Particles matter: Transformation of suspended particles in constructed wetlands

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Processes removing faecal indicator organisms in constructed wetlands

Abstract

Treatment of municipal wastewater by wastewater treatment plants strongly reduces the numbers of faecal indicator organisms and pathogens, but in discharged treated wastewater numbers are still high enough to be a major anthropogenic input of faecal indicator organisms and pathogens into receiving surface waters, potentially threatening recreational purposes and drinking water production. The natural purification capacity of wetlands has long been recognized and man-made constructed wetlands are widely used to improve the water quality of treated wastewater before discharge into receiving surface waters. Although the removal efficiencies of constructed wetlands are well-studied, the importance of different processes involved in the removal of faecal indicator organisms and pathogens are often not analysed. In this study we monitored the monthly numbers and removal of several bacterial, protozoan and viral faecal indicator organisms in a full scale constructed wetland receiving treated municipal wastewater. In addition we performed small scale experiments to estimate the importance of individual processes influencing pathogen dynamics including sedimentation, predation, UV irradiance, mortality and external input. The results showed substantial removal of faecal indicator organisms during residence in the constructed wetland, with total removal efficiencies ranging between 96 and 99% for bacterial faecal indicator, 89% for a faecal protozoan indicator and 73 and 88% for the viral indicators. Zooplankton predation and biofilm retainment appeared to be the most important processes governing the removal of faecal indicator organism removal in the constructed wetland, whereas the effects of sedimentation, inactivation and UV-radiation are of minor importance. No significant reintroduction of faecal indicator organisms by waterfowl or other warm blooded animals was observed, although very high abundance of waterfowl were present in the constructed wetland during freezing periods.
Introduction

Wastewater discharge is one of the major anthropogenic inputs of faecal pathogens into surface waters (Arnone and Walling 2007; Tchobanoglous et al. 2004). Although treatment of raw wastewater by wastewater treatment plants (WWTPs) strongly improves the hygienic water quality (Wen et al. 2009; Tchobanoglous et al. 2004; Wery et al. 2008) treated wastewater still contains high numbers of faecal pathogenic organisms (Seviour and Nielsen 2010), compromising the hygienic quality of receiving surface waters (Holeton et al. 2011; Tchobanoglous et al. 2004). To reduce the faecal pathogen load of surface water, additional polishing of treated wastewater is often desirable. Several technical methods have been developed to remove pathogenic organisms, including UV treatment, slow sand filtration, membrane filtration and addition of reactive oxygen species (Gomez et al. 2006). These technical methods are, however, relatively expensive and energy consuming, and can produce harmful transformation products that are discharged into receiving surface waters (Watson et al. 2012; Tchobanoglous et al. 2004). Alternatively, constructed wetlands (CWs) are widely used biological systems for polishing treated wastewater and it has been demonstrated that these wetlands are sustainable and low cost systems for improving the hygienic quality of treated wastewaters (Molleda et al. 2008; Vymazal 2005; Kadlec and Wallace 2008). Although the effects of CWs on removing faecal indicators have been studied extensively (Kadlec and Wallace 2008; Vymazal, 2005 Reinoso et al. 2011; Molleda et al. 2008; Karim et al. 2004; Ghermandi et al. 2007, Diaz et al. 2010), the majority of these studies focus only on the net removal efficiencies, without quantifying the different processes involved. The processes involved in the removal of faecal indicator or pathogenic organisms from treated wastewater during residence in CWs include sedimentation, predation, natural mortality, biofilm retention, UV inactivation, resuspension and population growth (Brookes et al. 2004; Chabaud et al. 2006; Stott and Tanner 2005; Kadlec and Wallace 2008).

The aim of this study was to quantify the effects of these different processes on the removal of faecal bacterial and viral indicators in constructed wetlands. To meet this aim, monthly water samples were collected from different compartments of a full-scale surface flow constructed wetland (unvegetated ponds and reed beds) receiving treated municipal wastewater. Numbers of faecal indicators for bacterial pathogens, *Escherichia coli*, Enterococci and *Bacteroidales* (Gerba 2000), indicators for protozoa pathogens, *Clostridium perfringens*, and indicators for viral pathogens (Payment and Franco 1993; Gerba 2000), *Bacteroides* phages (Ebdon et al. 2012; Gerba 2000), were analyzed. Furthermore, samples were also analyzed for zooplankton abundance and species composition to estimate the species specific predation capacity. Finally, additional laboratory and field experiments were conducted to estimate the effect of temperature, total predation, UV radiation and sedimentation.
Material and methods

Treatment plant and constructed wetland

This study was carried out in a full scale surface flow constructed wetland (CW) located in Grou, The Netherlands. The CW was built in 2006 and primarily receives a constant hydraulic loading of 1200 m$^3$ day$^{-1}$ treated municipal wastewater. The inflow of the constructed wetland leads treated waste water through a series of three unvegetated ponds and four parallel reed beds, before being pumped into receiving surface water (channel) (Fig. 4.1).

