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BACTERIOPHAGES – POTENTIAL FOR APPLICATION IN WASTEWATER TREATMENT PROCESSES

Withey, S., Cartmell, E.*, Avery, L. M. and Stephenson, T.

School of Water Sciences, Cranfield University, Cranfield, Bedfordshire, UK, MK43 0AL.

*** Corresponding Author**

Email e.cartmell@cranfield.ac.uk;

Tel +44 (0) 1234 754053; Fax +44 (0) 1234 751671;

School of Water Sciences, Cranfield University, Cranfield, Bedfordshire, UK, MK43 0AL.

ABSTRACT

Bacteriophages are viruses that infect and lyse bacteria. Interest in the ability of phages to control bacterial populations has extended from medical applications into the fields of agriculture, aquaculture and the food industry. Here, the potential application of phage techniques in wastewater treatment systems to improve effluent and sludge emissions into the environment is discussed. Phage mediated bacterial mortality has the potential to influence treatment performance by controlling the abundance of key functional groups. Phage treatments have the potential to control environmental wastewater process problems such as: foaming in activated sludge plants; sludge dewaterability and digestibility; pathogenic bacteria; and to reduce competition between nuisance bacteria and functionally important microbial populations. Successful application of phage therapy to wastewater treatment does though require a fuller understanding of wastewater microbial community dynamics and interactions. Strategies to counter host specificity and host cell resistance must also be developed, as should safety considerations regarding pathogen emergence through transduction.

KEY WORDS

Bacteriophages; Wastewater; Sludge, Pathogens; Sludge Dewatering; Sludge Digestion.

1. Introduction

Viruses are small infectious particles, typically 20-200 nm consisting of a nucleic acid core (single or double stranded RNA or DNA) enclosed by a protein coat (capsid) and in some cases a lipid envelope (Singleton and Sainsbury, 2002). Bacteriophages (phages) are viruses that infect prokaryotes. Like all viruses, phages are obligate intracellular parasites, which have no intrinsic metabolism and require the metabolic machinery of the host cell to support their reproduction.

Contact with the host cell occurs by passive diffusion. Phage adsorption and entry are mediated by specific receptors such as carbohydrates, proteins and lipopolysaccharides on the surface of the host cell (Marks and Sharp, 2000). The specificity of interaction between phage attachment structures and host–cell surface receptors influences the bacterial host range. Host range is generally assumed to be narrow for aquatic phages (Alonso *et al*. 2002). However, broad host-range (polyvalent) cyanophages are widely isolated (Suttle, 2000) and polyvalent phages have been isolated from sewage treatment plants (Jensen *et al*. 1998).

Two categories of bacteriophages are recognised; temperate and virulent. During lytic infection, virulent phages inject their nucleic acid into the host cell following attachment. Expression of the phage genome directs the cellular machinery of the host to synthesise new phage capsule material. The resulting phage progeny are released by fatal cell lysis enabling the lytic cycle to continue as new cells are infected. The number of progeny released (burst size) varies from 50-200 new phage particles (Wommack and Colwell, 2000). In contrast, during lysogenic infection temperate phage nucleic acid recombines with the host cell genome forming a dormant prophage. The prophage is reproduced in the host cell line and confers immunity from infection by the same type of phage. Stress conditions such as ultraviolet light or chemical mutagens can induce a switch to the lytic cycle (Jiang and Paul, 1998).

Bacteriophages are highly abundant in the aquatic environment ranging from 10^4 ml^{-1} to in excess of 10^8 ml^{-1} (Bergh *et al*. 1989). Numbers are typically 3-10 times greater than the bacterial counts, although there is substantial variation between ecosystems (Weinbauer, 2003). A relationship with bacterial numbers and activity implies that the majority of aquatic viruses may be phages. Furthermore, numerous viral abundance studies show seasonal (Bergh *et al*. 1989;

Cochran and Paul, 1998; Hofer and Sommeruga, 2001) and diel variations (Jiang and Paul, 1994; Weinbauer *et al*. 1995), and are particularly responsive to plankton blooms (Bratbak *et al*. 1990; Hennes and Simon, 1995). Wommack and Colwell (2000) summarised studies concluding that addition of concentrated viral particles tended to decrease bacterial populations by 20-40 %. Consequently, carbon transfer to higher organisms could be reduced thus influencing the entire aquatic food web (Noble and Furhman, 1997). Furthermore, aquatic viruses may have a role in determining the diversity of bacterial communities through control of selected species competing for resources (Hewson *et al*. 2003).

Indeed, phage species richness is immense (Rohwer, 2003). Jiang *et al*. (2003) demonstrated that the genetic diversity of marine bacteriophage is also substantially greater than that of host bacteria. Phages are subject to environmental constraints associated with aquatic ecosystems. For example, Womack *et al*. (1999) and Weinbauer *et al*. (1995) observed that depth influenced marine viral species distribution at Chesapeake Bay and in the northern Adriatic, respectively. In contrast, phage distribution off the coast of Southern California did not appear to be limited by the changing physical and chemical differences in the water column (Jiang *et al*. 2003). The ecology of prokaryotic viruses is reviewed in depth by Weinbauer (2003).

Since their discovery by Twort (1915) and independently by d'Herelle (1917), the bacteriocidal properties of phage have raised interest in their potential use in the control of medical conditions. The history of phage therapy is reviewed in depth by Chanishvilli *et al*. (2001) and Sulakvelidze *et al*. (2001). In brief, phages were used widely in the early 20th century to treat human and animal illness with varying degrees of success. In the West and US, research into phage therapy declined following inconsistent results and as a consequence of the discovery of antibiotics in the 1940s. Phage therapy research continued in Eastern Europe where phage treatments against a

wide array of bacteria including staphylococci, pseudomonads, *Proteus* spp. and enteric pathogens were produced (Weber-Dabrowska *et al*. 2000; Chanishvilli *et al*. 2001). There has been a renewed interest in phage therapy over the past two decades, partly as a consequence of increasing antibiotic resistance in bacteria. Much recent work has focussed on animals as models for human infection or veterinary applications (Smith and Huggins 1987; Biswas *et al*. 2002; Huff *et al*. 2002; Matsuzaki *et al*. 2003).

