VIRAL INDICATOR REMOVAL IN A FULL-SCALE MEMBRANE BIOREACTOR (MBR) – IMPLICATIONS FOR WASTEWATER REUSE

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Abstract

The aim of this study was to assess the potential removal efficacy of enteric viruses in a full-scale membrane bioreactor (MBR) wastewater reuse system, using a range of indigenous and 'spiked' bacteriophages (phages) of known size and morphology. Samples were taken each week for three months from nine locations at each treatment stage of the water recycling plant (WRP) and tested for a range of microbiological parameters (n=135). Mean levels of faecal coliforms were reduced to 0.3 CFU/ 100ml in the MBR product and were undetected in samples taken after the chlorination stage. A relatively large reduction (5.3 log) in somatic coliphages was also observed following MBR treatment. However, F-RNA and human-specific (GB124) phages were less abundant at all stages, and demonstrated log reductions post-MBR of 3.5 and 3.8, respectively. In 'spiking' experiments, free-swimming 'spiked' phages (MS2 and B14) displayed post-MBR log reductions of 2.25 and 2.30, respectively. The removal of these 'free-swimming' phages, which are smaller than the membrane pore size (0.04 µm), also highlights the possible role of the membrane biofilm as an effective additional barrier to virus transmission. The findings from this study of a full-scale MBR

system demonstrate that the enumeration of several phage groups may offer a practical and conservative way of assessing the ability of MBR to remove enteric viruses of human health significance. They also suggest that virus removal in MBR systems may be highly variable and may be closely related on the one hand to both the size and morphology of the viruses and, on the other, to whether or not they are attached to solids.

Keywords

Full-scale membrane bioreactor; Wastewater reuse; Virus removal efficacy; Bacteriophages; Phage morphology; Spiking experiments

1. Introduction

A key objective of all municipal wastewater recycling operations is to minimise the onward transmission of human enteric pathogens. Those of potential human health significance in secondary wastewater effluents include oocysts of *Cryptosporidium parvum*, cysts of *Giardia lamblia* and a range of enteric bacteria and viruses (De Luca *et al*., 2013). Removal of enteric viruses normally represents a more challenging objective in water and wastewater treatment systems than the removal of enteric bacteria, primarily because most viruses are significantly smaller than bacteria, but also because they can normally more-readily pass through widely used biological treatment processes, such as activated sludge and trickling filters (Shang *et al*., 2005). Therefore in some circumstances effluents of these biological treatment processes may be subjected to additional 'tertiary' treatment to reduce further the levels of enteric viruses (and other pathogens) in the final effluent. Tertiary treatment technologies include sand filtration, ultraviolet and ionising radiation and, more commonly, chemical disinfection with chlorine, ozone or peracetic acid (Taghipour, 2004; Koivunen and Heinonen-Tanski, 2005; Zanetti *et al*., 2006; De Luca *et al*., 2008; Chen and Wang, 2012). However, the

addition of tertiary processes to a treatment plant inevitably increases capital and operational costs. Further, chemical disinfection processes can generate disinfection by-products that are potentially harmful to the environment and human health (Wert *et al*., 2007; Chen and Wang, 2012), such as trihalomethanes, haloacetic acids, N-Nitrosodimethylamine, bromate and chlorite.

The term 'membrane bioreactor' (MBR) refers to water and wastewater treatment processes that combine a permselective membrane with a biological process (Judd, 2011). In MBR systems, separation of solids is achieved without the need for secondary sedimentation (De Luca *et al*., 2013). Instead, removal of solids is achieved by the membrane. The small pore size of the membrane (0.03-0.40 µm) also results in the physical removal of a wide variety of microorganisms. In recent years, MBR technology has emerged as an alternative to conventional activated sludge treatment (van Nieuwenhuijzen *et al*., 2008). In part this is because activated sludge effluents have been shown to contain levels of enteric organisms that may pose an unacceptable hazard to human health, particularly when indirect or even direct reuse (for potable or non-potable uses) is proposed (Koivunen and Heinonen-Tanski, 2005; Simmons and Xagoraraki, 2011; Zhang and Farahbakhsh, 2007).

