Title: Detection, fate and inactivation of pathogenic Norovirus employing settlement and UV treatment in wastewater treatment facilities.

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Abstract

It is accepted that discharged wastewaters can be a significant source of pathogenic viruses in receiving water bodies contributing to pollution and may in turn enter the human food chain and pose a risk to human health, thus norovirus (NoV) is often a predominant cause of gastroenteritis globally. Working with NoV poses particular challenges as it cannot be readily identified and detection by molecular methods does not assess infectivity. It has been proposed that the infectivity of NoV may be modelled through the use of an alternative virus; F-specific RNA (FRNA) bacteriophages; GA genotype and other FRNA bacteriophages have been used as a surrogate in studies of NoV inactivation.

This study investigated the efficiency of novel pulsed ultraviolet irradiation and low pressure ultraviolet irradiation as a potential pathogen inactivation system for NoV and FRNA bacteriophage (GA) in secondary treated wastewaters. The role of UV dose and the impact of suspended solids concentration on removal efficiency were also examined. The study also investigated the role of settlement processes in wastewater treatment plants in removing NoV. While NoV inactivation could not be determined it was found that at a maximum UV dose of 6.9 J/cm^2 (6900 mJ/cm^2) an average 2.4 log removal of FRNA bacteriophage (GA) was observed; indicating the potential need for high UV doses to remove NoV if FRNA bacteriophage prove a suitable indicator for NoV. The study found that increasing concentrations of suspended solids impacted on PUV efficiency however, it appears the extent of the impact may be site specific. Furthermore, the study found that settlement processes can play a significant role in the removal of FRNA bacteriophage, thus potentially NoV.

1.0 Introduction

The release of treated and untreated effluent to water-bodies is a global occurrence and can be a significant source of pathogenic viruses. Norovirus (NoV) is one such pathogenic virus, which has garnered significant attention particularly in the public health sector and shellfish industry. Noroviruses (NoVs) are small single stranded RNA viruses that are members of the Caliciviridae family. The species is subdivided into five separate genotypes, Genogroups I (GI), II (GII), III (GIII), IV (GIV) and V (GV) (da Silva *et al.,* 2007). Typically GI and GII predominate (da Silvia *et al.,* 2007; Scallan *et al.,* 2011; Ahmed *et al.,* 2014). As diagnostic methods for NoV infection have improved there has been growing recognition of its importance in response to growing incidence of NoV infection. NoV is now accepted as the most common aetiological agent of sporadic acute gastroenteritis in many communities, posing a particular risk to young, elderly and immune-compromised individuals (Nordgren *et al.,* 2009; CDC, 2012). NoV also accounts for a high proportion of community and hospital outbreaks of acute self-limiting gastroenteritis infection (Lopeman *et al.,* 2004; da Silvia *et al.,* 2007; Nordgren *et al.,* 2009; Ahmed *et al.,* 2014). Direct person to person transmission of NoV and transmission in contaminated indoor environments (hospitals, long term care facilities and cruise ships) have been established as important factors in the spread of infection.

Food and waterborne transmissions however are also of concern and in this context discharges of human effluent containing large numbers of virus particles (10^{11} viruses/gram of faeces) is a concern (Atmar *et al.,* 2008). Inadequate wastewater treatment can result in the persistence of viruses and microorganisms leading to environmental pollution, economic impacts and risk to human health (da Silva *et al.,* 2007; Cheng *et al.,* 2012). To date most technology development and regulation related to wastewater treatment has focused on organic carbon, suspended solids (SS), nutrient and general microbial removal from discharged wastewaters. While wastewater treatment plants (WWTPs) can contribute to virus removal it is widely accepted that specifically designed process are required to fully remove or inactivate viruses (Ottoson *et al.,* 2006; Nordgren *et al.,* 2009; EPA, 2016). However, molecular methods for the detection of NoV do not reliably differentiate between active and inactivated viruses which are a major limitation in the evaluation of the effectiveness of methods such as ultraviolet irradiation (UV) which does not remove the virus but rather inactivates it.

There is a need for robust RNA based identification tools to critically evaluate, not just the genomic viral load, but distinguish between non-infectious and infectious NoV (Gibson *et al.*, 2012; Vinjé, 2015). Advances in molecular applications have permitted increased investigation into infection rates associated with the specific NoV genogroups and their pattern of distribution (Franck *et al.*, 2015). However, the sensitivity and quantitative ability of RT-qPCR is overshadowed by its inability to distinguish between infectious and non-infectious viral loads.

Surrogates, such as those from the Caliciviridae family have been studied as possible indicators to determine the level of NoV viral contamination. It is widely agreed that their use may allow adequate assessment of the environmental persistence of NoV (Dore & Lees, 1995; Flannery *et al.*, 2012). Surrogates suggested for NoV detection include the murine calicivirus, feline calicivirus, tulane virus and the male-specific or F-specific RNA (FRNA) bacteriophages (Dore *et al.*, 2000; Karst *et al.*, 2003; Cannon *et al.*, 2006; Hirneisen & Kniel, 2013; Kniel, 2013). The FRNA bacteriophage (GA) is a human-specific phage strain that lies within the Levivirus genogroup II. It is morphologically and physio-chemically similar to NoV and it may also be cultivated i.e. infectivity can be determined, thus it has been suggested as a robust choice of NoV surrogate and was used in this study.

Current European regulations do not require monitoring of viral loads in treated wastewater which can be an intense source of pathogenic viruses. WWTPs generally comprise a number of stages including primary treatment, (generally removal of larger solid matter), secondary treatment, (generally biological treatment of wastewater) and tertiary treatment (further treatment of wastewater or removal of targeted pollutants). It is widely accepted that primary and secondary wastewater treatment systems do not fully remove viruses or FRNA bacteriophages (Nordgren *et al.*, 2009; Ottoson *et al.*, 2006). Tertiary treatment such as pathogen removal is increasingly crucial in order to comply with strict regulations, such as those that may necessitated by implementation of the Water Framework Directive (WFD - 2000/60/EC), Surface Waters Regulations (S.I. No. 272 of 2009) and the European Community Shellfish Waters Directive (2006/113/EC) or to enable opportunities for wastewater reuse. Ultraviolet (UV) disinfection is frequently preferred in the wastewater treatment sector (where pathogen removal technologies are deployed) as it limits production of harmful by products that require further processing and is a cost-effective option both terms of capital costs and running costs (Moghadam & Dore, 2012**;** EPA, 2016). UV

processes are divided into three main categories (1) low pressure/low intensity lamps (2) low pressure/ high intensity lamps (3) medium pressure/high intensity lamps. These systems vary with respect to operating pressure and output level and it is low and medium pressures that are employed in the treatment of wastewater (Whitby & Scheible, 2004; Leong *et al.* 2008). A further consideration is the upstream performance of a WWTP which can have significant impacts on the efficacy of downstream UV technologies. While turbidity and suspended solids can have a negative effect on UV disinfection by decreasing transmissivity (the transmission of UV light through the water body) these factors are not routinely considered when monitoring UV performance.

Low pressure (LP) UV disinfection results in photochemical damage to the DNA or RNA of the microorganism thus inhibiting reproduction leading to eventual cell death. It is worth noting however, that photoreactivation or 'dark repair' can occur post UV disinfection in wastewater, albeit mainly in bacteria strains (Guo *et al.,* 2011; Goosen & Moolenaar, 2008). Recently pulsed UV (PUV) light technology has been investigated as an alternative to existing technologies. PUV offers the capability to better control discharge energy and UV dosage (J/cm^2 / mJ/cm^2) by varying the operating voltage of the unit or the frequency of discharges (Garvey *et al.,* 2010; 2015).

