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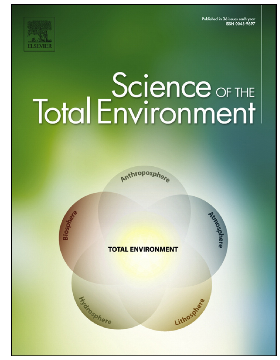

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Presence of bacteria and bacteriophages in full-scale trickling filters and an aerated constructed wetland

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Abstract

Aerated Constructed Wetlands are a state-of-the-art design that provides a different physical and chemical environment (compared to traditional passive wetland designs) for the wastewater treatment processes and, thus, may have different pathogen removal characteristics. In order to establish the fate of bacterial and viral indicators, a field study was carried out at a Sewage Treatment Works (STW) in the UK for a 5-month period. The STW consists of primary and secondary sedimentation and trickling filters (TF) as the biological stage. A large pilot aerated Vertical Flow Constructed Wetland (AVFCW) was constructed at the STW as tertiary stage receiving ¼ of the total flow rate. Effluent quality of the AVFCW complied with national and international standards for environmental discharge and reuse. For the first time, two sets of bacterial (Faecal coliforms, E.coli and intestinal enterococci) and viral indicators (Somatic coliphages, F-RNA specific bacteriophages and human-specific B. fragilis GB124 phages) were simultaneously investigated in an AVFCW. High elimination rates were detected (up to 3.7 and 2.2 log reduction for bacteria indicators an phages, respectively) and strong correlations between the two sets were found. The superior efficiency of the aerated Constructed Wetlands in microbiological contamination removal compared to passive wetland systems was established for the first time, which may have implications for process selection for wastewater reuse. This field study therefore provides new evidence on the fate of bacteriophages and a first indication of their potential use for performance evaluation in TF and aerated Constructed Wetlands.

Keywords: aerated constructed wetland; trickling filter; bacteriophages; indicator bacteria; wastewater reuse, sewage treatment works .

1. Introduction

Traditionally, the primary goal of wastewater treatment is to reduce the load of pollutants that have an immediate environmental impact on receiving waters such as organic matter and nutrients. Wastewater of human origin also contains various pathogenic microorganisms that pose a threat to public health, especially when discharged to surface waters or reused with inadequate previous treatment. Therefore, when wastewater is to be reused or there are concerns of direct public exposure (e.g. bathing waters), it is necessary to reduce the numbers of these microorganisms below a certain level before the final discharge or reuse of the treated effluent (Stefanakis and Akratos, 2016). Sanitary efficiency, i.e., the elimination of pathogenic microorganisms from wastewater, is a growing concern because of global moves to recycle treated wastewaters where possible. Specifically, the removal of enteric viruses is a major challenge in wastewater treatment, given that most viruses are smaller than bacteria and can pass through conventional biological treatment processes, e.g., activated sludge systems and trickling filters (Shang et al., 2005) and have low minimum infection doses. Thus, an additional ‘tertiary’ treatment stage is often necessary to further enhance the elimination of enteric bacteria and viruses.

Constructed Wetlands (CW) have been widely used as an efficient, cost-effective and sustainable wastewater treatment technology (Stefanakis and Tsihrintzis, 2012a; Stefanakis et al., 2014; Wu et al., 2015). Continuous research and optimization resulted in a variety of advanced phytoremediation designs, one design being promoted is wetlands with artificial aeration, characterized by enhanced efficiency, high effluent quality and reduced footprint (Boog et al., 2014). CWs have also been applied as a polishing stage, i.e., to upgrade existing conventional treatment plants (Butterworth et al., 2013). Trickling filters (TF) are in use for many decades, providing good effluent quality along with robust operation and relatively low energy consumption (Daigger and Boltz, 2011; Metcalf and Eddy, 2014). The combination of TF and CW has been proposed as an integrated system for wastewater management (Maheesan et al., 2011; Kim et al., 2014) and has been applied in the UK (Gardner et al., 2013).

The performance of wastewater treatment systems is usually evaluated using pollutant indicators that have an impact on the receiving environment, i.e., organic matter (BOD_5 / COD) and nutrients (nitrogen, phosphorus). However, as more stringent standards are continuously adopted for the safe effluent reuse and human exposure, more focus is given on the microbiological aspects of wastewater treatment. The sanitary quality of wastewater through the treatment path is mainly assessed using traditional indicator microorganisms such as Total and Faecal Coliforms. Various physical (sedimentation, filtration, adsorption), chemical (UV oxidation, biocides excretion) and biological (predation, biolysis, natural die-off) processes have been reported as pathogen removal mechanisms in CW and TFs (Stevik et al., 2004; Vacca et al., 2005; Wand et al., 2007; Stefanakis et al., 2014; Wu et al., 2015; Stefanakis and Akratos, 2016). Generally, pathogens survive best in dark, acidic, anoxic conditions with high concentrations of organic matter with plenty of attachment sites for protection. Predation is also believed to play an important role in bacterial removal in CWs (Vacca et al., 2005; Wand et al., 2007), but the actual role of the different predators (e.g., protozoa, bacteriophages) has not been studied in detail.

Bacteriophages are strain specific viruses that attack and infect bacteria and are considered to be the most abundant and diverse biological entity on earth (Withey et al., 2005; Shapiro and Kushmaro, 2011). They have been suggested as potential indicators of feacal contamination, especially of enteric viruses, in conventional treatment systems (IAWPRC, 1991; Montazeri et al., 2015; Purnell et al., 2015; Amarasiri et al., 2017; McMinn et al., 2017; Dias et al., 2018), given that human pathogenic viruses monitoring on a frequent basis is a challenging, costly and time-consuming task. There is still limited knowledge and understanding regarding the relationships between the removal of bacterial indicators and the enteric viruses' indicators, such as somatic coliphages, *F*-specific bacteriophages and human-specific phages, while the removal processes have not yet been fully elucidated.

Related data from field studies in full-scale CW facilities are limited; only few studies report the removal of indigenous phages (e.g., somatic coliphages or F-specific phages) in passive wetland systems such as Free Water Surface CW (Yousefi et al.,

2004) and Subsurface CW of horizontal (Thurston et al., 2000; Reinoso et al., 2008; Williams et al., 1995) or vertical flow (Torrens et al., 2009). Aerated CW, which is a state-of-the-art design modification, could offer more aerated and air scoured conditions within the bed that may in theory promote the removal of enteric pathogens compared to the more anoxic and organic matter rich environments found particularly in HSF wetlands. To date no published literature on this topic has been identified and the elimination rate of pathogens remains largely unexplored in Aerated CW, while no studies were found on the fate of bacteriophages.

Therefore, this field study evaluates the efficiency of a full-scale treatment facility in the UK, comprising of Trickling Filters and an experimental Aerated Constructed Wetland, where - for the first time - the fate of a set of bacterial indicators and enteric phages is simultaneously investigated under real operating conditions. Specific objectives are to present the behaviour of each treatment system regarding the various parameters (e.g., organics, nutrients, pathogenic bacteria and viral indicators), to investigate the potential health impact of the treated effluent to evaluate the potential use of bacteriophages as bio-indicators of the treatment and to provide better understanding of the removal mechanisms of bacterial pathogens in these treatment systems.

2. Materials and Methods

2.1. Facility description

The field study took place at the STW of Petersfield, Hampshire, UK (51°00'00.5''N, 0°54'19.6''W) for a 5-month period (March to July) and evaluated to fate of various physicochemical parameters and microbiological indicators. Petersfield is a rural town with a population above 20,000 inhabitants with livestock farms and light industry. The STW consists of preliminary treatment (screening and grit removal), iron salt addition ($FeSO_4$) for phosphorus (P) precipitation, primary treatment (two sedimentation tanks of diameter 15 m each; PST), secondary treatment (10 trickling filters of diameter 24 m each; TF) and two secondary sedimentation tanks (SST) of diameter 23 m each (Oliver et al., 2005). The average wastewater inflow through the primary and secondary stages was 4,750 ± 1,080 m³/d over the study period. There is no final disinfection step before the final discharge to the adjacent River Rother.