A range of studies, performed at both pilot-scale and within full-scale municipal wastewater plants, have demonstrated that microbial removal in MBR systems is more effective than in conventional activated sludge treatment systems (Arraj *et al*., 2005; Ottoson *et al*., 2006; Francy *et al*., 2012; Marti *et al*., 2011). Further, MBR systems have been shown to remove microorganisms that are greater in size than the membrane filter pores. The dimensions of faecal indicator bacteria (>0.5µm x >2.0µm), the spores of bacterial indicators (1-5µm), helminth eggs (>20µm x 25µm) and protozoa, including oocysts of *Cryptosporidium* and

Giardia (>4 µm), all exceed the membrane pore size, and should be removed by exclusion (Marti *et al*., 2011). Ueda and Horan (2000) observed greater than 5 log removal rates for faecal coliform bacteria and spores of sulphite-reducing *Clostridium* spp. in an MBR pilot plant with a nominal pore size of 0.4 µm. These relatively high removal rates for bacteria and protozoa have also been observed in a number of other studies (Ottoson *et al*., 2006; Zhang and Farahbakhsh, 2007; Zanetti *et al*., 2010).

However, most viruses of human health significance are smaller than the pore sizes used in MBR treatment systems. Noroviruses, sapoviruses, rotaviruses, enteroviruses, and hepatitis A and E viruses have diameters of approximately 30 nm, while the diameter of larger viruses, such as adenoviruses, ranges from 60 to 90 nm (van Regenmortel *et al*., 2000). Although viruses are clearly smaller in size than the membrane pores used, high removal rates of viruses have been reported for MBR (Winnen *et al*., 1996; Ueda and Horan, 2000; Farahbakhsh and Smith, 2004). The removal of viruses is thought to be primarily influenced by the development of a biofilm on the membrane, and by virus adsorption to this biomass (Da Silva *et al*., 2007; Wong *et al*., 2009; Hirani *et al*., 2014; van den Akker *et al*., 2014). Viruses capable of infecting bacteria (bacteriophages or phages) have long been proposed as models for the removal of enteric viruses in treatment systems (IAWPRC, 1991). Indeed, phages may be a more appropriate indicator of the presence of enteric viruses in water and wastewaters than the bacterial indicators that continue to be widely used (Jofre *et al*., 1986; Gantzer *et al*., 1998; Purnell *et al*., 2011; Ebdon *et al*., 2012; Jofre *et al*., 2014) because of their similarity to these viruses in terms of structure, morphology, size and resistance to inactivation.

Several studies have considered the removal of indigenous phages, namely somatic coliphage, F-specific RNA phages, and phages of *Bacteroides* species in water and wastewater treatment systems. Studies have demonstrated that MBR systems remove phages more effectively than conventional activated sludge treatment processes. For example, a recent study by De Luca *et al*. (2013) demonstrated that reductions in levels of somatic coliphages and F-RNA specific phages were 2.7 and 1.7 log higher as a result of MBR treatment than by conventional activated sludge treatment. Zanetti *et al*. (2010) observed that, despite the smaller diameter of F-RNA specific phages (21-30nm), their levels in the permeate were lower than those of somatic coliphages (30-100nm). Research conducted by Gantzer *et al*. (2001) supports these findings, demonstrating that F-specific RNA phages have a greater tendency to adsorb to solids and the membrane, and are therefore removed in greater numbers.

In addition to monitoring the concentration of indigenous phages in MBR systems, 'spiking trials' have been conducted using phage such as MS2 from the family *Leviviridae* (an F-specific RNA phage that has been extensively used to assess the removal efficacy of viruses in treatment systems) (Shang *et al*., 2005; Hijnen *et al*., 2010; Marti *et al*., 2011). This is a relatively small virus (20-25 nm), and as a result it has been recommended as a potential pathogenic virus surrogate in treatment efficacy studies (Marti *et al*., 2011). Results of previous phage spiking studies have shown MS2 removal by MBR systems to range from 1.0 log to 5.9 log (Madaeni *et al*., 1995; Ueda and Horan, 2000; Hirani *et al*., 2010). Hirani *et al*. (2010) suggested that differences in virus removal between different MBR systems may be attributed to variations in membrane pore size between these systems. Differences in biomass and the length of time the mature biofilm takes to form, may also contribute to the variance in results obtained from spiking trials. Shang *et al*. (2005) demonstrated a 0.8 log removal of

spiked MS2 by adsorption to biomass, and a 2.1 log removal by biofilm formed during 21 days of filtration.

Removal of enteric viruses by MBR treatment has also been investigated (Ottoson *et al*., 2006; Francy *et al*., 2012) and results from comparative studies suggest that MBR treatment removes enteric viruses more effectively than conventional secondary treatment (Oota *et al*., 2005; Zhang and Farahbakhsh, 2007). Ottoson *et al.* (2006) demonstrated that human virus genomes were not removed as effectively as phages, with 1.8 and 1.1 log removals for enteroviruses and noroviruses, respectively, but the authors concluded that the differences were probably related to the use of different detection methods. For example, phages are normally cultured, whilst human viruses are typically detected using culture-independent molecular approaches (e.g., PCR), which are based on the detection of nucleic acids, rather than a complete, infectious particle (virion). Therefore, the detection of nucleic acids from damaged organisms in MBR effluents may lead to an underestimation of virus removal efficiency, and hence may overestimate the potential risk to human health of these effluents.

