Appl Microbiol Biotechnol
DOI 10.1007/s00253-015-6882-9

ENVIRONMENTAL BIOTECHNOLOGY

Selective elimination of bacterial faecal indicators in the *Schmutzdecke* of slow sand filtration columns

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Received: 22 June 2015 / Revised: 23 July 2015 / Accepted: 25 July 2015

Abstract Slow sand filtration (SSF) is an effective low-tech water treatment method for pathogen and particle removal. Yet despite its application for centuries, it has been uncertain to which extent pathogenic microbes are removed by mechanical filtration or due to ecological interactions such as grazing and competition for nutrients. In this study, we quantified the removal of bacterial faecal indicators, *Escherichia coli* and *Enterococcus faecalis*, from secondary effluent of a wastewater treatment plant and analysed the microbial community composition in compartments of laboratory model SSF columns. The columns were packed with different sand grain sizes and eliminated 1.6–2.3 log units of faecal indicators, which translated into effluents of bathing water quality according to the EU directive (<500 colony forming units of *E. coli* per 100 ml) for columns with small grain size. Most of that removal occurred in the upper filter area, the *Schmutzdecke*. Within that same zone, total bacterial numbers increased however, thus suggesting a specific elimination of the faecal indicators. The analysis of the microbial communities also revealed that some taxa were removed more from the wastewater than others. These results accentuate the contribution of biological mechanisms to water purification in SSF.

Keywords Wastewater reuse · Bacteria removal · Ecology of slow sand filtration · *Schmutzdecke*

Electronic supplementary material The online version of this article (doi:10.1007/s00253-015-6882-9) contains supplementary material, which is available to authorized users.

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Introduction

Globally increasing fresh water stress has stimulated a search for new sources of water supply and other approaches to close water budget gaps (Al Salem and Abouzaid 2006; Shannon et al. 2008; WHO 2006). One option is the reclamation and reuse of domestic wastewater, in particular for irrigated agriculture, which accounts for about 75 % of freshwater consumption worldwide. An estimated one billion people consume agricultural products grown with treated or untreated wastewater. In order to avoid outbreak of diseases amongst farm workers and consumers of pathogen-contaminated uncooked crops and produce, the WHO recommends thresholds for faecal indicators in water for various agricultural utilizations, e.g. generic *Escherichia coli* numbers of $<10^3$ per 100 ml for uncooked root crops (Hespanhol and Prost 1994; WHO 2006). While various wastewater treatment technologies that aim at reduction of microbes are available, the water volumes, often together with the country's economic situation, necessitate the implementation of simple and low-cost treatment technologies.

Published online: 12 August 2015

Slow sand filtration (SSF) is such a technology applicable for the removal of bacterial, eukaryotic and even viral pathogens from wastewater (Bauer et al. 2011; Farooq and Alyousef 1993; Hijnen et al. 2004; Timms et al. 1995). This method, established for drinking water purification in the 19th century and still in operation in cities such as London and Zürich, has been shown to be reliable for purification of waters with higher microbial loads such as secondary effluents from domestic wastewater treatment plants (Ausland et al. 2002; Elliott et al. 2008; Langenbach et al. 2009; Langenbach et al. 2010; Logsdon et al. 2002; Sadiq et al. 2003). Despite its long track record, the underlying mechanisms for pathogen removal involved in SSF are still insufficiently understood. Elimination of retained or immobilized microbial cells may be due to abiotic stress or biotic processes such as competition for nutrients with indigenous microorganisms as well as predation by protozoa, *Bdellovibrio*-and-like organisms, and viruses (Bomo et al. 2004; Chabaud et al. 2006; Haig et al. 2015; Schijven et al. 2003; Stevik et al. 2004; Wand et al. 2007).

The most effective removal compartment of a SSF system is the *Schmutzdecke* (German for 'dirt cover'; plural *Schmutzdecken*), a biofilm-like layer developing on top of the sand filter bed in which more than 90 % of pathogen indicator bacteria and coliphages are retained (Adin 2003; Hendricks 1991; Wotton 2002). It is assumed that formation of this structure is initiated through sorption of organic matter to sand particles. Thus, a nutrient-rich zone with concomitant microbial growth is generated in which further material may be trapped by physical straining (mechanical filtration) or adsorption (Wotton 2002). The microbial complexities of the *Schmutzdecke* have hampered comprehensive characterizations, with previous studies targeting individual aspects such as microscopic structure or presence and abundances of microbial taxa (Devadhanam and Pillay 2008; Wakelin et al. 2011).

With the aim of identifying relevant processes for pathogen removal in SSF, various compartments of laboratory-scale SSF columns (feed-water, supernatant, *Schmutzdecke*, depth-resolved sand and water samples of the filter bed, and effluent) were analysed by means of faecal indicator counting, confocal laser scanning microscopy, quantitative PCR, T-RFLP fingerprinting and DNA sequencing of prokaryotic and eukaryotic small subunit (SSU) ribosomal RNA (rRNA) genes. Our results highlight that physical retention of microbes is only one feature of SSF; biological factors particularly in the *Schmutzdecke* are also important for the overall purification process. Evidence is provided for selective elimination of bacterial taxa. The findings highlight that the ecology of SSF is an important yet intricate contribution to wastewater purification; a better understanding of these microbial interactions might foster applications of such low-cost purification systems.

