Particles matter: Transformation of suspended particles in constructed wetlands

Mulling, B.T.M.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 3

Changes in the planktonic microbial community during residence in a constructed wetland

Abstract

Suspended particles are a major constituent of municipal wastewater and generally contain high levels of bacteria, including human pathogens. Discharge of these particles of anthropogenic nature can have profound effects on receiving aquatic ecosystems and mitigation of these effects requires additional polishing of treated municipal wastewater. Previously it was shown that surface flow constructed wetlands are effective in improving water quality by reducing the numbers of faecal indicator organisms. However, faecal indicator organisms represent only a minor fraction of the total planktonic bacterial community and knowledge on the effects of these constructed wetlands on the composition and functioning of the entire planktonic bacterial community is limited. The aim of this descriptive study was therefore to identify changes in the planktonic bacterial community during residence of treated municipal wastewater in a full-scale surface flow constructed wetland. To this purpose water samples were taken in which the bacterial community composition and functioning was analyzed using FISH, DGGE and BIOLOG. Surprisingly, the bacterial abundance at the inflow of the constructed wetland was relatively low compared with receiving surface waters. However, the inflowing bacterial community showed high metabolic activity and functional diversity. During residence in the constructed wetland the bacterial abundance doubled, but decreased in metabolic activity and functional diversity. Shifts in the community composition indicate that these changes are related to turn-over of the bacterial community. The planktonic bacterial community in the effluent of the constructed wetland closely resembled natural bacterial communities in urban and agricultural ditches. Based on these observations we conclude that constructed wetlands are capable to mitigate possible impacts of the particle load in treated wastewaters by transforming the anthropological bacterial community to a bacterial community resembling more “natural” surface waters.
Introduction

Discharges of municipal wastewater is a major source of anthropological impacts on aquatic ecosystems and therefore it is common to treat municipal wastewater before discharge. Treatment of raw municipal wastewater by wastewater treatment plants (WWTPs) strongly improves water quality, but treated municipal wastewater generally still contains high levels of nutrients, organic matter and high densities of heterotrophic bacteria originating from the WWTP (Tchobanoglous et al. 2004; Seviour and Nielsen 2010). The discharge of treated wastewater can therefore still have a profound impact on receiving surface waters (Seviour and Nielsen 2010; Holeton et al. 2011) and mitigation of this impact requires additional polishing of treated municipal wastewater. To this purpose, several techniques are available, but for the polishing of municipal treated municipal wastewater free surface constructed wetlands (CWs) are often favored (Kadlec and Wallace 2008). Free surface CWs improve the water quality by reducing nutrient concentrations, decreasing the numbers of faecal indicator organisms and improving the oxygen regime (Sundaravadivel and Vigneswaran 2001; Vymazal 2005; Kadlec and Wallace 2008; van den Boomen and Kampf 2012).

The removal of particular matter from the water is another purification process occurring is these systems, but is only profound in CWs receiving relative high concentrations of suspended particles while CWs receiving low concentrations of suspended particles are seemingly ineffective in removing suspended particles (Ghermandi et al. 2007; van den Boomen and Kampf 2012). However, all studies on particle dynamics in CW analyze bulk concentrations of suspended matter only, while suspended particles consist of particles of highly variable type and origin (Wotton 1994) and includes not only dead organic and inorganic particles, but also zooplankton, phytoplankton and bacterioplankton. Especially bacteria are regarded as important players in many biological processes and biochemical cycles in aquatic ecosystems (Kalff 2002) and the anthropogenic nature of the bacterial community in treated municipal wastewater may therefore influence the functioning of receiving aquatic ecosystems. It is expected that residence in the different functional compartments of a CW could strongly change the nature and composition of the planktonic bacterial community. However, besides detailed observations on the removal of bacterial faecal indicator organisms (Kadlec and Wallace 2008; Vymazal 2005; Reinoso et al. 2011; Moleda et al. 2008; Karim et al. 2004; Ghermandi et al. 2007, Diaz et al. 2010), knowledge about changes in the planktonic bacterial community during residence in CWs is virtually none existing. Therefore the aim of this descriptive study was to identify changes in the planktonic bacterial community during residence in a surface flow CW.