The unvegetated ponds are open water systems without vegetation with an average depth of 1.35 m, volume between 360 and 440 m$^3$ each and total hydraulic retention time (HRT) of 17.9 h (Fig 4.1; Table 4.1). The four reed beds each have an average water depth of 40 cm, approximate volume of 443 m$^3$, are covered with *Phragmites australis* and receive ca. 300 m$^3$ treated wastewater day$^{-1}$ with an average HRT of 23.6 h. The total HRT of the CW was 41.5 h. HRTs were calculated from the residence time distribution obtained by a tracer experiment using lithium chloride (van den Boomen et al. 2012). The average HRT was determined at 50% passage of the lithium chloride load.

Table 4.1 Dimensions and hydraulic retention time (HRT) of constructed wetland, Aqualân in Grou, The Netherlands. The length, width and volume of the ponds were manually determined and calculated, the length, width and volume of the reed beds were calculated from the construction blueprints. The HRT’s were determined by a tracer experiment.

<table>
<thead>
<tr>
<th></th>
<th>Pond 1</th>
<th>Pond 2</th>
<th>Pond 3</th>
<th>Total</th>
<th>Bed 1-4</th>
<th>Total</th>
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<tbody>
<tr>
<td>Length (m)</td>
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<td>55</td>
<td>55</td>
<td>165</td>
<td>110</td>
<td>110</td>
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<tr>
<td>Average width (m)</td>
<td>7.6</td>
<td>8.1</td>
<td>8.1</td>
<td>7.9</td>
<td>11.5</td>
<td>46</td>
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<tr>
<td>Average depth (m)</td>
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<td>1.31</td>
<td>1.43</td>
<td>1.35</td>
<td>0.4</td>
<td>0.4</td>
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<tr>
<td>Surface area (m$^2$)</td>
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<td>446</td>
<td>446</td>
<td>1304</td>
<td>1265</td>
<td>5060</td>
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<tr>
<td>Volume (m$^3$)</td>
<td>362</td>
<td>388</td>
<td>441</td>
<td>1191</td>
<td>443</td>
<td>1771</td>
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<tr>
<td>Hydraulic loading (m$^3$ day$^{-1}$)</td>
<td>1200</td>
<td>1200</td>
<td>1200</td>
<td>1200</td>
<td>300</td>
<td>1200</td>
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<tr>
<td>HRT (h)</td>
<td>17.9</td>
<td></td>
<td></td>
<td></td>
<td>23.6</td>
<td>41.5</td>
</tr>
</tbody>
</table>

Sampling

Water samples were taken monthly from January till December 2010, between 09.00 and 11.00 am at the in- and outflow of each compartment (unvegetated ponds and reed beds; Fig. 4.1). Sampling moments for the wetland sampling locations were not corrected for the hydraulic residence time which could in combination with possible daily variation in faecal indicator organism concentrations influence the removal efficiency calculations. In the middle of the unvegetated ponds and reed beds samples were taken for zooplankton
analyses (Fig. 4.1). For zooplankton identification and counting, two types of water samples were taken from April until December 2010. For larger zooplankton taxa water samples (10 L) were filtered over a 30 µm filter. For Ciliophora and Amoebozoa smaller than 30 µm water samples (3 L) were fixated with Lugol and left for settling for 5 days. In addition, sedimentation traps were placed at twelve locations in the unvegetated ponds (5, 4 and 3 locations in first, second and third unvegetated pond respectively (Fig. 4.1)). At each location eight sedimentation traps with a height of 33 cm, a diameter of 5 cm and a volume of 650 mL, were placed on top of the sediment. After 29, 49, 93 and 168 h two sedimentation traps (total volume of 1.3 L) from each of the twelve locations were retrieved, the content was well mixed and transferred in 2 L glass bottles. Samples were taken from these bottles to determine the concentration of *E. coli* in the same manner as normal water samples (paragraph 2.3). Prior to further analysis all samples were stored at 4°C.

**Fig. 4.1** Map of the WWTP in Grou, the Netherlands (0) with a sedimentation tank (1) which discharges treated municipal wastewater into a constructed wetland consisting of unvegetated ponds (2), reed beds (3) and an ecological buffer zone (4) which is in open connection with the receiving channel (5). Sampling points were located at; PONDS-IN (a), PONDS-OUT (b), REED-BEDS-OUT (c). At twelve locations in the unvegetated ponds (2) sedimentation traps were placed indicated with white dots. Zooplankton samples were collected in the middle of the unvegetated ponds and reed beds indicated by the open circles.
Faecal indicator organisms

