THE DARK SIDE OF VIRUS REMOVAL BY WASTE STABILISATION PONDS

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ABSTRACT

Adequate sewage treatment is vital for maintaining New Zealand's economy (aquaculture, tourism) and lifestyle (mahinga kai and recreation). Viruses are particularly problematic because they can be infectious at very small doses, shellfish concentrate them as they filter-feed, and they can survive for long times in the environment. Virus removal mechanisms in waste stabilisation ponds (WSPs) are likely to be complex. Sunlight is an important mechanism, but the focus of this study is what removal occurs in the absence of direct sunlight, at night and at depths in the pond where there is no sunlight penetration. Here, we report on the development of two mesocosms which allowed us to compare the removal rates of *Escherichia coli* with those of MS2 phage, the latter being commonly used as a virus model, with and without solar radiation (referred to as 'light' and 'dark' conditions). The second mesocosm configuration, which had efficient exchange of dissolved oxygen and pH between the light and dark sections, resulted in similar levels of removal of MS2 and *E. coli* and in both the light and dark sections. The level of removal was 1 and 2 log, respectively.

KEYWORDS

Virus removal, waste stabilisation ponds, *E. coli*, bacteriophage

1 INTRODUCTION

Adequate sewage treatment is vital for maintaining New Zealand's economy (aquaculture, tourism) and lifestyle (mahinga kai and recreation). In addition to removal of oxygen-demanding organic matter and suspended solids, pathogen removal is increasingly important. Viruses are particularly problematic because they can be infectious at very small doses, shellfish concentrate them as they filter-feed, and they can survive for long times in the environment.

Virus removal mechanisms in waste stabilisation ponds (WSPs) are likely to be complex, reflecting the diverse environment that exists within the pond. Sunlight, interacting with increases in pH and dissolved oxygen (DO) (which fluctuate throughout the day because of algal photosynthesis), has been identified previously as the key microbial removal mechanism. However, the potential importance of light-independent or "dark" virus removal mechanisms in WSP has largely been overlooked. Although these dark virus removal mechanisms may be expected to dominate at night, they probably occur all the time at depths in the ponds where solar ultraviolet (UV) light penetration is too low for virus inactivation, or when solar UV light is reduced e.g. in winter and when it is overcast.

In this study we monitored the removal of virus and *Escherichia coli* in wastewater mesocosms with part of the mesocosm exposed to sunlight and part in the dark. The main challenge was to ensure that the natural diurnal variation in DO and pH occurred in the dark chamber, at the same time ensuring that the virus and *E. coli* that were added to the light and dark chambers remained in the respective chambers.

2 METHOD

2.1 EXPERIMENTAL PROCEDURE

Wastewater was collected from a local WSP, using a 63 μM pore sieve to remove larger particulate matter. The wastewater was stored in the dark at 4°C and used within 8 h. Propagation of MS2 phage was undertaken according to the procedures in Debartolomeis and Cabelli (1991), as described previously (Wall *et al.*, 2008). MS2 phage was assayed by overlay pour plating (APHA, 1998) on host *E. coli* HS(pFamp)R (Debartolomeis and Cabelli, 1991). *E. coli* J6-2 cells were cultured at 37˚C in BHI (Brain Heart Infusion) broth (BBL, Sparks MD, USA), washed, resuspended in saline solution, and stored at 4˚C prior to injection. Before inoculation, tests with rhodamine dye indicated complete mixing occurred within 2 min (data not presented).

The mesocosms were filled with the wastewater, and the microorganisms inoculated at dusk, and a sample taken after 10 min. Further samples were taken during daylight at dawn (around 07:00), 09:00, noon, 15:00 and dusk (around 18:00) over three days and analysed for *E. coli*, and MS2. Background *E. coli* and F-RNA phage levels were determined in the WSP wastewater before inoculation. . Samples were analysed for *E. coli* by pour plating (APHA, 1998) onto Chromocult® Coliform Agar ES media (Merck, Darmstadt, Germany) and incubating at 44.5˚C (±0.5˚C) for 20 (±4) h. As a measure of overall bacterial activity, heterotrophic plate counts (HPC) were determined (APHA, 2005) on samples collected at dusk, 10 min after inoculation, then at noon on days 1, 2, 3 and dusk on day 3. Chlorophyll (APHA, 2005a), ammonia (APHA 2005b) and biological oxygen demand over five days (BOD_5) (EN 1998) were measured before inoculation and at the end of the experiments. The experiments took place in April (mid-autumn) and May (late autumn).