To meet this aim, we set up a sampling campaign in a full scale surface flow CW consisting of unvegetated ponds and reed beds, that receives municipal wastewater with low concentrations of suspended particles (3.6 mg L$^{-1}$; Mulling et al. 2013; van den Boomen and Kampf 2012; Ghermandi et al. 2007) and *Phragmites australis*. Water samples were taken at five points in the CW and structural and functional changes in the bacterial community were described using FISH, DGGE and BIOLOG. In addition, we analyzed water samples from six
different types of surface waters for comparisons between the bacterial communities in the CW and more natural bacterial communities.

**Material and methods**

*Site descriptions and sampling*

This study was conducted at seven locations in The Netherlands (Fig. 3.1). The main location was a full scale surface flow constructed wetland (CW) located in Grou that was built in 2006 and receives a constant hydraulic loading of 1200 m$^3$ day$^{-1}$ of treated primarily municipal wastewater. After inflow into the CW, the treated wastewater flows through three in series connected unvegetated ponds, four parallel reed beds and a collection pond before being pumped into receiving surface water (Fig. 3.1). The ponds are open water systems without vegetation with an average depth of 1.35 m, a volume between 360 and 440 m$^3$ each and a combined hydraulic retention time (HRT) of 17.9 h (Fig. 3.1). The reed beds are covered with *Phragmites australis* and have an average water depth of 40 cm, an approximate volume of 443 m$^3$, and each receive a hydraulic loading of $\pm 300$ m$^3$ day$^{-1}$ with an average HRT of 23.6 h. The total HRT of the CW is 41.5 h (Mulling et al. 2013).

Samples were taken in June 2010 as point samples 10-20 cm below the water surface at five different locations in the CW: at the in- and outflow of the unvegetated ponds and at the outflow of the reed beds (further referred to as PONDS-IN; PONDS-OUT; REED-BEDS-OUT respectively) and in the middle of these compartments (further referred to as PONDS and REED-BEDS respectively) (Fig. 3.1). The six reference surface waters used for comparison differed in water quality and typology. Locations could be described as an artificial fen created by sand excavation and fed by rain and groundwater, an agricultural ditch next to grassland with dairy cows, an urban ditch located next to an apartment building in Amstelveen (The Netherlands), an excavated recreational peat lake, an urban river flowing into the city of Amsterdam and a canal in the centre of Amsterdam (Fig. 3.1).
Fig. 3.1 Left: Sampling locations in the Netherlands. Right: Map of the WWTP in Grou, the Netherlands (0) with a sedimentation tank (1) which discharges treated municipal wastewater into a CW consisting of unvegetated ponds (2), reed beds (3) and an ecological buffer zone (4) which is in open connection the receiving channel (5). Sampling points were located at; PONDS-IN (a), PONDS (b), PONDS-OUT (c), REED-BEDS (d), REED-BEDS-OUT (e).

Measurements and analyses: Bacterial abundance and size

For the determination of bacterial abundance, water samples (3×50 mL) were fixated with 37% formaldehyde (end concentration: 10% v/v) within 30 min after sampling. After one hour of incubation, the samples were filtered over 0.2 µm polycarbonate filters (Ø47mm; Sartorius Stedim Biotech, Göttingen, Germany) and stored at -20°C till further analyses. Bacteria were labelled using fluorescence in situ hybridization (FISH) according to Glockner et al. (1996), with a general bacteria probe mix, EUB338mix (Daims et al. 1999). After hybridization the filters were mounted on microscope slides and imbedded in anti-bleaching media (4:1), Citifluor (AF1; Citifluor Ltd., Leicester, UK) and Vectashield (Vectorshield Laboratories, Inc., Burlingame, USA). From each filter 5 - 10 randomly chosen images, each composed from the average of 16 frames, were taken using a confocal laser scanning microscopy (Nikon A1, Tokyo, Japan) at 600× magnification. The images were converted to binary images, after which the bacteria were sized and counted automatically using ImageJ (Collins 2007) with a diameter size threshold of 0.2 µm. Bacterial counts and average size are grouped per sampling location, resulting in 30 technical replicas for the CW samples and 15 for the surface waters samples. The data of several of the sites were not normally divided, therefore the data was statistically tested for differences between sites with Kruskal-Wallis tests using PAST (Hammer et al. 2001).
Measurements and analyses: Community-level physiological profiling (CLPP)