Material and methods

SSF column design and analyses of bulk parameters

Laboratory-scale SSF columns can be viewed as good surrogates for full-scale systems (Haig et al. 2014). Our experimental setup consisted of three sand filter columns (labelled C1, C2, C3) with a diameter of 5 cm and a height of 210 cm (Fig. S1). Each column was made of two tubes of clear acrylic that were joined 5 cm above the sand level and protected from light by a cover of insulation foam. This segmental column design allowed for better maintenance during the start-up phase and between experiments. Sampling ports were located 2 cm above and 5, 10 and 25 cm below the sand bed surface. The filter beds in each column consisted of sieved, washed and dried 0/2-building sand from a quarry (Freudlsperger, Sprotta, Germany) and was supported by a gravel layer. The sand in the three beds had grain sizes (d_{10}) of 0.25 mm (C1), 0.4 mm (C2) and 0.63 mm (C3) of relatively high uniformity, i.e. a coefficient of uniformity (C_u) of 1.6 each. Grain sizes and C_u were determined by sieving according to DIN 18123 (1996) with 500–1000 g of dried sand samples. The filter porosity (p) and the hydraulic conductivity (k_f) were determined as described in Langenbach et al. (2009): C1, p= 37.6 %, k_f=6.2×10^{-4} m/s; C2, p=38.9 %, k_f=1.6× 10^{-3} m/s; C3, p=39.9 %, k_f=4.3×10^{-3} m/s.

Peristaltic pumps were used to continuously feed the columns with secondary effluent of an activated sludge wastewater treatment plant with denitrification and biological phosphorus elimination (Langenreichenbach, Germany). The hydraulic loading rate was 0.05 m/h. The minimum supernatant level of 30 cm in the columns was controlled by an outflow weir. SSF feeding water was renewed on a weekly basis. While used as inflow, it was stored in a 120-l barrel protected from light, cooled to 4–8 °C and continuously stirred (MAXIMR1, IKA, Staufen, Germany) at 300 rpm.

Electrochemical analyses of filter influent and effluent (temperature, dissolved oxygen, redox potential, pH and conductivity; 50-ml sampling size) as well as analyses of turbidity, total suspended solids (TSS), biochemical oxygen demand (BOD) (500-ml sampling size), chemical oxygen demand (COD) and total organic carbon (TOC) (10-ml sampling size) were carried out using standard methods as described in Langenbach et al. (2009), with results listed in Table 1.

Determination of faecal indicator removal

During the first phase of operation (4 weeks), the *Schmutzdecke* (Fig. S2A) built up and only the wastewater parameters listed in Table 1 were recorded. Once TSS removal and reduction of turbidity were stable, additional sampling for microbiological analyses was carried out over a period of 10 weeks (n=12). Samples (100 ml) for cultivation-based microbiological enumerations were taken from the filter influent and effluent using pipettes and from the sampling ports with syringes. They were collected in sterile centrifuge tubes and analysed directly for *E. coli* and intestinal *Enterococci* numbers by standardized membrane filtration and plate-based cultivation techniques (EN ISO 9308–1 and EN ISO 7899–2, respectively). In brief, adequate sample volumes and dilutions were chosen such as to yield approximately 10–50 colony forming units (CFUs). Samples were passed through a sterile membrane filter with a pore size of 0.45 μm (GN-6 Metricell, PALL, East Hills, USA) in an autoclaved filtration apparatus. For *E. coli* determination, cultivation occurred sequentially on CASO agar and TBA agar followed by an Indole test. Pink colonies were counted as *E. coli*. Selective cultivation for determination of intestinal *Enterococci* was carried out first on Slanetz-Bartley agar and then on bile-esculin agar. Colonies with a characteristic brownish-black colour that had appeared red on Slanetz-Bartley agar were counted as intestinal *Enterococci*. All statistical analyses were carried out as described before (Langenbach et al. 2010). In brief, bacteria concentrations were log10-transformed and checked for normal distribution using the Kolmogoroff–Smirnov test in SPSS. The software SPSS was also used to calculate arithmetic mean (μ), standard deviation (σ), standard error and 95 % confidence intervals. Faecal indicator removal in log units was calculated from these mean values. 90th percentile values were calculated as described in the EU bathing water directive (EU 2006):

$$90\text{th percentile} = \text{antilog}\,(\mu + 1.282\sigma)$$

Table 1 Bulk characteristics of filter influent and effluents from three laboratory-scale slow sand filter columns with sand grain sizes (d_{10}) of 0.25 mm (column C1), 0.4 mm (column C2) and 0.63 mm (column C3)

	Influent	Effluent		
		C1	C2	C3
TSS (mg/l)	8.2±2.7	0.4±0.3	0.5±0.2	0.4±0.3
TSS removal (%)		94	94	94
Turbidity (NTU)	3.6±4.5	1.3±0.8	0.9±0.3	1.0±0.4
Turbidity reduction (%)		64	77	73
Dissolved oxygen (mg/l)	10.8±0.7	5.2±2.0	5.0±1.3	5.1±1.2
BOD_5 (mg O_2/l)	2.0±3.2	1.9±1.2	2.0±1.4	1.8±1.4
COD (mg O_2/l)	35.8±3.5	31.5±2.7	30.7±2.4	32.9±4.3
TOC (mg/l)	10.3±2.1	9.3±1.9	8.3±1.5	9.5±1.7
pH	7.5±0.2	7.1±0.2	7.2±0.1	7.2±0.2
Redox potential (mV)	272±23	258±38	259±27	259±24
Conductivity (mS/cm)	1380±67	1360±83	1370±87	1350±69
Temperature (°C)	4±1	21±2	21±2	21±2