The objectives of this study were to examine the effectiveness of using conventional low pressure UV irradiation and novel high-intensity pulsed UV disinfection in removing NoVs (via a FRNA bacteriophage (GA) surrogate) from secondary treated wastewater. The study also focused on the role of settlement processes in removing NoV within WWTPs and the impact of SS on UV system performance.

2.0 Materials and Methods

2.1 Source of wastewater

Samples for this study were taken from three municipal activated sludge WWTPs (WWTP 1, 2 and 3), whereby WWTP 1 was used for the majority of the study and the remaining two WWTP were used to ascertain the effect of SS on disinfection performance. WWTP 1 comprised primary and secondary treatment systems with phosphorus removal via ferric sulphate addition and tertiary treatment by sand filtration. WWTPs 2 and 3 comprised primary and secondary treatment systems with WWTP 2 also featuring a LP UV system. The WWTPs ranged in size from 21,000 population equivalent (WWTP 1) to 250 (WWTP 2) and

3,500 (WWTP 3). All received municipal wastewater from combined storm and municipal sewers.

2.2 Pulsed UV irradiation

Secondary effluent was sampled at an effluent sampling point in WWTP 1. Samples were transported to the laboratory and were stored at constant temperature (10 °C) prior to processing. A bench–scale pulsed power source (PUV – 1, Samtech Ltd., Glasgow) was used to power a low pressure (60 kPa) xenon-filled flashlamp (Heraeus Noblelight XAP type NL4006 series constructed from a clear UV transparent quartz tube) which produced a high intensity beam of polychromatic pulsed light. The lamp was placed 10 cm above a sterilised aluminium flow- through vessel which pumped secondary effluent through the vessel at the desired flow rate (Figure 1). The system when operating at 900 V and 5.6 pulses per second (PPS) - gave a maximum dose of 6.9 J/cm^2 (6900 mJ/cm^2) at a hydraulic residence time (HRT) of 120 s. The PUV system allowed for the input voltage and the pulse rate to be varied between 300 and 900 V and 0.1 and 10 PPS.

As a control, wastewater batches (secondary effluent and distilled water; 7 L) were "spiked" with known quantities of NoVs (genogroup I (GI) and genogroup II (GII)) and FRNA bacteriophage (GA) to accurately measure log reduction. These were processed using the bench-scale novel PUV system in the same conditions as used on the effluent samples and the results compared against the "spiked" concentrations.

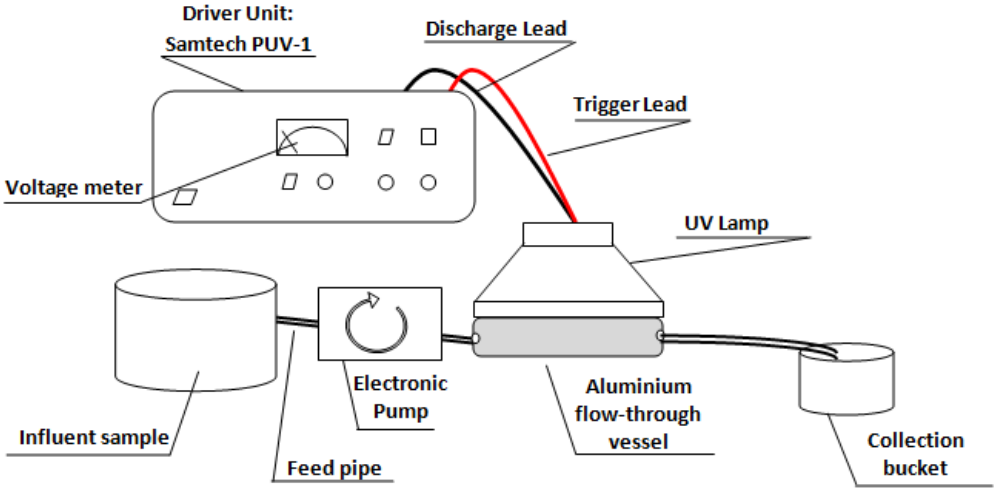


Figure 1 Schematic of the bench-scale PUV experimental set-up.

To ascertain the effect various wastewater parameters (suspended solids, total organic carbon and total inorganic carbon) had on PUV viral inactivation in secondary effluent four experimental trials (Trials 1 – 4) were carried out on separate days (where the number of runs carried out on each day varied between 1 (trial 1) and 3 (trials 2, 3 and 4), examining FRNA bacteriophage (GA; infectivity assay) inactivation/removal. The effect of SS on PUV efficiency was investigated over 10 trials in total in terms of GA bacteriophage removal. During Trials 2, 3, and 4 secondary effluent (7 L) was spiked with a known concentration of mixed liquor suspended solids (MLSS) and seeded with a known concentration of FRNA bacteriophage (GA). Samples were collected pre and post treatment. The flow through the PUV system was controlled to give hydraulic residence times of 60 s HRT and a UV dosage of 3.4 J/cm^2 (3400 mJ/ cm^2)and 120 s HRT resulting in a UV dosage of 6.9 J/cm^2 (6900 mJ/ cm^2).

Batches (25 L) of secondary treated wastewater samples were collected from three separate WWTPs – 1, 2 and 3. Five litres from each batch was then seeded with a known quantity of GA bacteriophage and pumped through the PUV system at two HRTs; 60 s HRT (3.4 J/cm^2; 3400 mJ/ cm^2) and 120 s HRT (6.9 J/cm^2; 6900 mJ/ cm^2)**.** Influent and effluent samples to and from the PUV system were collected and analysed in each case. For comparison, separate 5 L samples from each batch of the aforementioned 25 L samples were filtered using 0.1 - 0.2 µm filter removing all SS. The same procedure for the unfiltered samples was then applied to the filtered samples and the results were compared to the unfiltered samples.

2.3 Comparison between PUV and low pressure UV systems

WWTP 2 comprised a LP UV treatment system (AquaPRO, UV12GPM-HTM) which treated wastewater from a secondary treatment process with a clarifier. During this study grab samples (1 L) were collected at both the influent and effluent points of the LP UV system to investigate pathogen removal. In order to draw a direct comparison, 6 x 5 L grab samples of the wastewater influent to the LP UV system were collected for processing via the bench-scale PUV system. These samples were taken concurrently with the aforementioned 1 L samples.

The grab samples for the PUV system were immediately stored in a refrigerator at 4 °C, transported to the laboratory and were processed via the bench-scale PUV system within 24 hours. The operating details of the LP UV and PUV systems are outlined in Table 1.

Following this, three categories of pathogen inactivation/removal were examined via LP UV and PUV; *E. coli*, total coliforms and FRNA bacteriophage (GA). The comparison between the LP UV and PUV systems were carried out in duplicate on three consecutive days. The LP UV system was operated at three flow rates (resulting in three hydraulic residence times (HRT's); 0.23 m^3 /hr, 0.52 m^3 /hr and 0.85 m^3 /hr. These resulted in UV dosage rates of 0.117 J/cm^2 (117 mJ/cm^2), 0.0516 J/cm^2 (51.6 mJ/cm^2) and 0.0316 J/cm^2 (31.6 mJ/cm^2), respectively.

Table 1. Operational detail of LP UV and PUV .