While the removal efficacy of viruses in pilot MBR treatment systems has previously been reported, published data from full-scale MBR treatment systems remain limited, at a time when interest in this technology is increasing rapidly. According to Dahl (2010), the limited availability of empirical data on the operational efficacy of full-scale MBR treatment systems means that their potential role in the disinfection of waters and wastewaters is yet to be fully recognised. The aim of this project was therefore to investigate for the first time the behaviour of a range of enteric phages (both indigenous and ‘spiked’) in a full-scale MBR treatment (with subsequent GAC treatment) system for wastewaters intended for direct non-potable reuse in order to elucidate whether the approach may provide new insights into the

removal of enteric viruses in such systems. The treatment processes investigated are particularly interesting because they comprise the largest UK example of a community equivalent scale reuse system designed to treat raw municipal wastewater for direct non-potable reuse within a high-profile setting (namely, the Queen Elizabeth Olympic Park, London).

2. Material and methods

2.1 The membrane bioreactor water recycling plant

The Old Ford WRP treats raw municipal wastewater mined from the Northern Outfall Sewer to provide 574 m^3/day non-potable supply of water to the Queen Elizabeth Olympic Park, London for the purposes of parkland irrigation, venue toilet flushing and rain water harvesting top up (Hill and James, 2014). The raw sewage is predominantly domestic and light commercial with surface drainage inputs from a large catchment population of approximately 360,000. The Old Ford WRP takes a small proportion of the flow from the Northern Outfall Sewer for treatment. The process comprises a pre-treatment stage with gross solid removal through underground septic tanks followed by 1 mm rotating screens for particulate matter removal (hair and fibres). Screened sewage flows to the membrane bioreactor (MBR) which consists of an above ground activated sludge tank operating at 7 g/L mixed liquor suspended solids and segregated in to anoxic and aerobic zones. A separate cross-flow membrane tank holds three racks of aerated ultra-filtration membranes (nominal pore size of 0.04 µm (Siemens Water Technologies Memcor Ltd)) which are periodically cleaned in place. The reclaimed water undergoes post-treatment in the form of granular activated carbon (GAC), primarily for colour removal, and chlorination (0.3 to 1.5 mg/l chlorine residual) before entering a dedicated 3.65km distribution network.

2.2 Monitoring programme

The potential removal efficacy of viruses at the Old Ford WRP was determined by monitoring background levels of indigenous phages (somatic coliphage, F-specific RNA phages and phages capable of infecting GB124, a human-specific strain of *Bacteroides fragilis*) at each stage of the WRP (so as to try to reflect the wide range of sizes, morphologies and adsorption that are characteristic of common waterborne enteric pathogenic viruses, including noroviruses and adenoviruses). Samples were taken each week (15 sampling occasions), over a three month period from nine sampling points, located after each stage of the Old Ford WRP treatment system (Figure 1). In addition, the MBR system was also challenged with high-titre suspensions of phages MS2 and GB124 (B-14). All samples were transported to the University of Brighton laboratory, in the dark, at 4^{o}C, and were analysed within four hours of collection.

2.3 Quantification of faecal indicator organisms

Faecal coliforms were enumerated by membrane filtration on mFC agar, in triplicate, according to standard methods (Anon., 2000) and results were expressed as colony-forming units per 100 ml (CFU/100ml). Somatic coliphage, F-specific RNA phages and human-specific GB124 phages were quantified by enumerating plaque-forming units (PFU/100ml), in triplicate, according to standardised double-agar-layer methods (Anon, 2001[a-c], respectively). Host strain WG5 (*E. coli*) was used for somatic coliphage enumeration, WG49 (*S. typhimurium*) was used for F-specific RNA phages, and GB124 (*Bact. fragilis*) was used for the detection of phages active against this human specific gut bacterium.

2.4 Phage isolation, purification and concentration

Plaques enumerated in the MBR product were picked for phage isolation. These phages were then purified and concentrated by a plate propagation method described elsewhere (Carey-Smith *et al*. 2006; Fard *et al*. 2010). In brief, cores of agar, containing a distinct single plaque, were picked using sterile glass Pasteur pipettes and suspended in 200 µl of phage buffer (19.5 mM Na2HPO4, 22 mM KH2PO4, 85.5 mM NaCl, 1 mM MgSO4, 0.1 mM CaCl2) (Puig and Girones, 1999; Diston *et al*., 2014) in microcentrifuge tubes (Fisher Scientific, UK). These phage suspensions were then left overnight at 4°C to allow phage diffusion into the buffer. The suspensions and dilutions were retested (using the double agar-layer method) to purify and confirm the presence of phages. This process was repeated three times to obtain purified phage.

