Studies on the processes of bacteria elimination in constructed wetlands

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Summary

The bacteriologic purification efficiency of three multistage subsurface flow constructed wetlands with vertical and horizontal reed beds was analyzed by culture independent methods. All wetlands are located in Germany and treat domestic wastewater of 100-1,000 population equivalents. Microscopic enumerations of the wastewater bacteria at different purification stages revealed that bacteria removal efficiency of the vertical beds was high (between 93% and 99.5%), whereas the contribution of the horizontal reed beds to the bacteriological purification of wastewater was low and erratic (average of 46%). However, when assessed with standard cultivation procedures, the purification efficiency of the horizontal reed beds was higher than that of the vertical reed beds (98% vs. 95%). Comparison of the data obtained by microscopic counts with standard cultivation revealed that the fraction of culturable bacteria was especially low in the effluent of the horizontal reed beds. Consequently, the purification capacity of the horizontal beds was significantly overestimated by the standard cultivation procedures.

Moreover, a PCR-based approach for rapid, sensitive and reliable detection of four waterborne, VBNC forming enteropathogenic bacteria (*Campylobacter jejuni*, *Escherichia coli* O157:H7, *Helicobacter pylori* and *Yersinia enterocolitica*) was tested in the wastewater samples. The highest sensitivity was obtained with the protocol for *Y. enterocolitica* with 5 cells per 100 ml of treated wastewater and 200 cells per 100 ml of settled wastewater. The lowest sensitivity was obtained for *E. coli* O157:H7 with 250 target cells per 100 ml of treated wastewater and 10,000 target cells per 100 ml of settled wastewater. All PCR methods could be performed within 12 hours and were specific and reproducible in a background of 10^{11} non-target cells per 100 ml of wastewater.

None of the investigated pathogenic bacteria were detected in the investigated constructed wetlands. Therefore, in order to study defined aspects of the processes of pathogen elimination in vertical beds, the fate of a pathogenic *Y. enterocolitica* strain was investigated in a lab-scale fixed-bed reactor. The reactor was constructed and operated in order to simulate a reed bed for wastewater treatment. The reactor was percolated with primary settled wastewater amended with the pathogenic strain of *Y. enterocolitica*. The TaqMan assay was selected and optimized for tracing *Y. enterocolitica* in the fixed-bed reactor. The assay displayed low detection limits (200 target cells ml^{-1} of wastewater and 400 target cells g^{-1} of filter medium). After three weeks of operation biofilm formation in the reactor bed was low: only 0.16% of organic matter accumulated in the filter and bacterial densities varied between 10^6-10^7 cells g^{-1} of dry weight filter medium. Despite of low biofilm formation in the filter bed, wastewater bacteria were retained at a nearly constant rate (97%). The retention of *Y. enterocolitica* by percolation amounted to 99.8%. A significant elution of bacteria from

the reactor filter bed was not detected. Effluent bacterial populations were constituted by a sub-population of the not retained influent bacteria. A similar retention mechanism seemed to have taken place in the full-scale reed beds. These assumptions are supported by the following data:

- Cultivation and in situ hybridization indicated that the physiologic activity of the filter medium bacteria was lower than the physiologic activity of both the influent and effluent bacteria.
- The morphology of the effluent bacteria was rather similar to a sub-population of the influent bacteria and not to the morphology of filter medium bacteria
- The amounts of influent and effluent bacteria were nearly proportional

After 8 days of percolation with *Y. enterocolitica*, the pathogen accumulated in detectable amounts in the filter bed. *Y. enterocolitica* concentration was highest in 3 cm depth (10^4 cells g^{-1} dw) and decreased to 10^2 cells g^{-1} dw in 30 cm depth. However approximately 99% of the total retained *Y enterocolitica* cells were eliminated in the reactor filter bed. The autochthonous wastewater bacteria were eliminated by approximately 96%. Abiotic factors like low moisture and organic matter content, and relatively high temperatures (25ºC) probably contributed strongly to bacteria elimination in the reactor column. In the full-scale plants, the physical factors were more favorable for bacteria survival in the filter beds, since moisture and organic matter content were higher and temperatures in general lower. On the contrary, antagonistic relationships between bacterial species and predation probably played a more significant role in bacteria elimination in the full-scale filters than in the lab-scale filter. In both the constructed wetlands and the fixed-bed reactor protozoa were almost only detected in the aqueous phase and in spots of the filter beds with high bacterial density. The results derived from the analysis of the full-scale and lab-scale systems suggest that, for long-term operation, the vertical flow operation modus combined with low biofilm formation can constitute a feasible strategy for reliable bacteriologic wastewater purification in subsurface flow constructed wetlands

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Zusammenfassung

Die bakteriologische Reinigungsleistung dreier mehrstufiger Pflanzenkläranlagen mit bewachsenen Bodenfiltern wurde mit kulturunabhängigen Methoden ermittelt. Alle Pflanzenkläranlagen befinden sich im deutschen Raum und reinigen häusliches Abwasser von 100 bis 1.000 Einwohnergleichwerten. Direkte mikroskopische Zählungen der Abwasserbakterien nach jeder Reinigungsstufe zeigten, dass die bakteriologische Reinigungsleistung der vertikalen Bodenfilter hoch war (zwischen 93% und 99,5%). Im Gegensatz dazu war die Reinigungsleistung der horizontalen Bodenfilter unregelmäßig und im Allgemeinen niedriger (durchschnittlich 46%). Die Ergebnisse von standardisierten Kultivierungsverfahren ergaben, dass die durchschnittliche Reinigungsleistung der untersuchten horizontalen Bodenfilter mit 98% höher als die der vertikalen Bodenfilter (95%). Eine Vergleichsanalyse der mit beiden Methoden ermittelten Ergebnisse zeigte jedoch, dass der Anteil an kultivierbaren Bakterien im Ablauf der Horizontalfilter sehr stark abnahm. Demzufolge wurde die Reinigungsleitung der untersuchten horizontalen Bodenfilter mit Standardkultivierung stark überschätzt.

Darüber hinaus wurde an den Abwasserproben ein PCR-Ansatz für die spezifische und sensitive Detektion von vier enteropathogenen Bakterien mit Relevanz in der Abwasserhygiene (*Campylobacter jejuni*, *Escherichia coli* O157:H7, *Helicobacter pylori* und *Yersinia enterocolitica*) getestet. Die höchste Sensitivität wurde mit der Methode zur Detektion von *Y. enterocolitica* ermittelt. Sie betrug 5 Zielzellen pro 100 ml behandeltem Abwasser und 200 Zielzellen pro 100 ml vorgeklärtem Abwasser. Die niedrigste Sensitivität wurde für *E. coli* O157:H7 ermittelt (250 Zielzellen pro 100 ml behandeltem Abwasser und 10,000 Zielzellen pro 100 ml vorgeklärtem Abwasser). Alle PCR Methoden konnten innerhalb von 12 Stunden durchgeführt werden und waren bei einem Hintergrund von 10^{11} Nicht-Zielzellen pro 100 ml Abwasser spezifisch und reproduzierbar. Die untersuchten pathogenen Bakterien wurden in den Pflanzenkläranlagen nicht detektiert. Um dennoch definierte Aspekte der Eliminationsprozesse von pathogenen Bakterien in bewachsenen Bodenfiltern zu untersuchen wurde ein Labormaßstabreaktor gebaut und betrieben. Die Bau- und Betriebsparameter wurden so ausgewählt, dass der Reaktor als Simulation eines bewachsenen Bodenfilters zur Abwasserreinigung fungierte. An dem Reaktor wurde das Schicksal eines in das Abwasser eingeführten enteropathogenen Bakteriums (*Y. enterocolitica*) verfolgt. Für die Detektion von *Y. enterocolitica* im Reaktor wurde die TaqMan PCR ausgewählt und optimiert. Die Sensitivität der Methode war 200 Zielzellen pro ml Abwasser und 400 Zielzellen pro g Filtermaterial. Nach einer Betriebszeit von drei Wochen war die Biofilmbildung im Reaktorkörper gering: 0,16% organisches Material wurde im Filterkörper akkumuliert und die Bakteriendichte variierte zwischen 10^6 und 10^7 Zellen pro g

Trockengewicht des Filtermaterials. Die zulaufende Bakterien wurden jedoch mit einer fast konstanten Rate zurückgehalten. *Y. enterocolitica* wurde zu 99,8% zurückgehalten, während die allgemeine Reduktion von Bakterien bei 97% lag. Eine relevante Auswaschung von Bakterien aus dem Filterkörper wurde nicht festgestellt. Vielmehr setzten sich die Bakterienpopulationen im Ablauf des Reaktors aus einer Subgruppe nicht zurückgehaltener Zulaufbakterien zusammen. Ähnliche Vorgänge schienen in den realen vertikalen Bodenfiltern stattgefunden zu haben. Diese Annahmen werden durch die folgenden Daten unterstützt:

- Kultivierung und in situ Hybridisierung gaben Hinweise darauf, dass die physiologische Aktivität der Bakterien im Filtermaterial niedriger war, als die physiologische Aktivität der Bakterien im Zu- und Ablauf der Filter.
- Die morphologischen Eigenschaften der Bakterien im Ablauf der Filterbetten waren einer Subpopulation der zulaufenden Bakterien (und nicht den Filterbakterien) ähnlich.
- Die Mengen an zu- und ablaufenden Bakterien waren annähernd proportional.

Nach 8 Tagen Beschickung mit *Y. enterocolitica*, konnte das pathogene Bakterium in nachweisbaren Mengen im Filterbett detektiert werden. Die Konzentration war in 3 cm Tiefe am höchsten (10^4 Zellen pro g Trockengewicht) und nahm bis auf 10^2 Zellen pro g Trockengewicht in 30 cm Tiefe ab. Allerdings wurden ca. 99% der gesamten zurückgehaltenen *Y. enterocolitica* Zellen im Reaktorkörper eliminiert. Die zurückgehaltenen autochthonen Abwasserbakterien wurden zu ca. 96% eliminiert. Abiotische Faktoren wie niedriger Feuchtigkeits- und Organikgehalt und relativ hohe Temperaturen (25ºC) trugen wahrscheinlich stark zur Bakterienelimination im Reaktorkörper bei. In den realen bewachsenen Bodenfilter unterstützten die abiotischen Faktoren das Überleben von Bakterien in den Bodenfiltern in größerem Umfang, da sowohl die Feuchtigkeit als auch der Anteil organischen Materials höher und die Temperatur im Allgemeinen niedriger war. Im Gegenteil spielten antagonistische Beziehungen zwischen den verschiedenen Bakterienarten und Fraßdruck vermutlich eine größere Rolle bei der Elimination der Abwasserbakterien in den bewachsenen Bodenfiltern als im Reaktor. Sowohl in den Pflanzenkläranlagen als auch im Reaktor wurden Protozoen fast nur in der wässrigen Phase und an Stellen der Filterkörper detektiert, die eine hohe Bakteriendichte aufwiesen.
Die in den beschriebenen Untersuchungen gewonnenen Erkenntnisse deuten darauf hin, dass vertikale Bodenfilter mit niedrigem Biofilm eine gute Strategie für die langfristige und zuverlässige bakteriologische Abwasserreinigung durch bewachsene Bodenfilter darstellen können.

Table of Contents

Abbreviations

ATCC	American Type Culture Collection, Rockville, MD, U.S.A
(d)ATP	(deoxy)adenosine triphosphate
bp	base pairs
BSE	back scattered electrons
CCUG	Culture Collection University of Goteborg, Department of Clinical Bacteriology, Goteborg, Sweden
Co	DNA copy numbers determined by TaqMan PCR
CSIC	Consejo Superior de Investigación Científica, Madrid, Spain
CSPD	(3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.3.1.^{3,7}]decan}-4-yl)phenyl phosphate).
Ct	fluorescence threshold cycle value
(d)CTP	(deoxy)cytosine triphosphate
Cy3	5,5'-disulfo-1,1'di(γ-carbopentynyl)-tetramethyl-inolocarbocyanine
D10	effective sand grain size 10
D60	effective sand grain size 60
DAPI	4´,6-diamino-2-phenylindole
DIG	digoxigenin-3-O-methylecarbonyl-ε-aminocaproate-N-hydroxysuccinimide ester
dNTP	deoxynucleoside triphosphate
DOC	dissolved organic carbon
DSM	Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany
dw	dry weight
EDX	energy dispersive X-ray spectroscopy
EHEC	enterohemorrhagic *Escherichia coli*
FISH	fluorescence in situ hybridization
(d)GTP	(deoxy)guanidine triphosphate
HRB	horizontal reed beds
IPTG	isopropyle thiogalactoside
kb	kilobase pairs
LB	Luria Bertani

LOI	weight loss on ignition
MPIMG	Max-Planck-Institut für Molekulare Genetik, Berlin, Germany
MPN	most probable number
MTP	municipal wastewater treatment plant
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
p.e.	population equivalents
ppm	parts per million
REM	raster electron microscopy
rpm	rotations per minute
RTP	rural treatment plant
SDS	sodium dodecyl sulphate
SEM	scanning electron microscope
SSI	Statens Serum Institut, Copenhagen, Denmark
STEC	shiga toxigenic *Escherichia coli*
THB	Todd Hewitt broth
TOC	total organic carbon
TSB	Tryptic soy broth
(d)TTP	(deoxy)thyimidine triphosphate
UC	Universidad de Cantábria, Departamento de Biologia Molecular, Santander, Spain
(d)UTP	(deoxy)uridine triphospahte
ULB	Université Libre de Bruxelles, Faculté de Sciences, Génétique des Procaryotes, Brussels, Belgium
UV	ultra violet rays
V	Volt
VRB	vertical reed beds
ZELMI	Zentralinstitut für Elektronenmikroskopie, Berlin Technical University, Germany

I. INTRODUCTION

In the European community, people take the access to clean and abundant water for granted. However, as described by the European Commission for Environment and Water Quality, “all human activities put a burden on water quality and quantity. All polluted water, whether polluted by households, industry or agriculture, returns back, one way or another, to the environment and may cause damage to human health and environment”.

Improperly treated wastewater is a major cause of illness and death particularly in developing countries (Chaudhuri &Sattar, 1990) but developed nations, with high hygienic standards and good sanitary infrastructures, also suffer from waterborne disease outbreaks (Hänninen et al., 2003; Ljungstrom & Castor, 1992). Efficient wastewater sanitation and the separation of wastewater and drinking water sources are thus of great importance for public health affairs worldwide.

Modern wastewater treatment plants are based on the activated sludge technology and in some cases disinfection, displaying a reliable wastewater purification performance. However, rural populations are frequently not connected to these treatment facilities. In order to provide decentralized and economic alternatives for wastewater treatment in rural areas the constructed wetland technology has been developed in the last decades.

The definition of a constructed wetland is “a designed and man-made complex of saturated substrates, emergent and submergent vegetation, animal life, and water that simulates natural wetlands for human use and benefits” (Hammer, 1989).

Constructed wetland systems are designed to maximize the physical, chemical and biological abilities of natural wetlands to reduce the pollutants and microbial pathogens in wastewater. There are basically two types of constructed wetland design: the free water phase and the subsurface flow constructed wetlands. The free water surface wetland typically consists of a basin or channels with a barrier to prevent leakage, soil to support the roots of the emergent vegetation, and water at a relatively shallow depth flowing through the systems. The water surface is exposed to the atmosphere, and the intended flow path through the system is horizontal. Subsurface flow wetlands are comprised of beds filled with porous media and planted with emergent vegetation such as cattails (*Typha spp*.), bulrush (*Scirpus*

spp.) and reeds (*Phragmites spp*.). The water level in the bed remains below the top of the media. The flow path through the operational system can be vertical or horizontal. Design and operation are chosen in order to enable saturation of the porous media in the horizontal flow beds, whereas the porous media in the vertical flow beds are unsaturated. Vertical flow beds should mainly promote aerobic depletion of organic compounds and nitrification, whereas the horizontal flow beds should promote denitrification.

The subsurface flow constructed wetlands emerged as a wastewater treatment technology in Western Europe. Based on the research of Seidl in the 1960s (Seidl, 1966), and of Kikuth in the late 1970s (Kikuth, 1977), these systems have several advantages over the free water phase type: reduction of odors and insects as well as reduction of the risk of exposure of persons and animals to hazardous substances and microbial pathogens. In addition, it is believed that the filter media provide greater available surface area for wastewater treatment than the free water phase concept, thus accelerating treatment responses. As a result, by 1990 about 500 of these systems were set in operation in Germany, Denmark, Austria and Switzerland (Reed, 1993). Subsurface flow systems include on-site single-family units as well as larger systems treating sewage of 1,000 population equivalents or more. This form of treatment plant allows a decentralized treatment of wastewater and requires reduced residue disposal as well as low investment and maintenance costs, making them an attractive alternative for wastewater treatment in rural areas.

The mechanisms of pollutant removal in subsurface flow constructed wetlands include both aerobic and anaerobic microbiological remediation, sorption, sedimentation, volatilization, and chemical transformation (White, 1995). In ideal case, treated wastewater should be free of organic and inorganic pollutants. Moreover, it should contain only a small amount of bacteria and no pathogenic microorganisms. In fact, if suitably operated, subsurface flow constructed wetlands display satisfactory results for the elimination of organic and inorganic compounds and their performance is well documented (Kadlec & Knight, 1996; Demin et al., 2002; Jenssen et al., 2005). Biological oxygen demand, total suspended solids, total nitrogen, and phosphorus, are significantly reduced when wastewater flows through the vegetated subsurface. However, the hygienic quality of wastewater purified by constructed wetlands is not so strictly, if ever, controlled as that of conventional

municipal sewage treatment systems. Consistent data on the hygienic safety of these treatment plants are often scarce.

Sewage can cause illness by direct application onto crop fields and subsequent food contamination (Linnemann et al., 1984). Moreover, surface water and groundwater are continuously used as sources of drinking water (Ibekwe, 2002; Klinger, 2002; Ritter, 2002). Since treated wastewater is discharged into receiving bodies or infiltrates into the soil, it cannot be ruled out that improperly treated wetland run-offs may ultimately contaminate drinking water resources. Additional risk factors associated with wetland systems are the lack of wastewater disinfections, the common practice of promoting the treatment sites as recreational areas, and the role of animals as disease carriers. Hence, the quality and reliability of microbiological purification of constructed wetland systems should be more strictly controlled.

The processes of elimination of microorganisms in subsurface flow constructed wetlands can be divided into two main steps: i) the microbes are removed from wastewater by immobilization in the porous media of the vegetated beds, and ii) the microbes are eliminated in the beds.

i) As for the first purification step, bacteriological investigations on the microbial removal capacity of constructed wetlands were performed in the last years. By means of conventional culture methods, the studies were based on the detection of standard indicator bacteria (Kadlec & Knight, 1996; Hagendorf et al, 2000, Hill & Sobsey, 2001; Arias et al., 2003). Only occasionally pathogens like *Salmonella* (Hill & Sobsey, 2001, Pundsack et al., 2001), *Clostridium spp*. (Barrett et al., 2001), coliphages (Barret et al., 2001, Quinonez-Diaz, 2001), *Giardia spp.* and *Cryptosporidium spp*. (Thurston et al. 2001) and *Campylobacter* (Hagendorf et al., 2000) were also investigated. However, traditional indicators (*E. coli*, coliforms and fecal enterococci) may sometimes have shorter life times than pathogens: Stenström & Carlander (2001) reported a survival time of 252-396 days for *Clostridum perfringens* in the sediment of a free water surface constructed wetland, whereas *E. coli* and fecal enterococci only survived 27-53 days under the same conditions; Karapinar and Gönül (1991) reported that, when incubated in sterile spring water, *Y. enterocolitica* grew in the first three weeks and survived 64 weeks, whereas after 1 week of incubation the number of *E. coli* in water started to decrease and after 13 weeks no *E. coli* was detected.

Additionally, limitations intrinsic to the cultivation-based methods may hamper accurate detection of pathogens: interference of background flora, impossibility to discriminate between pathogenic and nonpathogenic strains and the very long cultivation steps (Rompre et al., 2002). A good example is the isolation of *Yersinia enterocolitica* from water and sewage samples, for which the usual procedure comprises a 21 days pre-enrichment step at 4°C, followed by a second cultivation for at least 48 h. Putative *Yersinia*-colonies must be submitted to biochemical tests for identity confirmation. Further biochemical tests are necessary for the speciation of the genus *Yersinia* and serological tests for the identification of pathogenic serovars (Schulze, 1996).

Standard cultivation-based detection methods are based on the assumption that living cells are basically able to grow/replicate. However, the ability of bacteria to persist in the environment in a viable but nonculturable (VBNC) state is widespread (Byrd et al., 1991; McKay, 1992; Colwell, 2000). When in this state, bacteria are still viable in the sense that they exhibit metabolic activity and respiration, but can no longer be recovered as colony forming units (CFU) when plated on conventional growth media. The VBNC state is a survival strategy adopted by microorganisms when exposed to environmental stress such as low nutrient conditions and unfavorable temperature (Roszak & Colwell, 1987; Barer et al., 1997, Oliver, 2005). VBNC formation has been reported for a number of important bacteria in water hygiene such as *Campylobacter jejuni* (Cappelier and Federighi, 1998), *Yersinia enterocolitica* (Smith et al., 1994), *Vibrio cholerae* (Colwell et al., 1996), enteropathogenic *E. coli* (Pommepuy et al., 1996), *Helicobacter pylori* (Adams et al., 2003), and *E. faecalis* (Signoretto et al., 2000). Pathogenic bacteria that converted to VBNC forms may still be virulent. This phenomenon has been observed for *Campylobacter* spp. (Talibart et al., 2000), *Vibrio vulnificus* (Oliver & Bockian, 1995), *Shigella dysenteriae* (Rahmann et al., 1996), *Legionella pneumophila* (Steinert et al., 1997), and *E. faecalis* (Pruzzo et al., 2002). Hence, cultivation may be in many cases unable to detect active, pathogenic bacteria in environmental samples. In this context culture independent, rapid and specific tests are required as reliable alternatives to cultivation for water and wastewater quality assessment studies.

ii) The second purification step consists in the elimination of bacteria once they were retained in the porous media of the vegetated beds. This aspect is often neglected in

the investigations. Once retained in the filter, bacteria, if not eliminated, can travel long distances in the soil (Celico et al., 2004; Stevik et al., 2004) and be eventually reintroduced in wastewater by resuspension (Stenstrøm & Carlander, 2001). Moreover, allochthonous bacteria can survive for long periods in soil-like environments (Celico et al., 2004; Matos et al., 2005) under unfavorable conditions. This fact raises the question if pathogenic bacteria retained in the vegetated beds can possibly adapt and survive, constituting a cause of soil and water contamination. Therefore, this issue should be a matter of more concern.

Several factors are thought to contribute to the elimination of pathogens in these systems: besides of abiotic factors like temperature, pH, moisture content, composition of the filter media and its organic content (Campbell et al., 1976; Reddy et al., 1981; Kristiansen, 1981; Tate, 1978), several biotic factors are pointed out to influence the elimination of pathogenic bacteria in filter systems. The most relevant biotic factors are the survival capacity of each species (Karapinar & Gönul, 1991; Terzieva & McFeters, 1991; Stenström & Carlander, 2001), antagonistic relationships (Rudolfs et al., 1950; Thompson et al., 1990), and predation (Acea et al., 1988; Decamp et al., 1998; Borno et al., 2004; Stevik et al, 2004). However, there is little information on the contribution of each of these factors to the overall reduction of bacteria in constructed wetland systems.

In conclusion, there is a strong need for systematic analyses on the removal processes and the fate of pathogenic bacteria percolating through subsurface flow constructed wetlands and to assess the health impact of these treatment plants. Investigations should however not be based on culture methods in order to avoid bias of culturability.

In the 1980’s the development of nucleic acid based technology like PCR (Mullis et al., 1992) and fluorescent in situ hybridization (Pace et al., 1986; Olsen et al., 1986) have opened new possibilities to study complex microbial systems without the need for cultivation. Nucleic acid based technologies provide knowledge on the absolute composition of microbial communities, on their abundance and structure. The dominant technique in environmental molecular biology is PCR. Up to date, PCR has been applied to innumerable environmental samples providing new insight in the dynamics of the natural microbial communities, thus constituting a potentially good alternative to cultivation for the analysis of environmental samples.

AIMS

The aim of this work was to provide more insights into the processes of bacteria elimination in subsurface flow constructed wetlands.

The wastewater purification capacity of three subsurface flow constructed wetlands should be evaluated. Several factors associated with the elimination process should be studied in more detail: the contribution of each type of reed bed to the general elimination performance, the influence of the operation time on the elimination performance and the fate of the bacteria immobilized in the vegetated beds. In particular the elimination of potential enteropathogenic bacteria associated with waterborne disease outbreaks should be tracked.

All investigations should be carried out with culture-independent techniques. The potential of nucleic acid technologies, especially of PCR, as a feasible alternative to cultivation, should be evaluated. For the development of the culture independent approach four pathogenic bacteria should be used: *Campylobacter jejuni*, *Helicobacter pylori*, *Escherichia coli* O157:H7, and *Yersinia enterocolitica* serogroup O:3. These four pathogens have two features in common: all have been associated with waterborne diseases (Blaser et al., 1982; Thompson & Gravel, 1986; Mead & Griffin, 1998; Brown, 2000) and all can form VBNC forms (Smith et al., 1994; Cappelier & Federighi, 1998; Kolling & Matthews, 2001; Nilsson et al, 2002).

Finally, a laboratory-scale simulation of a subsurface flow wetland bed should be constructed and operated in order to study in more detail the mechanisms governing the removal and elimination or survival of enteropathogens in the vegetated beds.

II. MATERIAL AND METHODS

1 Treatment systems

1.1 Full scale systems

Four full-scale treatment systems were investigated: the municipal wastewater treatment plant (MTP) Ruhleben and the rural treatment plants (RTPs) Wiedersberg, Ettenbüttel and Ließen. All wastewater treatment facilities are located in Germany. Ruhleben is located in the city of Berlin. Wiedersberg, Ettenbüttel and Ließen are located in rural areas in the states of Saxony, Lower Saxony and Brandenburg, respectively.

1.1.1 Rural treatment plants (RTPs)

The rural treatment plants investigated in this work are reed bed systems. A diagrammatic representation of the RTPs investigated is shown in Figures 1, 2 and 3. All vertical flow and horizontal flow beds are planted with reed (*Phragmites australis*).

At the RTP Wiedersberg (Figure 1) primary settling takes place in a multi-compartment septic tank.

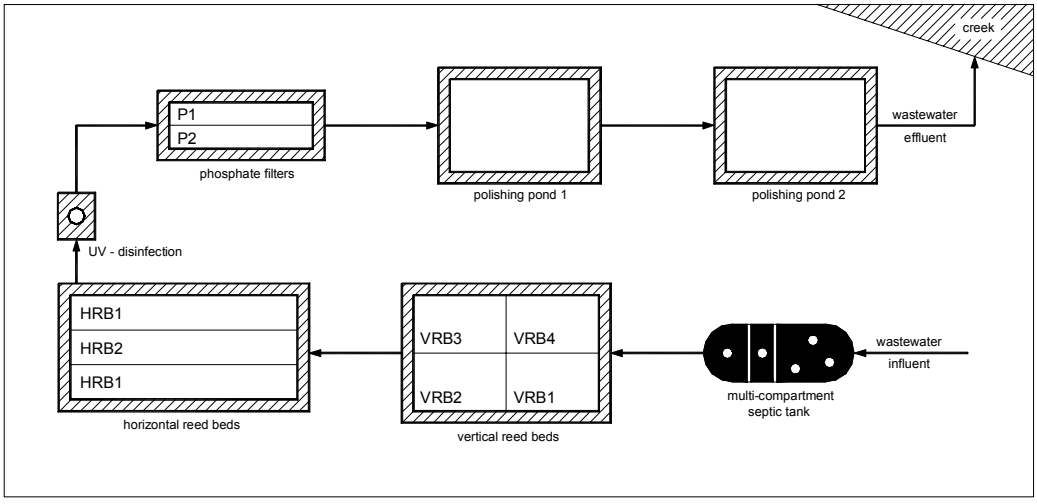


Figure 1: Diagrammatic representation of the rural treatment plant Wiedersberg. Arrows indicate the path of wastewater during purification.

The second wastewater purification step consists of percolation through four vertical flow and subsequently three horizontal flow reed beds. Further

purification/disinfection is carried out by a UV-lamp. Additionally, phosphorus removal is enhanced by a phosphorus precipitation filter. Treated wastewater flows into two polishing ponds and remains there for two to three days before it is discharged into a receiving stream.

At the RTP Ettenbüttel (Figure 2) primary settling takes place in wastewater lagoons 1 and 2. Wastewater lagoon 3 was not in operation during the period of investigation. Afterwards, settled wastewater is percolated through the vertical reed beds VRB1 and VRB2 and directly discharged into a receiving stream.

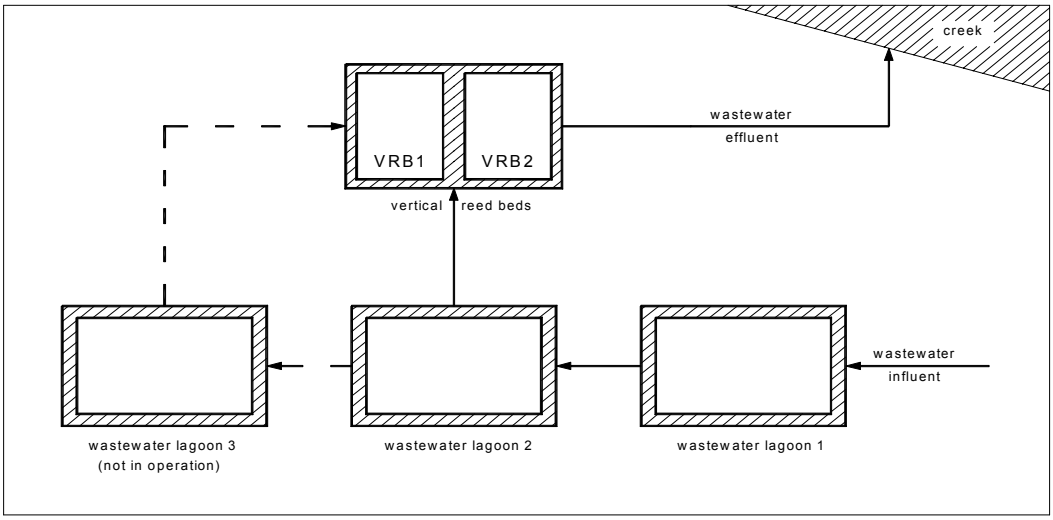


Figure 2: Diagrammatic representation of the rural treatment plant Ettenbüttel. Arrows indicate the path of wastewater during purification.

At the RTP Ließen (Figure 3) primary settling takes place in an Imhofftank. A screen has previously removed bigger solid waste particles. The primary settled wastewater percolates through the vertical flow and subsequently through the horizontal flow reed beds. Treated wastewater infiltrates into the ground by means of an infiltration shaft.

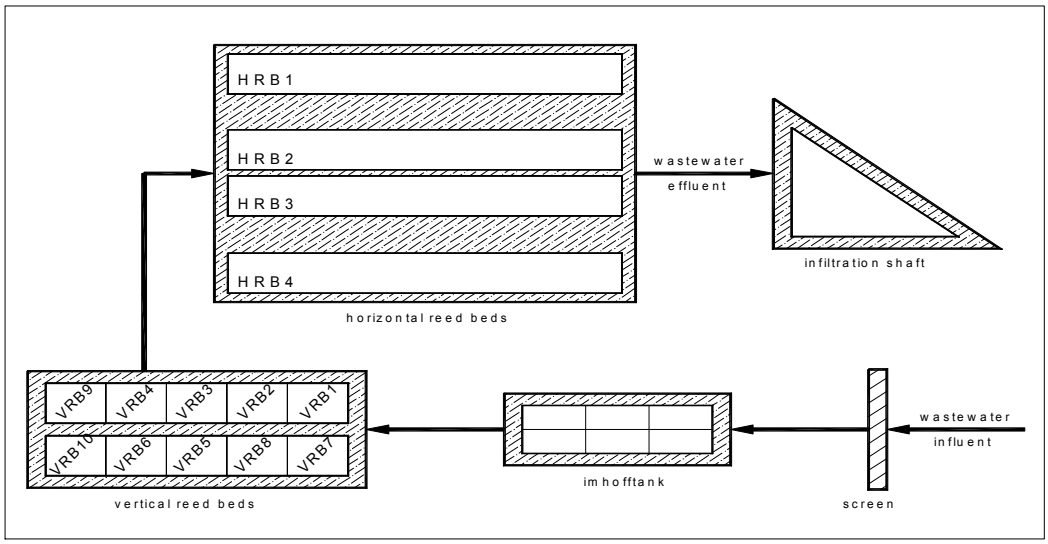


Figure 3: Diagrammatic representation of the rural treatment plant Ließen. Arrows indicate the path of wastewater during purification.

The main technical and operational data of each of the investigated RTPs are summarized in Table 1.

Table 1: Technical data and operation parameters of the rural treatment plants.

	RTP Wiedersberg [a)]	RTP Ettenbüttel [b)]	RTP Ließen [c)]
Year of construction	November 1998	1970's (lagoons) 1999 (reed beds)	1994
Population equivalents (p.e.)	145	1,000	150
Primary settling	Multi-compartment settling tanks	Wastewater lagoons	Imhofftank
Vertical reed beds (VRB)	4 x 116 m^2 0.5 m depth	2 x 1,125 m^2 0.5 m depth	10 x 60 m^2 n.f.
Seizing VRB	2.9 m^2/p.e.	2.25 m^2/p.e.	4 m^2/p.e.
Horizontal reed beds (HRB)	2 x 270 m^2 0.6 m depth	n.p.	4 x 174 m^2 n.f.
Seizing HRB	3.7 m^2/ p.e.	n.p.	4.6 m^2/ p.e.
Discharge site	creek	creek	infiltration in the soil
Type of irrigation	intermittent, discontinuous	intermittent, continuous	intermittent, discontinuous
Wastewater charge	15 – 35 m^3/d	184 m^3/d	8 m^3/d
Type of wastewater	domestic sewage infiltration water rainwater	domestic sewage rainwater	domestic sewage rainwater

n.p. = not present; n.f. = data not found; Data sources: [a)] Rustige & Platzer (2001); [b)] Kayser (2003); [c)] Platzer (1997)

The reed beds of the RTP Ließen (Table 1) have been in operation 4 years longer than in the RTP Wiedersberg and Ettenbüttel. Ettenbüttel is the biggest plant, treating wastewater of 1,000 population equivalents, whereas the RTPs Wiedersberg

and Ließen have smaller dimensions and less population equivalents connected (145 in Wiedersberg and 150 in Ließen). Dimensioning of the vertical reed beds varies between 2.25 m^2 per population equivalent in the RTP Ettenbüttel to 4 m^2 per population equivalent in the RTP Ließen. The dimensioning of the horizontal reed beds is also more generous in the RTP Ließen: 4.6 m^2 per population equivalent versus 3.7 m^2 per population equivalent in the RTP Wiedersberg. The reed beds of the three treatment plants are intermittently irrigated. The vertical reed beds are discontinuously irrigated, i.e. only three of four reed beds are in operation at any given time. The fourth reed bed is set out of operation for approximately one week. All three RTPs treat combined sewage (mainly domestic wastewater and rainwater). The hydraulic application rate is lower in Ließen than in Wiedersberg and Ettenbüttel.

Table 2 to Table 4 summarize the average values for the standard chemical parameters determined for the wastewater derived from the investigated RTPs at different purification stages.

Table 2: Mean routine parameters of the main components of the rural treatment plant Wiedersberg.

	COD mg l^{-1}	BOD_5 mg l^{-1}	TOC mg l^{-1}	Total N mg l^{-1}	NH_4-N mg l^{-1}	N_{org} mg l^{-1}	N_{anorg} mg l^{-1}
Influent	463	215	134	75	31	40	35
Influent VRB	232	135	115	38	27	10	28
Effluent VRB*	21	< 5	8	31	1	6	25
Effluent HRB	22	< 5	13	21	2	5	16
Final effluent	17	< 5	7	22	0.2	6	16

VRB = vertical reed beds; HRB = horizontal reed beds; *: the outlet of the vertical beds is the inlet of the horizontal beds; Data source: Hagendorf et al. (2002).

Table 3: Mean routine parameters of the main components of the rural treatment plant Ettenbüttel.

	COD mg l^{-1}	BOD_5 mg l^{-1}	Total N mg l^{-1}	NH_4-N mg l^{-1}	NO_2-N mg l^{-1}	NO_3-N mg l^{-1}	PO_4-P mg l^{-1}
Influent	825.0	467.5	94.0	63.6	0.128	0.506	11.9
Influent VRB*	175.5	76.81	36.0	15	0.061	0.23	4.82
Effluent VRB	33.4	5.31	9.3	1-2	0.080	10.38	2.11

VRB = vertical reed beds; the influent to the VRB is the effluent of the wastewater lagoons. Data source: Kayser (2003).

Table 4: Mean routine parameters of the main components of the rural treatment plant Ließen.

	COD mg l^{-1}	BOD_5 mg l^{-1}	DOC mg l^{-1}	Total N mg l^{-1}	N_{org} mg l^{-1}	N_{anorg} mg l^{-1}
Influent	417	237	n.d.	n.d.	n.d.	n.d.
Influent VRB	396	187	45-48	65-95	10-20% of total N	0-5
Effluent VRB*	n.d.	161	19-40	0.7-1.1	n.d.	20-50% of total N
Effluent HRB	n.d.	59	20-28	n.d.	n.d.	n.d.

n.d. = not determined; *: the outlet of the vertical beds is also the inlet of the horizontal beds. Data source: Platzer (1997). VRB = HRB = horizontal reed beds

1.1.2 Municipal wastewater treatment plant (MTP)

The municipal wastewater treatment plant (MTP) Ruhleben is a conventional activated sludge plant including mechanical and biological purification stages. It is basically composed of a screen, a grit chamber, rectangular tanks for primary settling, aeration tanks with anaerobic, anoxic and aerobic zones and three different final clarifiers. The MTP Ruhleben purifies the wastewater of 1.3 millions population equivalents. Wastewater influent capacity amounts to 240,000 m^3 d^{-1}. Table 5 summarizes the mean analytical parameters for the influent and effluent of the MTP Ruhleben.

Table 5: Mean routine parameters for influent and effluent of the MTP Ruhleben.

	COD mg l^{-1}	BOD_5 mg l^{-1}	Total P mg l^{-1}	Total N mg l^{-1}	NH_4-N mg l^{-1}	NO_3-N mg l^{-1}
Influent	881	350	11.4	66.9	41.9	-
Effluent	44	5	0.35	11.74	0.64	7.8

1.2 Lab-scale system

In order to investigate the elimination of pathogenic bacteria in reed beds a lab-scale model of a vertical filter bed was constructed for continuous operation under controlled conditions. The lab-scale fixed-bed reactor was operated for 29 days. Construction parameters and hydraulic load were adjusted in order to simulate a vertical flow reed bed of the RTP Wiedersberg type. During operation, the removal of microorganisms from the influent and their persistence or death in the filter were

investigated. The elimination of the pathogen *Y. enterocolitica* was studied in more detail.

1.2.1 Construction data

The reactor, a Plexiglas hollow cylinder with the dimensions of 10 cm inner diameter and 90 cm height (Figure 4), was filled with a 10 cm pebble layer, on the top of which a 45 cm sand layer was placed.

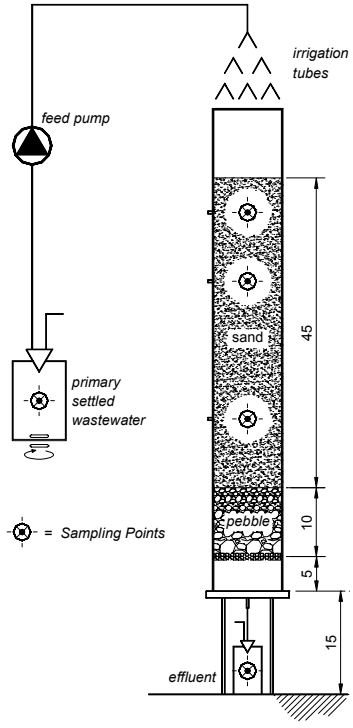


Figure 4: Diagrammatic representation of the fixed-bed reactor. Values are given in cm.

Four pebble diameters were used (from the bottom to the top): 16-32 mm, 8-16 mm, 4-8 mm and 2-4 mm. The reactor contained 3,500-cm^3 white quartz sand with a 0/2 mm grain spectrum and a clay and silt content of less than 5%. Bulk density was determined by weighing defined volumes of dry sand and amounted to1.53 g cm^{-3}. Before being used as filling material for the reactor, sand and pebbles were saturated with water and autoclaved twice at 121°C for one h. After sterilization excess water was removed and sand and the 2-4 mm pebbles were dried at 80°C to the previous weight. Sterility control consisted of mixing 1 g of sand and 10 g of small pebbles with 1 ml sterile saline (0.9% NaCl solution), vigorous shaking and plating of

serial dilutions on full medium agar plates (see Table 6 for composition). Plates were incubated for 3 days at room temperature. The sand and pebbles were held by a Plexiglas support plate. The plate was perforated with 10 mm wide pores for water passage. Influent percolating through the reactor was collected in a sterile glass bottle. The Plexiglas column was perforated along the longitudinal axis with 1 cm diameter holes for filter media sampling, corresponding to 10, 20 and 40 cm depth.

1.2.2 Operation conditions

Influent was fed to the reactor using a peristaltic pump controlled by an automatic time and volume controller. Irrigation was performed top-to-bottom. Wastewater was distributed uniformly over the reactor surface by using several irrigation tubes with small diameter. During all the experiments the irrigation set for the feed was 20 ml h^{-1}, corresponding to a hydraulic loading rate of 61 l m^{-2} d^{-1}.

The reactor was accommodated with sterile tab water for 8 days. Thereafter, irrigation was carried on for 20 days with primary settled wastewater collected at the RTP Ließen. During the last 8 days of operation, the influent was amended with an overnight culture of *Yersinia enterocolitica* DSM 11502 to a final concentration of 10^5-10^6 cells per milliliter of influent, as determined by total cell counts. Afterwards the reactor was sacrificed for collection of larger amounts of filter medium.

Primary settled wastewater was stored at 4°C in the dark and kept at approximately 15°C during the feed. The influent vessel was changed every day.

2 Sample collection

All samples taken at the full-scale treatment plants were transported at 4°C and processed within 12 h. The samples derived from the fixed-bed reactor and the batch assays were immediately analyzed or frozen at -20°C.

2.1 Rural treatment plants

Wastewater samples were taken with a sampling bucket from the influent (Ettenbüttel), the upper 50 cm wastewater layer of the primary settling tanks or the effluent of the wastewater lagoons, from the effluent of each set of reed beds, and (for Ließen) from the final effluent at the outlet. Effluent samples of the reed beds were mixed samples derived from all vertical or all horizontal beds.

The RTP Wiedersberg was sampled in the period between July 1999 and January 2001, the RTP Ettenbüttel was sampled between August 1999 and January 2001

and the RTP Ließen was sampled between May and December 2001. Wiedersberg was sampled 12 times (corresponding to 36 samples), Ettenbüttel 8 times (corresponding to 24 samples) and Ließen 5 times (corresponding to 15 samples). Samples were taken at different times of the day between 10 a.m. and 16 p.m. Once Wiedersberg was sampled in the same day (May 23rd 2000) at 10:00 a.m. and 15:30 p.m. Each time a total of 5 x 500 ml primary settled wastewater and 5 x 2,000 ml effluent of each set of reed beds were collected. Each sample was a mixture of 4–10 independent bucket contents. Primary settled wastewater used to feed the fixed-bed reactor was collected in larger amounts (10 x 3 L) from the RTP Ließen. All wastewater samples were transferred to 1 L or 5 L sterile glass bottles to enable air penetration.

