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Full Length Research Paper

The impact of bacteriophages in bacteria removal associated with soba stabilisation station efficiency

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The existence of bacteriophages in wastewater of Soba Stabilisation Station was determined by isolating and identifying methods for their activities against *Escherichia coli* and *Staphylococcus aureus* isolated from the anaerobic, facultative and maturation ponds. The general viable count of the bacteria showed an average of 2.0 x 10^6 cfu/ml. In broth media the affection of the bacteriophage interactions with bacteria showed increasing of bacteriophages with concomitant decrease in bacteria due to culture clearance, where the readings of the turbidity for the first and second infection showed statistical significant of light transmission among *E. coli* phages' samples due to place of sample collections as follows: from the anaerobic and facultative ponds P > 0.05, facultative and maturation P < 0.05 and anaerobic and maturation P > 0.05. Whilst, the *S. aureus* phages samples' light transmission from the anaerobic and facultative P < 0.05, facultative and maturation P < 0.05 and anaerobic and maturation P > 0.05. On solid media, the affection of the bacteriophage was recognised by the phage plaque formation on bacterial cultures. The linear equations of phages' densities and distributions according to their wavelength were y = 0.0008x + 0.0303 for *E. coli* phage and y = -0.0102x + 0.2438 for *S. aureus* phage. This study concluded that phages naturally present where their hosts present and naturally destroyed bacteria that aided to recover from polluted environment.

Key words: Bacteriophage, *Escherichia coli*/ general viable count, light transmission, linear equations, stabilisation station, *Staphylococcus aureus*.

INTRODUCTION

Anaerobic treatment of wastewater is a valuable technology with a broad variety of purposes. Although the progression of efficient novel reactor types, relatively little is known about the structure and function of the microbial communities involved in the process. There are many sewage/wastewater treatment systems available but Waste Stabilisation Pond (WSP) systems seem to be the most common system widely recommended for developing countries. The WSP system is indeed an effective and a very economical means of treating wastewater. They are not systems only reserved for poor nations with hot climates but also many Western European countries with widely varying temperature conditions use them very successfully (Mara et al., 1992). Well-designed WSP are extremely efficient in the removal of excreted pathogen from wastewater (Davis, 2005). Wastewater treatment systems are thought to contain from 17 to 268 different bacterial species (Wagner and Loy, 2002). The numbers of phages and their host bacteria are an important factor in the replication of phages, evidence has been presented that successful phage replication requires at least 104 host bacteria per ml (Goyal et al., 1987).

Furthermore, aquatic viruses have a role in determining the diversity of bacterial communities through control of selected species competing for resources (Hewson and Fuhrman, 2003). Phage adsorption and entry to their bacterial hosts is mediated by specific receptors such as carbohydrates, proteins and lipopolysaccharides on the surface of the host cell (Marks and Sharp, 2000). Phages can be isolated from different environments such as Arctic sea ice (Borriss et al., 2003), marine ecosystems (Sullivan et al., 2003), the dairy industries (Sillankorva, 2008), and wastewater (Oliveira et al., 2009) and the current data indicates that roughly 1031 bacteriophages are existing worldwide (Serwer et al., 2007).

Since bacteriophages are natural infectious for some of the functionally important bacteria in wastewater they enhanced bacteria removal from wastewater (Khan et al., 2002). The occurrence of phages according to host type is most natural treatment and keeps the environment

clean by their influences over to kill the bacteria. In the present study, an attempt has been made in soba Stabilisation station to analyse the degree of degradation of bacteria by their phages. The incidence of phages in water samples generally indicates pollution by human or animal faeces (IAWPRC, 1991; DWAF, 1996; Leclerc, 2000; Schaper and Jofre, 2000).

Fecal pollution is typically indicated by the presence of indicator organisms, including fecal coliform bacteria that can be originate from human, domestic animal, or wildlife sources and may present exaggerated problems in inhabit areas due to warm temperatures and heavy rainfall (Toranzos and McFeters, 1997) . Information on the origin of faecal pollution is important because it gives an indication of the pathogens that may be expected, the risk of infection, and the treatment that may be needed to control the transmission of the disease (Scott et al., 2002). The importance of the bacteriophages in wastewater ponds resembles in the grazing of free bacteria.

