacterial biomass due to methodical problems as an incomplete desorption of the bacteria from filter material particles or small cell volume estimation is possible. A significant contribution of algal biomass, especially in the deeper layers can be ruled out due to the absence of light in the filter body and also the biomass of protozoa can be assumed to be very low (Wieltschnig et al., 2003).

Calculation of bacterial production with the commonly used conversion factor (determined for sea water) reported by Simon and Azam (1989), which was confirmed by Buesing and Marxsen (2005) for freshwater sediments resulted in theoretical doubling times of the bacterial community below one hour for the deeper layers of the filter body, which seem to be unrealistically low. However, the values for the upper layers were with a magnitude of 2–5 hours in the typical range of active bacterial communities. The use of the conversion factor (CF) determined by Michel and Bloem (1993) for soil bacteria resulted in theoretical doubling times ranging from 17-97 h, which can be regarded as rather high. This CF was performed with selected cultured bacterial strains and does not reflect the natural ecological situation. Thus the set-up of empirical conversion factor experiments is necessary to determine the appropriate conversion factors for the investigated constructed wetlands in order to assess the magnitude of the carbon flux through the bacterial community in these systems.

4.1.4.2 Distribution of the microbial biomass in the filter body

The main part of the microbial biomass and activity was accumulated in the first 10 cm of the filter layer. Table 4.3 shows the distribution of the microbial biomass in the planted and unplanted PSCW. The proportion of bacterial biomass to microbial biomass increased markedly below a depth of 20 cm indicating that bacteria become relatively more important in the deeper strata of the PSCW. Mainly as a result of the better oxygen and nutrient supply the biomass concentrates in the first centimeters. These results are in agreement with Ragusa et al. (2004) who found a similar decrease for proteins, EPS (extrapolymeric substances), bacteria and TOC within the first 5 cm of a constructed wetland microcosm. Thus we can assume that also the majority of the microbial degradation processes take place only in the uppermost centimeters.

4.1.4.3 Influence of plants on the microbial biomass

Roots and rhizomes in constructed wetlands have been found to stimulate bacteria by the release of oxygen and root exudates (Stottmeister et al., 2003). Münch et al. (2005) reported that this rhizosphere-effect of *Phragmites australis* in a constructed wetland stimulates bacteria up to a distance of 50 mm from the root surface. For the PSCWs the density of the plants varied from 40 to 130 stems per square-meter. In contrast to a comparable outdoor wetland these plant densities are rather low. This fact could be an explanation why no difference in the amount of microbial biomass and production between planted and unplanted PSCWs was detected. However our results are in agreement with Larsen and Greenway (2004) who also found no difference between a planted and an unplanted gravel bed considering EPS. For a detailed assessment of the rhizosphere-effect a determination of the root- and rhizomes-density as well as measurements of the oxygen release and analysis of the plant specific root exudates would be necessary.

4.1.4.4 Microbial biomass in constructed wetlands in comparison to soils

For the chosen sandy filter material the immobilisation surface area for biofilm growth was low in the beginning of the PSCW set-up, due to the missing clay and silt fraction within the filter body, which is crucial for biofilm development. However, after two years of operating the system, an accumulation of fine material in the top layer of the filter body occurred and due to the good nutrient and oxygen supply the microbial biomass was quite high compared with other soils due to the accumulation of fine material in the top layer of the filter body and because of the good nutrient and oxygen supply (table 4.5).

Table 4.5: Comparison of the results for microbial biomass and production from this study with other studies investigating soils.

Method (Unit)	**Sample type** This study [I]	Soil
Substrate induced respiration ($\mu l\ CO_2\ g\ soil^{-1}\ h^{-1}$)	3.1 – 49.4	5.3 [1)] – 70.1 [2)]
Microscopic direct counts (10^8 cells $g\ soil^{-1}$)	1.2 – 190	0.01 [3)]- 105 [4)]
Leucine incorporation ($nmol\ Leucine\ ml\ soil^{-1}\ h^{-1}$)	0.28 – 22.3 [II] 0.05 - 3.87 [III]	0.02 [5)] – 1.5 [5)]
C Fumigation-extraction ($\mu g\ C\ g\ soil^{-1}$)	<6 [IV] – 2042	61 [6)] – ~1484 [7)]
N Fumigation-extraction ($\mu g\ N\ g\ soil^{-1}$)	<6 [IV] - 422	3 [6)] – 346 [8)]
ATP ($nmol\ ATP\ g\ soil^{-1}$)	<0.05 [V] – 13.8	0.15 [9)] – 32.3 [10)]
C/N ratio of the biomass (-)	<3.1 [VI] - 11.2	4.2 [6)] – 1250 [11)]

[1)] Barajas-Aceves (2005); [2)] Bailey et al. (2002a); [3)] Kepner and Pratt (1994); [4)] Torsvik et al. (1990) [5)] Uhlirova and Santruckova (2003); [6)] Sparling and West (1989); [7)] Sparling and West (1988); [8)]Turner et al. (2001); [9)] Prevost et al. (1991); [10)] Dyckmans et al. (2003); [11)] Joergensen and Raubuch (2003).

[I] Maximum and Minimum values from planted and unplanted systems.
[II] Conversion factor = CF by Simon and Azam 1989.
[III] CF by Michel and Bloem 1993.
[IV] Detection limits: 6 µg C / N $g\ soil^{-1}$soil for C- / N-fumigation.
[V] Detection limit of 0.05 nmol $g\ soil^{-1}$.
[VI] This value was sometimes below the molar C/N ratio of proteins (3.1) which is not plausible. The low C/N ratio compared with other soils is a result of the high biomass N values, which are most probably overestimated due to methodical bias because of the high nitrogen load of the wastewater applied on the investigated filter material.

Thus the grain size of 0.06–4 mm seems to be a good compromise between providing optimal conditions for microbial growth and adsorption of wastewater compounds on one side and maintaining ideal hydraulic conditions to prevent clogging of the filter system on the other side.

4.1.5 Conclusions

The detailed characterization of the microbial biomass in vertical flow constructed wetlands revealed high values for microbial biomass in the top 10 cm of the filter body due to the high nutrient content and the good oxygen supply. In this study we could observe no significant differences in the quantity of the microbial biomass and the general purification performance between planted and unplanted vertical flow constructed wetlands. We demonstrated that the microbial biomass is quite high compared with natural soils. The results of our study also indicate that there is still a need to further adapt common methods from soil and aquatic microbial ecology to the specific conditions of subsurface flow constructed wetland, e.g. by determining specific conversion factors for the calculation of bacterial carbon production and microbial biomass. A description of the microbial biocoenosis under varying operation conditions is required to understand the reactions of the system to changing environmental conditions. It will not only be necessary to analyse the bacterial diversity but also to examine their physiological activity at the same time. A fundamental understanding of the system will finally help us to improve the performance of constructed wetlands by providing a scientific basis to enable it to find the optimal design and the way of operating the system.

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4.2 Publication II: Bacterial carbon utilization in vertical subsurface flow constructed wetlands

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Abstract

Subsurface vertical flow constructed wetlands with intermittent loading are considered as state of the art and can comply with stringent effluent requirements. It is usually assumed that microbial activity in the filter body of constructed wetlands, responsible for the removal of carbon and nitrogen, relies mainly on bacterially mediated transformations. However, little quantitative information is available on the distribution of bacterial biomass and production in the “black-box” constructed wetland. The spatial distribution of bacterial carbon utilization, based on bacterial ^{14}C-leucine incorporation measurements, was investigated for the filter body of planted and unplanted indoor pilot scale constructed wetlands as well as for a planted outdoor constructed wetland. A simple mass balance approach was applied to explain the bacterially catalyzed organic matter degradation in this system by comparing estimated bacterial carbon utilization rates with simultaneously measured carbon reduction values. The pilot scale constructed wetlands proved to be a suitable model system for investigating microbial carbon utilization in constructed wetlands. Under an ideal operating mode, the bulk of bacterial productivity occurred within the first 10 cm of the filter body. Plants seemed to have no significant influence on productivity and biomass of bacteria as well as on wastewater TOC removal.

Keywords

Leucine incorporation, phospholipid fatty acids, organic matter degradation, bacteria, fungi, vertical flow constructed wetlands

4.2.1 Introduction

The use of constructed wetlands (CWs) for treating different kinds of wastewater is popular and has been well documented during the last thirty years (e.g. Kadlec et al., 2000, Haberl et al., 2003). In particular, subsurface vertical flow CWs with intermittent loading are considered as state of the art and achieve treatment efficiencies comparable to conventional wastewater treatment plants regarding the removal of organic matter, total suspended solids, ammonium and fecal indicator bacteria. All types of constructed wetlands combine characteristics of both natural wetlands and artificial systems, as well as they are hybrids between terrestrial and aquatic ecosystems. They have characteristics of a natural wetland like the typical helophytic vegetation and regular flooding. Due to the missing clay and silt fraction, the grain size distribution of subsurface flow CWs is not comparable to a natural wetland and additionally it is an engineered system with a controlled water regime which brings extremely high loads of organic and inorganic nutrients into the system. Moreover, municipal wastewater can contain a multitude of persistent organic compounds derived from domestic applications, such as active ingredients from pharmaceuticals and personal care products. If the system is

operated in an intermittent loading mode the filter body is well aerated so that no large anoxic zones occur.

The purification performance of this nature-oriented technique is a result of the interaction between microbes, plants and the sandy filter material itself. The transformation and mineralization of degradable organic pollutants is mainly performed by microorganisms. Optimization of nutrient removal processes thus requires a more detailed understanding of microbial processes in constructed wetlands in order to gain a scientific basis for the design and operation of these kinds of wastewater treatment facilities (Flood et al., 1999). A sizeable fraction of organic matter which is degraded and assimilated by microbes in a nutrient rich environment is utilized for cellular growth. Consequently, the microbial secondary production constitutes a significant route of carbon and nutrient fluxes in ecosystems (Buesing and Gessner, 2006). Bacteria play a key role in organic carbon processing in river sediments (Fischer and Pusch, 1999) and natural wetlands (Murray and Hodson, 1985). Beside heterotrophic bacteria, also fungi are widely recognized as crucial mediators of carbon, nutrient and energy flow in ecosystems, especially in river sediments and soils, while in wetlands they dominate only on standing dead shoots of emergent plants (Buesing and Gessner, 2006). In CWs, only little information is available on the importance of the different microbial groups taking part in organic matter degradation, but it is usually assumed that these wastewater treatment systems rely heavily on bacterially mediated transformations for the removal of carbon and nitrogen (Flood et al., 1999). Despite of the high relevance of bacteria, the distribution and quantification of bacterial biomass and production in constructed wetlands has not been investigated sufficiently. Up to now, to the best of our knowledge, only few papers describing the microbial biomass in subsurface flow CWs (e.g. Larsen and Greenway, 2004) and two papers dealing with bacterial production in this system (Baptista et al., 2003; Tietz et al., 2007, *see chapter 4.1*) have been published, but a detailed estimate of bacterial organic matter degradation performance has not been provided in any of these publications.

In this study we present for the first time a quantitative calculation on bacterial carbon utilization, based on bacterial ^{14}C-leucine incorporation measurements, a common and well established technique for the determination of bacterial heterotrophic production, which has been applied for a variety of different ecosystems (Kirchman et al., 1985, Kirschner and Velimirov, 1999; Pulido-Villena and Riche, 2003; Uhlirova and Santruckova, 2003). For this reason this technique was chosen to investigate the spatial distribution of the bacterial production in the sandy filter body of subsurface vertical flow CWs. Data were derived from planted and unplanted indoor pilot scale constructed wetlands (PSCWs) and from an outdoor CW with comparable design and operation. A simple model was developed to explain the bacterially catalyzed organic matter degradation in this system by comparison of the estimated bacterial carbon utilization rates with simultaneously measured carbon reduction values.

4.2.2 Material and Methods

4.2.2.1 Description of the investigated CW systems

The main investigations were carried out at eight indoor PSCWs with a surface area of 1 m^2 each. The sampling campaigns were distributed over a period from 1.5 to 3.5 years after operation start of the plant. The 50 cm main filter layer consists of a sandy substrate with a grain size of 0.06-4 mm (d_{10} = 0.2 mm) with an adjacent 10 cm layer underneath with a grain size of 4–8 mm preventing fine particles being washed out into the drainage layer (15 cm thickness; grain size 16–32 mm). Each PSCW received 15 L of mechanically settled municipal wastewater four times per day (hydraulic loading rate of 60 L m^{-2} d^{-1}) which resulted in an organic load of approximately 20 g COD m^{-2} d^{-1}, corresponding to a specific

surface area of 4 m² per person. The wastewater was distributed equally over the surface area using a sprinkler system that was constructed to ensure a homogenous distribution even for coarser sandy substrate. Due to the intermittent loading mode, oxygen concentrations in all depths were always above 2.5 mg L^{-1} with an average of 4.1 during loading and 8.5 mg L^{-1} between loadings (Tietz et al., 2007b, *see chapter 4.1*). Six PSCWs were planted with *Miscanthus sinensis giganteus* growing under a 1000 W mercury-vapour lamp and an additional UV-lamp specific for plant growth (Osram, Vienna, Austria) whereas the other two were unplanted. Tracer experiments (with potassium chloride) revealed a hydraulic retention time of the systems of 2.5-3 days. A more detailed description of the PSCWs is given by Tietz et al. (2007b, *see chapter 4.1*).

To evaluate the results derived from the PSCWs, samples were taken from an experimental full-scale outdoor subsurface vertical flow CW located in Ernsthofen (Lower Austria). The CW was two years old at the beginning of the sampling campaigns and the samples were taken over a period of one year. The CW has the same design and configurations as the PSCWs except that it is planted with *Phragmites australis.* The wetland consists of three beds with a surface area of 18 m^2 each. The vertical flow beds are loaded four times a day with mechanically settled municipal wastewater. The wastewater was distributed via a network of parallel pipes (diameter 5 cm) with an opening every 75cm (representing a standard distribution system). This enabled a nearly equal wastewater distribution over the surface of the filter body. The hydraulic loading rate of the investigated filter bed 2 was 43 L m^{-2} d^{-1}. The organic load applied was 27 g COD m^{-2} d^{-1}, corresponding to a specific surface area of 3 m² per person. A more detailed description of this system is given by Langergraber et al. (2007).

4.2.2.2 Sampling

Vertical profiles

For depth profiles, samples were taken from seven different depths (0-1, 1-5, 5-10, 10-20, 20-30, 30-40 and 40-50 cm) of all CWs. PSCW samples from deeper layers (10 to 50 cm) originated from one big core taken at each sampling occasion, using a drill corer (inner diameter: 10 cm). Quadruplicate samples from the three upper strata (0–10 cm) were removed by a syringe cut at the top (inner diameter: 2.5 cm). The four samples were combined to one composite sample for each depth layer. For the outdoor CW, triplicate samples from all depths were taken by a drill corer (inner diameter: 10 cm) and combined to composite samples. All PSCW samples were taken at a distance of 20 cm from the center of the filter body and in 20 cm distance from the wastewater pipes for the outdoor CW, respectively.

Five sampling campaigns with a complete depth profile were performed for the planted and unplanted PSCWs during May 2005 to February 2007. On eight additional occasions, samples were taken from the three uppermost layers of planted and unplanted PSCWs only, because preliminary results revealed that the main bacterial productivity was located within the top 10 cm. For the outdoor CW, three sampling campaigns took place in May and September 2005 and May 2006, when outdoor temperatures were comparable to indoor conditions.

For analysis of phospholipid fatty acids (PLFA) two sampling campaigns took place for the PSCWs and the outdoor CW. The samples were immediately transported to the laboratory (< 2 hours) and analyzed. All analytical results were calculated on the basis of oven-dry (105℃, 48 h) weight of soil.

Horizontal profiles

On four occasions the horizontal distribution of bacterial abundance and production was determined for the 0-1 cm and the 40-50 cm layer of the PSCWs at distances of 0-10, 10-20, 20-30, 30-40 and 40-50 cm from the center of the filter body.

4.2.2.3 Chemical characteristics of the wastewater and the filter body

Influent and effluent samples from the PSCWs were taken monthly for analysis of organic carbon (COD, TOC), according to the German standard methods (German Standard Methods for the Examination of Water, Wastewater and Sludge, 1993). Wastewater from the outdoor CW were analyzed weekly for COD and monthly for TOC. Temperature in the filter body was measured online with Pt100 sensors and showed no differences between 10, 30 and 50 cm depth.

Total organic carbon of the sandy filter material from the PSCWs was measured 18 times during the investigation period (August 2005 – November 2006) by combustion at 900°C with a C/N-analyzer Vario Max (Elementar; Hanau, Germany). TOC was calculated from the difference between the total carbon (TC) and inorganic carbon (TIC). For calibration L-glutamine acid was used. The applied protocol is described in Sleytr et al. (2007; see annex V). TOC accumulation rates were calculated from the difference between the averaged TOC concentration at the end and at the beginning of the study period.

4.2.2.4 Determination of bacterial biomass by microscopic direct counts and measurement of cell volume

Samples for microscopic direct counts were preserved in formaldehyde (4 % final conc. in phosphate buffered saline) and stored at 4 °C (max. for four weeks). Additional measurements proved no decrease of bacterial numbers during this period. Representative samples for cell volume estimations were immediately processed after preservation. Diluted and homogenized samples were stained with the fluorescent dye SYBR Green I (10000 x final dilution in DMSO; Molecular Probes; Leiden, Netherlands), following the protocol of Weinbauer et al. (1998) with slight modifications made by Tietz et al. (2007, *see chapter 4.1*). The stained samples were filtered on a 0.2 µm aluminium oxide filter (Anodisc; Whatman; Middlesex, UK). The cells were enumerated by using an epifluorescence microscope (Zeiss Axioplan; Göttingen, Germany) at a magnification of 1600 x. From two filters per sample 30 randomly chosen microscopic fields with 10-20 bacteria were counted. Additionally, the cell volumes of 600 cells were determined for representative samples by the use of an AxioCam MRc digital camera with the corresponding AxioVision software (Zeiss; Göttingen, Germany). A detailed description of the calculation of the cell volume is given elsewhere (Tietz et al., 2007, *see chapter 4.1*). Bacterial cell numbers were converted to bacterial biomass-C by the average bacterial cell carbon content.

4.2.2.5 Bacterial secondary production (BSP)

For the estimation of bacterial secondary production the incorporation rate of radiolabeled leucine was measured according to a modified protocol after Buesing and Gessner (2003). Alternatively, the protocol of Wieltschnig et al. (2003) was also tested, but resulted in comparatively higher blank values (data not shown). Several pre-investigations were performed to check the validity of the method, as proposed by Kirschner and Velimirov (1999) to be prerequisite for sediments.

Influence of label

For comparison, ^{14}C and ^{3}H labeled leucine was applied to check if the label has a significant influence on the observed incorporation rates.

Substrate saturation

With the chosen method, the concentration of the added radiolabeled leucine must lead to saturation of the incorporation rates in order to obtain quantitative estimates of bacterial production (Simon and Azam, 1989). If the rate limiting step is the incorporation of leucine into bacterial proteins, then at saturation conditions radiolabeled leucine uptake is directly proportional to the bacterial protein synthesis rate and production and no internal isotope dilution occurs. If the uptake of external leucine into the bacterial cells is the rate limiting step, then at saturation conditions the measured leucine incorporation rates may be biased through the intracellular production of non-radioactive leucine. In this case a factor for this internal isotope dilution has to be assumed (Simon and Azam, 1989). In this study we tested leucine concentrations between 1.25 and 40 µM with a fixed concentration of 1.25 µM ^{14}C-leucine (ARC; St. Louis, MO, USA; specific activity 325 mCi mmo L^{-1}) diluted between 2 x and 32 x with non-radioactive leucine.

Medium for the homogenous distribution of ^{14}C-leucine

As the water content of the sandy filter material is rather low (15–28 % for the upper 5 cm and 6–18 % for the lower 45 cm) compared to river sediments, natural wetlands or most soils types, additional water was added to achieve an equal distribution of the label within the sample. It was shown that no difference in radioactive substrate uptake rates occurred neither when a slurry is made nor when the radioactive substrate is added via injection or pore water replacement (Dobbs et al., 1989). In ecological studies mostly autoclaved distilled water has been used or better, filter sterilized natural water of the respective environment (e.g. Fischer and Pusch, 1999). In the present study we tested filter sterilized influent and effluent of the PSCWs (prefiltration through 0.45 µm pore-size cellulose nitrate and subsequent filtration through 0.2 µm pore-size cellulose acetate syringe filters) as well as autoclaved distilled water.

Linearity over time

Additional preliminary tests showed that the incorporation of ^{14}C-leucine was linear for at least 2 hours and therefore 1 hour was chosen as the appropriate incubation time.

Extraction efficiency

For the extraction of the radiolabeled proteins from the sandy filter material an alkaline extraction method was used. The extraction efficiency was tested in 3 consecutive loops of repeated extraction of the same sample.

As conclusion of the performed pre-investigations, the following protocol was used for all further investigations. Four replicate samples, consisting of 1 g fresh weight sediment, and one blank were amended with 40 µM ^{14}C-leucine and 1 ml of autoclaved distilled water in 10 ml centrifugation vials. Blanks were stopped immediately with trichloroacetic acid (TCA, 5 % final conc.) and samples after an incubation period of 1 hour at in situ temperature (between 20 and 25 °C). Three washing steps with 5 % TCA, 40 mM non-radioactive leucine and 80 % ethanol followed to remove leucine not incorporated in the protein fraction. This procedure resulted in blank values always less than 0.1 % of the sample values. After an alkaline extraction of the proteins with 0.6 M NaOH, 25 mM EDTA and 0.4 % SDS in a boiling water bath for 1 h and subsequent incubation over night at room temperature, the samples were centrifuged (16.000 x g, 10 min). An aliquot was transferred into a scintillation

vial, scintillation cocktail (Ultima Gold, Canberra Packard) was added and the radioactivity was measured with a liquid scintillation counter (Canberra Packard SC 1900TR). Counts were automatically corrected for quenching using a stored standard curve and a machine counting efficiency program. Leucine incorporation rates were converted to carbon production using the theoretical conversion factor of 1550 g C (mol leucine)$^{-1}$ recommended by Simon and Azam (1989) and empirically derived conversion factors of 529 g C (mol leucine)$^{-1}$ (Michel and Bloem, 1993). Both of these factors yielded unrealistically high estimates of bacterial production, a mass balance approach was followed to calculate appropriate conversion factors for the investigated CWs.

4.2.2.6 Analysis of Phospholipid Fatty Acids (PLFA)

For analysis of PLFAs the filter material was sieved (2 mm pore size) and stored at -20℃ until further analyses were conducted. PLFA were extracted from filter body samples according to the procedure of Bligh and Dyer (1959) as described by Frostegård et al. (1991). 1.5 g of fresh filter material was extracted twice in test tubes containing a mixture of chloroform / methanol / citrate buffer (pH 4.0) at room temperature over night. Crude extracts of lipids were collected after phase partitioning by adding chloroform and deionised water and dried under a gentle stream of N_2. Total lipids were resolved in chloroform and separated into different lipid classes using solid phase extraction columns containing 500 mg of silica. Neutral lipids, glycolipids and phospholipids were obtained by sequential elution using chloroform, acetone and methanol, respectively. Ester-linked PLFAs were subjected to a mild alkaline trans-methylation to produce fatty acid methyl esters (FAMEs), which were analyzed on a Hewlett Packard 5890 II GC equipped with a flame ionization detector. Analytical separation of the FAMEs was accomplished in split mode using a HP Ultra 2 (50 m x 0.20 mm) (Agilent). The carrier gas was helium and the column temperature was held at 70 ℃ for 2 min and subsequently ramped from 70 to 160 ℃ at 15℃ min $^{-1}$, then to 280℃ at 2.5℃ min $^{-1}$. The injector temperature was held at 250 ℃, the flame ionization detector temperature at 300 ℃. Individual compounds were id entified from their relative retention time by comparison with commercially available individual FAMEs and bacterial FAMEs standards (Supelco, Sigma Aldrich). Concentrations of individual compounds were obtained based on the GC response relative to that of the internal standard 19:0 fatty acid. The fatty acids were named according to the nomenclature described by Frostegård et al. (1993). A total of 28 different PLFA were detected and quantified. The sum of the following 12 PLFAs were considered to be mainly bacterial origin: i15:0, a15:0, 15:0, i16:0, 16:1ω7c, 16:1ω7t, i17:0, a17:0, 17:0, cy17:0, 18:1ω7c and cy19:0. The PLFA 18:2ω6,9 was taken as fungal biomarker (Frostegård and Baath, 1996).

4.2.2.7 Statistical analysis

For statistical analysis SPSS 14.0 for Windows was used. Differences between samples were tested for significance using Students T-test.

4.2.3 Results

4.2.3.1 TOC concentrations in the applied wastewater and in the filter body

TOC concentrations of the wastewater applied to the PSCWs amounted to an average of 152 mg TOC L^{-1} and were reduced efficiently by about 97 % (ranging from 93.3 to 98.6 %) (table 4.6). TOC removal rates calculated on a daily basis made up 8.88 (5.04–12.10) g TOC m^{-2} d^{-1}. Similar values were observed for the outdoor CW with a removal rate of 8.24 (4.92-10.60) g TOC m^{-2} d^{-1} and a TOC removal efficiency of 95 % (94.4-97.5). TOC concentrations of the filter layer of the PSCWs ranged from 0.2 to 20.88 mg C (g DW)$^{-1}$ over depths and time (table 4.6). About 38 % of the TOC in the whole filter body was concentrated in the first 5 centimeters mainly as a result of deposition of suspended solids. During the investigation period, the TOC input from wastewater led to a significant accumulation of organic matter ($r = 0.60$; $p < 0.01$) in the top 5 cm (table 4.6), while no significant accumulation could be observed in the layers below. Because too few data were available, no difference between planted and unplanted CWs could be calculated. The investigated outdoor CW exhibited similar values except for the first centimeter, where the TOC content was two times higher than in the PSCWs, presumably due to a higher organic input by the denser vegetation of the outdoor CW (data not shown).

Table 4.6: TOC concentrations, removal rates and accumulation rates in the wastewater and in the filter body of the PSCWs. Results of planted and unplanted CWs were pooled because too few data were available for each type (n.s: no significant TOC accumulation occurred).

	TOC concentration (mg TOC L^{-1})			**TOC removal** (g TOC m^2 d^{-1})
Wastewater	Average	Min	Max	Average rate
Influent (n = 24)	152	51	272	8.88 (5.04 – 12.10)
Effluent (n = 100)	4.6	2.8	8.1	
	Substrate TOC concentration (mg TOC g DW^{-1})			**TOC accumulation** (µg TOC gDW m^2 d^{-1})
Depth of the filter body	Average	Min	Max	Average rate
0-1 cm (n = 18)	12.37	5.89	20.88	25.8
1-5 cm (n = 18)	5.11	3.13	8.75	5.6
5-10 cm (n = 18)	2.97	1.71	4.90	n.s.
10-20 cm (n = 18)	1.29	0.31	2.97	n.s.
20-30 cm (n = 18)	0.87	0.33	3.02	n.s.
30-40 cm (n = 18)	1.05	0.15	2.78	n.s.
40-50 cm (n = 18)	0.62	0.20	1.99	n.s.

4.2.3.2 Pre-investigations to check the validity of the ^{14}C-leucine incorporation technique

Influence of label

For four depths the influence of the added label (^{14}C-leucine in comparison to ^{3}H-leucine) was tested. No significant differences were observed in the 0-5, 10-20 and 30-40 cm depth-layers ($p > 0.1$), in the 20-30 cm depth a slightly higher average value was observed with the ^{14}C-method (figure 4.4). However, the rates achieved within this depth layer can be neglected because they account only for a small percentage of the rates in the uppermost 20 cm. Therefore the ^{14}C-leucine method was used for all further investigations due to economical reasons.

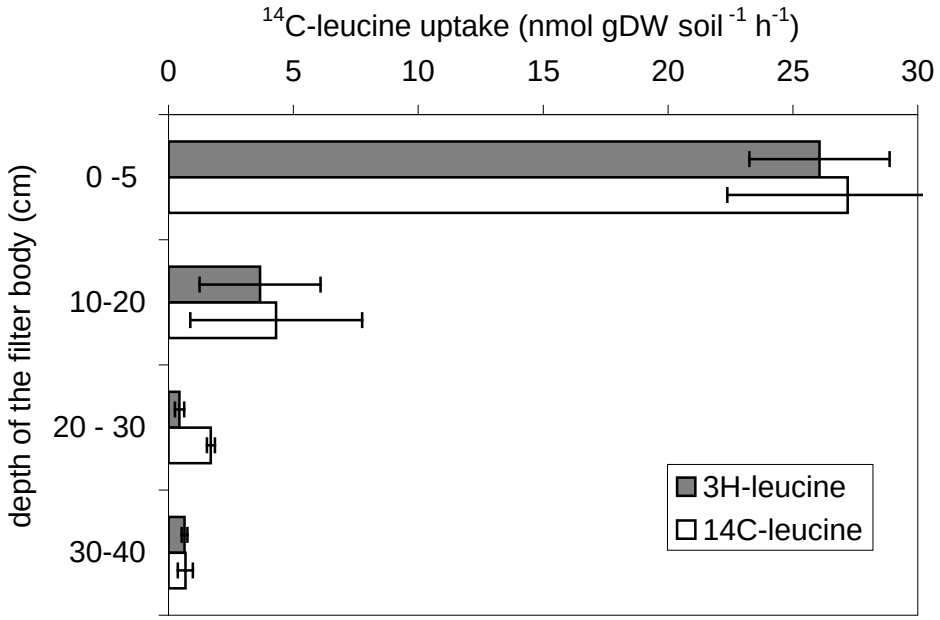

Figure 4.4: Comparison of ^{3}H-leucine and ^{14}C-leucine uptake into bacterial proteins in four different depths of one PSCW. (Bars depict the mean of 4 replicates ± standard deviation.)

Substrate saturation

Increasing ^{14}C-leucine concentrations between 1.25 and 40 µM were added to samples from two different depths (0-1 and 30-40 cm) of a planted PSCW and in both cases the uptake followed the Michaelis-Menten kinetics and saturation (V_{max}) was achieved at concentrations of ≥ 30 µM. Figure 4.5 shows the substrate saturation data for the 0-1 cm depth. Based on these results a 40 µM solution, consisting of 1.25 µM ^{14}C-leucine and 38.75 µM non-radioactive leucine, was used for all further experiments. Dilution of the purchased radioactive leucine with non-radioactive leucine up to a ratio of 50 x does not affect the validity of the results, when high incorporation rates are measured, but significantly reduces the costs (Kirschner and Velimirov 1999).