*E. coli* and enterococci numbers were determined by membrane filtration according to standardized procedures NEN-EN-ISO-9308-1 (2000) and NEN-EN-ISO-7899-2 (2000), respectively. *C. perfringens* numbers were determined by placing filtered samples (Cellulose Nitrate; Ø47mm) on Triptose Sulfite Cyclocerine medium (TSC-medium) under anaerobic conditions for 24 ± 2 h at 45 ± 1°C sequentially for confirmation onto Mobility-Nitrate reduction medium (BN-medium) and Lactose-Gelatine medium (LG-medium) and incubated at 37 ± 1°C for 24 ± 2 h. This method is based on standardized procedures ISO/TC-147/SC4/WG5 (1995), ISO-6461-2 (1986) and NEN-EN-ISO-8199 (2007). *Bacteroidales* were quantified by qPCR (van der Wielen and Medema, 2010). In short, 100 ml of a water sample was filtered over a 25 mm polycarbonate filter (0.22 µm pore size). DNA was isolated using a FastDNA spin kit for soil (Qbiogene, US) according to the supplier’s protocol. Subsequently, primer sets Allbac 296F and Allbac 412R were used with the Taqman probe Allbac375Bhqr to quantitatively determine the 16S rRNA gene copy numbers of *Bacteroidales* spp in the water samples using a real-time PCR apparatus. For determination of the plaque forming units of *Bacteroides* phages, plates with *Bacteroides* host cells were incubated with water samples according to standardized procedure (ISO-10705-4 2001). Two different host strains of *Bacteroides* were used to enable differentiation between general and human specific *Bacteroides* phages. For the concentration of general *Bacteroides* phages *Bacteroides fragilis* was used as host (ISO-10705-4 2001), whereas for the human specific *Bacteroides* phages *Bacteroides* strain HB13 was used as host (Payan et al. 2005).

Zooplankton

The abundance of larger zooplankton taxa (>30 µm) was determined by a two stepped inverted light microscope (Olympus; IX70-71; minimal magnification of 60×) counting procedure. First samples are left to settle for 15 min before counting zooplankton. Subsequently, samples were fixated with lugol, left for another 15 min to settle and recounted. The difference between the first and second enumeration was interpreted as the number of living organisms. For counting the number of Ciliophora and Amoebozoa smaller than 30 µm, water samples (3 L) were fixated with Lugol and left to settle for 5 days. Overlying water was carefully removed and the remaining sample (4-5 mL) was transferred into a smaller container. Numbers were determined at a magnification of 400-600× using an inverted light microscope.

Laboratory incubation

20 L water samples were collected at the inflow of the CW (PONDS-IN) of which halve was filtered through 5 µm polycarbonate membranes filters (Ø47mm; Nuclepore™, California USA) on location to remove the zooplankton. Three 1 L samples of both the filtered and unfiltered samples were incubated at 10 and 20°C in presence and absence of UV radiation (Exoterra Repti Glo 40 Watt; 57% PAR, 33% UVA, 10% UVB; 5 cm distance to
Faecal indicator organisms

water surface). All samples were incubated under a 16/8 light/dark-cycle. All 24 h samples were taken from each incubation and transferred onto Coliscan® S Easygel® (Micrology Laboratories, Goshen, IN, USA) plates, according to the manufacturer’s instructions. The plates were incubated at 36°C, and after 20 h of incubation, plates were photographed. The number of colony forming units (CFU) of E. coli were counted with ImageJ 1.44 software (http://rsb.info.nih.gov/ij/) using the Cell Counter plugin.

Data analyses

To obtain monthly removal rates for both the unvegetated ponds as reeds beds, the outflow numbers (log_{10}) were subtracted from the inflow numbers (log_{10}). The removal data was normally distributed and to test if average removal of the indicator organisms differed significantly from zero one sample t-tests were performed (PAST; Hammer et al. 2001). To test differences in the removal of indicator organisms between the ponds and reed beds paired t-test were performed (PAST; Hammer et al. 2001).

Sedimentation fluxes (±s.e.) were calculated by linear regression analyses over the four sampling moments at each of the twelve sampling points using SPSS (version 17.0).

Conversion factors between abundance and predation capacity were gathered from literature (Table 4.2). Several low abundance zooplankton species of groups including several Amoebozoa species, Ceriodaphnia and Rotifera sp. are expected bacteriovores (Knight and Waller 1992; Abrantes and Goncalves 2003), but the predation capacity for these zooplankton species is not described in literature and were therefore estimated using taxonomically related species (Table 4.2). Correlations between the predation capacity of zooplankton species and log removal of faecal indicators were calculated using SPSS (version 17.0). For normally distributed data a Pearson’s correlation test was performed, otherwise a Spearman’s rho test was performed.

Results

Removal of faecal indicators in constructed wetlands

The average number of faecal bacterial indicators ranged between 10^3 and 10^6 CFU L⁻¹ at PONDS-IN (Fig 4.2a-c). The number of C. perfringens at PONDS-IN showed no large monthly changes throughout the year, but a trend of lower numbers in summer compared with winter was observed (Fig 2c). E. coli and enterococci at PONDS-IN showed strong variations between consecutive months throughout the year (Fig 4.2a,b). 16S rRNA gene copies of Bacteroidales at PONDS-IN ranged between 10^8 and 10^{11} gene copies L⁻¹ (Fig 4.2d) and similar to the other bacterial indicators Bacteroidales showed lower numbers in summer compared with winter (Fig 42d). The number of the faecal viral indicator B. fragillis phages at PONDS-IN was around 10^3 plaque forming units (PFU) L⁻¹, whereas Bacteroides strain HB13 phages ranged between 10^1 and 10^3 PFU L⁻¹ (Fig 4.2e,f).
Both bacteriophages showed relative high numbers in summer (July/August). Most faecal indicators also showed relative stable outflow numbers (REED-BEDS-OUT) throughout the year, with similar seasonal fluctuations as seen at the inflow (PONDS-IN) (Fig 4.2). However, high numbers of *E. coli* and enterococci at the outflow were found in January, February, August and September.