Confocal laser scanning microscopy

For confocal laser scanning microscopy (CLSM) analysis, samples of the *Schmutzdecke* and sand bed at various depths were collected from all columns in the 8th week of the 10-week monitoring period. They were removed via the ports in 10-ml syringes, pushed out of the syringes, cut longitudinally into slices and mounted in 2-mm deep cover well chambers (Molecular Probes, Eugene, OR, USA) for staining and subsequent CLSM with a TCS SP1 (Leica, Solms, Germany) attached to an upright microscope. The instrument was controlled by the CONFOCAL software, v2.61. Nucleic acid staining was done with Syto9 (Molecular Probes), glycoconjugates were stained with *Aleuria aurantia* AAL lectin (Vector Laboratories, Burlingame, CA, USA) labelled with Alexa568 (Molecular Probes). Images were recorded using 20× NA 0.5 and 40× NA 0.8 water immersible lenses. For excitation, the laser lines at 488 nm (reflection, Syto9), 561 nm (AAL-568) and 633 nm (chlorophyll *a* autofluorescence) were employed. Emission signals were recorded in the range of 480–495 nm (reflection), 500–550 nm (Syto9), 575–625 nm (AAL-568) and 650–750 nm (chlorophyll *a*). Three-dimensional data sets were projected using Imaris version 7.0 (Bitplane, Zürich, Switzerland).

DNA extraction

Water and sand samples were taken over the depth profile of the filters in order to assess feed-water, column supernatant, *Schmutzdecke*, sand layers and effluents of the three filters on 3 subsequent days in the 8th week of the 10-week monitoring period. The water samples (25 ml) were taken as described for cultivation-based enumeration of faecal indicators, concentrated on 0.2 μm filters and stored at −20 °C. The concentrates were pooled prior to analysis. Sand samples of 2 g each were taken directly from the sampling ports with a sterile spatula and also frozen until used. Triplicate DNA extracts were derived from each filter or freshly thawed ~0.4–0.6 g of wet sand aliquot as previously described (Winderl et al. 2008) and subsequently pooled. DNA was dissolved in 35 μl of elution buffer (Qiagen, Hilden, Germany) and stored frozen (−20 °C) until further analyses. The yield and purity of extracted nucleic acids were checked by means of UV quantification (ND-1000 Spectrophotometer, NanoDrop Technologies; Thermo Scientific, Wilmington, USA).

T-RFLP fingerprinting

Terminal restriction fragment length polymorphism (T-RFLP) analyses were performed on samples from each of the three columns. Triplicate DNA extracts were used as templates in PCR amplifications of 16S and 18S rRNA genes using a FAM (fluorescein)-labelled forward primer. Analysis of bacterial 16S rRNA genes was done using the bacterial-specific primer pair Ba27f-FAM/Ba907r (Biomers, Ulm, Germany), followed by *Msp*I (Fermentas, St. Leon-Rot, Germany) digestion of 80 ng of purified amplicons (Pilloni et al. 2011). Eukaryotic 18S rRNA genes were amplified using the primer combination Euk20f-FAM/Euk516r, and 40 ng of amplicons were digested using *Bsh*1236I (Fermentas) (Euringer and Lueders 2008). Thirty electropherograms each of 16S and 18S rRNA genes were analysed per column with identical influent.

All T-RFLP data were processed and analysed using the T-REX online T-RF (terminal restriction fragments) analysis software (Culman et al. 2009) available at http://trex.biohpc.org. Background noise filtering (Abdo et al. 2006) was with default factor 1.2, and the clustering threshold for aligning peaks across the samples was set to 0.5 using the default alignment method. Relative T-RF abundance was inferred from peak heights. For reduction of data complexity, T-RFs that occurred in less than 10 % of the samples were excluded from further analysis. There was no occurrence of highly abundant but unique T-RFs in any given sample, as determined by visual inspection of raw versus binned data sets. Processed data were subjected to the additive main effects and multiplicative interaction model (AMMI) analysis of the T-REX software using a data matrix constructed based on the presence/absence of peaks. The abundance of dominant T-RFs was plotted over depth. Diversity in fingerprints was estimated by calculating the Shannon index (*H'*) as $H' = -\Sigma\, p_i \ln p_i$, where p_i is the relative frequency of a specific T-RF (Hill *et al.* 2003).

Cloning, sequencing and phylogenetic analyses

In order to validate the phylogenetic placement of the T-RFs, bacterial clone libraries of 96 clones each were constructed from two water and two sediment samples of SSF column C1 (supernatant, *Schmutzdecke*, 10 cm sand and effluent) using the primer pair Ba27f/Ba907r. Additionally, 3 eukaryotic clone libraries of 32 clones each from the supernatant, the *Schmutzdecke* and the effluent of column C1 were prepared with the primer pair Euk20f/Euk516r. These samples were chosen to best describe the overall diversity present in the filter, as they differed most according to the AMMI analysis (see Results). Plasmid isolation and Sanger sequencing of 384 bacterial and 96 eukaryotic clones using standard M13 primers were outsourced (GATC-Biotech, Konstanz, Germany). Analysis of trace files, vector clipping and quality filtering were performed using the DNA Baser software (Heracle BioSoft S.R.L., Pitesti, România). In silico T-RF predictions and phylogenetic relationships were analysed using the ARB software package (Ludwig et al. 2004) and SILVA database project (Quast et al. 2013).