For the functional diversity and bacterial activity BIOLOG GN2 plates (Biolog, Inc., Hayward CA, USA) were used, a cell culturing method in which the microbial community is tested for the capacity to utilize 95 different carbon substrates. From each location water samples (3×50 mL) were taken and inoculation of the BIOLOG plates occurred within 8 h. Prior to inoculation, samples were filtered over a pre-washed nitrate-cellulose filter with pore size 5.0 µm (Ø47 mm; Sartorius Stedim Biotech, Göttingen, Germany) and sonicated for 2 min. This pretreatment was performed to remove large grazers and acquire a homogeneous sample. The BIOLOG plates were incubated at 15°C with a light/dark cycle of 14/10 h. Following the manufacturer’s protocol a plate reader (VersaMax™ Microplate reader, Molecular devices, Sunnyvale, USA) was used to determine the utilization of each carbon source every 24 h over a four days incubation period. The average well color development (AWCD) was calculated by subtracting the absorption of the blank from the signal in each substrate absorption before averaging all 95 different substrates. Differences between the AWCD after 96 h of incubation were analyzed with an ONEWAY-ANOVA using PAST (Hammer et al. 2001). For the community metabolic diversity, the absorption from the blank was subtracted from the absorption of each substrate, than substrates with a signal higher absorption than 0.2 in at least two of the three replicas per site were defined as being utilized. Data was converted to binary data representing that the carbon source was used or not. Principal component analyses were performed on the community metabolic diversity profiles from the different locations using PAST (Hammer et al. 2001).

Measurements and analyses: Bacterial community composition

Species composition was analyzed by DGGE for the general bacterial community and methane oxidizers as an example of a specific functional group. This functional group was chosen as an example because generally methane is abundant in WWTP effluent and large community shifts were expected caused by changes in oxygen levels throughout the CW (Kadlec and Wallace 2008; Oremland 1988; Conrad 2007).

For determination of the total DNA content samples were filtrated on site over 0.2 µm cellulose nitrate membrane filters (Ø25mm; Whatman NC 20) and stored at -20°C prior to DNA extraction. DNA extraction was performed using PowerWater® DNA Isolation Kit (MO BIO laboratories Inc., Carlsbad, USA) according to the manufacturer’s instructions. For analyses of the total bacterial community general bacterial primers (F357GC and R518) were used which amplify the variable V3 region of 16S rDNA (Muyzer et al. 1993). PCR was conducted with the following cycling conditions: Initial denaturation: 94°C, 5 min Cycling step: 94°C, 30 sec, 54°C, 30 sec, 72°C, 1 min; 35 cycles; Final elongation 72°C, 8 min Reaction volumes were 50 µL containing: 1 µL of template DNA (5 ng µL⁻¹), 8.75 µL PCR-grade water (Applichem, Darmstadt, Germany) 25 µL 2× premixture (Epicentre, Madison, USA), 5 µL primer GCA189 (5 pmol µL⁻¹), 5 µL primer 661Nd (5 pmol µL⁻¹), 5 µL BSA (1:5, Biolabs, Ipswich, USA) and 0.25 µL taq-polymerase (Invitrogen, New York, USA). Methanotroph
specific pmoA were amplified using a nested design which has been previously described (Steenbergh et al. 2010; Lin et al. 2005). The nested PCR was performed to obtain enough pmoA product for the analysis and to make sure only pmoA of MOB was amplified since also ammonium oxidizers have a similar gene which potentially could be amplified by the first primer-set (ammonia monooxygenase-α subunit) (Holmes et al. 1995). The first PCR amplification used the primers A189 and A682 (Holmes et al. 1995) and consisted of 35 cycles: Initial denaturation: 94°C, 5 min Cycling step: 94°C, 1 min, 56°C, 1 min, 72°C, 1 min; 35 cycles; Final elongation 72°C, 6 min. Reaction volumes of the first round were 25 µL containing: 1 µL of template DNA (5 ng µL⁻¹), 3.88 µL PCR-grade Water (Applichem, Darmstadt, Germany) 12.5 µL 2× premixture (Epicentre, Madison, USA), 2.5 µL primer a682 (5 pmol µL⁻¹), 2.5 µL primer a189 (5 pmol µL⁻¹), 2.5 µL BSA (1:5, Biolabs, Ipswich, USA) and 0.125 µL taq-polymerase (Invitrogen, New York, USA). Before the nested PCR was performed samples were diluted 5 till 200 times depending on the intensity of the bands on a 1% agarose gel. For the nested PCR primer set GCA189 and mb661_nd (Lin et al. 2005) was used and consisted of 25 cycles: Initial denaturation: 94°C, 5 min Cycling step: 94°C, 30 sec, 56°C, 30 sec, 72°C, 30 sec; 25 cycles; Final elongation 72°C, 5.5 min. Reaction volumes of the nested PCR were 50 µL containing: 1 µL of (diluted) template DNA from the first round, 8.75 µL PCR-grade Water (Applichem, Darmstadt, Germany) 25 µL 2× premixture (Epicentre, Madison, USA), 5 µL primer GCA189 (5 pmol µL⁻¹), 5 µL primer 661_nd (5 pmol µL⁻¹), 5 µL BSA (1:5, Biolabs, Ipswich, USA) and 0.25 µL taq-polymerase (Invitrogen, New York, USA). All PCRs were performed in a MBS 0.2 S thermocycler (ThermoHybaid, Ashfort, UK).

