Australian Water Recycling
Centre of Excellence

Project Report

Pasteurisation for the Production of Class A Water

A report of a study funded by the
Australian Water Recycling Centre of Excellence

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About the Australian Water Recycling Centre of Excellence

The mission of the Australian Water Recycling Centre of Excellence is to enhance management and use of water recycling through industry partnerships, build capacity and capability within the recycled water industry, and promote water recycling as a socially, environmentally and economically sustainable option for future water security.

The Australian Government has provided $20 million to the Centre through its National Urban Water and Desalination Plan to support applied research and development projects which meet water recycling challenges for Australia's irrigation, urban development, food processing, heavy industry and water utility sectors. This funding has levered an additional $40 million investment from more than 80 private and public organisations, in Australia and overseas.

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EXECUTIVE SUMMARY

Victorian Department of Health (2013) Guidelines for validating treatment processes for pathogen reduction – Supporting Class A recycled water schemes in Victoria require revalidation of the recycling systems producing Class A water in Victoria, including Class A water production at Melbourne Water's Western Treatment Plant (WTP). This has proved challenging due to lack of filtration. Suspended particles present in the wastewater can protect pathogens from UV and chlorination disinfection processes. This project investigated the feasibility of using pasteurisation rather than UV and free chlorination disinfection processes for the production of Class A water from unfiltered secondary effluent. Pasteurisation has, however, not been used for wastewater disinfection in Australia and there is no Australian data available to inform regulatory authorities as to the reliability and efficacy of pasteurisation for the disinfection of unfiltered Australian municipal wastewater. This project provided validation data to inform the Department of Health's decision making processes regarding the use of currently available wastewater pasteurisation technology for the production of Class A water. The knowledge and expertise gained during this project was used to develop a protocol for the validation of pasteurisation for wastewater recycling for use by future proponents of pasteurisation technology.

Literature Review:

Based on reviewed scientific literature, the suggested surrogates (i.e., microorganism that are enumerated to estimate target pathogen reduction) and target pathogens for inclusion in this project were *E. coli*, faecal streptococci (*Enterococcus*), FRNA bacteriophage, *Cryptosporidium*, *Giardia*, adenovirus, coxsackievirus and *Ascaris*. It was recommended that the laboratory-scale testing compare these key pathogens / surrogates in a secondary treated wastewater matrix at temperatures and times relevant to the full-scale system. The Pasteurization Technology Group (PTG) pasteurisation pilot plant that was used in this project operates as a High Temperature Short Time (HTST) system, with contact times in the contact chamber of less than one minute at flows greater than 500 L/minute. This, and past performance in US trials of demonstration units, was used to inform the design of the laboratory-scale experiments. The laboratory testing experiments were designed to verify that the matrix does not cause any unexpected protective effect towards the pathogens of interest, and also to verify the selection of candidate surrogates (*E. coli*, faecal streptococci (*Enterococcus*), FRNA).

A major research gap identified in the literature review was the available data for *Cryptosporidium* and *Giardia*. The available literature results suggest that *Cryptosporidium* is highly temperature sensitive and *Giardia* has similar or higher temperature sensitivity compared with enteric bacteria. Similarly, there are limited data for helminths, although based on inactivation in sludge as a conservative measure, the heat sensitivity of *Ascaris* is similar to that of environmental *E. coli*. Human enteric viruses, including hepatitis A and the enteroviruses (poliovirus, coxsackievirus etc), appear to have similar temperature sensitivity to *E. coli* and *Pseudomonas*.

The literature data suggested that native *E. coli* is a reasonable surrogate for organisms of interest such as enterococci, *E.coli*, Coxsackievirus, Adenovirus, *Cryptosporidium* and *Ascaris*, with FRNA (MS2, an FRNA bacteriophage that infects *E.coli*) phage and faecal streptococci (*Enterococcus)* conservative surrogates of pathogen inactivation (for all pathogens of concern). Somatic coliphage appear to be relatively heat resistant and are likely to be too conservative as a surrogate, but could possibly be used as a process indicator (in the absence of challenge testing using spiked microorganisms).

Laboratory trials:

The temperature inactivation experiments were consistent with literature values and in some instances suggested higher sensitivity to temperature in the case of human enteric viruses. For the organisms tested, FRNA were the most heat resistant, followed by enterococci and *E. coli. Ascaris* and adenovirus 2 showed some survival at 55°C, but coxsackievirus B5 and *Cryptosporidium* were highly temperature sensitive, being rapidly inactivated even after brief time exposures to 55°C. The results are summarised in Table A.

The effect of different water quality was evaluated using MS2 phage. There was no evidence for any difference in temperature inactivation for phage spiked into Pond 2 (turbidity 8.5 NTU, TOC 21.2 mg/L) or Pond 10 water (turbidity 1.8 NTU, TOC 10.7 mg/L).

Experiments were conducted to compare the heat inactivation of the laboratory strains with wild isolates for *E. coli* and FRNA. The wastewater isolates of *E. coli* showed similar sensitivity to temperature compared with the laboratory strain. The native FRNA appeared to be slightly less heat sensitive compared with MS2, particularly at 75°C for 30 seconds exposure, although both the native FRNA and MS2 were completely inactivated after 60 seconds at 75°C.

Table A: Log reduction values (LRV*) achieved at 55°C, 65°C and 75°C, at various contact times.

Organism	Temperature (°C)								
	55			65			75		
	Contact time (seconds)			Contact time (seconds)			Contact time (seconds)		
	5	30	60	5	30	60	5	30	60
MS2	0		0.1			0.8	1	6	**>7****
Enterococci					0.6	2	2	**>6**	**>6**
E. coli				1.0	2	**>6**	**>6**	**>6**	**>6**
Coxsackievirus		5	6	**>7**	**>7**	**>7**	**>7**	**>7**	**>7**
Ascaris	~0	~0	0.9	**>2**	**>2**	**>2**	**>2**	**>2**	**>2**
Adenovirus	~0	2	**>8**	**>8**	**>8**	**>8**	**>8**	**>8**	**8**
Cryptosporidium		**>3**			**>3**		**>3**		

* LRV = $\log_{10}$ (organism number before treatment) - $\log_{10}$ (organism number after treatment)
** when the organism number after treatment is zero, the detection limit is substituted for the organism number and the LRV is expressed as “greater than” the calculated value (>).

Due to its higher heat resistance, MS2 was recommended as a surrogate in the pilot trial challenge tests.

Pilot plant trials:

The Pasteurization Technology Group (PTG) pasteurisation pilot plant tested in this study is composed of heat exchangers, stack heater (also termed the waste heat recovery unit (WHRU)) and pipeline contactors (also termed contact chamber). The pasteurisation performance of the contact chamber and of the entire plant was tested in this project.

Contact chamber tests

Pilot testing of the PTG pasteurisation demonstration unit contact chamber using Eastern Treatment Plant (ETP) water showed that at temperatures between 75°C and 69°C with a contact chamber contact time of 30 seconds (flow at ~1100 L/min) can achieve log reductions values (LRVs) between 5.0 ± 0.5 and 0.9 ± 0.1 respectively for the chosen heat resistant surrogate (MS2). The trial also showed that doubling the contact time by halving the flow rate can increase the contact chamber LRV at 72°C from 2.4 ± 0.1 to 4.0 ± 0.3.

The MS2 LRV achieved during the pilot plant contact chamber challenge tests at 75°C and a contact time of 30 seconds (5.0 ± 0.5,) was found to agree with the MS2 LRV achieved in the laboratory trials for pond 2 and pond 10 water at this contact time and temperature combination (5.6 ± 0.1). The pilot trial LRVs and the lab trials LRVs at this temperature and contact time combination were not found to be statistically significantly different (t-test P = 0.2).

Entire plant tests

In addition to testing of the system contact chamber, investigation of inactivation of bacteria was also conducted in this project by testing of native *E.coli* levels across the entire plant. These tests were also conducted with MS2 coliphage injection to confirm the higher heat resistance of MS2 than native *E.coli* that was determined in laboratory trials.

Testing of the entire pasteurisation process, including the heat exchangers, with sampling of influent and effluent to/from the plant showed that an *E.coli* LRV of 2.9 ± 0.3 can be achieved at 68°C at a contact time of 254 seconds (at ~1,100 L/min). MS2 inactivation at this temperature was found to be 0.8 ± 0.2. The higher heat sensitivity of native *E.coli* than MS2 at the pilot plant scale confirmed the laboratory scale results (native *E.coli* LRV = 1.2, MS2 LRV = ~0.1, at 65 °C and 30 seconds contact time). The trend in *E.coli* LRVs at varying temperatures indicated that a minimum temperature of approximately 72°C is required for complete inactivation of the native *E.coli* in this water (present at ~ 5 LV).

Comparison of plant performance with previous studies

The LRV results achieved during the current work (0.9, 2.4 and 5.0 at 69°C, 72°C and 75°C respectively) are considerably different to those achieved for the contact chamber in past pasteurisation trials at Ventura, California (Carollo, 2012) where LRVs of 5.5, 7.0 and 7.2 were reported at 72°C, 73°C and 79°C respectively, using the same strain of MS2 (ATCC 15597-B1) and

similar contact times. There was, however, considerable agreement between the current results and those in other US, California, studies at Graton and Santa Rosa (LRVs of 4.5 to 5.3 at approximately 75°C, at contact times between 15 and 40 seconds). The observed difference between the Ventura trial data and the other trial data may possibly be attributable to the use of high seed doses in the Ventura trial, which can cause artificially high LRVs (USEPA, 2005). Another possible cause of the differences observed is a difference in water quality. The results of a 2007 wastewater pasteurisation trials suggests that water quality plays a role in pasteurisation disinfection kinetics, particularly with regard to coliform disinfection (Carollo, 2012).

Effect of feed water quality

The ETP water on the challenge test days had lower COD, TOC, EC and pH values (44 to 70 mg/L, 13 to 16 mg/L, 730 to 890 µS/cm, pH 6.6 to 6.9, respectively) than the WTP water used in the laboratory trials (84 to 223 mg/L, 9 to 29 mg/L, 1700 to 2150 µS/cm, pH 7.3 to 8.2, respectively), The WTP VSS values (2 to 11 mg/L) were within the range of values found in ETP water samples (2 to 33 mg/L). The turbidity and SS of ETP water (2.3 to 9 NTU, 13 to 24 mg/L, respectively) was generally higher than that of WTP water (1.8 to 2.5 NTU, 4 to 12 mg/L). These differences between ETP and WTP water, however, are not expected to be sufficient to influence the heat sensitivity of pathogens. The protective effect of salt, for example, is very small at the salt content of the ETP and WTP waters (less than 1 g/L or 0.1 % w/v). Similarly, the pH of ETP water is not sufficiently low to induce the protective effect associated with acid stress.

It was found that there was generally poor correlation between the tested ETP water quality parameters on the challenge test days and the LRV achieved on these days. The order of correlation coefficients (R^2) from highest to lowest was: SS (0.60) > Ca (0.42), > Turbidity (0.38) > TOC (0.33) > pH (0.31) > VSS (0.27) > COD (0.20) > UVT (0.16) > Alkalinity (0.09) > EC ($8x10^{-5}$). Only two of the parameters showed a trendline with a negative slope that would be indicative of a decrease in LRV with increase in the parameter (Alkalinity and COD). More data is required to establish whether or not there is a correlation between LRV and the tested water quality parameters, but this lack of clear correlation is consistent with these parameters having no influence over the range of values tested in these trials.

Plant process control

This project experienced major delays in the installation of the pilot plant, leaving only 8 weeks for the challenge testing and continuous operation. This period was further reduced by delays in the installation of a safety feedwater gate valve without which continuous operation was not deemed to be safe (3 weeks), a plant shutdown due to the malfunction of an important plant component (burner fan motor, 2 weeks), and a process logic control (PLC) related issue that shut down the plant during continuous operation. This prevented the planned collection of process control related data during long periods of continuous operation.

The longest continuous period of operation was ~36 hours. The observed fluctuations of temperature and flow rate over a 24-hour period of continuous operation at 75°C and 845 L/min were the same as over the much shorter challenge test sample collection periods at temperatures between 66°C and 75°C, and flows between 570 and 1,100 L/min – 0.2% for temperature and 2% for flow (and contact time).

Gas usage and running costs

Due to the brevity of the trial period, it was not possible to determine the gas usage and cleaning requirements for the process.

Validation LRV

The PTG pasteurisation pilot plant contact chamber was validated at 1,100 L/min, 30 seconds contact time The achieved average LRVs and the bottom 5th percentile LRV required by the Department of Health (Guidelines for validating treatment processes for pathogen reduction – Supporting Class A recycled water schemes in Victoria, 2013) for the heat resistant surrogate chosen, MS2, using a total of 24 sample data points at each temperature are shown in Table B.

Table B: Average and Bottom 5th percentile MS2 LRVs for 1,100 L/min, 30 seconds contact time.

Temperature (°C)	Average LRV	SD	Bottom 5th Percentile LRV
75	5.0	0.5	4.0
72	2.4	0.1	2.1
69	0.9	0.1	0.7

Validation Protocol:

The knowledge and expertise gained during this project was used to draft a protocol for the validation of pasteurisation for production of Class A water. This is appended at the end of this report (Appendix A).

Recommendations:

This study has shown that pasteurisation can be used to reduce the levels of pathogens in wastewater to achieve Class A water quality standards. Challenge tests conducted over short periods of operation showed that operation of the pilot plant at 1,100 L/min (contact time of 30 seconds) and a temperature of 75°C can achieve an average log reduction value of 5.0 of MS2. The bottom 5th percentile LRV was found to be 4.0 under these conditions.

Major project delays reduced the pilot plant trial period such that pasteurisation performance over extended periods of continuous operation could not be evaluated. This prevented evaluation of the

reliability of the pilot plant and the cost of operation (gas use, power use and cleaning requirements). Favourable economics for pasteurisation have been demonstrated in Ventura, California, where a 400 gallon per minute (gpm) (1,500 L/min)) demonstration system has been constructed and tested. These favourable economics need to be confirmed with further testing in Australia. This testing should include consideration of the fouling potential of the feedwater and the influence of fouling on the effectiveness of the process and its energy efficiency.

Further research is also required to confirm the poor correlation between the tested water quality parameters and the effectiveness of the pasteurisation process for municipal wastewater over long periods to confirm that there are indeed no matrix effects that can render the pasteurisation process less effective for municipal wastewater.

Although the current study deals with the pasteurisation treatment of municipal wastewater, this technology can also be applied to other wastewaters, such as stormwater or wastewater from food processing. This would, however, require careful consideration of the microbial and chemical composition of each wastewater and the potential pathogen protective effects that may arise as a result of the chemical composition. Furthermore, the economic feasibility for the treatment of different wastewater may vary considerably. The process is expected to be most economical when it utilises a source of waste heat such as the waste heat from on-site electricity generation.

1. INTRODUCTION

1.1. Background and Relevance

Pasteurisation is a well-established process having been invented in 1862 by Louis Pasteur and Claude Bernard. It is a process generally applied to food, particularly liquids such as milk. This is done by forcing milk with a starting temperature of 4°C between metal plates or through pipes heated on the outside by hot water, with the result that the milk is heated to 71.7°C (161°F) for 15–20 seconds. Rapid cooling then follows and the shelf life of refrigerated milk is extended by two to three weeks. A similar process is proposed for disinfection of recycled water and penetration of heat into fine particles is likely to be very rapid making the presence of particles less likely to significantly interfere with pathogen inactivation than is typical of traditional disinfection methods such as UV or chlorination. Cows' milk can be treated by pasteurisation despite its complex composition: 3.9 g/100mL fat which can influence resistance to heat uptake, 2.6 g/100mL casein, 0.6 g/100mL whey protein, 4.6 g/100mL lactose, 0.7 g/100mL ash and 12.7 g/100mL total solids (Juffs and Deeth, 2007). As a result, pasteurisation of a less complex fluid, such as secondary treated effluent containing particles, is likely to be extremely effective.

In order for pasteurisation to be readily accepted by the water industry, it must gain approval from the Australian state health regulators. While pasteurisation has been proven to remove viruses to California's "Title 22" standards for filtered secondary effluent, it has not been proven for unfiltered water or to remove protozoa and helminths. In addition, the virus surrogate used to prove removal in the US will need approval by the Australian health departments. By proving this process at laboratory and pilot scale, a validation process will be developed which is aimed to satisfy the Australian health departments, the Australian Guidelines for Water Recycling (AGWR) (2006) and the Victorian Department of Health Draft Guidelines for Validating Treatment Processes for Pathogen Reduction, Supporting Class A Water Recycling Schemes in Victoria (2013). Additionally, the proposal sought to identify the advantages and disadvantages of this technology over competing disinfection processes. This was not achieved due to time limitations. The process is commercially available and has been proven to be effective at full-scale (2 ML/d demonstration site in Ventura, California), so it can be readily implemented once barriers to approval are removed and its performance characteristics are verified and known. The work will be used to assess the suitability of the process for future increases in Class A recycled water production at Melbourne Water's Western Treatment Plant and for decentralised recycled water schemes more typical of integrated water management.

1.2. Project Objectives

This project aims to reduce treatment costs and energy requirements and to simplify control of disinfection processes by proving a new treatment process, pasteurisation, under rigorous conditions required by the Australian Departments of Health. The key aims of this project are:

- To demonstrate to the satisfaction of the Australian Departments of Health that pasteurisation can be used to produce Class A water from secondary treated wastewater containing small particles. Disinfection targets are virus, protozoa, helminths and bacteria.
- To develop a validation protocol that can be recommended by the DoH for future users of pasteurisation and also be included in “NatVal” output.
- To demonstrate and evaluate the usefulness and feasibility of pasteurisation for existing wastewater treatment plants, identifying the requirements for variable flows, turbidity, and temperature for use in the production of Class A water under varying conditions of flow and temperature.

2. LITERATURE REVIEW

2.1. Scope

The primary purpose of this review of the scientific literature on pasteurisation is to identify potential indicator organisms for the pasteurisation of secondary effluent. This review builds upon a literature review conducted by Pasteurization Technology Group (PTG) and Carollo Engineers (PTG Title 22 Report, 2006) prior to trials of this disinfection technology in Ventura, California, and also serves to inform current laboratory work which aims to experimentally confirm the literature findings regarding the suitability of the selected organisms to be used as indicators for enteric viruses, protozoa (*Cryptosporidium*), helminths (*Ascaris*) and bacteria in the pilot trials.

2.2. Introduction

The literature review was conducted by Paul Monis of AWQC SA Water and utilised PubMed, Current Contents and Google Scholar databases. Keywords used in searches included pasteuris(z)ation, temperature, thermal, inactivation, wastewater, water and specific organisms (eg *Cryptosporidium*, helminths, viruses, surrogates).

In brief, most of the literature was found to be focused on pasteurisation of food (primarily milk, juices, shellfish). There has been no reported application of pasteurisation for domestic secondary treated wastewater at any scale and few comparisons of surrogates and pathogen inactivation in a pasteurisation system. There has been application of pasteurisation to sludge, but no systematic comparisons of pathogens with indicators. For general information – some studies have calculated the thermal death time (D), which is the time required for 1 $\log_{10}$ surrogate/pathogen inactivation at a given temperature, which is also referred to as T_{90} in some papers or TFL (time for 1 log inactivation) in the Bertrand et al. 2012 paper. The PTG pasteurisation plant/process is essentially a high temperature (>60°C) short time (HTST) pasteurisation process, with a contact time of ~5 minutes, including ramp up and ramp down. The literature reviewed has focused on processes with temperatures >50°C and, due to the lower number of studies that deal with HTST pasteurisation, considers longer contact times to assess the *relative* heat sensitivity of the organisms of interest.

2.3. Pasteurisation inactivation of bacteria

Only one study was found that reported on pasteurisation of sludge. This was at laboratory scale and resulted in 6.2 $\log_{10}$ inactivation for faecal coliforms and 2.7 $\log_{10}$ inactivation for enterococci after 60 minutes at 80°C (Bonjoch and Blanch, 2009). The nature of the WWTP producing the sludge was not specified. This paper was not clear on how the pasteurisation was performed or what controls were used to account for thermal ramp times (the time it takes for the sample to change from the initial temperature to the target temperature). Another paper assessed HTST pasteurisation of *E. coli* in

raspberry puree, showing 3 – 4 $\log_{10}$ inactivation after 15 – 30 sec at 65 or 75°C (Baert et al., 2008).