(Day)	**LP UV**			**PUV**
(1)				
Flow Rate (m^3/hr)	0.23	0.52	0.85	0.04
UV Dose - J/cm^2 (mJ/cm^2)	0.117 (117.0)	0.0516 (51.6)	0.0316 (31.6)	3.4 (3447.8)
(2)				
Flow Rate (m^3/hr)	0.220	0.529	0.818	0.04
UV Dose - J/cm^2 (mJ/cm^2)	0.122 (122)	0.050 (50.7)	0.0328 (32.8)	3.4478 (3447.8)
(3)				
Flow Rate (m^3/hr)	0.209	0.526	0.810	0.04
UV Dose - J/cm^2 (mJ/cm^2)	0.1284 (128.4)	0.051 (51)	0.0331 (33.1)	3.4478 (3447.8)

2.4 Fate of both NoV and FRNA bacteriophage (GA strain) in wastewater treatment plants

To ascertain the fate of NoV and FRNA bacteriophage through WWTP1, 2 L batch samples of wastewater were collected from five sampling points at the WWTP; raw influent (R), primary clarifier (PT), the activated sludge reactors and the secondary clarifier (ST) on two separate sampling days. The activated sludge reactor was divided into two samples; the sample as taken from the reactor (Sample A) and a sample (Sample AS) representing the clarified portion was collected following 24 hours of settlement in a graduated cylinder. In all cases samples were transported at 4 ^oC before immediate processing (detailed below).

2.4 Measurement of wastewater characteristics

Suspended solids (SS) were measured in accordance with standard methods (APHA, 2005). Total organic carbon (TOC) and total inorganic carbon (TIC) were measured using the Biotector TOC, TN, TP Analyser (Biotector Analytical System Ltd., Cork, Ireland) in accordance with standard methods. Results were expressed in mg/L. Wastewater samples were tested for iron and manganese levels by Environmental Laboratory Services (ELS) Ltd Acorn Business Campus, Mahon Industrial Park, Blackrock, Cork, Ireland. Five litre batch samples were collected; 2 L of each sample was filtered through a 0.1 µm membrane cassette (Pall's Omega™ polyethersulfone [PES]; Fitzhenry *et al.,* 2014). One x 75 mL sample of both unfiltered and filtered effluent from each site was held in a storage vial at the correct pH at 4 °C. All samples were collected after two days and couriered to ELS for metals analysis. The LOQ of the tests were 5 µg/L and 1 µg/L for iron and manganese respectively.

2.4 Extraction procedure for NoV and FRNA bacteriophage (GA) analysis

Harvested samples were concentrated from 40 mL to 500 µL using a virus adsorption-elution method previously described in Fitzhenry *et al.* (2014) and Flannery *et al.* (2013). The resulting 500 µL underwent RNA extraction employing NucliSENS miniMAG extraction platform and the corresponding NucliSENS magnetic extraction reagents. Samples were then stored at -80 °C prior to further processing. The use of a non-related virus (Mengo virus) was also employed as an 'internal process control' (IPC) to determine the extraction efficiency (Costafreda *et al.,* 2006). A negative RNA extraction control, molecular grade water (Bioline) was additionally processed alongside the samples.

2.5 Microbiological analysis

Samples were processed using both molecular and cultivation-dependent analyses. NoV GI and GII analysis was carried out via molecular methods only while FRNA bacteriophage (GA) was processed by both molecular analysis and microbiological culture analysis.

2.5.1 Microbiological culture analysis

FRNA bacteriophage (GA)

A standardised plaque assay coupled with an in-situ hybridisation assay was employed to determine infectious FRNA bacteriophage (GA) and to distinguish between genotypes (Sundram *et al.,* 2006). *Salmonella enterica* serovar Typhimurium WG49 was used as a host strain in order to enumerate the FRNA bacteriophage (GA) using a double - layer overlay

plaque assay as previously outlined in Flannery *et al.* (2013). Briefly, 1 mL of appropriately diluted sample was added to 1 mL of host culture (*Salmonlla Typhimurium* WG49) and 2.5 mL of molten tryptone – yeast glucose agar and held at 45 °C. This mix was then poured onto hardened tryptone – yeast glucose agar plates and left to solidify before being transferred to a 37 °C incubator for 18 ± 2 hours inverted. Once the incubation period had passed, the plates were removed and characteristic plaques were counted by visual inspection, where each plaque was assumed to originate from one GA bacteriophage. The results were expressed as plaque forming unit (pfu)/mL. The Limit Of Detection (LOD) for this test was 1 pfu/mL. Additionally an internal positive culture control was run alongside the environmental samples to ensure the assay was performed to capacity.

Escherichia coli (E. coli) enumeration in wastewater

Total coliforms were enumerated using the Quanti – Tray Colisure test, IDEXX based on the most probable number method (MPN).The standardised five-tube, three-dilution MPN; ISO-TS 16649) was used to assess the presence/reduction of *E. coli* in UV influent and UV treated effluent wastewater samples. Appropriate $\log_{10}$ dilutions of samples were inoculated into 10 mL volumes of minerals modified glutamate broth (MMGB; CM0607, Oxoid) and were incubated at 37 °C for 24 ± 2 h. The presence of *E. coli* was subsequently confirmed by sub-culturing tubes indicating acid production onto Tryptone Bile Xglucuronide (TBX) agar (CM0945, Oxoid) at 44 °C for 22 ± 2 h. The limit of detection (LOD) of the assay was 20 *E. coli*/100 mL.

2.5.2 Microbiological molecular analysis

A one step Reverse Transcriptase Quantative Polymerase Chain Reaction (RT-qPCR) was employed to enumerate NoV GI, NoV GII and FRNA bacteriophage (GA). During this process, extracted RNA was reverse transcribed into its complementary double stranded plasmid DNA (dsDNA) sequence and was quantified. Standard curves for quantification of the three targets (NoV GI, NoV GII and FRNA bacteriophage (GA) were constructed using dsDNA corresponding to each virus and primer/probe sets and thermal cycling protocol, as outlined in detail in Flannery *et al.* (2012; 2013; Table 2). Briefly to create a standard curve, a 10-fold serial dilution series, ranging from 10^5 down to 10^1 copies (of each target) μL^{-1} was generated and analyzed in duplicate by real-time PCR using Applied Biosystems AB7500 qPCR instrument with the corresponding primer/probe sets (Table 2). Five microlitres of sample RNA were added in duplicate to the wells of a 96 – well optical reaction plate with 20

µL one-step mastermix (Invitrogen). NoV GI, GII and GA FRNA bacteriophage were quantified by comparing the Cq values to the appropriate standard curves in copies per mL, and then adjusted to reflect the volume of RNA analysed (expressed as genome copies g_1 HP or genome copies 100 mL_1 wastewater). The Limit Of Quantification (LOQ) for this test was 125 copies/100 mL and the Limit Of Detection (LOD) was 25 copies/100mL. For extraction efficiency (determined using the IPC; Mengo virus; Table 2) previously described forward (Mengo209) and reverse (Mengo110) primers and probe (Mengo147) were used (Pinto *et al.*, 2009) and was performed as outlined in detail in Flannery *et al.* (2012). The Cq value of the sample was compared to a standard curve obtained by preparing log dilutions from the same batch of Mengo virus as was used to spike samples for analysis. This was expressed as percentage extraction efficiency and samples with an extraction efficiency of less than 1 % were not accepted.

Table 2: The suite of RT-qPCR primers and probes utilized in this study.