After DNA extraction and amplification DGGE analysis was performed according to (Bodelier et al. 2005), with minor adjustments. The PCR products were separated on a 0.6 mm thick vertical gel consisting of 6% (w/v) polyacrylamide (37.5:1 acrylamide:bisacrylamide) and a linear gradient of the denaturants urea and formamide, increasing from 30% at the top of the gel till 70% at the bottom of the gel. The 100% denaturant was defined as 7 M urea with 40% v/v formamide. The gels were loaded with 45 µL of PCR product and 0.20 µL loading dye per µL PCR product. In total three clone ladders described by (Steenbergh et al. 2010) as mix nr.2 were loaded, one at each side of the gel and one in the middle to be able to calibrate the different gels. Electrophoresis was performed in a buffer containing 40 mM Tris, 40 mM acetic acid, 1 mM EDTA (pH 7.6) (0.5× Tris- acetate-EDTA buffer) for 17 h at 100 V (temp. 60°C). Gels were stained for 1 h in 0.1µL mL⁻¹ Ethidiumbromide in 0.5× TAE-buffer. The bands were visualized by UV-light and then photographed. Images were analyzed using Phoretics 1D Advanced (5.20, Biosystematica, Llandysul, UK). The lanes were manually detected and 90% of the lane width was analyzed. Bands were manually detected using the pixel intensity graph. The retardant factor (rf) was calibrated using the ladder. The bands were converted to binary data reflecting if bands were either present or not present. From the DGGE band data dendograms were constructed based on the Euclidean distance using PAST (Hammer et al. 2001).
Measurements and analyses: Carbon and BOD5 measurements

Total carbon (TC), inorganic carbon (IC) and total organic carbon (TOC) concentrations were analysed using a total organic carbon analyser (Schimadzu, TOC-Vcph; Kyoto, Japan). To determine the concentrations of dissolved organic carbon (DOC) water samples were vacuum filtered over pre-washed 0.2 µm cellulose nitrate membrane filters (Whatman NC 20) and analysed with the same method as described for the TOC. Biochemical oxygen demand (BOD5) was analysed according to standardized methods (NEN-EN-1899-1 1998).

Results

Bacterial abundance

The planktonic bacterial abundance at all sites ranged between \(10^6\) and \(10^7\) cells mL\(^{-1}\) (Fig. 3.2). At the inflow of the constructed wetland, the average bacterial abundance (±s.e.) was \(3.2 \times 10^6 \pm 4.8 \times 10^5\) cells mL\(^{-1}\) (Fig. 3.2). Between PONDS-IN and PONDS the abundance remained the same, but in the second halve of the unvegetated ponds (between PONDS and PONDS-OUT) the bacterial abundance increase to an average of \(4.9 \times 10^6 \pm 2.8 \times 10^5\) cells mL\(^{-1}\) (p<0.001) (Fig. 3.2). The same pattern was observed during residence in the reed beds, with no significant change in the bacterial abundance in the first halve of the reed beds, but an significant increase in the second halve of the reed beds to \(7.9 \times 10^6 \pm 7.8 \times 10^5\) cells mL\(^{-1}\) (p<0.001) (Fig. 3.2). The average bacterial abundance in the surface waters (comparison sites) showed high variation between sites, ranging from \(2.2 \times 10^6 \pm 6.3 \times 10^5\) cells mL\(^{-1}\) in the canal, to \(1.2 \times 10^7 \pm 6.7 \times 10^5\) cells mL\(^{-1}\) in the artificial fen (Fig. 3.2).
Metabolic activity and functional diversity