An experimental aerated vertical flow constructed wetland (AVFCW) was built in 2013 (Fig. 1) and receives only a quarter of the inflow (i.e., 1,250 ± 17 m³/d), resulting in a hydraulic load of 1.08 m/d. The pilot AVFCW is used to test the aerated constructed wetland technology and represents one of the first aerated constructed wetlands in the UK installed to provide effluent polishing, especially for spikes of ammonia nitrogen occasionally detected in the STW. The pilot AVFCW is a saturated downflow wetland (L = 29 m, W = 40 m, D = 0.9 m), split into two beds for maintenance purposes. The SST effluent is applied on top of the pilot AVFCW surface through a pipe network with 6 surface distribution points per bed. The treated effluent is collected through a network of perforated laterals along the base of the bed that connects to a main collection header pipe. Aeration lines (i.e., driplines with 0.5'' diameter) are placed on the base and artificial aeration is continuously provided in a uniform grid pattern using a mechanical air compressor (5.5 kW) that provides an average air flow of 300 m³/day. The aeration lines network is connected via a main manifold line (2.5'' diameter) to the air compressor. Treated water flows by gravity from the pilot AVFCW through a level control chamber to the final discharge point. The pilot AVFCW base is lined with HDPE membrane (1.5 mm) and the bed is filled with medium gravel (size 8-15mm, thickness 70 cm) and planted with *Typha latifolia*. Water level is maintained 5 cm above the gravel layer surface.

<<Insert Figure 1>>

2.2 Sampling programme

Duplicate samples were taken on a bi-weekly basis (8 sampling occasions, each time at 10am) over a five-month period (March – July 2015) at five sampling points (Fig. 1); raw wastewater (RAW), influent (TFI) and effluent (TFO) of the trickling filters and influent (CWI) and effluent (CWO) of the pilot AVFCW. Over the last four sampling campaigns, samples were also taken from the nearby river that receives the final mixed treated effluent, i.e., 75% from CWI and 25% from CWO; one sample 100 m upstream (RU) and one 100 m downstream (RD) the discharge point. Physicochemical parameters, i.e., temperature, pH and electrical conductivity (EC), were measured onsite immediately after sampling. Analyses for BOD_5, COD, Total Suspended Solids (TSS), ammonia nitrogen (NH_4^+-N), nitrate (NO_3^--N), phosphate (PO_4^{-3}-P) and sulphate (SO_4^{-2}) were carried out immediately after sampling at the onsite laboratory (University of Portsmouth). Samples were also kept in dark at 4ºC, transported to the University of Brighton laboratory and analyzed within three hours of collection for a series of microbiological indicators: indicator bacteria, i.e., *Escherichia coli* (*E.coli*), Faecal Coliform (FC) and Intestinal Enterococci (IE) and viral indicators, i.e., somatic coliphages (SC), *F*-RNA specific phages and phages capable of infecting GB124, a human-specific strain of *Bacteroides fragilis*.

2.3. Physicochemical analyses

Physicochemical parameters (temperature, pH, EC) were measured using WTW Inolab series instruments. For BOD_5 determination, respirometric bottles were used following Standard Methods (APHA, 2012); the other chemical parameters were measured colourimetrically using the Palintest™ 7100 photometer and the following methods using the Palintest™ supplied reagents; COD, (method 80, 81 or 82 depending on concentration), ammonia (method 4), nitrate (method 23), orthophosphate (method 28) and sulphate (method 32).

2.4. Quantification of bacterial and viral indicators

Faecal coliforms were enumerated by membrane filtration on mFC agar in triplicates with different dilutions (ISO, 2000a). For *E.coli*, TBX medium was used and for intestinal enterococcus SB agar (ISO, 2000a;b Caplin et al., 2008; Vergine et al., 2017). Results for indicator bacteria were expressed as colony forming units per 100 ml (CFU/100 ml). Somatic coliphages, *F*-RNA specific phages and human-specific *B. fragilis* GB124 phages were quantified in triplicates by enumerating plaque-forming units (expressed as PFU/100 ml) on modified Scholten's media, tryptone yeast glusoce media and *Bacteroides* phage recovery media, respectively, according to standardized double-agar-layer methods (ISO, 2001a-c). Host strain WG5 (*Escherichia coli*) was used for somatic coliphage enumeration, WG49 (*Salmonella typhimurium*) for F-specific phages, and GB124 (*B. fragilis*) was used for the detection of phages active against this human-specific gut bacterium. The methodology has been previously described (Harwood et al., 2013).

2.5. Data Interpretation and Statistical Analyses

All microbiological data were log_{10} transformed and zero concentration values were treated as log_{10} of 1 (i.e., 0). The log_{10} transformed microbial data and untransformed physicochemical data was tested for normality using the Anderson-Darling (AD) test. This showed a complex pattern, with many parameters being normal within treatment stages but, assuming a significance level of 0.1, a number of parameters within stages and most of the combined stage data were significantly different from normality. Out of the microbial groups of the combined stage, only the log_{10} SC was normal. It was therefore decided to use non parametric tests that do not require normality to analyse the data to allow comparison between all groups and stages. Central tendency is therefore assessed by the median, variability by the inter quartile range (IQR), associations by Spearmans Rank order correlation ($-1 \leq r_S \leq 1$) and differences between locations by the Krushall Wallis test. All statistical analyses were undertaken using Minitab v17.

3. Results and discussion

3.1. Overall performance

Fig. 2 presents the variations of the various parameters over the study period in each treatment stage, while respective removals are shown in Table 1. Each Box–Whisker box shows the inter quartile ranges, the median is shown as the horizontal line across the box and the whiskers the 95% confidence limits, while outliers are shown by stars. A gradual removal of all parameters is observed along the treatment stages. Sedimentation removes most of the suspended solids. Organic matter (BOD_5 and COD; 99.5% and 97.7%, respectively) and ammonia (99.5%) are almost completely removed in the system, with the TF and the pilot AVFCW accounting for the majority of NH_4^+-N removal. In terms of areal load removal, the STW removed 3.3 g BOD_5/m²/d, 10.6 g COD/m²/d and 0.33 g NH_4^+-N/m²/d. Low effluent nitrate concentration (< 6 mg/L) indicates that denitrification takes also place.

The combined system of the TF and the SST managed to remove 95% of BOD_5, 82% of COD, 93% of NH_4-N and 69% of PO_4-P from the primary effluent, figures which are in line with what is reported in literature (Naz et al., 2014; Abou-Elela et al., 2017). The relatively high rate of nitrification in the TF system could be attributed to the low hydraulic load (1.08 m/d) applied in the TF, which allows for enhanced nitrification

(Lessard and Le Bihan, 2003). The addition of the pilot tertiary AVFCW in the treatment train further improved the secondary effluent quality. The pilot AVFCW removed 76% of BOD_5, 22% of COD and 89% of NH_4-N, providing a final effluent of high quality. Especially for ammonia, results confirmed that artificial aeration enhances aerobic conditions and, thus, nitrification (Boog et al., 2014; Stefanakis et al., 2014).

P removal reached 91% (or 96 mg PO_4^{-3}-P/m²/d) in the system and most of it took place in the PST, mainly due to the upstream addition of $FeSO_4$. It is noticeable that the performance of the AVFCW is limited. It is widely known that adsorption and precipitation is the main P removal mechanism in CW, directly related to the physicochemical characteristics of the substrate media (e.g., Al, Fe, Ca oxides content, mineralogical composition etc.), while plant uptake is generally considered negligible (Vymazal, 2007; Garcia et al., 2010; Stefanakis et al., 2014; Wu et al., 2015). The gradual and relatively fast saturation of the filter media is the main reason for the overall low P removal rates in CW, while in VF systems the short contact time between the wastewater and the media due to the vertical drainage further limits these removal mechanisms (Stefanakis and Tsihrintzis, 2012a). For example, Paing et al. (2015) studied the efficiency of 169 full-scale VF wetlands and reported a gradual decrease in P removal, i.e., 47% in the first operational year, 30% between 2-6 years and 9% between 6-12 years. This is why a gravity filter filled with a reactive media has been proposed as a polishing stage after VF wetlands to enhance P removal (Brix and Arias, 2005; Stefanakis and Tsihrintzis, 2012b; Adera et al., 2018). In the present study, the inflow P was already low (median 1.2 mg/L) and no special filter media was used in the AVFCW, which was already in operation for almost three years. These could explain the low efficiency, while the negative performance observed in few sampling campaigns could be attributed to the release of P adsorbed onto the media, as also reported elsewhere (Paing et al., 2015).