Consequently, they decrease the turbidity of the effluent as well as the Biochemical Oxygen Demand (BOD), the dry mass percentage and the pathogenic bacteria (95% and 50%) respectively, (Gerardi et al., 1995). Because phage mediated bacterial mortality, the potential application of phage techniques in wastewater treatment systems was used to improve effluent of wastewater emissions into the environment with the minimum numbers of hazardous bacteria. In addition, bacteriophages in combination with biological sludge stabilisation processes have the potential to influence treatment performance by controlling the abundance of key functional bacteria groups to reduce specific pathogenic bacterial strains, (Withey et al., 2005). Eventually, 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.

MATERIALS AND METHODS

Determination of the number of bacteria in samples

Ten samples were taken from each of the inlet, outlet, surface and bottom by screening the anaerobic, facultative and maturation ponds of Soba Stabilisation Station respectively to determine the existence of bacteriophages and their corresponding bacteria in the wastewater. Serial dilutions were prepared and they were incubated at 37^{O}C in agar medium for overnight and examined for bacterial colonies count.

The isolation of bacteriophages

Preliminary enrichment

To prepare a sample of 5 ml of crude sewage, or polluted water, 1 ml of chloroform was added, mixed and centrifuged, then 1 ml of the supernatant liquid was transferred to 5 ml of a broth culture of bacteria in the exponential phase (this was prepared by adding 1 ml of an overnight culture to 4 ml of broth and incubated for 24 h at 37^{O} C) (Harrigan and McCance, 1993).

Isolation of phage

After incubation, the bacteria were removed by centrifugation. The supernatant liquid was transferred to another centrifuge tube; 1 ml of chloroform was added, mixed and centrifuged again. One millilitre of the supernatant liquid was transferred to 9 ml of quarter-strength Ringer's solution; a decimal dilution series was prepared. The dilutions were examined for the presence of phages by using Miles and Misra surface drop technique {to inoculate "Lawn" cultures of bacteria *E- coli* 0.1 ml of 24 h of its culture was spread over the surface of a nutrient agar plate}. The plates were incubated at 37^{O}C overnight. After incubation the presence of phage was shown by a clear area or by several small areas (plaques) (Harrigan and McCance, 1993).

Preparation of phage culture

An isolated pure phage culture from the plaques was made by transferring an isolated plaque with a sterile needle to an actively growing culture of bacteria. The culture was incubated until clearing occurred. A control bacterial culture (not infected by phage) was incubated with the phage culture. The pure phage stock was freed of bacteria and cell debris by centrifugation followed by filtration through a bacteriological filter (membrane filters) (Harrigan and McCance, 1993).

Storing phage culture

Suspending liquid, which contains a small amount of protein-gelatine (Oxoid) was added to the phage culture to inactivate the phage growth (Harrigan and McCance, 1993).

RESULTS AND DISCUSSION

Wastewater stabilisation station ponds

Wastewater has been treated to protect human and ecological health from waterborne diseases. Despite the improvement in effluent quality, point source discharges continue to be a significant contributor to degradation of surface water quality. In addition, Wastewater treatment systems reduce environmental impacts in the receiving water, but create other life cycle impacts for the microbial community. The general concept of design is to establish the combination of unit processes in the right sequence such that each may; operate at optimum efficiency, produce the required product, achieve the lowest capital and operating cost.

The Wastewater stabilisation Ponds were designed according to the standard practices similar to other anaerobic, facultative and maturation ponds suggested in Metcalf and Eddy (1991). The WSP is connected in series, where the first pond is the anaerobic pond, the

