MECHANISMS OF VIRUS REMOVAL IN WASTE STABILISATION PONDS

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ABSTRACT

Globally waste stabilisation ponds (WSP) offer a sustainable and economical method of treatment for wastewater. The 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.

The virus (and indicator organisms) removal efficiencies of WSP in the presence and absence of sunlight was investigated. The effect of controlled pH and DO was also studied.

The results demonstrated that by increasing pH and DO levels, even in the absence of light, efficient removal of viruses was achieved: die off rate (k) ln -5.5 to -9.5 day^{-1}. Results over two summers showed a similar removal rate for virus (4-5 log) but lower for indicator organisms when the temperature was lower during the second summer. Only *E. coli* showed a significant negative correlation between sunlight and removal rate.

By maintaining WSP with high pH and DO levels efficient removal of virus can be achieved using these sustainable, economic treatment methods.

KEYWORDS

Waste stabilisation ponds, viruses, removal rates, mechanisms, pH, Dissolved oxygen (DO), light, temperature.

1 INTRODUCTION

Wastewater treatment is a requirement worldwide to protect both public health and the environment from anthropogenic activities. The treatment methods used are based on a long timeline of technologies stretching back to the 19th century (Metcalf & Eddy, 1972). There is a drive for more cutting edge and advanced technologies to treat wastewater but are they always necessary? Many of the treatment technologies developed in the 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 is based on the retention time of the ponds, the amount of sunlight and the temperature of the ponds. Treatment efficiency is monitored mostly by measuring the physico-chemical parameters such as biological oxygen demand (BOD), total suspended solids (TSS) and chemical oxygen demand (COD). It is assumed that in the pond due to the long retention times and the sunlight presence pathogenic organisms will be destroyed. Although there is no doubt that sunlight plays a major role in degradation of microbial pathogenic organisms in environmental situations the normal high presence of algae within WSP may prevent the penetration of sunlight very deep into the pond. So, how crucial is sunlight on the removal mechanisms? Are there other mechanisms present?

Previous research as shown that there are a number of mechanisms involved independently or combined in disinfection in WSP and the main factors are summarized in Table 1 (Davies-Colley, 2005).

Table 1: Proposed main mechanisms in disinfection in WSP's

Factor	Mechanism(s)	Microorganism affected	Ponds where active
Temperature	Affects rate of removal processes e.g. enzyme activity increases as temperature increases.	Bacteria, viruses, protozoan parasites, helminth worms.	Anaerobic, facultative, maturation.
Hydraulic retention time (HRT)	Affects extent of removal, longer HRT longer time for operation.	Bacteria, viruses, protozoan parasites, helminth worms.	Anaerobic, facultative, maturation.
Toxins	Algal and bacterial toxins can be toxic to other bacterial (and viruses?).	Bacteria (others?).	Facultative, maturation.
Sedimentation	Settlement of pathogenic organism (e.g. helminth ova).	Helminth worms, protozoan parasites.	Anaerobic, facultative, maturation.
	OR settlement of particulate matter with pathogenic organism attached.	Protozoan parasites, helminth worms (bacteria, viruses?).	Anaerobic, facultative, maturation.
Biological disinfection	Ingestion by higher organisms e.g. flagellates.	Bacteria, viruses (protozoan parasites?).	Facultative, maturation.
Sunlight	DNA damage by solar UV-B radiation.	Bacteria*, viruses, protozoan parasites.	Facultative, maturation.
	OR photo-oxidation (DO sensitive) (range of wavelengths).	Bacteria (protozoan parasites?).	Facultative, maturation.

Table adapted from Davies-Colley, 2005. * DNA damage caused by UV-B radiation can be repaired in bacteria, lethal effect is seen when the repair mechanism is overwhelmed.

Although temperature has a role in increased disinfection within WSP's its action is likely to be secondary to other factors. Increased temperature reduces survival of microorganisms within the environment due to increased action of processes within the pond such as enzymatic activity and chemical activity. To see a direct effect by temperature the value would be too high to see in the natural environment (normally over 45°C). Also, different organisms respond differently to temperature increases and some can survive well at elevated temperatures and may grow (Enriquez et al., 1995; Rose et al., 2001).

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 (Davies-Colley, 2005). Remediation of short-circuiting can be achieved by introducing baffles into the pond.

Microorganisms such as algae and bacteria can produce extracellular materials, which have a toxic effect on other organisms present within a system (oufdou et al., 2001). At present there is insufficient evidence as to the importance of this mechanism within WSP and more research is required in this area.

Larger organisms, such as helminth ova can be sedimented out of the water fraction within a pond and accumulate in the sludge. There is some evidence that protozoan parasite (oo)cysts also sediment to the sludge layer within WSP's but as their settling velocities are low, particularly for *Cryptosporidium* oocysts it is thought that attachment to particulate matter must aid their sedimentation (Grimason et al., 1993; Medema et al., 1998). 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 (Bolster et al., 2006).

Within WSP's a range of organisms co-exist and survive by gaining nutrients from the wastewater and ingestion potentially of microorganisms (Davies-Colley, 2005). Previously, ingestion of protozoan parasites has been demonstrated in water and wastewater (Stott et al., 2001; Stott et al., 2003). If high levels of predators are present in WSP it is likely that ingestion of pathogens, by intention or when attached to particulate matter, may play an important role in removal.