Filter media samples were taken from the vertical reed bed 1 of Wiedersberg and vertical reed bed 2 of Ließen. Each time, a soil core of 3 cm diameter and 40-50 cm depth was taken from the filter beds and a total of six mixed samples corresponding to 0-4, 4-8, 8-12, 12-16, 16-20 and 40-44 cm depth were taken with a sterile scoop from the interior of the core and transferred to sterile 50 ml Falcon tubes.

2.2 Municipal treatment plant

The MTP Ruhleben was sampled twice. Each time a total of 12 x 10 ml primary settled wastewater and 12 x 60 ml treated wastewater was collected. Primary settled wastewater was transferred to sterile 50 ml Falcon tubes. Treated wastewater was transferred to 500 ml sterile glass bottles.

2.3 Laboratory fixed-bed reactor

Influent and effluent wastewater samples (each 100 ml) were taken every two days. Influent samples were taken at two different times: immediately after the influent was connected to the feed pump and after 24 h, before exchange for a new influent vessel.

Filter medium samples were taken every 3 or 4 days before addition of *Y. enterocolitica* to the influent and 1, 3, 5, and 7 days after addition of *Y. enterocolitica*. 2 x 500 mg filter media samples were taken with sterile scoops from each 10, 20 and 40 cm depth. At the end of the experiment the reactor was opened. Mixed samples of approximately 50 g of filter medium were removed from the interior of the core in 3, 10, 20, 30, and 40 cm depth. Samples were placed in sterile 50 ml Falcon tubes.

3 Determination of physical and chemical parameters of the reactor

3.1 Determination of the hydraulic residence time

The mean hydraulic residence time in the fixed-bed reactor was determined in the first operation phase, during accommodation with sterile tab water. After three days of accommodation, the reactor was fed for 12 h with a 100 mg l^{-1} sodium bromide solution (Merck) prepared with sterile tab water. Afterwards, the column was irrigated again with sterile tab water. The effluent samples (15-20 ml) were collected in glass bottles every hour during 24 h. The bromide content in the water samples was measured according to the manufacturer's instructions. The bromide and the reference electrode were from Mettler-Toledo. The calibration curve was performed with standard sodium bromide solutions (1, 2, 5, 10, 20, 50, and 100 mg l^{-1}).

3.2 Determination of organic carbon content in wastewater samples

Total and dissolved organic carbon was measured in the influent and effluent reactor samples according to DIN 38409 DEV H3. The organic carbon was oxidized with O_2 at 1020°C. The resulting CO_2 was quantitatively reduced with H_2 to CH_4. CH_4 was quantified by flame ionization detection.

3.3 Sand sieve analysis

A sand sieve analysis was performed for the sterilized sand used as reactor filter medium according to DIN 19683. Briefly, sand samples were passed through a set of sieves of appropriate mesh sizes. Sieves were arranged in downwards decreasing mesh diameters. The sieves were mechanically vibrated. The weight of sediment retained was measured and converted into the percentage of the total sediment sample.

3.4 Determination of the interstitial space in the sand column

The interstitial space in the filter bed was determined empirically by filling a miniature column of known volume with sand and by adding water until saturation was reached. Sand and water were stirred carefully in order to remove air from the pores. The volume of water supported by the sand was equaled to the pore space and porosity was defined as the percentual volume occupied by the water.

3.5 Weight loss on ignition and water content of filter media

Weight loss on ignition (LOI) and water content were determined for the reactor filter medium in different depths according to the Standard Methods for the Examination of Water and Wastewater (1995). 10 g samples were taken from each 3, 10, 20, 30 and 40 cm depth at the end of the experiment. The blank sample was 10 g sterilized filter medium. Samples were weighed in porcelain crucibles and incubated over night at 105°C, cooled in a desiccator and weighed again. Afterwards samples were ashed for 3 h at 550°C in a muffle furnace, cooled in the desiccator and reweighed. The percentage of LOI was calculated according to the equation:

$$\% \text{ LOI} = 100 \times \text{Wd-Wa} / \text{Wd-Wc} \quad \textbf{(1)}$$

where Wd is the sample weight after drying at 105°C, Wa is the weight of the ashed sample, and Wc is the weight of the crucible. The percentual water content (% H_2O) of the samples was calculated according to the equation:

$$\% H_2O = 100 \times (\text{Ws-Wd} / \text{Ws}) \quad \textbf{(2)}$$

where Ws is the initial wet weight of the sample.

3.6 X-ray fluorescence spectrometric analysis of filter media

Quantitative assessment of the elemental composition of the filter media samples was performed by X-ray fluorescence spectrometry with a Philips PW 1404 fluorescence spectrometer. Samples (0.6 g) were taken after reactor sacrification in 10, 20 and 40 cm depth and the blank sample consisted of sterilized filter medium. After mixing with 3.6 g Spectromelt A12 (Merck) the samples were placed in Pt/Au-crucibles and fused at 1,200°C for 6 min. After cooling samples were analyzed with the software analysis program Oxyquant (Philips) according to the manual's instructions.

4 Scanning electron microscopic analysis (SEM) of filter media

Scanning electron microscopy was applied to the filter medium of the fixed-bed reactor in order to i) visualize its surface and ii) to carry out an element analysis of the grains by energy dispersive X-ray analysis.

4.1 Sample preparation

2 g filter media collected at the end of the experiment (depths 10, 20 and 40 cm) and a blank sample (2 g sterilized filter medium) were placed in sterile glass petri dishes and covered with 3.7% (v/v) formaldehyde diluted with phosphate buffered saline (PBS, per liter: 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0,24 g KH_2PO_4; pH 7.4). Samples were fixed at room temperature. After 1.5 h incubation formaldehyde was carefully removed and samples were subjected to dehydration using a 20%, 30%, 40%, 50%, 70% and 96% (v/v) ethanol series. Changing of the ethanol solution was performed carefully using a pipette. Samples were air dried and kept at room temperature. Before analysis, samples were consolidated to aluminum carrier dishes (Plano) and coated with elemental carbon.

4.2 SEM analysis

The SEM analysis was performed at the Zentralinstitut für Elektronenmikroskopie of the Berlin Technical University, Germany, under the supervision of Dr. Jörg Nissen.
SEM analysis of the prepared samples was performed with a Hitachi S-2700 scanning electron microscope, equipped with a back scattered electron (BSE) detector, and digital image processing (4096 x 4096 pixel, 12 Bit/pixel) from DIPS (Point Electronic). Samples were visualized at different magnifications.

4.3 Energy dispersive X-ray analysis (EDX)

Energy dispersive X-ray analysis was performed with the prepared filter media samples during SEM analysis using the Hitachi S-2700 scanning electron microscope and the IDFix software analysis system (SamX) according to the manual's instructions. Additionally, a point elemental analysis of two filter medium grains of unusual appearance in the BSE image was performed and compared with the overall composition of the sample. Finally, a mapping analysis was performed for the elements Si, Al, Ca, Fe, K, Mn, Na and Ti.

5 Culture media

Table 6 shows the composition of the media used for cultivation of the reference strains and environmental/collected samples. Unless otherwise stated, culture plates were obtained by adding 15-17 g Bacto Agar (Difco) per liter of culture medium. pH was adjusted with HCl or NaOH and media were sterilized at 121°C for 20 min.

Table 6: Composition and reference of the culture media.

Medium	Composition per liter deionized water	Reference
Artificial wastewater	160 mg peptone, 100 mg meat extract, 30 mg urea, 7 mg NaCl, 4 mg $CaCl_2 \times 7H_2O$, 2 mg $MgSO_4 \times 7H_2O$, 28 mg K_2HPO_4	http://europa.eu.int/smartapi/cgi/sga_doc
Blood free *Campylobacter* selective agar base	20.0 g peptone, 3.0 g casein hydrolyzate, 4.0 g charcoal, 5.0 g NaCl, 1.0 g sodium desoxycholate, 0.25 g sodium pyruvate, 0.25 g Fe_2SO_4, 12.0 g agar	Oxoid
Luria Bertani medium (LB)	10.0 g tryptone, 5.0 g yeast extract, 5 g NaCl, pH 7.2	Sambrook et al. 1989
Heterotrophic plate counts medium (HPC)	10.0 g peptone, 10.0 g meat extract, 5.0 g NaCl, pH 7.2	DIN 38411 T.5 1979
Todd Hewitt broth (THB)	9.0 g beef heart digest, 11.0 g pancreatic digest of casein, 3.0 g soy bean peptone, 2.0 g dextrose, 2.5 g $NaCO_3$, 0.5 g NaH_2PO_4, 2.0 g NaCl, pH 7.8	Difco
Yersinia agar base	20.0 g peptone, 2.0 g yeast extract, 20.0 g mannitol, 2.0 g sodium pyruvate, 1.0 g NaCl, 0.003 g neutral red, 0.001 g crystal violet, pH 7.4	Oxoid
Yersinia selective supplement	15 mg cefsulodine, 4 mg irgasan, 2.5 mg novobiocine	Oxoid
YPD medium	20 g peptone, 10 g yeast extract, 0.2% (w/v) glucose, pH 5.8	Zhu & Hannon 2000

6 Microorganisms, culture conditions and storage

Microbial strains used in this work and their sources are listed in Table 7. *Campylobacter jejuni* subsp. *jejuni* DSM 4688 and *Campylobacter coli* DSM 4689 were grown at 37°C in blood free *Campylobacter* selective agar base for 48 h under microaerophilic conditions in Anaerocult C mini bags (Merck). Cultures were stored in Microbank vials at -80°C. *Y. enterocolitica* DSM 11502 was grown overnight in tryptone soy broth, in HPC culture medium or in artificial wastewater with gentle shaking at room temperature or at 37°C. Cultures were plated on HPC medium and incubated for 24 h at room temperature or on supplemented *Yersinia* selective agar basis and incubated for 48 h at 4°C, respectively. *S. cervisiae* was grown on YPD culture medium with shaking at 30ºC. *Enterococcus faecalis* OG1X was grown overnight in THB medium at 37°C with shaking. For selection of the plasmid pMV158 culture plates were supplemented with 4 µg ml^{-1} of tetracycline. *E. coli* JM109 was

grown in LB medium at 37°C with shaking. All other *E. coli* strains listed in Table 8 were grown on LB agar supplemented with the respective antibiotics.

Table 7: Microorganisms used.

Name	Investigated plasmids	Source
Campylobacter jejuni subsp. *jejuni*		DSM 4688
Campylobacter coli		DSM 4689
Escherichia coli O157:H7		GFEA
Escherichia coli JM109		Promega
Helicobacter pylori		CCUG 17874
Yersinia enterocolitica		DSM 11502
Enterococcus faecalis OG1X	pMV158	CIB
Escherichia coli SCS1	RP4	MPIMG
Escherichia coli K12	pULB2432	ULB
Escherichia coli JE2571	R751	MPIMG
Escherichia coli DH5α	R388	UC
Escherichia coli JE723	pJE723	MPIMG
Saccharomyces cervisiae L40cc		MDC

CCUG = Culture Collection University of Goteborg, Department of Clinical Bacteriology, Goteborg, Sweden; CIB = Centro de investigaciones Biológicas, Madrid, Spain; DSM = Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; GFEA = German Federal Environmental Agency; MDC = Max-Delbrück Centrum, Berlin, Germany; MPIMG = Max-Planck-Institut für Molekulare Genetik, Berlin, Germany; UC = Universidad de Cantábria, Santander, Spain; ULB = Université Libre de Bruxelles, Faculté de Sciences, Genetique des Procaryotes, Brussels, Belgium.

Table 8: Antibiotics used for the cultivation of plasmid strains.

Host strain	Investigated plasmids	Antibiotic concentration (μg ml^{-1})
E. coli SCS1	RP4	Kan 40, Tc 10, Ap 30
E. coli JE2571	R751	Tp 20
E. coli DH5α	R388	Tp 20
E. coli K12	pULB2432	Ap 100
E. coli JE723	pJE723	Gent 10
E. faecalis OG1X	pMV158	Tc 4

Ap = ampiciline; Gent = gentamycine; Kan = kanamycine; Tc = tetracycline; Tp = trimethoprime

7 Sample processing

Table 9 gives an overview of the preparation of the different samples and of the corresponding methods applied.

Table 9: Sample preparation and methods for analysis of wastewater and filter media.

Treatment system	Sample	Quantity	Preparation	Analytical method
RTP	Influent / effluent	2 ml	Native processing	PCM
	Influent / effluent	2 ml	Fixation	FISH
	Influent / effluent	500 ml/ 2,000 ml	Native processing Spiking [(a)]	DNA extraction, PCR
	Filter media	1 g	Detachment, fixation	TCC, FISH, EM
MTP	Influent / effluent	1 ml	Fixation	TCC
	Influent / effluent	10 / 60 ml	Native processing Spiking [(a)]	DNA extraction, PCR
Reactor	Influent / effluent	2 ml	Native processing	Cultivation
	Influent / effluent	2 ml	Fixation	PCM, TCC, FISH, EM
	Influent / effluent	50 ml	Native processing	TOC/DOC measuring
	Influent / effluent	2 ml	Native processing Spiking [(a)]	DNA extraction TaqMan PCR
	Filter media	500 mg	Native processing Spiking [(a)]	DNA extraction TaqMan PCR
	Filter media	500 mg	Detachment, native processing /fixation	HPC / TCC
	Filter media	10 g	Native processing	Weight loss on ignition
	Filter media	3 g	Native processing	X-ray fluorescence spectrometry
	Filter media	1 g	Fixation, dehydration	SEM analysis EDX analysis

(a): a part of the samples was spiked with pure cultures of reference strains prior to further processing. DOC = Dissolved Organic Carbon; EDX analysis = Energy Dispersive X-ray spectroscopy; EM = Epifluorescence Microscopy; FISH = Fluorescence in situ Hybridization; HPC = Heterotrophic Plate Counts; PCM = Phase Contrast Microscopy; PCR = Polymerase Chain Reaction; SEM = Scanning Electron Microscopy; TaqMan PCR = Quantitative Real Time 5' fluorogenic TaqMan PCR; TCC = Total cell counts; TOC = Total Organic Carbon.

8 Phase contrast microscopy

All native wastewater samples were examined by phase contrast microscopy with a Zeiss Axioskop or Zeiss Axioplan 2 (Zeiss) at a 400X, 630X or 1,000X magnification. Agar-covered slides were used for sample immobilization if necessary. A semi-quantitative analysis based on directly identifiable morphological traits of bacteria and eucaryotes was performed. Micrographs were taken with a MC80 (Zeiss) camera and a 100 Asa color film (Kodak). Negatives were developed and scanned for printing.

Agar covered microscopy slides were prepared as follows: 3.3 g Bacto agar (Difco) were diluted in 100 ml distilled water and agitated for 10 min at 500 rpm min^{-1}. Afterwards agar was allowed to settle down, water was decanted and the agar was diluted again in 100 ml distilled water. The procedure was repeated five times. At the end of the washing steps, the 100 ml agar solution was autoclaved for 20 min at

121°C and cooled down to approximately 50 °C. A thin layer of liquid agar was applied to the slides and allowed to solidify.

9 Epifluorescence microscopy

Epifluorescence microscopy was performed with a Zeiss Axioplan 2 (Zeiss) fitted with a 100 W high pressure bulb and a Zeiss light filter set no. 01 for DAPI (excitation 365 nm, dichroic mirror 395 nm, suppression 397 nm), no. 09 for Oregon Green 488 and Fluos (excitation 450-490 nm, dichroic mirror 510 nm, suppression 520 nm) and a HQ light filter 41007 (AF Analysentechnik) for Cy3-labeled probes (excitation 535-550 nm, dichroic mirror 565 nm, suppression 610-675 nm). To avoid bleaching effects of the fluorescent markers, the Fluos-labeled cells were counted, followed by the Cy3 labeled cells. DAPI counts were performed at last. Micrographs were taken with a MC80 (Zeiss) camera and a Kodak EES 1600 reversal film (Kodak) - and negatives were developed and scanned for printing - or with a Colorview 12 digital camera (1300 $\times$ 1030 Pixel) and the analysis software AnalySIS, both from Soft Imaging System GmbH.

10 Detachment of microorganisms from filter media

10.1 RTP filter media

1 g wet weight samples was mixed with 3 x 5 ml sterile tab water in a sterile 15 ml Falcon tube and gently inverted by hand for 3 x 1 min. The supernatants were pooled together and centrifuged at 1,000 rpm for 5 min. After the pellet was discarded the supernatant was fixed as described in section 12. Before microscopy samples were concentrated by centrifugation at 14,000 rpm for 2 min.

10.2 Reactor filter media

In a first step 500 mg samples were mixed with 3 x 1 ml modified Neff's amoeba saline (20 mM NaCl, 310 µM $MgSO_4$ x $7H_2O$, 270 µM $CaCl_2$ x $2H_2O$, 9.5 mM Na_2HPO_4 and 9.5 mM KH_2PO_4) in sterile 15 ml Falcon tubes and inverted by hand for 1 min. Supernatants were transferred to another centrifuge tube. Then samples were processed according to the method described by Böckelmann et al. (2003) with modifications: pellets were dispersed with 1.5 ml pyrophosphate solution (7.5 mM $Na_4O_7P_2$ x 10 H_2O and 0.5 g Tween 80 per liter) containing 5 mM EDTA and 0.1% SDS. Samples were shaken on a rotatory shaker (Eppendorf mixer 5432) for 45 min

and subsequently sonicated in an ultrasonic water bath (Bandelin Sonorex RK1028 Transistar) for 20 min. After sonication samples were shaken by hand and allowed to settle for 5 min. The third step consisted of centrifugation at 14,000 rpm for 1 min, discarding of the supernatant and resuspending of the pellet in 1.5 ml modified amoeba saline. Finally, all supernatants were pooled to a final volume of 4.5 ml. 1 ml was frozen at -20°C without further processing, 1.8 ml was fixed and the remaining suspension was used for cultivation of heterotrophic bacteria.

11 Cultivation of wastewater and filter medium bacteria

Bacteria from the influent, the effluent and the filter samples of the fixed-bed reactor were plated with the Eddy Jet Instrument (IUL Instruments) according to the manufacturer's instructions. 100 µl of sample dilution series were spread onto each plate. Each dilution was plated in triplicate. Determination of colony forming units per ml (CFU ml^{-1}) of original sample was performed according to the manufacturer's instructions.

11.1 Cultivation on *Yersinia* selective agar

Serial dilutions of the influent and effluent reactor samples were plated on *Yersinia*-selective culture plates composed of *Yersinia* agar base and *Yersinia* selective supplement (Oxoid). Control plates were streaked with appropriate dilutions of a pure culture of *Y. enterocolitica* DSM 11502. Incubation was performed at 4°C for 48 h in the dark.

11.2 Cultivation on HPC plates

The heterotrophic plate counts were determined according to DIN 38411 T.5. Samples from the influent and the effluent and microbial suspensions detached from the filter medium in 10, 20 and 40 cm depth were serially diluted with sterile saline. Appropriate dilutions were plated on HPC plates, incubated at room temperature for 24 to 72 h and the colony numbers were determined per ml of native sample.

11.3 Proof of culturability for detached bacteria

5 ml of overnight cultures of *Y. enterocolitica* DSM 11502 and *E. coli* JM109 were grown on HPC and LB medium, respectively, to an OD_{600} = 1. Afterwards 1 ml of each culture was subjected to the treatment described in 10.2 but the first step with amoeba saline was omitted. Culturability test consisted of plating triplicate serial

dilutions of the native and the treated bacteria on the respective LB or HPC agar plates. The plates were incubated for 48 h at room temperature for *Y. enterocolitica* and at 37°C for *E. coli*.

12 Fixation procedure

Fixation was performed according to the protocol of Amann et al. (1990) with the following modification: 37% formaldehyde was added at a final concentration of 3.7% to the native samples in order to minimize cell disruption. 2 x 1,800 µl liquid samples from each the influent, the effluent and microbial suspensions were dispersed from the filter media, mixed with 200 µl of 37% formaldehyde and incubated for 1.5 h at 4°C. Afterwards they were centrifuged at 17,000 × g for 1 min, washed twice with 1 ml PBS and resuspended in 500 µl storage buffer consisting of a 1:1 mixture of 96% ethanol and PBS. Fixed samples were kept at -20°C until analyzed.

13 Determination of total cell counts

Native or fixed wastewater samples, microbial suspensions dispersed from the filter media and pure cultures were 10-fold serially diluted, filtered through polycarbonate filters (0.2 µm pore size, Millipore), and stained with DAPI (4´,6-diamino-2-phenylindole, Sigma) at a final concentration of 1 µg ml^{-1}. Enumeration was performed by means of epifluorescence microscopy. For statistical evaluation, at least 10 microscopic fields (125 by 125 µm) and a minimum of 1,000 cells were chosen randomly and enumerated.

14 DNA extraction

DNA was extracted from pure cultures of *Y. enterocolitica* and *E. coli* JM109, from the settled and treated wastewater of the treatment plants Wiedersberg, Ettenbüttel and Ruhleben and from the liquid and solid samples of the fixed-bed reactor. Two different extraction procedures with different lysing principles were tested for their efficiency with the wastewater samples from the RTP Wiedersberg and Ettenbüttel.

14.1 Preparation of pure cultures

Overnight cultures (5 ml) of *Y. enterocolitica* and *E. coli* JM109 were grown in HPC culture medium at room temperature to an OD_{600} = 1. Thereafter 1 ml of each culture was transferred to a Lysing Matrix E Tube of the FastDNA Spin Kit for Soil (Bio 101)

and centrifuged at 14,000 rpm for 1 min. The supernatant was discarded and the pellet was used for DNA extraction.

14.2 Preparation of treatment plant wastewater

For each sample, the original volume was reduced to 500 µl by two centrifugation steps at 4°C: i) 14,000 rpm for 30 min, resuspension in 5 ml phosphate buffered saline (PBS, per liter: 8.00 g NaCl, 0.20 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, pH 7.4) and ii) 14,000 rpm for 5 min. The pellet was washed twice in PBS before resuspension in 500 µl sterile distilled water. The total 500 µl were used for DNA extraction.

As a positive control, half of the samples of the municipal treatment plant (6 x 10 ml settled and 6 x 60 ml treated wastewater) were spiked before centrifugation with 5- to 10-fold dilution series (1 to 1,000 cells per ml) of *C. jejuni* DSM 4688 and *Y. enterocolitica* DSM 11502 cultures, respectively.

14.3 Preparation of reactor samples

Replicate samples of 1 ml wastewater from the influent and the effluent of the fixed-bed reactor were vigorously vortexed twice for 30 s and centrifuged at 14,000 rpm for 2 min. The pellets were resuspended in 1 ml PBS and transferred to SpinTubes of the FastDNA Spin Kit for Soil (FastDNA Kit). After centrifugation at 14,000 rpm supernatant was discarded and pellets were used for DNA extraction.

Filter medium samples (500 mg) were transferred to Lysing Matrix E Tubes of the FastDNA Kit. 1 ml PBS was added and samples were vigorously vortexed for 30 s. After centrifugation at 14,000 rpm for 2 min, supernatant was discarded and samples were DNA extracted without further preparation.

Additionally, in order to determine the recovery rate of the extraction procedure *for Y. enterocolitica*, replicate samples of 5 g were removed after reactor sacrification from 10, 20 and 40 cm depth and amended with each 200 µl of serial dilutions of *Y. enterocolitica.* The final inoculum concentrations were 10^3, 10^5 and 10^7 *Y. enterocolitica* cells per g wet weight of filter medium. Spiked filter medium samples were incubated for 30 min without shaking. After incubation, 500 mg of each sample were prepared and DNA-extracted in the same way as non-spiked samples. *Y. enterocolitica* cells used for inoculation were grown in HPC medium until an OD_{600} = 1, serially diluted with sterile saline and enumerated microscopically after DAPI staining.

14.4 DNA extraction procedures

DNA from the wastewater of the RTP Wiedersberg and Ettenbüttel was extracted with two different, ready to use DNA extraction protocols: the FastDNA Kit complemented with the FastDNA instrument and protocol D of the QIAMP DNA Mini Kit (Qiagen), both according to the manufacturer's instructions. The double amount of reagents was used for each extraction with the QIAMP DNA Mini Kit. DNA was eluted with 100 µl DNAse free, sterile distilled water. Protocol D was used alone and supplemented by proteinase K treatment. In this case, after the precipitation step, Proteinase K (Roche Diagnostics) was added to a final concentration of 50 µg ml^{-1}. After incubation for 1.5 h at 37°C, DNA was extracted with a 25:24:1 (v/v) phenol-chloroform-isoamyle-alcohol mixture and eluted with 100 µl DNAse free, sterile distilled water.

Samples derived from the MTP Ruhleben and from the fixed-bed reactor were extracted only with the FastDNA Kit. DNA from the settled and treated wastewater of the MTP was eluted in 50 µl and 200 µl DNAse free, sterile distilled water, respectively. All other DNA extracts were eluted in 100 µl DNAse free sterile distilled water.

15 Determination of DNA extraction yield and purity

DNA extraction yield was determined i) photometrically at a wavelength of 260 nm and ii) with the E.A.S.Y. Win 32 software (Herolab). An optical density of 1 at 260 nm equaled to 50 µg DNA per milliliter solution. For the computer-based determination 30 µl DNA were loaded onto a 1% agarose gel. A 1 Kb Plus DNA Ladder (Invitrogen) was included as molecular size marker. Gel electrophoresis was carried out at 90 V for 1.5 to 2.5 h. DNA was stained in a 0.5 µg ml^{-1} ethidium bromide solution, visualized under UV light and recorded with the E.A.S.Y. package, consisting of a E.A.S.Y. 429K camera, a ICU-1 integration unit, a SU-1 saving unit and the EASY Analysis software.

For DNA purity assessment, the optical density of all DNA extracts was measured photometrically at 260 nm and 280 nm. The 260/280 nm ratio was taken as an indication for DNA purity. Values ranging from 1.9 to 2.1 indicate high purity; higher ratios indicate contamination with RNA, lower ratios with proteins and/or carbohydrates.

16 PCR analysis of the wastewater samples

The method of the Polymerase Chain Reaction (PCR) was used for four purposes: i) to amplify specific DNA sequences of five model pathogenic bacteria in pure cultures and wastewater samples of the RTP Wiedersberg and Ettenbüttel; ii) to amplify plasmid specific DNA sequences in the wastewater samples of the RTP Wiedersberg; iii) to produce digoxigenine labeled oligonucleotide probes for Southern hybridization and iv) to confirm the identity of bacteria colonies isolated from wastewater samples.

Prior to specific PCR detection of the target bacteria or plasmids, the amplification feasibility and integrity of the extracted DNA was assessed by a PCR assay targeting a 1.5 kb sequence of the 16S rDNA of *Eubacteria*. PCR with the primers 27f and 1492r (Table 10) was carried out according to the protocol of Lane (1991). 1 µl of the DNA extracts was applied per assay.

16.1 Detection of the selected pathogenic bacteria

16.1.1 Preparation of pure cultures for PCR

Serial dilutions of *Y. enterocolitica* overnight cultures were heated at 95°C for 5 min for cell lysis and applied to PCR without further preparation. Dilutions were prepared with sterile bidistilled water. *E. faecalis* was submitted to rapid alkaline lysis prior to PCR: overnight cultures were serially diluted with sterile saline and centrifuged at 14,000 rpm for 2 min at 4°C. The supernatant was discarded and 20 µl of lysis solution (50 mM NaOH and 0.25% SDS) was added to the pellet. Aliquots were incubated for 15 min at 94°C and diluted 10-fold with sterile bidistilled water prior to PCR. Autoclaved cells of *E. coli* O157:H7 were applied to PCR without further preparation. *C. jejuni*, *C. coli* and *H. pylori* were subjected to enzymatic lysis: a colony was picked and treated by 1 mg ml^{-1} lysozyme solution for 15 min at room temperature. Proteinase K was added to a final concentration of 200 µg ml^{-1} and aliquots were incubated for 30 min at 60°C followed by 15 min in a water bath at 100°C.

16.1.2 PCR assays

PCR detection of the selected model bacteria was performed with pure cultures and DNA extracts obtained from the RTP Wiedersberg and Ettenbüttel and the MTP Ruhleben. The primers used for the detection of the model pathogens and the PCR

product sizes are listed in Table 10. In order to determine the sensitivity of each PCR one part of the DNA extracts was additionally spiked with dilutions of the pure cultures prior to PCR. PCR primers were synthesized by MWG Biotech, all other reagents and *Taq* polymerase were from Promega.

Table 10: PCR primers for the detection of target bacteria.

Name	Sequence	Target organism	Product size	Reference
CF03 CF04 CF02	5´-gctcaaagtggttcttatgcnatg-3´ 5´-gctgcggagttcattctaagacc-3´ 5´-aagcaagaagtgttccaagttt-3´	*Campylobacter jejuni/ C. coli*	CF03/CF04: 340-380 bp CF03/CF02: 180-220 bp	Waage et al. 1999(a)
Y1 Y2 P1 P2	5´-ggaatttagcagagatgcttt-3´ 5´-ggactacgacagactttatct-3´ 5´-tgttctcatctccatatgcatt-3´ 5´-ttctttctttaattgcgcgaca-3´	*Yersinia enterocolitica*	Y1/Y2: 0.3 kb P1/ P2: 0.6 kb	Lantz et al. 1998
ACT1 ACT2	5´-cttgctagagtgctgatta-3´ 5´-tcccacactctagaatagt-3´	*Helicobacter pylori*	542 bp	Thoreson et al. 1995
VS1 VS2 VS4 VS5 VS8 VS9	5´-catagtggaacctcacgacgca-3´ 5´-tttgccgaaaacgtaaagcttca-3´ 5´-gggcagttattttgctgtgga-3´ 5´-tgttgccgtattaacgaaccc-3´ 5´-ggcggattagacttcggcta-3´ 5´-cgttttggcactatttgccc-3´	*E. coli* O157:H7	VS1/VS2: 80 bp VS4/VS5: 120 bp VS8/VS9: 150 bp	Sharma et al. 1999
E1 E2	5´-atcaagtacagttagtct-3´ 5´-acgattcaaagctaactg-3´	*Enterococcus faecalis*	941 bp	Dutka-Malen et al. 1995
27f 1492r	5´-agagtttgatymtggctcag-3´ 5´-tacggytaccttgttacgactt-3´	*Eubacteria*	1.5 kb	Lane 1991

m = c:a; n = a:g:c:t; y = c:t; bp = base pairs; kb = kilobase pairs.

All PCRs (Table 10) were performed in a final volume of 50 µl. *Y. enterocolitica* serogroup 0:3 was detected by a multiplex PCR described by Lantz et al. (1998) comprising the amplification of a 0.3-kb *Yersinia sp.* specific 16S rDNA fragment (primers Y1 and Y2) and a 0.6-kb *yadA* gene fragment (primers P1 and P2) encoded by the virulence plasmid pYV. *Helicobacter pylori* was detected with the primers ACT1/ACT2 directed to a specific region of the 16S rRNA gene according to Thoreson et al. (1995) except that dTTP was used in the nucleotide mix instead of dUTP. PCR detection of *E. coli* O157:H7 was performed with the primers described by Sharma et al. (1999) for the fluorogenic 5' nuclease PCR detection of shiga toxigenic *E. coli* strains. An 80-bp sequence of the shiga toxin encoding gene *stx1* and a 120-bp sequence of the shiga toxin encoding gene *stx2* were amplified with the primer pairs VS1/VS2 and VS4/VS5, respectively. *stx1* and *stx2* identify shiga toxigenic *E. coli* strains. A further 150-bp fragment of the intimine encoding gene *eaeA* which is - additionally to *stx1* and *stx2* - present in the enterohemorrhagic

strains was amplified with the primer pair VS8/VS9. Each reaction mixture contained 1 x PCR buffer with 60 mM KCl and 10 mM Tris-HCl (pH 8.3), 200 µM dNTPs, 5 mM $MgCl_2$ and 100 nM of the VS1/VS2 primers, 150 nM of the VS4/VS5 primers or 200 nM of the VS8/VS9 primers. Except for the first denaturation step, which was performed for 5 min at 95°C, the PCR amplification profile was applied to the reaction mixtures without further modifications. *E. faecalis* was detected by amplification of a 941-bp species-specific 16S rDNA fragment as described by Dutka-Malen et al. (1995) with modifications: the reactions contained 0.40 µM of each primer, 500 µM dNTPs, 3.5 mM $MgCl_2$, 1 x PCR buffer with 60 mM KCl and 10 mM Tris-HCl (pH 8.3) and 1 U *Taq* DNA polymerase. All PCR assays directed to *Y. enterocolitica, Helicobacter pylori, E. coli* O157:H7, and *E. faecalis* were performed with 1, 2, 5, 10, and 12 µl of template DNA. PCR was carried out with a Primus 96^{plus} Thermocycler (MWG Biotech). For the detection of *C. jejuni/coli* a semi-nested PCR directed to the intergenic space between the virulence-associated *flaA* and *flaB* genes was performed according to Waage et al. (1999a). For the first reaction with primers CF03 and CF04 (product size 340 to 380 bp), parallel PCRs with 1, 2, 5, 10, and 12 µl template DNA were carried out. The second reaction (product size 180 to 220 bp) with primers CF02 and CF03 was performed with 1-2 µl PCR sample from the first reaction. For the analysis of the results 30 µl of the PCR products were loaded onto 1.0-3.0% agarose gels according to the expected product size length and subjected to electrophoresis for 1 to 2 h at 60 to 80 V. DNA was visualized by ethidium bromide staining (0.5 µg ml^{-1}) under UV light. A 100-bp DNA ladder (Promega) was included as molecular size marker.

16.2 Synthesis of digoxigenin labeled oligonucleotide probes

Digoxigenine (DIG) labeled probes were used for detection of plasmids in wastewater by Southern Hybridization. Labeling was carried out with the PCR DIG Probe Synthesis Kit (Roche Diagnostics). The incompatibility groups and replication types tested and the corresponding template plasmids are listed in Table 11. Incorporation of DIG-11-dUTP during PCR was performed according to The DIG System User's Guide for Filter Hybridization (Roche Diagnostics). The DIG-probes targeted DNA sequences specific to plasmids of different incompatibility (Inc) groups or to the family of the rolling circle replicating plasmid pMV158. The primer set

pMVoriT targeting the plasmid pMV158 and its relatives was developed and tested in this work.

Table 11: Plasmid groups tested.

Inc group / Replication type	Template plasmid	Host	Gene/gene locus
IncPα	RP4	*E. coli* SCS1	trfA2 IncP*oriT*
IncPß	R751	*E. coli* JE2571	trfA2 IncP*oriT*
IncW	R388	*E. coli* DH5α	IncW*oriT*
IncN	pULB2432	*E. coli* K12 lab strain	IncN*rep*
IncQ	pJE723	*E. coli* JE723	IncQ*oriV* IncQ*oriT*
rolling circle	pMV158	*E. faecalis* OG1X	pMV*oriT*

Inc = incompatibility.

The bacterial strains harboring the target plasmids were cultured on agar plates as described in 0. Prior to PCR, colonies of the *E. coli* host strains were picked with a sterile toothpick, diluted in 50 µl sterile bidistilled water and heated at 95° C for 15 min. *E. faecalis* was submitted to an alkaline lysis as previously described. PCR amplification was carried out with the primers listed in Table 12 according to the amplification profiles described in Götz et al. (1996).

Table 12: PCR primers, targets and product size.

Primers	Sequence	Annealing temp. (ºC)	Target site	Product size (bp)	Reference
trfA2	fw: 5´-cgaaattcrtrtgggagaagta-3´ rv: 5´-cgyttgcaatgcaccaggtc-3´	57	replication protein	241	Götz et al. 1996
IncPoriT	fw: 5´-cagcctcgcagagcaggat-3´ rv: 5´-cagccgggcaggataggtgaagt-3´	57	origin of transfer	110	Götz et al. 1996
IncWoriT	fw: 5´-tctgcatcattgtagcacc-3´ rv: 5´-ccgtagtgttactgtagtgg-3´	51	origin of transfer	317	Götz et al. 1996
IncNrep	fw: 5´-agttcaccacctactcgctccg-3´ rv: 5´-caagttcttctgttgggattccg-3´	55	replication protein	164	Götz et al. 1996
IncQoriV	fw: 5´-ctcccgtactaactgtcacg-3´ rv: 5´-atcgaccgagacaggccctgc-3´	57	origin of replication	436	Götz et al. 1996
IncQoriT	fw: 5´-ttcgcgctcgttgttcttcgagc-3´ rv: 5´-gccgttaggccagtttctcg-3´	57	origin of transfer	191	Götz et al. 1996
pMVoriT	fw: 5´-ctacctgtcccttgctgat-3´ rv: 5´-gcagtgccgaccaaaacc-3´	55	origin of transfer	142	This work

Each assay was performed in 50 µl and contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, each 200 µM dATP, dCTP and dGTP, 130 µM dTTP, 70 µM DIG-11-dUTP, 4 µM of each primer, 2 U *Taq* Polymerase and 1 µl of cell suspension. Amplification programs consisted of 94ºC for 5 min, followed by 35 cycles of amplification divided in 1 min of denaturation at 94ºC, 1 min of primer annealing at the temperature according to Table 12 and 1 min of primer extension at 72ºC, followed by a 10 min final extension step at 72ºC.PCR product yield and the success of labeling were estimated according to the manufacturer's instructions. PCR products were purified with the Wizard SV Gel and PCR Clean-UP System (Promega). For product size confirmation and yield estimation 10 µl of the PCR products were loaded onto 2-3% agarose gels according to the expected product size, submitted to electrophoresis for 3 h at 60 volt and stained with ethidium bromide.

16.3 Amplification of plasmid specific DNA sequences in wastewater

The DNA extracts derived from the influent and effluent of the reed beds in the RTP Wiedersberg were PCR amplified with the primers and programs described in 16.2. The reaction composition differed in two aspects: the PCR deoxynucletide mix contained 200 µM of each dATP, dCTP, dTTP and dGTP and no plasmid DNA isolation was carried out. Instead, 1 µl of whole community DNA extract from the influent and effluent of the vertical reed beds and 10 µl DNA from the effluent of the horizontal reed beds were used as templates. 10 µl of the PCR products were loaded onto 3% agarose gels, submitted to gel electrophoresis for 3 h at 60 volt. Plasmids were detected by Southern hybridization of the PCR products followed by chemiluminescence detection of the DIG-labeled hybrids.

16.4 Colony identity proof by PCR

16.4.1 Yersinia enterocolitica serogroup 0:3

Wastewater samples derived from the influent and effluent of the fixed-bed reactor were plated on the supplemented *Yersinia* agar basis plates (see 11.1). The identity of the bacterial colonies was further investigated with the PCR protocol directed to *Y. enterocolitica* serogroup 0:3. After examination of the colony morphology with the help of Prof. Dr. Elke Haller (Charité Medical Institute of the Humboldt University of Berlin) all putative *Yersinia* colonies ("bull eye" appearance) on the effluent plates

and 25% of the putative *Yersinia* colonies on the influent plates were picked randomly with a sterile toothpick and resuspended in 50 µl sterile saline. A *Y. enterocolitica* pure culture plate was taken as reference. Picked colonies were frozen at -20°C before analysis. Colony suspensions were diluted 1:50 with sterile saline before application to PCR. PCR for the identification of *Yersinia enterocolitica* serogroup 0:3 was performed as described before using 1 µl of the colony suspensions per reaction.

16.4.2 Enterococcus faecalis

A total of 25 fecal streptococci colonies isolated from the investigated wastewater samples according to ISO 7899-2 (courtesy of Dr. Regine Szewzyk, Federal Environmental Agency, Department of Berlin) were picked randomly with a sterile toothpick and resuspended in 50 µl sterile saline. Prior to PCR, colony suspensions were treated with lysis solution as previously described and 1 µl of the lysates was applied to PCR directed to *E. faecalis*.

17 Real-Time 5' fluorogenic TaqMan PCR

The Real-Time TaqMan PCR method was selected in order to detect *Y. enterocolitica* in the influent, effluent as well as in different depths of the filter bed of the percolated fixed-bed reactor.

TaqMan PCR was carried out according to the protocol developed by Sen (2000) for the detection of *Y. enterocolitica* in blood samples. The primers and fluorescent probe were designed in order to amplify a 201-bp fragment spanning the nucleotides 47 to 247 of the *Yersinia enterocolitica* 16S rRNA gene.

17.1 TaqMan assay

Prior to TaqMan PCR the samples derived from the fixed-bed reactor were prepared and the DNA extracted as described in 14, followed by DNA yield and purity assessment as described in 15. Afterwards DNA was five to ten-fold serially diluted with sterile bi-distilled water. For each analyzed sample, replicates of two to three appropriate DNA dilutions were applied to 96-wells microtiter plates (Applied Biosystems). All reagents were from Applied Biosystems. PCR was carried out in 50 µl volumes containing 25 µl of universal Master Mix, 400 nM of each primer, 200 nM of the labeled TaqMan probe and 5 µl of template DNA. The forward (16SF) and reverse (16SR) primers had the sequences 5'-CGGCAGCGGGAAGTAGTTT-3' and

5'-GCCATTACCCCACCTACTAGCTAA-3', respectively. The TaqMan fluorescent probe YE1 (5'-FAM-AAGGTCCCCCACTTTGGTCCGAAG-TAMRA-3') is located between nucleotides 166 and 190 (reverse complement) of the 16S rRNA gene from *Y. enterocolitica*. FAM (6-carboxyfluorescein) was the reporter dye and TAMRA (6-carboxytetramethylrhodamine) was the quencher dye. The 3' end of the probe was phosphorylated to prevent extension by *Taq* Polymerase. Amplification program consisted of an initial single denaturation step at 95°C for 10 min to activate Ampli*Taq* Gold, followed by 45 cycles of a two-temperature profile consisting of 15 s at 95°C and 1 min at 60°C. PCR was performed with an AbiPrism 7000 sequence detector (Applied Biosystems), according to the manufacturer's instructions.

17.2 Preparation of the reference DNA

A reference DNA sample was applied to each TaqMan PCR assay and used by the software analysis program for result quantification. *Y. enterocolitica* DSM 11502 was grown on HPC medium until OD_{600} = 1 and enumerated by means of total cell counts. DNA was extracted and serial dilutions of DNA equivalent to 1-10^7 cells were applied to TaqMan PCR.

17.3 Post-PCR analysis

Data were analyzed with the SDS software (PE Biosystems). The software calculated the value for ΔRn using the equation $\Delta Rn = Rn^+ - Rn^-$. Rn^+ is the emission intensity of the reporter divided by the emission intensity of the quencher at any given time of the reaction. Rn^- is the emission intensity of the reporter divided by the emission intensity of the quencher of the same reaction prior to PCR amplification. ΔRn is the increase in emission intensity when the reporter dye is cleaved during polymerization. The ΔRn values were plotted on the *y*-axis, and the time, represented by the cycle number, was plotted on the *x*-axis. The threshold cycle (C_t) value is the noninteger calculation of the number of cycles required for the reporter dye fluorescence to become significantly higher than the background. The C_t values for each reaction were automatically calculated by the default parameters of the program. The correct size of the PCR products was verified by running a sample from each reaction on 2% agarose gels, followed by ethidium bromide staining.

18 Southern blotting

Southern Blotting and the subsequent chemiluminescent detection of the DIG-labeled DNA hybrids were carried out according to The DIG System User's Guide for Filter Hybridization (Roche Diagnostics).

18.1 DNA transfer and fixation

The agarose gels were denatured twice for 15 min in denaturing solution (0.5 N NaOH; 1.5 M NaCl) at room temperature with shaking, washed with bidistilled water and incubated in neutralizing solution (0.5 M Tris-HCl, pH 7.5; 3 M NaCl) twice for 15 min at room temperature. The DNA was transferred overnight to a positively charged Nylon Membrane (Roche Diagnostics) by capillary transfer with 20 x SSC solution (3 M NaCl; 300 mM sodium citrate; pH 7.0). Finally, the DNA was heat fixated on the membrane at 120°C for 20 min.