Target	Probe	Primer	Sequence (5` to 3`)	Reference
NoV GI	NVGG1p		VIC-TGGACAGGAGAYCGCRATCT-TAMRA	Svraka *et al.*, 2007
		Forward-QNIF4	CGCTGGATGCGNT TCCAT	
		Reverse-NV1LCR	CCTTAGACGCCATCATCATTTAC	da Silva *et al.*, 2007
NoV GII	QNIFS		FAM-AGCACGTGGGAGGGCGATCG-TAMRA	Loisy *et al.*, 2005
		Forward-QNIF2	ATGTTCAGRTGGATGAGRTTCTCWGA	
		Reverse-COG2R	TCGACGCCATCTTCATTCACA	Kageyama *et al.*, 2003
				Loisy *et al.*, 2005
FRNA Bacteriophage (GA)	Genogroup I- Probe		CAWGGTAGCGTCTCGCTAAAGACATT	Wolf *et al.*, 2008
		Forward-Genogroup I-	GTCCTGCTCRACTTCCTGT	
		Reverse - Genogroup I	CGGCTACCTACAGCGATAG	
Mengo virus	Mengo147		ATCACATTACTGGCCGAAGC	
		Forward-Mengo209	GAAGTAACATATAGACAGACGCA CAC	Costafreda *et al.*, 2006
		Reverse-Mengo110	GCGGGTCCTGCCGAAAGT	Pintó *et al.*, 2009

2.6 Calculation of log reductions

Log reductions of NoV GI, NoV GII and FRNA bacteriophage (GA) were calculated by determining the absolute of each target before and after treatment with bench-scale PUV and On-site LP UV, respectively.

2.7 Statistical analysis

In order to evaluate any differences between NoV GI and NoV GII concentrations in the environmental samples and between concentrations of infectious and total FRNA bacteriophage GA, minitab statistical software was used to carry out nonparametric tests

(MannWhitney Rank Sum Test) and Microsoft excel was used for correlation coefficient analysis.

3.0 Results and Discussion

3.1 Comparison of molecular and culture viral enumeration methods and potential the use of FRNA Bacteriophage (GA) as surrogate for NoV

In this study the effect of PUV disinfection on virus inactivation in wastewater was measured over the HRTs of 60 (UV dose – 3.4 J/cm^2; 3400 mJ/cm^2) and 120 seconds (s) (UV dose – 6.9 J/cm^2; 6900 mJ/cm^2), pulsed UV intensity and at varying SS concentrations. In parallel with molecular analysis targeting NoV GI and GII and GA bacteriophage via RT–qPCR, FRNA bacteriophage (GA) via infectivity assay pre and post PUV treatment (n = 10) were also studied as an indicator of infectivity. Results for the infectivity assay showed an average 2 $\log_{10}$ mean reduction of FRNA bacteriophage (GA) at a HRT of 120 s (UV dose – 6.9 J/cm^2; 6900 mJ/cm^2) over the entire study period. The starting GA bacteriophage copy concentrations (0 s) were significantly different to NoV GI and GII viral copy concentrations following PUV treatment at 60 s (p = 0.0002) and at 120 s (p = 0.0002; UV dose – 6.9 J/cm^2 (6900 mJ/cm^2) at a 95% confidence interval (Figure 2). The overall copy concentrations varied significantly between PUV treatment at HRT's of 60 s and 120 s (UV doses – 3.4 J/cm^2 (3400 mJ/cm^2) and 6.9 J/cm^2 (6900 mJ/cm^2), respectively, at a 95 % confidence interval (p = 0.0211). The average reduction of FRNA bacteriophage (GA), NoV GI and NoV GII as measured by RTqPCR was < 0.5 $\log_{10,}$ thus there was little or no reduction of copy concentrations at the maximum UV doses applied throughout the study (6.9 J/cm^2 (6900 mJ/cm^2); all stated PUV dosage rates relates to wavelengths < 300 nm; (Figure 2; Table 3). NoV GI results yielded significant differences pre and post PUV treatment at 60 s (p= 0.0409) and 120 s (p = 0.0298) and also between PUV treatments (60s and 120s; p= 0.0409) at 95 % confidence interval.

While the data indicates significant differences in the recorded copy concentrations pre and post PUV treatment, it is not representative of the reduction of infectious virus. Therefore in this incidence, RT-qPCR was not appropriate method of assessing the inactivation of NoV and FRNA bacteriophage (GA) via PUV post UV treatment. This may be possibly due to the fact that genomic debris resulting from UV treatment can result in false-positive PCR signals (Pecson *et al.,* 2010). As viable NoV concentrations could not be assessed post PUV treatment, findings in this study could not draw direct comparisons between FRNA

bacteriophage (GA) and NoV reductions. However, due to the high resistance properties of FRNA bacteriophage (GA) to PUV treatment and its morphological similarities to NoV, this would indicate that the use of this virus as a potential surrogate may have value and would warrant further analysis based on previous studies (Doré *et al.*, 2000; Flannery *et al.*, 2012).

Similar to this study, Baert *at al.* (2008) found RT-qPCR results were unable to distinguish between infections and non-infectious NoV, using murine norovirus (MNV) as a surrogate; no correlation was seen when comparing an infectivity assay and RT-qPCR for the detection of MNV examining virus inactivation using heat treatment. Conversely, when comparing infectivity assays and RT-qPCR, Bae and Schwab (2008) determined that MNV, FRNA coliphage MS2, and poliovirus (PV) all have the potential to be useful surrogates for human NoVs and are the most robust choice of NoV identification. In order to efficiently distinguish between infectious and non-infectious NoV, investigative focus has moved towards firstly the optimisation of targeting relevant gene sequences to permit the generation of specific primer and probes for infections/non-infectious identification, independent of culture based systems. This in itself is extremely challenging as only one small region is adequately conserved enough to construct specific genogroup oligonucleotide primers and probes (Green *et al.*, 2001; Katayama *et al.*, 2002; Zheng *et al.*, 2006; Vinjé, 2015).

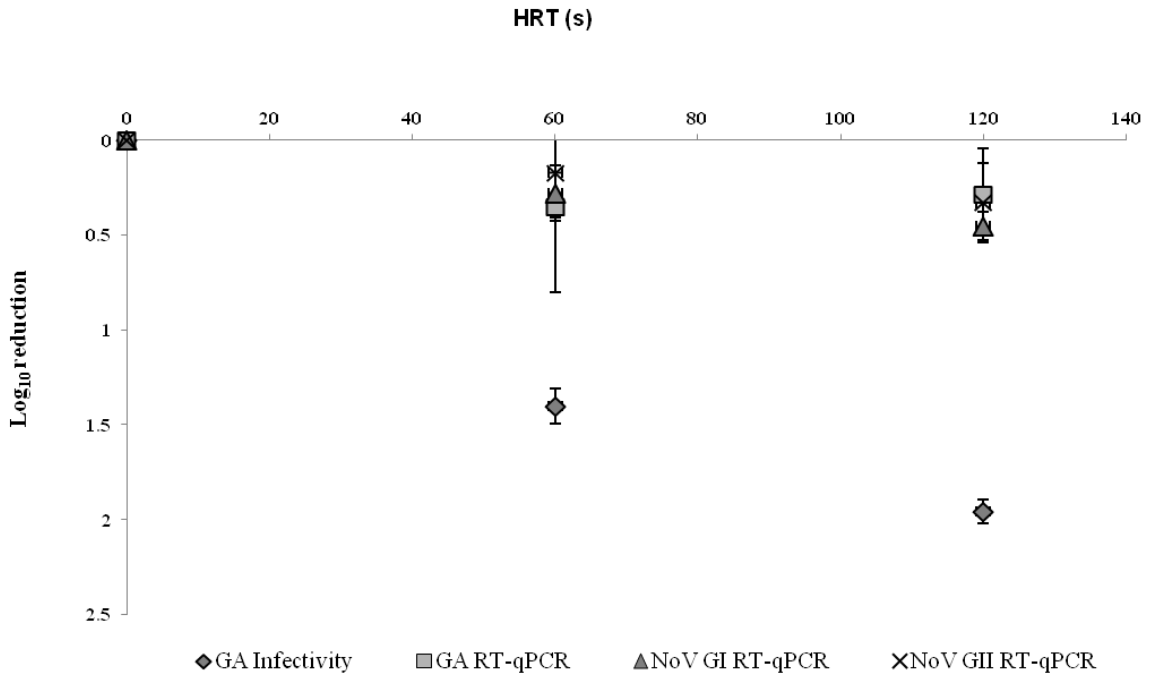


Figure 2. Average $\log_{10}$ reduction of viable FRNA bacteriophage (GA; infectivity assay; n = 10) and copy number of FRNA bacteriophage (GA) and norovirus GI and GII (RT-qPCR; n = 7) post PUV treatment in wastewater. (The average initial concentration was 10^6/100 mL).