Acknowlegement of the importance of bacteriophages in aquatic and terrestrial ecosystems (Weinbauer, 2003) has led to interest in wider environmental applications. The potential of phages to control bacterial infections in cultured fish (Nakai *et al*. 1999; Nakai and Park, 2002), in plants (Flaherty *et al*. 2000) and to control cyanobacterial blooms (Mole *et al*. 1997) have been studied. Schuch *et al*. (2002) reported on the isolation of a phage enzyme capable of lysing the biological warfare bacterium, *Bacillus anthracis*. Commercial production of a phage to kill *E. coli* O157:H7 in manure and to remove pathogens from carcasses and food preparation areas is already underway (Thiel, 2004). There is also the potential to use phage techniques to influence wastewater treatment. This is discussed further in this review together with the occurrence of phage in wastewater treatment and possible treatment limitations.

2. Occurrence of Bacteriophages in Wastewater Treatment Plants

Many studies report applications of bacteriophages as indicators or tracers for presence of bacteria in wastewater treatment systems. Nevertheless, their role in the microbial communities of wastewater treatment systems is poorly understood. Early studies (Dias and Bhat, 1965) indicated that *E. coli* phages (coliphage) were not functional in laboratory scale activated sludge systems. Within two hours of aeration coliphage abundance declined 10-fold from 2460 PFU ml^{-1}

to 230 PFU ml^{-1}, stabilising thereafter for a further 23 hours. Coliphages, infectious for Enterobacteria present in wastewater, are removed during activated sludge treatment (Bitton, 1999). However, coliform bacteria comprise only a small component of activated sludge microflora (Ewert and Paynter, 1980; Hantula *et al*. 1991). Little is known about the bacteriophages which infect the dominant, viable and functionally important components of the community. The few studies available to date, suggest that phages may be active components of activated sludge systems (Dias and Bhat, 1965; Ewert and Paynter, 1980; Hantula *et al*. 1999; Hertwig *et al*. 1999; Khan *et al*. 2002a and b; Thomas *et al*. 2002). Ewert & Paynter (1980) reported an increase in total phage concentration during an activated sludge process, suggesting active replication was occurring *via* host infection and lysis. Maximum phage concentrations in reactor mixed liquors (9.5×10^7 ml^{-1}) were significantly higher than concentrations in influent sewage (2.2×10^7 ml^{-1}). Ewert and Paynter (1980) noted that direct electron microscopy detected higher phage titres than by plaque assays highlighting that studies employing plaque assays to quantify the specific phage titres of the host used may not reflect the overall dynamics of the total phage population. Hantula *et al*. (1991) also demonstrated the presence of active phage-host relationships in activated sludge. The authors identified 49 indigenous phage-host systems, reporting high individual titres (up to 5×10^4 PFU ml^{-1}) which fluctuated indicating phage activity during the 5 week study period. In contrast, coliphage concentrations (<60 PFU ml^{-1}) were frequently below detection limits. Bacteriophages infectious for some of the functionally important bacteria in wastewater treatment plants have also been isolated. Khan *et al*. (2002b) isolated three phages from laboratory scale enhanced biological phosphorus removal (EBPR) reactors, which were infectious for *Brevibacterium epidermidis* and *linens* and *Rhodococcus erythropolis.* Phages appear to be active components of activated sludge systems and phage-mediated bacterial mortality has the potential to influence treatment performance by controlling the abundance of key functional groups.

A number of activated sludge treatment processes including phosphorus removal and nitrification are known to be unstable. Nitrification, (the microbial oxidation of ammoniacal nitrogen via nitrite to nitrate) can be a difficult process to maintain in activated sludge nutrient removal systems (Wanner *et al*. 2000). Due to the slow growth rate of nitrifying bacteria, effective nitrification requires a high sludge age in order to prevent biomass washout and wastage. Poor nitrification rates have been attributed to low specific activities of nitrifiers as a consequence of toxic inhibition by chemicals or ammonia in the influent wastewater (Burgess *et al*. 2002). Inhibition often occurs in wastewater treatment works (WwTWs) receiving high strength manufacturing and industrial wastewaters. However, nitrification failure has also been attributed to a low concentration or even absence of the nitrifying bacteria (Wanner *et al*. 2000). It is possible that low abundances of nitrifying bacteria could be the result of lytic infection by bacteriophages.

The impact of bacteriophages in WwTWs anaerobic digesters is unknown but it is likely that they do play a role in controlling strain composition and abundance. Studies employing laboratory anaerobic digesters have revealed rapid shifts in microbial composition (Zumstein *et al*. 2000; Casserly and Erijman, 2003). Continuous, long-term evolution of the anaerobic community, with successive shifts in the dominance of at least eight species, was not related to abiotic factors such as pH, temperature and feed composition, which all remained constant; internal biotic factors such as phage-host cycles and predation by protozoa were suggested as the causal agents for shifts in community structure (Zumstein *et al*. 2000).

3. Potential Phage Treatments

3.1 Pathogen Control

Sludge produced by biological wastewater treatment processes contains a diverse array of pathogenic bacteria, viruses, protozoan and metazoan parasites. In excess of 1 million and 6.8 million tonnes of dry sludge (DS) are produced annually in the UK and US respectively (DEFRA, 2002; Hettenbach *et al*. 1998). The beneficial reuse of this sludge by application to agricultural land is generally regarded as the best practical environmental option. The EU does not currently impose numerical standards for pathogen levels in sludge recycled to land. However, as a result of recent public concern over recycled sewage sludge, as a vector for the transmission of human diseases, revisions to guidelines in the UK have been proposed (DEFRA, 2002). Two categories of sludge product have been identified with specifications for microbiological standards. Conventionally treated sludge must attain a minimum 2 log reduction of *E. coli* abundance across the treatment process and a final product standard with a maximum admissible concentration (MAC) of 1×10^5 CFU g^{-1} DS *E. coli*. Treatment processes producing enhanced treated sludge must achieve a minimum 6 log reduction in *E. coli* abundance during treatment and a final end product with a MAC of 1×10^3 CFU g^{-1} DS *E. coli* and zero *Salmonella* in 2 g DS. US EPA regulations are similarly categorised in to Class A and Class B sludges. Class A pathogen reduction requirements are more stringent than those of class B sludges which are subject to application restrictions. Class A sludges are required to reduce pathogens to below the detectable limit (< 3 MPN Salmonella, < 1 PFU enteric viruses and < 1 viable helminth ovum per 4g DS). Treatment processes designed to achieve the pathogen reduction can incur substantial capital and operating costs.