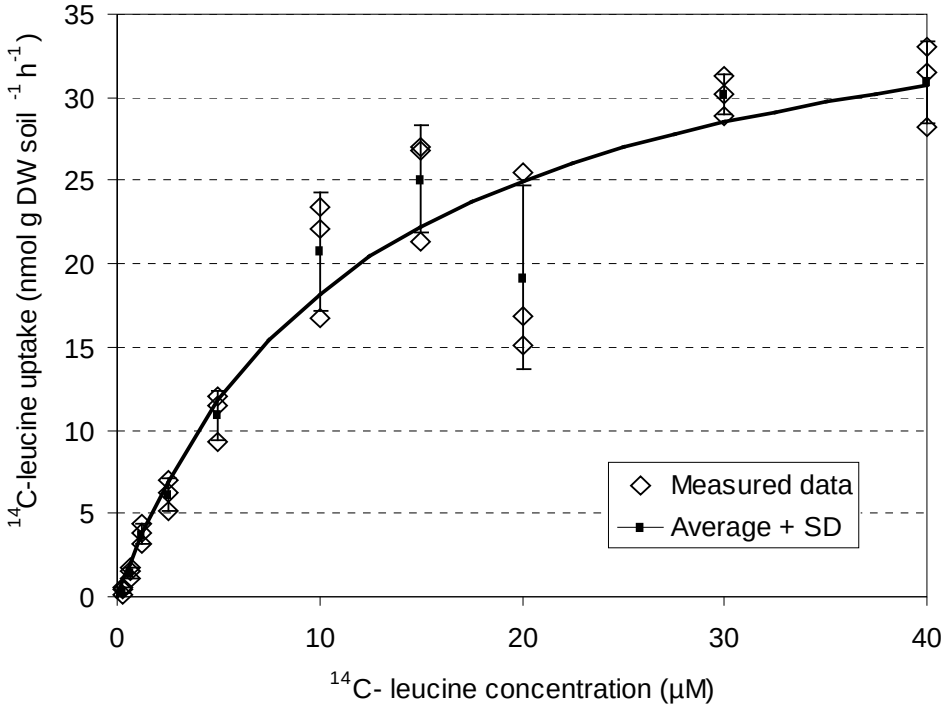

Figure 4.5: Michaelis-Menten kinetics of the uptake of ^{14}C-leucine into bacterial proteins of the 0-1 cm depth layer of a planted PSCW. (Triplicate measurements were performed for each concentration.)

Medium for the homogenous distribution of ^{14}C-leucine

When filter sterilized influent, effluent and sterile distilled water were compared as medium for administering the radioactive label to the sample slurry, a significant inhibition of the measured ^{14}C-leucine uptake rates was observed in case of the filtered influent. In a series of six different mixture ratios from 100 % distilled water to 100 % influent a significantly increasing inhibition up to 28.5 % was observed relative to the uptake rate observed at 100% autoclaved distilled water (figure 4.6). No inhibition occurred, when 100 % of effluent was added. For all further experiments autoclaved distilled water was used to measure non-inhibited uptake rates.

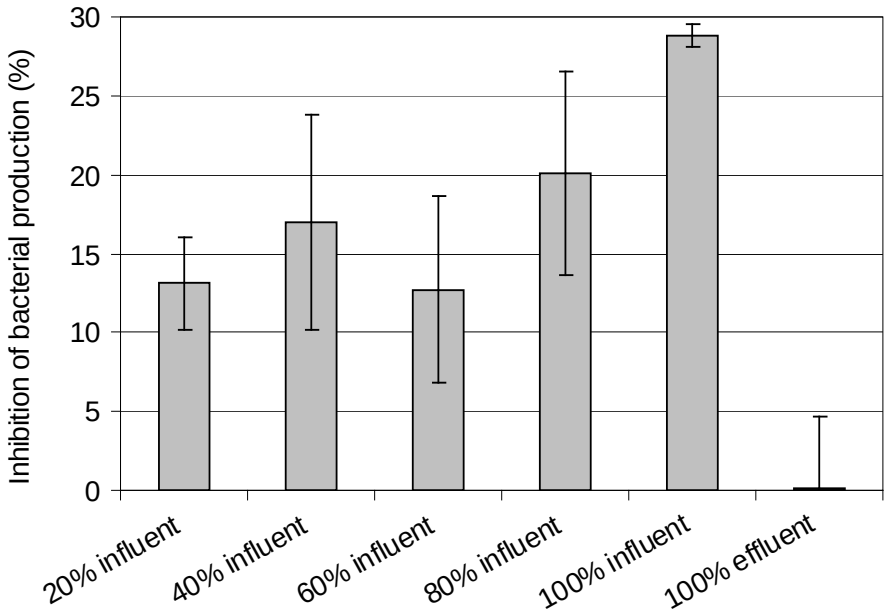

Figure 4.6: Inhibition of the bacterial ^{14}C-leucine incorporation in the 0-1 cm layer at different ratios of sterile filtered influent to sterile distilled water and 100 % effluent used for administering the radioactive leucine to the sample slurries. (Bars depict the mean of 4 replicates ± standard deviation.)

Extraction efficiency

The measured radioactivity in the bacterial proteins in the three consecutive extraction loops showed that 87.0-88.2 % of the incorporated label could be gained during the first run, 10.4-11.7 % in the second run and 1.3-1.5 % in the third run. In the further investigations only one extraction step was performed and the data were multiplied with a factor of 1.14.

4.2.3.3 Distribution of bacteria within the filter body of the PSCWs

4.2.3.4 Horizontal distribution of the bacterial production (BSP)

For the PSCWs ^{14}C-leucine uptake was measured in different distances from the center of the filter body, to check for horizontal distribution patterns. The top layer (0-1 cm) as well as the deepest layer (40-50 cm) was analyzed. Figure 4.7 shows that there were no significant differences between the different sampling points for the top layer with the exception of the samples taken from the center of the filter body ($p < 0.001$) and for the sample taken in 40-50 cm distance ($p < 0.05$). In consequence the areas from 0-10 and from 40-50 cm distance from the center were excluded as sampling locations for depth profiles.

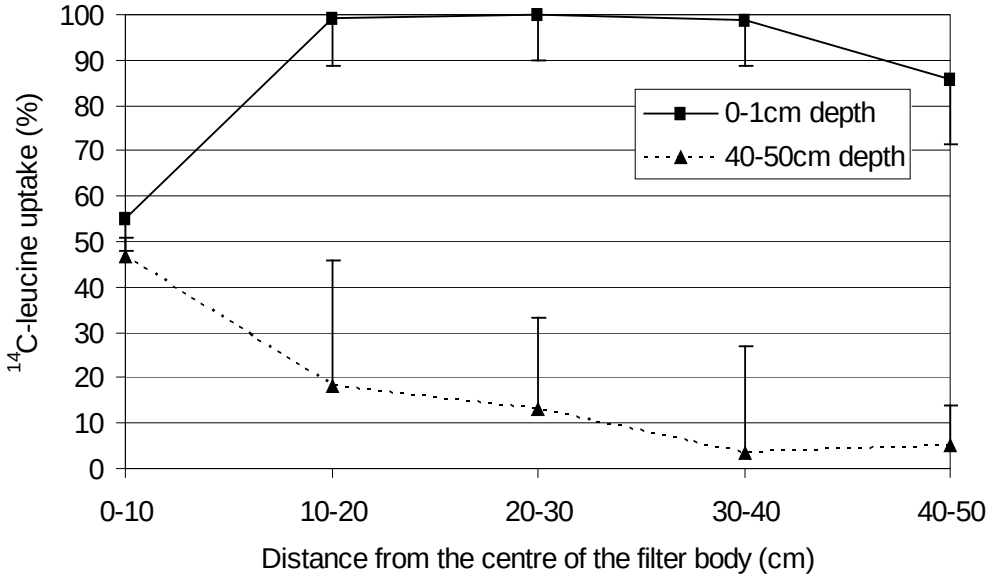

Figure 4.7: Horizontal distribution of the ^{14}C-leucine uptake within the filter body of the PSCWs (results pooled from planted and unplanted) in two different depths. (Means of 4 replicates ± standard deviation.)

4.2.3.5 Vertical distribution of ^{14}C-leucine uptake, bacterial numbers and biomass

For the three investigated CW-systems (planted and unplanted PSCWs, outdoor CW) the uptake of ^{14}C-leucine as well as the number of bacterial cells decreased with the depth of the filter body (figure 4.8 and 4.9). Bacterial biomass-C, calculated from cell numbers and average cellular carbon content (27.6 fg C cell^{-1}) ranged from 382 µg C g DW^{-1} in the top layer of the unplanted PSCWs to 1.7 µg C g DW^{-1} in the 30-40 cm layer of the planted PSCWs.

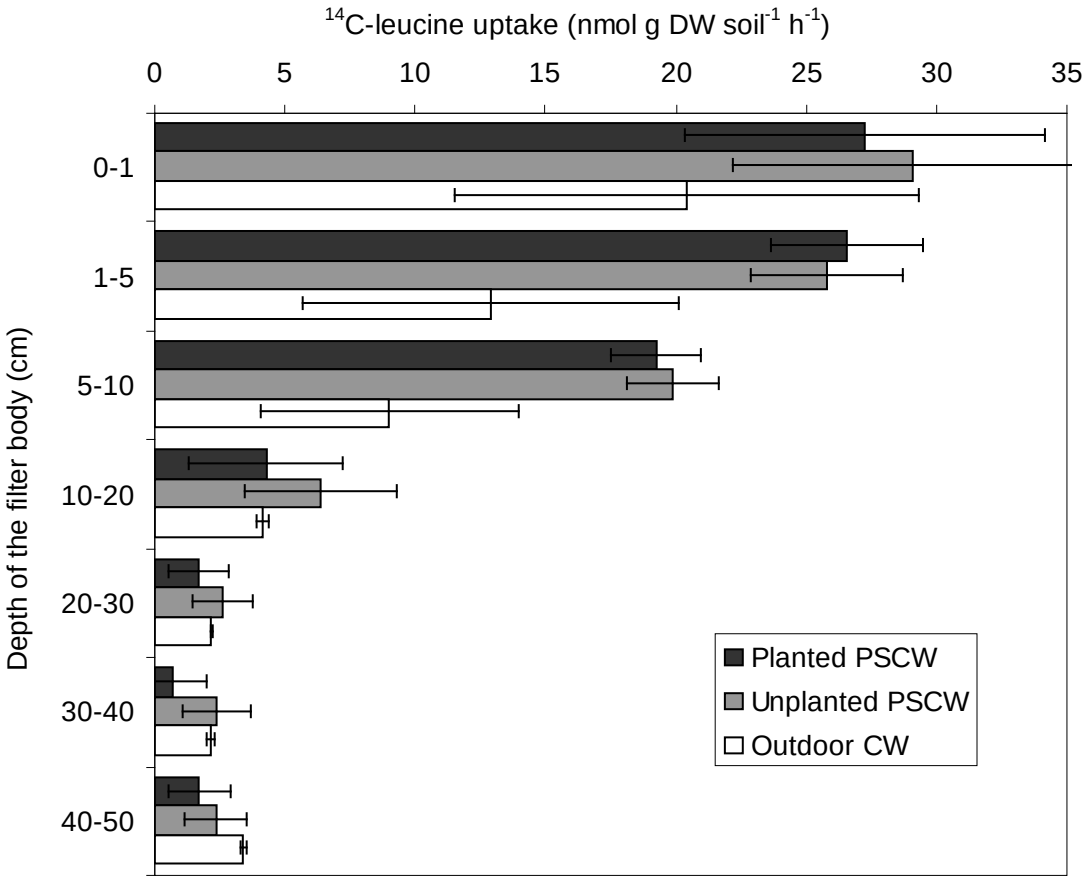

Figure 4.8: Variation of ^{14}C-leucine uptake over the depth of the filter body of the planted, unplanted PSCWs and the outdoor CW. (Means ± standard deviations of thirteen sampling occasions for 0-10 cm, five sampling occasions for 10-50 cm of the PSCWs and of three sampling occasions for the outdoor CW; at each sampling occasion 4 replicate samples were measured.)

The number of bacterial cells showed a much steeper decrease with the depth than ^{14}C-leucine uptake (figure 4.8 and 4.9). The ^{14}C-leucine uptake decreased only by 8 % within the first 5 cm of the PSCWs whereas bacterial numbers and PFLAs declined by about 70 %. However, bacterial production was significantly correlated with bacterial numbers, PLFAs, TOC ($r = 0.66$-0.87; $p < 0.05$).

In 20 to 50 cm depth ^{14}C-leucine incorporation amounted to less than 10% and bacterial numbers to less than 5 % of the values from the uppermost centimeter. The minimum and maximum values for bacterial abundance ranged from 1.02×10^8 cells $(g\ DW)^{-1}$ in 30-40 cm depth of the unplanted PSCWs, up to 1.88×10^{10} cells $(g\ DW)^{-1}$ in the top layer of the unplanted filter body. Maximal leucine incorporation was 36.0 nmol $(g\ DW)^{-1}\ h^{-1}$ in the top layer of the unplanted PSCWs, and decreased to a minimum of 0.37 nmol $(g\ DW)^{-1}\ h^{-1}$ in 30-40 cm of the planted PSCWs. Between the three investigated CW types no differences in the overall distribution patterns of bacterial biomass and productivity (decreasing values with depth) were observed. In case of bacterial abundance no statistically significant differences ($p > 0.05$ for all depths) between planted, unplanted PSCWs and the outdoor CW were recorded, although average values in the four deepest layers were between 22 % and 64 % higher in the outdoor CW compared to the two PSCW types (figure 4.9). In case of bacterial production, an on average 50 % lower ^{14}C-leucine uptake in 1-5 and 5-10 cm depth was observed in the outdoor CW.

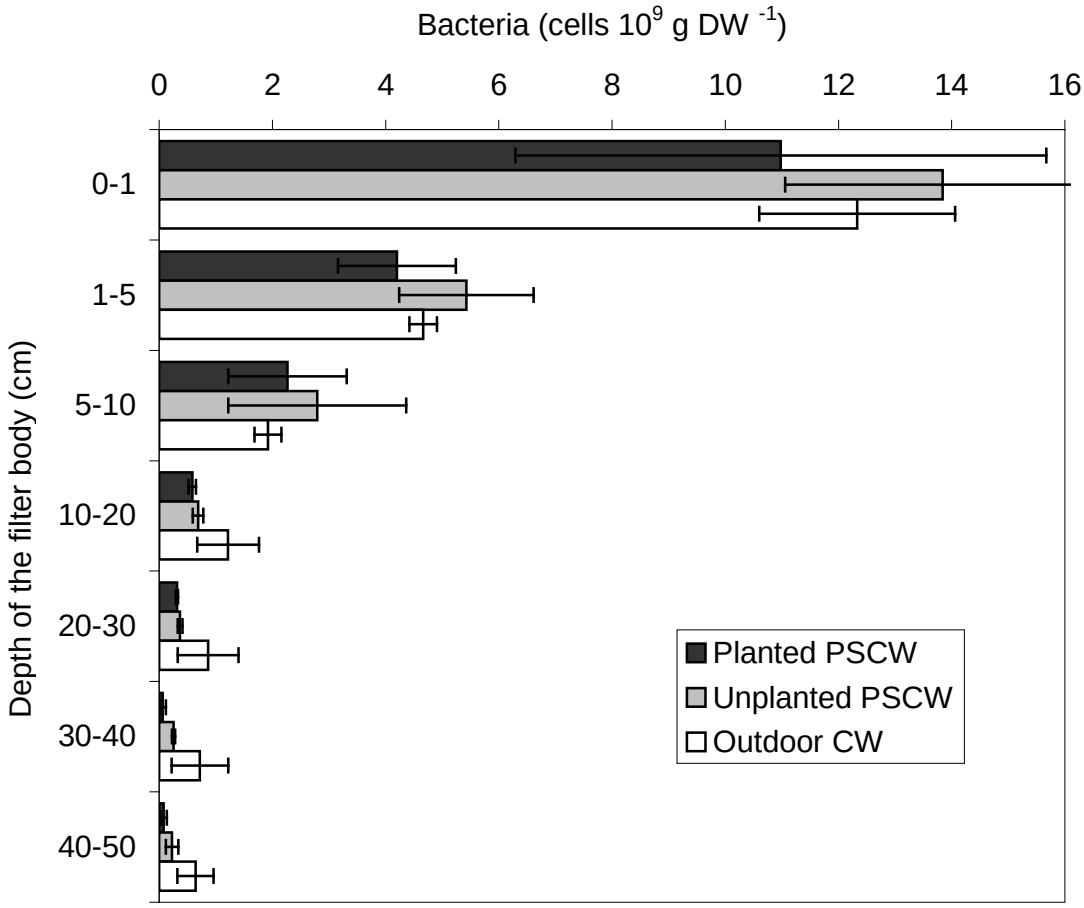

Figure 4.9: Abundance of bacterial cells in the filter body in different depths of the planted, unplanted PSCWs and the outdoor CW. (Means ± standard deviations of six sampling occasions.)

4.2.3.6 PLFA distribution

The sum of total PLFA concentrations in the investigated CWs decreased with depth from a maximum value of 513 nmol (g DW)$^{-1}$ in the uppermost cm to 14.7 nmol (g DW)$^{-1}$ in 10-20 cm depth. Below this depth most of the PLFAs were below detection limit. Therefore, no sum of total PLFAs is given in table 4.7 for these depth layers. Bacterial PLFAs varied between 332 nmol (g DW)$^{-1}$ in the top layer and 4.7 nmol (g DW)$^{-1}$ in the depth of 10-20 cm of the outdoor CW. The contribution of bacterial PLFAs to total PLFAs varied between 43 and 63 %, whereas the fungal PLFA accounted for only 2.3-7.2 %, indicating a low contribution of fungi to total microbial biomass. In all depths bacterial PLFA concentrations exceeded by far the fungal PLFA (table 4.7). The fungal / bacterial biomass ratio using the PLFA 18:2ω6,9 (fungal biomarker) and the sum of the bacterial PLFAs varied between 0.04 and 0.05 for the outdoor CW and between 0.09 and 0.17 for the indoor PSCWs. Average bacterial abundance, PLFAs and TOC showed highly significant linear correlations among each other over the seven depths ($r = 0.86\text{-}1.00$; $p \leq 0.01$).

Table 4.7: Bacterial and fungal phospholipid fatty acid (PLFA) concentrations in different layers of the investigated indoor and outdoor CWs (average values ± standard deviation).

	Indoor				Outdoor			
Depth (cm)	**Total PLFAs**	**Bacterial PLFAs** (nmol PLFA gDW $^{-1}$)	**Fungal PLFA**	**Fungal / bacterial ratio** -	**Total PLFAs**	**Bacterial PLFAs** (nmol PLFA gDW $^{-1}$)	**Fungal PLFA**	**Fungal / bacterial ratio** -
0-1	381.2 (± 179.5)	167.6 (± 81.4)	28.4 (± 16.8)	0.17	487.6 (± 35.6)	309.6 (± 22.3)	11.4 (± 1.3)	0.04
1-5	118.1 (± 45.1)	53.6 (± 18.7)	5.3 (± 3.3)	0.1	137.4 (± 42.8)	79.7 (± 28.7)	3.1 (± 0.63)	0.04
5-10	56.0 (± 23.8)	25.7 (± 10.6)	2.3 (± 0.8)	0.09	40.2 (± 15.6)	21.9 (± 8.3)	1.2 (± 0.11)	0.05
10-20	28.6 (± 5.3)	13.7 (± 2.4)	1.2 (± 0.1)	0.09	16.5 (± 2.4)	7.9 (± 3.1)	b.d.l.	-

b.d.l.: values below detection limit

4.2.4 Discussion

4.2.4.1 Reliability of the chosen ^{14}C-leucine incorporation method

Bacterial organic matter utilization in natural ecosystems is commonly measured using the incorporation rate of radioactive substrates into bacterial biomass and conversion to biomass-carbon production with theoretical or empirical conversion factors. This approach is an elegant way to assess net bacterial growth in complex samples and microbial communities, as the incorporation of the substrates, mainly leucine and thymidine, into bacterial protein and DNA respectively, is sensitive and specific for bacteria with the usually used short incubation times (10 min-1 hour) and the low concentrations in the nM to µM range (Kirchman et al., 1985). In this study we used and adapted the radioactive leucine incorporation method for estimation of bacterial carbon utilization in subsurface vertical flow CWs for the first time, as this approach is more sensitive and less variable than the thymidine method (Kirschner and Velimirov, 1999) and because it has been applied successfully in aquatic sediments (Buesing and Gessner, 2003) and soils (Baath, 1994).

Several key parameters of this method were carefully determined in preliminary experiments, such as; (i) linearity of incubation over time, (ii) Michaelis-Menten kinetics and the concentration at which leucine incorporation into bacterial biomass is saturated, (iii) the comparability between ^{3}H and ^{14}C label, (iv) the extraction efficiency of the proteins from the samples and (v) the appropriate medium for the homogenous distribution of the substrate in the sample.

An important feature for the reliability of the method, however, is the choice of an appropriate conversion factor for converting bacterial leucine uptake to biomass production and carbon utilization (Simon and Azam, 1989). This conversion factor can be a theoretical one, based on the assumptions of (i) a constant amount of leucine in the bacterial proteins (7.3 mol%), (ii) a constant amount of proteins in the bacterial biomass (62.5 %) and a constant amount of C in the bacterial biomass (53.8 %). Considering the molecular weight of leucine (131.2 g mol^{-1}) a theoretical conversion factor of 1550 g C mol^{-1}, as determined by Simon and Azam (1989) for coastal marine bacteria, has usually been applied for a broad variety of different environments. Their finding that an isotope dilution factor of two is necessary to compensate for intracellular isotope dilution of the added label leads to a conversion factor of 3100 g C mol^{-1}. Empirical conversion factors for soil or aquatic sediment samples are scarcely available in the literature mainly due to methodical problems. Within a conversion factor experiment, bacterial net growth has to be achieved by significantly reducing predators and phages and by the addition of nutrients to the batch cultures. From this follows an increase in bacterial numbers / biomass which is directly related to the incorporation of the radioactive substrate. This is possible for water samples but not for soil / sediment samples, where the separation of bacteria from their predators cannot be achieved by simple filtration. Therefore, alternative set-ups are used which have the major disadvantage that they mimic a situation far away from natural conditions. Michel and Bloem (1993) published an average conversion factor for soil bacteria of 529 g C mol^{-1} (range: 216–1024 g C mol^{-1}) based on the growth of five selected and previously isolated and cultured bacterial strains. Another approach was used by Buesing and Marxsen (2005), who separated bacteria from their aquatic sediments by sonication and reintroduced them to a batch culture of interstitial water (natural nutrient situation) spiked with sterile sediment particles. Their conversion factor of 1440 g C mol^{-1} was very similar to the theoretical factor of Simon and Azam (1989). However, also for this experimental set-up it can be assumed that a significant part of the bacterial community can not be detached from the sediment particles. In addition, sediment or soil bacteria are to a great extent organized in complex biofilms and disruption from their original consortium may lead to completely altered activity levels. Thus, bacterial growth in the culture may not be representative for the natural situation. In the course of this study we

made several attempts to design a conversion factor experiment using the natural bacterial populations by mixing one part of unaltered sample material with nine parts of sterilized samples amended with wastewater. Different methods for sterilization (gamma radiation, freeze-thawing, heating) were applied and different wastewater types (influent, effluent, artificial wastewater) were used, but in no case was bacterial net growth achieved. Only in the case of unnaturally high nutrient addition (yeast extract) was bacterial cell number increase observed. For all of these reasons we decided to calculate bacterial carbon turn-over from mass-balance calculations.

4.2.4.2 Mass balance calculation of bacterial C-fluxes

Based on the difference between C input and outflow, the accumulation rates of organic carbon in the systems, the calculated contribution of fungi and the spatial distribution of the leucine incorporation data a mass balance calculation was performed to estimate the bacterially mediated C-fluxes in the investigated subsurface vertical flow CWs (table 4.8). Moreover, a conversion factor for converting leucine incorporation rates to bacterial biomass-C production rates was assessed from this mass-balance calculation.

Table 4.8: Mass balance of TOC turnover rates and calculation of a conversion factor for converting bacterial leucine incorporation rates to bacterial biomass production.

	TOC (g TOC m^{-2} d^{-1})		
	Outdoor CW	**Indoor PSCWs planted**	**Indoor PSCWs unplanted**
Influent	8.65	9.15	9.15
Effluent	0.41	0.29	0.33
TOC input by plants	1.30 [1)]- 2.75 [2)]	0.65 - 1.30 [3)]	0
Accumulation in the sediment	2.95 [2)]	0.80	0.80
TOC available for microorganisms	6.59 – 8.04	8.71 – 9.36	8.02
TOC utilized by fungi	0.09-0.10 (1.3%)[4)]	0.27-0.29 (3.1%)[4)]	0.25 (3.1%)[4)]
Bacterial carbon demand	6.50 - 7.95	8.42 – 9.09	7.77
TOC respired by bacteria	3.25 - 3.98	4.21 – 4.55	3.89
TOC assimilated by bacteria	3.25 - 3.98	4.21 – 4.55	3.89
		Conversion factor (g C mol^{-1})	
leucine conversion factor	100.0 – 120.0	98 – 105.2	81.8

[1)] Kirschner and Velimirov (1999)
[2)] Nguyen (2000)
[3)] Calculated for a plant density of about 85 (40-130) stems m^{-2} based on the measurements by Kirschner and Velimirov (1999)
[4)] Estimation based on the assumption of the same percentage of fungal carbon consumption of total carbon consumption as the percentage of fungal biomass to total biomass

Based on the difference between C input and outflow, the accumulation rates of organic carbon in the systems, the calculated contribution of fungi and the spatial distribution of the leucine incorporation data, a mass balance calculation was performed to estimate the bacterially mediated C-fluxes in the investigated subsurface vertical flow CWs (table 4.8). Moreover, a conversion factor for converting leucine incorporation rates to bacterial biomass-C production rates was assessed from this mass-balance calculation.

The average input of organic carbon to the indoor CW estimated from the average TOC concentration of 152 mg L^{-1} in the 60 L of added wastewater per day, amounts to 9.15 g C m^{-}

^{2} d^{-1}. For the outdoor system the input of TOC by the influent wastewater amounted to 8.65 g C m^{-2} d^{-1}. The amount of carbon originating from algae growing on the surface of the investigated CWs is considered as negligible as no visible algae growth occurred. On the one hand, macrophyte growth on the planted CWs leads to light limitation, while on the other hand light penetration into the filter body is limited to a few mm by the filter body material itself. Additional input of organic carbon from plants was estimated to 0.65-1.30 g C m^{-2} d^{-1} for the planted indoor system, for the outdoor system this input was assumed to be in the range of 1.30–2.75 g C m^{-2} d^{-1} (for an explanation see table 4.8).

From the temporal changes of TOC concentrations an average daily TOC accumulation rate of 0.80 g TOC m^{-2} d^{-1} was assumed for both unplanted and planted CWs, because too few data are available for each system. These TOC accumulation rates are in the lower range of the organic matter accumulation rates given by Caselles-Osorio et al. (2007) for a horizontal subsurface flow constructed wetland loaded with municipal wastewater ranging from 0.38 to 7.8g organic matter m^{-2} d^{-1}, corresponding to 0.19–3.9 g C m^{-2} d^{-1}, assuming a 50 % carbon content. The TOC accumulation rates of the CWs investigated within this study have to be linked to the TOC influent-concentrations, which are rather low for a typical municipal wastewater (table 4.6). Therefore, it is reasonable that the accumulation rates of these systems are also relatively low. For example, much higher accumulation rates of organic matter (5.9-6.0 g organic matter m^{-2} d^{-1}; corresponding to 2.95–3.0 g C m^{-2} d^{-1}, assuming a 50 % carbon content) were reported by Nguyen (2000) and Tanner et al. (1998) for outdoor gravel bed CWs, which received dairy wastewater with typically much higher TOC concentrations than municipal wastewater.

The TOC-output of our systems by the effluent wastewater was 0.29–0.33 g C m^{-2} d^{-1} in case of the indoor and 0.41 g C m^{-2} d^{-1} for the outdoor CW. The TOC-removal efficiencies varying from 93.3 to 98.6 % are in the range of other vertical flow constructed wetlands (e.g. Weedon, 2003). In total, between 8.0 (unplanted) and 8.7–9.4 g C m^{-2} d^{-1} (planted indoor) and between 6.6 and 8.0 g C m^{-2} d^{-1} (planted outdoor) are available for microbial utilization .

Analyses of PLFAs in environmental samples are usually used to provide a fingerprint of the microbial community structure. Relative ratios of bacterial and fungal biomass can also be determined by applying this technique, but a conversion to absolute biomass values tends to produce uncertainties (Baath and Anderson, 2003) and is therefore rarely reported. By using conversion factors from the literature (Frostegård and Baath, 1996; Baath and Anderson, 2003) we tried to make a rough estimation of fungal biomass by translation of the fungal PLFA-biomarker (18:2ω6,9) into ergosterol. With typical ergosterol-contents of fungi ranging from 2.3 to 11.2 µg ergosterol mg^{-1} mycelium dry mass (Gessner and Chauvet, 1993) the ergosterol values were further converted to fungal biomass. The comparison of the calculated fungal biomass with the bacterial biomass revealed a contribution of fungal to bacterial biomass of maximally 1.3 % for the top 10 cm of the outdoor CW. For the PSCWs, the maximal contribution of fungal to bacterial biomass was reached in the uppermost cm with 3.1 % decreasing to 1.3% in the layers below. These results indicate that fungi play only a minor role in organic matter degradation of vertical flow constructed wetlands. Assuming the same percentage of carbon-consumption for fungi as for bacteria (1.3–3.1 %), about 7.8 (unplanted) to 8.4–9.0 g C m^{-2} d^{-1} (planted) remain for bacterial utilization (bacterial carbon demand, BCD) of indoor CWs and 6.5-8 g C m^{-2} d^{-1} in case of the outdoor CW (table 4.8). Assuming an average growth efficiency of the bacterial population of 50 % for nutrient rich environments (Del Giorgio and Cole, 1998), between 4.21 and 4.55 g C m^{-2} d^{-1} are respired and assimilated by bacteria in the indoor CWs and between 3.25 to 3.98 g C m^{-2} d^{-1} in the outdoor CWs, respectively. CO_2 emission rates of the same magnitude have been reported by Teiter and Mander (2005) for a subsurface vertical flow CW (e.g. 3.4–7.0 g CO_2-C m^{-2} d^{-1}). Methane emission from vertical flow systems with intermittent loading can be neglected due to the good oxygen supply down to the deepest areas so that no significant anoxic zones occur. Additionally, the fact that the PLFAs 16:1ω8 and 18:1ω8, which are typical for obligate methanotrophic bacteria (Gebert et al., 2004) were detected only in few samples at very low concentrations, indicates that methane is absent in this system.

Conversion factors for the leucine incorporation were calculated by dividing the total amount of assimilated bacterial carbon, by the total amount of incorporated leucine per m^2 per day. These mass balance based conversion factors range from 81.8 to 120 g C mol^{-1} and are much lower than the theoretical factor of 1550 g C mol^{-1} (Simon and Azam, 1989) or the empirical factors of 529 g C mol^{-1} proposed by Michel and Bloem (1993) or 1440 g C mol^{-1} proposed by Buesing and Marxsen (2005). The use of these factors from the literature would result in a 4.4-18.9 times higher bacterial carbon demand of 29-172 g C m^{-2} d^{-1}, being thus unrealistically high. Furthermore the application of the literature conversion factors would result in extremely high median specific bacterial growth rates (µ) in the range of 0.13 to 0.57 h^{-1} corresponding to median doubling times of 1.7–7.5 hours for the planted indoor system. When applying the mass balance based factor, doubling times of 11–135 hours are calculated with median values of 33 and 35 h for the planted and the unplanted indoor system respectively and 75 h for the outdoor system. These values are of comparable magnitude to other soils (Uhlirova and Santruckova, 2003) and aquatic sediments (Bott and Kaplan, 1985; Kirschner and Velimirov, 1999).

Figure 4.10 displays a 3-dimensional profile of the bacterial carbon demand in the PSCWs. In the first centimeter at a distance of 20 to 30 cm from the center bacterial carbon demand reaches its highest values with 91.8 and 78.9 µg C cm^{-3} h^{-1} for planted and unplanted PSCWs respectively. Lowest values of 0.16 µg C cm^{-3} h^{-1} were observed in both PSCWs in 30-40 cm distance from the center in 30-40 cm depth. It becomes obvious that the overwhelming part (80 %) of the bacterial C utilization takes place in the upper 10 cm of the filter body. Of the median BCD of 8.8 g C $m^{-2}d^{-1}$ in the whole planted PSCWs, 0.83g C m^{-2} d^{-1} (9.4 %) are utilized by the bacteria in the first centimeter, 3.23 g C m^{-2} d^{-1} (36.7 %) in the 1-5 cm layer and 2.99 g C m^{-2} d^{-1} (34.0 %) in the 5-10 cm layer. Thus only about 20 % are metabolized in the layers below.