Radiation and ultraviolet (UV) light data were obtained from two local climate stations, 2.5 and 6 km from the site. The cumulative erythemal (skin burning) solar UV dose was determined from the Cliflo database (NIWA), as data for DNA damaging UV doses are not yet available (Richard McKenzie, pers. com, 2010). This gave an indication in the reduction of UV between the experiments, as the reduction in DNA damaging UV dose will be greater than the erythemal dose.

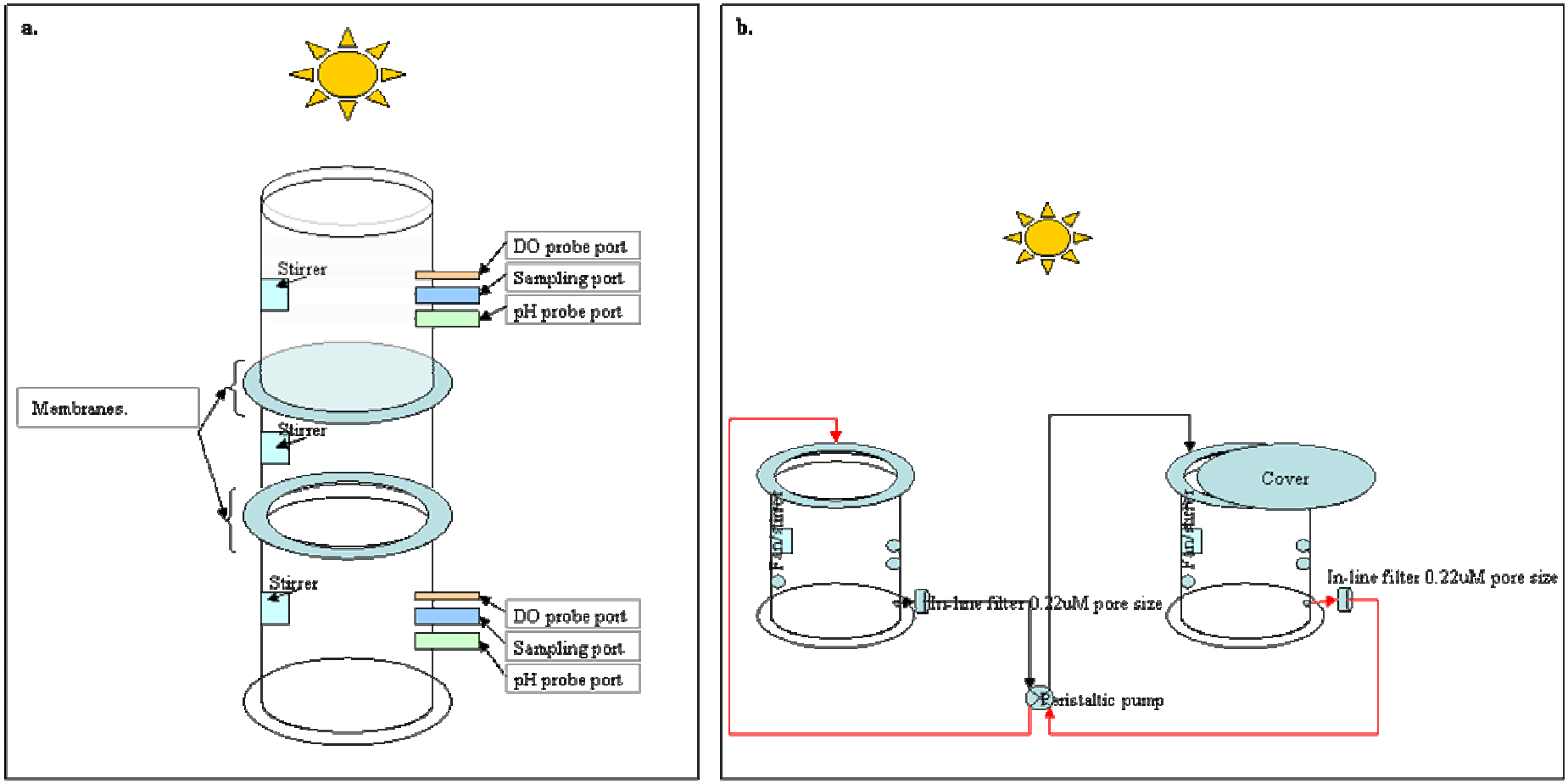
Variations in pH and DO in WSP were recorded at the local WSP just below the surface (light conditions) and above the sludge layer (dark conditions), to compare field data with those from the mesocosms.

