LOW COST SUSTAINABLE PATHOGEN REMOVAL IN WASTEWATER

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ABSTRACT

Removal of pathogens present in wastewater is an ongoing issue for wastewater water treatment operators. One approach to removal is installation of high technology, high cost solutions. Another approach is to assess and optimise lower technology but low cost sustainable treatment systems.

Waste stabilisation ponds (WSP) offer a sustainable and economical method of treatment for wastewater across the world. Pathogen removal mechanisms occurring within ponds are largely unknown and only a few studies have been conducted on virus removal. While it is clear that sunlight (UV) and temperature play a major role in removal of pathogens in WSP's there are other mechanisms present in these complex systems that also play their part in removal of pathogens such as viruses.

Our research investigates virus (and indicator organism) removal efficiencies of WSP, mechanisms of removal independent of sunlight such as attachment and settlement, enzyme action and predation. The research presented here is the attachment and settlement of indicator organisms (bacterial and viral) compared with pathogenic viruses in WSP.

KEYWORDS

Pathogens, wastewater treatment, sustainable, removal

1 INTRODUCTION

Wastewater treatment is fundamental to the protection of both public health and the environment. Most wastewater treatment systems employ high technology, which, are not affordable, or sustainable options for smaller communities or developing countries. Our research is aimed at the investigation of the effectiveness of low cost, sustainable wastewater treatments. One such treatment is oxidation ponds (waste stabilisation ponds). Waste stabilisation ponds (WSP) offer a sustainable and economical method of treatment for wastewater across the world. Pathogen removal mechanisms occurring within ponds are largely unknown and only a few studies have been conducted on virus removal. While it is clear that sunlight (UV) and temperature play a major role in removal of pathogens in WSP's there are other mechanisms present in these complex systems that also play their part in removal of pathogens such as viruses.

Our research is focused on investigating and optimizing pathogen removal efficiency in wastewater. The project focuses on the mechanisms of removal occurring during wastewater treatment. The research presented here is one part of the research focused on the attachment and settlement properties of viruses in WSP. The outcomes of this part of the research will be included in a detailed model of WSP removal efficiency for viruses.

Many treatment technologies developed early last century still provide effective treatment of wastewater and crucially for less developed countries or small communities, are able to do this with low costs and low maintenance required. Worldwide waste stabilisation ponds (WSP) are used to treat wastewater from a variety of sources (human, agricultural, abattoir waste). Their efficacy at nutrient and pathogen removal is based on the retention time of the ponds, the amount of sunlight received and pond temperature. Treatment efficiency is monitored, mostly, by measuring physico-chemical parameters such as biological oxygen demand (BOD), total

suspended solids (TSS) and chemical oxygen demand (COD). It is assumed that pathogenic organisms in the wastewater will be destroyed while in WSPs due to long retention times and sunlight. Although there is no doubt that sunlight plays a major role in degradation of microbial pathogenic organisms in environmental situations, the normal presence and high concentration of algae within WSP may prevent the penetration of sunlight to all depths of a pond. This raises questions of how sunlight interacts with wastewater constituents to bring about pathogen removal, and whether other mechanisms independently or in combination with sunlight contribute to pathogen removal.

Previous research has shown that there are a number of mechanisms involved independently or combined in disinfection in WSP [1]. The time wastewater spends in the WSP is important for effective disinfection to take place. Poor removal efficiencies in WSP have been attributed to short-circuiting where HRT has been drastically reduced [1]. Remediation of short-circuiting can be achieved by introducing baffles into the pond.

Sunlight inactivation of microorganisms is the most studied factor for disinfection in natural environments, including WSPs [2,3,4,5,6,7,8,9,10,11]. There is evidence, however, that other factors interact with sunlight to bring about the inactivation of microorganisms [1]. For sunlight disinfection there are three mechanisms appear to be involved [1]. 1) Photobiological DNA damage (UV-B, 300-320 nm), which is not dependent on other factors but organisms do have mechanisms for repair. 2) Photo-oxidative damage (UV-B (and UV-A possibly)), which react with oxygen to form photo-oxidising species that damage cell constituents (e.g. DNA). Again, organisms can have mechanisms for repair present within their cells. 3) Photo-oxidative damage by external action (UV-A, 320-400 nm) involving activation of materials within the WSP (e.g. humic acids) into photo-oxidising species such as singlet oxygen. These then damage external targets on cell surfaces, and are not able to be repaired. Another external factor is when increasing pH is coupled with sunlight exposure and brings about the die off of microorganisms [3,12], even though pH itself has been shown to have little effect on die off of microorganisms unless very high levels, for prolonged periods are achieved.