Table 3. Log_{10} reduction of infective FRNA bacteriophage (GA; n = 10) as tested by the infectivity assay and copy concentrations of FRNA bacteriophage (GA) and norovirus GI and GII (n = 7) as tested by RT-qPCR post PUV treatment in wastewater (60 s HRT: UV dose – 3.4 J/cm^2 (3400 mJ/cm^2), and 120 s HRT: UV dose 6.9 J/cm^2 (6900 mJ/cm^2); the average initial concentration was 10^6/100 mL).

Infectivity Assay		**RT-qPCR Assay**					
FRNA bacteriophage (GA)		FRNA bacteriophage (GA)		NoV GI		NoV GII	
Log_{10} removal at HRT ± standard deviation		Log_{10} removal at HRT ± standard deviation		Log_{10} removal at HRT ± standard deviation		Log_{10} removal at HRT ± standard deviation	
60s	120s	60s	120s	60s	120s	60s	120s
2.21± 0.16	3.14 ± 0.03	1.07± 0.27	1.07± 0.21	0.26± 0.27	0.15± 0.12	0.21± 0.27	0.78± 0.08
1.29± 0.12	1.72± 0.01	0.42± 0.60	0.27± 0.01	0.09± 0.36	0.46± 0.01	0.15± 0.03	0.30± 0.04
1.02± 0.13	1.60± 0.07	0.15± 0.23	0.11± 0.12	0.20± 0.10	0.40± 0.09	0.17± 0.01	0.34± 0.13
1.19± 0.02	1.49± 0.07	0.43± 0.56	0.23± 0.07	0.12± 0.07	0.20± 0.16	0.40± 0.45	0.21± 0.08
1.49± 0.05	2.30± 0.08	0.03± 0.10	0.19± 0.11	0.46± 0.15	0.70± 0.06	0.27± 0.05	0.50± 0.16
1.24± 0.15	1.80± 0.03	-0.43± 0.76	-0.19± 1.09	0.52± 0.01	0.70± 0.09	0.11± 0.32	0.30± 0.33
1.23± 0.01	1.40± 0.08	0.79± 0.65	-1.27± 2.31	0.32± 0.06	0.59± 0.04	-0.11± 0.55	-0.11± 0.65
1.68± 0.10	2.48± 0.08	-	-	-	-	-	
1.60± 0.12	1.95± 0.03	-	-	-	-	-	
1.06± 0.08	1.70± 0.15	-	-	-	-	-	

3.2 Impact of operating conditions on PUV irradiation

Overall, a linear relationship was observed between voltage and inactivation rate for any given HRT (Figure 3). FRNA bacteriophage (GA) concentration was reduced by 1.3 log_{10} at a UV dose of 1.2 J/cm^2 (1200 mJ/cm^2; 120 s HRT) when subjected to 900 V at 1 PPS. A reduction of 1.0 log_{10} was achieved at a UV dose of 0.55 J/cm^2 (550 mJ/cm^2; 120 s HRT) at 600 V and 1 PPS; whereas at 300 V and 1 PPS, a maximum virus reduction of 0.4 log_{10} was observed at a UV dose of 0.14 J/cm^2 (140 mJ/cm^2; 120 s HRT); thus demonstrating the impact of HRT on inactivation.

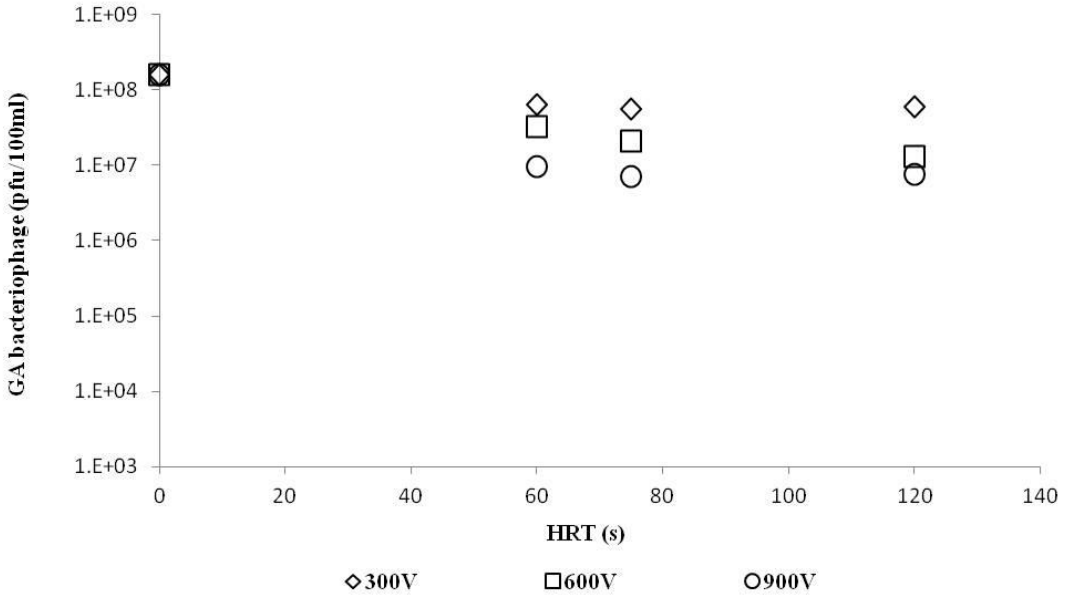


Figure 3. The effect of three specific voltages (300, 600 and 900 V) and three HRTs (60, 75 and 120 s) at 1 PPS on FRNA bacteriophage (GA) reduction.

3.3 Comparison of on-site LP UV and bench-scale PUV systems

Figures 4A, 4B and 4C summarizes the comparative reduction of *E. coli*, total coliforms and FRNA bacteriophage for the LP UV and PUV. A reduction in *E. coli*, total coliforms and FRNA bacteriophage concentrations was observed in response to increasing UV dose. Comparative reduction rates between LP UV and PUV under varying doses are illustrated in Table 4. Previous studies have observed the PUV showing a significantly greater inactivity potential compared to LP UV when using *E. coli* as a bacterial indicator organism (Li *et al.*, 2010). However in this study such a difference was not apparent at similar radiation dosages.

