Dynamics of antibiotic resistance genes and their relationships with system treatment efficiency in a horizontal subsurface flow constructed wetland

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HIGHLIGHTS

• ARG were detected in influent, effluent, and media biofilm of a constructed wetland.
• System operation time and temperature affect amounts of ARGs in the effluent.
• ARG abundance in media biofilm and effluent is related to system’s treatment efficiency.
• Constructed wetlands are alternatives to conventional treatment for ARG removal.

ABSTRACT

Municipal wastewater treatment is one of the pathways by which antibiotic resistance genes from anthropogenic sources are introduced into natural ecosystems. This study examined the abundance and proportion dynamics of seven antibiotic resistance genes in the wetland media biofilm and in the influent and effluent of parallel horizontal subsurface flow mesocosm cells of a newly established hybrid constructed wetland treating municipal wastewater. The targeted genes (tetA, tetB, tetM, ermB, sul1, ampC, and qnrS) encode resistance to major antibiotic classes such as tetracyclines, macrolides, sulfonamides, penicillins, and fluoroquinolones, respectively. All targeted antibiotic resistance genes were detectable in the tested mesocosm environments, with the tetA, sul1, and qnrS genes being the most abundant in the mesocosm effluents. After initial fluctuation in the microbial community, target gene abundances and proportions stabilized in the wetland media biofilm. The abundance of 16S rRNA and antibiotic resistance genes, and the proportion of antibiotic resistance genes in the microbial community, were reduced during the wastewater treatment by the constructed wetland. The concentration of antibiotic resistance genes in the system effluent was similar to conventional wastewater treatment facilities; however, the mesocosms reduced sulfonamide resistance encoding sul1 concentrations more effectively than some traditional wastewater treatment options. The concentrations of antibiotic resistance genes in the wetland media biofilm and in effluent were affected by system operation parameters, especially time and temperature. The results also revealed a relationship between antibiotic resistance genes abundance and the removal efficiencies of NO3–N, NH4–N, and organic matter. Correlation analysis between the abundance of individual antibiotic resistance genes in the mesocosms influent, effluent and wetland media biofilm indicated that depending on antibiotic resistance gene type the microbes carrying these genes interact differently with microbial communities already present on the wetland media.

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1. Introduction

The occurrence and spread of antibiotic resistant bacteria in the environment is a well-recognized concern to the extent where, besides antibiotic residues, the antibiotic resistance genes (ARGs) are being considered as pollutants themselves (Martínez, 2009). Anthropogenic residues contain ARGs that survive and contaminate natural environments, altering natural microbial communities and giving rise to the emergence of antibiotic resistance in the clinical/human setting (Schmieder and Edwards, 2012). However, the transport...
pathways, development, and environmental reservoirs of resistance determinants are not well understood and need further research (Martínez, 2008; Allen et al., 2010).

Municipal wastewater treatment is one major route by which ARGs from human settings are introduced into natural ecosystems (Servais and Passerat, 2009; Novo and Manaia, 2010). Numerous ARGs encoding resistance for major antibiotic groups, such as tetracycline, aminoglycosides, macrolides, sulfonamides, and β-lactams, have been found both in activated sludge and effluents from wastewater treatment plants (X.X. Zhang et al., 2009). Traditional wastewater treatment facilities (i.e. using activated sludge processes) have also been recognized as possible concentration points and reservoirs for antibiotic resistant bacteria owing to their high concentrations of microbial biomass and the abundance of nutrients (Guardabassi et al., 2002). In wastewater treatment facilities the mainly mobile genetic element (i.e. plasmid) borne ARGs are selected, enriched, and transferred to other bacterial species (Guardabassi et al., 2002; Szczepanowski et al., 2009). While antibiotic resistance in conventional wastewater treatment plants has received attention, antibiotic resistance in alternative wastewater treatment systems, such as constructed wetlands, has been mostly overlooked.

Constructed wetlands (CW) are engineered systems designed and constructed to harness the processes that occur in natural wetlands for the treatment of wastewater. With relatively low setup and maintenance costs and relatively good wastewater purification efficiencies, CWs have proven to be effective alternatives or useful complements to traditional wastewater treatment systems (Scholz and Lee, 2005).

Various types of CWs are often combined in sequence as hybrid systems to enhance wastewater treatment efficiencies; the treatment performance of CWs is mainly based on the combined action of microbes and filter material which may be complemented by plants (Truu et al., 2009). Although the removal of both medical and veterinary antibiotics in CWs have been targeted in recent years (Conkle et al., 2008; Hijosa-Valsero et al., 2011; Hussain et al., 2012), the presence and removal of ARG-carrying microbes within such systems have received less attention. Currently, antibiotic resistance studies in CWs have mostly been limited to testing the susceptibility of fecal indicator bacteria (Escherichia coli, Enterococcus) isolates to antibiotics (Helt et al., 2012; Sidrach-Cardona and Bécares, 2012) which covers only a very small fraction of bacterial community. However, little research has been done to estimate the extent and types of antibiotic resistance genes in the CWs whole microbial communities.