Once purified, 5 ml of phage buffer were added to plates exhibiting near complete-lysis of the host bacterium. These plates were left at room temperature for 1 h and 'swirled' using an orbital shaker (Stuart™) to promote phage diffusion into the buffer. The liquid and top agar-layer were then scraped into a 50 ml centrifuge tube (Fisher Scientific, UK), mixed briefly using a Whirlimixer™, and left at room temperature for a further thirty minutes. Bacterial debris and the top agar-layer were removed from the suspension by centrifugation at 3000xg for twenty minutes. The supernatant was then filtered through a 0.22 µm polyvinylidene difluoride membrane syringe-driven filter, and stored in light-tight glass bottles at 4 °C in the dark. The titre of the suspension was determined by testing ten-fold dilutions (10^{-1}-10^{-8}) using the spot test assay. The process was repeated until a minimum titre of 1 x 10^{8} PFU/ml was achieved with all phage suspensions.

2.5 Transmission electron microscopy (TEM)

All phages were examined by transmission electron microscopy (TEM) to determine their morphology. To view the phage under TEM, the phage suspensions were negatively stained. This was achieved by mixing the phage particles with an electron-dense solution of a metal salt of high molecular weight and small molecular size, into which the particles were embedded. As a result of this process, phages appeared white on a dark background (Ackermann, 2009). Uranyl acetate (UA) stain (pH 4.0-4.5) was used to stain the phage suspensions. One drop (10 µl) of previously prepared high-titre phage suspension was applied to 200 mesh Formvar/Carbon copper electron microscope grids (Agar Scientific, UK). After two minutes, any excess suspension was removed using Whatman No. 1 filter paper (Whatman, UK). One drop (10 µl) of UA stain (1 % w/v, previously filtered through a 0.22 µm filter unit) was then applied to the grid for one minute. Excess stain was removed again with Whatman No. 1 filter paper, and the grids were then left to dry. Grids were subsequently viewed under the TEM (Hitachi-7100) at 100 kV.

2.6 Spiking trials

The system was challenged with high-titre suspensions of two phages, namely MS2 and phages of GB124 (B-14). It is important to note that the addition of 'free-swimming' (unattached) phages into the treatment system may not provide results that reflect normal operational conditions, as phages have been shown to adsorb readily and rapidly to suspended sediments, facilitating their removal by MBR technology (Marti *et al*., 2011). Therefore, both 'free-swimming' phages and phages previously mixed into mixed liquor solids were spiked in the system before the MBR and the removal of phages by the membrane was determined using regression analysis to model the curve, followed by integration.

3. Results

In total, 135 samples (15 from each sampling point) were analysed for levels of faecal coliforms, somatic coliphages, F-RNA phages and phages capable of infecting *B. fragilis* strain GB124 over a period of three months.

3.1 Faecal coliforms

Mean levels of faecal coliforms at the nine sampling points through the Old Ford WRP are presented in Figure 2. Mean numbers were reduced to 0.27 CFU/ 100ml after MBR, and to 0.17 CFU/100ml after GAC treatment. Removal rates of 6.81 and 6.83 log were recorded after MBR and GAC, respectively. Following chlorination, faecal coliforms were undetected (<1 per 100 ml) in all samples.

3.2 Indigenous bacteriophages

Figure 3 demonstrates the mean number of indigenous phages recorded at each stage of the Old Ford WRP system. Somatic coliphage predominated throughout much of the system, with levels as high as 1.23×10^6 PFU/100ml observed in the raw wastewater. A relatively large reduction in somatic coliphage numbers was observed following MBR treatment (5.34 log). In contrast, F-RNA and *B. fragilis* GB124 phages were detected at lower levels throughout, and demonstrated log reductions through the MBR stage of 3.5 and 3.8, respectively. Following MBR treatment, somatic coliphages were the only phages detected (F-RNA and *B. fragilis* GB124 phages being undetected in all samples).

3.3 Results of transmission electron microscopy

All phage plaques obtained from the MBR product (i.e., only somatic coliphages) were processed and viewed by TEM to determine their morphology. All these phages re-infected their bacterial host, positively identifying them as viable lytic phages. These phages were

then successfully propagated and concentrated to a high titre (10^{11}), stained and viewed under the TEM. All micrographs demonstrated a single phage morphology (Figure 4), indicating them to be members of the family *Microviridae*. *Microviridae* are a non-tailed family of non-enveloped virions that demonstrate icosahedral symmetry (Ackerman, 2011). They are relatively small phages, with diameters of between 25- 27nm.