Development of phage treatment of sludge may provide long term and cost effective control of potentially pathogenic bacteria (e.g. *E. coli* and *Salmonella*). Successful phage treatment of wastewater bacterial pathogens would be dependent on the prevalence and diversity of pathogen

species within wastewater. It would be virtually impossible to produce phage targeted at all pathogenic serotypes. For example, there is a high diversity of *E. coli* serotypes (Singleton and Sainsbury, 2002) and around 2400 known *Salmonella* serotypes exist (Popoff and LeMinor, 1992). However, wastewater treatment conditions intrinsically reduce the numbers of some pathogenic bacteria. Therefore, there is potential for phage treatment to be used successfully in combination with biological sludge stabilisation processes to reduce the abundance of specific pathogenic bacterial strains such as *E. coli* O157. Indeed, research into phage therapy for reduction of this pathogen in animal reservoirs is already underway (Bach *et al*. 2003).

3.2 Improving Dewaterability

Sludge dewatering is an important process in wastewater sludge treatment with sludge volume reduction promoting savings in equipment capacity as well as downstream treatment costs. Microbial exopolysaccharide (EPS) is responsible for binding microbial cells and particulate matter together (Wingender *et al*. 1999), thus influencing formation, settling and water binding ability of activated sludge flocs (Bura *et al*. 1998). High levels of microbial EPS, which can contain up to 99 % water by weight (Costerton *et al*. 1981), can inhibit the dewaterability of waste activated sludge (WAS) (Kang *et al*. 1989; Sanin and Vesilind, 1994) and anaerobically digested sludge (Houghton and Stephenson, 2002). Excessive production of EPS by species such as *Zoogloea* and *Thauera*, which form zoogleal clusters or flocs, has been implicated in dewatering problems of WAS. Lajoie *et al*. (2000) observed a negative correlation between abundance of zoogleal clusters and dewaterability.

Bacteriophages carry polysaccharide depolymerase enzymes (PDE) (Adams and Park, 1956) which are secreted during attachment of phage to the bacterial host. The enzymes degrade the bacterial exopolysaccharide capsule (secondary receptor) allowing the phage to bind to primary

receptor sites in the outer membrane (Hughes *et al*. 1998). This characteristic has attracted interest in their application for control of biofilm formation (Tait *et al*. 2002). Doolittle *et al*. (1995) observed lytic infection of *E. coli* biofilms by phage T4 (a well-studied member of the lytic T-even phages) and Skillman *et al*. (1999) utilised phage PDE to specifically denature *Enterobacter agglomerans*. Hanlon *et al*. (2001) demonstrated the ability of bacteriophages to diffuse through commercial alginate gels and purified *Pseudomonas aeruginosa* EPS. Phage treatment reduced the viscosity of commercial alginate gels by up to 40 % compared to controls, while application of phage to intact *P. aeruginosa* biofilms at a ratio of 1000 : 1 (phage : bacteria) resulted in a 99 % reduction in cell density. Alginate degradation may be attributed to both the presence of phage-borne enzymes and the release of bacterial alginate depolymerise enzymes following cell lysis. Similarly, Hughes *et al*. (1998) showed that *Enterobacter agglomerans* bacteriophage SF153b isolated from sewage and applied to *E. agglomerans* 53b biofilms degraded the EPS matrix and subsequently lysed infected cells.

Application of bacteriophages which selectively target strains such as *Zoogloea* and *Thauera*, could provide additional benefits in reducing EPS levels and improving sludge dewaterability. Enzyme treatments have already been shown to improve sludge dewaterability by the hydrolysis of water binding polymers (Thomas *et al*. 1993) and phage-borne polysaccharide enzymes have the potential to be exploited for similar purposes.

Unfortunately, phage treatment of biofilms and flocs is not without its constraints. The complex floc architecture may serve as a refuge habitat inhibiting the delivery of phage to target cells; bacterial cells situated within intact biofilms have been shown to be less amenable to infection than suspended cells (Hanlon *et al*. 2001). In addition, phage-borne depolymerase enzyme specificity is high (Reiger-Hug and Stirm, 1981) and minor changes in EPS structure and

composition may render the glycocalyx unsusceptible to depolymerase attack. Hanlon *et al*. (2001) reported that degradation of commercial alginate was phage specific. *Escherichia coli* phage Lambda and staphylococcal bacteriophage failed to reduce gel viscosity in contrast to *P. aeruginosa* phage GL1. Similarly, the high specificity of phage SF153b glycanase to *E. agglomerans* prevented the degradation of *Serratia marescens* EPS (Hughes *et al*. 1998). The probability of isolating a phage which bears an enzyme specific to the bacterial EPS (secondary receptor) and which, is capable of infection by the primary receptor, may be low (Lindberg, 1977). Mixed species flocs would contain a complex mixture of EPS from a range of bacterial species. The efficacy of phage treatment of multi – species biofilms has not received a great deal of attention (Tait *et al*. 2002).

Wastewater treatment systems are thought to contain from 17 to 268 different bacterial species (Wagner and Loy, 2002). However, a range of diversity surveys analysing more than 750 16S rRNA sequences have revealed that these communities tend to be dominated by the β –, α – and γ – Proteobacteria (Juretschko *et al*. 2002; Wagner and Loy, 2002) (Figure 1). Fluorescent in-situ hybridisation (FISH) studies have indicated that a small number of species dominate the microflora. For example, Juretschko *et al*. (2002) reported that operational taxonomic units related to *Z. ramigera* and the close relatives *Azoarcus* and *Thauera* comprised 17 % and 16 % of all detectable cells in activated sludge from an industrial wastewater treatment plant. Similar levels of abundance (up to 10 %) were reported for *Zoogloea ramigera* by Rossello–Mora *et al*. (1995) while Lu *et al*. (2001) found slightly lower concentrations of *Z. ramigera* in mixed liquor supernatant (4.2×10^4 MPN ml^{-1}) and solids (2×10^7 MPN g^{-1}) representing 5.9 % and 1.5 % of the total abundance of aerobic bacteria respectively. It is apparent that a small number of bacterial species dominate floc microflora and hence the contribution to EPS. Thus, despite high

bacterial diversity, it is possible that only a small number of different phage species would be required for efficient degradation of EPS.

3.3 Improving Digestibility

Municipal wastewaters generated in the UK and Europe are commonly treated by conventional activated sludge processes. This involves the removal and transformation of organic pollutants in wastewater into cellular biomass, carbon dioxide and water by an aerobic microbial community (Weemaes and Verstraete, 1998). Assimilation efficiencies for aerobic microorganisms are high (Mayhew and Stephenson, 1997) resulting in the production of excess biological sludge (e.g. WAS). The subsequent processing of which is an important and complex problem.