18.2 DNA-DNA hybridization and chemiluminescent detection

For the DNA hybridization Southern blots were submitted to a pre-hybridization step for 2 h at 42°C in 20 ml of DIG Easy Hyb (Roche Diagnostics) pre-hybridization solution. After discarding the pre-hybridization solution the membrane was hybridized overnight at 42°C. The hybridization solution consisted of 5 ml pre-hybridization solution containing approximately 20 ng ml^{-1} of the DIG labeled oligonucleotide probe. Probes were synthesized as described in 16.2. After the hybridization step the membrane was washed twice for 5 min at room temperature in 2 x wash solution (0.75 M NaCl, 0.075M sodium citrate, 0.04% (w/v) N-lauroyl-sarcosine, 0.4% (v/v) Blocking Reagent (Roche Diagnostics)) containing 0.1% (w/v) SDS and afterwards twice for 15 min in 0.5 x wash solution containing 0.1% SDS. The chemiluminescent detection of the DIG labeled DNA hybrids was performed with the DIG Luminescent Detection Kit (Roche Diagnostics) and CSPD (3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.3.1.^{3,7}]decan}-4-yl)phenyl phosphate). After the hybridization step the membrane was equilibrated for 1 min in washing buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5; 0.3% (v/v Tween 20) and incubated in blocking solution (1% w/v Blocking Reagent (Roche Diagnostics) dissolved in washing buffer without Tween 20) for 30-60 min with shaking at room temperature. The Anti-Digoxigenin-Antibody (750 U ml^{-1} alkaline phosphatase conjugated with Fab-Fragment (Roche Diagnostics)) was diluted 1:10^5 in blocking solution and used for the next incubation step for 30 min at room temperature, after which the membrane was washed twice

with washing buffer for 15 min. The membrane was equilibrated for 2 min in detection buffer (100 mM Tris-HCl, 100 mM NaCl, pH 9.5) and for 5 min in 0.5 ml of a 1:100 CSPD solution in detection buffer. The membrane was covered with Lumi-Film (Roche Diagnostics) for 20 min in the dark and developed with developer and fixation solution for X-ray films (Tetenal) according to the standard procedures.

19 Fluorescence in situ hybridization

Fixed samples of pure cultures, wastewater, and filter media suspensions were subjected to in situ hybridization with the fluorescent oligonucleotide probes listed in Table 13.

Table 13: Fluorescent probes used for whole cell in situ hybridization.

Probe	Target-organism	FA (%)	Labeling	Reference
S-D-Bact-0338-a-A-18[c] (EUB338) [a,b,c]	Domain *Bacteria*	30/35	Cy3, OG	Amann et al. 1990
S-*-BactP-0338-a-A-18[c] (EUB338-II) [a,b,c]	*Planctomycetales*	30/35	Cy3, OG	Daims et al. 1999
S-*-BactV-0338-a-A-18 (EUB338-III) [a,b,c]	*Verrucomicrobiales*	30/35	Cy3, OG	Daims et al. 1999
S-D-Bact-0338-a-S-18[c] (non-EUB338) [a,b c]	Negative control	30/35	Cy3, OG	Amann et al. 1990
S-Sc-aProt-0019-a-A-17[c] (ALF1b) [a,b c]	α-subclass of *Proteobacteria*	35	Cy3	Manz et al. 1992
S-Sc-bProt-1027-a-A-19 (BETA42a) [a,b d]	β-subclass of *Proteobacteria*	35	Cy3	Manz et al. 1992
S-Sc-gProt-1027-a-A-17 (GAM42) [a,b d]	γ-subclass of *Proteobacteria*	35	Cy3	Manz et al. 1992
S-D-Arch-0915-a-A-20 (Arch915) [a,b c]	Domain *Archaea*	35	Cy3	Stahl & Amann 1991
S-P-CyFla-0319-a-A-18 (CF319a) [a,b c]	*Cytophaga-Flexibacter-Bacteroides*	35	Cy3	Manz et al. 1996
S-P-CyFla-0319-b-A-18 (CF319b) [a,b c]	*Cytophaga-Flexibacter-Bacteroides*	35	Cy3	Manz et al. 1996
S-P-Pla-0046-a-A-18 (Pla46) [a,b c]	*Planctomycetales*	35	Cy3	Neef et al. 1998
EUK1209 [a, e]	Domain *Eukarya*	30/35	OG	Giovanonni et al. 1988
EUK502 [a,e]	Domain *Eukarya*	30/35	OG	Amann et al. 1990
Y.ent.16S-184[c]	*Yersinia enterocolitica*	30/35	Cy3	Trebesius et al. 1998

FA = formaldehyde; OG = Oregon Green; [a] = *Escherichia coli* numbering (Brosius et al. 1981); [b] = probe nomenclature as described by Alm et al. (1996); [c] = 16S rRNA probes; [d] = 23S rRNA probes; [e] = 18S rRNA probes.

Probes were either labeled with Oregon Green 488 or with Cy3. Hybridization was performed in a humid chamber at 46°C as described by Manz et al. (1994). Probe

working solutions had a concentration of 50 ng DNA per µl. The fluorescent probe and the hybridization buffer (0.9% NaCl, 20 mM Tris/HCl (pH 7.2), 0.03% SDS, and 30 or 35% formamide) were gently mixed in a ratio of 1:10 (v/v) to obtain a final oligonucleotide concentration of 5 ng DNA per µl. For the probes specific to the β- and the γ-subclass of the *Proteobacteria*, competitor γ- and β-probes were used, respectively, in order to improve the hybridization specificity. Competitor probes were not labeled with a fluorescent marker and were applied in 5-fold excess in relation to the labeled probe. The liquid samples were pipetted on the wells of the Teflon-coated hybridization slides, air dried at 46°C and covered with 10 µl or 20 µl of probe in hybridization buffer. For hybridization of biofilms grown on microscopic slides the probe solutions (30 µl) were directly applied onto the slides in marked areas to enable multiple hybridization on a single slide. All slides were placed in 50 ml Falcon tubes and hybridization took place at 46°C in the dark for 1.5 h to 12 h. Following hybridization the slides were washed with pre-warmed (46°C) washing buffer (20 mM Tris/HCl, 0.01% SDS, and 125 mM or 88 mM NaCl, corresponding to 30 or 35 % formamide stringency, respectively). Microscopic analysis was carried out as described in section 9.

III. RESULTS

Properly designed reed bed systems are reported to significantly reduce concentrations of microorganisms in wastewater. However, these results are derived solely from cultivation approaches. Therefore the wastewater purification potential of reed beds was investigated during a cooperation project involving several interdisciplinary research groups. The aim of the present work was i) to apply culture independent techniques to assess the general and the specific removal of microorganisms from wastewater; ii) to compare the results with those obtained with standard cultivation detection techniques, which were performed by one of the collaborators (Federal Environmental Agency) and iii) to study the processes leading to retention and elimination of bacteria in reed beds.

General bacteria removal performance was assessed by direct microscopic enumerations after DAPI staining. Specific elimination performance of bacterial groups was studied by phase contrast microscopy and fluorescence in situ hybridization. PCR based methods were optimized and applied to wastewater samples in order to detect and quantify pathogenic bacteria. The presence of plasmids belonging to different incompatibility groups was investigated in the wastewater samples of the RTP Wiedersberg at different purification stages. Finally, the microbial communities living in the reed beds of an 1.5 years old and a 6 years old treatment plant were investigated.

Finally, in order to investigate in more detail the processes of retention and elimination of pathogenic bacteria in filter bed systems a lab scale fixed bed reactor was constructed and operated under defined conditions for 20 days. The reactor influent consisted of primary settled wastewater amended with a pathogenic strain of *Y. enterocolitica*. General bacteria removal efficiency was assessed by a combination of total cell counts and heterotrophic plate counts. The removal of *Y. enterocolitica* from the influent and its persistence or elimination in different depths of the filter was tracked using quantitative Real Time 5'-fluorogenic TaqMan PCR.

A) PCR based detection of six pathogenic bacteria in wastewater

Application of cultivation-based methods to detect pathogenic bacteria in environmental samples like wastewater presents several drawbacks such as lack of specificity, failure to detect VBNC forms and long incubation times. The aim of this investigation was to develop a new approach for rapid, specific and quantitative detection of pathogenic bacteria in wastewater. The approach should enable the detection of bacteria independently of their physiological state and constitute a feasible alternative to cultivation for routine assessment of wastewater quality. The adequacy of the approach was evaluated for wastewater derived from different sources. The selected approach comprised three basic steps: i) concentration of the native samples by centrifugation, ii) rapid genomic DNA extraction and iii) PCR detection of 5 selected wastewater relevant pathogenic bacteria.

1 Evaluation of two methods for extraction of total community DNA

Prior to DNA extraction, the wastewater samples derived from the rural treatment plants (RTPs) Wiedersberg and Ettenbüttel were subjected to a centrifugation step, reducing the original volume by 5,000-fold for the settled wastewater samples and by 20,000-fold for the treated wastewater samples. The concentration step should enable the extraction of larger sample volumes and hence to obtain lower detection limits by using highly concentrated DNA in the PCR assays. However, the quality of the template DNA is a crucial factor for successful and reproducible PCR. It could not be ruled out that DNA contained PCR inhibitors. Therefore the quality of the extracted DNA was accurately tested. The DNA extraction protocol should be rapid and not include laborious purification steps. Therefore, two rapid, ready-to-use methods for the extraction of genomic DNA were evaluated for their adequacy to extract pure, inhibitor-free DNA from the wastewater samples of the rural treatment plants Wiedersberg and Ettenbüttel.

The selected methods differed basically in the lysis procedure: cell lysis by the QIAMP DNA Mini Kit is based on lysozyme/SDS/EDTA treatment, whereas cell lysis with the FastDNA Kit was based on bead beating using an EDTA- and SDS-containing lysis buffer. The QIAMP DNA Mini Kit has been developed for Gram-positive bacteria and the FastDNA Kit for soil bacteria. Quality criteria for the extracted DNA were yield, purity and integrity.

1.1 Yield and purity of the extracted DNA

Replicates of 16 native wastewater samples were processed with both the QIAMP DNA Mini Kit and the FastDNA Kit. For each treatment plant, the influent of the reed beds (settled wastewater) and its effluent were investigated. DNA purity was assessed photometrically by the 260/280 nm ratio and DNA was quantified with the E.A.S.Y. Win 32 software after gel electrophoresis and ethidium bromide staining. The intrinsic error of the quantification method was estimated by applying the DNA marker in duplicate on the gel and by quantifying both lanes against each other. The intrinsic error, which can be originated by possible limitations of the software, was estimated to 5%. Results are shown in Table 14 and Table 15.

Table 14: Extraction yield and DNA purity obtained with the two DNA extraction protocols for the wastewater samples of the RTP Wiedersberg. Values are means of duplicate extractions and are expressed in ng of extracted DNA per ml of native wastewater sample.

Sample	Yield FastDNA ng DNA/ml	Purity 260/280 nm	Yield QIAMP ng DNA/ml	Purity 260/280 nm
Settled WW 1	327	1.9	222	2.0
Settled WW 2	97	2.1	61	1.8
Settled WW 3	125	2.0	94	2.3
Settled WW 4	146	2.1	106	2.1
Effluent HRB 1	33	1.9	failed	-
Effluent HRB 2	12	2.2	10	1.5
Effluent HRB 3	18	1.9	9	1.3
Effluent HRB 4	24	1.9	18	1.7

WW = wastewater; HRB = horizontal reed beds

Table 15: Extraction yield and DNA purity obtained with the two DNA extraction protocols for the wastewater samples of the RTP Ettenbüttel. Values are means of duplicate extractions and are expressed in ng of extracted DNA per ml of native wastewater sample.

Sample	Yield FastDNA ng DNA/ml	Purity 260/280 nm	Yield QIAMP ng DNA/ml	Purity 260/280 nm
Settled WW 1	185	1.9	159	1.8
Settled WW 2	179	2.1	82	1.9
Settled WW 3	142	1.9	105	1.7
Settled WW 4	179	2.2	184	1.7
Effluent VRB 1	12	1.9	8	1.5
Effluent VRB 2	17	2.0	14	1.6
Effluent VRB 3	10	2.2	7	1.7
Effluent VRB 4	20	1.9	15	1.3

WW = wastewater, VRB = vertical reed beds

DNA extraction yield with the Fast DNA kit ranged from 97 ng to 327 ng and from 142 ng to 185 ng per ml of settled wastewater derived from the RTP Wiedersberg (Table 14) and Ettenbüttel (Table 15), respectively. Treated wastewater yielded 12 ng to 33 ng per ml of native sample for the samples derived from Wiedersberg and 10 ng to 20 ng for the samples derived from the RTP Ettenbüttel. The 260/280 nm ratio ranged between 1.9 and 2.2, indicating low degree of contamination of the DNA with proteins and/or carbohydrates.

Except for one sample (Ettenbüttel settled WW 4) the extraction yield obtained with the QIAMP DNA Kit was lower. DNA yield ranged from 61 ng to 222 ng and from 82 ng to 184 ng per ml of settled wastewater from the RTP Wiedersberg and Ettenbüttel, respectively. Treated wastewater yielded 7 ng to 15 ng per ml of treated wastewater for the samples of the RTP Ettenbüttel. For one of the Wiedersberg samples (effluent HRB 1) extraction completely failed. This was true for two independent DNA extractions. For the other samples, DNA yield varied between 9 and 18 ng per ml of wastewater. For both treatment plants, the 260/280 nm ratio of the DNA extracted with the QIAMP DNA Kit was satisfactory for the settled wastewater (1.7 to 2.3) but very low for the treated wastewater DNA extracts (1.3 to 1.7), indicating contamination of the DNA with proteins and/or carbohydrates. Additional steps with Proteinase K and a subsequent phenol-chloroform-isoamyle alcohol extraction improved purity of the DNA but increased extraction time and resulted in DNA loss.

1.2 Proof of DNA integrity

As a measure of integrity of the extracted DNA a PCR amplifying a 1.5 kb sequence of the 16S rDNA of *Eubacteria* was performed with each extracted DNA. Amplification of the 16S rDNA was successful for all DNA extracts and resulted in a fragment of the expected size but product yield was lower when the DNA was extracted with the QIAMP DNA Mini Kit. This fact may indicate partial inhibition of the *Taq* Polymerase. Moreover, products obtained with the QIAMP DNA Mini Kit were sometimes accompanied by a smear-like appearance, which usually occurs by nonspecific polymerization.

After testing the efficiency of both extraction protocols with the wastewater samples derived from the treatment plants Wiedersberg and Ettenbüttel, all further samples derived from the rural treatment plants, the municipal treatment plant and the

percolated fixed-bed reactor were extracted with the FastDNA Kit followed by the above mentioned DNA quality and yield control tests.

2 Optimization of the PCR detection protocols

PCR assays targeting specific DNA sequences of five wastewater relevant pathogenic bacteria (*C. jejuni*, *E. coli* O157:H7, *H. pylori*, *E. faecalis* and *Y. enterocolitica*) were chosen for testing the sensitivity and reproducibility of the PCR based approach. Since some of the selected PCR protocols were developed for samples other than wastewater, the original DNA preparation (e.g. pre-enrichment, DNA extraction) differed from the ones carried out in this work. Each protocol was tested first with pure cultures, applied afterwards to the wastewater DNA extracts and adapted for optimal sensitivity. The sensitivity of each PCR detection method was determined by spiking the wastewater DNA extracts with defined cell numbers of reference strains.

2.1 Adaptation and optimization of the PCR protocols

The reaction composition of the PCR assays targeting *E. coli* O157:H7 and *E. faecalis* were significantly modified. The original protocol for the detection of *E. coli* O157:H7 had been developed for the fluorogenic 5'-nuclease assay and was adapted to a classic PCR assay in this work. The reaction composition of the original PCR protocol targeting the 16S rDNA of *E. faecalis* was modified for higher sensitivity. The protocols for the detection of *C. jejuni*/*coli*, *Y. enterocolitica* serogroup 0.3 and *H. pylori* were applied without modification. All amplification profiles were applied without modifications.

2.2 Determination of PCR sensitivity and method detection limit

Three different kinds of PCR experiments were carried out for the target pathogens. First, the sensitivity of each PCR protocol was tested in pure cultures. Second, PCR sensitivity in the wastewater samples was determined by spiking replicates of the DNA extracts with increasing numbers of target cells prior to PCR. Prior to the spiking steps, control PCRs were performed in order to ensure that the non-spiked DNA extracts delivered negative PCR results for the respective target bacterium. The investigated target cell range comprised 4 orders of magnitude. In each PCR assay, the vials were spiked with 1, 5, 10, 50, 100, 1,000 or 10,000 target cells, which had been previously enumerated by total cell counts.

The detection limit of the PCR assays was defined as the lowest number of spiked cells that gave a visible PCR product. For instance, if a product was obtained with 100 spiked cells but not with 50, 100 cells were defined as the PCR detection limit. Finally, the effect of increasing quantities of DNA on PCR sensitivity and specificity was assessed with increasing DNA amounts in parallel PCR assays, each vial containing the same target cell number.

The sensitivity of the PCR assays was different in pure cultures (Table 16), ranging from 1 cell per assay for *Y. enterocolitica* to 100 cells per assay for *E. coli* O157:H7 and *E. faecalis.* PCRs performed with different amounts of background DNA (1 to 12 µl) showed that no more than 1 µl of DNA extracted from the settled wastewater and 10 µl of DNA extracted from the treated wastewater could be applied to PCR. These DNA amounts had been extracted from an average of 10^{10} and of 10^{9} non-target wastewater microorganisms, respectively. Comparison between the sensitivity of the PCR assays with pure cultures and spiked DNA extracts (Table 16) revealed that sensitivity of the PCR protocols targeting *C. jejuni*, *E. faecalis*, *H. pylori* and *E. coli* O157:H7 decreased 5- to ten-fold when applied to the wastewater DNA extracts.

Table 16: Sensitivity of the PCR assays in the spiked DNA extracts and pure culture. PCRs were performed with 1 µl DNA from the settled wastewater and 10 µl DNA from the treated wastewater. Values are expressed in cells per assay.

	Y. enterocolitica	*H. pylori*	*C. jejuni*	*E. coli* O157:H7	*E. faecalis*
DNA settled WW	10	50	100	500	1000
DNA treated WW	10	50	100	500	1000
Pure cultures	1	10	10	100	100

WW = wastewater

The detection limit of each of the PCR methods was defined as the lowest target cell number spiked in the highest non-inhibitory DNA background giving a positive result. Table 17 shows the detection limits of the PCR methods. The PCR methods had different sensitivity when applied to the wastewater DNA extracts: 5 cells of *Y. enterocolitica*, 25 cells of *H. pylori*, 50 cells of *C. jejuni*, 250 cells of *E. coli* O157:H7 and 500 cells of *E. faecalis* per 100 ml of treated wastewater and 200 cells of *Y. enterocolitica,* 1,000 cells of *H. pylori*, 2,000 cells of *C. jejuni/coli*, 10,000 cells of *E. coli* O157:H7 and 20,000 cells of *E. faecalis* per 100 ml of settled wastewater.

Table 17: Detection limit of the PCR detection methods for pathogenic bacteria. Values were converted to cells per 100 ml of wastewater.

Sample source	Cell counts [a] cells/100ml	*Y.enterocolitica* cells/100 ml	*H. pylori* cells/100 ml	*C. jejuni* cells/100 ml	*E. coli* [b] cells/100ml	*E. faecalis* cells/100 ml
Wiedersberg settled WW	2.8×10^{11}	200	1,000	2,000	10,000	20,000
Wiedersberg effluent HRB	1.0×10^{9}	5	25	50	250	500
Ettenbüttel settled WW	2.4×10^{11}	200	1,000	2,000	10,000	20,000
Ettenbüttel effluent VRB	4.9×10^{8}	5	25	50	250	500

WW = wastewater; HRB = horizontal reed beds; VRB = vertical reed beds; [a]: determined by DAPI staining, values are the mean of 7 (Wiedersberg), and 6 (Ettenbüttel) independent measurements; [b] = *E. coli* O157:H7

The detection limit for each method (Table 17) was constant for all analyzed samples derived from the same sampling site. The PCR methods were especially sensitive for treated wastewater samples: in a background of 10^8 to 10^9 cells as few as 5 to 500 cells of the target pathogens could be reliably detected. Moreover, the detection limits of the PCR methods were the same for the settled and treated wastewater of both investigated plants.

3 Extension of the new approach to municipal wastewater

The adequacy of the PCR approach for the detection of pathogenic bacteria in more polluted wastewater was evaluated for the municipal treatment plant (MTP) Ruhleben. DNA was extracted from the wastewater samples with the FastDNA Kit and the spiking assays were conducted in the same manner as for the RTPs. The sensitivity of the new approach was tested with the detection protocols for *C. jejuni*/*coli* and *Y. enterocolitica* (Table 18).

Table 18: Comparison of the PCR sensitivity in samples of the MTP Ruhleben and the RTPs Wiedersberg and Ettenbüttel. Values are expressed as target cell numbers per 100 ml native wastewater sample.

Treatment plant	Samples	Background [b] cells/100 ml	*C. jejuni* cells/100 ml	*Y. enterocolitica* cells/100 ml
RTPs	Settled WW	10^{11}	2,000	200
MTP	Settled WW	10^{11}	25,000	2,500
RTPs	Treated WW [a]	10^{8}-10^{9} [c]	50	5
MTP	Treated WW	10^{7}	2,000	200

WW = wastewater; [a] treated wastewater was the effluent of the horizontal reed beds in Wiedersberg and the effluent of the vertical reed beds in Ettenbüttel [b] mean order of magnitude of cell counts determined by DAPI staining in 7 measurements for Wiedersberg, 6 in Ettenbüttel and 2 in Ruhleben [c] value for Wiedersberg and Ettenbüttel, respectively

The background flora in the wastewater samples of the MTP Ruhleben (10^{11} cells per 100 ml of settled wastewater and 10^{7} cells per 100 ml of treated wastewater) was in the same magnitude for the settled wastewater of the RTPs and lower than in the treated wastewater of the RTPs (10^{11} cells per 100 ml of settled wastewater and over 10^{8} cells per 100 ml of treated wastewater, Table 18). The detection limits determined for both PCR methods targeting *C. jejuni/coli* and *Y. enterocolitica* in the wastewater samples of the MTP were constant for all analyzed samples. However, their sensitivity decreased in comparison to the detection limits determined with the same protocols in the wastewater derived from the RTPs: 200 cells of *Y. enterocolitica* and 2,000 cells of *C. jejuni/coli* per 100 ml treated wastewater and 2,500 cells of *Y. enterocolitica* and 25,000 cells of *C. jejuni/* per 100 ml settled wastewater.

4 Recovery rate of the PCR methods

In order to determine the recovery rates of the PCR based methods the half of the native samples derived from the MTP Ruhleben were spiked with *C. jejuni* and *Y. enterocolitica* before processing. Table 19 shows the sensitivity determined for spiked samples in comparison with spiked DNA extracts.

Table 19: Recovery of spiked target bacteria in wastewater of the MTP. Values are expressed as target cell numbers required for positive PCR results in 100 ml of native wastewater.

Sample	Spiking	*C. jejuni* cells/100 ml	*Y. enterocolitica* cells/100 ml
Settled WW	Native samples	25,000	2,500
	DNA extracts	25,000	2,500
Treated WW	Native samples	2,000	200
	DNA extracts	2,000	200

WW: wastewater

The spiked native samples were processed in the same manner and together with the non-spiked samples. DNA was extracted from the pellets after centrifugation and PCR was performed as described before. PCR detection in the non-spiked native samples yielded negative results for the target bacteria. Sensitivity of the detection method was the same when the samples were spiked with the target bacteria before centrifugation or in the DNA extracts (Table 19), confirming accuracy of the concentration and DNA extraction steps.

The new PCR based approach enabled the detection of all target bacteria within 12 hours and showed specificity and reproducibility for the investigated pathogens.

B) Studies on the bacteria elimination performance of reed beds

The RTPs Wiedersberg and Ließen are multi-stage treatment plants composed of primary settling, vertical flow and horizontal flow reed beds. The RTP Ettenbüttel is also a multi-stage treatment plant composed of wastewater lagoons followed by vertical reed beds. The pilot plant Wiedersberg has other components (UV lamp, phosphorus precipitation filter, polishing ponds) which were not subject of this investigation. In this work, focus was laid upon the bacterial retention capacity of reed beds.

1 General bacteria reduction performance of the reed beds

To assess the general bacterial reduction performance the bacteria in the influent and effluent of the treatment plant components were enumerated by total cell counts using DAPI staining and epifluorescence microscopy.

1.1 RTP Wiedersberg

Bacterial concentrations in the wastewater samples of the RTP Wiedersberg are depicted in Figure 5. In November 1999 two sampling campaigns were performed (November 15th and 29th). In May 23rd 2000 the RTP was sampled twice at the same day, (10:00 a.m. and at 3:30 p.m) and the depicted values are the average of the two independent measurements in November 1999 and in May 2000. During the period of investigation, bacterial concentrations in the primary settled wastewater ranged from 3.30 x 10^7 to 7.10 x 10^9 cells ml^{-1}. The average bacterial concentration was 1.74 ± 2.57 x 10^9 cells ml^{-1}. After percolation through the vertical reed beds, bacterial concentrations decreased to 2.80 x 10^5– 6.20 x 10^7 cells ml^{-1} (October 1999 and August 2000, respectively). The average concentration of the effluent of the vertical reed beds was 8.02 ± 17.20 x 10^6 cells ml^{-1}. After passing through the horizontal beds, wastewater had bacterial concentrations between 6.40 x 10^4 (November 2000) and 5.40 x 10^7 cells ml^{-1} (August 2000). The average value was 6.24 ± 15.10 x 10^6 cells ml^{-1}. Value scattering of bacterial concentrations was higher in the effluent of the horizontal beds than in the settled wastewater and the effluent of the vertical beds. For the analyzed samples no clear seasonal variation of the bacterial concentrations was detected.

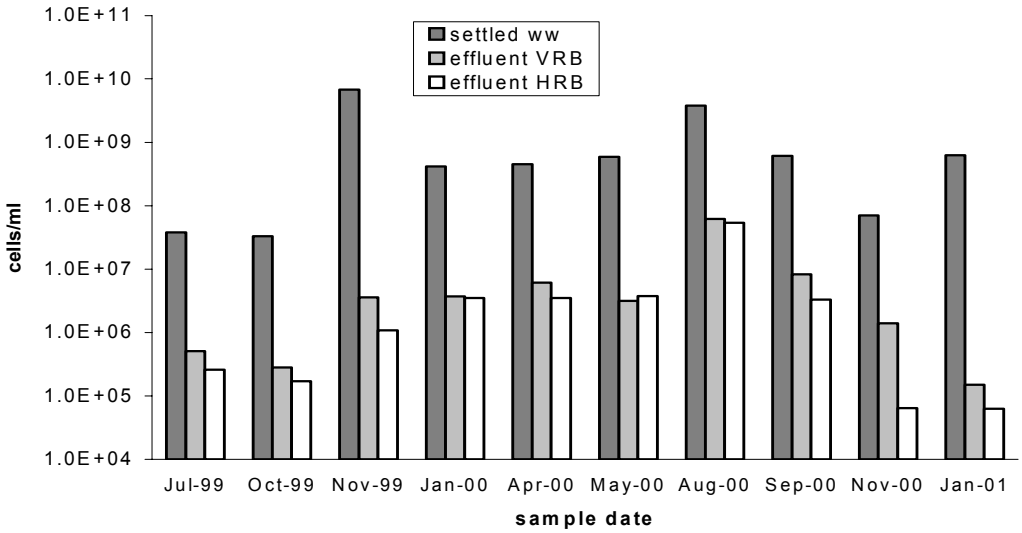


Figure 5: Bacterial concentrations determined by total cell counts in the influent and effluent of the reed bed filters of the RTP Wiedersberg. ww = wastewater; VRB = vertical reed bed; HRB = horizontal reed bed.

Bacterial removal rates were determined for the vertical and the horizontal reed beds (Table 20). The capacity of both filter sets to remove bacteria from the respective influent varied strongly: whereas the vertical beds removed 98.00% to 99.97% of influent bacteria, removal rates of the horizontal filter were erratic and in general low.

Table 20: Bacterial reduction rates of the filter beds in the RTP Wiedersberg.

Date	Total counts cells/ml settled ww	Total counts cells/ml effluent VRB	Total counts cells/ml effluent HRB	Reduction (%) VRB	Reduction (%) HRB	Reduction (%) VRB+HRB
12.07.99	3.80×10^7	5.10×10^5	2.60×10^5	98.66	49.02	99.32
18.10.99	3.30×10^7	2.80×10^5	1.70×10^5	99.15	39.29	99.48
15.11.99	6.50×10^9	1.90×10^6	1.20×10^6	99.97	36.84	99.98
29.11.99	7.10×10^9	5.30×10^6	9.50×10^5	99.93	82.08	99.99
18.01.00	4.20×10^8	3.70×10^6	3.50×10^6	99.12	5.41	99.17
11.04.00	4.50×10^8	6.10×10^6	3.50×10^6	98.64	42.62	99.22
23.05.00 (1)	5.70×10^8	2.40×10^6	3.10×10^6	99.58	-29.17 a)	99.46
23.05.00 (2)	6.10×10^8	3.90×10^6	4.40×10^6	99.36	-12.82 a)	99.28
22.08.00	3.80×10^9	6.20×10^7	5.40×10^7	98.37	12.90	98.58
19.09.00	6.10×10^8	8.30×10^6	3.30×10^6	98.64	60.24	99.46
28.11.00	7.00×10^7	1.40×10^6	6.40×10^4	98.00	95.43	99.91
09.01.01	6.23×10^8	5.03×10^5	4.83×10^5	99.92	3.98	99.92

ww = settled wastewater; VRB = effluent of the vertical reed beds; HRB = effluent of the horizontal reed beds; (1) = sample collected at 10:00 a.m.; (2) sample collected at 3:30 p.m.; a) = negative values represent an increase in bacterial concentration after passage through the filter

There was no reduction of wastewater bacteria in May 2000 (Table 20). In the remaining sampling campaigns, bacteria reduction rates varied between 3.98% and 95.43%. Mean bacterial removal rates were 99.11 ± 0.66% for the vertical beds. For the horizontal beds removal rates were 42.78 ± 30.74% excluding the values for May 2000. The cumulative bacteria reduction potential rate of the vertical and horizontal reed beds of the RTP Wiedersberg (Table 20) varied between 98.58% and 99.99%, with an average of 99.48 ± 0.42%. The highest removal rate was observed in November 1999 and the lowest in August 2000.

1.2 RTP Ettenbüttel

At the RTP Ettenbüttel wastewater purification is carried out by two wastewater lagoons followed by a set of vertical reed beds. Although the main subject of the investigations was the elimination performance of the reed beds bacterial reduction in the extensive wastewater lagoons (2 x 1,100 m^2) preceding the reed beds was also determined. Figure 6 shows the bacterial concentrations in the influent wastewater, the effluent of the lagoons and the effluent of the vertical reed beds during the period of investigation (August 1999 to January 2001).

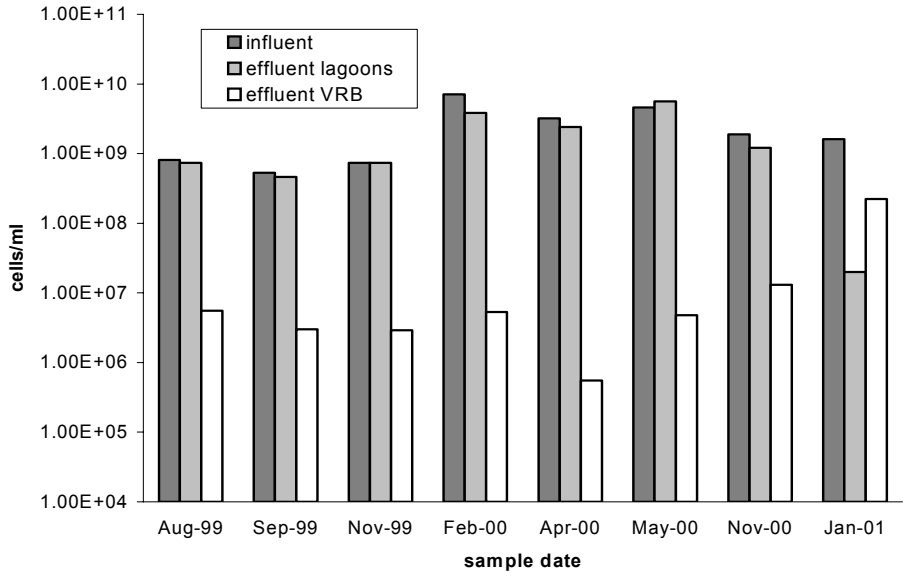


Figure 6: Bacterial concentrations determined by total cell counts in wastewater samples of the RTP Ettenbüttel. VRB = vertical reed beds.

Between June and October 2000 the vertical reed beds were set out of operation. Consequently no sampling campaign took place in that period.

The average bacterial concentration in the influent wastewater of Ettenbüttel (Figure 6) amounted to 2.81 ± 2.36 x 10^9 cells ml^{-1}. After treatment by the lagoons the bacterial concentrations did not decrease significantly (average value 1.87 ± 1.94 x 10^9 cells ml^{-1}). The highest concentration in the effluent of the wastewater lagoons was determined for May 2000 (5.60 x 10^9 cell ml^{-1}) and the lowest for January 2001 (1.99 x 10^7 cells ml^{-1}). Bacterial reduction by percolation through the vertical reed beds was higher: average concentration in the effluent of the beds was 3.59 ± 8.21 x 10^7 cells ml^{-1}. The highest concentration in the effluent of the reed beds was determined in January 2001 (2.22 x 10^8 cells ml^{-1}), where outflowing bacteria were more abundant than the inflowing, and the lowest concentration in April 2000 (5.50 x 10^5 cells ml^{-1}).

Bacteria reduction rates were very low and irregular in the wastewater lagoons (Table 21): in May 2000 no reduction in bacterial numbers occurred and concentration in the lagoon effluent was higher than in the influent samples; for the other samples reduction varied between 1.35% and 46.48%, with the exception of January 2001, where reduction was 98.76%. The average bacterial reduction rate was 26.22 ± 36.14%.

Table 21: Bacterial reduction rates of the components of the RTP Ettenbüttel.

Date	Total counts cells/ml influent	Total counts cells/ml effluent WWL	Total counts cells/ml effluent VRB	Reduction (%) WWL	Reduction (%) VRB	Reduction (%) WWL+VRB
27.08.99	8.10 x 10^8	7.30 x 10^8	5.50 x 10^6	13.21	99.35	99.43
01.09.99	5.30 x 10^8	4.60 x 10^8	3.00 x 10^6	9.88	99.25	99.32
04.11.99	7.40 x 10^8	7.30 x 10^8	2.90 x 10^6	1.35	99.60	99.61
14.02.00	7.10 x 10^9	3.80 x 10^9	5.30 x 10^6	46.48	99.86	99.93
03.04.00	3.20 x 10^9	2.40 x 10^9	5.50 x 10^5	25.00	99.98	99.98
16.05.00	4.60 x 10^9	5.60 x 10^9	4.80 x 10^6	-21.74 [a)]	99.91	99.90
13.11.00	1.90 x 10^9	1.20 x 10^9	1.30 x 10^7	36.84	98.92	99.32
15.01.01	1.60 x 10^9	1.99 x 10^7	2.22 x 10^8	98.76	-1015.58 [a)]	86.13

WWL = wastewater lagoons; VRB = vertical reed beds; [a)] = negative values represent an increase in bacterial concentration after treatment

For the vertical reed beds, except for January 2001, where an increase in bacterial numbers after percolation was registered (Table 21), reduction rates were high, varying between 98.92% and 99.98%. The mean reduction rate was 99.55 ± 0.40% excepting the value for January 2001. Cumulative bacteria reduction performance of

the wastewater lagoons and the vertical beds varied in all but one case between 99.32% and 99.98%. In January 2001 total elimination was low with 86.13%. Average value for total elimination of bacteria was 97.95 ± 4.79%.

1.3 RTP Ließen

The RTP Ließen was studied between May and December 2000. Wastewater was collected from 4 sites: from the influent and effluent of the reed beds and further in the sewerage system, before infiltration into the ground. The bacterial concentrations determined by total cell counts in the different wastewater samples are depicted in Figure 7. The standard deviation was calculated for the measured values (n = 5). Although the statistic significance of these values is low, they are presented here in order to give an indication of the value scattering. Results show a decrease in bacterial concentrations after percolation through the filters and again an increase when the samples were collected at the outlet site.

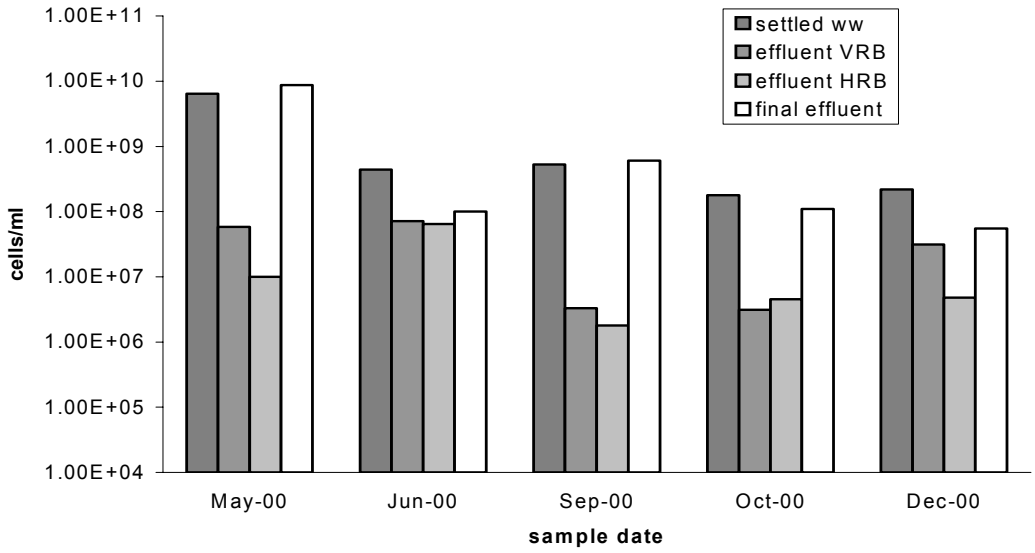


Figure 7: Bacterial concentrations determined by total cell counts in the wastewater samples of the RTP Ließen. ww = wastewater; VRB = vertical reed beds; HRB = horizontal reed beds.

The influent to the vertical reed beds (settled wastewater, Figure 7) had an average bacterial concentration of 1.55 ± 2.71 x 10^9 cells ml^{-1}. Highest concentration was determined in May (6.40 x 10^9 cells ml^{-1}) and the lowest in October (1.80 x 10^8 cells ml^{-1}). After percolation through the vertical reed beds bacterial concentration averaged 3.33 ± 3.10 x 10^7 cells ml^{-1}. Highest and lowest bacterial concentrations

were registered in June and October (7.10×10^7 and 3.10×10^6 cells ml^{-1}, respectively). After treatment by the horizontal reed beds reduction in bacterial concentrations was lower: concentrations varied between 1.80×10^6 (September) and 6.50×10^7 cells ml^{-1} (June), and the average concentration was $1.72 \pm 2.69 \times 10^7$ cells ml^{-1}. Bacterial concentrations in the treated wastewater increased by 1 to 2 orders of magnitude between the outlet site of the horizontal reed beds and the infiltration shaft: concentrations increased up to 8.70×10^9 cells ml^{-1} and the average bacterial concentration in the final wastewater was $1.92 \pm 3.80 \times 10^9$ cells ml^{-1}, which is in the range of the concentrations determined for the primary settled wastewater. In the samples from May and September final bacterial concentration was even higher than in the settled wastewater.

Percolation through the vertical reed beds reduced bacterial numbers by 83.86% to 99.38% (Table 22). Mean reduction rate was 93.30 ± 7.73%. The bacteria reduction rate of the horizontal reed beds varied strongly: removal rates ranged from failure in October (more bacteria in the effluent than in the influent) over very low removal rates e.g. 8.45% to a maximum of 84.52% in December.

Table 22: Bacterial reduction rates for the RTP Ließen. Total cell counts were determined by DAPI staining.

Date	Total counts cells/ml settled ww	Total counts cells/ml VRB	Total counts cells/ml HRB	Total counts cells/ml effluent	Reduction (%) VRB	Reduction (%) HRB	Reduction (%) VRB+HRB
May-00	6.40×10^9	5.80×10^7	1.00×10^7	8.70×10^9	99.09	82.76	99.84
Jun-00	4.40×10^8	7.10×10^7	6.50×10^7	1.00×10^8	83.86	8.45	85.23
Sep-00	5.30×10^8	3.30×10^6	1.80×10^6	6.10×10^8	99.38	45.45	99.66
Oct-00	1.80×10^8	3.10×10^6	4.50×10^6	1.10×10^8	98.28	-45.16 [a)]	97.50
Dec-00	2.20×10^8	3.10×10^7	4.80×10^6	5.50×10^7	85.91	84.52	97.82

ww = wastewater; VRB = vertical reed beds; HRB = horizontal reed beds; [a)] = negative values represent an increase in bacterial concentration after percolation

Changes in bacterial removal efficiency of the horizontal beds in Ließen were erratic (Table 22). In every case bacterial removal efficiency of the horizontal reed beds was always lower than of the vertical reed beds. The cumulative bacteria reduction rate achieved by the combination of vertical and horizontal reed beds ranged from 85.23% to 99.84% and averaged 96.01 ± 6.12%.

Because of the regrowth in the sewerage system the net bacteria reduction in the RTP Ließen was very low and very inconstant, ranging from complete failure (May and September) to 77.27% in June.

2 Specific elimination performance of the reed beds

After studying the general removal performance of the reed bed filters for bacteria the aim of this investigation was to study qualitative aspects of retention of microorganisms in the reed beds. Shifts in the wastewater microbial consortia after purification by the reed beds were investigated by means of phase contrast microscopy, fluorescence in situ hybridization and PCR. Preferential removal of defined morphologic forms was assessed by phase contrast microscopy. FISH was used in order to ascertain if changes in microbial populations due to percolation would be reflected at the phylogenetic level. Finally, the optimized PCR based detection methods developed in this work were used in order to detect pathogenic bacteria and eventually its elimination by the reed beds.

2.1 Phase contrast microscopic analysis

Phase contrast microscopic analysis was applied to the fixed samples of the influent and effluent of the reed beds. A 400-fold magnification was used. At least 10 microscopic fields were analyzed per sample and a semi-quantitative analysis was carried out. Evaluation was carried out based upon directly identifiable morphologic traits. The relative abundance of each identified microbial group is given in Table 23 to Table 25 according to the legend. Special focus was laid upon protozoa, algae, sulfur bacteria and filamentous bacteria because they are easily recognizable and may be indicators of certain operation conditions. The collective term “other bacteria” comprises all small (<1 µm in diameter), mostly rod-shaped bacteria without striking morphologic traits.

2.1.1 RTP Wiedersberg

Phase contrast microscopic analysis of the influent and effluent of the reed bed systems is summarized in Table 23 (a and b). Results shown for May 23rd refer to the 10:00 a.m. samples. First obvious observations are i) the big variety of bacteria in the settled wastewater in contrast to the treated wastewater (Figure 8) and ii) the strong reduction of the large bacterial species (e.g. *Chromatium* sp. and *Thiothrix* sp.) and of protozoa and algae after passing the reed beds.

Table 23: RTP Wiedersberg: microbial diversity in the settled (4.a) and treated (4.b) wastewater. Abundance is expressed as cells per optical field at 400X magnification.