3.4 Results of spiking trials

MS2 phages and B14 phages were spiked into the membrane tank at titres of 2 x 10^{12} and 1 x 10^{8}, respectively. The experiment was undertaken twice, first using 'free-swimming' phages ('first protocol') and secondly using phages that had previously been spiked into the MBR mixed liquor and which were therefore likely to be bound to the mixed liquor suspended solids ('second protocol'). While the results of the first protocol provided valuable insights into the removal of 'free-swimming' phages by the membrane, this spiking protocol is unlikely to have effectively mimicked normal operational conditions within the system, hence the inclusion of the modified second protocol. Figures 5 and 6 show levels of MS2 phages and B14 phages detected in the MBR product for both spiking protocols. Both MS2 and B14 phages were removed by the membrane to a greater extent when initially associated with solids (second protocol). The recorded removal of 'free-swimming' MS2 and B14 phages was 2.25 and 2.30 log, respectively and the recorded removal of MS2 and B14 phages associated with mixed liquor solids was 2.3 and 8.0 log, respectively. Although an 8.0 log removal of B14 was recorded, the level of phages fell below the detection limit of the method used in the MBR product and the log removal in reality is likely to be considerably lower.

4. Discussion

The log removal values for faecal coliforms and phages reported in this study are consistent with the findings of other recent studies, which have shown greater phage removal in MBR systems, in comparison with conventional activated-sludge treatment. Not only were somatic coliphages recorded at the greatest concentration of all phage groups investigated but they were also demonstrated to be the only phage group that was detected in the MBR effluent. Clearly a direct comparison of the removal rates of the phage groups studied is problematic since their concentrations in the raw wastewater varied. However, the findings do suggest that somatic coliphages may represent a useful conservative model by which to assess virus removal in MBR systems. Although the removal rates from this study should be treated with caution, they appear to be consistent with the findings of both Gantzer *et al.* (2001) and Zanetti *et al.* (2010), who demonstrated that F-RNA phages were removed in greater numbers than somatic coliphages as a result of their greater tendency to adsorb to solids. In our study, plaques of somatic coliphages detected in the MBR product were propagated and the resulting phage concentrated to a high titre and viewed by transmission electron microscopy. The observation that all somatic coliphages isolated from the MBR product were identified as belonging to the *Microviridae* family, which is composed of relatively small un-tailed phages (25 and 27nm) may support the hypothesis that that tailed phage families (namely, *Myoviridae* and *Siphoviridae*) may be more susceptible to adsorption to solids and/or damage within the MBR process. The detection of only a single family of somatic coliphage in the MBR product suggests that viral morphology may be an important factor in their removal by MBR membranes. However, given the low numbers of phage detected in the MBR product, further research is recommended to ascertain whether morphological characteristics played a role in their apparent resilience to the treatment process.

Although efforts were made in the second spiking protocol to model the attachment of phages to particles prior to filtration through the MBR membrane, the protocol used is unlikely to have achieved the level of attachment that has previously been observed in MBR systems. Indeed, other studies have suggested that spiking phages into environmental matrices is unlikely to reproduce the conditions of the system (Guzmán *et al*., 2007). One reason for this could be that levels of phage may exceed the number of available binding sites resulting in limited attachment levels. While the spiking trials may not have effectively mimicked normal operational conditions, the experiments did allow removal of 'free-swimming' (unattached) phage by the membrane to be assessed. Significantly, 'free-swimming' phages were successfully removed by the membrane, even though these phage groups investigated were smaller than the membrane pore size. Other authors have demonstrated that phage removal in the absence of solids may be highly dependent on the formation of the biofilm (Ueda and Horan, 2000).

Within the constraints of the experimental design, our study demonstrated virus removal in a full-scale MBR wastewater treatment system as high as 5.3 log. This is comparable with that achieved in reverse osmosis (RO) treatment processes where removal rates between 1.4 and greater than 7.4 log have been recorded.

5. Conclusions

The microbial removal values recorded in a full-scale MBR wastewater system were greater than those commonly reported for conventional activated sludge treatment. Somatic coliphages were shown to represent a potential conservative model by which to assess virus removal in MBR systems, but importantly the research also demonstrates the potential benefits of studying a range of enteric phages (with a diverse range of sizes and

morphologies) to assess the virus removal performance of treatment technologies. Therefore this study provides first evidence that a 'toolbox' approach to wastewater treatment process monitoring, in which relatively low-cost methods are used to detect a range enteric phages, may form the basis of a revised monitoring paradigm that more effectively protects human health within a risk-based integrated approach to wastewater reuse. However, further research is recommended to elucidate more fully the relationship between phages that may be used to monitor treatment systems at relatively low-cost and specific enteric viral pathogens of human health significance. Such studies may demonstrate whether, under specific circumstances, phage models may represent an acceptable low-cost substitute for viral pathogen enumeration in support of a quantitative microbial risk assessment (QMRA) approach to managing the risk to human health of future wastewater reuse systems.

