Universität für Bodenkultur Wien

Department für Wasser, Atmosphäre, Umwelt, Universität für Bodenkultur Wien

Institut für Siedlungswasserbau, Industriewasserwirtschaft und Gewässerschutz

BACTERIAL REMOVAL PROCESS IN DIFFERENT DESIGNS OF SUBSURFACE VERTICAL FLOW CONSTRUCTED WETLANDS

Dissertation zur Erlangung des Doktorgrades an der Universität für Bodenkultur Wien

Betreuer:
Univ.Prof. Dipl.-Ing. Dr.nat.techn. Raimund Haberl

Eingereicht von:
DI Kirsten Sleytr
Matrikelnummer: 9340604

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This work is dedicated to my parents Henriette and Uwe.

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Abstract

Constructed wetlands (CWs) are among the most popular wastewater treatment systems for decentralized solutions in Austria. All over the world CWs are widely used for the treatment of municipal wastewaters, which are characterized by high concentrations of nutrients and pathogens. CWs are simple in operation and maintenance and offer a lot of benefits to the environment beside a high capacity of treating wastewater. They can be seen as an artificial habitat for many organisms, birds, insects, macrophytes, bacteria and other living organisms which play an important role for the treatment efficiency.

In addition to the ability to eliminate nutrients, pollutants and toxic substances, the removal of pathogens is an important aspect of wastewater treatment. In the filter body and the root zone area of CWs far-reaching elimination of pathogenic germs take place, which can exceed the elimination capacity of conventional, biological (activated sludge) wastewater treatment plants.

Although not regulated in the Austrian effluent standards hygienisation processes in CWs are and will get more important to evaluate and understand. The basic knowledge about the processes of pathogen removal in wetlands is currently inadequate. By dealing with bacterial removal most studies only compare the influent and effluent of the CWs. But wastewater purification and bacterial removal take place inside the filter bed caused to interactions between plants, soil and micro-organisms.

There is still a lack of knowledge of complete understanding of many microbiological and pathogen removal mechanisms. Therefore CWs are often seen and described as “black boxes”. The aim of the presented study was to bring light into the “black box” in terms of bacterial removal processes, the influence of different designs on the treatment efficiency of CWs and bacterial diversity patterns. The present work is linking up the results from various techniques of different disciplines to characterize the system CW by understanding the interactions of the sandy substrate, plants, microorganisms, hydraulic retention time (HRT) and the ratio of dissolved oxygen during the infiltration processes, shifts of the grain size over time and other parameters. Besides influent and effluent sampling, the focus was to investigate the filter body in different depths of ten indoor pilot scale CWs (PSCWs) with different designs and of an outdoor full scale CW (FSCW) in Austria.

Indicator organisms such as faecal coliforms (Escherichia coli, total coliforms), Enterococci and a number of heterotrophic bacteria have been analysed from the influent and effluents of the constructed wetlands as well as from water and substrate samples at different depths within the system. The investigated CWs show a high removal rate for indicator organisms; with a log removal for HPC of 2.85, for E. Coli 4.35, for total coliforms 4.31, and for Enterococci 4.80, respectively. Most of the elimination processes took place in the first 10 to 20 cm of the main layer. Higher log removal rates could only be achieved by combining systems like two stage VF CWs.

Two pathogens - Clostridium perfringens spores and Pseudomonas aeruginosa – have been investigated from the PSCWs and it was found out that these systems are capable for significantly reduction. Differences in the removal efficiency from C. perfringens spores and Pseudomonas aeruginosa between planted and unplanted PSCWs could be observed whereas unplanted systems showed higher removal rates. This effect can be linked to the longer HRT in the planted PSCWs.

The microbial biomass is mainly responsible for the degradation performance in vertical flow (VF) CWs. It was found out that microbial biomass was quite high compared with natural soils caused to the high nutrient level in the used wastewater.

Very few information is available on the microbial diversity within CWs. For this purpose, the common molecular fingerprinting technique “terminal restriction fragment length polymorphism” (T-RFLP, or TRF) was used to characterize the microbial communities; PSCWs and the FSCW that were operated under similar conditions, were investigated. It was revealed that both systems are colonized by similar populations showing only little variation in their composition over the filter depth. 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 Removal Processes
Indicator Organisms
Bacterial Diversity
Treatment Optimisation

Zusammenfassung

Vertikal durchströmte Bodenfilter, auch Pflanzenkläranlagen genannt, sind ein weltweit sehr beliebtes und häufig eingesetztes System zur Reinigung von häuslichen,- und industriellen Abwässern. In Österreich werden solche Anlagen meist im dezentralen Raum verwendet, wo eine Abwasserentsorgung via Kanal und zentraler Anlage technisch sehr aufwändig und damit sehr teuer ist. Pflanzenkläranlagen kennzeichnen sich durch Einfachheit in Betrieb und Wartung aus. Neben ihrer Eigenschaft Abwasser effizient zu klären bieten sie eine Reihe von anderen Vorteilen wie z.B.: Habitat für viele Mikroorganismen (spielen die wichtigste Rolle beim Klärvorgang), Vögel, Insekten und Makrophyten.

Neben der Eigenschaft, Nährstoffe, Schadstoffe und toxische Substanzen zu beseitigen, ist die Elimination von Pathogenen ein wichtiger Aspekt der Abwasser-Behandlung. Im Filterkörper und Wurzelbereich der Pflanzenkläranlagen kann es zu einer effizienten Beseitigung von pathogenen Keimen kommen, die sogar die Eliminationskapazität von herkömmlichen, biologischen (Belebtschlamm) Abwasserreinigungsanlagen überschreiten kann.

Obwohl Pflanzenkläranlagen ein gut untersuchtes System darstellen, sind viele Prozesse die während der Abwasserreinigung im inneren des Filterbeets stattfinden immer noch nicht adäquat untersucht und beschrieben – vergleichbar mit einer “Black Box”.

Die meisten Studien über Eliminationsprozesse von Bakterien beruhen auf Vergleichen von Zulauf und Ablauf einer Pflanzenkläranlage. Die wesentlichen Eliminationsprozesse finden jedoch innerhalb des Filterbettes statt; hervorgerufen durch physikalische, chemische und biologische Prozesse und Wechselwirkungen zwischen Pflanzen, Algen, Bakterien, Mikroorganismen und dem Bodenkörper.

Um Licht in die „black box“ zu bringen, lag der Schwerpunkt dieser Arbeit darin, Hygienisierungsabläufe zu untersuchen, zu beschreiben und bakterielle Eliminationsprozesse zu ermitteln. Dazu wurden unterschiedliche Designs von „indoor“-Pflanzenkläranlagen im Versuchsmaßstab konstruiert und mit vollmaßstäblichen „outdoor“-Anlagen verglichen. Untersucht wurden unter anderem: verschiedene Indikatororganismen und zwei Pathogene im Zu,- Ablauf und in verschiedenen Substrattiefen, Hydraulische Aufenthaltszeit, Sauerstoffgehalt in vier Tiefen, Veränderung der Substratverschiebung über die Projektszeit von zwei Jahren, diverse chemische und physikalische Parameter im Abwasser und Substrat. Ziel war, über verschiedene Disziplinen und Techniken das System Pflanzenkläranlage zu charakterisieren, Reinigungs- und Eliminationsprozesse zu beschreiben.

Die Anlagen zeigten eine hohe Eliminationsleistung in Bezug auf die untersuchten Organismen und anderen Parameter; erreicht wurde eine durchschnittliche log-Elimination (bepflanzten Plots mit feinsandigem Substrat) von heterotrophen Bakterien mit 2.85, für E. Coli 4.35, für gesamt-Coliforme 4.31 und für Enterokokken 4.80. Bei den unbepflanzten Plots war die Eliminationsraten für die zwei untersuchten Pathogenen Clostridium perfringens Sporen und Pseudomonas aeruginosa höher, was durch eine längere hydraulische Aufenthaltszeit erklärt werden kann. Die Ergebnisse zeigen, dass es zu vergleichsweise höheren Reduktionen der untersuchten Bakterien im vergleich zu anderen Kläranlagensystemen kam.

Abwassserinhaltsstoffe (z.B. Nährstoffe und Pathogene Keime) werden im Wesentlichen von mikrobieller Biomasse, die im Filterkörper der untersuchten vertikal durchströmten Pflanzenkläranlagen angesiedelt ist, abgebaut. Analysen der mikrobiellen Biomasse zeigten, dass diese im Vergleich zu natürlichen Böden relativ hoch war – bedingt durch die hohe Nährstoffzufuhr des Abwassers.

Noch immer sind wenig Informationen über die mikrobielle Diversität in Pflanzenkläranlagen vorhanden; zu diesem Zweck wurde die gebräuchliche molekulare Fingerprint Technik “terminal restriction fragment length polymorphism” kurz T-RFLP adaptiert und mit dieser die mikrobiellen Lebensgemeinschaften im Filterkörper und im Ablauf untersucht. Eins der interessanten Ergebnisse war, dass es zu einem Diversitätsverlust im Ablauf der bepflanzten

Anlagen kam.

Schlagwörter
Vertikal durchströmte bepflanzte Bodenfilter
Pflanzenkläranlagen
Keimelimination
Indikatororganismen
Bakterielle Diversität

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List of abbreviations

BOKU	University of Natural Resources and Applied Life Sciences Vienna
BOD_5	Biological oxygen demand in 5 days
COD	Chemical oxygen demand
CWs	Constructed Wetlands
DO	dissolved oxygen
DW	dry weight
EHEC	enterohaemorrhagic Escherichia coli
FSCW	full scale constructed wetlands
FWF	Austrian Science Fund
HLR	Hydraulic loading rate
HRT	hydraulic retention time
HSSF CWs	horizontal subsurface flow constructed wetlands
IWA	International Water Association
NH_4-N	Nitrate
PE	population equivalent
P_{tot}	total Phosphorus
PSCWs	indoor pilot-scale subsurface vertical flow constructed wetlands
SFCWs	Surface flow constructed wetlands
SSFCWs	sub surface flow constructed wetlands
T-RFLP	Terminal restriction fragment length polymorphism (method)
TRFs	Terminal restriction fragments (parameter) (peaks in a T-RFLP profile)
TOC	total organic carbon
VF CWs	vertical flow constructed wetlands
VSSF CWs	vertical subsurface flow constructed wetlands
WHO	Word Health Organisation

1 Preface

The present 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).

This work was carried out between May 2003 and May 2006 within the project "Characterisation of microbial biocoenosis to optimise removal efficiency and des ign of subsurface flow constructed wetlands for wastewater treatment" funded by the Austrian Science Fund (FWF, project no.: P16212-06).

The main part of the practical work was done in the laboratory of the Institute for Sanitary Engineering and Water Pollution Control. Beside that additionally analyses were carried out in cooperation with following institutes:

- Department of Bioresources Working Group Environmental Molecular Analytics: Austrian Research Centre, Seibersdorf
- Department of Water, Atmosphere and Environment, Institute of Waste Management, University of Natural Resources and Applied Life Sciences, Vienna
- Department of Biotechnology, Institute of Applied Microbiology, University of Natural Resources and Applied Life Sciences, Vienna
- Clinical Institute for Hygiene and Medical Microbiology, Medical University of Vienna

The scientific outcome of the presented work comprised:

Two published peer reviewed papers in the journals Science of the Total Environment and Ecological Engineering and one paper published in peer reviewed proceedings of an IWA conference as first author:

Sleytr, K., Tietz, A., Langergraber, G., Haberl, R.(2007). Investigation of bacterial removal during the filtration process in constructed wetlands. Science of the Total Environment 380(1-3): 173-80. (see Appendix 1)

Sleytr, K., Alexandra Tietz, A., Langergraber, G., Haberl, R., Sessitsch, A. (2009) Diversity of abundant bacteria in subsurface vertical flow constructed wetlands. Ecological Engineering 35: 1021-1025 (see Appendix 2)

Sleytr, K., Langergraber, G., Haberl, R. (2008). Investigations to characterize bacterial removal processes in different subsurface vertical flow constructed wetlands. Proceedings of the 11th IWA Specialized Group Conference on "Wetland Systems for Water Pollution Control", Volume I, page 260-267 [11th IWA Specialized Group Conference on "Wetland Systems for Water Pollution Control", Indore-Ujjain, India, 1-7 November 2008] (see Appendix 3)

Two published peer reviewed paper in the journal Science of the Total Environment and Desalination as co-author.

Tietz, A., Kirschner, A., Langergraber, G., Sleytr, K., Haberl, R. (2007). Characterisation of microbial biocoenosis in vertical subsurface flow constructed wetlands. Science of the Total Environment 380(1-3): 163-172. (ses Appendix 4)

Langergraber, G., Leroch, K., Pressl, A., Sleytr, K., Rohrhofer, R., Haberl, R. (2009). High-rate nitrogen removal in a two-stage subsurface vertical flow constructed wetland. Desalination 246: 55-68 (see Appendix 5)

In this dissertation work all pertinent research was summarised in the following framework article: including introduction, a list of research topics, materials and methods, the scientific and practical relevance of the research discussed in results and conclusion; this work concludes with the five papers listed above.

2 Introduction

In many countries, excreta and wastewater contain, beside pollutants and high amount of nutrients, high concentrations of pathogens. Therefore excreta-related infections are increasing. A lack of proper treatment methods and wastewater management of excreta is responsible for adverse health and environmental effects. Human excreta have been implicated in the transmission of many infectious diseases, including cholera typhoid, types of viral hepatitis, polio, schistosomiasis and a variety of helminth infections. Most of these excreta-related illnesses occur in children living in poor and developing countries. (WHO, 2006)

Liquid wastes such as untreated raw sewage or industrial waste are the major sources of pollutants in developing countries. Municipal sewage and industrial wastewaters containing readily biodegradable organic matter, inorganic and organic chemicals, toxic substances and disease causing agents are frequently discharged into aquatic environments (oceans, rivers, lakes, wetlands) without treatment. This uncontrolled practice results in contamination of water that becomes unsuitable for human consumption, irrigation, fish production or recreation. (Kivaisi, 2001)

Often raw sewage is drained into open ditches to which animals and humans have open access. Therefore it is often used to irrigate crops and can cause many diseases like e.g. diarrhoea. In a global sense of meaning adequate wastewater treatment becomes absolutely necessary as water resources are not everywhere available.

For wastewater treatment in rural and decentralized areas constructed wetlands (CWs) become more important in technical, environmental and sustainable context. Effluents from small treatment plants are often discharged in small and ecological sensitive recipients and areas. It cannot be excluded that the treated effluent gets into groundwater or bathing waters and cause contamination through pathogens. Therefore it is very important to apply well-known wastewater treatment systems that have the potential to hygienisise the water to a certain level to minimize the risk of infections.

But also wastewater in so called developed countries like Austria show high contents of nutrients and pollutants, which make wastewater treatment a must; regulations exist and are strictly executed.

A wide range of treatment technologies for domestic wastewater exists: Activated sludge based wastewater treatment plants, many alternative technologies like fixed film systems or wastewater ponds, ditches and wetland systems.

CWs can provide high treatment efficiency with low energy consumption and low construction costs as described in Kadlec at al. (2000). These qualities make CWs quite attractive for implementation all around the world. Additional benefits include their tolerance against fluctuations of flow, the facility of water reuse and recycling and the provision of habitat for many wetland organisms. (Langergraber and Haberl, 2001)

CWs, also defined as engineered wetlands, can permit an effective, economical and ecological treatment solution for industrial and municipal wastewater. They are widely used all over the world in different climates for the treatment of wastewater mostly in small communities, in decentralized regions and in developing countries. There are many fields of application for these systems (Bavor et al., 1995), e.g. for:

- Biological stage for small waste water treatment plants (up to 50 population equivalent (PE)) and for municipal wastewater treatment plants up to a size of 1000 PE
- Treatment of industrial wastewater
- To treat grey water (water from kitchen, bath and showers excluding wastewater from toilets)
- Treatment of contaminated surface water (for urban and rural run off management)
- Land fill and mining leachate treatment
- To optimize effluents of existing treatment plants
- Groundwater recharge

Although, most people in Austria are serviced by centralized municipal sewage treatment systems, many decentralized regions (including high elevated refuges) are not. These individuals must use onsite wastewater treatment plants to control nutrient and pathogen contamination of groundwater and surface water. CWs became a very popular treatment method due to relatively low cost installation and easy maintenance in these areas.

In addition to the ability to eliminate nutrients, pollutants and toxic substances, the removal of pathogens is an important aspect of wastewater treatment. In the filter body and the root zone area of CWs far-reaching elimination of pathogenic germs take place, which can exceed the elimination capacity of conventional, biological (activated sludge) wastewater treatment plants. The basic knowledge about the processes of pathogen removal in wetlands is currently inadequate. The mechanisms of bacterial removal in these systems are not yet scientifically clear and this complicates the optimum technical implementation.

A broad range of designs for these systems exist and have been studied for removal of nutrients, pollutants, microorganisms, indicators and pathogens. Most of the studies (e.g. Payment et al., 2001; Quinonez-Diaz et al., 2001; Manios et al., 2002; Hench et al. 2003, Song et al., 2008 Torrens et al., 2009a and 2009b) only investigated the in- and the outflow patterns of the wastewater treatment plant.

So there is still a lack of knowledge of complete understanding of many microbiological and pathogen removal mechanisms and therefore CWs are often seen and described as “black boxes”. The aim of the presented study was to bring light into the “black box” in terms of bacterial removal processes and diversity patterns and their influence on the treatment efficiency in the sandy filter body of the different designs of the investigated CWs. Beside influent and effluent sampling, the focus was to investigate the filter body in different depths.

3 Objectives and aims

Previous investigations concerning bacterial removal processes with CWs focused mainly on the inflow- and outflow values of bacterial parameters. The sandy filter body of CWs are therefore often described as „black boxes“.

To bring more light into the „black box“, the present study investigates the occurrence and fate of indicator organisms and two selected pathogens. The complexity of different processes in vertical flow CWs was linked with the abundance of bacteria, inflow and outflow patterns related to the measured physical, chemical and microbiological parameters.

The major objectives of this work were:

1) to show, if vertical flow CWs are able to eliminate different kinds of bacteria and nutrients at a higher level than other treatment systems.
2) comparing bacterial removal efficiency of different constructed wetland designs (outdoor, indoor, full scale, pilot scale, planted and unplanted, saturated and unsaturated drainage filter layer, stone and fibre drainage filter layer, different loading rates, material and grain size for the filter body) to give advise for optimal design regarding to higher bacterial removal efficiency.
3) describing the change of diversity from abundant bacteria during the filtration process.
4) comprehensive literature- analyses

Summary of the scientific outcome linked with the major objectives:

ad 1 and 2:

Sleytr, K., Tietz, A., Langergraber, G., Haberl, R.(2007). Investigation of bacterial removal during the filtration process in constructed wetlands. Science of the Total Environment 380(1-3): 173-80. (see Appendix 1)

Sleytr, K., Langergraber, G., Haberl, R. (2008). Investigations to characterize bacterial removal processes in different subsurface vertical flow constructed wetlands.

Proceedings of the 11th IWA Specialized Group Conference on "Wetland Systems for Water Pollution Control", Volume I, page 260-267 [11th IWA Specialized Group Conference on "Wetland Systems for Water Pollution Control", Indore-Ujjain, India, 1-7 November 2008] (see Appendix 3)

Langergraber, G., Leroch, K., Pressl, A., Sleytr, K., Rohrhofer, R., Haberl, R. (2009). High-rate nitrogen removal in a two-stage subsurface vertical flow constructed wetland. Desalination 246: 55-68 (see Appendix 5)

Ad 3:

Sleytr, K., Alexandra Tietz, A., Langergraber, G., Haberl, R., Sessitsch, A. (2009)

Diversity of abundant bacteria in subsurface vertical flow constructed wetlands. Ecological Engineering 35: 1021-1025 (see Appendix 2)

Tietz, A., Kirschner, A., Langergraber, G., Sleytr, K., Haberl, R. (2007). Characterisation of microbial biocoenosis in vertical subsurface flow constructed wetlands. Science of the Total Environment 380(1-3): 163-172. (see Appendix 4)

4 Background

4.1 The system constructed wetland

Constructed wetland treatment systems are engineered systems that have been designed and constructed to utilize the natural processes involving wetland vegetation, soils, and their associated microbial assemblages to assist in treating wastewater. They are designed to take advantage of many of the cleaning processes that occur in natural wetlands, but do so within a more controlled environment.

CWs can be built with a much greater degree of control than natural systems, thus allowing the establishment of experimental treatment facilities with well-defined composition of substrate, type of vegetation, and flow pattern. In addition, constructed wetlands offer several additional advantages compared to natural wetlands including site selection, flexibility in sizing, and most importantly, control over the hydraulic pathways and retention time (Brix, 1993).

Many different CWs systems exist, but they can be generally classified into two main types: surface flow (see figure 4.1) and subsurface flow constructed wetlands (Kadlec and Knight, 2000) that are again subdivided into horizontal flow (figure 4.2) and vertical flow constructed wetlands (figure 4.3), depending on the direction of the water flow through the porous medium (soil or gravel).

A broad range of CWs sizes exist and vary from small single-family systems to big municipal facilities up to a capacity for much more than 1.000 person equivalents (PE).

Also the used filter materials can differ from system to system. The most often used materials for the filtration body are gravel and sand with almost no clay and silt content to avoid clogging.

CWs are highly complex natural-based and divers systems like natural wetlands, marches, swamps and natural soils. A lot of microorganisms like bacteria, protozoa and algae are attached to the soil and play the key role in the treatment process. Different Plants and macrophytes are part of the design. Concerning on their natural habitat, climate and use of the constructed wetlands, engineers can select out of a wide range of possible planting solutions.

Plants, suitable for use in CWs, should be able to grow in water or under hypertrophic water-logged conditions and be well adapted to local climatic conditions, diseases and waste water pollutants. Moreover plants must be readily propagated, establish easily, and spread and grow rapidly. Currently, the most used plants in CWs are common reed (Phragmites australis), rushes (Juncus spp.), bulrushes (Scirpus spp.), narrow-leaved cattail (Typha angustifolia L.), broad-leaved cattail (Typha lati- folia L.), yellow flag (Iris pseudacorus L.), sweet flag (Acorus calamus L.), reed grass (Glyceria maxima) and Carex spp. (Barbera et al., 2009)

Regarding the importance of the need of planting in constructed wetlands the literature shows many controversial results. In general plants have been observed to exhibit little to no effect on the removal of indicator organisms e.g. faecal coliforms in constructed wetlands (Tanner et al., 1995). But also several studies have shown the important role of macrophytes (e.g. in Brix, 1996 and 1997). This topic will be discussed in chapter 6.3.

4.1.1 Surface flow CWs

The surface flow wetland technology is strongly related with natural wetlands where 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 enhances the process rates of the system and therefore decreases the surface requirement of the treatment system (Kadlec et al., 2000; Langergraber and Haberl, 2001)

Surface flow constructed wetlands (SFCWs) or reed beds are shallow basins with soil or other medium to support the roots of the macrophytes and are characterized by the horizontal flow of wastewater across the roots of the plants and an exposed surface to the atmosphere. They are no longer used as much due to the large land-area requirements to purify water up to 20 m^2 per person, and the increased smell and poor purification performance in winter times.

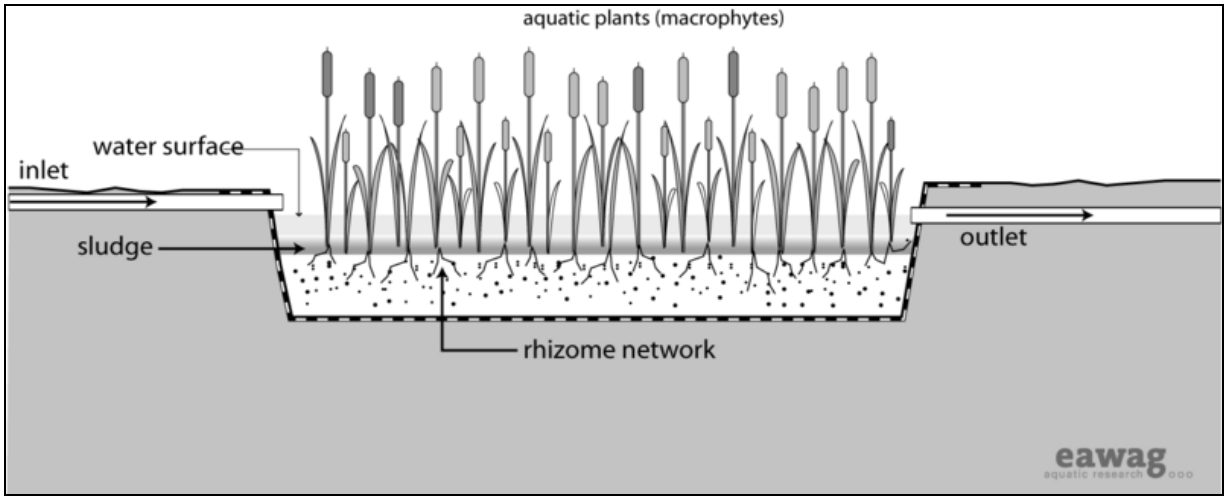

Figure 4.1: Scheme of a surface flow constructed wetland with emergent macrophytes (Tilley et al., 2008, slightly modified)

The lists below gives an overview of the advantages and disadvantages of surface flow CWs as reported in the literature (Kadlec, 1995, Greenway and Woollley, 1999, Van de Moortel et al., 2009, Tilley et al., 2008, Thullen et al., 2005, Perkins and Hunter, 2000)

Advantages:

+ Aesthetically pleasing like natural wetlands and provides animals a valuable habitat and potentially a recreation area for people

+ High reduction in BOD and solids; moderate pathogen removal e.g. up to 94% for faecal coliforms and Enterococci

+ Can be built and repaired with locally available materials (in developing countries)

+ If constructed with slope no electrical equipment is required and therefore operation and maintenance costs are low

+ If used correctly there should be no problems with flies or odours

+ financial advantage is the extremely low base cost of operation

Disadvantages:

- Large land areas are required which can mean high capital costs

- The removal of total Nitrogen is only about 36.6 % in the SFCWs due to nitrification-limiting conditions (compared to about 96.7% SSFCWs) depending on the retention time

- Surface flow constructed wetlands may facilitate mosquito breeding

- It needs long start up time to work at full capacity

- If the treatment plant is not fenced it can be an area for transmitting diseases due to free excess for humans and animals, especially in developing countries (effluent water is often used to water animals and for drinking water for humans)

4.1.2 Subsurface flow CWs

Horizontal Flow CW

Among the treatment wetlands, horizontal subsurface flow constructed wetlands (HSSF CWs) are a widely applied concept. Pre-treated wastewater flows horizontally through the artificial filter bed; usually consisting of a matrix of sand or gravel and the helophyte roots and rhizomes. This matrix is colonised by a layer of attached microorganisms that forms a so-called biofilm. Purification is achieved by a wide variety of physical, chemical and (micro)biological processes, like sedimentation, filtration, precipitation, sorption, plant uptake, microbial decomposition and nitrogen transformations. (Rousseau et al., 2004)

HSSF CWs have successfully been used for treatment various types of wastewater for more than four decades. Most systems have been designed to treat municipal sewage but also effectively used for wastewaters from agriculture, industry and landfill leachate. (Vymazal, 2009)

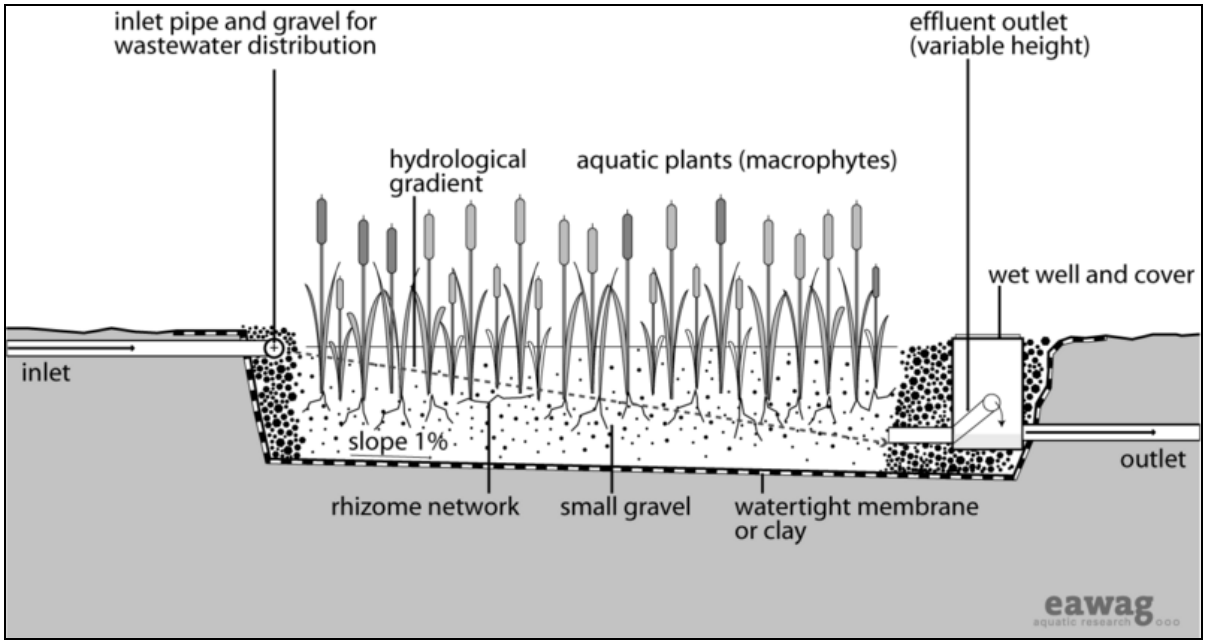

Figure 4.2: Scheme of a horizontal subsurface flow constructed wetland (Tilley et al., 2008, slightly modified)

The lists below gives an overview of the advantage and disadvantage of HSSF CWs as reported in the literature (Tilley et al., 2008, Vymazal et al., 2009)

Advantages:

+ Compared to free water, surface flow systems, the contact area of water with bacteria and substrate is much higher, which decreases the area requirement of sub-surface flow CWs

+ High reduction in nutrients, suspended solids and pathogens

+ Does not have the mosquito problems of the SFCWs

+ Can be built and repaired with locally available materials and the construction can provide short-term employment to local labourers

+ It is possible to maintain the system without electrical energy

Disadvantages:

- To prevent clogging of the filter material, the use of mechanically pre-treated wastewater, which contains lower content of particulates, is required
- Requires expert design and super vision
- Moderate capital cost depending on land, liner, fill, etc.; low operating costs

Vertical flow CWs

In vertical subsurface flow constructed wetlands (VSSF CWs) the wastewater is loaded onto the surface of a planted filter bed (Figure 4.3). The recommended surface are is about 3 m^2 per PE and can vary depending on the used filter material a depth. Microorganisms, attached to the filter substrate and the roots of the plants, remove or transform most of the pollutants. To have great performance, the filter should be not saturated or covered with water in order to secure a high oxygen level in the filter. This can be achieved with intermittent loading of the wastewater so the filter pores can be filled with air again. The bed is planted with different species of water tolerable plants like the common reed (Phragmites australis). The treated wastewater is collected in a system of passively aerated drainage pipes placed in the bottom of the filter.

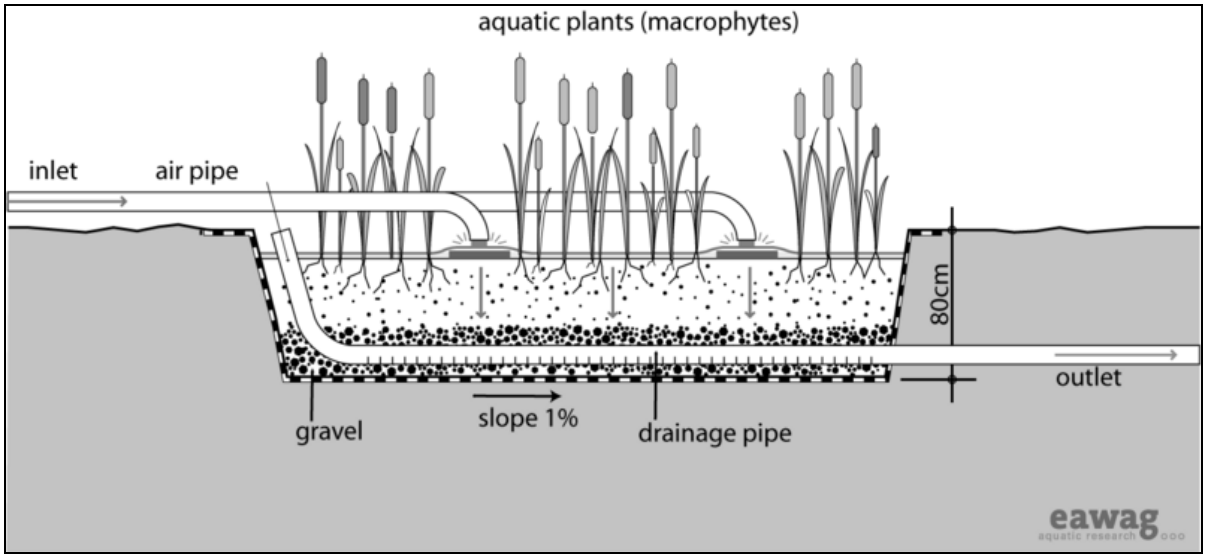

Figure 4.3: Subsurface vertical flow constructed wetlands (Tilley et al., 2008, slightly modified)

The lists below gives an overview of the advantage and disadvantage of sub surface, vertical flow CWs as reported in the literature (Blazejewski and Murat-Blazejewska, 1997, Platzer and Mauch, 1997, Laber, 2001, Langergraber et al., 2003, Cooper et al., 2005, Tilley et al., 2008)

Advantages:

+ Less clogging than in a HSSF CWs
+ Requires less space than a SF CWs and don't have the mosquito problems
+ VSSF CWs with intermittent loading are used widely due to their high level of efficiency in the removal of BOD, ammonia nitrogen, suspended solids and pa thogens; so they can achieve a higher quality effluent than HSSF CWs (e.g. Torrens et al., 2008)
+ Construction can provide short-term employment to local labourers (can be an important fact for developing countries)

Disadvantages:

- Although less than in HSSF CWs, clogging of the surface is one of the biggest operational problem of VSSF CWs. Substrate clogging summarizes several processes, which lead to a reduction of the infiltration capacity of the substrate surface (Blazejewski and Murat-Blazejewska 1997, Platzer and Mauch, 1997). Most removal processes require aerobic conditions. Clogging hinders oxygen transport which results in rapid failure of the treatment performance. To prevent clogging the construction and size of the settling has to ensure that the mean concentration of suspended solids after settling does not exceed 100 mg/l and that the organic load should not exceed 20 g COD.m/d/1. Primary mechanical pre-treatment of the wastewater is required to prevent clogging processes (Langergraber et al., 2003; Cooper et al., 2005).
- Requires expert design and super vision and not all parts and materials may be available locally (important for developing countries)
- Moderate capital cost depending on land, liner, fill, etc.; low operating costs
- The distributing system (mostly designed with pipes) requires more complex engineering and maintenance

4.2 Pathogens in wastewater

Wastewater contains human, animal and plant pathogens capable of causing viral, bacterial, or parasitic infections. There are several routes whereby sewage pathogens may affect human health, including direct contact, contamination of food crops, zoonoses, and vectors. The range and numbers of pathogens in municipal wastewater vary with the level of endemic disease in the community, discharges from commercial activities, and seasonal factors.