More work has been conducted examining pasteurisation in milk (Dumalisile et al., 2005). The report of Dumalisile assessed the level of inactivation that occurred during the ramp time. The bacteria *E coli*, *Acinetobacter*, *Chryseobacter* and *Pseudomonas* and the yeast *Candida* were all shown to be heat sensitive, with 2 – 3 $\log_{10}$ inactivation achieved during the 3-minute ramp time from ambient to 63°C, with 5 minutes at this temp resulting in >4 $\log_{10}$ inactivation (Dumalisile et al., 2005). *Bacillus cereus* (presumably spores) were resistant at this temperature with approx. 0.5 $\log_{10}$ inactivation measured after 5 minutes and 1 $\log_{10}$ inactivation measured after 40 minutes at 63°C. From the methods described (Dumalisile et al., 2005) the authors used nutrient broth for bacterial culture, so did not specifically enrich for *Bacillus* spores or encourage sporulation, but the low inactivation suggests that the *Bacillus* culture was predominantly spores. With a higher inoculum, most organisms showed higher inactivation, with 3-4 $\log_{10}$ inactivation during the 3-minute ramp time and an additional 1-2 $\log_{10}$ inactivation after 5 minutes at 63°C, with a total of 6 $\log_{10}$ inactivation after 15 minutes, including ramp time (Dumalisile et al., 2005). An exception was *E. coli*, which appeared more resistant when starting with a higher inoculum, with approximately 0.5 $\log_{10}$ inactivation following the 3 minutes ramp, 1 $\log_{10}$ inactivation after 5 minutes exposure, 3 $\log_{10}$ inactivation after 15 minutes, and 6 $\log_{10}$ inactivation after 25 minutes at 63°C (Dumalisile et al., 2005).

Work with total coliforms at the City of Ventura's Water Reclamation Plant using sand filtered secondary effluent (PTG Title 22 Validation Report, 2006) showed that 68°C resulted in greater than 4-5 $\log_{10}$ inactivation of total and faecal coliform, with all samples showing "not detected".

Additional experiments were described for *Bacillus coagulans*, a variety of lactic acid bacteria, *Staphylococcus aureus* and *Listeria monocytogenes*. These all appeared to be sensitive to 63°C (approx 3 $\log_{10}$ inactivation for a 5-minute exposure), suggesting that the *B. coagulans* did not form spores under the conditions used to prepare that culture. The resistance of *Bacillus* spores has been separately confirmed, with significant inactivation not being observed until temperatures of 100°C or more were used (e.g., 7 $\log_{10}$ inactivation for 60 minutes at 100°C, 7 $\log_{10}$ inactivation for 2 minutes at 130°C) (Novak et al., 2005). One important result from this study was that the suspension medium did not impact inactivation, with similar results for distilled water, skim milk and brain heart infusion broth (Novak et al., 2005).

Thermal inactivation of bacteria in raw sewage has also been described, although it is not clear if the impact of ramp times were considered (Moce-Llivina et al., 2003). The results were consistent with those of Dumalisile et al. (2005). Inactivation for *E. coli* and faecal coliforms was 6 $\log_{10}$ at 60°C for 30 minutes (Moce-Llivina et al., 2003). Under the same conditions, inactivation of faecal streptococci was 3.4 $\log_{10}$, which is a greater inactivation than that reported in sludge for enterococci. This study included anaerobic spores (sulphate reducing clostridia), which were resistant to heat treatment (0.1 $\log_{10}$ inactivation after 30 minutes at 60°C).

Another study assessed laboratory based pasteurisation of biowaste from a biogas plant (Sahlstrom

et al., 2008). The waste was a combination of household waste, food industry and abattoir waste. The ramp time was 14-20 minutes, inactivation during this period was not measured. The data in this study are poorly presented, $\log_{10}$ inactivations are not provided, ranges of bacterial counts are presented for pre- and post-heat treatment, rather than averages. At 70°C for 30 minutes there was >4-5 $\log_{10}$ inactivation for enterococci, coliforms and *E. coli*. Enterococci were more resistant at lower temperatures, with 1–5 $\log_{10}$ inactivation at 55°C after 60 minutes, compared with >4-5 $\log_{10}$ inactivation for the coliforms. This study also confirmed the heat resistance of *C. perfringens* (Sahlstrom et al., 2008). Pathogens (*Salmonella typhimurium*, *Campylobacter jejuni*, *L. monocytogenes*, *E. coli* O157) were spiked into the biowaste. Inactivation data were not presented, but the text suggested inactivation similar to that of *E. coli* / coliforms.

A comparison of environmental and laboratory strains of *E. coli* found that environmental strains were more resistant (eg D value of 4.4 for the laboratory strain versus 7.1 for environmental) to a temperature of 55°C (Lang and Smith, 2008). The environmental *E. coli* had similar decimal reduction times compared with temperature resistant strains of *Salmonella*. At higher temperatures (HTST conditions), high inactivation was achieved, with >8 $\log_{10}$ reduction after 0.17 minutes at 70°C. This was much higher than that reported by Baert et al. (2008) in raspberry puree. In the Lang & Smith study, the medium affected inactivation, with tryptone soy broth providing a protective effect compared with sludge supernatant (Lang and Smith, 2008). This is different to the results presented by Sahlstrom et al. (2008), where there was no difference in inactivation in distilled water, skim milk or a brain heart infusion broth. Possibly the tryptone soy broth was more complex than the media in the Sahlstrom study, affording some protection from the effects of heat.

A summary of the achieved total $\log_{10}$ inactivation from literature studies that deal with pasteurisation for inactivation of bacteria is shown in Table 2.1. From these results, *E. coli* is a good candidate for further testing as an indicator for enteric bacterial pathogens, as it is more resistant to heat inactivation than four of the listed organisms (see highlighted rows in Table 2.1). Only one organism can be seen to be more resistant than *E. coli* – *Enterococcus* - and thus would be a good candidate for further testing as a conservative indicator for enteric bacterial pathogens. The available data also shows that HTST conditions are effective at inactivating *E. coli*. Aerobic spores are considered to be too conservative as an indicator for bacteria, viruses or protozoa.

Table 2.1: Summary of pasteurisation log_{10} inactivation values for bacteria, ("ns" stands for not specified).

Organism	Total Log_{10} inactivation	Temp (C)	Time (min)	Ramp (min)	Ramp Log_{10} inactivation	T90 (min)	Method	Matrix	Inoculum (log_{10})	Reference
Acinetobacter baumannii	>6	63	15	3	2.97		water bath	milk	6	Dumalisile
Acinetobacter baumannii	5.11	63	10	3	2.97		water bath	milk	6	Dumalisile
Acinetobacter baumannii	4.35	63	5	3	2.97		water bath	milk	6	Dumalisile
Acinetobacter baumannii	>4	63	5	3	3.13		water bath	milk	4	Dumalisile
Bacillus cereus	7.53	150	0.5	ns	ns		ns	dH2O	ns	Novak
Bacillus cereus	7.53	150	0.5	ns	ns		ns	Brain heart infusion	ns	Novak
Bacillus cereus	7.37	130	2	ns	ns		ns	skim milk	ns	Novak
Bacillus cereus	7.62	100	60	ns	ns		ns	skim milk	ns	Novak
Bacillus cereus	0.21	78	60	ns	ns		ns	skim milk	ns	Novak
Bacillus cereus	0.39	72	90	ns	ns		ns	skim milk	ns	Novak
Bacillus cereus	0.28	63	40	3	0.03		water bath	milk	6	Dumalisile
Bacillus cereus	0.86	63	40	3	0.46		water bath	milk	4	Dumalisile
Bacillus coagulans	>6	63	15	3	3.82		water bath	milk	6	Dumalisile
Bacillus coagulans	5.31	63	10	3	3.82		water bath	milk	6	Dumalisile
Bacillus coagulans	4.26	63	5	3	3.82		water bath	milk	6	Dumalisile
Bacillus coagulans	>4	63	10	3	2.15		water bath	milk	4	Dumalisile
Bacillus coagulans	2.84	63	5	3	2.15		water bath	milk	4	Dumalisile
Campylobacter jejuni	>5	70	30	ns	ns		water bath	biowaste	5	Sahlstrom
Campylobacter jejuni	>5	55	30	ns	ns		water bath	biowaste	5	Sahlstrom
Chryseobacter meningosepticum	>6	63	15	3	4.03		water bath	milk	6	Dumalisile
Chryseobacter meningosepticum	4.91	63	10	3	4.03		water bath	milk	6	Dumalisile

Organism	Total Log_{10} inactivation	Temp (C)	Time (min)	Ramp (min)	Ramp Log_{10} inactivation	T90 (min)	Method	Matrix	Inoculum (log_{10})	Reference
Chryseobacter meningosepticum	4.31	63	5	3	4.03		water bath	milk	6	Dumalisile
Chryseobacter meningosepticum	>4	63	10	3	2.87		water bath	milk	4	Dumalisile
Chryseobacter meningosepticum	3.24	63	5	3	2.87		water bath	milk	4	Dumalisile
E. coli	>3.6	80	30	ns	ns		oven	sludge	6	Moce-Livinia
E. coli	3.7	75	0.25	ns	ns		water bath	raspberry puree	6	Baert
E. coli	>8	70	0.17	ns	na		water bath	centrifuged sludge supernatant	8	Lang
E. coli	>5.4	70	60	ns	ns		water bath	biowaste	4.1-5.4	Sahlstrom
E. coli	>5.4	70	30	ns	ns		water bath	biowaste	4.1-5.4	Sahlstrom
E. coli	>8	70	0.17	0	na		water bath	Tryptone soy broth	8	Lang
E. coli	3	65	0.5	ns	ns		water bath	raspberry puree	6	Baert
E. coli	>6	63	25	3	0.20		water bath	milk	6	Dumalisile
E. coli	5.06	63	20	3	0.20		water bath	milk	6	Dumalisile
E. coli	1.44	63	10	3	0.20		water bath	milk	6	Dumalisile
E. coli	0.95	63	5	3	0.20		water bath	milk	6	Dumalisile
E. coli	>4	63	10	3	2.38		water bath	milk	4	Dumalisile
E. coli	3.56	63	5	3	2.38		water bath	milk	4	Dumalisile
E. coli	6	60	30	ns	ns		water bath	raw sewage	6.7	Moce-Livinia
E. coli	>5.4	55	60	ns	ns		water bath	biowaste	4.1-5.4	Sahlstrom
E. coli NCTC 9001 (lab strain)	7 to 8	55	30	0	na	4.4	water bath	Tryptone soy broth	8	Lang
E. coli O148 (environmental)	4 to 7	55	30	0	na	7.1	water bath	Tryptone soy broth	8	Lang
E. coli O158 (environmental)	5 to 8	55	30	0	na	5.9	water bath	Tryptone soy broth	8	Lang
E. coli	1 to >5.4	55	30	ns	ns		water bath	biowaste	4.1-5.4	Sahlstrom
E. coli NCTC 9001 (lab strain)	8	55	20	0	na	2.1	water bath	centrifuged sludge supernatant	8	Lang

Organism	Total Log_{10} inactivation	Temp (C)	Time (min)	Ramp (min)	Ramp Log_{10} inactivation	T90 (min)	Method	Matrix	Inoculum (log_{10})	Reference
E. coli O148 (environmental)	8	55	20	0	na	2.4	water bath	centrifuged sludge supernatant	8	Lang
E. coli O158 (environmental)	8	55	20	0	na	2.6	water bath	centrifuged sludge supernatant	8	Lang
Enterococci	2.66	80	60	ns	ns		ns	sludge	6.91	Bonjoch
Enterococci	0.18	60	90	ns	ns		ns	sludge	6.91	Bonjoch
Enterococci	>5.4	70	60	14-20	ns		water bath	biowaste	4.1-5.4	Sahlstrom
Enterococci	>5.4	70	30	14-20	ns		water bath	biowaste	4.1-5.4	Sahlstrom
Enterococci	1 to >5.4	55	60	14-20	ns		water bath	biowaste	4.1-5.4	Sahlstrom
Enterococci	1 to >5.4	55	30	14-20	ns		water bath	biowaste	4.1-5.4	Sahlstrom
Faecal coliforms	6.24	80	60	ns	ns		ns	sludge	8.5	Bonjoch
Faecal coliforms	>5.4	70	60	ns	ns		water bath	biowaste	4.1-5.4	Sahlstrom
Faecal coliforms	>5.4	70	30	ns	ns		water bath	biowaste	4.1-5.4	Sahlstrom
Faecal coliforms	5.47	60	90	ns	ns		ns	sludge	8.5	Bonjoch
Faecal coliforms	6.2	60	30	ns	ns		water bath	raw sewage	6.7	Moce-Livinia
Faecal coliforms	>5.4	55	60	ns	ns		water bath	biowaste	4.1-5.4	Sahlstrom
Faecal coliforms	1 to >5.4	55	30	ns	ns		water bath	biowaste	4.1-5.4	Sahlstrom
Faecal streptococci	>2.7	80	90	ns	ns		oven	sludge	5	Moce-Livinia
Faecal streptococci	>1.4, <1.8	80	30	ns	ns		oven	sludge	5	Moce-Livinia
Faecal streptococci	3.4	60	30	ns	ns		water bath	raw sewage	5.7	Moce-Livinia
Pseudomonas putida	>6	63	20	3	4.02		water bath	milk	6	Dumalisile
Pseudomonas putida	5.85	63	15	3	4.02		water bath	milk	6	Dumalisile
Pseudomonas putida	5.25	63	10	3	4.02		water bath	milk	6	Dumalisile
Pseudomonas putida	>4	63	15	3	2.69		water bath	milk	4	Dumalisile

Organism	Total Log_{10} inactivation	Temp (C)	Time (min)	Ramp (min)	Ramp Log_{10} inactivation	T90 (min)	Method	Matrix	Inoculum (log_{10})	Reference
Pseudomonas putida	3.85	63	10	3	2.69		water bath	milk	4	Dumalisile
Salmonella	>5	70	30	ns	ns		water bath	biowaste	5	Sahlstrom
Salmonella	>5	55	60	ns	ns		water bath	biowaste	5	Sahlstrom
Salmonella	<5	55	30	ns	ns		water bath	biowaste	5	Sahlstrom
Salmonella Oranienburg	8	55	30	0	na	3.6	water bath	Tryptone soy broth	8	Lang
Salmonella Oranienburg	7	55	20	0	na	2.9	water bath	centrifuged raw sludge supernatant	8	Lang
Salmonella Senftenberg 775W	4	55	30	0	na	7.7	water bath	Tryptone soy broth	8	Lang
Salmonella Senftenberg 775W	7	55	20	0	na	3.2	water bath	centrifuged raw sludge supernatant	8	Lang
Staphylococcus aureus	>6	63	20	3	2.05		water bath	milk	6	Dumalisile
Staphylococcus aureus	4.62	63	15	3	2.05		water bath	milk	6	Dumalisile
Staphylococcus aureus	4.13	63	10	3	2.05		water bath	milk	6	Dumalisile
Staphylococcus aureus	3.44	63	10	3	1.92		water bath	milk	4	Dumalisile
Staphylococcus aureus	>4	63	15	3	1.92		water bath	milk	4	Dumalisile

2.4. Pasteurisation inactivation of protozoa

Cryptosporidium has been shown to be sensitive to temperature, particularly above 40°C. One of the earliest reports used mouse infectivity to assess heat inactivation. The *Cryptosporidium* species was not specified, but since the *Cryptosporidium* were from an infected calf and infected mice, they were most likely *C. parvum*. The study was not quantitative, but showed that warming calf faeces, caecal contents or ileal scrapings from 9°C to 55°C over a period of 15 – 20 minutes completely inactivated oocysts (Anderson, 1985). Inactivation most likely occurred once the temperature exceeded 45°C, because no reduction in mouse infectivity was detected during the ramp time from 9°C to 45°C (9 minutes). Incubation of oocysts at 45°C in ileal scrapings resulted in complete inactivation after 5 minutes, with 20 minutes required for oocysts in caecal contents. It was not clear if this difference was due to a matrix effect or due to differences in oocyst numbers in the different matrices. Another study using mouse infectivity to measure temperature inactivation of *C. parvum* oocysts in water demonstrated complete inactivation after 1 minute at 72°C and 2 minutes at 64°C (Fayer, 1994). The oocyst dose to each mouse was 1.5×10^5. The estimated reduction in infectivity was at least 4 $\log_{10}$, based on oocysts age (1 month) and direct oocyst isolation from experimentally infected calves. A finer-scale study using a temperature of 71.7°C showed complete inactivation (measured by mouse infectivity) in milk and water after 5 seconds (Harp et al., 1996). In the study by Harp et al. (1996), the ID_{50} in the infant mice was shown to be 100 oocysts, so infectivity reduction was estimated to be at least 3 $\log_{10}$. Using an *in vitro* cell culture infectivity assay, similar results were demonstrated for flash pasteurisation of oocysts in cider, with 3 $\log_{10}$ inactivation for 5 seconds at 70°C and 4.8 $\log_{10}$ inactivation for 5 seconds at 71.7°C (Deng and Cliver, 2001). The conditions tested were similar to HTST pasteurisation. Sensitivity to high temperature was demonstrated for *C. parvum*, *C. muris* and a *Cryptosporidium* spp isolated from a chicken, with complete inactivation after 15 seconds at 60°C or 30 seconds at 55°C using a dose of 10^6 oocysts into mice for *C. parvum* and *C. muris* or 2-week-old chickens for the *Cryptosporidium* spp. This shows that temperature sensitivity is common to both intestinal and gastric species of *Cryptosporidium* (Fujino et al., 2002).

Comparable time points are not available for bacteria, but based on the sensitivity of oocysts to heat, bacterial indicators such as *E. coli* could be used as a conservative indicator for *Cryptosporidium* inactivation.

There is little information available regarding heat inactivation of *Giardia*. An early study, using excystation, determined that the thermal death point for *Giardia muris* cysts was 54°C after 10 minutes (>5 $\log_{10}$ inactivation), with 10 minutes at 50°C or 52°C causing at least 2 $\log_{10}$ inactivation (Schaefer et al., 1984). Vital dye staining using propidium iodide has been shown to correlate with excystation for temperature inactivation but not chlorine/chloramine exposure (Sauch et al., 1991). Using excystation, a 5 minutes exposure at 56°C caused 2 $\log_{10}$ inactivation for *G. muris* (Sauch et al., 1991), which is much lower than that reported by Schaefer et al. (1984) at 54°C. These results are similar to those reported in an earlier study, which reported 1–2 $\log_{10}$ inactivation following a 10

minute exposure at 50°C or 60°C and greater than 3 $\log_{10}$ inactivation following 10 minutes at 70°C (Ongerth et al., 1989). The Ongerth study showed comparable inactivation rates for *G. duodenalis* (human pathogen) and *G. muris* (rodents host) and comparable results for excystation and vital dye staining (using fluorescein diacetate or ethidium bromide) for temperatures ≥60°C, but at lower temperatures (≤50°C), the vital dye staining appeared to overestimate viability by 20 – 40%. Vital dye staining using the Live/Dead BacLight kit (a combination of the dyes SYTO9 and propidium iodide) has also been shown to correlate with animal infectivity for chemical and heat (60°C) inactivation (Taghi-Kilani et al., 1996). A potential issue with the use of excystation methods is the endpoint measurement that is used. As noted by Schaefer et al. (1984), counting 100 cysts demonstrated no excystation after exposure to 50 or 52°C, but scanning slides containing 100,000 cysts exposed to these temperatures identified the presence of motile trophozoites, indicating that some cysts had successfully excysted and that the trophozoites were still active.

The Australian guidance for issuing and rescinding boil water advisories states that *Cryptosporidium parvum* is inactivated in less than 1 minute once temperatures exceed 70°C (NHMRC, 2011). Although data are more limited for *Giardia*, it is generally more sensitive to environmental pressure than *Cryptosporidium* (Sattar et al., 1999) and it is likely that it would at least be as sensitive to thermal inactivation as *Cryptosporidium*. The high sensitivity of protozoa to heat inactivation suggests that *E. coli* may be a good indicator of this class of organisms (compare the highlighted *E. coli* row in Table 2.1 with the highlighted rows in Table 2.2).

Table 2.2: Summary of pasteurisation log_{10} inactivation values for Protozoa.