During residence in the unvegetated ponds, numbers of most faecal indicators significantly decreased (p<0.05), with average log removal efficiencies of 0.29 ± 0.09, 0.48 ± 0.17 and 0.30 ± 0.08 log_{10} CFU for respectively *E. coli*, enterococci and *C. perfringens*, 0.33 ± 0.06 log_{10} gene copies of *Bacteroidales*, and 0.54 ± 0.16 log_{10} PFU of *B. fragillis* phages (Fig. 4.3). With an average log removal of 0.17 ± 0.14 log_{10} PFU, numbers of *Bacteroides* HB13 phages did not decrease significantly during residence in the unvegetated ponds (Fig. 4.3). Residence in the reed beds caused significant reduction in numbers of all faecal indicators.
Faecal indicator organisms

(p<0.05), with an average log removal of $1.19 \pm 0.29$, $0.88 \pm 0.20$ and $0.64 \pm 0.10 \log_{10}$ CFU of *E. coli*, enterococci and *C. perfringens*, respectively, $1.67 \pm 0.08 \log_{10}$ gene copies of *Bacteroidales*, and $0.63 \pm 0.10$ and $0.49 \pm 0.12 \log_{10}$ PFU of *B. fragilis* phages and *Bacteroides* HB13 phages, respectively (Fig. 4.3). Although for all faecal indicator organisms a trend of higher removal in the reed beds compared with the unvegetated ponds was observed, this difference was only significant for *E. coli*, *C. perfringens* and *Bacteroidales* (p<0.05; Fig 4.3). Over the total constructed wetland the average removal for bacterial faecal indicators ranged between 96 and 99%, for protozoan faecal indicator was 89% and viral indicator between 73 and 88%.

**Fig. 4.3** Average removal of faecal indicator organisms (±s.e.; n=6-12) in the unvegetated ponds (black bars) and the reed beds (white bars).

**Fig. 4.4** The sedimentation fluxes of *E. coli* in the unvegetated ponds. Error bars represent uncertainty in calculated (linear regression) sedimentation fluxes.
Sedimentation

*E. coli* numbers in the sedimentation traps varied strongly between sampling moments and resulted in large error margins in the calculated sedimentation fluxes of *E. coli*, especially in the first unvegetated pond (Fig. 4.4). No significant sedimentation of *E. coli* was observed in the unvegetated ponds. Significant sedimentation of suspended matter (accounting for 10% of total inflow) was observed in the same sedimentation traps (van den Boomen et al. 2012), indicating proper functioning of the sedimentation traps.

Bacteroides phages

The ratio between bacteriophages infecting *B. fragillis* and *Bacteroides* HB13 was on average (±s.e.) 0.61 ±0.04, 0.61 ±0.04 and 0.53 ±0.07 at respectively PONDS-IN, PONDS-OUT and REED-BEDS-OUT. The ratio between bacteriophages infecting *B. fragillis* and *Bacteroides* HB13 showed no significant changes during residence in the unvegetated ponds or the reed beds, which indicates no input of faecal matter from warm blooded animals (including waterfowl) into the CW and a comparable removal rate of bacteriophages infecting *B. fragilis* and *Bacteroides* phages infecting *Bacteroides* strain HB13.

Zooplankton abundance and predation capacity

In the unvegetated ponds the average abundance of zooplankton (bacteriovores) was $2.2 \times 10^5 \pm 8.2 \times 10^4$ individuals L$^{-1}$ (±s.e.), the average abundance in the reed beds was similar with $2.1 \times 10^5 \pm 6.1 \times 10^4$ individuals L$^{-1}$ (Table 4.2). At both locations in the CW, >99% of the total zooplankton individuals identified were classified as bacteriovores. The zooplankton community showed seasonal variation with peak abundances in May, August and November and were observed in both the unvegetated ponds and reed beds (data not shown). The most abundant group of organisms in the unvegetated ponds and reed beds were Amoebozoa smaller than 30µm, followed by the Ciliophora smaller than 30 µm (Table 4.2). The community of zooplankton taxa larger than 30µm was dominated by Ciliophora species including *Vorticella* (Table 4.2). On average the abundance of Ciliophora as well as the bacteriovore species richness was higher in the reed beds compared with the unvegetated ponds (Table 4.2). The estimated average predation capacity of the total zooplankton community in the unvegetated ponds was $1.3 \times 10^7 \pm 4.9 \times 10^6$ bacteria h$^{-1}$ L$^{-1}$ (±s.e.), which was not significantly different in the reed beds where an average predation capacity of $2.1 \times 10^7 \pm 8.0 \times 10^6$ bacteria h$^{-1}$ L$^{-1}$ was estimated (Table 4.2). In both the unvegetated ponds and the reed beds the Ciliophora were in general the main contributors to the zooplankton community predation capacity (>90%) with the largest group being small Ciliophora (>73%). The small Amoebozoa were a stable but minor contributor (4%) to the community predation capacity (Table 4.2).