Many of the pathogens in excreta and wastewater can survive in the environment long enough to be transmitted to humans through contact with excreta or wastewater or consumption of contaminated products irrigated with wastewater. Viruses for example can survive 50-120 days, bacteria like salmonella spp 30-60 days, protozoa up to 180 days and helminths eggs many month up to years (measured in selected environmental media at 20-30°C) (WHO, 2006)

In many countries excreta-related infections are increasing. A lack of proper treatment methods and wastewater management of excreta is responsible for adverse health and environmental effects. Human excreta have been implicated in the transmission of many infectious diseases, including cholera typhoid, types of viral hepatitis, polio, schistosomiasis and a variety of helminth infections. Most of these excreta-related illnesses occur in children living in poor and developing countries. Overall, WHO estimates that diarrhoea alone is responsible for 3.2% of all deaths worldwide. In addition to diarrhoea, WHO estimates that each year, 16 million people contact typhoid and over one billion people suffer from intestinal helminth infections (WHO, 2000, 2003b, 2003c, 2004b). The risk of infection through different pathogens is summarized in table 4.1 below.

Table 4.1: Risk of infection through waterborne, reference pathogens reported from the WHO (Westrell, 2004 in WHO, 2006)

Pathogen	Incidence (per 100 000 people)	Under-reporting	Morbidity (%)	Excretion (per gram faeces)	Duration (days)	ID_{50}
Salmonella	42–58	3.2	6–80	10^{4-8}	26–51	23 600
Campylobacter	78–97	7.6	25	10^{6-9}	1–77	900
EHEC	0.8–1.4	4.5–8.3	76–89	10^{2-3}	5–12	1 120
Hepatitis A virus	0.8–7.8	3	70	10^{4-6}	13–30	30
Rotavirus	21	35	50	10^{7-11}	1–39	6
Norovirus	1.2	1562	70	10^{5-9}	5–22	10
Adenovirus	300	–	54	–	1–14	1.7
Cryptosporidium	0.3–1.6	4–19	39	10^{7-8}	2–30	165
Giardia	15–26	20	20–40	10^{5-8}	28–284	35
Ascaris	15–25	–	15	10^{4}	107–557	0.7

Source: Westrell (2004).

***Incidence (per 100.000 people)**: yearly number of reported cases divided by the total population, expressed per 100 000 people*

***Underreporting**: number of how many more cases exist in the community (The real incidence of rotavirus is for example estimated to be 21 x 35 = 754 per 100.000)*

***Morbidity (%)**: is a measure of the percentage of people that will acquire symptoms when infected*

***Excretion (per gram faeces)**: numbers of pathogens that are excreted during infection*

***Duration (days)**: days that infected person excretes pathogens*

***ID_{50}**: is the dose, or number of pathogens, at which 50% of a population will be infected.*

In Europe regulations exist to control pathogen risk and arise from land application of biosolids and are based on the concept of multiple barriers to the prevention of transmission. The barriers could be treatment to reduce pathogen content and vector attraction, restrictions on crops grown on land to which biosolids have been applied, and minimum intervals following application and grazing or harvesting. (Godfree and Farrell, 2005)

Wastewater treatment in general and with CWs can reduces the number of pathogens in wastewater. The mechanisms for microbial inactivation are not fully understood for all processes by now but will be discussed more in detail in chapter 6.1.

Table 4.2 gives an overview of wastewater relevant pathogens, diseases and symptoms. Although the employees of modern wastewater treatment systems have to follow high hygienic standards to avoid health impacts, there is still a chance to get infected. Smaller natural systems like CWs are often located in small communities or villages and be operated and maintained by laymen. This personal often underestimates the hygienic risk while handling with wastewater. So it is very important to know about the risks of pathogenic potential of wastewater.

Table 4.2: Overview of selected pathogens in wastewater and t heir disease and symptoms (WHO, 2006)

Group	Pathogen	Disease and symptoms
Bacteria	*Aeromonas* spp.	Enteritis
	Campylobacter jejuni/coli	Campylobacteriosis – diarrhoea, cramps, abdominal pains, fever, nausea, arthritis; Guillain-Barré syndrome
	Escherichia coli (EIEC, EPEC, ETEC, EHEC)	Enteritis
	Plesiomonas shigelloides	Enteritis
	Salmonella typhi/paratyphi	Typhoid/paratyphoid fever – headache, fever, malaise, anorexia, bradycardia, splenomegaly, cough
	Salmonella spp.	Salmonellosis – diarrhoea, fever, abdominal cramps
	Shigella spp.	Shigellosis – dysentery (bloody diarrhoea), vomiting, cramps, fever; Reiter's syndrome
	Vibrio cholerae	Cholera – watery diarrhoea, lethal if severe and untreated
	Yersinia spp.	Yersiniosis – fever, abdominal pain, diarrhoea, joint pains, rash
Viruses	Enteric adenovirus 40 and 41	Enteritis
	Astrovirus	Enteritis
	Calicivirus (including norovirus)	Enteritis
	Coxsackievirus	Various: respiratory illness; enteritis; viral meningitis
	Echovirus	Aseptic meningitis; encephalitis; often asymptomatic
	Enterovirus types 68–71	Meningitis; encephalitis; paralysis
	Hepatitis A virus	Hepatitis – fever, malaise, anorexia, nausea, abdominal discomfort, jaundice
	Hepatitis E virus	Hepatitis
	Poliovirus	Poliomyelitis – often asymptomatic, fever, nausea, vomiting, headache, paralysis
	Rotavirus	Enteritis
Parasitic protozoa	*Cryptosporidium parvum*	Cryptosporidiosis – watery diarrhoea, abdominal cramps and pain
	Cyclospora cayetanensis *Entamoeba histolytica*	Often asymptomatic; diarrhoea, abdominal pain Amoebiasis – often asymptomatic; dysentery, abdominal discomfort, fever, chills
	Giardia intestinalis	Giardiasis – diarrhoea, abdominal cramps, malaise, weight loss
Helminths	*Ascaris lumbricoides* (roundworm)	Ascariasis – generally no or few symptoms; wheezing, coughing, fever, enteritis, pulmonary eosinophilia
	Taenia solium/saginata (tapeworm)	Taeniasis
	Trichuris trichiura (whipworm)	Trichuriasis – Unapparent through vague digestive tract distress to emaciation with dry skin and diarrhoea
	Ancylostoma duodenale / Necator americanus (hookworm)	Itch, rash, cough, anaemia, protein deficiency
	Schistosoma spp. (blood fluke)	Schistosomiasis, bilharzia

Source: Adapted from Ottosson (2003).

4.2.1 Microbiological parameters and indicator organisms

Indicator micro-organisms of faecal pollution are e.g. faecal coliforms, Escherichia coli, Enterococci and Clostridium perfringens. They are often used as parameter organisms to describe water quality. Coliforms are part of the intestinal flora of mammals and other animals. The quantity of coliforms that is daily excreted (in faeces) by an average human, varies between 10×10^6 and 40×10^6 (CFU/100 ml). All coliforms may exist as saprophytic organisms, with the exception of those belonging to the genus Escherichia, the origin of which is exclusively intestinal. The great quantity of E. Coli present in the human digestive tract, together with the fact that it is not usually found in other environments, cause this bacterium to be considered one of the best available faecal contamination indicators. (Molleda 2008)

Thus, the presence of this bacterium in water proves a recent faecal contamination and the possible existence of pathogens. The use of the indicator bacterial group Enterococci is frequently suggested as an alternative to coliforms. Their advantage over E. Coli lies in their greater resistance and their inability to grow in any environment, such as soil, water, and others (Vera et al., 2006; Ryu et al., 2007).

C. perfringens is an anaerobic spore-forming sulphite reducing bacterium that is found in high concentrations in human and animal faeces. Its spores are very resistant to environmental conditions, while its vegetative forms do not seem to multiply in aquatic environments (Vera et al., 2006; Payment and Franco, 1993). Winward et al. (2008) report a significant correlation between Pseudomonas aeruginosa and total coliforms in the effluents. This can be interpreted that these bacteria are removed or inactivated by similar processes. Total coliforms seem to be the best indicators for the presence of Pseudomonas aeruginosa. (Molleda 2008)

Human pathogenic protozoa are present in water in resistant forms (cysts and oocysts) which protects them from environmental stress. Protozoal dispersion forms are much more resistant to disinfection processes than bacteria. The resistance to disinfectants of Giardia cysts, combined with the small infective dose needed for infection (10 cysts) and their viability for 1–3 months in water, make them one of the main vectors for contagion.

Giardia may cause several gastrointestinal disorders and episodes of malnutrition arising from deficient absorption of fats (Vera et al., 2006; Gomez-Couso et al., 2005). Cryptosporidium is related to a wide range of symptoms, especially severe and chronic in the case of extremely weak patients, and is even able to cause death (Vera et al., 2006; Bonadonna et al., 2002; Ottoson et al., 2006; Reinoso et al., 2007). A great variety of helminth eggs may be found in wastewaters.

The most important is the group of nematodes, known as intestinal worms, especially those belonging to the genera Ascaris, Ancylostoma, Necator, Trichuris, and others. Their main epidemiological characteristics are long persistence in the environment, a minimal infective dose, limited immune response and the ability to remain viable in soils over long periods of time (Vera et al., 2006; Amahmid et al., 2002).

In Molleda et al (2008)

4.2.1.1 Indicator organisms

The number of pathogens in wastewater varies as a function of numerous factors including geographic location, socioeconomic status, sanitary conditions and season. Nevertheless, nearly all domestic sewage contains pathogens, and the larger the contributing population, the less variable the concentration.

Indicator organisms ideally have the same properties in terms of tenacity and behaviour in the environment as the pathogenic organisms. They don't need to be pathogenic, but can be, but to deal with them is far less dangerous.

The use of human pathogens for experimentation is often not possible due to difficulty in

laboratory culture, enumeration and occupation health risk to researchers. It is therefore usually necessary to use model organisms for undertaking experimentation. A model organism is essentially a tracer, which has behavioural characteristics similar to those of the pathogen of interest. In particular, a model organism should have the same or greater resistance to environmental stressors. The most common faecal indicators used with environmental samples are bacteria belonging to the coliform group. Bacteriophages and Clostridium perfringens could be better suited as models for human pathogens.

For technical reasons it is often not possible to assess all pathogenic microorganisms in wastewater. So the detection of so-called indicator organisms or model organisms is used to describe epidemic and hygienic quality of wastewater during the wastewater treatment process. Table 4.3 summarises the most common indicators for human pathogens like bacteria, viruses and protozoa.

Petterson and Ashbolt (2003)

Table 4.3: Appropriate indicator and model organisms for human pathogens (Petterson and Ashbolt (2003) slightly modified)

	Pathogen examples	**Indicator and model organsims**	**Details to the indicators**
Bacteria	*Shigella*, enterotoxigenic *E. Coli*, *Campylobacter*, *Vibrio cholerae* (Cholera)	*E. Coli,* intestinal Enterococci	The thermotolerant coliform/E. Coli group of bacteria have been used for more than 100 years as a model for pathogenic bacteria. Behaviour of E. Coli, intestinal Enterococci (not total coliforms) under environmental conditions is expected to reflect ennot environmental bacteria such as Legionella.
Viruses	Adenovirus, Rotavirus, Enteroviruses, Hepatitis A, B	Bacteriophages – somatic coliphages or F-RNA coliphages	Bacteriophages are viruses that infect bacteria, and considered non- pathogenic to humans, and can be r eadily cultured and enumerated in the laboratory. Generally present in faeces of warm-blooded animals, but certain strains may be human specific.
Protozoa	*Cryptosporidium* oocysts, *Giardia* cysts	*Clostridium perfringens,*	*Clostridium perfringens* is a spore forming bacteria, which is highly resistant to environmental conditions. It has been shown to be a useful model for *Cryptosporidium* oocysts and *Giardia* cysts. Aerobic (*Bacillus*) spores could also be used, but likely to grow in treatment systems and slough off surfaces providing misleading numbers.
		Particle counter	Particle - counter Protozoan pathogens are generally larger in size than those belonging to the other groups. Studies have been successfully undertaken using particles of similar size (e.g. fluorescent beads, or total particles 5- 20µm) as a model for oocysts.

4.3 Guidelines and laws

In the year 2008 about 1.16 million m³ of wastewater was produced in Austria. 92.8% of the population is connected to sewer systems and wastewater treatment plants. Because of many rural settlements 100% connection is quite unrealistic to reach. This is the reason for more than 500 CWs in use, most of them placed in decentralized regions and small in size (5-50PE).

Bundesministerium für Land- und Forstwirtschaft, Umwelt und Wasserwirtschaft (2010)

The most relevant legal standards, guidelines and laws relevant for construction and effluent emissions of CWs and others related to wastewater and drinking water in Austria are listed in this chapter.

4.3.1 Legal standards relevant for wastewater emission from wastewater treatment systems in Austria

- **Commission Directive 98/15/EC** amending **Council Directive 91/271/EEC (Kommunale Abwasserrichtlinie (91/271/EWG):** concerning urban waste-water treatment; its objective is to protect the environment from the adverse effects of urban waste water discharges and discharges from certain industrial sectors and concerns the collection, treatment and discharge of: domestic waste water and mixture of water
- **Bundesgesetzblatt BGBL 1996:** 186. Verordnung des Bundesministers für Land- und Forstwirtschaft über die allgemeine Begrenzung von Abwasseremissionen in Fließgewässer und öffentliche Kanalisationen (**AAEV**) [in German]
- **Bundesgesetzblatt BGBL 210/1996. 1. Abwasser Emissionsverordung für kommunales Abwasser (Austrian regulation for emissions from domestic wastewater)**: Verordnung des Bundesministers für Land,- und Forstwirtschaft über die Begrenzung von Wasseremissionen aus Abwasserreinigungsanlagen für Siedlungsgebiete [in German]

  Table 4.4 gives an ov erview of the required effluent concentrations for wastewater parameters from wastewater-treatment plants. The CWs in the present study could fulfil the required effluent concentrations as summarized in Table 6.6.

Table 4.4: Required effluent concentrations for wastewater parameters from wastewater-treatment-plants

	MAX. ROHZULAUFFRACHTEN ENTSPRECHEND			
Parameter mg/l bzw. %	> 50—500 EGW_{60}	> 500—5 000 EGW_{60}	> 5 000—50 000 EGW_{60}	> 50 000 EGW_{60}
BSB_5 f)	25	20	20	15
CSB f)	90	75	75	75
TOC	30	25	25	25
NH_4-N a)	10	5	5	5
Gesamt-N-Entfernung a)	c)	c)	mind. 70%	mind. 70%
Gesamt-N-Entfernung b)	c)	c)	mind. 60%	mind. 60%
Gesamt-P	c)	1,5 d)	1,0 e)	1,0 e)
PO_4-P	c)	1,0 d)	0,8 e)	0,8 e)

4.3.2 Guidelines for subsurface flow constructed treatment wetlands

Long times constructed wetlands have been built after rules of thumb. As CWs are very common they have been developed and described in technical descriptions. If CWs are constructed today, the use of guidelines and orders is recommended. In Austria the **ÖNORM B 2505** summarizes the state of the art and is used for designing subsurface flow constructed wetlands, their application, dimensioning, installation and operation, maintenance and inspection.

- **ÖNORM B 2505, version 1 (1997):** required area 4 m^2/PE
- **ÖNORM B 2505** - Bepflanzte Bodenfilter (Pflanzenkläranlagen) – Anwendung, Bemessung, Bau und Betrieb (Subsurface flow constructed wetlands – Application, dimensioning, installation and operation, maintenance and inspection). Vienna, Austria: Österreichisches Normungsinstitut; 2009 [in German]; required area 5 m^2/PE
- **DWA-A 262 (2006):** Arbeitsblatt ATV-A262 der Abwassertechnischen Vereinigung (ATV); Grundsätze für Bemessung, Bau und Betrieb von Pflanzenbeeten für kommunales Abwasser bei Ausbaugrößen bis 1000 Einwohnergleichwerte. [in German]. CWs build after ATV-A262 normally have a size up to 1000 person equivalent (PE).
- **ATV (1997):** commented that CWs are divided into two types depending on their size. The so called very small CWs (in German „Kleinkläranlagen“) are described in **DIN 4261 (2010)** (up to 8 m^2/d, that means up to a level of 50 PE) and as small CWs (in German „Kleine Kläranlagen“) (from 50 up to 1000 EGW).

Wastewater effluents are a major source of faecal contamination of aquatic ecosystems and cause severe disturbance in their ecological functioning. Despite the fact that raw wastewater also carries large quantities and a wide variety of faecal micro-organisms (including pathogens for humans), the reduction of bacteriological pollution in wastewater has not been a priority so far in Europe and, at present, there are no European directives regarding the bacteriological quality of treated wastewater. Bacterial indicators of faecal contamination and enteric viruses are present in high concentrations in raw wastewater. The primary treatment of settling is not very efficient in removing the microbiological pollution (faecal coliform removal of 10-60% and 20-50%). Although vertical flow constructed wetlands, designed after technical standards, show high removal efficiencies up to 99.9% (for indicator organisms and pathogens) this still can mean high, absolute levels of pathogens in the effluent which can negatively effect the environment. (George et al., 2002)

Effluent standards only exist for nutrients and pollutants (see guidelines above) – however, as there are no effluent standards for indicator organisms and pathogens in CWS effluents existing by now, relevant laws and guidelines concerning water quality in general were used in this study. The aim was to have levels for comparing pathogen contamination in the different CWs effluents and their treatment-efficiency.

- **ÖWAV Regelwerk (ÖWAV-Arbeitsbehelf Nr. 11) Empfehlung für Bewässerungswasser** (Wien 2003) [in German]
- **ÖNORM M 6230-1: Badegewässer – Anforderungen an die Wasserbeschaffenheit** [in German]

Table 4.5: Microbiological investigation parameters adapted from ÖNORM M 6230-1

Parameter	Guide value (cfu)	Extreme value (cfu), (RL 76/160/EWG)
Total coliforms	500 in 100 ml	10000 in 100 ml
Faecal coliforms	100 in 100 ml	2000 in 100 ml
Salmonella	0 in 1000 ml	0 in 1000 ml
Escherichia coli	100 in 100 ml	2000 in 100 ml
Enterococci	50 in 100 ml	400 in 100 ml

- **Richtlinie 2006/7/EG** des europäischen Parlaments und des Rates vom 15. Februar 2006 über die Qualität der Badegewässer und deren Bewirtschaftung und zur Aufhebung der RL 76/160/EWG [in German]

Table 4.6: Quality levels for fresh water (adapted from Richtlinie 2006/7/EG)

Parameter	excellent quality	Good quality	Adequate quality
Intestinal Enterococci (cfu/100 ml)	200 (*)	400 (*)	330 (**)
Escherichia coli (cfu/100 ml)	500 (*)	1 000 (*)	900 (**)

- **Wasserrechtsgesetz WRG 1959 idF BGBL I Nr 123/2006**: [in German]

Einbringungsbeschränkungen und -verbote

§ 32a. (1) Der Bundesminister für Land- und Forstwirtschaft, Umwelt und Wasserwirtschaft kann zum Schutz der Gewässer (§ 30), insbesondere zur Erreichung der gemäß §§ 30a, c und d festgelegten Umweltziele mit Verordnung sowohl die Einbringung bestimmter Stoffe in Oberflächenwasserkörper oder Kanalisationen als auch die direkt (ohne Bodenpassage) vorgenommene Einbringung in Grundwasserkörper im allgemeinen Interesse an der Reinhaltung der Gewässer sowie in Erfüllung gemeinschaftsrechtlicher Verpflichtungen verbieten. Solche Verbote gelten nicht für

a) Haushaltsabwässer aus Einzelobjekte in Streulage außerhalb von Schutz- und Schongebieten (§§ 34, 35, 54),

- **Bundesgesetzblatt BGBL 2001**; 304. Verordnung: Trinkwasserverordnung - TWV [in German]
- **Bundesgesetzblatt BGBL 2006**; 96. Verordnung: Qualitätszielverordnung Chemie Oberflächengewässer – QZV Chemie OG [in German]
- **Bundesgesetzblatt BGBL 2010;** 98. Verordnung: Qualitätszielverordnung Chemie Grundwasser – QZV Chemie GW [in German
- **Bundesgesetzblatt BGBL 2010**; 99. Verordnung: Q ualitätszielverordnung Ökologie Oberflächengewässer – QZV Ökologie OG [in German]

5 Material and methods

5.1 Pilot scale indoor system

The study has been carried out at the technical laboratory and 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, Austria (BOKU). Ten parallel-operated indoor pilot-scale subsurface vertical flow constructed wetlands (PSCWs) have been investigated (Figure 5.1).

Figure 5.1: Front view of the PSCWs at BOKU-University

Figure 5.2 shows a vertical cross-section of a PSCW. The PSCWs have a surface area of 1 m^2 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 (see the distribution system in figure 5.3). 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 every 6 hours with 15 litres) and 240 mm/d (8 loadings per day), respectively.

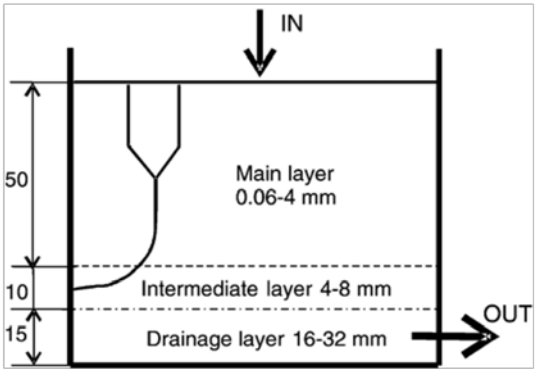

Figure 5.2: Vertical cross-section of a PSCW showing height (in cm) and grain size of the layer

Figure 5.3: Wastewater distribution system for the PSCWs

Table 5.1 shows a plan view of the different designs of the PSCWs tested. Eight PSCWs are planted with *Miscanthus gigantea* (common reed *Phragmites australis* did not grow under indoor-conditions) whereas two PSCWs (Plots 2 and 3) are unplanted. Four PSCWs have been operated with a saturated drainage layer to increase the retention time (Plot 6–9). In plot 8 and 9 special glass-fibre filters were used for as a drainage layer (Figure 5.4).

Water content and dissolved oxygen have been measured on-line in Plots 1–5. In Plots 1–5 sampling systems to collect water from different depths have been installed; four systems in each plot allow sampling of water from different depths: 10, 20, 30, and 40 cm, respectively. The sampling systems consist of a polyethylene column 20°cm diameter that is ending in an open funnel (Figure 5.5). The layer's 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 PSCW (Langergraber, 2007b) have been analysed.

Table 5.1: Drawing of the top view from all ten PSCWs describing their design characteristics (grain size, Plot-number, planting and drainage layer status)

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 5.4: Special glass-fibre filters for the drainage layer (Plot 9 and 8)

Figure 5.5: Collecting systems for the investigation for the effluents in different depths (Plot 1–5)

5.2 Outdoor full scale constructed wetland (FSCW)

The experimental, FSCW is located at the site of the wastewater treatment plant Ernsthofen (Lower Austria; coordinates: longitude: 14.482693; latitude: 48.127522) (figure 5.6: summer time; 5.7: winter time). A 3-chamber unit is used for mechanical pre-treatment. The plant consists of three parallel-operated subsurface vertical flow beds with a surface area of about 20 m^2 (4 m x 5 m) each and is operated with intermittent loading (hydraulic loading rate: 64.7 mm/day; 4 loadings a day). It was constructed according to the Austrian design standards (ÖNORM B 2505, 2005) and is equivalent to the PSCWs-design (see figure 5.2): 50 cm main layer of sandy substrate (grain size 0.06-4 mm; d_{10} = 0.2 mm; d_{60} = 0.8 mm), 10 cm intermediate layer with a gravel size of 4-8 mm and 15 cm drainage layer (gravel size 16- 32 cm). The distribution system consisted of 4 pipes (distance between the pipes 1 m) with holes (8 mm) every 75 cm. The beds have been planted with common reed (*Phragmites australis*). Figure 5.6 and 5.7 show the difference between summer and winter times.

The organic loads applied were 20, 27 and 40 g COD m^2/d, which corresponds to a specific surface area requirement of 4, 3 and 2 m^2 per PE COD, respectively. The organic load of 20 g COD m^2 /d has been found to be the limit up to which subsurface vertical flow beds with sandy substrate for the main layer can be operated in temperate climates without clogging problems (e.g. Winter and Goetz, 2003). In this study the results of bed 2 (3 m^2/PE COD) are used for the investigations.

The experimental plant was operated automatically using a LabView® program over a period of 20 months (including two winters). The pore water content in different depths of the main layer and effluent flow rates have been recorded on-line. (Langergraber, 2007a)

After 2 years of operation the first bed of the full scale outdoor CW was divided and became a two-stage CW system. It consists of two VF beds with intermittent loading operated in series with a surface area of approximately 10 m^2 for each stage. The first stage uses a grain size of 2–3.2 mm for the 50 cm main layer and has a drainage layer that is impounded; the second stage uses a grain size of 0.06–4 mm (d_{10} = 0.2 mm; d_{60} = 0.8 mm) and a conventional drainage layer. All beds were planted with common reed (*Phragmites australis*). This two-stage system was operated with an organic load of 80 g COD m^2 /d for the first stage (1 m^2 per PE), that is 40 g COD m^2 /d for the whole system (2 m^2 per person equivalent). The two-stage system was loaded intermittently every 3 h with mechanically pre-

treated wastewater (16.2 mm per loading). In the start-up period from May until August 2005, all beds were operated with lower organic load. From September 2005 to May 2007 the two-stage system was operated with the target load of 40 g COD m^2 /d. Langergraber et al., (2009)

Figure 5.6: Overview of the FSCWs in Ernsthofen, Lower Austria in summer times

Figure 5.7: Overview of the FSCWs in Ernsthofen, Lower Austria in winter times

5.3 Wastewater

For the indoor and outdoor system pre-treated wastewater was used for the loadings. Table 5.2 shows the average concentration of the measured parameters in the influent.

Table 5.2: Mean influent concentrations for chemical and microbiological parameters

Chemical parameter	COD	BOD_5	TOC	NH_4-N	TP
mg/l	367	150	160	42	6,6

Indicator organsims	HPC	E. Coli	Total coliforms	Enterococci
Log CFU/ml	6,22	6,59	6,99	6,06

Bacterial direct counts	MDC
Log cells/ml	6,03

5.4 Methods

This study tries to bring light into the „black box“ CS by using a wide range of methods.

5.4.1 Sampling

Soil- and wastewater samples were taken from the FSCW planted with *P. australis*. Bacterial communities in the rhizosphere of this plant are of great interest because of their potential for bioremediation of industrial effluent (Chaturvedi et al., 2006). Additionally, samples were taken from all indoor PSCWs. Six of the eight plots, with a surface area of $1m^2$ each, were planted with *M. sinensis giganteus*, whereas two beds were unplanted. A detailed description of the two sampling sites can be found in chapter 5.1 and 5.2.

Wastewater samples were collected from the inflow and outflows from the FSCW and the PSCWs.

5.4.1.1 Water samples

Water samples (influent: mechanically pre-treated municipal wastewater and the effluent from all PSCWs) were collected every month over a two years period. The effluent-wastewater was collected within two hours in two litre sterile Schott-bottles and analysed within two hours for the indicator organisms and the physical parameters. The grab samples were diluted serially. For the chemical parameters samples were fixed and stored in the fridge.

For analysing chemical and physical standard parameters and indicator organisms, water samples have been taken from the influent and the effluent of the system and at different depths (four effluents from 10cm, 20cm, 30cm and 40cm from the PSCWs Plot 1-5 (see collection systems in Figure 5.5). The samples were analysed for heterotrophic bacteria, faecal coliforms (E. Coli, total coliforms) and Enterococci. The grab samples were diluted serially.

5.4.1.2 Soil samples

To measure the substrate-attached bacteria, soil samples were taken: with a syringe (Figure 5.8) for samples in 0-10 cm depths, with a sampling-needle (conical, 1.5–3 cm in diameter) and a drill (10 cm in diameter) for all other depths (see Figure 5.9). The position of the sampling-spots (FSCW and PSCW) varied with every sampling date over the whole sampling period of two years. At every sampling date two (PSCWs) to five (FSCW) substrate samples were taken on different spots. The samples were separated into seven different depth (0-1, 1-5, 5-10, 10-20, 20-30, 30-40 and 40-50 cm) and the amount of each depth was mixed together (pooled sample per depth). From this pooled sample 10 g well-mixed wet soil per depth was placed into a sterile 200 ml plastic container. 90 ml of sterile de-ionized water was added. For separation of microorganisms from soil and particles the samples were shaken (shaker: KS 501D, Janke&Kunkel, IKA®- Labortechnik) for 30 min and then sonicated in an ultrasonication bath (Branson 5510, 135 W, 42 kHz) for 1 minute. After 5 minutes settling, 1

ml of solution was taken and serially diluted. This was found to be the most effective technique to determine the indicator organisms, inspired by Craig et al. (2002)

Figure 5.8: Syringe cut at the top to take samples from depths of 0-10 cm of the CW filter body

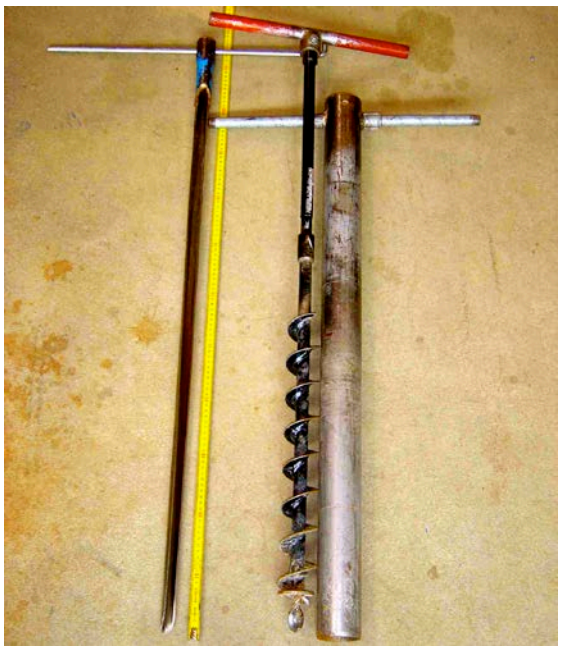

Figure 5.9: Sampling needle and sampling drill for the soil samples for all depths

Samples for microbial community analyses were collected four times from different depths of the filter bed of the FSCW and from different PSCWs with the instruments described above. Samples taken at any place from the filter bed with no reference to plants are referred to as "bulk soil" in contrast to samples, which were directly removed with a sterile spoon from roots and rhizomes ("rhizosphere soil") of *P. australis* (FSCWs) and *M. sinensis giganteus* (PSCWs). Rhizosphere samples were sampled at the same time and the same depths from 10 to 20 cm. Rhizosphere soils from the planted PSCWs and the FSCWs were compared; further more the differences between the rhizosphere soils and bul k soil from the two unplanted PSCWs (Plot 2 and 3). The bulk soil samples were used to investigate potential differences in bacterial diversity from seven different depths (0–1, 1–5, 5–10, 10–20, 20–30, 30–40 and 40–50 cm), between the planted and unplanted PSCWs and the FSCW.

Total organic carbon (TOC) and dry weight (DW) are measured from the substrate samples. For 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 NO_x 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 (NO_x and CO_2) pass through the drying-tubes and the CO_2 absorber. CO_2 is absorbed and NO_x passes through the detector (TCD = Temperature Current Detector), which needs also helium as the reference. After detection of NO_x 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 VariaMAX-Software 4.3 D. For calibration l-glutamine acid was used. The area below the peaks is integrated and the result is given in % of the 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 h and weighed. The percent dry weight was then calculated from these results.

Important Sample experiences:

Sampling is an essential key factor for serious research results. Within the sampling campaigns with different sampling tools like drill and needle (figure 5.9) it was observed that a cross contamination took place with the sampling needle for soil samples (Figure 5.10).

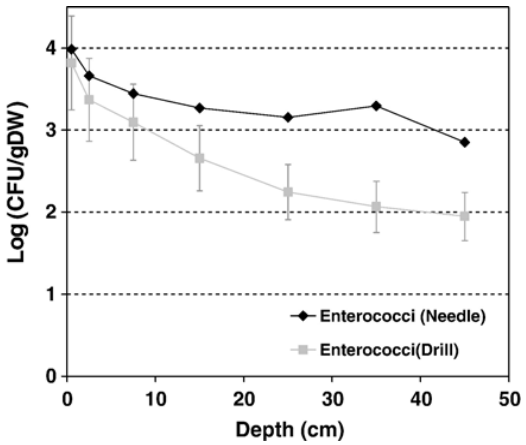

Figure 5.10: Different results with needle and drill sampling devices; error bars show the standard deviation (n = 8).