Quantitative PCR

For the sand filter C1, quantitative PCR (qPCR) of 16S and 18S rRNA genes were performed using three independent DNA extracts with 1:10, 1:20 and 1:40 dilutions each. qPCR targeting the 16S rRNA gene was done with the primer pair 519f/907r as in Winderl et al. (2008). Quantification of the 18S rRNA gene was achieved by using the Euk20f/Euk516r primer, 2 μl of DNA template per reaction, an annealing temperature of 55 °C, and a *Bodo* sp. 18S rRNA gene amplicon as qPCR standard essentially as described before (Euringer and Lueders 2008; Winderl et al. 2008).

Nucleotide sequence accession numbers

All cloned sequences of partial 16S rRNA and 18S rRNA genes obtained in this study have been deposited with GenBank under the accession numbers JX564253–JX564534 and KP686479–KP686567.

Results

Filter performance and removal of indicator bacteria

SSF columns ameliorated the wastewater characteristics, removing 94 % of total suspended solids and reducing turbidity by more than 70 % irrespective of sand grain sizes. The effluents' dissolved oxygen concentrations were around 3 mg/l higher than the respective BOD_5 values, thus indicating mostly aerobic conditions throughout the columns (Table 1). Removal of the faecal indicator *E. coli* amounted to 1.6–2.2 log units or 97.4–99.4 %. Intestinal *Enterococci* removal was similar, ranging from 1.7 to 2.3 log units or 98.1–99.5 % (Table 2). The effluents of filter columns C1 and C2 (sand of d_{10}=0.25 mm and d_{10}=0.4 mm) always complied with the WHO recommendation for unrestricted irrigation water of $<10^3$ CFU *E. coli* per 100 ml (WHO 2006) and even fulfilled the requirements for excellent bathing water quality of <500 CFU *E. coli* per 100 ml according to the EU bathing water directive (EU 2006). The effluent of C3 (sand of d_{10}= 0.63 mm) only occasionally reached irrigation water quality or good bathing water quality. Quantifying *E. coli* and *Enterococci* abundances along the columns' flow path demonstrated the importance of the *Schmutzdecke* in faecal indicator removal. Although encompassing <10 % of total filter

Table 2 Removal of faecal indicator bacteria in three laboratory-scale slow sand filter columns of different grain sizes [(d_{10}) of 0.25 mm (C1), 0.4 mm (C2) and 0.63 mm (C3)] at a hydraulic loading rate of 0.05 m/h

Compartment	*Escherichia coli* (CFU/100 ml)		Intestinal *Enterococci* (CFU/100 ml)	
	Mean	90th percentile	Mean	90th percentile
Supernatant	4840	26,700	2550	6870
Effluent C1	30	159	14	30
Effluent C2	52	221	16	41
Effluent C3	126	1360	49	447

length, the *Schmutzdecke* accounted for >90 % of total faecal indicator removal (Fig. 1).

Microscopic examinations

As a first approach to describe the structure-function relationship in our SSF systems, we carried out CLSM analyses of samples taken from various depths of all columns. The three *Schmutzdecken* had essentially the same vertically structured partition. The surface layers were of flocculate texture and were strongly labelled with the nucleic acid stain Syto9 (Fig. S2B), whereas the deeper zones were more compact and showed extensive EPS glycoconjugate labelling with interspersed small Syto9-stain patches (Fig. S2C). These observations demonstrated that the surfaces of the *Schmutzdecken* consisted mostly of microbes present as single cells and small aggregates whereas in deeper zones, extensive biofilms dominated. Confined to the *Schmutzdecken*, there were also several isolated microbial algae, as shown by their chlorophyll *a* signals. Their abundance gradually decreased from the top layer downwards; they may have been present due to light contamination in the inflow or at the top of the columns. In the upper sand layers below the *Schmutzdecken*, biomass was present mostly in form of biofilms as judged by the strong label for EPS matrix (Fig. S3A and B), while micro-colonies dominated in lower zones (Fig. S3C and D). Overall, signal intensities for biomass decreased with depth, while the ratio of isolated micro-colonies versus extended biofilm matrices increased.

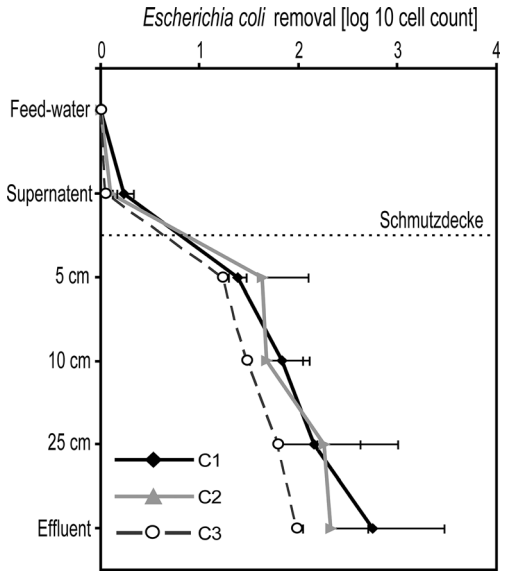

Fig. 1 *Escherichia coli* removal during percolation of waste water through slow sand filtration columns C1, C2 and C3 [grain sizes (d_{10}) of 0.25 mm (C1), 0.4 mm (C2) and 0.63 mm (C3)]

Prokaryotic communities and distributions in the SSF columns

To explore further the biological landscape where faecal indicator removal in SSF columns took place, we characterized the microbial community structures and overall abundances in the influent, various compartments of the sand columns and effluents via SSU rRNA gene analyses. T-RFLP profiles were used to evaluate bacterial species richness by computing the Shannon index (H') as well as discerning community similarities and differences through AMMI analysis. These analyses were supported by sequencing of clone libraries and by qPCR. First, the results for the bacterial communities are described.