Organism	Total Log_{10} inactivation	Temp (C)	Time (min)	Ramp (min)	Ramp Log_{10} inactivation	T90 (min)	Method	Matrix	Inoculum (log_{10})	Reference
Cryptosporidium parvum	>4	72.4	1	ns	ns		Thermal cycler	distilled water	5	Fayer
Cryptosporidium parvum	>3	71.7	0.08	ns	ns		Lab pasteurizer	distilled water	8	Harp
Cryptosporidium parvum	>3	71.7	0.08	ns	ns		Lab pasteurizer	milk	8	Harp
Cryptosporidium parvum	>4	64.2	5	ns	ns		Thermal cycler	distilled water	5	Fayer
Cryptosporidium parvum	>3	55	0.5	ns	ns		water bath	distilled water	7	Fujino
Cryptosporidium muris	>3	55	0.5	ns	ns		water bath	distilled water	7	Fujino
Cryptosporidium sp (chicken)	>3	55	0.5	ns	ns		water bath	distilled water	7	Fujino
Giardia muris	2	60	10	ns	ns		ns	distilled water	ns	Ongerth
Giardia muris	>5	54	10	ns	ns		ns	distilled water	ns	Schaefer
Giardia muris	5	50	10	ns	ns		ns	distilled water	ns	Schaefer
Giardia muris	1	50	10	ns	ns		ns	distilled water	ns	Ongerth
Giardia duodenalis	2	60	10	ns	ns		ns	distilled water	ns	Ongerth
Giardia duodenalis	1	50	10	ns	ns		ns	distilled water	ns	Ongerth

2.5. Pasteurisation inactivation of viruses

A key review is that of Bertrand et al. (2012), which used literature data to calculate TFL (time for 1 $\log_{10}$ inactivation, also called decimal reduction time or *D*-value) to compare inactivation of different viruses (Bertrand et al., 2012). Using TFL calculated from studies measuring temperatures between 0-50°C, the order of temperature sensitivity in a simple matrix (most sensitive first) was calicivirus > echovirus > rotavirus > FRNA > coxsackievirus > astrovirus > poliovirus > murine norovirus (MNV) > hepatitis A (HAV) > PRD1 > PhiX174. By definition, simple matrices included synthetic media without suspended matter, artificial seawater, drinking water and groundwater. The order was similar for complex matrices, with calicivirus > echovirus > rotavirus > FRNA > coxsackievirus > astrovirus > poliovirus > adenovirus > MNV > HAV > PRD1 > PhiX174. Complex matrices included surface waters, seawater, wastewater, soil, dairy products, food and urine. Both of these lists were compiled from experiments measuring virus infectivity by cell culture. The phage PhiX174 appears to be the most temperature resistant virus from these calculations. However, the TFL appears to be affected by the temperature, with some viruses (eg poliovirus, HAV) changing the order of sensitivity compared with other viruses when studies assessing inactivation between 50-100°C were used to calculate the TFL. In complex matrices, somatic phage followed by FRNA had the highest TFL calculated using higher inactivation temperatures. The review suggests that detection of viruses by PCR is inappropriate for measuring heat inactivation. The TFLs were larger and the rank was different. This is likely due to the different mechanisms of inactivation, with heat most likely affecting critical virus proteins required for cell adhesion or virus replication, rather than affecting nucleic acid (detected by PCR).

Hepatitis A is covered in the PTG Title 22 Validation report but relevance of this to Australian wastewater treatment plants needs to be agreed. HAV is much more resistant to temperature compared with bacteria, requiring 33-37 minutes for 4 $\log_{10}$ inactivation at 65°C, compared with >4 $\log_{10}$ inactivation after 5 minutes at 63°C for bacteria (Bidawid et al., 2000). In contrast with bacteria and oocysts, the medium affected HAV inactivation, with higher fat content (eg cream versus skim milk) reducing inactivation from heat (Bidawid et al., 2000). The temperature affected the rate of inactivation and impact by the medium. At lower temperature (65°C) the protective effect of the cream was highest during the initial temperature exposure, decreasing such that the times to achieve 4 or 5 $\log_{10}$ inactivation were similar for the different media (2.7x longer exposure time required for 1 $\log_{10}$ inactivation, 1.19x exposure time for 3 $\log_{10}$ inactivation, 1.03x exposure for 5 $\log_{10}$ inactivation for cream versus skim milk). At higher temperature (eg 69°C), the difference in inactivation between the different media was more similar over time for the different $\log_{10}$ inactivations measured (1.5x longer exposure time required for 1 $\log_{10}$ inactivation, 1.8x exposure time for 3 $\log_{10}$ inactivation, 1.3x exposure for 5 $\log_{10}$ inactivation for cream versus skim milk).

Poliovirus appears to be more temperature sensitive than HAV, with >5 $\log_{10}$ inactivation after 30 minutes at 55°C (Strazynski et al., 2002), in comparison with 33 minutes at 65°C in skim milk for 4

$\log_{10}$ inactivation for HAV. Poliovirus also appear to be affected by the nature of the suspension medium, with higher inactivation in water compared with milk (1.1 $\log_{10}$ inactivation vs 0.56 $\log_{10}$ inactivation for water vs milk following 15 s at 72°C), which is similar to the observations with HAV with increased protection with increasing fat content. Studies using dry heat inactivation for a range of viruses showed that poliovirus was most sensitive, followed by adenovirus and polyomavirus (Sauerbrei and Wutzler, 2009). The dry heat test dried the viruses onto stainless steel before starting the inactivation experiments. The lowest temperatures tested was 75°C for 60 minutes, with 4 $\log_{10}$ inactivation for poliovirus and 0.7 $\log_{10}$ inactivation for adenovirus. DNA viruses appear to be more heat resistant compared to RNA viruses. Longer incubation time (2 hours) at 85°C was required to achieve significant inactivation. It should be noted that dry heat is less efficient at inactivating poliovirus compared with moist heat.

Murine norovirus has been used as a surrogate for human noroviruses (HNV) (Hewitt et al., 2009). Feline calicivirus (FCV) has also been assessed but appears to be less stable so is not suitable (Topping et al., 2009). Based on inactivation times compared with HAV, MNV was more temperature sensitive in milk but more stable in water (Hewitt et al., 2009). In the absence of a cell culture assay for HNV, a molecular assay was used to compare MNV and HNV. In general, MNV inactivation did not correlate with HNV inactivation at different temperatures or in different matrices (Hewitt et al., 2009). Assay conditions may play a large role in determining virus response to heat. Bidawid et al. (2000), using a plaque assay, reported times of 11-15 minutes for 2 $\log_{10}$ inactivation, 23 minutes for 3 $\log_{10}$ inactivation and 33 minutes for 4 $\log_{10}$ inactivation of HAV in milk at 65°C. Hewitt et al, (2009) also used a plaque assay but reported times of 2 minutes for 2.3-2.7 $\log_{10}$ inactivation and 5 minutes for ≥3.5 $\log_{10}$ inactivation in water or milk at 63°C. A difference between the studies was the method of heat delivery. The Bidawid study used a U-shaped microcapillary immersed in a water bath, whereas the more recent Hewitt study used 100 µL volumes in tubes in a thermal cycler. Thermal cyclers have well characterised and rapid thermal ramping. A microcapillary system could also be assumed to facilitate more rapid heat transfer so it is unclear if differences in thermal ramping could account for the extra inactivation reported in the Hewitt study. Another difference was the virus density, with the Hewitt study measuring less than 4 $\log_{10}$ inactivation and the Bidawid study measuring at least 5 $\log_{10}$ inactivation, suggesting at least a 1 or 2 $\log_{10}$ difference in inoculum. It is known that microbial density can affect disinfectant efficacy, so it is interesting to speculate that virus density could have an effect on thermal stability. A finding of the Hewitt et al. (2009) study was that PCR-based analysis of virus reduction grossly underestimated thermal inactivation, which was most pronounced at 72°C, where after 1 minute both MNV and HAV had >3.5 $\log_{10}$ inactivation by cell culture but 0.2-0.5 $\log_{10}$ inactivation by PCR. These conditions suggest that HTST will be highly effective. The results of MNV inactivation in raspberry puree (Baert et al., 2008) were similar to those reported by Hewitt for inactivation of MNV in milk.

Tulane virus (TV) is a potential surrogate for human noroviruses, showing comparable sensitivity with MNV to temperatures that would be used for pasteurisation (Hirneisen and Kniel, 2013). However, TV

has only recently been discovered from rhesus macaques and so are not likely to be readily available in Australia and also not naturally present in wastewater, making them poor candidates as a surrogate.

A recent study comparing MNV and MS2 phage (a member of the FRNA) demonstrated that MS2 was substantially more resistant to 60°C than MNV, with a TFL of 44-46 minutes compared with 2-2.5 minutes for MNV in different types of milk (Jarke et al., 2013). Interestingly, there was no protective effect observed in the presence of milk fat (0.3% - 3.5%). Fifty percent sucrose had a protective effect for MNV (TFL of 24 vs 1.3 for no sucrose), whereas the presence of sucrose slightly reduced the TFL for MS2. Different levels of NaCl had no effect on the TFL for MNV, but 10% NaCl increased the TFL for MS2 from 45 minutes to 54.6 minutes. The PTG Title 22 report (Carollo, 2006) suggests that MS2 is a suitable conservative indicator for human enteric viruses.

There are limited data available for heat inactivation of adenoviruses, with the only study available on natural viruses assessing dry heat, which is less effective compared with moist heat for other viruses. A study has reported on the moist heat inactivation of adenovirus 5 constructs (developed as a vector for vaccine production), showing >8 $\log_{10}$ inactivation after 10 minutes at 70°C and approximately 6 $\log_{10}$ inactivation after 5 minutes at 50°C (Maheshwari et al., 2004). The inactivation kinetics at 50°C suggested rapid inactivation within the first 10 minutes, with significant tailing after that.

The Ventura report (Carollo, 2012) claimed MS2 4 $\log_{10}$ inactivation = polio 5 $\log_{10}$ inactivation. This is not completely correct, as the value was negotiated between the Californian Department of Health and the project team to use this as a conservative measure. This is highly conservative since from published data MS2 2.8 $\log_{10}$ inactivation = poliovirus 5.4 $\log_{10}$ inactivation (poliovirus is 1.93x more sensitive, whereas the conservative measure uses a sensitivity factor of 1.25x).

A major gap in the existing literature is direct comparison between the enteric viruses of interest in Australia for the production of reuse water with the proposed candidates. It is also possible that the chemistry of the wastewater could affect the response of viruses or phage to temperature. It would, therefore, be prudent to compare potential surrogates such as somatic or FRNA phage with enteric viruses such as adenovirus and enteroviruses. Based on the study of Moce-Llivina et al. (2003), coxsackievirus may be a suitable surrogate for enteroviruses (eg poliovirus and HAV).

Comparison of the data in Table 2.3 with the *E. coli* data in Table 2.1 indicates that *E. coli* may be more heat sensitive than many viruses, and a more conservative indicator organism such as faecal streptococcus (*Enterococcus),* or *FRNA* may be more appropriate for viruses. A total $\log_{10}$ inactivation of 6 was achieved for *E. coli* at 60°C for 30 minutes (see Table 2.1) while the total $\log_{10}$ inactivation under these conditions is less for most viruses (see Table 2.3).

Table 2.3: Summary of pasteurisation log_{10} inactivation values for viruses.

Organism	Total Log_{10} inactivation	Temp (C)	Time (min)	Ramp (min)	Ramp Log_{10} inactivation	T90 (min)	Method	Matrix	Inoculum (log_{10})	Reference
Adenovirus 5 constructs	>8	70	10	ns	ns		water bath	water, cell lysate	>7	Maheswari
Adenovirus 5 constructs	6	50	5	ns	ns		water bath	water, cell lysate	>7	Maheswari
Coxsackie virus	ns	50	ns	ns	ns	0.005	ns	complex	ns	Bertrand
Culturable Coxsackie B4	5.1	60	30	ns	ns		water bath	raw sewage, spiked	8	Moce-Livinia
Culturable Coxsackie B5	4.8	60	30	ns	ns		water bath	raw sewage, spiked	8	Moce-Livinia
Culturable Echovirus 6	4.3	60	30	ns	ns		water bath	raw sewage, spiked	8	Moce-Livinia
Culturable Enterovirus	>1.7	60	30	ns	ns		water bath	raw sewage	ns	Moce-Livinia
Culturable EV1	4.4	60	30	ns	ns		water bath	raw sewage, spiked	8	Moce-Livinia
Culturable EV2	4.3	60	30	ns	ns		water bath	raw sewage, spiked	8	Moce-Livinia
Feline calicivirus	ns	50	ns	ns	ns	0.0008	ns	complex	ns	Bertrand
Feline calicivirus	ns	50	ns	ns	ns	0.0032	ns	simple	ns	Bertrand
FRNA phage	2.1	60	30	ns	ns		water bath	raw sewage	5	Moce-Livinia
FRNA phage I	ns	50	ns	ns	ns	0.0079	ns	complex	ns	Bertrand
FRNA phage I	ns	50	ns	ns	ns	0.0316	ns	simple	ns	Bertrand
FRNA phage all	ns	50	ns	ns	ns	0.0251	ns	complex	ns	Bertrand
FRNA phage MS2	2.8	60	30	ns	ns		water bath	raw sewage, spiked	9	Moce-Livinia
FRNA phage MS2	0.8	60	30	ns	ns	45.14	Thermal cycler	SM buffer	5	Jarke

Organism	Total Log_{10} inactivation	Temp (C)	Time (min)	Ramp (min)	Ramp Log_{10} inactivation	T90 (min)	Method	Matrix	Inoculum (log_{10})	Reference
FRNA phage MS2	1.1 to 1.5	60	30	ns	ns	38.02	Thermal cycler	SM buffer 50% sucrose	5	Jarke
hepatitis A	5	80	0.68	ns	ns		water bath	milk (skim, full)	ns	Bidawid
hepatitis A	5	80	1.24	ns	ns		water bath	cream	ns	Bidawid
hepatitis A	2.22	72	1	ns	ns	<0.3	Thermal cycler	milk	5.7	Hewitt
hepatitis A	>3.5	72	1	ns	ns	<0.3	Thermal cycler	water	5.7	Hewitt
hepatitis A	4	65	33-37	ns	ns		water bath	milk	ns	Bidawid
hepatitis A	3.35	63	5	ns	ns	1.1	Thermal cycler	milk	5.7	Hewitt
hepatitis A	>3.5	63	5	ns	ns	0.6	Thermal cycler	water	5.7	Hewitt
hepatitis A	ns	50	ns	ns	ns	0.0016	ns	complex	ns	Bertrand
hepatitis A	ns	50	ns	ns	ns	0.0063	ns	simple	ns	Bertrand
murine norovirus	3	75	0.25	ns	ns		water bath	raspberry puree	6	Baert
murine norovirus	>3.5	72	1	ns	ns	0.5	Thermal cycler	milk	5.5	Hewitt
murine norovirus	>3.5	72	1	ns	ns	<0.3	Thermal cycler	water	5.5	Hewitt
murine norovirus	2	65	0.5	ns	ns		water bath	raspberry puree	6	Baert
murine norovirus	>3.5	63	5	ns	ns	0.7	Thermal cycler	milk	5.5	Hewitt
murine norovirus	3.13	63	5	ns	ns	0.9	Thermal cycler	water	5.5	Hewitt

Organism	Total Log_{10} inactivation	Temp (C)	Time (min)	Ramp (min)	Ramp Log_{10} inactivation	T90 (min)	Method	Matrix	Inoculum (log_{10})	Reference
murine norovirus	4	60	5	ns	ns	1.35	Thermal cycler	PBS	6 to 7	Jarke
murine norovirus		60		ns	ns	24.15	Thermal cycler	PBS 50% sucrose	6 - 7	Jarke
murine norovirus	ns	50	ns	ns	ns	0.0013	ns	complex	ns	Bertrand
murine norovirus	ns	50	ns	ns	ns	0.005	ns	simple	ns	Bertrand
phage infecting *B. fragilis*	0.4	60	30	ns	ns		water bath	raw sewage	4.7	Moce-Livinia
phage infecting *B. fragilis*	ns	50	ns	ns	ns	0.0032	ns	complex	ns	Bertrand
phage MY2	0.5	60	30	ns	ns		water bath	raw sewage, spiked	9	Moce-Livinia
phage phiX174	2.1	60	30	ns	ns		water bath	raw sewage, spiked	9	Moce-Livinia
phage SR51	1	60	30	ns	ns		water bath	raw sewage, spiked	9	Moce-Livinia
phage B40-8 infecting *B. fragilis*	4	75	0.25	ns	ns		water bath	raspberry puree	5.7	Baert
phage B40-8 infecting *B. fragilis*	4	65	0.5	ns	ns		water bath	raspberry puree	5.7	Baert
phage B40-8 infecting *B. fragilis*	0.4	60	30	ns	ns		water bath	raw sewage, spiked	9	Moce-Livinia
phage phiX174	ns	50	ns	ns	ns	0.01	ns	complex	ns	Bertrand
phage phiX174	ns	50	ns	ns	ns	0.0398	ns	simple	ns	Bertrand
poliovirus	ns	50	ns	ns	ns	0.0005	ns	complex	ns	Bertrand
poliovirus	ns	50	ns	ns	ns	0.002	ns	simple	ns	Bertrand
culturable polivirus 1	>5	72	0.5	ns	ns		ns	water	5 - 6	Strazynski
culturable polivirus 1	>5	72	0.5	ns	ns		ns	milk	5 - 6	Strazynski

Organism	Total Log_{10} inactivation	Temp (C)	Time (min)	Ramp (min)	Ramp Log_{10} inactivation	T90 (min)	Method	Matrix	Inoculum (log_{10})	Reference
culturable polivirus 1	1.1	72	0.25	ns	ns		ns	water	5 - 6	Strazynski
culturable polivirus 1	0.6	72	0.25	ns	ns		ns	milk	5 - 6	Strazynski
culturable polivirus 1	5.4	60	30	ns	ns		water bath	raw sewage, spiked	8	Moce-Livinia
culturable polivirus 1	>5	55	30	ns	ns		ns	water	5 - 6	Strazynski
culturable polivirus 1	>5	55	30	ns	ns		ns	milk	5 - 6	Strazynski
Simian rotavirus	ns	50	ns	ns	ns	0.004	ns	complex	ns	Bertrand
Simian rotavirus	ns	50	ns	ns	ns	0.0158	ns	simple	ns	Bertrand
Somatic coliphages	0.8	60	30	ns	ns		water bath	raw sewage	6.7	Moce-Livinia
Somatic coliphage	ns	50	ns	ns	ns	0.0316	ns	complex	ns	Bertrand
Somatic coliphage SC12	0.5	60	30	ns	ns		water bath	raw sewage, spiked	9	Moce-Livinia
Somatic coliphage SS13	0.3	60	30	ns	ns		water bath	raw sewage, spiked	9	Moce-Livinia

2.6. Helminths

The PTG Title 22 report (Carollo, 2006) did not consider helminths. Compared with viruses there has been limited work studying the temperature inactivation of helminths. *Ascaris suum* has been shown to be >4 $\log_{10}$ inactivated following 15 minutes at 55°C in waste from a biogas plant (Sahlstrom et al., 2008). Anaerobic sludge digestion at 51-56°C, resulted in >2 $\log_{10}$ inactivation for *A. suum* within 2 hours (Popat et al., 2010). Another study of thermal treatment of sludge found >2 $\log_{10}$ inactivation after 45 minutes at 61-62.5°C and >2 $\log_{10}$ inactivation after 15 minutes at 65-66.5°C (Paulsrud et al., 2004). Thermal treatment of digester sludge assessed inactivation over a finer time-scale, showing approximately 1.5 $\log_{10}$ inactivation after 15 minutes at 55°C and 1.5 $\log_{10}$ inactivation after 10 minutes at 53°C (Aitken et al., 2005). These values are higher than those in the Popat study. Inactivation rates of *Ascaris* at pasteurisation temperatures within shorter time-scales is a knowledge gap that will need to be addressed for this project, particularly if the pilot-scale system will be using short contact times with the wastewater.

Comparison of the data in Table 2.4 with the *E. coli* data in Table 2.1 indicates that *E. coli* may be more heat sensitive than *Ascaris suum*, and a more conservative indicator organism such as faecal streptococcus (*Enterococcus),* or *FRNA* may be more appropriate for helminths. A total $\log_{10}$ inactivation of >5.4 was achieved for *E. coli* at 55°C for 60 minutes (see Table 2.1) while the total $\log_{10}$ inactivation under these conditions is less for *Ascaris suum* (see Table 2.4).

Table 2.4: Summary of pasteurisation log_{10} inactivation values for helminths.

Organism	Total Log_{10} inactivation	Temp (C)	Time (min)	Ramp (min)	Ramp Log_{10} inactivation	T90 (min)	Method	Matrix	Inoculum (log_{10})	Reference
Ascaris suum	>3	55	15	14-20	ns		water bath	biowaste	4	Sahlstrom
Ascaris suum	>3	70	15	14-20	ns		water bath	biowaste	4	Sahlstrom
Ascaris suum	0.7	51.1	60	ns	ns		lab digester	sludge	4	Popat
Ascaris suum	1	55.5	60	ns	ns		lab digester	sludge	4	Popat
Ascaris suum	>2	61 - 62.5	45	ns	ns		fullscale?	sludge	ns	Paulsrud
Ascaris suum	>2	65 - 66.5	15	ns	ns		fullscale?	sludge	ns	Paulsrud
Ascaris suum	1	51	30	ns	ns	32	lab reactor	digester sludge	5	Aitken
Ascaris suum	2.2	51	60	ns	ns	32	lab reactor	digester sludge	5	Aitken
Ascaris suum	1.5	55	15	ns	ns	10	lab reactor	digester sludge	5	Aitken

2.7. Conclusions

Based on reviewed scientific literature, the suggested surrogates and pathogens for inclusion in this project are listed in Table 2.5.

Table 2.5: List of pathogens and indicators suggested for lab and pilot scale testing.