5.4.2 Particle size analyses

Using particle size analyses one determines a proportion on a mass basis of different size mineral particles in soil matrix (Gee and Bauder 1986).

The particle size analyses consist of dry sieving (for particles > 2 mm), wet sieving (for smaller than 2 mm), and pipette approach based on the principle of sedimentation or Stokes' Law for the smallest one (< 0.063 mm) (ÖNORM L1061, 2002).

To determine the grain size distribution after 3 years operation from planted and unplanted PSCWs and FSCWs soil profiles at three different points per plot in two replications from 7 different depths (0-1, 1-5, 5-10, 10-20, 20-30, 30-40 and 40 -50cm) were sampled. The analyses have been done with the original substrate (project start) and samples taken after 3 years of operation from the different depths.

5.4.3 Dissolved Oxygen measurements

Most bacteria, which are responsible for nitrifying, need a certain amount of oxygen – so dissolved oxygen (O_2) was online measured every 10 min in with five Electrochemical O_2 Sensors (EC- sensor-OM-E200302 3600 Analyzer for Oxygen; Orbi- sphere) at four different depths (5, 10, 20, 40 cm) of the main PSCW layer. The sensors were used in planted and unplanted PSCWs over a time of 2 years (from the year 2004 to 2006).

5.4.4 Tracer studies with potassium chloride (KCl)

For investigating the hydraulic retention time (HRT), tracer experiments with potassium chloride (KCl, 60%) were performed at 2 unplanted and 5 planted PSCWs. About 15 grams of KCl were dissolved in 15 litres of wastewater with about 2,7ms/cm (pure wastewater had about 0.8 ms/cm electrical conductivity) and initially charged to the plots (adequate to one loading rate). Measurements were done with a WTW electrical conductivity probe and data was recorded every 10 minutes by using a data logger.

HYDRAULIC RESIDENCE TIME: the hydraulic residence time (HRT) of a treatment wetland is the average time that water remains in the wetland, expressed as mean volume divided by mean outflow rate. If short-circuiting develops, effective residence time may differ

significantly from the calculated residence time.

HYDRAULIC LOADING RATE: hydraulic loading rate (HLR) refers to the loading on a water volume per unit area basis. [loading = (parameter concentration)(water volume/area)].

5.4.5 Chemical parameters

The following standard chemical paramters have been measured every month over a period of 2 years from all PSCWs and FS CWs: BOD_5, COD, TOC, NH_4-N P_{tot}. The detailed sampling is described in chapter 5.4.1.

The grab-samples from the influent and effluent have been analysed according to the German Standard Methods (1993) for organic matter (COD (DIN 38409-T41) and BOD_5 (DIN EN 1899-T1), TOC (DIN EN 1484)), ammonia nitrogen (NH_4-N (DIN 38406-T5)) and total phosphorus (P_{tot} (DIN EN ISO 6878)) in the laboratory of the Institute.

5.4.6 Physical parameters

Following standard physical parameters have been measured: pH-value (in water and soil), Temperature in °C (air temperature in,- and outdoor and soil temperature in 4 different depth with the O_2-probes), dried matter, annealing loss

5.4.7 Microbiological parameters

E. Coli, total Coliformes, Enterococci, Heterotrophic bacteria

Faecal 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 faecal coliforms and E. Coli Chromocult Coliform® agar (MERCK; ISO 16649) plus Cefsulodin (10 µg/ml) (CC+) was used. The incubation lasted 24 h at 37 C (Byamukama et al. 2000)

Chromocult agar was developed for the simultaneous detection of total coliforms and E. Coli (Figure 5.11) due to the inclusion of two chromogenic substrates. Chromocult agar for the identification and enumeration of human faecal 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 (ISO 7899-2, 2000). The incubation lasted 48 h at 37°C (Figure 5.12).

The number of heterotrophic bacteria (heterotrophic plate count, HPC) was determined by the pour plate method (ISO 6222, 1999) with yeast extract agar (MERCK). The incubation lasted 72 h at 22°C.

For better illustration, the numbers of total coliforms, *E. Coli*, Enterococci and heterotrophic bacteria were converted to $\log_{10}$ values and expressed as $\log_{10}$ CFU/ml.

In Sleytr et al (2007)

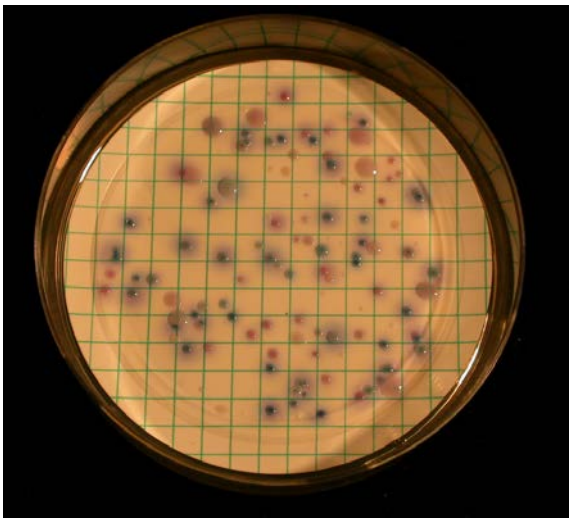

Figure 5.11: Plate count method to count total coliforms (one pink spot is one colony) and *E. Coli* (blue colonies)

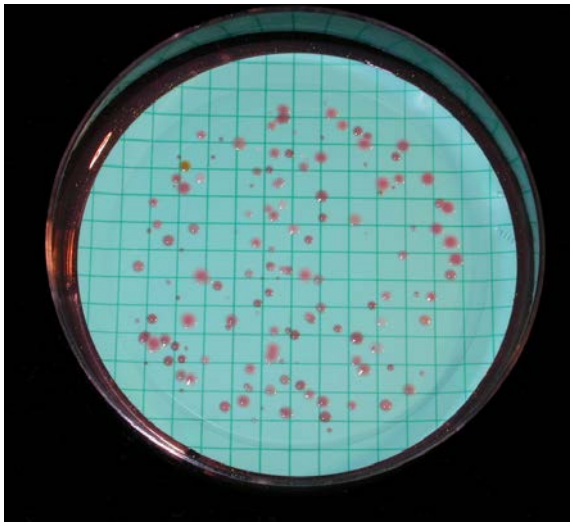

Figure 5.12: Plate count method to count total Enterococci (pink/brown colonies)

Clostridium perfringens, Pseudomonas aeruginosa

Model organism for human pathogens:

Clostridium perfringens is a spore forming bacteria, which is highly resistant to environmental conditions. It has been shown to be a useful model for Cryptosporidium oocysts and Giardia cysts. Aerobic (Bacillus) spores could also be used, but likely to grow in treatment systems and slough off surfaces providing misleading numbers. (Petterson and Ashbolt 2003)

Clostridium is the most widely occurring pathogenic bacterium and is found within human and animal intestinal tracts (Karpiscak et al 2001). Spores of Clostridium perfringens possess high heat resistance and when these spores germinate and return to active growth, they can cause gastrointestinal disease. As these indicator organisms are quite common to describe drinking water quality we decided to use this parameter to characterize the Outlets concerning to the hygienic safety.

Pseudomonas aeruginosa is another organism that is commonly used as an indicator for wastewater quality due to its direct pathogenic effect on humans and the higher resistance than coliforms (Ghermandi et al. 2007).

Clostridium perfringens spores (according to the protocol of EN ISO WD 6461-2, 1986) and Pseudomonas aeruginosa (according to the protocol of EN ISO 12780, 1986) were analyzed from the inlet and outlets from all PSCWs with a grain size 0,063-4mm (plot 1-8) (analysis have been carried out by the clinical Institute for Hygiene and Medical Microbiology (Medical University of Vienna). Two sampling campaigns took place and mean values were calculated. Sleytr et al (2008)

Bacterial direct count with DNA dying with DAPI

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 10 min and filtered through a griddled blackened polycarbonate membrane filter (25 mm diameter, 0.45 µm 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). Sleytr et al (2007)

5.4.8 Bio molecular parameters

DNA isolation

DNA extractions of the soil samples were carried out with the PowerSoil DNA isolation kit; for the wastewater samples the UltraCleanTM Water DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) was used, following the manufacturer's recommendations.

Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP analysis is a technique used to study complex microbial communities based on variation in the 16S rRNA gene. T-RFLP analysis can be used to examine microbial community structures and community dynamics in response to changes in different environmental parameters or to study bacterial populations in natural habitats. It has been applied to the study of complex microbial communities in diverse environments such as soil, marine and activated sludge systems. (Osborn, 2010)

Samples for analysing T-RFLP were taken from the bulk soil of planted and unplanted indoor PSCW, planted outdoor FSCW, rhizosphere soil from the planted PSCW and the planted outdoor FSCW, from inflow indoor and outdoor and the outflow of unplanted and planted PSCW and the planted outdoor FSCW. In Table 5.3 details are listed.

T-RFLP analysis was done according to a modified protocol after Hackl et al. (2004) and Sessitsch et al. (2002). Twenty-four TRF community profiles derived from twelve bulk soil samples (ten planted and two unplanted), four rhizosphere soil samples and eight wastewater samples were analysed. For the wastewater samples the impact of the CW passage on changes in bacterial diversity of the outflow was analysed. All samples were compared and standardized to the lowest quantity, according to the method of Dunbar et al. (2001). Terminal restriction fragments (TRFs are the peaks in a T-RFLP profile) of 50–500 base pairs (bp) in length and with heights of ≥50 fluorescence units (FU) were included in the analysis. TRFs 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 (numbers of TRFs with intensities equal or more than 50 fluorescence units) 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 TRFs with intensities higher than 500 FU were designated as highly abundant TRFs.

Table 5.3: List of different soil and wastewater samples. (Slightly modified from Sleytr et al., 2009)

Sample	number of samples	Sample type	Depth (cm)
FSCW	1	Bulk soil, *Phragmites a.*	0-1
FSCW	2	Bulk soil, *Phragmites a.*	1-5
FSCW	2	Bulk soil, *Phragmites a.*	5-10
FSCW	1	Bulk soil, *Phragmites a.*	10-20
FSCW	1	Bulk soil, *Phragmites a.*	20-30
FSCW	1	Bulk soil, *Phragmites a.*	30-40
FSCW	1	Bulk soil, *Phragmites a.*	40-50
PSCW	2	Bulk soil, *Miscanthus s. g.*	5-10
PSCW	2	Bulk soil, unplanted	1-10
FSCW	2	Rhizosphere soil, *Phragmites a.*	10-20
PSCW	2	Rhizosphere soil, *Miscanthus s. g.*	10-20
Inflow	2	Wastewater indoor	inflow
Inflow	2	Wastewater outdoor	inflow
Outflow	2	Wastewater indoor, *Miscanthus s. g.*	outflow
Outflow	1	Wastewater indoor, unplanted	outflow
Outflow	1	Wastewater outdoor, *Phragmites a.*	outflow

5.4.9 Statistics

For data analyses the Spearman's rank correlation was used to test 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).

6 Results and Discussion

Many physical, chemical, microbiological and other processes, which are responsible for nutrient, pollutant and pathogen removal, are running in parallel in vertical flow CWs. It would go beyond the scope of this work to describe all mechanisms responsible for removal processes, so selective methods have been combined to help bringing light into the "black box". One focus was to obtain detailed knowledge about removal efficiency of indicator organisms and pathogens in different designs of planted and unplanted indoor pilot-scale vertical flow constructed wetlands (PSCWs) and a full-scale outdoor plant (FSCW). A very controversial discussed topic about the need of planting in CWs will be described and pointing out why plants play an important role for these wastewater treatment systems. CWs can be seen as artificial and very complex systems with a surprisingly high diverse biozoenosis. Diversity patterns from different depth in filter body, inlet and outlet, will describe CWs as very dense populated habitats. The more diverse a system is, the better the performance can be.

Results have been compared with data from the literature and are discussed. More detailed results can be found in the published papers in Appendix 1- 5.

For a better overview, the most interesting topics and results are summarized and discussed in 4 parts:

1) Removal mechanisms and key factors for bacterial elimination during the filtration process in CWs (chapter 6.1, see also in Appendix 1and 3)

2) Comparison of different designs of CWs regarding bacterial removal efficiencies. (chapter 6.2, see also in Appendix 1 to 5)

3) The role of plants for CWs and importance for bacterial removal in these systems. (chapter 6.3, see also in Appendix 1 to 4; Literature review)

4) Diversity of abundant bacteria in VFCWs: how diverse are CWs compared to their design and to other systems? Describing the change of diversity from abundant bacteria during the filtration process. (chapter 6.4, see also in Appendix 2)

6.1 Removal mechanisms and key factors for bacteriological elimination during the filtration process in CWs

Beside a high rate of nutrient reduction (microbial communities are responsible for the majority of the removal processes) the effective control of human pathogens and microbial pollutants become more and more relevant within wastewater treatment.
The pathogen removal performance of a CW is based on a combination of physical, chemical and microbiological processes in the filter body. Different technical constructions and operation systems (surface flow, horizontal or vertical subsurface flow, different filter materials, etc.) allow optimizing the cleaning performance.

6.1.1 Physical mechanisms

Many physical parameters like temperature, adsorption of bacteria to particles, aggregate formation, sedimentation and filtration can have removal effects.

To get an idea how long pathogens can survive in their environment Table 6.1 gives a short summary about the die-off of selected microorganisms and viruses in soil. (WHO, 2006)

Table 6.1: Die-off of selected pathogens in soil, expressed as T_{90} values (adapted from WHO, 2006)

	T_{90} (days for 90% inactivation)
Salmonella	35 ± 6
EHEC	25 ± 6
Rotavirus	30 ± 8
Hepatitis A virus	75 ± 10
Giardia	30 ± 4
Cryptosporidium	495 ± 182

Adsorption

Adsorption and survival are the main factors defining the behaviour of microorganisms in soil. Gantzer et al. (2001) e.g. studied these factors in somatic coliphages, F-specific RNA phages and faecal coliforms. He showed a massive adsorption for all the microorganisms (61–86%). Therefore, for the soil and wastewater used, faecal coliforms had a high adsorption capacity in equilibrium conditions. He found out, that temperature had a significant effect on the survival of microorganisms in wastewater (Table 6.2). It was less if the microorganisms were adsorbed onto soil. The moisture content of soil also affected survival and the T_{90} values of faecal coliforms and somatic coliphages were two and three times higher, respectively, at 15% than at 35% moisture content.

Temperature

Gantzer et al. (2001) observed that the survival characteristics of faecal coliforms in wastewater is a function of temperature and is shown in Table 6.2. In wastewater, temperature had a significant effect on the survival of this microorganism ($P = <0.05$). T_{90} decreased as temperature increased.

Table 6.2: Survival of faecal coliforms in wastewater depending on temperature (slightly modified from Gantzer et al., 2001)

	°C	T_{90} (days for 90% inactivation)
Faecal coliforms	8	6,8
	16	3,2
	22	2,3

Lab based inactivation experiments with wastewater done by Boutilier et al. (2009) showed that E. Coli concentrations dropped below detection limits after 20 days at room temperature (average 24.6°C) and 67 days at refrigerated temperature (average 7.7°C). Lower removal rates reported in Boutilier et al. (2009) can be explained with outdoor effects of solar radiation, sedimentation or vegetation.

Table 6.3 shows the survival characteristics of faecal coliforms in soil as a function of temperature. Temperature had a smaller effect than in wastewater. However, as in wastewater, T90 increased as temperature decreased. In addition, the T90 for faecal coliforms was significantly higher in soil than in wastewater for all temperatures ($P = <0.01$). The T90 values for faecal coliforms are similar for all temperatures ($P = >0.1$). (Gantzer et al., 2001)

Table 6.3: Survival of faecal coliforms in soil (moisture 25%) in function of temperature (slightly modified from Gantzer et al., 2001)

	°C	T_{90} (days for 90% inactivation)
Faecal coliforms	8	17,2
	16	13,5
	22	12,0

Dissolved oxygen (DO)

Most of the relevant removal processes in CWs (e.g. carbon reduction and nitrification) take place under aerobic conditions. A positive influence on pathogen removal due to higher concentrations of DO in anoxic wastewater stabilisation ponds was found out by Almasi and Pescod (2000). Investigations at a pilot scaled vertical flow CW showed that the highest oxygen consumption takes place in the first 30 cm of the filter layer and can influence biological purifying processes. Further more DO concentration was one of the most important factors for the occurrence of filter clogging which cause degradation of removal efficiency (Wozniak et al., 2007). Therefore the oxygen supply in the filter bed gets very important to understand.

The DO measurements aimed to describe the different conditions within the filter bed and during the different stages of saturated and unsaturated phases during the wastewater charging times. No difference could be detected between the planted and unplanted PSCWs. Throughout the whole filter bed DO concentration ranged between 8.7 – 9.3 mg O_2 /l in between the loadings and between 7.8 – 9.3 mg O_2 /l during the loadings. The PSCWs in this study showed no significant change in the DO concentration all over the entire filter bed with time and depth. Figure 6.1 shows a typical DO concentration curve of 24 hours from a planted PSCW after two years operation. The minima occur after loadings but aerobic conditions could be measured in the whole filter bed. An intermittent loading of 60 mm/day and the use of fine, sandy material (0.06-4 mm grain size) could provide oxygen transfer to take place and support sufficient aerobic microorganisms to grow.

Since 50% microbial biomass and metabolic activity is located in the upper layer (0-10cm) (Tietz et al., 2007) each loading step (interval 6 hours) will initiate a significant increase in oxygen demand (see Figure 6.1). A stable oxygen concentration beginning in a depth of 20 centimetres and deeper is maintained. This reflects a more balanced nutrient supply and metabolic activity in these layers. In Tawfik et al. (2004) the removal rate of E. Coli under aerobic conditions (DO from 3.3 to 8.7 mg/l) was significantly higher than under anaerobic conditions. Therefore the concentration of DO in the filter bed can play an important role for pathogen (or indicator) removal efficiency beside adsorption to bio films, sedimentation and other die-off-processes.

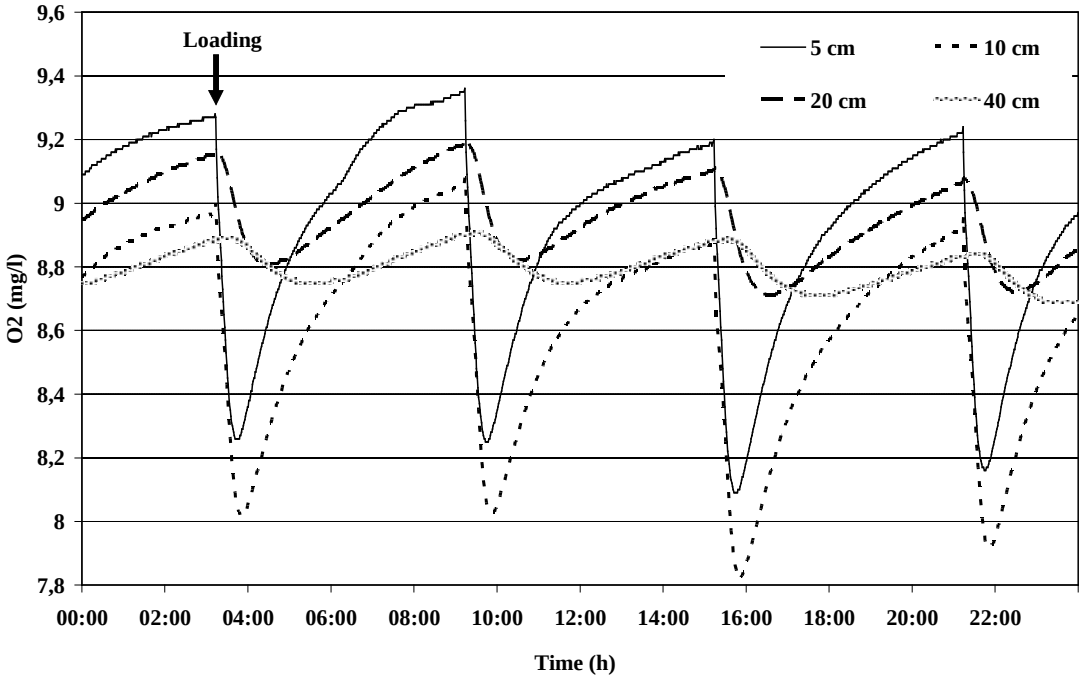

Figure 6.1: Time series of the DO concentrations over 24 hours in different depths in a planted PSCW (temperature 19°C).

Hydraulic retention time (HRT)

It is often reported (e.g. Green et al. 1997, Sleytr et al., 2008, Warren et al., 2000) that longer retention time increases bacterial removal and can play a key role for inactivation of pathogens.

In this study the HRT was determined from tracer experiments which were performed to describe different flow pattern and to evaluate the mean residence time for a conservative tracer in a range of the planted and unplanted PSCWs. The mean HRT was 4.1 days for both systems. The unplanted systems (mean value 4.5 days ± 0.71) had a 0.6 days longer HRT than the planted ones (mean value 3.9 days ± 0.57). This could be explained with plant effects, e.g. that the tracer is able to take some hydraulic shortcuts along the roots. However, no negative effect on the removal rates could be observed. As described in García et al. (2003) for HF beds, a HRT over three days does not result in a significantly higher inactivation ratio and therefore a lower microbial concentration in the effluents. A minimum retention time is a key parameter for predicting removal of bacteria in infiltration filters (Ausland et al., 2002).

Moisture, water content and hydraulic loading rate

In studies with the transport of *E. Coli* in sand columns with constant and changing water contents it was found out that constant unsaturated flow is probably the best for removal pathogenic bacteria as it results in lower maximum concentrations of E. Coli and greater cell removal in comparison with saturated and variable unsaturated flow. (Powelson and Mills, 2001)

Higher hydraulic (intermittent) loading rates showed a reduction in disinfection capacity, which is reported in Torrens et al. (2009a)

Increasing the wastewater loading rate can result in lower purification and is shown in several experiments with intermittent loading (e.g. Tanner et al., 1995, Torrens et al., 2009b)

To calculate the correlation of measured bacterial parameters in the main layer with DW (%) and TOC (mg/g DW) from 112 samples the Pearson correlation was used and is shown in Table 6.4. All Correlations are statistically significant.

Table 6.4: Correlations of measured bacterial parameters in the main layer with DW (%) and TOC (mg/g DW) (n=112)

Parameter	HPC	E. Coli	Total Coliforms	Enterococci
R^2 (DW)	0.624	0.574	0.626	0.510
R^2 (TOC)	0.728	0.854	0.952	0.741

6.1.2 Chemical mechanisms

Rhizosphere exudates

Activities of all enzymes (phosphatase, urease, and cellulase) were significantly correlated with root activity in SSF CWs planted with *Phragmites australis* by Kong et al. (2009); enzyme activities were significantly higher in the top layer of the substrate than in the deeper layers, there were generally no significant differences between the deeper layers (deeper than 15 cm) - root activity was significantly correlated with removal efficiencies of all contaminants.

Oxidation and solar radiation (more important for surface flow systems)

Zdragas et al. (2002) investigated the seasonal change in the reduction of coliform population and studied the effect of solar radiation on the ability of a combination of surface and subsurface flow constructed wetland to reduce the concentration of total coliforms and the relation to *Salmonella spp..* He found out that solar radiation has a lethal effect on coliforms, which means that the climate can influence the ability of a constructed wetland to disinfect municipal wastewater.

Further disinfection (chlorination and ozonation)

Although some types of constructed wetlands have a high removal performance for indicator organisms and pathogens they rarely reach the low concentrations which are required in some regulations for bathing water and drinking water. Options to achieve a higher disinfection level could be e.g. chlorination, ozonation and UV-irradiation of the treated wastewater. Chlorination of treatment plant effluents is often performed in hot climate and developing countries, but has the disadvantage of possible damage of the environment.

Ultraviolet disinfection is considered as one of the best alternatives to chlorination to improve the microbiological quality of the treated wastewater, George et al (2002)

6.1.3 Biological mechanisms

Predation (protozoan and/or viral)

Constructed wetlands can show a high diverse biota that also can participate in the treatment process as shown in Vymazal et al. (2001a). It is also shown that predation is participating in removal mechanism in HFCWs. Stott et al. (2001) found out that predation by free-living ciliated protozoa, which are commonly found in constructed wetlands, can be a dominant mechanism for the removal of cryptosporidium oocysts. Impact of protozoan grazing on bacterial community structure in soil microcosms was also investigated by Ronn et al. (2002).

Wand et al (2007) concluded that predation is the dominant mechanism of bacterial removal in planted and unplanted constructed wetlands.

High copepods concentration in surface flow constructed wetlands are correlated with high removal performance for indicators and pathogens (e.g. *E. Coli*, *Clostridium p*.) showed by Song et al. (2008)

Competition, inactivation and natural die-off

Some pathogens and indicator organisms are not able to survive out of their hosts or natural habitat for long (see table 6.2).

Bio film formation and antibiosis by plants and other microorganisms can also play a role for removal of bacteria during the filtration process.

6.2 Comparison of different designs of CWs regarding bacterial removal efficiencies

Many different CWs have been described according to their indicator organisms and pathogen removal efficiency. Most of the studies (e.g. Payment et al. 2001, Quinonez-Diaz et al. 2001, Torrens et al. 2009, Manios et al. 2002, Song et al. 2008) investigated the in- and the outflow of the wastewater treatment plant. Depending on the system (e.g. surface flow, subsurface horizontal or vertical flow CWs) and design factors like the used filter media, retention time, plant species, loading rate etc. bacterial removal efficiency can be quite different.

Changes in temperature, different bioactivity levels, disparate levels of plant and microbial life and patterns of hydraulic flow can contribute significantly to shifts in removal rates in the same CW. Therefore, experimental data reporting CW treatment performance cannot be truly compared without references to the respective biological activity levels and mixing conditions at the time of the sampling. Microbial activity levels will also change with root development and system maturation. (Werker et al., 2002)

Table 6.5 gives an overview of removal efficiencies and removal rates in wastewater treatment systems and different designs of CWs. Indicator organisms (E. Coli, faecal coliforms, total coliforms, Enterococci and Clostridium perfringens spores) are summarise according to the literature and own data. Detailed descriptions of the systems can be found in the literature listed in Table 6.5.

Table 6.5: Maximum log removal rates (log_{10}) and removal efficiencies (%) from different indicator organisms and pathogens of different CW designs according to the literature

Treatment system	E. Coli	faecal coliforms	total coliforms	Enterococci	Clostridium p. spores
SF CWs	99.8% g) 92% c) 96% l)	86-94% a) 91% c)	87.9% g) 84% l)	99.5% g) 89% l)	99.9% g) 78% l)
HSSF CWs	5.4 log b) 96.6 % h)	4.2 log e) 3.4 log b)	4 log e) 2.81 log b)	2.4 log e)	2.4 log e)
VF PSCW	4.35 log d)	3.7 log f)	4.3 log d)	4.8 log d) 2.8 log f)	3.47 log d)
VSSF CWs	3.7 log b) 1.5 log j)	2.2 log e) 1.5 log j)	3.55 log b) 2.9 log e)	3.62 log b) 2.4 log e)	2.4 log e)
Two-stage VF CWs	3.31 log m)	1.5 log b) 4.2 log e)	2 log b) 4.2 log e) 3.42 log m)	1.3 log b) 1.5 log e) 3.36 log m)	2.9 log e)
Activated sludge (biological)	2.06 log k)	2.4 log k) 5.7 log i)	2.2 log k) 5.4 log i)	3.3 log k)	1.27 log k)

a) Perkins and Hunter (2000)
b) Cabello et al (2002)
c) Hill and Sobsey (2001)
d) Sleytr et al. (2007)
e) Barrett et al (2001)
f) Hench et al. (2003)
g) Stenström and Carlander (2001)
h) Warren et al. (2000)
i) Zhang and Farahbakhsh (2007)
j) Torrens et al. (2009b)
k) Kazmi et al. (2008)
l) Reinoso et al. (2008)
m) Langergraber et al. (2009)

6.2.1 Activated sludge treatment systems

Faecal coliforms, faecal streptococci, human enteric viruses, clostridium perfringens (pathogens and faecal indicator bacteria) occurrence and removal were studied by Payment et al. (2001): Faecal coliforms were the most numerous of the indicator bacteria with removal averaged of 25%. Faecal streptococci removal was 29%, while E. Coli removal was 12%. Clostridium perfringens removal averaged 51%. Giardia cysts levels were not markedly different throughout the study period, and 76 % of the cysts were removed by treatment. Cryptosporidium oocyst counts were erratic, probably due to the methods, and removal was 27%. Human enteric viruses were detected in all samples of raw and treated wastewater with no removal observed (0%). Overall, the plant did not perform well for the removal of faecal indicator bacteria, human enteric viruses, or parasite cysts.

Zhang and Fa rahbakhsh found out that activated sludge tertiary treatment could show removal efficiency for faecal coliforms up to 5.7 log and for total coliforms up to 5.4 log (due to the use of ferrous chloride for phosphorus removal) which is very high according to other systems investigated for removal efficiency in the literature (e.g. in Wen et al., 2009)

6.2.2 Surface flow CWs

The removal efficiency for bacteria from SFCWs was investigated by e.g. Green et al. (1997) for bacteria like E. Coli and total coliforms and showed 1.5 to 2.1 log removal rates (less than 1000 cfu E. Coli/100 ml in the effluent) with a retention time of 24 hours and more.

The removal rate of enteric bacteria (faecal coliforms and faecal streptococci) in surface flow constructed wetlands showed an removal efficiency of 85-94%. The rate was negatively correlated with the flow rate (retention time) by Perkins and Hunter (2000)

Occurrence and die-off of indicator organisms in the sediments in two SF CWs was investigated by Stenström and Carlander (2001) and showed for coliforms 87.9% reduction, for E. Coli 99.989 %, for faecal Enterococci 99.5%, for Clostridium 99.9% and for coliphages 50%. Log Concentrations (log10/g DW) for coliforms was 3-5, for E. Coli 3-4, for clostridium 5.5-5 and for coliphages 3.5-5 in the deposited material in the SF CWs.

In Song et al. (2008) indicator MOs and pathogen removal in in SF CWs was for E. Coli 99,9% (log removal 3.03) for faecal streptococci 99,8% (log 2.72) for total coliforms 99,8 % (log 2.75) for Salmonella spp. 97,5% (log 1.6) and for C. perfringens 77,2% (log 0.64).

Reinoso et al. (2008) found out, that the highest removal of indicator bacteria (total coliforms, E. Coli, faecal streptococci and Clostridium perfringens) occurred in a SF stabilization pond, reaching 84%, 96%, 89% and 78%, respectively. The greatest removal of protozoan pathogens (Cryptosporidium and Giardia) and coliphages was found in the SSF wetland, 98%, 97% and 94%. In contrast, the SF wetland was most efficient in the removal of pathogenic parasites when considering superficial removal rates. Seasonal differences in organism removal were not statistically significant during the studied period.

6.2.3 Horizontal sub surface flow CWs

E. Coli reduction level in HSSF CWs and VSSF CWs was quite similar ranging between 5.4 – 5.5 log removal rate. Different types of pilot scale CWs with two different filter materials (mixture of expanded clay; washed sand with a grain size of 0-2mm) were tested by Baeder-Bederski et al. (2005). The role of HRT (3-5 d) in different granular medium in microbial removal showed a log removal rate for faecal coliforms from 0,1 - 3,4 and 0,9 – 2,6 for somatic coliphages.

Horizontal flow CWs: Faecal coliforms removal efficiency above 3 log units; 4 log only achieved in those units operated at retention times above 7 days (effluent from different wetland units were on average less than 10.000 fcu/100ml); less than 2.000fcu/100ml could only be reached with combined systems with alternating open water and planted zones. Okurut and van Bruggen (2000)

In the study from Barrett et al. (2001) an onsite HSSF CW (0.3m depth gravel substrate, HRT of 2.25 d and loading rate 51.5l/m^2/d) showed following log removal efficiencies for different microbial indicators: for total and faecal coliforms 0.5. -2.6; for Enterococci 0.1-1.5; for Clostridium perfringens -0.3-1.2 and for coliphages -0.3-2.2.

Green et al. (1997) shows a significant correlation between retention time in HSSF CWs and bacterial removal E. Coli up to 3.11 log and total Coliforms up to 2.81 log.

6.2.4 Vertical sub surface flow CWs

The efficiency of subsurface flow reed bed treatment with sequential loading and continuous loading was a statistically significant with a log reduction of 3.56 and 4.25 for E. Coli, 3.2 and 3.88 for coliforms and 3.85 and 4.2 for total aerobic bacteria, shown by Duggan et al. (2001)

Torrens et al. (2009b) found out that the removal of microbial indicators in VSSF CWs and intermittent loaded sand filters depends mainly on the water retention time in the filter, which in turn depends on the depth of the filter, the hydraulic loading rate and the dose volume per batch. The presence of plants did not significantly affect the removal of indicator microorganisms in our study, which indicates that the presence of Phragmites is of minor

importance for the removal of microorganisms in VSSF CWs intermittently dosed. No significant differences were found between the two kinds of sand tested (crushed and river sand). Low temperatures did not limit the removal of indicator microorganisms in these systems. A mean removal rate of 1.5 log removal for E. Coli and 1.6 log removal for faecal coliforms show a much lower removal efficiency than other investigated VSSF CWs (e.g. Sleytr et al., 2007).

Hench et al. (2003) showed that bacterial pathogens (*Salmonella, Shigella, Yersinia)* were removed with slightly less efficiency than sanitary indicator organisms ranging from 1.5 - 2.3 log reduction.

Quinonez-Diaz et al. (2001) report about removal rates of indicator bacteria (total and faecal coliform), coliphages and Cryptosporidium of more then 90%.

Molleda et al. (2008) describes up to 100% removal efficiency in summer times for Clostridium p. and maximum values in spring and autumn at 99,9% for E. Coli and total coliforms in Winter times 97% for faecal streptococci.