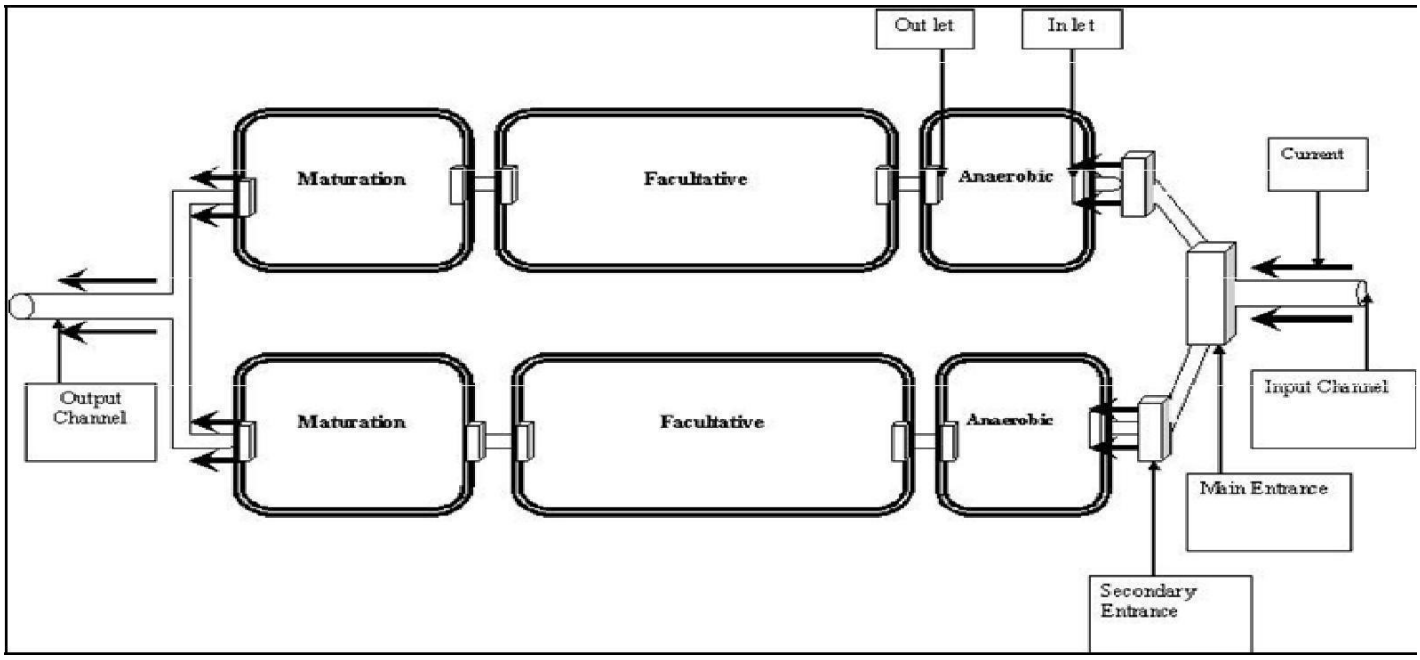

Figure 1. Pattern of soba stabilisation station ponds.

second is the facultative pond, and the last pond is the maturation pond (Figure 1) shows the double pond system. The ponds cover an area of 30,000 m^2 and the depths were thoroughly measured in this study to be on average of 2.00, 1.75 and 1.50 m for the anaerobic, facultative and maturation pond, respectively. The volume of the anaerobic ponds is 20, 000 m^3, the facultative ponds is 15,000 m^3 and the maturation pond is 10,000 m^3.

Bacteria general viable count

Viable counts can estimate bacterial numbers in their original source. When this method has been applied on wastewater ponds, bacterial numbers expected to be eliminated successively Pedersen et al. (2008), Hallbeck and Pedersen, (2008), respectively.

The average general viable count of the station was determined to be 2.0 x 10^6 cfu/m (Figure 2). This is in accordance with results of Mara et al. (1992), Mara and Pearson (1998, 1999) and Miguel and Mara (2004). This wastewater cannot be used for the irrigation of the restricted crops such as vegetables according to the report of the World Health Organisation (1989) which confirmed that the wastewater to be used for irrigating restricted crops is 1000˚C /100 ml and the number become 200˚C /100 ml in public lawns. The increasing of the bacteria numbers especially at the outlet of maturation pond (2.56 x 10^6) may be related to the irregular cleaning and maintenance operations Parr and Horan (1994) stated that many wastewater treatment stations in developing countries do not function properly for three principal reasons of wastewater treatment such as processes failure, lack of technical knowledge and failure to consider all relevant local factors at the pre-design stage and inappropriate discharge standards.

Effect of bacteriophages against bacteria in liquid media

In broth media, the effect of the bacteriophages extraction toward *E. coli* and *S. aureus* was recognised by measuring their culture turbidity.

Light-scattering methods (primarily turbidity measurement) techniques were used to follow the growth of liquid suspension cultures. Spectrophotometer was used to measure the amount of light that is not scattered by a cell suspension. The advantages of these techniques over colony counting are convenience, speed and the fact that the analysis is non-destructive. Light scattering follows the classic Law of Beer and Lambert (which applies to the absorption of light by coloured samples) reasonably well. As the cell density increases, the measured absor-bance increasingly because light scattered from one microbe is re-scattered by another in such a way that the light is redirected back into the photodetector.