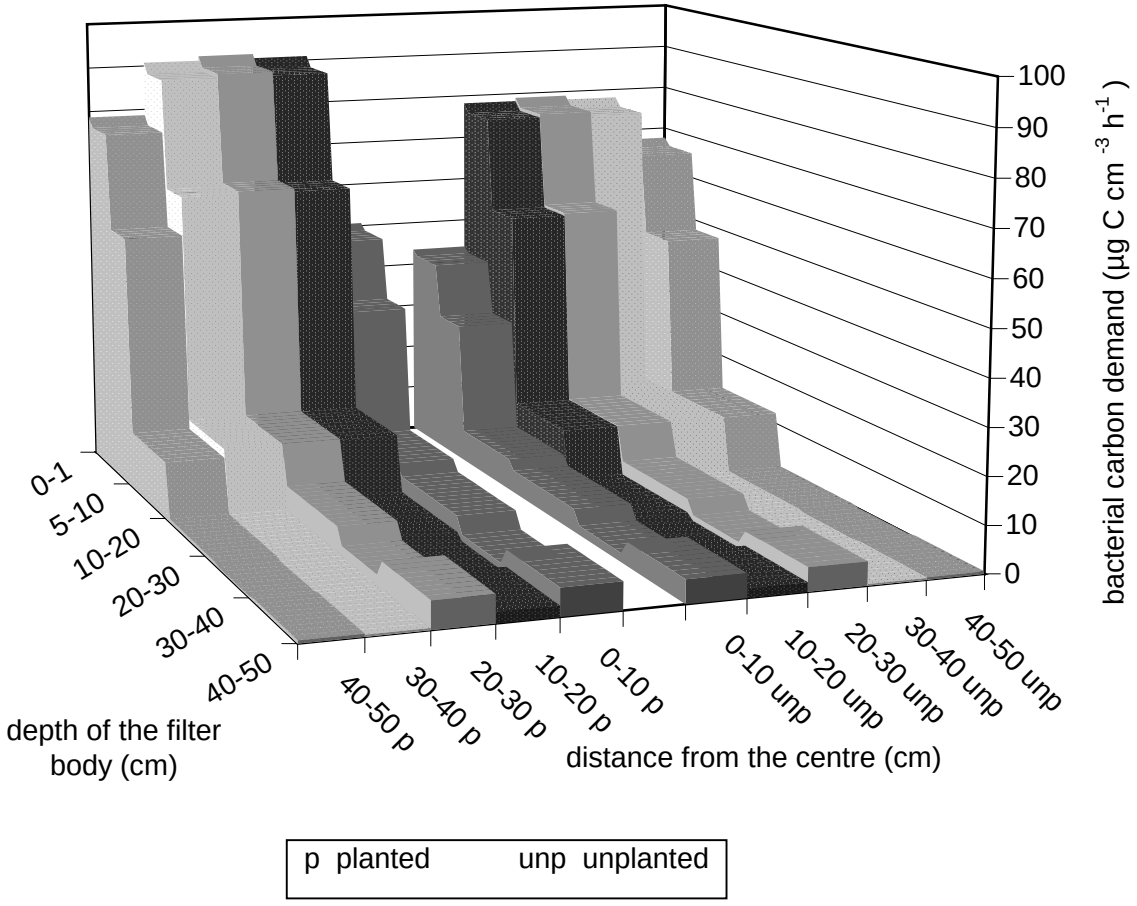

Figure 4.10: Spatial distribution of the bacterial carbon demand within the filter body of the planted PSCW.

4.2.4.3 Spatial distribution of bacterial productivity and biomass

For the estimation of the total amount of carbon utilized by bacteria it was necessary to analyze the spatial distribution of the bacterial secondary production (BSP) within the filter body. Due to a hydraulically optimized loading system (sprinkler system) the wastewater is distributed rather equally across the surface of the PSCWs. Thus, bacterial productivity did not vary much within one depth layer (figure 4.7). Only the outermost areas (40-50 cm distance from the center), which receive a little less wastewater showed a lower productivity and in the central 10 x 10 cm area of the PSCWs the productivity was reduced because the wastewater splashes on the surface of the filter body during each hydraulic loading and washes away the fine filter material and therefore lowers bacterial biomass and activity. In the 40-50 cm depth, ^{14}C-leucine uptake was significantly higher in the center of the filter body than in the outer areas, most probably due to the downward translocation of fine filter material by the higher hydraulic pressure in the center of the PSCWs. In full-scale systems however, such effects are often prevented by the use of bouncing plates. These results also indicate the importance of an equal wastewater distribution for an optimal utilization of the filter body.

The rapid decrease of bacterial biomass and productivity with depth is most likely a consequence of the lower nutrient content in the deeper parts of the filter body (figure 4.8, 4.9 and table 4.7), as indicated by the low TOC-concentrations in these layers. Oxygen concentrations are not considered as limiting for bacterial metabolism, as the whole filter body has always been aerobic. Similar patterns of microbial biomass decrease with depth were reported by Tietz et al. (2007, *see chapter 4.1*) and by Ragusa et al. (2004) who found a sharp decrease of the protein-content and the number of viable cells between 1 and 4.5 cm depth of the filter body of a constructed wetland microcosm.

The slighter decrease of bacterial C production rates compared to bacterial abundance within the first 5 cm can be explained by the accumulation of wastewater bacteria, on the surface of the filter body. These bacteria are not adapted to the conditions outside their intestinal environment and therefore they die off or at least significantly reduce their metabolic activity, but they are still included in bacterial cell counts, TOC and partially in PLFA measurements. Furthermore, inhibiting substances in the wastewater which adsorb to the sediment particles additionally impede bacterial metabolism (see chapter 4.2.3.2 and figure 4.6). In deeper filter layers this effect is reduced due to the degradation of wastewater pollutants during the filter passage.

4.2.4.4 Comparison of bacterial productivity and biomass in the planted, unplanted PSCW, and in the full scale outdoor CW

In general, all three investigated systems showed similar values and trends for bacterial biomass-, BSP-; PLFA- and TOC-measurements. Significantly lower BSP values (~50 %) were found within the 1-10 cm layer of the outdoor CW in comparison to the PSCWs. This difference may be a consequence of different wastewater composition or due to less oxygen in the outdoor CW caused by impeded diffusion of oxygen into the filter body covered by a thick layer of plant litter. Due to better growing conditions for the macrophytes of the outdoor CW (e.g. light conditions) in comparison to the indoor CWs a low plant density has been developed in the PSCWs (Tietz et al., 2007, *see chapter 4.1*). Therefore, it can be assumed that also the below ground plant biomass (roots and rhizomes) was higher in the outdoor CW than in the PSCWs. The higher average bacterial biomass values in the four deepest layers of the outdoor CW could be a consequence of the better and deeper root penetration in this part of the filter body. The allocation of oxygen and especially the release of root exudates (low molecular weight organic acids) can compensate for lower oxygen and nutrient concentrations in these deeper layers. It has been reported (Armstrong and Armstrong, 1991) that macrophytes like *Phragmites australis* effectively transport oxygen via convection through their roots. Their results indicate that oxygen released from plant roots has an influence on the micro-environment around the root tissues. However, oxygen release from roots is low compared to the oxygen demand for degradation of organic matter and nitrification, and to the amount of oxygen brought into the system via convection and diffusion. Therefore the effect of oxygen release on the treatment performance can be neglected especially in subsurface vertical flow CWs with intermittent loading (Langergraber, 2005). But nevertheless a stimulating effect on bacteria by roots in constructed wetlands has been reported by Münch et al. (2005). However, as the contribution of the four lowest layers to total carbon turn-over is low (about 20 %) and the main part of carbon is metabolized by the microbial community in the uppermost layers, the effect of stimulation of bacterial C turn-over through plants can be considered as negligible in the investigated CWs. For a more substantive assessment of the influence of plants on the microbial community, information on the root density and experiments on the interaction between bacteria and roots or rhizomes would be useful.

4.2.5 Conclusions

- The measurement of radioactive leucine incorporation into bacterial biomass has proved to be a useful tool to reveal the spatial patterns of bacterial productivity within the filter body of a vertical subsurface flow constructed wetland. As the use of any published conversion factor for converting leucine incorporation into carbon production yielded unrealistically high values we chose a mass balance approach to estimate this factor indirectly. For future applications also direct estimates should be assessed, either by performing adequate empirical experiments or by determining the parameters necessary for calculating a theoretical conversion factor.
- In vertical subsurface flow CWs with intermittent hydraulic loading and optimized distribution of the wastewater across the surface, the bulk of the microbial productivity (> 80 %) takes place within the first 10 cm of the filter body, although significant inhibitory substances from wastewater were obviously present in this layer. Bacteria are obviously dominating productivity and carbon degradation while fungi seem to play a minor role in this ecosystem. However, when concluding that vertical subsurface flow CWs could be built with a much shallower design, it has to be taken into consideration that a minimum depth > 10 cm may be required for a stable and robust operation performance as well as for physical filtration processes. This is of special relevance because one of the main advantages of vertical subsurface flow CWs compared to technical treatment systems, such as activated sludge technology is their stable operation performance.
- In this study, plants seemed to have little influence on bacterial productivity and biomass as well as on TOC removal rates. Whether plants increase organic matter accumulation in the uppermost layers leading to an earlier clogging of planted wetlands remains to be investigated under different hydraulic loadings, operating conditions and with different types of wastewater and plant species.

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4.3 Publication III: Diversity of ammonia oxidizing bacteria in a vertical flow constructed wetland

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Abstract

Vertical flow constructed wetlands (VFCWs) with intermittent loading are very suitable for nitrification. Ammonia oxidizing bacteria (AOB) are the limiting step of nitration. Therefore the AOB community of a full-scale VFCW, receiving municipal wastewater, was investigated within this study. The diversity of the functional gene encoding the α–subunit of the ammonia monooxygenase (*amoA*), present only in AOB, was assessed by denaturing gradient gel electrophoresis (DGGE). Only very few *amoA* sequence types dominated the wetland filter substrate, nevertheless a stable nitrification performance could be observed. During the cold season the nitrification was slightly reduced, but it has been shown that the same AOB could be identified. No spatial AOB pattern could be observed within the filter body of the VFCW. The most prominent bands were excised from DGGE gels and sequenced. Sequence analyses revealed two dominant AOB lineages: *Nitrosomonas europaea* / "Nitrosococcus mobilis" and *Nitrosospira*. Species of the *Nitrosomonas* lineage are commonly found in conventional wastewater treatment plants (WWTPs). In contrast, members of the *Nitrosospira* lineage are rarely present in WWTPs. Our observations indicate that the AOB community in this VFCW is similar to that found in horizontal flow constructed wetlands, but differs from common WWTPs regarding the presence of *Nitrosospira*.

Keywords

amoA, bacterial diversity; constructed wetland; denaturing gradient gel electrophoresis (DGGE); *Nitrosomonas*; *Nitrosospira*

4.3.1 Introduction

Wastewater treatment by constructed wetlands (CWs) is a low cost technique which achieves treatment efficiencies comparable to conventional wastewater treatment plants (WWTPs). CW technology has been developed in the seventies and since then many investigations regarding the efficiencies and reliability of the system have been conducted (e.g. Kadlec et al., 2000). However, limited information on the characteristics of the microbial community is available.

It is well known that the elimination of easily degradable organic wastewater compounds is a consequence of a combination of chemical, physical and biological processes. The main force that propagates the transformation and mineralization of degradable organic pollutants is done by microbes. Transformation and elimination of nitrogen compounds is one of the major goals of wastewater treatment plants. Due to the optimal oxygen conditions in the filter body of an intermittently loaded vertical flow constructed wetland (VFCW) ammonium is converted to nitrate by nitrifying bacteria. The limiting step of this process is ammonia oxidation to nitrite by ammonia oxidizing bacteria (AOB) which is subsequently oxidized to nitrate. Because anoxic zones with sufficient organic carbon to promote heterotrophic denitrifiers is generally lacking in VFCWs, nitrogen is emitted into the environment mainly as

nitrate. Since AOB are key players in nitrification in wastewater treatment process they were chosen for diversity analysis in VFCWs.

Cultivation-dependent analysis of environmental AOB diversity is time consuming due to the slow growth rates of these microorganisms (Purkhold et al., 2003). The diversity of the functional gene encoding the α–subunit of the ammonia monooxygenase (*amoA*), only present in AOB has been used and proved to be a suitable marker for AOB in several soil analysis (e.g. Avrahami et al., 2003).

Only two studies have looked at the diversity of AOB in horizontal flow CWs by DGGE (Ibekwe et al., 2003, Truu et al., 2005). Up to now, no work has been done to characterize the AOB and their spatial distribution in a VFCW. Therefore it was the aim of this study to identify the most important AOB in a VFCW to obtain a more detailed understanding of nitrogen degrading processes.

4.3.2 Materials and Methods

4.3.2.1 Description of the sampling site

Filter substrate samples were taken from a 2.5 years old experimental subsurface VFCW located in Ernsthofen (Lower Austria). The three beds of the wetland are planted with *Phragmites australis* and have a surface area of 18 m^2 each. The 50 cm deep filter body consists of sand with a grain size of 0.06-4 mm supported by a drainage layer made of coarse gravel with a diameter of 16-32 mm. Tracer experiments (with potassium chloride) revealed a hydraulic retention time of the system of 2.5-3 days. The VFCW beds are loaded four times a day with mechanically settled municipal wastewater. The hydraulic loading rate of filter bed two – the bed on which the investigations took place – was 43 L m^{-2} d^{-1}. The organic load applied was 27 g COD m^{-2} d^{-1} (i.e. a specific surface area of 3 m^2 per person). The temperature in the filter bed was measured online with Pt100 sensors in 10, 30 and 50 cm depth. In addition, the influent and effluent temperature, and the air temperature have been measured online.

4.3.2.2 Sampling

Samples from the filter layer were taken from three individual sampling points within filter bed 2. Samples from same depths were mixed for one composite sample. The sampling took place in November 2005 and in May 2006. The air temperature varied between 9.1℃ at sampling date one and 29.6℃ at sampling date two. Samples were taken from seven different depths of the soil (0-1 cm, 1-5 cm, 5-10 cm, 10-20 cm, 20-30 cm, 30-40 cm and 40-50 cm). Samples from deeper layers were taken by a drill corer, whereas samples from the three upper layers were collected by inserting a syringe cut at the top. Filter substrate samples were taken, sieved (pore size < 2mm) and stored at -20℃ until DNA extraction. Additionally, wastewater samples were taken for chemical and microbiological analyses.

4.3.2.3 Chemical and microbiological analyses of the wastewater

The chemical analysis of the influent and effluent were done on a weekly basis using cuvette tests. The following parameters have been analyzed: biological oxygen demand in 5 days (BOD_5), chemical oxygen demand (COD), and the nitrogen compounds ammonium, nitrate and nitrite. The number of heterotrophic bacteria (HPC) was determined by the pour plate

method with yeast extract agar. Total Coliforms (TC) and *Escherichia coli* (*E. coli*) were enumerated by the membrane filtration method using Chromocult agar. For Enterococci (EC) determination the membrane filters were incubated on Slanetz and Bartley agar. All culture media were purchased from Merck, Darmstadt, Germany.

4.3.2.4 Measurement of TOC and pH in the substrate samples

Total organic carbon (TOC) of the filter substrate samples was measured by combustion at 900°C with a Vario Max C/N-analyzer (Elementar, Hanau, Germany). The pH was measured with a pH-probe after a 24 hour extraction of 10 g of filter material with 25 ml of 0.01 M $CaCl_2$.

4.3.2.5 Molecular biological analyses in the filter substrate

DNA extraction

DNA extractions for filter substrate samples were performed with an UltraClean Power Soil DNA kit (MoBio Laboratories, Inc. Carlsbad, CA, USA) by the bead beating method (FastPrep™ FP120, Bio-101, Vista, CA, USA) following the manufacturer's recommendations. Cell lysis was performed at a speed setting of 5.5 m s^{-1} for 25 s. Extractions of the 2nd sampling date were performed in duplicates and the DNA was pooled afterwards. DNA was checked on a 1.5 % agarose gel to see if enough DNA was present. Extracted DNA was stored at -20°C. Dilutions (10 and 100 fold) of extracted crude DNA were used as templates for subsequent PCR amplification.

Polymerase chain reaction (PCR)

PCR amplification was performed in a total volume of 50 µl by using Thermocyler TGradient from Biometra (Göttingen, Germany). PCR reaction mixes and thermal profile applied have been described previously (Hornek et al., 2005). Following primer combinations were used to analyse the AOB: amoA-1F-GC (Clamp-GGGGHTTYTACTGGTGGT (Stephen et al., 1996) in combination with amoA-2R (CCCCTCKGSAAAGCCTTCTTC (Rotthauwe et al., 1997) or inosine (I) containing reverse primer amoAr-i (CCCCTCIGIAAAICCTTCTTC (Hornek et al., 2005). No-template-control PCR reactions were performed using ultra-pure water (Merck, Darmstadt, Germany). All PCR products were analyzed by agarose gel electrophoresis.

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE analyses of PCR products were performed applying the D-gene system (Biorad, Hercules, CA, USA). Polyacrylamide gels (8 % of a 37:1 acrylamide - bisacrylamide mixture in 0.5 × TAE buffer) were made with a gradient maker (Biorad) according to the manufacturer's guidelines. Denaturing gradients (100 % defined as 7 M urea and 40 % formamide (Muyzer et al., 1993)) of urea and formamide were ranging from 0 % to 70 %. Gels were run for 4.5 hours at 200 V in 1 × TAE buffer at a constant temperature of 55°C. The gels were stained for 30 minutes in 1 × TAE buffer containing a 1:10.000 dilution of SYBR green (Molecular Probes Europe, Leiden, Netherlands).

Sequencing of DGGE bands

Selected bands were recovered, sequenced and currently submitted to BLAST (http://www.ncbi.nlm.nih.gov) to allocate available partial amoA sequences. Sequences were deposited at GeneBank under following accession numbers: DQ812982 and DQ812983.

4.3.3 Results and Discussion

4.3.3.1 Chemical and physical conditions in the filter substrate

The pH ranges from 6.1 in the 0-1 cm stratum to 7.7 in 40-50 cm. TOC ranges from 26 mg g $DWsoil^{-1}$ (DW = dry weight) in the top layer and decreases with depth down to 0.3 mg g $DWsoil^{-1}$ in the deepest layer. TOC accumulates especially in the 0-1 cm layer due to the high amount of biomass derived from the plants and from deposition of suspended solids from wastewater. As a result of the relatively warm influent (minimum temperature during the winter was 5.4℃) the temperature in the filter body never fell below 1.5℃. Due to the intermittent loading modus enough oxygen is able to diffuse into the filter material to provide optimal conditions for nitrification.

4.3.3.2 Removal efficiencies of carbon- and nitrogen-compounds and bacteria

Chemical analysis of the wastewater was performed weekly between September 2005 and June 2006 (table 4.9). The treatment efficiency was high for C-compounds, ammonium-nitrogen and bacteria (table 4.10). During the winter season the ammonium removal decreased from 98.7 % during the warmer period to 84.5 % during winter. These removal efficiencies are comparable with other VFCW (e.g. Weedon, 2003).

Table 4.9: Mean values (MV) ± standard deviation (SD) and maximum and minimum values of chemical properties of the used wastewater.

	Influent				Effluent			
	n	MV±SD	Max.	Min.	n	MV±SD	Max.	Min.
Temp. (℃)	40	11.0±4.1	18.8	5.4	48	8.3±4.5	16.7	2. 6
BOD_5 (mg L^{-1})	39	343±62	460	180	33	10±18	86	2
COD (mg L^{-1})	40	513±97	630	268	34	32±32	153	18
NH_4-N (mg L^{-1})	40	60.9±10.9	74.5	36.1	34	6.8±15.1	52.9	0.02
NO_2-N (mg L^{-1})	40	n.d.*	0.02	n.d.*	34	n.d.*	0.54	n.d.*
NO_3-N (mg L^{-1})	40	n.d.**	0.53	n.d.**	34	51.9±18.3	71.6	6.4

* all values below the detection limit of <0.015 mg NO_2-N L^{-1};
** all values below the detection limit of <0.1 mg NO_3-N L^{-1}

Table 4.10: Medians of removal efficiencies of chemical and microbiological characteristics.

	HPC (log)	***E. coli*** (log)	**TC** (log)	**EC** (log)	**BOD_5** (%)	**COD** (%)	**NH_4-N** (%)
Removal efficiency	2.81	3.53	3.38	3.66	97.0	93.8	88.8

4.3.3.3 DNA extraction and PCR

Filter substrate samples of the VFCW were taken from various depths. PCR-amplifiable DNA was gained, except for the lowest layer at a depth of 40-50 cm taken in November. For the investigation of the spatial pattern of the AOB from the VFCW, the functional gene, *amoA* was used. The most common primer set targeting the *amoA* gene (amoA-1F and amoA-2R) produced no amplicon for the VFCW samples. By using the primer combination amoA-1F and the inosine containing primer amoAr-i a specific PCR-product of correct length (approx. 500 bp) could be obtained. Both primer sets target the same position of the *amoA* gene, but the amoA-2R contains degenerate bases and amoAr-i contains universal bases. Depending on the AOB present in the investigated environment one primer set works better than the other. PCR products obtained with primer combination amoA-1F-GC and amoAr-i were further subjected to DGGE analysis.

4.3.3.4 Analysis of filter substrate samples by DGGE

Although all AOB use ammonia as their sole energy source the distribution patterns of distinct species depend on their physiological properties determined by environmental parameters (Koops and Pommerening-Roser, 2001). Consequently, we investigated which AOB might be responsible for ammonium consumption in this VFCW. Two sampling campaigns (November and May) were started (a) to investigate probable nitrification problems due to low temperature in November and (b) to test the reproducibility independently. DGGE analyses allows to separate PCR products of the same length depending on their base composition. The DGGE band pattern obtained from samples from different depths of the filter body is shown in figure 4.11.

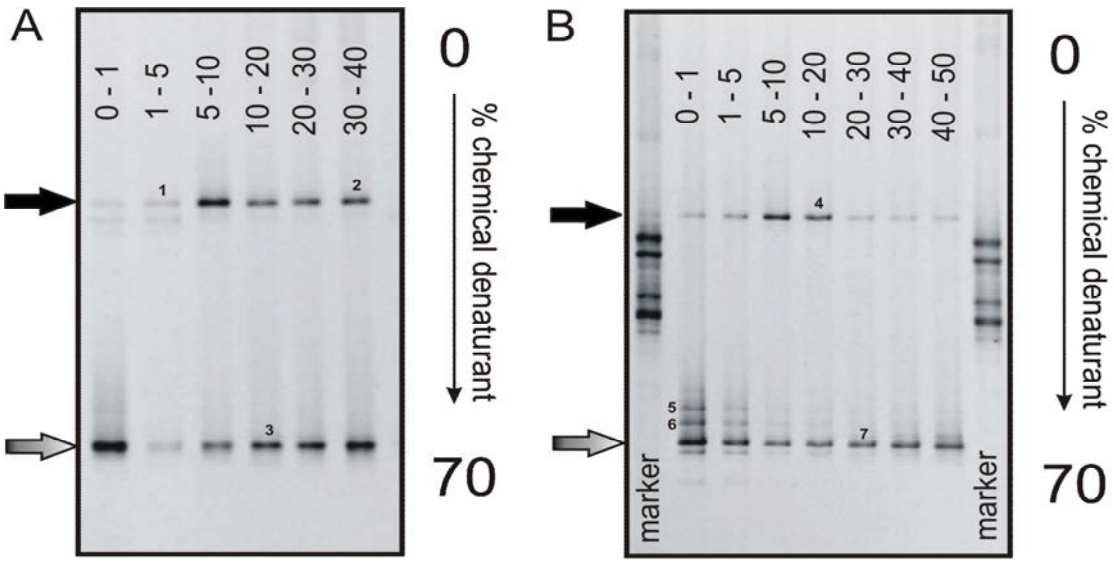

Figure 4.11: DGGE of *amoA* amplicons from substrate samples of various depths (in cm) of the VFCW in Ernsthofen (conditions: 8 % PAA, denaturant 0-70 %, 4.5 h, 200 V, 55℃ buffer temperature). Analyzed samples from November (A) and May (B). Numbers indicate sequenced bands (table 4.11).

Generally, only very few dominant AOB (2-7 bands) seemed to inhabit the filter body of the CW. The band pattern at a depth lower than 5 cm revealed only two bands at both sampling periods, in contrast the top layers (0-1 cm and 1-5 cm) showed more than two bands. Surprisingly, identical mobility of the two most dominant *amoA* sequences (figure 4.11; indicated by black and grey arrows) was observed throughout the various depths at both time points. Both dominant *amoA* bands were present independent of filter substrate depth and sampling date. This indicates that AOB are present under varying environmental conditions including colder temperatures. Further, the presence of AOB in the deeper zones of the main layer indicates that aerobic conditions are prevailing in the whole filter.

To retrieve some basic phylogenetic information on the AOB community structure DGGE banding patterns of the *amoA* gene can be used (Nicolaisen and Ramsing, 2002). The spreading of the bands in the gel is due to the *amoA* sequences obtained from different organisms which differ in their base composition. The uppermost bands (figure 4.11; black arrow) are *amoA* sequences derived from species of the *Nitrosomonas* lineage. The second dominant bands (figure 4.11; grey arrow) are likely to be a *Nitrosospira* – like sequences. To confirm this assumption seven dominant bands (figure 4.11) were excised and sequenced.

All sequences retrieved with the *amoA* primers fell into the beta-AOB lineages (table 4.11). The sequences can be assigned to the *Nitrosomonas europaea* “Nitrosococcus mobilis” lineage and to the “Cluster 0” of the *Nitrosospira* lineage. Band 5 and 6 produced no legible sequences. It can only be assumed that they belong to the lineage of *Nitrosospira*.

Table 4.11: Occurring AOB lineages in the VFCW.

Band (Fig. 1.)	Sequence affiliations to cultured AOB	AOB Lineage	Ecophysiological parameters of AOB lineage*	AOB lineage commonly found in*/**
1, 2, 4	*Nitrosococcus* sp. Nm107[#] DQ812982[$]	*Nitrosomonas europaea* / "Nitrosococcus mobilis"	halotolerant or moderate halophilic; no urease activity; high affinity for ammonia (30-61 µM)	eutrophic aquatic environments like e.g. Wastewater treatment plants
3, 7	*Nitrosospira* sp. Nsp5[##] DQ812983[$]	*Nitrosospira* "Cluster 0"	no salt requirement	soil and freshwater

according to *(Koops and Pommerening-Roser, 2001) and **(Koops et al., 2003);
BLAST search: [#] E value = 0, Score 837, Identities 98 %; [##] E value = 4e-138, Score = 498 bits, Identities 95 %;
[$] accession number

Further allocation of available partial *amoA* sequences (band 1, 2 and 4) revealed sequence affiliation to the *amoA* sequence of cultured AOB *Nitrosococcus* sp. Nm107 from activated sludge obtained from a rendering plant (Purkhold et al., 2000). Molecular and cultivation-based studies have shown that *Nitrosomonas* species are commonly present in WWTP, (e.g. Koops et al., 2003). In contrast species of the *Nitrosospira*-lineage occur rarely in WWTP and seem to play a minor role in the nitrification process.

In addition to *Nitrosococcus* sp. Nm107, also *Nitrosopira sp*. Nsp5 related sequence affiliations were found (band 3 and 7) in the investigated VFCW. These results are in accordance with previous phylogenetic analyses of cloned *amoA* sequences obtained from a horizontal flow bed (Ibekwe et al., 2003). They demonstrated sequence affiliations within the *Nitrosomonas europaea* /"*Nitrosococcus moblis*" and the *Nitrosospira* lineage Cluster 0 and 3. Others found 16S rRNA sequences affiliated with the *Nitrosospira*-lineage Cluster 0 in horizontal beds of wetland plants (Abd El Haleem et al., 2000).

4.3.4 Conclusions

It has been hypothesized that a diverse ammonia oxidizing bacteria (AOB) community may be critical for maintaining stable nitrification (Daims et al., 2001) in wastewater treatment plants or constructed wetlands. In this study only very few *amoA* sequence types dominated the CW, nevertheless a stable nitrification performance could be observed. AOB can nitrify ammonium in the wastewater of the VFCW. This VFCW nitrifies also under colder conditions and it has been shown that the AOB community diversity was not influenced by strong temperature changes.

Our data indicate that AOB belonging to *Nitrosomonas europaea* / "Nitrosococcus mobilis" and to *Nitrosospira* lineage were well established in the filter body of the VFCW up to a depth of 50 cm. According to the studies of Ibekwe et al. (2003) and Abd El Haleem et al. (2000) it could be demonstrated that the AOB community of VFCW is similar to horizontal flow CWs.

Detected AOB may account principally for the good nitrification as indicated by the observed high ammonium removal rates. But recently it was shown that the ability to oxidize ammonia is not only restricted to bacteria but could be also accomplished by specific archaea (Crenarchaeota) (Könneke et al., 2005). Truu et al. (2005) found a very high diversity of archeae in a horizontal flow CW which could be further evidence for the importance of archaea in CW. More studies are required to reveal the possible importance and function of crenarcheota for wastewater treatment.

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5 Manuscript

5.1 Diversity of abundant and active bacteria in subsurface vertical flow constructed wetlands

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Abstract

Microorganisms are mainly responsible for the transformation and mineralization of degradable organic pollutants within constructed wetlands (CWs). There is still a lack of knowledge considering microbial community composition within CWs. In order to elucidate the diversity of bacteria inhabiting subsurface vertical flow CWs, the molecular fingerprint technique “Terminal Restriction Fragment Length Polymorphism” (T-RFLP) derived from total community DNA, was applied. By the construction of a bacterial clone library, based on the 16S rRNA, highly active community members were identified. A comparison of the bacterial communities from a full-scale outdoor vertical flow CW with planted and unplanted indoor pilot-scale vertical flow CWs, running under similar conditions, revealed that both systems are colonized by similar populations showing only little variation in their composition over filter depth. The most abundant active bacteria belonged to the Actinobacteria and Betaproteobacteria as well as to unknown bacterial phylogenetic groups. A comparison of bulk soil from an unplanted CW with the rhizosphere soil from the planted CWs revealed differences in the bacterial composition, demonstrating the influence of the plants on the rhizosphere community. A comparison of the wastewater before and after the CW passage demonstrated, that the bacterial diversity was clearly reduced only within the planted outdoor system.

Keywords

Vertical flow constructed wetlands, bacterial community composition, active populations, 16S rRNA, T-RFLP, clone library

5.1.1 Introduction

Constructed wetlands (CWs) have received increased attention and are implemented in a variety of geographic regions as a result of the wide range of benefits in creating and maintaining wetlands. The potential for achieving improved water quality while creating valuable wildlife habitat has lead to a growing interest in the use of constructed wetlands for treating and recycling wastewater. CWs are engineered systems which have been modelled on natural wetlands taking advantage of the processes by which nutrients are decomposed and retained. By designing a CW it is important to create an environment which is optimal for processes responsible for the wastewater purification. The complex microbial community mainly associated with the filter material and roots or rhizomes, developed in interaction with the wastewater, is mainly responsible for the purification performance of the system. Investigations of microbial community composition and diversity in natural or created habitats

are important for characterization of such habitats, since microbes are key players in many environmental processes (Nogales et al., 2001). Therefore a detailed characterization of the microbial community is of great interest for a better understanding of the requirements of microorganisms, which will help to lead to the development of optimized systems.