PSCWs and FSCWs from the presented study

In most CWs studies dealing with bacterial removal (e.g. Decamp and Warren, 2001 and Thurston et al., 2001), only the influent and effluent concentrations of bacteria (expressed in colony forming units (CFU)) are compared. These investigations do not give any information regarding in which zone of the sandy substrate the majority of the bacterial contamination is eliminated. So in this project bacterial removal processes in the main layer of subsurface vertical flow CWs could be investigated and described with simple methods - this enables a better design regarding hygienic safety.

Therefore indicator organisms such as faecal coliforms (Escherichia coli (E. Coli), total coliforms), Enterococci and heterotrophic bacteria (heterotrophic plate counts, HPC) are analysed from the influent, the effluent and specific for this study at different depths of the sandy main layer (water and substrate samples). In addition, dry matter content (DW) and total organic carbon (TOC) have been analysed for the samples taken from the main layer of the filter bed.

To give an overview of mean values (n = 23) for the influent and effluent concentrations, and removal efficiencies of the PSCW (Plot 4) with a sandy main layer (grain size of 0.06–4 mm) are shown in Table 6.6. Bacterial indicators of faecal contamination measured in the mechanically pre-treated municipal wastewater showed comparable concentrations as reported by George et al. (2002) who showed typical abundances of total and faecal coliforms (FC) in raw sewage of 107–109 ml−1 and 106–108 ml−1, respectively. The removal efficiency for the indicator parameters is quite high compared to the literature (e.g. Ottova et al., 1997 and Thurston et al., 2001).

Table 6.6: Influent and effluent concentrations, and removal efficiencies for chemical and bacteriological parameters for a PSCW (Plot 4) with a main layer gravel size of 0.06–4 mm (mean values ± standard deviation; n = 23) (Sleytr et al., 2007)

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±1.3	4.1±1.35	0-47.4
Indicator organisms	**Log CFU / ml**		**Log**
Heterotrophic plate count (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 (MDC)	8.08±1.18	6.03±0.55	2.05

*Limit of detection.

Figure 6.2 shows the log removal rates of the bacterial parameters of all 10 indoor PSCWs. Significantly 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).

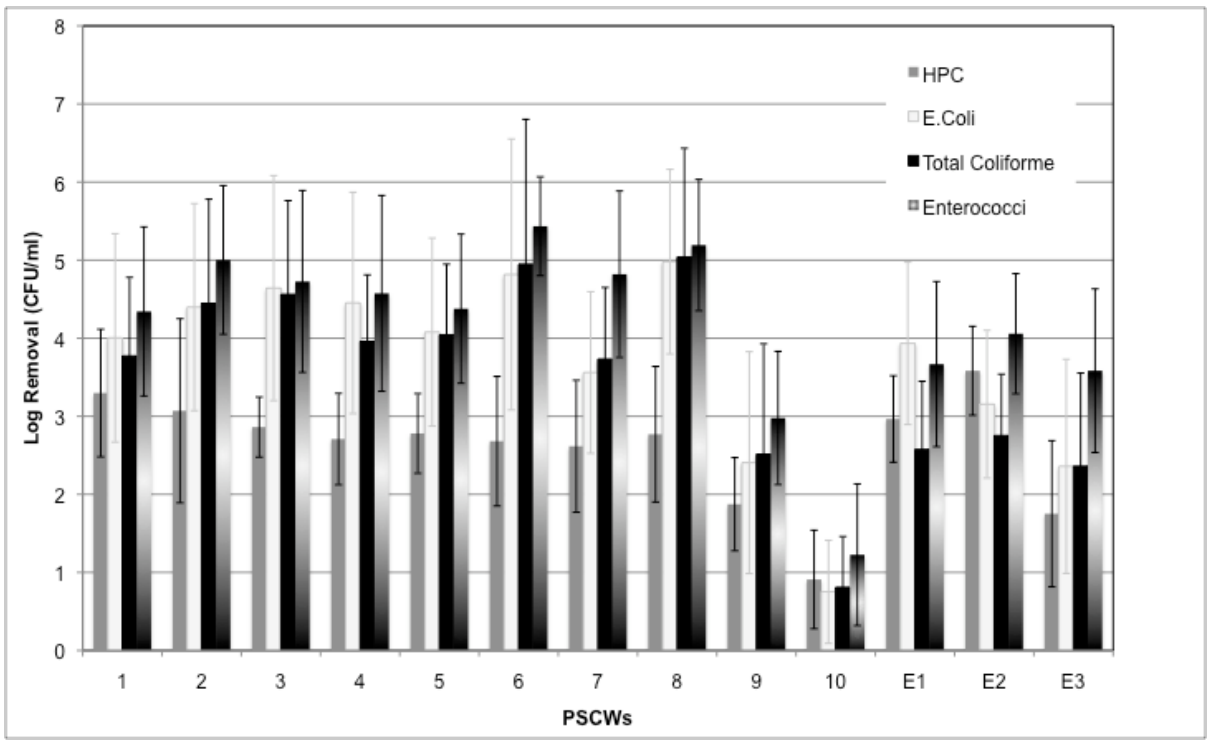

Figure 6.2: Log removal rates of bacterial parameters for all 10 PSCWs (1-10) and 3 full scale outdoor CWs in Ernshofen (E1-E3)

The PSCWs from this study show higher removal efficiency compared to similar systems described in the literature. 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.

6.2.4.1 Two stage VF CWs

Another possible design of CWs is the two-stage system, which was optimized within this study. It is consisting of two vertical flow beds with intermittent loading (40 g COD m^2/d) and operated in series. The first stage uses sand with a grain size of 2–3.2 mm for the main layer and has a drainage layer that is saturated; the second stage uses sand with a grain size of 0.06–4 mm and a conventional drainage layer.

In conclusion it can be said that a higher effluent quality could be reached with the two-stage system as compared to the single-stage VF CW system designed with 4 m2 per person. The higher effluent quality could be reached although the two-stage CW system was operated with the double organic load resulting in half the specific surface area requirement (2 m^2 per person). Table 6.7 shows the influent (In) and 2 effluents (Out 1 and Out 2) concentration form the two-stage CWs in Ernsthofen, Austria and the removal rates for HPC, E. Coli, total coliforms and Enterococci. (Langergraber et al., 2009)

Table 6.7: Log influent and effluent concentrations form the two-stage full scale CWs in Ernsthofen, Austria and r emoval rates for HPC, E. Coli, total coliforms and Enterococci (Langergraber et al., 2009 slightly modified)

	HPC per ml					*E. coli* per 100 ml				
Parameter	Log concentrations			Log removal		Log concentrations			Log removal	
Location	In	Out 1	Out 2	Out 1	Out 2	In	Out 1	Out 2	Out 1	Out 2
No. of analysis	8	8	8	8	8	8	8	8	8	8
Median value	6.32	5.65	3.43	0.68	2.88	6.52	5.82	3.46	0.65	3.06
Mean value	6.33	5.62	3.45	0.71	2.87	6.18	5.65	3.20	0.87	3.31
Standard deviation	0.10	0.18	0.39	0.14	0.38	0.57	0.54	0.41	0.54	0.41
95% confidence interval	0.15	0.26	0.56	0.21	0.55	0.82	0.78	0.59	0.78	0.59
Minimum	6.10	5.14	4.56	0.44	3.85	4.23	3.87	3.76	0.34	4.45
Maximum	6.60	5.92	2.51	0.99	1.79	6.66	6.30	2.15	2.73	2.85

	Total coliforms per 100 ml					Enterococci per 100 ml				
Parameter	Log concentrations			Log removal		Log concentrations			Log removal	
Location	In	Out 1	Out 2	Out 1	Out 2	In	Out 1	Out 2	Out 1	Out 2
No. of samples	8	8	8	8	8	8	8	8	8	8
Median value	6.93	6.23	3.70	0.63	3.18	6.11	5.25	2.80	0.85	3.30
Mean value	6.56	5.98	3.49	0.93	3.42	5.94	5.10	2.76	1.03	3.36
Standard deviation	0.65	0.56	0.39	0.57	0.40	0.40	0.44	0.30	0.43	0.28
95% confidence interval	0.94	0.81	0.56	0.82	0.57	0.57	0.63	0.43	0.62	0.41
Minimum	4.26	4.03	4.06	0.46	4.36	4.54	3.58	3.23	0.62	4.00
Maximum	7.00	6.49	2.60	2.93	2.90	6.26	5.63	2.11	2.53	2.89

6.2.4.2 Microbiological quality described with the indicatior Organisms Clostiridium perfringens sporen and Pseudomonas aeroginosa

Winward et al. (2008) report a significant correlation between Pseudomonas aeruginosa and total coliforms in the effluents. This can be interpreted that these bacteria are removed or

inactivated by similar processes. Hence, total coliforms seems to be the best indicator for the presence of Pseudomonas aeruginosa.

In other studies (e.g. Medema et al., 1997) the die off rates for E coli, faecal Enterococci and C perfringens spores in surface water were determined and showed that C.perfringens spores were 3-4 times more persistent than other indicators. Sleytr et al. (2007) measured high log removal rates for for the PSCWs for indicator parameters (E. Coli 4.35, total coliforms 4.31, Enterococci 4.80 and log 2.85 for Heterotrophic bacteria (HPC)).

Results shown in Figure 6.3 reveal the different removal efficiency from the investigated pathogens between the different designs of the PSCWs. Overall the PSCWs showed higher removal efficiency for the investigated pathogens compared to e.g. in Barett et al. (2001) who measured a Clostiridium perfringens log removal rate of 2.2 to 2.4 in VFCWs.

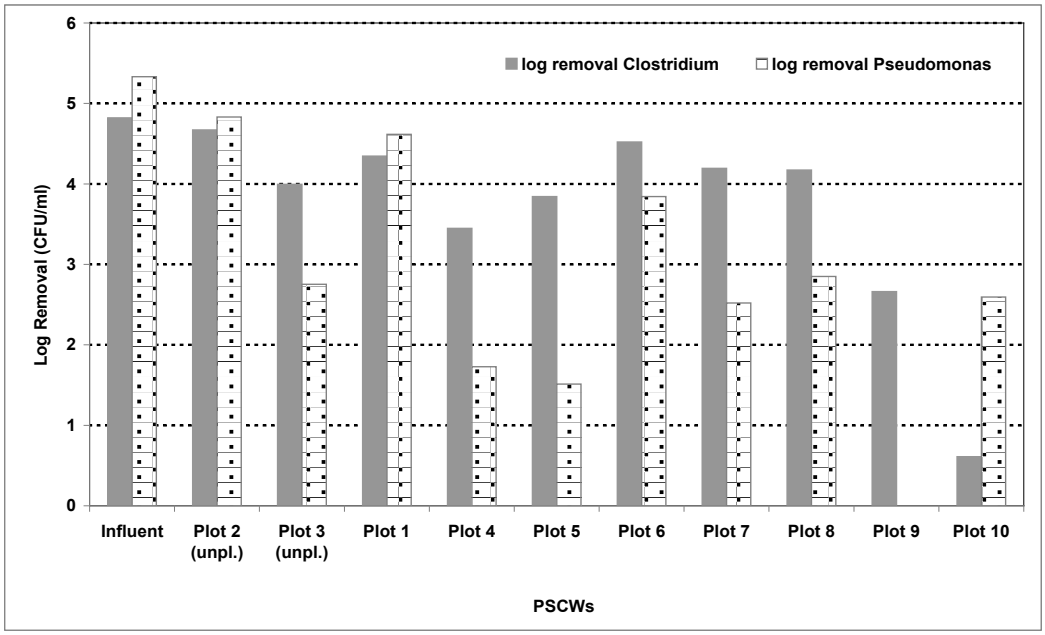

Figure 6.3: Log removal rates of bacterial parameters for all 10 PSCWs (1-10) and 3 full scale outdoor CWs in Ernshofen (E1-E3)

6.3 The role of plants for CWs and importance for bacterial removal in these systems

Concerning their natural habitat, climate and use of the constructed wetlands, engineers can select out of a wide range of possible planting solutions. Many species of plants are used for constructed wetlands like common reed (*Phragmites australis*), cattail (*typha*), rushes (*Juncus*), bulrushes (*Scirpus sp.*), sedges (*Carex sp.*), flat sedge (*Cyperus sp.*) and many others.

This chapter will discuss the relevance of planting for the design and performance of CWs.

6.3.1 Are Plants relevant for bacterial removal?

Regarding the importance of the need of planting in constructed wetlands the literature shows many controversial results. In general plants have been observed to exhibit little to no effect on the removal of indicator organisms e.g. faecal coliforms in constructed wetlands (Tanner et al., 1995). But also several studies have shown the important role of macrophytes (e.g. in Brix, 1997).

No Relevance

No significant difference in the removal efficiency of vertical flow CWs planted with *phragmites australis* and unplanted ones was observed in Torrens et al (2009a and 2009b).

Investigated mesocosms also planted with *Phragmites australis* did not exhibit an effect on pathogen removal as described in Van Loon et al. (2002). A higher removal efficiency has been described for planted constructed wetlands e.g. in Wand et al (2007); Rivera et al. (1995) and Decamp and Warren (2001).

Many studies did not detect significant differences between planted and unplanted systems (Vacca et al., 2005, Karathanasis et al., 2003, Tanner et al., 1995) for total coliforms, faecal coliforms, faecal streptococcii and heterotrophic bacteria and *clostridium perfringens*. Soto et al. (1999)

Removal of indicator microorganisms with different substrates (best results in gravel bed) showed a removal rate of 3.3 log for E. Coli and 3.0 log for faecal coliforms. No significant difference in the performance of planted and unplanted reed beds (subsurface horizontal flow) have been investigated by Manios et al. (2002).

Relevant

Natural wetlands

In Kansiime and Bruggen (2001) natural wetlands were investigated and it was found out that coliforms were better removed from the water in the papyrus-dominated vegetation compared to that of *Miscanthidium*.

Significant seasonal differences were evident, with the vegetated systems (cattail and fescue) showing a much better performance during the warmer months of the year and the unplanted systems performing best during winter months. In spite of unusually high loading rates, the vegetated systems also showed statistically greater annual removal of BOD and TSS than the unplanted system. Karathanasis et al. (2003)

SSHF CWs

A higher removal efficiency has been described for planted constructed wetlands e.g. in Wand et al (2007); Rivera et al. (1995) and Decamp and Warren (2001).

In Hench et al. (2003) for faecal coliforms, Enterococci, *Salmonella, Shigella, Yersinia* and coliphage greatest microbial reductions were observed in planted mesocosms compared to those lacking vegetation. Increased dissolved Oxygen and reduction of all investigated micro organisms also were observed in vegetated wetlands (horizontal sub surface flow sysstes with pea gravel and a depth of 45-60cm, planted with a combination of cattails (*Typha latifolia*), rush (*Juncus effussu*) and bulrush (*Scirpus validus*); loading 3 times a day with 16l each loading). Bacterial pathogens (*Salmonella, Shigella, Yersinia)* were removed with slightly less efficiency than sanitary indicator organisms ranging from 1.5-2.3 log reduction. Hench et al. (2003).

In another study with horizontal subsurface flow CWs the planted beds generally performed better than unplanted beds although the type of hydrophytes use was not significant. For the removal of faecal and total coliforms the CWs planted with *Phragmites australis* were slightly more efficient than those planted with Typha. The sandy substrate showed a slightly higher removal efficiency than gravel; overall not significant. In planted beds total coliforms were reduced up to 91%, faecal coliforms up to 90%. In the unplanted beds both organisms only showed a maximum reduction of 35% as reported in Rivera et al. (1995).

Plants and their rhizosphere can play an important role in increasing faecal indicator removal efficiency in pilot scale subsurface flow CWs (about 1 m^2 surface area; 0.6m^3 planted with *Scirpus lacustris*; hydraulic load 4-7cm/d; HRT 4-8d). They show significantly higher pathogen removal efficiency (reaching up to 99.999%) compared to other treatment systems. Some researchers have found an improvement in wastewater treatment in presence of macrophytes (Tilley et al., 2003); removal rates in horizontal subsurface flow CW planted

with *Phragmites australis* (gravel filter body with 5-10mm grain size) were significantly greater than that in the unplanted ones (removal rate of 96.6% of E. Coli) as reported in Warren et al. (2000).

SSVF CWs

Investiagations of removal efficiencies have been done by Sleytr et al. (2008); a higher log removal rate for *Clostiridium perfringens* sporen & *Pseudomonas aeroginosa* in unplanted pilot scale constructed wetlands could be discovered.

Results from VF CWs in the presented study

In the present study no significant difference was observed in the performance from planted and unplanted PSCWs comparing the effluents (see Figure 6.2).

But the absolute counts from different indicator organisms in planted and unplanted PSCWs in seven depths show different results (Table 6.4). HPC numbers from the planted PSCWs show a lower, not significant value at nearly all depths compared to total coliforms, E. Coli and Enterococci. 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. Similar result occurred for E. Coli. Only the result of the Enterococci shows a statistically significant difference between planted and unplanted PSCWs ($p < 0.05$; $n = 12$). (Sleytr et al. 2007)

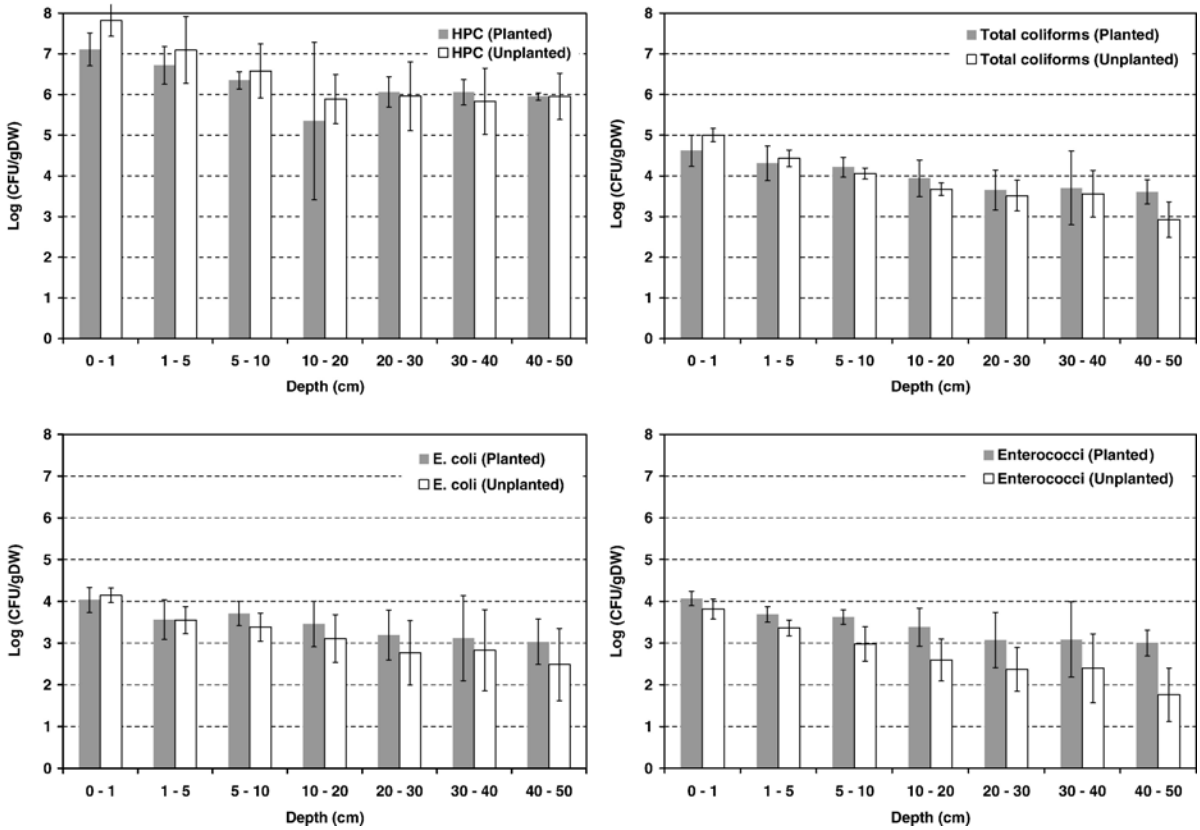

Figure 6.4: Heterotophic plate count, Total coliforms, E. Coli and Enterococci at different depths of the main layer of planted and unplanted PSCWs; error bars show the standard deviation (planted: n = 35; unplanted: n = 12).

Results shown in Table 6.8 reveal the different removal efficiency from the two investigated pathogens between planted and unplanted PSCWs. The effect of slightly better removal efficiency in the unplanted Plots can be linked up to a longer HRT (4.5 days; 0.6 days longer than the planted PSCWs; see chapter 6.1.1) and is most interesting. Overall the PSCWs showed higher removal efficiency for the investigated pathogens compared to e.g. in Barett et al. (2001) who measured a C lostiridium perfringens log removal rate of 2.2 to 2.4 in VFCWs.

Table 6.8: Mean log removal values of Clostiridium perfringens sporen & Pseudomonas aeroginosa in the effluents of planted and unplanted PSCWs (Sleytr et al., 2008)

	Clostridium perfringens	Pseudomonas aeroginosa
mean value all Outlets	4.15±0.39	3.08±1.24
mean value planted Outlets	4.09±0.39	2.85±1.02
mean value unplanted Outlets	4.33±0.48	3.79±1.47

6.3.2 Benefits of planting

The following arguments from the literature point out the benefits of planting and are listed below:

Physical Effects

- Plants can improve the physical filter effect through the rhizomes and roots (Reinhofer et al., 2007)
- They reduce wind velocity which effects better settlement (in surface flow constructed wetlands) and minimize the risks of erosion through stabilizing the surface of the used sediment (Brix, 1997)
- Through dense subterranean growth plants can cause stabilization of the filter surface by reduction of surface erosion (Brix, 1996)
- The vegetation cover protects the surface from erosion and shading prevents algae growth.
- Keeping the surface open by the movement of plants (Brix, 1996)
- The roots of plants can prevent clogging in sub-surface flow systems (Laber, 2002)
- Plants can influence the microclimate and equalizing the temperature by shading in summer and covering the surface in winter times (Mayer, 1994)
- Excessive algae growth in surface flow constructed wetlands can be prevented by the use of plants creating shading (Brix, 1996)
- Reduction of the effluent by evaporation of the plants is possible (Reinhofer et al., 2007)

Biological and other effects

- Plants can increase bacterial density by providing favourable conditions for microorganisms in the soil, so-called “rhizosphere effects” (e.g. changes in environmental conditions in the rhizosphere by the excretion root exudates through organic acids) (Knight et al., 1999).
- Deploying a large growth surface for microorganisms. These biofilms are responsible for the majority of microbiological processes in the Constructed wetlands occur (Brix, 1996).
- Supply of antibiotics (e.g., Schoenoplectus sp.) and other organic Compounds (Brix, 1996)
- Transport of oxygen by plants into the root zone (Reddy and D’Angelo, 1997) Oxygen can also be brought into the system via convection and diffusion. The excretion of photosynthetic oxygen into the rhizosphere by the plant roots can influence the bio-chemical cycles by altered redox status and increase the aerobic degradation (Brix, 1996).
- Improved cleaning performance of the wetland (in comparison with unplanted soil filters, etc.) (Flamisch, 1995) Only in planted, but not in unplanted soil filters, the

removal of oils and fats has been demonstrated.

- Direct recording and storage of nutrients: the inclusion of Nitrogen and phosphorus by the plants can be harvested annually by 50 to 150 kg phosphorus per hectare and 1000 to 2500 kilograms of nitrogen per hectare from the system will be removed. If the plants are not harvested, these substances retain and are not removed from the system. The same applies to heavy metals and non or poorly degradable compounds (Flamisch, 1995).
- For a constructed wetland treating municipal wastewater a potential nutrient uptake of about 1.9% of the influent nitrogen and phosphorus load can be expected. For lower loaded systems the potential uptake is significantly higher, e.g. 46% of the nitrogen load for treatment of grey water. (Langergraber, 2005a)
- Uptake of heavy metals and organic pollutants are described in many studies (e.g. Bragato et al., 2006, Khan et al., 2009, Lesage et al., 2007)
- Plants are an essential component of the design of constructed wetlands. (Brix, 1997)
- Plants in CWs provide additional value creation through high value habitat for many other plants and animals (Flamisch, 1995). CWs are a natural technology and should therefore have a near-natural appearance, which fits much better in landscapes.
- The majority of a large and diverse sample of wetland plant species enhanced year-round COD removal compared to gravel-only systems, especially at low temperatures. Certain species performed better than others, whereas a few species provided no appreciable benefit, suggesting that appropriate species selection potentially can increase treatment wetland efficiency throughout the year in cold regions. In general, species of the Cyperaceae (sedge) and Juncaceae (rush) families performed best while most species of the Poaceae (grass) family had poorer performance, with the notable exception of Deschampsia cespitosa, which was one of the top species. (Taylor et al., 2010)
- In developing countries plants from CWs can be harvested and used for fodder and building materials (e.g. roofs)

This list was modified from Reinhofer et al. (2007)

To conclude this chapter Table 6.9 gives a summary of the major roles of macrophytes in CWs and makes unplanted CWs unimaginable

Table 6.9: Summary of the major roles of macrophytes in constructed treatment wetlands (Brix, 1997)

Macrophyte property	Role in treatment process
Aerial plant tissue	• Light attenuation → reduce growth of phytoplankton • Influence on microclimate → insulation during winter • Reduce wind velocity → reduce risk of resuspension • Aesthetic pleasing appearance of system • Storage of nutrients
Plant tissue in water	• Filtering effect → filter out large debris • Reduce current velocity → increase rate of sedimentation, reduces risk of resuspension • Provide surface area for attached biofilms • Excretion of photosynthetic oxygen → increases aerobic degradation • Uptake of nutrients
Roots and rhizomes in the sediment	• Stabilising the sediment surface → less erosion • Prevents the medium from clogging in vertical flow systems • Release of oxygen increase degradation (and nitrification) • Uptake of nutrients • Release of antibiotics

6.4 Diversity of abundant bacteria in vertical flow constructed wetlands

This study focuses on a comparative overview of diversity patterns in such systems by a first experimental setup. The aim was to investigate the usefulness of a molecular method to characterize potential differences in microbial diversity within and between pilot-scale CWs (PSCWs) and full-scale CWs (FSCWs) as done by Ansola et al. (2003). Possible differences between planted and unplanted CWs; within the filter bed depth profile and between the rhizosphere soils of *Miscanthus sinensis giganteus* and *Phramites australis*, were investigated. Changes in diversity pattern between the inflow and outflow effluent were surveyed to get an overview of the distribution of microorganisms within vertical flow constructed wetlands.

6.4.1 Phylotype richness from all samples

Phylotype richness (S) as shown in table 6.10 was calculated for all community profiles. The phylotype richness (numbers of TRFs with intensities ≥50 fluorescence units) ranged between twenty-nine and s eventy-eight and peaks ≥500 FU varied from zero to six. No differences between the individual layers and between the phylotype richness of rhizosphere soil and non-rhizosphere soil of the outdoor FSCW were detected, with exception of a lower diversity in the uppermost layer (FSCW 5; 0–1 cm). Between the outflows and t he corresponding inflows no clear differences regarding the bacterial diversity were observed. In the unplanted PSCW, the diversity was higher in the outflow than the inflow, whereas in the FSCW the opposite was observed. The TRF-profiles from outdoor bulk soil samples (FSCW) and indoor bulk soil samples (PSCW) implicate a similar community in both CWs (no data shown). The intensity of some peaks differed strongly between the out- and the indoor system. However samples from the seven different depths showed similar results, except the uppermost layer of the filter bed (like for S), which showed higher TRFs.

Table 6.10: Phylotype richness (S) of the filter body and wastewater samples from twenty-four profiles of the full-scale constructed wetland (FSCW) and the pilot-scale constructed wetlands (PSCWs); calculated from standardized fluorescence intensities. (Sleytr et al., 2009)

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

6.4.2 TRF community profiles from the wastewater samples

Figures 6.5 and 6.6 show the TRF-profiles from the inflows and outflows of the PSCW and FSCW, respectively. From the forty peaks occurring in the PSCW in-flow only ten peaks were found in the planted PSCW out-flow, whereas fifteen of the forty peaks were detected in the unplanted PSCW outflow (Figure 6.5). The bacterial diversity in the outflow of the planted PSCW was not clearly reduced, and for the unplanted system even an increase of the diversity in the outflow was observed. On the other hand figure 6.6 shows the TRF-profiles from the FSCW in and outflow. The FSCW shows a clear reduction of the bacterial diversity after the filter bed passage; from fifty-nine peaks in the in-flow to thirty-three peaks in the outflow, whereas only twenty-eight peaks were identical within the FSCW in- and outflow. The intensive peak from 70 to 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 bed samples (most abundant in the uppermost layer), but missing in the inflow samples. This fact suggests that this peak is originated from a soil-borne bacterium, which was washed out from the filter bed. Another reason could be that they may be insignificant members of the inflow but then find the CW an ideal habitat where they proliferate. Similarly, the peaks at 233 and 239 bp were more intensive in the outflow samples of both the planted and unplanted PSCWs but almost not detectable in the inflow (Figure 6.5). This suggests that these peaks were also derived from soil bacteria rather than from wastewater ones. In contrast, the most intensive peaks at 187 bp (<4000 FU) from the indoor inflow was not detected in neither the PSCW soil samples (data not shown) nor the outflow effluent samples (Figure 6.5), which suggests that this peak represented a wastewater bacterium that was completely killed off in the CWs.

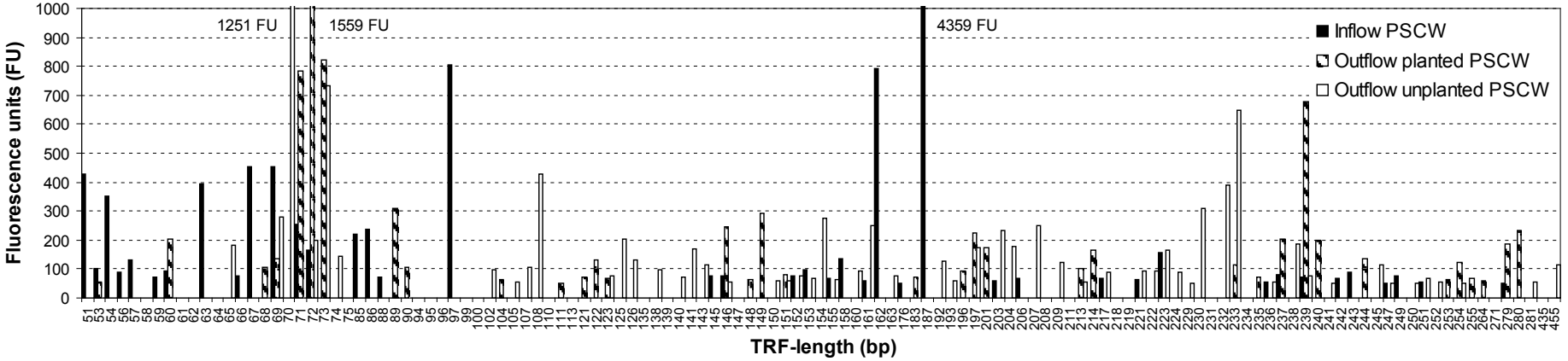

Figure 6.5: Comparison of the inflow (T-RFLP profile pooled from two samples), the planted outflow (two samples) and unpl anted outflow (one sample) TRF profiles from the indoor PSCW. (Sleytr et al., 2009)

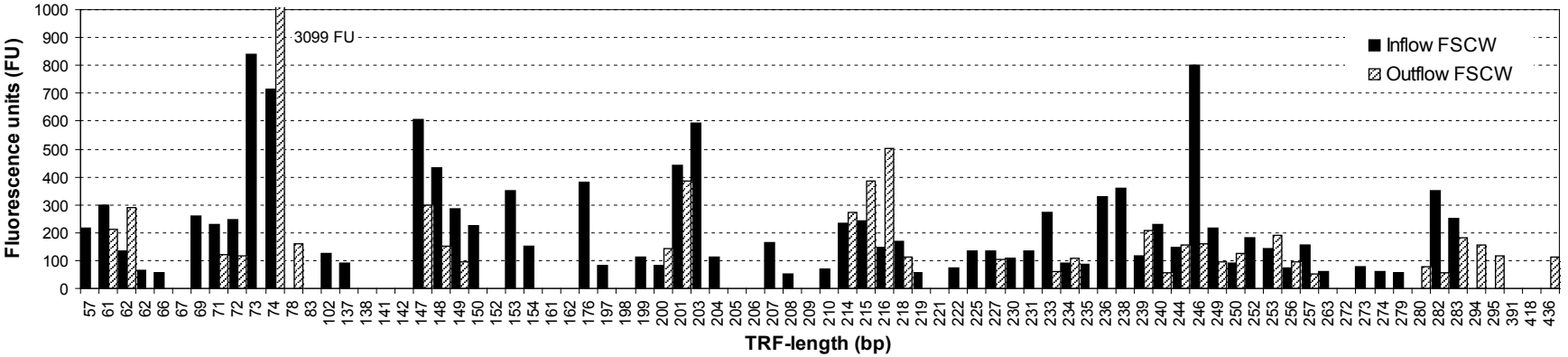

Figure 6.6: Comparison of the inflow (TRF profile pooled from two samples) and the outflow (one sample) TRF profiles from the outdoor FSCW. (Sleytr et al., 2009)

6.4.3 Discussion

Community fingerprinting offers a useful tool 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 has been demonstrated previously by Dunbar et al. (2000). T-RFLP analysis has the advantage of analytic consistency and a high throughput capability (Hartmann and Widmer, 2006).

Community profiles of the soil and wastewater samples obtained within the present study, demonstrated a rather high bacterial diversity, which is typical for complex environmental habitats. A comparison between the bacterial diversity in the filter bed of the outdoor and the indoor systems revealed no clear differences, although the two systems are exposed to different temperatures, are planted with different helophytes, and treat municipal wastewater with different bacterial compositions (Figure 6.5 and 6.6, inflow). Both systems are, however, very similar with respect to physiochemical factors such as pH, grain size distribution, as well as nutrient-, oxygen-, and water-content (unpublished data), which may have promoted the development of similar microbial communities.