Sedimentation is an important pathogen removal mechanism in WSPs. Larger organisms, such as helminth ova can sediment out of the aqueous phase within a pond and accumulate in the sludge. There is some evidence that protozoan parasite (oo)cysts also sediment to the sludge layer within WSPs but as their settling velocities are low, particularly for *Cryptosporidium* oocysts, it is thought that attachment to particulate matter must aid their sedimentation [13,14]. The evidence for bacterial and viral sedimentation is not so clear but if they attach to particulate matter within the pond, sedimentation may occur. Again, more research in this area is required as different bacteria and viruses will have different attachment properties related to their surface charges [15].

Our research aims to establish a more complete view of virus removal in WSP to enable more efficient removal of viruses (and other pathogens). By understanding the mechanisms involved more completely indications as to problems occurring within ponds can be established. Ultimately a model of removal in WSP will allow both the efficient running of WSP and aid in design of new ponds. Doing this will provide a low cost, sustainable method of wastewater treatment for the future.

2 MATERIALS AND METHODS

2.1 EXPERIMENTAL SET UP

WSP was collected from a local wastewater treatment plant (WWTP). Immediately after collection, pH and dissolved oxygen (DO) were measured and recorded. The WSP was transported to the laboratory and stored in the dark at <8°C, until ready for use (24 hours). To enhance any attachment and settlement in these sets of experiments the WSP was concentrated by centrifuging twice at 2,200 xg for 10 minutes. The pellets from these centrifugation steps were recombined with unconcentrated WSP. To assess the background levels of indicator organisms (*E. coli* and fRNA bacteriophage (phage)) and viral pathogens, samples of concentrated WSP were analysed for these determinands prior to the experiment. Prior to the experiment the pH and DO were measured and recorded in the concentrated WSP. Total solids (TS) were also performed on concentrated WSP.

For the experiments, two beakers were filled with concentrated WSP to 200 mL and one beaker was filled with filter sterilized deionized water to 200 mL. Beakers were covered in foil to prevent light inactivation occurring. To each of the three beakers, a mixture of *E. coli*, fRNA phage, Rotavirus (RoV), Echovirus (EcV) and Adenovirus (AdV) was added to give a final concentration of 10^5 cells per mL in each beaker.

The WSP was mixed using overhead stirrers, and after 5 minutes an initial sample was taken and analysed for *E. coli*, fRNA phage, RoV, EcV and AdV. The samples were taken at a fixed position in the beaker, mid way from the top. The beakers were kept stirring continually for 4 hours and a sample was taken at the same position in the beaker (measured distance down the beaker). Samples were again analysed for the microbial organisms described above.

After this sampling occasion the stirring was stopped and the samples left to settle for 44 hours. After this time, samples were taken at the same position as above and analysed for microbial organisms described above. The beakers were left for a further 5 days (one week in total from the start of the experiment) and again sampled as described above.

After the final sampling occasion, the remaining liquid fraction, from each beaker, was aspirated off to leave the settled solids fraction. Total solids analysis was performed on the remaining liquid fractions and dry weight analysis on the settled solids fraction.

2.2 INOCULUM PREPARATION

The bacterial tracer *E. coli* J6-2 was cultured in Brain Heart Infusion (BHI) broth (BBL, Sparks, MD, USA) at 37˚C for 18hr. The cells were then washed in saline twice by centrifuging at3000rpm, 15min. After each spin the supernatant was aspirate off and the pellets resuspended in saline solution. The final suspension was stored at 4˚C prior to injection 2 [16].

MS2 phage [17] was used as a viral tracer. Propagation of MS2 phage was by harvesting confluent plaques on host *E. coli* HS(pFamp)R, centrifuging and filtering to remove debris and stored at -20°C [18].

EcV, RoV and AdV were prepared by growing stock viruses at a known concentration in cell lines until confluent growth was achieved (normally 3-5 days). Infected cell lines were then washed and treated to freeze-thaw cycles before storing at -80°C for use in experiments. The titre of the stock prepared was calculated using TCID50 procedure [19].

EV, RoV and AdV were combined into a glass Schott bottle and shaken gently (120rpm) overnight at 4°C on a platform shaker. Before the injection mixture was prepared, the virus mixture was sonicated in a water bath at room temperature for 2 minutes, and then shaken on a platform shaker for 30 minutes at 4°C. After shaking, the virus mixture was sonicated again for 2 minutes at room temperature just prior to adding to inoculum mixture.

Prior to addition to the beakers, *E. coli*, MS phage (fRNA phage) and the virus mixture was combined into a sterile beaker. Appropriate dilutions of each microorganism were added to the inoculum mix to give an end concentration in the WSP beakers of 10^5 cells per mL.