Molecular fingerprinting techniques to characterize microbial communities, like “Denaturing Gradient Gel Electrophoresis“ (DGGE) (Muyzer et al., 1993), “Terminal Restriction Fragment Length Polymorphism” (T-RFLP) (Liu et al., 1997), or “Single Strand Conformation Polymorphism (SSCP) (Schwieger and Tebbe, 1998), are culture-independent tools, which have been applied to analyse the bacterial diversity in a wide range of environmental habitats. These techniques can be used to generate profiles of the total bacterial community (based on the amplification of phylogenetic markers such as the 16S rRNA gene), or by amplifying functional genes the diversity of physiological groups, like ammonia oxidizing bacteria (e.g. Schmidt et al., 2007) or sulphate reducing bacteria (e.g. Sercu et al., 2006), can be characterized.

By sequence analysis of 16S rRNA genes the most dominant species inhabiting the system are identified resulting in a more detailed description of the community structure. Analyses of 16S rDNA libraries can reveal the existence of yet undescribed and uncultivated bacteria, which may play important roles in pollutant degradation or other community processes, which are necessary to provide sustainable conditions for the system.

DNA-based community analysis detects bacteria irrespective of their viability or metabolic activity (Sessitsch et al., 2002), whereas RNA-based sequence analyses are more suitable for describing the metabolically active members of a population, as the amount of rRNA produced by cells roughly correlates with the growth activity of bacteria (Wagner, 1994). Since metabolically active cells usually contain higher numbers of ribosomes than inactive cells, a 16S rRNA library generated from total extracted RNA is considered to reflect predominantly the diversity of the metabolically active members of the community (Nogales et al., 2001). It can be assumed that metabolically active bacteria contribute more to the processes responsible for wastewater purification than metabolically inactive bacteria.

Only few publications are available trying to elucidate the complex microbial diversity within the filter body of CWs created to purify municipal wastewater. Truu et al. (2005) for example analyzed the microbial community structure within a horizontal flow CW and found a decrease in bacterial diversity with increasing filter body depth. Ibekwe et al. (2007) assessed the bacterial diversity of a free water surface flow constructed wetland by DGGE in combination with a bacterial clone library, which revealed that Gamma-, Beta- and Deltaproteobacteria were the dominant groups within this system. Walsh et al. (2002) created a clone library based on 16S rDNA sequences derived from a surface-flow wetland, which showed that Proteobacteria were the dominating group in this system. Due to the great diversity within these systems other studies focussed on distinct functional groups relevant for the wastewater purification process like ammonia oxidizing bacteria (Tietz et al., 2007a, *see chapter 4.3*; Gorra et al., 2007), or methanotrophic bacteria (De Journett et al., 2007). However, due to the variety of CW designs and the different wastewater types no study dealing with the bacterial community composition within a subsurface vertical flow CW with intermittent loading receiving municipal wastewater has been published yet.

The efficiency of CWs concerning the removal of microorganisms, especially fecal indicator bacteria, is a topic that has been thoroughly investigated by conventional culture-based plate techniques (e.g. Sleytr et al., 2007, see annex V). Vacca et al. (2005) analyzed the microbial diversity of Enterobacteriaceae before and after the passage of a pilot-scale subsurface vertical flow CW by PCR-SSCP and showed a strong removal of bacterial diversity after the CW passage, but with big taxon-specific differences. However, until now it has not been investigated, if bacteria found in the effluent of CWs, are either typical wastewater bacteria which pass the system, or bacteria which are washed out from the soil fraction of the CW. Therefore not only the bacterial diversity of the filter body has been investigated within this study, but also community fingerprints of the in- and outflows of the CW systems have been analyzed.

The major tasks of this study were to make a brief inventory of the most dominant and metabolically active bacteria inhabiting the filter material and to assess potential vertical distribution pattern of bacterial diversity within the filter body of a pilot-scale indoor and an outdoor full-scale subsurface vertical flow CW.

5.1.2 Material and Methods

5.1.2.1 Sampling sites

Samples were taken from a two years old experimental full-scale subsurface vertical flow constructed wetland (FSCW) located in Ernsthofen (Lower Austria). The plant consisted of three beds with a depth of 0.5 m and a surface area of about 18.5 m^2 each. All of them were planted with *Phragmites australis.* Additionally samples from a two years old indoor pilot-scale subsurface vertical flow constructed wetland (PSCW) were collected. Six of the eight PSCWs with a surface area of 1 m^2 each were planted with *Miscanthus sinensis giganteus*, whereas two beds were unplanted. The indoor as well as the outdoor plant are loaded four times a day with pre-settled municipal wastewater, with a total organic load of 20 g and 27 g COD m^{-2} d^{-1}, respectively. A detailed description of the two sampling sites is given elsewhere (Langergraber et al., 2007a, 2007b, see annex IV).

5.1.2.2 Sample collection

Samples for microbial community analyses were collected four times from different depths of the filter body of the full-scale outdoor plant and from the indoor PSCWs, respectively (table 5.1 and 5.2). Samples taken at any place from the filter body with no reference to plants are referred as “bulk soil” in contrast to samples, which are directly removed from roots and rhizomes (“rhizosphere soil”) of *Phragmites australis* and *Miscanthus sinensis giganteus*, respectively. Samples up to a depth of 10 cm were taken with a syringe (inner diameter 2.5 cm); samples from deeper layers were collected using a drill corer (inner diameter 10 cm). Additionally five times wastewater samples were collected from the in- and outflow of the PSCWs and four times from the FSCW, respectively.

Table 5.1: Overview of the sampling campaigns for analyses of the bacterial diversity of the indoor PSCWs.

Sampling Date	Sample name	Sample depth (cm)	Sample type	Planting	Air temp. (°C)
March 2005	PSCW 1	5-10	Bulk soil	*Miscanthus s. g.*	21
	PSCW 2	30-40	Bulk soil	-	
	Inflow 1		Wastewater		
May 2005	PSCW 3	5-10	Bulk soil	-	21
	PSCW 4	10-20	Rhizosphere soil	*Miscanthus s. g.*	
Nov 2005	PSCW 5	10-20	Rhizosphere soil	*Miscanthus s. g.*	17
	PSCW 6	1-5	Bulk soil	-	
	Inflow 3		Wastewater		
	Outflow 2		Wastewater	*Miscanthus s. g.*	
Dec 2005	Outflow 4		Wastewater	*Miscanthus s. g.*	16
	Outflow 5		Wastewater	-	

Table 5.2: Overview of the sampling campaigns for analyses of the bacterial diversity of the outdoor FSCW.

Sampling Date	Sample name	Sample depth (cm)	Sample type	Planting	Air temp. (℃)
May 2005	FSCW 1	10-20	Rhizosphere soil	*Phragmites a.*	
	FSCW 2	5-10	Bulk soil	*Phragmites a.*	11
	Inflow 2		Wastewater		
	Outflow 1		Wastewater	*Phragmites a.*	
Nov 2005	FSCW 3	1-5	Bulk soil	*Phragmites a.*	
	FSCW 4	10-20	Rhizosphere soil	*Phragmites a.*	12
	Outflow 3		Wastewater	*Phragmites a.*	
Dez 2005	FSCW 5	0-1	Bulk soil	*Phragmites a.*	
	FSCW 6	1-5	Bulk soil	*Phragmites a.*	
	FSCW 7	5-10	Bulk soil	*Phragmites a.*	
	FSCW 8	10-20	Bulk soil	*Phragmites a.*	9
	FSCW 9	20-30	Bulk soil	*Phragmites a.*	
	FSCW10	30-40	Bulk soil	*Phragmites a.*	
	FSCW11	40-50	Bulk soil	*Phragmites a.*	
	Inflow 4		Wastewater		

DNA extractions were performed immediatly after sample taking (see chapter 5.1.2.3). For all samples listed in table 5.1 and 5.2 T-RFLP analyses were performed. Additionally the soil samples taken in May 2005 were used for the construction of a clone library. These samples were preserved immediately after sampling with RNAProtect Bacteria Reagent (Qiagen, Hilden Germany) and stored at 4℃ for RNA isolation . The other sample was stored at -20℃ until the DNA extraction was performed (*see chapter 5.1.2.5*).

5.1.2.3 Terminal restriction fragment length polymorphism (T-RFLP) of the community DNA

DNA extractions of soil samples (initial soil weight of 1g) were performed with the PowerSoil DNA isolation kit (MoBio Laboratories, Inc. Carlsbad, CA, USA) by the bead beating method (FastPrep™ FP120, Bio-101, Vista, CA, USA) following the manufacturer's recommendations. DNA from the water samples was extracted by using the Ultra Clean Water DNA Isolation Kit (MoBio Laboratories, Inc.). For wastewater analyses 50ml to 100ml of the inflow and 3l to 6l of the outflow, respectively, were filtered using the filters provided by the DNA isolation kit. The filters were cut into pieces and transferred into tubes for bead beating. After a centrifugation step the supernatant was processed according to the protocol of the manufacturer. The extracted DNA was checked on a 1.5 % agarose gel to control the amount and quality of the extracted nucleic acid. DNA- isolates were stored at -20℃ until further processing.

TRFLP analyses were performed according to a modified protocol after Hackl et al. (2004). The PCR to amplify 16S rRNA genes was performed by using the primers 8f (5′-AGAGTTTGATCMTGGCTCAG-3′) (Lane, 1991), labeled with 6-carboxyfluorescein (MWG Inc., Ebersberg, Germany) at the 5' end, and 1507r (5′-TACGGTTACCTTGTTACGACTT-3′) (Wilson et al., 1990). Reactions were done in a volume of 50 µl, which contained 10 to 20 ng of extracted DNA, 1x reaction buffer (Invitrogen / GIBCO Inc., Carlsbad, CA, USA), 200 µM (each) dATP, dGTP, dCTP, dTTP, 3 mM $MgCl_2$, 150 nM of each primer, and 2U of *Taq* DNA polymerase (GIBCO Inc.). Amplifications were performed in a PTC-100 thermocycler (MJ Research, Miami, FL, USA) with an initial denaturing step of 5 min at 95 ℃ followed by 30 cycles of 30 s at 95 ℃, 1 min of annealing at 54 ° C, and 10 min of extension at 72 ℃. Three

amplification products were prepared from each DNA sample and were then pooled to reduce PCR bias. 200 ng of fluorescently labeled PCR amplification products were digested with the restriction enzyme *AluI* (GIBCO Inc.) and subsequently purified by passage through Sephadex G-200 columns. Aliquots of 5 µl were mixed with 2 µl of loading buffer (diluted five times in deionized formamide; Sigma-Aldrich; St. Louis, USA) and 0.3 µl of the DNA fragment length standard (Rox 500; PE Applied Biosystems Inc., Foster City, CA, USA). Mixtures were denatured for 2 min at 92℃, and fluo rescently labeled TRFs were then detected using an ABI 373A automated DNA sequencer (PE Applied Biosystems Inc.) in the GeneScan mode. Lengths of labeled fragments were determined by comparison with the internal standard using the GeneScan 2.5 software package (PE Applied Biosystems Inc.).

5.1.2.4 Analyses of T-RFLP profiles

The DNA quantity was analyzed for twenty-six T-RFLP community profiles. Due to a low quantity of peaks two profiles were eliminated (PSCW 2 and outflow 3). The remaining twenty-four profiles were compared and standardized to the lowest quantity, according to the method of Dunbar et al. (2001). Terminal restriction fragments (T-RFs) of 50 to 500 bp in length and with heights of ≥ 50 fluorescence units were included in the analysis. T-RFs that differed by less than 0.5 bp in different profiles were considered identical and were clustered. Fragment length and peak height were used as parameters for profile comparison.

The phylotype richness (S) was calculated from standardized profiles of individual samples as the total number of distinct TRF sizes from 50 to 500 bp according to Dunbar et al. (2001). Numbers of T-RFs with intensities higher than ≥ 500 FU were designated as highly abundant T-RFs.

5.1.2.5 Construction of a clone library based on the 16S rRNA

From the fixed out- and indoor CW samples the extraction was accomplished with the RNeasy Mini Kit (Qiagen Inc.) according to the protocol of the manufacturer. Samples were first lysed and homogenized in the presence of a buffer, which immediately inactivates RNases to ensure isolation of intact RNA. Before elution of the RNA with RNAase free water 27 U DNAase I per RNeasy mini column were added to degrade the DNA of the extracted nucleic acids. For reverse transcription (RT-PCR) the Revert RevertAid First Strand cDNA Synthesis Kit (Fermentas Inc.) was applied. The resulting cDNA was used as template for a PCR with the forward primer 8f and the reverse primer 1507r to amplify 16S rRNA genes. PCR reactions were performed on a Mastercycler gradient (Eppendorf Inc., Hamburg, Germany) and started with 15 min of 95℃ for the ac tivation of the HotStar Taq polymerase (Qiagen Inc.), followed by 31 cycles each consisting, of 40 sec for 94℃, 45 sec for 50℃ and 70 sec at 72℃ for denaturation, annealing, and ext ension steps, respectively.

The 16S rRNA PCR products were cloned into pGEM-T Easy Vector System II (Promega Inc., Madison, WI, USA). Ligation products were transformed into competent *E. coli* JM109 cells according to the protocol of the manufacturer. About hundred colonies per sample (if available) were collected and suspended in ultra-pure, sterile water and boiled for 10 min to release DNA followed by centrifugation and stored at -20℃. A PCR with the pUC / M13 forward primer (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') and the pUC / M13 reverse primer (5'-TGCAGGCGGCCGCACTAGTGATT-3') for the amplification of small parts of the plasmid and the inserted 16S rDNA was performed as described above. PCR products where loaded on a 1.6 % agarose gel to check if they contained an insert of correct length.

5.1.2.6 Screening of the clone library by ARDRA (Amplified Ribosomal DNA Restriction Analysis)

For a screening of the library the ARDRA method was chosen to make a pre-selection of the clones, to select only different inserts for sequence analysis. A total of 347 clones with a correct insert size were used for the ARDRA. PCR products were digested with the restriction enzymes *Hin*6I (Fermentas Inc., St. Leon-Rot, Germany) and *Bsu*RI (*Hae* III) (Fermentas Inc.) individually. After incubation for 4-5 h at 37°C the fragments were separated by electrophoresis for 60 min at 80 V in 1.6 % agarose gels and the digestion patterns were visually analyzed for similarities. Clones with identical ARDRA patterns were grouped and were referred as ARDRA groups. Clones derived from the four samples were pooled, to represent the variety of the system CW.

5.1.2.7 Sequencing of 16S rRNA genes

From each of the thirty ARDRA groups containing at least two clones, one representative was chosen for a partial sequencing reaction by using the pUC / M13 forward primer. Additionally thirty-three clones with unique ARDRA patterns were selected randomly for sequencing. Sequencing was performed on a sixteen capillary sequencer ABI PRISM 3100 genetic analyzer (Applied Biosystems Inc.) by using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc.) according to the instructions of the manufacturer. A BLAST search of the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) identifying the nearest, mainly cultured relatives of the sequenced clones has been conducted with partial 16S rRNA sequences. Sequences that did not associate with any of the sequences deposited in the NCBI database were defined as “unknown” taxa.

5.1.2.8 Prediction of theoretical terminal fragment lengths (T-RFs) from 16S rRNA sequences

For sequences that could be assigned to a phylogenetic group by performing a BLAST search the theoretical fragment length of their corresponding T-RF was calculated by determining the number of base pairs from the beginning of the sequence (forward primer) to the restriction site of the tetrameric restriction enzymes *Alu*I.

5.1.2.9 NCBI accession numbers

The sequences were deposited at GeneBank under accession numbers EU370806 to EU370868.

5.1.3 Results

5.1.3.1 T-RFLP community profiles from the filter body samples

24 T-RFLP community profiles derived from twelve bulk soil samples (ten planted and two unplanted), four rhizosphere soil samples and 8 wastewater samples were analyzed. The soil samples were used to investigate potential differences in bacterial diversity between the

different depth layers, between the pilot-scale indoor and the full-scale outdoor CW, and between rhizosphere soils compared to unplanted filter body samples. For the wastewater samples the impact of the CW passage on changes in bacterial diversity of the outflow was analyzed.

Phylotype richness (S) as shown in table 5.3 was calculated for the bulk soil samples (planted and unplanted), for the rhizosphere soil samples (indoor and outdoor) and for the wastewater samples (inflow and outflow). The phylotype richness (numbers of T-RFs with intensities ≥ 50 fluorescence units) ranged between 29 and 78t and peaks ≥ 500 FU varied from 0 to 6. With exception of a lower diversity in the uppermost layer of the outdoor plant (FSCW 5; 0-1cm), no differences between the individual depth layers and between the phylotype richness of rhizosphere soil and non-rhizosphere soil were detected. Between the outflows and the corresponding inflows no clear differences regarding the bacterial diversity were observed, with exception of a higher S in the unplanted indoor outflow compared to the corresponding inflow and a lower S in the outdoor outflow in comparison to the inflow.

Table 5.3: Phylotype richness (S) of the filter body samples (calculated from standardized fluorescence intensities).

Sample	Sample type	Depth (cm)	S	Σ P ≥ 500 (FU)[1)]
FSCW 5	Bulk soil, *Phragmites a.*	0-1	36	5
FSCW 3/6	Bulk soil, *Phragmites a.*	1-5	50/47	0/4
FSCW 2/7	Bulk soil, *Phragmites a.*	5-10	29/54	4/3
FSCW 8	Bulk soil, *Phragmites a.*	10-20	47	4
FSCW 9	Bulk soil, *Phragmites a.*	20-30	51	3
FSCW 10	Bulk soil, *Phragmites a.*	30-40	46	5
FSCW 11	Bulk soil, *Phragmites a.*	40-50	49	2
PSCW 1	Bulk soil, *Miscanthus s. g.*	5-10	49	2
PSCW 3/6	Bulk soil, unplanted	1-10	56/56	1/4
FSCW 1/4	Rhizosphere soil, *Phragmites a.*	10-20	37/40	6/2
PSCW 4/5	Rhizosphere soil, *Miscanthus s. g.*	10-20	63/49	1/4
Inflow 1/3	Wastewater indoor		35/40	4/5
Inflow 2/4	Wastewater outdoor		78/44	0/4
Outflow 1	Wastewater outdoor, *Phragmites a.*		39	2
Outflow 2/4	Wastewater indoor, *Miscanthus s. g.*		39/33	3/6
Outflow 5	Wastewater indoor, unplanted		58	2

[1)] Fluorescence units

Figure 5.1 shows the T-RFLP-profiles pooled from eleven outdoor samples (FSCW) and five indoor samples (PSCW) to enable a comparison of the two CW systems. In the FSCW profiles 73 peaks are present, whereas the PSCW profiles contain 68 peaks. 63 peaks occurred in the outdoor as well as in the indoor system, which implicates a similar community in both CWs. But the intensity of some peaks differed strongly between the out- and the indoor system. Whereas the peaks at 66, 102, 138, 162, 203, 204 and 252 bp showed a higher intensity in the PSCWs, the peaks at 67-74, 206, 207, 234-240, 250, 256 and 391 bp were more intense in the FSCW.

Samples from seven different depths (0-1, 1-5, 5-10, 10-20, 20-30, 30-40 and 40-50 cm) were used to investigate potential differences of TRF profiles over the depth of the filter body of the planted outdoor FSCW. Figure 5.2 shows three TRF profiles as examples to reveal potential diversity variances with increasing filter body depth; one from the uppermost layer (0-1 cm - FSCW 5), one from the middle of the filter body (10-20 cm - FSCW 8) and the deepest layer (40-50 cm - FSCW 11). The different T-RFLP patterns display similar results, except the uppermost layer of the filter body (FSCW 5), which shows higher T-RFs at 231,

249, 250, 273 and 391 bp. Nevertheless 21 of the 66 peaks within the 7 profiles occurred in all depths (e.g. the peaks between 69-74 bp and the peaks at 231, 233 and 236 bp). Additional 15 peaks were only absent in one or two depth layers. These results reveal a rather equal distribution pattern of the diversity over the depth of the filter body.

Figure 5.3 compares the T-RFLP-profiles pooled from two rhizosphere samples from the outdoor CW, with two rhizosphere samples from the indoor CW and with two samples taken from the unplanted PSCWs. These three sample types show some differences in their T-RFLP-profiles. Only twenty-two peaks of the 79 peaks occurring in any of the three profiles were present in all three sample types. The indoor rhizosphere profile shows higher peaks at 66, 138, 204, 225 and 250 bp, whereas in the outdoor rhizosphere profile higher peaks appear at 69, 231, 239 and 391 bp. In general higher peaks were found in the rhizosphere samples.

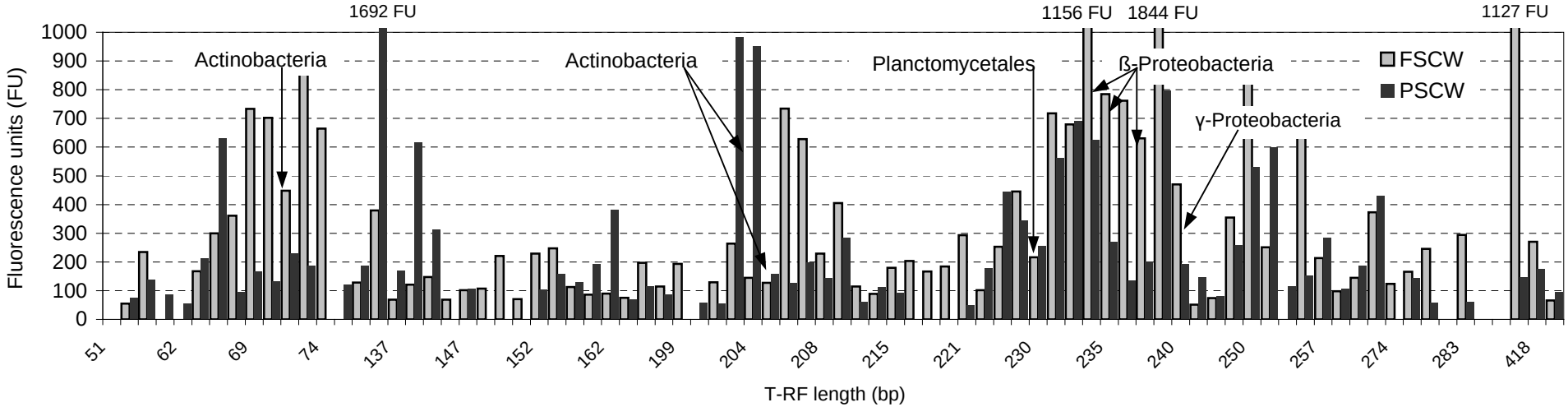

Figure 5.1: Comparison of the outdoor FSCW (*Phragmites australis*) and the planted indoor PSCWs (*Miscanthus sinensis giganteus*). (TRF profiles from outdoor and the indoor systems were pooled.)

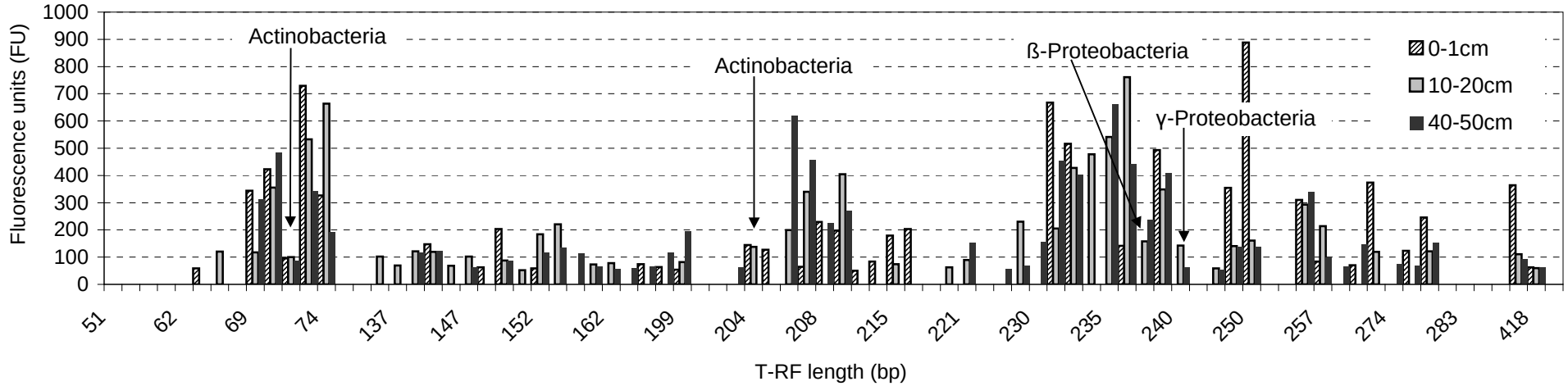

Figure 5.2: Comparison of bulk soil samples from the planted outdoor FSCW taken from 0-1 (FSCW 5), 10-20 (FSCW 8) and 40-50 cm (FSCW 11) depth.

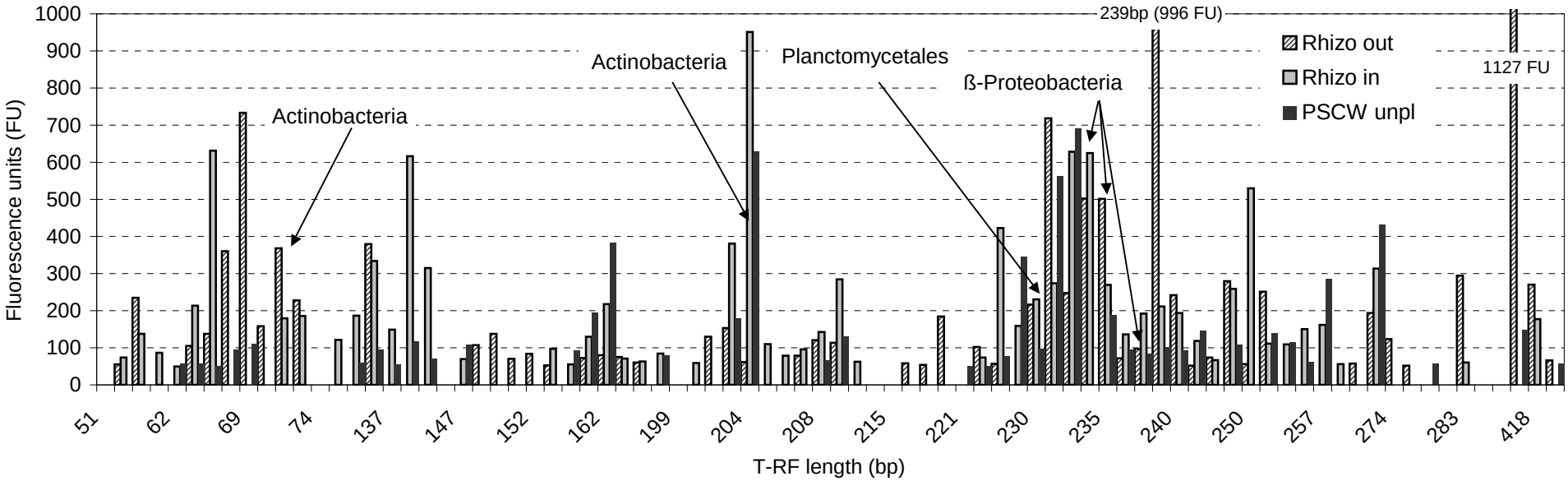

Figure 5.3: Comparison of rhizosphere soil from the planted outdoor CW (FSCW 1+4), the planted indoor CWs (PSCW 4+5) and the non-rhizosphere soil from the unplanted indoor CWs (PSCW 3+6). (TRF profiles from the corresponding sample types were pooled).

5.1.3.2 Sequences analyses of the 16S rRNA based clone library

From two samples of the full scale outdoor system (rhizosphere soil = FSCW 1, bulk soil = FSCW 2) and two samples of the pilot scale indoor system (rhizosphere soil = PSCW 4, bulk soil = PSCW 3) a clone library based on the 16S rRNA was constructed. 63 sequences consisting of 400 to 700 nucleotides were derived from partial sequencing of the clones originating from the four filter body samples. Table 5.4 summarizes the results of the BLAST analysis, which enables the phylogenetic assignment of the sequences.

Figure 5.4 shows a crude estimation of the active community composition of the constructed wetland samples obtained by calculating the proportion of each of the five phylogenetic groups based on the number of identified members of each group multiplicated by the number of representatives per ARDRA group. Actinobacteria (40 %) were the dominant, active phylogenetic group within this system, followed by Proteobacteria (17 %), Firmicutes (6%) and Planctomycetales (5%). 30 % of the community could not be assigned to any phylogenetic group (unknown species). Additionally the groups Chloroflexi and Bacteroidetes were detected.

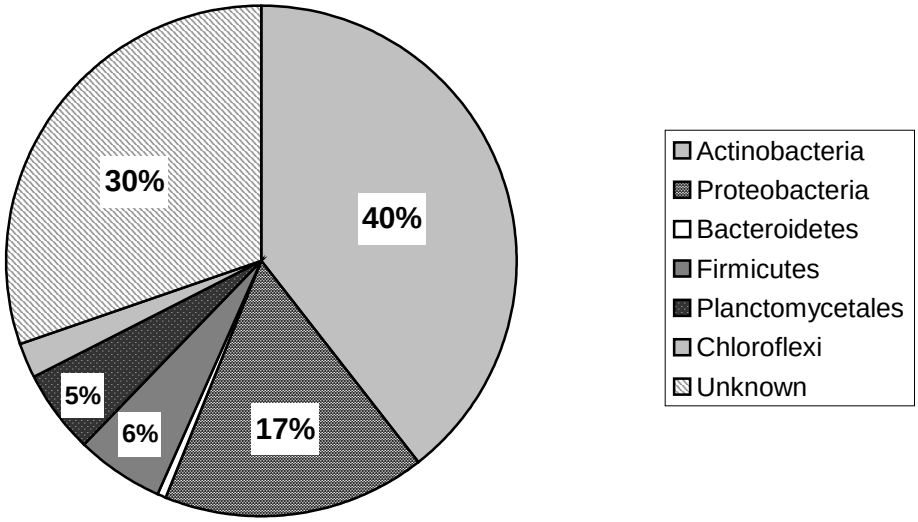

Figure 5.4: Composition of highly metabolically active bacteria present in the filter material of the constructed wetlands based on 16Sr RNA analysis.

The bacterial genera *Leucobacter, Nocardioides, Mesorhizobium, Leptothrix, Nitrospira, Thauera, Bacillus, Clostridium, Xanthomonas, Aeromonas* and *Nostocoida* have been identified with at least 95.5 % homology to their closest NCBI database match.

5.1.3.3 Affiliation of theoretically predicted fragment lengths to the T-RFLP profiles

Determining the theoretically predicted T-RF lengths (results are shown in table 5.4) of the clones which could be assigned to any phylogenetic group enables an identification of some peaks within the T-RFLP soil community profiles (this was only possible if the partial sequence included the region with the forward primer). In the T-RFLP soil community profiles (figure 5.1-5.3) the clones are assigned to their corresponding peaks according to their theoretical fragment length. These affiliations of phylogenetic groups to T-RF peaks showed

that peaks around 72 bp occurred in all samples with the exception of the unplanted PSCWs, and with a higher intensity in the outdoor profiles than in the indoor profiles (figure 5.1-5.3). These peaks are supposedly originated from Actinobacteria, which dominated the clone library. Also the peaks at 203/204 bp, which were present in all soil samples, but with a higher intensity within the indoor samples (figure 5.1), could be the result of the occurrence of Actinobacteria. The peak around 234 bp, present in all samples with the exception of the uppermost soil layer from the outdoor plant (FSCW 5 – 0-1 cm), presumably belongs to the Betaproteobacteria. Furthermore the peaks at 235-238 bp, which were also present in all samples with rather high intensities in the profiles derived from the outdoor plant, could belong to Betaproteobacteria. The peak at 239 bp, with a greater intensity in the outdoor samples than in the indoor samples, is a result of the existence of Proteobacteria (Beta- or Gammaproteobacteria). The peak at 240 bp, occurring in four of the eleven outdoor samples and in four of the five outdoor samples, seemed to be originated from Gammaproteobacteria. Additional peaks at 230/231 bp could be assigned to the phylum Planctomycetales.