23.a: Settled wastewater

Date	*Thiothrix* sp.	*Spirillum* sp.	*Chromatium* sp.	Filamentous bacteria	Other bacteria	Protozoa	Algae
Jul 99	●●●●	●●●●	●●●●	○	●●●●●	●●	●●
Oct 99	○	●●●	●●●	○	●●●●●	○	○
Nov 99 (1)	●●●●	●●●	●●●	○	●●●●●	●●	●●
Nov 99 (2)	●●	●●	●●	●●●●	●●●●●●	●●●	●●●
Jan 00	●●	●●	●●	○	●●●●●●	●●●	●●●
Apr 00	●●	●●	●	○	●●●●●	●●	●●
May 00 (3)	●●	●●	●●	○	●●●●●	●●	●●●
Aug 00	●●	●●●	●●●●	○	●●●●●	●●	●●
Sep 00	●●	●●	●●●●	●●	●●●●	●●	●●●
Nov 00	●●	●●	●●	●●●	●●●●●	●●●	●●●
Jan 01	●●	●●	●●	●●●	●●●●●	●●	●●

○ = not detected; ● = only sporadically detected; ●● =2-5 cells; ●●● = 6-20 cells; ●●●● = 21-50 cells; ●●●●● = 51-100 cells; ●●●●●● = >100 cells; (1) collected the 15th November 99; (2) collected the 29th November 99; (3) collected at 10:00 a.m.

23.b: Effluent of the horizontal reed beds

Date	*Thiothrix* sp.	*Spirillum* sp.	*Chromatium* sp.	Filamentous bacteria	Other bacteria	Protozoa	Algae
Jul 99	●	●	●	○	●●	○	●
Oct 99	●	●	●	○	●●	○	○
Nov 99 (1)	●	●	●	○	●●	○	○
Nov 99 (2)	●	●	●	○	●●	○	●●
Jan 00	●	●	●	○	●●	○	○
Apr 00	●	●	●	○	●●	○	○
May 00	●●	●●	●●	○	●●	●	●●
Aug 00	●	●	●	○	●●	●●	●
Sep 00	●	●	●	○	●●	●●	●
Nov 00	●	●	●	○	●●	●	●
Jan 01	●	●	●	○	●●	●	●

○ = not detected; ● = only sporadically detected; ●● =2-5 cells; ●●● = 6-20 cells; ●●●● = 21-50 cells; ●●●●● = 51-100 cells; ●●●●●● = >100 cells; (1) collected the 15th November 99; (2) collected the 29th November 99; (3) collected at 10:00 a.m.

Protozoa (mainly small flagellates of the *Bodo* sp. type) were almost always present in the settled wastewater samples but were only detected in the effluent samples between May 2000 and January 2001 (Table 23). Algae were very numerous in the settled wastewater and were strongly reduced, occurring irregularly and in small amounts in the effluent. Filamentous bacteria occurred only in November 1999 and from September 2000 to January 2001 and were never detected in the effluent

samples. Small rod-shaped bacteria (“other bacteria”) were predominant in the treated wastewater samples.

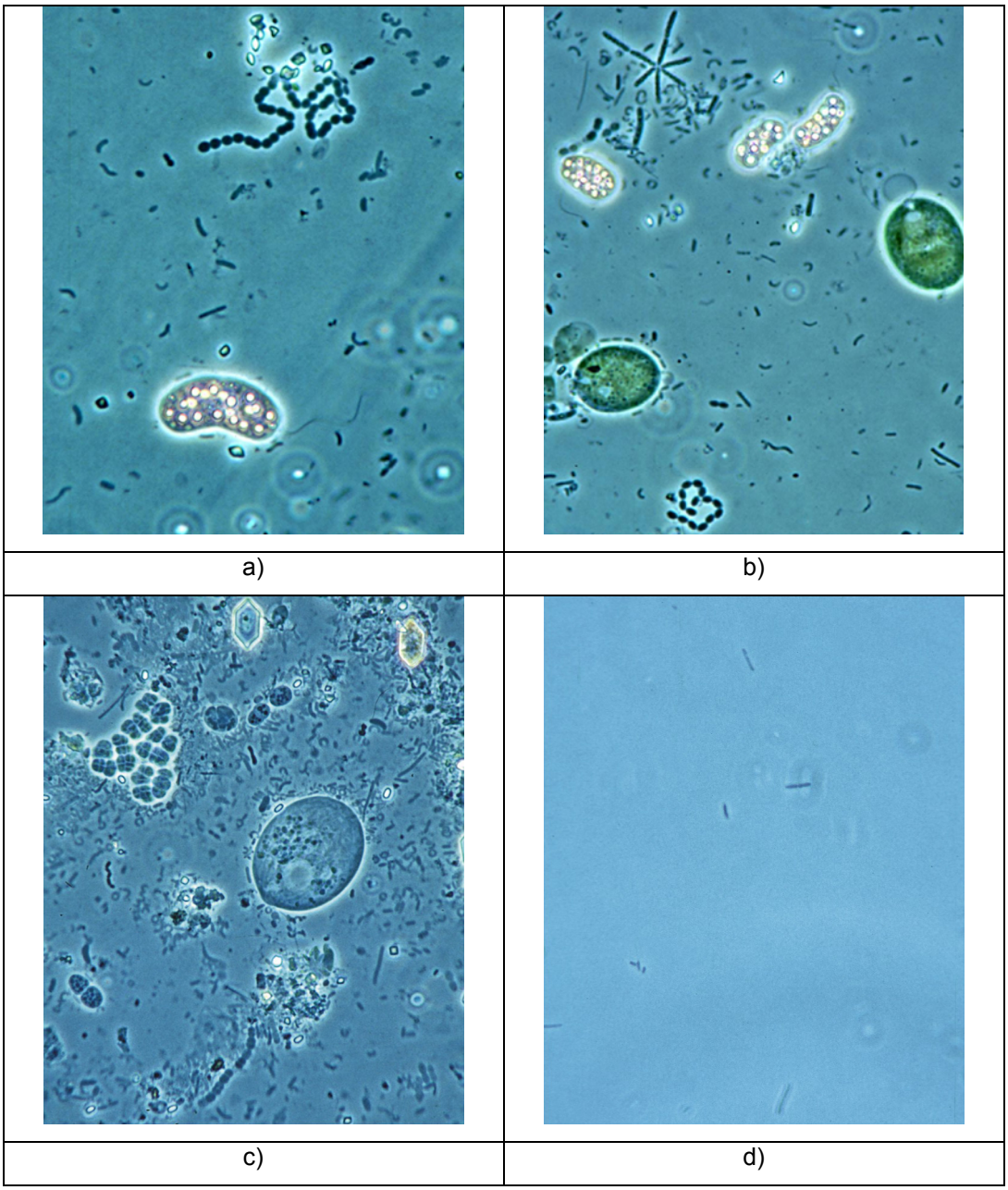


Figure 8: Morphological variety of the microorganisms in the settled wastewater (a,b,c) and the effluent of the horizontal reed beds (d) in the RTP Wiedersberg. Pictures were taken at a 1000X magnification.

2.1.2 RTP Ettenbüttel

Phase contrast microscopic analysis of the influent and effluent of the reed beds of the RTP Ettenbüttel is summarized in Table 24 (a and b). A high diversity and

abundance of bacteria was observed in the wastewater lagoons. Algae were very numerous.

Table 24: RTP Ettenbüttel: microbial diversity in the settled (24.a) and treated (24.b) wastewater. Abundances are expressed as cells per optical field at 400X magnification.

24.a: Settled wastewater

Date	*Thiothrix* sp.	*Spirillum* sp.	*Chromatium* sp.	Filamentous bacteria	Other bacteria	Protozoa	Algae
Aug 99	●●●	●●●●	●●	●●	●●●●●●	●●	●●●
Sep 99	●●	●●●	●●●	●●	●●●●●	●●●	●●●
Nov 99	●	●●●	●●●	●●	●●●●●	●●	●●●
Feb 00	●	●●●	●●	●●	●●●●●	●●●	●●●
Apr 00	●●	●●	●●	●●	●●●●●●	●●●	●●●
May 00	●●●	●●	●●	●●	●●●●●	●●●	●●●
Nov 00	●●●	●●	●●●●●●	●●●●●	●●●●●	●●	●●●
Jan 01	●●●	●●	●●●●●	●●●●●	●●●●●	●●	●●●●

○ = not detected; ● = only sporadically detected; ●● =2-5 cells; ●●● = 6-20 cells; ●●●● = 21-50 cells; ●●●●● = 51-100 cells; ●●●●●● = >100 cells

24.b: Effluent of the vertical reed beds

Date	*Thiothrix* sp.	*Spirillum* sp.	*Chromatium* sp.	Filamentous bacteria	Other bacteria	Protozoa	Algae
Aug 99	●	●	●	●	●	○	○
Sep 99	●	●	●	●	●●	●	○
Nov 99	●	●	●	●	●●	○	○
Feb 00	●	●	●	●	●●	○	○
Apr 00	●	●	●	●	●●	○	○
May 00	●	●●	●	●	●●	●	●●●
Nov 00	●	●●	●●	●●●	●●	●●	●●●
Jan 01	●	●	●	●●●	●●	●●●	●●●

○ = not detected; ● = only sporadically detected; ●● =2-5 cells; ●●● = 6-20 cells; ●●●● = 21-50 cells; ●●●●● = 51-100 cells; ●●●●●● = >100 cells

In November 2000 and January 2001 abundance of *Chromatium* sp. and filamentous bacteria increased both in the settled and in the treated wastewater of Ettenbüttel (Table 24). Microscopy of the effluent of the vertical reed beds showed a strong reduction of bacterial numbers and diversity. Algae and protozoa were strongly reduced from August 1999 to April 2000. Afterwards algae occurred in higher numbers (effluents of May 2000, November 2000 and January 2001). Protozoa numbers also increased from May 2000 to January 2001.

2.1.3 RTP Ließen

The microscopic observations of the wastewater samples in the RTP Ließen (Table 25 – a to c) revealed that diversity and abundance of microorganisms in the settled wastewater were similar to Wiedersberg and Ettenbüttel.

Table 25: RTP Ließen: microbial diversity in the settled (25.a) and treated (25.b) wastewater and from the final effluent (25.c). Abundances are expressed as cells per optical field at 400X magnification.

25.a: Settled wastewater

Date	*Thiothrix* sp.	*Spirillum* sp.	*Chromatium* sp.	Filamentous bacteria	Other bacteria	Protozoa	Algae
May 00	●●●	●●	●●●	●●●	●●●●●	●●●	●●●
Jun 00	●●●	●●	●●●	●●●	●●●●●	●●	●●●●
Sep 00	●●	●●	●●●	●●●	●●●●●	●●●	●●●
Oct 00	●●●	●●	●●	●●●	●●●●●●	●●●	●●●
Dec 00	●●	●●	●●	●●●	●●●●●●	●●●	●●●

○ = not detected; ● = only sporadically detected; ●● =2-5 cells; ●●● = 6-20 cells; ●●●● = 21-50 cells; ●●●●● = 51-100 cells; ●●●●●● = >100 cells

25.b: Effluent of the horizontal reed beds

Date	*Thiothrix* sp.	*Spirillum* sp.	*Chromatium* sp.	Filamentous bacteria	Other bacteria	Protozoa	Algae
May 00	●	●	●	●	●●●	●	●●
Jun 00	●	○	●	●	●●●	●	●●
Sep 00	●	●	●	●	●●●	●	●●
Oct 00	●	●	●	●	●●●	●	●●
Dec 00	●	●	●●	●	●●●	●	●●

○ = not detected; ● = only sporadically detected; ●● =2-5 cells; ●●● = 6-20 cells; ●●●● = 21-50 cells; ●●●●● = 51-100 cells; ●●●●●● = >100 cells

25.c: Final effluent

Date	*Thiothrix* sp.	*Spirillum* sp.	*Chromatium* sp.	Filamentous bacteria	Other bacteria	Cocci	Protozoa	Algae
May 00	○	●	○	●●●	●●●●●●	●●●	●●●	●●●
Jun 00	○	●	○	●●●	●●●●●●	●●●	●●●	●●●
Sep 00	○	●	○	●●●	●●●●●●	●●	●●●	●●●
Oct 00	○	●	○	●●●	●●●●●●	●●	●●●	●●●
Dec 00	○	●	○	●	●●●	●	●	●●

○ = not detected; ● = only sporadically detected; ●● =2-5 cells; ●●● = 6-20 cells; ●●●● = 21-50 cells; ●●●●● = 51-100 cells; ●●●●●● = >100 cells

The effluent of the horizontal reed beds in Ließen (Table 25.b) contained in most cases all the morphotypes detected in the settled wastewater, but the abundance was lower. The final effluent (Table 25.c) showed a different morphology pattern: *Thiothrix* sp. and *Chromatium* sp. were not present, filamentous bacteria and other bacteria without striking morphological traits increased significantly and coccoid shaped forms appeared in significant amounts. Protozoa and algae numbers also increased significantly.

2.2 FISH analysis of bacteria in the wastewater samples

In order to study the influence of soil percolation on the bacterial composition of wastewater, the influent and effluent of the vertical reed beds of the RTPs Wiedersberg and Ließen were subjected to a whole cell in situ hybridization. The vertical reed beds were selected because they displayed the highest bacterial removal rate in the treatment plants studied. Moreover, since the RTP Wiedersberg and Ließen have very similar construction differing in the time of operation, results obtained could give insights in changes in removal patterns due to long-term operation. The vertical beds V1 of Wiedersberg and V2 of Ließen were selected for the analysis because very similar filter medium material was used for the beds. The analyzed wastewater samples were collected in January 2000 and August 2000 in Wiedersberg and in September 2000 and October 2000 in Ließen.

Proteobacteria are the most common phylogenetic group in wastewater. Therefore, the changes in the relative abundances of the α-, β- and γ-*Proteobacteria* were determined in order to track eventual changes in population composition during percolation. In situ hybridization was performed with the fluorescent oligonucleotide probes EUB338, ALF1b, BETA42a and GAM42a. The percentage of hybridized cells was determined as the ratio between the fluorescent signals obtained with the fluorescent probe and the fluorescent signals obtained by DAPI staining.

For both analyzed plants, hybridization patterns of the influent and effluent of the vertical reed beds did not vary significantly in the two sampling campaigns (Figure 9 and Figure 10). For the RTP Wiedersberg (Figure 9), the percentage of EUB-hybridized cells decreased after percolation (76% and 79% in the influent samples of January and August vs. 54% and 56% in the respective effluents).

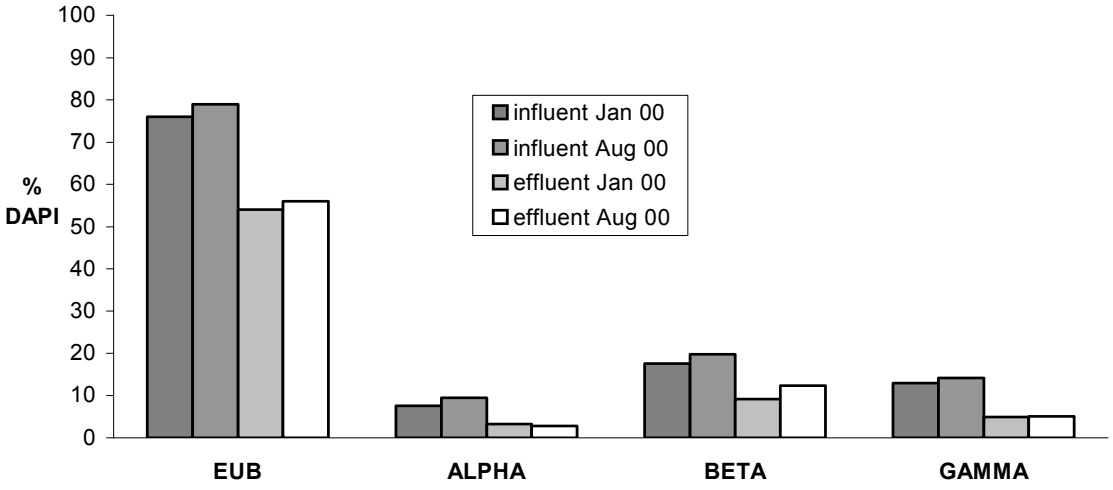


Figure 9: FISH analysis of the influent and effluent of the vertical reed beds of Wiedersberg. EUB = *Eubacteria*; ALPHA = α- *Proteobacteria*; BETA = β-*Proteobacteria*; GAMMA = γ-*Proteobacteria.*

Interestingly, percentage of EUB338 signals increased after percolation through the vertical reed beds of the RTP Ließen (Figure 10): the influent samples showed 77% and 71% of hybridized cells in September and October, whereas the respective effluent samples contained 78% and 75% of hybridized cells.

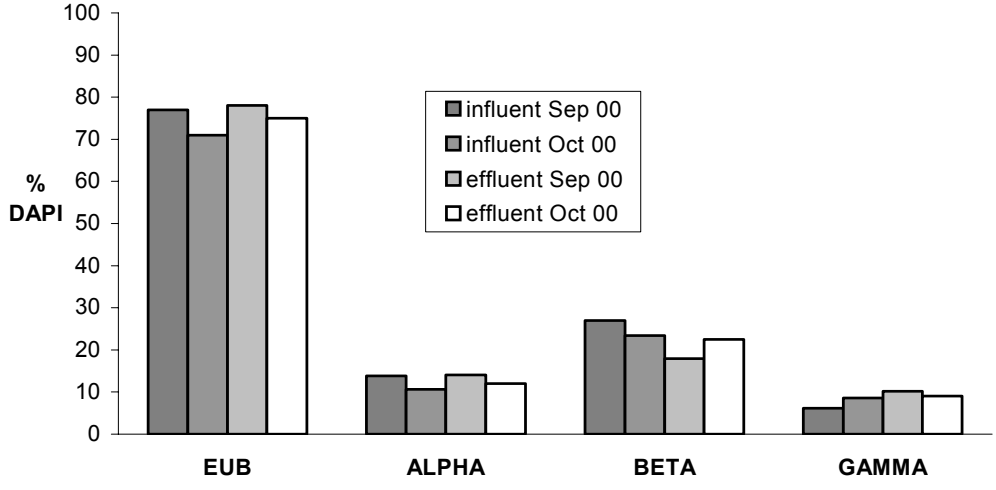


Figure 10: FISH analysis of the influent and effluent of the vertical reed beds of Ließen. EUB = *Eubacteria*; ALPHA = α- *Proteobacteria*; BETA = β-*Proteobacteria*; GAMMA = γ-*Proteobacteria*.

The probe-conferred signal intensity was lower in all effluent samples than in the respective influents, but the difference was more pronounced in the wastewater samples of Wiedersberg than in the samples of Ließen. As for the three subclasses of the *Proteobacteria*, the β-subclass was predominant in all samples of Wiedersberg and Ließen. In Wiedersberg BETA42a probe conferred signals accounted for 17.5% (January) and 20% (August) in the influent of the vertical beds and 9% (January) and 12% (August) in the effluent. *γ-Proteobacteria* were more abundant than α-*Proteobacteria* and were present as 13% (January) and 14% (August) in the influent of the beds and 5% in the respective effluent. ALF1b conferred signals accounted only for 8% (January) and 9.5% (August) in the influent of the beds and 3% in the respective effluents. Figure 11 shows an organic flock in the wastewater samples of Wiedersberg populated with mainly *γ-Proteobacteria*.

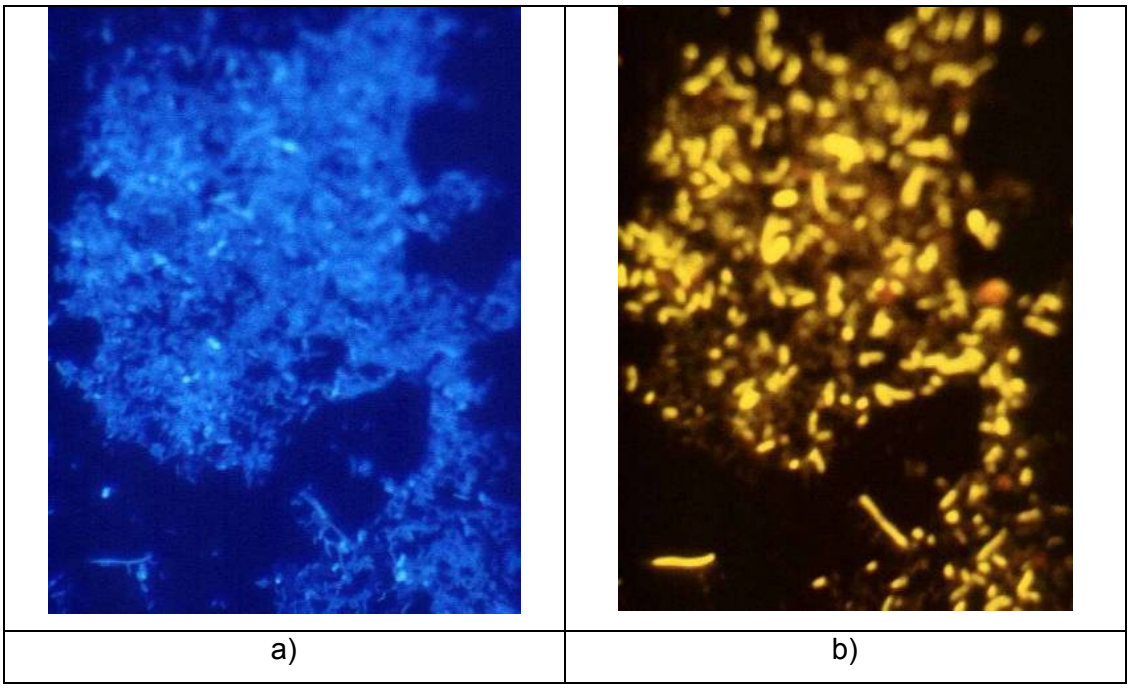


Figure 11: Micrographs obtained with the probe GAMMA42a (a) against DAPI staining (b) in the settled wastewater of Wiedersberg.

In the Ließen samples (Figure 10) the β-subclass of *Proteobacteria* accounted for 27% (September) and 23% (October) of total cell counts in the influent samples and 18% (September) and 22.5% (October) in the effluent samples. The hybridized α-*Proteobacteria* were more abundant than γ-*Proteobacteria*: 14% (September) and 11% (October) vs. 6% (September) and 8.5% (October) in the influent of the vertical

beds and 14% (September) and 12% (October) vs. 10% and 9% in the respective effluents.

2.3 PCR detection of the model pathogens

The influent and effluent wastewater of the reed beds from the RTP Wiedersberg and Ettenbüttel were investigated for the presence of the model pathogens *Y. enterocolitica* serogroup 0:3, *Campylobacter jejuni*, *Helicobacter pylori*, enterohemorrhagic *E. coli* and *E. faecalis*. DNA was extracted from 500 ml settled wastewater and 2,000 ml effluent of the reed beds and the PCR based detection described in Section A was applied to the DNA extracts. DNA extracts spiked with serial dilutions of the model bacterium were always analyzed simultaneously with the respective nonspiked DNA sample in order to exclude errors in the execution and to verify the invariability of the detection limit. Each PCR was repeated at least once.

In samples displaying positive PCR results for the target bacteria, numbers of target cells were estimated by the dilution to extinction procedure: DNA was 1:5 serially diluted until the PCR result was negative. Results were defined as the interval between the dilution showing no PCR result and the neighboring dilution giving a positive result. By application of the PCR based detection method *Campylobacter jejuni*, *C. coli*, *Helicobacter pylori*, enterohemorrhagic *E. coli* and *E. faecalis* were detected neither in the influent nor in the effluent of the reed beds of the RTPs Wiedersberg and Ettenbüttel (Table 26).

Table 26: Detection of the model pathogens in the unspiked wastewater samples of the RTP Wiedersberg and Ettenbüttel

Sampling site	*Yersinia* sp.	*Y. enterocolitica*	*H. pylori*	*C. jejuni*	*E. coli* O157:H7	*E. faecalis*
Wiedersberg influent VRB	-	-	-	-	-	-
Wiedersberg effluent HRB	+	-	-	-	-	-
Ettenbüttel influent VRB	+	-	-	-	-	-
Ettenbüttel effluent HRB	+	-	-	-	-	-

VRB = vertical reed beds; HRB = horizontal reed beds

However, in 4 samples (2 settled wastewater and the respective treated wastewater samples) from the Ettenbüttel and 1 sample (treated wastewater) from the

Wiedersberg wetland (Table 26) a 0.3-kb fragment corresponding to the 16S rDNA of *Yersinia sp.* was amplified, whereas PCR directed to the 0.6 kb-fragment of the virulence plasmid pYV was negative. (Date: Ettenbüttel February 14th and April 3rd and Wiedersberg August 22nd 2000). In these samples, *Yersinia* concentrations were between 200 and 1000 cells per 100 ml of settled wastewater and between 50 and 250 cells per 100 ml of treated wastewater samples.In contrast to *C. jejuni/coli, Y. enterocolitica, H. pylori and EHEC*, which are usually only sporadically detected in wastewater, fecal streptococci, to which *E. faecalis* belongs, are common in domestic wastewater. However, *E. faecalis* was not detected by PCR in the investigated wastewater samples. Detection limit of the PCR method for *E. faecalis* was 200 cells ml^{-1} in the settled wastewater and 5 cells ml^{-1} in the treated wastewater. A further approach was carried out in order to estimate the abundance of this species, since fecal streptococci colonies are usually easily isolated from wastewater: a total of 25 fecal streptococci colonies isolated from the investigated wastewater samples were picked randomly and submitted to a colony identity proof as described in MATERIAL & METHODS (16.4.2). From the analyzed colonies only 20% gave a positive result with the species specific PCR, indicating that *E. faecalis* may constitute only a minor fraction of the total numbers of cultured fecal streptococci, thus supporting the results obtained by PCR.

3 Fate of plasmids during the purification process in the RTP

The goal of this investigation was to assess if conjugative or mobilizable plasmids could pass percolation through the reed beds in detectable amounts. At the RTP Wiedersberg settled wastewater and effluent of the vertical and horizontal reed beds were examined for the presence of plasmids assigned to incompatibility groups IncPα, IncPß, IncW, IncN and IncQ. Additionally, a primer pair for the detection of plasmids assigned to the family of the mobilizable plasmid pMV158 was developed and applied to the wastewater samples.

Plasmid detection consisted of PCR amplification of plasmid specific sequences in the DNA extracted from the wastewater samples, followed by Southern hybridization with digoxigenin-dUTP-labeled oligonucleotide probes and finally chemiluminescence detection of the DNA-DNA hybrids with CSPD.

Table 27 shows the results obtained. Plasmids assigned to the incompatibility group IncPα were detected in the influent and effluent of the vertical reed beds. All other

investigated plasmids were not detected. The effluent of the horizontal reed beds gave negative results for all plasmids.

Table 27: Detection of plasmids by Southern hybridization in the wastewater of the RTP Wiedersberg

Inc group/ Replication type	Template plasmid	Primers	Sample	Detection
IncP	RP4, R751	trfA2	settled ww	+
		IncPoriT	effluent VRB	+
			effluent HRB	-
IncW	R388	IncWoriT	settled ww	-
			effluent VRB	-
			effluent HRB	-
IncN	pULB2432	IncNrep	settled ww	-
			effluent VRB	-
			effluent HRB	-
IncQ	pJE723	IncQoriV	settled ww	-
		IncQoriT	effluent VRB	-
			effluent HRB	-
rolling circle plasmids	pMV158	pMVoriT	settled ww	-
		pMVrep	effluent VRB	-
			effluent HRB	-

VRB = vertical reed beds; HRB = horizontal reed beds

4 Comparison of microbial populations in a young and an old reed bed

In order to assess if microbial consortia of clogged filters differed from non-clogged ones the vertical reed beds of Wiedersberg and Ließen were compared. The RTP Ließen was 4 years longer in operation and the reed beds showed first signs of clogging, whereas vertical reed beds in Wiedersberg displayed good hydraulic parameters.

Each of the RTPs was sampled twice: The vertical reed bed 1 of Wiedersberg was sampled in May and September 2000 and the vertical reed bed 2 of Ließen was sampled in July and October 2000. Each time, two soil cores were removed from the vertical reed beds of each of the RTPs and separated in 6 samples of different depth. Bacterial density was determined by total cell counts after detachment of microorganisms from the filter medium. Afterwards bacterial composition was analyzed for the upper layer of both reed beds by whole cell fluorescence in situ hybridization.

4.1 Total cell counts in different depths of the vertical reed beds

Reed bed cores with a depth of 50 cm were taken and microorganisms originating from mixed samples of 0-4 cm, 4-8 cm, 8-12 cm, 12-16 cm, 16-20 cm, and 40-44 cm

depth were dispersed and fixed. The dispersion method was gentle and possibly did not detach bacteria more tightly adsorbed to the filter. After DAPI staining total cell counts were determined for each sample of Wiedersberg and Ließen for two independent measurements (Figure 12).

DAPI staining combined with epifluorescence microscopy proved be a good tool to observe soil bacteria in suspension. Except for the upper layer of the vertical filter in Ließen neither large bacteria nor protozoa could be found in the soil samples. Interestingly, hyphae forming fungi were not observed in significant amounts in any of the filter samples.

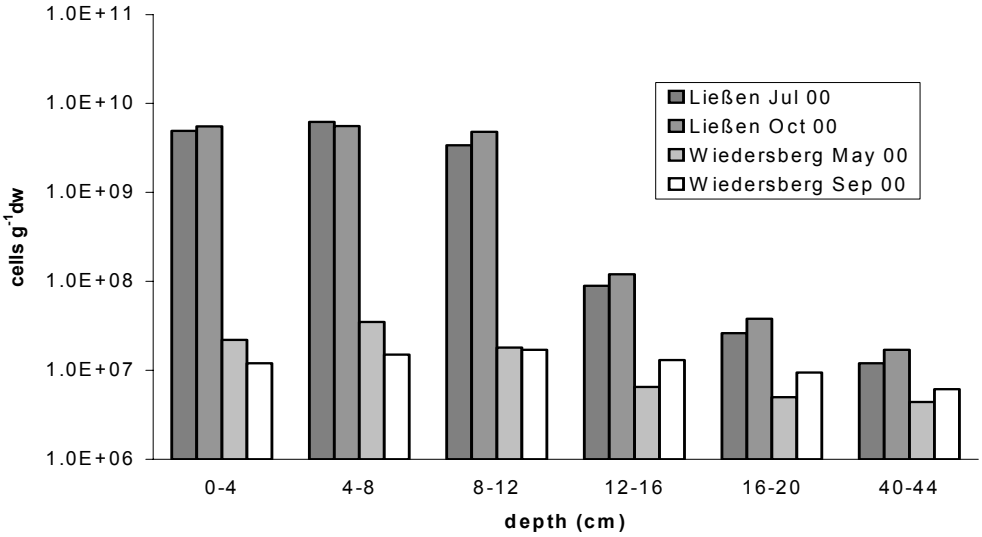


Figure 12: Bacterial density determined by total cell counts in different depths of the vertical beds of Wiedersberg and Ließen. Values were determined by total cell counts after DAPI staining.

For each of the vertical beds bacterial densities did not vary considerably in the two analyzed samples (Figure 12). Between 0 and 12 cm depth bacterial density of the vertical bed in Wiedersberg averaged 10^7 bacterial cells per g of dry weight: 1.70 x 10^7 cells g^{-1} dw in 0-4 cm depth , 2.50 x 10^7 cells g^{-1} dw in 4-8 cm depth and 1.75 x 10^7 cells g^{-1} dw in 8-12 cm depth. Below 12 cm bacterial density was lower: 9.75 x 10^6 cells g^{-1} dw in 12-16 cm depth, 7.20 x 10^6 cells g^{-1} dw in 16-20 cm depth, and 5.25 x 10^6 cells g^{-1} dw in 40-44 cm depth. In the upper layers the highest density was determined in May whereas in the lower layers the highest density was determined in September. In the vertical reed bed of the RTP Ließen (Figure 12) bacterial densities

were significantly higher than in Wiedersberg. From 0 to 12 cm depth mean densities averaged 10^9 cells g^{-1} dw, with 5.2 x 10^9 cells g^{-1} dw in 0-4 cm depth, 5.90 x 10^9 cells g^{-1} dw in 4-8 cm depth and 4.10 x 10^9 cells g^{-1} dw in 8-12 cm depth. Below 12 cm depth bacterial densities decreased to mean values of 1.05 x 10^8 cells g^{-1} dw in 12-16 cm depth and 3.20 x 10^7 cells g^{-1} dw in 16-20 cm depth. In 40-44 cm depth bacterial density was only slightly lower (1.45 x 10^7 cells g^{-1} dw) than in 16-20 cm depth. Comparison of the densities determined for both filter beds showed that bacterial density in the upper 12 cm in Ließen was two orders of magnitude higher than in Wiedersberg. Between 12 and 16 cm the difference between both reed beds was lower (one order of magnitude), while below 16 cm bacterial densities differed by less than one order of magnitude.

4.2 FISH analysis of bacterial populations in the vertical reed beds

The clogging layer (0 to 4 cm) of the vertical reed beds was analyzed by whole cell fluorescence in situ hybridization. Bacteria detached from the samples were fixed and subsequently hybridized with the fluorescent labeled probes EUB338, ALF1b, GAM42a, BET42a, Arch915, CF319a and CF319b and Pla46. The values depicted in Figure 13 are expressed as percent values of probe conferred signals in relation to DAPI conferred signals. The hybridization patterns of both clogging layers did not vary significantly in the two sampling campaigns, although samples were taken in different seasons of the year. For both reed beds and in both independent hybridizations, only a small proportion of the bacteria detected by DAPI produced a signal with the probe EUB338: EUB338-hybridized cells constituted 23% (July) and 25% (October) of total cells in the samples from Ließen and 17% (May) and 19% (September) in the samples from Wiedersberg (Figure 13). Signals with the Arch915 probe accounted for less than 0.1% of total cell counts in all samples and were not included. In Ließen the maximum abundance was recorded for β-*Proteobacteria* (average of 0.9% of total cell counts), followed by the α- and γ-subclass with an average of 0.6% and 0.5% of total cell counts, respectively. Hybridizable members of the *Cytophaga-Flavobacterium-Bacteroidetes* group were present as 0.3% of total cell counts. Members of the *Planctomycetales* accounted for less than 0.1% of total cell counts and were not represented in Figure 13.

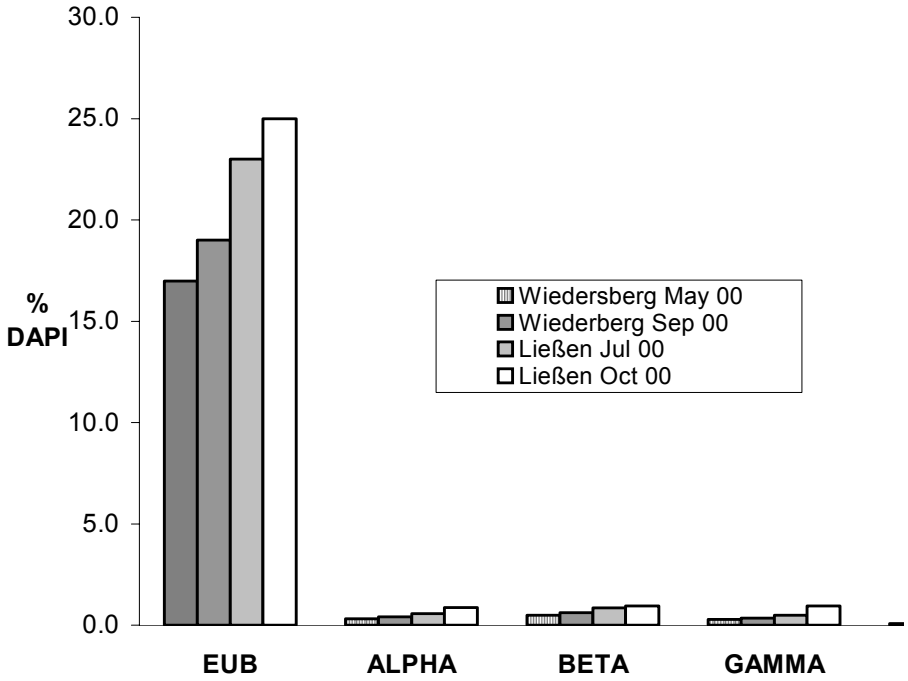


Figure 13: Abundance of bacteria assigned to different phylogenetic groups in the upper layer of the vertical reed beds Wiedersberg and Ließen. EUB = *Eubacteria*; ALPHA = α- *Proteobacteria*; BETA = β-*Proteobacteria*; GAMMA = γ-*Proteobacteria*; CF = *Cytophaga-Flavobacterium-Bacteroidetes*

In Wiedersberg β-*Proteobacteria* were also most abundant (average of 0.5%), followed by the α-*Proteobacteria* (average of 0.4%) and the γ-*Proteobacteria* (average of 0.3%). The *Cytophaga-Flavobacterium-Bacteroidetes* were present in very low amounts (0.1% of total cell counts) and no signals were detected with the Pla46 probe.

4.2.1 Estimate of probe conferred signal intensity

For all investigated filter medium samples, probe conferred signals were weak. Therefore, probe conferred signals with EUB338 were compared with Fluos-labeled and Cy3-labeled non-EUB338 probe. The reason for using both nonEUB338 probes is that Fluos conferred signals are always weaker than Cy3-signals. In each hybridization slide, the analyzed sample was hybridized twice: with Cy3-EUB338 and Fluos-nonEUB338 and in a separate well only with the Cy3-labeled non-EUB338 probe. The fluorescence intensity produced by all three probes was compared. Background fluorescence, produced by both the Fluos- and the Cy3-labeled non-

EUB probe was much weaker than the signals conferred by the Cy3-labeled EUB338 probe. Consequently it was possible to clearly distinguish positive, weak hybridization signals from background fluorescence.

C) Elimination of bacteria in a lab scale fixed-bed reactor

A lab scale fixed-bed reactor was constructed and operated under defined conditions for 20 days. The reactor influent consisted of primary settled wastewater collected from one of the investigated RTPs. In the last 8 days of operation, the influent was amended with the reference strain *Y. enterocolitica* DSM 11502.

Bacteria removal efficiency was assessed by a combination of total cell counts and heterotrophic plate counts. Retention of organic matter in the filter bed was determined by TOC and DOC measurements. Biofilm formation was investigated by weight loss on ignition, EDX analysis and fluorescence spectrometry on one side and by bacteria enumeration and cultivation on the other side. SEM was used for biofilm and bacteria visualization on the filter medium. Fluorescence in situ hybridization was applied to the liquid and filter medium samples in order to estimate the metabolic activity of the bacterial communities in general and of *Y. enterocolitica* in particular at different purification stages.

Using two eukaryotic FISH probes the numbers and localization of protozoa at different purification stages were investigated. Finally, the removal of *Y. enterocolitica* from the influent and its persistence or elimination in different depths of the filter was tracked using quantitative Real Time 5'-fluorogenic TaqMan PCR.

1 Physical and chemical properties of the fixed bed reactor

The physical properties of the filter medium affect filtration capacity decisively. Therefore, the dimensions, elemental composition, organic matter and water content of the filter medium as well as the porosity of the filter bed and the mean hydraulic residence time were determined.

1.1 Dimensions and chemical composition of the filter medium grains

1.1.1 Dimensions

The dimensions and uniformity of the sand grains composing the filter medium were determined by means of a sand sieve analysis. Figure 14 shows a granulometric curve of the analyzed sand. Clay (1%), silt (1.6%) and coarse sand (1.6%) were present in low percentages. Fine and medium size sand amounted together to 96.0% of the total mass of the sample. Medium sand (64.0%) was the dominant fraction. The parameters effective size 10 (d10) and effective size 60 (d60) were calculated

by interpolation of the curve and are defined as the sand size, at which a maximum of 10% or 60% of the sample weight has smaller grain size. d10 and d60 values were 0.084 and 0.300 mm in diameter, respectively.

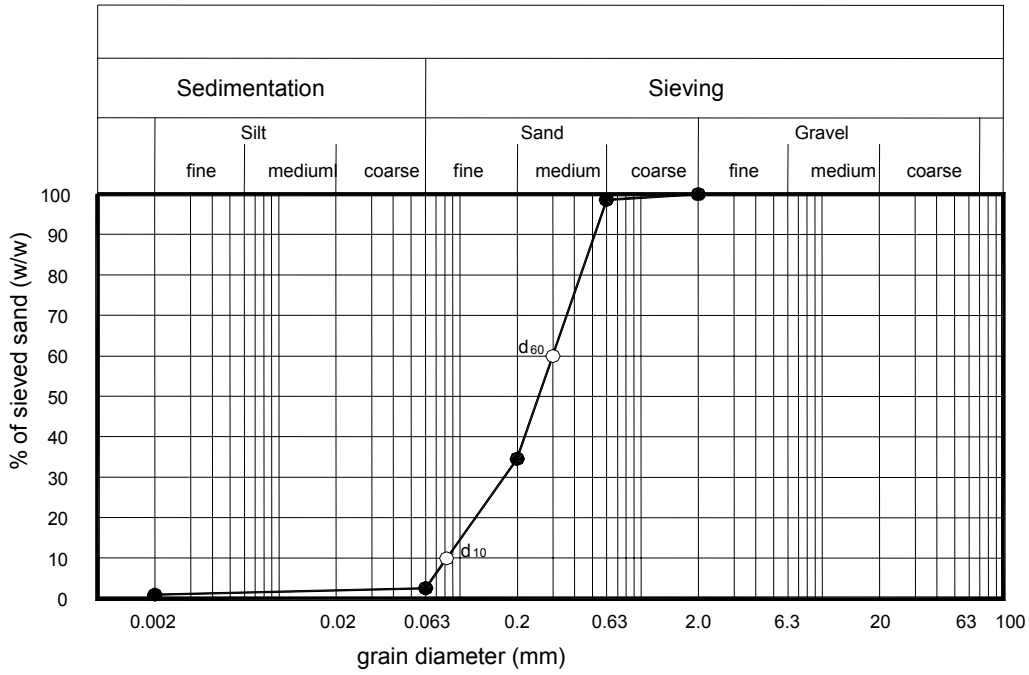


Figure 14: Cumulative arithmetic curve of the grain size distribution of the reactor filter medium.

Uniformity coefficient U gives a measure of the homogeneity of the sand grain sizes and is defined as the arithmetic quotient between d60 and d10 (U=d60/d10). The filter medium used for the reactor filter bed has a U of 3.57. Filter media with U values below 5 are considered uniform and pores are larger than in non-uniform media (U>15).

1.1.2 Elemental composition

The aim of this experiment was: i) to determine the chemical composition of the sand used as filter medium in the fixed-bed reactor and ii) to investigate if deposition of substances during operation had changed the chemical – and hence the adsorption – properties of the porous medium. Special focus was laid upon mono- and divalent cations. The original chemical composition of the filter medium was determined with the sterilized sand. After operation, samples were taken from 3, 10, 20, 30, and 40

cm depth and analyzed. Elemental composition was determined by means of X- ray spectrometry and EDX analysis. X-ray spectrometry makes an absolute quantification of the chemical elements in a sample but gives no information on its molecular structure. In its turn, EDX analysis displays only relative abundances of the main components of a sample but enables the spatial localization of the detected elements in the analyzed area, so that it is possible to draw conclusions on the molecular aggregates present.

X-ray spectrometric analysis of the sterilized filter medium (Table 28) showed that the highest sand fraction consisted of silicium (91.8%), indicating, together with the analysis of the morphology of the sand grains, that the analyzed sand was mostly composed of quartz.

Table 28: Elemental composition of the filter medium after operation. Values are given as oxidation products and were obtained by X-ray fluorescence spectrometry.

Sample depth	SiO_2 %	Al_2O_3 %	Fe_2O_3 %	CaO %	Na_2O %	K_2O %	TiO_2 %	P_2O_5 ppm	Ba ppm	Zr ppm	Sr ppm
3 cm	89.2	1.88	0.42	1.29	0.29	0.66	0.15	270	437	121	30
10 cm	90.0	2.03	0.50	1.13	0.35	0.67	0.18	260	299	170	29
20 cm	87.6	1.93	0.48	1.14	0.31	0.71	0.24	260	296	201	25
30 cm	89.2	1.95	0.48	1.23	0.31	0.69	0.20	320	272	211	28
40 cm	90.8	1.79	0.44	1.11	0.29	0.67	0.20	180	295	147	27
Control	91.8	1.83	0.42	1.16	0.26	0.65	0.16	220	250	116	26

Control was sterilized filter medium. Values are given in percentage (w/w) or in ppm (mg/kg) of sample dry weight.