Scale	Pathogens	Surrogate	Conservative surrogates
Laboratory	*Cryptosporidium* *Giardia* Adenovirus Coxsackievirus *Ascaris*	*E. coli*	*Enterococcus* FRNA
Pilot	Adenovirus	*E. coli*	*Enterococcus* FRNA

It is recommended that the laboratory-scale testing compare these key pathogens / surrogates in the selected test water (or waters) at temperatures and times relevant to the full-scale system. It is understood that the pilot plant will operate as a HTST system, but the anticipated operational temperature range and contact time at the target temperature needs to be agreed upon to inform the design of the laboratory-scale experiments. The lab testing will verify that the matrix does not cause any unexpected effect on the temperature sensitivity of the pathogens of interest, and also verify the selection of candidate surrogates (*E. coli*, faecal streptococci (*Enterococcus*), FRNA).

Two experimental designs are proposed. The first will use a thermal cycler for temperature inactivation experiments. The advantages of this approach are that it allows the use of indigenous organisms in the test water without the need for spiking (unless the test organism is not present) and it also provides rapid heating and cooling of the test sample. The disadvantage is that the sample volume is small (0.1 mL). The second approach is to heat a larger volume of sample (eg 30 mL) on a heated plate with a stirrer and to add a small volume of test organism, which is then well mixed, avoiding any effect of ramp time on the measurement of inactivation. The disadvantage of this approach is that the sample will require some dilution to allow rapid cooling. Both of these approaches allow easy control of sample temperatures that could be replicated elsewhere without the need for specialist equipment.

The available inactivation data for the organisms of interest over a range of 55-65°C is summarised in Table 2.6. The metadata from the Bertrand et al. (2012) paper has been excluded because the calculated T_{90} values do not match other literature, suggesting either an error in their calculation, or an error in conversion of their data (no units were provided for their Log_{10} TFLs, assumed to be in minutes).

Table 2.6: Summary of inactivation data for temperatures 55 – 65°C.

Organism	Log_{10} Inactivation	Temp. (°C)	Time (min)	T_{90} (D)	Matrix
E. coli lab	7 - 8	55	30	2.1 – 4.4	sludge supernatant / medium
E. coli wild	4 - 8	55	30	2.4 – 7.1	sludge supernatant / medium
Salmonella	4 - 8	55	30	2.9 – 7.7	sludge supernatant / medium
Campylobacter	>5	55	30		biowaste
Enterococci	>5.4	55	30		biowaste
Cryptosporidium	>3	55	0.5		water
Giardia	>5	54	10		water
Ascaris suum	>3	55	15		biowaste
Ascaris suum	1.5	55	10	10	sludge
Poliovirus 1	>5	55	30		milk / water
E. coli	6	60	30		sewage
Faecal streptococci	3.4	60	30		sewage
Somatic coliphage	0.8	60	30		sewage
FRNA MS2	2.8	60	30		sewage
FRNA MS2	0.8	60	30	45	buffer
Murine Norovirus	4	60	5	1.3	buffer
Coxsackie / Enterovirus	4.3 – 5.1	60	30		sewage
E. coli	3.6 – 5.1	63	5 - 20		milk
Pseudomonas	3.9 – 5.9	63	5 - 15		milk
Cryptosporidium	>4	64.2	5		water
Ascaris suum	>2	61 – 62.5	45		sludge
Hepatitis A	>3.35	63	5	0.6 – 1.1	milk / water
Murine Norovirus	>3.13	63	5	0.5 – 0.9	milk / water

A major research gap is that available data for *Cryptosporidium* and *Giardia* is only for inactivation in distilled water or cider. The available results suggest that *Cryptosporidium* is highly temperature sensitive and *Giardia* has similar or higher sensitivity compared with enteric bacteria. Similarly, there are limited data for helminths, although based on inactivation in sludge as a conservative measure, the heat sensitivity of *Ascaris* is similar to that of environmental *E. coli*. Human enteric viruses, including hepatitis A and the enteroviruses (polio, coxsackie etc), appear to have similar temperature sensitivity compared with *E. coli* and *Pseudomonas*.

Based on the available literature, native *E. coli* may be a reasonable surrogate for most organisms of interest, with FRNA (MS2) phage and faecal streptococci (*Enterococcus)* conservative indicators of pathogen inactivation (for all pathogens of concern). Somatic coliphage appear to be relatively heat

resistant and are likely to be too conservative as a surrogate, but could possibly be used as a process indicator (in the absence of challenge testing using spiked microorganisms).

The matrix can also have an influence on the efficiency of heat inactivation, with dry heat less effective than moist heat for inactivation of viruses and inactivation in sludge generally less effective than inactivation in liquids. The impact of the liquid composition on inactivation efficiency is difficult to predict, with the mechanisms likely to vary between microorganisms, especially viruses. The few studies that have examined matrix effects are not directly relevant to wastewater, focussing on high sugar or fat concentrations for the pasteurisation of foods or beverages.

3. LABORATORY-SCALE TESTING

3.1 Scope

The primary purpose of the laboratory-scale testing was to verify the temperature inactivation of the pathogens and surrogates identified from the literature review (Table 2.5). Temperatures were selected based on the literature values for pathogen/surrogate inactivation and also on the temperatures likely to be used in the pilot system. Times were chosen to be conservative compared with the contact times predicted for the pilot system.

3.2 Methods

3.2.1. Design of inactivation experiments

Experiments were conducted to determine:

(1) Effect of temperature and time: 55°C, 65°C and 75°C, 5 sec, 30 sec, 60 sec

(2) Effect of water type: L55E Pond 2 (P2) and L55E Pond 10 (P10)

(3) Effect of organism strain (where possible): laboratory strain and isolates from wastewater.

Controls included: ambient temperature control, temperature ramping control (from room temperature to target temperature with a nominal 1 sec hold at the target temperature).

Inactivation experiments were conducted in 0.2 mL tubes on a Palmcycler thermal cycler (Corbett Research, Sydney, Australia). Sample volumes were 120 µL. Samples were diluted into the relevant volume of sterile reagent-grade water to provide the required volume for microbiological analysis.

3.2.2. Male specific coliphage (MS-2)

Male-specific coliphage were analysed following the double layer overlay technique described in APHA Method 9224C, with the exception that 1 mL (rather than 10 x 1 mL) of sample was analysed. The *E. coli* host was strain ATCC 700891 and the MS2 used for experiments was ATCC 15597-B1. This method detects total male-specific coliphage (FRNA and FDNA).

For isolation of indigenous phage, 1 mL of wastewater was processed using the phage plating method. Phage were harvested from plaques by scraping off the top layer of agar from the plate, vortexing the agar with tryptone soy broth (Oxoid) and centrifuging the sample to pellet cells / debris / agar. The supernatant was used to inoculate *E. coli* cultures and the process was repeated to obtain the spike used for the native FRNA experiments. To check for the presence of FDNA, samples were plated with or without RNase, with the difference in count attributed to the effect of the RNase on inactivating FRNA (this technique is also called a differential plaque count, the FRNA are indirectly counted by subtracting the FDNA count from total F-phage). Although the number of FDNA appeared

to be low in P2 and P10 waters, they increased after phage isolation / propagation, requiring the use of differential plaque counts to enumerate native FRNA for the heat inactivation experiments.

3.2.3. *Escherichia coli*

E. coli were enumerated by the Australian Water Quality Centre's NATA accredited laboratory using a method based on AS 4276.7, which analyses a 100 mL sample volume using membrane filtration / culture. The culture medium used was MI agar, which is a selective chromogenic medium that allows for the selection and differentiation of coliforms and *E. coli*.

3.2.4. Enterococci

Faecal streptococci and *Enterococcus* were enumerated by the Australian Water Quality Centre's NATA accredited laboratory using a method based on AS 4276.9, with the addition of glucosidase agar for confirmation of *Enterococcus*. Similar to the *E. coli* method, 100 mL of sample was filtered through a membrane and the membrane was placed onto m-Enterococcus agar, which is a chromogenic agar. Presumptive colonies were further tested using glucosidase agar and other culture media to confirm identity as *Enterococcus* spp or faecal streptococci.

3.2.5. Human enteric viruses

Virus stocks were prepared by infecting cells in culture. Confluent cell monolayers in 175 cm^3 flasks were rinsed with Phosphate Buffered Saline (PBS) and infected with respective virus at a concentration of approximately 1 multiplicity of infection, diluted in 3 mL media without foetal bovine serum (FBS). The flasks were incubated at 37°C in a $C0_2$ incubator for 90 minutes with rocking every 10 minutes, after which the inoculum was replaced with 15 mL of complete media. Infected flasks were incubated at 37°C in a $C0_2$ incubator until >90% cell monolayer destruction, due to cytopathic effect (CPE), was observed. One to three freeze-thaw steps were performed to release virus particles from host cells. The supernatant was centrifuged at 4°C and 10,000 g for 10 minutes to remove cell debris. Further purification was accomplished by filtering supernatant through a 0.2 µm Acrodisc syringe (Pall Life Sciences, USA). All viral stocks were titrated using the plaque assay method as described below and stored in 1 mL lots at -80°C.

Coxsackie B5 (CB5) (ATCC VR-185) was cultured in buffalo green monkey kidney (BGM) cells; adenovirus 2 (Ad2) was obtained from the National Institute of Allergy and Infectious Diseases (NIAID) and cultured in human lung adenocarcinoma epithelial (A549) cells. All viral stocks were titrated using a previously described plaque assay method (Kahler et al., 2011). Overnight confluent cell monolayers were washed and infected with 100 or 200 µl of serially diluted (10^{-1} – 10^{-6}) supernatant or sample as described above. Following infection, inoculum was removed and cells

were washed and overlaid with 2 or 4 ml of a 1:1 mix of 2% SeaPlaque Agarose (Lonza Rockland, Inc, USA) and 2X MEM plus 10% FBS. Agarose was allowed to set, and plates were inverted and incubated at 37°C for 3 or 10 days for CB5 or Ad2 respectively. After appropriate incubation time, cells were fixed with 1% formalin for 30 minutes. The overlaid agar was removed and cells were stained with 0.2% crystal violet and rinsed with distilled water to visualise the plaques. Levels of infectious virus were reported as PFU per mL.

3.2.6. *Cryptosporidium* infectivity

A cell culture assay was used to measure oocyst infectivity. This method was a simplified version of the assay described by King et al. (2015). In brief, oocyst counts were determined by fluorescence microscopy prior to temperature exposure experiments. Set numbers of oocysts were processed by the infectivity assay. Infectious oocysts were detected using the focus detection method, which uses a specific fluorescent antibody to detect infection of host cells by *Cryptosporidium*. The % infectious oocysts was determined using the number of infectious oocysts detected by cell culture and the total number of oocysts applied to cell culture. A limitation of this method is the number of oocysts that can be applied to a single cell culture well and the number of infectious oocysts that can be counted in a single cell culture well. The total number of oocysts applied across multiple cell culture wells was used to allow calculation of maximum log removal values (LRVs) for large numbers of oocysts.

3.2.7. *Giardia* excystation

Viable *G. duodenalis* cysts were purchased from BTF (Sydney, Australia). Cysts were stained using Easystain (BTF, Sydney, Australia) and visualised using fluorescence microscopy or by flow cytometry using a FACS Calibur (Becton Dickinson). A method for the excystation of *G. muris* (Schaefer et al., 1984) was trialled for *G. duodenalis*. Staining cysts using SYTO9 and Propidium Iodide (PI) was also trialled (this method is used for bacterial live/dead staining and PI has historically been used as a viability stain for cysts). Both heat treated and non-heat treated cysts were subjected to excystation and vital dye staining methods.

3.2.8. Helminths

The collection of fertile *Ascaris suum* eggs was performed using the method described by Jeska et al. (1986). Pig intestines were recovered from slaughtered animals by the Dept Primary Industries, Vic, Australia, and sent to AWQC. Worms were sorted to select intact females based on size and rinsed in egg laying solution consisting of phosphate buffered physiological saline (pH 7.3), 0.0015 N sodium hydroxide and 11 mM glucose. Females were placed in 75 cm^2 cell culture flasks with egg laying medium and 125 mg/L gentamycin sulfate and incubated at 37°C for several days. Released eggs

were collected daily and kept at 4°C until required, with replacement of fresh egg laying medium and removal of dead worms as required. Enumeration was performed by microscopy.

Following temperature inactivation experiments, eggs were shipped to James Cook University and stored at 4ºC. Batches of approximately 200-300 eggs in 25 µl were mixed with 225 µl 0.1N sulphuric acid (Sigma-Aldrich) in a 90 well microplate (Corning). The plate was incubated in the dark at 22ºC and the well volume was topped up with distilled water as required. After 40 days, eggs were visually assessed for development at 100-400 X magnification with an CKX41 microscope (Olympus). The developmental stage of 60-100 eggs were assessed and categorized as unfertilized (dark and elongated with incomplete eggshells), pre-larval and developed larva. The eggs that reached larval stage were considered viable and percentage calculations excluded unfertilized eggs. Viability was calculated using the following equation:

$$\%\text{viability} = \text{larva}/(\text{pre-larval} + \text{larva})$$

3.3. Results

3.3.1. Water quality data

Water was collected from Western Treatment Plant Ponds 1, 2 and 10 from the L55E system and Ponds 1, 2 and 10 from the L25W system. The results are summarised in Table 3.1 and Table 3.2. Water collected from L55EP2 and L55EP10 was used in initial temperature inactivation experiments for phage and bacteria, with L55EP2 being used for later experiments.

Table 3.1: Water quality data for Western Treatment Plant Pond water collected in April 2014.

April 2014	L55EP1[1]	L55EP2	L55EP10	L25WP1[2]	L25WP2	L25WP10
E coli (cfu/100 mL)	600	71000	7	140000	4500	42
Faecal coliforms (cfu/100 mL)	1000	71000	29	230000	4500	42
Enterococci (cfu/100 mL)	130	48000	9	10000	350	5
FRNA (pfu/mL)	165	30	680	95	1	<1
pH	7.67	7.28	8.17	7.49	7.66	7.88
Turbidity (NTU)	32.4	8.49	1.79	39.6	9.44	9.82
Ammonia (mg/L)	68.4	67.8	<0.5	68.8	3.118	11.45
nitrate+nitrite (mg/L)	3.2	0.007	21.7	0.007	13.1	4.8
Phosphate (mg/L)	10.9	10.9	8.88	11.6	8.05	8.91
TKN (mg/L)	69.4	63.8	1.59	73.1	4.68	12.9
DOC (mg/L)	15.3	15.3	8.9	15.2	10	10.1
TOC (mg/L)	27.4	21.2	10.7	44.9	13.3	14.5
BOD (mg/L)	55	14	<2	41	6	17
COD (mg/L)	174	104	223	225	92	131
conductivity (EC) (µScm)	2060	2150	1860	2140	1700	1900
TDS (mg/L)	1100	1200	1000	1200	940	1000
SS (mg/L)	68	12	4	50	6	17
VSS (mg/L)	48	11	2	42	5	11

[1] L55E indicates the L55E pond system, P1, P2, P10 indicates Ponds 1, 2 and 10 respectively

[2] L25W indicates the L25W pond system, P1, P2, P10 indicates Ponds 1, 2 and 10 respectively

Table 3.2: Water quality data for Western Treatment Plant Pond water collected in May 2014.

May 2014	L55EP1[1]	L55EP2	L55EP10	L25WP1[2]	L25WP2	L25WP10
E coli (cfu/100 mL)	58000	520000	6	140000	800	98
Faecal coliforms (cfu/100 mL)	97000	520000	15	140000	1000	98
Enterococci (cfu/100 mL)	1200	23000	0	5600	200	25
FRNA (pfu/mL)	-	-	-	-	-	-
pH	-	-	-	-	-	-
Turbidity (NTU)	-	-	-	-	-	-
Ammonia (mg/L)	60.76	63.01	0.195	61.4	7.927	10.83
nitrate+nitrite (mg/L)	0.01	0.007	20.9	0.009	5.76	4.89
Phosphate (mg/L)	9.87	10.9	8.36	13.2	3.14	7.55
TKN (mg/L)	65.7	65.5	1.73	67.7	9.94	13.5
DOC (mg/L)	18.1	17.1	8.4	15.9	8.9	8.7
TOC (mg/L)	27.4	29.5	9.5	41	10.2	10.6
BOD (mg/L)	46	24	<2	29	8	6
COD (mg/L)	153	127	84	159	72	90
conductivity (EC) (µScm)	2020	2170	1700	2060	1690	1730
TDS (mg/L)	1100	1200	940	1100	930	950
SS (mg/L)						
VSS (mg/L)	-	-	-	-	-	-

[1] L55E indicates the L55E pond system, P1, P2, P10 indicates Ponds 1, 2 and 10 respectively

[2] L25W indicates the L25W pond system, P1, P2, P10 indicates Ponds 1, 2 and 10 respectively

3.3.2. F-RNA

Initial experiments investigated the inactivation of MS2 phage spiked into L55EP2 (P2) and L55EP10 (P10) water. Replicate experiments were conducted but the volumes used in the thermal cycler tubes precluded the use of technical replicates for sample analysis. No inactivation was observed for 55°C (up to 60 seconds exposure time) so this temperature was not further investigated.

Initial experiments were conducted using different May 2014 samples of P2 and P10 water. To investigate variability, triplicate inactivation experiments were conducted using a June 2014 sample of P2 water. The results of pasteurisation treatment at 65°C are shown in Figures 3.1, 3.2 and 3.3. The results of pasteurisation treatment at 75°C are shown in Figures 3.4, 3.5 and 3.6.

Comparison of Figures 3.1 (P2 water) and 3.2 (P10 water) shows similar MS2 inactivation in P2 & P10 waters at 65°C.

Native male-specific phage were cultured from the L55E system pond water and used for inactivation experiments. The isolation process appeared to enrich for FDNA phage, so the FRNA were indirectly counted by subtracting the FDNA count from total F-phage. Comparison of Figure 3.3 to Figures 3.1 and 3.2 shows that the native FRNA was slightly more heat resistant than MS2 at 65°C. The FDNA proved to be more heat resistant than FRNA. A 60-second contact time at 65 °C did not affect the FDNA numbers (results not shown).

At 75°C, the observed inactivation of MS2 after 30 seconds contact time was approximately 6, with no apparent difference between P2 and P10 water results (see Figures 3.4 and 3.5). The inactivation rate was poorer for spiked native FRNA than for MS2 at 30 seconds (MS2 LRV = 1.5, FRNA LRV = 6) (see Figure 3.6). The unspiked FRNA LRV remained low at 60 seconds due to the low starting phage number (71). This contact time did not, however, kill all the phages present in the unspiked sample. Two plaques were detected after 60 seconds treatment at 75°C, indicating that the native population of phages contained some individuals with higher heat resistance.

Plaque counts from plates with RNase (which will kill FRNA) showed that inactivation of FDNA at 75°C (60 seconds) ranged from 0.3 – 0.6 LRV. Interestingly, the inactivation of male-specific phage in the unspiked sample and the spiked native FRNA was similar at 5 seconds and 30 seconds. The unspiked sample result at 60 seconds showed some suggestion of tailing (suggesting that there is a fraction of resistant phage, most likely FDNA).

The numbers of native F-RNA in Pond 2 or Pond 10 water are unlikely to be high enough to allow the measurement of > 2 LRV, but the number should be sufficient to validate inactivation at 65°C.

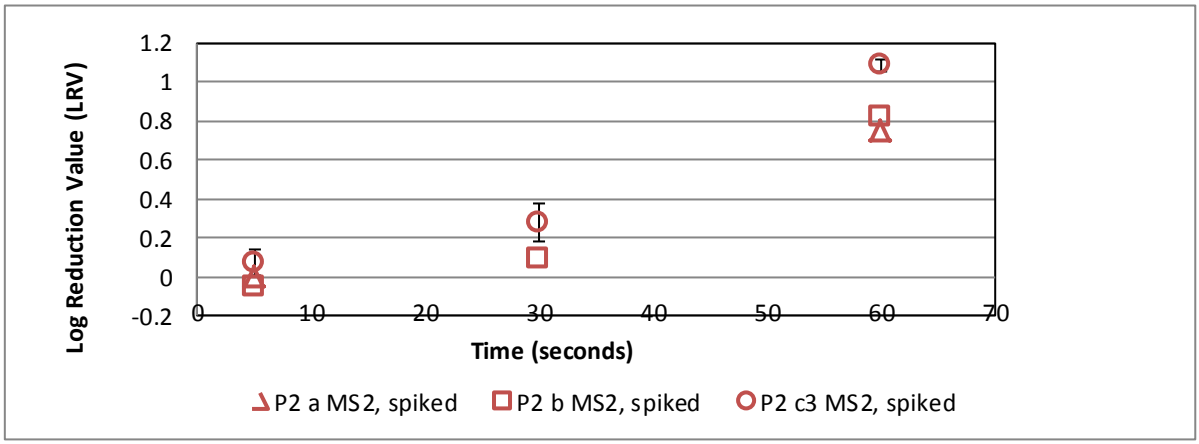

Figure 3.1: Laboratory pasteurisation treatment of MS2 in Pond 2 (P2) water at 65°C, in two replicate experiments involving spiking of cultured MS2 in different May 2014 samples of water (a and b), and in a July 2014 sample of water (c3, triplicate experiment, error bars represent on standard deviation).