This research had three goals. First, to study the abundance and dynamics of seven ARGs (tetA, tetB, tetM, ermB, sulI, ampC, and qnrS) and their proportion in microbial communities in the influent, effluent, and wetland media biofilm of horizontal subsurface flow filter mesocosms (HSSF MCs) of a newly established pilot scale hybrid CW treating municipal wastewater. Second, to evaluate correlations between environmental factors and ARG abundance as well as ARG proportions in community in both the effluent water and wetland media biofilm. Finally, to assess the relationships between ARG removal and wastewater purification efficiency in the HSSF MCs.

2. Materials and methods

2.1. Site description and sampling

150-day municipal wastewater treatment experiment was conducted from June to November 2009 in Nõo village, Estonia. Nõo village is the center of a parish that has a permanent population of about 1500 people. Small meat processing and dairy industries are situated near the village. The village's activated sludge wastewater treatment facility treats domestic municipal wastewater combined with the effluents from the dairy and meat industries. This study was conducted in an unplanted hybrid CW system fed with raw wastewater pumped from the inlet of the activated sludge treatment plant. The pilot system consisted of a septic tank (2 m³), followed by six parallel vertical subsurface flow mesocosms (total area 6 m²), a collection well, and 21 parallel HSSF MCs (each MC cell: length — 1.5 m, width — 0.2 m, depth — 0.6 m). A detailed description of the system is given by Nurk et al. (2009). The three HSSF MCs used in this study were filled with light expanded clay aggregates (LECA) with 2–4 mm particle size forming the wetland media and providing high surface area environment for microbial biofilm attachment. The hydraulic loading rate was ≤20 mm d⁻¹ and the wastewater retention time in the HSSF MCs was 1.2 days. The HSSF MC influent was pretreated wastewater which had passed through the septic tank and the vertical subsurface flow mesocosms. The characteristics of the influent to the HSSF MCs and the difference in respective parameter values (removal efficiencies) between influent and effluent of the studied mesocosms are shown in Table 1.

Sampling began after 26 days of regular operation of the CW system and samples were collected five times during the five-month trial period. Between the second and the third samplings partial clogging with biomass occurred in pipes connecting the collection well and HSSF MCs causing uneven distribution of wastewater to the parallel MC cells. The clogging was removed by dismantling, wash-out and reattachment of the pipes, as soon as it was detected. The clogging had a short term effect on the system reflected in higher deviation between parallel MCs wastewater treatment efficiency on third samplings (Table 1); by the fourth sampling this effect was not perceivable any more. Grab samples of wastewater were taken from the collection well located before the inlet to the HSSF MCs and from the outlets of each HSSF MC. During each sampling event also five subsamples from the wetland media (25–35 cm depth) of each HSSF MC were collected at even spacing along the longitudinal axis; all mesocosm media subsamples were mixed to form a composite sample. Water temperature and pH inside the mesocosms were measured during each sampling. The water temperature in mesocosms

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Wastewater quality parameters of HSSF MCs influent with means and standard deviations (given in parentheses, n = 3) of treatment efficiencies (TE) and pH differences (ΔpH) between influent and effluent of HSSF MCs at each sampling.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days of HSSF MCs operation</td>
<td>Conc.</td>
</tr>
<tr>
<td>Day 26</td>
<td>NO₂⁻</td>
</tr>
<tr>
<td>Day 45</td>
<td>NO₂⁻</td>
</tr>
<tr>
<td></td>
<td>NH₄⁺</td>
</tr>
<tr>
<td></td>
<td>N_{total}</td>
</tr>
<tr>
<td></td>
<td>BOD₅</td>
</tr>
<tr>
<td></td>
<td>TOC</td>
</tr>
<tr>
<td></td>
<td>ΔpH</td>
</tr>
</tbody>
</table>

a. Conc.— water quality parameter concentrations in mg l⁻¹, except pH.
b. Treatment efficiency in %.
ranged between 14.3 °C and 18.6 °C during first 94 days and dropped to 3.0 °C at day 150 of the experiment.

In the laboratory the collected samples were divided into subparts: the wastewater and wetland media samples for molecular analyses were stored at –80 °C until DNA extraction; samples for chemical analyses were held at –7 °C. Concentrations of N_\text{tot}, NH_4–N, NO_2–N, NO_3–N, and total organic carbon (TOC) were measured using Dr. Lange cuvette tests (Hach-Lange, Germany). The seven-day biochemical oxygen demand (BOD_7) was measured in accordance with Standard Methods for the Examination of Water and Wastewater (APHA, 1998).

2.2. DNA extraction

A portion of the collected wetland media was crushed using a mortar and pestle and DNA was extracted from 10 g of the crushed material using a PowerMax Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer’s protocol. As recommended by the manufacturer, the extracted DNA was purified with 5 M NaCl and 100% ethanol, and resuspended in 80 μl of 10 mM Tris buffer.