The motility of the *E. coli* phages gives a positive linear equation; meanwhile the negative equation indicates the non-motile *S. aureus* phages (Figures 3 and 4). The ability of bacteriophages to survive in an environment is influenced by their capacity for motility. The longer introduced phages are retarded in the station ponds, the

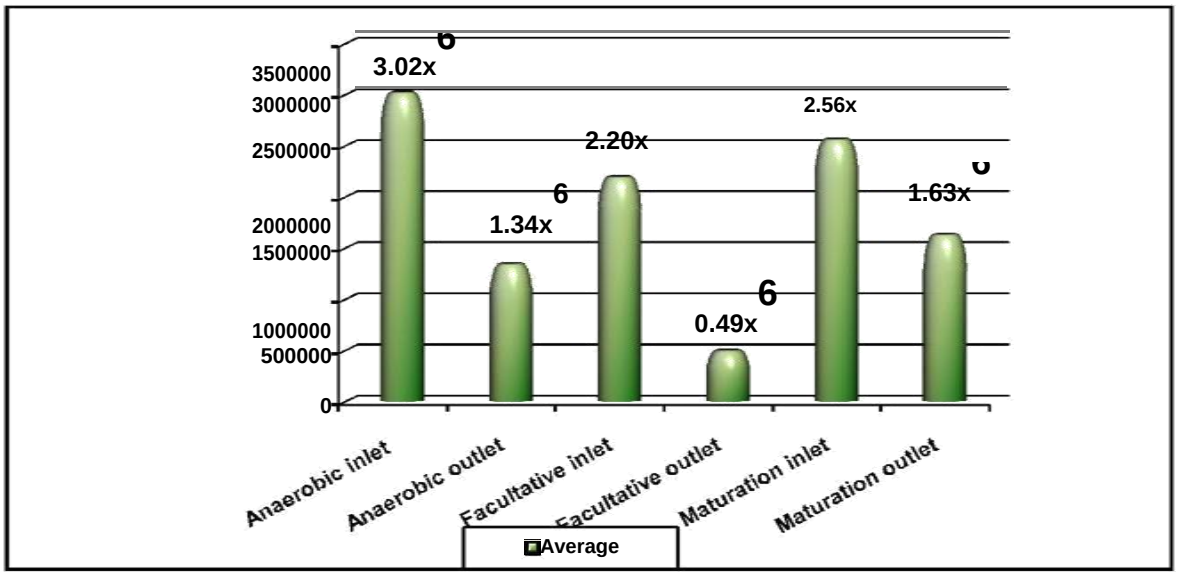

Figure 2. General viable count.