However, differences between the two rhizosphere soils (*P. australis* and *M. sinensis giganteus*) were found, and those were more clearly distinguishable than the differences between the rhizosphere and the bulk soil samples of the respective systems. This indicates an influence of the plant species on the rhizospherebacteria, which has been frequently reported for soil systems (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.

For the bacterial diversity no clear correlation between the depth of the filter bed and the existence of distinct bacterial groups could be observed. Similarly, phylotype richness did not change with depth, with the exception of the 0–1cm layer, which showed a reduced bacterial diversity. In a recent study, more than 50% of the microbial biomass and bacterial activity could be found in the first cm of the filter bed of the PSCWs and about 95% within the first 10cm (Tietz et al., 2007b). This indicates that although the lower layers contain a lower biomass, they are probably 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 layer of the filter bed (50–60 cm). The filter bed of the CWs can be imagined as a sink for bacterial species, but additionally it can also be a source of bacteria. The number of bacteria in the wastewater is substantially reduced by the CWs; bacterial removal rates range from 2.0 log units determined by total microscopic direct counts, up to 4.8 log units for Enterococcus (Sleytr et al., 2007).Nevertheless it seems that the diversity was not so strongly reduced; but this does not seem to be accompanied by a corresponding reduction in the bacterial community diversity. A clear reduction of the bacterial diversity between the in- and outflow was only evident in the FSCW (a planted outdoor system).

Generally, the removal efficiency is considered to be a result of both chemical (e.g. adsorption), physical (e.g. filtration and sedimentation) and biological mechanisms. Examples of the latter are possible antimicrobial effect of root exudates, predation by nematodes and protists, lytic bacteria and viruses, retention in biofilms, and natural die-off (Vacca et al., 2005). The study showed that CWs operated under similar conditions had communities with similar diversities. However, the diversity and composition of the rhizosphere communities seemed to be influenced by the plant species. While the microbial biomass generally decreases with depth of the bed, the results suggest that the microbial community composition show little variation with depth.

By analysing the sequence of the 16S rRNA genes, the most dominant species inhabiting the system can be identified, resulting in amore detailed description of the community structure. 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.

Further research will give more precise information on the time dynamics of the microbial populations and the effect of different wastewater qualities.

7 Summary and Conclusion

CWs are practical and sustainable systems, easy to build and maintain but the processes, which are responsible for all different kind of pollutant and bacterial removal mechanisms are rather complex.

The aim of the present study was to describe the “black box” CW with the focus on bacterial removal rates within the filter body of VFCWs. Further more bacterial diversity and changes regarding to different designs and operations were characterized.

It has to be said that basic research, as done in this work, only can show a small part of a full spectrum of such a big variety of processes taking place in VFCWs.

May this study inspire to look deeper into the fascinating world of nature in a manmade, sustainable wastewater treatment system.

7.1 Efficiencies of bacterial removal during the filtration process in constructed wetlands. (see Appendix 1)

- The PSCWs show a high removal rate for indicator organisms; the log removal for HPC was 2.85, for E. Coli 4.35, for total coliforms 4.31, and for Enterococci 4.80, respectively.
- Most of the elimination processes take place in the first 10 to 20 cm of the main layer.
- There is no significant difference in the performance from planted and unplanted PSCWs comparing the effluents ($p < 0.05$; $n = 23$).
- The only significant difference between planted and unplanted systems was measured for Enterococci in the main layer of the filter ($p < 0.05$; $n = 11$). In unplanted systems the number of Enterococci is significantly lower than in the planted systems.
- TOC and DW showed a statistically significant 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 unpathogenic antibiotic resistant E. Coli.

7.2 Investigations to characterize bacterial removal processes in different subsurface vertical flow constructed wetlands. (see Appendix 3)

- CWs are very dynamic and diverse systems where a lot of complex processes are running and influencing each other. CWs are close to natural systems and can remove and transform nutrients and pathogens on an efficient performance level.
- For treating wastewater with SSVF CWs the use of the suitable grain size, hydraulic dosing rate and distribution methods was shown to be very important. The combination of these factors used in this study showed a very high bacterial removal performance according to the literature.
- DO concentrations can play a key role for pathogenic removal. The measured DO concentration in 4 depths (5, 10, 20, 40cm) ranged between 8.7 – 9.3 mg O2 /l which indicates stable conditions for a high pathogen removal efficiency.
- Constructed wetlands are capable of significantly reducing total and faecal coliforms, Enterococci, C. perfringens spores and Pseudomonas aeruginosa. Differences in the

removal efficiency from C. perfringens spores and P seudomonas aeruginosa between planted and u nplanted PSCWs could be obs erved whereas unplanted systems showed higher removal rates. This effect is very similar to the significantly higher removal rate of Enterococci described in Sleytr et al. (2007) and can be linked to the longer HRT in the planted CWs.

- There is still a l ack of knowledge to understand all the other processes in the filter bed of different kinds of CWs, but with this study some important questions could be answered.

7.3 Diversity of abundant bacteria in subsurface vertical flow constructed wetlands. (see Appendix 2)

- Constructed wetlands (CWs) have received an increased attention in the recent years. As a result of the wide range of benefits in creating and maintaining wetlands they are implemented in a variety of geographic regions. When designing CWs it is important to create an environment which is optimal for reactions responsible for the wastewater purification.

- The complex microbial community mainly associated with the filter material or roots, created by interaction with the wastewater, is mainly responsible for the degradation performance of the system.

- Investigations of the microbial community composition and di versity in natural or constructed habitats are important for their characterization of such habitats, since microbes are the key factors in many environmental processes (Nogales et al., 2001).

- Until now it has not been investigated whether bacteria found in the effluent of CWs are typical wastewater bacteria that pass the system or bacteria that are washed out from the soil particles in the CW. Therefore community fingerprints of the in- and outflows of the CW have been investigated in the present study.

- This study was created to make a brief inventory to assess various patterns of bacterial diversity within the filter bed of indoor vertical flow PSCWs, an outdoor FSCW, and the inflow and outflow effluent. Possible differences between planted and unplanted CWs; within the filter bed depth profile and between the rhizosphere soils of Miscanthus sinensis giganteus and *Phramites austrialis*, were investigated. For this purpose, the common molecular fingerprinting technique “terminal restriction fragment length polymorphism” (T-RFLP, or TRF) (Liu et al., 1997; Ishida et al., 2006) was used to characterize the microbial communities within the CWs. This culture-independent tool has been applied to analyse the bacterial diversity in a wide range of environmental habitats and is one of the easiest and cheapest molecular analyses available. T-RFLP profiles, based on the amplification of the phylogenetic marker gene of the 16S rRNA, display the complexity of the investigated bacterial communities.

7.4 Characterisation of microbial biocoenosis in vertical subsurface flow constructed wetlands. (see Appendix 4)

- The detailed characterisation 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.

- No significant differences in the quantity of the microbial biomass and the general

purification performance between planted and unplanted vertical flow constructed wetlands could be observed.

- It was demonstrated that microbial biomass is quite high compared with natural soils.
- There is still a need for further adaption of common methods from soil and aquatic microbial ecology to the specific conditions of subsurface flow constructed wetland, e.g. by determining specific con- version factors for the calculation of bacterial carbon production and microbial biomass.
- A description of the microbial biocoenosis under varying operational 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 the physiological activity at the same time.
- Fundamental understanding of the system will finally help to improve the performance of constructed wetlands by providing a scientific basis to enable it to find the optimal design and way of operating the system.

(Tietz et al., 2007)

7.5 High-rate removal through two-stage subsurface vertical flow constructed wetlands (see Appendix 5)

A two-stage CW system (first stage – grain size 2–3.2 mm for the 50 cm main layer, impounded drainage layer; second stage – grain size 0.06– 4 mm for the 50 cm main layer, conventional drainage layer) was operated with an organic load of 40 g $CODm^2$ / d on the whole system (a design area requirement of 2 m^2 per person), resulting in an effective load of 80 g $CODm^2$ / d on the first stage. The results of the investigations can be summarized as follows:

- Very low and stable effluent concentrations were measured for organic matter and ammonia nitrogen in the effluent of the two-stage CW system, as well as high removal rates for microbial parameters.
- The effluent concentrations of the two-stage system for BOD5, COD and NH4+-N were observed to be lower than the ones of the single-stage VF bed designed and operated according to the Austrian design standard ÖNORM B 2505.
- A whole year round operation of the two-stage system was maintained, without clogging tendencies even during very cold winter. It was shown that it is possible to operate the two-stage CW system without any problems even during long winters.
- The Austrian effluent standards could be met during the whole investigation period.
- Additionally, by using the two-stage CW system it was possible to reach about 53% nitrogen elimination and a high nitrogen removal rate in average of 986 g Nm 2 yr 1 (maximum more than 1’400 g Nm2 / yr) without recirculation. The nitrogen removal rate is very high compared to reported literature values of 630 g Nm2 / yr for VF beds.
- In conclusion it can be said that a higher effluent quality could be reached with the two-stage system as compared to the single-stage VF CW system designed with 4 m2 per person. The higher effluent quality could be reached although the two-stage CW system was operated with the double organic load resulting in half the specific surface area requirement (2 m2 per person).

8 Outlook

CWs can offer a sustainable solution for wastewater treatment and have been used ever since the 1960s. These systems will get more important in a changing world in which freshwater resources will run shortly or are short already. Good design is important for proper treatment and has therefore an effect on elimination processes. Nutrients, pollutants and pathogens are eliminated on a high level.

In many years of research and practical experience, a lot of applications and know how has been generated. However, a number of basic aspects, such as what exactly is going on in CWs, are still not adequately understood. Although CWs seem to be simply working, inside the filter body many different components are interacting and a lot of complex processes take place.

Elimination processes and mechanisms are still not completely investigated and therefore further research and development work has to be done with respect to: e.g.

- Optimization of the design parameters such as specific area, retention time, filter material and filter body depth with regard to pathogen removal
- Accumulation behaviour of pollutants and pathogens in the filter body over a long period
- Monitoring of microorganisms and their contribution to elimination processes
- Mechanisms of wastewater disinfection
- Possibility for complete disinfection of wastewater by means of CWs
- Interaction of the above mentioned parameters with others such as temperature, water quality with regard to bacterial removal

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10 Selected Publications

10.1 Appendix 1

Sleytr, K., Tietz, A., Langergraber, G., H aberl, R.(2007). Investigation of bacterial removal during the filtration process in constructed wetlands. *Science of the Total Environment* **380**(1-3): 173-80.

10.2 Appendix 2

Sleytr, K., Alexandra Tietz, A., Langergraber, G., Haberl, R., Sessitsch, A. (2009)
Diversity of abundant bacteria in subsurface vertical flow constructed wetlands. *Ecological Engineering* **35**: 1021-1025

10.3 Appendix 3

Sleytr, K., Langergraber, G., Haberl, R. (2008). Investigations to characterize bacterial removal processes in different subsurface vertical flow constructed wetlands.
Proceedings of the 11th IWA Specialized Group Conference on "Wetland Systems for Water Pollution Control", Volume I, page 260-267 [11th IWA Specialized Group Conference on "Wetland Systems for Water Pollution Control", Indore-Ujjain, India, 1-7 November 2008]

10.4 Appendix 4

Tietz, A., Kirschner, A., Langergraber, G., Sleytr, K., Haberl, R. (2007). Characterisation of microbial biocoenosis in vertical subsurface flow constructed wetlands. *Science of the Total Environment* **380**(1-3): 163-172.

10.5 Appendix 5

Langergraber, G., Leroch, K., Pressl, A., Sleytr, K., Rohrhofer, R., Haberl, R. (2009). High-rate nitrogen removal in a two-stage subsurface vertical flow constructed wetland. *Desalination* **246**: 55-68

Appendix 1

Sleytr, K., Tietz, A., Langergraber, G., H aberl, R.(2007). Investigation of bacterial removal during the filtration process in constructed wetlands. *Science of the Total Environment* **380**(1-3): 173-80.

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Investigation of bacterial removal during the filtration process in constructed wetlands

Kirsten Sleytr *, Alexandra Tietz, Günter Langergraber, Raimund Haberl

Institute of Sanitary Engineering and Water Pollution Control, BOKU, University of Natural, Resources and Applied Life Sciences, Vienna, Muthgasse 18, A-1190 Vienna, Austria

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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 faecal coliforms (*Escherichia coli*, total coliforms) and enterococci, and a number of heterotrophic bacteria (heterotrophic plate counts) have been analysed from the influent and effluent of the constructed wetlands as well as at 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 (a log removal rate of 2.85 for HPC, 4.35 for *E. coli*, 4.31 for total coliforms and 4.80 for enterococci was observed). In general no significant difference in the removal efficiency of planted and unplanted vertical flow beds could be measured. Only enterococci measured in the substrate samples of the main layer of the filter could a statistically significant difference be observed.

Keywords: Bacterial removal; Indicator organisms; Constructed wetlands; Subsurface vertical flow; Wastewater treatment

1. Introduction

Wastewater discharges are the major source of faecal 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. Perkins and Hunter 2000; Kadlec et al., 2000; Langergraber and Haberl, 2001; Hench et al., 2003). 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 generally classified into two main types: surface flow 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 where 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 enhances the process rates of the system

* Corresponding author. Tel.: +43 1 36006 5822; fax: +43 1 3689949.
E-mail address: kirsten.sleytr@boku.ac.at (K. Sleytr).

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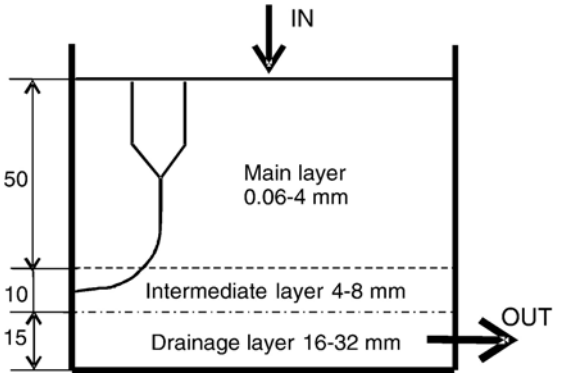

Fig. 1. Vertical cross-section of a PSCW showing height (in cm) and grain size of the layers.

and therefore decreases the surface requirement of the treatment system (Kadlec et al., 2000; Langergraber and Haberl, 2001).

In most studies dealing with bacterial removal (e.g. Decamp and Warren, 2001; Thurston et al., 2001) only the 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 faecal 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 at different depths of the sandy main layer (water and substrate samples). In addition, 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.

2. Material and methods

2.1. 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, Austria (BOKU). Ten parallel operated indoor pilot-scale subsurface vertical flow constructed wetlands (PSCWs) have been investigated.

Fig. 1 shows a vertical cross-section of a PSCW. The PSCWs have a surface area of 1 m^2 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. Fig. 2 shows a top view of the different layouts of the PSCWs tested. 8 PSCWs are planted with *Miscanthus gigantea* (common reed *Phragmites australis* did not grow under indoor-conditions) whereas two PSCWs (Plots 2 and 3) are unplanted. Four 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 Plots 1–5.

In Plots 1–5 sampling systems to collect water from different depths have been installed; four systems in each plot allow sampling of water from different depths: 10, 20, 30, and 40 cm, respectively. The sampling systems

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

Fig. 2. Fig. 2 Top view from the Indoor PSCWs at the Institute of Sanitary Engineering, BOKU.

consist of a polyethylene column 20°cm diameter that is ending in an open funnel (Fig. 1). The layer's 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 PSCW (Langergraber, 2007) have been analysed.

2.2. Sampling

For analysing the indicator organisms, samples have been taken from the influent and the effluent of the system and at different depths. The samples were analysed for heterotrophic bacteria, faecal coliforms (*E. coli*, total coliforms) and enterococci.

Water samples (mechanically pre-treated municipal wastewater and the effluent from all PSCWs) were collected in 2 l sterile Schott-bottles and analysed within 2 h. 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 de-ionized water was added. For separation of microorganisms from soil and particles the samples were shaken (shaker: KS 501D, Janke&Kunkel, IKA®-Labortechnik) for 30 min and then sonicated in an ultrasonication bath (Branson 5510, 135 W, 42 kHz) for 1°min. After 5°min settling, 1°ml of solution was taken and serially diluted. This was found to be the most effective technique, inspired by Craig et al. (2002).

2.3. Indicator organisms

Faecal 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 faecal coliforms and *E. coli* Chromocult Coliform® agar (MERCK; ISO 16649) plus cefsulodin (10 μg/ml) (CC^+) was used. The incubation lasted 24 h at 37 °C (Byamukama et al., 2000).

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 faecal 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 (ISO 7899-2, 2000). The incubation lasted 48 h at 37 °C.

The number of heterotrophic bacteria (heterotrophic plate count, HPC) was determined by the pour plate method (ISO 6222, 1999) with yeast extract agar (MERCK). The incubation lasted 72 h at 22 °C.

For better illustration, the numbers of total coliforms, *E. coli*, enterococci and heterotrophic bacteria were converted to $\log_{10}$ values and expressed as $\log_{10}$ CFU/ml.

2.4. 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 10 min and filtered through a griddled blackened polycarbonate membrane filter (25 mm diameter, 0.45 μm 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).

2.5. Substrate samples

Total organic carbon (TOC) and dry weight (DW) are measured from the substrate samples. For 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 NO*x* 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 (NO*x* and CO_2) pass through the drying-tubes and the CO_2 absorber. CO_2 is absorbed and NO*x* passes through the detector (TCD = Temperature Current Detector) which needs also helium as the reference. After detection of NO*x* 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 VariaMAX-Software 4.3D. For calibration L-glutamine acid was used. The area below the peaks is integrated and the result is given in % of the 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 h and

Table 1
Influent and effluent concentrations, and removal efficiencies for chemical and bacteriological parameters for a PSCW (Plot 4) with a main layer gravel size of 0.06–4 mm (mean values±standard deviation; $n=23$)

Parameter	Influent	Effluent	Removal
Chemical parameters	mg/l		%
COD	367±113	<20[a]	>94.5
BOD_5	150±61	<3[a]	>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±1.3	4.1±1.35	0–47.4
Indicator organisms	Log CFU/ml		Log
Heterotrophic plate count (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 (MDC)	8.08±1.18	6.03±0.55	2.05

[a]Limit of detection.

weighed. The percent dry weight was then calculated from these results.

2.6. Chemical parameters

Samples from the influent and effluent have been analysed according to the German Standard Methods (1993) for organic matter (COD (DIN 38409-T41) and BOD5 (DIN EN 1899-T1), TOC (DIN EN 1484)), ammonia nitrogen (NH_4–N (DIN 38406-T5)) and total phosphorus (TP (DIN EN ISO 6878)) in the laboratory of the Institute.

2.7. Dissolved oxygen

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

2.8. Data analyses

For data analyses the Spearman's rank correlation was used to test 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).

3. Results and discussion

3.1. Removal efficiency

The mean values ($n=23$) for the influent and effluent concentrations, and removal efficiencies or the PSCW (Plot 4) with a sandy main layer with a grain size of

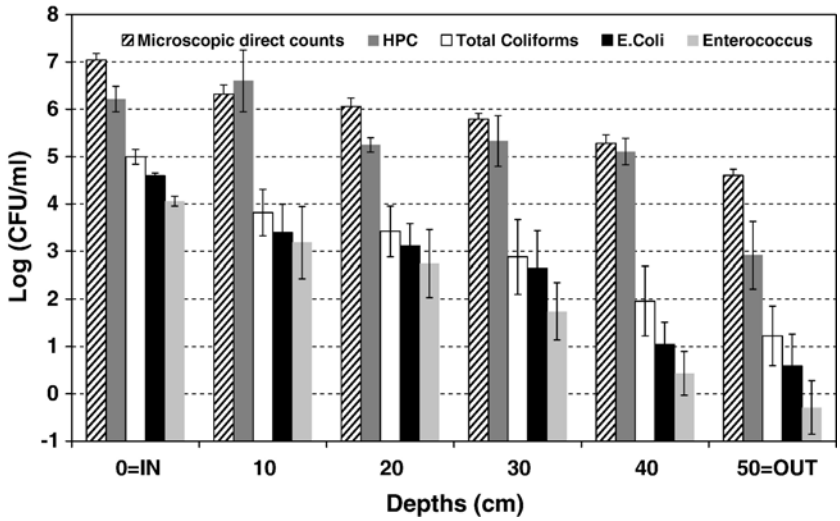

Fig. 3. Fig. 3 Bacterial parameters of the influent and effluent of the PSCWs and of water samples from different depths; error bars show the standard deviation ($n=6$).

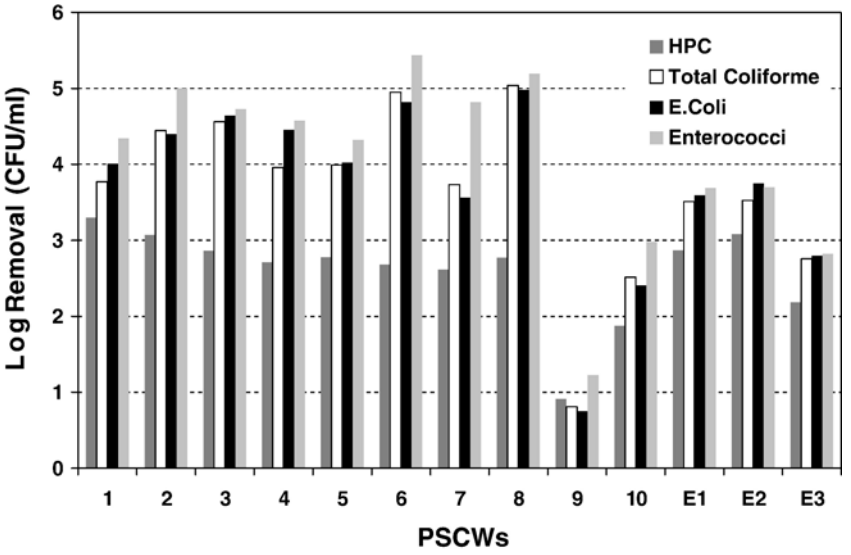

Fig. 4. Log removal rate of bacterial parameters for all Plots ($n=23$) and for the outdoor systems; error bars show the standard deviation (E1–E3; $n=7$).

0.06–4 mm are shown in Table 1. Bacterial indicators of faecal contamination measured in the mechanically pre-treated municipal wastewater showed comparable concentrations as reported by George et al. (2002) who showed typical abundances of total and faecal 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).

Fig. 3 shows bacterial parameters in the influent and effluent of Plot 1 (mean values; $n=6$) 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 approximately 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.

Fig. 4 shows the log removal rates of the bacterial parameters of Plots 1 through 10 as well as for the investigated outdoor plants (E1–E3). Lower bacterial removal in Plots 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.

The concentration of the bacterial parameters in the effluent from these plants has a greater 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 ($p>0.05$; $n=23$). 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.

3.2. Measurements at different depths of the main layer

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. Fig. 5 shows the difference from the results from log

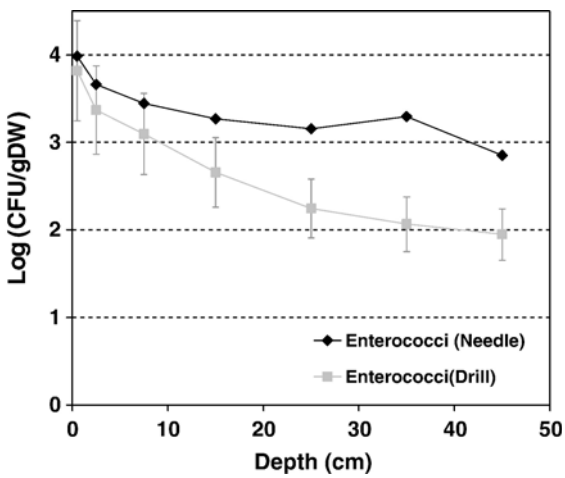

Fig. 5. Different results with needle and drill sampling devices; error bars show the standard deviation ($n=8$).

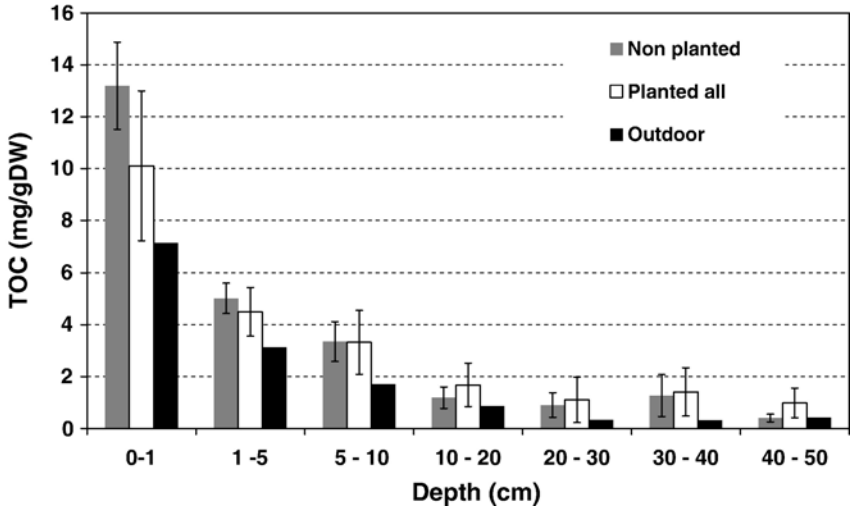

Fig. 6. TOC at different depths of the main layer; error bars show the standard deviation (Indoor: $n=12$; Outdoor: $n=7$).

(CFU/g DW) enterococci with the needle and the drill. The difference has been shown to be statistically significant at 20–50 cm depth ($p>0.05$; $n=8$).

As the PSCWs are intermittently loaded the main layer is always unsaturated. Dissolved oxygen is an important parameter for aerobic biological processes in

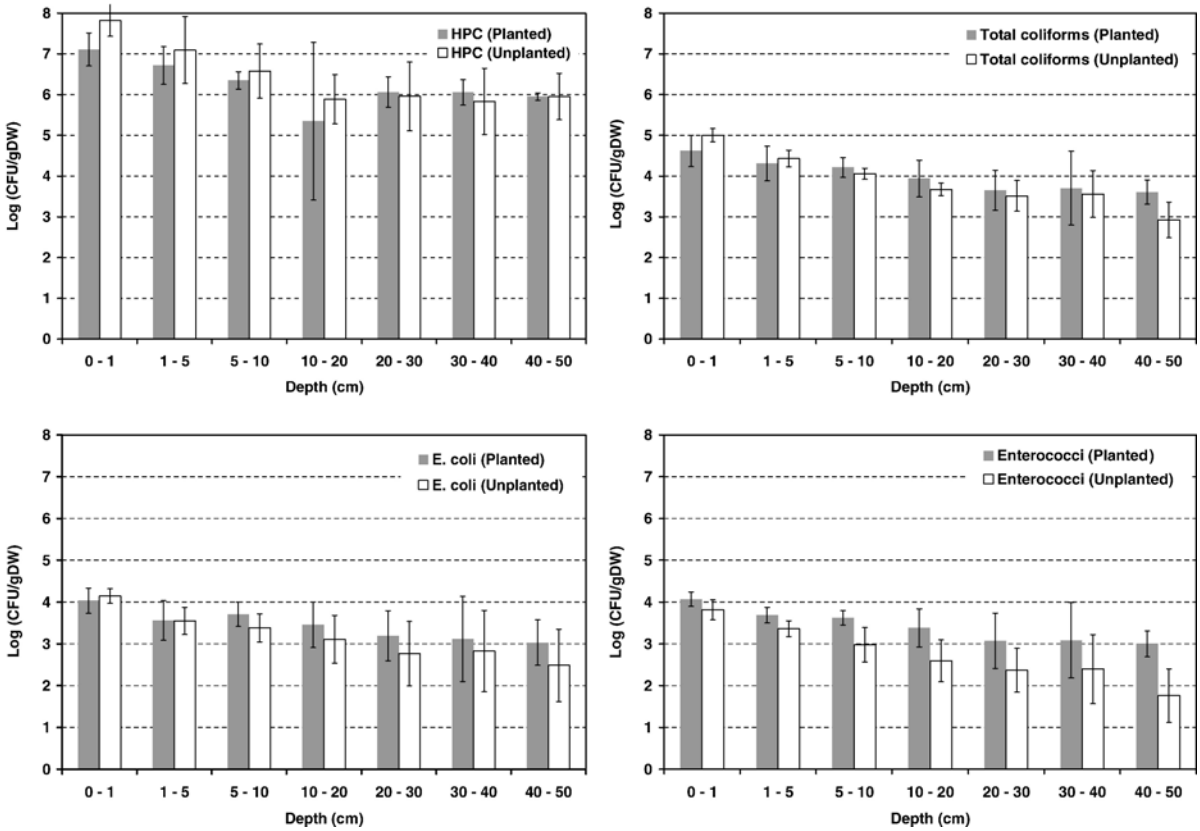

Fig. 7. Heterotophic plate count, Total coliforms, *E. coli* and Enterococci at different depths of the main layer of planted and unplanted PSCWs; error bars show the standard deviation (planted: $n=35$; unplanted: $n=12$).

Table 2
Correlations of measured bacterial parameters in the main layer with DW (%) and TOC (mg/g DW); (n=112)

Parameter	HPC	*E. coli*	Total coliforms	Enterococci
R^2 (DW)	0.624	0.574	0.626	0.510
R^2 (TOC)	0.728	0.854	0.952	0.741

wastewater treatment and was measured at four different depths within PSCW. 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, and outdoor) at seven different depths. Fig. 6 compares the TOC measurements in planted and unplanted PSCWs (n=12) with the outdoor systems (n=7). The decrease of TOC with increasing depths can be clearly seen. No statistically significant difference can be observed between the systems (p>0.05).

Fig. 7 shows vertical profiles of HPC, total coliforms, *E. coli* and enterococci counts (per g dry weight of the sandy substrate) at seven different depths of planted and unplanted PSCWs, respectively. Each bar represents the mean value of 35 measurements (12 for unplanted), the intervals the standard deviation. There is no continuous decline as may be expected but a minimal increase at 30–40 cm depth. This is more obvious for the unplanted PSCWs. The reason for this, as described earlier, was an error introduced by the sampling device (Fig. 5). This fact can also explain the high standard deviations at the four last depths.

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 significant value at nearly all depths (Fig. 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. Similar result occurred for *E. coli*. Only the result of the enterococci shows a statistically significant difference between planted and unplanted PSCWs (p<0.05; n=12).

A high concentration of indicator organisms has been measured in the main layer of the filter at all investigated depths. The values were between 0.5 and 8 (log CFU/g DW) for all parameters. This could give an indication at which depth the retention of the investigated 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 enterococci values could a statistically significant difference between planted and unplanted PSCWs be measured in the main layer.

Table 2 shows the correlations of the measured bacterial parameters 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.

To calculate the correlation between the parameters the Pearson correlation was used and all correlations in Table 2 are statistically significant (n=112).

4. Conclusions

From this study it can be concluded that:

- The PSCWs show a high removal rate for indicator organisms; with a log removal for HPC was 2.85, for *E. coli* 4.35, for total coliforms 4.31, and for enterococci 4.80, respectively.
- Most of the elimination processes take place in the first 10 to 20 cm of the main layer
- There is no significant difference in the performance from planted and unplanted PSCWs comparing the effluents (p>0.05; n=23).
- The only significant difference between planted and unplanted systems was measured for enterococci in the main layer of the filter (p<0.05; n=11). In unplanted systems the number of enterococci is significantly lower than in the planted systems.
- TOC and DW showed a statistically significant 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 unpathogenic antibiotic resistant *E. coli*.

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Appendix 2

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Short Communication

Diversity of abundant bacteria in subsurface vertical flow constructed wetlands

Kirsten Sleytr[a,*], *Alexandra Tietz*[a], *Günter Langergraber*[a], *Raimund Haberl*[a], *Angela Sessitsch*[b]

[a] *University of Natural Resources and Applied Life Sciences (BOKU), Institute of Sanitary Engineering and Water Pollution Control, Muthgasse 18, A-1190 Vienna, Austria*

[b] *Austrian Research Centers GmbH – ARC, Department of Bioresources, 2444 Seibersdorf, Austria*

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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 concerning 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.

A comparison of the bacterial communities from a full-scale outdoor vertical flow CW with planted and unplanted indoor pilot-scale vertical flow CWs, operated under similar conditions, revealed that both systems are colonized by similar populations showing only little variation in their composition over filter depth. A comparison of bulk soil from an unplanted CW with the rhizosphere soil from the outdoor and indoor CWs showed 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 within the planted outdoor system only.

1. Introduction

Constructed wetlands (CWs) have received an increased attention in the recent years. As a result of the wide range of benefits in creating and maintaining wetlands they are implemented in a variety of geographic regions. When designing a CW it is important to create an environment which is optimal for reactions responsible for the wastewater purification. The complex microbial community mainly associated with the filter material or roots, created by interaction with the wastewater, is mainly responsible for the degradation performance of the system. Investigations of the microbial community composition and diversity in natural or constructed habitats are important for their characterization of such habitats, since microbes are the key factors in many environmental processes (Nogales et al., 2001).

This study focuses on a comparative overview of diversity patterns in such systems by a first experimental setup. The aim was to investigate the usefulness of a molecular method to characterize potential differences in microbial diversity within and between pilot-scale CWs (PSCWs) and a full-scale CWs (FSCWs) as done by Ansola et al. (2003). Possible differences between planted and unplanted CWs; within the filter bed depth profile and between the rhizosphere

* *Corresponding author*. Tel.: +43 1 36006 5806; fax: +43 1 3689949.
E-mail address: kirsten.sleytr@boku.ac.at (K. Sleytr).

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soils of *Miscanthus sinensis giganteus* and *Phramites australis*, were investigated. Furthermore, changes in diversity pattern between the inflow and outflow effluent were surveyed to get an overview of the distribution of microorganisms within vertical flow constructed wetlands.