Anaerobic digestion, of WAS with primary sludge, has traditionally been used to stabilise sludge and reduce odour emissions and pathogens. In addition, the reduction in sludge volume reduces post-digestion processing, transport and re-use costs, while energy can be recovered in the form of methane. In the UK, mesophilic anaerobic digestion (MAnD), is the most common sludge stabilisation process with 54 % of UK sludge treated in this manner (Gendebien *et al*. 1999).

Unfortunately however, WAS can be difficult to digest because the organic material is largely compartmentalised within microbial cells resistant to lysis (Weemaes and Verstraete, 1998). WAS may contain up to 70 % bacterial biomass (Lehne *et al*. 2001) and the hydrolysis and solubilisation of these residual cells is considered to be the rate limiting step of anaerobic digestion (Eastman and Ferguson, 1981; Gujer and Zehnder, 1983).

The problem of poor WAS digestibility has received a great deal of attention in recent years. A wide range of treatments have been examined for their potential to improve WAS digestibility

during MAnD (Muller, 2001). Digestibility has been improved by mechanical (Dohanyos *et al*. 2000; Winter, 2002), thermal (Li and Noike, 1992; Kepp *et al*. 2000), chemical (Ray *et al*. 1990; Lin *et al*. 1997) and ultrasonic (Rooksby *et al*. 2002) pre-treatments. In all cases the treatments are designed to disintegrate microbial cells and convert high molecular weight refractory organic material to low molecular weight biodegradable components prior to their reduction in sludge digestion.

Similarly, phage mediated cell lysis could be developed as a pre-treatment for WAS to aid anaerobic digestion. There is potential for targeted phage treatment to simultaneously aid dewaterability and digestibility of WAS. Phages, infectious for numerically dominant strains in activated sludge such as *Zoogloea*, *Azoarcus*, *Thauera* and *Sphaerotilus*, could be used to lyse a significant proportion of the bacterial biomass, enhancing substrate availability to the anaerobic microflora during MAnD. Microbial community structure varies considerably between wastewater treatment plants and such disparity could hinder the development of generalised phage treatments for improving digestibility, necessitating tailor – made treatments for different plants.

Another potential application of phage in anaerobic digestion is the control of sulphate reducing bacteria (SRB). The presence of oxidised sulphur compounds in wastewater promotes growth of species such as *Desulfovibrio* and *Desulfosarcina* during sludge stabilisation by anaerobic digestion. SRB can compete against acidogenic and acetogenic bacteria and methanogenic archaea for substrates (Kalyuzhnyi and Fedorovich, 1998), thus affecting degradation processes and leading to reduced methane yields (O'Flaherty *et al*. 1998; Ranade *et al*. 1999). Furthermore, the associated sulphide production contributes to corrosion and odour problems and can lead to precipitation of trace metals (Stephenson *et al*. 1994). Thus, inoculation with lytic phage targeted

towards SRB genera (which comprise up to 20 % of the anaerobic reactor total cell count - Merkel *et al*. 1999), may aid control of nuisance microbes in digestion degradation processes.

3.4 Control of filamentous bacteria in activated sludge

Settlement of sludge formed during activated sludge processes is essential if clean effluents are to be produced. Bulking and foaming of activated sludge due to the over proliferation of filamentous bacteria is a common operational problem in many nutrient removal plants (Mamais *et al*. 1998; Madoni *et al*. 2000). The filamentous bacteria responsible include the genera *Microthrix*, *Gordona*, *Nocardia*, *Corynebacteria*, *Dietzia*, *Rhodococcus* and *Skermania* (Soddell, 1999). The filamentous flocs formed have high surface area: mass ratios producing poor sludge settling properties, which result in bulking. In addition, hydrophobic cell surfaces (Lemmer, 1986) and the production of extracellular polymers (Khan and Forster, 1988) promote the stabilisation of bubbles (air, water and microbial cells), which cause foaming. Subsequent pollution of the clarifier effluent with suspended solids has a negative impact on plant productivity and efficiency.

Various methods have been developed to maintain the correct balance between floc forming bacteria and the filamentous microorganisms responsible for solids separation problems. However, none of the existing methods are totally adequate. Disinfection can result in the mortality of important functional groups such as the nitrifiers, while the efficacy of selectors has been mixed (Eikelboom, 1994; Davoli *et al*. 2002; Martins *et al*. 2004).

Recently, Thomas *et al*. (2002) highlighted the potential for bacteriophages to control foam formation in activated sludge plants. From six activated sludge samples, the authors isolated 17 phages (primarily Siphoviridae) capable of lysing foam-forming bacteria. Host range studies

were carried out on 47 species from the genera *Dietzia* (1), *Gordonia* (8), *Mycobacterium* (4), *Nocardia* (14), *Rhodococcus* (16), *Skermania* (1), *Streptomyces* (1), *Tsukamurella* (1) and a suspected new genus (1). Fifteen phages were found to be polyvalent with varying spectra of genera and species lysed. Twelve isolates showed broad host ranges and were infectious for two or three of the genera *Nocardia*, *Gordonia* and *Rhodococcus*, however, none were able to lyse all members of one genus. None of the phages were able to lyse certain hosts (*Dietzia, Skermania, Streptomyces* and the suspected new genus). It is evident from this study that a large reservoir of polyvalent phages may exist. With further research, this pool could potentially be exploited to provide an effective long-term control of bulking and foaming problems.

3.5 Control of non-phosphate accumulating bacteria

Biological phosphate removal by phosphorus accumulating organisms (PAOs) is widely used to prevent eutrophication in aquatic systems receiving treated effluent. Anaerobic-aerobic reactor configurations (enhanced biological phosphate removal or EBPR systems) are designed to select for PAOs. During the anaerobic phase, PAOs degrade polyphosphate and take up carbon sources from influent wastewater, releasing orthophosphate which is then re-absorbed during subsequent aerobic growth. Phosphate is removed from the system through discharge of excess sludge (Mino *et al*. 1998). However, some non-PAO organotrophic ('G') bacteria can compete under anaerobic conditions (Cech and Hartman, 1993; Wanner *et al*. 2000). They obtain energy for substrate uptake through glycogen catabolism rather than by hydrolysis of polyphosphate (Satoh *et al*. 1994). The dominance of 'G' bacteria is believed to be responsible for process failure in EBPR systems (Wanner *et al*. 2000; Whang and Park, 2002).

