A mass balance approach to the fate of viruses in a municipal wastewater treatment plant during summer and winter seasons
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ABSTRACT

In contrast to previous discussion on general virus removal efficiency and identifying surrogates for human pathogenic viruses, this study focuses on virus retention within each step of a wastewater treatment plant (WWTP). Additionally, the influence of weather conditions on virus removal was addressed. To account for the virus retention, this study describes a mass balance of somatic coliphages (bacterial viruses) in a municipal WWTP, performed in the winter and summer seasons of 2011. In the winter season, the concentration of coliphages entering the WWTP was about 1 log lower than in summer. The mass balance in winter revealed a virus inactivation of 85.12 ± 13.97%. During the summer season, virus inactivation was significantly higher (95.25 ± 3.69%, p-value < 0.05), most likely due to additional virus removal in the secondary clarifier by insolation. Thus, a total removal of coliphages of about 2.78 log units was obtained in summer compared to 1.95 log units in winter. Rainfall events did not statistically correlate with the concentrations of coliphages entering the WWTP in summer.

INTRODUCTION

Viruses, somatic coliphages and human adenoviruses have been proposed as useful indicators for testing microbial water quality (Bosch 1998; Silva et al. 2011). Both inhabit the gastro-intestinal tracts of humans and multiply inside their respective host cells: somatic coliphages in the bacterium Escherichia coli (E. coli) and adenoviruses in human cells. In contrast to adenoviruses, somatic coliphages may also multiply in various environments depending on the presence of their hosts and favourable environmental conditions (e.g. temperature, availability of substrates) (Sillankorva et al. 2004).

The main sources for the spread of human viruses in the environment are effluents of wastewater treatment plants (WWTPs) and combined sewer systems. In rivers receiving high loads of wastewater, human pathogenic viruses may be found at concentrations up to 10–100 infectious units per litre (IU/l), in contrast to less than 1–10 IU/l in surface waters with low contamination (Botzenhart 2007). The amount of viruses entering and leaving WWTPs has already been assessed in several studies, e.g. Lodder & De Roda Husman (2005) or Muniesa et al. (2012). However, so far, there has been little discussion on the contribution of specific steps in WWTPs to the retention of viruses and how weather conditions may influence virus removal in these processes. Rainfall events, for example, may lead to higher pathogen loads entering the wastewater treatment facilities, but their specific impact in wastewater treatment is difficult to evaluate. According to Carducci & Verani (2015) only one out of four monitoring campaigns revealed a significant lower removal of human adenovirus on rainy days as compared to dry days, which was unexpected.

Viruses infecting bacteria (bacteriophages) are very similar to human pathogenic viruses with regard to their surface structures, physical characteristics, tenacities and elimination properties. Especially bacteriophages infecting bacteria of the human gut (e.g. somatic coliphages) are very similar to human viruses, but much easier to monitor. Somatic coliphages have been suggested to be early
indicators of faecal pollution of waters additionally to the classical indicator bacteria (E. coli and intestinal enterococci); these phages are especially useful for monitoring the presence of contaminations with human viruses (Bosch 1998; Selinka et al. 2011).

For our study, concentrations of all somatic coliphages (subsequently referred to as total somatic coliphages or coliphages) are chosen as indicators for the putative presence of human pathogenic viruses, since these bacteriophages can be detected along the different processes in WWTPs (Guzmán et al. 2007). A mass balance of total somatic coliphages was calculated for a municipal WWTP in order to quantify the virus retention within each of the individual processes of the plant as well as to assess the influence of weather conditions (summer and winter of 2011) on virus removal.

**METHODS**

*In vitro* growth potential of the somatic coliphage PhiX174

The presence of the host E. coli in wastewater suggests the possibility for somatic coliphages to multiply. Therefore, a preliminary *in vitro* test of the growth potential was conducted in a static culture with the E. coli strain WG5 (DSM 18455, ATCC: 700078) and the somatic coliphage PhiX174. Synthetic wastewater (SWW, DIN 38412-26:1994-05 1994) and modified Scholten Bouillon (MSB, ISO 10705-2 2001) were used to evaluate the effect of nutrient availability on the growth of PhiX174. The cultures were incubated at 20 °C and 10 °C, corresponding to the average wastewater temperatures in summer and winter, respectively. The SWW was used with slight modifications: distilled water was used instead of tap water and the pH was kept at 7.8 with the addition of a carbonate buffer (98 mg/l NaHCO₃). For the static culture of E. coli and PhiX174, a multiplicity of infection (ratio between bacteria and phage) of approximately one was used, as previously assessed in the WWTP (data not shown).