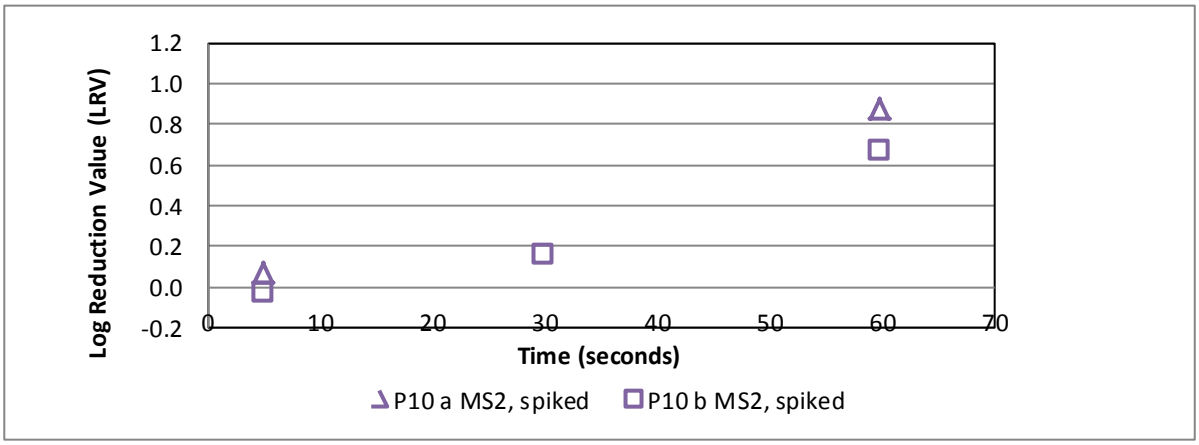

Figure 3.2: Laboratory pasteurisation treatment of MS2 in Pond 10 (P10) water at 65°C, in two replicate experiments involving spiking of cultured MS2 in different May 2014 samples of water (a and b).

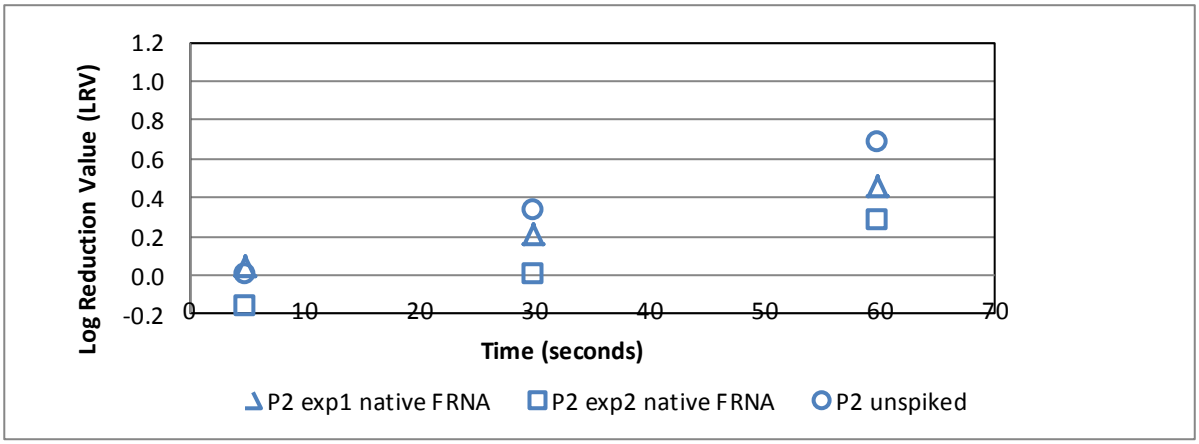

Figure 3.3: Laboratory pasteurisation treatment of native FRNA in Pond 2 (P2) water at 65°C, in two replicate experiments involving spiking of cultured native FRNA (exp1 and exp2), and without spiking (P2 unspiked).

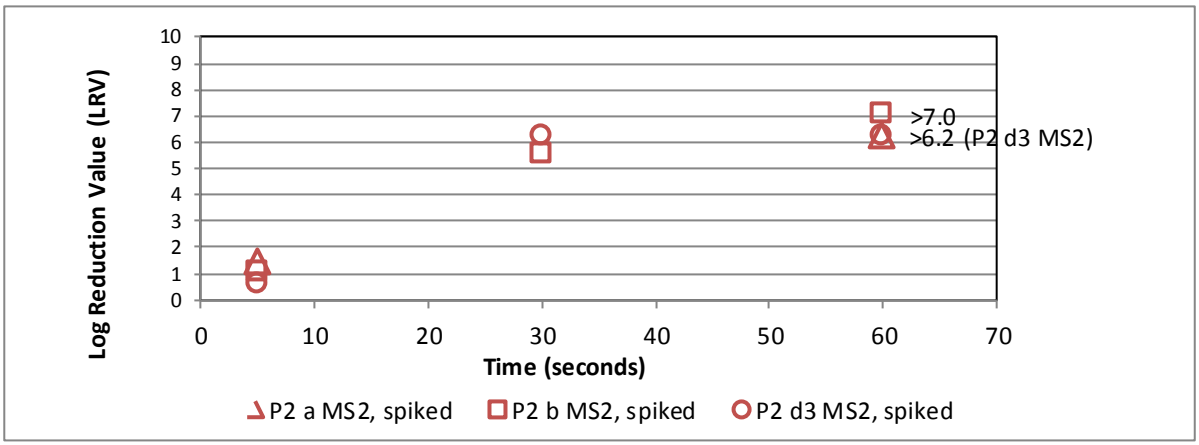

Figure 3.4: Laboratory pasteurisation treatment of MS2 in Pond 2 (P2) water at 75°C, in two replicate experiments involving spiking of cultured MS2 in different May 2014 samples of water (a and b), and in a July 2014 sample of water (d3).

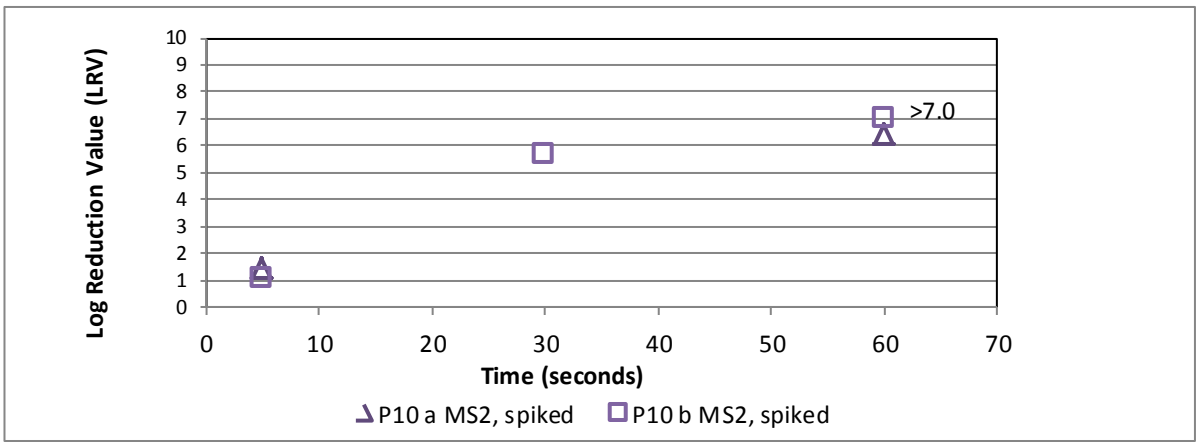

Figure 3.5: Laboratory pasteurisation treatment of MS2 in Pond 10 (P10) water at 75°C, in two replicate experiments involving spiking of cultured MS2 in different May 2014 samples of water (a and b).

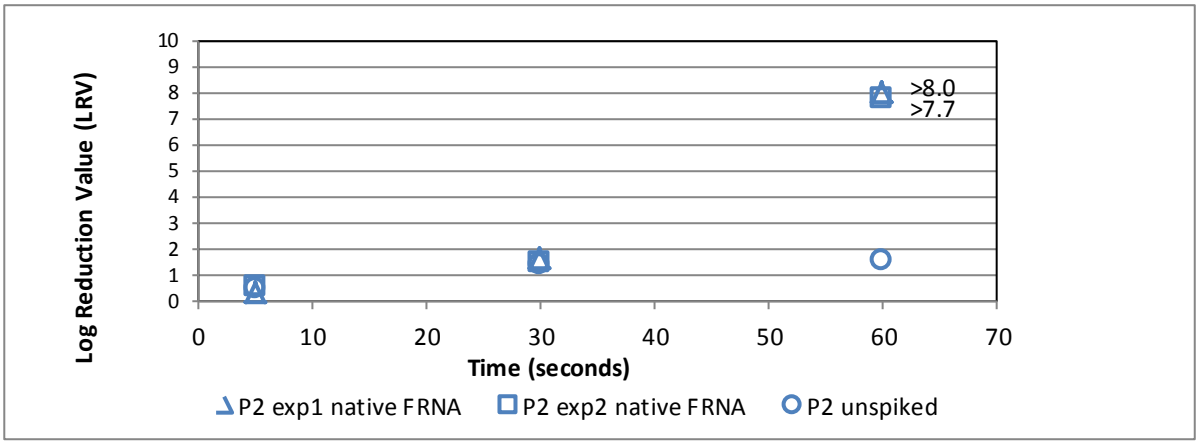

Figure 3.6: Laboratory pasteurisation treatment of native FRNA in Pond 2 (P2) water at 75°C, in two replicate experiments involving spiking of cultured native FRNA (exp1 and exp2), and without spiking (P2 unspiked).

3.3.3. E. coli

Similar inactivation levels were observed for laboratory-cultured *E. coli* spiked into Pond 2 (P2) water (Figure 3.7(a)) and Pond 10 (P10) waters (Figure 3.7(b)), indicating that the water matrix has little or no effect on *E.coli* LRV. Poor inactivation was observed at 55°C; with 0.3 LRV after 5 seconds with a maximum of 0.47 LRV after 60 seconds. The laboratory *E. coli* was completely inactivated (>6 LRV) after 60 seconds at 65°C and during the ramp to 75°C. Inactivation at 65°C (30 seconds) ranged from 1.8 – 3.2 LRV.

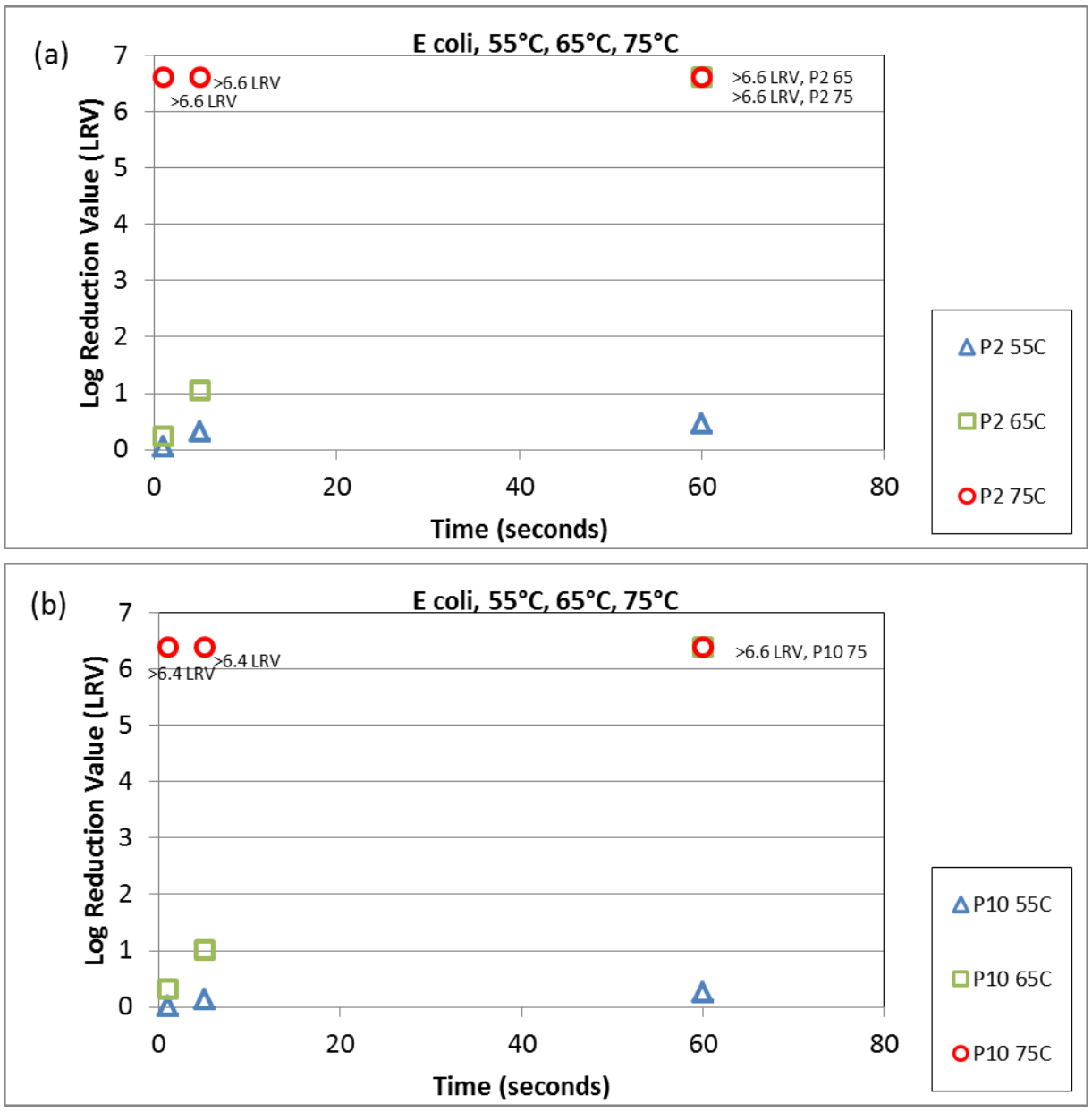

Figure 3.7: Laboratory pasteurisation treatment of *E.coli* in (a) Pond 2 water (P2), and (b) Pond 10 (P10) water.

The numbers of native *E. coli* in P2 would allow validation of 4 – 5 LRV at 65°C, so are probably too low to allow measurement of LRV at 65°C for contact times >30 s. The numbers are insufficient in P10 to use the native bacteria in validation studies.

E. coli were isolated from P10 for comparison with inactivation of the laboratory strain at 65 °C. Isolates were taken from P10 because there was too much overgrowth from other bacteria in P2 water. The results are shown in Figure 3.8.

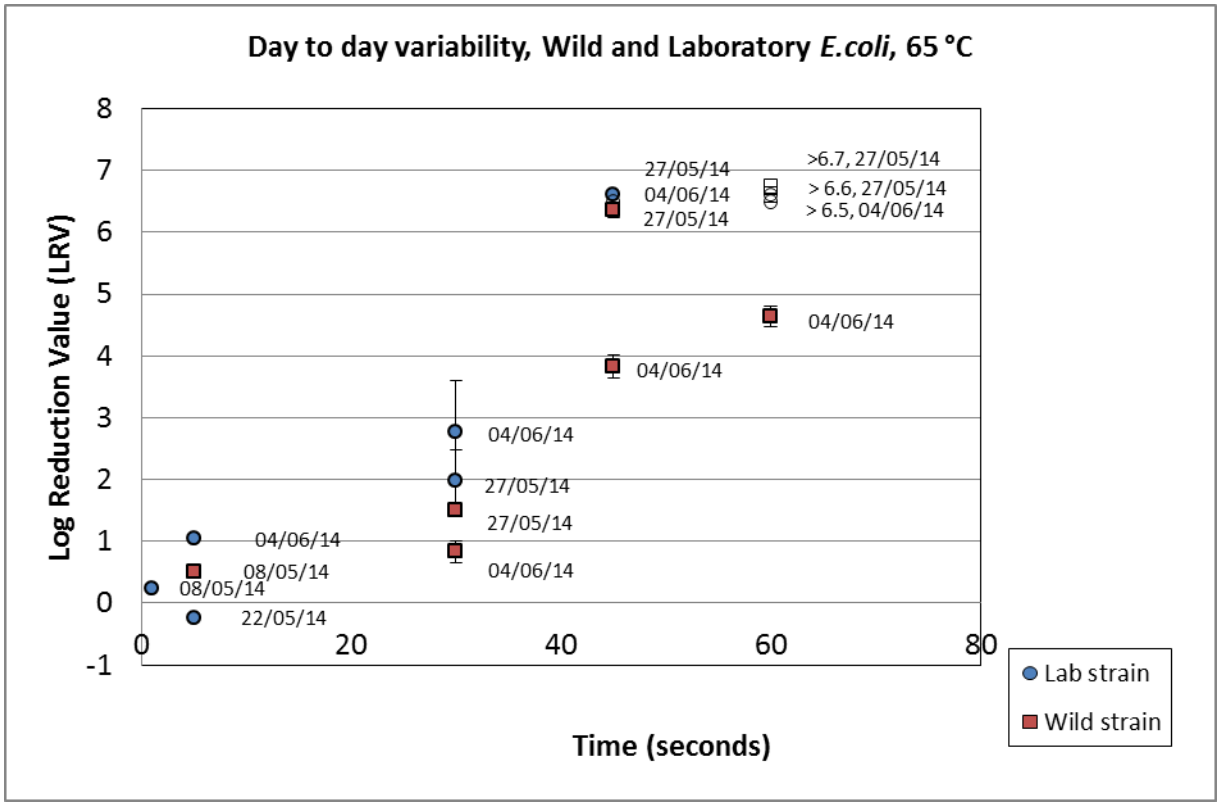

Figure 3.8: Pasteurisation treatment of *E.coli* in P2 water. Error bars represent one standard deviation for three replicate experiments. Dates signify date of assay. Empty symbols signify that the LRV was greater than the shown value.

The wastewater isolated wild strain generally had higher heat resistance compared with the laboratory strain. Replicate experiments at 65°C using P2 water have shown LRVs for the laboratory strain of 2.0 – 2.8 LRV after 30 seconds and >6.5 LRV after 60 seconds, while for the wastewater isolates, the LRVs were 0.8 – 1.5 LRV after 30 seconds and 4.6 – 6.7 LRV after 60 seconds.

The results show that both the laboratory and wild strains of *E.coli* are less heat resistant than MS2, and that the native wild *E.coli* are less heat resistant than the native FRNA (compare the 30 seconds contact time data in Figures 3.8 with that in Figure 3.3).

3.3.4. Enterococci

Triplicate inactivation experiments at 65°C and 75°C were conducted in P2 water using cultured enterococci wastewater isolates. The results are shown in Figure 3.9.

Experiments at 65°C found 0.6 LRV after 30 seconds and 2.1 LRV after 60 seconds. Inactivation was more variable, higher and faster at 75°C, with 1.8 LRV after 5 seconds, 3.2 LRV after 15 seconds and >5.9 LRV for 30, 45 and 60 seconds.

The numbers of enterococci in P2 would be sufficient to validate inactivation at 65°C but the numbers in P10 would be insufficient.

The results show that enterococci are more heat resistant than *E. coli* but less resistant than MS2.