It may be possible to target phage treatment towards bacteria which out-compete PAOs. The exact strains responsible for treatment failure have not been identified and 'G' bacteria appear to

be phylogenetically diverse (Kong *et al*. 2002). *Amaricoccus* species were thought to be implicated in process failure (Maszenan *et al*. 1998) but subsequent reports have shown that although abundant in EPBRs, pure cultures did not display the metabolic characteristics described above (Kong *et al*. 2002). If 'G' bacteria responsible for EBPR failure can be identified, selective targeting of those species with lytic bacteriophages has the potential to selectively reduce interspecific competition contributing to the long term stability of EBPR processes.

4. Limitations to Phage Treatments

General limitations to phage treatment have been addressed in a number of reviews (e.g. Barrow and Soothill, 1997; Sulakvelidze *et al*. 2001; Duckworth and Gulig 2002). However, many of these are not relevant to the use of phage in WwTWs. This review lists potential limitations for applications in wastewater treatment in Table 1, with some limitations outlined in more detail in the following section.

4.1 Host Specificity

The WwTW operator must identify which phage preparation to add to address a specific process failure or limitation. Microbial analysis of the system microflora is likely to be required because community structure can vary substantially between wastewater treatment plants. Manz *et al*. (1994) used subclass specific probes to demonstrate differences in the community structure between municipal and dairy waste activated sludge plants. Dairy waste batch reactor samples were dominated by cells which hybridised with a cytophaga-flavobacterium-specific probe but in contrast, municipal sewage was dominated by ß-proteobacteria. To date, studies of community structure using culture-independent molecular techniques have provided snapshots of microbial composition in wastewater treatment plants. Temporal changes in community structure are

poorly understood and may form an obstacle to the successful application of strain specific phages.

When target bacteria have been identified, phage infective for those species must then be selected. Host specificity is central to selection of suitable phage for particular WwTW applications. Narrow host specificity was implicated in the failure of some early phage treatments (Sulakvelidze *et al*. 2001). More recent attempts to control meat spoilage using phages lytic for *Pseudomonas* strains were also unsuccessful, with only 57.2 % of the 1023 strains isolated from beef being phage sensitive (Greer and Dilts, 1990). This study highlights the potential impracticality of attempting to develop phage treatments for generalised or broad-spectrum control of bacterial populations.

In contrast, although most phages in aquatic environments are thought to have highly specific host ranges (Wommack and Colwell, 2000), theory might suggest otherwise. In diverse microbial communities, where each species is present at low abundance, polyvalent phages would be expected to be at a competitive advantage relative to highly specific phages (Wilkinson, 2001). Contact by random diffusion is much more likely to result in successful infection and lifecycle closure if the phages show relaxed receptor specificity. A number of workers have isolated polyvalent bacteriophages from sewage. Of six *Yersinia ruckeri* phages isolated by Stevenson and Airdrie (1984), all but one lysed over 50 % of 49 *Y. ruckeri* strains from four serovars. All six phages lysed 13-100 % of eight *Y. enterocolitica* strains and 26-57 % of a range of 27 species from other bacterial genera. Hantula *et al*. (1991) found that approximately 10 % of phages isolated from activated sludge were polyvalent and suggested that broad host ranges may be common in natural systems. Later, Jensen *et al*. (1998) reported that 90 % of bacteriophages from collections isolated from freshwater and sewage were polyvalent. For example, bacteriophage

SN-T was capable of lysing members of six genera including strains of *Pseudomonas*, *Escherichia*, *Proteus* and *Shigella*. More recently, Thomas *et al*. (2002) found that 15 out of 17 phages isolated from activated sludge had broad host ranges (section 3.4). Khan *et al*. (2002a) demonstrated that all eight phages they isolated from activated sludge were polyvalent and 50 % formed plaques on both Gram positive and Gram negative bacteria. It has been suggested that observed narrow host-specificity may be an artefact of phage enrichment and isolation techniques (Jensen *et al*. 1998). To minimise modifications to phage during enrichment, it would be prudent to explore multiple host isolation techniques *vs*. single host methods.

Phages used therapeutically have traditionally been thought to exhibit a narrow host range. If this is true of phages associated with wastewater bacteria, the potential of phage treatments is more likely to lie in selective targeting of particular problematic strains (Hughes *et al*. 1998). If (as studies described above suggest) polyvalency is prevalent among wastewater phages, there may be potential for their use in ameliorating problems caused by a range of different bacterial species and strains. Despite the potential advantages of polyvalent phages, broader host range could lead to phage influencing not only the target strains, but also beneficial degradative bacteria. Further research to determine the true extent of polyvalency of wastewater and activated sludge phages would undoubtedly aid their use in wastewater treatment.

4.2 Phage isolation and production

It is essential for the success of any phage therapy that suitable phage can be both isolated and enriched to produce sufficient numbers for the application. Phage enrichment typically involves the inoculation of mixed environmental samples and growth media with a single host strain. Following overnight incubation, the bacteriophages produced by lytic infection of the host are detected by plaque assay on lawns of the original isolation host. This relies on the ability to

isolate a suitable host bacterium which may not be straight forward given that only 1-20 % of bacteria in the environment are culturable *in vitro* (Andreotola *et al*. 2002). However, advances in culturability have occurred in the last few years (Connon and Giovannoni, 2002; Rappe *et al*. 2002; O'Sullivan *et al*. 2004). Repeated phage purification using just one host strain may increase specificity for that strain potentially narrowing the host-range of the phage. Jensen *et al*. (1998) found that phages fell into two groups according to isolation method. Where a single host bacterium (*Sphaerotilus natans*) was used during isolation, phages displayed a similar efficiency of plating (EOP) on both the isolation host and an alternative host (*Pseudomonas aeruginosa*). In addition, phage DNA was insensitive to type II endonucleases. Where a multiple host isolation technique was employed (*E. coli* plus *Pseudomonas aeruginosa* or *Sphaerotilus natans*), EOP was lower on the alternative host than the isolation host and phage DNA was sensitive to both type I and II restriction endonucleases. Since DNA from phage isolated on a single host did not prevent cleaveage of phage lambda DNA, it appears that modifications to the viral DNA were responsible for the insensitivity to restriction enzymes. The authors argued that multiple-host isolation techniques may be more effective at isolating polyvalent phages by avoiding the selection bias of single host methods.