2.2 MESOCOSM CONFIGURATIONS

Two mesocosm configurations were constructed and tested. The first configuration comprised three 300 mm diameter pipe sections stacked on top of each other separated by molecular weight cut off membranes (300 K Omega membrane, Pall Life Sciences, USA) at 300 mm and 700 mm above the base. A submersible propeller pump provided mixing in each chamber. The pH and dissolved oxygen (DO) probes (pH and LDO HQd probe series field kit, Hach, Germany) were fixed in the top (light) and bottom (dark) sections to measure the exchange between the sections (Figure 1).

The second configuration used two separate chambers (300 mm high x 300 mm diameter). The DO and pH were maintained at similar levels in the two chambers by circulating wastewater between the two mesocosms with membrane filters (45 mm diameter, 300 K Omega membrane, Pall Life Sciences, USA) inserted into the circulation lines to keep the inoculated microorganisms in the separate chambers (Figure 2). The dark chamber was covered with black polyethylene to exclude light. At night, the sunlight mesocosm was covered to reduce heat loss, and the circulation pump was turned off.

Figure 1: Mesocosm set up for a) Experiment 1 b) Experiment 2



3 RESULTS AND DISCUSSION

3.1 WASTEWATER CHARACTERISATION

Diurnal variation in DO and pH were measured in the local WSP over three days and showed a similar range to that measured in the mesocosm, even at depth (Dark) (Table 1). Variation in WSP wastewater also occurs from day to day, so the water quality parameters BOD_5, ammonia and chlorophyll a were measured between the experiments and at the end of the experiments. Ammonia is a potential virucidal agent, but at the concentrations measured in these experiments (Table 1) it is unlikely to have this effect. BOD_5 was similar in both experiments. While the concentrations of chlorophyll a were lower in April, than in May, the effects of photosynthetic activity (measured as variation in DO and pH) appeared greater in April. It is likely that this was because of the warmer temperatures and consequently greater levels of photosynthetic activity.

Table 1: Chemical concentrations at the beginning and end of the experiments

	Ammonia-N mg/L	**BOD_5mg/L**	**Cholorophyll a µg/L**
Experiment 1 Day 0	7	16	200
Experiment 1 Day 3	8.2	12	127
Experiment 2 Day 0	11	18	245
Experiment 2 Day 3 (Light)	9.1	15	277
Experiment 2 Day 3 (Dark)	9.2	17	242.8

The experiments were designed so that the conditions in the chamber without light ('dark') would follow diurnal variations in pH and DO (associated with algal photosynthesis) and changes in temperature. Only the direct effect of solar radiation was removed in the 'dark' chamber. While temperature was similar in both chambers in both configurations (Figure 2), the low level of diffusion across the membrane in configuration 1 gave steady pH (Table 2) and falling DO in the dark mesocosm (Table 2). The second configuration provided better mixing of DO and pH into the dark section, but the maximum DO concentration in the light chamber was still 3.7 mg/L higher than in the dark chamber (Table 2).