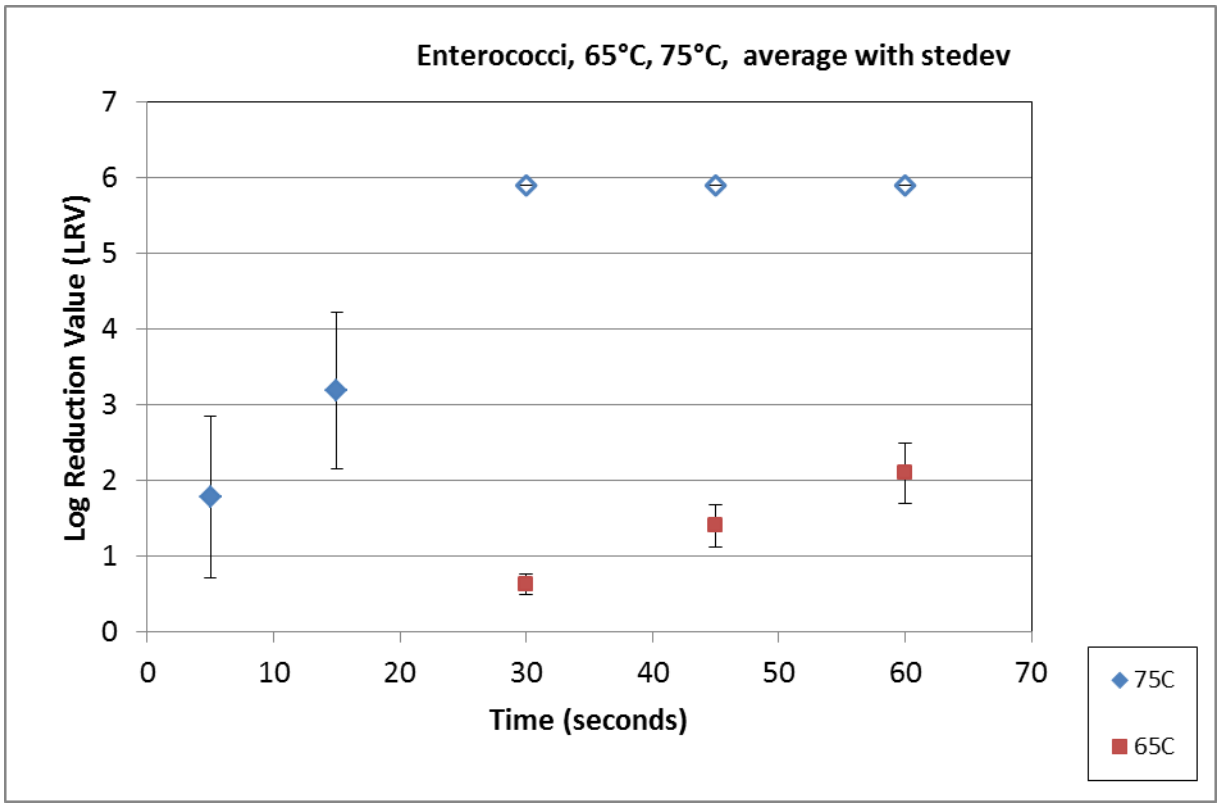

Figure 3.9: Laboratory trial results for enterococci, P2 water. Error bars represent standard errors from triplicate experiments. Empty symbols signify that the LRV was greater than 5.9.

3.3.5. *Cryptosporidium*

Cryptosporidium was found to be highly heat sensitive. Complete inactivation was observed even at 55°C for 15 seconds. Initial experiments were conducted at 55°C in milliQ water and with P2 and P10 water. No inactivation was observed for the ramp rate controls, while 2.5 - >2.6 LRV was observed after 30 seconds exposure. The data suggested that sub-lethal exposure to the elevated temperature affected the infectivity assay (possibly by affecting the excystation rate), causing what appeared to be more foci to be detected after exposure to 55°C for 1 second, compared to the room temperature control. The assay relies on infection to be initiated once oocysts are in contact with host cells, if this does not happen then it is possible that some foci are the result of infection by stray *Cryptosporidium* lifecycle stages released from the oocyst prematurely.

Additional experiments using P2 water have demonstrated >2.9 LRV after 15 seconds & 30 seconds at 55°C, after 15 seconds at 65°C and after 5 seconds at 75°C. Combining the technical replicates to calculate LRV for all oocysts exposed to a given temperature increased the measured LRV to >3.45. A repeat experiment at 65°C for 15 seconds demonstrated >3.8 LRV (cumulative inactivation for all of the infectious oocysts treated in the experiment). A repeat of the 55°C 15 second exposure failed to reproduce the previous result. However, the results in this experiment were inconsistent for replicates, suggesting a technical issue had occurred.

The heat inactivation results were as expected, with the literature showing that *Cryptosporidium* oocysts are highly temperature sensitive with >3 LRV after 30 seconds at 55°C.

3.3.6. *Giardia*

Attempts were made to establish a *Giardia* cyst viability assay to verify published data on the sensitivity of cysts to heat. While the purchased cysts could be readily observed by microscopy and also detected by flow cytometry, the published excystation assay did not appear to promote release of trophozoites and the vital dye staining did not show any detectable difference between heat treated and control cysts. It is possible that the cysts did not maintain viability during this time, even though they were stored at 4°C. The purchase of fresh cysts would have caused excessive delays to the completion of the laboratory studies, as well as additional cost. Considering that the assay was not already established at AWQC and the additional time / cost required to establish the assay and then conduct the experiments, advice was sought from the PAC and it was decided to not continue the verification of temperature inactivation of *Giardia* cysts. The high heat sensitivity of *Giardia* and *Cryptosporidium* have already been discussed (see Section 2.4).

3.3.7. Viruses

Virus inactivation studies used Adenovirus 2 (Ad2) and Coxsackie B5 virus (CB5). Cultured laboratory strains of these viruses were spiked into P2 water. Preliminary experiments were conducted without experimental replication at temperatures of 55°C, 65°C and 75°C for 1, 30 and 60 second exposures (Figure 3.10).

Adenovirus 2: Complete inactivation (>7.6 LRV) was observed for Ad2 at 65°C and 75°C for all time exposures and for 55°C after 60 seconds. There was negligible inactivation during temperature ramping at 55°C, with 2.1 LRV after 30 seconds at 55°C. Replicate experiments were conducted at 55°C, confirming the low inactivation of Ad2 during ramping and suggesting >7.8 LRV after 60 s (Figure 3.11).

Coxsackie B5: The inactivation of CB5 was 5 LRV with a 1 second hold at 65°C (inactivation occurring primarily during ramping up and down from ambient to 65°C and back again). CB5 was completely inactivated for all times at 75°C (>7 LRV) and for 30 seconds and 60 seconds at 65°C. Although CB5 appeared to be more resistant than Ad2 at 65°C, at 55°C there was 2.5 LRV after 1 second and 5.6 LRV after 60 second. Replicate experiments with CB5 at 55°C suggest that it is more rapidly inactivated than Ad2, with 5 LRV after 15 seconds exposure (Figure 3.11), but that there is a proportion of the virus population able to survive at 55°C after 60 seconds, with a maximum of 6 LRV measured.

The results from the human enteric virus testing suggest that they will be readily inactivated at 65°C and above and that high inactivation will be achieved at 55°C for contact times of 60 s or more.

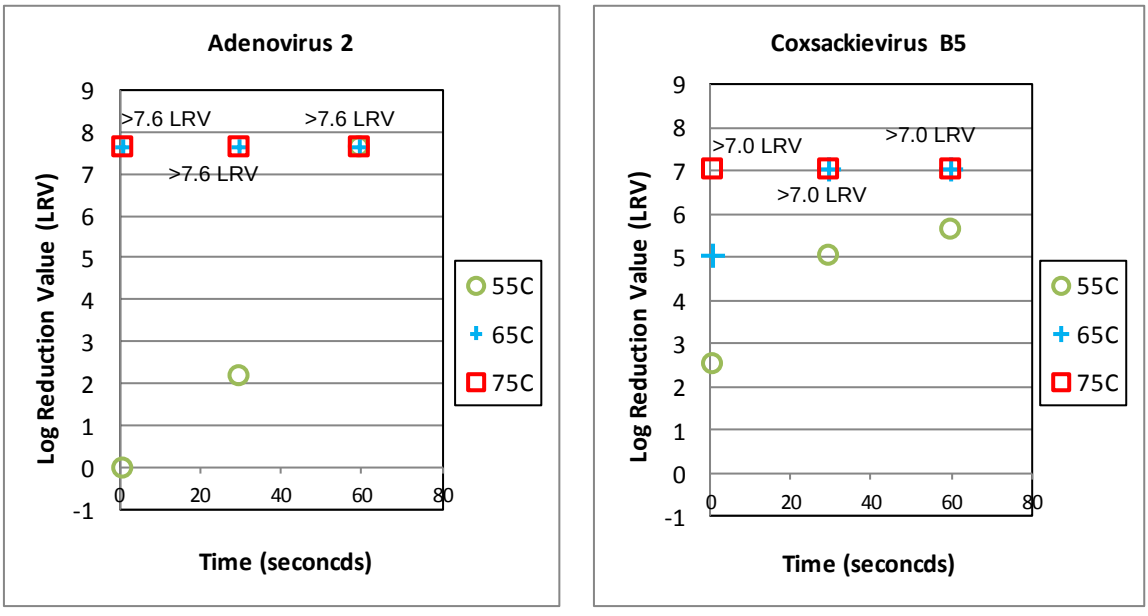

Figure 3.10: Virus inactivation in P2 water, Ad2: Adenovirus (left), CB5: Coxsackie B5 (right).

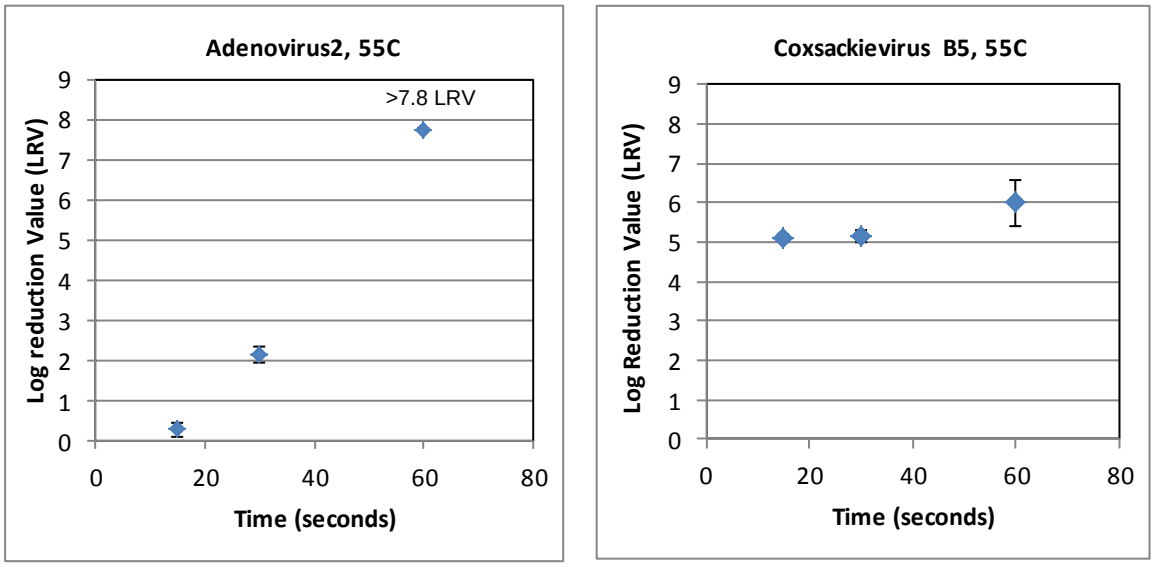

Figure 3.11: Virus inactivation at 55°C in P2 water, Ad2: Adenovirus inactivation (left), CB5: Coxsackie B5 (right). Error bars are standard deviation from triplicate experiments.

3.3.8. Helminths

After several attempts, live *Ascaris suum* were obtained from pig intestines and eggs were harvested from the worms over several days. The absolute concentration of eggs was not large, limiting the LRV that could be measured. The time required for the viability analysis was also large, limiting the work that could be completed in a single experiment. Eggs were suspended in P2 water and exposed to 55, 65 or 75°C for 1, 5, 30 or 60 seconds. Complete inactivation (>1.7 - >2.0 LRV) was observed for all time exposures at 75°C and all but the 1 second time exposure (ramping control) at 65°C (Figure 3.12). An inactivation of 0.9 LRV was observed for 55°C after 60 seconds, but no inactivation was observed for contact times of 30 seconds or lower.

As with the other pathogens, the inactivation of *Ascaris* eggs was higher compared with the proposed surrogate organisms tested in these trials. There are no comparable studies for either waste matrix or inactivation time, and the closest published study reported >3 LRV after 15 min at 55°C (Sahlstrom, 2008).

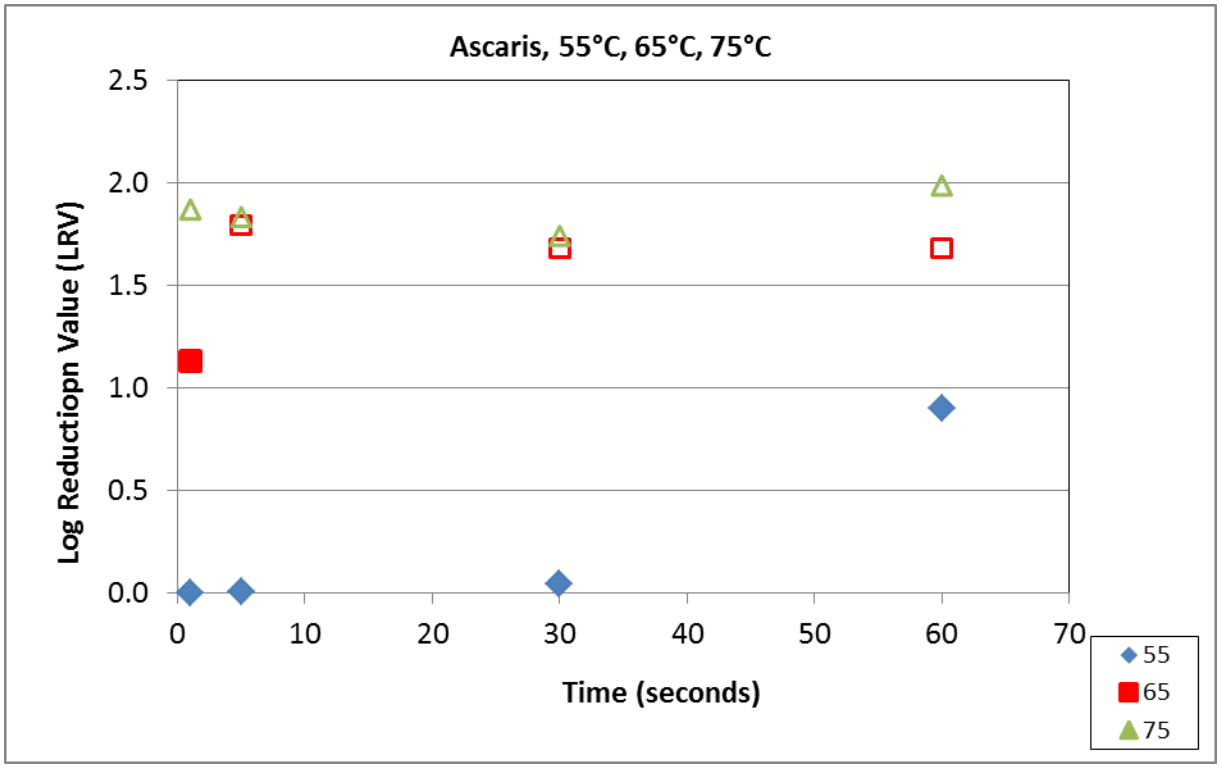

Figure 3.12: Heat inactivation of *Ascaris suum* eggs suspended in P2 water. Empty symbol signify that the LRV was greater than the shown value.

3.4. Conclusions

The temperature inactivation experiments were consistent with literature values and in some instances suggested higher sensitivity to temperature in the case of human enteric viruses.

The effect of different water quality was evaluated using MS2 phage and *E.coli*. There was no evidence for any difference in temperature inactivation for phage or *E.coli* spiked into P2 (8.5 NTU) or P10 water (1.8 NTU), suggesting that turbidity at these levels had no effect, at least at these levels.

A summary of the results is shown in Table 3.3. For the organisms tested, MS2 were the most heat resistant, followed by enterococci and *E. coli. Ascaris* and adenovirus 2 showed some survival at 55°C. Coxsackievirus B5 and *Cryptosporidium* were highly temperature sensitive, being rapidly inactivated even after brief time exposures to 55°C. Due to its higher heat resistance, MS2 was recommended as surrogate in pilot trial challenge tests.

Experiments were conducted to compare the heat inactivation of the laboratory strains with wild isolates for *E. coli* and FRNA. The wastewater isolates of *E. coli* showed similar sensitivity to temperature compared with the laboratory strain. The native FRNA appeared to be less heat sensitive compared with MS2, particularly at 75°C for 30 seconds exposure (Native FRNA LRV = ~1.5, MS2 LRV =~6, see Figures 3.4 to 3.6).

The results for the native FRNA were confounded by the presence of FDNA, which were enriched during the isolation process for the FRNA. The FDNA did not appear to impact upon the results of the MS2 spiking experiments, so are unlikely to affect the pilot trials. However, some monitoring for FDNA is recommended in case the numbers in P2 have changed between the time of the lab trials and pilot trials.

Table 3.3: Log reduction values (LRV*) achieved at 55°C, 65°C and 75°C, at various contact times.

Organism	Temperature (°C)								
	55			65			75		
	Contact time (seconds)			Contact time (seconds)			Contact time (seconds)		
	5	30	60	5	30	60	5	30	60
MS2	0		0.1			0.8	1	6	>7**
Enterococci					0.6	2	2	>6	>6
E. coli				1.0	2	>6	>6	>6	>6
Coxsackie virus		5	6	>7	>7	>7	>7	>7	>7
Ascaris	~0	~0	0.9	>2	>2	>2	>2	>2	>2
Adenovirus	~0	2	>8	>8	>8	>8	>8	>8	8
Cryptosporidium		>3			>3		>3		

* LRV = $\log_{10}$ (organism number before treatment) - $\log_{10}$ (organism number after treatment)
** when the organism number after treatment is zero, the detection limit is substituted for the organism number and the LRV is expressed as “greater than” the calculated value (>).

3.5. References

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4 PILOT PLANT TESTING AND VALIDATION

4.1. Introduction

The Pasteurization Technology Group (PTG) Pasteurisation Demonstration Unit tested is composed of heat exchangers, stack heater (also termed the waste heat recovery unit (WHRU)) and pipeline contactors as shown diagrammatically in Figure 4.1.

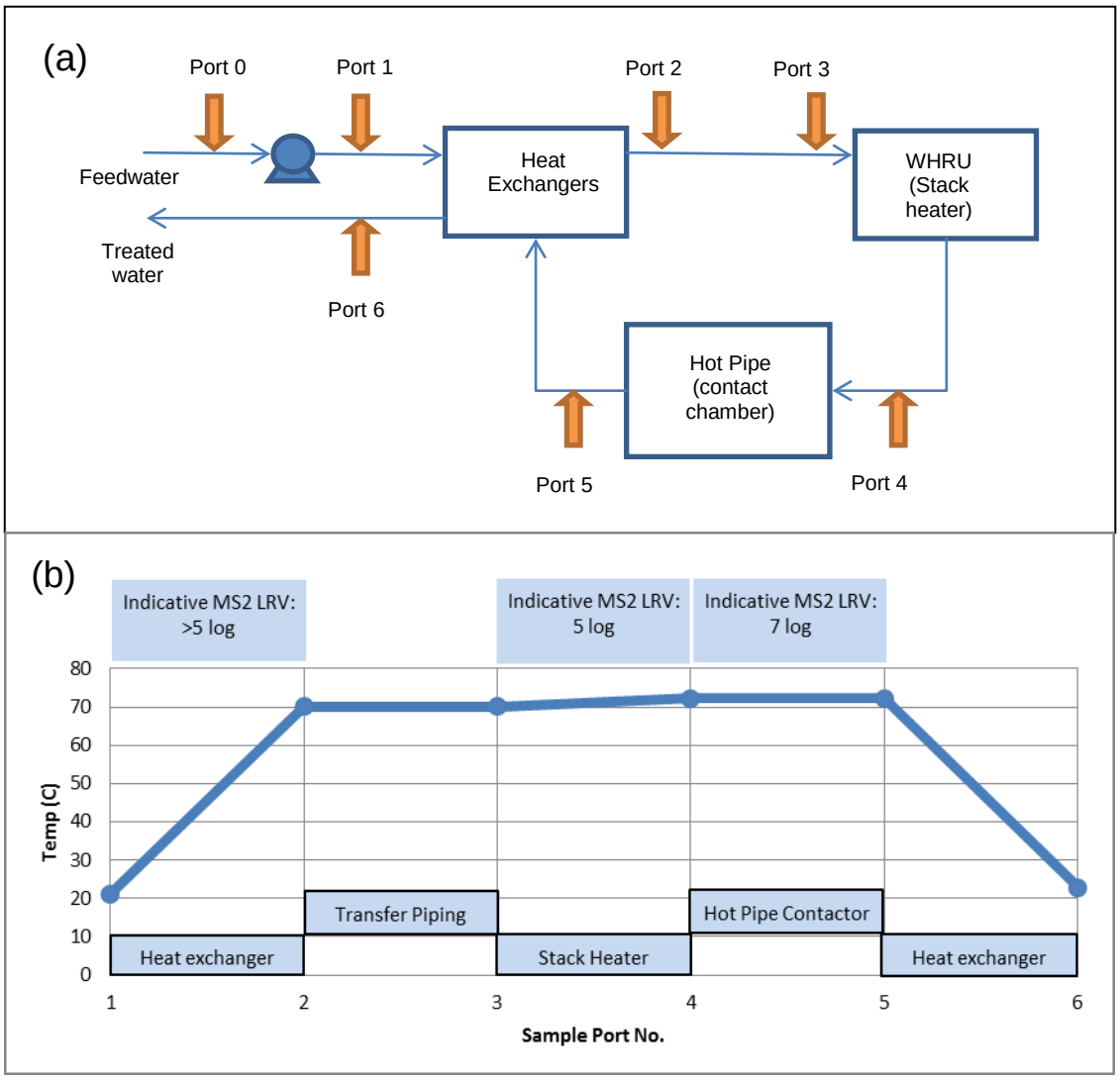


Figure 4.1: The Pasteurization Technology Group (PTG) Pasteurisation Demonstration Unit. (a): process stages and sampling ports, and (b): Indicative MS2 reductions across different sampling ports at 1,060 L/min feed flow rate (30 seconds contact time) as determined in previous trials at Ventura, California (Carollo Engineers, 2012).