Successful application of phage to WwTWs is likely to require addition of high concentrations of phage because of the potentially large volume of wastewater to be inoculated. Large scale production of phages was carried out between 1950 and 1970 (Marks and Sharp, 2000). Sergeant and Yeo (1966) produced *E. coli* phage μ2 at concentrations above 10^{13} PFU cm^{-3} in 150 L tanks which equated to 75 mg phage per L culture. The authors found that the optimum time for phage addition to the bacterial host cultures was that which allowed lysis to take place just before CO_2 evolution reached maximum levels. Increasing aeration also improved yields. Previous studies

yielded up to 200 mg L^{-1} of phages MS2 and T2 respectively, at smaller culture volumes (Seigel and Singer, 1953; Strauss and Sinsheimer, 1963).

4.3 Host Resistance

It is evident through chemostat studies that bacteria can rapidly become resistant to phage infection (Fuhrman, 1999). A stable equilibrium is reached where most bacteria are resistant to their co-occurring phages and only a small phage-sensitive host pool remains (Lenski, 1987). It has been theorised that the metabolic cost of resistance gives sensitive strains a competitive growth advantage or that low abundance reduces risk of phage-host contact (Gill and Nealson, 1972; Chao *et al*. 1977). Through chemostat studies, Lenski and Levin (1985) concluded that, while the evolution of a phage resistant host strain was inevitable, emergence of new phage variants which exploit different receptor sites to counter resistance, was rare.

Evidence of host resistance in activated sludge is contradictory. Ewert & Paynter (1980) observed phage-sensitivity in only three out of 48 (unidentified) dominant bacterial isolates from mixed liquor and sewage and Hantula *et al*. (1991) observed approximately 15 % sensitivity in activated sludge isolates. In contrast, Khan *et al*. (2002a) reported that 60 % of bacterial isolates from activated sludge were phage sensitive. Fuhrman (1999) suggested that phage resistance in bacterial hosts may not be prevalent in aquatic ecosystems, particularly where oligotrophic conditions prevail. Middelboe *et al*. (2001) used a modelling approach to predict that in a mixed bacterial culture, host cell resistance would develop following infection and lysis of the dominant bacterial strain. They hypothesised that viral lysis would only have temporary effects upon total and strain-specific bacterial abundances as the proportion of resistant clones would increase and replace the sensitive population. They also predicted, however, that non-specific protozoal grazing (which is prevalent in wastewater treatment systems) may allow for recovery of sensitive

host populations. This is thought to be due to the reduced fitness of resistant bacteria in comparison to sensitive bacteria which results in faster recovery and multiplication of sensitive populations after grazing. This is corroborated by Simek *et al*. (2001) who recorded increased viral infection of reservoir bacteria as a consequence of flagellate grazing. The problem of resistance is likely to complicate the development of potential phage treatments. However, it has been observed that numerous phage species can infect a single bacterial strain (e.g. Wolf *et al*. 2003). There is potential to exploit this phenomenon to counter development of resistance.

4.4 Decay and loss of infectivity

Viral decay and loss of infectivity may reduce the efficacy of phage treatment of wastewater. Studies of coliphage fate have demonstrated a reduction in viral particles during wastewater treatment (Tanji *et al*. 2002a and b), although this is perhaps not surprising since coliform bacteria do not feature strongly in the active microflora of WwTWs (Fig. 1; Ewert and Paynter, 1980; Hantula *et al*. 1991). Removal of viruses during activated sludge treatment occurs by viral adsorption to sludge flocs (Wellings *et al*. 1976; Tanji *et al*. 2002b) and more than 97 % of coliphages may be associated with suspended particles (Ketratanakul and Ohgaki, 1989), which are transferred to sludge during settlement. Poor phage penetration into sludge flocs may therefore limit phage treatment efficacy as phage die-off may occur before contact with the host cell is made (section 3.2). The importance of phage penetration of flocs is currently unclear.

Ingestion of viral particles by bacteria, protozoa and metazoa may also contribute to phage loss in treatment systems (Kim and Unno, 1996). Environmental stresses, particularly solar radiation and starvation, may also promote reduction in phage numbers (Wommack *et al*. 1996; Davies-Colley *et al*. 1999) and have deleterious effects on phage replication (Kokjohn *et al*. 1994). In WwTWs, penetration of sunlight is likely to be limited by turbidity, and oligotrophic conditions are

unlikely to occur. Hantula *et al*. (1991), however, reported low *in-situ* infectivity in activated sludge samples, with phage titre rising to maximal abundance several weeks following their initial emergence. Since the precise generation times and densities of the hosts were unknown, slow infection may have been a result of either slow absorption kinetics or slow host lifecycles. Thus, inactivation or loss of introduced bacteriophages may be exacerbated by low *in vivo* infectivity.

Insufficient host cell concentration may also contribute to phage decline, particularly in the case of pathogens, where the target bacterium, e.g. enterohaermorrhagic *E. coli* (Grant *et al*. 1996) are not among the dominant species of the wastewater or sludge. Payne and Jansen (2001) developed a model to predict the outcome of phage treatments on the basis of replication and density-dependent properties of phage-host interactions. Major factors identified which could determine the outcome of phage treatment included: (i) whether the concentration of phage in the inoculum exceeded a threshold level sufficient to cause net reduction in bacterial numbers without subsequent replication (inundation); (ii) whether the phage inoculum exceeded a bacterial clearance threshold and could bring about complete clearance of the bacteria by inundation and (iii) whether phage could persist long enough to undergo active replication (active therapy - dependent on timing of inoculation and bacterial numbers exceeding a threshold level sufficient to allow proliferation). Temporal changes in the abundance of target bacterial populations are inherently likely. Such shifts in population abundance as a consequence of changing environmental conditions, in addition to competition, predation and development of host resistance within the treatment system should be taken into account in order to optimise the phage treatment strategy. Payne and Jansen (2001) suggested that for successful treatment, phage inoculation should coincide with a bacterial population density sufficient to support phage replication. Based on this approach, there are two potential treatment strategies for wastewater:

1) Application of high concentrations of phage as an inundative treatment to ensure a rapid 'passive' kill by primary infection of target bacteria; or 2) Low-level application of phages for long-term control of target populations where continuous removal is sustained by actively replicating phage populations. Continuous active therapy would be dependent on the bacterial concentration in the treatment system, but the exact threshold required to sustain active phage populations is not clearly understood. Wiggins and Alexander (1985) observed three phage-host systems *in vitro*: 80α-*S. aureus*, SPβ cI-*Bacillus subtilis* and T4-*E. coli*. The authors observed that minimum bacterial densities of $1 - 1.5 \times 10^4$ CFU ml^{-1}, 3×10^4 CFU ml^{-1} and 7×10^3 CFU ml^{-1} respectively were required before phage replication occurred. Studies of phages in natural systems have also suggested the presence of such a proliferation threshold. Suttle and Chan (1994) found that when marine *Synechococcus* concentrations exceeded 10^3 CFU ml^{-1}, the abundance of co – occurring cyanophages increased substantially, indicating active phage replication above a critical host abundance. In contrast, Kokjohn *et al.* (1991) demonstrated that *P. aeruginosa* bacteriophages could replicate at low host cell densities (10^2 ml^{-1}) *in vitro*. Recently, Kasman *et al*. (2002) argued that there is no replication threshold of host cells *per se*, independent of the phage population and adsorption constant. Rather, the rate of infection is entirely dependent on the host cell density in accordance with the theoretical kinetics of colloidal particles subject to Brownian motion.

If indeed a threshold host level for replication exists, in activated sludge systems total bacterial cell densities exceed 10^9 CFU ml^{-1}. Likely target strains in WwTWs occur at cell densities in the region of 4×10^4 MPN ml^{-1} for *Zoogloea ramigera* (Juretschko *et al*. 2002) and for *Sphaerotilus natans* and relatives of the β-Proteobacteria at approximately 3×10^8 MPN ml^{-1} (Snaidr *et al*. 1997).

Density of non-host cells may also be important in determining the success of phage treatment of wastewater. Wilkinson (2001) highlighted competitive hindrance by non-host species as a barrier to the success of phage treatments. In diverse and densely populated ecosystems including activated sludge and biofilms, non-host strains behave as 'decoys'. Phage particles collide and adsorb briefly to non-host cells thus inhibiting phage contact and lytic infection of the host strain. This could further explain low infection rates in activated sludge communities observed by Hantula *et al*. (1991). It may also be theorised that highly specific phages will be at a competitive disadvantage to polyvalent phages. The overall ecosystem effect of decoy hindrance is to dampen the classic Lotka-Volterra predator-prey oscillations, preventing near extinction of the host species (Wilkinson, 2001). Clearly, the presence of decoys in the diverse communities of treatment systems may hinder the success of phage treatments.

Continuous loss of phages in WwTWs may mandate frequent re-application while low infectivity could constrain the practicality of some phage treatments if prolonged treatment and consequently storage times are required.

4.5 Transduction

Horizontal transfer of genetic material between bacterial cells can occur through temperate transducing phages. Transduction arises when host DNA is mistakenly packaged into the phage capsid during production of the viral progeny (Wommack and Colwell, 2000). Progeny adsorb and introduce genetic material into new recipient cells. Infection does not give rise to lysis because the phage particles do not contain the complete phage genome required for replication.

Bacterial virulence factors are frequently encoded by bacteriophages, which can confer virulence by transduction (Saunders *et al*. 2001). For example, the conversion of non-pathogenic *E. coli*

strains to enterohaemorrhagic strains through phage-mediated transfer of Shiga toxin genes (Stx I and II) has been demonstrated (Muniesa *et al*. 2000; Müller *et al*. 2002). Phages encoding the Stx II gene and which are infectious for *E. coli* 0157: H7 are abundant in raw (influent) urban sewage (Muniesa and Jofre, 1998; Muniesa and Jofre, 2000). Their importance in the horizontal transfer of Stx II genes among enterobacteriaceae in sewage is unknown. However, generalised transduction by broad host-range phages isolated from sewage has been demonstrated (Jensen *et al.* 1998). Bacteriophage SN-T and SN-1 lysates produced by infection of phototrophic *P. aeruginosa* PAO1 were capable of inducing arginine auxotrophy in *P. aeruginosa* PAO303 following infection. The authors highlighted the potential role of polyvalent bacteriophages in dissemination of genetic information between a diverse range of host species. Similarly, Hertwig *et al*. (1999) demonstrated that mixed *Yersinia* bacteriophages isolated from raw sewage were capable of the generalised transduction of small (4.3 and 5.8 kb) plasmids isolated from non – pathogenic *Y. enterocolitica* strains. However, the *Yersinia* phages were incapable of transducing larger (72 kb) virulence plasmids from pathogenic *Y. enterocolitica* to benign strains. In this case the phage particles (40-60 kb) were probably not large enough to package the virulence plasmid.

Bacteriophage therapy requires rapid lysis of host cells thus temperate phage are generally not selected. However, little is known about the biology of phages associated with many WwTWs bacteria and demonstrating the inability of a phage to increase bacterial virulence is not easy. It would be preferable to avoid phages capable of lysogeny or with genetic material consistent with ability to display lysogeny (Goodridge and Abedon, 2003). Horizontal transfer of virulence determinants may result in the emergence of new pathogens with obvious associated health implications, thus assessing sensitivity of non-target organisms to phage may be important, particularly if phage targets are pathogenic bacteria.

Molecular biology may provide long-term solutions to many of the potential limitations of wastewater applications of phages. For example, genetic modifications to alter host specificity could abate some of the barriers to phage applications in WwTWs, however, further research and risk assessments would be required before use of engineered phage could be implemented for wastewater treatment.

5. Discussion

The re-awakening of interest in the use of phages to control bacterial infections has spread from the medical sector laterally into the fields of agriculture, aquaculture and the food industry. Non-clinical applications of phage therapy have raised interest in their potential for control of wastewater treatment processes. Indeed, Thomas *et al.* (2002) have already begun investigations into phage biocontrol in wastewater treatment and this review has highlighted aspects of wastewater treatment where phage-induced bacterial lysis might be harnessed in order to deliver improvements in sludge dewaterability and digestibility, to control foaming and to control levels of specific bacterial pathogens.

It remains to be seen whether phages also have the potential to be used to optimise wastewater treatment processes. Through long-term phage-mediated control of the microbial composition of treatment systems, it may be possible to alleviate problems such as competition between glycogen and polyphosphate accumulating bacteria in activated sludge systems and between sulphate reducing bacteria and acidogenic, acetogenic and methanogenic microbes in anaerobic digestion units.