Residence in the unvegetated ponds had no effect on the high Average Well Color Development (AWCD) of 1.6 observed at PONDS-IN (Fig. 3.3), but during residence in the reed beds a significant decrease in maximum AWCD (to 1.2) was observed ($p<0.001$) (Fig. 3.3). The AWCD at the outflow of the reed beds was comparable the AWCD of the urban ditch samples, which were relatively high when compared to the other surface waters, ranging from 1.2 and 0.7 after 96 h incubation.

After 96 h incubation, all three samples in the unvegetated ponds showed a high functional diversity, with 93, 92 and 91 of the 95 available substrates being utilized at PONDS-IN, PONDS and PONDS-OUT respectively (Fig. 3.3). At REED BEDS and REED-BEDS-OUT the number of utilized carbon substrates decreased to 85 and 87 out of 95 respectively. The surface waters all showed lower diversity in carbon substrate utilization compared with the CW, ranging from 81 out of 95 in the urban ditch to 64 out of 95 in the peat lake. The principal component analyses (PCA) of the functional diversities showed separation of several locations: the unvegetated ponds, reed beds, the flowing water surface waters and the stagnant waters like the artificial fen and peat lake (Fig. 3.4). The first principal component explained 41% of the variance in functional diversity of carbon utilization and was mainly determined by the utilization of carboxylic acids and amino acids (Table 3.1). The second principal component explained 14% of the variance and was composed of a variety of carbon source types (Table 3.1).

Fig. 3.3 Community level physiological profiling (Biolog) average well color development (AWCD) and community metabolic diversity in the CW (PONDS-IN – REED-BEDS-OUT) and varies types of surface waters.
Chapter 3

Fig. 3.4 Principal component analysis of the functional carbon utilization diversity in the unvegetated ponds (black triangles), reed beds (black circles) and comparison sites (open squares).

Table 3.1 Carbon source loads determining the principal component axis.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>PC 1</th>
<th>PC 2</th>
<th>Carbon source</th>
<th>PC 1</th>
<th>PC 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrates</td>
<td></td>
<td></td>
<td>Amino acids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i-Erythritol</td>
<td>0.22</td>
<td></td>
<td>L-Threonine</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Lactulose</td>
<td>0.20</td>
<td>0.35</td>
<td>L-phenylalanine</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>Carboxylic acids</td>
<td></td>
<td></td>
<td>L-Ornithine</td>
<td>0.21</td>
<td>0.20</td>
</tr>
<tr>
<td>Sebacic Acid</td>
<td>0.24</td>
<td></td>
<td>Amides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Keto Valeric Acid</td>
<td>0.23</td>
<td>0.20</td>
<td>Glucuronamide</td>
<td>0.21</td>
<td>-0.26</td>
</tr>
<tr>
<td>α-Keto Butyric Acid</td>
<td>0.21</td>
<td>0.20</td>
<td>L-Alaninamide</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>p-Hydroxy-Phenylacetic Acid</td>
<td>0.21</td>
<td>0.20</td>
<td>Amines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Itaconic Acid</td>
<td>0.21</td>
<td>0.20</td>
<td>Phenethylamine</td>
<td>-0.29</td>
<td></td>
</tr>
<tr>
<td>D-Glucosaminic Acid</td>
<td>0.20</td>
<td>0.35</td>
<td>Methyl ester</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dicarboxylic acids</td>
<td></td>
<td></td>
<td>Succinic Acid Mono-methyl Ester</td>
<td>-0.40</td>
<td></td>
</tr>
<tr>
<td>Succinamic Acid</td>
<td>0.24</td>
<td>-0.26</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Carbon and BOD$_5$**

The total carbon (TC) concentration in the CW ranged between 60 to 100 mg L$^{-1}$ and did not show changes during residence in the CW (Fig. 3.5). The average inorganic fraction in all samples was 54 ±3 mg L$^{-1}$ (Fig. 3.5). The organic carbon fraction was approximately 20 mg L$^{-1}$ throughout the CW and mainly consisted on dissolved organic carbon (DOC), with a marginal fraction of carbon present in the form of particulate organic carbon (POC) (Fig. 3.5). The average biological oxygen demand (BOD$_5$) at RED-BEDS-OUT (1.55 ±0.12 mg L$^{-1}$) was significantly lower than at PONDS-IN (3.20 ±0.60 mg L$^{-1}$) (p<0.05). The ratio between the biological oxygen demand (BOD$_5$) and the DOC (BOD$_5$/DOC) decreased from 0.14 to 0.08 during residence in the unvegetated ponds and was 0.07 at REED-BEDS-OUT.