The predation capacity of the two most abundant zooplankton groups in the unvegetated ponds, small Ciliophora and Amoebozoa showed seasonal variation, with periods of low (June, July, Sep, Dec) and periods of high (August, November) predation capacity corresponding with the dynamics of *E. coli* removal (Fig 4.5). The predation capacity
Faecal indicator organisms

of small Amoebozoa showed a significant positive correlation with the log removal of E. coli in the unvegetated ponds (0.88; p<0.05). Some of the other zooplankton species, with lower contributions to the community predation capacity, did also show significant correlations between predation capacity and the log removal of certain specific faecal indicators as well. In the unvegetated ponds Nauplius-larvae correlated positively with the removal of C. perfringens (0.86; p<0.05) and Bacteroides HB13 phages (0.89; p<0.05), while Vorticella correlated negatively with the log removal of E. coli (-0.78; p=<0.05). In the reed beds, the correlations were generally weaker compared with the unvegetated ponds, with no significant correlations with the removal of bacterial or protozoan faecal indicators. Significant negative correlations were observed between the predation capacity of Ciliophora sp. (-0.84; p=<0.05) and Chydorus (0.96; p<0.05) with the removal of B. fragilis phages. Significant positive correlations were observed in the reed beds between Vorticella and Ceriodaphnia correlations and the removal of Bacteroides HB13 phages (-0.82; p=<0.05).

![Graph showing monthly E. coli (log_{10} CFU) removal and predation capacity of Ciliophora and Amoebozoa smaller than 30 µm.]

Fig. 4.5 Monthly E. coli (log_{10} CFU) removal and predation capacity of Ciliophora and Amoebozoa smaller than 30 µm.
Table 4.2 Bacteriovore zooplankton abundance (s.e.) in the unvegetated ponds and reed beds, and the calculated predation capacity.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Conversion factor</th>
<th>Unvegetated ponds</th>
<th>Predation factor</th>
<th>Abundance</th>
<th>Estimate predation capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conversion factor</td>
<td></td>
<td>(Bacteria ind.⁻¹ h⁻¹ L⁻¹)</td>
<td>Reference</td>
<td>(ind. L⁻¹)</td>
</tr>
<tr>
<td>Amoebozoa</td>
<td>&lt;30 µm</td>
<td>2</td>
<td>187942 (78849)</td>
<td>[1]</td>
<td>375885 (157697)</td>
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<tr>
<td>Arcellinida Arcella</td>
<td>200</td>
<td>[1] †</td>
<td>139 (67)</td>
<td></td>
<td>27751 (13313)</td>
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<td>Arcellinida Diffugia</td>
<td>284</td>
<td>[1] †</td>
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<td>Arcellinida Euglypha</td>
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<td>[1] †</td>
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<td>Gymnamoebae</td>
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<td>4 (2)</td>
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<td>Ciliophora</td>
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<td>388</td>
<td>32039 (12513)</td>
<td>[3,4]</td>
<td>12430938 (4854850)</td>
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<tr>
<td>Ciliophora sp.</td>
<td>950</td>
<td>[4,5,6,7,8,9,10,11]</td>
<td>255 (101)</td>
<td></td>
<td>242203 (95977)</td>
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<td>Vorticella</td>
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<td>Ceriodaphnia</td>
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<td>0 (0)</td>
<td>‡</td>
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<td>Nauplius-larvae</td>
<td>33</td>
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<td>Pompholyx</td>
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<td>†</td>
<td>27 (16)</td>
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<td>Synchaeta</td>
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<td>Total</td>
<td></td>
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<td>13164311 (4863663)</td>
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<tr>
<th>Faecal indicator organisms</th>
<th>Abundance (ind. L⁻¹)</th>
<th>Estimated predation capacity (Bacteria h⁻¹)</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amoebozoa</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;30 µm</td>
<td>160450 (54941)</td>
<td>320900 (109882)</td>
<td>4 (3)</td>
</tr>
<tr>
<td>Arcellinida Arcella</td>
<td>107 (52)</td>
<td>21328 (10389)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Arcellinida Diffugia</td>
<td>21 (10)</td>
<td>6077 (2744)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Arcellinida Euglypha</td>
<td>3 (2)</td>
<td>500 (357)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Gymnamoebae</td>
<td>7 (4)</td>
<td>2719 (1472)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>Ciliophora</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;30 µm</td>
<td>49507 (21325)</td>
<td>19208619 (8273956)</td>
<td>73 (13)</td>
</tr>
<tr>
<td>Ciliophora sp.</td>
<td>910 (420)</td>
<td>864225 (399434)</td>
<td>16 (10)</td>
</tr>
<tr>
<td>Vorticella</td>
<td>661 (621)</td>
<td>255162 (239594)</td>
<td>3 (3)</td>
</tr>
<tr>
<td><strong>Cladocera</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ceriodaphnia</td>
<td>42 (41)</td>
<td>62504 (61786)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Chydorus</td>
<td>10 (3)</td>
<td>15951 (5338)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Daphnia sp.</td>
<td>20 (12)</td>
<td>87363 (52708)</td>
<td>2 (1)</td>
</tr>
<tr>
<td>Daphnia ambigua</td>
<td>10 (10)</td>
<td>45500 (45500)</td>
<td>1 (1)</td>
</tr>
<tr>
<td><strong>Copepoda</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Harpacticoida</td>
<td>&lt;1 (0)</td>
<td>11 (8)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Nauplius-larvae</td>
<td>216 (156)</td>
<td>7117 (5164)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Nematoda</td>
<td>28 (13)</td>
<td>581 (277)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>Rotifera</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brachionus</td>
<td>6 (6)</td>
<td>5500 (5500)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Conochilus</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Keratella quadrata</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Polyarthra</td>
<td>1 (1)</td>
<td>179 (179)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Pompholyx</td>
<td>&lt;1 (0)</td>
<td>211 (211)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Rotifera sp.</td>
<td>68 (27)</td>
<td>13571 (5370)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Synchaeta</td>
<td>1 (1)</td>
<td>325 (325)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>212367 (60890)</td>
<td>20918343 (8023309)</td>
<td></td>
</tr>
</tbody>
</table>
Laboratory incubations