Abundance of *E. coli* and sensitivity to PhiX174 in activated sludge

The abundance of E. coli relative to the total cultivable bacteria in activated sludge was determined by the plating method and expressed in colony forming units per litre (CFU/l). E. coli was grown on chrome coliform agar (CCA; Roth, CL45.1) and incubated overnight at 37 °C, while total cultivable bacteria were grown on R2A agar (Difco) and incubated at 20 °C for 44 h. E. coli colonies were distinguished from other colonies growing on the CCA by their blue to dark-violet colour, resulting from the enzymatic cleavage of chromogenic substrates. The blue-coloured colonies were used in a spot test to evaluate the sensitivity of E. coli to PhiX174. The spot test was performed according to Carlson (2005) and modified for an application in 24-well plates (Brand).

**WWTP and samples**

Samples were collected from a municipal WWTP with a capacity of about 500,000 population equivalents, receiving an average flow of 78,983 m³/d in winter and 62,401 m³/d in summer. The WWTP includes an activated sludge tank in which a pre-denitrification step is followed by biological phosphorus removal and nitrification. The average concentrations of total suspended solids (TSS) in the activated sludge tank were 4.0 g/l in winter and 3.4 g/l in summer. A total sludge retention time of about 12.1 d in winter and 10.9 d in summer was maintained constant during all investigations. Biosolids from the activated sludge were concentrated (216 m³/d with 67 g/l TSS) and anaerobically degraded under mesophilic conditions (37 °C) in a digester operating with a retention time of about 19 d.

To account for the fate of total somatic coliphages in the WWTP, samples were collected in February and in August/September of the year 2011. Taking samples in different seasons enabled us to consider the effects of weather conditions. Samples were taken at the influent and effluent of the primary clarifier, the activated sludge tank and the effluent of the secondary clarifier. The concentrations of coliphages were measured in single grab samples nearly every working day for 6 weeks in summer (28 samples) and 4 weeks in winter (16 samples). The winter sampling period included 13 d with rainfall events, with a total amount of rain of 25 mm and an average wastewater temperature of 11.6 ± 0.2 °C. In the summer, both the number of days with rainfall and the total amount of rain were higher, 19 d with 68 mm of rain, as was the average wastewater temperature, 18.9 ± 0.2 °C. The sampling time, assessed in a diurnal measurement cycle (every 2 h), operated at all sampling locations in summer (data not shown), was set to 8 a.m. and corresponded to the highest daily concentrations of coliphages in the effluent of the primary clarifier. Additionally, samples were taken in winter in the influent and effluent of the digester. Furthermore,
concentrations of human adenoviruses present in the WWTP samples were determined every working day during a 1 week period in summer (five samples).

Quantification of coliphages

The samples were analysed for virus concentration in both the mixed liquor and in the aqueous phase of the samples immediately after collection. The aqueous phase was obtained after separating the flocs by centrifuging the samples at 4,000 g for 10 min at 4 °C (Sigma 2K15). The presence of somatic coliphages in wastewater and test phage PhiX174 was determined according to ISO 10705-2 (2001) and expressed in plaque-forming units per litre (PFU/l). This standardized ISO method is based on the cultivation and enumeration of somatic coliphages in the presence of E. coli using the double-agar-layer technique.

Quantification of adenoviruses

The adenoviruses were quantified by a cultivation-independent method: the quantitative polymerase chain reaction (qPCR). Therefore, DNA extraction from wastewater samples was performed with the NucliSens Magnetic Bead Extraction Kit (Biomerieux, France) according to the manufacturer’s instructions. Then the viral nucleic acids were directly subjected to qPCR or stored at −80 °C. Human adenoviruses were quantified by Taqman qPCR on an ABI Prism 7500 sequence detection system (Applied Biosystems, Germany), as described by Hernroth et al. (2002) and Bauer et al. (2011), with additional controls to exclude putative inhibitory effects of environmental matrices. Each test consisted of duplicate sets of undiluted and diluted (1:10) samples, supplemented by samples with spiked adenoviral DNA to assess the degree of putative inhibitory effects. The human adenovirus-specific primers, probes and quantification of the Ad41 plasmid standards were previously described by Hernroth et al. (2002). The qPCR detection limits for adenoviruses stayed in the range of 1–10 copies/ml, depending on the original sample volumes.