Wastewater samples were centrifuged for 15 min at 4000 rpm and DNA from the pellet was extracted using the PowerSoil DNA Extraction Kit (Mo Bio Laboratories, Inc.). The manufacturer’s protocol was modified by replacing 10 min of vortex treatment with homogenization of the samples using Precellys® 24 (Bertin Technologies, France) at 5000 rpm for 20 s.

DNA for the qPCR standard preparation from reference strains E. coli LH1035 ALU1 and Pseudomonas mendocina PC1 was extracted according to the manufacturer’s instructions or, when a higher transformation rate proved necessary, using electroporation ( Sharma and Schimke, 1996). Plasmid-DNA was extracted using a QiAprep Spin Miniprep Kit (Qiagen, CA, USA), and controlled with nucleotide sequencing using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 377 DNA Sequencer (Perkin-Elmer, Waltham, MA, USA) with M13-primers (Fermentas). The quantity and quality of all DNA extracts were determined via spectrophotometry (NanoDrop 1000 and Infinite M200).

2.3. Preparation of standards for qPCR calibration

Using gene-specific primer sets, seven ARGs (representing the main antibiotic classes) that are commonly found in water environments were quantified: tetracycline resistance encoding tetA, tetB and tetM, fluoroquinolone resistance encoding qnrS, sulfonamide resistance encoding sul1, macrolide resistance encoding ermA, and β-lactamase resistance encoding ampC (Table 2). Additionally, primer set L-V6/R-V6 was used for 16S rRNA gene quantification and primer set λ7403FL/λ7512R, targeting λ bacteriophage DNA (MBL Fermentas, Lithuania) and serving as an internal standard, for qPCR inhibition measurement (Table 2). For standard curve creation, target gene tetB, tetM, ermA, ampC, sul1, and qnrS fragments were PCR-amplified from environmental samples using the respective primers. For tetA and 16S rRNA gene fragment amplifications bacterial strains E. coli LH1035 ALU1 and P. mendocina PC1 were used.

The 25 μl volume reaction mixture contained 1 × PCR buffer with (NH_4)_2SO_4 (75 mM Tris–HCl, pH 8.8; 20 mM (NH_4)_2SO_4; 0.01% Tween 20), 2.5 mM MgCl_2, 0.2 mM of each deoxynucleoside triphosphate, 0.8 μM of both forward and reverse primers, 0.04 U μl\(^{-1}\) of Taq DNA polymerase (Fermentas), and approximately 50 ng of DNA template. The reaction mixture was preheated at 95 °C for 3 min, followed by 30 thermal cycles of 45 s at 95 °C, 45 s at the primers’ specific annealing temperature (Table 2), and 1 min at 72 °C; the final extension was carried out at 72 °C for 10 min.

The PCR reactions were performed with an Eppendorf Mastercycler IPCR machine and PCR fragments were analyzed by agarose gel electrophoresis and visualization using ethidium bromide staining. PCR-products were cloned using an InSt/Aclone PCR cloning kit (Fermentas), applying either transformation according to the manufacturer’s instructions or, when a higher transformation rate proved necessary, using electroporation ( Sharma and Schimke, 1996). Plasmid-DNA was extracted using a QiAprep Spin Miniprep Kit (Qiagen, CA, USA), and controlled with nucleotide sequencing using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 377 DNA Sequencer (Perkin-Elmer, Waltham, MA, USA) with M13-primers (Fermentas). The quantity and quality of plasmid DNA extracts were determined using spectrophotometry (NanoDrop 1000 and Infinite M200). The copy numbers of standard plasmids were calculated according to the plasmid (2886 bp) plus insert lengths (Table 2), assuming a molecular mass of 660 Da for a base pair. Finally, standard DNA stock solutions of 10⁶ copies of plasmid μl\(^{-1}\) were prepared and 10-fold serial dilutions ranging from 10⁶ to 10⁵ target gene copies (with additional dilutions of 50 and 25 target gene copies) were used for creating standard curves.

2.4. Quantitative PCR conditions and analyses

The qPCR assays were performed on the real-time PCR system Rotor-Gene® Q (Qiagen). The optimized reaction mixture contained 5 μl Maxima SYBR Green Master Mix (Fermentas), 0.4 μl of forward and reverse primer, 1 μl template DNA, and 3.6 μl sterile distilled water. 