Average *E. coli* numbers at the start of the lab incubations were 213±5 and 233±7 CFU mL\(^{-1}\) in respectively the unfiltered and filtered (5 µm) samples (Fig 4.6). The different treatments caused no significant difference between treatments in the pH (8.69 ±0.05) and dissolved oxygen (7.76 ±0.11 mg L\(^{-1}\)) until the end of the incubation period (data not shown). Incubation of the samples under field conditions (Fig. 4.6a) resulted in a constant decrease of *E. coli* numbers over time resulting in 72 ±6 CFU mL\(^{-1}\) after 69 h incubation. Removal of predation pressure by filtration of the samples (5 µm) before incubation (Fig 4.6c) resulted in an increase of *E. coli* numbers. Exposure to UV radiation of filtered samples (Fig 4.6b) caused a decrease in *E. coli* numbers to 35 ±19 CFU mL\(^{-1}\) after 69 h incubation. Incubation of filtered samples at a higher temperature (20°C; Fig 4.6d) resulted in an initial increase of *E. coli* to 311 ±7 CFU mL\(^{-1}\), but was followed by a steady decrease in the following days to 144 ±27 CFU mL\(^{-1}\) after 69 h of incubation.

![Fig. 4.6](image_url)  
*Fig. 4.6 E. coli numbers (CFU; ±s.e.; n=3) in incubated WWTP effluent (Grou) over time. Zooplankton [+]: unfiltered water; Zooplankton [-]: 5µm filter water; UVR [-]: only white light; UVR [+]: white light and UV exposure; temperature: 10 or 20°C.*
Discussion

Removal of faecal indicator organisms

The removal efficiencies for E. coli and enterococci observed in this study were relatively low compared to other studies (Molleda et al. 2008; Reinoso et al. 2008; van den Boomen and Kampf 2012). Bacterial pathogens were more efficiently removed than protozoa and viral pathogens, which seem to be related to the differences in inflow concentrations. However, the low numbers of faecal bacterial, protozoan and viral indicators in the effluent of the constructed wetland still demonstrated that the constructed wetland is a suitable tool to reduce the potential threat of treated wastewater for water recreation and drinking water production.

For all faecal indicator organisms the average removal tended to be higher in the reed beds than in the unvegetated ponds, which can only partially be explained by the larger hydraulic retention time in the reed beds. In the next paragraphs we try to explain the difference in removal efficiency of the indicator organisms between the unvegetated ponds and reed beds by tentatively comparing the relative importance of sedimentation, predation, UV exposure, natural mortality and biofilm trapping for the removal efficiency.