The silicium fraction, together with lower percentages of aluminum (1.83%), calcium (1.16%), iron (0.42%), potassium (0.65%), sodium (0.26%), and titanium (0.16%) made up 96.28% of the total dry mass of the filter material (Table 28). Manganese and magnesium concentrations were below the detection limit (0.02% for Mn and 0.10% for Mg). Phosphorus, barium, strontium, and zirconium were present in very low amounts. All further analyzed elements were below the detection limits of the method, which varied between 6 and 40 mg per kg of dry mass. Analysis of the filter medium in different depths after operation showed no significant changes in the elemental composition of the sand in any of the depths, indicating that adsorption of the investigated elements to the filter medium grains must have occurred only in non-detectable amounts.

EDX analysis of the filter medium supported the results obtained by fluorescence spectroscopy. Figure 15 shows a scanning electron microscopic image (SEM) of the

analyzed area of the control sample. Additionally to the whole area analysis, two sand grains of different appearance in the SEM image (sand grain 1 and sand grain 2, Figure 15) were analyzed.

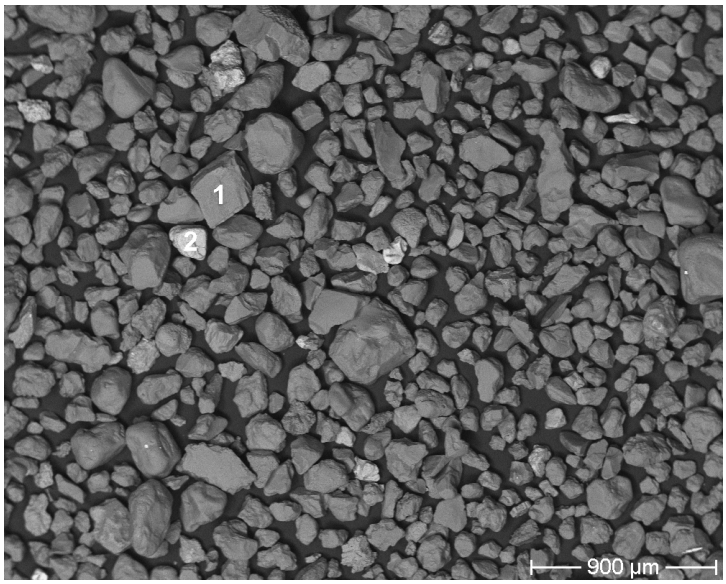


Figure 15: Scanning electron microscopic image of the filter medium.

In comparison with the average of the whole sample, grain 1 had a significantly higher amount of aluminum and potassium, a small increase in sodium and less silicium and oxygen (Figure 16.a). This combination is typical for feldspar.

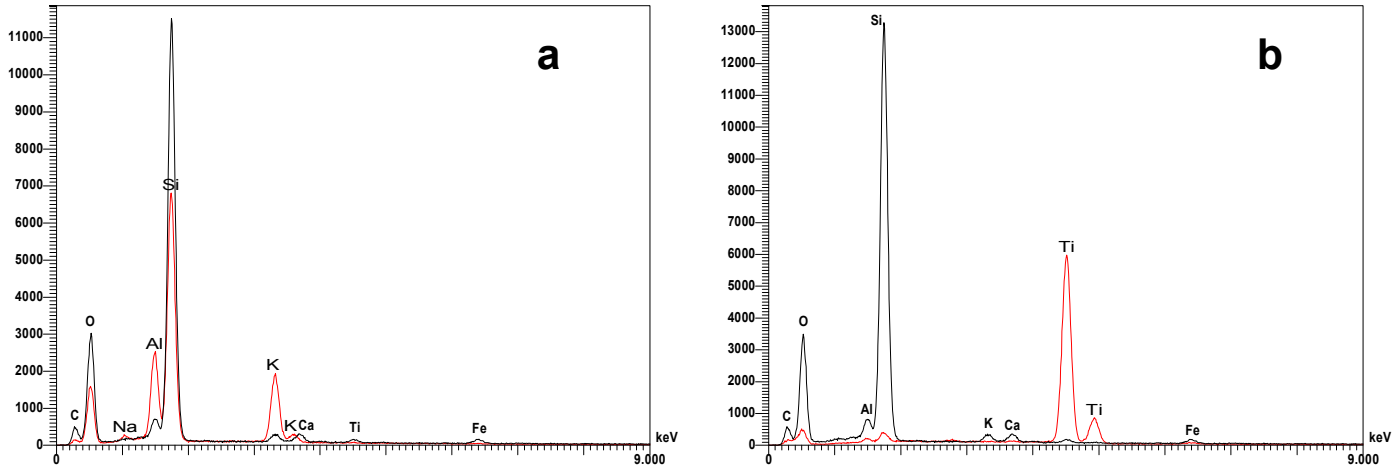


Figure 16: Comparison of the elemental compositions of the whole sample area (black) with: (a) grain 1 and (b) grain 2 (in red).

Sand grain 2 (Figure 16.b) was mostly composed of titanium, being probably a titanium oxide. Titanium oxides were rare in the analyzed sample but feldspar occurred more frequently. EDX analysis of the filter medium samples after operation

(Figure 17) confirms the X-ray spectrometric results: there was no significant difference in element composition between the blank sample (Figure 16, black line) and the samples taken after 20 days of operation in 10, 20 and 40 cm depth (Figure 17, red lines).

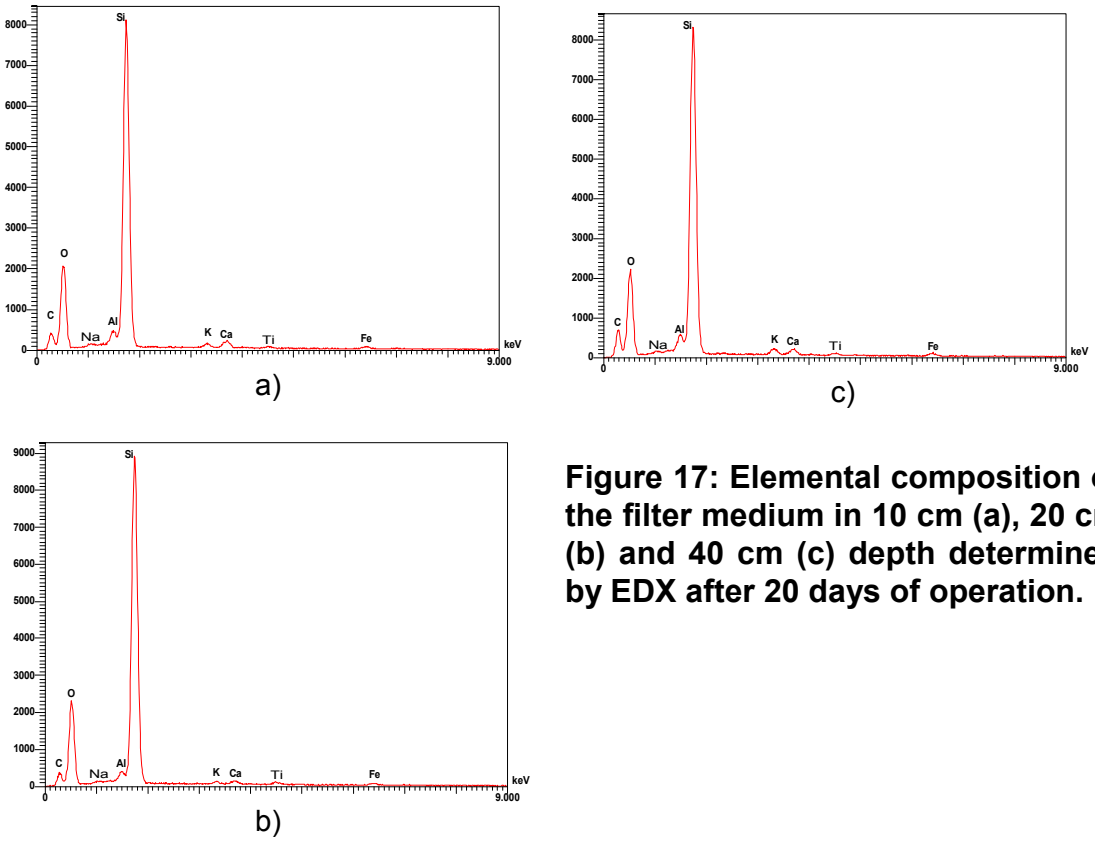


Figure 17: Elemental composition of the filter medium in 10 cm (a), 20 cm (b) and 40 cm (c) depth determined by EDX after 20 days of operation.

1.2 Water and organic matter content

Organic matter and water content of the filter medium were determined by weight loss on ignition measurements. The water content in different depths of the filter bed after operation is shown in Table 29. The moisture content of the filter medium increased significantly with depth of the column (Table 29): at 40 cm depth the water content in the reactor bed (16.5%) was over two and a half times higher than at the surface (6.2%). The water content of the sterilized filter medium (control) was neglectable (< 0.5%).

Table 29: Water content in the reactor filter bed measured in different depths.

Sample depth	Water content (%)
3 cm	6.2
10 cm	8.5
20 cm	13.3
30 cm	15.0
40 cm	16.5

As for the organic matter content, in order to evaluate the degree of homogeneity of biomass formation in the filter, triplicate samples were taken from each depth and analyzed (Figure 18). The control sample, consisting of sterilized filter medium, contained very small amounts of organic matter (average of 0.072%). During operation, the filter medium accumulated little amounts of organic matter. Values obtained with the triplicate samples were very close to each other. Results indicate that the distribution of organic matter along the depth of the filter bed occurred uniformly. The average percentual fraction of organic matter in the different depths was (from 3 cm to 40 cm) 0.160%, 0.178%, 0.155%, 0.151% and 0.162%.

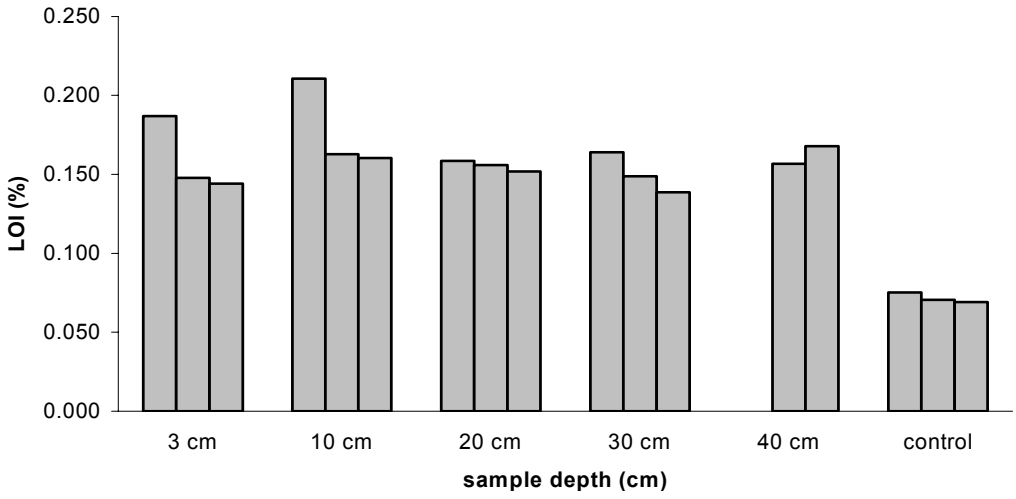


Figure 18: Organic carbon content of the filter medium in triplicate samples. Percentage values are given in relation to sample dry weight. Measurement failed for one sample in 40 cm depth.

1.3 Residence time of water in the filter bed

The residence time of wastewater percolating through the reactor strongly influences the contact time between bacteria and filter surface. Therefore a pulse of the tracer

NaBr was given to the filter bed and the concentrations of Br^- were measured every hour in the effluent.. Calibration of the bromide electrode was performed with standard NaBr solutions and the following correlation was deduced by exponential regression analysis:

$$y = 0.1631e^{-0.05x} \quad (1)$$

where y is the bromide concentration given in mg l^{-1} and x is the measured potential difference given in mV. The calculated correlation R^2 was 0.9881. The bromide concentration in the influent was 100 mg l^{-1}. Only after 24 h bromide concentrations higher than 1% (4.52 mg l^{-1}) could be detected. From the total bromide given in the pulse, only 7.2% were detected in the effluent, indicating that the bulk mass of the bromide pulse remained in the filter bed for at least 24 h.

2 Filtration performance of the reactor

The wastewater purification potential of reed bed systems depends greatly on the capacity to retain bacteria and organic matter in the filter bed. If the lab scale fixed bed reactor should simulate a vertical reed bed, it was necessary to verify its filtration performance and compare it with the investigated full-scale systems. Filtration performance was evaluated by determining the capacity for retaining bacteria and organic matter. The influent and the effluent of the reactor were regularly sampled. Retention of organic matter was determined by TOC and DOC measurements. Filtration of bacteria was assessed by a combination of several methods. The overall retention of bacteria in the filter was determined by total cell counts. The removal of culturable heterotrophic bacteria was assessed by heterotrophic plate counts on HPC medium. Phase contrast microscopy gave an insight into the changes in composition of the microbial populations after percolation through the filter bed. Finally, by means of whole cell fluorescent in situ hybridization with eubacterial and eukaryotic fluorescent probes active bacteria and protozoa were enumerated.

2.1 Retention of organic carbon

As a standard parameter for wastewater purification efficiency the removal of total and organic carbon from the influent of the fixed bed reactor was assessed. The concentrations of TOC and DOC in the influent and effluent samples were measured according to DIN 38409 DEV H3. Samples were taken after 3, 7, 12, 18 and 20 days of operation. The results obtained are presented in Figure 19. The TOC and DOC

contents of the influent wastewater decreased during operation (from 226.22 to 145.88 mgl^{-1} and from 150.47 to 99.78 mgl^{-1}, respectively), although the values were higher at day 20 than at day 18 (145.88 vs. 99.59 mgl^{-1} for TOC and 99.78 vs. 73.64 mgl^{-1} for DOC).

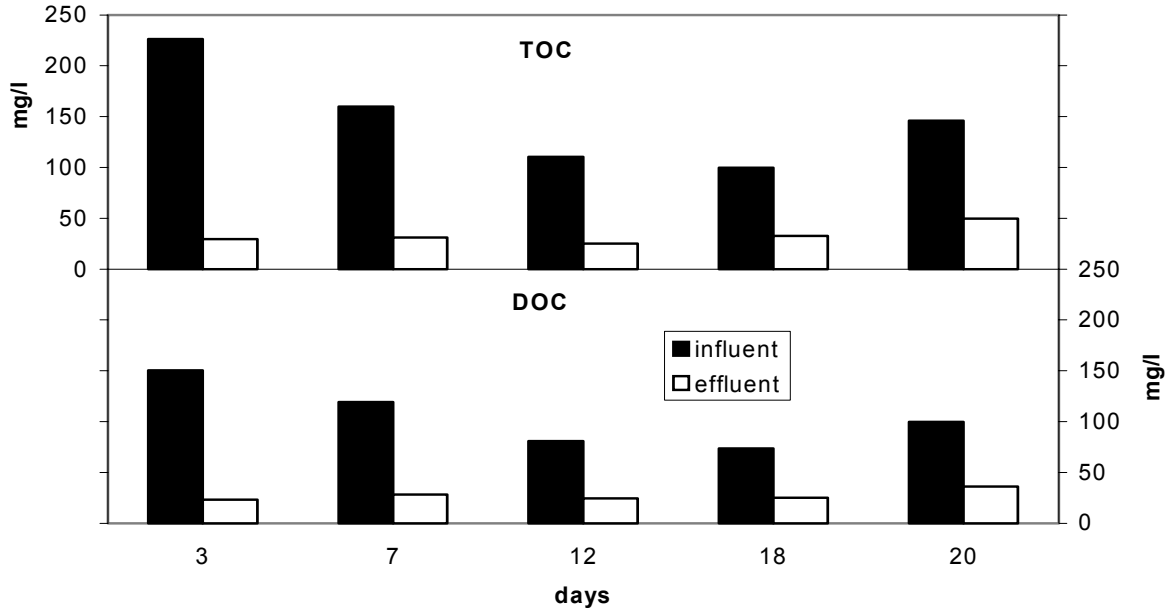


Figure 19: Total and dissolved organic carbon contents in the influent and effluent of the fixed bed reactor. TOC = total organic carbon; DOC = dissolved organic carbon.

TOC removal in the reactor decreased during operation from 87% to 66%. The mean removal rate was 76%. For DOC the removal pattern was similar: removal rate was 85% at day 3 and decreased to 67% at day 20. Mean DOC removal rate was 72%.

2.2 Retention of microorganisms

Removal of total bacteria from the influent wastewater was determined by total cell counts and heterotrophic plate counts. Enumerations were performed with 1 ml sample taken every second day. Figure 20 and Figure 21 depict the variations in total cell counts and heterotrophic plate counts in the reactor influent and effluent during operation with wastewater. When determined with total counts, bacterial concentrations in the influent and effluent wastewater were approximately constant. The influent concentrations ranged from 4.31×10^8 to 1.12×10^9 cells ml^{-1}. The average concentration for the 10 measurements was $9.72 \pm 2.05 \times 10^8$ cells ml^{-1}.

Effluent concentrations varied between 1.52 x 10^7 and 4.18 x 10^7 cells $ml^{-1}$and the average value was 2.50 x ± 0.83 x 10^7 cells ml^{-1}.

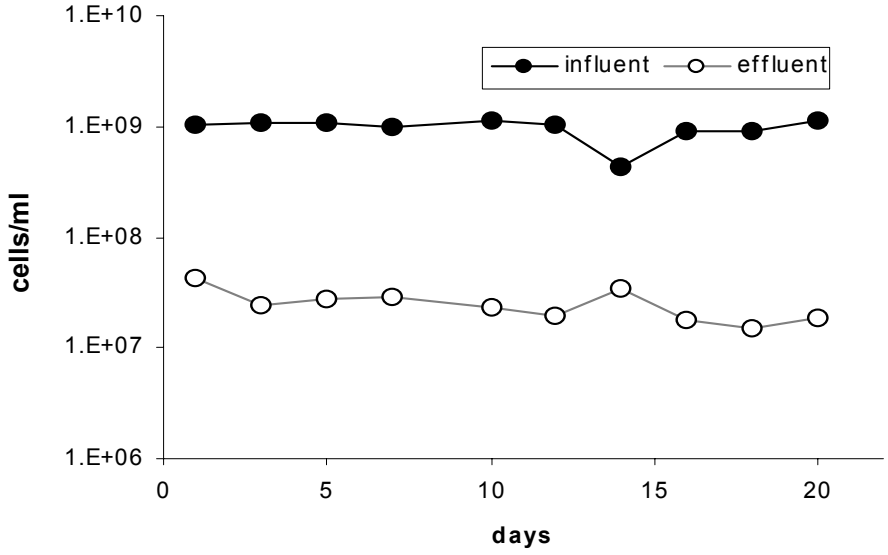


Figure 20: Total cell counts in the influent and effluent of the fixed bed reactor

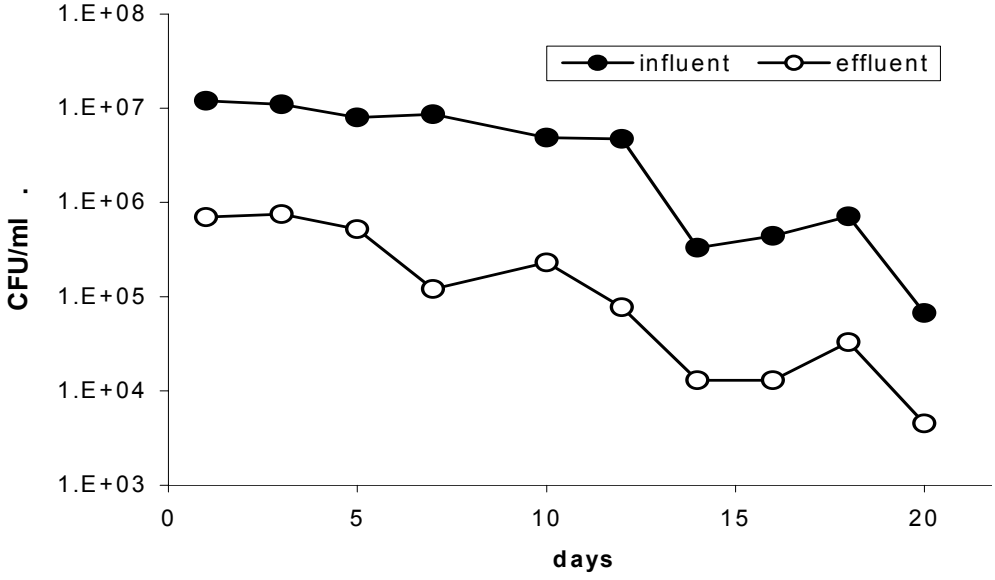


Figure 21: Heterotrophic plate counts in the influent and effluent of the fixed bed reactor. CFU = colony forming units

At day 14 influent and effluent concentrations suffered a change in the general pattern (Figure 20): influent bacterial numbers were lower and effluent numbers were higher than for the other measurements. This measurement coincided with the utilization of a new stored influent bottle.

In contrast to the results obtained by total cell counts, the amounts of culturable bacteria decreased significantly during the 20 days of operation (Figure 21). Culturable bacteria decreased from 1.2 x 10^7 to 6.7 x 10^4 CFU ml^{-1} in the influent and from 7.0 x 10^5 to 4.5 x 10^3 CFU ml^{-1} in the effluent. There was a steady decline in bacterial concentrations. The decline was proportional for the influent and effluent samples. A more pronounced decline in plate counts was observed for days 14 and 20.

The percentage of culturable cells in the influent and effluent of the reactor was calculated for each sampling day as the percentual ratio between heterotrophic plate counts and total cell counts. Values depicted in Figure 22 show that the proportion of culturable bacteria was higher in the effluent than in the influent of the reactor. Culturability decreased in the influent wastewater from 1 % to 0.006 % between days 1 and 20. For the effluent samples the highest percentual culturability was at days 3 and 5, with 3% and 2%, decreasing to 0.02 % at day 20. Mean culturability values were 0.5% for the influent samples and 0.9% for the effluent samples.

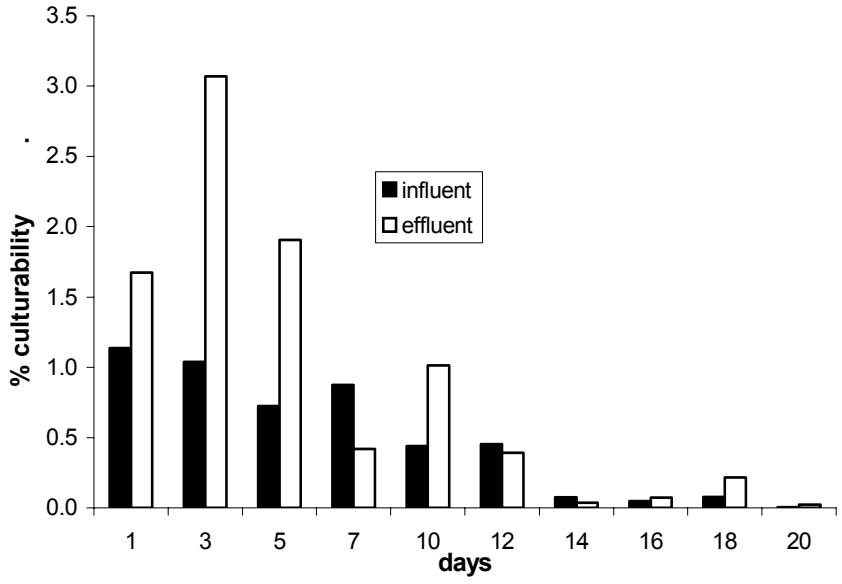


Figure 22: Variations in bacterial culturability in the influent and effluent samples of the fixed bed reactor.

The percentual removal rates of bacteria from the influent wastewater samples were determined based on total cell counts and on heterotrophic plate counts (Table 30). Highest and lowest retention rate of bacteria in the filter bed were 98.4% and 91.9% as determined by total cell counts and 98.6% and 93.2% as determined by plate counts. Average retention rate was 97.1 ± 2.0% for total cell counts and 95.5 ± 2.0% for plate counts. Except for day 14, where bacterial removal amounted to 91.9%, removal rates determined by total cell counts were very constant, ranging between 96.4% and 98.4%.

Table 30: Bacteria removal rates from the influent of the fixed bed reactor as determined by total cell counts and heterotrophic plate counts.

Sampling day	% Removal total counts	% Removal plate counts
1	96.0	94.2
3	97.7	93.2
5	97.5	93.5
7	97.1	98.6
10	98.0	95.3
12	98.1	98.4
14	91.9	96.1
16	98.1	97.1
18	98.3	95.3
20	98.4	93.3

There was no correlation between the bacterial removal rates determined for the filter beds and the organic content of the influent wastewater. There was a positive correlation between the concentrations of culturable bacteria in the influent and the respective effluent concentrations (Figure 23).: the linear correlation determined by regression analysis was given by the equation $y = 0.076x^{0.9656}$, where y is the effluent and x the influent bacterial concentration determined by heterotrophic plate counts. The correlation coefficient was 0.9032. Hence, the amount of bacteria passing through the filter was positively correlated with their concentration in the influent wastewater. A correlation was not determined for the values obtained by total cell counts since the range of bacterial concentrations was too narrow for analysis.

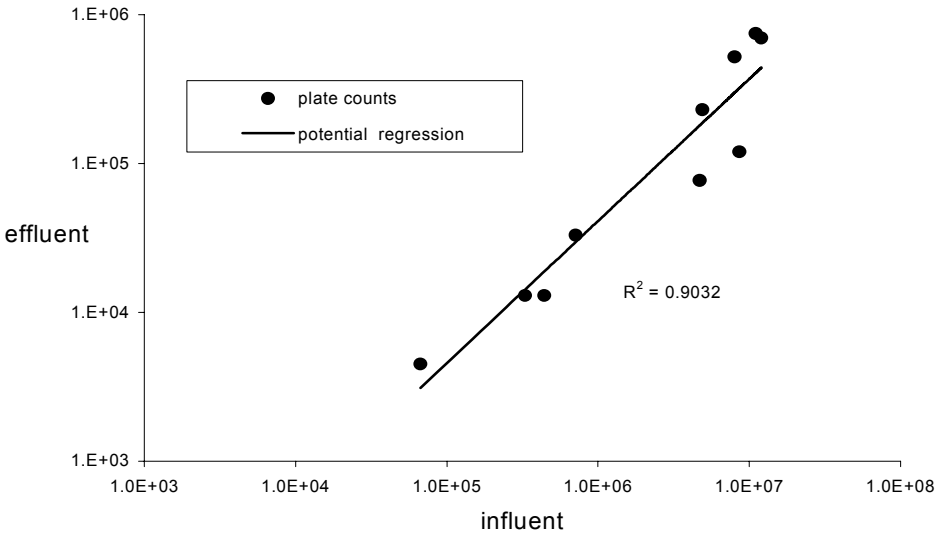


Figure 23: Correlation between influent and effluent concentrations of culturable bacteria.

Whole cell fluorescence in situ hybridization was performed with the eubacterial probes EUB, EUB-II and EUB-III and the eukaryotic probes EUK1209 and EUK502 for the fixed influent and effluent samples from days 12, 14, 16, 18, and 20 (Table 31). EUB probes were Cy3-labeled and EUK probes were Oregon Green 488-labeled. Although EUK1209 and EUK502 target all eukaryotic cells, the reason for application of these probes was to enumerate protozoa. Hybridized algae and other eukaryotic organisms were not considered. Although the fixed samples were two-fold concentrated in relation to the native ones, protozoan numbers were too low to allow a statistic enumeration in relation to DAPI signals. Higher sample concentration increased background too much, making identification of protozoa even more difficult. Therefore, the whole wells of the FISH microscopic slides were enumerated at a 600-fold magnification and the values are given as hybridizable protozoa per ml of native sample.

Table 31: Hybridizable cells in the influent and effluent of the reactor.

Sampling day	EUB influent (%)	EUB effluent (%)	EUK influent (cells ml^{-1})	EUK effluent (cells ml^{-1})
12	45	12	1.53×10^4	0
14	54	21	n.d.	n.d.
16	48	18	n.d.	n.d.
18	51	23	2.15×10^3	0
20	53	23	8.80×10^3	0

EUB = signal conferred by the fluorescent probe EUB338; EUK = signals conferred by the fluorescent probes EUK1209 and EUK502. Percentual values are given in relation to total cell counts, concentration values are given as total hybridizable cells per ml of native sample; n.d. = not determined

FISH results (Table 31) revealed a decrease in the amount of hybridizable cells (EUB to DAPI signal ratio) during percolation through the filter. The percentage of total cells giving a signal with the EUB probe mixture varied from 45 to 54% in the influent and from 12 to 23% in the effluent samples. Probe conferred signal intensity of the effluent cells was significantly lower than that of the influent cells. Mean hybridizable eubacterial cells amounted to 50.2% of total bacterial cells in the influent and 19.4% in the effluent. The percentage of hybridizable cells varied more strongly for the effluent samples. Hybridizable protozoan cells in the influent samples decreased from 1.53×10^4 cells ml^{-1} at day 12 to 2.15×10^3 cells ml^{-1} at day 18 and 8.80×10^3 at day 20. No EUK signals were detected in the analyzed effluent samples. In order to rule out errors in the hybridization performance or signal quenching by unknown substances, 2 fixed effluent samples were spiked with fixed cells of *Saccharomyces cerevisiae* and hybridization was repeated. *S. cerevisiae* cells gave a very bright signal with the EUK probes.

2.3 Qualitative analysis of the retention performance of the filter

Fixed influent and effluent samples of the fixed bed reactor were qualitatively analyzed by phase contrast microscopy at a 600X magnification. Diversity of microorganisms in the influent was very high: protozoa (mostly flagellates), unicellular algae, *Chromatium sp.*, *Thiothrix sp.*, *Spirillum sp.*, cocci, filamentous bacteria and rods of different sizes were identified in all samples. Protozoa numbers in the influent samples varied between10^5 and 10^6 protozoan cells ml^{-1}. In the effluent samples the morphology of microorganisms was much more uniform: only small rods, spirilla and filamentous bacteria were observed, whereas bigger cells (>2-3 µm in length) were almost absent. Protozoa and algae were not or only very sporadically detected in the effluent samples.

The utilization of a new storage bottle of wastewater at day 14 of operation coincided with a decline in plate counts and a reduction of the removal efficiency for bacteria. To search for possible reasons the influent samples of day 12 (taken from a previous storage bottle) and 14 were analyzed in more detail after DAPI staining. Influent bacteria from day 12 contained many phosphate granules. *Chromatium sp.* were present in high numbers, as well as *Thiospirillum sp.* and filamentous bacteria. Bacteria with phosphate granules were also abundant at day 14, but the relative amounts of *Chromatium sp.* had decreased and no *Thiospirillum sp.* was observed. Small filamentous bacteria were more abundant. Microcolonies of uniform appearance constituted by small rods of approximately 1 µm in diameter and without phosphate granules were a striking feature in the sample from day 14. Protozoa were more abundant at day 14: in 20 analyzed microscopic fields, 8 protozoa were detected, versus 4 at day 12. In general, small bacteria were more abundant in the samples from day 14, which could explain a reduction in the removal efficiency.
Microbial composition in wastewater at day 14 differed significantly from the previous one utilized for reactor percolation. The pronounced decline in culturability of the sample suggests that microbial populations shifted towards more nonculturable species than before.

3 Microbial colonization of the filter bed

Investigations on the formation of a microbial mat in the filter bed included ex situ visualization and enumeration of the bacteria detached from the filter medium and visualization of the filter medium surface by scanning electron microscopy. For ex situ analysis microorganisms were dispersed from the filter medium with a two-step detachment procedure as described before. Enumerations were performed by total cell counts, heterotrophic plate counts and whole cell fluorescence in situ hybridization. For scanning electron microscopy whole filter bed cores were dehydrated and fixed before analysis.

3.1 Diversity of microorganisms in the filter bed

Microscopic observations of the microorganisms present in the filter matrix were performed with a 1000X magnification. Microorganisms were detached from the samples collected between day 12 and 20 in 10, 20 and 40 cm depth. Most bacteria were very small (< 2µm in length) and displayed no striking morphological traits. Both features made it difficult to localize them among small organic particles. DAPI

staining proved more suitable for visualization than phase contrast microscopy. The most characteristic bacteria of the influent, e.g. *Chromatium sp.*, *Thiothrix sp.* and *Spirillum sp.* were absent. Protozoa (flagellates) were detected only in 10 cm depth at day 20. Algae or other eukaryotic organisms were not detected.

3.2 Determination of total and culturable bacterial in the filter medium

During reactor operation single filter media samples were taken for bacterial enumeration from 10, 20 and 40 cm depth. At the end of the experiment replicate samples were taken from 3, 10, 20, 30, and 40 cm depth. Microbial suspensions derived from detachment from the filter medium samples were fixed for determination of total cell counts, microscopic analysis and FISH and used without fixation for plating and subsequent determination of heterotrophic plate counts. Every sample was enumerated at least twice.

Figure 24 shows the distribution of bacteria along the depth of the fixed-bed reactor. Results are expressed as total cell counts per g of dry weight sample (cells gdw^{-1}). The lowest bacterial density was observed for all depths at day 0, i.e. during accommodation with tab water and one day before percolation with wastewater. After 3 days of operation bacterial density increased significantly (from 10 to 40 cm) from 4.7×10^5 cells gdw^{-1}, 3.6×10^5 cells gdw^{-1} and 3.8×10^5 cells gdw^{-1} at day 0 to $4. \times 10^7$ cells gdw^{-1}, 5.73×10^7 cells gdw^{-1} and 4.7×10^6 cells gdw^{-1} at day 3. Afterwards densities fluctuated between 10^6 and 10^7 cells gdw^{-1}. Values did not differ significantly along the depth. In 10 cm, the highest density was determined for day 7 with 4.8×10^7 cells gdw^{-1}. In 20 cm depth the highest bacterial density was determined for day 3 with 5.7×10^7 cells gdw^{-1}. This value was also the highest bacterial density determined in the reactor bed. For 40 cm depth, the highest bacterial density was determined for day 18 with 2.1×10^7 cells gdw^{-1}. After 20 days of operation bacterial densities were very similar in the three analyzed depths: 4.0×10^7 cells gdw^{-1} in 10 cm, 1.5×10^7 cells gdw^{-1} in 20 cm and 1.1×10^7 cells gdw^{-1} in 40 cm.

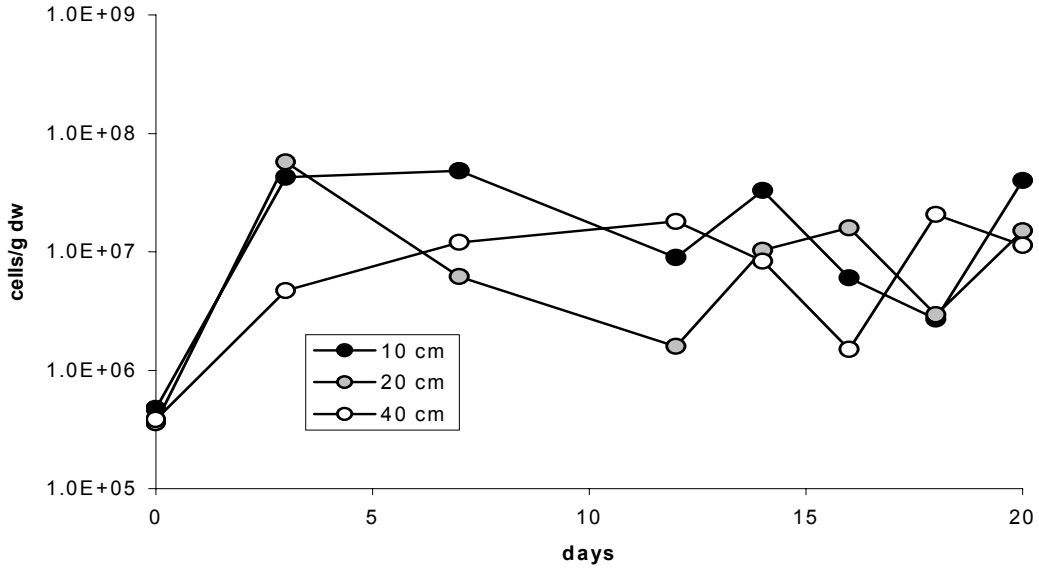


Figure 24: Variations in bacterial density as determined by total cell counts in different depths of the filter bed.

If bacterial density in the filter medium was determined by cultivation instead of total cell counts a totally different pattern was observed. Figure 25 shows the density of culturable bacteria. Colony forming units per gram of dry sample (CFU gdw^{-1}) were very low at day 0: 1.3 x 10^1, 2.6 x 10^1 and 3.3 x 10^1 CFU gdw^{-1} in 10, 20 and 40 cm, respectively. The highest bacterial densities were determined after 3 days in 10 (6.2 x 10^5 CFU gdw^{-1}) and 20 cm (4.6 x 10^5 CFU gdw^{-1}) and after 10 days in 40 cm (6.6 x 10^5 CFU gdw^{-1}). From day 10 on, bacterial density of culturable cells decreased in all analyzed depths, reaching final concentrations of 5.4 x 10^3 CFU gdw^{-1} in 10 cm, 6.3x 10^3 CFU gdw^{-1} in 20 cm and 7.5 x 10^2 CFU gdw^{-1} in 40 cm at day 20. Additionally, the average colony size decreased for all analyzed depths during the 20 days, and the incubation time increased 3-fold.

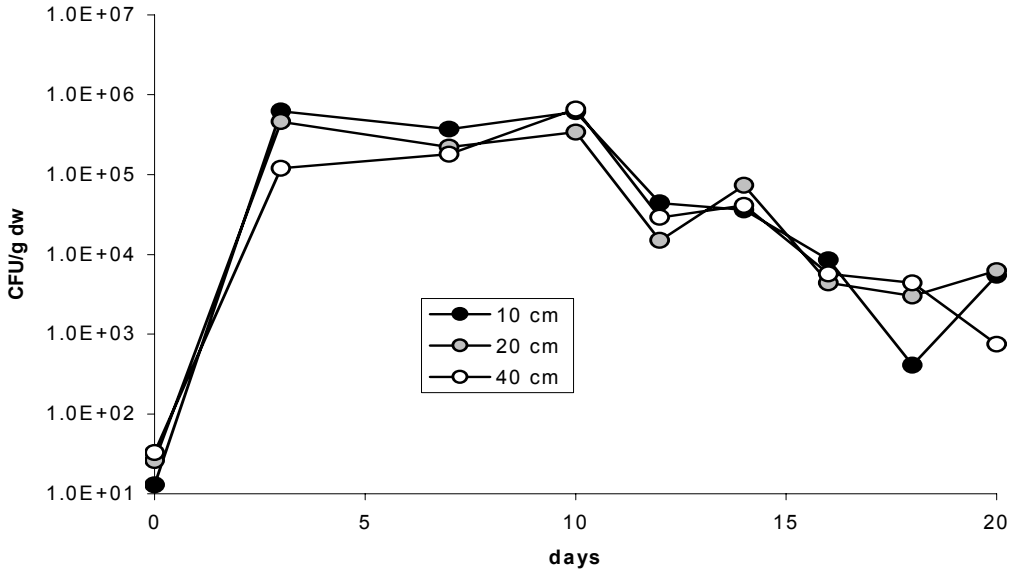


Figure 25: Variations in bacterial density as determined by heterotrophic plate counts in different depths of the filter bed. CFU/g dw = colony forming units per g of dry weight sample.

The proportion of culturable bacteria in the filter medium samples was calculated (Figure 26).

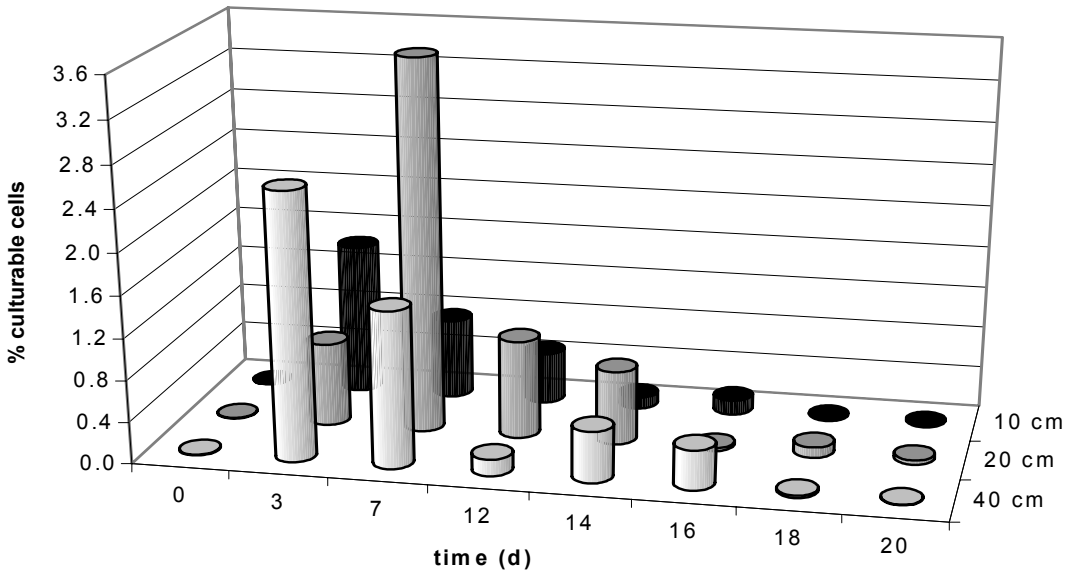


Figure 26: Changes in culturability of the bacterial populations colonizing the filter bed in different depths.

In order to determine the degree of homogeneity of bacterial distribution in each depth of the reactor bed, triplicate samples were randomly collected from 3, 10, 20, 30, and 40 cm depth after reactor sacrification and bacterial densities were determined for each sample by cultivation (Figure 27). The gray columns depict the average value of the three independent measurements, whereas the black whiskers show the highest and lowest bacterial densities determined for each depth.

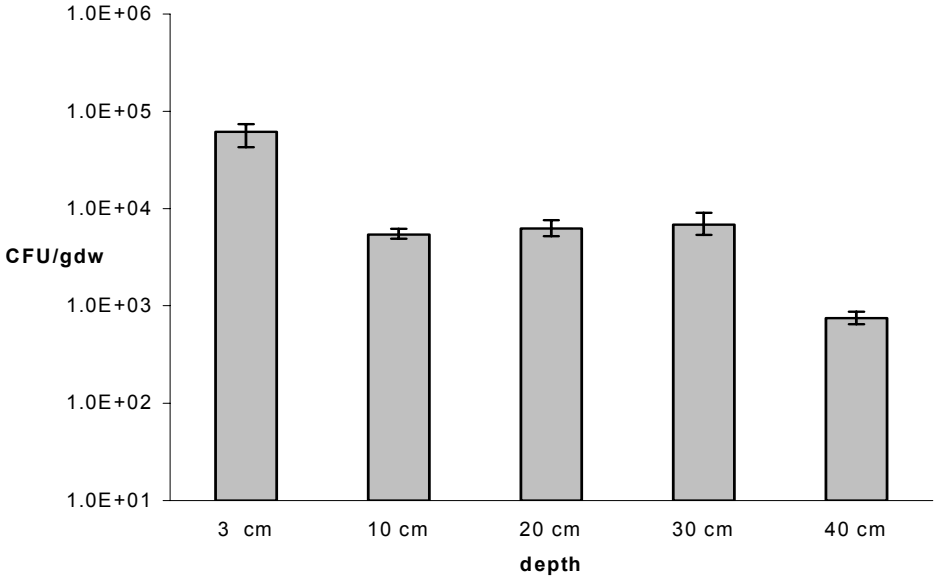


Figure 27: Variation of bacterial density at different spots in the same layer as determined by heterotrophic plate counts. Gray columns show the average density, whiskers show the highest and lowest densities obtained for the triplicate measurements. CFU = colony forming units.