<<Insert Figure 2>>

<<Insert Table 1>>

Fig. 3 presents the levels of FC, *E.coli* and IE at different sampling points of the STW in the same manner as Fig. 2, while Fig. 4 depicts the cumulative log reduction of bacterial and viral indicators based on each treatment stage. High elimination rates of bacterial indicators were observed in the system; 3.47 log unit reduction for FC, 3.58 for *E.coli* and 3.65 for IE (Table 1). As Fig. 3 shows, the major portion of the microbiological indicators was removed in the two biological treatment stages (TF and AVFCW), while median effluent values of the pilot AVFCW were 1.61, 1.15 and 0.48 log for FC, *E.coli* and IE, respectively (respective concentrations of 78, 17 and 7 CFU/100 mL).

The removal of FC (0.39 log or 58%) in the PST is similar to previously reported values (Curtis, 2003). The combined system of TF and SST achieved 1.83, 1.95 and 1.99 log removal (all > 99%) for FC, *E.coli* and IE, respectively. In general, lower removal rates (20-90%) of feacal indicators are reported in literature for traditional TF filled with natural rocks (Yahya et al., 2000; Curtis, 2003; Bitton, 2005). As the important role of the biofilm support media was gradually realized in promoting bacterial removal via filtration, adsorption and desorption (Lucena and Jofre, 2010; Stevik et al., 2004), new materials (i.e., plastic- and sponge-based support media) instead of the typical rock-based media are used to provide an improved environment for biofilm development and enhance the removal of bacteria indicators (Bressani-Ribeiro, et al., 2018). For example, Naz et al. (2014) reported a higher than 3.5 log removal of FC in TF with polystyrene, plastic, rubber and stone media, while Wasik and Chmielowski (2017) report coliform removal rates higher than 98%.

<<Insert Figure 3>>

<<Insert Figure 4>>

Fig. 3 also presents the levels of SC, F-RNA and GB-124 phages at the different sampling points, while the cumulative log reduction of all bacteriophage groups through the different treatment stages is shown in Fig. 4. SC is the most abundant phage group in raw wastewater (1.32 x 10^6 PFU/100 mL) and throughout the STW compared to *F*-RNA (2.3 x 10^4 PFU/100 mL) and GB-124 phages (0.74 x 10^4 PFU/100 mL). This is also elsewhere reported for activated sludge plants and TF plants (Dias et al., 2018). This is a first indication that SC could be a potential useful conservative indicator for virus removal assessment in TF and aerated CW, which is also proposed in other studies (Dias et al., 2018). Slightly lower SC levels (1.23 x 10^6 PFU/100 mL) and slightly higher *F*-RNA phages (1.88 x 10^4 PFU/100 mL) are reported for raw wastewater treated in MBR unit, but significantly higher GB-124 phages (2.71 x 10^4 PFU/100 mL) compared to this study (Purnell et al., 2015). Overall, the median log removals in the STW are 1.90, 2.16 and 1.62 for SC, *F*-RNA and GB-124 phages, respectively, which are lower compared to bacterial indicators (3.47 and 3.55 for FC and *E.coli*, respectively), as was also found in other studies for activated sludge and TF plants (Dias et al., 2018).

The primary treatment generally had low removal rates of less than 0.4 log unit reductions for all indicators, with the lowest median removal of 0.19 for the phage group GB-124. Bacteriophages have the tendency to adsorb on solids surfaces, as also elsewhere observed (Zhang and Farahbakhsh, 2007), which could explain this removal. The two bioreactors (i.e., TFs and AVFCW) showed a more or less similar efficiency, with the combined system of TF and STT presenting higher log removals of indicator bacteria and phages, except for the F-RNA group log for which the AVFCW showed a higher removal. The TF combined with the SST as one-unit process gave higher removals of bacterial indicators (almost 2 log units; 1.83 to 1.99) compared to phage groups (less than 1 log units). The AVFCW gave a further 1.2 to 1.3 log unit reductions in bacterial indicators, but a varied removal of phage groups (0.6 for SC and GB-124 compared to 1 log unit for FRNA). After the treatment, SC were the only phages constantly detected, since F-RNA and GB124 phages were often undetected in the effluent water. F-RNA phages showed the highest log removal among the phages (2.16), possibly due to their greater tendency to adsorb onto solids (Zanetti et al., 2010; Purnell et al., 2015).

As previously mentioned, 25% of the STW hydraulic load was treated through the pilot AVFCW. The majority (75%) of the inflow was discharged after the secondary sedimentation, i.e., the SST. This means that the final STW effluent is a mixture of the SST (i.e., CWI sample) and pilot AVFCW effluents (i.e., CWO sample). Table 2 presents average values for the tested physicochemical and microbiological parameters in the mixed STW effluent and the river receiving it. The water upstream the STW discharge point is of very good quality; all parameters measured were below the respective values of the AVFCW effluent (CWO). Downstream the STW discharge point, all pollutant concentrations are elevated, typically higher than the wetland effluent concentrations. Especially for microbiological parameters, FC and *E. coli* are almost 23 and 49 times, respectively, higher than in the upstream water.

The same was also found for bacteriophages. These elevated pollution levels should be attributed to the mixed effluent discharged to the river. It should also be noted that the presence of all three different bacteriophage groups was ascertained in natural water (i.e., RU sample), showing that these groups can be found in nature, as it is already known (Withey et al., 2005; Shapiro and Kushmaro, 2011), since they co-live with their host (bacteria) that are present in most, if not all, water bodies (Clokie et al., 2011). It is reported that in most studied natural ecosystems a ratio of ten phages for every bacterial cell is detected (Suttle, 2007). They have been suggested as potential indicators of feacal contamination, especially of enteric viruses, in conventional treatment systems (IAWPRC, 1991; Montazeri et al., 2015; Purnell et al., 2015; Amarasiri et al., 2017; McMinn et al., 2017; Dias et al., 2018), Among them, SC were the most abundant, a finding also elsewhere reported (McMinn et al., 2017).

<<Insert Table 2>>

3.2. STW evaluation

TF and CW are two low-tech alternatives to treatment methods that require mechanical equipment and energy. CW in particular are a near-nature passive technology with multiple environmental and economic advantages (Stefanakis et al., 2014). The combination of TF with an aerated CW bed is a novel design for STW; no similar study was found in the literature. However, TF have been combined with passive CW systems. Maheesan et al. (2011) tested a combination of TF-passive VFCW treating light domestic wastewater (influent BOD_5 195 mg/L, COD 570 mg/L, TSS 113 mg/L, NH_4-N 21.6 mg/L and P 6.3 mg/L) and reported good removal rates (89.7% for BOD_5, 88.7% for COD, 75.6% for TSS, 97.1% for NH_4-N and 72.7% for P); however, the system examined in the current study performed better, although it received higher pollutant loads. Another setup was tested by Kim et al. (2014); a TF followed by two partially saturated passive VFCWs showed comparable results to the present study, but the pilot AVFCW bed has much smaller footprint compared to the two VFCWs tested (Kim et al., 2014). Similar results (with the exception of NH_4-N) were also found for a pilot system comprising TF, $FeCl_3$ injection and a passive VFCW for domestic wastewater treatment (Kim et al., 2015a;b); however this pilot received approximately 1/3 of the hydraulic load received by the studied STW. A combination of TF and horizontal subsurface (HSF) CW was also tested by Vucinic et al. (2012), showing comparable results. However, the hydraulic load applied was much lower (0.035 - 0.144 m/d) compared to the load applied to the pilot AVFCW in the present study (1.08 m/d), while lower effluent concentrations were achieved in the present study for BOD_5 and ammonia. Again, the footprint of the proposed wetland system is higher than that of the present study. In another study, a pilot HSF CW was used as tertiary stage receiving secondary effluent from a TF under lower hydraulic load (0.36 m/d) (Toscano et al., 2015). This pilot did not reach the same effluent quality with the studied AVFCW, especially for NH_4-N, while the area demand was three times higher compared to the present study.