Sedimentation

Observations with the sedimentation traps showed that sedimentation of E. coli in the unvegetated ponds is an unimportant process in the removal of E. coli, despite 10% sedimentation of the inflowing suspended matter (van den Boomen et al. 2012). The low sedimentation rate of E. coli is in concurrence with a study of Boutilier et al. (2009), who concluded that sedimentation contributed less than 3% of E. coli removal in wastewater treatment wetlands. The importance of sedimentation is mainly dependent on the association of microorganisms to suspended matter as “free living” microorganisms exhibit very low sedimentation rates (Brookes et al. 2004; Medema et al. 1998; Boutilier et al. 2009; Jin et al. 2004). In our study, the laboratory experiments showed that the filtration of WWTP effluent over a 5µm filter did not influence the E. coli concentration, indicating that the majority of E. coli cells was indeed either free living or associated with particles smaller than 5µm. The constructed wetland in this study receives very low concentrations (3.6 mg L⁻¹) of suspended matter (Ghermandi et al. 2007). Low concentrations of inflowing suspended matter probably prevent substantial aggregation of faecal organisms like E. coli with suspended matter, thereby reducing the importance of sedimentation for the removal of faecal microorganisms in this constructed wetland. Due to the dimensions of the reed beds no sedimentation traps could be placed and a no good estimation of sedimentation rates E. coli in the reed beds could be made. The inflow of suspended matter is however low and a substantial increase is sedimentation of faecal indicator organisms is unlikely.
Predation on the microbial consortia by zooplankton was dominated by Ciliophora (and to lesser extent Amoebozoa) in both the unvegetated ponds and the reed beds. The importance of Ciliophora to the total predation rate on particles, including microorganisms, has been shown by several studies (Albright et al. 1987; Sanders et al. 1989; Vaque et al. 1994). Ciliophora comprise a large and diverse group of organisms which differ in size, behaviour and occurrence (Brusca and Brusca 2003). Ciliophora prefer to ingest freely suspended rather than attached bacteria (Albright et al. 1987). They can be either non-selective or selective grazers, some preferring rod-shaped coliforms (incl. *E. coli*) over coccus-shaped enterococci (Epstein and Shiaris 1992). Several other studies have indicated that bacterial grazing by zooplankton is depending on the size of both bacteria and grazers (Porter et al. 1983; Knoechel and Holtby 1986; Hart and Jarvis 1993). The correlations between the removal of an individual faecal indicator and specific zooplankton species as shown in this research seem to support selective predation of zooplankton species on faecal microorganisms. The high correlations between the removal of faecal indicators and the abundance of the most numerous zooplankton species suggest that predation by zooplankton is an important contributor to the removal of the faecal indicator organisms in the unvegetated ponds. These findings in the field were supported by laboratory experiments which demonstrated that *E. coli* removal is strongly reduced when the entire zooplankton community is removed. Removal of faecal indicator organisms is used to predict the behavior of faecal pathogens like *Campylobacter*, Norovirus, Enterovirus and *Cryptosporidium*. Because zooplankton species might selectively predate on certain microbial species, it remains uncertain whether faecal pathogens are removed by the zooplankton community to the same extent as the faecal indicators measured in this study. Additional laboratory experiments are required to demonstrate the effect of predation on other faecal pathogen species. Although the abundance of zooplankton was similar in the unvegetated ponds and the reed beds throughout the year, substantial less correlations were found in the reed beds between faecal indicator removal and zooplankton abundance. This does not directly mean that the predation on faecal organisms by zooplankton is not occurring in the reed beds, but the contribution of predation on the total removal of faecal microorganisms in the reed beds is probably lower, as lower numbers of inflowing faecal indicator organisms make them a small source of food and other processes including biofilm retention may be more important. In general, the predation capacity of the zooplankton community in both the unvegetated ponds and reed beds exceeded the total number of faecal indicator organisms (excluding the gene copies of *Bacteroidales*) with two orders of magnitude, indicating that the predation capacity of the zooplankton community is a driving force for the removal of faecal indicator organisms and pathogens in constructed wetlands.
**UV exposure**

The influence of UV radiation on the removal of the faecal indicator organisms in treated wastewater during residence in constructed wetlands was shown by the laboratory experiments to be potentially strong and has been proven to effectively decrease the concentration of bacterial and viral organisms in surface waters, wastewater and other media (Garvey et al. 1998; Sommer et al. 2000; Craik et al. 2001; Brookes et al. 2004; Tchobanoglous et al. 2004; Whitman et al. 2004; Oteiza et al. 2005). Sinton et al. (2002) found that the inactivation of bacteria was ten times higher in sunlight than in darkness and concluded that this effect is mainly caused by UV-B radiation. However, we do not expect this to be an important factor in the constructed wetland itself: based on the calculated light attenuation coefficient estimated from the average DOC content of the water (Morris et al. 1995) at PONDS-IN, the penetration of UV radiation ranges from 15 to 35 cm for UV-A (380 nm) and from 3 to 6 cm for UV-C (250 nm). Because of this low penetration of especially UV radiation of low wavelength (UV-C), which is most harmful for organisms, only a minor contribution to the disinfection of faecal microorganisms is expected for UV radiation in the unvegetated ponds (average depth 2 m). The reed beds are covered by a thick layer of vegetation most of the year which will lower UV irradiance strongly (Boutilier et al. 2009) and reduce the disinfection contribution of UV radiation in the reed beds, even with a shallow average water depth of 30 cm.

**Inactivation of faecal indicators**

The inactivation of microorganisms is another factor contributing to the reduction in numbers of faecal indicator organisms in constructed wetlands. Several studies (Boutilier et al. 2009; Darakas 2002) show that *E. coli* can survive twice as long at temperatures around 10°C compared with 20°C and that 10°C is actually the optimal survival temperature for *E. coli* (Darakas 2002). Similar results were observed in our study where incubation at 20°C caused a substantial reduction in *E. coli* numbers, whereas incubation at 10°C did not result in a decrease of *E. coli*. The contribution of inactivation to the removal of faecal indicator organisms in constructed wetlands is however dependent on individual indicator organisms (*E. coli*, enterococci, Bacteroides-phages, etc), temperature, organic matter, redox conditions, etc and is therefore difficult to estimate. The temperature dependence on the natural mortality may contribute to higher removal efficiencies in summer compared with winter. Khatiwada and Polprasert (1999) estimated a contribution to coliform removal by temperature modulated death in pond systems be relatively minor (6.5% at 20°C).