Sunlight is the most important factor in disinfection within the environment, including WSP's (Alkan et al., 1995; Altherr et al., 2008; Davies-Colley et al., 1999; Kayombo et al., 2002; King et al., 2008; Maïga et al., 2009; Mausezahl et al., 2009; McGuigan et al., 2006; Sinton et al., 1999; Sinton et al., 2002). There is evidence, however, that other factors are involved (Davies-Colley, 2005). Within sunlight disinfection there are 3 mechanisms involved (Davies-Colley, 2005). 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.

Increase in pH coupled with sunlight exposure also has an increasing effect on die off of microorganisms (Curtis et al., 1992; Davies-Colley et al., 1999). pH itself has been shown to have little effect on die off of microorganisms unless very high levels, for prolonged periods are achieved.

There is a scarcity of information on the disinfection properties of wastewater treatments for virus removal and those that have concentrated mostly on the indicator organisms for prediction of virus removal (Carducci et al., 2009; Hewitt et al., 2011). Virus numbers in wastewater can vary greatly and the removal efficiencies also vary and can be less than 1 log reduction. Hewitt et al. (2011) monitored influent and effluent levels of Adenovirus and Enterovirus in a variety of wastewater treatment works in New Zealand (including WSP's). Adenovirus levels decreased from 1.0-4.08 $\log_{10}$ infectious units per litre in the influent to 0.7-3.2 $\log_{10}$ infectious units per litre in the effluent. Enterovirus levels decreased from 0.7-5.2 $\log_{10}$ plaque-forming units (pfu) per litre to 0.7-2.15 $\log_{10}$ pfu per litre in the effluent. Little difference was found between wastewater treatment technologies. A study on WSP's in India found with a detention time of 2.7 to 17.2 days virus removal efficiencies of 88-98% could be achieved (Rao et al., 1981).

The use of indicator organisms was devised in the early 20th century to predict the potential for presence of pathogenic organisms in a sample. The organism of choice from the start was *E. coli* but recently questions have been raised as to its applicability in prediction of the wide variety of pathogenic organisms that may be present in wastewater. This has been especially true for the viruses and so new indicators have been suggested and tested including bacteriophages (viruses that infect bacteria) due to their relatively easy, rapid and cheap methods for analysis. Although the bacteriophage may prove to be more similar in their removal rates to viruses than *E. coli* there is still the question of using just one bacteriophage to predict the wide variety of

pathogenic viruses that can be present in wastewater. It is recognized, however, that the use of indicator organisms is still required for routine monitoring of treatment efficiencies. The problem with trying to use pathogenic organisms is that they are only present in high numbers when an infection or outbreak is occurring and at other times may only be present in very low numbers or absent (Davies-Colley, 2005). Also the choice of pathogen to test for is vast and the methods for identification and enumeration are often costly and time consuming. Indicator organisms are present consistently and at relatively high levels in wastewater and faecally contaminated environments (Bitton, 2011).

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 MESOCOSM CONSTRUCTION

Large containers (mesocosms) were constructed to hold WSP water for the experiments (Photograph 1). Identical mesocosms held 21.5 litres of pond water and ports were included to allow for placement of *in-situ* probes for continuous monitoring of the pH, temperature and DO in the mesocosms during the experiments. Sample ports were included to allow sampling to take place from the centre of the mesocosms with minimum disturbance to the mesocosm during the experiment. A small pump was situated within the mesocosms to allow for slow turnover of the pond water and prevent settlement occurring.

Photograph 1: Mesocosms constructed for experimental work. Peristaltic pumps with molecular weight cut-off in-line filters were used to control pH and DO levels in the dark mesocosms.

To enable control of the pH and DO between the light and dark mesocosms peristaltic pumps were used that pumped pond water from the light mesocosm to the dark mesocosm and visa versa at a rate of one turnover per 12 hours (Photograph 1). In-line molecular weight cut-off filters were used to prevent transfer of materials (and injected microorganisms) between the mesocosms and only allow transfer of pH and DO (photograph 1).

During experiments one of the pair of mesocosms was covered to prevent light entering the mesocosm (photograph 1).

2.2 EXPERIMENTAL SET UP

WSP water was collected on the morning of the start of the experiment from a local wastewater treatment works. The wastewater treatment works comprised a pre-screen and grit removal before passing to the pond system. Pond water was collected using a pump and then passed through a 63 μM pores size sieve to removal large predators such as *Daphnia*. In previous experiments the *Daphnia* were found to bloom and consume all of the algae present in the mesocosms. The sieved pond water was placed into sterile containers transported to the laboratory and stored in the dark at <8°C until ready for use.