Overall, the presence of the aerated CW improved the STW efficiency in terms of the physicochemical parameters using a much smaller footprint (3-6 times). This is a very important finding, since lower area demand is translated to lower material volumes (e.g., earthworks, gravel, HDPE liner etc.), hence lower investment costs and is particularly advantageous if there are space constraints for a STW. At the same time, the aerated wetland design provides increased performance consistency and can reach performance levels that have been unobtainable in passive wetlands with less performance variability. The blower and the plastic aeration lines cost is minimal compared to the overall costs savings and the operation and maintenance complexity does not increase significantly.

The major portion of inflow TSS is removed in the two sedimentation stages, with respective load removal rates of 2.7 and 0.4 g TSS/m²/d for PST and SST. After the addition of iron upstream the PST, the remaining phosphorus is removed in the PST and the TFs. The pilot AVFCW bed practically did not remove any phosphorous, given the already very low influent concentration and the fact that after few years of operation, the adsorption capacity of the substrate media is gradually depleted (Stefanakis and Tsihrintzis, 2012b). The effluent quality of the pilot AVFCW indicates the good performance and the increased nitrification potential of the AVFCW technology, due to the aerobic conditions that enhance both organic matter biodegradation and nitrification. Improved performance with artificial aeration of CWs is also elsewhere reported (Foladori et al., 2013; Boog et al., 2014; Stefanakis et al., 2014; Hou et al., 2018), which explains the increasing interest in aerated CW over the last few years.

In general, the proposed treatment scheme of the studied STW proved to be capable of providing a high quality effluent. The final effluent (after the pilot AVFCW) had pollutants concentrations below the limits of the Urban Wastewater Treatment Directive - UWTD (Council Directive, 1991), which has been adopted in the UK (Statutory Instrument, 2003), allowing for the final discharge to surface waters. The WHO limits are also met for unrestricted irrigation of (WHO, 2006). It should be noted that the secondary effluent (CWI sample) did not meet the UWTD standard for Total Nitrogen, neither the WHO limit for *E.coli*.

3.3. Microbiological dynamics

Control of pathogens in the STW outflow is a crucial factor for maintaining ecosystem good health status and, thus, protect human health. However, it has not yet gained the attention it deserves (Wu et al., 2016). Regarding passive wetland systems, summarized information can be found on the removal of bacterial indicators (e.g., Vymazal, 2005; Garcia et al., 2010; Stefanakis and Akratos 2016; Wu et al., 2016). Nevertheless, typically the efficiency of the examined system in terms of common bacterial indicators is only reported. Removal of bacteria is usually not the main target in the design of CW systems, although CW have been proved to be efficient in the removal of microbiological contamination (Stefanakis and Akratos, 2016). The tested experimental STW was found capable of providing a high effluent quality in terms of bacteria and phages removal too. Median effluent concentrations of FC, *E.coli* and IE after the tertiary treatment stage (i.e., the pilot aerated wetland) were 41, 14 and 3 CFU/100 mL, below the WHO guidelines for reusing treated wastewater in agriculture (WHO, 2006), which eliminated the need for the final disinfection step. The secondary effluent of the STW (i.e., after the secondary sedimentation) did not fulfill these criteria. This is an important finding, since the addition of the aerated wetland as tertiary treatment stage improved the final effluent quality not only in terms of physicochemical parameters but of microbiological indicators too. This means that a final chlorination/dechlorination step after the AVFCW is not required, i.e., a chemical process can be avoided. Physicochemical methods such as ozonation or UV radiation are effective and useful, but can be expensive to install and operate, especially at small STW sites, while chlorination is cheap and effective but brings concerns about disinfection by-products and health risks associated with chemical management and storage (Mezzanotte et al., 2007). Therefore, aerated CW should be further investigated regarding their potential as a cheaper and easy-to-handle method that can limit the needs and costs for a disinfection stage and is especially appropriate for small sites, with additional benefits for the general effluent quality.

All studies found in published literature on aerated CW (either of horizontal or vertical flow) almost exclusively focus on organic matter degradation and nitrification capacity of this wetland type and not on its sanitation efficiency. While more than 30 recent publications (published within the last four years) were found in the literature on aerated CW, only one reports bacteria and virus removal. It should also be mentioned that most of these studies are laboratory experiments or small-size trial beds. Moreover, no publication was found reporting the fate of bacteriophages in aerated CW systems. This confirms the fundamental knowledge gap in this field and highlights the necessity for more research. The lack of data regarding microbiological indicators in aerated CW could be explained, considering that these systems are a new development in wetland technology and the main interest currently is to optimize their performance and operational parameters (e.g., aeration equipment/schedule).

Only one study was found reporting *E.coli* removal in aerated CW (Headley et al., 2013); aerated HSF and VF beds treating primarily treated domestic wastewater showed more than double *E.coli* removal compared to conventional (passive) CW systems. Reported log reduction was 3.3 and 2.1 for the aerated HSF and VF beds, respectively, lower than the present study (3.6), while the VF bed showed a much higher areal load removal rate than the HSF bed (7.2 and 1.0 x 10^9 MPN/m²/d, respectively). The present study also demonstrated the improved removal capacity of aerated wetland systems to reduce pathogens. The achieved areal load removal rates for FC, *E. coli* and IE in the pilot AVFCW were 1.0 x 10^7, 3.3 x 10^6 and 7.2 x 10^5 CFU/m²/d, respectively. These results indicate the ability of artificially aerated CW to provide a high effluent quality almost free of pathogens under a smaller footprint compared to passive wetland systems.

The exact mechanism(s) that result in this improved performance are not yet clear (Wu et al., 2016; Alufasi et al., 2017). This could be possibly attributed to the added air in the bed, since this is the main modification of aerated wetlands compared to passive systems. Few previous studies indicate a correlation between aerobic conditions (i.e., which is the case with the artificially aerated wetlands) and bacterial removal; for example, a 8-10 fold increase in bacterial die-off rate constant is reported for bacterial indicators after a 2-hr aeration was applied (Fernández et al., 1992). In general, enteric bacteria are either facultative or obligate anaerobs, hence, aerobic conditions do not favour their longevity, while anaerobic environments prolong coliform survival (Vymazal, 2005). Higher efficiency of VF CWs compared to HF CWs is also reported in terms of bacterial removal, which again implies that aerobic environment (i.e., oxidation) enhances the removal of bacteria (Winward et al., 2008). The effect of aeration conditions is also implied by the enhanced pathogen removal rates observed in CWs planted with *Phragmites australis* than with *Typha latifolia*, considering that common reed is known to provide higher oxygen release rates in its rhizosphere (Werker et al., 2002; Wu et al., 2014). Moreover, as also indicated by Headley et al. (2013), artificial aeration and the respectively increased concentration of dissolved oxygen within the saturated wetland bed probably alters the characteristics and the composition of the microbial ecology and the trophic structure, which also enhances the development and growth of natural predator groups that prey on bacteria (Wand et al., 2007). However, a future detailed characterization of the microbial ecology patterns and composition in aerated wetlands is required to provide a deeper understanding of the fundamental processes leading to increased bacterial removal by artificial aeration.