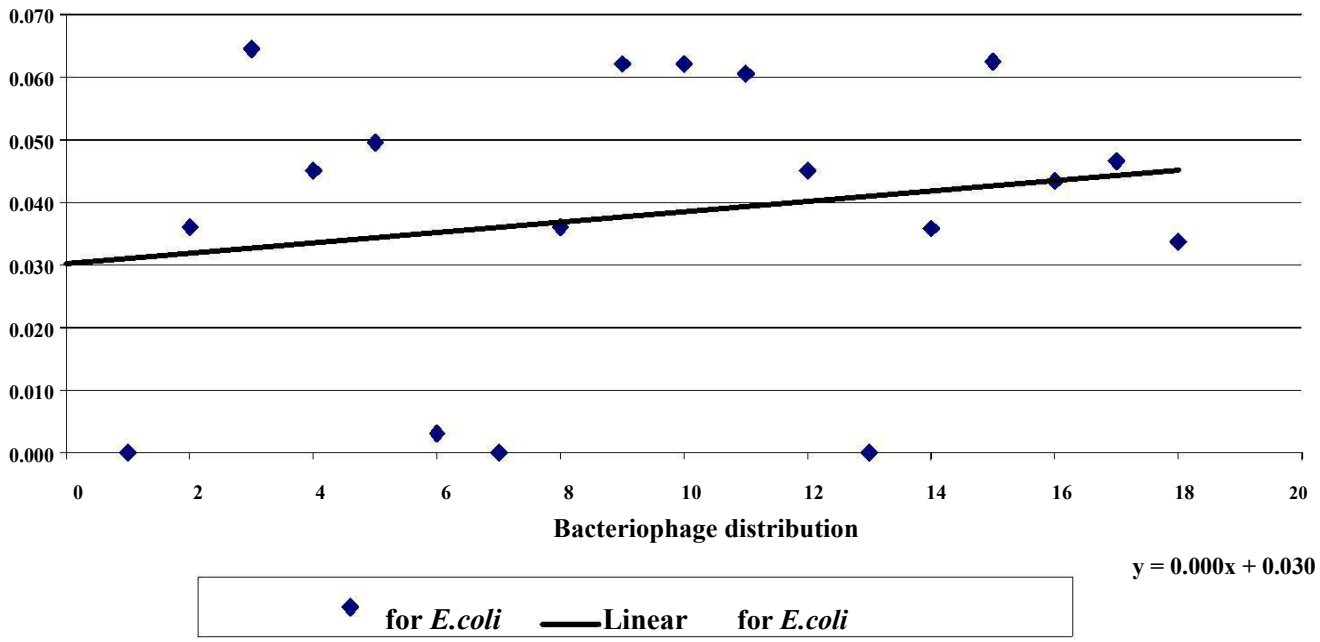

Figure 3. Wavelength distribution among *E. coli* bacteriophages' samples.

greater the chance that their elimination of bacteria will occur.

Beer Lambert equation with wavelength 260 nm was used for measuring the culture turbidity to know the bacteriophage replication and the bacteria mortality; the study shows that there was a reversible relationship between the increasing of bacteriophages and declining of bacteria due to culture clearance. The maximum activity of extracted phages was shown in the facultative pond (Figure 5).

For wastewater treatment in Soba Stabilisation Station, the reduction in bacteria was 3.02×10^6 -1.63×10^6 (Figure 2), while the light absorbency by the *E. coli* bacteriophage was 1.0 AU (Absorbency Unit) in the anaerobic pond – 0.5 AU in the maturation pond and the *S. aureus* Bacteriophage was 0.6 - 0.3 AU (Figure 5), this reduction pattern agrees with earlier findings by Ottoson (2005).

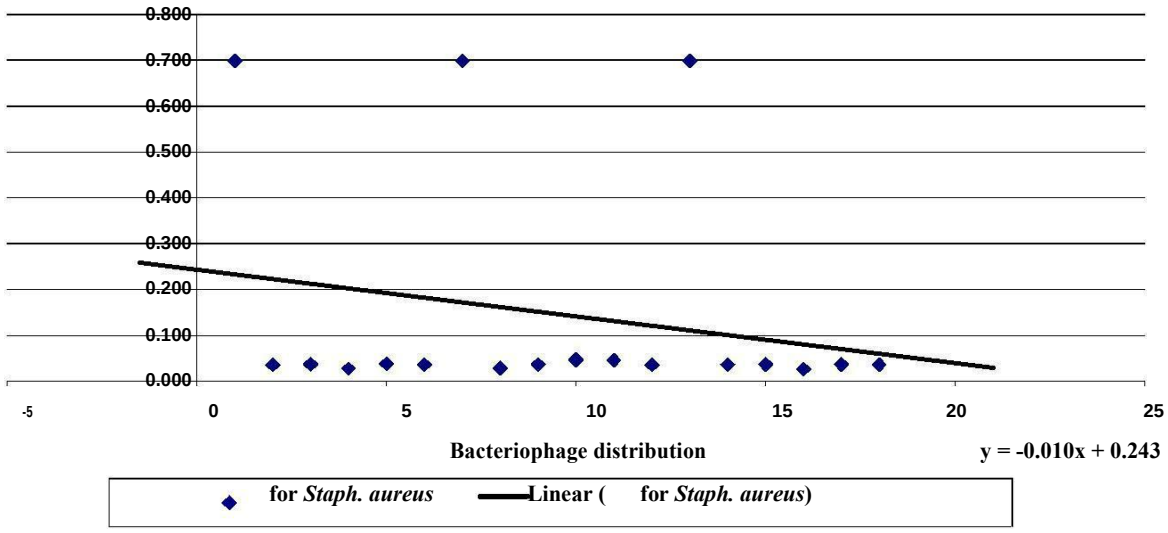

Figure 4. Wavelength distribution among *S. aureus* bacteriophages' samples.