Dataloggers were programmed to measure the pH, DO and temperature within each mesocosm at 30 minute intervals throughout the experiment. Just before dusk the mesocosms were filled with the pond water. Into each mesocosm 50 mL of the injection mix (see section 2.3.1 for preparation) was added aseptically. Mesocosms were left to fully mix and at dusk a sample was taken from each mesocosm using 50 mL sterile syringe. Samples were stored at <8°C in the dark until analysed (less than 12 hours). Samples were taken for microbial analysis at dawn, solar noon and dusk on each day of the experiment. In addition samples were taken for microbial indicators *E. coli* and MS2 phage at 09:00 and 15:00. No sampling was conducted between dusk and dawn on each day.

In order to use an experimental set-up relevant to real situations measurements were taken at WSP ponds over time. Temperature, pH and DO were measured at the top and bottom of the ponds for periods of at least 3 days during different seasons.

2.3 ANALYSIS

2.3.1 INJECTION PREPARATION

An injection mixture was prepared from laboratory strains of test microorganisms to give a final concentration in each mesocosm of at least 1 x 10^4 cells per mL. This was to enable a 4 log reduction in counts to be obtained in the experiment as this is normally the requirements used for assessing performance in a wastewater treatment.

The bacterial tracer *E. coli* J6-2 (Sinton, 1980) is a non-pathogenic, lactose negative, nalidixic acid-resistant derivative of *E. coli* K-12. Cells were cultured in Brain Heart Infusion (BHI) broth (BBL, Sparks, MD, USA) at 37˚C, washed, resuspended in saline solution and stored at 4˚C prior to injection.

MS2 phage (Goyal and Gerba, 1979) was used as a viral tracer. MS2 phages are icosahedral and approximately 26 nm in diameter. The host strain was *E. coli* HS(pFamp)R (Debartolomeis and Cabelli, 1991), which is resistant to ampicillin and streptomycin sulphate. 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.

Echovirus 7 was prepared by growing stock Echovirus 7 (ATCC) 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 -70°C for use in experiments. The titre of the stock prepared was calculated using TCID50 procedure (see below).

2.3.2 METHODS OF ANALYSIS

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). 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 (American Public Health Association (APHA), 1998). After incubation plaques were enumerated by eye and the number of MS2 phage (pfu) present per mL calculated. The detection limit was 1 pfu per mL.

Echovirus 7 was analysed using end point titration assay (tissue culture infectious dose fifty (TCID50) procedure (Karber, 1931), 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.

Previous studies did not demonstrate any infectivity of *E. coli* J6-2 by MS2 phage (Sinton et al., 2010) and so little effect on the *E. coli* added to the mesocosms by MS2 phage was expected.

Samples were taken of the pond water before the start of the experiment and from each mesocosm at the end of the experiment and sent for algal analysis (Cawthron Instititute, New Zealand).