Table 2

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’–3’</th>
<th>Target gene</th>
<th>Amplicon size (bp)</th>
<th>Annealing temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-V6</td>
<td>GAACCGGAGAAGACTTACC</td>
<td>16S rRNA</td>
<td>111</td>
<td>54</td>
<td>Hummelen et al. (2010)</td>
</tr>
<tr>
<td>R-V6</td>
<td>ACAACACCAGTGACGAC</td>
<td></td>
<td>96</td>
<td>60</td>
<td>Börjesson et al. (2009)</td>
</tr>
<tr>
<td>tetA-F2</td>
<td>TGAATCTCCCAGGCGCTG</td>
<td>tetA</td>
<td>101</td>
<td>60</td>
<td>Börjesson et al. (2009)</td>
</tr>
<tr>
<td>tetA-R2</td>
<td>GAACCCGAGCGGCCTGACG</td>
<td></td>
<td>88</td>
<td>58</td>
<td>Peak et al. (2007)</td>
</tr>
<tr>
<td>tetB-F1</td>
<td>AGTCGGCTTGTAGCTCCTG</td>
<td>tetB</td>
<td>239</td>
<td>58</td>
<td>Heuer et al. (2008)</td>
</tr>
<tr>
<td>tetB-R1</td>
<td>TGGTGGGTATAGGGGTAATGTA</td>
<td>sul1</td>
<td>139</td>
<td>60</td>
<td>Knapp et al. (2010)</td>
</tr>
<tr>
<td>tetM-F</td>
<td>GTTCTTCTTGGATACCTAACATCACR</td>
<td>tetM</td>
<td>67</td>
<td>60</td>
<td>Volkmann et al. (2004)</td>
</tr>
<tr>
<td>tetM-R</td>
<td>CCATACTATACTTTCTCTCCTC</td>
<td>ampC</td>
<td>169</td>
<td>52</td>
<td>Guilard et al. (2011)</td>
</tr>
<tr>
<td>Sul1-F1</td>
<td>AAAACACCTGCCCGTTCR</td>
<td></td>
<td>239</td>
<td>58</td>
<td>Nõlvak et al. (2012a)</td>
</tr>
<tr>
<td>Sul1-R1</td>
<td>AAAACACCTGCCTCCGTCR</td>
<td></td>
<td>139</td>
<td>60</td>
<td>Knapp et al. (2010)</td>
</tr>
<tr>
<td>ermB-F</td>
<td>AAAATGCACCCCATCACA</td>
<td>ermA</td>
<td>67</td>
<td>60</td>
<td>Volkmann et al. (2004)</td>
</tr>
<tr>
<td>ermB-R</td>
<td>TTTCGCGCTCCTTGTCTG</td>
<td></td>
<td>169</td>
<td>52</td>
<td>Guilard et al. (2011)</td>
</tr>
<tr>
<td>Lak2-FF</td>
<td>GGAATCTTCTGAGCTAACAA</td>
<td>ampC</td>
<td>109</td>
<td>62</td>
<td>Nõlvak et al. (2012a)</td>
</tr>
<tr>
<td>Lak2-R</td>
<td>CATGACCCAGCTGACGAC</td>
<td></td>
<td>109</td>
<td>62</td>
<td>Nõlvak et al. (2012a)</td>
</tr>
<tr>
<td>QnrS-F</td>
<td>GTAAGATTGAAGTACCTTCT</td>
<td>qnrS</td>
<td>109</td>
<td>62</td>
<td>Nõlvak et al. (2012a)</td>
</tr>
<tr>
<td>QnrS-R</td>
<td>AAAACACCTGACCTAATTCT</td>
<td></td>
<td>109</td>
<td>62</td>
<td>Nõlvak et al. (2012a)</td>
</tr>
<tr>
<td>λ7403FL</td>
<td>caccttGACGCGAGATCATAATAAGTGGT</td>
<td>Bacteriophage λ DNA</td>
<td>109</td>
<td>62</td>
<td>Nõlvak et al. (2012a)</td>
</tr>
<tr>
<td>λ7512-R</td>
<td>ATCGTATGCAGCCTCAACACTGC</td>
<td></td>
<td>109</td>
<td>62</td>
<td>Nõlvak et al. (2012a)</td>
</tr>
</tbody>
</table>

* Sequence of artificial tail added to the primer sequence and enabling hairpin formation is denoted in lowercase; can be used as LUX™ primer when appropriate fluorophore is attached to the primer.
water for a total volume of 10 µl. The optimized reaction conditions were: 2 min at 50 °C; 10 min at 95 °C; and a sequence of 45 cycles of 15 s at 95 °C, 30 s at the annealing temperature of the primer pair used (Table 2) and 30 s at 72 °C. Immediately after the real-time PCR assay, melting curve analyses was performed by ramping up the temperature from 60 °C to 95 °C (0.35 °C/3 s) with continuous fluorescence recording. All qPCR reactions from samples and standards were run in triplicate and the cycle threshold was set manually to ΔRn = 0.1 in every qPCR run-file to ensure comparability of individual runs.

For qPCR data analyses Rotor-Gene Serier software, version 2.0.2 (Qiagen) and the LinRegPCR program, version 11.0 (Ruijter et al., 2009) in combination with a three-step outlier removal process were used as described by Nõlvak et al. (2012b). The target gene copy numbers, representing the abundance of ARGs in the tested samples, were deduced from the standard curves; these were calculated as geometric means of three parallel amplifications of each sample and presented as gene copy numbers per ml (copies ml⁻¹) for water samples and gene copy numbers per gram of dry weight (copies g⁻¹) for wetland media. Antibiotic resistance encoding functional genes were normalized against 16S rRNA genes, representing the proportion of ARGs in bacterial communities, using amplicon-specific amplification efficiencies and Ct values, as proposed by Ruijter et al. (2009). The amplification efficiencies of the target genes from the sampled HSSF MCs influent, effluent, and wetland media are shown in Table 3. Since the amplification efficiencies differed significantly between targeted genes, the direct summation and comparison of absolute copy numbers was avoided in this study (Nõlvak et al., 2012b). The proportions of tetM in the microbial community could not be calculated owing to the generally low quality of associated amplification datasets.