In all three columns, bacterial species richness increased moderately from that in the common influent (H'=3.52) to an observed maximum (H'≈4) at 5–10-cm sand layer depth (Table S1). The diversity index associated with the pore water was similar to that of the influent. The AMMI biplot of all T-RFLP patterns (explaining 74 % of total variability) showed that bacterial communities differed across the compartments rather than between columns, despite the variations in sand grain dimensions (Fig. 2a). Notably, the *Schmutzdecke* communities clustered closer to those on the sand grains than to the ones in the surrounding water, which were similar throughout the flow path. The compartmentally different distribution of bacterial taxa was particularly apparent for T-RFs of overall high relative abundance. For example, a T-RF of 492 bp, representing clones affiliated to candidate division OD1, was most abundant in the sand samples (up to 14 % of the total), while others were detected only in water samples, e.g. T-RFs of 186 and 523 bp, comprising ~5 and 10 % of the total abundances and representing uncultured *Gemmata* (a

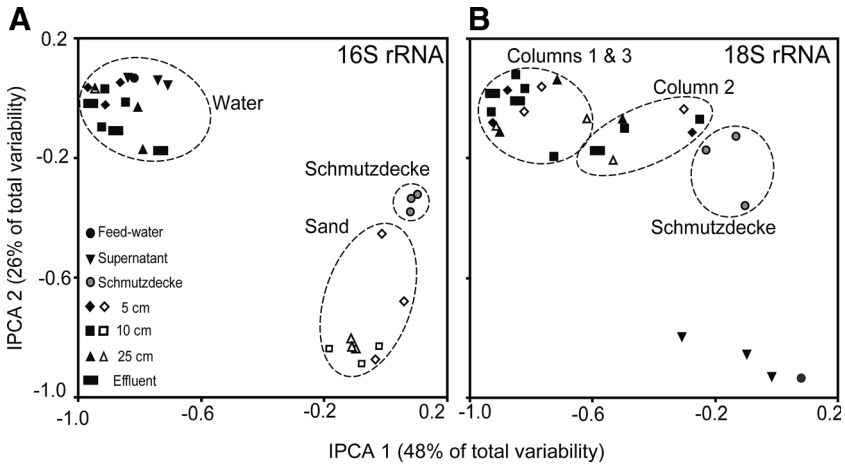

Fig. 2 AMMI biplot with the interaction principal component axes (IPCA 1 and IPCA 2) of the bacterial 16S rRNA gene (**a**) and eukaryotic 18S rRNA gene (**b**) T-RFLP fingerprints. Sample points clustering to a specific compartment are *highlighted with a circle. Filled symbols* represent water samples, *open symbols* represent sand samples and the *grey circle* represents *Schmutzdecke* samples

genus within the *Planctomycetes*) and candidate division OP11, respectively (Fig. 3).

Since the T-RF analysis revealed inter-compartment rather than inter-column differences of the bacterial communities, we focused further analyses on only one column, C1. 16S rRNA gene libraries from the influent, the *Schmutzdecke*, 10 cm sand and the effluent yielded a total of 373 high quality (>800 bp) sequences comprising members of 15 different bacterial phyla (Fig. 4). As expected from the T-RFLP analyses, the relative abundances of the dominant taxonomic groups