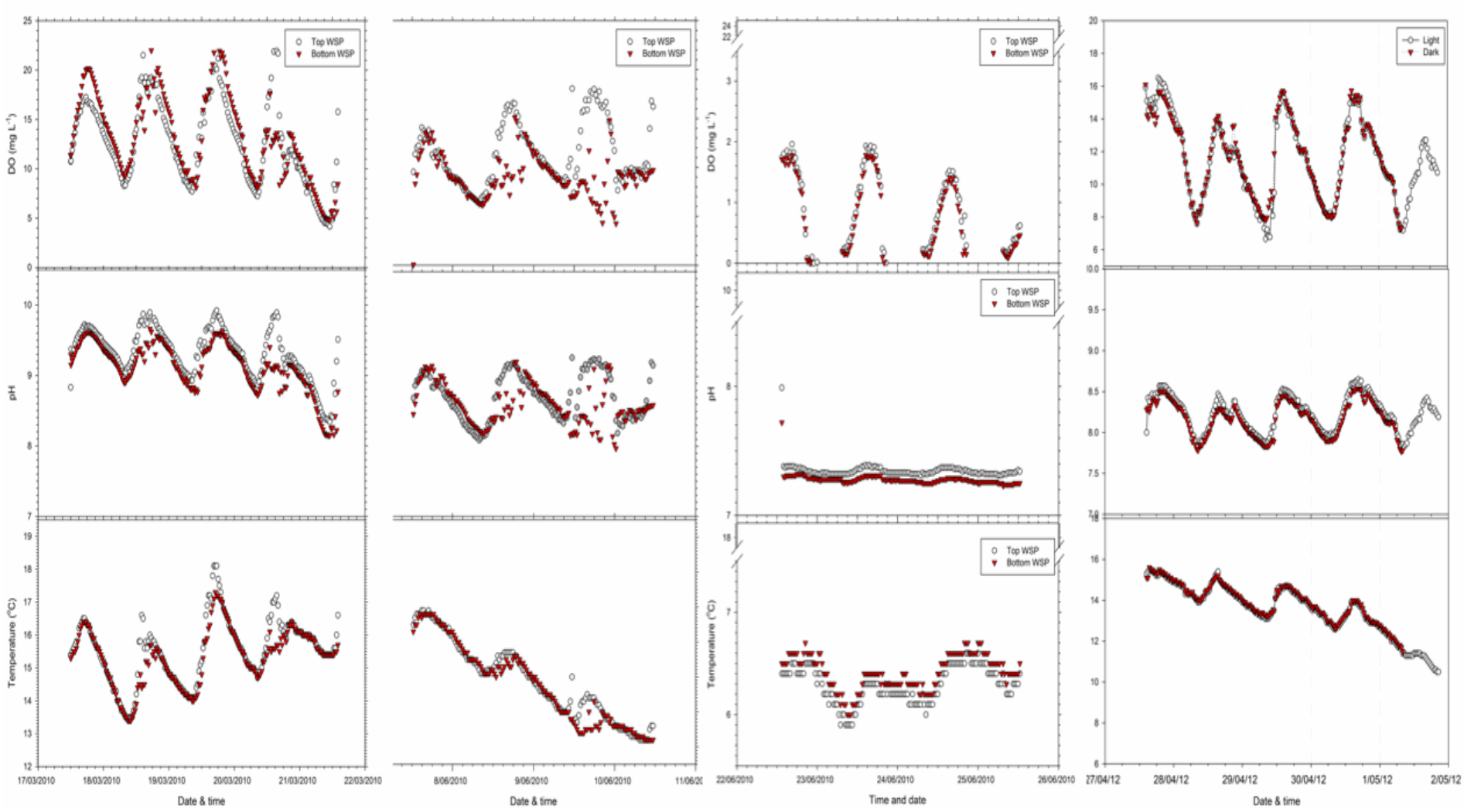
3 RESULTS AND DISCUSSION

3.1 PHYSICAL PARAMETERS IN WSP

The readings taken at WSP ponds showed that diurnal fluctuations of pH, DO and temperature occurred at both the top and bottom of the ponds (Figure 1). Few studies have looked at the diurnal fluctuations that occur in WSP at the top and bottom of the ponds, most have recorded the variations occurring at the surface of the ponds where most activity is assumed to take place due to the effect of sunlight. An overseas study of the diurnal variations in WSP also found diurnal fluctuations occurring throughout the depth profile of the pond (Kayombo et al., 2002). The key driver of the fluctuations of pH and DO in the ponds is due to sunlight activating algal photosynthesis (Davies-Colley et al., 1999; Kayombo et al., 2002). In a pond system that has good mixing, which is desirable to enhance treatment efficiency it is logical that the pH, DO and temperature changes occurring at the surface of the pond will be seen, to a lesser extent, at pond depth.

In our mesocosm studies by allowing pH and DO transfer between light and dark mesocosms biochemical parameters remained constant between light and dark (pH, DO, temperature (Table 2)). In addition this system enabled the diurnal fluctuations in pH and DO seen in WSP's to be mimicked in the light and dark mesocosms.