Calculations

Based on a mass balance of the WWTP (Figure 1) the following values were calculated:

(1) Theoretical enrichment of coliphages in the activated sludge tank (X_{AS,\text{theoretical}}) within one sludge age (in PFU/l)

\[
X_{AS,\text{theoretical}} = X_{PC,\text{out}} \cdot (\text{SRT/HRT}) - X_{SC,\text{out}}
\]

(2) Coliphages inactivated in the activated sludge tank (in %)

\[
X_{AS,\text{inactivated}} = \left(\frac{X_{AS,\text{theoretical}} - X_{AS}}{X_{AS,\text{theoretical}}}\right) \cdot 100
\]

(3) Coliphages released in the effluent of the secondary clarifier (in %)

\[
X_{\text{not-removed}} = \left(\frac{X_{SC,\text{out}}}{X_{PC,\text{in}}}\right) \cdot 100
\]

(4) Coliphages removed in surplus sludge (in %)

\[
X_{\text{removed,SS}} = \left(\frac{(\text{HRT/SRT}) \cdot X_{AS,\text{theoretical}}}{X_{PC,\text{out}}}\right) \cdot 100
\]

Statistical analysis

The StatGraphics Software X64 was used for the following statistical analyses: (i) unpaired t tests (different variance) to compare virus inactivation in the activated sludge system in summer and winter and (ii) multiple regression analyses to evaluate correlations between the concentration of somatic coliphages in the effluent of the primary clarifier, the daily wastewater inflow and meteorological parameters (rainfall, sunshine hours and temperature) in summer. The data collected in winter were insufficient to perform multiple regression analyses.
RESULTS AND DISCUSSION

Growth of bacteriophage PhiX174 in vitro and in activated sludge

After 48 h of growth at 20 °C in a rich nutrient medium (MSB) with E. coli WG 5, the concentration of phage PhiX174 increased by about 4.20 logPFU/l. On the other hand, in experiments with SWW, a comparatively poor nutrient medium, PhiX174 reached titers of about 3.15 logPFU/l under the same conditions. The lag phase in phage replication lasted 8 h in MSB, whereas it took 24 h before an increase in phage titers could be measured in SWW at 20 °C. According to Sillankorva et al. (2004), the host growing conditions (e.g. nutrient availability and temperature) strongly influence the multiplication of phages. In our assays, a decrease in temperature from 20 to 10 °C increased the lag phase in SWW wastewater from 24 to 144 h. It seems likely that the growth of E. coli at sub-optimal conditions of nutrient availability (SWW as compared with MSB) and temperature (10 °C/20 °C instead of 37 °C) has contributed to the slower growth of PhiX174 in the present study.

Withey et al. (2005) discovered higher coliphage concentrations in the activated sludge than in the primary clarifier, which implies that phages can multiply in the activated sludge tank. Our results do not support their findings, since, as shown in Figure 2, the concentration of coliphages in the primary clarifier (7.87 ± 0.43 logPFU/l) was similar to the one in the activated sludge tank (7.53 ± 0.46 logPFU/l). Possible explanations are either a decreased multiplication ability of the somatic coliphages due to low concentrations of the host E. coli or a high number of resistant E. coli strains. Both possibilities would decrease the chance of the phage to multiply in the WWTP, so it was important to identify the most likely reason. The comparison of total bacterial count on R2A agar and E. coli grown on CCA agar established that only about 3% of the bacteria in the activated sludge were E. coli. The subsequent spot test revealed about 72% of these E. coli to possibly be infected by PhiX174. These results suggest that a very low host concentration was responsible for the somatic coliphages’ lack of multiplication in the activated sludge (also compare Jofre (2009)). Although in the spot test most of the E. coli strains were sensitive and therefore can be infected by the phage for multiplication, it was more likely for the phage to be adsorbed by other bacteria, than to find their host in the activated sludge.

Winter season mass balance of coliphages in the WWTP

Based on the scheme of the WWTP and the sampling points presented in Figure 1, a mass balance for the WWTP was compiled using the arithmetic average concentrations of coliphages determined in the winter season (Figure 2). Digester data were not included in this balance. Considering coliphages as nanoparticles adsorbed by sludge flocks (Gerba et al. 1978), an enrichment of coliphages was expected to occur in the activated sludge tank due to recirculation. However, the expected concentration of coliphages in the activated sludge tank (X_{AS, theoretical}; see Equation (1)), calculated to be 2.26 × 10^6 PFU/l, was significantly higher than the measured data, 2.57 × 10^7 PFU/l, suggesting a putative inactivation of 85.12 ± 13.97% of the coliphages (see Equation (2)). According to scientific discourse, predation by bacteria and protozoa (Kim & Unno 1996) as well as enzymatic degradation (Elliott et al. 2011) contributes to the reduction of virus particles. From the coliphages entering the WWTP, only 1.43 ± 1.13% were discharged to surface waters via secondary effluents (see Equation (3)) and 14.47 ± 13.97% were moved by the surplus sludge in the digester (see Equation (4)).