Table 5.4: Assignment of phylogenetic groups and the closest sequence matches of clones from the four different constructed wetland samples and their predicted T-RF sizes.

Sample site	Predicted T-RF size (bp)	Closest relative / Accession Nr.	Identities (%)	No. of representatives / ADRA gr.	Phylogenetic group
FSCW 1, PSCW 3, PSCW 4	72	Uncultured Actinobacteria clone AKYH710 / AY921660	88-97	8, 4, 8	Actinobacteria
FSCW 2	72	*Leucobacter chromiireducens* / AJ781046	98	2	Actinobacteria
FSCW 1, PSCW 4	204	Uncultured Actinobacterium clone T4114 / EU029419	94-95	3, 1	Actinobacteria
PSCW 4	202	*Nocardioides sp.* / AF416746	96	2	Actinobacteria
FSCW 2	204	Uncultured Actinobacterium clone SI-2F_H07 / EF221417	92	4	Actinobacteria
FSCW 1	205	Uncultured Actinobacterium clone DOK_BIODYN / DQ828194	93	1	Actinobacteria
FSCW 1	-	Uncultured Actinobacterium clone SI-1F_B01 / EF221257	92	1	Actinobacteria
FSCW 1	-	*Mycobacterium nonchromogenicum* / X52928	95	8	Actinobacteria
FSCW 1	-	*Microbacterium oxydans* / DQ417333	95	3	Actinobacteria
PSCW 4, FSCW 1	-	Uncultured *Actinobacterium* clone FI-2F_D11 / EF220416	92-93	1, 5	Actinobacteria
PSCW 4, FSCW 1	-	Bacterium Ellin504 / AY960767	91-94	2, 7	Actinobacteria
PSCW 4	-	Uncultured Actinobacterium clone FB-2_E09 / EF220335	95	1	Actinobacteria
PSCW 4	-	Uncultured Actinobacterium clone AKYH1301 / AY921795	96	1	Actinobacteria
FSCW 1	-	Uncultured Rubrobacteridae bacterium clone EB1043 / AY395362	92	1	Actinobacteria
PSCW 3	-	Uncultured Actinobacterium clone FI-2F_B06/ EF220390	94	5	Actinobacteria
FSCW 1, FSCW 2	112	*Mesorhizobium sp.* R50 / EF690387	96-97	2, 1	Alphaproteobacteria
FSCW 2	-	Bacterium Ellin331 / AF498713	97	4	Alphaprotebacteria
PSCW 3	-	Alphaproteobacterium BAC29 / EU180509	98	1	Alphaprotebacteria
PSCW 3	-	Uncultured Alphaproteobacterium clone pGA021 / DQ146475	98	1	Alphaprotebacteria
FSCW 1	-	Uncultured *Candidatus Odysella sp*. / EU305601	90	1	Alphaprotebacteria
FSCW 1	-	Uncultured Bacteroidetes bacterium clone Skagen114 / DQ640739	93	1	Bacteroidetes
FSCW 2	155	*Nirospira marina* / X82559	96	2	Betaproteobacteria
FSCW 1	234	Uncultured *Leptothrix sp*. clone MoB-G4-114 / EF016846	96	1	Betaproteobacteria
FSCW 1	235	Uncultured Betaproteobacterium clone nsc054 / DQ211409	96	1	Betaproteobacteria

FSCW 2	238	Uncultured bacterium FukuN108 / AJ289984	96	3	Betaproteobacteria
FSCW 2	-	*Thauera terpenica* / AJ005818	96	2	Betaproteobacteria
PSCW 3	-	Uncultured Betaproteobacterium clone Fl-1F_E09 / EF220515	98	2	Betaproteobacteria
PSCW 3	-	Uncultured Burkholderiaceae bacterium / AM420125	95	2	Betaproteobacteria
PSCW 3	75	*Xanthomonas sp*. R-20819 / AJ786786	95	2	Gammaproteobacteria
FSCW 2	240	*Xanthomonas sp*. / DQ177466	96	2	Gammaproteobacteria
FSCW 1	-	Uncultured Gammaproteobacterium / AM905315	94	1	Gammaproteobacteria
PSCW 4	-	*Aeromonas hydrophila* / AF468055	98	1	Gammaproteobacteria
PSCW 3	205	Uncultured *Proteobacterium* clone / EF018749	93	2	Proteobacteria
PSCW 3	64	Uncultured Clostridiaceae bacterium / AB089030	92	2	Firmicutes
PSCW 3	74	*Bacillus* sp. SD43 / DQ462180	96	2	Firmicutes
PSCW 4	192	*Clostridium lituseburense* / M59107	98	2	Firmicutes
FSCW 1	-	*Clostridium* sp. clone EHFS1 / EU071510	96	1	Firmicutes
FSCW 2		Oscillospiraceae bacterium NML 061048 / EU149939	95	3	Firmicutes
PSCW 4	188	Uncultured Planctomycetacia bacterium clone SI-2M_E08 / EF221562	93	1	Planctomycetales
FSCW 2	214	Uncultured Planctomycetacia bacterium clone / AB265889	94	2	Planctomycetales
FSCW 1	230	Uncultured Planctomycetacia bacterium clone GASP-WC1S1 / EF074378	90	1	Planctomycetales
FSCW 2, PSCW 4	639	Uncultured Planctomycetacia bacterium clone AKYG1836 / AY921993	91-95	1, 2	Planctomycetales
FSCW 1, FSCW 2	-	*Nostocoida limicola III* strain Ben225 / AF244752	95-96	1, 1	Planctomycetales
PSCW 4	-	Uncultured Chloroflexi bacterium clone G33 / EF998952	93	7	Chloroflexi

5.1.4 T-RFLP community profiles from the wastewater samples

Figure 5.5a and 5.5b show the T-RFLP-profiles from the inflows and outflows of the PSCWs and FSCW, respectively. From the 40 peaks occurring in the indoor inflow only ten peaks were found in the planted outflow, whereas 15 of the 40 peaks were detected in the unplanted outflow (figure 5.5a). The bacterial diversity within the outflow of the planted PSCWs was not clearly reduced, and for the unplanted system even an increase of the diversity within the outflow was observed. On the other hand the T-RFLP-profiles from the in- and outflow of the outdoor FSCW displayed a clear reduction of the bacterial diversity after the filter body passage; from 78 peaks in the inflow to 39 peaks in the outflow, whereas only 18 peaks were identical within the in- and outflow of the FSCW (figure 5.5b). The very intensive peak from 70-73 bp was detected in all outflow profiles (in highest intensities in the FSCW outflow) and also in the FSCW profiles derived from the outdoor filter body samples (most abundant in the uppermost layer), but missing in the inflow samples. This fact suggests that this peak is originated from a typical soil bacterium, which was washed out from the filter body. Results from the predicted fragment lengths of the soil T-RFLP-profiles (table 5.4) show that the peak at 72 bp is probably originated from an Actinobacterium. The most intensive peak at 187 bp (<4,000 FU) from the indoor inflow has been not detected in any of the PSCW soil samples, which implicates that this peak is originated from a wastewater bacterium, which rapidly dies off within the filter body of the CW and therefore this peak was also not present in any outflow. Additionally the peaks at 233 and 239 bp, respectively (supposed to be originated from Betaproteobacteria), were also more intensive in the outflow of the unplanted and planted PSCWs, respectively, but almost not detectable in the inflow and therefore these peaks are supposed to be originated from bacteria typical for the filter body.

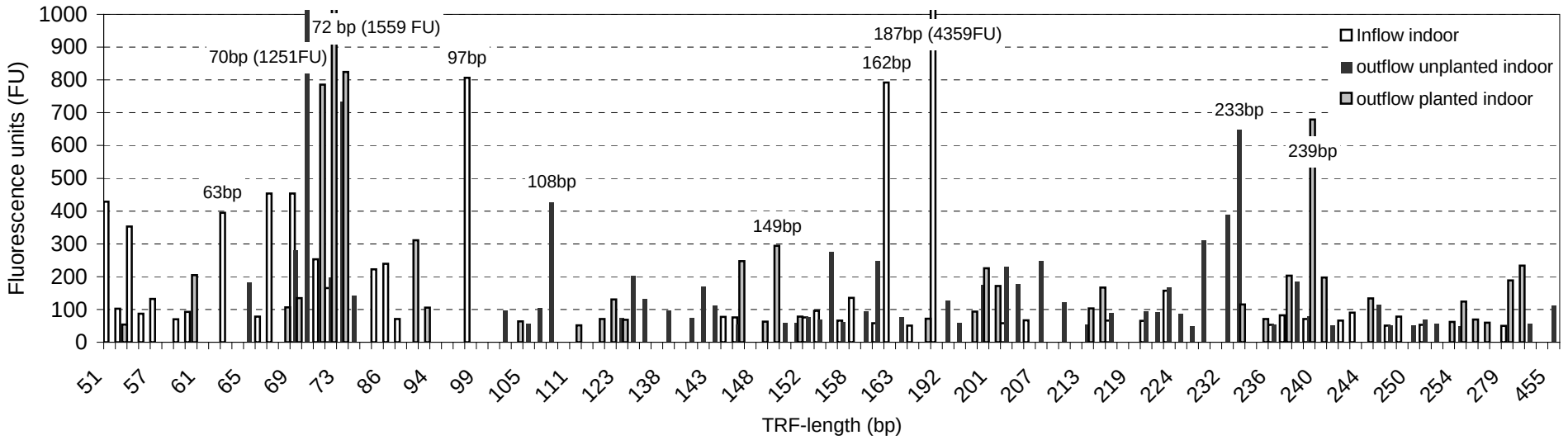

Figure 5.5a: Comparison of the inflow (inflow 3), the planted outflow (outflow 2) and unplanted outflow (outflow 5) from the indoor PSCWs.

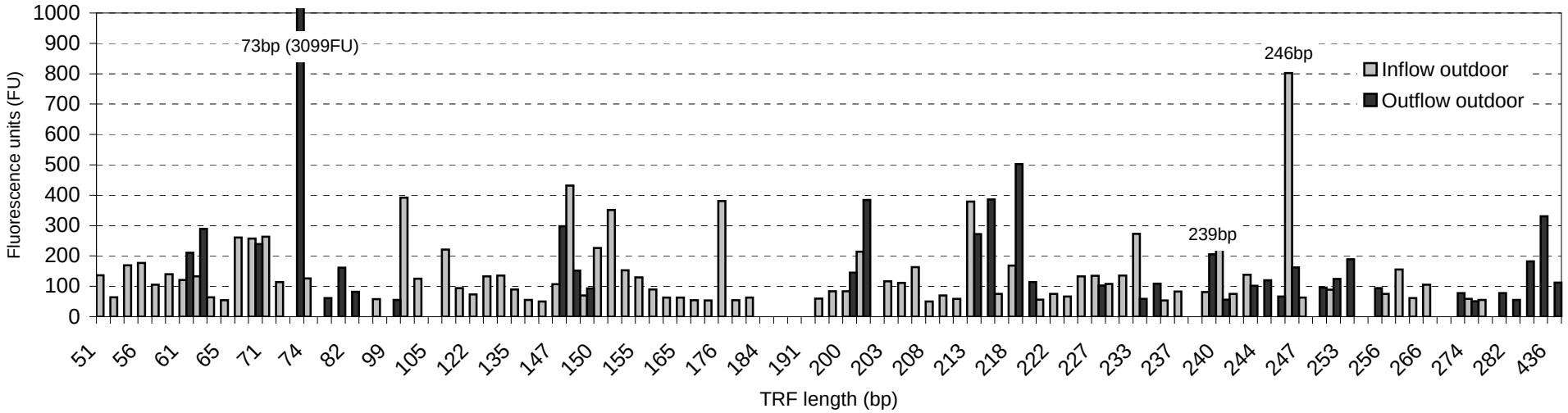

Figure 5.5b: Comparison of the inflow (inflow 2) and the outflow (outflow 1) from the outdoor FSCW.

5.1.5 Discussion

Community fingerprinting in combination with the identification of highly active community members offer a powerful way to investigate functionally important microorganisms of an environmental habitat. Fingerprint techniques provide information on the diversity but with a resolution, which is surely not satisfactory to describe the full microbial diversity in complex habitats (Smalla et al., 2007). However the rather high detection sensitivity of the T-RFLP method was demonstrated previously by Dunbar et al. (2000), showing that populations comprising between 0.1 and 1 % of a bacterial soil community could be detected in T-RFLP profiles. While T-RFLP-analysis has the advantage of analytic consistency and a high throughput capability (Hartmann and Widmer, 2006), clone libraries offer the highest degree of phylogenetic resolution available (Kent et al., 2003). RNA-based analyses allow the identification of actively growing bacteria further pinpointing to the functionally important fraction of a community.

Community profiles of the soil and wastewater samples obtained within this study demonstrate a rather high bacterial diversity, which is typical for complex environmental habitats. A comparison between the bacterial diversity of the filter body of the outdoor and the indoor plants revealed no clear differences (figure 5.1), although the two systems are exposed to different temperatures, are planted with different macrophytes, and despite that they both receive municipal wastewater, which has a different bacterial compositions (figure 5.5a/b). But despite these differences important chemical and physical factors, like nutrient-, oxygen- and water-content, pH and grain size distribution are very similar in both systems (unpublished data) and therefore favour the development of similar microbial communities. However, differences between the two rhizosphere soils (indoor and outdoor) were found, which were more pronounced than the variations between the rhizosphere samples in general and the unplanted soil (figure 5.3). This indicates an influence of the plant species on rhizosphere bacteria, which has been frequently reported in soils (e.g. Smalla et al., 2001; Kowalchuk et al., 2002). Nevertheless, Zul et al. (2007) reported clear differences in the community composition in soils from lysimeters without plants, compared to populations in planted lysimeter soils, whereas no influence of plant species composition on bacterial diversity could be discerned.

No correlation between the depth of the filter body the bacterial diversity have been observed, although for few bacteria a decrease or increase with filter body depth was observed (figure 5.2). Similarly, phylotype richness did not change with depth, with the exception of the 0-1 cm layer, which showed a reduced bacterial diversity (table 5.3). In a recent study it has been demonstrated, that more than 50 % of the microbial biomass and bacterial activity could be found in the first cm of the filter body of the PSCWs and about 95 % within the first 10 cm (Tietz et al., 2007b, *see chapter 4.1*). This indicates that although lower layers contain lower biomass, they are supposed to be composed of similar populations as the biomass in upper layers. In contrast to these results, Truu et al. (2005) found a higher bacterial diversity in the upper layers (0-10 cm) of a horizontal subsurface flow CW in comparison to the deeper horizon of the filter bed (50-60 cm).

The filter body of the CW can be regarded as a sink for bacterial species, but additionally it can be also a source for bacteria. The number of bacteria in wastewater is massively reduced by passing through the CW; bacterial removal rates range from 2.0 log units determined by total microscopic direct counts, up to 4.8 log units for Enterococci (Sleytr et al., 2007, see annex V). Nevertheless it seems that the diversity was not so strongly reduced; only for the planted outdoor system a clear reduction of the bacterial diversity between the in- and outflow was evident. Generally the removal efficiency is considered to be a result of a combination of chemical and physical factors, including mechanical filtration and sedimentation, biological mechanisms like antimicrobial activity of root exudates, predation by nematodes and protists, activity of lytic bacteria or viruses, retention in biofilms and natural die-off (Vacca et al., 2005).

Our study demonstrated that Actinobacteria and Betaproteobacteria are the dominant and physiologically active groups in the CWs analyzed. Furthermore, a high number of clones showed extremely low homologies to known bacteria, indicating that they belong to novel yet undescribed genera or species. The theoretically predicted fragment lengths of 72 and 204/205 bp from representatives of the phylogenetic phylum Actinobacteria, which dominated the clone library, were detected in all soil samples. Also the predicted fragment lengths of 234 and 238 bp, probably originated from Betaproteobacteria, the second most common phylum within the clone library; were found within the community profiles of almost all soil samples with partially very high intensities. The T-RFLP profiles were based on the 16S rDNA, whereas the clone library was based on the 16S rRNA, representing the physiological active part of the community. These results indicate that the most abundant active bacterial phyla (Actinobacteria and Betaproteobacteria) were also abundant in the community DNA. Nogales et al. (2001) prepared two clone libraries from a biphenyl-polluted soil; one from rRNA and one from rDNA, and reported that only about 29 % of the cloned sequences in the rDNA library were identical to sequences in the rRNA library. The phyla Actinobacteria and Betaproteobacteria are very common in soils (e.g. Gremion et al., 2003; Axelrood et al., 2002). Janssen (2006) analyzed the results obtained from 32 libraries of 16S rRNA and 16S rRNA genes, prepared from a variety of soils and summarized that the dominant phyla are occurring in the following ranking: Acidobacteria > Actinobacteria > Proteobacteria > Verrucomicrobia > Bacteroidetes > Firmicutes > Chloroflexi > Planctomycetales. With the exception of the absence of Acidobacteria and Verrucomicrobia all these phyla could have been detected within the filter body of the CWs investigated within this study in a similar composition. However, these groups, with the exception of Acidobacteria and Verrucomicrobia, are also the most abundant ones in conventional activated sludge wastewater treatment plants (e.g. Kapley et al. 2007). Considering the origin of the nearest mainly cultured relatives of the 44 sequenced clones, which could be assigned to a phylogenetic group by performing a BLAST search, 22 clones were originally isolated in soils, six clones were originated from wastewater treatment systems and two from rhizosphere samples. As CWs are hybrids between terrestrial and aquatic ecosystems but with extremely high loads of organic and inorganic nutrients, also the identified physiological active genera display the intermediate position of this habitat. The genera *Mesorhizobium* and *Nocardioides* are typical for soils, whereas *Leptothrix* and *Aeromonas* are common in meso- to euthrophic aquatic habitats. The genera *Nitrospira* and *Nostocoida* are typical for wastewater and activated sludge. These results imply that CWs are nutrient-rich soil-like systems, where also typical wastewater bacteria are physiologically active.

5.1.6 Conclusion

Our study showed that CWs running under similar conditions are colonized by similar populations, however microbial communities are influenced by the plants. While biomass decreases with depth of the filter body, microbial populations show little variation in their composition. The most important, actively growing community members belong to the Actinobacteria and the Betaproteobacteria, as well as yet unidentified bacteria. Further research will give more precise information on the behaviour of microbial populations with time or the effect of different wastewater qualities. Modern techniques such as stable isotope probing could link phylogenetic assignment with metabolic activity and give more information on the various microorganisms involved in wastewater purification.

5.1.7 Acknowledgements

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5.1.8 References

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6 Summary and conclusions

As with any technology, science is the foundation, and engineering makes sure that the technology works as designed (Moharikar et al., 2005). To obtain a more detailed knowledge of the microorganisms, mainly responsible for the purification performance of a CW, quantitative and qualitative aspects of the microbial community inhabiting the filter body of planted and unplanted indoor pilot-scale vertical flow constructed wetlands (PSCWs) and a full-scale outdoor plant (FSCW) were investigated. In the following chapter the results obtained within this study are summarized.

6.1 Treatment performance of the investigated VFCWs

The evaluation of the purification performances of the investigated CWs was not a main focus within this study; however they are important to ensure that the investigations of the microbial community were carried out on properly functioning wastewater treatment systems. Therefore the treatment performances of the outdoor and the indoor CWs are only summarized shortly:

During the whole investigation period the systems worked properly without relevant disturbances. Table 4.2 and 4.9 show the influent and effluent concentrations of the measured chemical and microbiological wastewater parameters for the investigated in- and outdoor systems, respectively. The influent-concentrations are slightly lower and more variable for the PSCWs than for the FSCWs, but all of them are in the typical range for mechanically pre-treated municipal wastewater. Additionally the corresponding removal efficiencies are listed within table 4.2 and 4.10 for the indoor and the outdoor system, respectively. The removal efficiencies for carbon compounds (monitored via the parameters COD, BOD_5 and TOC) were constantly high ranging from 94-98 % on average for the in- and the outdoor CWs. The removal rate for ammonium for the indoor plant was constantly high about 99 %, due to the stable nitrification performance of the plant. The outdoor system also displayed a stable nitrification rate varying from 84.5 % during winter to 98.7 % during the warmer period. The removal rates for total nitrogen within these systems were very low (about 8% for FSCW; 13% for PSCWs), due to the unfavourable conditions for denitrification in vertical flow CWs (the filter body displayed a good oxygen supply up to the deepest layers). For the indoor PSCWs additionally the removal rate for total phosphorus (TP) was monitored over three years after operation start. The TP-removal rate showed a strong decrease from 47 % in the first year to 30 % in the second year and to 0 % in the third year due to the diminished adsorption capacity of the filter material. For the indoor system there were no significant differences regarding the removal efficiency of the planted and unplanted indoor systems for all investigated parameters. (For an explanatory note about the comparison of the planted an unplanted PSCWs see chapter 6.2.)

In general the indoor systems as well as the investigated filter bed of the outdoor CW were able to fulfil the stringent effluent requirements given by the Austrian regulation for emissions from domestic wastewater for wastewater treatment plants designed for <500 persons (max. NH_4-N effluent concentrations of <10 mg L^{-1}, required only for a water temperature >12℃; COD and BOD_5-effluent concentrations of <25 mg $L^{-1}$and <90 mg L^{-1}, respectively) (1.AEVkA, 1996).

For a more detailed description of the treatment performance of the outdoor VFCW investigated within this study during different temperatures and under varying organic loads see Langergraber et al. (2007a).

6.2 Microbial and bacterial biomass distribution patterns in the filter body of vertical flow constructed wetlands

Four different approaches, three direct methods (ATP, C-FE and PLFA - analyzing specific compounds and fractions, of the microbial biomass) and one indirect method (SIR - measuring physiological activities of microorganisms), have been applied to quantify the microbial biomass in seven different depths of the indoor pilot-scale systems and the outdoor CW. Additionally the bacterial abundance and biomass was determined within the different depth layers by a direct microscopic approach.

The results of these investigations revealed high values for microbial biomass in the top 10 cm of the filter body due to the high nutrient content and the good oxygen supply. As shown in table 4.4 the three methods ATP, C-FE and SIR gave similar results, although they are based on completely different measurement principles and therefore comprise also different aspects of the microbial biomass (microbial / bacterial biomass, active / living / total biomass). For the results of the PLFA-analyses no correlations with other methods for the determination of biomass can be made, because the PLFA-content was not analyzed within the same samples.

Generally the SIR-method produced highest values, followed by the ATP-approach and the measurement of the C-FE, although the SIR-method comprises only the physiological active part of the microbial community. These differences are presumably a consequence of the conversion factors (CFs) taken from the literature to convert all results into µg biomass-C. These CFs, a result from microbial biomass measurements with different methods in various types of soils, are obtained by correlating different methods with one reference method for biomass determination (mostly the C-FE method is used as reference) (e.g. Anderson and Domsch, 1978). This CF-dilemma making the problem evident that no direct “perfect” reference method for biomass determination in soil-like ecosystems is available. Therefore absolute values have to be considered carefully and comparisons between different systems should be done only by non-converted results (ATP-concentrations, C-flush, PLFA-concentrations, oxygen demand / CO_2 production). A comparison of the results obtained from the ATP-, C-FE and the SIR-measurements with data from soils (see table 4.5) revealed that the microbial biomass of the filter material of the PSCWs is comparable to natural soil ecosystems, although the specific surface area of the used sandy filter material available for biofilm growth was lower, especially in the beginning of the set-up of the PSCWs, due to absence of fine particles (<0.06 mm). But the good nutrient and oxygen supply of the microbial community inhabiting the filter body of VFCWs (with intermittent loading) provides optimal conditions for the development of an abundant microbial population.

Despite the problems with CFs taken from the literature for a direct comparison of all four “microbial-biomass methods” a conversion into biomass-C is indispensable. For a conversion of the results from the ATP-, C-FE-, and the SIR-method see Tietz et al. (2007b; chapter 4.1). For the transformation of the PLFA-concentrations into biomass-C a factor, which was determined by Leckie et al. (2004) for mineral soil, as well as by Bailey et al. (2002) for 20 different soils, was applied:

$$1 \text{ nmol PLFA} \mathrel{\hat{=}} 2{,}4 \text{ µg biomass-C}$$

In the following the planted indoor and outdoor systems are compared regarding the distribution of microbial and bacterial biomass within the filter bodies.

The results from the four methods applied to measure the microbial biomass (MB) and the microscopic approach applied to determine the bacterial biomass (BB) showed a very similar strong decrease of total microbial and bacterial biomass with depth (see table 6.1, figure 4.2 and figure 4.2). In average 58-59 % of the total microbial biomass within the whole filter body of the planted PSCWs and the outdoor system were located within the uppermost cm. 94 %

and 88 % of the microbial biomass were situated within the first 10 cm of the PSCWs and the outdoor CW, respectively.

The bacterial biomass determined by microscopic counts and biovolume measurements of the cells accounted only for about 14% of the total microbial biomass in the first 10 cm of the filter body of the planted PSCWs and for about 35 % within the outdoor CW, respectively. In Tietz et al. (2007b, see chapter 4.1) slightly different values are given, because the results obtained from the PLFA-measurements, which were performed after publication, are not included in these calculations. One reason for the discrepancy between microbial and bacterial biomass can be the overestimation of microbial biomass, due to the not suitable conversion factors for the transformation of the results from the three different methods applied to measure the biomass into microbial carbon. But due to the fact that the four methods yielded rather similar absolute values for microbial biomass (differing on a factor of max. 2) it seems to be more realistic that the bacterial biomass has been underestimated due to methodical problems, like incomplete displacement of the cells from the filter material and incomplete disaggregation of bacteria / filter material complexes whereby substrate particles can mask bacteria. In contrast to the results obtained by the microscopic approach the measurements of bacterial PLFAs resulted in a more realistic percentage of about 44 % of bacterial PLFAs on total PLFAs for the planted indoor PSCWs and of 63 % for the outdoor CW, respectively. Calculating the proportion of bacterial biomass on total microbial biomass within the uppermost 10 cm of the planted filter bodies by combining the results from the PLFA measurements and the microscopic approach resulted in 17-31 % BB for the indoor system and 34-54 % BB for the outdoor system, respectively.

Tietz et al. (2007b, see chapter 4.1) hypothized that fungi could be mainly responsible for the difference between microbial and bacterial biomass, however Tietz et al. (2008, see chapter 4.2) demonstrated that fungi play only a minor role within this system. Although it has to be noted that the measurement of the PLFA 18:2ω6,9 as a fungal biomarker gives only a crude estimation of the fungal biomass within the system.

The proportion of microbial biomass-C on total organic carbon (TOC) showed a decrease with increasing filter body depth (with an exception of the 40-50 cm layer where the proportion increased again slightly). Within the three uppermost layers (0-10 cm) the microbial biomass accounted for 11-13 % of the total TOC within the planted PSCWs, and about 6-8 % in the outdoor system, respectively. These values decreased down to around 3 % in deeper layers of the in- and outdoor systems. Therefore it can be concluded that dead organic material (detritus, refractory material) accumulates slightly also in deeper regions of the filter body, although these accumulation was not detectable within this study (see table 4.6), due to a too low number of samples in combination with a high variation within the measured TOC-values.

Furthermore the TOC - content showed high correlations with microbial biomass (table 4.4), reflecting the indicator function of this rather simple parameter as a measure for microbial biomass within constructed wetlands.

Table 6.1: Microbial and bacterial biomass within the planted indoor PSCWs and the outdoor FSCW.

	Planted indoor PSCWs						Outdoor FSCW					
Depth	Microbial biomass*	Standard deviation	MB within depth layer	Bacterial biomass**	Standard deviation	BB within depth layer	Microbial biomass*	Standard deviation	MB within depth layer	Bacterial biomass**	Standard devotion	BB within depth layer
(cm)	(µg MB-C g DW^{-1})		%	(µg BB-C g DW^{-1})		%	(µg MB-C g DW^{-1})		%	(µg BB-C g DW^{-1})		%
0-1	1484	473	58.3	461	60	67.7	1005	293	58.5	543	284	68.5
1-5	618	285	24.3	138	20	20.3	346	108	20.1	159	73	20.1
5-10	303	173	11.9	52	4.0	7.7	167	130	9.7	56	34	7.0
10-20	42	27	1.7	24	8.5	3.6	64	44	3.7	20	12	2.5
20-30	26	32	1.0	2.4	0.4	0.4	28	32	1.6	5.5	7.8	0.7
30-40	17	19	0.7	1.4	0.7	0.2	41	34	2.4	4.0	5.6	0.5
40-50	55	72	2.2	1.6	0.1	0.2	68	87	4.0	5.2	7.3	0.7

* Mean values of the ATP-, the fumigation-extraction-, the SIR- and the PLFA-measurements (three sampling campaigns were performed for each method)

** Mean values of the results obtained from the microscopic direct count method and the measurement of the bacterial PLFAs (mean values of three sampling campaigns performed for each method)

MB-C Microbial biomass-carbon

BB-C Bacterial biomass-carbon

Comparison of the microbial biomass in planted and unplanted VFCWs:

The results obtained in this study showed no significant differences between the magnitude and distribution of microbial and bacterial biomass within planted and unplanted systems. But it has to be noted critically that the plant growth on the indoor PSCWs was rather poor, due to the indoor conditions (despite extra lamps for plant growth there was not enough light; no wind; high nutrient loads). For the PSCWs the density of plants varied from 40-130stems m^{-2}. In contrast to these results the full-scale outdoor plant investigated within this study, covered with *Phragmites australis,* displayed plant densities of about 150-300 stems m^{-2} (unpublished data). *Miscanthus sinensis giganteus,* the macrophyte species used for the indoor CWs, is most commonly cultivated species for biomass production across Europe (Maciorowski and Kołtoniak, 2006), but not common as vegetation for CWs. Therefore no comparative studies about the application and performance of *Miscanthus* within CW systems are available. But due to preliminary experiments, which revealed that *Miscanthus sinensis giganteus* exhibited a better indoor growth-performance than *Phragmites australis* (most common for CWs in Austria; also used for the outdoor CW in this study), *Miscanthus* was chosen for the PSCWs. Thus the conclusion that no significant differences between planted a planted systems were found within this study has to be regarded with caution.

A comparison of the unplanted indoor systems and the planted outdoor CW is also only permissible under restricted conditions due to the different climatic conditions. The indoor CWs showed generally slightly higher microbial biomass mean values (with the only exception of the PLFA-concentrations, which showed higher values for the outdoor system), as well as higher BSP-values within the uppermost 10 cm of the filter body, despite the higher organic load of the outdoor systems compared to the indoor CWs (indoor: 20g COD m^{-2} d^{-1}; outdoor: 27g COD m^{-2} d^{-1}). This can be explained mainly by the different

climatic conditions (higher annual average temperature indoor) and optionally by potential differences in the composition of the applied wastewater. In depths from 20-50 cm the outdoor system revealed higher bacterial biomass values, which can be a result of the stimulating effects of roots and rhizomes on bacteria (e.g. Münch et al., 2005).

6.3 Bacterial carbon utilization within vertical flow constructed wetlands

Constructed wetlands (as well as natural wetlands) are typically a major sink for carbon. Easy biodegradable C is degraded by microorganisms and metabolized or assimilated for biomass growth. Refractory organic material originated from the wastewater, or evolved from decomposition processes is accumulated within CWs or washed out. VFCWs are very suitable systems for carbon removal (see chapter 6.1). For a better understanding of the mechanisms and the spatial distribution of these degradation processes within the filter body of VFCWs, the (heterotrophic) bacterial secondary production (BSP) was investigated.