For 3 cm depth, plate counts varied between 4.3 x 10^4 and 7.4 x 10^4 CFU gdw^{-1}, for 10 cm between 4.9 x 10^3 and 6.2 x 10^3 CFU gdw^{-1}, for 20 cm between 5.2 x 10^3 and 7.6 x 10^3 CFU gdw^{-1}, for 30 cm between 5.4 0 x 10^3 and 9.0 x 10^3 CFU $gdw^{-1}$and for 40 cm between 6.5 and 8.7 x 10^2 CFU gdw^{-1} (Figure 27). Mean densities were (from 3 to 40 cm): 6.2 x 10^4, 5.4 x 10^3, 6.3 x 10^3, 6.9 x 10^3 and 7.5 x 10^2 CFU gdw^{-1}, indicating that variations in bacterial concentrations within the same horizontal layer were not significant.

3.3 Amount of hybridizable microorganisms in the filter medium

The percentage of viable eubacterial cells was determined by whole cell fluorescence in situ hybridization with the oligonucleotide probes EUB338, EUB338-II and EUB338-III. Hybridization with the eukaryotic probes EUK1209 and EUK502 was simultaneously performed in order to localize protozoa in the filter bed: Results are shown in Table 32. All EUB signals in the filter medium samples were very weak. Therefore, parallel assays with Oregon Green- and Cy3-labeled EUB338 and nonEUB338 probe mix were performed. Comparison of the signal intensities with both fluorescent labels revealed that the probe conferred signals, although weak, were clearly higher than background fluorescence produced by either Cy-3-labeled or Oregon Green-labeled nonEUB probes. Signals were brighter if the EUB338 probes were labeled with Cy3. All results shown in Table 32 for EUB-conferred signals refer to Cy3 signals in relation to DAPI signals. Oregon Green labeled EUK probes gave a very bright signal, so that EUK and EUB hybridizations could be performed in a single well. Since protozoa were very rare, the whole hybridization well was enumerated as described previously in 2.2 for hybridization of wastewater samples.

Table 32: Hybridizable bacteria and protozoa in the filter medium samples.

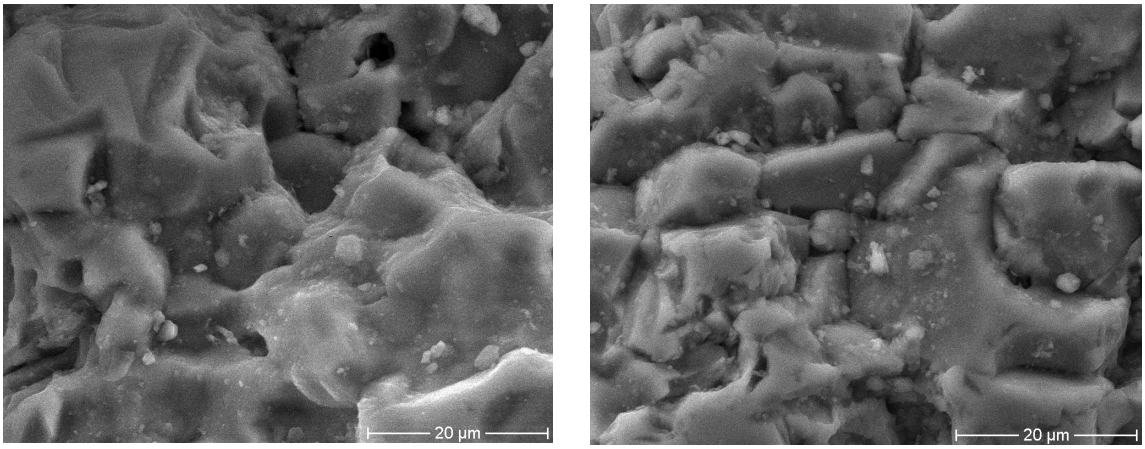
day	14		18		20	
probe	EUB %	EUK cells gdw^{-1}	EUB %	EUK cells gdw^{-1}	EUB %	EUK cells gdw^{-1}
10 cm	10	0	15	0	15	6.04×10^4
20 cm	10	0	7	0	17	0
40 cm	13	0	6	0	32	0

EUB = signal conferred by the fluorescent EUB338 probe mix; EUK = signals conferred by the fluorescent probes EUK1209 and EUK502. Percentual values are given in relation to total cell counts, concentration values are given as total hybridizable cells per g of dry weight sample

Hybridizable bacteria (Table 32) varied between 6% and 32% in the different samples. In the samples derived from 10 cm depth hybridizable bacteria increased from 10% at day 14 to 15% at day 18 and remained constant at day 20. For 20 and 40 cm, hybridizable bacteria decreased at day 18 (from 10 to 7% and from 13 to 6%, respectively) and increased significantly at day 20 to 17% and 32%, respectively. Protozoa were only found at day 20 in 10 cm depths and amounted to 6.04×10^4 cells gdw^{-1} of filter medium.

3.4 SEM visualization of the filter medium surface

In order to study the extent of biofilm formation after 20 days of operation the surface of the filter medium was visualized by scanning electron microscopy in 10, 20 and 40 cm depth. For each analyzed sample, 10 microscopic fields were scanned in order to localize microcolonies or greater organic aggregates on the surface of the filter. Figure 28 and Figure 29 show the surface of the filter medium at the indicated magnification. After 3 weeks of operation, the surface of the filter grains appeared to be very smooth and free from organic matter (Figure 28). Biofilm formation on the exposed surfaces of the sand grains was very reduced or inexistent. Bacteria colonizing the filter medium were therefore probably located in less exposed areas like the cracks and fissures of the filter grains, as shown in Figure 29.



a) b)

Figure 28: Scanning electron microscopic image of the filter medium surface in 10 cm (a) and 20 cm (b) depth after 20 days of operation

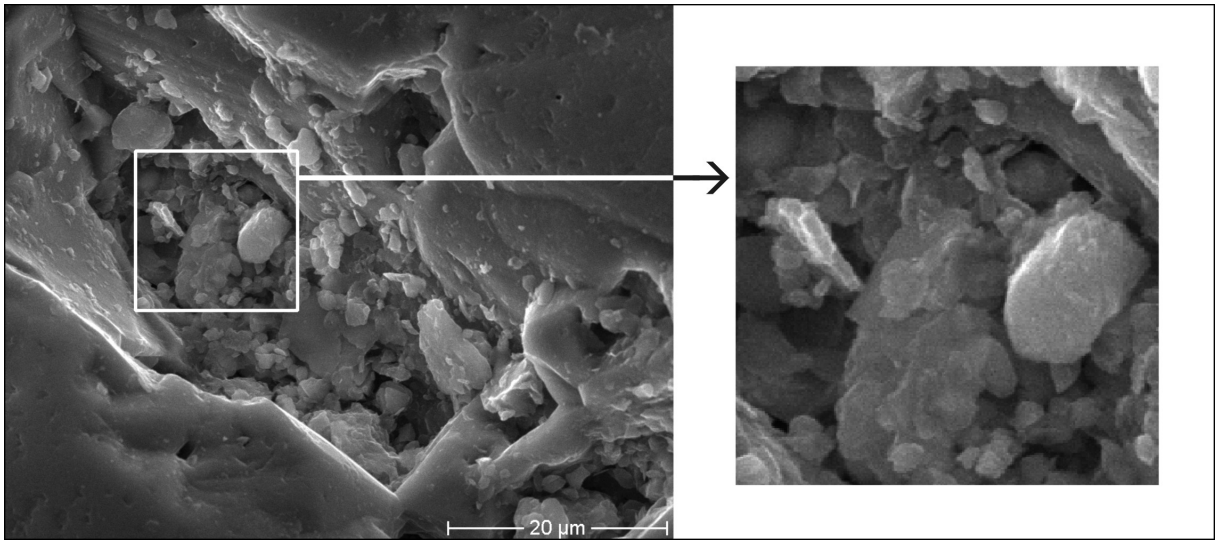


Figure 29: Amplification of a fissure in a filter medium grain derived from 40 cm depth after 20 days of operation.

3.5 Calculation of the bacterial density on the filter medium surface

In order to estimate the expected density of bacteria on the surface of the filter medium the surface area was calculated based on the granulometric curve shown in Figure 14, the bulk density of the sand and the specific density of quartz.

The total surface area available for bacteria in 1 cm^3 of filter medium (TA) was calculated as the sum of the partial areas of each filter medium fraction (PA) determined by sand sieve analysis (TA = ΣPA). Each PA was calculated according to the equation:

$$PA = Ap \times PV / Vp \qquad (2)$$

where PV is the total effective volume of each sand fraction in 1 cm^3 of filter medium, Vp is the average volume of the filter medium particles in each sand fraction and Ap is the average area of the particle in each sand fraction. Determination of the partial areas obtained by different sand grain dimensions (Table 33) was carried out based on the average radius of each diameter interval determined by the granulometric curve depicted in Figure 14. The surface area per cm^3 of filter medium sample was calculated as follows:

The volume (Vp) and surface area (Ap) of a filter medium particle was calculated according to the equations:

$$Vp = 4/3 \times \pi \times r^3 \quad (3)$$

$$Ap = 4 \times \pi \times r^2 \quad (4)$$

where Vp and Ap are the volume and surface area of one particle and r the respective radius. Thereby it was assumed that filter medium particles had a spherical shape (although clay has a rather platy shape) and were not fractured. If both factors would be concerned (shape of clay and fissures), the calculated area would be greater. Therefore the area of the filter medium particles available for bacteria was calculated by underestimation. The specific density of quartz is 2.65 g cm^{-3}. The bulk density (i.e. weight of 1 cm^3 sample) of the filter medium was empirically determined and amounted to 1.52 g cm^{-3} of filter medium sample. Thereby, 1 cm^3 filter medium sample contained 0.57 cm^3 of sand (effective sand volume). The remaining 0.43 cm^3 were pore space. Partial volumes for each sand fraction were determined as the product of their abundance by the total effective sand volume in 1 cm^3 of sample. Partial areas for each sand fraction were determined by multiplying the number of filter medium particles of each fraction by the area of one particle.

The partial areas determined for the different sand fractions are listed in Table 33. According to these calculations the average total area per cm^3 of filter medium sample is 495 cm^2.

Table 33: Volume and area occupied by components of the filter medium according to their abundance as determined by sand sieve analysis. Partial areas and volumes are given in relation to 1 cm^3 of sample.

Sand components	Average diameter D (mm)	Abundance in sample A (% w/w)	Partial volume [2] PV (mm^3)	Partial area [2] PA (mm^2)
clay [1]	0.0010 [1]	1.0	5.7	34,200
silt	0.0325	1.6	9.12	1,684
Fine sand	0.1315	32.0	182.4	8,322
Medium sand	0.4150	64.0	364.8	5,274
Coarse sand	1.315	1.6	9.1	42

[1] = clay dimensions are defined as <0.002 mm; [2] = partial volume/area defined as the fraction of total sand volume/area in 1cm^3 of filter medium

Bacterial densities determined by total cell counts in the filter medium samples collected after reactor sacrification were 4.00 x 10^7 cells g^{-1} dw in 10 cm, 1.50 x 10^7 cells g^{-1} dw in 20 cm and 1.13 x 10^7 cells g^{-1} dw in 40 cm depth. The average bacterial density in the reactor column was therefore 2.21 x 10^7 cells g^{-1} dw or 3.35 x 10^7 cells per cm^3 of filter medium sample. This equals to a distribution pattern of 6.77 x 10^4 bacterial cells per cm^2 of filter medium surface. The highest magnification utilized for scanning electron microscopy observations delivered an image of 60 x 60 µm side length. According to the calculated distribution pattern an average of 0.24 cells per 60 x 60 µm scanning field would be expected.

4 Tracking the fate of a pathogen in the reactor

This investigation had two aims: on one side the elimination and survival of a wastewater relevant pathogenic bacterium in the reactor should be studied and compared to the survival behavior of autochthonous wastewater bacteria. On the other side the potential of the TaqMan assay for accurate and reproducible detection of bacteria in reed bed-like systems should be evaluated.

Y. enterocolitica was added to the reactor influent during the last 8 days of operation. The TaqMan assay selected for the detection of *Y. enterocolitica* in wastewater and filter medium samples had been developed for blood samples. Therefore preliminary assays were required to assess the applicability and sensitivity of the method for wastewater and filter medium samples.

Removal rates of the pathogen during passage through the filter were determined by TaqMan PCR. Results were compared with those obtained by selective cultivation of *Y. enterocolitica*. The TaqMan assay was also applied to filter medium samples in order to study the accumulation and distribution of *Y. enterocolitica* in the reactor filter bed during operation.

In order to estimate the fraction of metabolically active target cells the results obtained by TaqMan PCR were compared with fluorescence in situ hybridization directed to *Y. enterocolitica.*

4.1 Growth potential of *Y. enterocolitica* in wastewater

Y enterocolitica is a gastrointestinal pathogen and naturally adapted to nutrient-rich environments. The domestic wastewater used for percolation of the reactor had low carbon content (see Figure 19). Therefore the survival potential of this organism under similar conditions to those existing in this wastewater was evaluated. Artificial

wastewater medium, with a DOC of approximately 100 mg l^{-1}, was used as growth medium. HPC medium has a DOC of approximately 10,000 mg l^{-1}, which is usual for cultivation of *Y. enterocolitica*. Triplicate assays were prepared for each medium. Incubation was carried out at room temperature on a rotary shaker at 120 rpm. Optical density was measured every 30 min in the first 10 hours of the experiment (Figure 30). *Y. enterocolitica* density did not exceed 2.5 x 10^8 cells per ml in artificial wastewater (Figure 30). Exponential growth began approximately 3 hours after inoculation and was verified for this time interval by regression analysis of the measured bacterial concentrations. The average doubling time during exponential growth of *Y. enterocolitica* amounted to 70 min. *Y. enterocolitica* final density was higher in HPC medium and reached 1.27 x 10^9 cells ml^{-1}. Exponential growth began 2 h after inoculation. The average doubling time in the exponential growth phase was 43 min. Results suggest that *Y. enterocolitica* can survive and replicate under the nutrient conditions present in wastewater.

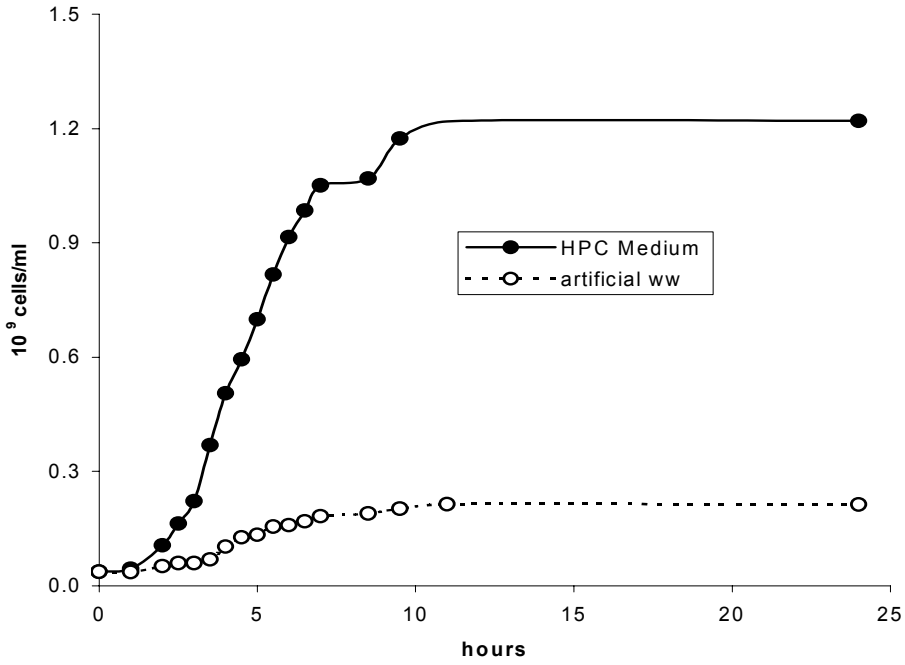


Figure 30: Growth curve of *Y. enterocolitica* in artificial wastewater and HPC culture medium. Each value is the mean of 3 replicate assays. ww = wastewater.

4.2 Preparation of the external standard for TaqMan quantification

Prior to application in the TaqMan assay the DNA yield obtained by extraction of pure cultures of *Y. enterocolitica* with the Fast DNA kit was determined. The genome

size of *Y. enterocolitica* is estimated to 4,400 kb, which is equivalent to 4.8 fg DNA. *E. coli* JM109 was co-extracted as a positive control. *E. coli* genome size is approximately 4,600 kb long corresponding to a weight of 5.0 fg. Triplicate HPC overnight cultures of both strains were grown until the stationary phase and 10^8 cells from each culture(determined by direct cells counts) were extracted with the FastDNA Kit (Table 34). DNA was quantified with the E.A.S.Y. Win 32 software program. The extraction yield was calculated as the ratio between the extracted DNA and the theoretical DNA amount of 10^8 cells of each of the bacterial strains. It was assumed that each cell contained only one chromosome copy.

For *E. coli*, 10^8 cells yielded between 325 and 360 ng DNA (Table 34). Mean extraction yield was 328 ng DNA per 10^8 cells. Based on the *E. coli* genome size the theoretical DNA amount of 10^8 *E. coli* cells is 500 ng. Hence the percentage of extracted DNA varied between 60% and 72% of the theoretical value. Mean extraction yield was 66% of total genomic DNA. For *Y. enterocolitica* DNA extraction yielded between 280 and 350 ng per 10^8 cells, corresponding to 58% to 73% of the estimated theoretical value assuming one chromosome per cell. Mean extraction yield was 65%.

Table 34: DNA yields of *Y. enterocolitica* and *E. coli* JM109.

Extraction	*E. coli* ng DNA	% of total DNA	*Y. enterocolitica* ng DNA	% of total DNA
1	325	65	280	58
2	300	60	350	73
3	360	72	310	65

Percentage of total DNA was calculated based on the ratio between the extracted DNA and the theoretical DNA amount of 10^8 cells.

The external standard required for quantification by the SDS software was generated from *Y. enterocolitica* genomic DNA extracted with the FastDNA Kit from stationary overnight culture. Cell concentration was determined by total cell counts. DNA was extracted and quantified with the E.A.S.Y. Win 32 software. Figure 31 shows the calibration curve obtained by computer analysis for the TaqMan assay.

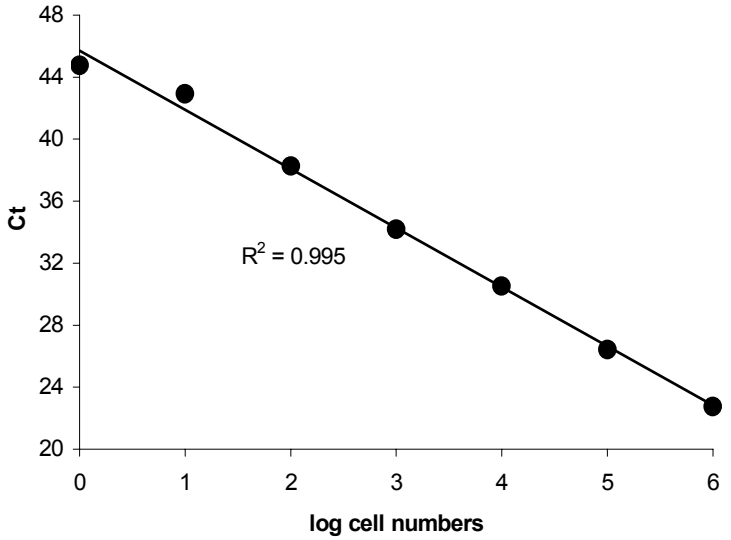


Figure 31: Calibration curve obtained with the *Y. enterocolitica* external standard. log cell numbers = logarithm of the *Y. enterocolitica* cell numbers per assay; Ct = fluorescence threshold value.

Before application to TaqMan PCR the *Y. enterocolitica* genomic DNA was 10-fold serially diluted spanning 7 orders of magnitude and corresponding to 1 to 10^6 cells of the original culture. When applied to TaqMan PCR, 3.1 fg of extracted DNA –- corresponding to1 cell of *Y. enterocolitica* –- were quantitatively detected and were therefore set as the detection limit of the assay. TaqMan detection of *Y. enterocolitica* was log proportional over the 7 orders of magnitude and the correlation coefficient determined by the SDS software was R^2 = 0.9950.

4.3 Influence of the filter medium on the accuracy of the DNA extraction

The influence of the filter medium on the DNA extraction yield was evaluated in batch assays. Furthermore, it should be assessed if the amount of extracted DNA was proportional to the amount of sample. For this purpose two 500 ml Erlenmeyer flasks were filled with 10 g sterilized filter medium and 35 ml sterile wastewater medium amended with 7.2 x 10^7 *Y. enterocolitica* cells per ml in the stationary growth phase. The sand formed a fine layer on the bottom of the flask and was completely submerged by the liquid medium, providing optimal contact conditions and a uniform distribution of the cells. After 1 h of incubation duplicates of 50 mg, 100 mg, 250 mg, and 500 mg filter medium were collected and excess liquid was removed. The DNA

was extracted from the filter medium samples, appropriately diluted and subjected to TaqMan PCR.

Parallel duplicate filter medium samples (200 mg) were collected for direct enumerations: after removal of excess liquid, *Y. enterocolitica* cells were detached from the filter medium and enumerated by total cell counts after DAPI staining.

TaqMan quantification of the 8 samples confirmed reproducibility of the DNA extraction for replicate samples: single values for assays 1 and 2 varied between 4.5 % and 12.5 % from the calculated average value. However, the DNA extraction yield was proportionally higher for greater amounts of samples. Therefore, in order to exclude results biased due to DNA extraction, always the same amount of sample was extracted (500 mg).

As for the influence of the filter medium on the DNA extraction yield, enumerations of the adsorbed *Y. enterocolitica* cells delivered a mean density of 7.29 x 10^6 *Y. enterocolitica* cells g^{-1} of wet weight sample. The average value obtained by TaqMan PCR was 2.08 x 10^6 cells g^{-1} of wet weight, suggesting that there was no negative interference of the filter medium on the DNA extraction yield.

4.4 DNA extraction of the reactor samples

Environmental samples have levels of chemical and genetic complexity not normally encountered in tissue and/or pure culture samples which may affect the ability of TaqMan PCR to quantify DNA and/or influence the DNA extraction itself. Therefore, the DNA yield obtained with the FastDNA Kit on one side and the sensitivity of the TaqMan assay on the other side were determined for the samples derived from the fixed bed reactor.

All spiked and nonspiked wastewater and filter medium samples designated for TaqMan PCR analysis were extracted with the FastDNA kit. For each extraction, an additional vial containing 100 µl of a *Y. enterocolitica* DSM 11502 and/or *E. coli* JM109 overnight culture was extracted as a proof of accuracy of the extraction.

Adequacy of the FastDNA Kit for the extraction of DNA derived from domestic and municipal wastewater has already been confirmed (see Section A). Therefore the reactor influent and effluent samples were extracted without further modifications, followed by the described quality control tests.

However, during loading onto the agarose gels for quantification of the extraction yield with the E.A.S.Y. Win 32 software, samples sometimes escaped from the gel

pockets, resulting in sample loss and hindering quantification. This phenomenon is normally a sign for ethanol residues derived from DNA washing steps during extraction. Attempts to eliminate the ethanol (e.g. by elongating the silica drying step after ethanol washing and/or by performing drying at higher temperature) did not eliminate the effect. Finally, vacuum drying of the DNA after elution from the Spin Column and posterior resuspension in sterile bidistilled water proved the only suitable way to remove all ethanol residues. The extraction yield obtained for each filter medium sample is shown in Figure 32, together with the respective cell numbers. The DNA extraction yield from 10 and 20 cm varied between 86 and 262 ng g^{-1}dw for 10 cm and between 90 and 275 ng g^{-1}dw for 20 cm depth. DNA amounts extracted from filter medium samples at 40 cm depth was always lower than from 10 and 20 cm and did not exceed 52 ng g^{-1}dw. Bacterial density in the filter medium varied between 10^6 and 10^7 cells g^{-1}dw filter medium in all depths.

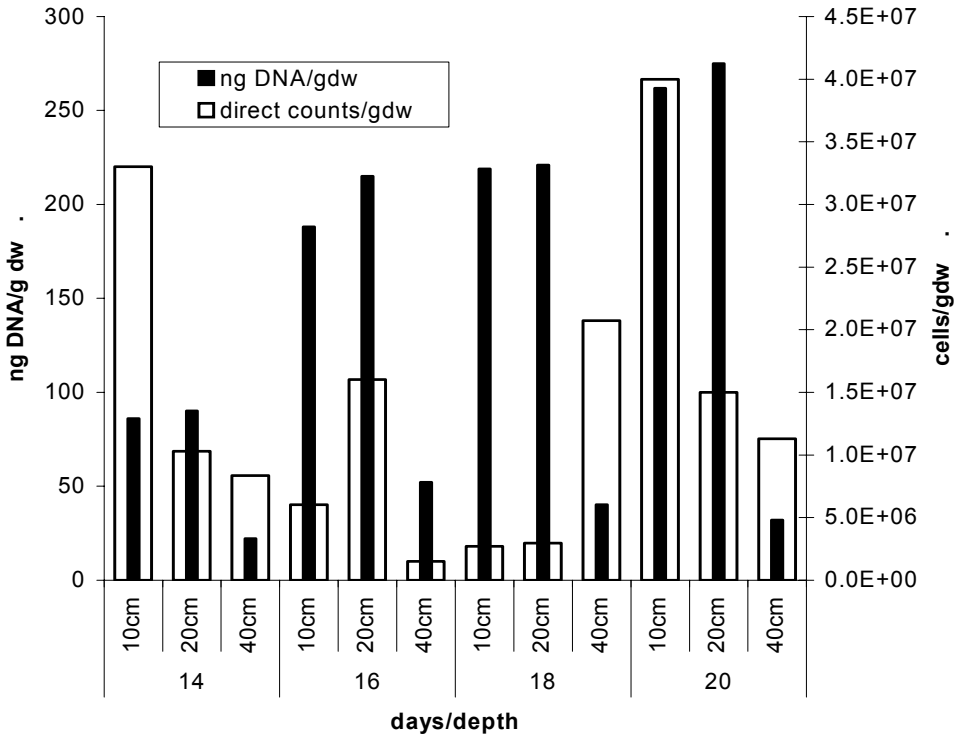


Figure 32: Correlation between total cell counts and extracted DNA from the filter medium. dw = dry weight; total counts were determined by DAPI staining and enumeration.

In order to obtain a rough estimate of the DNA extraction efficiency in the different depths, the quotient between the DNA yield and the total cell numbers determined by total cell counts (Figure 32) was calculated for each sample. The quotient yielded 30

fg DNA per cell for the samples in 10 cm depth, 21 fg DNA for the samples in 20 cm depth and 10 fg DNA per cell for the samples withdrawn from 40 cm depth.

For DNA quality assessment, amplification feasibility of the extracted DNA was evaluated by applying 1 µl DNA to the PCR with the primers 27f and 1492r directed to the 16S rDNA eubacterial gene. All DNA extracts obtained from the reactor samples showed good amplification potential with the primers.

4.5 Sensitivity of the TaqMan assay in the reactor samples

The sensitivity of the TaqMan assay in the reactor samples was determined based on the parameters related to the DNA extraction, on the required dilutions and on the sensitivity of detection. The sensitivity of the method in the wastewater samples was 200 *Y. enterocolitica* cells per ml of influent or effluent (assuming 1 chromosome per cell). For the filter medium samples, 400 cells per g of sample wet weight was the lowest target cell number required for positive results. Since the FastDNA Kit allows the extraction of a maximum of 500 mg of wet sample the sensitivity of the whole detection procedure, when referred to dry weight of sample, varied slightly with the moisture content of the filter sample, being higher for the samples with lower water content (Table 35).

Table 35: Sensitivity of the TaqMan based method for the filter medium samples.

Sample depth	Water content %	Sensitivity (cells g^{-1} dw)
3 cm	6.2	426
10 cm	8.5	438
20 cm	13.3	462
30 cm	15.0	470
40 cm	16.5	480

4.6 Reduction of *Y. enterocolitica* in the influent vessel

The decrease of cell numbers of *Y. enterocolitica* in the influent vessel during the 24 h of usage as reservoir was determined. *Y. enterocolitica* was added to the influent vessel between days 13 and 20. Influent wastewater samples were collected 1, 3, 5, and 7 days immediately after addition of *Y. enterocolitica* and 24 h later. TaqMan results obtained with the extracted DNA were compared for both time points. Results depicted in Table 36 shows a mean reduction of 85.0% in the *Y. enterocolitica* concentration after 24 h permanence in the influent wastewater vessel. Highest and

lowest reductions were detected for day 18 and 20 with 92.1% and 80.4%, respectively.

Table 36: Reduction of *Y. enterocolitica* in the influent vessel.

Sampling day	C_o cells ml^{-1}	C_{24} cells ml^{-1}	Elimination %
14	8.46×10^5	1.31×10^5	84.5
16	6.27×10^5	1.07×10^5	82.9
18	1.12×10^6	8.86×10^4	92.1
20	9.51×10^5	1.86×10^5	80.4

C_o = concentration of *Y. enterocolitica* after addition to influent wastewater; C_{24} = concentration of *Y. enterocolitica* after 24 h permanence in the influent vessel.

4.7 Net reduction of *Y. enterocolitica* by percolation

The reduction of *Y. enterocolitica* by percolation was tracked by TaqMan PCR. The concentration of the model pathogen in the influent vessel was determined immediately after addition (C_0) and 24 h later (C_{24}) before exchange for new influent vessel. For the calculation of the removal rates of *Y. enterocolitica* the average value between C_0 and C_{24} was calculated (Table 37). The removal rates were calculated as the percentual difference between the average concentration of *Y. enterocolitica* in the influent vessel and its concentration in the effluent after 24 h.

As shown in Table 37, the reduction of *Y. enterocolitica* by percolation was very constant: the lowest average reduction rate was 99.7% and the highest 99.9%.

Table 37: Reduction of *Y. enterocolitica* by percolation as determined by TaqMan PCR.

Sampling day	Influent C_0 cells ml^{-1}	Influent C_{24} cells ml^{-1}	Influent ½ (C_0+C_{24}) cells ml^{-1}	Effluent C_{24} cells ml^{-1}	Mean reduction %
14	8.46×10^5	7.28×10^5	7.87×10^5	1.15×10^3	99.8
16	6.27×10^5	5.40×10^5	5.83×10^5	2.93×10^2	99.9
18	1.12×10^6	9.64×10^5	1.04×10^6	2.38×10^3	99.8
20	9.51×10^5	8.19×10^5	8.85×10^5	2.35×10^3	99.7

Influent C_0 = concentration of *Y. enterocolitica* in the influent vessel immediately after addition; Influent/Effluent C_{24} = concentration of *Y. enterocolitica* in the influent vessel/effluent after 24 h of permanence

4.8 Culturability of *Y. enterocolitica* before and after percolation

The concentrations of culturable *Y. enterocolitica* cells in the influent and effluent wastewater samples were assessed by a combination of cultivation on supplemented *Yersinia* agar base and subsequent colony PCR. Samples were taken at day 14 and 20 of operation. Cultivation of the influent samples (Table 38) resulted in high amounts of background flora (6.20×10^5 CFU ml^{-1} at day 14 and 8.30×10^5 CFU ml^{-1} at day 20). The same occurred for the effluent samples, which had total colony numbers of 2.94×10^4 CFU ml^{-1} and 3.35×10^4 at day 14 and 20, respectively. The colonies grown on the *Yersinia* selective agar base were numerous and displayed strikingly different morphologic traits, e.g. color, size, shape, aspect of the rim and shine. Only the presumptive *Yersinia spp*. colonies, characterized by the typical "bull-eye" appearance, were analyzed with the multiplex PCR directed to *Y. enterocolitica* serogroup 0:3 with the primers Y1/Y2 and P1/P2. Colony PCR revealed concentrations of culturable *Y. enterocolitica* of 1.08×10^4 CFU ml^{-1} (day 14) and 2.85×10^4 CFU ml^{-1} (day 20) in the influent and 1.20×10^1 CFU ml^{-1} (day 14) and 1.98×10^1 CFU ml^{-1} (day 20) in the effluent (Table 38).

Table 38: Culturable bacteria in the wastewater samples on *Yersinia* agar.

Sample	Background CFU ml^{-1}	*Y. enterocolitica* CFU ml^{-1}
influent day 14	6.20×10^5	1.08×10^4
effluent day 14	2.94×10^4	1.20×10^1
influent day 20	8.30×10^5	2.85×10^4
effluent day 20	3.35×10^4	1.98×10^1

The percentage of culturable *Y. enterocolitica* cells (cfu vs. direct, species-specific amplification of the wastewater DNA by TaqMan PCR) revealed 1.3% resp. 3.0% in the influent samples and 1.0% resp. 0.8% in the effluent (Table 39).

Table 39: Culturability of *Y. enterocolitica* before and after percolation.

Sample	Detection with TaqMan PCR cells/ml	Detection with cultivation CFU/ml	% culturable *Y. enterocolitica*
Influent day 14	8.46×10^5	1.08×10^4	1.3
Effluent day 14	1.15×10^3	1.20×10^1	1.0
Influent day 20	9.51×10^5	2.85×10^4	3.0
Effluent day 20	2.35×10^3	1.98×10^1	0.8

4.9 FISH analysis of *Y. enterocolitica* before and after percolation

Whole cell fluorescence in situ hybridization was selected for estimating the metabolic activity of *Y. enterocolitica* cells in the influent and the effluent of the reactor. A species-specific *Y. enterocolitica* fluorescent probe was used in combination with the EUB338I, EUB338II and EUB338III probes and DAPI staining. Hybridizable *Y. enterocolitica* cells were enumerated in the influent and effluent samples of the fixed bed reactor.

Hybridization was performed with wastewater samples collected at days 12, 14, 16, 18, and 20. Hybridization with the samples collected at day 12 served as proof of specificity of the probe and stringency of the hybridization, since no *Y. enterocolitica* was added to the influent from day 13 on.

Hybridization with samples derived from day 12 (Table 40) confirmed the specificity of the probe in the investigated wastewater samples and the stringency of the hybridization conditions.

Between days 14 and 20 *Y. enterocolitica* probe conferred signals varied between 2.9% and 3.2% (mean 3.0%) of total hybridizable eubacteria in the influent samples. All effluent samples showed no hybridization signals with the *Y. enterocolitica* probe. Total hybridizable eubacteria ranged from 45% to 54% (mean 50%) in the influent sample and from 12% to 23% (mean 19.4%) in the effluent samples. Signal intensity was lower in the effluent samples than in the influent samples.

Table 40: Proportion of hybridizable *Y. enterocolitica* cells in the wastewater samples.

sampling day	Influent samples		Effluent samples	
	%Yer/DAPI	%EUB/DAPI	%Yer/DAPI	%EUB/DAPI
12	0	45	0	12
14	2.9	54	0	21
16	3.0	49	0	18
18	3.2	51	0	23
20	3.1	53	0	23

Yer = *Y. enterocolitica* probe conferred signals; EUB = probe conferred signals with the mixture EUB338I, EUB338II and EUB338III; DAPI = total cell counts determined after DAPI staining.

In order to verify if absence of fluorescence with the *Y. enterocolitica* probe in the effluent samples was an artifact, fixed cells of a *Y. enterocolitica* overnight culture were spiked in the effluent samples prior to hybridization. Probe conferred signals

were very bright, leading to the conclusion that absence of fluorescence in the effluent wastewater samples was not due to fluorescence inhibitors in the samples.

4.10 Accumulation of *Y. enterocolitica* cells in the reactor bed

TaqMan PCR was applied to the filter medium samples in order to verify if *Y. enterocolitica* could persist in the filter bed. Single filter medium samples were taken in 10, 20 and 40 cm depth after 1, 3 and 5 days of *Y. enterocolitica* addition. At the end of the experiment replicate filter medium samples were taken from 3, 10, 20, 30, and 40 cm depth. Results presented in Table 41 show that quantifiable amounts of *Y. enterocolitica* were detected after 8 days of percolation. After one day of addition no target cells were detected; after 3 and 5 days TaqMan showed a weak but not reliably quantifiable signal for 10 and 20 cm depth. A not reliably quantifiable signal was set as less than the equivalent of 1 *Y. enterocolitica* cell per TaqMan assay. After 8 days of percolation *Y. enterocolitica* densities were 2.83×10^4 cells g^{-1}dw in 3 cm, 1.86×10^3 cells g^{-1}dw in10 cm, 1.50×10^2 cells g^{-1}dw in 20 cm and 7.53×10^2 cells g^{-1}dw in 30 cm depth. Detection in all samples derived from 40 cm depth delivered negative results.

Table 41: Detection of *Y. enterocolitica* in the filter medium samples.

Sampling day	Sample depth	*Y. enterocolitica* cells g^{-1}dw
14	10	-
	20	-
	40	-
16	10	+
	20	+
	40	-
18	10	+
	20	+
	40	-
20	3	2.83×10^4
	10	1.86×10^3
	20	1.50×10^2
	30	7.53×10^2
	40	-

- = no signal in the TaqMan assay; **+** = positive, not quantifiable signal

4.11 Recovery rate of *Y. enterocolitica* in the filter medium samples

The recovery rate of the target bacterium *Y. enterocolitica* was estimated by spiking assays. The correlation between the extent of recovery of the target bacterium and i) the sample depth, and ii) the target cell numbers was investigated. The aim was to obtain additional information on the reason for the negative results in 40 cm depth (see Table 41). At the end of the experiment (day 20) triplicate filter medium samples (each 5 g) were taken from 10, 20 and 40 cm and spiked with cells from a stationary phase culture of the reference strain *Y. enterocolitica*. DNA was extracted without method modifications. Results shown in Figure 33 are the mean of 3 independent PCR measurements.

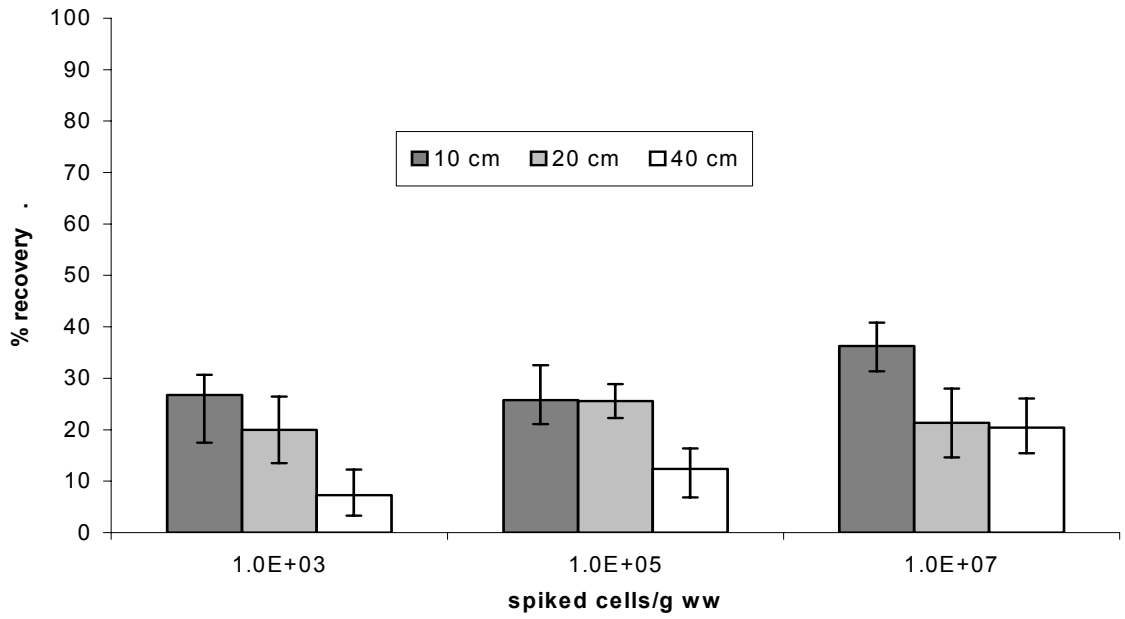


Figure 33: Recovery of spiked *Y. enterocolitica* cells in the filter medium samples by TaqMan PCR. Columns represent the means of three independent measurements. Whiskers represent maximum and minimum values. ww = wet weight.

For 10 cm depth, the recovery rate of *Y. enterocolitica* was 27% for 10^3 cells per g, 26% for 10^5 cells per g and 36% for 10^7 cells per g of sample (Figure 33). For 20 cm depth, recovery rates did not vary significantly with increasing concentrations of spiked cells. For 10^3 cells g^{-1} wet weight mean recovery rate was 20%. For 10^5 cells g^{-1} wet weight mean recovery was 26% and for 10^7 cells g^{-1} wet weight mean recovery value as 21%. For 40 cm depth recovery rate was especially low for low

target cell numbers with mean value of 7% and increased to 12% and 21% for 10^5 and 10^7 cells of *Y. enterocolitica* g^{-1} wet weight, respectively.

4.12 Search for inhibitors in the DNA extracts derived from 40 cm depth

In order to assess if low extraction yield was the cause of low recovery of *Y. enterocolitica* in the reactor samples in 40 cm depth an ex situ DNA extraction was tested. Ex situ DNA extraction usually delivers less DNA than in situ extraction procedures. However, if inhibitors in the filter matrix at 40 cm depth caused the low DNA yield with the in situ extraction, previous detachment of the microorganisms followed by extraction might deliver better results.

Detachment of the microorganisms from filter medium samples derived from 40 cm depth at day 20 was performed as described. Parallel samples derived from 10 and 20 cm depth served as control. The microbial suspensions were transferred to Lysing Matrix E Tubes of the FastDNA Kit and DNA was extracted. The ex situ lysis proved not adequate to improve DNA recovery from the filter medium samples, since it resulted in DNA loss: no DNA could be detected in the 40 cm samples. Additionally, DNA amounts extracted from 10 and 20 cm were lower than with the direct extraction.

In order to investigate if TaqMan results for the samples in 40 cm were influenced by co-extracted substances, DNA extracts derived from 40 cm depth were spiked with exactly the same amounts of target cells as used for the calibration curve and the efficiency of the TaqMan assay in the spiked samples was compared with the polymerization efficiency with the reference DNA. Figure 34 shows the correlation between the spiked cell numbers and the correspondent fluorescence threshold cycle (Ct) value in the assay. Additionally, the efficiency of each TaqMan PCR assay (Table 42) was calculated according to the equation:

$$E = 10^{(-1/S)-1} \qquad (5)$$

where E is the amplification efficiency and S is the slope of the equation determined by linear regression for the values depicted in Figure 34.

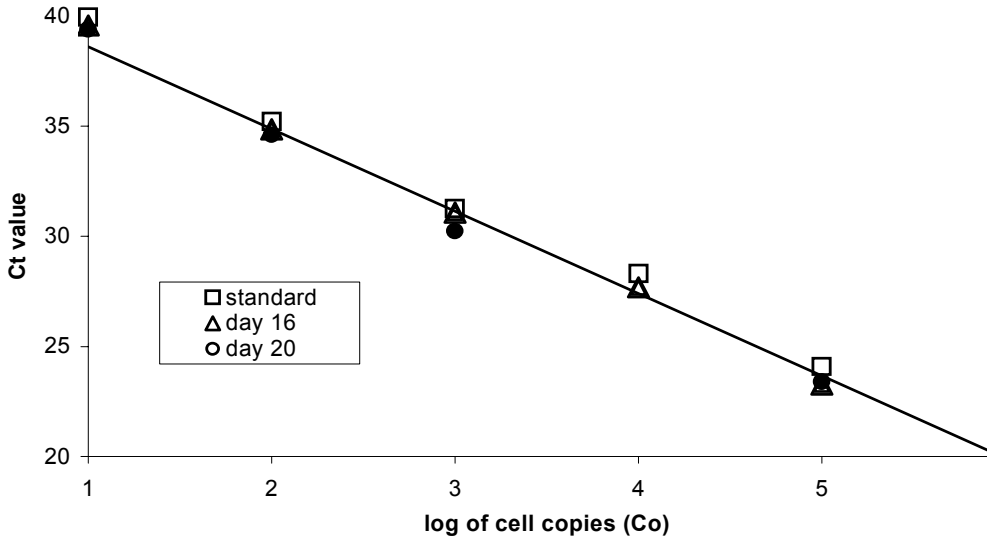


Figure 34: Correlation between target cells and Ct values for spiked samples derived from 40 cm depth. Ct = fluorescence threshold cycle.