3.4. The role of bacteriophages in bacterial removal

Current knowledge dictates that the removal of pathogenic bacteria in CW takes place through a variety of physical, chemical, and biological mechanisms such as filtration, UV radiation by sunlight, antimicrobial exudates of plant roots, predation by protozoa, activity of biolytic bacteria, and natural die-off (Stevik et al., 2004; Wand et al., 2007; Stefanakis et al., 2014; Wu et al., 2016). However, detailed fundamental knowledge on bacterial removal processes is still limited. Particularly, the role of biological factors, namely predation by other microorganisms, still remains largely unknown. Predation is attracting interest in dealing with bacterial populations, since most bacterial pathogens are food for other microorganisms such as protozoa and bacteriophages (Vacca et al., 2005; Wand et al. 2007; Stefanakis et al., 2014). The grazing activity probably depends on the target bacteria-prey characteristics (e.g., concentration), the specific characteristics of the predator (morphology, physiology, feeding strategy, etc.), and physicochemical parameters (e.g., temperature, redox conditions) (Shapiro et al., 2010); however, their significance and the main principles that regulate their activity are not well understood yet.

Bacteriophages are viruses known for their biolytic activity and the pressure they can apply on microbial communities, reducing fecal coliforms and pathogens (Ottova et al., 1997; Vacca et al., 2005; Vymazal, 2005; Shapiro and Kushmaro, 2011). They get adsorbed onto the host cells and kill the bacterial cell or integrate its genome into the host genome. Their entry into the host cell depends on specific receptors present on the host cell surface, e.g., proteins, carbohydrates and lipopolysaccharides (Marks and Sharp, 2000). Bacteriophages are commonly used as human enteric virus removal indicators since the direct detection and enumeration of pathogenic viruses is a costly and time consuming process. Bacteriophages are natural predators of bacteria, specific and precise in their predation activity and highly specific for feacal pollution (Vacca et al., 2005; Stefanakis et al., 2014). The role of phages in wastewater treatment processes is considered very important due to their predation power, considering also that they are not pathogenic or toxic to humans. Predation is known as the main mechanism for pathogen removal in CW, but the exact role of bacteriophages and the competition with other predators (e.g., protozoa) is still under discussion (García et al., 2013; Stefanakis et al., 2014). Moreover, still today little is known regarding their exact influence on the treatment performance (Shapiro and Kushmaro, 2011).

Existing knowledge on bacteriophages comes from conventional wastewater treatment methods, mainly activated sludge systems and MBR (Withey et al., 2005; Zhang and Farahbakhsh, 2007; Goldman et al., 2009; Purnell et al., 2015; Dias et al., 2018). Most of the studies imply their use as indicators of the survival and release of pathogenic viruses into the environment. It is reported that in activated sludge the number of bacteriophages is the highest compared to any other environment (Shapiro and Kushmaro, 2011). In CW, the lytic action of bacteriophages has been suggested as a mechanism for bacterial loss (Thurston et al., 2001). They are the least investigated group in CW and their use as pollution indicators has been only fragmentarily discussed (Thurston et al., 2001; Abdulla et al., 2007), e.g., to estimate their role in *E.coli* removal (Withey et al., 2005). A positive correlation between the bacteriophages and the classic bacterial indicators is reported in few studies for passive CW (Yousefi et al., 2004; Abdulla et al., 2007).

Limited information exists in the literature about the fate of bacteriophages in CW and it mostly comes from passive horizontal flow systems, while no study was found on aerated CW. The study by Torrens et al. (2009) is the only one that examined the efficiency of two passive VFCW in terms of microbial indicators removal, including bacteriophages. Authors report a 0.5 and 2 log removal of FC and *E.coli*, respectively, and 0.4-1.5 log and 0.2-1 log removal of SC and *F*-specific bacteriophages, respectively. Both figures are lower than the ones found in the present study (Fig. 4). The log reduction of bacteriophages was always lower than that of the bacterial indicators, as also found in this study. It is also interesting that SC removal was higher than that of *F*-specific phages, while the opposite was found in the examined pilot AVFCW (Fig. 4). O'Luanaigh and Gill (2010) examined two HSF CW, operating as secondary and tertiary treatment stages and tested three bacteriophages (MS2, ΦX174 and PR772). Total Coliforms (TC) and *E.coli* removals were 1.8 (98.5%) and 1.4 log (96%), respectively, in the secondary HSF bed and 1.3 log (94.6%) and 1.7 log (97.7%), respectively, in the tertiary HSF bed. A high recovery rate was reported for bacteriophages ΦX174 and MS2 in both beds, indicating the low efficiency of HSF systems to remove viral microorganisms.

Thurnston et al. (2001) examined two subsurface flow CW treating secondary sewage effluent and potable groundwater. TC removal rates reached 98.8% and FC 98.2%, while coliphage removal was 95.2%. Detected coliphage and FC effluent concentrations were 4.7 PFU/mL and 45 CFU/100 mL, respectively, both higher than the present study. Hench et al. (2003) reported $16x10^2$ and 31.6 PFU/mL coliphages concentration in the influent and effluent, respectively, of a subsurface flow CW (98% removal rate). Significant reductions of FC and enterococci are also mentioned (>99%), but the effluent quality had to be post-treated in order to reach FC standards. Surface flow (SF) CW for stormwater treatment have been found capable in removing SC and *F*-RNA bacteriophages, but extended survival rates are reported (Yousefi et al., 2004). Higher efficiency (41 and 19 times during winter and summer) of SSF CW compared to SF CW is also reported by Adhikari et al. (2013) for the removal of bacteriophage P22 from livestock drainage. Similar results are reported by Reinoso et al. (2008); higher removal rates of coliphages were found in a SSF CW (94%) compared to a SF CW, for an influent concentration of 4.86 log.

In general, the comparison of the results of the present study with these few existing studies previously mentioned, i.e., passive wetland systems of different types, provides a good indication of the higher treatment capacity of the tested pilot AVFCW. Although operating as tertiary treatment stage, the pilot AVFCW showed similar or even higher removal capacity of the SC and *F*-RNA groups.

The collected data were analyzed as a pooled data set, i.e., considering measurements for all treatment stages for each indicator, to determine the correlations between bacterial indicators and bacteriophages across the STW. Table 3 presents Spearman's correlation coefficients in wastewater samples. There were significant differences in each indicator group counts with location (KW: H>26 and p <0.0001) and there were strong correlations (p>0.0001) between all bacterial and viral indicators (Table 3). The two strongest correlations between FC and SC and FC and GB-124 are shown in Figure 5, with the counts by location (i.e., sampling point) shown by the different symbols. There is a clear linear component to this association, which suggests a similar log-linear die-off rate for these organisms.

<<Insert Table 3>>

<<Insert Figure 5>>

Strong correlations between bacteriophages and bacterial indicators were found. The highest coefficients were found for SC-FC (0.89), SC-*E.coli* (0.88) and SC-IE (0.84). This strong positive correlation is the first indication that SC could be potentially used as indicators for the investigated bacteria removal in aerated CW and TF. A positive correlation between bacteriophages and classic bacterial indicators (*E. coli*) is also implied for passive wetland systems (Yousefi et al., 2004; Withey et al., 2005; Abdulla et al., 2007). A high correlation rate between coliphages and FC (0.82), *Salmonella* sp. (0.82), *Shigella* sp. (0.89), *Vibrio* sp. (0.83) and *Pseudomonas* sp. (0.97) has also been reported (Abdulla et al., 2007), which again implied the possibility to use SC as pollution indicators.