Figure 2 Temperatures in mesocosms during the first and second experiment

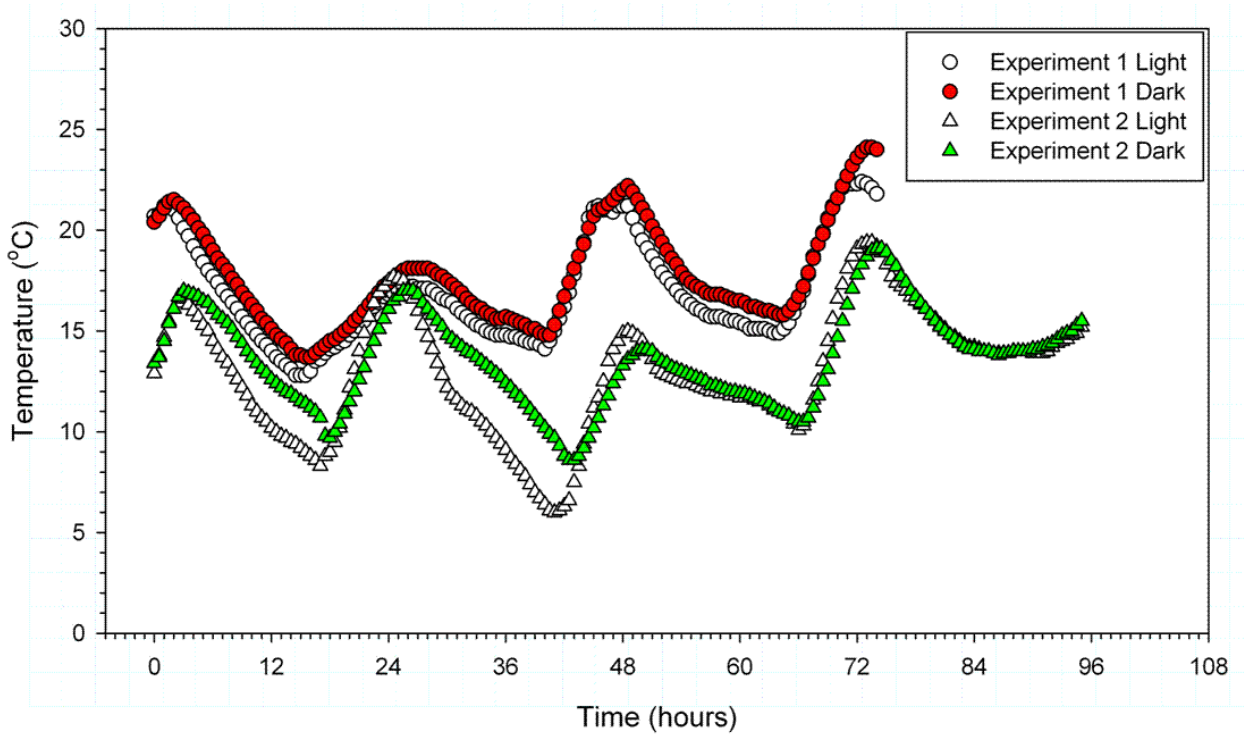


Table 1: Comparison of physiochemical parameters under light and dark conditions of experiment 1 and 2

		Temperature °C	**DO mg/L**	**pH**
Experiment 1	**Light**	12.8-22.4 (17)*	10.7-22 (16.2)	8.7-10.1 (9.3)
	Dark	13.7-24.1 (17.8)	0-13.3 (4.5)	7.6-8.7 (8)
Experiment 2	**Light**	6-19.4 (13)	9-16.3 (12.2)	8.4-8.9 (8.6)
	Dark	8.6-19.1 (13.6)	8.9-12.6 (10.6)	8.3-8.7 (8.5)
Local WSP[#]	**Light**	13.3-18.0 (15.4)	4.2-21.9 (12.7)	8.3-9.9 (9.3)
	Dark	13.3-17.2 (15.3)	4.8-22.0 (13.2)	8.2-9.7 (9.1)

- Figures in brackets denote mean values
- [#] Measured prior to experiments (March 2010).

3.2 REMOVAL OF MS2 PHAGE AND *E. COLI*

The greatest removal of *E. coli* occurred under sunlight conditions in Experiment 1, achieving a 4 log removal, compared with a 2 log removal under the other experimental conditions (Table 3). Sunlight exposure in Experiment 1 also removed the greatest concentration of MS2 phage, a 6 log removal compared with a 1 log removal recorded in Experiment 2. The ability to vary pH and DO in the "dark" experiments resulted in higher removals of *E. coli* compared with other published studies, where removals of *E. coli* were negligible under constant pH conditions (Davies-Colley *et al.*, 2003; Davies-Colley *et al.*, 2005; Davies-Colley *et al.*, 1997). The 1 log removal of MS2 phage in the dark mesocosm in Experiment 2 was consistent with previous work undertaken at pH 7.5 (Davies-Colley *et al.*, 1997), but was not consistent with our other experiments. A general reduction in bacterial activity over the length of both experiments, as indicated by HPC removal, indicated that conditions of Experiment 1/Light, Experiment 2/Light and Experiment 2/Dark gave greater removal of bacteria than conditions of Experiment 1/Dark.