The presence of qPCR inhibitors was evaluated by mixing 1 µl of environmental DNA with 1 µl of 10⁶ copies of the bacteriophage λ standard plasmid. To determine the recovery rates of the λ bacteriophage DNA, comparison of qPCR amplification results with the standard curve was done as described by Keller et al. (2002). When the detected quantity of λ bacteriophage DNA (used as internal standard for inhibition estimation) differed from 100% of the added amount, the quantification data (outliers removed) were corrected using the corresponding efficiency factor.

### 2.5. Statistical analysis

The statistical analyses were carried out using the Statistica 10.0 program. The differences in amplification efficiencies of the targeted ARGs and 16S rRNA in the HSSF MC environments were estimated using unequal variance t-test. The ARG concentrations of three parallel HSSF MCs were calculated as geometrical means due to non-normality of the data. Spearman’s rank correlation coefficient was calculated using triplicate mesocosms data from all sampling days and was used to evaluate the extent to which water quality parameters and wastewater purification efficiencies correlated with target gene concentrations and relative abundances. In all cases, statistical significance was determined at the 95% confidence level. A linear mixed model was fitted to the data (package nlme in R) and, as a first step, test for a presence of trend in time for all seven target genes was performed. For those genes that did not exhibit the time trend, the equality of the intercepts was tested further. The p-values for multiple testing were corrected using Bonferroni correction. Prior to the mixed model analysis ARG abundance values were log-transformed. Spearman’s rank correlation coefficient was used to test the presence of monotonic trend in individual ARG abundance data. Partial Spearman correlation analysis was applied to reveal the impact of temperature on the relationships between the abundance of ARG genes and environmental parameters.

### 3. Results

#### 3.1. Dynamics of ARG abundances and proportions in bacterial communities in HSSF MCs influent

The 16S rRNA gene and targeted ARG abundances in HSSF MCs influent showed increases during the first 64 days, followed by slight decreases during next 30 days and strong declines on day 150 of the experiment (Fig. 1). The bacterial 16S rRNA gene concentration in the water entering the HSSF MCs was at the level of 10⁶–10⁷ copies ml⁻¹ during the first 94 days, but dropped rapidly by day 150. The most abundant ARGs were tetA, qnrS, and sul1, while tetM and tetB were detected in the lowest quantities during the first 94 days of the experiment. Although all targeted ARGs were detectable in the influent water during the course of the experiment, amounts of tetA, tetM, and ampC genes remained under the quantification limit of the current qPCR assay in the day 150 sample.

The proportion of ARG-carrying microbes in the HSSF MCs influent was stable during the entire course of the experiment (Fig. 2). The most pronounced changes, in the range of 4% in the bacterial community, were recorded in the proportion of the ermB gene. The tetB-, ampC-, and qnrS-carrying microbes formed the lowest proportions (in most cases less than 0.03%) in the studied communities.

#### 3.2. Dynamics of ARG abundances and proportions in HSSF MCs media biofilm and effluent

The HSSF MCs media biofilm and effluent showed similar overall dynamics of target gene abundances over the sampling period. During the first 64 days that the constructed wetland was in operation, ARG and 16S rRNA concentrations in wetland media biofilm (WMB) and effluent generally increased (Fig. 1). Similarly to the influent, the predominant ARGs in WMB and effluent were tetA and sul1 genes. The systematic difference between abundances of different ARGs in WMB and effluent was confirmed by the linear mixed model. According to this analysis, in case of WMB the abundance of tetA and sul1 genes was higher than the ermB gene, and the abundance of tetM and ampC was significantly lower than the abundance of these three genes. Although only a small quantity of qnrS was present in the WMB, this gene was among the most abundant of the ARGs in the mesocosms effluent. The least abundant ARGs in both the effluent and WMB were the tetM and tetB genes. While 16S rRNA, sul1 and ermB concentrations increased during whole study period (Spearman R = 0.53...0.75, p < 0.05), tetB and tetM abundances showed opposite trend (Spearman R = −0.69...−0.82; p < 0.05) in

### Table 3

<table>
<thead>
<tr>
<th>PCR efficiency</th>
<th>Target genes</th>
<th>16S rRNA **</th>
<th>tetA***</th>
<th>tetB***</th>
<th>tetM***</th>
<th>sul1***</th>
<th>ermB**</th>
<th>ampC</th>
<th>qnrS**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water n = 60</td>
<td>1.88 (±0.04)</td>
<td>1.87 (±0.07)</td>
<td>1.90 (±0.06)</td>
<td>1.52 (±0.08)</td>
<td>1.74 (±0.04)</td>
<td>1.61 (±0.13)</td>
<td>1.91 (±0.05)</td>
<td>1.90 (±0.05)</td>
<td></td>
</tr>
<tr>
<td>WMB n = 45</td>
<td>1.80 (±0.10)</td>
<td>1.74 (±0.08)</td>
<td>1.78 (±0.06)</td>
<td>1.28 (±0.06)</td>
<td>1.67 (±0.05)</td>
<td>1.51 (±0.12)</td>
<td>1.88 (±0.07)</td>
<td>1.84 (±0.12)</td>
<td></td>
</tr>
</tbody>
</table>

Statistically significant (* p < 0.01, ** p < 0.001, *** p < 0.0001) difference between target gene amplification efficiencies of water and WMB samples.
the WMB. In case of effluent only tetB and qnrS abundances decreased in time (Spearman R = −0.63...−0.64; p < 0.05).