Only a few publications are available where the authors have tried to elucidate the complex microbial diversity within the filter body of CWs created to treat municipal wastewater. Truu et al. (2005) analysed the microbial community structure within a horizontal flow CW and found a decrease in bacterial diversity with increasing filter bed depth. Due to the great bacterial diversity within these systems, other studies focused on distinct functional groups relevant for the degradation process in wastewater like ammonia oxidizing bacteria (Tietz et al., 2007a; Gorra et al., 2007), or methanothrophic bacteria (DeJournett et al., 2007). However, no study dealing with the total bacterial community composition within a subsurface vertical flow CW with intermittent loading with municipal wastewater has been published yet.

The efficiency of CWs concerning the removal of microorganisms, especially faecal indicator bacteria, is a topic that has been thoroughly investigated by conventional culture-based plate count techniques (Sleytr et al., 2007). Until now it has not been investigated whether bacteria found in the effluent of CWs are typical wastewater bacteria that pass the system or bacteria that are washed out from the soil particles in the CW. Therefore community fingerprints of the in- and outflows of the CW have been investigated in the present study.

This study was created to make a brief inventory to assess various patterns of bacterial diversity within the filter bed of indoor vertical flow PSCWs, an outdoor FSCW, and the inflow and outflow effluent. For this purpose, the common molecular fingerprinting technique "terminal restriction fragment length polymorphism" (T-RFLP, or TRF) (Liu et al., 1997; Ishida et al., 2006) was used to characterize the microbial communities within the CWs. This culture-independent tool has been applied to analyse the bacterial diversity in a wide range of environmental habitats and is one of the easiest and cheapest molecular analyses available. T-RFLP profiles, based on the amplification of the phylogenetic marker gene of the 16S rRNA, display the complexity of the investigated bacterial communities.

2. Materials and methods

2.1. Sampling

Soil- and wastewater samples were taken from a 2-year-old experimental full-scale subsurface vertical flow CW (FSCW) with a surface area of 20 m^2, located outdoor in Ernsthofen (Lower Austria; coordinates: longitude: 14.482693; latitude: 48.127522) planted with *P. australis*. Bacterial communities in the rhizosphere of this plant are of great interest because of their potential for bioremediation of industrial effluent (Chaturvedi et al., 2006). Additionally, samples were taken from a 2-year-old indoor pilot-scale vertical flow CW (PSCW; coordinates: longitude: 16.223213; latitude: 48.150339). Six of the eight PSCWs units, with a surface area of 1 m^2 each, were planted with *M. sinensis giganteus*, whereas two beds were unplanted. All systems were loaded intermittently four times a day with municipal wastewater with an organic load of 20 g COD/(m^2 day) for the indoor system and 27 g COD/(m^2 day) for the outdoor CW. A detailed description of the two sampling sites is given in Langergraber et al. (2007).

Samples for microbial community analyses were collected four times from different depths of the filter bed of the FSCW and from different PSCWs. Samples taken at any place from the filter bed with no reference to plants are referred to as "bulk soil" in contrast to samples, which were directly removed with a sterile spoon from roots and rhizomes ("rhizosphere soil") of *P. australis* and *M. sinensis giganteus*. Rhizosphere samples were sampled at the same time and the same depths from 10 to 20 cm. The bulk soil samples were used to investigate potential differences in bacterial diversity from seven different depths (0–1, 1–5, 5–10, 10–20, 20–30, 30–40 and 40–50 cm), between the PSCW and the FSCW, and between rhizosphere soils compared to unplanted PSCW filter bed samples. Wastewater samples were collected two times from the inflows to the FSCW and the PSCWs, respectively, once from the outflow from the FSCW and once from three different PSCWs outflows.

2.2. Terminal restriction fragment length polymorphism (T-RFLP or TRF) of the community DNA

DNA extractions of the soil samples were carried out with the PowerSoil DNA isolation kit; for the wastewater samples the UltraClean™ Water DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) was used, following the manufacturer's recommendations.

T-RFLP analysis was done according to a modified protocol after Hackl et al. (2004) and Sessitsch et al. (2002).

Twenty-four profiles were compared and standardized to the lowest quantity, according to the method of Dunbar et al. (2001). TRFs of 50–500 base pairs (bp) in length and with heights of ≥50 fluorescence units (FU) were included in the analysis. TRFs 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 TRFs with intensities higher than ≥500 FU were designated as highly abundant TRFs.

3. Results

3.1. TRF community profiles from the filter body samples

Twenty-four TRF community profiles derived from twelve bulk soil samples (ten planted and two unplanted), four rhizosphere soil samples and eight wastewater samples were analysed. For the wastewater samples the impact of the CW passage on changes in bacterial diversity of the outflow was analysed.

Phylotype richness (S) as shown in Table 1 was calculated for all community profiles. The phylotype richness (num-

Table 1 – Phylotype richness (S) of the filter body and wastewater samples from twenty-four profiles of the full-scale constructed wetland (FSCW) and the pilot-scale constructed wetlands (PSCWs); calculated from standardized fluorescence intensities.

Sample [#]	Sample type	Depth (cm)	$S = \sum P \geq 50$ (FU)[a]	$\sum P \geq 500$ (FU)[a]
FSCW [1]	Bulk soil, *Phragmites a.*	0–1	36	5
FSCW [2]	Bulk soil, *Phragmites a.*	1–5	50; 47	0; 4
FSCW [2]	Bulk soil, *Phragmites a.*	5–10	29; 54	4; 3
FSCW [1]	Bulk soil, *Phragmites a.*	10–20	47	4
FSCW [1]	Bulk soil, *Phragmites a.*	20–30	51	3
FSCW [1]	Bulk soil, *Phragmites a.*	30–40	46	5
FSCW [1]	Bulk soil, *Phragmites a.*	40–50	49	2
PSCW [1]	Bulk soil, *Miscanthus s. g.*	5–10	49	2
PSCW [2]	Bulk soil, unplanted	1–10	56; 56	1; 4
FSCW [2]	Rhizosphere soil, *Phragmites a.*	10–20	37; 40	6; 2
PSCW [2]	Rhizosphere soil, *Miscanthus s. g.*	10–20	63; 49	1; 4
Inflow [2]	Wastewater indoor		35; 40	4; 5
Inflow [2]	Wastewater outdoor		78; 44	0; 4
Outflow [2]	Wastewater indoor, *Miscanthus s. g.*		39; 33	3; 6
Outflow [1]	Wastewater indoor, unplanted		58	2
Outflow [1]	Wastewater outdoor, *Phragmites a.*		39	2

[a]Fluorescence units.

bers of TRFs with intensities ≥50 fluorescence units) ranged between twenty-nine and seventy-eight and peaks ≥500 FU varied from zero to six (data shown in Table 1). No differences between the individual layers and between the phylotype richness of rhizosphere soil and non-rhizosphere soil of the outdoor FSCW were detected, with exception of a lower diversity in the uppermost layer (FSCW 5; 0–1 cm). Between the outflows and the corresponding inflows no clear differences regarding the bacterial diversity were observed. In the unplanted PSCW, the diversity was higher in the outflow than the inflow, whereas in the FSCW the opposite was observed.

The TRF-profiles from outdoor bulk soil samples (FSCW) and indoor bulk soil samples (PSCW) implicate a similar community in both CWs (no data shown). The intensity of some peaks differed strongly between the out- and the indoor system. However samples from the seven different depths showed similar results, except the uppermost layer of the filter bed (like for S), which showed higher TRFs.

3.2. *TRF community profiles from the wastewater samples*

Figs. 1 and 2 show the TRF-profiles from the inflows and outflows of the PSCW and FSCW, respectively. From the forty peaks occurring in the PSCW inflow only ten peaks were found in the planted PSCW outflow, whereas fifteen of the forty peaks were detected in the unplanted PSCW outflow (Fig. 1). The bacterial diversity in the outflow of the planted PSCW was not clearly reduced, and for the unplanted system even an increase of the diversity in the outflow was observed. On the other hand Fig. 2 shows the TRF-profiles from the FSCW in- and outflow. The FSCW shows a clear reduction of the bacterial diversity after the filter bed passage; from fifty-nine peaks in the inflow to thirty-three peaks in the outflow, whereas only twenty-eight peaks were identical within the FSCW in- and outflow. The intensive peak from 70 to 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 bed samples (most abundant in the uppermost layer), but missing in the inflow samples. This fact suggests that this peak is originated from a soil-borne bacterium, which was washed out from the filter bed. Another reason could be that they may be insignificant members of the inflow but then find the CW an ideal habitat where they proliferate. Similarly, the peaks at 233 and 239 bp were more intensive in the outflow samples of both the planted and unplanted PSCWs but almost not detectable in the inflow (Fig. 1). This suggests that these peaks were also derived from soil bacteria rather

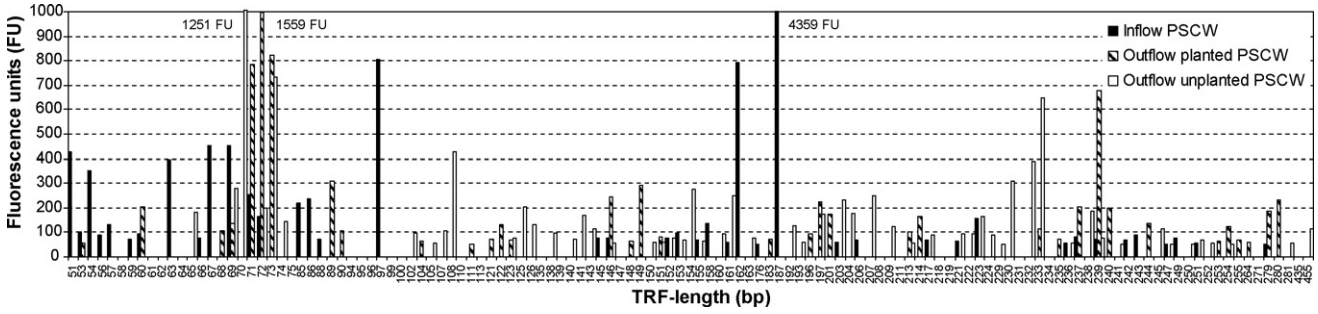

Fig. 1 – Comparison of the inflow (T-RFLP profile pooled from two samples), the planted outflow (two samples) and unplanted outflow (one sample) TRF profiles from the indoor PSCW.

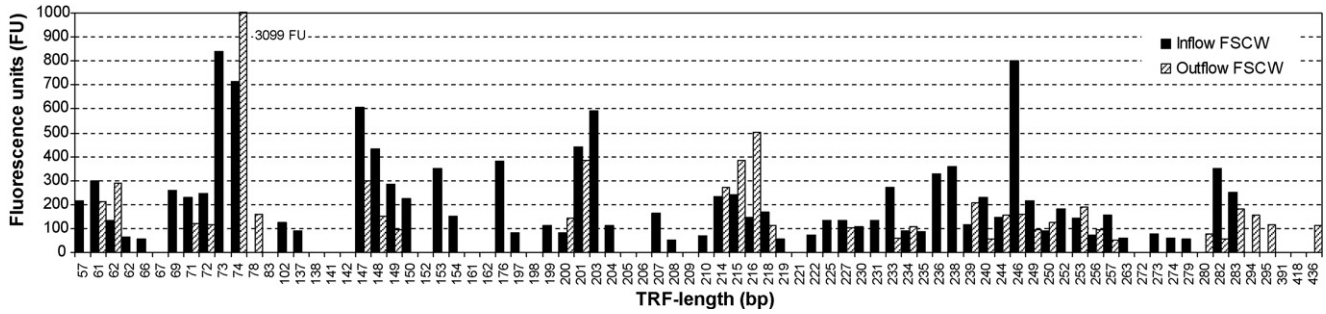

Fig. 2 – Comparison of the inflow (TRF profile pooled from two samples) and the outflow (one sample) TRF profiles from the outdoor FSCW.

than from wastewater ones. In contrast, the most intensive peaks at 187 bp (<4000 FU) from the indoor inflow was not detected in neither the PSCW soil samples (data not shown) nor the outflow effluent samples (Fig. 1), which suggests that this peak represented a wastewater bacterium that was completely killed off in the CWs.

4. Discussion

Community fingerprinting offers a useful tool 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 has been demonstrated previously by Dunbar et al. (2000). T-RFLP analysis has the advantage of analytic consistency and a high throughput capability (Hartmann and Widmer, 2006).

Community profiles of the soil and wastewater samples obtained within this study, demonstrated a rather high bacterial diversity, which is typical for complex environmental habitats. A comparison between the bacterial diversity in the filter bed of the outdoor and the indoor systems revealed no clear differences, although the two systems are exposed to different temperatures, are planted with different helophytes, and treat municipal wastewater with different bacterial compositions (Figs. 1 and 2, inflow). Both systems are, however, very similar with respect to physiochemical factors such as pH, grain size distribution, as well as nutrient-, oxygen-, and water-content (unpublished data), which may have promoted the development of similar microbial communities. However, differences between the two rhizosphere soils (*P. australis* and *M. sinensis giganteus*) were found, and those were more clearly distinguishable than the differences between the rhizosphere and the bulk soil samples of the respective systems. This indicates an influence of the plant species on the rhizosphere bacteria, which has been frequently reported for soil systems (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.

For the bacterial diversity no clear correlation between the depth of the filter bed and the existence of distinct bacterial groups could be observed. Similarly, phylotype richness did not change with depth, with the exception of the 0–1 cm layer, which showed a reduced bacterial diversity. In a recent study, more than 50% of the microbial biomass and bacterial activity could be found in the first cm of the filter bed of the PSCWs and about 95% within the first 10 cm (Tietz et al., 2007b). This indicates that although the lower layers contain a lower biomass, they are probably 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 layer of the filter bed (50–60 cm).

The filter bed of the CWs can be imagined as a sink for bacterial species, but additionally it can also be a source of bacteria. The number of bacteria in the wastewater is substantially reduced by the CW; bacterial removal rates range from 2.0 log units determined by total microscopic direct counts, up to 4.8 log units for *Enterococcus* (Sleytr et al., 2007). Nevertheless it seems that the diversity was not so strongly reduced; but this does not seem to be accompanied by a corresponding reduction in the bacterial community diversity. A clear reduction of the bacterial diversity between the in- and outflow was evident only in the FSCW (a planted outdoor system). Generally, the removal efficiency is considered to be a result of both chemical (e.g. adsorption), physical (e.g. filtration and sedimentation) and biological mechanisms. Examples of the latter are possible antimicrobial effect of root exudates, predation by nematodes and protists, lytic bacteria and viruses, retention in biofilms, and natural die-off (Vacca et al., 2005).

The study showed that CWs operated under similar conditions had communities with similar diversities. However, the diversity and composition of the rhizosphere communities seemed to be influenced by the plant species. While the microbial biomass generally decreases with depth of the bed, the results suggest that the microbial community composition show little variation with depth. Further research will give more precise information on the time dynamics of the microbial populations and the effect of different wastewater qualities. By analysing the sequence of the 16S rRNA genes, the most dominant species inhabiting the system can be identified, resulting in a more detailed description of the community structure. 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.

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Appendix 3

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Investigations to characterize bacterial removal processes in different subsurface vertical flow constructed wetlands

Kirsten Sleytr*, Guenter Langergraber, Raimund Haberl

Institute for Sanitary Engineering and Water Pollution Control, University of Natural Resources and Applied Life Sciences, Vienna (BOKU), Muthgasse 18, A-1190 Vienna, Austria.
kirsten.sleytr@boku.ac.at
* corresponding author

Abstract
The aim of this study was to investigate bacterial removal efficiencies of different vertical flow constructed wetlands (CWs) including pilot- and full-scale, outdoor and indoor, and planted and unplanted systems to gain a more detailed insight of the processes in the sandy substrate. Various techniques from different disciplines have been combined to characterize the system "constructed wetland". From the influents and effluents of the CWs chemical and physical standard parameters, indicator organisms, *Clostridium perfringens* spores and *Pseudomonas aeruginosa* have been analysed. In 7 depths of the main layer indicator organisms have been measured from substrate samples. Additionally, a sieving analysis has been performed to show the translocation of the substrate over time and to link this to other processes. Hydraulic retention time and the ratio of dissolved oxygen during the infiltration processes were measured. The investigated systems show a high removal rate for indicator organisms (e.g. a log removal rate of 4.35 for E. coli, 4.31 for Total Coliforms, 4.80 for Enterococci, 3.65 for *Clostridium perfringens* spores and 3.03 for *Pseudomonas aeruginosa* respectively). Differences between planted and unplanted systems could be measured.

Keywords
bacterial removal, *Clostridium perfringens* spores, Dissolved Oxygen DO, *Pseudomonas aeruginosa*, indictor organisms, vertical flow constructed wetlands

INTRODUCTION

Constructed wetlands (CWs) are among the most popular wastewater treatment systems for decentralized solutions in Austria. They are simple in operation and maintenance and offer a lot of benefits to the environment beside a high capacity of treating wastewater. They can be seen as an artificial habitat for many organisms, birds, insects, macrophytes, bacteria and other living organisms which play an important role for the treatment efficiency.

Although not regulated in the Austrian effluent standards hygienisation processes in CWs are and will get more important to evaluate and understand. All over the world CWs are widely used (Brix et al., 2007) for the treatment of municipal wastewaters, which are characterized by high concentrations of nutrients and pathogens (Barrett et al., 2001, Zdragas et al., 2002). By dealing with bacterial removal (e.g. Decamp and Warren, 2000; Thurston et al., 2001) most studies only compare the influent and effluent of the CWs. But wastewater purification and bacterial removal took place inside the filter bed caused to interactions between plants, soil and micro-organisms which still have not been described in various studies.

The results presented in this study were carried out within the project "Characterisation of microbial biocoenosis to optimise removal efficiency and design of subsurface flow CWs for wastewater treatment". Tietz et al (2007) characterized the microbial biomass in vertical flow (VF) CWs. It was found that microbial biomass was quite high compared with natural soils caused to the high nutrient level in the used wastewater. Indicator organisms such as faecal coliforms (*Escherichia coli*, total coliforms) and Enterococci, and a number of heterotrophic bacteria (heterotrophic plate counts,

HPC) have been analysed from the influent and effluents of the constructed wetlands as well as from water and substrate samples at different depths (Sleytr et al., 2007). The investigated CWs show a high removal rate for indicator organisms; with a log removal for HPC of 2.85, for *E. coli* 4.35, for total coliforms 4.31, and for Enterococci 4.80, respectively. Most of the elimination processes took place in the first 10 to 20 cm of the main layer (Sleytr et al. 2007). Removal rates in other studies, investigating similar CWs systems ranged between log 0.5-4.25 for different indicator parameters like e.g. *E. coli*, total coliforms, total aerobes and enterococci (e.g.in Green et al., 1997, Perkins et al. 2000; Barrett et al., 2001; Ausland et al. 2002; Manios et al. 2002; Arias et al., 2003; García et al. 2003). Higher log removal rates up to 5.5 for *E. coli* can only be achieved by combining systems like VF and horizontal flow (HF) filters as described in Baeder-Bederski et al. (2005). Very few information is available on the microbial diversity within CWs: Truu et al. (2005) described the bacterial community composition and Vymazal et al. (2001) the biota of wastewater, vegetated beds and effluents. Sleytr et al (submitted) investigated bacterial communities from indoor and outdoor systems that were operated under similar conditions. It was revealed that both systems are colonized by similar populations showing only little variation in their composition over the filter depth. 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.
The aim of the present work was to link up the results from various techniques of different disciplines to characterize the system CW by understanding the interactions of the sandy substrate, plants, microorganisms, hydraulic retention time and the ratio of dissolved oxygen during the infiltration processes, shifts of the grain size over time and other parameters.

METHODS

Investigated systems

Indoor pilot-scale VF CWs (PSCWs) have been operated at the technical lab hall of the Institute at BOKU University. Six of the eight PSCWs, with a surface area of 1 m2 each, were planted with *Miscanthus sinensis giganteus*, whereas the two beds were unplanted. The PSCWs were loaded intermittently four times a day (60mm/day) with mechanically pre-treated municipal wastewater with an organic load of 20 g COD/(m².day). The filter beds were constructed according to the Austrian design standards (ÖNORM B2505, 2005). The 50 cm main layer consisted of sandy substrate (grain size 0.06–4 mm; d_{10} = 0.2 mm; d_{60} = 0.8 mm). A detailed description of the PSCWs is given in Sleytr et al. (2007). Additionnaly, an experimental full-scale subsurface vertical flow CW (FSCW) located outdoor in Ernsthofen (constructed in spring 2003 in lower Austria) planted with *Phragmites australis*, was investigated. The construction of the FSCW was the same as for the PSCWs, the FSCW had an surface area of about 20 m² and was operated with an organic load of 27 g COD/(m².day) (Langergraber et al., 2007). For both systems the same sandy substrate was used for the main layer.

Sample collection and analyses

Wastewater and soil samples were taken from the FSCW and all PSCWs. The following standard chemical and physical parameters have been analysed according to the German standard methods (German Standard Methods for the Examination of Water, Wastewater and Sludge, 1993): organic matter (BOD5, COD, OC), total suspended solids (TSS), ammonia- (NH_4-N), nitrate nitrogen (NO_3-N) and total phosphorus (TP).
The indicator organisms faecal coliforms (*Escherichia coli*, total coliforms), Enterococci, and a number of heterotrophic bacteria (HPC) were taken from the inlet and the different outlets from both systems; used methods and detailed results from wastewater and soil analysis are described in Sleytr et al. (2007). Clostridium perfringens spores (according to the protocol of EN ISO WD 6461-2, 1986) and *Pseudomonas aeruginosa* (according to the protocol of EN ISO 12780, 1986) were analyzed from the inlet and outlets from all PSCWs (analysis have been carried out by the clinical

Institute for Hygiene and Medical Microbiology (Medical University of Vienna). Two sampling campaigns took place and mean values were calculated.
Substrate samples were taken with a special drill (10cm in diameter) from the 7 different depths of 0-1, 1-5, 5-10, 10-20, 20-30, 30-40 and 40-50cm for sieving analyses. Sieving analyisis were performed for substrate samples with the original substrate and samples taken after 3 years of operation from the different depths. Mean values from 2 sieving analysis are shown.
Dissolved oxygen (DO) was online measured every 10 min with electrochemical DO sensors (ECsensor OM-E200302 3600 Analyzer for Oxygen; Orbisphere) at four different depths (5, 10, 20, 40 cm) of the main layer of the PSCWs. The sensors were used in planted and unplanted PSCWs over a time of 2 years.
Tracer experiments with potassium chloride (KCl) were performed at 2 unplanted and 5 planted PSCWs. Measurements with a WTW electrical conductivity probe were recorded every 10 minutes by using a data logger.

RESULTS AND DISCUSSION

Sieving analysis

Figure 1 shows the grain size distribution shifts from 3 representative depths (0-5, 20-30 and 40-50) of the FSCW after 3 years of operation and compare them with analysis done for samples of original substrate (project start). Figure 2 and Figure 3 show the same comparison for the planted and the unplanted PSCWs, respectively.

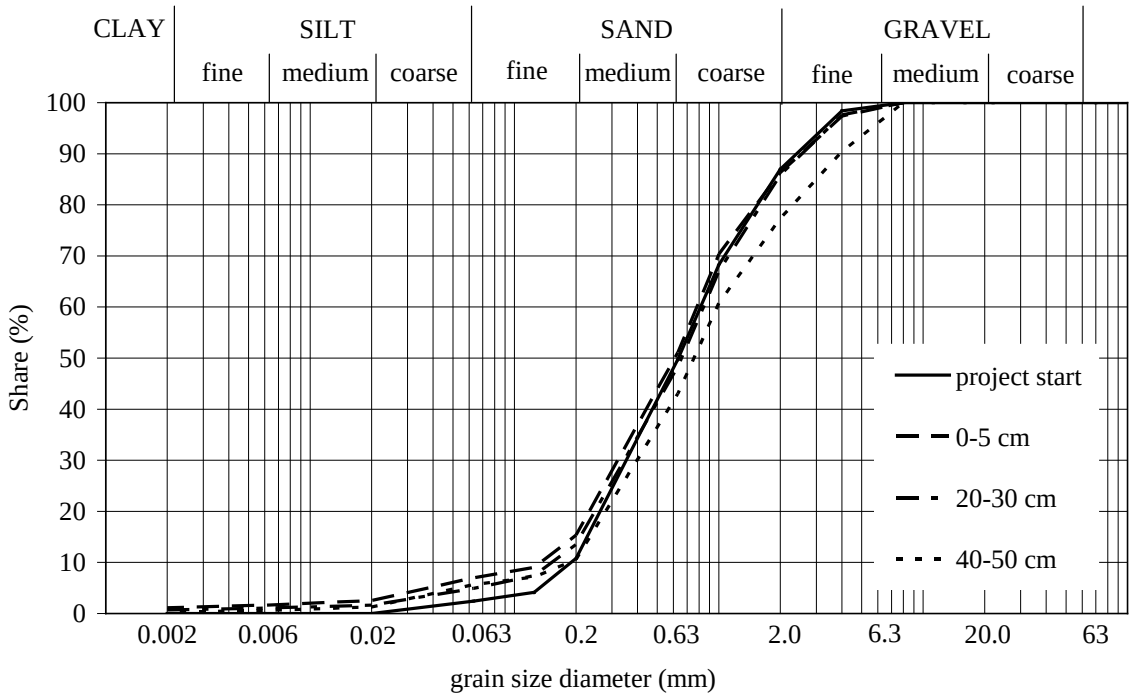

Figure 1: Grain size distribution from the FSCW in 3 depths after 3 years time of operation compared to the original substrate.

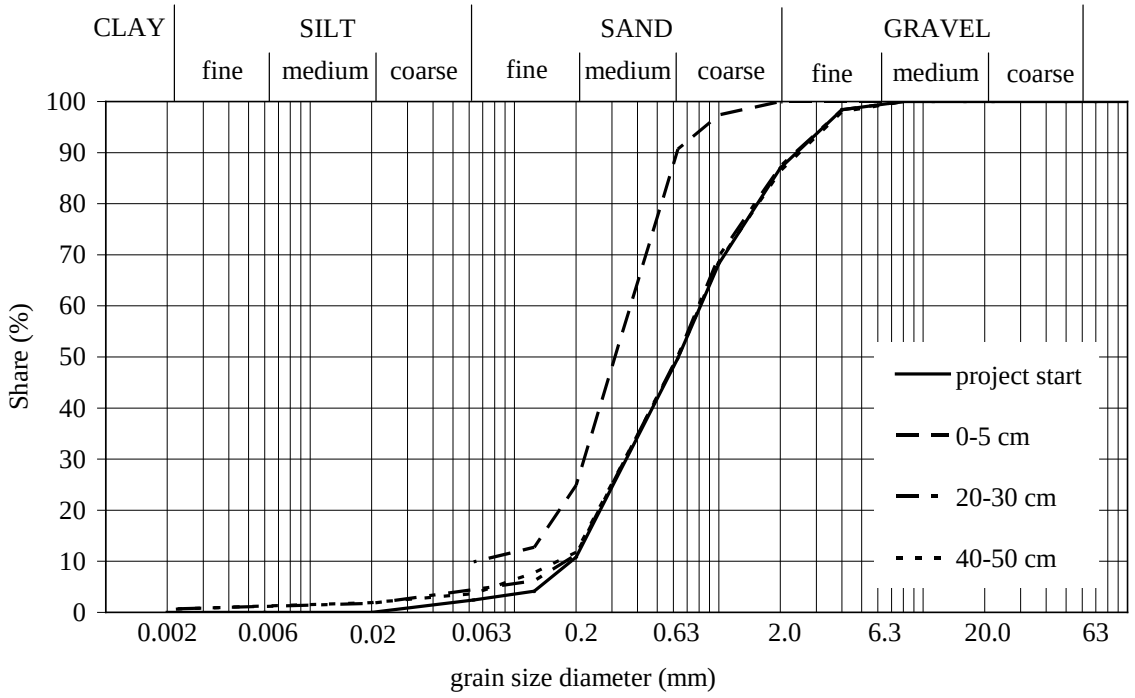

Figure 2: Grain size distribution from the planted PSCW in all 3 depths after 3 years time of operation compared to the original substrate.

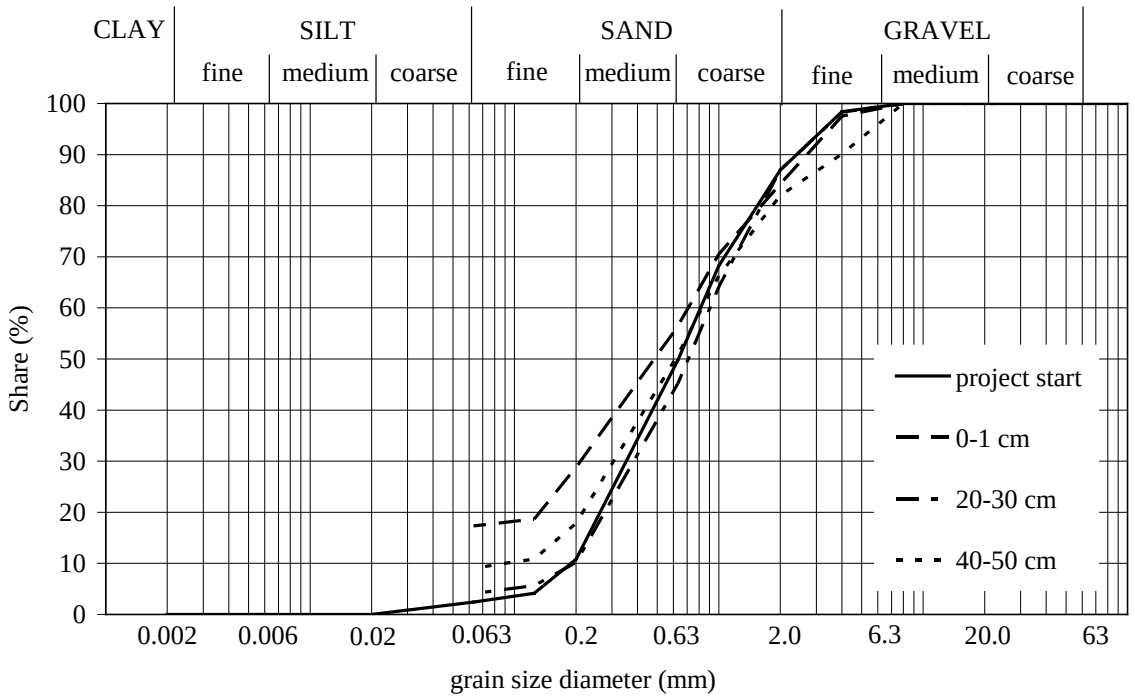

Figure 3: Grain size distribution from the unplanted PSCW in all 3 depths after 3 years time of operation compared to the original substrate.

Table 1: d_{10} and d_{60} obtained from the sieving analysis for the original substrate (project start) and the samples taken after 3 years of operation.

Sample	d_{10} (mm)	d_{60} (mm)
Project start	0.20	0.80
FSCW 0-5 cm	0.15	0.79
FSCW 20-30 cm	0.17	0.80
FSCW 40-50 cm	0.20	1.00
PSCW planted 0-5 cm	0.07	0.36
PSCW planted 20-30 cm	0.20	0.80
PSCW planted 40-50 cm	0.19	0.80
PSCW unplanted 0-5 cm	< 0.06	0.70
PSCW unplanted 20-30 cm	0.20	0.90
PSCW unplanted 40-50 cm	0.10	0.80

After three years operation a shift of the grain size distribution especially in the top layer (0-5 cm) could be observed. In general, d_{60} ranged between 0.36 and 0.79 mm; d_{10} ranged between 0.07 and 0.20 mm. The fraction of fine material increased over time in all layers of all investigated CWs. It was observed that algae growth took place on the top and suspended solids from the wastewater discharges accumulated in the upper layer (0-10 cm) of the main layer which shows an even higher fraction of fine material compared to deeper layers. More than 50 % of microbial biomass and bacterial activity could be found in the first centimeters and about 95 % in the first 10 cm of the filter layer (Tietz et al. 2007) which shows the importance of this area of CWs. The shift of the grain size distribution in the planted indoor PSCW (Figure 2) is most interesting but it could not be clarified if the plants (*Miscanthus sinensis giganteus*) plays the key role together with the "indoor" effect. However the FSCW planted with *Phragmites australis* didn't show this effect (Figure 3).

Hydraulic retention time (HRT)

The hydraulic retention time (HRT) was determined from tracer experiments which were performed to describe different flow pattern and to evaluate the mean residence time for a conservative tracer in a range of the planted and unplanted PSCWs. The mean HRT was 4.1 days for both systems. The unplanted systems (mean value 4.5 days ± 0.71) had a 0.6 days longer HRT than the planted ones (mean value 3.9 days ± 0.57). This could be explained with plant effects, e.g. that the tracer is able to take some hydraulic shortcuts along the roots. However, no negative effect on the removal rates could be observed. As described in García et al. (2003) for HF beds, a HRT over three days does not result in a significantly higher inactivation ratio and therefore a lower microbial concentration in the effluents. A minimum retention time is a key parameter for predicting removal of bacteria in infiltration filters (Ausland et al., 2002).

Dissolved oxygen

Most of the relevant removal processes in CWs (e.g. carbon reduction and nitrification) take place under aerobic conditions. A positive influence on pathogen removal due to higher concentrations of DO in anoxic wastewater stabilisation ponds was found out by Almasi and Pescod (2000).
Investigations at a model scaled vertical flow CW showed that the highest oxygen consumption takes place in the first 30 cm of the filter layer and can influence biological purifying processes. Further more DO concentration was one of the most important factors for the occurrence of filter clogging which cause degradation of removal efficiency (Wozniak et al., 2007).
Therefore the oxygen supply in the filter bed gets very important to understand.
The DO measurements we aimed to describe the different ratios within the filter bed and during the different stages of saturated and unsaturated phases during the wastewater charging times. No difference could be detected between the planted and unplanted PSCWs. Throughout the whole

filter bed DO concentration ranged between 8.7 – 9.3 mg O_2 /l in between the loadings and between 7.8 – 9.3 mg O_2/l during the loadings. The PSCWs in this study showed no significant change in the DO concentration all over the entire filter bed with time and depth.
Figure 4 shows a typical DO concentration curve of 24 hours from a planted PSCW after 2 years operation. The minima occur after loadings but aerobic conditions could be measured in the whole filter bed. With an intermittent loading of 60 mm/day and the use of fine, sandy material (0.06-4 mm grain size) we could provide oxygen transfer to take place and support sufficient aerobic micro organisms to grow.