2.3 EXPERIMENTAL ANALYSIS

At each time point samples were taken from the same point in each beaker (same distance from the top of the beaker). Samples were analysed for each of the microorganisms inoculated.

Liquid fraction: The sample removed from the beaker was firstly analysed immediately for fRNA phage and *E.coli*. Aliquots were frozen at -80°C until ready for analysis for EcV, RoV and AdV.

To assess the attached cells, the liquid fraction was centrifuged at a low speed [20]. Briefly, the sample was centrifuged at 2,500 xg for 5 minutes at room temperature and the supernatant (S1) aspirated from the pellet and stored in a sterile glass universal bottle. The pellet was resuspended in 10 mL filter sterile deionised water and centrifuged at 2,500 x g for 5 minutes at room temperature. The resulting supernatant (S2) was aspirated from

the pellet and stored in a sterile glass universal bottle. The pellet (P1) was resuspended in 10 mL filter sterile deionised water.

All fractions, S1, S2 and P1 were analysed immediately for fRNA phage and *E.coli* or aliquots frozen at -80°C until ready for analysis for EcV, RoV and AdV.

Settled solids fraction: At the end of the experiment (1 week) the settled solids were collected in a glass vessel and the weight recorded. The settled solids were resuspended 1:1 with an elution solution (3% beef extract, 3% Tween 80, 0.5M NaCl, pH 9.0) and then sonicated in a water bath for 4 minutes at room temperature. The resulting solid suspension was analysed immediately for fRNA phage and *E.coli* and aliquots frozen at -80°C until ready for analysis for EcV, RoV and AdV. Dry weight analysis was performed on the remaining solid suspension.

2.4 ANALYSIS METHODS

E. coli was analysed by pour plating 1 mL aliquots (or dilutions there of) into selective agar (Brilliance™ *E. coli*/coliform Selective Agar, Oxoid, UK). Plates were incubated at 44°C for 20± 4hours. After incubation, typical colonies were enumerated by eye and the number of *E. coli* (cfu) present per mL calculated. The detection limit was 1 cfu per mL.

MS2 phage was analysed using double layer overlay assay method with *E. coli* HS(pFamp)R as the host [21]. Plates were incubated at 35°C for 20±4 hours. After incubation, plaques (clearing zones) were enumerated by eye and the number of MS2 (fRNA) phage (pfu) present per mL calculated. The detection limit was 1 pfu per mL. Previous studies did not demonstrate any infectivity of *E. coli* J6-2 by MS2 phage [22] and so little effect on the *E. coli* by MS2 phage was expected in the experiments.

Samples for virus analysis were treated with chloroform prior to extraction to remove bacterial contamination. Briefly, samples (0.5ml) were sonicated in an ultrasonic waterbath for 2minutes at room temperature. Chloroform (0.5ml) was added to each sample and the tubes shaken at 220 rpm on an orbital shaker for 15min at RT. The tubes were then centrifuged at 10,000g for 20 minutes and the upper aqueous phase aliquoted to a new tube. Samples were store at –80°C until further analysis.

Viral DNA was extracted from the chloroform-extracted samples using the High Pure Viral Nucleic Acid Kit (Roche, Mannheim, Germany) according to the manufactures instructions and eluted with 50µl elution buffer. Each sample was then analysed for viral pathogens via real time quantitative PCR using specific primer sets for each organism (Donaldson et al., 2002; Pang et al., 2004; Wolf et al., 2007 and Heim et al., 2003).

Echovirus 7 was also analysed using end point titration assay (tissue culture infectious dose fifty (TCID50) procedure [23], in 96-well plates (Becton Dickinson Labware, N.J.), which, gave a measure of viability. The number of Echovirus 7 (pfu) per mL was calculated. Detection limit was 7 pfu per mL.

Total solids of the concentrated WSP and liquid fraction remaining at the end of the experiment (500ml) were dried at 103-105°C in glass beakers until dry and a constant weight was achieved. Total solids were then calculated as mg per L. Dry weights of the settled solid fraction were calculated by drying 5 g of the settled solids at 103-105°C until a constant weight was achieved. The dry weights were then calculated as g dry weight.

3 RESULTS

The results have demonstrated a significant difference in the behavior of indicator organisms compared with viral pathogens (Table 1). Bacterial indicator, *E. coli*, was markedly different to both the viral indicator (MS2 (fRNA) bacteriophage) and the viral pathogens tested. Attachment of *E. coli* occurred within 4 hours and was at the highest prior to cessation of stirring. Between 18 and 35% of *E. coli* attached to particulate matter present in WSP. Thus, was variable within the replicates of WSP tested but is still much higher than the viral indicator and pathogens tested (<1%). The results initially indicted a high rate of settlement as the numbers of attached

and unattached *E. coli* present in the liquid fraction reduced over the experimental time. However, when the settled fraction was analysed there appeared to be only a small fraction that had settled, 0.5-0.8% (accounting for numbers present in settled fraction compared with initial numbers inoculated). It is hypothesized that this is due to die off occurring during the course of the experiment. Further experiments are underway to further investigate this. When the proportions of *E. coli* present in the liquid fraction compared with the settled fraction after one week it is clear that there is a high proportion settled (mean of 9,758 cfu mL^{-1} in settled solid fraction compared with mean of 15 and 21 cfu mL^{-1} in the attached and unattached liquid fraction, respectively).