The proportions of ermB and sul1 genes were predominant of the tested ARGs in the WMB, although they decreased over the study period, stabilizing by day 64 at a level of about 0.5% (Fig. 2). In the effluent bacterial community, ermB exhibited the most strongly pronounced and highly fluctuating relative abundance. Overall, the proportions of sul1 and other target ARGs were more stable in the effluent. In both the effluent and WMB communities, tetB, ampC, and qnrS genes were present in low proportions and remained below 0.005% (except for tetB in the WMB).

3.3. Differences between HSSF MCs influent and effluent 16S rRNA and ARG copy numbers and their proportions in bacterial communities

The differences between target gene abundances and their proportions in HSSF MCs influent and effluent are shown in Table 4. In most cases, the target ARGs showed a reduction in copy numbers during treatment in HSSF MCs throughout the study period (Table 4A). The actual decrease varied with time and was different for different genes and for parallel mesocosms within the same sampling event. On day 26, increases in 16S rRNA, tetB, sul1, and qnrS gene copy numbers in effluent samples was observed as compared to the influent. On days 45, 64, and 94, all ARGs (except for tetM on day 64) and 16S rRNA copy numbers in effluent decreased compared to the influent. On day 150, ARGs and 16S rRNA copy numbers increased in the effluent compared to the influent except for ermB and qnrS.

In general, the proportions of different ARGs decreased in mesocosm effluent bacterial communities (compared to the influent) during the treatment process (Table 4B). The exceptions were ermB on day 26 and day 150, and sul1 and ampC on day 150.

3.4. Factors related to ARG abundances and proportions in communities of HSSF MCs media biofilms and effluent

Data analysis showed strong correlation between ARG abundance dynamics in the influent and effluent for the tetA, tetB, and sul1 genes (Fig. 3). Strong correlations were also found between ARG concentrations in effluent and WMB for tetA, tetB, and qnrS. A weak correlation was observed between tetM abundance dynamics of influent and WMB and no correlations were found between respective gene abundances in the effluent and in the influent and WMB. Additionally, qnrS abundance in the influent was not related to either WMB or effluent qnrS gene copy numbers, but correlation was found between qnrS abundances in the WMB and the effluent. No such relationships were found for ermB and ampC concentrations or any targeted ARG proportions within the bacterial community.

Statistical analyses revealed a number of significant correlations between targeted gene copy numbers in the HSSF MCs effluent and media biofilm and influent quality parameters and duration of system operation (Table 5). Both positive and negative relationships were found between most of the measured gene abundances (except for tetA and ampC) in the WMB with the duration of mesocosms operation. Additionally, negative correlation between time of mesocosm operation and the proportion of ermB-carrying bacteria in the WMB was found (r = −0.67, p < 0.05). In the effluent, negative correlations for tetB and qnrS with days of operation were found. The temperature in the treating filter significantly affected the abundances of 16S rRNA and ARGs, mainly in the effluent, but in some cases (16S rRNA, tetB, tetM, and qnrS) also in the WMB (Table 5). The proportion of tetB-carrying bacteria within the effluent community (r = 0.60, p < 0.05) was also related to the interior temperature of the mesocosms. The influent pH was negatively correlated with sul1 and ampC abundances (Table 5) and positively correlated with ermB proportion (r = 0.53, p < 0.05) in the WMB. Partial Spearman correlation analysis was applied to ascertain the importance of mesocosms’ interior temperature and duration of mesocosm operation on abundance of ARG genes which were related to the both variables. Only in case of tetB abundance in the effluent the correlation with the mesocosms’ temperature (R = 0.55, p < 0.05) was significant when duration of mesocosm operation was taken into account as a covariable in the analysis.

3.5. Relationship of ARG abundances and proportions in bacterial communities with treatment efficiency

Data analyses showed different relationships between 16S rRNA gene copy numbers in the WMB and in the effluent to the parameters
characterizing treatment efficiency of the HSSF MCs (Table 5). A number of significant correlations between ARG abundances in the WMB and effluent and the removal efficiencies for nitrogen (NH₄₋N, NO₃₋N and NO₂₋N) and organic matter (decrease in BOD₅ and TOC) during the treatment process in HSSF MCs was revealed. Negative correlations were observed between proportions of tetA- and tetB-carrying bacteria in the effluent and the decrease in BOD₅ (r = −0.60, p < 0.05), and between the proportion of ermB in the effluent and the decrease in TOC (r = −0.54, p < 0.05). Although a positive correlation between NO₂₋-N and the tetA proportion within the microbial communities of the mesocosms effluent (r = 0.63, p < 0.05) was observed, decreases in effluent Ntot and NO₃₋-N were negatively correlated with the proportion of qnrS-carrying bacteria in microbial communities of the mesocosms effluent (r = −0.52, p < 0.05 and r = −0.62, p < 0.05, respectively). When temperature was taken into account as a covariable in correlation analysis, the decrease in tetB abundance in effluent was strongly related to removal efficiency of organic matter (BOD₅, r = −0.69, p < 0.001).