Moreover, no significant correlations were found between the removal of bacterial indicators and bacteriophages and temperature variations. However, considering that the study took place in spring and early summer months, it did not focus on the effect of temperature (i.e., the temperature range covered was 12.2 – 19.5°C; Fig. 2). The effect of temperature on pathogens removal in CW still remains a controversial issue in published literature with contradicting results reported by various authors (Reinoso et al., 2008; Wu et al., 2016; Alufasi et al., 2017). For example, it has been found that increased temperatures enhance the removal of indicator bacteria in HF CW, but had no effect on bacteria removal performance in VFCW (Winward et al., 2008). Increased temperature enhances the activity of non-pathogenic organisms, such as grazing protozoa, thus increasing pathogen removal via grazing (Weber and Legge, 2008). On the other hand, reduced oxygen solubility to water and natural die-off of macrophytes at lower temperatures limit dissolved oxygen concentration in the root zone (Rivera et al., 1997). Moreover, it is reported that enteric viruses and coliphages have a longer survival time and more frequent occurrence at lower temperatures, while they decay faster at higher temperatures (Bertrand et al., 2012). Nevertheless, other factors seem to play a more significant role in pathogens removal such as the presence/status of plants, the hydraulic regime, the hydraulic retention time, the water composition and of course the artificial aeration, which is the case in our study.

It can be assessed that bacteriophages can be a useful tool for performance evaluation in terms of removal of bacteria from municipal wastewater. This is also supported by the fact that bacteriophages are more resistant to treatment than the bacterial indicators. SC appear as a good indicator of microbiological quality and microbial removal efficiency of aerated CW, considering that this group is always detectable and more abundant in both wastewater and surface water (Table 1). Similar suggestions have already been formulated for conventional -mechanical treatment methods such as activated sludge and membrane bioreactors (Zhang and Farahbakhsh, 2007; De Luca et al., 2013; Purnel et al., 2015; Yahya et al., 2015). Further research should investigate the effect of phages morphological characteristics in their resilience to the examined treatment systems.

4. Conclusions

The field study on a full-scale STW with TF and an experimental AVFCW delivered an effluent that fulfils the legal criteria for environmental discharge and reuse, even without a final disinfection step. For the first time, three bacterial indicators and three bacteriophage groups were evaluated in an aerated CW, with respective removals in the STW system reaching 3.5log and 2log. Strong correlations were detected between bacteria and bacteriophages implying the role of phages as bacteria predators and their potential use as microbial removal indicators for TF and aerated CW. Finally, the superior efficiency of aerated CW in microbiological contamination removal compared to passive wetland systems is demonstrated for the first time.

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Figure 1. Schematic representation of the treatment train and sampling points at Petersfield Sewage Treatment Works in South UK (PST; primary sedimentation tanks, TF; trickling filters, SST; secondary sedimentation tanks, AVFCW; aerated vertical flow constructed wetland, RU; river upstream, RD; river downstream).

Figure 2. Box–Whisker plots of pollutants and physicochemical parameters during the field study period at each sampling point; RAW (raw wastewater), TFI (SST effluent), TFI (TF effluent), CWI (SST effluent), and CWO (CW effluent).

Figure 3. Box–Whisker plots of log values for indicator bacteria (FC, *E.coli* and IE) and viral indicators (SC, F-RNA and GB-124 phages) at each sampling point; RAW (raw wastewater), TFI (SST effluent), TFI (TF effluent), CWI (SST effluent), and CWO (CW effluent).

Figure 4. Cumulative $\log_{10}$ reduction of bacterial indicators (FC, *E.coli* and IE; log conversion of concentration; CFU/100 mL) and viral indicators (SC, F-RNA and GB-124 phages; log conversion of concentration; PFU/100 mL) through the series of PST, TF, SST and the pilot AVFCW.

Figure 5. Scatter plots of the associations between SC and GB-124 phage groups and three bacterial indicators for the pooled data set of each parameter.

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Table 1. Concentrations and removal of physicochemical and microbiological indicators (Median ± Inter Quartile Ranges) in each treatment stage and across the Petersfield STW system (PST; primary sedimentation tanks, TF; trickling filters, SST; secondary sedimentation tanks, AVFCW; aerated vertical flow constructed wetland).

Parameter	Raw wastewater	STW Effluent	Median removal (%)				
			PST	TF	SST	AVFCW	Total
BOD_5 (mg/L)	379 ± 93	2.1 ± 2.6	59.4	81.2	71.8	75.5	99.5
COD (mg/L)	1260 ± 610	49.0 ± 18	67.2	55.1	47.0	22.3	96.1
TSS (mg/L)	294 ± 55	9.2 ± 4.6	64.2	12.6	87.4	5.6	97.0
NH_4^+-N (mg/L)	47 ± 31	0.22 ± 0.76	45.4	86.0	40.6	89.1	99.6
PO_4^{-3}-P (mg/L)	12.9 ± 7.7	1.12 ± 0.65	65.9	58.7	3.8	0.0	91.0
Microbial group	**Raw wastewater**	**STW Effluent**	**Median removal (Log_{10})**				
Log_{10}FC, CFU/100 mL	5.08 ± 0.14	1.61 ± 0.28	0.39	1.23	0.60	1.25	3.47
Log_{10}E.Coli, CFU/100 mL	4.73 ± 0.28	1.15 ± 0.25	0.34	1.22	0.73	1.29	3.58
Log_{10}IE, CFU/100 mL	4.13 ± 0.23	0.48 ± 0.74	0.35	1.19	0.80	1.31	3.65
Log_{10}SC, PFU/100 mL	5.91 ± 0.67	4.01 ± 0.36	0.33	0.73	0.24	0.60	1.90
Log_{10}FRNA, PFU/100 mL	4.36 ± 1.01	2.20 ± 1.31	0.32	0.66	0.18	1.00	2.16
Log_{10}GB124, PFU/100 mL	3.62 ± 0.70	2.00 ± 0.83	0.19	0.69	0.17	0.57	1.62

Table 2. Median (± Inter Quartile Ranges) of physicochemical and microbiological parameters measured in the SST effluent (CWI sample location), the pilot AVFCW effluent (CWO sample location), the combined effluent (75% of CWI and 25% of CWO) and the RU and RD river sampling points (SST; secondary sedimentation tanks, AVFCW; aerated vertical flow constructed wetland, CWI; constructed wetland inflow, CWO; constructed wetland outflow, RU; river upstream, RD; river downstream).

Parameter	CWI sample	CWO sample	Combined effluent	RU	RD
pH (-)	7.9 ± 0.2	7.8 ± 0.6	7.9 ± 0.65	7.8 ± 0.3	7.8 ± 0.2
EC (μS/cm)	842 ± 125	831 ± 99	839 ± 147	515 ± 123	688 ± 94
SO_4^{-2} (mg/L)	74.5 ± 19.0	70.0 ± 16.8	73.3 ± 22.7	31.0 ± 2.8	48.5 ± 13.0
BOD_5 (mg/L)	6.7 ± 4.8	2.1 ± 1.8	5.5 ± 4.3	0.9 ± 0.7	2.5 ± 0.9
COD (mg/L)	66.5 ± 47.8	49.0 ± 12.8	61.9 ± 35.8	24.0 ± 7.0	578 ± 58
TSS (mg/L)	9.8 ± 3.1	9.2 ± 4.6	9.6 ± 4.9	9.5 ± 1.4	18.3 ± 9.6
NH_4^+-N (mg/L)	2.3 ± 3.1	0.2 ± 0.4	1.8 ± 2.3	0.10 ± 0.12	0.4 ± 0.3
NO_3^--N (mg/L)	5.9 ± 1.5	5.0 ± 1.9	5.7 ± 2.8	3.1 ± 1.4	5.3 ± 1.7
PO_4^{-3}-P (mg/L)	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 1.9	0.2 ± 0.1	1.5 ± 0.2
Log_{10}FC, CFU/100 mL	2.86 ± 0.57	1.61 ± 0.28	2.56 ± 0.41	1.34 ± 1.28	2.75 ± 1.15
Log_{10}E.Coli, CFU/100 mL	2.44 ± 0.38	1.15 ± 0.25	2.01 ± 0.26	0.83 ± 0.45	2.20 ± 0.72
Log_{10}IE, CFU/100 mL	1.79 ± 0.80	0.48 ± 0.74	1.42 ± 0.43	0.39 ± 0.79	1.33 ± 0.40
Log_{10}SC, PFU/100 mL	4.60 ± 0.39	4.01 ± 0.36	4.45 ± 0.43	2.66 ± 1.51	4.23 ± 0.33

Log_{10}FRNA, PFU/100 mL	3.20 ± 0.69	2.20 ± 1.31	2.93 ± 0.75	2.00 ± 1.5	2.96 ± 0.51
Log_{10}GB124, PFU/100 mL	2.57 ± 0.14	2.00 ± 0.83	2.38 ± 0.34	0.00 ± 1.28	2.15 ± 0.79

Table 3. Spearmans correlation coefficients ($r_{s)}$ and differences *p* (i Krushall Wallis test; in brackets) between microbiological parameters (log_{10} values) for pooled data set per parameter across the STW.