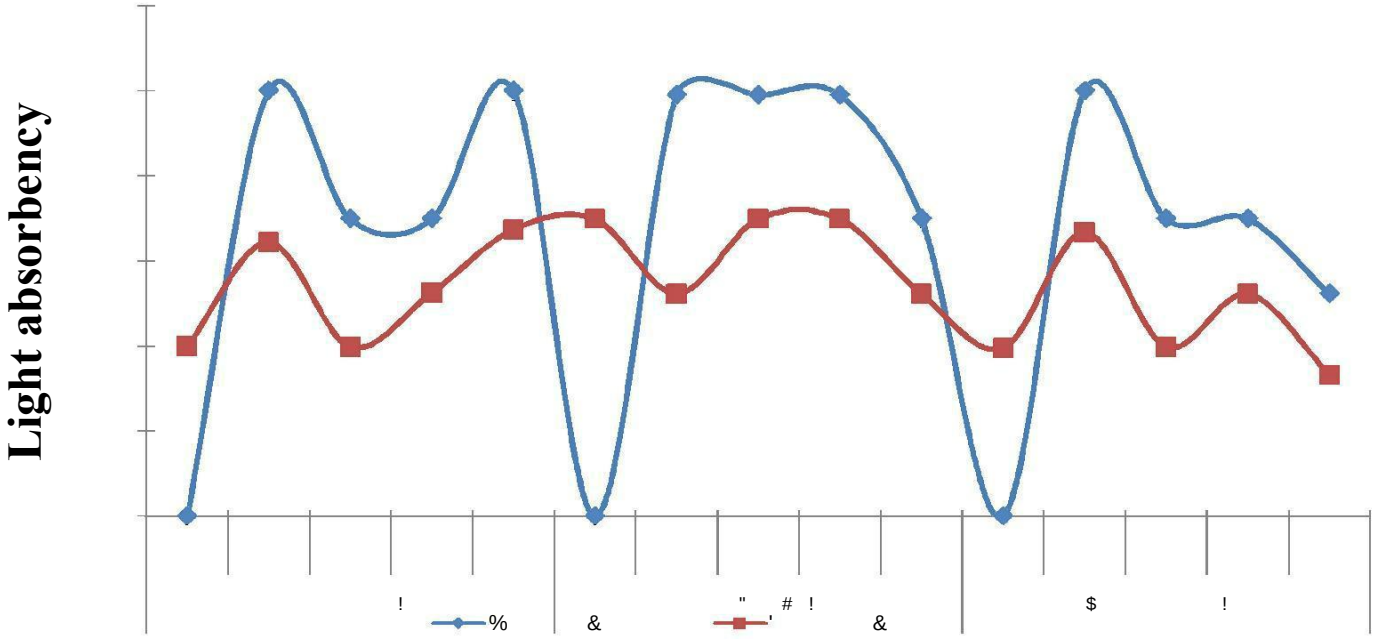

Figure 5. Spectrophotometer Absorbency for culture turbidity.

Effect of isolated bacteriophages against bacteria on solid media

On solid media, the plaque assay for bacteriophage showed pure phage colonies on bacterial lawns. Phage plaques were seen clearly with naked eyes after 48 h incubation period as colourless colonies at the medium and these colonies unstained with Gram stain (Figure 6). The efficiency of isolated phage from wastewater against *E. coli* and *S. aureus* showed remarkable inhibition of growth of the bacteria at both solid and liquid media, the difference of the effect between two bacteria at solid and liquid media might be due to physio-chemical changes and difference in motility of these two bacteria.

The presence of *E. coli* phage in wastewater might be explained by natural inheritance of these organisms in the intestinal tract of both human and animals due to the natural presence of their bacterial host and these confirm the isolation of *E. coli* phage from sewage by Furuse (1987). According to the behaviour of the bacteriophages

Figure 6. Bacteriophage plaques on Solid medium (Eosin Methelyne Blue).

in their lytic cycle, they play an important role in the removal of bacteria from the wastewater. This was proved by the spectrophotometer measures that matched the general viable count of bacteria observed in the present study. 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). Phage 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. It remains to be seen that phages also have the potential to optimize wastewater treatment processes. With a greater understanding of the microbial ecology of wastewater treatment systems, phage treatments may become effective solutions to wastewater treatment problems and optimisation (Withey et al., 2005). Thomas et al. (2002) have already investigated that biocontrol in wastewater treatment by using phages and it was explained that the wastewater treatment by phage induced bacterial lysis controls foaming and also specific bacterial pathogens. 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 in control of wastewater treatment systems. Success would also depends on identification of harmful bacteria, effective isolation and unbiased enrichment of phage and the ability of phage to remain infective *in situ*. The application of phage for wastewater treatment systems requires complete understanding of the microbiology of wastewater and aquatic habitats. In general, until this has been achieved, many potential phage treatments will remain speculative and current perceptions regard phage is in a positive light.

Conclusion

The study concluded that successful application of phages to wastewater treatment systems requires the understanding of microbial dynamics and interactions as regarding safety considerations to avoid host cell resistance and pathogen emergence through transduction.

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