Measurements of the incorporation rate of the radioactive labeled amino acid leucine revealed the distribution patterns of BSP within the filter bodies of the indoor and the outdoor CWs:

Horizontal distribution patterns of BSP:

An evaluation of the variation of the BSP within two different depth layers of the PSCWs (0-1 and 40-45 cm) revealed that the BSP is not fluctuating much within one depth (see figure 4.7), mainly due the hydraulic optimized sprinkler system (see figure 3.2) which enabled an equal distribution of the wastewater across the one square meter surface. Only the areas most far away from the center (= influent) exhibited a little lower productivity, because they received less wastewater. Directly under the sprinkler the productivity was lower within the uppermost centimetre, because biomass and fine material was washed away by the strong wastewater flush in the center and translocated to deeper layers, where it caused an increase of BSP. These findings emphazise the importance of an efficient hydraulic loading system to enable a utilization of the whole filter body.

Vertical distribution patterns of BSP:

The BSP decreased with increasing filter body depth (see figure 4.8), but not as strong as the number of bacteria (see figure 4.9), or as the microbial biomass. In comparison to the bacterial biomass, which decreased about 70 % within the uppermost 5 cm of the PSCWs and the FSCW, the BSP decreased only about 3 % in the planted PSCW, about 11 % in the unplanted PSCWs and about 37 % in the FSCW, respectively. Explanations for this finding can be the accumulation of wastewater bacteria, which die off because they are not adapted to this environment and secondly the influent comprises substances, which can inhibit the BSP (up to 30 % of the productivity can be inhibited; see figure 4.6). But in general the outdoor and all indoor systems displayed similar vertical distribution patterns of BSP within the filter bodies. BSP values were significantly lower within the 1-10 cm layer of the outdoor CW compared to the PSCWs. This is may be a consequence of different wastewater compositions, or possibly less oxygen is able to diffuse into the outdoor CW due to impeded oxygen diffusion as a result of a thick layer of plant litter, which covers the outdoor filter body. But nevertheless about 80 % of the BSP took place within the first 10 cm of all investigated filter bodies. Of course the BSP is related to the bacterial abundance, but this correlation can be affected by diverse environmental factors and therefore the direct measurement of the

bacterial production is indispensable for a correct determination of the bacterial mediated carbon utilization. About 9 % of the total organic carbon (TOC) fraction, which was utilized by bacteria within the system, were metabolized within the 0-1 cm filter body layer of the planted CW, about 37 % within the 1-5 cm layer and about 80 % of this TOC was metabolized within the 0-10 cm layer. The outdoor system displayed rather similar results.

Furthermore it was shown that bacteria obviously dominate the productivity and carbon degradation within this ecosystem, while fungi seem to play a minor role.

Additionally a simple mass balance calculation of bacterial C-fluxes within vertical flow CWs was performed based on an estimated carbon input (influent - TOC, TOC input by plants vegetation) and output of the system (effluent - TOC; CO_2 emission rates have been estimated only theoretically), estimated accumulation rates (TOC - accretion within the filter body), an assumed average growth efficiency (for eutrophic environments), and the spatial distribution of the BSP within the filter body. From this approach a theoretically factor to convert the leucine incorporation rates into a biomass-C production rate was calculated. Conversion factors (CF) taken from the literature resulted in unrealistic low doubling times of the bacterial biomass and were therefore considered as not suitable for this system. Experiments carried out to determine a CF specific for this system unfortunately failed (see chapter 4.2.4.1). But for an exact assessment of the BSP the ascertainment of a system-specific CF will be essential. The theoretically calculated CF revealed median doubling times of 33-35 h for the bacteria of the PSCWs. This simple mass balance approach showed, that the planted indoor PSCWs had a bacterial carbon demand (the amount of carbon needed for biomass growth and respiration) of about 8.8 g C m^{-2} d^{-1} (calculated for an average wastewater TOC-load of 9.15 g C m^{-2} d^{-1}). Within the planted indoor PSCWs about 86 % of the total carbon input of the system (including the organic carbon emitted by the vegetation) were utilized by bacteria for biomass growth and respiration (bacterial carbon demand), underlining the role of bacteria within the carbon removal of VFCWs.

6.4 Estimation of bacterial diversity in vertical flow constructed wetlands

Within Tietz et al. (2007a, see chapter 4.3) and chapter 5.1 (manuscript) a crude characterization of the diversity of the bacteria inhabiting the filter body of the outdoor FSCW and the indoor PSCWs was done.

Tietz et al. (2007a) investigated the first time for a full-scale vertical flow constructed wetland (FSCW) the diversity of ammonia oxidizing bacteria (AOB), performing the rate-limiting step of nitrification. Additionally the CW has been analyzed for potential spatial variations within the filter body. The application of the molecular fingerprint technique DGGE (Denaturing Gradient Gel Electrophoresis), to display the diversity within the functional gene *amoA*, present only in ammonia oxidizers, proved to be a very suitable approach to elucidate the diversity of AOB within the investigated CWs.

During the cold and the warm season samples were taken from seven different depths from the filter body of the FSCW, to reveal potential vertical spatial distribution patterns. The profiles obtained from the two sampling campaigns showed no significant differences, with the only exception of a slightly higher diversity within the tow uppermost layers (0-5 cm). It can be concluded that the presence of the AOB species is not influenced by strong temperature changes, but no conclusions about their physiological status can be drawn from these results. Yan et al. (2005) investigated the distribution of AOB within a CW planted with *Typha latifolia* and showed that the quantity of AOB decreased with increasing depth. Truu et al. (2005) detected fifteen different AOB populations within a horizontal flow CW, but only two dominant AOB were present in all depths. Additionally they observed no clear spatial pattern

regarding the abundance of AOB. From the results of this study no quantitative information concerning the AOB distribution within the filter body can be drawn.

The presence of AOB down to the bottom of the filter body of the CW (50 cm depth) indicates, that ammonia oxidation takes place within all depth layers and aerobic conditions are prevailing within the whole filter body. Although only very few *amoA* sequence types dominated the wetland filter material, the investigated FSCW displayed a very efficient and stable nitrification performance around the whole year.

The distribution patterns of distinct species depend on their physiological properties determined by environmental parameters (Koops and Pommerening-Roser, 2001), like the ammonium concentration (especially the free ammonia), oxygen concentration and pH. The two dominant bands within in the DGGE-gel were present within all analyzed samples, and could have been affiliated to the AOB lineages *Nitrosomonas europaea / "Nitrosococcus mobilis*" and *Nitrosospira. Nitrosomonas spp.* is a typical AOB present within most WWTP and adapted to high ammonia concentrations. The genera *Nitrosospira* occurs in some WWTPs, but has been detected in all available studies, which investigated the species diversity of the AOB community within CWs (Gorra et al., 2007; Ibekwe et al., 2003; Abd El Haleem et al., 2000) and is the most common AOB within soils (e.g. Horz et al., 2004).

The establishment of links between species diversity, functional diversity and ecosystem processes is central to our understanding of ecosystem function. It has been hypothized by Siripong and Rittman (2007) that the coexistence of different nitrifiers (within a WWTP), who perform the same task, implies functional redundancy, which may allow communities to maintain physiological capabilities when conditions change. Within some WWTPs AOB communities are dominated by only one species (e.g. Juretschko et al., 1998), compared to plants with a several AOB species (e.g. Daims et al., 2001). An explanation for these differences in the diversity could be the frequency of disturbances and changes within the system. A higher degree of slight disturbances can "force" the system to adapt to these varying conditions by establishing different pathways to maintain this process (e.g. nitrification) under different environmental conditions. The filter body of a constructed wetland has rather stable conditions, with the exclusion of the surface layer, which is exposed to all changes caused by the environment or by the wastewater composition. Therefore the uppermost filter layer has a protective and buffering function for the deeper filter body regions. In agreement with this hypothesis is also the fact that the 0-1 cm and the 1-5 cm layer comprised three to seven different AOB sequences, instead of only two bands (see figure 4.11). Webster et al. (2005) demonstrated that *Nitrosospira* species within a soil mesocosm were able to outcompete *Nitrosomonas*, which is traditionally considered to grow faster than *Nitrosospira* strains at high ammonium concentrations. It seems that the ecosystem "filter body of a VFCW" provides enough microniches for the stable establishment of at least two ammonia oxidizing community, which are sufficient to guarantee a stable nitrification performance.

The possible role of ammonia oxidizing archaea (Crenarchaeota) within the system CW is still to be questioned.

In chapter 5.1 (manuscript) the community profiling technique T-RFLP (Terminal Restriction Fragment Length Polymorphism) targeting the 16S rDNA was employed to reveal potential spatial distribution patterns of bacterial diversity within the filter body of the investigated CWs. Additionally it was used to evaluate the effect of the CW passage on the species composition of the wastewater. Furthermore a brief inventory of the most abundant highly active bacterial species inhabiting the filter bodies of the indoor PSCWs and the outdoor FSCW was performed by constructing a clone library based on the 16S rRNA.

Molecular-fingerprint techniques, such as T-RFLP analysis, have been proven to be sensitive and powerful tools to characterize complex bacterial communities and are useful tools for the rapid comparisons of community structures. T-RFLP community profiles derived from different depths of the filter bodies of the PSCWs (planted / unplanted / rhizosphere

samples), FSCW (planted / rhizosphere samples) and from wastewater samples (inflow and outflow samples), were analyzed and checked for differences regarding their bacterial community composition.

Unfortunately the number of replication analyses per sample type was too low (ranging from 0-1 replications per sample type) to carry out a statistical analysis of the measured profiles. Due to the low number of replications some of the conclusions drawn from these results have to be considered more as assumptions or speculations than as assured facts.

A comparison of the T-RFLP profiles from the in- and outdoor system revealed that the filter bodies were colonized by similar populations, but with varying abundances of specific bacterial groups (figure 5.1): 86% of the total peaks obtained from the in and outdoor profiles could have been detected in both systems. Although the in- and the outdoor systems were exposed to different climatic conditions, were covered by a different vegetation, and received wastewater with potential variations regarding its composition, a rather equal bacterial community composition developed within the filter bodies. But it seems that factors like the used filter material, as well as design and operation mode of the CW, which were equal for the in- and outdoor systems; are mainly responsible for the formation of the bacterial community. The comparison of the community profiles derived from this study with differently designed and operated CWs receiving other types of wastewater would be of great interest to characterize, which aspects are the key factors for the development and constitution of the bacterial population. But despite these similarities of the in- and outdoor CW populations also some differences could have been revealed. Distinct bacterial groups were found in both systems (in- and outdoor), but with a differently pronounced abundance (indicated by the T-RFLP peak intensity). By the prediction of the theoretical T-RFs lengths from the 16S rRNA sequences obtained from the clone library an identification of some of these peaks was possible, although we have to keep in mind that an individual T-RF may correspond to fifteen or more species (Kent et al., 2003). Due to this uncertainty the peaks within the T-RFLP profiles have been assigned to bacterial groups only of the phyla level. The assignments revealed that the group of Actinobacteria was much more abundant in the indoor systems than in the outdoor system (figure 5.1), whereas the group of Betaproteobacteria was rather dominant in both systems but with a clearly higher abundance in the outdoor system.

The T-RFLP profiles from a vertical depth profile taken from the filter body of the outdoor CW revealed a rather equal distribution pattern of the bacterial diversity over the depth (figure 5.2). The most dominant peaks were found in all seven depths of the filter body. Very few peaks showed a pronounced negative or positive correlation with depth. Also the phylotype richness of the filter body samples from the outdoor CW showed that the degree of bacterial diversity did not change with increasing filter depth (table 5.3).

Rhizosphere samples displayed no significant higher phylotype richness than the rest of the filter body (see figure 5.3 and table 5.3). Only about 50% of the total peaks derived from the in- and outdoor rhizosphere T-RFLP profiles occurred within both sample types, suggesting that the rhizosphere communities of *Miscanthus* and *Phragmites* have a different composition due to the plant specific rhizosphere-effect. In general the peak heights within the rhizosphere profiles where higher compared to bulk soil profiles, indicating a higher abundance of the existent bacterial groups. But it has to be considered that the sampling protocol used for the rhizosphere samples was rather simple and did not include a washing step of the roots and rhizomes and therefore bacteria from the rhizoplane could remain on the plant surface and were not included into the T-RFLP-analysis. Additionally the number of analyzed samples is too low to draw definite conclusions from these investigations about the influence of *Phragmites* and *Miscanthus* on the bacterial diversity within their rhizospheres. Samples around roots and rhizomes were only included in these analyses to take into account the potential heterogeneity of the system. Furthermore it should be questioned how relevant rhizosphere bacteria are for the purification process of a CW in general. Moreover the fact that the removal efficiencies of the planted and unplanted PSCWs were not significant different (see table 4.9), indicates that bacteria of the rhizosphere are not relevant for the purification performance of the investigated CWs. The assumption that plants have no

influence of the bacterial diversity of the bulk soil is supported by the work of Baptista et al. (2008), who investigated the community composition of a planted and an unplanted horizontal subsurface-flow laboratory-scale constructed wetland and ascertained that the microbial communities in both wetlands were typically no more similar than if they had been randomly assembled from a common source community.

A comparison of the phylotype richness of the inflows and the corresponding outflows showed no reduction of bacterial diversity within the wastewater before and after the filter body passage for the indoor PSCWs. These findings are in accordance with the results obtained by Ibekwe et al. (2007), who found a higher bacterial diversity in the outflow compared to the inflow of a free water surface CW. But for the outdoor FSCW investigated within this study a lower diversity was measured within the outflow compared to the inflow of the system (figure 5.5b). For verification of these results and to answer the question if the macrophytic vegetation is responsible for this findings further investigations are necessary. But as obvious within figure 5.5a and 5.5b although the phylotype richness is not clearly reduced, the bacterial community composition within the in- and outflow of the systems is very different. E.g. within the planted PSCWs only 25% of the bacteria were able to pass the filter body. These results demonstrated that the filter body also emitted bacteria, which were not existent or not abundant within the inflow.

Sequences analyses of clones from the 16S rRNA clone library revealed that the phylum Actinobacteria was the most abundant group of active bacteria followed by Betaproteobacteria and unidentified species. Additionally representatives of the phylogenetic groups Firmicutes, Planctomycetales, Chloroflexi and Bacteroidetes were detected. All of these groups are typical for natural soils as well as for WWTPs. Ibekwe et al. (2007) analyzed the bacterial population within a free water surface CW and revealed that Gammaproteobacteria were the dominant group with in the rhizosphere followed by Betaproteobacteria; whereas they found mainly unidentified species and Deltaproteobacteria within the sediment. Walsh (2002) reported that Betaproteobacteria were the most dominant group within a surface flow wetland. A comparison of Janssen (2006) who analyzed the results of 32 clone libraries obtained from different types of soils revealed that Acidobacteria followed by Actinobacteria and Proteobacteria are the most abundant bacterial groups in soils. Within most AS - WWTPs Proteobacteria (mainly Betaproteobacteria) are the dominant group (Snaidr et al., 1997; Wagner et al., 2002; Jiang et al., 2004; Lozada et al., 2004). The absence of Acidobacteria within the investigated CWs displays a big difference to soil ecosystems. The nearest mainly cultured relatives of the sequenced clones were mainly originally isolated from soils, AS - WWTPs and rhizosphere samples. These results underline the intermediate position of vertical flow CWs between terrestrial / aquatic and natural / engineered ecosystems.

The results obtained from this study demonstrated that VFCW with intermittent loading consisting of a sandy filter material provide optimal conditions for the development of an abundant, productive and diverse bacterial population, which is concentrated in the top 10 cm of the filter body and seems to be rather unaffected by the vegetation of the plant. For microbial degradation processes a filter body thickness of 10 cm would be sufficient. But for physical filtration processes, for an adequate development of the vegetation and to obtain optimal hydraulic retention times (about 2-4 days), a thickness of about 50 cm is advisable for a stable and robust plant operation. Although VFCWs are a simple and efficient alternative to conventional wastewater treatment plants, but there is still some potential left for an optimization of the system (see chapter 6.6).

6.5 Additional results from the project "Characterization of microbial biocoenosis to optimize removal efficiency and design of subsurface flow constructed wetlands for wastewater treatment"

Another focus of this project was to apply the results obtained from the measurements of microbial biomass for the filter body of the PSCWs to calibrate the parameter biomass within the multi-component reactive transport module CW2D (Reactive transport module for constructed wetlands two dimensional) (Langergraber and Simunek, 2005). This model was developed to simulate transport and reactions of the main constituents of municipal wastewater in subsurface flow constructed wetlands. This enables an increase of the quality of the simulation results obtained from the model. In Langergraber et al. (2007; see annex IV) the measured data for microbial biomass were compared with simulation results using different heterotrophic lysis rate constants. The measured microbial biomass data have been used to calculate biomass COD values needed for comparison with numerical simulations using the multi-component reactive transport module CW2D. Simulated microbial biomass COD in the first centimeter of the filter body varied between 3,400 and 5,600 mg COD g DW^{-1} (the range of the measured values) when using heterotrophic lysis rates between 0.35 and 0.25 d^{-1}. When comparing measured and simulated biomass COD in different depths of the main layer simulations seem to overpredict biomass COD in the 1-5 cm depth and underpredict biomass COD in the 5-10 cm depth. This could be an indication that the influence of biomass growth on the hydraulic properties has to be included into modelling considerations.

Yet another part of the project was the assessment of the removal efficiency of bacteria in general and of specific indicator bacteria for fecal contamination (Coliforms, *E. coli*, Enterococci) within VFCWs. The outcome of these investigations will be published within the thesis of Kirsten Sleytr with the working title "Bacterial removal efficiencies and processes in different designed constructed wetlands". First results of these analyses, published in Sleytr et al. (2007; see annex V), revealed that the investigated systems in general achieved very high removal efficiencies for fecal bacterial indicator organisms (4.4 log units for *E. coli*, 4.3 log units for total Coliforms and 4.8 log units for Enterococci) and high reduction rates for bacteria in general (2.9 log units for culturable heterotrophic bacteria). No significant differences in the removal rates of planted and unplanted VFCWs were observed. According to the biomass distribution within the filter body of the CWs it was shown that also the main part of the processes of elimination of bacteria takes place in the first 10 to 20 cm of the filter body.

6.6 Outlook

CW technology has become a well-accepted simple nature-orientated technique for decentralized wastewater treatment. By applying CWs within different climates for the treatment of various kinds of wastewater there is still potential left for an optimization of the system. The results of this study provides a coarse insight into the microbial community of VFCWs but within the field of microbial ecology of CWs there are also still some open questions:

- By knowing the behaviour and requirements of the species, which are the „key players" within degradation processes, it is possible to support their development and activity by providing optimal conditions for their establishment. For an identification of these "key players" the application of modern techniques such as stable isotope probing, FISH - MAR (fluorescence in situ hybridization - microautoradiography) or analyses of functional genes are necessary to link the phylogenetic assignment with

metabolic activity of the microorganisms to obtain more information on the various bacteria involved in wastewater purification process. To examine the in situ performance of the "key players" they should be investigated in CW systems with different environmental conditions (like climate, type of applied wastewater, hydraulic conditions).

- Further investigations about the correlation between the phylogenetic diversity of a functional group and its degradation performance should be done. The physiological activity of specific functional bacterial groups should be evaluated by monitoring the bacterial population of CWs under varying temperatures, different hydraulic loading rates, higher and lower nutrient loads and within different design types of CW.
- The intermittent loading mode provides optimal aerobic conditions for nitrification and carbon decomposition within VFCWs, but under non-watersaturated conditions total removal of nitrogen hardly takes place. Further research is necessary to explore and furthermore establish alternative ways of nitrogen removal (which already have been demonstrated to occur within CW), like the anammox process (Paredes et al., 2007b) or the completely autotrophic nitrogen-removal over nitrite (CANON) (Sun and Austin, 2008).
- Furthermore the role of microorgansims within the degradation of specific wastewater compounds like pathogens and partially biodegradable micro-pollutants (e.g. PPCPs = pharmaceutical and personal care products, EDC = endocrine disrupting chemicals, PAH = polycyclic aromatic hydrocarbons) has to be investigated more detailed.
- A further assessment of the capability of CWs to remove (or retain) pathogens from wastewater should not only focus on reduction rates of bacteria, but should also take a look on the removal of viruses and protozoan parasites (like *Giardia* and *Cryptosporidium*). The application of tracer studies, using phages (e.g. coliphages MS2, PRD1; bacteriophages P22, T7; phages labeled with a fluorescent dye) or artificial microspheres as biotracers, is necessary for a better understanding of the removal processes taking place within CW.
- To obtain a more detailed understanding of the biological processes causing soil clogging.

Generally there are only few possibilities to control and regulate the environmental conditions for microorganisms in VFCWs in comparison to an activated sludge tank. But changes of the intervals between hydraulic loadings, the level of recirculation of the effluent and the adjustment of the level of water-saturation within the filter body provide ways to vary the conditions (in particular the oxygen content and the nutrient supply) within VFCWs. Also constructional variations (e.g. the use of filter material, thickness of the filter body) are limited, because the maintenance of ideal hydraulic conditions to obtain optimal hydraulic retention times within the system has priority.

Further research in the field of CWs is needed on the following topics:

- Improvement of the removal efficiency of CWs (mainly due to an enhanced adsorption capacity of the filter material) regarding non - biodegradable compounds like phosphor, heavy metals and POPs (persistent organic pollutants).
- Long-term-studies are needed to understand the behaviour and purification performance over a long operation period.
- Reduction of the specific area demand by optimizing the operation and design of the CWs.

Despite the experience, which has been developed during years of practical application and research, a number of fundamental aspects of the function of constructed wetlands are not yet adequately understood. Stottmeister et al. (2003) remarked some aspects, which enable the application of CWs on a broader scale:

- Technological limits of wetlands of large size (>1,000 PE) and the problems, which are to be expected with such CWs.
- Extent to which the plant biomass in wetlands can subsequently be put to viable economic use, for example, as a source of energy or as raw material for the paper industry, etc.
- CWs are very effective for the removal of pathogenic microorganisms from wastewater. But in the case of reuse of the plant effluent (e.g. for irrigation) strict requirements regarding especially hygienic aspects have to be fulfilled (e.g. WHO guidelines, 2006). Additionally aquifer recharge by localized percolation of wastewater treated by CWs through the soil (e.g. in remote areas) is an issue that requires further investigations. A better understanding of removal mechanisms and the identification of correlations between different indicators can significantly contribute to better frame the opportunities offered by CW in water reuse schemes (Ghermandi et al., 2007b).
- The pressing need for an improved water quality in developing countries is an area where the usage of constructed wetlands to solve water and wastewater problems could be especially beneficial. This is a specific field, which needs further research and development work.

Achieving a better understanding of the complex interactions involved will enable the basic scientific aspects to be optimally combined with the technical possibilities available (Stottmeister et al., 2003).

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ADRA	Amplified rDNA Restriction Analysis
ADT	Adenosine diphosphate
AMP	Adenosine monophosphate
Anammox	Anaerobic ammonia oxidation
AMO	Ammonia monooxygenase
*amo*A	Gene encoding the α–subunit of the ammonia monooxygenase
AOA	Ammonia oxidizing archaea
AOB	Ammonia oxidizing bacteria
AS	Activated sludge
AS-WWTP(s)	Activated sludge wastewater treatment plant(s)
ATP	Adenosine triphosphate
$BOD_{(5)}$	Biological oxygen demand (in 5 days)
BSA	Bovine serum albumin
BSP	Bacterial secondary production
Bp	Base pairs
C	Carbon
cDNA	Complementary desoxyribonucleic acid
CF	Conversion factor
C-FE	Chloroform fumigation-extraction
COD	Chemical oxygen demand
CO_2	Carbon dioxide
CW(s)	Constructed wetland(s)
^{14}C	Radioactive carbon isotope
CW2D	Reactive transport module for constructed wetlands two dimensional
DAPI	4',6-diamidino-2-phenylindol
DGGE	Denaturing gradient gel electrophoresis
DNA	Desoxyribonucleic acid
DO	Dissolved oxygen
DMSO	Dimethylsulfoxide
dsDNA	Double stranded DNA
DW	Dry weight
d_{10}	Hazen's effective grain size in mm, relative to which 10% of the sample is finer
d_{60}	Hazen's effective grain size in mm, relative to which 60% of the sample is finer

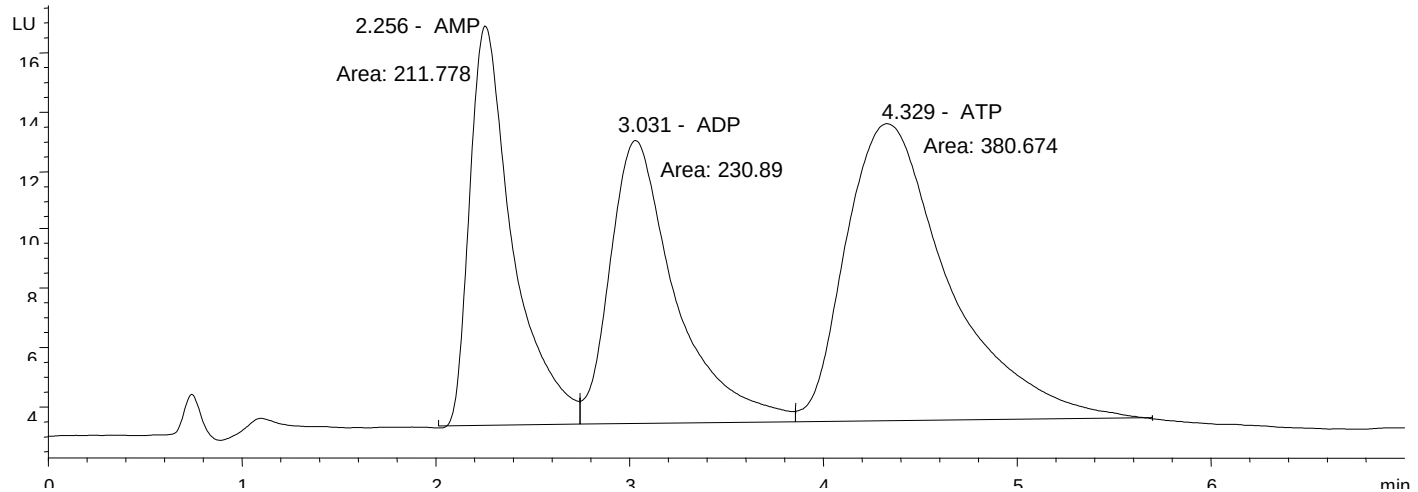
EC	Enterococci
EDTA	Ethylene diamine tetracetic acid
EPS	Extrapolymeric substances
FAMES	Fatty acid methyl esters
FE-C	Fumigation extraction to determine carbon-biomass
FE-N	Fumigation extraction to determine nitrogen-biomass
FISH	Fluorescence in situ hybridization
FSCW(s)	Full scale constructed wetland(s)
^{3}H	Tritium (radioactive hydrogen isotope)
HAO	Hydroxylamine oxidoreductase
HCl	Hydrochloric acid
HFCW(s)	Horizontal flow constructed wetland(s)
HPC	Heterotrophic plate count
HPLC	High-performance liquid chromatography
ISO	International organization for standardization
LEU	Bacterial production measurement via incorporation of radioactive labeled leucine
M	Mol per litre
MB	Microbial biomass
MDC	Microscopic direct counts
MPN	Most probable number
MV	Mean value
N	Nitrogen
NaOH	Sodium hydroxide
NH_3	Ammonia
NH_4 (-N)	Ammonium (-nitrogen)
NO_3 (-N)	Nitrate (-nitrogen)
NOB	Nitrite oxidizing bacteria
NOX	Nitrite oxidoreductase
Pt senors	Platinum resistance temperature sensors
PAA	Polyacrylamide
PE	Population equivalent based on 60g of BOD_5 per person
PE_{COD}	Person equivalent COD
PCR	Polymerase chain reaction
PL	Phospholipid
PLEL	Ether-linked phospholipids
PLFA	Phospholipid fatty acid
PSCW(s)	Pilot scale constructed wetland(s)

RNA	Ribonucleic acid
S	Phylotype richness
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SFCW(s)	Surface flow constructed wetland(s)
SSFCW(s)	Subsurface flow constructed wetland(s)
SIR	Substrate induced respiration
SSCP	Single strand conformation polymorphism
ssDNA	Single stranded DNA
SSFCW(s)	Subsurface flow constructed wetland(s)
TAE	Tris-acetate-EDTA
TC	Total Coliforms
TKN	Total Kjeldahl nitrogen
TOC	Total organic carbon
TON	Total organic nitrogen
TP	Total phosphor
T-RFLP	Terminal restriction fragment length polymorphism
TSS	Total suspended solids
US EPA	United states environmental protection agency
UV	Ultra violette light
VFCW(s)	Vertical flow constructed wetland(s)
WWTP(s)	Wastewater treatment plant(s)

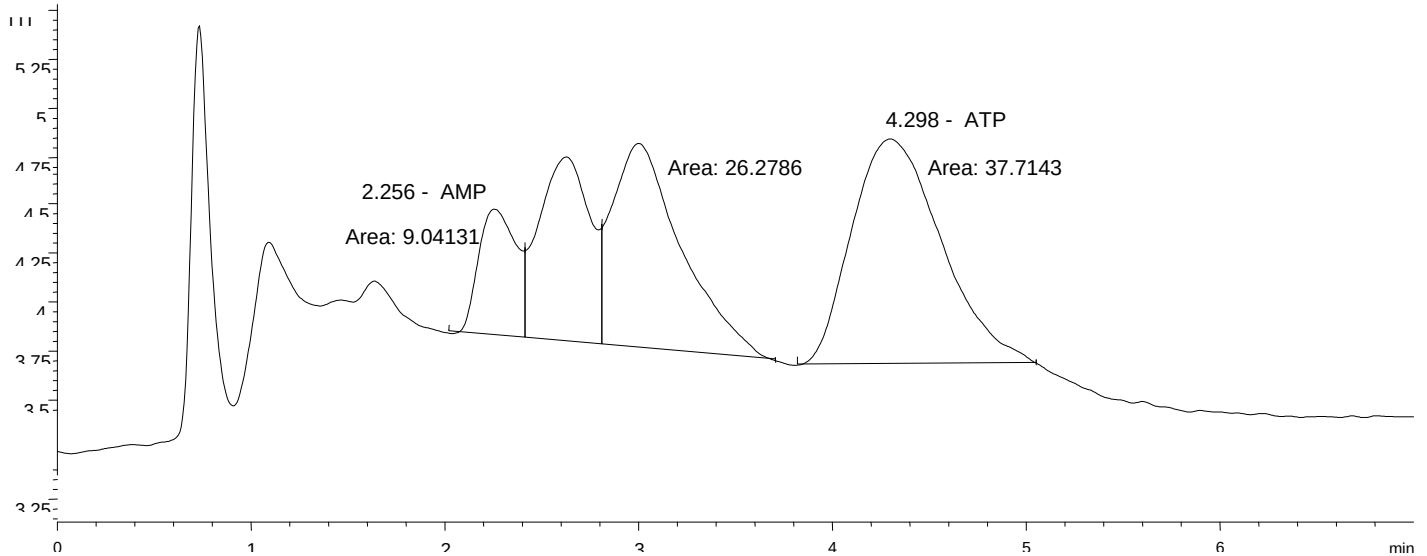
11 Annex

Annex I: HPLC-chromatograms of the three adenylates (AMP, ADP and ATP) measured from a standard-mix (a) and within a filter body sample of the indoor CW (b).

a)