4. Discussion

The capability of the wastewater treatment process to remove ARGs is pivotal, as treatment facility effluent provides a direct release of ARGs to the environment. The final stage of the municipal wastewater treatment process in a newly established hybrid CW system was studied to characterize the dynamics of ARG abundance and the proportions of ARGs in the bacterial community. Also, the factors affecting their fate (amounts released into environment) and the relationships with wastewater treatment efficiency of the studied CW mesocosms was analyzed.

Contrary to findings at a conventional wastewater treatment plant where tetA and tetB genes were detected only in the water phase, but not in activated sludge (T. Zhang et al., 2009), all the targeted ARGs were detectable in both water phases (influent and effluent) and in the WMB in this study. The dynamics of inflow 16S rRNA and all tested ARG abundances was similar during the test period and the proportions of ARGs in the bacterial community were quite stable. An exception was the varying proportion of ermB, a gene belonging to the group of ARGs that is easily transferred by plasmids and transposons from one host to another (XX. Zhang et al., 2009).

A stabilization of 16S rRNA abundances in the WMB during the first 94 days of the experiment was observed. This coincides with the 75–100 day stabilization phase of the CW microbial community detected with other methods (Truu et al., 2009; Weber and Legge, 2011). The ARG abundances did not show such distinct phases, but a stabilization trend was obvious in the cases of sul1 and qnrS proportions in the bacterial community of the HSSF MCs media biofilm.

The 16S rRNA concentration in the system effluent (10⁷ copies ml⁻¹) was consistent with previously reported 16S rRNA concentrations in activated sludge treatment plant effluents (Munir et al., 2011; Czekalski et al., 2012; Gao et al., 2012). TetA encoding tetracycline resistance and sul1 encoding sulfonamide resistance were the most abundant ARGs in all three studied system media. This is not surprising, as likewise to some other European Union countries (Boxall et al., 2003), tetracycline and sulfonamide have been among the most commonly used antibiotics in human and veterinary medicine in Estonia (Estonian State Agency of Medicines). The concentration of tetA and sul1 genes in HSSF MCs effluent was, in most cases, comparable to concentrations of these genes observed in the effluents of conventional wastewater treatment plants (T. Zhang et al., 2009; Munir et al., 2011; Gao et al., 2012), but sul1 gene concentrations were two orders of magnitude lower than those noted by Czekalski et al. (2012) in an activated sludge sewage treatment plant effluent. The proportions of sul1 genes have been reported in the range of 0.02–7.7% within the microbial communities of the effluents of conventional wastewater treatment facilities (Munir et al., 2011; Czekalski et al., 2012; Gao et al., 2012). In our study, the sul1 proportions were firmly within the bottom quarter of this range. Other ARGs were detected in lower concentrations; comparison data for most of these ARGs even from conventional treatment facilities is not abundant in the literature. These results indicate that, in addition to...
general wastewater treatment efficiency, CWs offer a commensurate alternative to conventional wastewater treatment facilities with respect to ARG removal. Czekalski et al. (2012) suggested that resistance to important new antibiotic classes, such as fluoroquinolones, is generally less prevalent than resistance to older antibiotics. However, in this study qnrS genes encoding fluoroquinolone resistance were the third most abundant ARG in the system effluent, with concentrations comparable to tetA genes. This finding suggests that the resistance to newer antibiotic classes is actually widespread and has been underestimated. In this study, the gene detected in lowest amounts was tetM; other studies have shown this to occur quite frequently (Auerbach et al., 2007; T. Zhang et al., 2009). These outcomes may be partly the result of lower quality of tetM amplification datasets (compared to those for other target genes) and low amplification efficiencies of the primer pair used.

A significant drop in all target gene abundances in water samples (both influent and effluent) on day 150 was probably caused by a period of low air temperatures (below 0 °C) that occurred before the last sampling. Such low temperatures can affect the microbial community, especially in non-insulated system components (such as the collection well before HSSF MCs where the influent samples were taken).