![Fig. 3.5 Average concentration of total carbon (TC), organic carbon (TOC) and dissolved organic carbon (DOC) in different locations in the CW. N=3-5; ±s.d.](image)

**Bacterial community composition**

The denaturant gradient gel electrophoresis (DGGE) of general bacteria resulted in 22 bands at PONDS-IN (Fig. 3.6). This relative high number of bands was reduced to 11 at PONDS and to 10 at PONDS-OUT. At the end of the reed beds the number of bands decreased further to 7. Although four bands at PONDS IN were also present at REED-BEDS-OUT, only one band present at PONDS-IN remained present at all locations in the CW. The number of bands observed in most of the surface waters ranged between four (agricultural ditch, canal) and six (peat lake), with the artificial fen as the only exception with 13 bands. Cluster analyses of the band patterns showed a strong separation of PONDS-IN and the artificial fen from all other sites. The surface waters (except the artificial fen) clustered closely together and also include the REED-BEDS-OUT. The other sites of the CW (ponds, PONDS-OUT and REED-BEDS) clustered together and showed a lower Euclidean distance from the comparison sites compared to PONDS-IN (Fig. 3.6).
The \( \text{CH}_4 \) oxidizing bacteria showed similar numbers of dominant bands throughout the CW ranging from 4 (reed beds) to 7 (PONDS-IN) (Fig. 3.7). The band composition was however highly variable: dominant bands at PONDS-IN were, for example, all replaced by other dominant bands at PONDS. Similar shifts in band patterns were observed between the other locations in the CW. Besides the agricultural ditch (1 dominant band) the surface waters showed similar numbers of dominant bands ranging between 3 (urban ditch) and 7 (artificial fen), but showed few mutual bands between different surface waters. This high diversity of dominant bands between sites was reflected in the cluster analyses of the \( \text{CH}_4 \) oxidizing bacteria band patterns (Fig. 3.7).

**Fig. 3.6** Euclidean distance dendrogram of the general bacteria DGGE in the CW and various types of surface waters (comparison sites).

**Fig. 3.7** Euclidean distance dendrogram of \( \text{CH}_4 \) oxidizing bacteria in the CW and various types of surface waters (comparison sites).
**Discussion**

*Bacterial community at the inflow of the CW*

The bacterial abundances observed were all in range of previously determined bacterial abundances in surface waters (Sanders et al. 1992; Glöckner et al. 1999), but the bacterial abundance at the inflow of the CW was on the low side in comparison with the comparison surface waters, while a relative high bacterial abundance was expected in the treated wastewater. Usually a large fraction of planktonic bacteria is associated with particles (Grossart et al. 1998) and the low inflow of suspended particles probably causes a relative low number of bacteria to enter the CW. Nonetheless, the metabolic activity and functional diversity of the bacterial community at the inflow of the CW was very high in comparison with the surface water samples, indicating that the bacterial community at the inflow of the CW consisted of a high percentage of active bacteria. The presence of a metabolic active and functional divers microbial community is important for the functioning of WWTPs (Tchobanoglous et al. 2004) and is therefore well maintained by providing favorable conditions (oxygen, temperature, resources). Previous research showed that 80% of the bacteria in activated sludge from WWTPs is metabolic active (Nielsen and Nielsen 2002) against 8 to 47% in the water column of lakes (Haglund et al. 2002). This high metabolic active fraction in activated sludge was indeed reflected in the high activity of the bacterial community at the inflow of the CW.