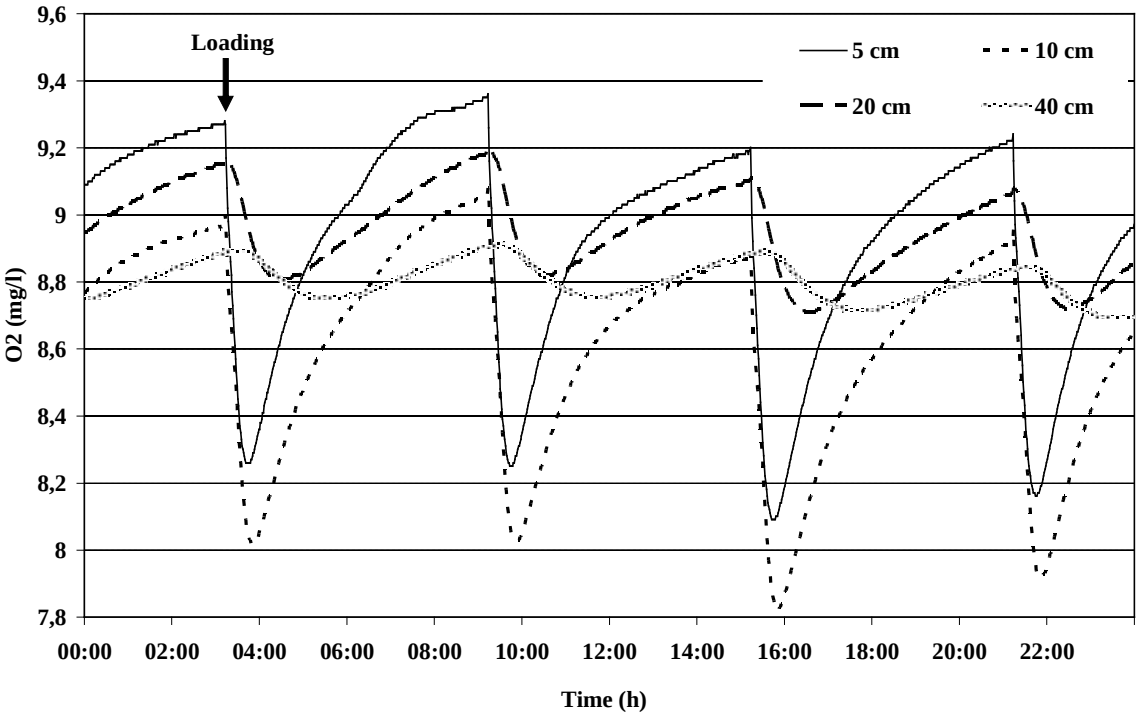

Figure 4: Time series of the DO concentrations over 24 hours in different depths in a planted PSCW (temperature 19°C).

Since 50% microbial biomass and metabolic activity is located in the upper layer (0-10cm) (Tietz et al., 2007) each loading step (interval 6 hours) will initiate a significant increase in oxygen demand (see Figure°4). On the other hand beginning in a depth of 20 and at a depth of 40 or more centimetres stable oxygen concentration is maintained. This reflects a more balanced nutrient supply and metabolic activity in these layers
In Tawfik et al. (2004) the removal rate of E. coli under aerobic conditions (DO from 3.3 to 8.7°mg/l) was significantly higher than under anaerobic conditions.
Therefore the concentration of DO in the filer bed can play an important role for pathogen (or indicator) removal efficiency beside adsorption to bio films, sedimentation and other die of processes.

Microbiological quality
Clostridium is the most widely occurring pathogenic bacterium and is found within human and animal intestinal tracts (Karpiscak et al 2001). Spores of *Clostridium perfringens* possess high heat resistance and when these spores germinate and return to active growth, they can cause gastrointestinal disease. As these indicator organisms are quite common to describe drinking water quality we decided to use this parameter to characterize the Outlets concerning to the hygienic safty. *Pseudomonas aeruginosa* is commonly used as an indicator of wastewater quality due to its direct pathogenic effect on humans and the higher resistance than coliforms (Ghermandi et al. 2007). In

other studies (e.g. Medema et al., 1997) the die off rates for E coli, faecal enterococci and *C perfringens* spores in surface water were determined and showed that *C.perfringens* spores were 3-4 times more persistent than other indicators.
Sleytr et al. (2007) measured high log removal rates for for the PSCWs for indicator parameters (*E. coli* 4.35, total coliforms 4.31, Enterococci 4.80 and log 2.85 for Heterotrophic bacteria (HPC)).
Figure 5 shows the mean log removal rates from *C. perfringens* spores and *Pseudomonas aeruginosa* from the inlfluent (all PSCWs get the same influent) and the effluents (Effluent 2 and 3 originate from unplanted PSCW). The values for *C. perfringens* ranged between 0.61 and 4.52 log (mean value log°3.65±1.21 cells/100ml) and for *Pseudomonas aeruginosa* between 1.51 and 4.83 log (mean value log°3.03±1.17 cells/100ml) for all effluents.

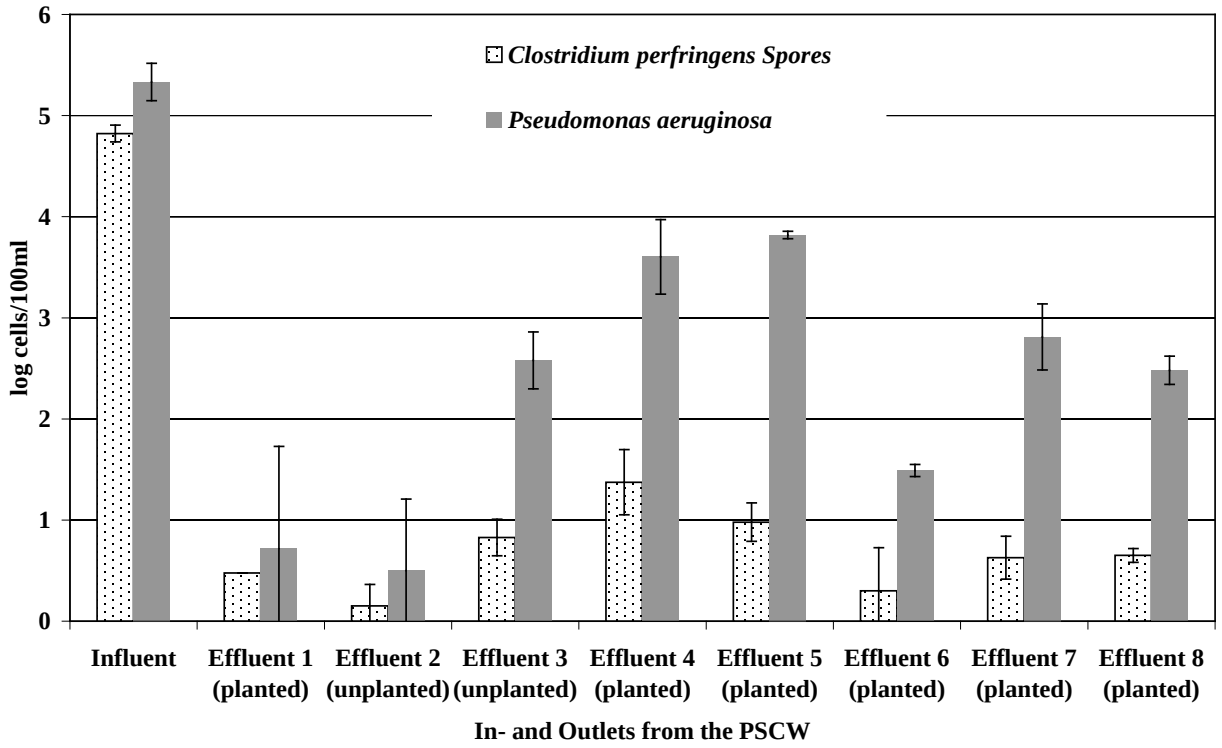

Figure 5: Mean Values from In- and Effluents from all PSCW 1-8(Plot 2 and 3 are unplanted) of *Clostiridium perfringens* sporen & *Pseudomonas aeroginosa*

Table°2: mean log removal values from *Clostiridium perfringens* sporen & *Pseudomonas aeroginosa*

	Clostridium perfringens	***Pseudomonas aeroginosa***
mean value all Effluents	**3.65**±1.21	**3.03**±1.17
mean value planted Effluents	**3.47**±1.30	**2.81**±1.10
mean value unplanted Effluents	**4.33**±0.48	**3.79**±1.47

Results shown in Table°2 reveal the different removal efficiency from the investigated pathogens between planted and unplanted PSCWs. This effect could be linked up to the longer HRT (4.5 days; 0.6 days longer than the planted PSCWs) and is most interesting. Overall the PSCWs showed higher removal efficiency for the investigated pathogens compared to e.g. in Barett et al. (2001) who measured a *Clostiridium perfringens* log removal rate of 2.2 to 2.4 in VFCWs.

CONCLUSIONS

- CWs are very dynamic and diverse systems were a lot of complex processes are running and influencing each other. This shows that CWs are close to natural systems and can remove and transform nutrients and pathogens on an efficient performance level.
- For treating wastewater with VF CWs the use of the suitable grain size, hydraulic dosing rate and distribution methods is very important. The combination of these factors used in this study showed a very high bacterial removal performance according to the literature.
- DO concentrations can play a key role for pathogenic removal. The measured DO concentration in 4 depths (5, 10, 20, 40cm) ranged between 8.7 – 9.3 mg O_2 /l which indicates stable conditions for a high pathogen removal efficiency.
- Constructed wetlands are capable of significantly reducing total and faecal coliforms, Enterococci, *C. perfringens* spores and *Pseudomonas aeruginosa*. Differences in the removal efficiency from *C. perfringens* spores and *Pseudomonas aeruginosa* between planted and unplanted PSCWs could be observed whereas unplanted systems showed higher removal rates (up to log 1 more). This effect is very similar to the significantly higher removal rate of Enterococci described in Sleytr et al. (2007) and can be linked to the longer HRT in the planted CWs.
- There is still a lack of knowledge to understand all the other processes in the filter bed of different kinds of CWs, but with this study some important questions could be answered.

ACKNOWLEDGEMENTS

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Appendix 4

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Characterisation of microbial biocoenosis in vertical subsurface flow constructed wetlands

Alexandra Tietz [a,*], Alexander Kirschner [b], Günter Langergraber [a], Kirsten Sleytr [a], Raimund Haberl [a]

[a] *Institute of Sanitary Engineering and Water Pollution Control, BOKU - University of Natural Resources and Applied Life Sciences, Vienna, Muthgasse 18, A-1190 Vienna, Austria*

[b] *Clinical Institute for Hygiene and Medical Microbiology, Department for Water Hygiene - Medical University of Vienna, Kinderspitalgasse 15, A-1090 Vienna, Austria*

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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 and bacterial activity 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 16–19% of the total microbial biomass. 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 with the exception of the reduction of enterococci. 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

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

* Corresponding author. Tel.: +43 1 36006 5814; fax: +43 1 3689949.
E-mail address: alexandra.tietz@boku.ac.at (A. Tietz).

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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 aerobic and anaerobic 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., 2002; 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 Šimůnek, 2005).

2. Material and methods

2.1. Description of the investigated constructed wetland 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 l mechanically settled municipal wastewater per square meter which results in an organic load of 20 g COD/m^2/d (i.e. a specific surface area of 4 m^2 per person). Six PSCWs are planted with *Miscanthus gigantea* 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 the operation started, 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 analysed 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.

2.2. Sampling

Samples were taken from different depths of the filter layer of the two years old planted and unplanted PSCWs. Soil samples from deeper layers were taken by a drill (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). Soil 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 analysed 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 analysis were conducted. Representative samples for cell volume estimation were stored no longer than one week.

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).

2.3. 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-h extraction of 10 g of soil 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).

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

The soil-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 (10,000 × dilution in DMSO; Molecular Probes; Leiden, NL). After incubation for 10 min 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 Eq. (1) given by Battin et al. (2001):

$$V = (w^2 \times \pi/4) \times (1-w) + (\pi \times w^3/6). \quad (1)$$

By applying the allometric model (Eq. (2)) describing the relationship between cell volume and cell carbon content from Norland (1993) we calculated the bacterial biomass C (C; [fgC]):

$$C = 120 \times V^{0.72}. \quad (2)$$

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).

2.5. 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; diluted 32 × 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 h. 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 recommended by Simon and Azam (1989) and an empirically derived conversion factor of 0.104 kg C/mol

leucine (Michel and Bloem, 1993). In both cases it was assumed that no intracellular isotope dilution occurred.

2.6. 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 soil samples were fumigated with ethanol-free chloroform for 24 h 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 analysed for total organic carbon by a Dohrmann Phoenix 8000 UV Persulfate TOC Analyzer. The soil microbial biomass C was calculated by division of the difference between the TOC concentrations of fumigated and non-fumigated soil extracts by a k_{EC}-value of 0.45 (Wu et al., 1990). For biomass N the extracts were analysed 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 (Brooks et al., 1985).

2.7. ATP measurements

ATP, ADP and AMP were measured after an extraction of the adenylates from soil 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 1 h 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 (Eq. (3)) given by Dyckmans et al. (2003):

$$1\ \text{nmol ATP} = 135\ \mu\text{g microbial biomass C}. \quad (3)$$

2.8. 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 soil were necessary to reach a maximal initial respiration rate. 50 g of soil (2 replicates) were put into nylon socks after a 1-h 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 (Eq. (4)) between CO_2 production and microbial biomass given by Anderson and Domsch (1978):

$$1\ \mu\text{g microbial biomass C/g soil} = (\mu\text{l } CO_2\text{/g soil/h}) \times 40.04 + 0.37. \quad (4)$$

2.9. Data analysis

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. 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).

3. Results

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 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.

3.2. Wastewater analysis

Results of the chemical and microbial wastewater analysis are shown in Table 2. The removal efficiencies are high as typically observed for vertical flow constructed wetlands (e.g. Weedon, 2003) and did not

Table 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 (°C)	25.8	17.9	20.9
DO (mg/l)	9.4	2.5	8.5 (between loadings) 4.1 (during loading)
TOC (mg/g DW soil)	20.0	0.2	10.1 (in 0–1 cm depth) 2.7 (in 40–50 cm depth)
TON (mg/g DW soil)	2.4	<0.01	1.2 (in 0–1 cm depth) 0.1 (in 40–50 cm depth)
C/N ratio	87	3.6	15
Water content (% of water sat.)	100	20	80–90 (in 0–1 cm depth during loading) 20–30 (in 0–1 cm depth between loadings) 55–65 (in 40–50 cm depth)

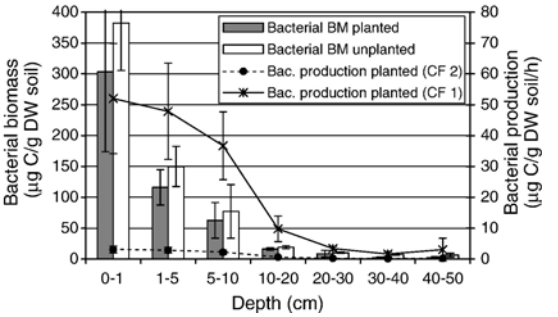

Fig. 1. Comparison of bacterial biomass determined by microscopic direct counts and volume measurements in planted and unplanted PSCWs and bacterial secondary production rates measured by incorporation of ^{14}C-labeled leucine in planted PSCWs calculated with a maximal (CF 1; Simon and Azam, 1989) and 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).

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, except for enterococci. This group of indicator bacteria was better removed in the unplanted system than in the planted with a difference in the removal efficiency of 0.87 log ($p<0.05$; $n=14$).

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 Fig. 1 the bacterial

Table 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	*n*	mg/l	*n*	%
COD	415 (141–1270)	46	<20 [a]	24	>95
BOD_5	133 (41–320)	24	<3 [a]	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 [a]	100	99
NO_3-N	<0.1 [a]	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	*n*	Log CFU/ml	*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 cell counts	Log cells/ml	*n*	Log cells/ml	*n*	Log removal
MDC	8.0 (7.9–8.2)	12	6.0 (5.8–6.2)	12	2.0

HPC = Heterotrophic plate count.
MDC = Microscopic direct counts.
[a] Limit of detection.

Table 3
Distribution of 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 to microbial biomass over the depth

Depth (cm)	Total microbial BM		Contribution of bacterial BM to microbial BM	
	Planted (%)	Unplanted (%)	Planted (%)	Unplanted (%)
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

biomass decreased very rapidly with the depth of the filter body and reached their lowest values in 30–40 cm with only 0.4% of the biomass in the 0–1 cm layer (Table 3). 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 (Fig. 1), which resulted in values with a large range reaching from 0.18 µg to 52 µg C/g DW soil/h. The values for bacterial biomass and production were significantly different between the different depths ($p<0.05$, $n=6$–13).

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 (Fig. 2). The average range of the contribution of bacterial biomass C to microbial biomass C was between 16 and 20% in the top 10 cm of the filter body and increased up to 51% in deeper layers (Table 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 in the top layer to 18 µg/g DW soil 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 ($r>0.84$; $p<0.001$, $n=26$–51) were found between the different methods to describe biomass and activity of the microbial community (Table 4). The microbial biomass at different depths was significantly different ($p<0.05$, $n=15$–40).

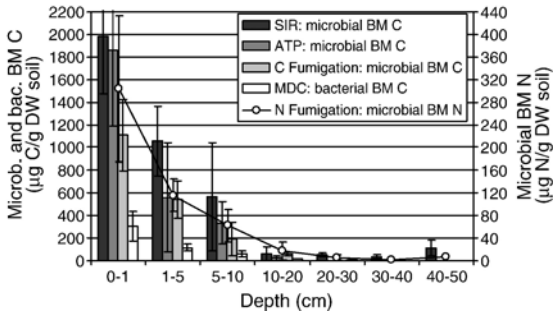

Fig. 2. Comparison of different methods used to characterise the microbial biomass C, -N and the bacterial biomass C determined via microscopic direct counts (MDC) and volume measurements of the cells in planted PSCWs. (Means±standard deviations of six replications for 0–10 cm and of three replications for 10–50 cm).

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 Fig. 3 was also not significantly different ($p>0.05$, $n=6$).

4. Discussion

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 (Figs. 1, 2 and 3). High correlation coefficients were observed between all methods used to characterise the microbial biomass (Table 4). The good correlation between TOC and the other methods shows the good indicator function for microbial biomass of this easy to measure method.

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 non-suitable conversion factors for the

Table 4
Spearman rank correlation coefficients of the different methods used to characterise the microbial biomass and productivity (FUM C/N = fumigation extraction, MDC = microscopic direct counts, SIR = substrate induced respiration, LEU = leucine incorporation, TOC = total organic carbon)

Method	FUM C	FUM N	MDC	LEU	SIR	TOC
ATP	0.94 (33)	0.97 (30)	0.95 (39)	0.92 (39)	0.92 (39)	0.91 (32)
FUM C		0.94 (39)	0.94 (33)	0.96 (33)	0.89 (33)	0.88 (26)
FUM 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)

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).

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 soil particles or a too 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 1 h 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 h 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.

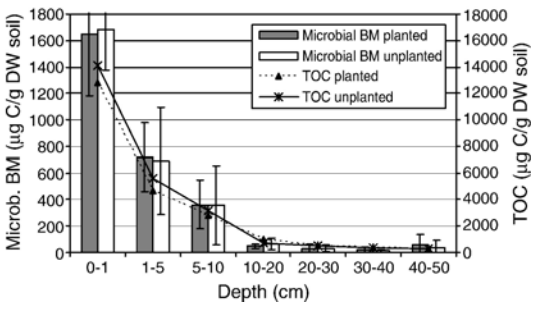

Fig. 3. TOC and microbial biomass C in different depths of planted and unplanted PSCWs. (Means ± standard deviations of six replications from microbial biomass determinations by fumigation extraction, SIR and ATP for 0–10 cm and of three replications for 10–50 cm).

4.2. 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 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 (extracellular polysaccharides), 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 centimetres.

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). Munch 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

Table 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 (µl CO_2/g soil/h)	3.1–49.4	5.3[1)]–70.1[2)]
Microscopic direct counts (10^8 cells/g soil)	1.2–190	0.01[4)]–63[5)]
Leucine incorporation (nmol Leucine/ml soil/h)	0.28–22.3[II] 0.05–3.87[III]	0.02[5)]–1.5[5)]
C Fumigation–extraction (µg C/g soil)	<6[IV]–2042	61[8)]–~1484[6)]
N Fumigation–extraction (µg N/g soil)	<6[IV]–422	3[8)]–346[9)]
ATP (nmol ATP/g soil)	<0.05[V]–13.8	0.15[10)]–32.3[7)]
C/N ratio of the biomass (−)	<3.1[VI]–11.2	4.2[8)]–1250[3)]

[1)]Barajas-Aceves (2005); [2)]Bailey et al. (2002), [3)]Joergensen and Raubuch (2003), [4)]Kepner and Pratt (1994); [5)]Uhlirova and Santruckova (2003); [6)]Sparling and West (1988); [7)]Dyckmans et al. (2003); [8)]Sparling and West (1989); [9)]Turner et al. (2001); [10)]Prevost et al. (1991).
[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/g soil for C- and N fumigation.
[V]Detection limit of 0.05 nmol/g soil.
[VI]This value was sometimes below the molar C/N ratio of proteins (3.1) which is not plausible. For an explanation see Section 4.4.

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.

4.4. Microbial biomass in constructed wetlands in comparison to other 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. 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 5). 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.

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.

5. Conclusions

The detailed characterisation 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 the 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 way of operating the system.

Acknowledgements

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Appendix 5

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High-rate nitrogen removal in a two-stage subsurface vertical flow constructed wetland

Guenter Langergraber[a]*, Klaus Leroch[b], Alexander Pressl[a], Kirsten Sleytr[a], Roland Rohrhofer[b], Raimund Haberl[a]

[a]*Institute of Sanitary Engineering and Water Pollution Control, University of Natural Resources and Applied Life Sciences, Vienna (BOKU), Muthgasse 18, A-1190 Vienna, Austria*
[b]*ÖKOREAL GmbH, Carl Reichert-Gasse 28, A-1170 Vienna, Austria*
Tel: +43-1-36006-5814; Fax: +43-1-3689949; email: guenter.langergraber@boku.ac.at

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Abstract

A two-stage constructed wetland (CW) system consisting of two vertical flow (VF) beds with intermittent loading operated in series has been investigated. The first stage uses sand with a grain size of 2–3.2 mm for the main layer and has a drainage layer that is impounded; the second stage uses sand with a grain size of 0.06–4 mm and a conventional drainage layer. The two-stage system was operated with an organic load of 40 g COD m^{-2} d^{-1} for the whole system, that is with a specific surface area requirement of 2 m^2 per person equivalent. The system was operated over a period of 2 years including two full winter seasons. No operational problems occurred, and the Austrian effluent standards for organic matter and ammonium nitrogen could be met during the whole investigation period. Additionally, average nitrogen removal efficiencies of 53% and average nitrogen elimination rates of 2.7 g Nm^{-2} d^{-1} and 986 g Nm^{-2} yr^{-1}, respectively, could be achieved without recirculation, thus allowing construction with energy-free loading systems. It can be concluded that a better effluent quality can be reached with such two-stage systems as compared to a conventional single-stage VF CW system with intermittent loading, even if the two-stage system is loaded with the double organic load and therefore has only half of the specific surface area requirement.

Keywords: Nitrogen removal; Surface area requirement; Two-stage constructed wetland system; Vertical flow

1. Introduction

Constructed wetlands (CWs) are engineered systems designed to improve water quality using the same processes that occur in natural wetlands [1]. Using subsurface vertical flow (VF) CWs with intermittent loading it is possible to fulfil the stringent Austrian effluent standards regarding nitrification [2]. For small plants

*Corresponding author.

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(less than 500 person equivalents) a maximum ammonia nitrogen effluent concentration of 10 mg/L has to be met at effluent water temperatures higher than 12°C. For organic matter effluent concentrations (90 mg COD/L and 25 mg BOD_5/L) and treatment efficiencies (85% and 95% for COD and BOD_5, respectively) have to be met the whole year around. For small plants there are no discharge limits for total nitrogen and phosphorus [3].

In this chapter results of the Austrian research project *Optimization of subsurface vertical flow constructed wetlands (Bepflanzte Bodenfilter)* are shown. The main goal of this project was to optimize the specific area requirement for intermittently loaded VF beds in Austria on field scale [4]. An additional goal was the optimization of nitrogen removal. Results of previous indoor experiments performed at the technical laboratory hall at BOKU had shown that a two-stage VF CW system at plot scale performed well for nitrogen elimination [5]. The chapter presents the results of the outdoor experiments carried out to validate the findings of these indoor experiments.

2. Materials and methods

2.1. Experimental set-up

The experimental site (Fig. 1) is located on the premises of the wastewater treatment plant Ernsthofen (Lower Austria), about 150 km west of Vienna. It consists of three independent planted beds operated in parallel [4]. One of the three beds was built as a two-stage system. This two-stage CW system consists of two VF beds with intermittent loading operated in series with a surface area of approximately 10 m^2 for each stage. The first stage uses a grain size of 2–3.2 mm for the 50 cm main layer and has a drainage layer that is impounded; the second stage uses a grain size of 0.06–4 mm (d_{10} = 0.2 mm; d_{60} = 0.8 mm) and a conventional drainage layer.

All beds were planted with common reed (*Phragmites australis*). The two-stage system

Fig. 1. The experimental CWs at the Ernsthofen WWTP in July 2006.

was operated with an organic load of 80 g COD m^{-2} d^{-1} for the first stage (1 m^2 per person equivalent), that is 40 g COD m^{-2} d^{-1} for the whole system (2 m^2 per person equivalent). The two-stage system was loaded intermittently every 3 h with mechanically pre-treated wastewater (16.2 mm per loading). In the start-up period from May until August 2005, all beds were operated with lower organic load. From September 2005 to May 2007 the two-stage system was operated with the target load of 40 g COD m^{-2} d^{-1}.

2.2. Sampling and analysis

Samples were taken weekly and analysed for BOD_5, COD, NH_4^+-N, NO_2^--N and NO_3^--N in the lab of the wastewater treatment plant using Dr. Lange cuvette tests (Hach-Lange, Germany). Organic N (N_{org}) was calculated using the N_{org}/COD ratio from reference samples analysed in the lab of the Institute at BOKU. The N_{org}/COD ratio turned out to be very stable over the investigation period and its median values were 0.026, 0.041 and 0.083 for the influent and the effluent of stage one, and the effluent of stage two, respectively. Furthermore, the microbial parameters heterotrophic plate counts (HPC), *Escherichia coli*, total coliforms and enterococci were analysed from reference samples in the lab of the Institute at BOKU.

3. Results and discussion

3.1. Influent concentrations and loads

The median value of the measured influent COD concentration over the investigation period was 505 mg/l (number of samples n = 88, 95% confidence interval (CI_{95}) = 18 mgl^{-1}) resulting in a median value of the organic load of 40.7 g COD m^{-2} d^{-1} (n = 88, CI_{95} = 1.85 g COD m^{-2} d^{-1}). The actual loading of the system was very close to the target value. The total Kjeldahl nitrogen (TKN) load was 9.80 gm^{-2} d^{-1} for the first stage.

The average organic load for the second stage was 13.1 g COD m^{-2} d^{-1} (N = 88, CI_{95} = 1.0 g COD m^{-2} d^{-1}). No clogging problems could be observed for the second stage, which is in accordance with the experience that no clogging occurs for organic loads less than 20 g COD m^{-2} d^{-1} [6].

3.2. Organic matter

Figure 2 shows the COD influent and effluent concentrations and effluent water temperature. The influent concentrations were constant until the start-up of a combined heat and power plant in January 2006. The unsteady release of cooling water from this plant during its start-up phase together with snowmelt in spring 2006 resulted in fluctuating influent flows to the wastewater treatment plant and therefore fluctuations in the influent concentrations. More constant influent concentrations occurred again after full operation of the combined heat and power plant from summer 2006.

Subsequently, Table 1 shows the COD concentrations for different effluent water temperatures. Effluent concentrations for COD were 20 and 38 mg/l for effluent water temperatures >8°C and <5°C, respectively; the COD removal efficiency was 95.9% (N = 88, CI_{95} = 0.6%) for the whole investigation period. The Austrian effluent requirements for COD (<90 mg/L) could be met.

The results for BOD_5 (Fig. 3 and Table 2) are similar to those for COD. The median value for the influent concentration was 340 mg/l (n = 85, CI_{95} = 14 $mg\,l^{-1}$) for the whole investigation period. Effluent concentrations were 4 and 12 mg/l for effluent water temperatures >8°C and <5°C, respectively; the removal efficiency was 98.7% (n = 85, CI_{95} = 0.4%) for the whole investigation period. As for COD the Austrian effluent requirements for BOD_5 (<25 mg/L) have been met.

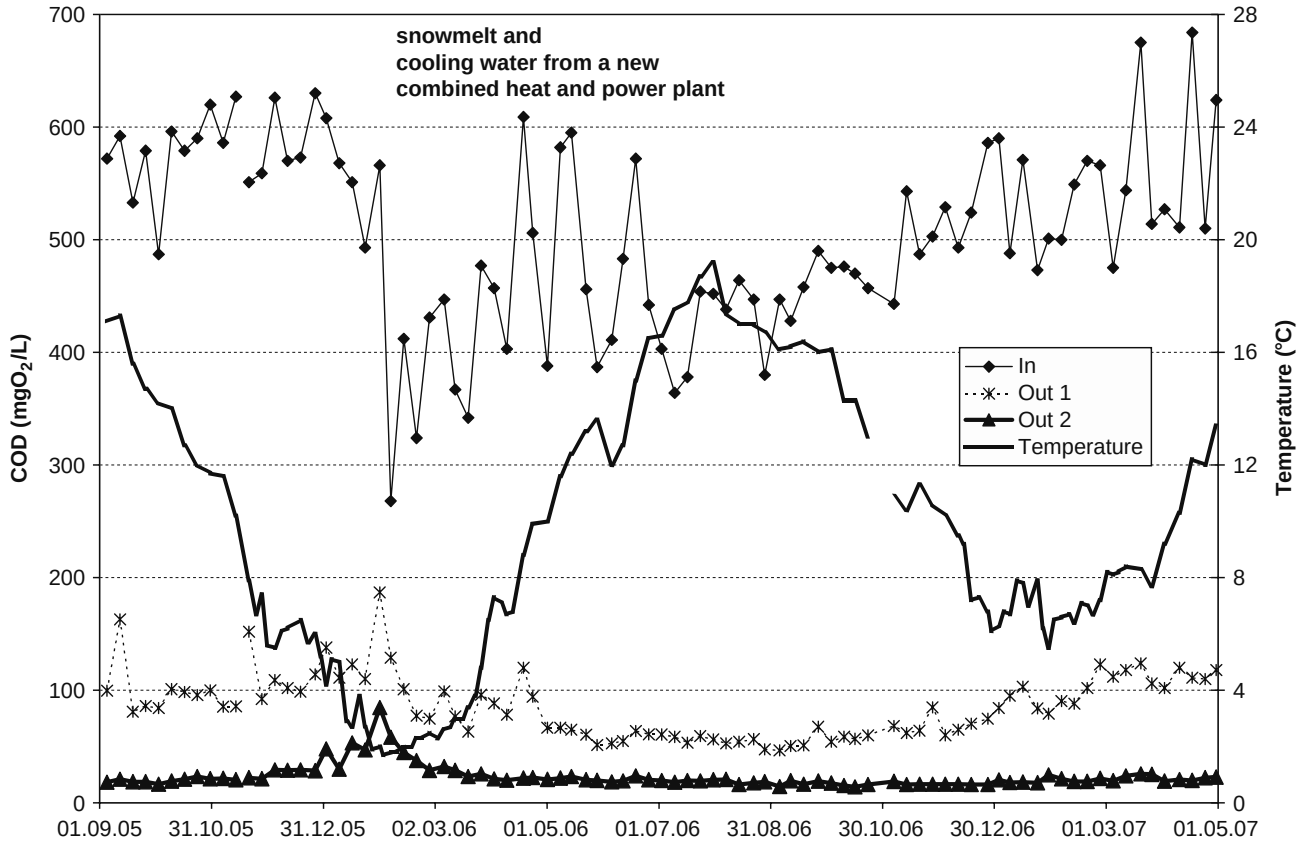

Fig. 2. Measured COD influent and effluent concentrations and effluent water temperature.