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Figures

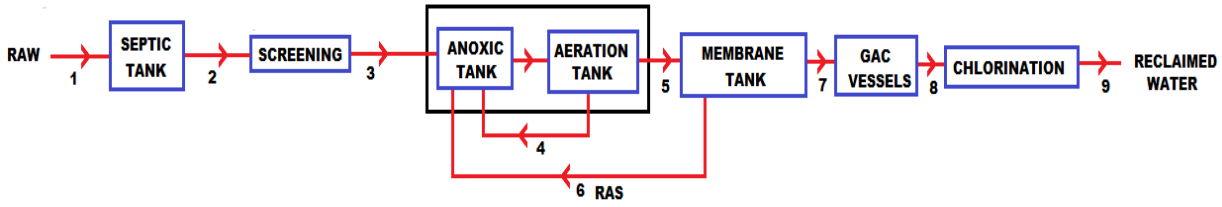

Figure 1. Sampling locations for weekly monitoring of surrogate levels at the Old Ford WRP

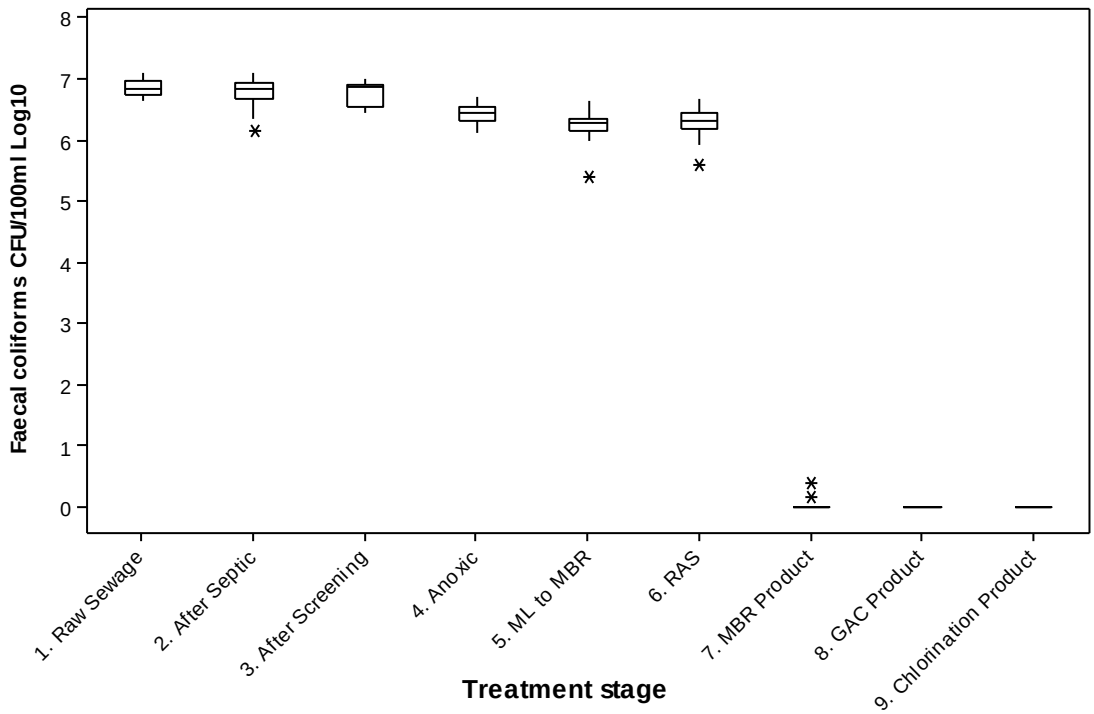

Figure 2. Mean numbers of faecal coliforms at each treatment stage in the Old Ford WRP. Outliers (observations >1.5 times the interquartile range) are represented by a *.