*Unvegetated ponds*

High concentrations of DOC at PONDS-IN provide a large pool of substrates for the heterotrophic bacterial community in the CW. The concentration of DOC remained the same during residence in the unvegetated ponds, but the ratio between the biological oxygen demand (BOD$_5$) and the DOC (BOD$_5$/DOC) decreased from 0.14 to 0.08. This indicates that the composition of the DOC changed and decreased in quality which has been often observed in other constructed wetlands (Kadlec and Wallace 2008), probably caused by a combination of removal of labile carbon substrates by metabolic uptake and abortion to POM, the formation of new DOC compounds in the system by degradation of POM and photochemical degradation of DOC (Lindell et al. 1995). However, the bacterial metabolic activity and functional diversity of the bacterial communities remained the same during residence in the unvegetated ponds and did not reflect the observed decrease in substrate quality. Moreover, the abundance of bacteria increased, indicating relative decrease in metabolic activity per cell. The DGGE analyses showed that the number of bacterial taxa decreased during residence in the unvegetated ponds, indication either partial turnover of bacterial community of shifts in the dominance between the original occurring taxa during residence in the unvegetated ponds.
These dynamics between bacterial species were well illustrated by the changes in the composition of methane oxidizing bacterial community in the unvegetated ponds, showing a shift towards a strong dominance of a type-Ia taxa which is regarded as a fast growing, but bad competitor (r-type) group of organisms (Steenbergh et al. 2010), indicating favorable conditions (Tanner et al. 1997). This primarily includes sufficient supply of organic matter (Conrad 2007) and low oxygen concentrations (Oremland 1988). Earlier research in this constructed wetland (van den Boomen et al. 2012) showed that the oxygen levels increase from almost anoxic at the PONDS-IN to 3 to 4 mg L\(^{-1}\) at PONDS-OUT, combined with high sedimentation fluxes of organic particles in the first part of the constructed wetland (van den Boomen et al. 2012).

**Reed beds**

In the reed beds, the bacterial metabolic activity and functional diversity decreased to levels comparable with the communities of the urban and agricultural ditches. Similar as in the unvegetated ponds, this decrease in activity coincided with an increase in bacterial abundance, even further reducing the relative bacterial metabolic activity per cell. The DGGE patterns showed that these changes are probably related to changes in the bacterial community composition. This is supported by the changes observed in the metabolic diversity were the utilization of carbon sources is reduced, especially (di)carboxylic acids and amino acids. The decreasing capacity of the bacterial community to utilize these type of carbon course is also the mean difference between CW influent and the comparison surface waters. The PCA analyses also showed that residence in the CW changes the bacterial functional diversity towards the urban and agricultural ditch systems. These two ditch systems closely resembled wetland ecosystems with relative high abundance of macrophytes, which indicates that the bacterial community at the outflow of the CW may largely consists of bacteria from originating from the last compartment, the reed beds. Surface areas in the reed beds, including reed stems, plant litter and sediment are generally colonized by biofilms which are not only known to retain high amounts of suspended particles and bacteria from the water column (Balzer et al. 2010; Chabaud et al. 2006; Stott and Tanner 2005; Eisenmann et al. 2001), but simultaneously also release large numbers of bacteria into the surrounding water column (McDougald et al. 2012; Picireanu et al. 2001). The large shift in the planktonic bacterial community characteristics during residence in the reed beds is therefore expected to be caused by strong exchange between water and solid surfaces. Although it is notable that there were small differences between the different measurements, both the general taxa composition and the metabolic activity of the bacterial community supports this strong shift in community composition during residence in the reed beds to resemble similar natural ecosystems.
Conclusion

This study showed that constructed wetlands receive a metabolic highly active bacterial community together with a high load of carbon substrates from the wastewater treatment plant. In the different functional compartments of the CW, changes in various biological, chemical and physical conditions occur, inducing many changes in the characteristics of the bacterial community. The planktonic bacterial community flowing out of the CW resembles communities of physically similar natural ecosystems. Constructed wetlands are therefore suitable to reduce the input and impact of anthropogenic bacterial communities discharged by wastewater treatment plants into receiving surface waters.

Acknowledgments

This work was financed by the Stichting WaterNet and supported by Foundation for Applied Water Research (STOWA), Witteveen+Bos, Wetterskip Fryslân and NIOO-KNAW. Special thanks go out to the people that supported this research, Richard Soeter, Paul Bodelier, Maxine Bogaert en Rinse van der Kooij.
Chapter 3

References


Planktonic bacteria


Steenbergh AK, Meima MM, Kamst M and Bodelier PLE (2010). Biphasic kinetics of a methanotrophic community is a combination of growth and increased activity per cell. FEMS microbiology ecology 71 (1).