In comparison, the viral indicator MS2 (fRNA) phage showed very low attachment (<1%) and throughout the experiment (analysed at 4 hours, 44 hours and 7 days) the majority (over 99%) were unattached to the WSP present. The viral pathogens tested showed comparable results to this and over 99% were found to be unattached to WSP.

In terms of settlement, again *E. coli* showed a marked difference to both viral indicator and pathogens tested. A significant number of *E. coli* were found present in the settled solids after 7 days. In comparison to the water control, significantly higher numbers were present in the settled solids. This also indicating that *E. coli* present in the settled solids survived longer than those in water.

MS2 phage was only present in the settled solids (after 7 days) in low numbers and only showed a very low settlement (0.6-1.4%). Again, the viral pathogens showed very similar settlement rates. Results of pathogenic viruses are not shown as they did not show any attachment or settling at all.

Table 1: Mean (n = 6) *E. coli* and MS2 phage attached and unattached in liquid fraction of WSP and in settled solid fraction. NT denotes not tested.

Time (hrs)	***E. coli* cfu mL^{-1}**			**MS2 bacteriophage pfu mL^{-1}**		
	Attached	Unattached	Settled	Attached	Unattached	Settled
4	416,667	731,556	NT	72	271,587	NT
44	1,579	2,008	NT	15	155,458	NT
168 (1 week)	15	21	9,758	7	21,687	361

4 DISCUSSION

The results presented demonstrate that there is a significant risk in relying on bacterial indicator organisms to predict viral pathogen removal efficiencies and behaviour in WSP.

Both bacteria and virus would not tend to settle out of the water column if present as discrete cells due to their small size and neutral buoyancy. Bacteria are generally thought to aggregate in water environments and thus can settle out of the water column faster than if present in the free form [24]. The attachment of bacteria or virus to other cells or particles present in WSP depends on various factors such as the charge of the cells and particles and the size of particles present. More complex factors also play a role, such as the phase of growth of the cells. For example, the dominant algal species Chlorella varies in its surface charge depending on the stage in its growth cycle; -1.6 $umVs^{-1}$ during log growth phase, to -1.4 $umVs^{-1}$ when in stationary phase [25]. There is a complex relationship that occurs in WSP that involves the formation of bacteria-algae biomass floccs, which, settle to the bottom of the pond. Here, the algae secrete a complex mixture of organic compounds which are utilized and broken down by the bacteria present as a carbon source, in turn releasing inorganic carbon which algae use in photosynthesis [26,27]. A study of attachment of *E. coli* to algal species showed only a low attachment [28]. It was hypothesised that one of the following occurred; die off of *E. coli*, temporary attachment, or attached *E. coli* were quickly settled out of the WSP. The results of the research presented here also demonstrated a variable attachment but also a high degree of settlement occurring for *E. coli*. The results of our research also

indicated that there may be a cytopathic effect from WSP as die off was observed (not attributable to light as the vessels were protected from light), possibly the algae (as indicated in another area of the whole research theme, not presented here).

There is a lack of information on the efficiency of virus removal by attachment and settlement in WSP. It is generally assumed that a high level of attachment of viruses occurs in WSP [1,29]. Recent research has brought this into question and the results demonstrate a more complex and variable picture [30,31]. In his thesis, Dr Verbyla indicated that if virus attachment did occur it was to particles <180 µm and thus too small to settle [32]. The research presented here shows a very low level of attachment occurring for both viral indicator (MS2 phage) and viral pathogens (enterovirus, rotavirus and adenovirus). Subsequent settlement was not significant at all.

5 CONCLUSIONS/RECOMMENDATIONS

The use of WSP for an effective natural wastewater treatment is a vital component for ongoing sustainable wastewater treatment. There is an urgent need, however to improve knowledge of the disinfection capabilities of these systems. Future research will further advance the effectiveness of these low cost, sustainable options for wastewater treatment. There is a need to investigate the treatment capacity of these systems for the virus pathogens (and bacterial pathogens) themselves as the reliance on one bacterial indicator is severely lacking. The results presented here demonstrate the wide differences that occur in removal mechanisms for bacterial and viral organisms.

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