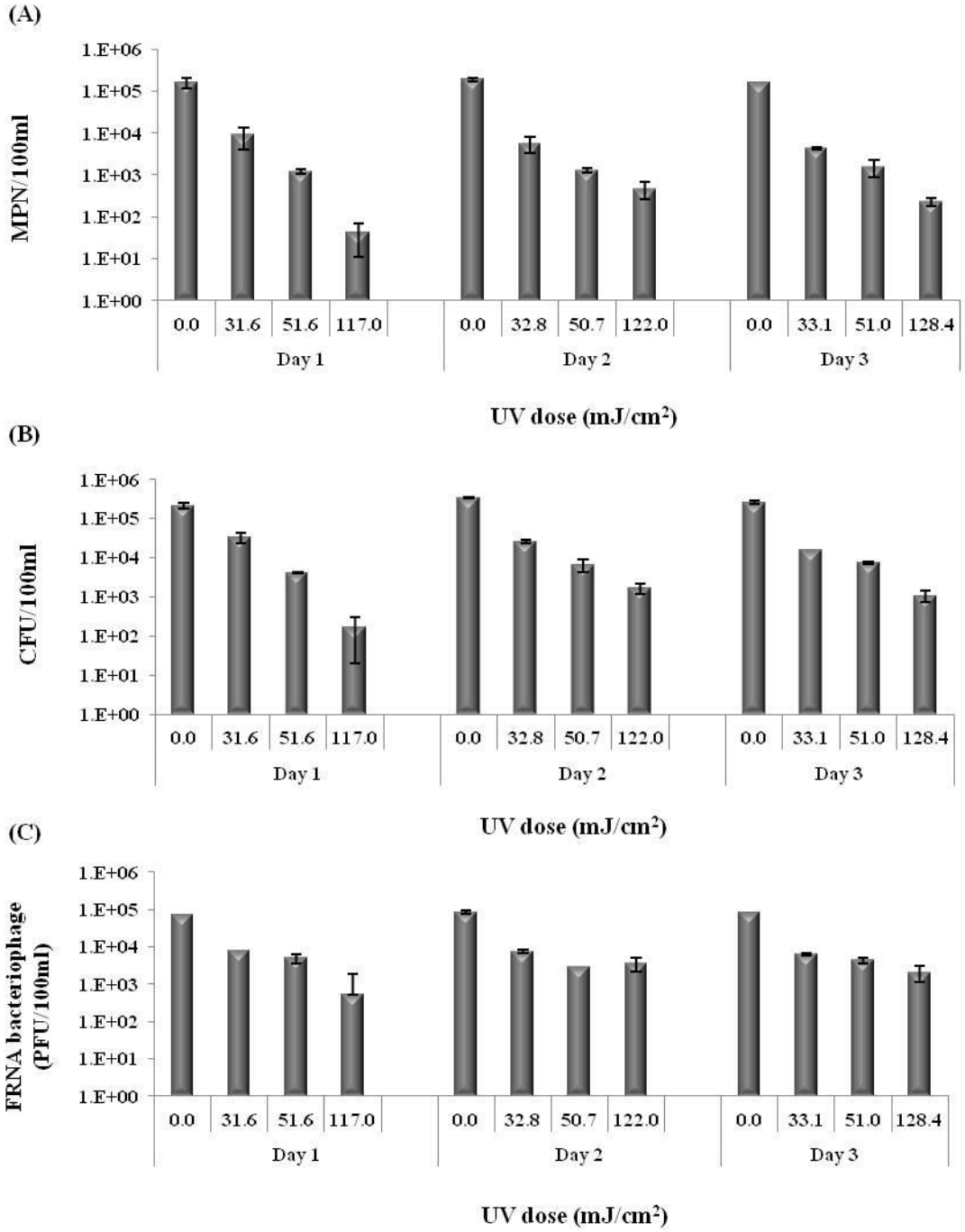


Figure 4. (A) *E. coli* reduction via LP UV over three separate trials **(B)** Total coliforms reduction via LP UV over three separate trials **(C)** FRNA bacteriophage reduction via LPUV over three separate trials.

Table 4. (A) Comparison of the operating conditions for the treatment of secondary treated wastewater employing on – site LP UV system (LP UV) and bench – scale PUV system (PUV).

Day	UV treatment	Flow Rate (m^3/hr)	HRT (s)	UV Dose - J/cm^2 (mJ/cm^2)
1	LP UV	0.228	3.9	0.117 (117)
	LP UV	0.52	1.7	0.05161 (51.61)
	LP UV	0.849	1.1	0.03161 (31.61)
	PUV	*0.04*	*60*	*3.4478 (3447.8)*
2	LP UV	0.22	4.1	0.12199 (121.99)
	LP UV	0.529	1.7	0.05073 (50.73)
	LP UV	0.818	1.1	0.03281 (32.81)
	PUV	*0.04*	*60*	*3.4478 (3447.8)*
3	LP UV	0.22	4.3	0.12841 (128.41)
	LP UV	0.529	1.7	0.05102 (51.02)
	LP UV	0.818	1.1	0.03313 (33.13)
	PUV	*0.04*	*60*	*3.4478 (3447.8)*

Table 4 (B) provides a comparison for indicative purposes and outlines the removal of *E. coli*, *Bacillus subtilis* (*B. subtilis*) and GA bacteriophage via PUV both in this study and in recent work by Uslu *et al.* (2014). In contrast to this study where the PUV was operated as a flow through system, Uslu *et al.* (2014) operated it as a static system. Recent studies employing PUV as a wastewater disinfection tool have indicated that in addition to pathogen removal/inactivation it also reduces the organic load of municipal wastewater effluent by reducing chemical oxygen demand (COD) and total organic carbon (TOC; Uslu *et al.*, 2016). Furthermore evidence suggests that the treatment with PUV enables the targeting and removal of pathogenic biofilm forming microbes, such as *Pseudomonas aeruginosa* and

Staphylococcus aureus, which are often resistant to common disinfection strategies (Garvey *et al.*, 2015). Additionally, its role in the breakdown and removal of industrial related pollutants such as azo dyes represent a novel application in the treatment of chemically contaminated wastewater and the protection of the environmental (Li *et al.*, 2016).

Table 4. (B) Microorganism reduction via PUV treatment

Uslu *et al.* (2014)						
PUV operating conditions	**Voltage:** 3,800 V **sample:** 8cm **Capacitance:** N/A **System set-up**: Static wastewater			**Lamp Distance from** **Discharge time:** 360 µs **Medium:** Treated municipal		
Microorganism	*E. coli*			*B. subtilis*		
Initial concentration	10^8 CFU/mL			10^9 CFU/mL		
SS (mg/L)	4			4		
Exposure time (s)	5	10	15	5	10	15
Broadspectrum Dose - J/cm^2(mJ/ cm^2)	3.6 (3600)	7.2 (7200)	10.9 (10900)	3.6 (3600)	7.2 (7200)	10.9 (10900)
Log_{10} removal (CFU/mL)	≈ 7 log	≈ 7 log	Complete reduction (undetected)	≈ 4 log	≈ 4 log	≈ 6 log
This study						
PUV operating conditions	**Voltage:** 900 V **sample:** 10.75cm **Capacitance:** 40µF **System set-up**: Flow-through wastewater			**Lamp Distance from** **Discharge time:** 28 µs **Medium:** Treated municipal		
Microorganism	*E. coli*			GA bacteriophage		
Initial concentration	10^5 MPN/100mL			10^7 PFU/mL	10^7 PFU/mL	10^6 PFU/mL
SS content (mg/L)	28			20	38	72
Exposure time (s)	60			120	120	120
Broadspectrum Dose - J/cm^2 (mJ/ cm^2)	18.8 (18800)			37.5 (37500)	37.5 (37500)	37.5 (37500)
Log_{10} removal	2 log_{10} MPN/100ml			2.48 log_{10} PFU/100mL	2.30 log_{10} PFU/100mL	1.95 log_{10} PFU/100mL

3.4 Impact of wastewater characteristics on PUV efficiency

Table 5 summarises the results of the trials carried out to determine the impact of wastewater characteristics on FRNA bacteriophage (GA) disinfection via the PUV. There was little evidence of TOC and TIC concentrations impacting on the overall log_{10} reduction of FRNA bacteriophage PFU (GA) decreased (SM 1) with mixed trends observed in each trial. Within trials 3 and 4 high correlations for each individual trial between SS concentration and FRNA bacteriophage (GA) PFU log_{10} reduction was observed. However, a relatively low statistical correlation (- 0.42) was observed between higher SS concentrations and decreasing log_{10} reductions of GA bacteriophage across all samples. Thus may indicate that FRNA log_{10} reductions may be influenced by factors other than the SS concentrations (Figure 5).