	FC	*E.coli*	IE	SC	F-RNA	GB-124
	Raw and treated wastewater after the pilot AVFCW					
FC	1					
E.coli	0.97 (0.000)	1				
IE	0.94 (0.000)	0.96 (0.000)	1			
SC	0.91 (0.000)	0.89 (0.000)	0.86 (0.000)	1		
F-RNA	0.82 (0.000)	0.81 (0.000)	0.77 (0.000)	0.86 (0.000)	1	
GB-124	0.92 (0.000)	0.89 (0.000)	0.90 (0.000)	0.86 (0.000)	0.78 (0.000)	1

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Presence of bacteria and bacteriophages in full-scale trickling filters and an aerated constructed wetland

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Highlights

- Trickling filters and an experimental aerated Constructed Wetland were investigated
- Effluent quality below discharge legal limits even without a final disinfection step
- 2-3.5log removal efficiency of three bacterial and three bacteriophages indicators
- Strong correlations imply phages use as performance indicators in aerated wetlands
- Aerated Wetlands outperform passive systems in microbiological contamination removal

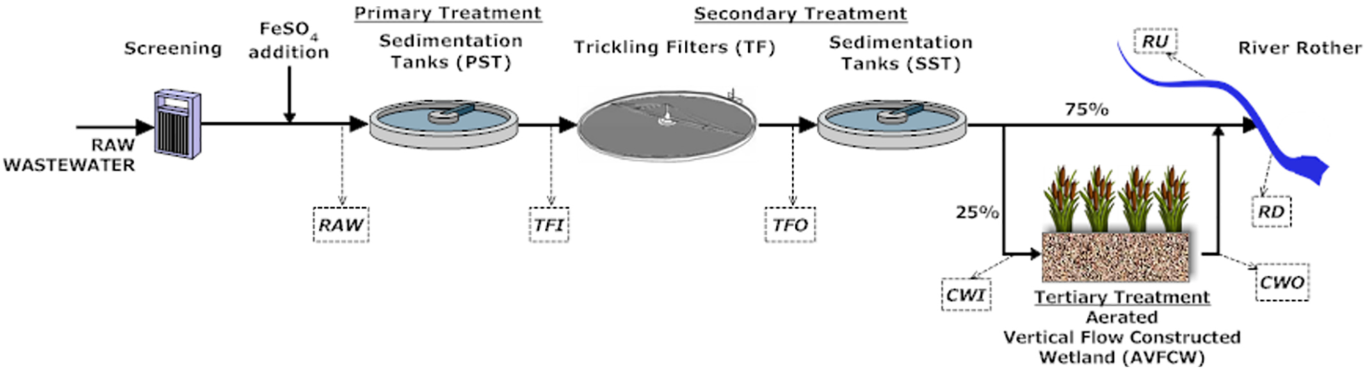

Primary Treatment
Secondary Treatment
Screening
FeSO4 addition
Sedimentation Tanks (PST)
Trickling Filters (TF)
Sedimentation Tanks (SST)
RU
River Rother
RAW WASTEWATER
75%
25%
RAW
TFI
TFO
RD
CWI
CWO
Tertiary Treatment
Aerated Vertical Flow Constructed Wetland (AVFCW)

Figure 1

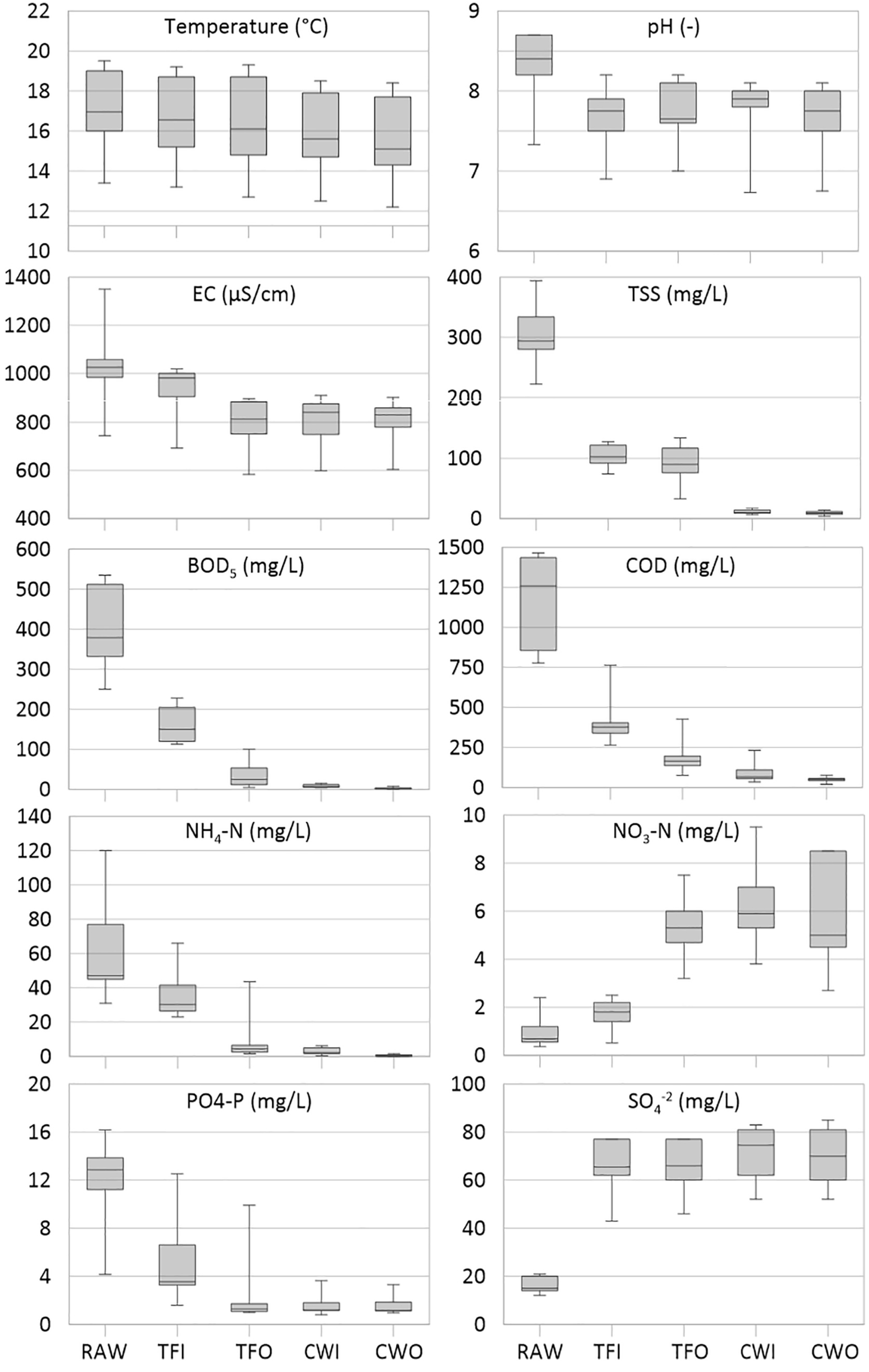

Temperature (°C)
pH (-)
EC (µS/cm)
TSS (mg/L)
BOD5 (mg/L)
COD (mg/L)
NH4-N (mg/L)
NO3-N (mg/L)
PO4-P (mg/L)
SO4-2 (mg/L)
RAW
TFI
TFO
CWI
CWO
RAW
TFI
TFO
CWI
CWO