Figure 1: pH, DO and temperature in WSP ponds in New Zealand

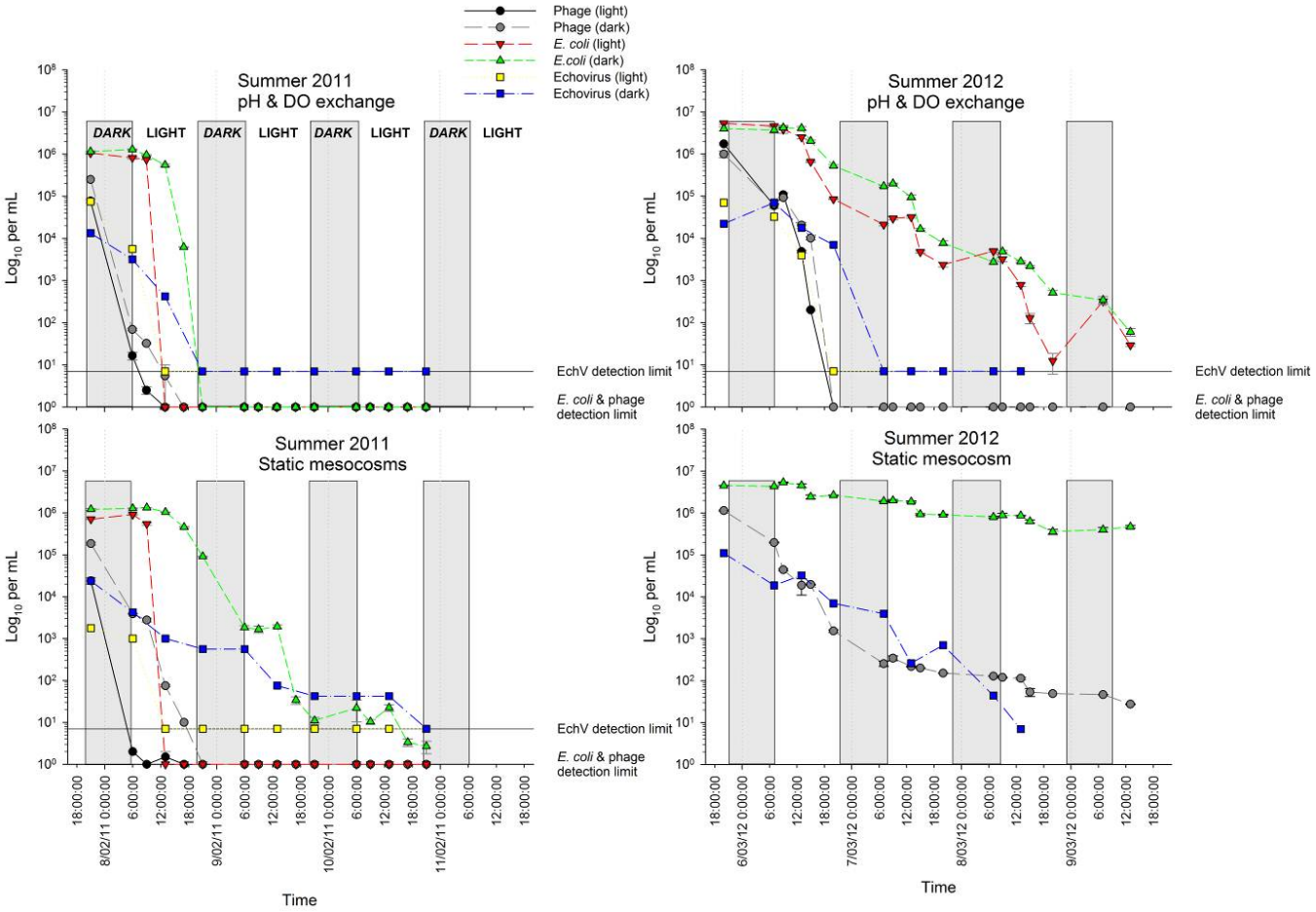


3.2 INFLUENCE OF PH AND DO ON REMOVAL EFFICIENCIES

The results of this study demonstrated that if the pH and DO levels in the WSP remain elevated similar levels of removal of indicator and virus can be achieved in the presence and absence of direct sunlight (Figure 2) *i.e* throughout the pond. Elevated pH has been shown in previous research to be important in pathogen removal, with increasing pH increasing pathogen removal rates related to sunlight (Davies-Colley et al., 2000). In this research when pH and DO was increased in the dark mesocosm (without sunlight) faster die off rates (*k*, ln per day) for all organisms tested was seen. MS2 phage die off rate increased from -11.8 to -16.2 per day, *E. coli* increased from -4.4 to -14.0 per day, and Echovirus increased from -2.4 to -9.5 during summer 2011. A similar trend was seen during summer 2012 experiment but to a lesser extent and all die off rates were lower whether in sunlight or not (Figure 2). Die off rates increased from -2.9 to -13.8 per day for MS2 phage, -0.6 to -3.0 per day for *E. coli*, and -3.6 per day to -5.5 per day for Echovirus.

The ability to equilibrate pH and DO between the dark and light mesocosms achieved similar removal rates in the light and dark mesocosms; 6 log reduction in *E. coli* during summer 2011 and 7 log during summer 2012, and 5 log reduction in MS2 phage during summer 2011 and 6 log during summer 2012, recorded in light and dark mesocosms (Figure 2). Echovirus 7 removal was slightly lower during both summers but still achieved high and comparable removal in light and dark mesocosms: 4 (dark) and 5 (light) log during summer 2011 and 5 log during summer 2012 (Figure 2).

Figure 2: Removal of indicator and virus in WSP in presence and absence of sunlight with pH and DO exchange over two summers (2011 and 2012)



3.3 VARIATION BETWEEN SUMMER EXPERIMENTS

When the rate of removal between the summer experiments was studied a slower removal rate was seen during the summer 2012 experiment (Figure 2). This can be related to the lower temperatures occurring during this experiment (Table 2) and not related to the sunlight or UV radiation occurring (as there was no significant relationship seen).

Table 2: Physico-chemical parameters during experiments

Date	Experiment	Temperature (°C)		pH		DO (mg/L)	
		Light	Dark	Light	Dark	Light	Dark
Feb-11	pH DO exchange	10.0 - 30.2 (19.1)	13.4 - 27.6 (19.6)	10.6 - 11.3 (11.1)	10.2 - 10.7 (10.4)	8.7 - 22.0** (14.2)	6.9 - 11.1 (9.1)
Feb-11	static system	10.1 - 21.9 (18.7)	13.7 - 23.9 (18.2)	9.3 - 11.3 (10.5)	9.0 - 10.2 (9.6)	10.9 - 22.0** (15.4)	6.4 - 9.1 (7.7)
Feb-12	pH DO exchange	7.8 – 24.5 (14.9)	6.0 – 24.5 (15.4)	9.4 – 10.5 (10.1)	9.6 – 10.2 (9.9)	8.4 – 22.0** (14.3)	6.6 – 14.7 (11.5)
Feb-12	static system		10.6 – 24.5 (16.4)		7.5 – 9.4 (7.9)		4.6 – 12.9 (5.8)

During summer 2011 experiment a similar comparable removal was seen in static mesocosms (no exchange of pH and DO) compared to exchange mesocosms for all parameters (Figure 2). This was most likely due to high temperatures occurring during the experiment enhancing removal in the dark static mesocosm (Table 2). A dark static mesocosm was set up at the same time as the exchange mesocosms during the summer 2012 experiment. A much lower removal of all *E. coli* compared to the exchange mesocosm was seen: 1 log removal in static compared to 7 log removal in exchange mesocosm (Figure 2). MS2 phage and Echovirus 7 results were similar in the static and exchange mesocosms indicating these organisms were more sensitive to the temperatures reached (Table 2 and Figure 2).