Efficiency of the assay for day 16 (Table 42) was lower than for the pure culture of the reference strain but the difference was within the range observed for replicate assays. Efficiency of the assay with DNA from day 20 was as high as for the external standard. It was concluded that no PCR inhibitors were present in the DNA extracts and that negative results in 40 cm depth of the reactor derived from insufficient DNA amount.

Table 42: Efficiency of the TaqMan assay in spiked DNA samples derived from 40 cm depth.

Sample	R^2	Efficiency
External standard	0.9947	0.851
day 16	0.9951	0.834
day 20	0.9919	0.852

5 Calculation of the numbers of cells eliminated in the reactor

Bacterial elimination in the fixed bed reactor was defined as the total number of bacteria that were added to the reactor and were detected neither in the effluent nor in the filter bed after operation. No differentiation between die-off and cell replication was made.

The net elimination was calculated according to:

$$\mathbf{T_{net} = \Sigma_{influent} - \Sigma_{effluent} - \Sigma_{soil}} \quad \mathbf{(6)}$$

$\mathbf{T_{net}}$ is the net elimination of bacteria in the fixed bed. $\mathbf{\Sigma_{influent}}$ is the estimate of the total number of influent cells that were added to the reactor during the time of operation. $\mathbf{\Sigma_{effluent}}$ is the estimate of the total of cell numbers enumerated in the effluent during the time of operation. $\mathbf{\Sigma_{soil}}$ is an estimate of the total cell numbers detected in the filter bed in the last day of sampling.

$\mathbf{\Sigma_{influent}}$ and $\mathbf{\Sigma_{effluent}}$ were calculated by multiplying the average bacterial concentration of all measurements (n = 10, expressed as cells ml^{-1}) by the daily hydraulic load (ml d^{-1}) and by the time of operation (20 d). $\mathbf{\Sigma_{soil}}$ was calculated by multiplying the average bacterial density at day 20 by the total weight of sand in the filter bed.

According to these estimates (Table 43) a total of 8.90 x 10^{12} cells were added to the reactor during the time of operation with wastewater and a total of 2.4 x 10^{11} bacteria were collected in the effluent after 20 days. In the last day of operation 1.2 x 10^{11} bacteria were enumerated in the filter bed.

Table 43: Net elimination of total bacteria and of *Y. enterocolitica* in the filter bed after 20 days of operation. Values for total bacteria were determined by total cell counts after DAPI staining; values *for Y. enterocolitica* were determined by TaqMan PCR.

	$\Sigma_{influent}$	$\Sigma_{effluent}$	Σ_{soil}	T_{net}
Y. enterocolitica	3.4×10^9	5.9×10^6	3.3×10^7	3.4×10^9
Total bacteria	8.9×10^{12}	2.4×10^{11}	1.2×10^{11}	8.5×10^{12}

$\Sigma_{influent}$ = total cell numbers in the influent; $\Sigma_{effluent}$ = total cell numbers in the effluent; Σ_{soil} = total cell numbers in the filter bed; T_{net} = net bacterial reduction in the filter bed.

If the bacterial numbers detected in the effluent and in the filter medium are subtracted from the influent bacterial it results in a difference of 8.5 x 10^{12} bacteria, which is equivalent to 96% net elimination of the influent bacterial numbers. The net elimination of *Y. enterocolitica* in the reactor was calculated according to the same principle as mentioned above (Table 43). A total of 3.4 x 10^9 *Y. enterocolitica* cells were detected in the influent by TaqMan PCR. The total number of *Y. enterocolitica* cells detected in the effluent was 5.9 x 10^6. For the calculation of the total *Y. enterocolitica* numbers in the filter medium, the *Y. enterocolitica* density in 40 cm

depth was taken as zero and the mean value was calculated taking into account the densities in 3, 10, 20 and 30 cm depth. According to this calculation there were on average 3.3 x 10^7 *Y. enterocolitica* cells in the filter column of the reactor. The subtraction of the total *Y. enterocolitica* cells in the effluent and the filter bed from the influent cells results in a difference of 3.36 x 10^9 cells *Y. enterocolitica* cells, corresponding to a net elimination of 98.8%.

IV. DISCUSSION

A) New methodologies for an old problem: how to monitor wastewater purification efficiency?

Detection of pathogenic bacteria by cultivation presents several limitations: interference of background flora, very long incubation steps and inability to distinguish pathogenic strains from their harmless environmental relatives (Rompre et al., 2002). Additionally, certain bacteria with relevance for public health (e.g. cyanobacteria producing cyanotoxins) are not quantifiable by cultivation. Others are basically culturable in standard media but may convert into a temporary or permanent nonculturable state (McKay, 1992; Collwell & Grimes, 2000), thus escaping cultivation. Finally, it has been suggested that bacteria associated with phyto- and zooplankton are removed from the free water phase thus invalidating cultivation methods to assess the microbiological quality of surface waters (Colwell, 2000; Signoretto et al., 2004; Signoretto et al., 2005). In water and wastewater quality assessment, the above mentioned limitations may lead to disease outbreaks, either due to failure in detecting pathogenic bacteria or due to insufficient prevention strategies because of result delay (Haugland et al., 2005). In this context, there is a growing interest from the scientific community and from the health authorities to include molecular techniques to the standard methods for the detection of pathogens in water and wastewater.

The goal of this study was to search for feasible alternatives to standard cultivation methods for routine microbiological assessment of wastewater. For this purpose a PCR-based approach was developed in this work and its potential for providing rapid, specific and reliable detection of pathogens in wastewater was evaluated. PCR has though, like every other technique, constraints. Especially in environmental samples like wastewater, some factors inhibit nucleic acid amplification (Catalan, 2003). Inhibition generally acts at one or more of three essential points: i) interference with cell lysis necessary for DNA extraction, ii) low DNA recovery due to nucleic acid degradation or capture, and iii) inhibition of *Taq* polymerase activity by co-extracted impurities in the DNA preparation or blocking of the target site by binding of non-target DNA to the target sequence (Rossen et al, 1992; Wilson, 1997; Guy et al., 2003). Each of these points was addressed in the development and evaluation of the PCR based approach presented in this work.

1 Yield and quality of the extracted DNA

After pre-concentration of the wastewater samples, the adequacy of two different DNA extraction protocols, the FastDNA Spin Kit for Soil and protocol D of the QIAMP DNA Mini Kit were tested with the wastewater samples of the constructed wetlands Wiedersberg and Ettenbüttel. The FastDNA Kit was developed for DNA extraction from soil. Cell lysis is based on mechanical disruption using ceramic and silica particles and EDTA and SDS are the active agents in the lysis buffer. Cell lysis by the QIAMP DNA Mini Kit is based on lysozyme/SDS/EDTA treatment.

From the numerous substances that inhibit the activity of the *Taq* Polymerase or influence the specificity of the reaction, humic acids are most frequently found (Wilson, 1997). Since these compounds are ubiquitous in soil, the FastDNA kit should also conveniently remove humic acids from wastewater. The kit should also conveniently lyse all types of cells in the wastewater samples, since microbial soil populations are composed of a high fraction of Gram-positive bacteria (Coyne, 1999). However, it was possible that the FastDNA Kit would degrade the DNA due to the high shear forces produced by the FastDNA instrument. Besides, because of the high proportion of Gram-negative bacteria in wastewater, it was possible that DNA liberated from more easily lysed microorganisms could become adsorbed to colloids, leading to an underestimation of the real amount of DNA (Frostegård et al., 1999). In this context, the degree of shearing was compared with the DNA extracted with the QIAMP DNA Mini Kit, with a different, less aggressive lysis principle.

DNA extraction yield was constant for replicate samples extracted with both kits. DNA amounts extracted from the settled wastewater samples were always higher than from the treated wastewater samples, correlating well with the results obtained by total cell counts of wastewater bacteria. However, the FastDNA Kit delivered higher amounts of DNA for the same samples than the QIAMP Mini Kit. For instance, mean extraction yield for the treated wastewater in Wiedersberg was 22 ng DNA per ml of wastewater with the FastDNA Kit and 12 ng DNA per ml of wastewater with the QIAMP Mini Kit.

All wastewater samples extracted with the FastDNA Kit delivered DNA with very good 260/280 nm ratios (between 1.9 and 2.2), indicating high purity of the DNA. The QIAMP Kit failed once to extract DNA from a treated wastewater sample and the 260/280 ratio was low (between 1.3 and 1.7) in the other samples.

The DNA fragmentation was evaluated with a PCR targeting the 16S rDNA of *Eubacteria*. The rationale was that if the 1.5 kb target DNA sequence could be amplified, the DNA was not significantly fragmented. Products were obtained with all DNA extracts, but product yield was higher with the extracts obtained with the FastDNA Kit than with the QIAMP Kit, confirming better adequacy of the FastDNA Kit.

Finally, the extent of recovery of two pathogenic bacteria from wastewater was evaluated. This test was a measure of the accuracy of the whole approach for quantifying pathogenic bacteria in wastewater. *Campylobacter jejuni* and *Yersinia enterocolitica* serogroup O:3 were spiked in the native wastewater samples, the DNA was extracted with the FastDNA Kit and recovery was determined by PCR. The same results were obtained for spiked DNA extracts and spiked samples, indicating that the recovery of the target DNA was very high. Thus, sample concentration, DNA extraction with the FastDNA kit followed by detection with the adapted PCR assays proved very suitable for quantitation of the target enteropathogens in wastewater. The incubation time of *Y. enterocolitica and C. jejuni* in wastewater was kept short in order to avoid cell replication, which would bias quantification. Provided that they survive, the extent of recovery for pathogenic bacteria with longer permanence in wastewater may be lower if they convert into viable but nonculturable (VBNC) states and/or if they form biofilms. An increase in the degree of cross-linking of the muropeptide, which is frequently observed for VBNC (Pisabarro et al., 1985, Costa et al., 1999; Signoretto et al., 2000), may increase the mechanical resistance of the cell wall. Additionally biofilms may provide a certain protection against lyses and adsorb the released DNA (Steinberger & Holden, 2005). Hence, potential loss of DNA due to insufficient lysis and/or biofilm was minimized by selecting a DNA extraction kit developed for soils. In soil environments Gram-positive bacteria predominate. Besides microbial communities in soil live in aggregates more protective against lysis than bacteria in aqueous environments.

The FastDNA Kit proved suitable for the purpose of this investigation: DNA extraction was easy to perform. DNA integrity and purity were high. Extent of DNA recovery from the target bacteria was very high in the settled and treated wastewater samples. The FastDNA kit was therefore used for the DNA extraction of all following wastewater samples.

2 PCR detection of model pathogens in wastewater

The PCR based approach was developed and evaluated in wetland wastewater. Four human pathogenic bacteria were selected as PCR targets: *Campylobacter jejuni*, *Helicobacter pylori*, *Escherichia coli* O157:H7, and *Yersinia enterocolitica* serogroup O:3. The standard fecal indicator *Enterococcus faecalis* was additionally investigated, since it is a common inhabitant of wastewater and has become increasingly a serious nosocomial pathogen (Murray, 1990; Moellering, 1991).

2.1 Campylobacter jejuni

Drinking water contaminated with *Campylobacter jejuni* has been the cause of several large outbreaks of enteritis (Blaser et al., 1982). This organism may contaminate drinking water through the feces of animals or infected humans. *C. jejuni* is not able to grow but may survive in the environment for several weeks at temperatures around 4ºC (Blaser et al., 1984).

2.2 *Yersinia enterocolitica*

Waterborne disease outbreaks caused by *Y. enterocolitica* have been reported in Canada and in Northern European countries (Thompson & Gravel, 1986; Ostroff et al, 1994). *Y. enterocolitica* has been isolated from wastewater effluents, river water and from drinking water (Bartley et al., 1982, Meadows & Snudden, 1982, Stathopoulos & Vayonas-Arvanitidou, 1990). This pathogen thrives at temperatures as low as 4ºC and is poorly correlated with traditional bacterial indicators (Sobsey and Olson, 1983).

2.3 *Helicobacter pylori*

Helicobacter pylori is a major human pathogen causing gastritis. It colonizes the stomach of more than 50% of humans (Brown, 2000). Besides the oral-oral way, waterborne transmission, probably due to fecal contamination, may be an important source of infection (Brown, 2000; Mazari-Hiriart et al., 2001).

2.4 *E. coli* O157:H7

E. coli O157:H7 is a causative agent of clinical illness that can end deadly (Mead & Griffin, 1998). This organism can live in the intestine of healthy cattle and disseminates quickly, whereby contaminated water can serve as vehicle for dissemination of the pathogen (Shere et al., 2002). Epidemiological data indicate

that *E. coli* O157:H7 is also transmitted to humans through contaminated water and direct contact with infected people or animals (Mead & Griffin, 1998).

2.5 *E. faecalis*

E. faecalis is a common indicator of fecal pollution in water and a normal inhabitant of wastewater. Moreover, this bacterium gained in importance in the last years as a nosocomial/opportunistic pathogen, causing infections in such varied systems like the gastrointestinal tract, the urinary tract, and the skin (Moellering 1991; Murray, 1990). Being resistant to many antimicrobial drugs (all commercially available aminoglycosides, penicillin, ampicillin, and vancomycin (Jha et al., 2005) it poses a serious health risk nowadays.

The PCR assays targeting the model bacteria were first adapted and optimized to wastewater. Spiking assays were carried out to quantify the target bacteria and to determine the detection limit of the methods in wastewater. With the spiking assays the sensitivity of the PCRs was verified each time, avoiding bias derived from the extraction performance and allowing sample-to-sample comparison.

The sensitivity of the PCR assays was different in pure cultures: under the given conditions of cell growth and DNA preparation 100 cells of *E. faecalis* were needed for a PCR product, whereas one cell of *Y. enterocolitica* gave a clearly visible product. It is possible that the mean chromosome copy number per cell was different for each model pathogen. The influence of the chromosome copy number on PCR sensitivity is discussed below in Section 4. In wastewater DNA extracts, the ratio between target DNA and background DNA was equivalent to one target cell in 10^5 - 1:10^9 non-target cells, depending on the provenance of the DNA and the PCR protocol. The sensitivity of the PCR protocols for these extracts was approximately one order of magnitude lower than for pure cultures.

Negative results were obtained for the target bacteria in the unspiked wastewater samples, proving that all PCR assays were specific for the target bacteria in all investigated samples.

Regardless of the origin of the wastewater, the sensitivity was constant for all settled wastewater samples, as well as for all treated wastewater.

According to the different sensitivities of the assays, the detection limits of the whole procedures for the detection of enteropathogenic bacteria in wastewater were

different. The method for *Y. enterocolitica* was the most sensitive (5 cells per 100 ml of treated wastewater and 200 cells per 100 ml of settled wastewater) and the method for *E. faecalis* was the least sensitive (500 cells per 100 ml of treated wastewater and 20,000 cells per 100 ml of settled wastewater).

3 Extension of the approach to municipal wastewater

For municipal wastewater, *C. jejuni/coli* and *Y. enterocolitica* serogroup O:3 directed PCR methods were used, because these pathogens have been often implicated in waterborne disease outbreaks in the northern European countries (Ostroff et al., 1994; Hänninen et al., 2003).

The sensitivity of the methods decreased for municipal wastewater: the detection limit for *C. jejuni* was 2,000 cells per ml of treated wastewater and 25,000 cells per ml of settled wastewater; whereas the detection limit for *Y. enterocolitica* was 200 cells per ml of treated wastewater and 2,500 cells per ml of settled wastewater. Both detection methods were thus 12.5 times less sensitive in the settled wastewater and 40 times less sensitive in the treated wastewater, although the DNA background was in the same range and DNA purity was high when assessed by the 260/280 nm ratio. Differences in sensitivity could possibly depend on partial inhibition of the *Taq* polymerase by inhibitory substances not detected by the quality control. Consequently, only DNA corresponding to 10^6-10^8 non-target cells could be applied to each PCR assay.

4 Comparison with other PCR based studies and techniques

Increase in sensitivity of PCR methods for the detection of pathogenic bacteria in water and wastewater is usually achieved by a pre-enrichment step. Besides of being time consuming, pre-enrichment presumes that the target cells will replicate in the chosen enrichment broth, which may not hold true. In the present work, the intention of the concentration step was to improve the detection limit by applying highly concentrated DNA to the PCR assays. Sample concentration prior to DNA extraction has been used for the detection of viruses in wastewater (Puig et al., 1994; Shieh et al., 1995). Concentration does not dilute potential PCR inhibitors and does not improve the target-to-non-target-cell ratio, but enables very quick sample processing. After sample concentration, a rapid DNA extraction followed by optimized PCR were carried out for the detection of the model pathogens.

Before comparison of the PCR methods developed in this work with those presented by other researchers it must be considered that sensitivities are not expressed in the same units. Whereas the sensitivities of the PCR methods developed in this work are given in total DAPI enumerated cells per 100 ml of sample, the sensitivities of the PCR works presented by other authors are given in CFU per 100 ml of sample. Since culturable cells constitute only a fraction of the total living cells in a bacterial population (Staley & Konopka, 1985), it must be assumed that the numbers of cells necessary for a positive result in a PCR assay were underestimated if they were determined by cultivation. When determined by total cell counts, it is possible that results are biased if the target cells employed for the determination of the PCR sensitivity contain more than one chromosome. It is still not clear under which circumstances cells possess more than one chromosome copy. DNA synthesis may sometimes be uncoupled from the cell cycle (Müller & Babel, 2003) and the ability to start uncoupled DNA synthesis may be a strain-specific attribute (Müller & Babel). The presence of two chromosomes in bacterial cells seems to be associated with carbon limitation (Ackermann et al., 1995; Herrmann et al, 1997). In a natural population or in a not synchronized culture, there will always be resting and growing cells with different amounts of chromosome copies (Kell et al., 1998). Trun & Gottesman (1990) observed that *E. coli* inherits only one chromosome when growth rates exceeded 60 min. In another experiment, stationary cultures of *E. coli* had 2-4 chromosome copies even when growth rates exceeded 6 hours (Claudia Keil, TU Berlin, personal communication).

In this work, the reference strains were harvested in the late stationary phase in order to minimize bias due to polyploidy. However, it is possible that the detection limits of the five PCR methods presented in this work were overestimated due to polyploidy. According to the above-mentioned works, the probable value for overestimation would be two- to four-fold.

Waage et al. (1999a,b) used PCR in combination with a nonselective pre-enrichment for the detection of *C. jejuni and Y. enterocolitica* in river water with fecal contamination. The detection limit was 3-15 CFU of *C. jejuni* and 8 to 17 CFU of *Y. enterocolitica* per 100 ml of river water. The above-mentioned sensitivity is comparable with the detection limits obtained in this work for wetland treated wastewater, although the investigated treated wastewater had a higher load of background flora (10^8-10^9 cells per 100 ml vs. 10^5 CFU per 100 ml in river water).

Moreno et al. (2003) detected *C. jejuni* by PCR in activated sludge samples from a municipal wastewater treatment plant (832,000 population equivalents). PCR detection limits were 10^3 CFU of *C. jejuni* per ml of activated sludge without pre-enrichment and 10^2 CFU when PCR was preceded by selective enrichment. The detection limit for settled municipal wastewater in our study was in the same order of magnitude (250 cells per ml) without pre-enrichment.

Mazari-Hiriart et al. (2001) used PCR to detect *H. pylori* in treated and untreated wastewater samples. The sensitivity obtained was 0.001 ng of DNA for a PCR targeting the 16S rDNA of *H. pylori*. The *H. pylori* genome has a weight of 1.52 fg. Hence, the detection limit of the assay, when expressed in target cells per assay, was 10^3 cells per assay for the 16S PCR assuming that the target cells contain a single chromosome. The sensitivity of the method presented in this work is 50 cells per assay.

No other protocols were found for the detection of *E. coli* O157:H7 with classic PCR in wastewater samples. Although real-time PCR does not suit the purpose of this work, since this technology is too complex and expensive for routine surveillance of wastewater, the sensitivity of the methods found in literature will be referred here. Ibekwe et al. (2002) and Spano et al. (2005) used multiplex fluorogenic Real-Time PCR without pre-enrichment for the detection of *E. coli* O157:H7 in dairy wastewater. The sensitivity achieved for the wastewater samples was similar in both papers (approximately 10^6 CFU per 100 ml) and lower than the sensitivity of the approach presented here (10,000 cells per 100 ml of settled and 250 cells per 100 ml of treated wastewater).

Lleo et al. (1999) used competitive PCR to quantify *E. faecalis* in lake water. The sensitivity of the assay was 200 cells per 100 ml of water, which is comparable to the sensitivity reported in this work. However, lake water contains less background flora and possibly fewer pollutants than wastewater. The sensitivity of the PCR-based detection method for *E. faecalis* was lower than for the other four enteropathogenic bacteria tested. In this work, *E. faecalis* constituted only a fraction (20%) of the total fecal enterococci present in wastewater. This observation was also made by Obiri-Danso & Jones (1999) and Harwood et al. (2004). A PCR protocol targeting the genus *Enterococcus* would be of advantage, since it facilitates the detection of fecal pollution in water. Cultivation of fecal enterococci is not very laborious and results are obtained in 1-2 days (ISO 7998-2, 1998) but the risk of drastic underestimation of the

enterococci numbers due to nonculturability (Lleo et al., 2005) must be taken into concern.

The methods presented in this work were especially sensitive for the detection of enteropathogenic bacteria in treated wastewater. Since surveillance of wastewater purification quality focuses on the quality of the effluent, the methods proved very adequate for this purpose.

5 Cultivation vs. PCR

There is controversy on which type of method, cultivation or PCR, is more adequate for wastewater and drinking water quality surveillance. Table 44 sums up the most common advantages and disadvantages of both approaches (from Bendinger et al., 2005; adapted).

Table 44: Advantages and disadvantages of cultivation and PCR

44.a) **Cultivation**

Advantages	Disadvantages
➢ Comparison with other works also in long-term aspect ➢ Inexpensive and easy to carry out	➢ Physiologic state influences detection, e.g. VBNC forms, spores ➢ non-culturable microbial species cannot be detected ➢ Lack of discrimination between pathogenic and non-pathogenic strains ➢ Very time-consuming for certain bacteria, no same-day-results ➢ Interference of background flora is possible

44.b) **PCR**

Advantages	Disadvantages
➢ High specificity and sensitivity ➢ No dependence on physiology ➢ Basically no interference of background flora ➢ Enables phylogenetic classification ➢ Enables etiological studies	➢ Possible detection of dead cells ➢ Lack of specificity in complex samples may occur ➢ Possible inhibition of the reactions ➢ No standard procedures until now ➢ Equipment more expensive

In fact, both cultivation and PCR have limitations and can deliver false positive and false negative results. In the case of cultivation, false positives are due to lack of specificity of the detection method. False negatives are usually related to interference of background flora and/or changes in culturability. In the case of

molecular biological methods, false negatives are usually due to inhibition of the amplification by one or more compounds, whereas false positives may be due to detection of dead cells/naked DNA or to lack of specificity of the primers. In the present study, all PCR assays were highly specific for the target bacteria. With regard to the occurrence of false positives, PCR will detect nonviable cells, provided intact target nucleic acids are available. It is though rather improbable that a significant fraction of dead cells or naked amplifiable DNA is present in the free wastewater, since in environmental samples with a substantial physiological activity, nucleic acid turnover will be fast (Lebuhn et al., 2004). Josephson et al. (1993) reported that, even when stored at 4ºC, DNA of dead *E. coli* cells was degraded in an environmental water sample.

Assuming that, despite of high nucleic acid turnover, some naked DNA would remain intact for amplification, its contribution to the PCR results would be lower than that of DNA derived from living cells: Uyttendale et al. (1999) reported a 1,000-fold decrease in PCR sensitivity for heat-killed cells of *E. coli* O157:H7. Besides, the authors reported that centrifugation and washing eliminated dead cells prior to DNA extraction. A centrifugation and washing step was also carried out in this work. Fach et al. (1999) reported an even lower sensitivity for dead cells with a PCR protocol directed to *Salmonella* spp: the threshold sensitivity was 10^2 CFU per ml of sample and 10^6 dead cells per ml of the same sample. Consequently, if PCR detects the DNA of dead cells of an enteropathogenic bacterium in the effluent of a treatment plant, this means that living cells are present in a previous purification stage at very high concentrations. Such results should be taken as a serious warning and not just as an artifact of the method.

In the same period of investigation the cooperation partner German Environmental Protection Agency investigated the wastewater samples of Wiedersberg and Ettenbüttel for the presence of *Campylobacter* spp., *Yersinia* spp. and *E. coli* O157:H7. The results were published by Hagendorf et al. (2004). Cultivation of *Campylobacter* spp. with the above-mentioned method detects not only the genus *Campylobacter* but also the genus *Arcobacter*. The settled wastewater contained 10^6 - 10^7 CFU of *Campylobacter/Arcobacter* spp. per 100 ml of sample in Wiedersberg and 10^4 - 10^6 CFU of *Campylobacter/Arcobacter* per 100 ml of sample in Ettenbüttel. Pathogenic strains of *C. jejuni* and *C. coli* were not detected. The results obtained by cultivation were thus consistent with the results obtained with the PCR approach.

The campylobacters/arcobacters isolated by cultivation were hygienically non-relevant environmental strains (Irmgard Feuerpfeil, Environmental Protection Agency Bad Elster, Germany, personal communication). This may however raise a typical problem of background interference: if the concentrations of harmless *Campylobacter/Arcobacter* strains are so high in wastewater samples, a campylobacteriosis outbreak might remain unrecognized because the high numbers of the environmental strain could mask an increase of the pathogen. *Campylobacter/Arcobacter* spp. were also detected in the treated wastewater of Wiedersberg. The mean concentration was 10^2 CFU per 100 ml of native sample. These values would be detectable by PCR assuming one chromosome per cell. Moreover, cultivation of *Campylobacter/Arcobacter* spp. is complex and takes at least 4 days; consequently PCR proved much more adequate than cultivation for detecting *C. jejuni* in wastewater.

PCR results for the detection of pathogenic *Y. enterocolitica* were consistent with cultivation: neither cultivation nor PCR detected pathogenic strains in the wastewater samples of Wiedersberg and Ettenbüttel. *Yersinia* spp. were detected by cultivation in very low amounts (maximum of 1 CFU per 100 ml). The PCR method detected higher amounts of *Yersinia* spp. in 5 samples (between 50 and 1,000 cells per 100 ml) but the virulence plasmid was never detected. Taking into account that the culture-dependent method for the detection of pathogenic *Y. enterocolitica* strains is very time-consuming, comprising of a 21-day enrichment step and further biochemical test for virulence factors, it would be advisable to use PCR instead of cultivation for the detection of this pathogen.

E. coli O157:H7 was never detected neither by cultivation nor by PCR. However, the PCR method was not as sensitive as for *C. jejuni* and *Y. enterocolitica*. A detection limit of 250 cells per 100 ml of treated wastewater, although low in comparison with literature, could be too high for this pathogen, since it has a very low minimum infective dose (10-100 cells can cause disease). Improving the sensitivity of the PCR assay could overcome this limitation. Cultivation could basically detect lower amounts of *E. coli* O157:H7, but the proportion of nonculturable cells in the wastewater samples could bias quantification significantly.

One of the constraints pointed out to molecular techniques is the lack of standardized methods that enable the comparison of the results from different

laboratories. Although this is true for the actual state of the art, it should not constitute a barrier to further research in the field of detection methods. These should be actualized in correspondence to new insights in the field of microbiology. The assays presented in this work show that PCR is a method with potential to substitute or complement cultivation in wastewater quality assessment.

B) Studies on bacteria elimination performance of reed beds

The wastewater purification potential of three subsurface flow constructed wetlands was investigated in a cooperation project with the German Environmental Protection Agency. In this work, culture independent techniques were applied to assess the efficiency of different reed beds to remove microorganisms from wastewater. For this purpose a PCR based approach for the detection of pathogenic bacteria in wastewater was developed as a feasible alternative to cultivation and is discussed in section A of this chapter. Defined aspects of the elimination of bacteria in the reed beds were studied in more detail, e.g. the purification performance of vertical and horizontal beds and the influence of the operation time and clogging on the bacterial removal efficiency. The mechanisms governing the removal of bacteria from wastewater were also investigated, as well as factors influencing their elimination in the beds. Finally, the fate of a pathogenic bacterium was studied in a lab-scale subsurface flow wetland.

1 Wastewater purification of reed bed systems – performance and health impact

The treatment plants Wiedersberg and Ließen are multistage treatment plants composed of primary settling, vertical and horizontal reed beds. The construction plan of the reed beds is very similar, so results obtained could deliver information on eventual changes in bacteria removal patterns after long-term operation. At the time of the investigations the reed beds in Wiedersberg were in operation since approximately one year, while the reed beds in Ließen were in operation since 6 years. The upper 5 cm layer (clogging layer or Schmutzdecke) of the filter beds in Ließen had a brownish color and oily consistence. The clogging layer in the Wiedersberg reed beds had no particular traits. The treatment plant Ettenbüttel is a multi-stage treatment plant composed of wastewater lagoons and vertical reed beds. The wastewater lagoons were constructed in the 1970’s and the vertical reed beds were constructed in 1999. At the beginning of the investigations the reed beds had been set in operation very recently and the clogging layer was not developed.

1.1 Reduction of total bacterial numbers in the vertical reed beds

The vertical reed beds in Ließen and Wiedersberg showed a high and constant reduction of bacteria. Mean bacteria elimination was 99% for Wiedersberg and 93%

for Ließen. However, irregularities in the hydraulic conductivity of the vertical beds were sometimes observed during the sampling campaigns in Ließen, indicating that the reed beds began to clog. Soil clogging is one of the most frequently reported problems in subsurface flow constructed wetlands (Langergraber et al., 2003, Kayser & Kunst, 2005). Clogging results from deposition of stable solids in the porous medium (Siegrist et al., 1987) and from the development of a biofilm in the porous media. Both processes change the hydraulic conductivity of the medium (Blazejewsky & Blazejewska, 1997; Winter, 2003). On one side, biofilm and organic matter restrict the pore size and can enhance straining (McDowell-Boyer et al., 1986). Consequently, removal of bacteria can be more effective in clogged filtration systems compared with unclogged ones (Baars, 1957; Blazejewsky & Blazejewska, 1997). On the other side, if the small channels are closed and water flows only through wider channels, the retention time in the filter will be shorter (Winter, 2003; Blazejewsky & Blazejewska, 1997) and straining and adsorption decrease, resulting in a poor filtration performance.

The clogging layer of the vertical reed beds in Ließen was thick and the microbial density was high. Until 12 cm depth bacterial concentrations in the bed were 100-fold higher than in Wiedersberg (10^9 cells per g of dry weight vs. 10^7 cells per g of dry weight in Wiedersberg). In this layer, protozoa were observed in substantial quantities. Almost no protozoa were detected in the clogging layer in Wiedersberg. The low protozoan numbers in Wiedersberg were probably related to the lower bacterial density since protozoa in soils have been associated with high bacterial densities (Decamp & Warren, 1998 Decamp et al., 1998; Ekelund et al., 2002; Rønn et al., 2002).

Despite of the different bacterial densities, there were no significant differences in the hybridization pattern of the clogging layer of both vertical beds. Signals conferred by the EUB338 probe accounted for 24% of all enumerated bacterial cells in Ließen and 18% in Wiedersberg. Only 0.25% (Ließen) and 0.06% (Wiedersberg) of total enumerated bacteria hybridized with the archaebacterial probe Arch915. Gram-positive bacteria could account for a considerable fraction of the cells that hybridized neither with the EUB338 nor with the Arch915 probes, since Gram-positive bacteria are very abundant in soil (Lindahl et al., 1997) and less permeable to hybridization probes than Gram-negative bacteria (Manz, 1993).

Signals conferred with the α-, β- and γ-*Proteobacteria* targeted probes were present in both samples in very low percentages and made up together 2.3% and 1.2 % of total cell counts for Ließen and Wiedersberg, respectively. This amounts to only 10% and 7%, respectively, of total EUB-hybridized cells. Cells belonging to the *Cytophaga-Flavobacterium-Bacteroides* group were only sporadically detected. The same occurred for bacteria hybridizing with the *Planctomycetales*-probe, which were detected only in Ließen. The analysis of the clogging layers of both vertical reed beds suggests that soil clogging in Ließen was rather due to a quantitative increase in the bacterial numbers and consequently in biofilm formation than to a development of a different type of bacterial community.

Bacteria elimination performance of the vertical reed beds in Ettenbüttel was irregular: either bacteria reduction was very high (99%) or no reduction in bacterial numbers took place. In May 2000 the effluent of the vertical reed beds contained higher amounts of *Spirillum* spp. and algae, indicating a loss in bacteria retention capacity in the beds. The beds clogged in May 2000 and the plant was set out of operation between May and November 2000 for regeneration of the beds (Kayser, 2003). In November 2000 and in January 2001 increasing amounts of filamentous bacteria were detected in the influent of the filters. The effluent of the beds contained more filamentous bacteria, protozoa and algae. Filamentous bacteria have been associated with pore clogging in filtration processes (Moghaddam et al., 2003, Lin et al., 2004). In January 2001 the beds did not reduce the influent bacterial numbers at all. These observations correlate well with the work of Kayser (2003), which reports changes in the hydraulic conductivity of the filters and a strong increase in ammonia concentration in the effluent of the reed beds for the same time. Ammonia concentrations reached a maximum of 29 mg/l at the end of January 2001. Finally the beds clogged in February-March 2001 and were set out of operation for regeneration (Kayser, 2003).

The vertical reed beds of the Ettenbüttel treatment plant have smaller dimensions than in the treatment plants Wiedersberg and Ließen. Consequently, hydraulic load was higher in Ettenbüttel than for the other plants. High hydraulic load has been associated with a reduction in the purification potential of wastewater if they increase the average water suction in an unsaturated filter medium resulting in significant bacterial transport (Smith et al., 1985).

Additionally, whereas the vertical reed beds of Wiedersberg and Ließen were operated discontinuously, i.e. the beds were set out of operation in a rotation modus, the vertical reed beds in Ettenbüttel were continuously in operation. The combination of high hydraulic loads with the lack of regeneration of the beds was possibly the cause of clogging and purification failure. Filamentous bacteria could have enhanced the clogging progress.

The investigations performed in this work indicate that vertical reed beds may be adequate for microbiological wastewater purification under the correct operation conditions. Clogging possibly resulted in loss of microbiologic purification performance. The type of inflowing bacteria may influence the susceptibility of a filter for clogging.

1.2 Reduction of total bacterial numbers in the horizontal reed beds

The horizontal reed beds in Wiedersberg and Ließen treated the effluent of the vertical reed beds. The efficiency of the horizontal reed beds in removing bacteria from the inflowing wastewater was low and erratic. For Wiedersberg, in one sampling campaign the effluent of the horizontal reed beds contained the same bacterial concentration as the influent. In the other cases bacteria retention performance varied between 5% and 95%. Like in Wiedersberg the horizontal reed beds in Ließen performed a low and irregular reduction of bacteria in the wastewater, ranging from failure to a reduction of 85%.

Several other factors may explain the poor bacteria removal efficiency of horizontal reed beds:

i) In a two-stage treatment system like Wiedersberg and Ließen, the larger bacterial species and those with higher affinity for the filter medium were probably removed in the first stage (vertical reed beds), and those remain which are “less filterable”.

ii) The adsorption rate of bacteria to porous media was suggested to increase linearly with cell concentration (Escher & Characklis, 1990). Since bacteria concentrations were lower in the influent of the horizontal reed beds in comparison to the influent of the vertical reed beds, low bacterial concentration could be a priori the reason for poor retention capacity of the horizontal reed beds. Hagendorf et al. (2004) investigated the removal of indicator bacteria in the treatment plants Wiedersberg and Ettenbüttel by means of standard cultivation methods. They did in fact find a correlation between the removal rates and the influent concentrations of the indicator bacteria: the removal was lower for low bacterial concentrations.

However, if the vertical reed beds were circumvented and the settled wastewater was directly treated by the horizontal reed beds, the elimination performance was significantly inferior to that achieved by the vertical beds (Hagendorf et al., 2004). This observation indicates that lower bacterial concentrations and lower affinity to the filter medium cannot alone explain the poor efficiency of the horizontal beds for bacteria removal.

iii) During unsaturated flow (case of the vertical reed beds) transport takes place in the smallest pores and straining can be more effective than during saturated flow in the horizontal reed beds (Stevik et al. 2004), where transport takes place in larger pores. This assumption was confirmed by Powelson & Mills (2001), who studied the influence of hydraulic conditions on the removal of *E. coli* in sand columns and observed that unsaturated flow was better than saturated flow for removal of bacteria in the columns.

iv) Being saturated, horizontal reed beds are probably more prone to short-circuit currents than vertical reed beds (Green et al., 1997). In the case of short-circuiting, wastewater circumvents treatment.

In the context of the cooperation project, the German Environmental Protection Agency analyzed the wastewater samples from Wiedersberg with conventional culture methods (Hagendorf et al., 2002). When the wastewater samples were investigated for heterotrophic plate counts the horizontal reed beds displayed higher elimination performance than the vertical reed beds (98.1 ± 3.7% vs. 94.9 ± 11.0%, respectively). The culturability of all wastewater samples was low when referred to total cell counts. This phenomenon is well known and applies to basically all environmental samples (Staley & Konopka, 1985; Brock, 1987). However, when the culturability of bacteria in the effluent of the vertical and horizontal beds is compared, it becomes clear that the high reduction values determined by cultivation for the horizontal reed beds were due to a decrease in culturability of the effluent samples (between 0.0006% and 0.2%, average of 0.04%) in comparison to the vertical beds (between 0.002% and 17.6%, average of 3.6%). It is not probable that an increase in the fraction of dead cells could account for the lower culturability in the effluent of the horizontal beds since phase contract microscopy showed bacterial cells with intact cell walls. Besides dead bacteria would probably persist for only a short time in wastewater. This rationale is confirmed by the observations of Decamp & Warren

(2001), who used BacLight probe to assess the viability of bacteria at several purification stages of four constructed wetlands. They concluded that 50% of the bacteria leaving the secondary treatment stage in constructed wetlands were physically intact and probably viable. The probable reason for the reduction of culturability during purification in the reed beds was rather a shift in the physiologic state of wastewater bacteria in response to nutrient depletion. If bacteria adapt to rather oligotrophic conditions, they may grow less well or not at all in the standard culture media for selection of heterotrophic plate counts. Additionally, it is possible that a fraction of the bacteria eluted from the saturated horizontal reed beds were adapted to microaerophilic or anaerobic conditions.

The investigations performed in this work show that the horizontal reed beds were not adequate to remove significant amounts of bacteria from wastewater and that the microbiological purification performance of the investigated treatment plants was overestimated by cultivation

1.3 Critical hygienic aspects: pathogens and broad-host-range plasmids

As mentioned in the INTRODUCTION, the studies on the removal of pathogenic bacteria in constructed wetlands are usually carried out by cultivation based-methods. If pathogens behave in the same manner as wastewater bacteria, low culturability in the effluent samples of wastewater treatment plants can lead to serious underestimation of pathogenic bacteria. Pundsack et al., (2005) reported very low culturability of *Salmonella choleraesius* in the effluent wastewater of subsurface flow constructed wetlands. This question is of high concern in this particular kind of treatment plants since they are operated in rural areas and run-offs have easy contact with ecologically sensitive areas and drinking water reservoirs (case of Wiedersberg) and no disinfections of wastewater are performed before release in the environment.

In this sense it was assessed if removal efficiency of the reed beds was different for autochthonous wastewater bacteria and allochthonous pathogens. The influent and effluent of the reed beds from Wiedersberg and Ettenbüttel were investigated with a PCR based approach for the presence of the model enteropathogens *Y. enterocolitica* serogroup O:3, *Campylobacter jejuni*/*coli*, *Helicobacter pylori* and enterohemorrhagic *E. coli*. The methods used and the detection limits are discussed in part A of this chapter. None of the tracked bacteria was detected either in the

influent or the effluent of the reed beds. Sporadically a 16S rDNA fragment of *Yersinia* spp. was amplified but the virulence plasmid pYV was never detected. Cultivation data were consistent with the PCR based results of this work (see part A of this chapter).

The fact that no target pathogens were present in the influent samples of the filters left the question open, whether these would have been eliminated to the same extent as autochthonous wastewater bacteria. In order to obtain more information on this question a lab scale fixed-bed reactor was constructed and the elimination of one of the previously investigated pathogens was studied in more detail.

The rapid dissemination of antibiotic resistance genes in bacterial populations as a consequence of the intensive use of antibiotics in medicine and agriculture can be partly attributed to plasmid-mediated horizontal transfer (Grohmann et al., 2003). Clinically relevant antibiotic resistance genes have been detected in municipal wastewater (Volkmann et al., 2004). Plasmids capable of being transferred and stably maintained in a wide range of bacteria, the so-called broad-host-range plasmids, are of special interest with respect to interspecies gene transfer. Most plasmids belonging to the incompatibility groups IncP, IncN, IncW are conjugative and many IncQ and rolling circle plasmids are mobilizable (Götz et al., 1996). The DNA derived from the influent and the effluent of the reed beds of the Wiedersberg plant was PCR amplified and the plasmid-specific DNA sequences were assayed by Southern hybridization. Only plasmids belonging to the IncP group were detected in the influent samples. Concentration of plasmid DNA was reduced during purification and was not detected in the effluent of the reed beds, indicating that the plasmid carrying bacteria were removed from wastewater to a considerable extent. IncP plasmids are widely distributed in Gram-negative bacteria, including health-relevant species like *Pseudomonas aeruginosa*, *Klebsiella aerogenes*, and *Bordetella bronchiosceptica* (Thomas, 1981). These plasmids were frequently detected in environmental systems (Götz et al., 1996; Izmakova et al., 2006).

1.4 Influence of other components of the plants on purification performance

1.4.1 Wastewater lagoons

Although the main goal of this investigation was to assess the bacteria removal capacity of reed beds, the performance of the wastewater lagoons in the treatment

plant Ettenbüttel was determined because the effluent of the lagoons was also the influent of the vertical reed beds. Therefore the first purification stage could have influenced the elimination performance of the beds. Bacteria reduction in the wastewater lagoons was highly irregular; in most cases the lagoons did not reduce the total numbers of bacteria. The mean bacterial concentrations in the influent and the effluent of the wastewater lagoons were 2.8 x 10^9 cells ml^{-1} and 1.9 x 10^9 cells ml^{-1}. However, the high amounts of autochthonous bacteria and protozoa, combined with longer retention times in the lagoons, could have increased the pressure of inter-specific competition and grazing, contributing to the elimination of the allochthonous bacteria introduced with the wastewater. This assumption is supported by the laboratory experiments performed with the percolated fixed-bed reactor: when introduced in the wastewater influent vessel, *Y. enterocolitica* numbers decreased by 85% after 24 h of residence. Kadlec & Knight (1996) reported similar reduction efficiencies of fecal indicator bacteria in the water phase of pilot and full-scale wetland systems: in general, the reduction values were in the range of 85-99% and depicted as a first order function against detention time.