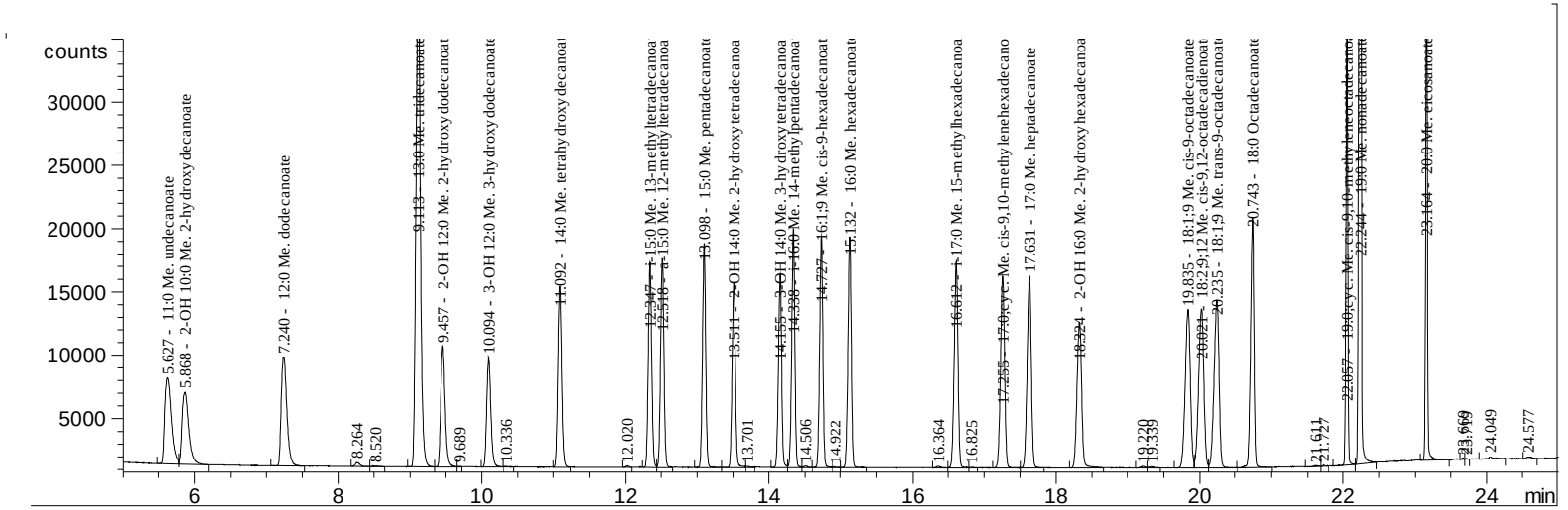


b)

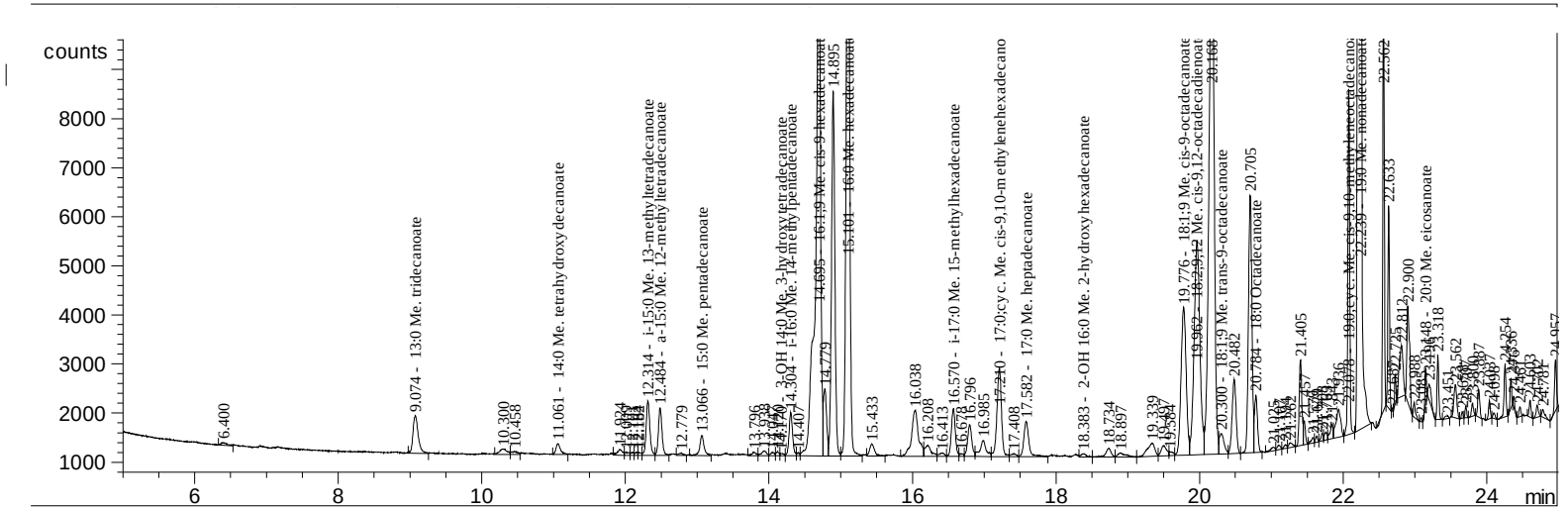


Annex II: HPLC-chromatograms of the FAMES measured from a bacterial FAMEs standard mix (Supelco, Sigma Aldrich) (a) and within a filter body sample of the indoor CW (b).

a)

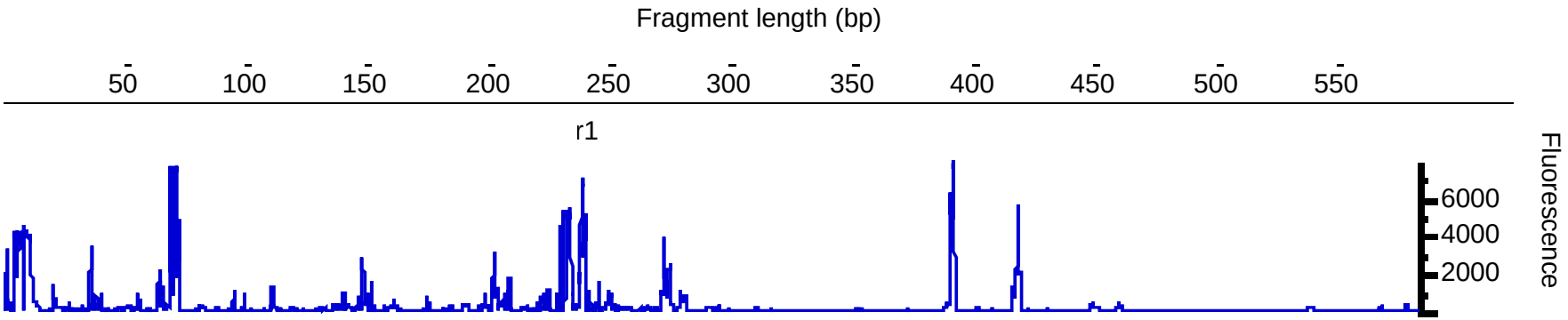


b)

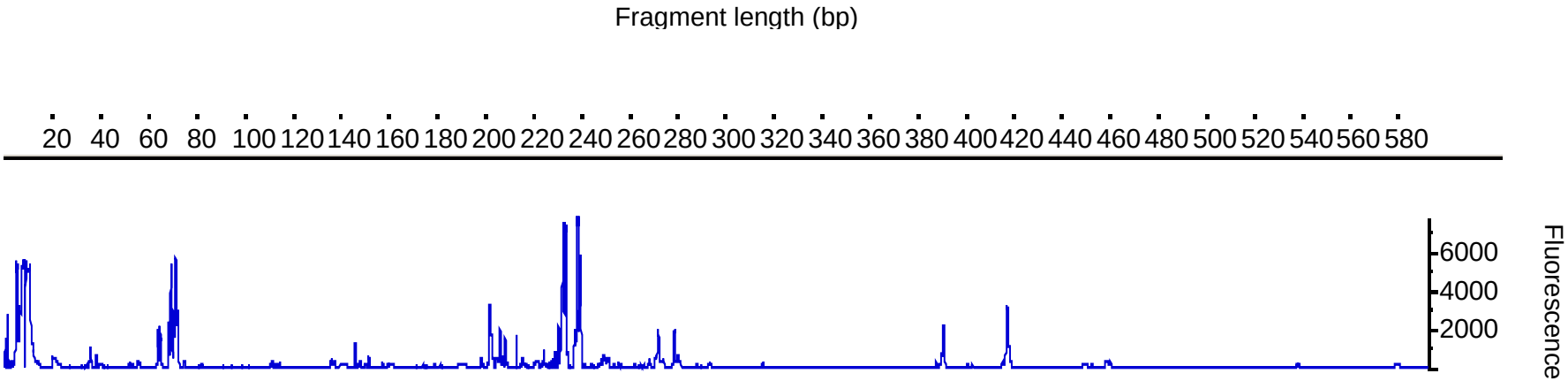


Annex III: T-RFLP- profiles (from a capillary electrophoresis) of the 16S rDNA from rhizosphere soil (a) and bulk soil samples (b) from the outdoor CW.

a)



b)



Annex IV: Comparison of measured and simulated distribution of microbial biomass in subsurface vertical flow constructed wetlands.

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Abstract
The multi-component reactive transport module CW2D has been developed to model transport and reactions of the main constituents of municipal wastewater in subsurface flow constructed wetlands and is able to describe the biochemical elimination and transformation processes for organic matter, nitrogen and phosphorus. It has been shown that simulation results match the measured data when the flow model can be calibrated well. However, there is a need for developing experimental techniques for the measurement of CW2D model parameters to increase the quality of the simulation results. Over the last years methods to characterize the microbial biocoenosis in vertical subsurface flow constructed wetlands have been developed. The paper shows measured data for microbial biomass and their comparison with simulation results using different heterotrophic lysis rate constants.

Keywords
CW2D, microbial biomass, numerical simulation, vertical subsurface flow constructed wetlands, wastewater treatment

INTRODUCTION

Constructed wetlands (CWs) are still often looked at as a "black box" in which water is purified. This is although there is much experience available in constructing and operating these systems. Numerical models can provide insight into the "black box" and are therefore means to increase the understanding of the complex processes in constructed wetland systems. Once reliable numerical models exist they can be used for evaluating and improving existing design criteria which are mainly based on rules of thumb using specific surface area requirements or simple first-order decay models (e.g. Kadlec et al., 2000).

Many models available are focusing on the description of seasonal trends (e.g. Wynn and Liehr, 2001) or using simplifications such as first-order reaction rates to describe the degradation of a substance along the flow path (Werner and Kadlec, 2000). Rousseau (2005) developed a mechanistic reaction model for subsurface horizontal flow constructed wetlands using tanks-in-series for modelling water flow. The model of Rousseau can therefore only be used for horizontal flow systems where saturated conditions occur.

The numerical model used in this study, HYDRUS-2D/CW2D (Langergraber and Šimůnek, 2006), uses mechanistic models for both water flow and reactions. This enables modelling both unsaturated and saturated water flow and therefore the model can be applied for subsurface vertical and horizontal flow constructed wetlands. CW2D is an extension of the variably-saturated water flow and solute transport program HYDRUS-2D (Šimůnek et al., 2006) and was developed to model transport and reactions of the main constituents of municipal wastewater in subsurface flow constructed wetlands. CW2D is able to describe the biochemical elimination and transformation processes for organic matter, nitrogen and phosphorus based on the mathematical formulation introduced by Henze et al. (2000) for the IWA Activated Sludge Models (ASMs).

The experience showed that the results of multi-component reactive transport simulations with CW2D match well with measured effluent concentrations when the water flow model can be calibrated. However, there is still a need for developing methods to measure CW2D model parameters

(Langergraber, 2003; Langergraber and Šimůnek, 2005). This is required for the calibration of the reactive transport part of the model and a precondition before the model can be used for evaluating design criteria. A number of measurement techniques have been developed to characterize the parameters of ASMs (Vanrolleghem et al., 1999; Henze et al., 2000). However, no experimental techniques are currently available to measure CW2D model parameters.

Over the last years methods to characterize the microbial biocoenosis in vertical subsurface flow constructed wetlands have been developed (Tietz et al., 2007). This paper shows how the measured data have been converted into parameters that can be used to compare the measured values with simulation results of CW2D. Simulation results with different rate constants for heterotrophic lysis are shown and compared with the measurements.

MATERIALS AND METHODS

The multi-component reactive transport module CW2D

The multi-component reactive transport module CW2D (Langergraber, 2001) was developed to describe the biochemical transformation and degradation processes in subsurface flow constructed wetlands. CW2D is incorporated into the HYDRUS-2D variably-saturated water flow and solute transport program (Šimůnek et al., 1999) and considers 12 components and 9 processes. The components include dissolved oxygen, organic matter (three fractions of COD with different degradability: CR, CS, CI = readily, and slowly biodegradable, and inert COD, respectively) ammonium, nitrite, nitrate, and nitrogen gas, inorganic phosphorus, and heterotrophic and autotrophic microorganisms. Organic nitrogen and organic phosphorus are modelled as nutrient contents of the organic matter. The processes considered in the model are hydrolysis, mineralization of organic matter, nitrification (modelled as a two-step process), denitrification, and lysis processes for microorganisms. The mathematical structure of CW2D is based on the mathematical structure of the ASMs (Henze et al., 2000). ASMs are based on mass balances for COD, nitrogen and phosphorus. For a detailed discussion of the CW2D module see Langergraber and Šimůnek (2005).

The indoor pilot-scale constructed wetlands (PSCWs)

The experiments have been carried out at indoor vertical flow pilot-scale constructed wetlands (PSCWs) in the technical laboratory hall of the Institute. Each PSCW had a surface area of 1 m² and was loaded intermittently 4 times per day. The 50 cm main layer of the 8 PSCWs sampled for the investigations consisted of a sandy substrate with a grain size of 0.06-4 mm. The organic load applied was 20 g COD.m^{-2}.d^{-1} (i.e. a specific surface area requirement of 4 m² per person), the hydraulic load about 60 mm/d. The PSCWs have been planted with *Miscanthus sinensis giganteus* (6) and unplanted (2), respectively.

Samples from the main layer have been collected from different depths (0-1, 1-5, 5-10, 10-20, 20-30, 30-40 and 40-50 cm) and have been analysed immediately for bacterial production and stored at 4℃ for analysis of microbial biomass and TOC within 10 days (Tietz et al., 2007). Water samples have been taken on a monthly basis and analysed in the lab of the Institute.

Methods for the determination of the microbial and bacterial biomass

As CW2D is based on mass balances for COD also biomass concentrations are expressed in terms of COD. A number of methods exist to determine the COD of the microbial biomass in activated sludge systems (e.g. Bullock et al., 1996; von Münch and Pollard, 1997; Contreras et al., 2002). However, no method exists for determining the COD of soil biomass. Therefore for the determination of microbial COD it was chosen to measure the C and N content of the biomass and calculate the biomass COD using conversion factors based on stoichiometry.

A number of methods have been applied to measure parameters to characterise microbial and bacterial biomass in a previous study. These methods include conversion of bacterial abundance determined by microscopic direct counts into biomass by measurement of the cell volume, fumigation-extraction for biomass-C and -N, ATP measurements for biomass-C, and substrate induced respiration (SIR) for biomass-C (Tietz et al., 2007).

Calculation of the theoretical biomass COD

As described above the biomass COD is calculated from the measured C and N content of the biomass. The conversion factor for microbial C and N into the COD of the biomass is derived from the reactions of the aerobic degradation of biomass.

Several chemical formula are used to describe the composition of biomass in wastewater systems (e.g. von Münch and Pollard, 1997; Contreras et al., 2002; Sötemann et al., 2005). For the calculation of biomass COD a simple biomass composition ($C_5H_7O_2N$) that is widely used for characterising biomass in activated sludge systems was chosen. It is assumed that the mineralization of biomass occurs in two steps: at first biomass is hydrolysed and in a second step oxidised.

Using the general formula for biomass hydrolysis given by Sötemann et al. (2005)

$$C_X H_Y O_Z N_A \Rightarrow \frac{Y+4X-2Z-3A}{24} C_6H_{12}O_6 + ANH_3 + \frac{Y-4X+2Z-3A}{4} H_2O + \frac{2Z+3A-Y}{4} CO_2 \quad (1)$$

hydrolysis of $C_5H_7O_2N$ results in

$$6 \cdot C_5H_7O_2N \Rightarrow 5 \cdot C_6H_{12}O_6 + 6 \cdot NH_3 - 18 \cdot H_2O \quad (2)$$

and the oxidation of the hydrolysis product $C_6H_{12}O_6$ in

$$C_6H_{12}O_6 + 6 \cdot O_2 \Rightarrow 6 \cdot H_2O + 6 \cdot CO_2 \quad (3)$$

Summarising the two reactions results in

$$C_5H_7O_2N + 5 \cdot O_2 \Rightarrow 2 \cdot H_2O + 5 \cdot CO_2 + NH_3 \quad (4)$$

as also given by e.g. von Münch and Pollard (1997).

Therefore for oxidising 113 g $C_5H_7O_2N$ 5 160 g O_2 are required resulting in an oxygen demand, i.e. COD, of 1.416 g O_2 per g $C_5H_7O_2N$. 113 g $C_5H_7O_2N$ biomass consist of 60 g C and 14 g N. Relating the oxygen demand to the biomass C and N results in 2.667 g O_2 per g biomass-C and 11.429 g O_2 per g biomass-N, respectively.

Using the formula $C_8H_{14}O_4N$ for biomass composition (Contreras et al., 2002) results in a similar COD of 1.489 g O_2 per g $C_8H_{14}O_4N$, and related to the biomass C and N the results are 2.917 g O_2 per g biomass-C and 20.0 g O_2 per g biomass-N, respectively.

Another possibility for calculating biomass COD would be to determine biomass COD from VSS measurements using given ratios for $C_5H_7O_2N$ and $C_8H_{14}O_4N$ of 1.42 and 1.49 mg COD/mg VSS, respectively. However, COD/VSS ratios have been only reported to be valid for activated sludge systems and pure cultures (Contreras et al., 2002).

RESULTS AND DISCUSSION

Measured microbial and bacterial biomass

Mean values and standard deviations for measured C and N content of the biomass in different depths of the main layer as reported by Tietz et al. (2007) are shown in Table 1. For all parameters characterising the biomass a similar decrease via depth in the vertical flow bed could be observed. There was no statistically significant difference between planted and unplanted PSCWs. Most of the biomass could be found in the top 10 cm of the main layer, the biomass concentrations measured in depths from 10 to 50 cm are very small.

Table 1: Measured biomass C and N content in different depths of the main layer (Tietz et al., 2007).

Depths	SIR		ATP		C fumigation		N fumigation	
	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.
cm	mg C/g DW		mg C/g DW		mg C/g DW		mg N/g DW	
0-1	1'831	315	1'912	755	1'287	85	359	85
1-5	979	295	596	334	401	26	199	26
5-10	522	104	358	218	241	27	73	27
10-20	57	*	8	7	5	*	6	*
20-30	49	*	3	1	2	*	6	*
30-40	31	*	4	1	3	*	2	*
40-50	106	*	4	2	3	*	7	*

* Not reported.

Calculated biomass COD

Table 2 shows the calculated biomass COD figures using the measured data from Table 1 and the biomass composition $C_5H_7O_2N$. In the first cm of the main layer the mean values are ranging from 3'400 to 5'100 mg COD/g DW, from 1 to 5 cm 1'100 to 2'600 mg COD/g DW, and from 5 to 10 cm 640 to 1'400 mg COD/g DW, respectively. Biomass COD calculated from C fumigation measurements resulted in the lowest values. No significant difference could be found when comparing the calculations using biomass measurements that are based on different methods (Figure 1).

Table 2: Calculated biomass COD in different depths of the main layer (mg COD/g DW).

Depths	SIR		ATP		C fumigation		N fumigation	
cm	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.
0-1	4'884	839	5'100	2'012	3'431	226	4'103	970
1-5	2'611	787	1'590	891	1'070	70	2'269	299
5-10	1'392	276	955	581	642	72	834	307
10-20	151	-	22	19	15	-	69	-
20-30	130	-	8	3	5	-	69	-
30-40	82	-	10	3	7	-	23	-
40-50	283	-	10	6	7	-	80	-

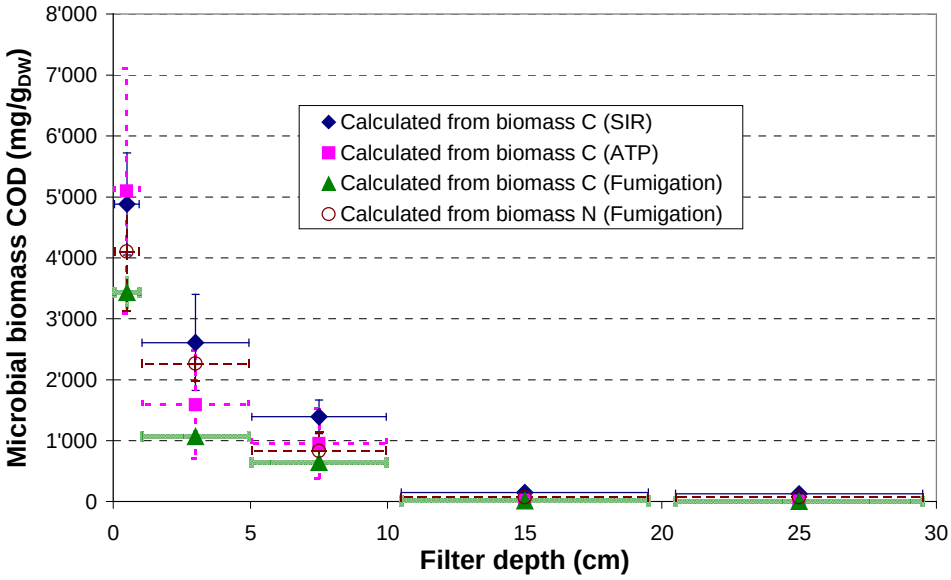

Figure 1: Calculated microbial biomass COD in different depths of the main layer.

Model set-up

To reduce the simulation time only a part of the PSCW cross section was considered for the numerical simulations. The width of the transport domain was 20 cm, and its depth 50 cm (the depth of the main layer), while the transport domain itself was discretized into 3 columns and 25 rows. This results in a two-dimensional finite element mesh consisting of 75 nodes and 96 triangular finite elements. An atmospheric boundary condition was assigned to the top of the system representing the influent distribution system, and a constant pressure head boundary condition (constant head of -2 cm) to the

bottom of the main layer. Parameters for the flow model, i.e. the parameters to describe the unsaturated hydraulic properties of the main layer, have been taken from Mollner (2005).

Simulation results

Heterotrophic organisms are the majority of the microbial community and therefore represent the main component of the total microbial biomass. Simulations have been carried out for different rate constants b_H for lysis of heterotrophic microorganisms. Lysis represents the sum of all decay and sink processes, and produces organic matter (CS, CR, and CI) as well as nutrients incorporated in the biomass (ammonium and inorganic phosphorus). The values for other rate constants of CW2D (as defined in Langergraber, 2001) have been kept constant: the maximum heterotrophic aerobic growth rate on CR was 2.4 d^{-1}, the maximum denitrification rate was 1.9 d^{-1}, the maximum autotrophic aerobic growth rate on NH_4^+ was 0.36 d^{-1}, the maximum autotrophic aerobic growth rate on NO_2^- was 0.40 d^{-1}, and the autotrophic rate constant for lysis was 0.036 d^{-1}.

The influent concentrations used for the simulations are shown in Table 3 that also shows the effluent concentrations for simulations with different rate constant for lysis of heterotrophic microorganisms. The effluent concentrations are median values of the last 5 days of a simulation time of 200 days. Steady-state conditions could be reached after this period. With increasing b_H values the COD effluent are increasing due to more decay processes of biomass resulting in CS and CI, respectively. Effluent concentrations of ammonia and nitrite nitrogen are not effected by different b_H values whereas nitrate nitrogen concentrations change due to different availability of readily biodegradable organic matter that is required for denitrification.

Table 3: CW2D influent concentrations and simulated effluent concentrations.

Parameter	**b_H**	**CR**	**CS**	**CI**	**COD**	**NH4-N**	**NO2-N**	**NO3-N**
Unit	(d^{-1})	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)
Influent	**-**	160	120	20	300	60	0.1	0.1
Effluent	**0.10**	0.2	2.9	28.9	32.0	0.060	0.019	50.6
	0.20	0.4	5.8	33.3	39.5	0.060	0.019	48.1
	0.25	0.5	7.5	34.4	42.4	0.061	0.019	48.6
	0.30	0.6	9.9	34.5	44.9	0.060	0.019	50.5
	0.35	0.7	12.5	33.8	47.0	0.061	0.019	53.0

CR, CS, CI: readily, and slowly biodegradable, and inert organic matter, respectively (Langergraber, 2001)

Table 4 shows the simulated microbial biomass COD in different depths of the main layer for different heterotrophic lysis rates. With increasing b_H the microbial biomass COD decreases. Using heterotrophic lysis rates between 0.25 and 0.35 d^{-1} results in microbial biomass COD between 5'600 and 3'400 mg COD/g DW for the first cm of the main layer. These values are in the range of the measured values (Table 2). Table 5 shows the simulated composition of the microbial biomass COD for b_H = 0.30 d^{-1}. As expected the main contribution to the microbial biomass COD comes from heterotrophic organisms (more than 90 % in the top layers).

Table 4: Simulated microbial biomass COD (mg COD/g DW) in different depths of the main layer for different heterotrophic lysis rates b_H.

Heterotrophic lysis rate b_H (d^{-1})		0.10	0.20	0.25	0.30	0.35
Depth	0–1 cm	8'191	6'466	5'557	4'634	3'384
	1-5 cm	6'454	5'017	4'402	3'867	3'223
	5-10 cm	3'811	2'069	1'245	480	227
	10-20 cm	910	374	198	191	192
	20-30 cm	219	171	149	133	103
	30-40 cm	84	64	61	63	61
	40-50 cm	44	38	38	37	34

Figure 2 compares calculated and simulated microbial biomass COD in different depths of the main layer for a heterotrophic lysis rate of b_H = 0.30 d^{-1}. For comparison only the calculated values from substrate induced respiration (SIR) are shown. The simulations over-predict biomass COD in 1-5 cm depth and under-predict biomass COD in 5-10 cm depth. This indicates that it might be required to include the influence of biomass growth (and volume) on the hydraulic flow properties of the sandy substrate. Biomass growth leads to reduced pore volume and therefore flow velocities are reduced resulting in longer contact times between the biomass and the wastewater constituents and therefore in higher degradation of organic matter in the upper 1 cm. Finally less organic matter is available in deeper zones of the main layer resulting in reduced biomass growth in 1-5 cm depth.

Table 5: Simulated composition of the microbial biomass COD (mg COD/g DW) in different depths of the main layer for b_H = 0.30 d^{-1}.

Depth	XH	XANs	XANb	Microbial biomass
0–1 cm	4'291	193	151	4'634
1-5 cm	3'549	168	150	3'867
5-10 cm	360	64	56	480
10-20 cm	108	43	41	191
20-30 cm	102	15	15	133
30-40 cm	57	3	3	63
40-50 cm	33	2	2	37

XH, XANs, XANb: heterotrophic organisms, *Nitrosomonas* spec., and *Nitrobacter* spec., respectively (Langergraber, 2001)

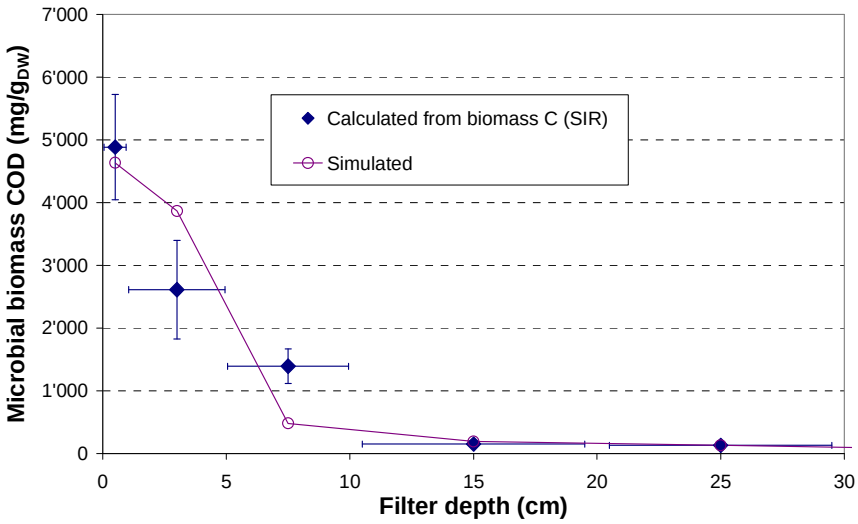

Figure 2: Calculated and simulated (b_H = 0.30 1/d) microbial biomass COD in different depths of the main layer.

SUMMARY AND CONCLUSIONS

This paper shows how measured data for microbial biomass in vertical subsurface flow constructed wetlands have been used to calculate biomass COD values needed for comparison with numerical simulations using the multi-component reactive transport module CW2D. Simulated microbial biomass COD in the first cm of the main layer are between 5'600 and 3'400 mg COD/g DW (the range of the measured values) when using heterotrophic lysis rates between 0.25 and 0.35 d^{-1}. When comparing measured and simulated biomass COD in different depths of the main layer simulations seem to over-predict biomass COD in 1-5 cm depth and under-predict biomass COD in 5-10 cm depth. This could be an indication that the influence of biomass growth on the hydraulic properties has to be modelled as well. However, up to now this is not possible with the HYDRUS-2D/CW2D software package.

ACKNOWLEDGEMENTS

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Annex V: Investigation of bacterial removal during the filtration process in constructed wetlands.

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Abstract
In this study, bacterial removal efficiencies of planted and unplanted subsurface vertical flow constructed wetlands are compared. Indicator organisms such as fecal Coliforms (*Escherichia coli*, total Coliforms) and Enterococci, and the number of heterotrophic bacteria (heterotrophic plate counts) have been analysed from the influent and effluent of the constructed wetlands as well as in different depths (water and substrate samples). Furthermore dry matter content and total organic carbon (TOC) have been analysed and correlated. The investigated systems show a high removal rate for indicator organisms (the log removal rate was 2.85 for HPC , 4.35 for *E. coli*, 4.31 for total Coliforms and 4.80 for Enterococci, respectively). In general no significant difference for the removal efficiency of planted and unplanted vertical flow beds could be measured. Only for Enterococci measured in the substrate samples of the main layer of the filter a statistically significant difference could be observed.

Keywords:
Bacterial removal, indicator organisms, constructed wetlands, wastewater treatment

INTRODUCTION

Wastewater discharges are the major source of fecal microorganisms, including pathogens, in rivers and coastal waters. The efficiency of constructed wetlands concerning the removal of microorganisms, especially indicator microorganisms like Coliforms and Enterococci, is a topic that has been thoroughly investigated (e.g. Kadlec et al., 2000; Langergraber and Haberl, 2001). Constructed wetlands are often used for treating wastewater in small communities and often discharge into pollution sensitive waters. In this case, contamination of the water by pathogens posing a risk to public health cannot be excluded (Hagendorf et al., 2000).

Constructed wetlands are classified into two main types: surface flow constructed wetlands and subsurface flow constructed wetlands that are again subdivided into horizontal flow and vertical flow constructed wetlands, depending on the direction of the water flow through the porous medium (soil or gravel). The surface flow wetland technology is strongly related with natural wetlands were the water flows over the soil surface from an inlet to an outlet point. Compared to surface flow systems the contact area of water with bacteria and substrate is much bigger in subsurface flow constructed wetlands. This enhance the process rates of the system and therefore decreases the area requirement of the treatment system. (Langergraber and Haberl, 2001)

However, in most studies dealing with bacterial removal (e.g. Decamp and Warren, 2001; Thurston et al., 2001) only influent and effluent of the constructed wetlands are compared with regard to their colony forming units (CFU). These investigations do not give any information regarding in which zone of the sandy substrate the majority of the bacterial contamination is eliminated.