Figure 2

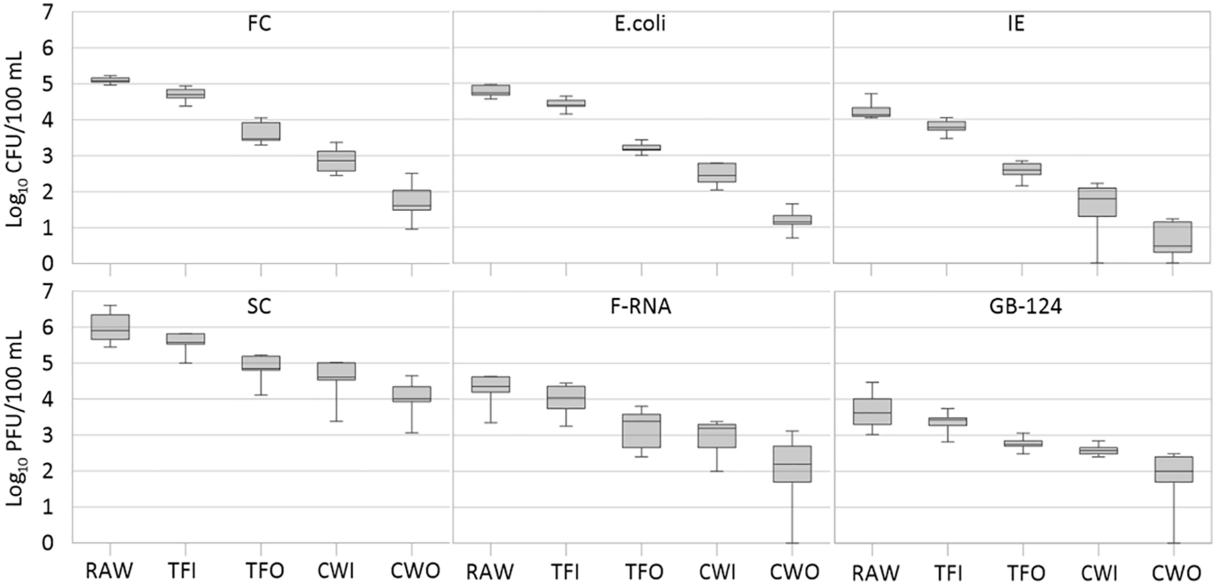

FC
E.coli
IE
SC
F-RNA
GB-124
Log10 CFU/100 mL
Log10 PFU/100 mL
0
1
2
3
4
5
6
7
RAW
TFI
TFO
CWI
CWO

Figure 3

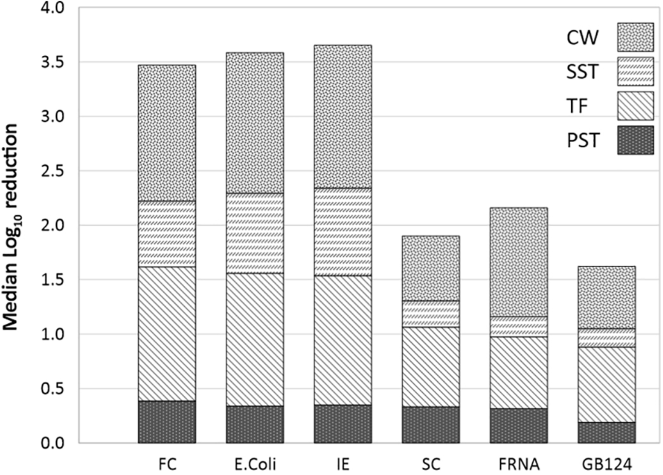
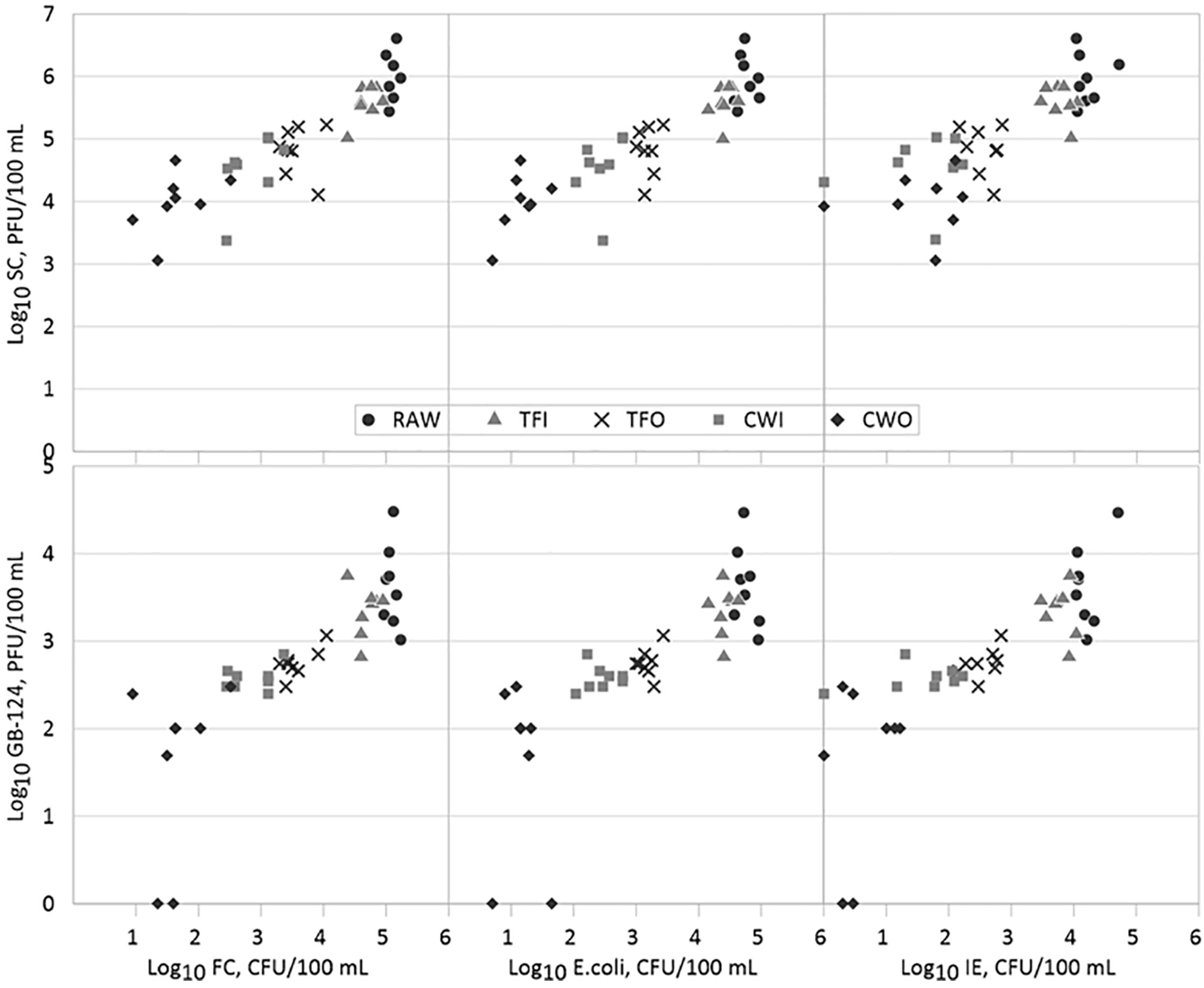

Median Log10 reduction
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
CW
SST
TF
PST
FC
E.Coli
IE
SC
FRNA
GB124

Figure 4



Log10 SC, PFU/100 mL
Log10 GB-124, PFU/100 mL
RAW
TFI
TFO
CWI
CWO
Log10 FC, CFU/100 mL
Log10 E.coli, CFU/100 mL
Log10 IE, CFU/100 mL

Figure 5