Table 3 Log removal of E. coli and MS2 phage

	Experiment 1		Experiment 2	
	Light	Dark	Light	Dark
E. coli	4.3	1.8	1.9	2
MS2 phage	6	0	1	1
HPC	1.5	0.9	1.3	1.3

The difference in DO between the dark and light chambers does not appear to have affected removal rates (Table 3), with the same level of removal of *E. coli* (2 log) and MS2 (1 log) recorded in light and dark chambers in configuration 2.

Temperature has been proposed as strongly influential in microorganism removal(Marais, 1974) this is not consistent with the results reported here. The temperatures in the two chambers in Experiment 1 were similar, but the removal of *E. coli* and MS2 differed (Table 3).

UVA, UVB and sunlight at wavelengths >550 nm are implicated in the removal of indicator bacteria (Davies-Colley *et al.*, 1997) and F-RNA phage (Davies-Colley *et al.*, 2005). In field experiments, it is not possible to ensure that the solar radiation dose is constant over a period of several days. Insolation, a measure of the solar radiation energy on a surface area, can be determined and used to compare microbial removal under the inevitably different sunlight conditions within and between experiments. However solar radiation excludes UV radiation, which has been identified as an important component of sunlight for bacteria and virus removal (Davies-Colley *et al.*, 2005, (Davies-Colley, 2005).

The following graphs (Figures 3 and 4) show removal in both the sunlight-exposed tanks plotted against the insolation received in the sunlight experiments (although, obviously, the dark tanks did not receive any insolation). While there were fewer 'bright sunshine hours' recorded from 13–15 April (16.3 hours) compared with the period from 4–6 May (26.4 hours) (Cliflo database), UV was at a higher level in April (10.2 KJ/m^2) as estimated from the cumulative erthymal dose (skin-damaging UV radiation), while in May UV was only at a level of 2.0 KJ/m^2 (Cliflo database). This is consistent with the greater removal of *E. coli* at 35 MJ/m^2, during April, compared with removal levels of *E. coli* and MS2 phage in May (Figure 3).