Following consultation with the Victorian Department of Health, prior to the commencement of this testing program, it was decided that validation testing of this pasteurisation test unit will concentrate on the disinfection performance of the pipeline contactors (contact chamber), i.e., testing across ports 4 and 5. The results of this testing are expected to be a very conservative estimate of the capability of the entire treatment unit since the time the water spends in the heat exchangers at elevated temperatures also contributes to the disinfection performance of the test unit (Carollo Engineers, 2012), i.e. the performance of the entire PTG pasteurisation unit is (or should be) greater than the performance of the contact chamber tested in these studies.

In addition to testing of the system contact chamber, investigation of inactivation of bacteria was also conducted in this project by testing of native *E.coli* levels across the entire plant, by sampling at ports 1 and 6 (see Figure 4.1). These tests were also conducted with MS2 coliphage injection at port 0 to confirm the higher heat resistance of MS2 relative to native *E.coli* as was determined in laboratory trials.

The heat transfer efficiency of the PTG pasteurisation system is dependent upon the use of a series of heat exchangers to transfer heat from disinfected water to undisinfected water. From an operations standpoint, the concern about heat exchangers is the potential for both mineral and biological fouling which can reduce heat transfer. As part of the pasteurisation trials in Ventura California, the research team reported biofouling as part of normal operation (~70 to ~74°C) and mineral fouling at temperatures of ~80°C and above (Carollo Engineers, 2012). However, the project team in Ventura noted that biofouling was readily controlled as part of monthly shock dosing of hypochlorite, and mineral fouling was controlled by a chemical cleaning process using citric acid. Past trials in Ventura, California have shown that mineral fouling is more prevalent and aggressive at or above 82°C than at 70 to 74°C. At 71°C and less, fouling was minimal and was primarily associated with biofouling, with estimated time between cleaning of 30 to 45 days (Carollo Engineers, 2012). As the pasteurisation process is not required to run at temperatures above 70 to ~74°C, mineral fouling is not anticipated to be problematic. Any future pasteurisation system will need to monitor biofouling to maintain efficient heat transfer and provide the target disinfection and minimize regrowth of coliform bacteria (Carollo Engineers, 2012). This monitoring involves the measurement of the difference in temperature (delta T) between the influent water and the effluent water at or near ports 1 and 6. A difference of more than 3°F (1.7°C) is seen as indicative of a decrease in heat transfer efficiency due to fouling. Fouling can also be monitored by measurement of the pressure difference across the heat exchangers (delta P) as a rise in delta T correlates with the increase in pressure differential across the plant heat exchangers.

4.2. Methods

4.2.1. Challenge tests

Challenge testing involved dosing of MS2 coliphage and sampling at different points along the treatment process path to determine the effectiveness of disinfection. Two different challenge test types were performed:

(1) Testing across the pasteurisation unit contact chamber (ports 4 and 5, see Figure 4.1). The purpose of this testing was to determine the effectiveness of the pasteurisation process conditions to inactivate the heat resistant surrogate, MS2, without the added disinfection offered by the extra contact time in the heat exchangers.

(2) Testing influent and effluent (ports 1 and 6, see Figure 4.1). The purpose of this testing was to determine the effectiveness of the entire treatment process to kill the native coliforms that can foul the heat exchangers, and to compare this to the inactivation of the heat resistant surrogate, MS2, to confirm at the pilot plant scale the relative heat sensitivity of MS2 and *E.coli* that was found in the laboratory trials.

The target flow rate and temperature conditions of the contact chamber (Ports 4 and 5) testing and the entire process (ports 1 and 6) testing are shown in Table 4.1.

Table 4.1: Target process conditions during challenge testing (L/min).

Test Label	Process stage	Temperatures (°C)	Flow rates (L/min)	Approximate contact time (minutes)
A	Contact chamber	75	1080	0.5
B		72	1080	
C		69	1080	
D		66	1080	
E		<30	1080	
F	Contact chamber	75	530	1
G		72	530	
H		69	530	
I		66	530	
J		<30	530	
K	Entire plant	68	1080	5
L		64	1080	
M		60	1080	
N		57	1080	

The pilot plant trial was conducted at Melbourne's Eastern Treatment Plant. The feedwater was sampled and analysed for total organic carbon (TOC), volatile suspended solids (VSS), electrical conductivity (EC), soluble calcium, pH, alkalinity, turbidity and UV transmittance (UVT) on the

challenge tests days. A correlation analysis was performed to determine if these water quality parameters have a measurable influence on the achieved LRV. A comparison of the water quality on the challenge test days and the quality of the water used in the laboratory trials was also performed (see Section 4.3.4).

For the contact chamber testing, the contact time at each flow rate was determined via fluorescence measurement using Rhodamine WT tracer injection at port 4 and sampling at port 5 at low water temperatures (19°C). For the entire process testing, the contact time was determined by injection of the tracer at port 0, with sampling at ports 1 and 6 at the same low temperature. The data from these studies was processed to determine the t_{10} value (i.e., the time required for the fastest 10% of the flow to pass through the system) at each flow rate according to the method outlined in Appendix D of the USEPA Guidance Manual for Disinfection Profiling and Benchmarking, 1999.

The contact chamber testing involved the injection of MS2 phage at port 4 and sampling at port 5. Once phage injection had commenced, samples were collected after a minimum of three times the contact chamber contact times. The pre-treatment MS2 concentration was determined by injection of the MS2 at port 0 and sampling at port 6 at ambient temperature (<30°C). The MS2 surrogate is known to be unaffected by these low temperatures. The high flow (~1100 L/min) tests of the contact chamber were performed on 4 separate days, taking 6 samples at each temperature, giving a total of 24 before-treatment and 24 after-treatment samples for the determination of the bottom 5^{th} percentile log reduction value (LRV) required for validation (see validation protocol, Appendix A). One batch of MS2 culture was used on each of these 4 days. The average MS2 concentration before treatment on the 4 separate days varied between 4.0 and 5.5 log. The heat in all samples from port 5 was quenched immediately by passing the sample through a spiral tube in an ice bath during sample collection. This reduced the temperature to between 40°C and 50°C. Inactivation of MS2 is negligible at temperatures less than 64°C. The samples were subsequently left in an ice bath for a further 5 minutes to ensure further cooling.

Testing of the performance of the entire process involved the injection of MS2 at a port prior to the feed pump (port 0, see Figure 4.1), and sampling at ports 1 and 6. Sampling was started after three times the entire plant contact time had elapsed. Six before-treatment and six after-treatment samples were taken at each test temperature. The port 1 results were used to determine the pre-treatment MS2 concentration. A separate batch of MS2 culture was used for each of the temperatures tested for pathogen inactivation between ports 1 and 6. The average MS2 concentration before treatment for the 4 separate temperatures varied between 4.1 and 4.5 log. Assays for MS2 and *E.coli* were performed according to the methods described in Sections 3.2.2 (p.33) and 3.2.3 (p.34).

4.2.2. Effect of turbidity tests

The native sediments in Eastern Treatment Plant (ETP) feedwater to the plant (turbidity of 2.1 NTU) were concentrated by centrifugation and the resulting pellet was resuspended in un-modified water. The resulting turbidity was 14.6 NTU.

E.coli were isolated by membrane filtration of 100 mL of water (or a dilution of the water in phosphate buffered saline so that single colonies could be observed) followed by culture on MI agar plates (as per Section 3.2.3). Plates were examined under a dissection microscope, a colony that appeared to originate from a particle was subcultured onto a MI agar plate. The subculture was then inoculated into 10 mL of Tryptone Soy Broth (TSB) and cultured with shaking overnight at 37°C. On the following day this culture was used to re-inoculate fresh 10 mL TSB bottles and these were incubated with shaking for 3-4 hours at 37°C until the culture reached an optical density of 0.6–0.8 absorbance units. To prepare samples for inactivation experiments, 10 mL of culture was pelleted by centrifugation and resuspended in 10 mL of 2.1 NTU or 14.6 NTU ETP pond water.

Inactivation experiments were carried out as described in Section 3.2.1. For these experiments temperatures of ambient (untreated control), 60°C and 65°C were investigated, using exposure times of 1 second (ramp control), 30 seconds and 60 seconds.

References:

USEPA Disinfection Profiling and Benchmarking Guidance Manual, 1999 EPA 815-R-99-013

Carollo Engineers, Testing Results of the Pasteurisation Demonstration Unit at the Ventura WRF, December 2012.

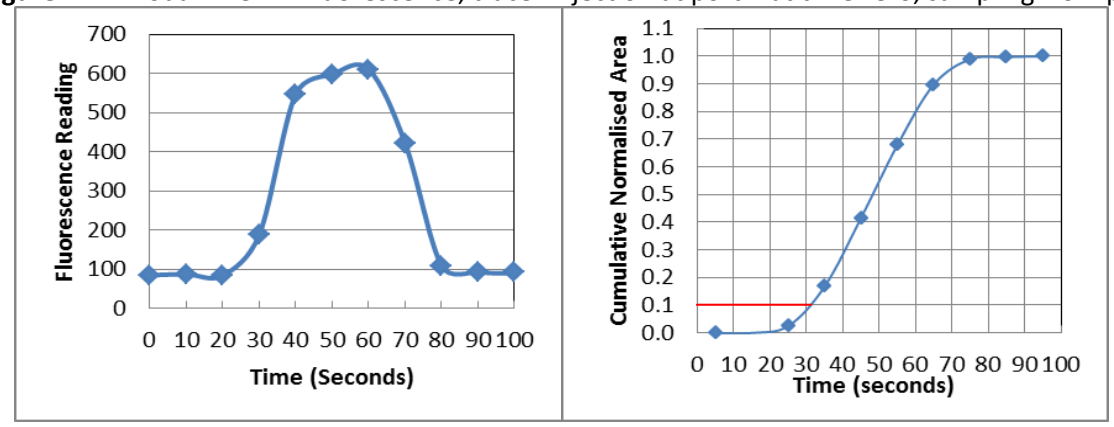
4.3. Results and discussion

The results pertaining to the determination of contact time are shown in Figures 4.2 to 4.4. The calculated contact times are shown in Table 4.2. The results pertaining to challenge testing of the contact chamber are shown in Tables 4.3 to 4.8, and summarised in Figure 4.5. The results pertaining to challenge testing of the entire process are shown in Tables 9 and 10, and summarised in Figure 4.6.

4.3.1. Contact time determination using Rhodamine WT tracer

The fluorescence readings and the resulting calculated cumulative normalised area for the tracer studies performed on the contact chamber (i.e., with tracer injection at port 4 and sampling at port 5) at 1,079 L/min and 530 L/min flow rates are shown in Figure 4.2 and Figure 4.3 respectively.

Figure 4.2: Rhodamine WT fluorescence, tracer injection at port 4 at time zero, sampling from port 5, 1,079 L/min.



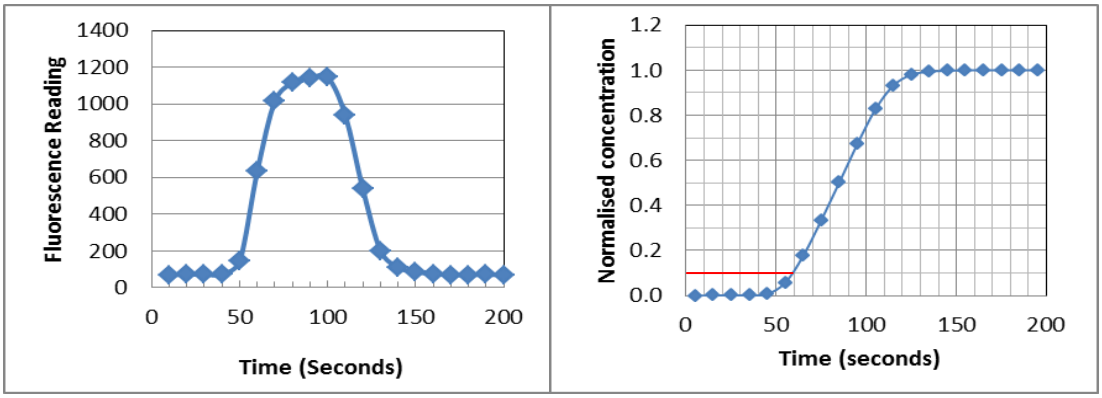


Figure 4.3: Rhodamine WT fluorescence, Tracer injection at port 4 at time zero, sampling from port 5, 530 L/min.

The fluorescence readings and the resulting calculated cumulative normalised area for the tracer studies performed on the entire process (i.e., with tracer injection at port 0 and sampling at ports 1 and 6, see Figure 4.1) at 1,079 L/min are shown in Figure 4.4. The time taken for the tracer to reach maximum fluorescence at port 1 (20 seconds) was subtracted from the port 6 times during the t_{10} calculation process.

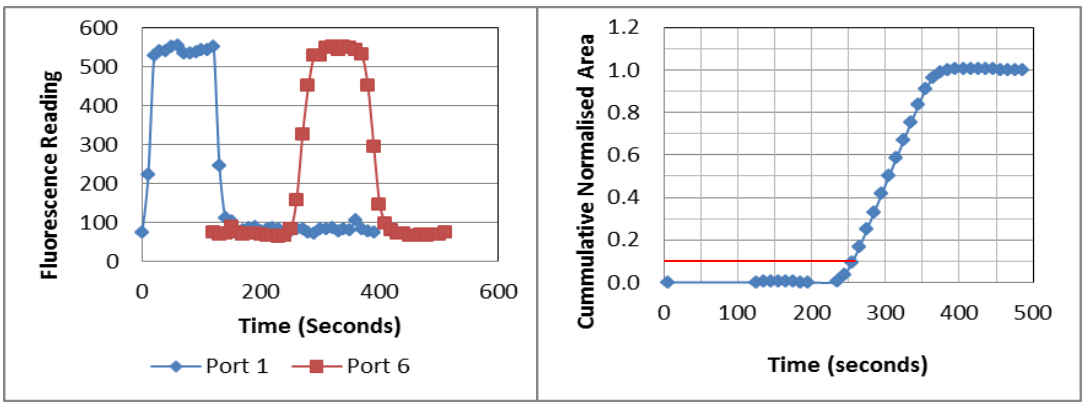


Figure 4.4: Rhodamine WT fluorescence, Tracer injection at port 0 (before pump), sampling from Ports 1 and 6, 1,079 L/min.

The calculated t_{10} values for the contact chamber (Ports 4 and 5) and for the entire process (ports 1 and 6) at the different flowrates are shown in Table 4.2.

Table 4.2: Calculated contact time (t_{10}, seconds) in the contact chamber (Ports 1 and 4) and in the entire process (ports 1 and 6) at high flow and low flow experiments.

Flow Rate (L/min)	Calculated t_{10} between ports that were tested	
	Ports 4 and 5	Ports 1 and 6
530	58	-
1,079	30	254

These results are in general agreement with those obtained by Carollo Engineers in previous pasteurisation studies. Based upon the corrected piping volumes the Ventura pilot "hot pipe" (contact chamber), the contact time at 280 gallons per minute (gpm) (1,060 L/min) was 25 seconds. For the Ventura testing at 281 gpm, the T_{10} between Ports 1 and 6 was determined to be approximately 220 seconds.

4.3.2. Contact chamber challenge tests

The results pertaining to challenge testing of the contact chamber are shown in Tables 4.3 to 4.8, and are summarised in Figure 4.5.

The low standard deviation (~0.2%) in the average temperature seen in Tables 4.3 to 4.7 indicates good control of temperature during the challenge test period (~5 minutes). The standard deviation in the flow was higher over this time period (~2%).

The standard deviation from the average microbial counts prior to treatment was generally below 2%, which is consistent with the variability in flow of approximately the same amount.

The standard deviation from the average microbial counts after treatment was generally larger than that of the before treatment counts, particularly for the higher temperatures. This is to be expected as these numbers are not only likely to be impacted by fluctuation in flow rate (i.e., contact time), but also by fluctuations in temperature and errors associated with the enumeration of low numbers of microorganisms (less than 10 at 75°C).

The standard deviation from the average LRV values was generally greater for the higher temperatures, reflecting the greater error in enumeration of low numbers of microorganisms, which are approaching the limit of detection. There was generally good agreement between the high flow (30 seconds contact time) LRVs obtained on four different days. The overall averages at each temperature are shown in Table 4.8.

The average LRV for the contact chamber tests are summarised in Figure 4.5. As expected, the LRV values at the lower flow rate (571 L/min, contact time of 58 seconds) were higher than those at high flow rates (~1,100 L/min, 30 seconds). This difference, however, was lower at 75°C and 66°C than at 72°C and 69°C. These results are in good agreement with the laboratory trials results which showed

LRV values less than 1 at both 30 and 60 seconds at 65°C, and a similarly high LRV at 30 and 60 seconds at 75°C (see Figure. 3.5, p.43). It is, however, noteworthy that the 75°C LRV at the lower flow rate was calculated from only 2 sample results since the starting MS2 concentration was lower than the other test runs, and this resulted in total inactivation for 4 of the 6 samples taken. This may have contributed to the low LRV as it is not known how high the LRV values would have been had the starting concentration been high enough to achieve a measurable LRV for all 6 samples.

Table 4.3: First high flow challenge test of contact chamber, count averages of 6 data points, all samples positive for MS2 plaques, conducted on 24-04-15.

Contact Chamber Temperature (°C)		Flow (L/min)		Before-Treatment Count* (Log pfu/mL)		After-Treatment Count (Log pfu/mL)		LRV	
Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
74.9	0.13	1109	26			-0.21	0.38	4.23	0.48
72.1	0.06	1110	28			1.81	0.11	2.21	0.20
69.1	0.04	1099	23			3.28	0.04	0.74	0.14
66.2	0.13	1089	23			3.22	0.15	0.80	0.25
28.7	0.15	1082	5	4.02	0.10				

* The pre-treatment MS2 concentration was determined by injection of the MS2 culture at low temperature (<30°C).

Table 4.4: Second high flow challenge test of contact chamber, count averages of 6 data points, all samples positive for MS2 plaques, conducted on 27-05-15.

Contact Chamber Temperature (°C)		Flow (L/min)		Before-Treatment Count* (Log pfu/mL)		After-Treatment Count (Log pfu/mL)		LRV	
Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
74.8	0.05	1054	26			0.15	0.17	5.28	0.32
72.3	0.19	1070	28			2.96	0.16	2.47	0.31
68.9	0.19	1063	165			4.59	0.15	0.84	0.30
28.7	0.34	1052	10	5.43	0.15				

* The pre-treatment MS2 concentration was determined by injection of the MS2 culture at low temperature (<30°C).

Table 4.5: Third high flow challenge test of contact chamber, count averages of 6 data points, all samples positive for MS2 plaques, conducted on 02-06-15.

Contact Chamber Temperature (°C)		Flow (L/min)		Before-Treatment Count* (Log pfu/mL)		After-Treatment Count (Log pfu/mL)		LRV	
Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
75.1	0.16	1105	25			0.43	0.30	4.99	0.32
72.3	0.19	1097	28			3.11	0.05	2.32	0.07
68.9	0.14	1101	27			4.40	0.03	1.02	0.05
28.3	0.30	1105	6	5.42	0.02				

* The pre-treatment MS2 concentration was determined by injection of the MS2 culture at low temperature (<30°C).

Table 4.6: Fourth high flow challenge test of contact chamber, count averages of 6 data points, all samples positive for MS2 plaques, conducted on 23-06-15.

Contact Chamber Temperature (°C)		Flow (L/min)		Before-Treatment Count* (Log pfu/mL)		After-Treatment Count (Log pfu/mL)		LRV	
Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
75.1	0.22	1072	25			-0.02	0.33	5.48.	0.35
72.3	0.05	1069	29			3.02	0.04	2.44	0.06
69.0	0.11	1068	22			4.45	0.03	1.00	0.05
28.5	0.18	1094	6.42	5.46	0.02				

* The pre-treatment MS2 concentration was determined by injection of the MS2 culture at low temperature (<30°C).

Table 4.7: Low Flow challenge test of contact chamber, count averages of 6 data points, all 72°C, 68°C and 66°C samples positive for MS2 plaques, 2 of 6 75°C samples positive for MS2 plaques, conducted on 22-04-15.