Table 5. PUV log_{10} reductions of infective FRNA bacteriophage (GA) in wastewater at two HRTs at a range of sample SS, TOC and TIC concentrations (mg/L; Trial 1 not included; 60 s HRT: UV dose – 3.4 J/cm^2 (3400 mJ/ cm^2) and 120 s HRT: UV dose 6.9 J/cm^2 (6900 mJ/ cm^2); the average initial concentration was 10^6/100 mL).

				Log_{10} reduction	
	Wastewater characteristics			**HRT**	
Trial	**SS (mg/L)**	**TOC (mg/L)**	**TIC (mg/L)**	**60 (s)**	**120 (s)**
2	57.5	8	24	2.2 ± 0.2	3.1 ± 0.0
	12.0	14	41	1.3± 0.1	1.8 ± 0.0
	18.6	20	38	1.0 ± 0.1	1.6 ± 0.1
	121.2	20	39	1.2 ± 0.0	1.5 ± 0.1
3	38.0	10	33	1.5 ± 0.1	2.3 ± 0.1
	105.0	8	32	1.2 ± 0.2	1.8 ± 0.0
	140.6	7	29	1.2 ± 0.0	1.4 ± 0.1
4	19.5	12	25	1.7 ± 0.1	2.4 ± 0.1
	72.0	16	26	1.6 ± 0.1	1.2± 0.0
	89.8	15	28	1.1 ± 0.1	1.7 ± 0.2

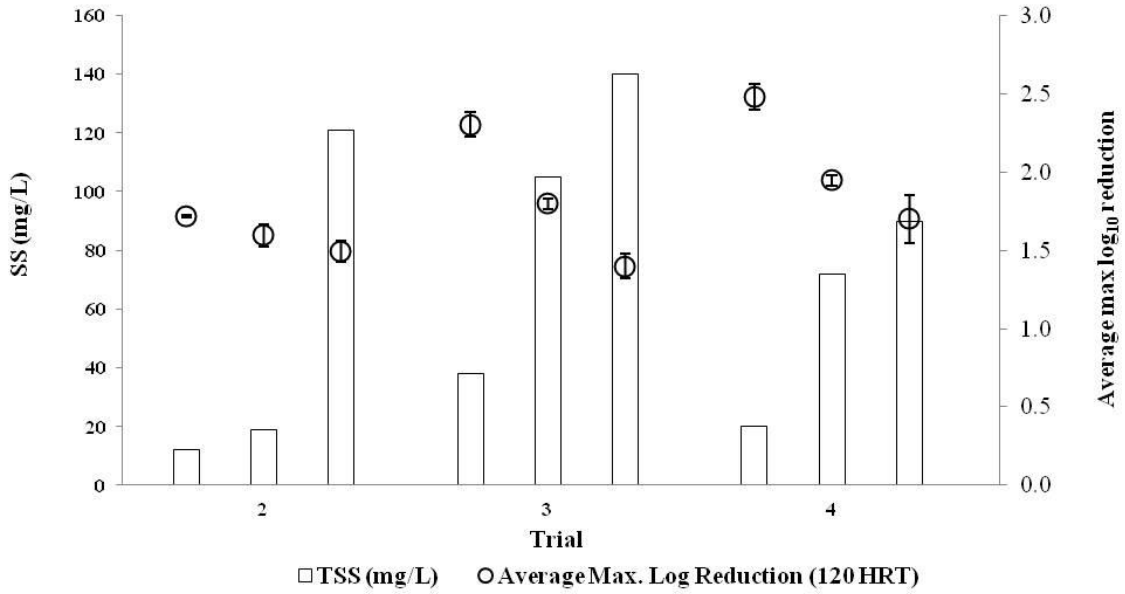


Figure 5. The relationship between SS and maximum $\log_{10}$ reduction (HRT 120 s– UV dose: 6.9 J/cm^2 (6900 mJ/cm^2) of GA bacteriophage via infectivity assay in three separate trials. (The average initial concentration was 10^6/100 mL).

During the study, the effluent wastewater from WWTPs 1, 2 and 3 was sampled with both filtered and unfiltered samples being subjected to PUV treatment. The SS concentrations of the treated wastewater at WWTPs 1, 2 and 3 were 19.5 mg SS/L, 8.7 mg SS/L and 16.0 mg SS/L, respectively; indicating the possibility of varying efficiency of secondary treatment in the respective WWTP's. Results indicate that the removal of SS within for any given WWTP resulted in improved PUV performance; however appeared to be unrelated to the initial SS (albeit the effluent samples all had relatively low SS concentrations). Following filtration of the secondary effluent obtained from WWTP 1, a 3.9 $\log_{10}$ reduction was recorded. Furthermore, virus inactivation to below the level of detection (< 1 pfu/mL) was achieved at a UV dosage of 3.4 J/cm^2 (3400 mJ/cm^2); HRT of 60 s; Table 6). Increased virus $\log_{10}$ reduction in filtered effluent compared to unfiltered effluent was also observed for WWTPs 2 and 3. However, complete viral removal was not achieved at a UV dosage of 6.9 J/cm^2 (6900 mJ/cm^2) and a maximum HRT (120 s) indicating that alternative environmental parameters were playing a role effecting UV efficacy.

Table 6. Comparison of maximum log_{10} reduction (120s HRT) of FRNA bacteriophages (GA) in filtered and unfiltered post-secondary treatment effluent treated with PUV at three separate WWTP sampling sites. (UV dose – 3.4 J/cm^2 (3400 mJ/cm^2) and 120 seconds (s) (UV dose: 6.9 J/cm^2 (6900 mJ/cm^2); the average initial concentration was 10^6/100 mL)

		Unfiltered Effluent		**Filtered Effluent**
		FRNA bacteriophages (GA)		**FRNA bacteriophages (GA)**
	HRT (s)	**(pfu/100ml)**	**HRT (s)**	**(pfu/100ml)**
WWTP Site 1	0	1.2E+07 ± 6.4E+05	0	2.3E+06 ± 1.8E+05
	60	2.5E+05 ± 4.4E+04	60	< 1 pfu/mL
	120	3.9E+04 ± 4.9E+03	120	< 1 pfu/mL
Maximum Log_{10} Reduction		**2.5 ± 0.1**		**6.4 ± 0.0**
WWTP Site 2	0	1.5E+07 ± 9.7E+05	0	1.5E+07 ± 1.1E+06
	60	2.7E+05 ± 6.7E+04	60	9.5E+04 ± 2.3E+04
	120	6.4E+04 ± 2.0E+04	120	7.0E+03 ± 1.4E+03
Maximum Log_{10} Reduction		**2.4 ± 0.1**		**3.3 ± 0.1**
WWTP Site 3	0	1.1E+06 ± 7.1E+04	0	1.1E+07 ± 4.6E+06
	60	3.7E+04 ± 7.5E+03	60	8.1E+04 ± 3.8E+04
	120	2.4E+04 ± 7.8E+03	120	3.8E+04 ± 3.4E+04
Maximum Log_{10} Reduction		**1.7 ± 0.2**		**2.5 ± 0.3**

Further analysis was carried out on these samples to determine if alternative contributing factors within the effluent which may have interfered with PUV efficiency. TOC and TIC were measured against FRNA bacteriophage (GA) log_{10} reduction (SM 1). A low statistical correlation was found between both variables however a slight trend was apparent between high TOC and TIC concentrations and decreasing virus log_{10} reductions (SM 1). In addition to this, sporadic checks for iron and manganese were carried out as literature indicated this play a role in inhibiting UV performance. No significant impact was recorded indicating that levels were insufficient enough to induce an effect (data not shown), which is in keeping with the characteristic municipal nature of the effluent.