Figure 3 Effect of insolation on E. coli removal

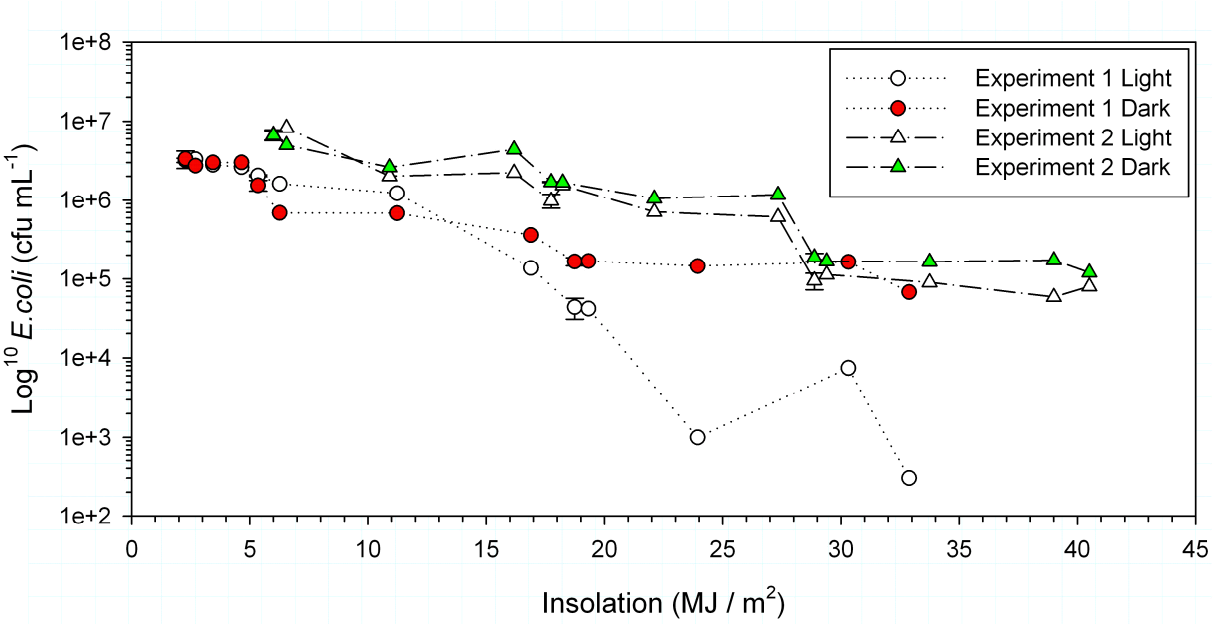
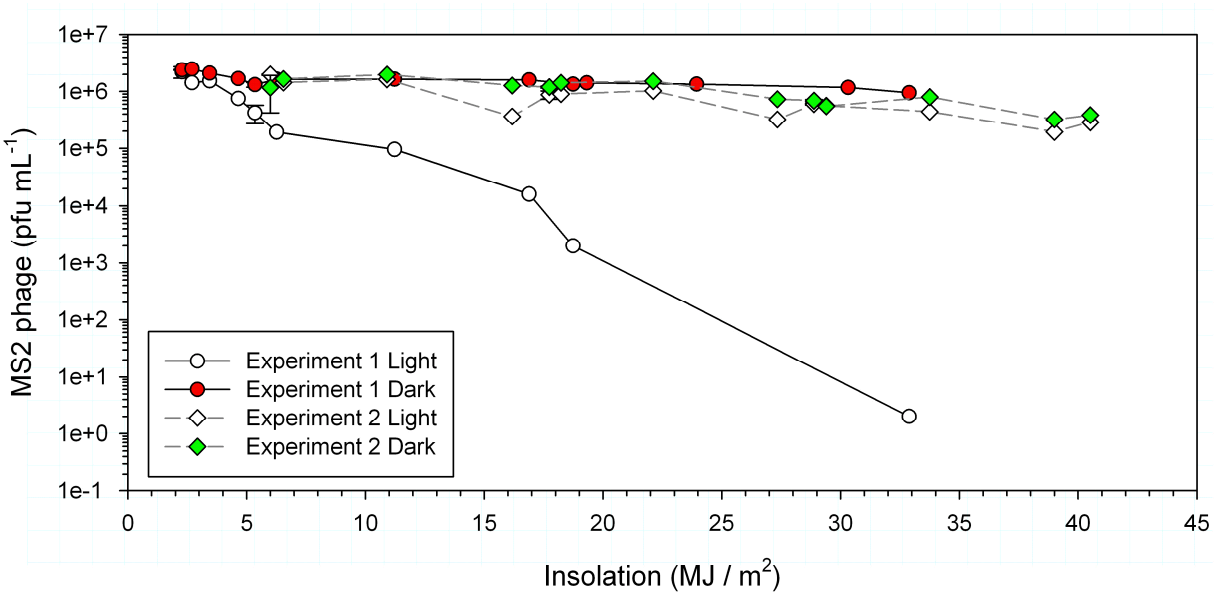


Figure 4 Effect of insolation on MS2 phage removal



As well as higher solar radiation, the critical conditions of pH of >10 and DO concentrations (>20mg/L) were reached (Curtis *et al.*, 1992) in Light/Experiment 1. This may account for the much greater removal of *E. coli* and MS2 phage in that experiment. At these elevated conditions of pH and DO, sunlight inactivation increases, probably through a photoxidative mechanism, further increasing the removal of F-RNA and *E. coli* (Davies-Colley *et al.*, 1999). While the mixing between chambers was better in the second configuration, the contents did not reach the critical pH of 9, varying only between 8.4–8.9. However, in the first experiment, pH in the light experiment reached 10.1. With conditions of lower DO and pH in Experiment 2, the lack of photo-oxidation may partially account for the lower removal of MS2 in Light/Experiment 2. F-RNA phage removal may be affected more by the longer wavelengths in sunshine (Davies-Colley *et al.*, 2005; Sinton *et al.*, 1999), but it would appear that conditions for photoxidation (high pH and DO concentrations) may also be important as lower removal occurred in the Light/Experiment 2 compared with Light/Experiment 1. DO may also be

important as MS2 removal was negligible in Dark/Experiment 1, but a 1 log removal of MS2 was achieved in Experiment 2 (Dark and Light) where DO did not fall below 8.9. Where critical DO and pH values were not reached, there appears to be another mechanism of virus removal, which needs further investigation, as it could be significant over the time period that wastewater spends in the WSP, which may be 20-33 days(Clark and Cameron, 2005).