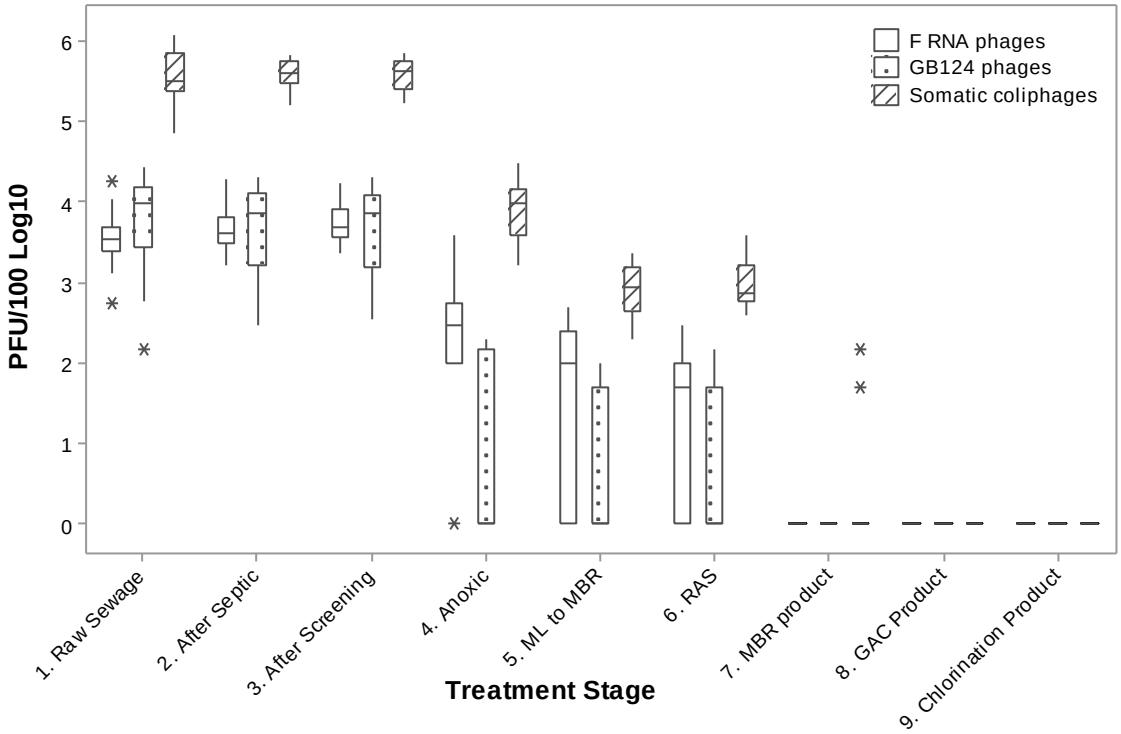

Figure 3. Mean numbers of bacteriophages at each treatment stage in the Old Ford WRP. Outliers (observations >1.5 times the interquartile range) are represented by a *.

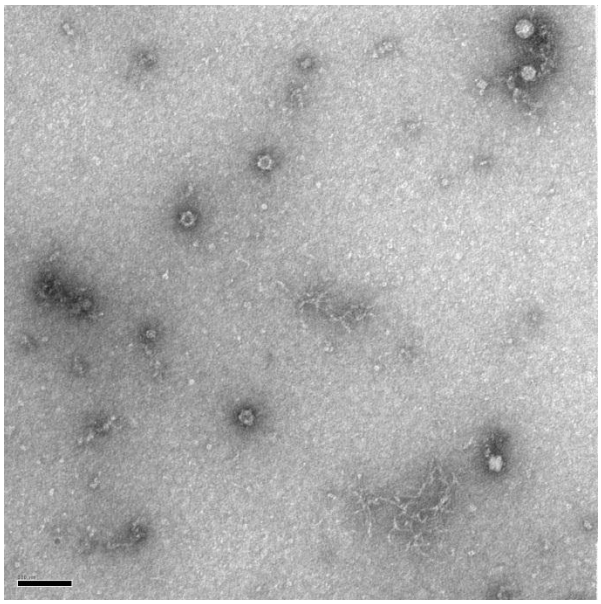

Figure 4. TEM micrograph with somatic coliphages present in MBR product belonging to the *Microviridae* family (bar=100nm)