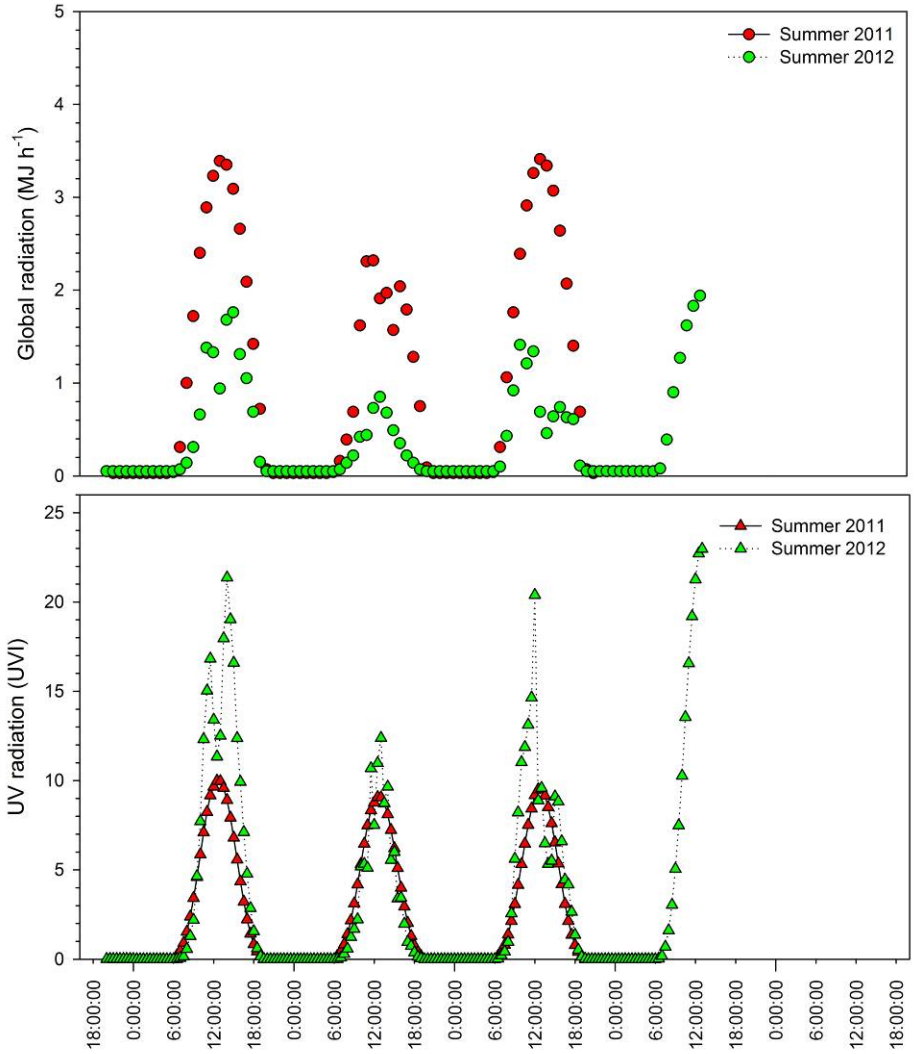
Ranges of temperature, pH and DO recorded during each experiment are presented in Table 2. Temperatures were consistent between light and dark mesocosms during experiments. High temperatures were obtained during the summer 2011 experiment on one day but the average temperatures obtained were within the normal range seen in WSP during summer months in NZ (January – March). Average temperatures were lower during the summer 2012 experiment compared to summer 2011 (Table 2). Similar ranges and means for pH were recorded for static and exchange mesocosms as well as between the summer experiments. The exception being during the summer 2012 experiment the dark static mesocosm remained lower than the summer 2011 experiment: mean 7.9 during summer 2012 compared to 9.6 during summer 2011 (Table 2). In the exchange mesocosms for both summers the mean pH remained over 10, which in previous research has been shown to be an important removal mechanism in WSP (Curtis et al., 1992). Previously elevated pH and DO have been shown to increase efficacy of sunlight inactivation (Davies-Colley et al., 1999) and in this experiment the same removal was observed in the dark mesocosms (Figure 2).

3.4 VARIATION IN SUNLIGHT AND ITS EFFECT ON REMOVAL EFFICIENCIES

Slower die off rates for all microorganisms tested was seen during the summer 2012 experiment (Section 3.2 and Figure 2). Comparing the amount of sunlight (global radiation) and amount of UV radiation occurring during the

summer experiments higher global radiation occurring during summer 2011 compared to summer 2012 (Figure 3). The UV radiation showed the opposite trend however, and higher UV radiation occurred during summer 2012 (Figure 3). Although there was a slightly higher log removal observed for E. coli, MS2 phage and Echovirus 7 during the summer 2012 experiment, there was only a significant (P = < 0.05) negative correlation seen between E. coli and UV radiation levels. Previous research has considered UV-B radiation to be an important factor in microbial removal efficiencies in WSP but other research has demonstrated that more than this range of wavelength of light are responsible for disinfection (Davies-Colley, 2005). The faster rate of die off of all microorganisms tested in this research also points to this fact as the fastest rates occurred when the global radiation (all wavelengths of light) was highest and not the UV range.