As depicted in Figure 2, no significant differences between the concentrations of coliphages in the mixed liquor samples of the primary clarifier effluent (6.89 ± 0.56 logPFU/l) and the activated sludge tank (7.16 ± 0.19 logPFU/l) were found in the winter sampling period. However, in contrast to the primary clarifier and the activated sludge, the concentration of coliphages in the effluent of the secondary clarifier (4.94 ± 0.19 logPFU/l) ranked about
1.95 log lower than in the primary clarifier. This is consistent with results of previous studies regarding the concentration of coliphages in the range from 6.5 to 7.72 log PFU/l for the primary clarifier (Contreras-Coll et al. 2002; Muniesa et al. 2012) and 4.37 to 5.5 log PFU/l for the secondary clarifier (Lodder & De Roda Husman 2005; De Luca et al. 2013). This reduction of coliphages in the primary clarifiers, as compared to the secondary, ranges from 1.1 log (Lodder & De Roda Husman 2005) to 3.3 log (Muniesa et al. 2012). As demonstrated in Figure 2 by the different concentrations of coliphages in the mixed liquor (black columns) and aqueous phase samples (white columns), adsorption of coliphages primarily took place on the flocs in the activated sludge tank. In this study the magnitude of coliphage adsorption to sludge, $3.82 \times 10^6$ PFU/gTSS, was considerably higher than the one reported by Lasobras et al. (1999), $1.5 \times 10^4$ PFU/gTSS. The amount of coliphages in the surplus sludge after centrifugation was $1.61 \times 10^6$ PFU/gTSS (Figure 2), whereas Mandilara et al. (2006) found only $3.3 \times 10^5$ PFU/gTSS. In the digester, operated under mesophilic conditions, we observed a phage reduction of about 1.6 log units (Figure 2), which was more than Lasobras et al. (1999), Guzmán et al. (2007) and Mandilara et al. (2006) reported: respectively 0.23 log, 0.90 log and 1.27 log units. In the present study, the log reduction in coliphages was slightly higher in the activated sludge system (1.95 log in winter and 2.78 log in summer) than in the digester (1.6 log).

Comparison of coliphages removal in winter and summer

Interestingly, as shown in Figure 3, summer concentrations of coliphages in the primary clarifier effluent ($7.87 \pm 0.43$ log PFU/l) and activated sludge ($7.53 \pm 0.46$ log PFU/l) were about 1 log higher than in winter, whereas concentrations of coliphages in the secondary clarifier effluent ($5.09 \pm 0.21$ log PFU/l) were similar to the ones measured in winter. Subsequently, the removal of coliphages in the WWTP increased from 1.95 log units in winter to 2.78 log in summer. These results are consistent with results from Lucena et al. (2004). Regarding the mass balance for the WWTP (Figure 1), a higher inactivation of coliphages was found in summer (95.25 ± 3.69%) than in winter (89.12 ± 13.97%), $p$-value = 0.014, $\alpha = 0.05$). Consequently, from the coliphages entering the WWTP in summer, only 0.22 ± 0.16% were discharged to surface waters via secondary effluent and 4.74 ± 3.69% were moved by the surplus sludge in the digester. In summer, the decrease in the concentration of coliphages of about 1 log in the secondary clarifier is most likely due to insolation.

Detection of adenoviruses in the WWTP

The concentrations of human adenoviruses entering and leaving the WWTP were determined during a 1 week testing period in the summer (data not shown). The WWTP reduced adenovirus concentrations with an average of $1.74 \times 10^7$ PCR units/l in the primary clarifier effluent to $1.15 \times 10^5$ PCR units/l in the secondary clarifier effluent, resulting in a total reduction of about 2.32 log units. This value is quite similar to the total reduction of coliphages (2.76 log) obtained in the same period. These results confirm the data by Kuo et al. (2010) and Hewitt et al. (2011) and support the suggestion that somatic coliphages and human adenoviruses behave quite similarly in the WWTP.

In the present study, the intensity of rainfall events in the summer was higher than in winter. However, no statistical correlation was found between the concentration of coliphages in the primary clarifier effluent and the intensity of the rainfall events conforming with Carducci & Verani’s (2013) recent results. While a detailed but not extensive sampling was done in our study (daily sampling for 4–5 weeks), more extended data collection on rainfall events is required to determine exactly how weather conditions influence the amount of viruses entering the WWTP and the removal in the different processes.
CONCLUSIONS

This work investigated the fate of somatic coliphages in a municipal WWTP using a mass balance approach. The findings suggest that virus inactivation in the activated sludge system is significantly higher in the summer (95.25 ± 3.69%) than in the winter (85.12 ± 13.97%). Virus removal took place mainly in the activated sludge tank by adsorption on the flocs within one hydraulic retention time. No statistical correlation was found between the concentration of coliphages in the primary clarifier effluent and the intensity of rainfall events during the summer and winter, despite the fact that bacteriophage concentrations entering the plant as well as the intensity of the rainfall events were higher in the summer than in the winter.

In order to improve water quality, the vulnerability of WWTPs to extreme climate events needs to be assessed more accurately to ensure better protection of the water bodies receiving wastewater effluents.

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