In the current study, the abundances of most targeted genes and the proportions of ARGs within the microbial community were lower in the final effluent than in the pretreated wastewater influent. Noted reductions for 16S rRNA and ARG (mostly tetA) concentrations in conventional wastewater treatment facilities have been between two to three orders of magnitude (Auerbach et al., 2007; T. Zhang et al., 2009; Gao et al., 2012). The somewhat lower ARG concentration reduction observed in HSSF MCs can be attributed to the fact that the results represent only the last stage of wastewater treatment in the CW as the pretreated wastewater influent had already passed through the septic and vertical flow filters. Further studies are needed to establish the ARG reduction capacity of the overall CW system. However, the HSSF MCs were more efficient in removing sul1-carrying bacteria than some conventional wastewater treatment plants where sul1 concentrations increased (Czekalski et al., 2012) or the sul1 proportion in the microbial community remained stable (Gao et al., 2012). Therefore, HSSF CWs may complement conventional wastewater treatment facilities particularly for reducing the quantities of sulfonamide resistant bacteria. The reduction

**Table 4**

<table>
<thead>
<tr>
<th>Days of HSSF MCs operation</th>
<th>ΔTGCN n = 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>tetA</td>
</tr>
<tr>
<td>26</td>
<td>1.67 × 10^7</td>
</tr>
<tr>
<td>(±0.21 × 10^7)</td>
<td>(±0.33 × 10^4)</td>
</tr>
<tr>
<td>45</td>
<td>−2.90 × 10^7</td>
</tr>
<tr>
<td>(±0.12 × 10^7)</td>
<td>(±0.06 × 10^4)</td>
</tr>
<tr>
<td>64</td>
<td>−2.38 × 10^7</td>
</tr>
<tr>
<td>(±1.32 × 10^7)</td>
<td>(±0.06 × 10^4)</td>
</tr>
<tr>
<td>94</td>
<td>−2.99 × 10^7</td>
</tr>
<tr>
<td>(±0.47 × 10^7)</td>
<td>(±0.01 × 10^4)</td>
</tr>
<tr>
<td>150</td>
<td>+4.43 × 10^6</td>
</tr>
<tr>
<td>(±0.25 × 10^4)</td>
<td>(±0.34 × 10^4)</td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th>Days of HSSF MCs operation</th>
<th>Fold differences n = 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>tetA</td>
<td>tetB</td>
</tr>
<tr>
<td>26</td>
<td>−97.40 (±107.02)</td>
</tr>
<tr>
<td>45</td>
<td>−5.97 (±3.32)</td>
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<tr>
<td>64</td>
<td>−2.63 (±2.02)</td>
</tr>
<tr>
<td>94</td>
<td>−10.90 (±4.72)</td>
</tr>
<tr>
<td>150</td>
<td>−5.21 (±3.97)</td>
</tr>
</tbody>
</table>

**Fig. 3.** Statistically significant Spearman’s rank correlations (marked with solid lines) between influent, wetland media biofilm (WMB), and effluent antibiotic resistance genes abundances of horizontal subsurface flow constructed wetland mesocosms (p < 0.05).
of tetA and tetB proportions in the effluent compared to the influent is consistent with previous studies targeting tetA genes in conventional treatment facilities (Munir et al., 2011; Gao et al., 2012).

Several factors, including system operation time and temperature, that affected the amount of certain ARGs in the CW effluent were detected. The pH of the pretreated wastewater influent affected only the sulI and ampC abundances and the ermB proportions in the WMB; this may support the claim that wastewater treatment conditions have different implications for different antibiotic resistance groups (Novo and Manaia, 2010). Further support for this hypothesis was provided by correlation analysis, which revealed different types of interactions between abundances of each of the studied ARGs in three studied system environments. The specific nature of these interactions needs to be established in further studies.

The results also revealed various correlations between wastewater treatment efficiency and the abundance of ARGs in HSSF MCs media biofilm and in the effluent, indicating that treatment efficiency plays a pivotal role in determining the amounts of ARGs released into the environment via CW effluent. Although efficient removal of NO2−N correlated with higher ARGs abundance in the HSSF MCs effluent, the extensive removal of NH4+=N and organic matter lowered the amounts of ARGs in CW effluent. Sidrach-Cardona and Bécares (2012) reported that the hydraulic design of CWs is important in total bacterial (and presumably ARGs) reduction in CW effluents; however, additional research is needed to determine how other factors may affect bacterial and ARG reduction.

5. Conclusions

All seven targeted ARGs were detectable in the influent, WMB, and effluent of the HSSF MCs within a pilot-scale hybrid constructed wetland for treating municipal wastewater. Target gene abundances and proportions stabilized in the WMB after initial fluctuations. The abundance of 16S rRNA and ARGs, and the proportions of ARGs in the microbial community, were generally reduced during the wastewater treatment process; ARG concentrations in system effluent were comparable to those observed for conventional wastewater treatment facilities. The HSSF MCs proved more efficient in reducing sulfonamide resistance encoding sulI concentrations compared to some traditional wastewater treatment options. Results showed that the ARG concentrations in HSSF MCs media biofilm and in the effluent were affected by system operation parameters, especially system maturity and temperature. The results also revealed a relationship between ARG abundance and the removal efficiencies of NO2−N, NH4+=N, and organic matter in the system. Significant relationships between individual ARG abundances in the HSSF MCs influent, media biofilm, and effluent indicated that the ARG-carrying microbes entering the system interact differently with the WMB. The nature of these different interactions remains to be established.

Acknowledgments

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References