Figure 3: Sunlight (global radiation and UV radiation during experiments. Top graph presents the global radiation readings taken during time the experiments and the bottom graph presents the UV readings taken during the time of the experiments by the NIWA CliFlo database.



3.5 ALGAL ABUNDANCE OCCURRING OVER SUMMER EXPERIMENTS

The algal abundance between the two experiments showed that during summer 2012 there was a higher abundance in terms of the number of species present (Figure 4). Also a higher abundance of potentially toxic

cyanobacteria was seen in summer 2012 (Figure 5). With respect to the impact of algal toxins on removal efficiencies in WSP the results are inconclusive and more research is required. The slower die off rates in the summer 2012 experiment, when algal levels and abundance was higher points to doubts in the importance of algal toxins on virus (and other organisms) removal. The literature also gives conflicting views on the impact of algal toxins on microorganism removal efficiencies (Oufdou et al 2001, Maynard et al 1999). Oufdou et al (2001) found that cyanobacteria present in WSP's were toxic to *E. coli* and other bacteria tested but Maynard et al (1999) dismissed the action of algal toxins as an important mechanism in WSP's. Further research is required in this area to elucidate the importance of algal toxins in disinfection mechanisms within WSP's.

One important note from this research is if the cyanobacteria leave the WSP and enter the receiving water (river system) there is a potential for their spread downstream to recreational areas. This could have major impacts on public health either directly from recreational activities or indirectly from shellfish gathering.