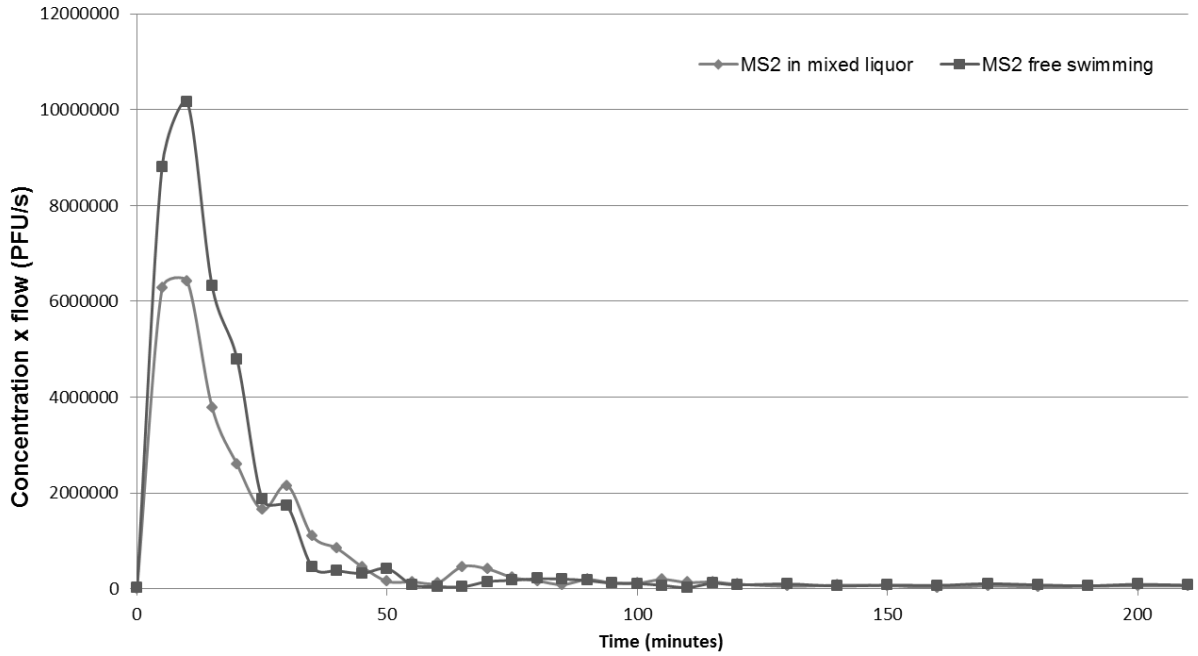

Figure 5. MS2 bacteriophages detected in MBR product with time following phage spiking

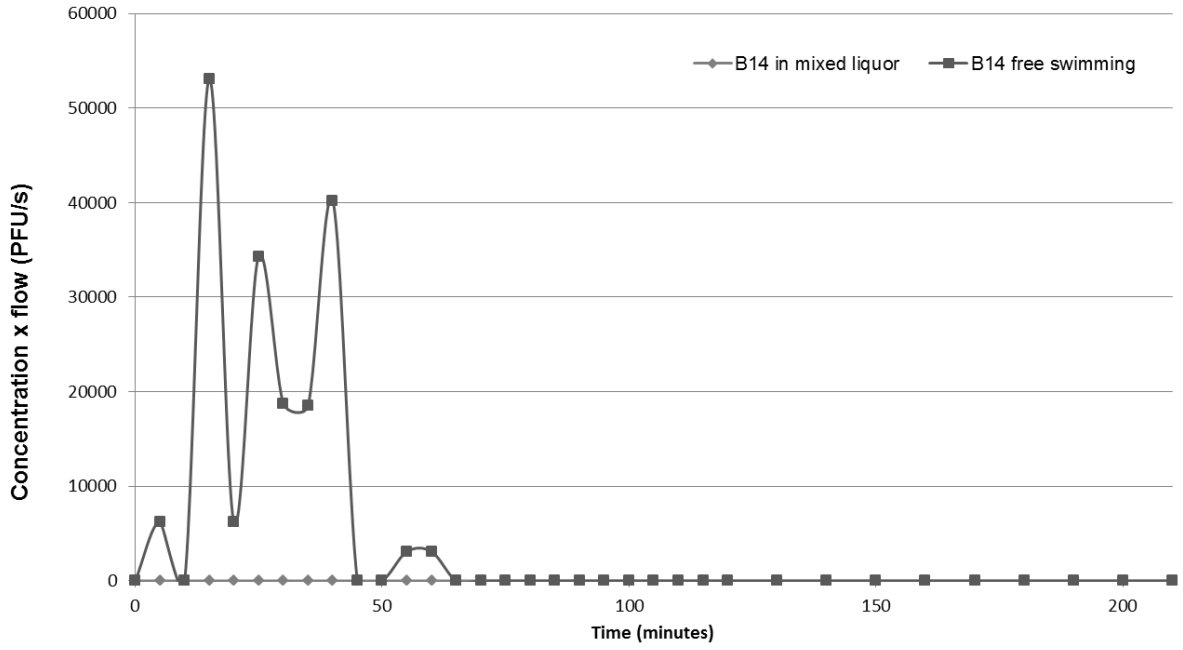

Figure 6. B14 bacteriophages detected in MBR product with time following phage spiking