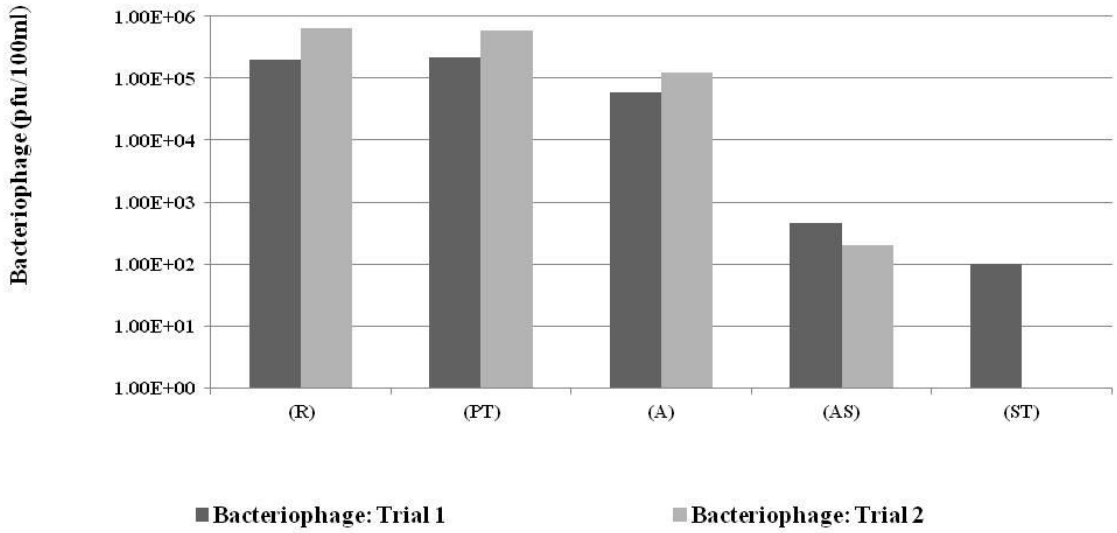
The effectiveness of UV disinfection in wastewater treatment relies heavily on the performance of upstream processes. While primary and secondary treatment of wastewater has demonstrated somewhat efficient removal of pathogens it is widely accepted that a suitable efficient robust tertiary treatment can increase these removal/inactivation rates greatly (Koivunen *et al.*, 2003). High concentrations of suspended solids in secondary treated wastewater, which can result from poor upstream process control or various stresses on

upstream processes, have a direct effect on the efficacy of UV disinfection systems. For example relatively high suspended solids concentrations can shield microbes from UV rays and result in bio-fouling of submerged UV lamp systems. Therefore UV is often used in conjunction with an alternative method of disinfection, such as pre-rapid sand filtration (Rajala *et al.,* 2003).

This study also highlights that SS can indeed play a key role in impacting PUV performance; however the magnitude of the impact may be WWTP specific and thus require site specific pre-treatment amendments (Table 6). It reaffirms the strong interaction between viral loads and suspended solids indicating the need for continuous monitoring of upstream applications to ensure no compact solids filter through to the tertiary stage, protecting viral particles and thus rendering the disinfection process inefficient (Gehr *et al.,* 2003; da Silva *et al.*, 2007; Flannery *et al.,* 2012). It should be noted that further study into the disposal of wastewater sludge may need to consider the presence of such viruses.

3.4 Fate and persistence of NoV through a WWTP using surrogate FRNA bacteriophage

The fate of FRNA bacteriophage was monitored through WWTP 1 and the results are summarized in Figure 6. Following the settling of the activated sludge sample (A), a greater than 2 $\log_{10}$ reduction of FRNA bacteriophage was observed in the clarified wastewater (AS) after settling.



591

592 **Figure 6.** Concentration of infectious FRNA bacteriophage (pfu/100mL) at selected sampling
593 locations in WWTP1 (R- Raw Influent; PT – Primary Treatment; A – Activated Sludge; AS –
594 Activated Sludge 'clarified wastewater post sludge settled'; ST – Secondary treatment; n= 2).
595 SS was also recorded (Trial 1: R = 180 mg/L; PT = 120 mg/L; A = 8427 mg/L; AS = 0; ST =
596 3) (Trial 2: R = 287 mg/L; PT = 150 mg/ml; A = 7767 mg/L; AS = 57; ST = 47; (The average
597 initial concentration was 10^5/100 mL).
598

599 Wastewater systems that treat both municipal wastewater and storm water are at high risk as
600 large rainfall occurrences can overwhelm the wastewater treatment system resulting in
601 inefficient treatment and solid release. These rainfall occurrences can be prevalent in the
602 winter months when the prevalence of NoV is traditionally high according to published
603 epidemiological studies (Galmore *et al.*, 2007). Nordgren *et al.* (2009) found that using
604 RTqPCR, NoV GI and GII exhibited seasonal variance with the highest concentrations
605 recorded in the winter months. The study monitored its prevalence through a WWTP over
606 one year targeting 8 particular treatment points during the process. They noted a 0.7 log
607 reduction in NoV during primary treatment in an activated sludge treatment plant. Similarly,
608 Flannery *et al.* (2012) noted a comparable seasonal trend indicating the concentrations of GI,
609 GII were higher in winter months, interestingly no seasonal variation were seen with respect
610 to FRNA bacteriophage (GA) concentration. Moreover, they noted that NoV GI and GII log
611 removal was 0.13 and 0.14 respectively following primary treatment. Comparison of NoV
612 and FRNA bacteriophage (GA) reduction during secondary treatment is difficult as often
613 varying secondary treatment methodologies are employed.
614

4.0 Conclusion

It is clear that the use of viral inactivation technologies is seen as an increasingly necessary part of the wastewater treatment processes. Key drivers for their implementation include legislation, public health, tourism and commercial demands for clean water (or reuse of wastewater). While there have been on-going technological and research developments in this sector it is not possible to recommend a single best solution. Thus on-site considerations are the key element in choosing a disinfection system for any given application. While no comparative link could be made between NoV GI, NoV GII and the surrogate FRNA bacteriophage (GA) in this study, several alternative studies have indicated a link using alternative surrogates which warrant further investigation under the conditions outlined above. Settling processes prior to treatment determines the fate of NoV and FRNA bacteriophage (GA) in a WWTP as greater than 3 $\log_{10}$ reduction of FRNA bacteriophage was observed in the clarified wastewater (AS) after settling occurred. As the SS concentration in the effluent sample increased, the maximum $\log_{10}$ reduction of FRNA bacteriophage (GA) PFU decreased for any given trial. It is worth noting that this trend varied and appeared to be site specific, therefore this study was unable to deduce SS allowable limits for efficient UV treatment.

This finding warrants further investigations as pre-treatment could greatly decrease the risk of viral-loaded effluent which may pass though tertiary treatment shielded by SS. Results indicate that the adsorption of viral particles to solids in wastewater occurs. As such, a twin tertiary treatment may be the best method of choice. Particularly in areas prone to turbid secondary treated wastewater where a barrier process such as tangential flow filtration system (TFF), filters the particulate matter and the flow through is treated with UV. Results highlight the importance of optimal performance of upstream primary and secondary WWTP processes highlighting how vital continuous monitoring is to ensure the SS are kept low. Results indicate that whilst SS may indeed affect LP UV and PUV treatment efficacy, additional/alternate environmental parameters may indeed play an unknown role.

5.0 Acknowledgements

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