Figure 4: Algal abundance during summer experiments

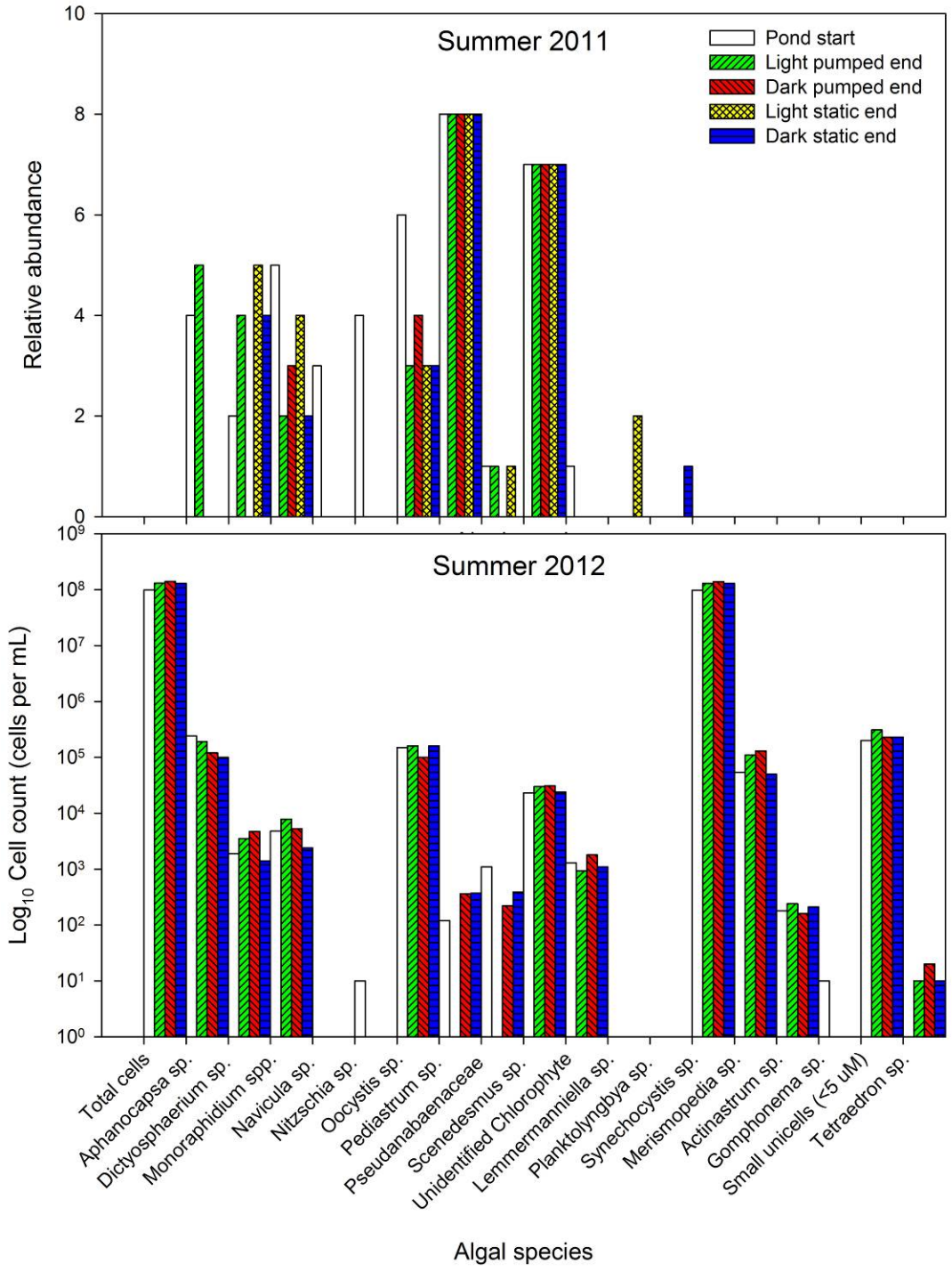
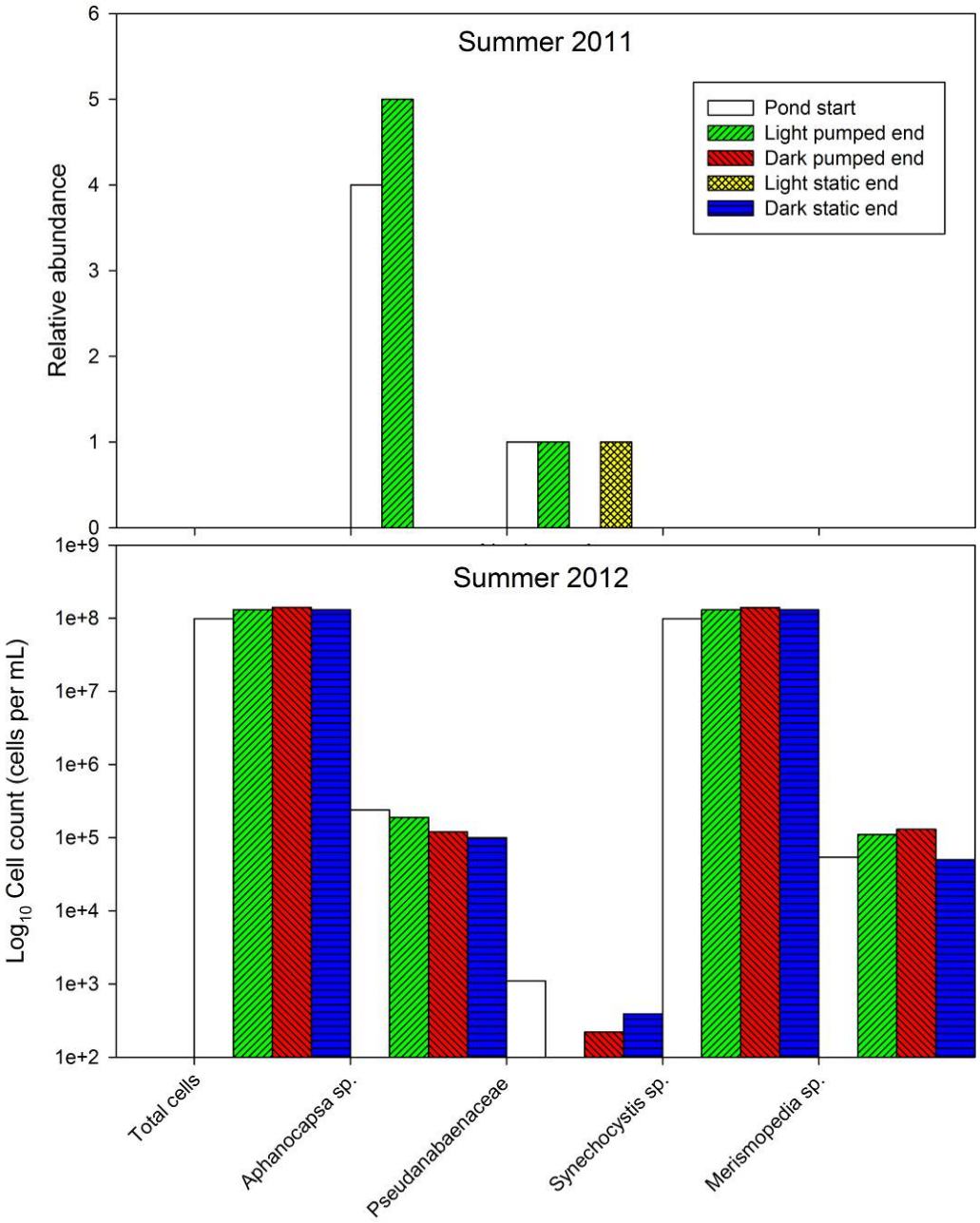


Figure 5: Potentially toxic cyanobacteria present over summer experiments



4 CONCLUSIONS

Virus removal in WSP has been demonstrated for Echovirus 7. The removal rates for Echovirus 7 were generally slower than MS2 phage but faster than *E. coli*. In these experiments *E. coli* appears to be a better indicator for Echovirus 7 than MS2 phage. This cannot however to be assumed to be true for other viruses as each have very different properties in terms of size and surface properties for example. Also DNA viruses such as Adenovirus has been shown to be much more resistant to disinfection e.g. UV than other RNA viruses like Echovirus. While it is clear sunlight plays a major role in removal of viruses the research presented here shows

that if a WSP is well mixed and pH and DO is elevated throughout the pond high removal can be achieved in the absence of direct sunlight.

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