**External input**

The number of *Bacteroides* phages indicated that there was no significant external input from June till December by warm blooded animals like birds. Several studies (Kadleček et al. 2010; Knowlton et al. 2002) have observed the attraction of large numbers of waterfowl to constructed wetlands during winter, especially in freezing periods. In this study generally
around five water birds were observed in the unvegetated ponds of the constructed wetland, but in January and February around 100-200 birds were counted in the ponds. The faeces of waterfowl can contain high numbers of faecal indicator organisms like *E. coli* and enterococci but also pathogens like *Campylobacter* (Moriart et al. 2011; Moriart et al. 2012; Meerburg et al. 2011; Benskin et al. 2009). On a daily base each individual bird can add $10^6-10^{10}$ CFU of both *E. coli* and Enterococci to the water (Moriart et al. 2011). Based on these numbers and assuming total mixing of the faeces, the waterfowl population approximately in the unvegetated ponds in wintertime could increase the abundance of *E. coli* and enterococci by 80-170 CFU L$^{-1}$. This addition of $10^1$ to $10^2$ CFU L$^{-1}$ is very minor in comparison with the average removal of $10^4$ CFU L$^{-1}$ and external input by waterfowl is negligible in the faecal indicator dynamics.

**Trapping in biofilm**

The trapping of bacteria and viruses in biofilms is another factor that could contribute to the removal of faecal microorganisms in reed beds, but was not addressed in this study. Biofilms are known to retain large quantities of small sized particles in the size range of viruses (0.1 µm), bacteria (1 µm) and parasitic protozoa (4.5 µm) (Stott and Tanner 2005; Balzer et al. 2010; Drury et al. 1993a; Drury et al. 1993b; Eisenmann et al. 2001). The reed beds in our study were more efficient in removing faecal indicator organisms from the water, which could only partially be explained by a difference in HRT. The submerged parts of the reed stems in the reeds beds in the CW form a large surface area for biofilm development and it is therefore expected that particle trapping by these biofilms is an important factor causing the difference in removal efficiencies between unvegetated ponds and reed beds.

**Conclusion**

This study showed that constructed wetlands significantly reduce the number of bacterial, protozoan and viral faecal indicators. As these organisms are used as an indication for removal of faecal pathogens it can be concluded that treatment of WWTP effluent by a constructed wetland results in lower numbers of faecal pathogens, effectively reducing the discharge of pathogens into receiving surface waters.

At the inflow of the constructed wetland *E. coli* cells were observed to be mostly planktonic (not associated with suspended matter) together with low concentrations of suspended matter for aggregation. Consequently, the contribution of sedimentation to the removal of these faecal indicators was low for *E. coli* and probably also for other faecal indicator organisms and pathogens. Planktonic bacterial cells and virus particles are however favorable for zooplankton predation which was observed to be an important process regulating the removal of faecal indicators in the unvegetated ponds. However, indications of selective grazing by individual zooplankton groups and species make the contribution of predation to pathogen removal variable and species specific and may therefore differ between faecal
indicator organisms and other pathogens. UV light was shown to have a strong potential impact on the removing of faecal indicator organisms in a laboratory experiment, but the dimensions and characteristics of the CW combined with high DOC concentrations in the treated wastewater, hamper penetration of UV light into the water column and thereby minimize the effects of UV light of the removal of faecal indicators. Although inactivation of faecal indicator organisms and pathogens in the CW was difficult to quantify, literature suggests a low contribution of inactivation to the total removal of faecal indicator organisms. Significant reintroduction of faecal indicator organisms by defecation by waterfowl and other warm blooded animals was not observed, but could be periodically significant in systems with low flow rates and a high surface area. In the reed bed systems, removal rates of faecal indicator organism were higher than in the unvegetated ponds. Here it is argued that particle trapping by biofilms growing on the reed stems is an important factor causing the difference in removal efficiencies between the two functional compartments in the CW. Based on these observations it can be concluded that the biological components of CW support the driving forces determining the capacity to remove “free living” faecal indicator organisms from treated wastewater.

**Acknowledgments**

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Chapter 4

References


Faecal indicator organisms


Faecal indicator organisms


Sinton LW, Hall CH, Lynch PA and Davies-Colley RJ (2002). Sunlight inactivation of fecal indicator bacteria and
bacteriophages from waste stabilization pond effluent in fresh and saline waters. Applied and Environmental Microbiology 68 (3), 1122-1131.


van den Boomen R, Kampf R and Mulling BTM A (2012). Research on suspended particles and pathogens in the Waterharmonica (constructed wetland). ISBN.978.90.5773.553.0 STOWA 2012-10