Table 1
Measured COD influent and effluent concentrations (mg/L) for different effluent temperatures

Temperature	>8°C			5–8°C			<5°C		
Location	In	Out 1	Out 2	In	Out 1	Out 2	In	Out 1	Out 2
No. of samples	54	54	54	21	21	21	11	11	11
Median value	492	66	20	566	99	22	431	99	38
Mean value	507	78	20	544	98	24	425	103	42
Standard deviation	79	27	3	58	17	7	94	35	18
95% confidence interval	21	7	1	25	7	3	56	20	11
Minimum	364	47	14	403	70	16	268	63	24
Maximum	684	163	26	630	138	48	566	187	85

3.3. Nitrogen parameters

Figure 4 shows the measured influent and effluent concentrations of ammonia nitrogen (NH_4^+-N) during the investigation period. Table 3 shows the NH_4^+-N concentrations for different effluent water temperatures. The median value for NH_4^+-N effluent concentrations of stage one was 13.2, 27.0 and 32.8 mg NH_4^+-N/l for effluent water temperatures >8°C, 5–8°C and <5°C,

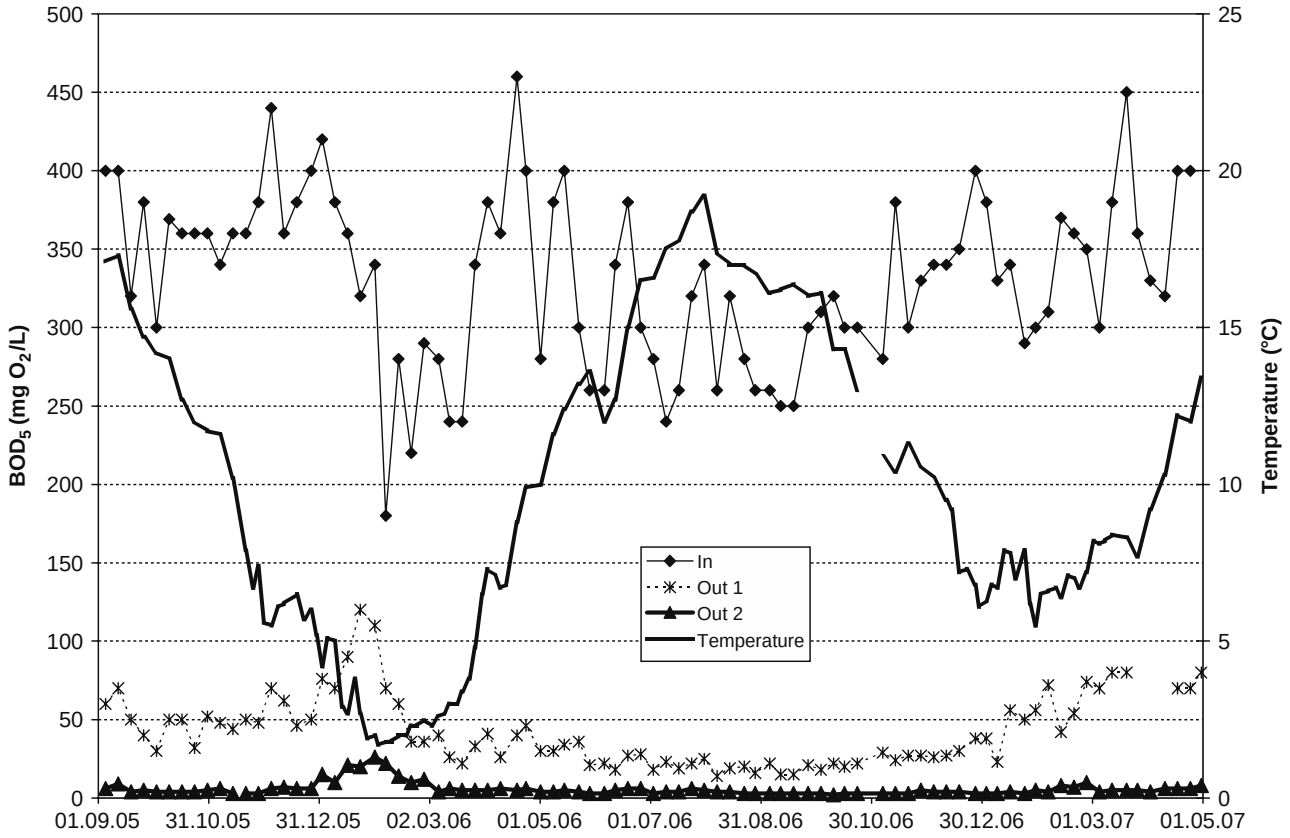

Fig. 3. Measured BOD_5 influent and effluent concentrations and effluent water temperature.

Table 2
Measured BOD_5 influent and effluent concentrations (mg/L) for different effluent temperatures

Temperature	>8°C			5–8°C			<5°C		
Location	In	Out 1	Out 2	In	Out 1	Out 2	In	Out 1	Out 2
No. of samples	51	52	54	21	20	21	11	11	11
Median value	320	28	4	360	52	5	280	40	12
Mean value	327	35	4	364	52	6	281	58	13
Standard deviation	53	19	1	36	17	3	57	35	8
95% confidence interval	14	5	0.4	15	7	1	33	20	5
Minimum	240	14	2	300	23	3	180	22	4
Maximum	460	80	9	440	76	15	360	120	26

respectively. The TKN elimination rate of the first stage is 6.65 $gm^{-2}\ d^{-1}$ and higher compared to the first stage of two-stage systems reported from France for treatment of raw wastewater where each of the three parallel operated beds is loaded for 3–4 days, before being rested for twice this amount of time [7].

Full nitrification could be observed for the overall two-stage system for effluent water temperatures >8°C. At temperatures <5°C the

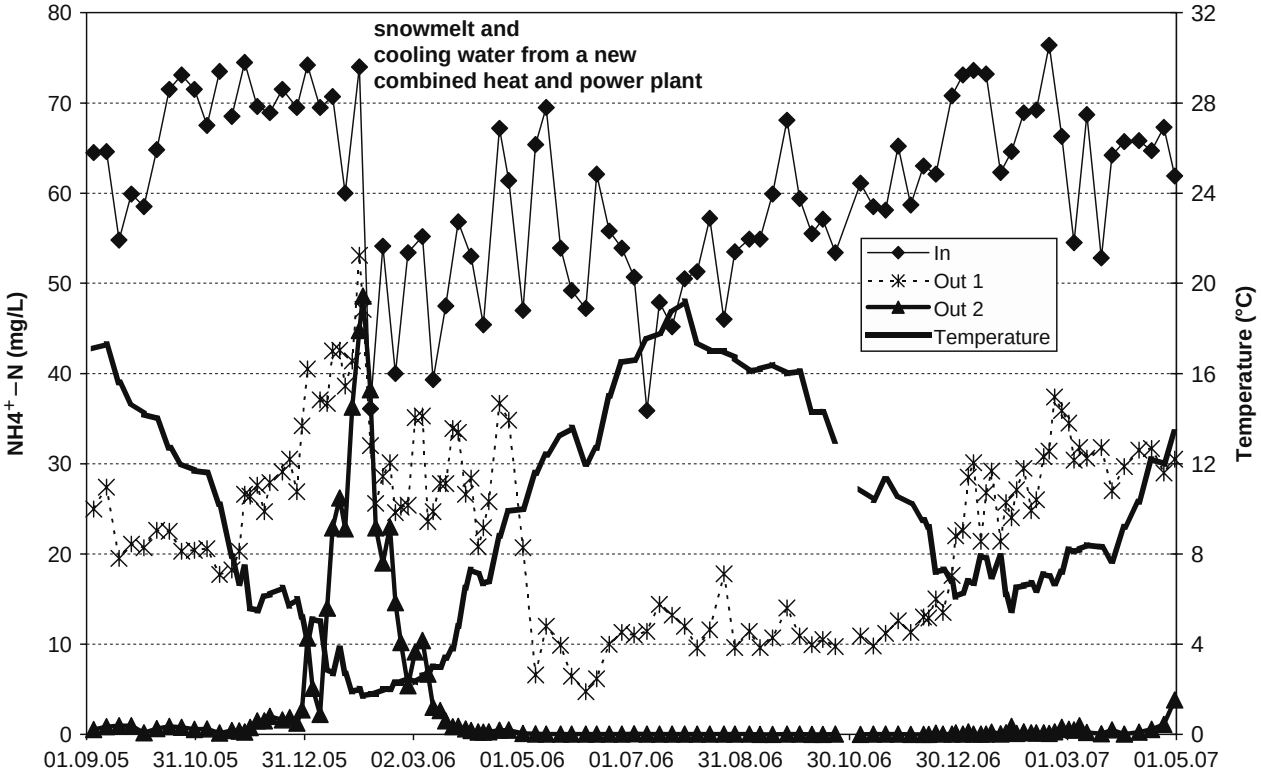

Fig. 4. Measured NH_4^+-N influent and effluent concentrations and effluent water temperature.

Table 3
Measured NH_4^+-N influent and effluent concentrations (mg/L) for different effluent temperatures

Temperature	>8°C			5–8°C			<5°C		
Location	In	Out 1	Out 2	In	Out 1	Out 2	In	Out 1	Out 2
No. of samples	54	57	57	21	39	40	11	22	22
Median value	59.7	13.2	0.03	69.5	27.0	0.30	54.1	32.8	14.3
Mean value	59.3	17.3	0.29	67.3	27.3	0.98	53.4	33.4	17.5
Standard deviation	8.1	8.5	0.57	7.9	5.7	1.86	12.2	8.1	14.5
95% confidence interval	2.2	2.2	0.15	3.4	1.8	0.58	7.2	3.4	6.04
Minimum	35.9	4.8	0.01	45.4	13.5	0.02	36.1	23.6	0.85
Maximum	73.5	36.7	3.82	76.4	40.5	10.7	74.0	53.1	48.6

median value of the NH_4^+-N effluent concentration of stage 2 was 14.3 mg NH_4^+-N/l. The Austrian effluent standard of 10 mg NH_4^+-N/l was exceeded only at effluent water temperatures lower than 5°C (far below the requested 12°C).

Tables 4 and 5 show the measured data for nitrite nitrogen (NO_2^--N) and nitrate nitrogen (NO_3^--N), respectively. NO_2^--N concentrations have been very low throughout the investigation period.

Table 4
Measured NO_2^--N influent and effluent concentrations (mg/L) for different effluent temperatures

Temperature	>8°C			5–8°C			<5°C		
Location	In	Out 1	Out 2	In	Out 1	Out 2	In	Out 1	Out 2
No. of samples	54	54	54	21	21	21	11	11	11
Median value	0.015	0.312	0.027	0.033	0.186	0.046	0.015	0.131	0.211
Mean value	0.020	0.379	0.035	0.030	0.228	0.081	0.015	0.210	0.269
Standard deviation	0.009	0.290	0.022	0.017	0.186	0.077	0.000	0.284	0.231
95% confidence interval	0.002	0.077	0.006	0.007	0.079	0.033	0.000	0.168	0.137
Minimum	0.015	0.064	0.015	0.015	0.055	0.027	0.015	0.033	0.062
Maximum	0.049	1.568	0.099	0.089	0.831	0.297	0.015	1.020	0.911

Table 5
Measured NO_3^--N influent and effluent concentrations (mg/L) for different effluent temperatures

Temperature	>87°C			5–8°C			<5°C		
Location	In	Out 1	Out 2	In	Out 1	Out 2	In	Out 1	Out 2
No. of samples	54	54	54	21	21	21	11	11	11
Median value	0.36	12.7	30.6	0.34	5.6	33.1	0.30	5.0	24.0
Mean value	0.37	13.9	30.9	0.34	6.7	32.7	0.30	4.4	21.1
Standard deviation	0.08	7.7	5.7	0.06	4.1	3.7	0.08	1.8	12.3
95% confidence interval	0.02	2.1	1.5	0.02	1.7	1.6	0.05	1.0	7.3
Minimum	0.23	2.6	20.7	0.25	1.2	25.3	0.16	1.0	1.8
Maximum	0.57	31.2	49.8	0.47	16.7	39.4	0.42	6.6	40.4

Figure 5 shows the total nitrogen (TN) influent and effluent concentrations during the investigation period, and Table 6 shows the TN concentrations for different effluent water temperatures. For the whole investigation period the median value of the TN elimination efficiency was 53.2% (N = 86, CI_{95} = 11.4%). For temperatures >8°C the TN effluent concentrations were stable. The median value of the TN elimination efficiency was 54.8% (N = 54, CI_{95} = 8.7%). The median value of the TN elimination efficiency was 56.9% (N = 21, CI_{95} = 6.6%) for effluent water temperatures between 5 and 8°C and dropped to a median value of 37.1% (N = 11, CI_{95} = 14.9%) for effluent water temperatures <5°C.

It can be assumed that nitrogen elimination in the two-stage CW system occurred due to the following facts related to the impounded drainage layer of the first stage:

- A nitrification of about 80% in the first stage guaranteed the presence of nitrate for denitrification in the impounded drainage layer.
- By using a grain size of 2–3.2 mm for the main layer of the first stage, no complete mineralization of the organic matter occurred. Therefore, enough organic matter was available

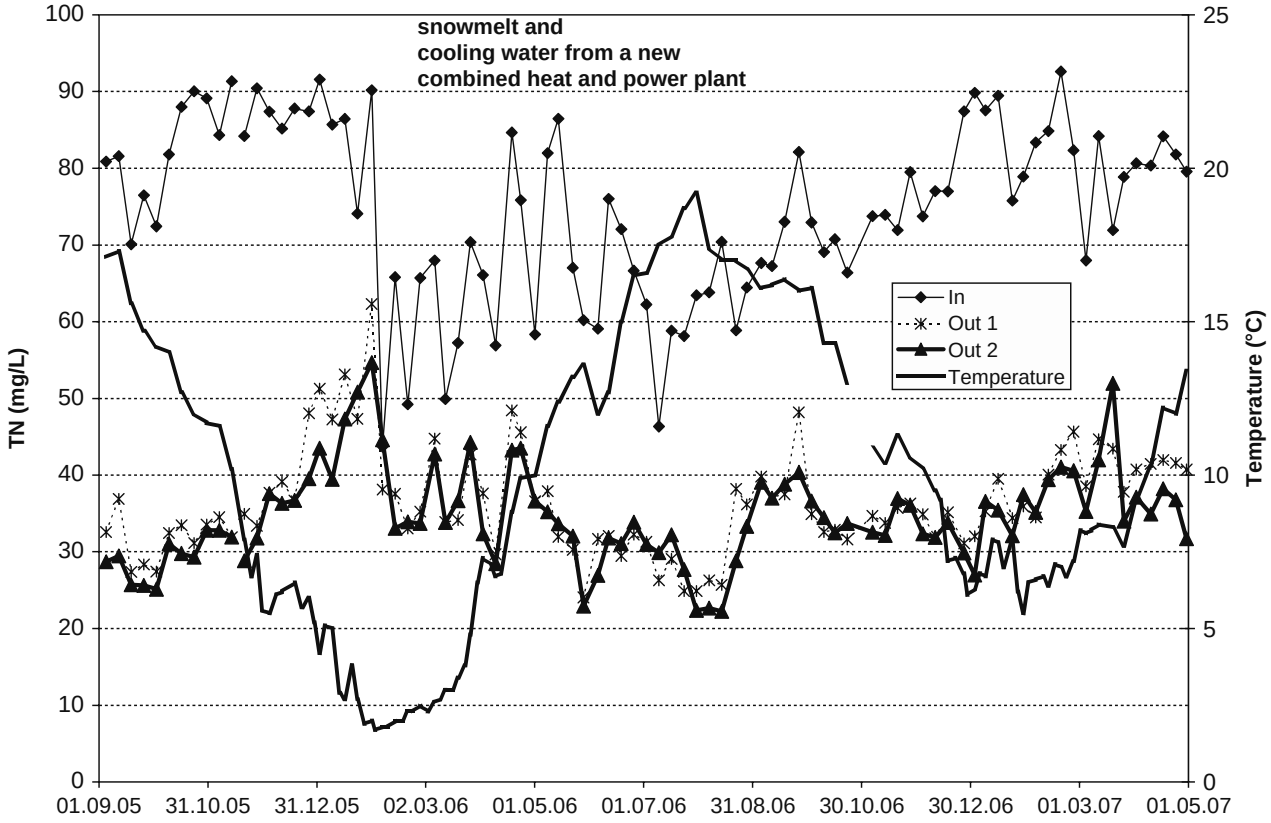

Fig. 5. Measured TN influent and effluent concentrations and effluent water temperature.

Table 6
Measured TN influent and effluent concentrations (mg/L) for different effluent temperatures

Temperature	>8°C			5–8°C			<5°C		
Location	In	Out 1	Out 2	In	Out 1	Out 2	In	Out 1	Out 2
No. of samples	54	54	54	21	21	21	11	11	11
Median value	73.4	33.7	32.4	85.2	37.6	36.3	65.8	38.1	42.7
Mean value	73.6	34.5	32.9	82.2	38.0	35.7	67.4	42.8	41.4
Standard deviation	9.8	5.9	5.7	8.9	5.1	4.4	16.8	9.7	7.6
95% confidence interval	2.6	1.6	1.5	3.8	2.2	1.9	9.9	5.7	4.5
Minimum	46.3	24.1	22.3	56.9	29.7	27.0	43.7	33.0	33.1
Maximum	91.3	48.4	52.0	92.6	48.1	44.3	91.6	62.3	54.7

for the denitrification in the impounded drainage layer.

- The impounded drainage layer increased the hydraulic retention time and therefore the contact time between the denitrifying micro-organisms and the substrates for denitrification (i.e., nitrate and organic matter).

The second stage of the two-stage CW system is required to guarantee full nitrification

and removal of the remaining organic matter. Additionally, the second stage is responsible for producing more stable effluent concentrations and thus making the two-stage CW system very robust.

3.4. Eliminated nitrogen

Figure 6 shows the eliminated nitrogen per m^2 filter surface area and day, Table 7 shows the referring data. The median value of the nitrogen

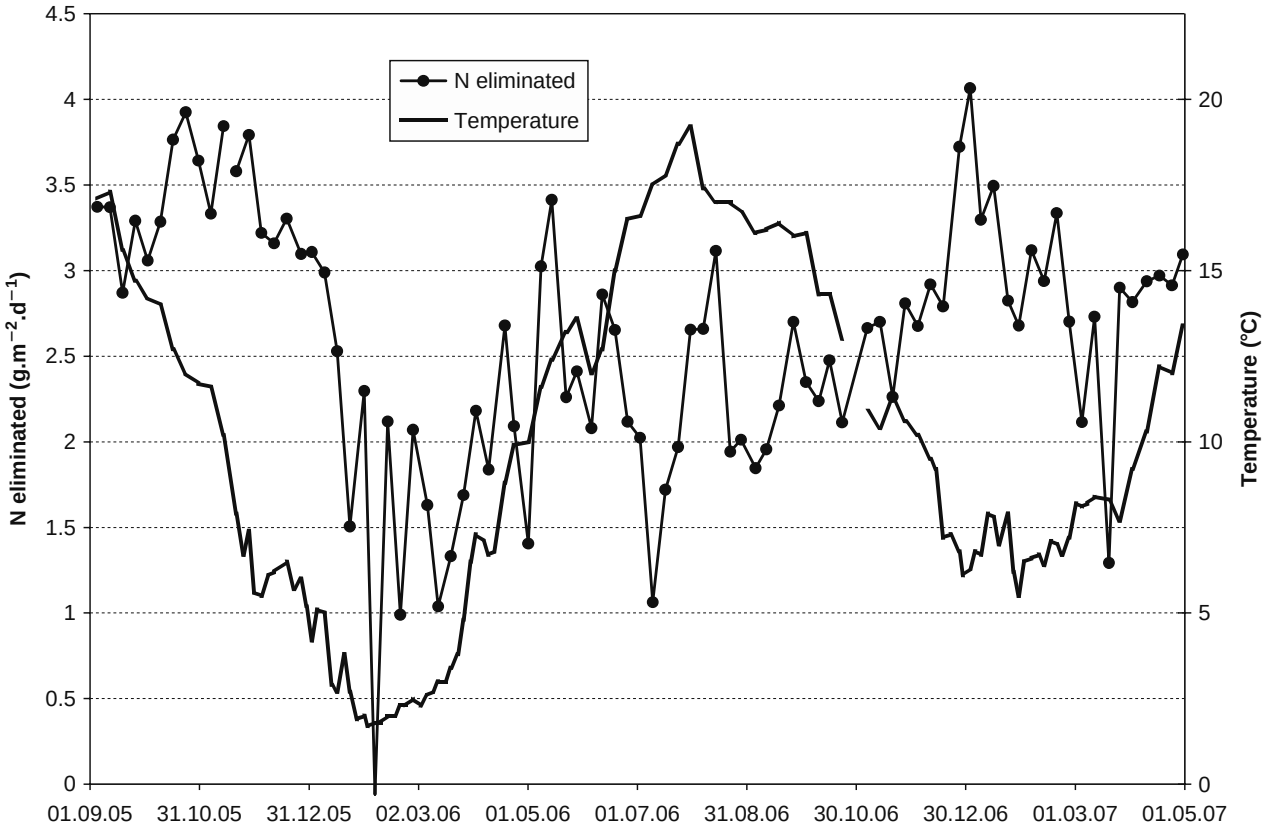

Fig. 6. Eliminated nitrogen.

Table 7
Nitrogen load and eliminated nitrogen (gm^{-2} d^{-1})

	Whole period		>8°C		5–8°C		<5°C	
Temperature	Load	Eliminated	Load	Eliminated	Load	Eliminated	Load	Eliminated
No. of samples	86	86	54	54	21	21	11	11
Median value	4.91	2.70	4.75	2.68	5.51	3.10	4.26	1.63
Mean value	4.84	2.60	4.76	2.63	5.31	3.01	4.36	1.69
Standard deviation	0.75	0.76	0.64	0.65	0.58	0.59	1.09	0.87
95% confidence interval	0.16	0.16	0.17	0.17	0.25	0.25	0.64	0.51
Minimum	2.83	−0.06	3.00	1.06	3.68	1.69	2.83	−0.06
Maximum	5.99	4.07	5.91	3.92	5.99	4.07	5.92	3.11

elimination rate over the whole experimental phase was 2.70 g Nm^{-2} d^{-1} or 986 g Nm^{-2} yr^{-1}, respectively, reaching a maximum of 4.07 g Nm^{-2} d^{-1} or 1′484 g Nm^{-2} yr^{-1}. The achieved elimination rates are very high as compared to literature values. A review reported mean nitrogen elimination rates of 250 g Nm^{-2} yr^{-1} for subsurface flow CWs with horizontal flow [8], the highest being 434 g Nm^{-2} yr^{-1} [9]. For VF beds elimination rates up to 576 g Nm^{-2} yr^{-1} have been reported [10].

3.5. Microbial contamination

The results for the microbial parameters are shown in Table 8 for HPC and *E. coli*, and in Table 9 for total coliforms and enterococci. A log removal rate of about three could be reached for all microbial parameters.

3.6. Comparison with a single-stage CW

In Figs. 7 and 8 the COD and NH_4^+-N effluent concentrations of the two-stage system

Table 8
Log influent and effluent concentrations and removal rates for HPC and *E. coli*

	HPC per ml					*E. coli* per 100 ml				
Parameter	Log concentrations			Log removal		Log concentrations			Log removal	
Location	In	Out 1	Out 2	Out 1	Out 2	In	Out 1	Out 2	Out 1	Out 2
No. of analysis	8	8	8	8	8	8	8	8	8	8
Median value	6.32	5.65	3.43	0.68	2.88	6.52	5.82	3.46	0.65	3.06
Mean value	6.33	5.62	3.45	0.71	2.87	6.18	5.65	3.20	0.87	3.31
Standard deviation	0.10	0.18	0.39	0.14	0.38	0.57	0.54	0.41	0.54	0.41
95% confidence interval	0.15	0.26	0.56	0.21	0.55	0.82	0.78	0.59	0.78	0.59
Minimum	6.10	5.14	4.56	0.44	3.85	4.23	3.87	3.76	0.34	4.45
Maximum	6.60	5.92	2.51	0.99	1.79	6.66	6.30	2.15	2.73	2.85

Table 9
Log influent and effluent concentrations and removal rates for total coliforms and enterococci

	Total coliforms per 100 ml					Enterococci per 100 ml				
Parameter	Log concentrations			Log removal		Log concentrations			Log removal	
Location	In	Out 1	Out 2	Out 1	Out 2	In	Out 1	Out 2	Out 1	Out 2
No. of samples	8	8	8	8	8	8	8	8	8	8
Median value	6.93	6.23	3.70	0.63	3.18	6.11	5.25	2.80	0.85	3.30
Mean value	6.56	5.98	3.49	0.93	3.42	5.94	5.10	2.76	1.03	3.36
Standard deviation	0.65	0.56	0.39	0.57	0.40	0.40	0.44	0.30	0.43	0.28
95% confidence interval	0.94	0.81	0.56	0.82	0.57	0.57	0.63	0.43	0.62	0.41
Minimum	4.26	4.03	4.06	0.46	4.36	4.54	3.58	3.23	0.62	4.00
Maximum	7.00	6.49	2.60	2.93	2.90	6.26	5.63	2.11	2.53	2.89

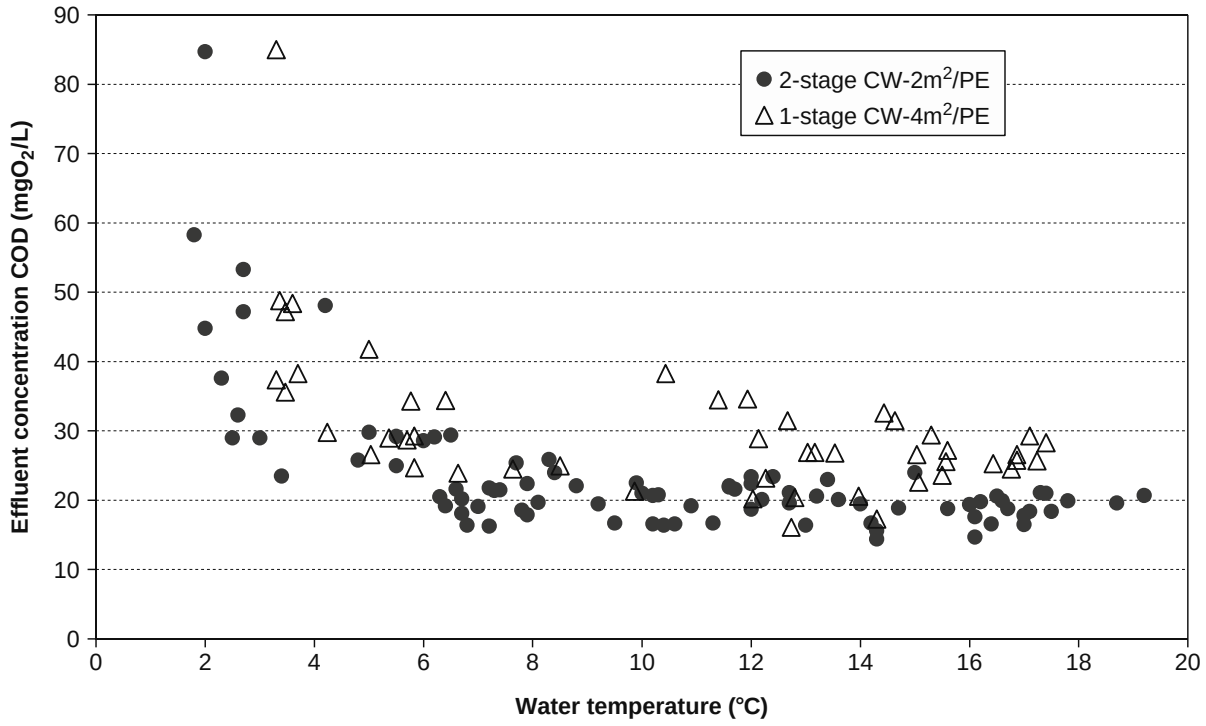

Fig. 7. COD effluent concentration vs. effluent water temperature.

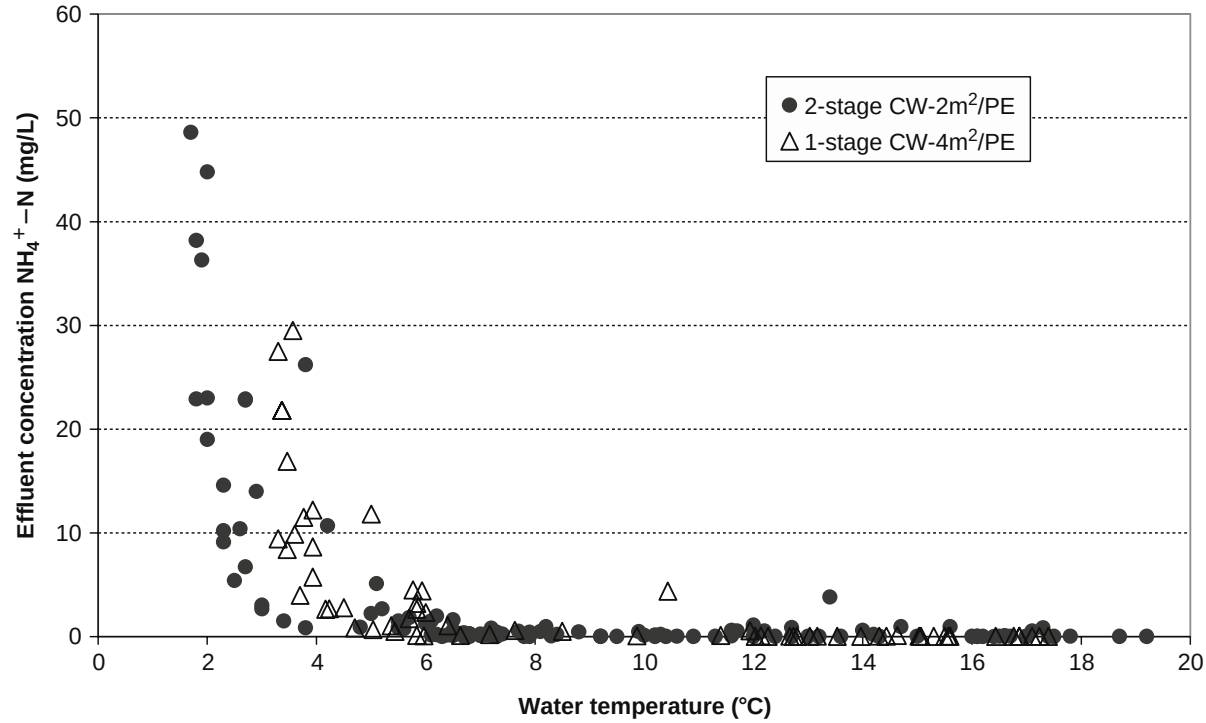

Fig. 8. NH_4^+-N effluent concentration vs. effluent water temperature.

and the single-stage VF bed are compared for different effluent water temperatures. The single-stage VF bed was designed and operated according to the Austrian design standards ÖNORM B 2505 [11] with an organic load of 20 g CODm^{-2} d^{-1} (i.e., 4 m^2 per person equivalent, with a 50-cm main layer using the same sand as for the second stage of the two-stage system, i.e., grain size of 0.06–4 mm, d_{10} = 0.2 mm, and d_{60} = 0.8 mm) [4]. The data shown in Figs. 7 and 8 are from April 2004 until March 2005. In general the two-stage system produced more stable and lower effluent concentrations as compared to the single-stage system, especially at low temperatures (<6°C).

Regarding the microbial parameters it could be shown that the effluent concentrations and removal rates of the single-stage VF bed and the two-stage system are equal, even when the hydraulic loading of the two-stage system is twice as high as the loading of the one-stage system, that is, the retention time is lower [5].

Tables 10 and 11 compare the COD, NH_4^+-N and TN loads and eliminated COD, NH_4^+-N and TN in the two-stage CW system and the single-stage VF bed, respectively. The elimination of

Table 10
COD, NH_4^+-N and TN loads and elimination (gm^{-2} d^{-1}) in the two-stage CW system

	COD		NH_4-N		TN	
Parameter	Load	Eliminated	Load	Eliminated	Load	Eliminated
No. of samples	86	86	86	86	86	86
Median value	32.46	31.03	3.99	3.86	4.91	2.70
Mean value	32.69	31.17	3.91	3.74	4.84	2.60
Standard deviation	5.37	5.42	0.62	0.81	0.75	0.76
95% confidence interval	1.13	1.15	0.13	0.17	0.16	0.16
Minimum	17.33	13.56	2.32	−0.14	2.83	−0.06
Maximum	44.23	42.93	4.94	4.93	5.99	4.07

Table 11
COD, NH_4^+-N and TN loads and elimination (gm^{-2} d^{-1}) in the single-stage VF bed

	COD		NH_4-N		TN	
Parameter	Load	Eliminated	Load	Eliminated	Load	Eliminated
No. of samples	49	49	55	55	40	40
Median value	17.16	16.39	2.14	2.06	2.65	0.85
Mean value	17.44	16.48	2.13	1.99	2.63	0.92
Standard deviation	0.53	0.55	0.05	0.07	0.06	0.11
95% confidence interval	1.91	1.98	0.18	0.27	0.20	0.36
Minimum	22.92	21.93	2.57	2.43	3.07	1.91
Maximum	12.66	11.17	1.67	1.13	2.12	0.30

the two-stage system was twice compared to the single-stage CW for COD and NH_4^+-N. The eliminated nitrogen was 2.70 g Nm^{-2} d^{-1} for the two-stage system compared to 0.85 g Nm^{-2} d^{-1} for the single-stage VF bed.

4. Conclusions

A two-stage CW system (first stage – grain size 2–3.2 mm for the 50 cm main layer, impounded drainage layer; second stage – grain size 0.06–4 mm for the 50 cm main layer, conventional drainage layer) was operated with an organic load of 40 g $CODm^{-2}$ d^{-1} on the whole system (a design area requirement of 2 m^2 per person), resulting in an effective load of 80 g $CODm^{-2}$ d^{-1} on the first stage. The findings of the investigations can be summarized as follows:

- Very low and stable effluent concentrations were measured for organic matter and ammonia nitrogen in the effluent of the two-stage CW system, as well as high removal rates for microbial parameters.
- The effluent concentrations of the two-stage system for BOD_5, COD and NH_4^+-N were observed to be lower than the ones of the single-stage VF bed designed and operated according to the Austrian design standard ÖNORM B 2505 [11].
- A whole year round operation of the two-stage system was maintained, without clogging tendencies even during the very cold winter of 2005/2006. It was shown that it is possible to operate the two-stage CW system without any problems even during long winters.
- The Austrian effluent standards [3] could be met during the whole investigation period.
- Additionally, by using the two-stage CW system it was possible to reach about 53% nitrogen elimination and a high nitrogen removal rate in average of 986 g Nm^{-2} yr^{-1} (maximum more than 1′400 g Nm^{-2} yr^{-1}) without recirculation. The nitrogen removal rate is very high compared to reported literature values of 630 g Nm^{-2} yr^{-1} for VF beds [8].

In conclusion it can be said that a higher effluent quality could be reached with the two-stage system as compared to the single-stage VF CW system designed with 4 m^2 per person. The higher effluent quality could be reached although the two-stage CW system was operated with the double organic load resulting in half the specific surface area requirement (2 m^2 per person).

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