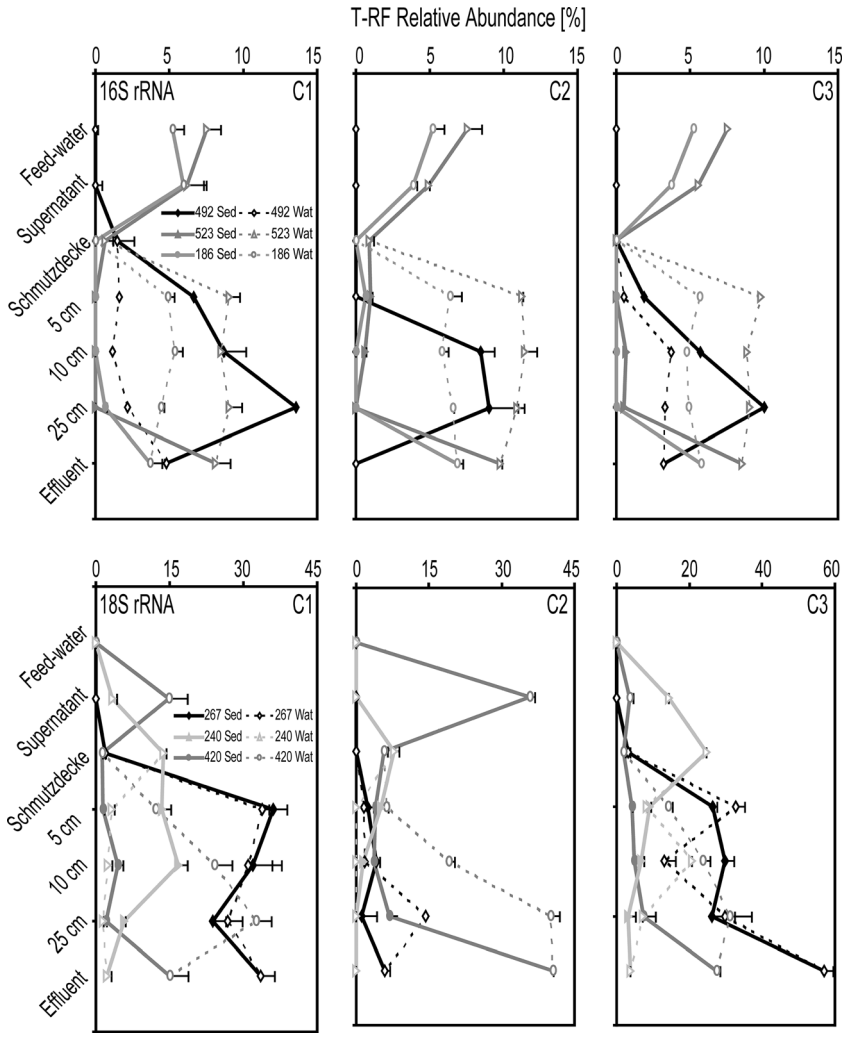

Fig. 3 Abundance of selected T-RFs especially contributing to the observed AMMI clustering of samples in Fig. 2 along the filter compartments. Depicted are abundances of the three most dominant T-RFs for the columns 1, 2 and 3 (C1–C3). The abbreviations *Wat* and *Sed* denote T-RFs present in water and sediment samples

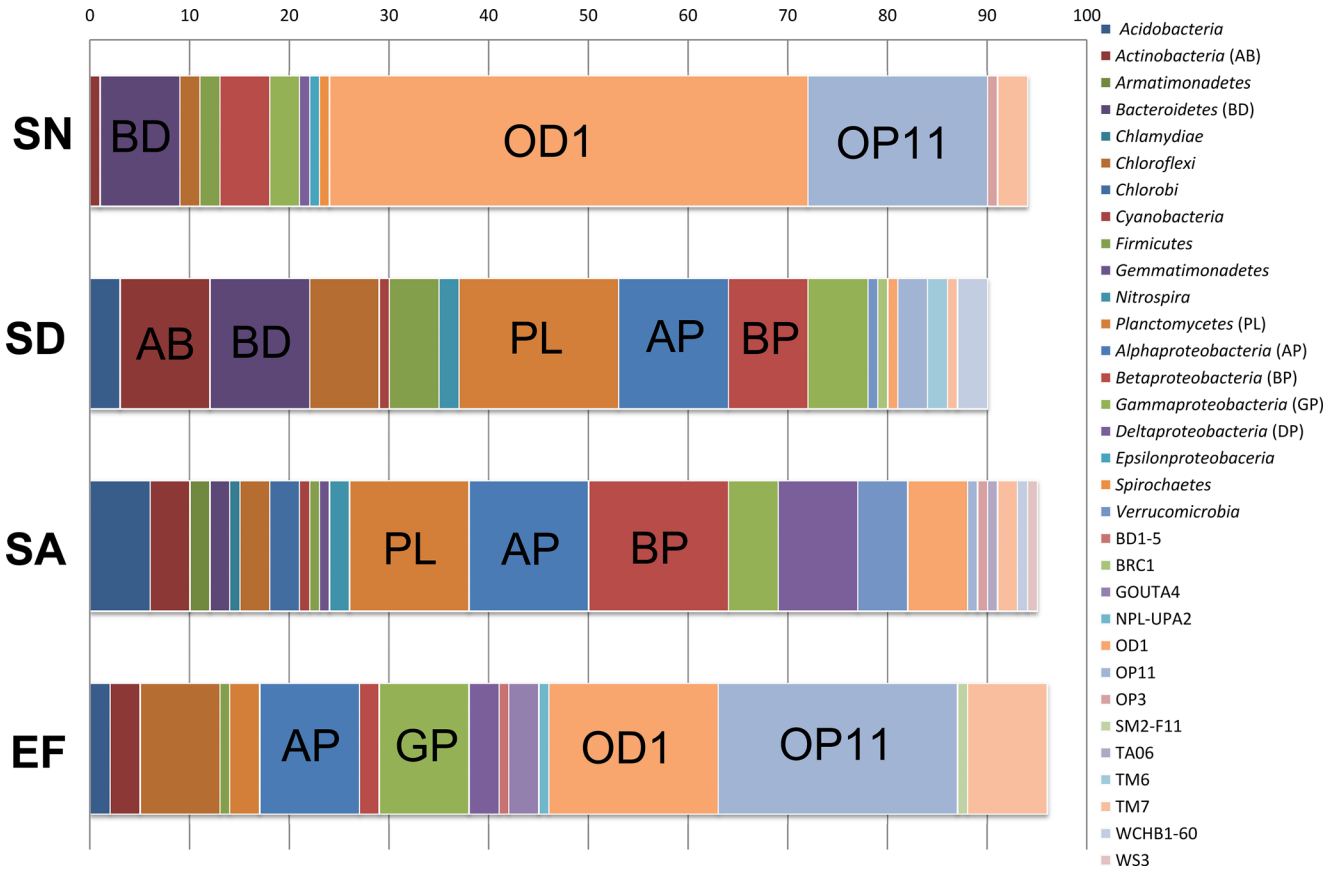

Fig. 4 Bacterial clone frequency in the four different compartments of the investigated slow sand filtration column (C1; d_{10}=0.25 mm). *SN* indicates the microbial community composition of the supernatant, *SD* of the *Schmutzdecke*, *SA* of the 5 cm sand layer and *EF* of the effluent