1.4.2 Sewerage system

The final effluent of the treatment plant in Ließen, which was collected before infiltrating in the soil, consistently contained at least as much bacteria as the settled wastewater. Consequently, there was no general bacteriological wastewater purification. The morphological pattern of the microorganisms in the settled wastewater and in the final effluent was different which is an argument against short-circuit currents and in favor of some kind of contamination in the canalization. The results presented here are the first studies on the wastewater treatment quality of the treatment plant Ließen and point out the need for more careful maintenance and surveillance of subsurface flow constructed wetlands.

2 Putative mechanisms of bacteria retention in the reed beds

Straining and adsorption are the two main processes responsible for immobilization of microorganisms moving through porous media (Stevik et al., 2004). Straining involves the physical blocking of movement through pores smaller than the bacteria. In porous media where the pores are larger than the bacteria, the dominant process of retention is adsorption. The factors affecting straining are the grain size of the porous media (Ausland et al., 2002), the size and shape of bacterial cells (Lawrence

& Hendry, 1996), the degree of water saturation (Smith et al., 1985) and clogging of the filter media (McDowell-Boyer et al., 1986). A well-graded filter medium has smaller pores than a poorly graded one. The smaller the pores and the larger the bacteria the stronger are straining forces. The water content of the medium determines if bacterial transport will take place in the greater or in the smaller pores, thus influencing straining. Finally, clogging reduces the pore sizes. The effect of clogging on bacterial retention has been discussed in 1.1.

Factors that influence adsorption of bacteria to porous media are very numerous. The surface charge of the medium is determined by its chemical composition and the presence of organic matter. The surface charge of medium, as well as its texture, interacts with the bacterial cell surface charge and influences greatly bacterial adsorption to the medium (DeFlaun & Mayer, 1983; Huysman & Vestraete, 1993). Cell surface components like fimbriae and pili are also thought to promote adsorption (Harvey, 1991). The pH and ionic strength of the wastewater may also interfere with adsorption (Goldschmidth et al., 1973). Higher temperatures are thought to increase adsorption of bacteria by changing the fluidity of the cell membrane and/or the physiology of the organisms (Fletcher, 1977). The flow rate through the porous medium influences the contact time between the media and the bacteria (Smith et al., 1985). The rate of adsorption has also been suggested to increase linearly with cell concentration because the number of collisions between the cells and the medium particles increase with concentration (Escher & Charaklis, 1990).

In a reed bed, if straining and adsorption are very effective, practically all inflowing bacteria are retained in the upper layers of the beds. During percolation through the beds, wastewater will elute soil bacteria from the filter, which will form the microbial population of the effluent of the reed beds. According to this model ("**shift model"**), the microbial composition of the effluent samples should be basically different from that of the influent. In a second retention model ("**reduction model**") straining and adsorption are weaker and will retain only a fraction of the influent bacteria. The type of bacteria, its size and adhesive properties of the cell surface and electrostatic interactions with the filter medium will determine which fraction will be retained and which fraction flows out. In this case, the effluent bacteria will constitute a sub-population of the influent bacterial community. In order to find indications on which retention model is predominant in the reed beds investigated, the shifts in the

microbial population after percolation were analyzed by phase contrast microscopy and fluorescence in situ hybridization.

Phase contrast microscopic analysis of the influent and effluent of the reed beds indicate a strong retention of bacteria in the reed beds of the RTP Wiedersberg: all large and typical microorganisms of the influent were strongly reduced, occurring only sporadically and in very small amounts in the effluent. The effluent of the filters at the treatment plants Ettenbüttel and Ließen contained in some cases more microorganisms typical for the influent (e.g. filamentous bacteria, protozoa and algae), probably derived from an increase in the permeability of the filter beds associated with channel formation in the sequence of clogging and/or short-circuiting. Short-circuiting occurs more frequently in horizontal than in vertical reed beds (Green et al., 1997).

Fluorescence in situ hybridization provides not only information on the phylogenetic affiliation of the bacterial groups in a system but also on their physiological state: the sensitivity of the FISH approach is strongly dependent on the number of ribosomes in the target cells and in its turn the ribosome content of cells correlates with the growth phase and the physiological state (DeLong et al., 1989; Wallner, 1993). Therefore the percentage of cells that hybridized with the eubacterial probe EUB338 reflected the physiological activity of bacterial cells in the influent and effluent of the reed beds. Moreover, *Proteobacteria* are the most common phylogenetic group in wastewater treatment systems (Manz et al., 1994; Snaidr et al., 1997). The differences in the relative abundances of the α-, β- and γ-*Proteobacteria* were taken as indication of the changes in the composition of bacterial populations as a result of percolation.

The percentage of EUB338 hybridized cells in the effluent of the vertical reed beds of Wiedersberg, as well as the lower probe conferred signal intensity, indicate that in general the effluent bacteria had less ribosomes/lower physiological activity than the influent bacteria. The fraction of EUB338 hybridized cells in the influent to the vertical reed beds in Ließen and Wiedersberg was in the same range (average of 74% and 77.5%, respectively). However, EUB338 hybridized cells were more abundant in the effluent of the reed beds of Ließen (average of 76.5% in Ließen vs. 55% in Wiedersberg). The high percentage of hybridized cells in the effluent of the vertical beds in Ließen and the higher signal intensity suggest that the formation of channels

decreased the retention time of bacteria in the filter. These data correlate well with the breakthroughs detected by phase contrast microscopy.

In Wiedersberg the hybridizable α-, β- and γ- *Proteobacteria* accounted together for 41% of total cell counts in the influent and 19% in the effluent of the vertical beds. The reduction pattern was similar for the three groups (thus supporting the **"reduction model"**): the α- *Proteobacteria* decreased in abundance from 8.5% to 3% of total cell counts, the β- *Proteobacteria* from 19% to 11% of total cell counts and the γ- *Proteobacteria* from 14% to 5% of total cell counts. Moreover, the reduction of the α-, β- and γ- *Proteobacteria* was in the same range as the reduction of total EUB338-hybridized eubacteria.

In Ließen the proportion of α-, β- and γ- *Proteobacteria* was not reduced by percolation: they made up 45% of total cell counts in the influent of the vertical reed beds and 43% in their effluent. The fraction of α- and γ- *Proteobacteria* increased slightly after percolation (on the average from 12% to 13% and from 7% to 10%, respectively). Only the β- *Proteobacteria* suffered a small reduction from 25% to 20%. This pattern correlates well with the phase contrast microscopic observations of the wastewater samples and suggests that permeability was higher in the vertical beds of Ließen in comparison to Wiedersberg.

In all cases, the morphological and hybridization pattern of the effluent of the reed beds was more similar to a fraction of the influent microorganisms than to the populations in the filters, indicating that the **"reduction model"** was predominant and a percentage of the influent bacteria escaped retention in the filter.

Size-selective filtration probably occurred in the vertical reed beds. This fact may have a detrimental effect on the hygienic safety of reed bed systems, since it is common that bacteria reduce their size as a consequence of starvation (Amy et al., 1983; Humphrey et al., 1983; Costa et al., 1999). Reduction in size will diminish straining during percolation and may increase the numbers of pathogens in the effluent.

3 Factors governing bacteria elimination in subsurface flow constructed wetlands

These investigations focused on the vertical reed beds, since the horizontal beds proved not adequate for bacteria removal. The vertical beds reduced the influent concentrations of bacteria by two orders of magnitude. Before discussing the factors

affecting bacteria elimination it will be discussed if there is a proof of elimination and not just retention and accumulation of bacteria in the vertical reed beds. Two observations point out that bacteria were truly eliminated. First, the morphology of the soil bacteria and their hybridization pattern was strikingly different from those of the wastewater bacteria. Secondly, if bacteria had just accumulated in the vertical filter, taking the time of operation, the hydraulic load, the dimensions of the filters and the mean bacterial concentrations in the influent and effluent of the vertical filter, then the vertical filters should have accumulated a mean of 10^{11} bacteria per g of soil (assuming they were distributed equally along the whole depth). In Ließen the highest bacterial density determined was 10^{9} cells per g of dw soil and concentration decreased with depth. In Wiedersberg the highest bacterial density was 10^{7} cells per g of dw soil and concentration also decreased with depth. Both results suggest that wastewater bacteria were truly eliminated in the filter matrix of the vertical reed beds.

Several abiotic factors affect the survival of bacteria in soil and soil-like environments like temperature, moisture content and organic matter. In general, the survival of bacteria decreases with temperatures around 20°C in comparison with temperatures around 5°C (Stenstrøm & Hoffner, 1982; Bitton, 1994). This may be in part explained by the fact that VBNC states may be formed at higher rates at lower temperatures (Smith et al., 1994; Mizunoe et al., 2000). In a temperate climate like in Germany, the temperature in the reed bed matrix will probably be low and support survival of bacteria. Longevity of bacteria is usually higher in moist than in dry soils (Bitton, 1994). Since the vertical reed beds were unsaturated, changes in the moisture content of the filter medium may have played a role in bacteria elimination. High concentrations of organic matter provide better conditions for the survival of bacteria (Dazzo et al., 1973; Acea & Alexander, 1988), in particular for gastrointestinal pathogens, which are adapted to higher nutrient conditions. Therefore, organic matter may enhance retention of bacteria in the matrix of the beds, but it may simultaneously increase their survival.

The biological elimination mechanisms of bacteria in reed beds include antimicrobial activity of root exsudates (Kickuth & Kaitzis, 1975; Axelrood et al., 1996), predation by protozoa, nematodes, rotifers and *Bdellovibrio* sp. (Decamp & Warren, 1998; Decamp et al., 1999) and lytic activity of viruses (Axelrood et al., 1996). The individual survival potential of each bacterial species is also an important biological factor affecting elimination (Stevik, 2004). Antagonistic relationships with other

bacteria (competition for nutrients and secretion of inhibitory substances) are also an important factor for the elimination of allochthonous bacteria in natural environments (Yates & Yates, 1988; Thompson et al., 1990). The relative importance of the various biotic factors will probably depend on the construction and operation of the reed bed systems. It is difficult to estimate the contribution of each of the biotic factors to the elimination of allochthonous bacteria in reed beds. If one of the factors is shut down, the others will be influenced by its absence. For instance, grazing by protozoa is selective: among other factors, Gram-negative bacteria are preferentially grazed in comparison to Gram-positive ones (Rønn et al., 2002) and medium-sized bacteria are preferred to large or small cells (Jürgens & Güde, 1994). Thus, if protozoa are removed from an investigated system, the shift in the bacterial populations resulting from their absence will probably affect grazing rates by other bacteria predators as well as the antagonistic relationships between the bacterial populations. Grazing rates of different predators were usually determined in two-variable predator-prey systems (Decamp & Warren, 1998; Decamp et al., 1998; Decamp et al., 1999) and may be very different in complex natural systems.

Significant amounts of protozoa were only detected in the clogging layer of the vertical filter in Ließen, suggesting that they only play a major role in bacteria elimination in well-developed clogging layers. Studies on protozoa grazing in reed bed systems were usually performed in horizontal flow reed beds (Decamp & Warren, 1996; Green et al., 1997; Decamp et al., 1998, Decamp et al., 1999). Decamp et al. (1999) found a higher quantity and diversity of protozoa in the first third of horizontal reed beds treating domestic sewage. They examined the interstitial fluid of the reed bed and enumerated app. 600 ciliates and 3,000 amoebae per ml of fluid. The authors attributed the higher numbers of protozoa in the first third of the filter to higher oxygen content and bacterial concentration. The dependence of protozoa concentration on bacterial density was discussed in 1.1. The life of protozoa in vertical reed beds may be restricted by low and variable moisture content of the matrix, since protozoan activity is restricted to water films and water-filled pores (Wright et al., 1993; Rønn et al., 1995). In contrast to the reed beds, protozoa were very abundant in the settled wastewater. Moreover, the influent canalization contained periodically thick microbial mats constituted by *Vorticella* sp. (Ulrich Szewzyk, personal communication). Hence, grazing by protozoa probably plays a very important role in all aqueous parts of the treatment plants.

Other bacteria predators, like rotifers and nematodes, are suggested to play a role in bacteria elimination in horizontal reed beds (Decamp et al., 1999, Vymazal et al., 2001). Rotifers and nematodes were not detected in the investigated filter medium samples. No data were found on the role of viruses, *Bdellovibrio* sp. and *Bdellovibrio*-like organisms on bacteria elimination in vertical reed beds. These organisms were not object of study in this work. Like protozoa, *Bdellovibrio* sp. predation seems to be confined to water films (Williams et al., 1995) and will probably be stronger in spots of higher bacterial density (e.g. clogging layer in Ließen) than in deeper layer of the filter beds.

The vertical beds in Wiedersberg had a thinner biofilm and significantly lower bacterial density than Ließen but displayed better bacterial retention and elimination rates. Hence, adsorption to mineral particles with low biofilm development and subsequent die-off by environmental stress may be a more efficient strategy for wastewater purification in vertical reed beds than antagonistic relationships with other microorganisms in a well-developed biofilm.

C) Tracking the fate of a pathogenic bacterium in a lab-scale fixed bed reactor

When functioning as a recipient for waste, soil may constitute a reservoir of enteric pathogens and play a role in the transmission of enteric diseases (Santamaria & Toranzos, 2003). Bacteria can travel long distances in soils (Celico et al., 2004; Stevik et al., 2004) and contaminate groundwater and drinking water resources. The goal of this study was to investigate the fate of an enteropathogenic bacterium introduced in a constructed wetland system. Since none of the target enteric pathogens was detected in the full-scale treatment plants during the period of investigation, a percolated fixed-bed reactor was constructed and operated with natural wastewater to simulate a subsurface flow constructed wetland. The vertical beds of the Wiedersberg constructed wetland displayed the best bacteria removal performance from wastewater. Therefore the hydraulic parameters and organic load of the reactor were adjusted to those practiced in the Wiedersberg treatment plant. Constant unsaturated flow was applied because these conditions were reported to be efficient for retaining bacteria in filter columns (Powelson & Mills, 2001). The reactor had no vegetation. Data on the role of plants in the elimination of bacteria in subsurface flow constructed wetlands is controversial: Manios et al. (2002) reported no differences in the performance of planted and unplanted soil filters. Vacca et al., (2005) suggested that an *E. coli* strain introduced in a planted filter could only survive in the rhizosphere. Bavor et al. (1989) predicted a longer retention time for the elimination of fecal coliform bacteria in planted beds than in unplanted beds. In the present experiment, natural wastewater was amended with a pathogenic strain of *Y. enterocolitica*. *Y. enterocolitica* was selected as model pathogen because it has been related to waterborne gastrointestinal diseases (Eden et al., 1977, Ostroff et al, 1994). Furthermore, *Y. enterocolitica* can form VBNC states - thus escaping cultivation – (Smith et al., 1994) and has a high survival capacity in the environment (Kapperud, 1981; Shayegani et al., 1981; Karapinar and Gönül, 1991). In order to circumvent the bias inherent to cultivation methods the adequacy of the TaqMan assay for tracking the fate of *Y. enterocolitica* at different purification stages in the reactor was evaluated.

1 General filtration performance of the fixed-bed reactor

Since clogging is one of the most important problems of filter beds for wastewater systems a filter medium should be selected for the reactor, which does not support

biofilm formation. This fact could affect significantly the retention capacity of the filter for bacteria, so this issue was thoroughly investigated. The medium used for package of the reactor column was composed of a very high fraction of medium sized quartz sand. Silt, clay and organic matter content, which could favor adsorption of organic matter to the porous medium (Gerba and Bitton, 1984), were very low. Cations and iron oxides were not detected at the filter medium surface. These molecules may increase the adsorption of bacteria by altering the surface charge of the medium surface (Mills et al., 1994). With a uniformity coefficient of 3.6, the filter medium was a uniform and poorly-graded medium, being more permeable to water and providing weak straining forces to bacteria percolating through the reactor. In conclusion, the physical and chemical characteristics of the filter medium predicted a low retention of bacteria and consequently low biofilm formation. In fact, during the whole experiment, no significant amount of organic matter was deposited on the mineral particles of the filter medium and weight loss on ignition only increased from 0.072% to a maximum of 0.178% in 10 cm depth after three weeks of operation.

However, total and dissolved organic carbon was efficiently removed form the percolating wastewater. Mean removal rates were 76% for total and 72% for dissolved organic carbon. These values are in agreement with those reported by other authors for simulated wetland systems (Castillo et al., 2001, Hunter et al., 2001). The decrease in the removal rates of TOC and DOC during operation was probably due to the successive collection of filter medium samples form the reactor column, which could have built channels in the filter bed.

Despite of the inert characteristics of the filter medium the bacteria filtration efficiency of the fixed-bed reactor was high. When determined by total cell counts, bacteria removal efficiency from the influent wastewater was 97% and in the same range of the removal rate in Wiedersberg. When determined by heterotrophic plate counts, removal efficiency was in the same range (96%). The proportion of culturable cells ranged from 0.006% to 1.1% in the influent samples and from 0.025% to 3.0% in the effluent samples. Culturability was higher in the effluent than in the influent samples probably because the proportion of phototrophic and/or fastidious bacteria in the influent samples was higher than in the effluent. The decrease in culturability of the influent wastewater bacteria with time correlated with the increase in the storage time of the wastewater. However, 50% of total enumerated bacteria in the influent samples gave a strong signal with the EUB probe mix, so it can be excluded that the

reduction in culturability was caused by a significant proportion of dead cells. Decrease in culturability of the influent samples during storage was possibly due to a shift in the microbial populations towards non-culturable bacterial species and/or towards lower culturability of the same species.

2 Microbial mat formation

The biofilm developed in the reactor bed during the three weeks of operation was examined in situ and ex situ. For the ex situ analysis the microbial communities were sequentially dispersed with amoeba saline and afterwards with a modification of the dispersion method for bacteria described by Böckelmann et al. (2003). Dispersion with amoeba saline has proved efficient for the recovery of protozoa from soil systems (Page, 1976; Rønn et al., 2002). Although protozoa were always present in the influent samples, these organisms were only sporadically detected in the upper layers of the reactor bed. Although it cannot be completely excluded that a fraction of the protozoa was damaged by dispersion, low protozoa numbers in he filter beds correlate well with the observations made for the reed beds of the investigated full-scale plants, where protozoa were only abundant in spots of high bacterial density.

In situ analysis of the biofilm by scanning electron microscopy confirmed that the biofilm on the surface of the filter medium was very thin and bacteria were not abundant. Morphology of the bacteria in the filter medium samples resembled much that of the full-scale vertical beds in the sense that they had no peculiar traits. After three weeks of operation, distribution of bacteria in the filter column was roughly uniform and fluctuated between 10^6 and 10^7 cells per g of dry weight. Since the biofilm was thin, the hydraulic conductivity was high. Oxygen and nutrients probably could have reached the deeper layers of the filter column. In this case, the whole depth of the filter (and not only the upper layers) was involved to a higher extent in the biological processes of wastewater purification than in a filter with a clogging layer. However, a stronger accumulation of bacteria in the upper layers of the reactor and a subsequent formation of a biofilm would have possibly occurred with longtime operation.

Results from cultivation and in situ hybridization suggest that the physiological activity of the filter medium bacteria was in general lower than in the influent and effluent of the reactor. The percentage of culturable cells decreased significantly in all analyzed depths of the filter column during the experiment. The decrease in culturability was probably partly derived from the decrease in culturability of the

influent samples and partly by a change in the physiology of the wastewater bacteria retained in the filter as an adaptation to new environmental conditions. The fraction of culturable bacteria showed a different growth behavior on the plates: average colony size was smaller and the required incubation time was longer, which could be a sign of starvation and a shift towards maintenance metabolism. In contrast to the wastewater samples, FISH signals with the EUB probe mix amounted to only 10-32% of the total cell counts and signals were weak in all depths of the filter.

3 Adequacy of TaqMan PCR for detecting *Y. enterocolitica* in the reactor samples

The 5' endonuclease (TaqMan) assay was selected to track the fate of *Y. enterocolitica* at different purification stages in the fixed-bed reactor because this technique is among the most specific and sensitive methods of quantitative PCR (Jebbink et al., 2003) and allows a high sample throughput. Although there is controversy on how representative DNA based results are for the analysis of an active bacterial population in soil systems, DNA was used as target instead of RNA because it is more robust and less prone to degradation than RNA (Lebuhn et al., 2004). So, using DNA, at least no large underestimations would occur. The risk of overestimation due to the amplification of DNA derived from dead cells was different for the wastewater and filter medium samples: wastewater is an environmental sample with substantial physiological activity. This general assumption was confirmed in this work by whole cell fluorescence in situ hybridization with the EUB338 probe mix. Consequently, nucleic acid turnover will be probably very fast in wastewater and no significant overestimation will occur (Bach et al., 2000). Besides, TaqMan quantification of *Y. enterocolitica* in the influent wastewater samples was in good agreement with the numbers of spiked cells, confirming the adequacy of the method for quantification of the pathogen in wastewater samples. When regarding the filter medium samples, we are facing a totally different environment. Predominance of small cells, weak hybridization signals with the EUB338 fluorescent probes, low culturability of the bacteria living in the filter medium and low biofilm formation indicated that most bacteria in the filter medium had very low metabolic rates and/or lived on the edge of starvation. Bacterial density was low and protozoa were only sporadically detected. Thus, the probability of contact between introduced and autochthonous bacterial species or between predators and preys, which could

help degrade and more rapidly recycle DNA from dead cells, was lower in the filter column than in the wastewater samples. Therefore, in this section, special focus was laid upon the adequacy of the TaqMan assay for the quantification of *Y. enterocolitica* in the filter medium and the problems of over- and underestimation are here discussed.

3.1 Batch assays

For the TaqMan quantification of *Y. enterocolitica* in the reactor samples an external DNA standard was prepared consisting of DNA extracted from pure cultures of *Y. enterocolitica*. The number of 16S rRNA gene copies in the genome of *Y. enterocolitica* is not known at present. Therefore, an empirical correlation was established between the DNA extracted from defined numbers of *Y. enterocolitica* cells and the fluorescence threshold cycles obtained by TaqMan PCR. For the calculations it was assumed that each *Y. enterocolitica* cell had one single chromosome. The influence of polyploidy on the sensitivity of PCR based methods was discussed in Section A. 4 of this chapter. All *Y. enterocolitica* cells used for spiking assays or production of external standards were harvested in the stationary phase because in most environments bacterial growth rates are usually low. Polyploidy, which could lead to overestimation of the sensitivity, is probably low in this growth phase (Bach et al., 2000) but cannot be ruled out (Ackermann et al., 1995; Herrmann et al, 1997; Müller & Babel, 2003). The TaqMan assay using the standard *Y. enterocolitica* DNA was quantitative and proportional over 7 orders of magnitude (R^2 = 0.9950) and the DNA equivalent to one target cell was clearly detected. The sensitivity achieved is in good agreement with that reported by Sen for the detection of *Y. enterocolitica* with the same TaqMan protocol but different DNA extraction method (Sen, 2000).

Moreover, prior to application to the filter medium samples, the reliability of TaqMan quantification was assessed for a simplified system composed of artificial wastewater, filter medium and *Y. enterocolitica* cells. Comparison with the external standard showed that the filter medium and the artificial wastewater had no influence on the efficiency and reliability of the DNA extraction and TaqMan quantification of *Y. enterocolitica.* Moreover, TaqMan PCR of the extracted DNA was accurate enough to reflect a two-fold variation in the target cell numbers.

3.2 Efficacy of the DNA extraction for the filter medium samples

Filter medium samples were withdrawn from different depths 1, 3, 5, and 7 days after addition of *Y. enterocolitica* to the influent (corresponding to sampling days 14, 16, 18, and 20) and the DNA was extracted for TaqMan PCR quantification of the model pathogen. Since especially low DNA recoveries were reported in soil and sediment samples (Moré, et al., 1994; Frostegård et al., 1999; Rose et al., 2003), the efficacy of the extraction was roughly estimated by calculating the quotient between the DNA yield and the cell numbers determined by total cell counts. The mean DNA yield decreased with depth from 30 to 10 fg DNA per cell. Assuming that each cell contained 1-2 chromosomes and a bacterial genome is estimated as weighing between 1 and 10 fg, the efficiency of the FastDNA kit for extracting DNA from the filter medium samples was high. The calculation of DNA recovery based on the cell numbers is obviously a rough estimation. Dispersion of bacteria from the medium may be incomplete and the DNA content of each single cell is variable. Besides of polyploidy, which has been discussed in Section A.4, bacteria can also reduce their DNA content as a response to starvation and form the so-called “dims” (Button & Robertson, 2001). However, despite of all variables that may influence the estimation of DNA recovery, a control is necessary in order to assure that no substantial underestimation was made due to low DNA recovery. The control of the qualitative and quantitative recovery of whole community DNA is frequently not or insufficiently performed in assays using PCR techniques for the study of the microbial diversity in environmental samples (e.g. Riemann et al., 2000; Yu et al., 2001; Calvo-Bado et al., 2003; Ellis et al., 2003).

3.3 Specific recovery of *Y. enterocolitica* from the filter medium samples

In order to determine the extent of recovery of the target bacterium in the filter medium, samples derived from day 20 were spiked with 10^3-10^7 cells of a *Y. enterocolitica* overnight culture per g of filter medium. The samples were processed as usual and subjected to quantification with the TaqMan assay. Recovery of the spiked cells of *Y. enterocolitica* revealed different patterns for the different depths. Recovery in 10 and 20 cm depth was similar (average of 26%) and the numbers of spiked cells had no significant influence on the recovery rate. Mumy & Findlay (2004) tested commercially available kits in different soils for their DNA extraction efficiency.

They reported that the Fast Prep kit delivered 28.3 ± 10.5% of the spiked DNA, which is within the range of the yield obtained for *Y. enterocolitica* DNA in this work.
In 40 cm depth, DNA recovery of the model bacterium was considerably lower than in the other depths (average of 13%). Moreover, the recovery rate seemed to be higher for higher amounts of inoculated cells. Result bias due to polyploidy was discussed in 3.1.

3.4 Trouble shooting for low recovery in 40 cm depth

Whole community DNA yield and the specific recovery rate of *Y. enterocolitica* were lower in filter medium samples derived from 40 cm depth than in the upper layers.
Several tests were carried out with the samples derived from 40 cm depth to search for substances that could interfere with the DNA extraction or its PCR amplification. Lorenz & Wackernagel (1987) reported that valency, and in particular divalent cations, significantly influenced the recovery of DNA from sand. X-ray spectroscopy and EDX analysis showed no differences in the elemental composition of the filter medium in 40 cm depth in comparison to 10 and 20 cm. However, it cannot be completely ruled out that undetected substances increased the affinity of the DNA to the filter medium in 40 cm depth. An ex situ lysis did not improve DNA yield. A special purification procedure for humic acids recommended by the manufacturer revealed that no detectable humic acids were present in the samples (data not shown). The DNA extracted from 40 cm depth could be amplified by classic PCR directed to the 16S rDNA from *Eubacteria.* Standard *Y. enterocolitica* DNA, when spiked in the DNA extracts, delivered the same amplification efficiency as standard DNA alone, indicating absence of co-extracted impurities. A fact that might have influenced the extent of cell lysis in the different layers was the higher moisture content of the filter medium in 40 cm depth (twice as high as in 10 cm depth): Frostegård et al. (1999) reported a more efficient lysis in soils that had been previously dried at room temperature.

On one side low recovery rates for the DNA of *Y. enterocolitica* probably lead to underestimation of its persistence in the filter medium. On the other side, amplification of DNA derived from dead cells might theoretically lead to overestimation. However, if naked DNA adsorbed to sand particles is available for amplification it is also available for degradation (Demanèche et al., 2001).

Additionally, observations made by Uyttendale et al. (1999) and Fach et al. (1999) suggested that PCR efficiency is much lower for the DNA of dead cells (see part A, section 1.3). Consequently, in this work, underestimation of *Y. enterocolitica* numbers by incomplete DNA recovery was probably much more significant than overestimation by amplification of DNA from dead cells.

Despite of the likely underestimation associated with the low DNA recovery from the filter medium samples, quantification of *Y. enterocolitica* by TaqMan PCR in the filter medium samples was considered more suitable than cultivation because i) the proportion of total culturable bacterial cells was very low and highly variable in these samples (between 0.003% and 4% of DAPI) and ii) *Y. enterocolitica* constituted only a small proportion of total influent cell numbers (1:100-1:1000). Therefore, the proportion of *Y. enterocolitica* numbers that would be detectable by cultivation was hardly predictable.

3.5 Sensitivity and efficiency of the TaqMan assay in the reactor samples

For the TaqMan assay, dilution of the template DNA is usually required for successful quantitative amplification; otherwise inhibition usually occurs (Bach et al., 2000, Harms et al., 2003). Especially for DNA derived from soil samples, up to 1,000-fold dilution of the DNA may be necessary. This constitutes a constraint when a DNA sequence should be targeted that is present in low concentrations in the total community DNA. The dilution factor is therefore an important parameter and may lead to false negative results if it decreases the number of target DNA copies below the detection limit of the assay.

In DNA extracted from wastewater a final dilution of 1:50 did not decrease amplification efficiency (R^2 = 0.99). For the filter medium samples the required dilution factor for R^2= 0.99 was 1:100. Hence, both dilution factors were low (see Bach et al., 2000; Stults et al., 2001), confirming the good quality of the extracted DNA and the applicability of the assay to a wide range of target cell concentrations.

For the determination of the detection limit of the whole method several factors were taken into account: the sensitivity of the selected TaqMan assay, the highest amount of filter medium sample extractable in a Fast Prep tube (500 mg of wet weight) and the required dilution of the template DNA for quantitative results.

For the wastewater samples, the detection limit of the TaqMan PCR was 200 *Y.* enterocolitica cells per ml of wastewater containing 10^8-10^9 total bacterial cells. For

the filter medium samples, the detection limit was 400 cells *Y. enterocolitica* in a background of 10^7 cells per g of filter medium. To the best of our knowledge no other works were performed on the TaqMan detection of *Y. enterocolitica* in comparable samples.

4 Survival potential of *Y. enterocolitica* in wastewater

The fate of *Y. enterocolitica* in the influent vessel of the fixed-bed reactor can be compared with its fate in the settling tanks or wastewater lagoons of the investigated full-scale treatment plants. In these systems, reduction of pathogens is caused by sedimentation, unfavorable wastewater chemistry, antagonistic relationships and predation (Green et al., 1997).

In order to estimate the contribution of the several factors on the survival capacity of *Y. enterocolitica* in wastewater the growth potential of this pathogen was first determined in sterile artificial wastewater. The results of the growth curves suggest that *Y. enterocolitica* is adapted to high nutrient conditions such as given in LB medium but can survive and replicate under nutrient conditions similar to wastewater. Although artificial wastewater and the natural wastewater used for the reactor had carbon contents in the same range, the type of nutrients and their accessibility for degradation were probably different. Due to the high variety of microorganisms present in the natural wastewater the more easily degradable carbon sources are probably depleted very quickly in the canalization and only more recalcitrant molecules will have a longer half-life. The nutrients of artificial wastewater (peptone and meat extract) are a priori more easily degradable than e.g. humic acids. It is though possible that *Y. enterocolitica* can survive well under the nutrient conditions present in natural wastewater, since this bacterium has been reported to survive in rivers and lake water (Shayegani et al, 1981; Karapinar and Gönül, 1991), which have less and less available carbon sources than wastewater.

When added to natural wastewater, decay of *Y. enterocolitica* in the influent vessel amounted to less than one order of magnitude after 24 h, whereas reduction of the *Y. enterocolitica* numbers by filtration amounted to two orders of magnitude or more. Hence filtration was by far the most important factor for the removal of *Y. enterocolitica* from wastewater. The same reduction pattern was observed by Arias et al. (2003) for *E. coli* introduced in experimental vertical flow filters preceded by sedimentation tanks. Sedimentation could not have accounted for the reduction of

the *Y. enterocolitica* numbers, since the wastewater was continuously stirred. The presence of pollutants in the natural wastewater may have contributed to the reduced survival of *Y. enterocolitica* in comparison to artificial wastewater. Due to the high bacterial concentration and diversity and high numbers of protozoa, predation and antagonistic relationships probably played an important role in the reduction of the *Y. enterocolitica* numbers when introduced into natural wastewater.

5 Reduction of *Y. enterocolitica* by percolation

Y. enterocolitica was removed from the influent by two orders of magnitude and hence to the same extent as the autochthonous wastewater bacteria, which were reduced by 97%. The influent contained an average of 10^5 *Y. enterocolitica* cells per ml and the effluent an average of 10^3 *Y. enterocolitica* cells per ml when determined by TaqMan PCR. Culturability of the target bacterium was lower in the effluent than in the influent samples: when cultivated on *Yersinia* agar base followed by PCR confirmation, 10^4 CFU of *Y. enterocolitica* were determined per ml of influent and only 10^1 CFU per ml of the effluent, thus confirming the observation made for the full-scale plants that underestimation of bacterial concentrations by cultivation is higher in the effluent than in the influent samples. FISH with the *Y. enterocolitica* specific fluorescent probe gave no signals in the effluent. When added to these samples, freshly spiked *Y. enterocolitica* cells gave very bright signals with the same probe, so the possibility of result bias by fluorescence quenching can be excluded. Together with the low culturability rates, this fact suggests that the metabolic activity of the target bacterium decreased significantly during percolation.

6 Putative mechanisms of bacterial retention in the fixed-bed reactor

Several observations suggest that the "**reduction model**" (see Section B. 2) played a predominant role in bacteria retention in the reactor bed:

i) There was a clear dependency between the amounts of culturable cells in the influent and their amounts in the effluent (R^2 = 0.90). This correlation was not determined for total cell counts because there was not a sufficiently wide range of bacterial concentrations in the influent samples. Hagendorf et al. (2004) made the same observation for the removal of indicator bacteria from the Ettenbüttel and Wiedersberg wetlands. Baeder-Bederski et al. (2005) reported the same concentration dependency for the removal of *E. coli* in pilot constructed wetlands.

ii) The morphologic pattern of the bacterial communities in the influent and effluent samples was very similar to those observed for the influent and effluent of the vertical reed beds of Wiedersberg (see Section B).
iii) FISH signals with the EUB probe mix were weak in all depths of the filter column, whereas they were strong in the influent and effluent wastewater samples.

7 Accumulation of *Y. enterocolitica* in the reactor filter bed

Y. enterocolitica accumulated in detectable quantities in the filter medium after 7 days of operation although the filter medium had a low organic carbon and moisture content, thus not promoting optimal conditions for survival. Based on the recovery experiments of *Y. enterocolitica* spiked in filter medium samples, probably only app. 20% of the total *Y. enterocolitica* DNA were recovered from 10 and 20 cm depth and less than 20% were recovered in 40 cm depth. So the real concentrations of *Y. enterocolitica* in the filter medium after operation were likely higher than determined. On one hand it is possible that survival and accumulation would be stronger with longer operation time and subsequent stronger biofilm formation. Biofilm formation could increase the adhesion properties of the medium and simultaneously improve the survival conditions for *Y. enterocolitica* by increasing the organic matter and water content of the environment. It has been suggested that allochthonous bacteria can survive for long periods in soil-like environments (Celico et al., 2004; Islam, 2004). On the other hand bacterial density would be higher in a thick biofilm. Matos et al. (2005) investigated the survival potential of an introduced pathogen (*Pseudomonas aeruginosa*) in rhizospheres of different microbial diversity. The capacity of *P. aeruginosa* to invade the rhizosphere was inversely related to the level of microbial diversity. So, with the development of a thicker biofilm during long-term operation, both can happen: i) the organic matter and water content of the biofilm provide better attachment and survival conditions for *Y. enterocolitica* in the filter bed or ii) the development of a more dense and complex microbial community will prevent colonization of the biofilm by the allochthonous pathogenic bacterium.

8 Factors governing bacteria elimination in the reactor

For the calculation of net elimination of wastewater bacteria and in particular of *Y. enterocolitica* in the reactor no differentiation was made between growth and death of cells. It was defined as the total number of influent bacterial (or *Y. enterocolitica*) cells that were added to the filter column and could be detected neither in the filter

column nor in the effluent. This value gives only a rough estimation of the magnitude of elimination, since i) the gradient of *Yersinia* in the filter was not applied to the calculations. Instead the average of the determined densities was used; ii) an eventual deposition of *Y. enterocolitica* in the support pebble layer was neglected. The error derived from this approximation is probably low since the surface area of the pebble layer is much smaller than of the filter column and the concentration of *Y. enterocolitica* in the effluent wastewater percolating through the pebbles was low.

Based on these calculations *Y. enterocolitica* was eliminated in the filter matrix to the same extent as the autochthonous wastewater bacteria (99% vs. 96%, respectively). There were no direct indications that *Y. enterocolitica* was preferentially eliminated or showed a better survival potential in the filter than the overall numbers of autochthonous bacteria. Among the wastewater bacteria, individual elimination rates were probably different, since for instance purple sulfur bacteria were never observed in the filter medium samples.

The factors influencing elimination of bacteria in porous media were presented in Section B. 3 of this chapter. Abiotic factors like temperature, moisture content and organic matter of the filter probably played a more dominant role in the elimination of wastewater bacteria and the allochthonous bacterium *Y. enterocolitica* than in the vertical reed beds of the full-scale plants. The laboratory had a constant room temperature of app. 25ºC. Temperatures around 20ºC favor elimination of bacteria in comparison with lower temperatures (Bitton, 1994; Stevik et al., 2004). The organic matter content of the reactor filter (average of 0.16%) was low in comparison with the full-scale reed beds, which amounted to 0.72-0.91% in Wiedersberg and 0.47-0.95% in Ließen (Winter, 2003). The low organic matter content of the reactor bed may have contributed positively to the elimination of wastewater bacteria and of *Y. enterocolitica*.

The moisture content of the reactor filter medium varied with depth from 6.2% in 3 cm depth to 16.5% in 40 cm depth. Winter (2003) measured moisture contents in 5, 20 and 60 cm depth of several vertical reed beds in Germany. The moisture content of the filter media increased immediately after each wastewater input and reached a mean value of 20% in all depths. The low moisture content of the reactor medium was probably due to the low content of silt and clay, as well as low organic matter content, which have a high water holding capacity (Bitton, 1994). In particular

biofilms can be constituted by more than 90% water (Bitton, 1994), thus providing good survival conditions for bacteria.

As for the biotic factors governing bacteria elimination of the reactor bed, antagonistic relationships with autochthonous bacteria probably played a stronger role in the water phase than in the filter column of the reactor since bacterial density was low in the filter. Nevertheless, competition for nutrients with filter medium bacteria may have contributed to the elimination of the wastewater bacteria. Protozoa were abundant in the influent wastewater but very rare in the filter. Probably water content and bacterial density of the medium were too low for proliferation of protozoa in the filter. They were only detected in the upper layer, where bacterial density was highest. Predation by *Bdellovibrio* sp. and the lytic activity of viruses were not investigated in the present work. Due to the low bacterial density and water content in the filter bed the contribution of *Bdellovibrio* sp. to bacteria elimination in the fixed-bed reactor was presumably similar to that of protozoa i.e. stronger in the aqueous phase and more negligible in the reactor bed.

Multicellular predators like nematodes and rotifers were not observed in the filter medium samples.

D) Conclusions and Outlook

This study focused on the hygienic implications of wastewater treatment by subsurface flow constructed wetlands. Moreover, the mechanisms underlying the retention and elimination of bacteria were studied in more detail. This knowledge should enable improvements in construction and operation of these treatment plants in order to achieve a high and reliable wastewater purification performance.

Especial focus was put on the analytic methods. Culture independent methods were used for the assessment of the general and the specific removal of bacteria in the reed beds. The PCR approach developed in this work for the detection of pathogenic bacteria in wastewater showed high potential for complementing or substituting culture dependent methods for wastewater quality surveillance. This approach should therefore be adapted to other wastewater relevant pathogens. Efforts should be made towards uniformization and standardization of the PCR methods in order to enable comparisons between results derived from different laboratories.

The new insights obtained in this work on wastewater purification performance of vertical and horizontal reed beds open new fields of investigation. It was clearly shown that horizontal reed beds are not adequate for bacteriological purification of wastewater. In contrast, vertical reed beds can achieve satisfactory purification performance if suitably designed, operated and maintained. Therefore, it would be of great interest to perform pilot-scale simulations of multistage constructed wetlands composed of primary settling followed by a sequence of vertical beds for secondary wastewater treatment (Figure 35). Hydraulic and organic load, type of porous media and operational conditions should be combined in order to assess the best combination of these parameters for high bacterial removal rates and simultaneously low extent of biofilm formation. Size fractionated filtration by using successively smaller porous media in the filter beds, combined with alternating irrigation/regeneration of the beds could overcome the problems inherent to clogging and provide enhanced filter performance: the first filter set should constitute a pre-filtration of larger organic particles and bacteria. The porous medium should have large pores to prevent clogging; the second set should contain smaller sized porous media in order to retain smaller bacteria that escaped filtration in the first stage.

The percolated fixed-bed reactor displayed high and constant removal rates of *Y. enterocolitica* at influent concentrations of 10^5-10^6 cells per ml of influent. Data obtained in this work suggest that the removal rates of the model pathogen and of the autochthonous wastewater bacteria may be dependent on their influent concentration. It would be of great interest to study this aspect in more detail and to assess concentration limits below which the pathogen escapes filtration. Furthermore, the effect of starvation and environmental stress on the retention of the pathogen in the filter could provide valuable information on the dynamics of dissemination of waterborne pathogens in the environment.

As shown in this work, reed beds for wastewater treatment can constitute a reservoir for pathogenic bacteria. Therefore, it would be of great importance to study the accumulation and survival of waterborne pathogens during long-term operation of reed beds. The TaqMan assay was suitable for the quantification of low cell numbers of *Y. enterocolitica* in the lab-scale reactor and should be further used in following experiments. Other DNA extraction kits besides the FastDNA Kit should be tested and the extent of DNA recovery should be quantified in order to minimize result bias due to DNA loss.

Observations made in this work suggest that environmental stress plays an important role in the elimination of wastewater bacteria percolating through vertical flow filters. The contribution of protozoan grazing to the general bacteria elimination in both the full-scale and the lab-scale systems seems to be higher in the aqueous phases and in spots of high bacterial density in the filters. The effect of suppressing/increasing protozoan grazing on the bacterial abundance and composition in the pilot-scale plant could give more detailed indications on the role of protozoa in modeling bacterial populations in these systems. Little data are available on the role of viruses and *Bdellovibrio* sp. on bacteria elimination in vertical reed beds. It is not possible to selectively remove these organisms from wastewater. Alternatively, the influence of the addition of higher numbers of *Bdellovibrio* sp. and viruses to the bacterial populations in pilot-scale reed beds should be investigated.

This work provided information on the potentials but also constraints and limitations of subsurface flow constructed wetlands for wastewater purification. In developing

countries, where lack of capital and infrastructures demand for low technical and financial input, this type of wastewater treatment may constitute a feasible alternative for wastewater treatment and could represent a major improvement of the existing standards of wastewater purification.

The knowledge obtained in this work on the elimination of bacteria in subsurface flow constructed wetlands may help improve their design and operation towards enhanced purification performance and hence contribute to prevent the spread of diseases.

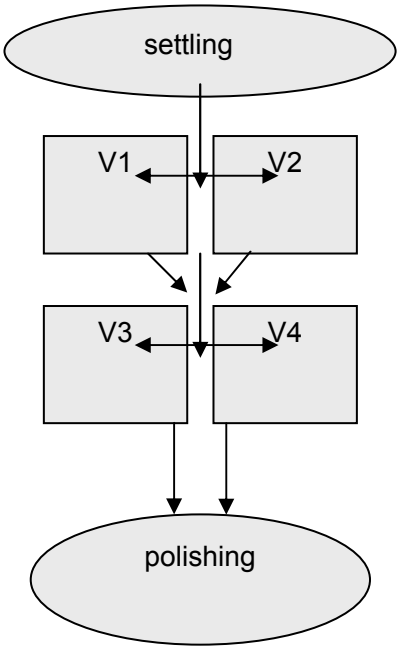


Figure 35: Schematic representation of the construction and operation plan for the multistage pilot-scale plant. V1/V2 and V3/V4 are alternately irrigated. V1/V2 have larger sand size, V3/V4 have smaller sand size.

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