The aim of the study is to show the removal efficiencies of subsurface vertical flow constructed wetlands comparing planted and unplanted systems. Indicator organisms such as fecal Coliforms (*Escherichia coli (E. coli)*, total Coliforms) and Enterococci, and heterotrophic bacteria (heterotrophic plate counts, HPC) are analysed from the influent and effluent of the system and different depths of the sandy main layer (water and substrate samples). In addition to the indicator organisms dry matter content and total organic carbon (TOC) have been analysed for the samples taken from the main layer of the filter bed. The expected result of the project is a simple method to describe bacterial removal in the main layer of subsurface vertical flow constructed wetlands to enable a better design regarding hygienic safety.

MATERIAL AND METHODS

Experimental design

The study has been carried out at the research facilities of the Institute of Sanitary Engineering and Water Pollution Control in the Department of Water, Atmosphere and Environment at the University of Natural Resources and Applied Life Sciences, Vienna (BOKU). 10 parallel operated indoor pilot-scale subsurface vertical flow constructed wetlands (PSCWs) have been investigated.

Figure 1 shows a vertical cross section of a PSCW. The PSCWs have a surface area of 1 m² each. The main layer has a depth of 50 cm and consists of sand with a grain size of 0.06-4 mm (for 8 PSCWs) and 1-4 mm (for 2 PSCWs), respectively. An intermediate layer of 10 cm thickness with a gravel size of 4-8 mm prevents fine particles to be washed out into the drainage layer (15 cm thick; gravel 16-32 mm). The PSCWs have been operated automatically and loaded intermittently with mechanically pre-treated municipal wastewater. The hydraulic loading rate for the PSCWs with a main layer of a grain size of 0.06-4 mm and 1-4 mm was 60 mm/d (4 loadings per day) and 240 mm/d (8 loadings per day), respectively.

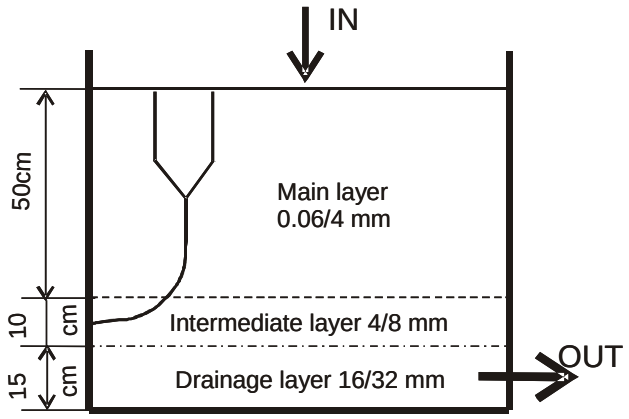

Figure 1: Vertical cross section of a PSCW

Figure 2 shows a top view of the different layouts of the PSCWs tested. 8 PSCWs are planted with *Miscanthus sinensis giganteus* (the further planted common reed *Phragmites australis* did not grow under indoor-conditions) whereas 2 PSCWs (Plot 2 and 3) are unplanted. 4 PSCWs have been operated with a saturated drainage layer to increase the retention time. Water content and dissolved oxygen have been measured on-line in Plot 1-5.

0.06/4 mm Plot 5 **planted**	0.06/4 mm Plot 4 **planted**	0.06/4 mm Plot 3 **unplanted**	0.06/4 mm Plot 2 **unplanted**	0.06/4 mm Plot 1 **planted**
1/4 mm Plot 10 **planted**	1/4 mm Plot 9 **planted** saturated drainage layer	0.06/4 mm Plot 8 **planted** saturated drainage layer	0.06/4 mm Plot 7 **planted** saturated drainage layer	0.06/4 mm Plot 6 **planted** saturated drainage layer

Figure 2: Top view from the Indoor PSCWs at BOKU

In Plot 1-5 sampling systems to collect water from different depths have been installed. 4 systems in each plot allow sampling of water from different depths: 10, 20, 30, and 40 cm, respectively. The sampling systems consist of a PE column with a diameter of 20 cm that is ending in an open funnel (Figure 1). The layers composition in the column is the same as in the PSCW (0.06-4 mm grain size).

In addition samples from an outdoor constructed wetland having the same design as the indoor plants (Langergraber, 2005) have been analysed.

Sampling

For analysing the indicator organisms samples have been taken from the influent and the effluent of the system and from different depths. The samples have been analysed for heterotrophic bacteria, fecal Coliforms (*Escherichia coli*, total Coliforms) and Enterococci.

The water samples (mechanically pre-treated municipal wastewater and the effluents from all PSCWs) were collected in 2 litres sterile Schott-bottles and analysed within 2 hours. The samples were diluted serially.
Substrate samples were taken with a sampling-needle (conical, 1.5-3 cm in diameter) and a drill (10 cm in diameter). 10 g well-mixed wet soil per depth was placed into a sterile 200 ml plastic container. 90 ml of sterile deionized water was added. For separation of microorganisms from soil and particles the samples were shaken (shaker: KS 501D, Janke&Kunkel, IKA®-Labortechnik) for 30min and after this sonicated in a ultrasonication bath (Branson 5510, 135W, 42KHZ) for 1min. After 5min settlement 1ml was taken and serially diluted. This was found of to be the most effective technique, inspired by Craig et al. (2002).

Indicator organisms
Fecal Coliforms and Enterococci in soil and water samples were enumerated by membrane filtration (0.45 µm-pore size, 47 mm diameter sterile cellulose nitrate filter, Satorius) and by the plate count method (colony forming units CFU/ml). For counting of fecal Coliforms and *E. coli* Chromocult Coliform® agar (MERCK; Norm: ISO 16649) plus cefsulodin (10µg/ml) (CC^+) was used. The incubation lasted 24 hours at 37°C.
Chromocult agar was developed for the simultaneous detection of total Coliforms and *E. coli* due to the inclusion of two chromogenic substrates. Chromocult agar for the identification and enumeration of human fecal Enterobacteriaceae does not need further biochemical tests for confirmation of identity (Finney et al., 2003).
Membrane-filter Enterococcus Selective Agar acc. to SLANETZ and BARTLEY (MERCK) was used to enumerate Enterococci. The incubation lasted 48 hours at 37°C.
The number of heterotrophic bacteria (heterotrophic plate count, HPC) was determined by the pour plate method with yeast extract agar (MERCK). The incubation lasted 72 hours at 22°C.
The numbers of total Coliforms, *E. coli*, Enterococci and heterotrophic bacteria were converted to $\log_{10}$ values and expressed as $\log_{10}$ CFU/ml because of better illustration.

Bacterial direct counts (DC)
For the determination of total cells in the influent and effluent samples the epifluorescent direct counting method was used. After finding the optimal (countable) dilutions, DAPI (4′, 6-diamidino-2-phenylindole) was used for dyeing the cells. Samples were incubated in the dark for 10min and filtered through a griddled blackened polycarbonate membrane filter (25mm diameter, 0.45µm pore size, Millipore). Bacteria were then viewed using an epifluorescence microscope (Axioplan, Zeiss), every square was counted and the mean was calculated. The numbers were expressed as $\log_{10}$ cells/ml (Taylor et al., 2002).

Substrate samples
Total organic carbon (TOC) and dry weight (DW) are measured from the substrate samples. For the TOC the C/N–Analyzer „Vario Max“ from Elementar was used. The principle of „Vario Max“ is the combustion of the sample at 900°C. Due to oxidation of organic and inorganic components of the sample NOx and CO_2 are produced during the combustion process.
The main combustion tube reaches 900°C, the second tube reaches 900°C as well and the reduction-tube works at 830°C. The carrier gas is helium. After burning the gases (NOx and CO_2) pass through the drying-tubes and the CO_2 absorber. CO_2 is absorbed and NOx passes through the detector (TCD = Temperature Current Detector) which needs also helium as the reference. After detection of NOx the CO_2 absorber is heated to 250°C and therefore CO_2 is desorbed and reaches the detector. The calculation from N-total and C-total is carried out by means of Vario Max-Software 4.3D. For calibration L-glutamine acid was used. The area below the peaks is integrated and the result is given in % of dry matter. The TOC is calculated by the difference between the total carbon (TC) and inorganic carbon (TIC).
To determine the DW of the soil a known weight of soil was placed in an oven at 105°C for 24 hours and weighed. The percent dry weight was then calculated from these results.

Chemical parameters
Samples from the influent and effluent have been analysed according to the German standards for organic matter (COD (DIN 38409-T41) and BOD5 (DIN EN 1899-T1), TOC (DIN EN 1484)), ammonia nitrogen (NH4-N (DIN 38406-T5)) and total phosphorus (TP (DIN EN ISO 6878)) in the laboratory of the Institute.

Dissolved oxygen

Dissolved oxygen O_2 was measured with 5 Electrochemical O_2 Sensors (EC-sensor-OM-E200302 3600 Analyzer for Oxygen; Orbisphere) in 4 different depths (5, 10, 20, 40cm) of the main layer. The sensors were used in planted and unplanted PSCWs.

RESULTS AND DISCUSSION

Data analyses

All data were related to g of DW (dry weight) soil. Spearman-Rank correlation was performed to test for the relationship between the investigated variables. Statistical significance was assumed at a probability level of $p < 0.05$. All statistical analyses were made with the software package SPSS 11.0 for Mac (SPSS Inc, Chicago, Illinois, USA).

Removal efficiency

The mean values (n=23) for the influent and effluent concentrations, and removal efficiencies for the PSCWs with a sandy main layer with a grain size of 0.06-4mm are shown in Table 1.

Table 1: Influent and effluent concentrations, and removal efficiencies for chemical and bacteriological parameters (mean values (n=23), 0.06-4 mm).

Parameter	**Influent**	**Effluent**	**Removal**
Chemical parameters	**mg/l**		**%**
COD	367±113	< 20	> 94.5
BOD_5	150±61	< 3	> 98.0
TOC	160±51	4.7±0.9	97.1
NH_4-N	42±8	0.20±0.02	99.5
TP	6.6±1.0	4.1±1.35	0-47.4
Indicator organisms	**Log CFU / ml**		**Log**
HPC	6.22±0.32	3.37±0.38	2.85
E. Coli	6.59±0.64	2.24±1.42	4.35
Total Coliforms	6.99±0.55	2.69±1.20	4.30
Enterococci	6.06±0.40	1.26±1.16	4.80
Bacterial direct counts	**Log cells / ml**		**Log**
Microscopic direct counts (DC)	8.08±0.43	6.03±0.82	2.05

Bacterial indicators of focal contamination measured in the mechanically pre-treated municipal wastewater show a comparable concentration with e.g. George et al (2002) who showed typical abundances of total and focal Coliforms (FC) in raw sewage of 10^7-10^9 ml^{-1} and 10^6-$10^8 ml^{-1}$, respectively. The removal efficiency for the indicator parameters is quite high compared to the literature (e.g. Ottova et al., 1997; Thurston et al., 2001)

Figure 3 shows bacterial parameters in the influent and effluent of Plot 1 (mean values) and for water samples in 4 different depths (10, 20, 30, 40 cm). The results show a typical distribution from the bacterial parameters with the depths. The removal efficiency was about 2.44 log for microscopic direct counts (DC), 3.3 log for HPC, 3.77 log for total Coliforms, 4.00 log for *E. coli* and 4.34 log for Enterococci.

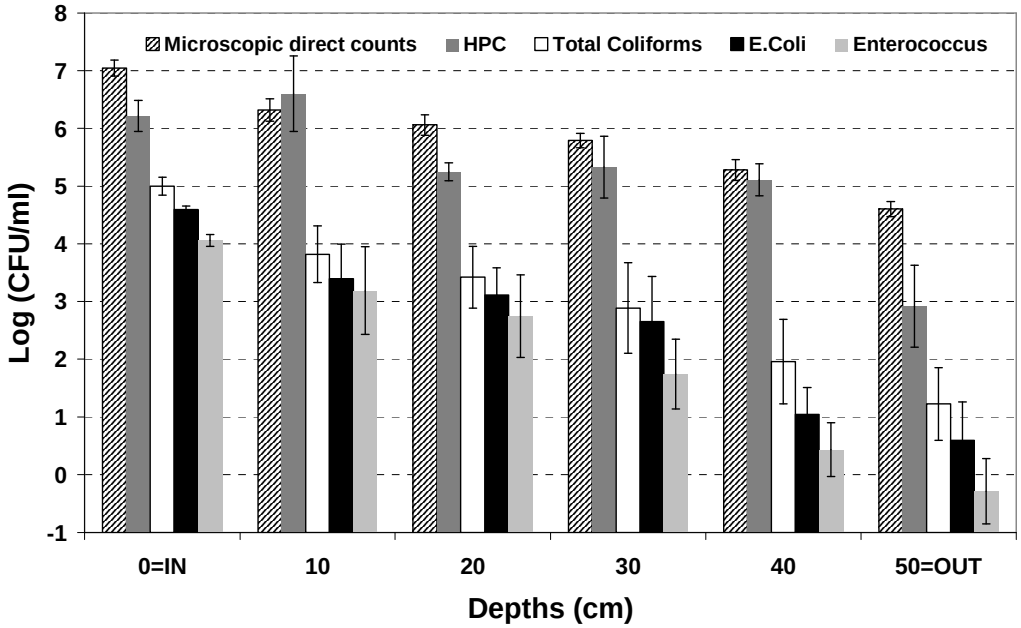

Figure 3: Bacterial parameters of the influent and effluent of the PSCWs and of water samples from different depths (n=23)

Figure 4 shows the log removal rates of the bacterial parameters of Plot 1 through 10 as well as for the investigated outdoor plants (E1-E3). Lower bacterial removal in Plot 9 and 10 can be explained by the bigger grain size (1-4 mm) and the higher hydraulic loading rate. These are similar results as in the study described by Ausland et al (2002). The removal rates for the outdoor plants (E1-E3) show a lower value in some parameters, because of different loadings and other factors according to outdoor systems.

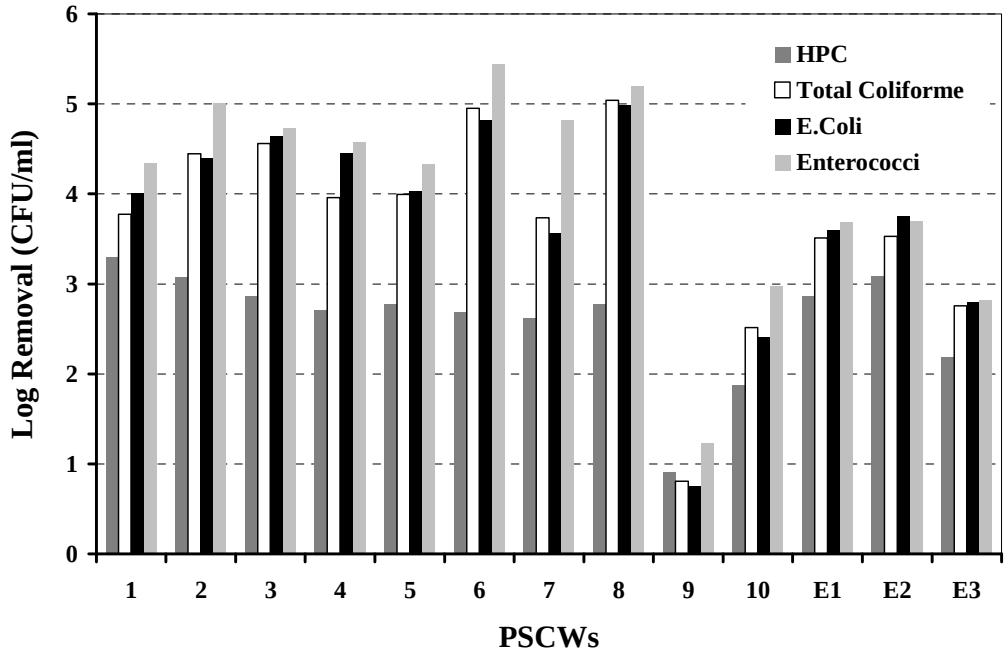

Figure 4: Log removal rate of bacterial parameters for all Plots and for the outdoor systems (E1-E3), (n=23).

The concentration of the bacterial parameters in the effluent from these plants has a great removal efficiency compared to similar systems described in the literature. There is no significant difference in the performance from planted and unplanted PSCWs comparing the effluents. Mean values of the

removal rate for the planted and unplanted systems are 2.05 log for DC, 2.85 log for HPC, 4.30 log for total Coliforms, 4.35 log for *E. coli* and 4.80 log for Enterococci.

Measurements in different depths of the main layer

For sampling the drill was found out to be the better sampling device, because contamination occurred due to sampling failures with the needle. It was found that the first cubic centimetres from the top of the sampled layer were displaced to a deeper level of the needle during sampling. Figure 5 shows the difference from the results from log (CFU/g DW) Enterococci with the needle and the drill. The difference has been shown to be statistically significant in 20-50 cm depth.

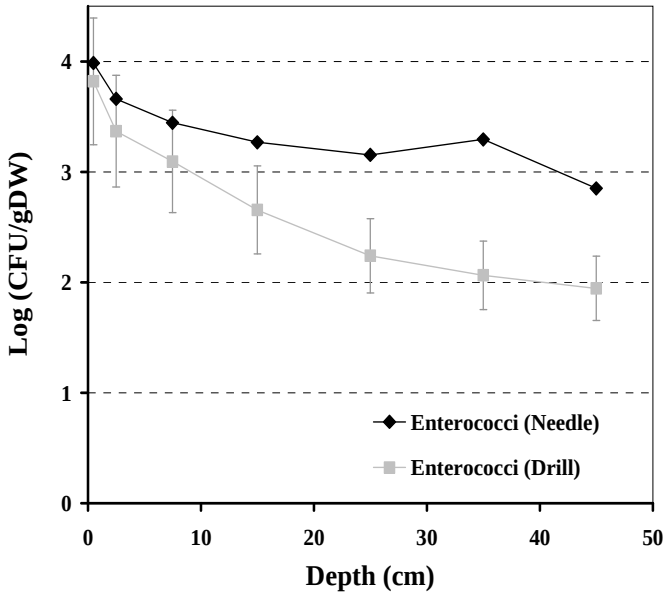

Figure 5: Difference from the results with needle and drill (n=15).

As the PSCWs are intermittently loaded the main layer is always unsaturated. Dissolved oxygen is an important parameter for aerobic biological processes in wastewater treatment and was measured in 4 different depths. Typical values for the minimum and maximum of the dissolved oxygen concentration during a loading interval were 7.8 and 9.3 mg/l, respectively. The changes are caused due to the intermittent loading, the amplitude of the changes is decreasing with depth. In the first 10 cm the drained wastewater has a bigger effect on the oxygen demand than in deeper layers of the main layer. But the measurement shows that there is still enough oxygen in the lower parts of the main layer.

TOC was analysed from the samples taken from the main layer of the filter from all systems (planted, unplanted, outdoor) in 7 different depths. Figure 6 compares the TOC measurements in planted and unplanted PSCWs with the outdoor systems. The decrease of TOC with increasing depths can be clearly seen. No difference can be observed between the systems.

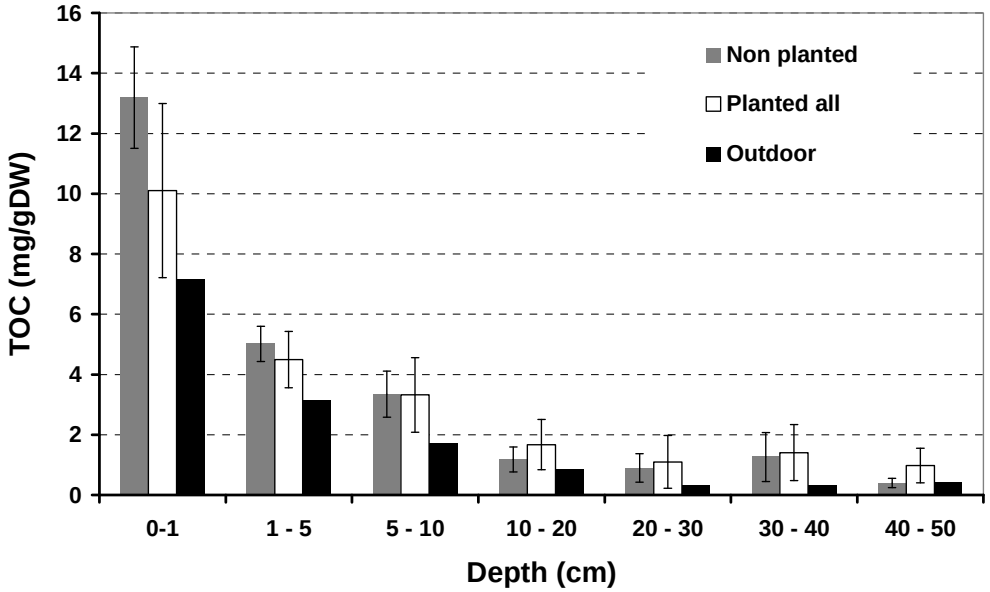

Figure 6: TOC in different depths of the main layer (n=23).

Figure 7, Figure 8, Figure 9 and Figure 10 show a vertical profiles of HPC, total Coliforms, *E. coli* and Enterococci counts (per g dry weight of the sandy substrate) in 7 different depths of planted and unplanted PSCWs, respectively. Each bar represents the mean value of 35 measurements (11 for unplanted), the intervals the standard deviation. There is no continuous decline as maybe expected but a minimal increase in 30-40 cm depth. This is more obvious for the unplanted PSCWs. The reason for that was described before and was caused by an error introduced by the sampling device (Figure 5). This fact can also explain the high standard deviations in the 4 last depths.

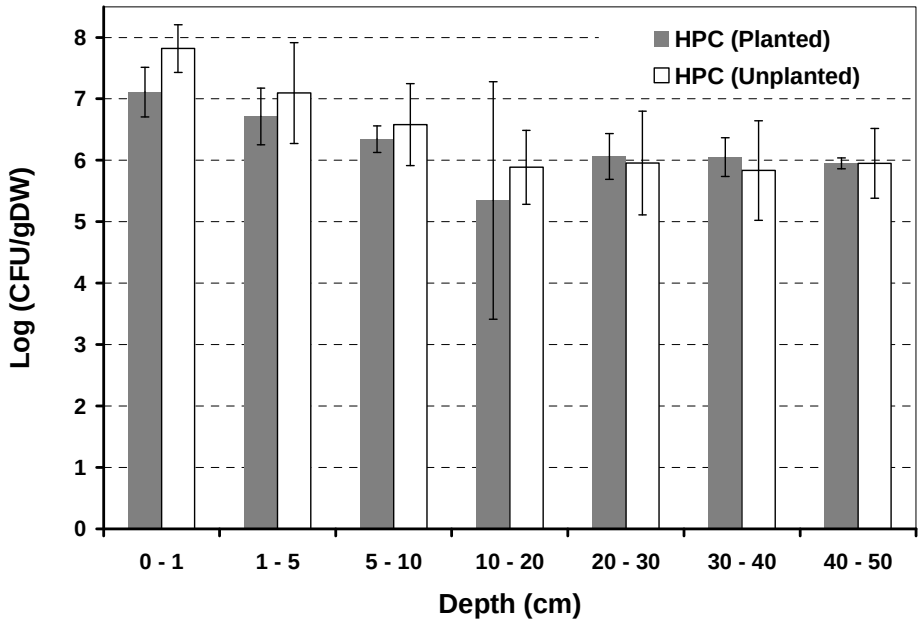

Figure 7: Heterotrophic plate count in different depths of the main layer of planted and unplanted PSCWs (n=23).

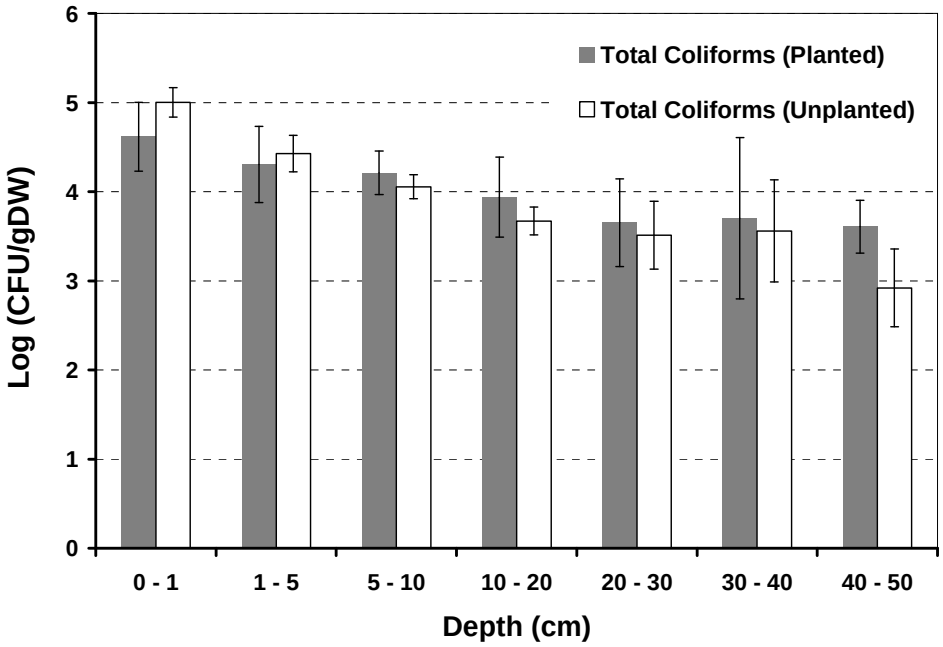

Figure 8: Total Coliforms in different depths of the main layer of planted and unplanted PSCWs (n=23).

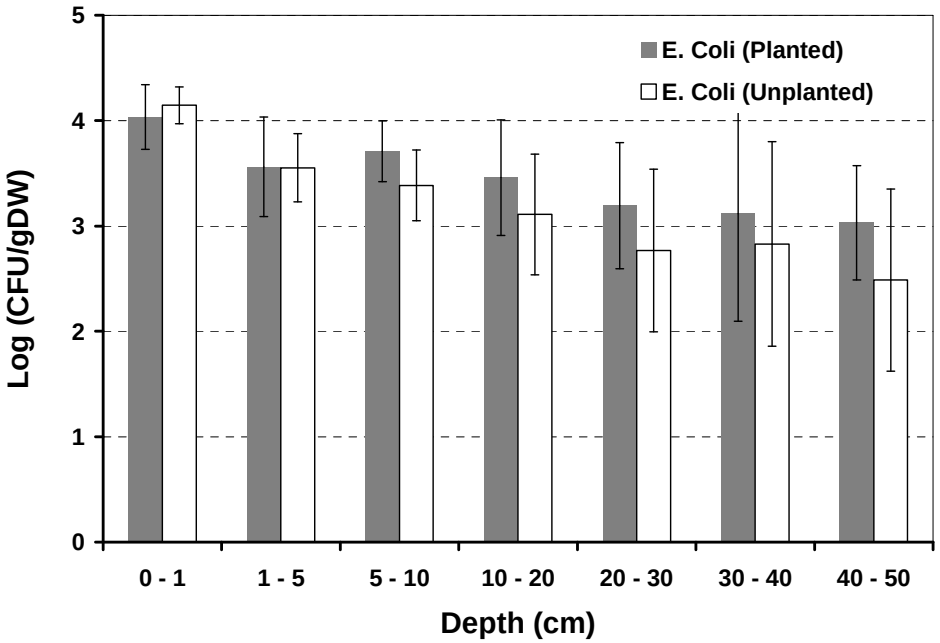

Figure 9: *E. coli* in different depths of the main layer of planted and unplanted PSCWs (n=23).

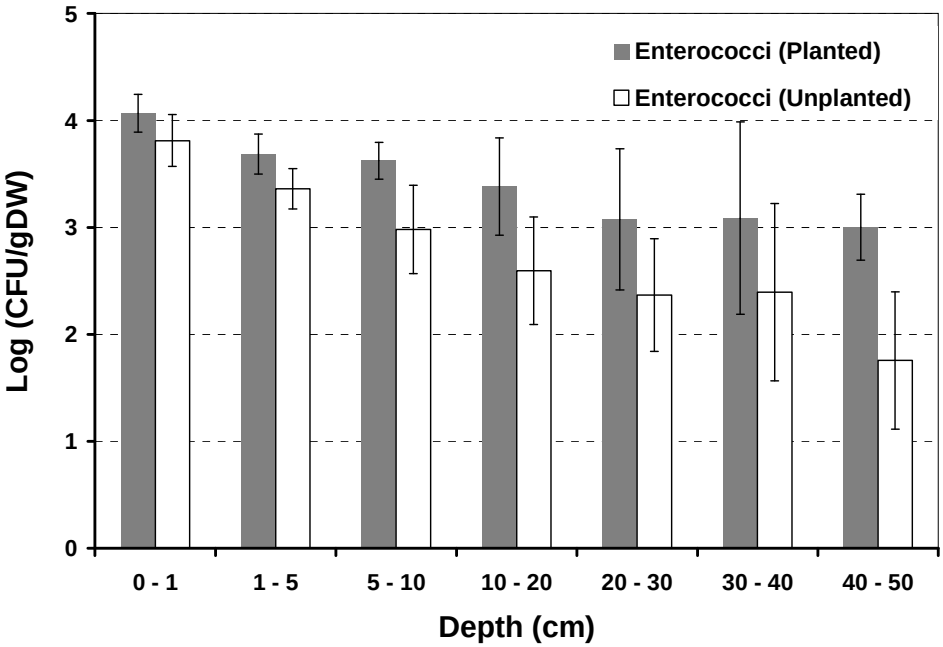

Figure 10: Enterococci in different depths of the main layer of planted and unplanted PSCWs (n=23)

Comparing the absolute counts from planted and unplanted PSCWs one can see the difference between HPC and total Coliforms. HPC numbers from the planted PSCWs show a lower, but not significantly value in nearly all depths (Figure 7). This is in contrast to the unplanted PSCWs. Total Coliforms show higher values in the planted PSCWs from the depth 5-10 to 40-50 cm. The same results as for total Coliforms have been found for *E. coli*. Only the results of the Enterococci shows a statistically significant difference between planted and unplanted PSCWs.
A high number of indicator organisms has been measured in the main layer of the filter in all investigated depths. The values were between 0.5 and 8 (log CFU/g DW) for all parameters. This could give an indication in which depth the retention of the invested organisms takes place. Since degradation of retained bacteria could occur during filter operation, it is important to note that simply comparing the post-operation densities of viable organisms at different depths does not necessarily equate exactly to a percent bacterial removal as a function of the depth (Stevik et al., 1999). Only for the Enterococci values a statistically significant difference between planted and unplanted PSCWs could be measured in the main layer.
The correlations of total Coliforms with DW (%) and TOC (mg/g DW), respectively. TOC shows a good correlation with the measured bacterial parameters and is also a parameter for microbial biomass. The result for all bacterial parameters is resumed in Table 2.

CONCLUSIONS
From this study it can be concluded that:

- The PSCWs show a high removal rate for indicator organisms (the log removal was for HPC 2.85, for *E. coli* 4.35, for total Coliforms 4.31, and for Enterococci 4.80, respectively)
- Most of the elimination process is taking place in the first 10-20 cm of the main layer
- There is no significant difference in the performance from planted and unplanted PSCWs comparing the effluents.
- The only significant difference between planted and unplanted systems was measured for Enterococci in the main layer of the filter.
- TOC and DW showed a high correlation with all measured bacterial parameters.
- To investigate bacterial fate in detail, bacterial tracer experiments have to be carried out e.g. by using an non-pathogenic antibiotic resistant *E. coli*.

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12 Curiculum vitae

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