differed in the four libraries. In the influent and effluent, a total of 67 and 41 %, respectively, of the clones were affiliated either with the candidate phyla OD1 or OP11, whereas less than 8 % of all clones from the *Schmutzdecke* and sand samples belonged to those groups. Sequences affiliated with the *Bacteroidetes*, a phylum that comprises common inhabitants of the human gut where they may hydrolyze complex organic molecules, were more abundant in the influent and the *Schmutzdecke* compared to the lower compartments. In contrast, members of the *Alphaproteobacteria* were of low abundance in the influent but made up around 12 % in the other clone libraries, while *Betaproteobacteria* and *Planctomycetes* were more abundant within rather than outside the filter bed. In the effluent, there were two sequences affiliated with bacterial predators, *Bdellovibrio*-and-like organisms (BALOs). In all four filter compartments, there were many obligate aerobes amongst those taxa whose phylogenetic placement indicated a particular energy metabolism, a finding which matched the stated assumption that oxic conditions prevailed throughout the filters (Table 1).

Based on qPCR analysis, 97.5 % of total bacterial 16S rRNA gene copies were eliminated by passage of the wastewater through filter C1 (Fig. 5). The 16S rRNA gene abundances increased first substantially from ca. 10^8 and 10^7 copies/ml in the influent and supernatant, respectively, to about 10^{11} copies/ml in the *Schmutzdecke*. Within the first 10 cm below the *Schmutzdecke*, abundances dropped to around 10^6 copies/ml and stayed at about that level through the lower portion of the column. 16S rRNA gene copy numbers in sand samples decreased gradually from 3×10^9 to 6×10^8 copies/g.

Eukaryotic communities and distributions in the SSF columns

Based on T-RFLP analysis, the eukaryotic diversity increased sharply from the influent (H'=1.71) to the *Schmutzdecke* (H' of 2.98, 2.81 and 2.58 in the columns C1, C2 and C3) and remained at levels slightly above those of the influent in the lower zones of the columns (Tab. S1). AMMI analysis revealed that the communities in the feed-water and column supernatants differed markedly from the other column compartments. The *Schmutzdecke* communities of the three columns clustered together, while the remaining compartments and the effluents of C1 and C3 were more similar to each other than to C2 (Fig. 2b). The eukaryote T-RFs of highest

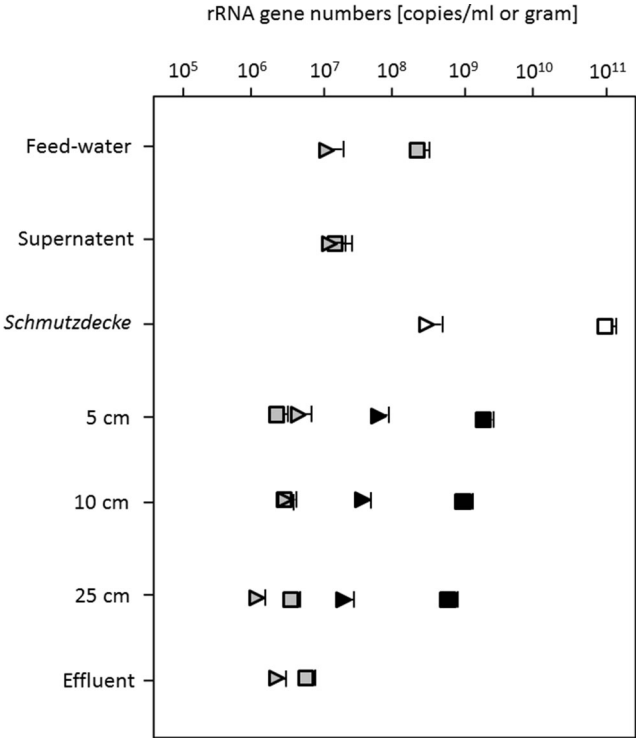

Fig. 5 Abundances of 16S rRNA genes (*squares*) and 18S rRNA genes (*triangles*) determined by qPCR of various filter compartments (column C1; d_{10}=0.25 mm). *Grey* symbols refer to water samples and *black* symbols refer to the sediment samples. Samples of the *Schmutzdecke* are considered neither water nor sediment sample and are shown in *white*

abundance in the columns were not found in the feed-water. They became abundant either in the supernatant (especially in column 2) or in the *Schmutzdecke* and compartment below. In particular, a 420 bp T-RF (*Cercozoa*, *Rhizaria*) was highly represented especially in the water samples from the filter beds of all three columns (up to 40 % of all T-RFs), and a 267 bp T-RF (*Oxythrichidae*, *Ciliata*) was increased up to about 40 and 60 % in sediment and water compartments of C1 and C3, respectively, but was less present in C2 (max. 14 %) (Fig. 3).

This picture was expanded by sequencing clone libraries of the influent, *Schmutzdecke*, and effluent of C1, the most effective column in indicator bacteria removal. In total, 89 high quality sequences were obtained (Table S2). The supernatant was dominated by closely related Alveolates of uncertain further classification (18 sequences), *Vorticellidae* (5 sequences), Chytrids (3 sequences) and heterotrophic flagellates of the genus *Heteromita* (*Cercozoa*, 3 sequences). The *Schmutzdecke* clone library consisted to ca. 50 % of sequences clustering with the *Cryptomycota* and other fungi. The remaining sequences affiliated mostly with Cercozoan sequences of uncertain further classification as well as diatoms, the presence of which had already been revealed microscopically. The effluent was dominated by closely related ciliate sequences affiliated with the *Oxytrichidae* (10 sequences). Based on in silico restriction analysis, those sequences would have yielded T-RFs of 269 bp rather than 267 bp found in the T-RFLP analysis. The 420 bp T-RF matched to the small subunit RNA sequence of the genus *Viridiraptor*. Overall, the intra-taxon similarities of the influent and effluent were higher than with the predominantly fungal-derived sequences in the *Schmutzdecke*, thereby the clone libraries mirrored the diversity indices as computed based on T-RFLP data.