There are significant barriers to the use of phage therapy in control of wastewater treatment systems, not least the paucity of understanding of microbial community dynamics and interactions during wastewater treatment. Success would also depend on accurate identification of problem bacteria, effective isolation and unbiased enrichment of phage and the ability of phage to penetrate flocs and remain infective *in-situ*. Strategies to counter host cell resistance must also be developed. Furthermore, safety considerations, such as risk of pathogen emergence through transduction must be assessed as should cost-benefit and reliability of treatments. Thus, substantial research is required before phage therapy can be applied successfully to WwTWs.

The application of phage therapy to wastewater treatment systems undoubtedly requires a more complete understanding of the microbiology of wastewater and aquatic habitats in general. Until this is achieved, many potential phage treatments will remain speculative. Indeed, failure to undertake research in this area will not only delay the development of beneficial applications of phage, but will also limit our knowledge of a component of modern microbiology critical to our understanding of bacterial pathogenesis and ecology. Current public perception regards phages in a positive light, particularly as an ally against bioterrorism and disease (Thiel, 2004), which already overcomes one important barrier to their use.

Despite some of the potential hinderances to the development of phage treatments for the wastewater industry, successful studies in other fields indicate that phage therapy deserves attention. With a greater understanding of the microbial ecology of wastewater treatment systems, phage treatments may become effective solutions to wastewater treatment problems and optimisation.

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Figure 1. Microbial community composition (% of total bacterial biovolume) of Kraftisried activated sludge determined by fluorescent in – situ hybridisation (adapted from Juretschko *et al*. 2002).

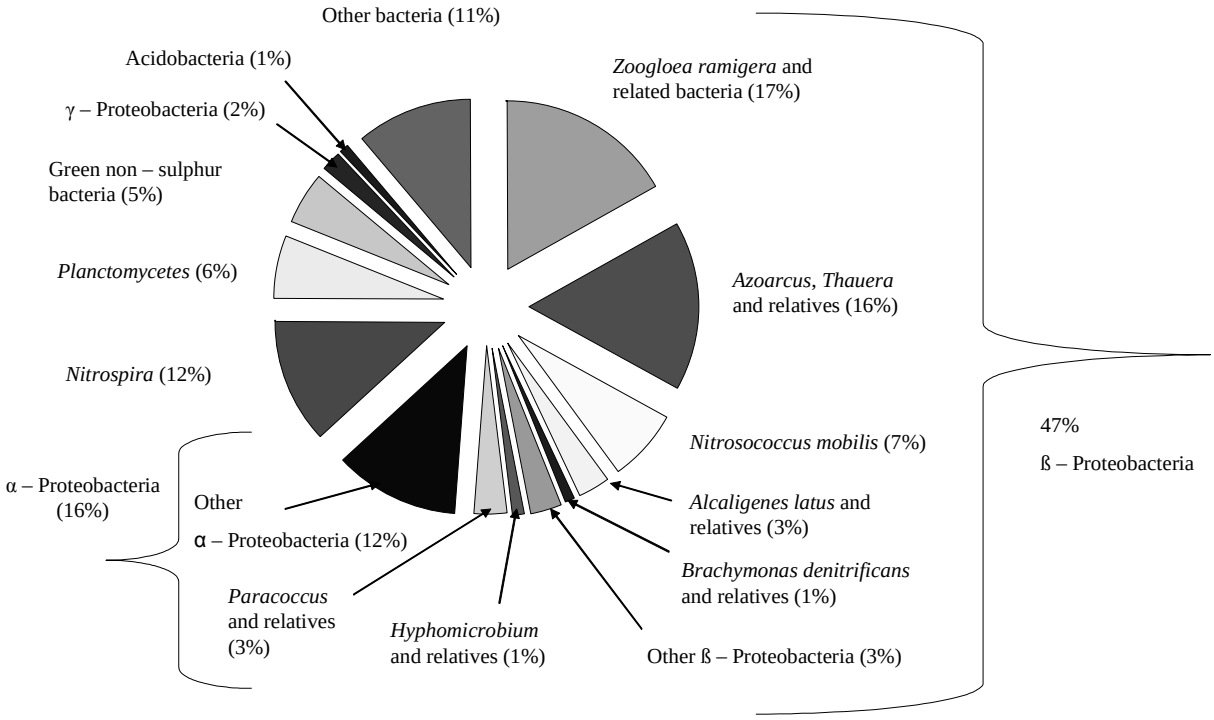

Table 1. Potential limitations of application of phage to wastewater treatment systems and possible solutions

Potential Limitation	Potential applications affected	Possible solution
Host Specificity:		
Narrow host range	All; particularly pathogen removal. Many target strains/species.	Phage cocktails; phage targeted towards dominant pathogen strains only. Use of polyvalent phages.
Inter-system variation in dominant microflora	All	Develop phage treatment targeted towards commonly dominant bacteria; assessment of microflora at individual WwTWs; use of phage cocktails and polyvalent phage for wider applications such as dewaterability.
Phage isolation and production:		
Identification of target bacteria / isolation of host bacteria	All, particularly 'problem' bacteria which have not been identified (e.g. 'G' bacteria) and may be unculturable *in vitro*	Further research into identification of bacteria responsible for process failure; isolation of alternative host.
Modification of phage during enrichment	All	Use multiple host methods of enrichment where possible.
Host resistance:		
Host resistance	All	Possible single dose treatment for clearance through inundation; treatment cycles with different phage; use of phage cocktails.
Decay and loss of infectivity:		
Loss of infectivity/ decline in phage numbers	All	High phage dosing. Isolation of phage from wastewater environment which are adapted to survive conditions; further research on conditions affecting phage survival in wastewater/ sludge; timing of application of treatment.
Poor penetration of sludge flocs	All	High phage dose. Extent of penetration unknown – further research to clarify.
Insufficient host bacteria	All, particularly pathogens which are present in relatively low numbers in WAS	High phage dose or repeated application for clearance through inundation. Use of polyvalent phage for broader host range. Further research on threshold host concentrations.
Inter-system variation in dominant microflora	All	Develop phage treatment targeted towards commonly dominant bacteria; assessment of microflora at individual WwTWs; use of phage cocktails and polyvalent phage for wider applications such as dewaterability.
Transduction:		
Transduction of genetic material	Primarily pathogen removal	Carefully select lytic phages; screen for genetic homology with known lysogeny genes; select narrow host range phage for highly virulent strains.

Bacteriophages - potential for application in wastewater treatment processes.

Withey, S.

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