4 CONCLUSIONS

We were able to design a configuration of mesocosms for WSP effluent experiments, which allowed the products of photosynthesis (primarily DO and pH) to be transferred to a dark chamber. Under these conditions, the removal of MS2 and *E. coli* was low but still significant (1 and 2 log removal, respectively). This removal rate appeared to be similar to that of HPC and indicates that mechanisms other than UV and solar radiation are active in WSPs. Monitoring these conditions in the field showed that high levels of DO and pH can occur naturally in a WSP. In future experiments, using the second configuration in summer conditions, it will be possible to determine whether the higher levels of DO and pH, which generate photoxidative by-products, are also a mechanism of removal in the dark sections of WSPs.

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REFERENCES

American Public Health Association (APHA) (2005a) 'Heterotrophic Plate Count (HPC) method'. In *Standard methods for the examination of water and wastewater* (22nd ed.). Washington D.C. American Public Health Association.

American Public Health Association (APHA) (2005b) 'Ammonia nitrogen 4500-NH3 F (modified)'. In *Standard methods for the examination of water and wastewater* (22nd ed.). Washington D.C. American Public Health Association.

American Public Health Association (APHA) (2005c). 'Chlorophyll a Fluorimetry 10200'. In *Standard methods for the examination of water and wastewater* (22nd ed.). Washington D.C. American Public Health Association.

BE EN (1998) 'Biological Oxygen Demand 18992-2': 1998/BS6068-2.64

Clark, S., Cameron, S., (2005). *Oxidation pond guidelines* Ministry for the Environment, Wellington.

Curtis, T.P., Mara, D.D., Silva, S.A., (1992). 'The effect of sunlight on faecal coliforms in ponds: implications for research and design'. *Water Science and Technology*, 26.

Davies-Colley, R.J., (2005). 'Pond Disinfection'. In A. Shilton (Ed.) *Pond Treatment Technology*. IWA, London. p. 100-131.

Davies-Colley, R.J., Craggs, R.J., Nagels, J.W., (2003). 'Disinfection in a pilot-scale "advanced" pond system (APS) for domestic sewage treatment in New Zealand'. *Water Science and Technology*, 48, 81-87.

Davies-Colley, R.J., Craggs, R.J., Park, J., Sukias, J.P., Nagels, J.W., Stott, R., (2005). 'Virus removal in a pilot -scale 'advanced' pond system as indicated by somatic and F-RNA bacteriophages'. *Water Science and Technology*, 51, 107-110.

Davies-Colley, R.J., Donnison, A.M., Speed, D.J., (1997). 'Sunlight wavelengths inactivating faecal indicator microorganisms in waste stabilisation ponds'. *Water Science and Technology*, 35, 219-225.

Davies-Colley, R.J., Donnison, A.M., Speed, D.J., Ross, C.M., Nagels, J.W., (1999). 'Inactivation of faecal indicator micro-organisms in waste stabilization ponds: interactions of environmental factors with sunlight'. *Water Research*, 33, 1220-1230.

Marais, G.v.R., (1974). 'Faecal bacterial kinetics in stabilization ponds'. *Journal of Environmental Engineering*, 100, 119-139.

Sinton, L.W., Finlay, R.K., Lynch, P.A., (1999). 'Sunlight inactivation of fecal bacteriophages and bacteria in sewage-polluted seawater'. *Applied and Environmental Microbiology*, 65, 3605-3613.

Wall, K., Pang, L., Sinton, L., Close, M., (2008). 'Transport and Attenuation of Microbial Tracers and Effluent Microorganisms in Saturated Pumice Sand Aquifer Material'. *Water, Air, & Soil Pollution*, 188, 213-224.