The numerical distribution of the 18S rRNA gene in the various compartments of column C1 followed a pattern similar to that observed for the prokaryotic counterpart (Fig. 5). Abundances in the influent, supernatant, pore water and effluent varied between 10^6 and 10^7 copies/ml, whereas the *Schmutzdecke* harboured 3.5×10^8 copies/ml, and 18S rRNA gene copy numbers in sand samples decreased gradually from 9×10^7 to 1.5×10^7 copies/g.

Discussion

SSF is a drinking water treatment technology which may also be adapted and optimized for hygienisation of domestic wastewater, but the relevant processes for pathogen removal are uncertain. In the present work, removal of faecal indicator bacteria (e.g. 1.6–2.2 log units for *E. coli*) was within the elimination range previously reported (0.3–3.5 log units) (e.g. Ausland et al. 2002; Elliott et al. 2008; Langenbach et al. 2009; Logsdon et al. 2002; Mwabi et al. 2012). Most of the removal in our columns occurred within the *Schmutzdecke* and the 5 cm beneath that layer. In filter bed depths below 5 cm, little absolute and relative elimination of indicator bacteria occurred. Therefore, simply increasing the filter depth may not be the most effective approach for optimizing pathogen removal in such treatment systems but rather to generate and maintain a highly effective *Schmutzdecke* (Seeger et al. submitted). The increased effectiveness in the lower portions of columns filled with finer sand was at least partially due to the sand particles' higher surface-to-volume ratio, hence more cell adhesion sites within same column volumes (Langenbach et al. 2010; Tan et al. 1992).

The *Schmutzdecke* apparently built up through sedimentation and subsequent microbial catabolism of particulate matter. The observed biofilm was rich in *Bacteroidetes* and fungal sequences affiliated with the *Cryptomycota*, which were presumably involved in the degradation of complex organic material (Jones et al. 2011). The habitat thus formed may not be suitable enough for many other microbes but rather constitutes a capture zone where they may face grazing by protozoa, hunting by BALOs and viral lysis (Haig et al.

2015; Wand et al. 2007), i.e. biotic pressures that outweigh their population maintenance (if any) through cellular replication. In fact, while total bacterial counts were increasing in the *Schmutzdecke*, numbers of the faecal indicator bacteria were decreasing. For some bacteria such as members of the candidate divisions OD1 and OP11, however, the *Schmutzdecke* might not constitute a physical straining barrier or a zone of enhanced predation, since sequences affiliated with those groups were highly abundant in the influent and effluent but not in the *Schmutzdecke*. Results by Miyoshi et al. (2005) suggest that OD1 and OP11 bacteria can be small compared to other bacterial cells, a feature that could help those organisms to escape grazing by predators or mechanical filtration (Corapcioglu and Haridas 1984; Pernthaler 2005).

Protozoan grazing in the sand columns was tracked, albeit indirectly, by the molecular and microscopic analyses presented here. Most prominent, abundant T-RFs of predatory eukaryotes such as the 267 bp T-RF (*Oxytrichidae*, *Ciliata*) first appeared in the supernatant and then proliferated within the filter. A decisive determination of the particular feeding mode, activity and prey of those protozoa is not possible solely based on our molecular analysis (Glücksman et al. 2010). We note, however, that *Oxytrichidae* are algae or bacterivorous filter or raptorial feeders (Verni and Gualtieri 1997) while the frequently detected Cercozoa include heterotrophic amoeboflagellates (Howe et al. 2009). The effluent contained a sequence affiliated with *Tetrahymena*, which are voracious feeders. Their bacterivorous activity, in particular in the upper filter zones, was indicated by the CLSM images which showed a decrease of relative EPS label with depth. Bacterial EPS production is one strategy against protozoan grazing (Pernthaler 2005).

While the importance of the *Schmutzdecke* is evident, the factors that govern the microbial community structure and dynamics are not yet fully apparent. Although all columns were operated in parallel and received the same influent over an extended time period, the community in the column with the intermediate sand grain size, C2, differed from those in C1 and C3, which were rather alike. Removal efficiencies, however, were similar in columns C1 and C2. In conclusion, our work evinces that the mechanism of faecal indicator removal in SSF systems is a combination of straining in the upper filter section, unsuitable habitat conditions, and enhanced predation. Indeed, a recent study using ^{13}C-labelled *E. coli* highlighted the importance of protozoan predation in the trophic network of a model slow sand filter (Haig et al. 2015). Similar studies may further aid the optimization of such treatment systems for improved and stable pathogen removal efficiencies.

Acknowledgments We would like to acknowledge the great technical support of Ute Kuhlicke and Michaela Blank. Furthermore, we would like to thank three anonymous reviewers for critically reading the manuscript and suggesting many improvements.

Conflict of interest The authors declare that they have no competing interests.

Compliance with ethical standards

Funding This work was supported by the German Federal Ministry of Education and Research (BMBF; Grant: 02WM0847), the Foundation of German Business (sdw), the European Commission (Grant Agreement No: FP7-KBBE-2012-6-311933, WATER4CROPS) and the Helmholtz Association.

Human and animal rights and informed consent This article does not contain any studies with human participants or animals performed by any of the authors.

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