Contact Chamber Temperature (°C)		Flow (L/min)		Before-Treatment Count* (Log pfu/mL)		After-Treatment Count (Log pfu/mL)		LRV	
Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
75.2	0.05	574	12			-0.31**	0.30	5.27.	0.43
72.3	0.11	571	11			0.85	0.19	4.11	0.32
69.2	0.13	569	10			3.34	0.03	1.63	0.16
66.2	0.11	578	10			4.36	0.18	0.61	0.31
25.10	0.06	573	41	4.96	0.13				

* The pre-treatment MS2 concentration was determined by injection of the MS2 culture at low temperature (<30°C). **Average of two data points as only two of the six samples were found to contain MS2 phage.

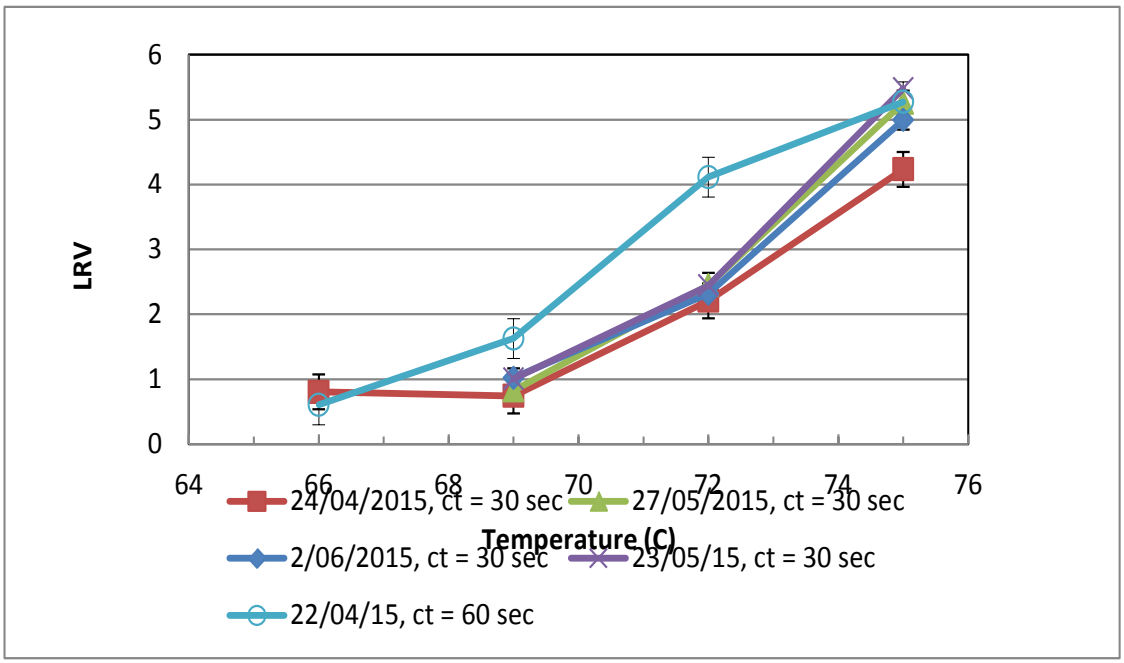


Figure 4.5: MS2 inactivation between ports 4 and 5, high flow challenge test (~1100 L/min) on 24-04-15, 27-05-15, 02-06-15 and 23-06-15 (contact time = ~30 seconds). Low flow challenge test on 22-04-15 (contact time =~60 seconds). The 571 L/min, 75°C data point represents the LRV calculated from the average of 6 before-treatment and only 2 after-treatment samples. All other data points represent the LRV calculated from the average of 6 before-treatment and 6 after-treatment samples. Error bars are one average standard deviation.

Table 4.8: Average high flow (~1,100 L/min, ~30 seconds contact time) challenge test of contact chamber conducted on 24/04/2015, 27/05/2015, 2/06/2015 and 23/06/2015, count average of 24 data points.

Temperature (°C)	Average LRV	SD
75	5.0	0.5
72	2.4	0.1
69	0.9	0.1

The LRV results achieved during the current work are considerably different to those achieved for the contact chamber in the Ventura study (Carollo, 2012) where LRVs of 5.5, 7.0 and 7.2 were reported at 72°C, 73°C and 79°C respectively, using the same strain of MS2 (ATCC 15597-B1). These results are compared to the results of the current study and to the results from other US studies by Carollo Engineers and PTG in Figure 4.6.

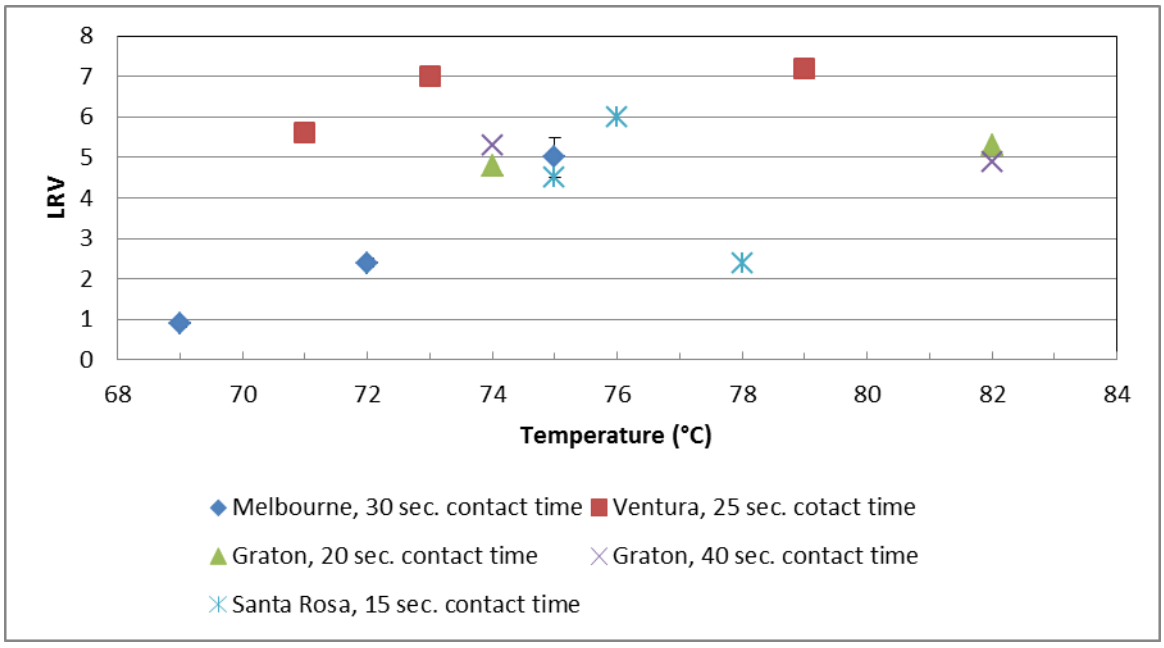


Figure 4.6: Comparison of current MS2 inactivation results to those from previous pasteurisation trials in the US.

Here it can be seen that there is some general agreement between the current study results and those for Graton and Santa Rosa. The observed difference between the Ventura trial data and the current trial data may possibly be attributable to the use of high seed doses in the Ventura trial, which can cause artificially high LRVs (USEPA, 2005). Also, the sampling protocol used at Ventura was also not fully documented, so it is unknown if the samples were adequately cooled to avoid continued pathogen inactivation after sampling.

Another possible cause of the differences observed in Figure 4.6 is a difference in water quality. The Ventura study (Appendix D, page D55, USEPA 2012) suggests that suspended solids and organic content of the feedwater may be protective at higher temperatures for bacteria (see Table 4.9 and Figures 4.7 and 4.8).

Table 4.9: Comparison of Unfiltered Effluent and Filtered Effluent BOD, Turbidity, and TSS RP&P Wastewater Pasteurisation System Validation Report Ryan Pasteurisation & Power.

Test Date	Water Quality	BOD, mg/L	Turbidity, NTU	TSS, mg/L
2/7/2006	Filtered	<2	3.1	<1
2/14/2006	Filtered	<2	0.4	<1
2/27/2006	Filtered	<2	0.7	2
3/7/2006	Filtered	<2	0.5	<2
3/28/2006	Filtered	<2	0.7	2
4/18/2006	Filtered	<2	0.4	<1
5/30/2006	Filtered	<2	0.6	1.2
3/14/2007	Filtered	<2	1.2	1.2
	Filtered Average	***<2***	***<1***	***<1.5***
6/20/2006	Unfiltered	7	3.8	7.9
3/20/2007	Unfiltered	3	3.3	5.5

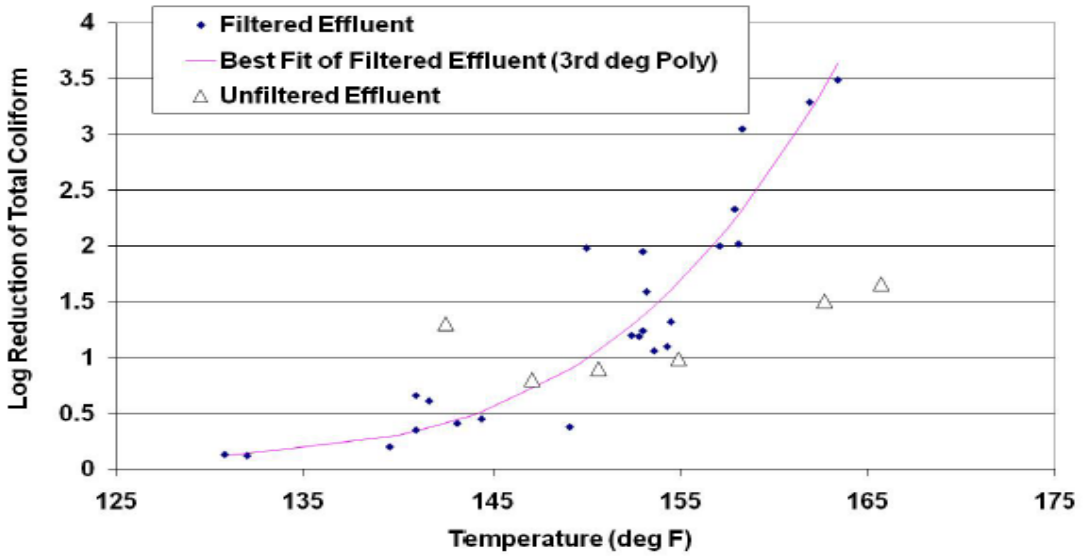


Figure 4.7: Disinfection of total coliforms in treated effluent (from Salveson et al., 2007).

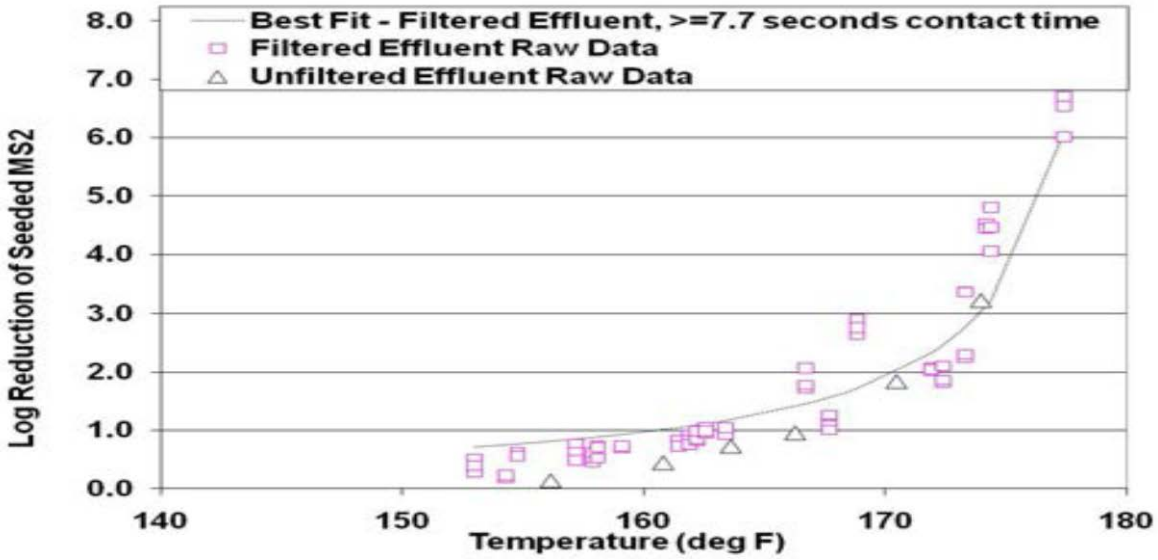


Figure 4.8: Disinfection of seeded MS2 in treated effluent (from Salveson et al., 2007).

The potential effects of water quality are further discussed in Section 4.3.4.

References:

USEPA, 2012 Guidelines for water reuse, EPA/600/R-12/618.

USEPA, Membrane Filtration Guidance Manual, EPA 815-R-06-009, November 2005

4.3.3. Whole-plant challenge tests

The results pertaining to challenge testing of the entire process are shown in Tables 4.10 and 4.11, and are summarised and compared to the contact chamber test results in Figure 4.9.

The MS2 LRV in the contact chamber was found to range from 0.9 at 66°C to 4.8 at 75°C. Since MS2 is more heat resistant than the pathogens of concern, this ensures that the expected LRV for the pathogens of concern is always greater than that of MS2. The choice of temperature, therefore, depends on the target pathogen, its concentration in the test water, and the desired final concentration of the target pathogen.

Biofouling on the effluent side of the heat exchangers can also lead to apparently poor disinfection performance despite good disinfection in the contact chamber. It has been found that faecal coliforms can colonise the effluent side of heat exchangers if a total kill of the colonising microbes is not achieved in the upstream contact chamber (Carollo Engineers, 2012). So, apart from the health risks and mitigation requirements, there is a need to achieve total kill of total and faecal coliforms to prevent biofouling of the heat exchangers. Similar concerns for regrowth are equally valid for UV disinfection and any oxidant based disinfection after the oxidant is quenched.

The level of native *E.coli* in the test water was found to be 5 Log (see Table 4.10). From Figure 4.9, it can be seen that to achieve a total kill of *E.coli* of 5 log across the entire treatment plant (ports 1 and 6) requires a temperature of at least 71.5°C, giving an MS2 LRV in the contact chamber (ports 4 and 5) of at least 2 LRV.

Table 4.10: Challenge test of entire pasteurisation unit, Native *E.coli* assays, count averages of 6 data points, conducted on 27-04-15.

Contact Chamber Temperature (°C)		Flow (L/min)		Before-Treatment Count (Log cfu/mL)		After-Treatment Count (Log pfu/mL)		LRV	
Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
68.3	0.1	1112	23	4.76	0.06	1.88	0.27	2.89	0.33
64.2	0.1	1114	19	4.75	0.05	3.27	0.12	1.48	0.16
60.2	0.2	1121	21	4.93	0.11	4.14	0.05	0.78	0.15
57.3	0.2	1122	19	4.96	0.20	4.55	0.06	0.41	0.27

Table 4.11: Challenge test of entire pasteurisation unit, MS2 assays, count averages of 6 data points, all samples positive for MS2 plaques conducted on 27-04-15.

Contact Chamber Temperature (°C)		Flow (L/min)		Before-Treatment Count (Log pfu/mL)		After-Treatment Count (Log pfu/mL)		LRV	
Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
68.3	0.1	1112	23	4.14	0.03	3.37	0.18	0.76	0.22
64.2	0.1	1114	19	4.16	0.04	4.18	0.04	-0.02	0.09
60.2	0.2	1121	21	4.41	0.03	4.44	0.07	-0.03	0.09
57.3	0.2	1122	19	4.45	0.03	4.45	0.02	0.00	0.05

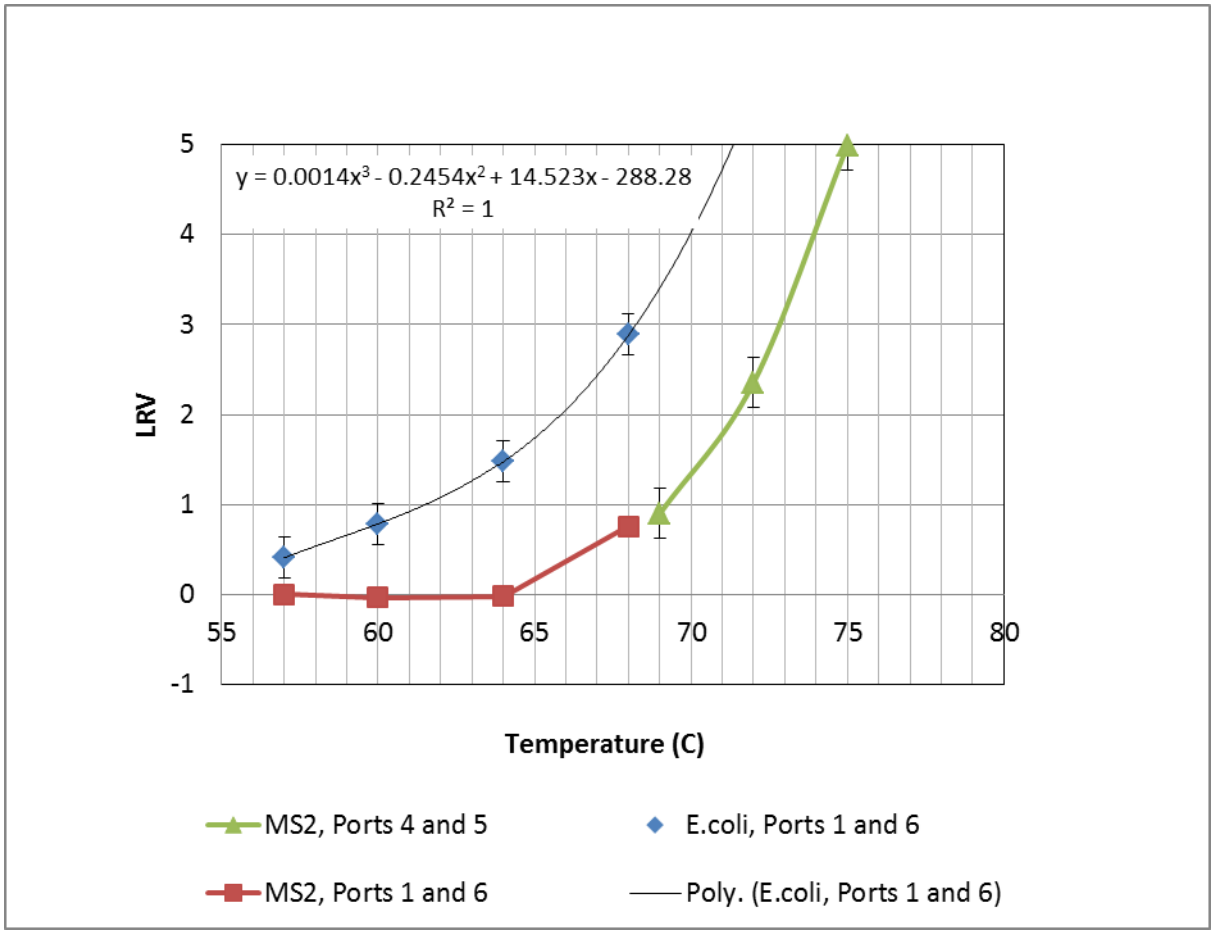


Figure 4.9: Comparison of disinfection performance at high flow rate, ~1,100 L/min) for the entire plant (ports 1 and 6, added MS2 and native *E.coli,* 254 seconds contact time) with the disinfection achieved in the contact chamber (ports 4 and 5, added MS2, 30 seconds contact time).

References:

Carollo Engineers, Testing Results of the Pasteurization Demonstration Unit at the Ventura WRF, December 2012.

4.3.4. Water quality on challenge test days

The Eastern Treatment Plant (ETP) water quality parameters that were measured during the challenge tests are shown in Table 4.12. A comparison of this data with water quality data for the Western Treatment Plant (WTP) water used in the laboratory trials is shown in Table 4.13.

Table 4.12: Water Quality on Challenge Tests Days.

Challenge test date	Target flow (L/min)	Temperatures tested (C)	Alkalinity (mg/L as $CaCO_3$)	pH	Ca (mg/L)	Turbidity (NTU)	SS (mg/L)	VSS (mg/L)	COD (mg/L)	TOC (mg/L)	EC (uS/cm)	UVT (%)
22/04/2015	355	75, 72,69, 66	36	6.8	17	3.5	13	12	60	13	830	50
24/04/2015	1100	75, 72,69, 66	60	6.6	16	3.5	16	6	65	14	850	44
27/04/2015	1100	68, 64, 60, 57	42	6.6	16	6.8	24	6	60	15	830	41
27/05/2015	1100	75, 72, 69	59	6.9	18	4.6	24	4	70	15	890	35
2/06/2015	1100	75, 72, 69	43	6.6	16	2.3	16	2	50	13	730	43
23/06/2015	1100	75, 72, 69	53	6.7	17	9	23	33	44	16	830	40

Table 4.13: Comparison of water quality parameters of ETP and WTP waters.

	ETP (on challenge test days)	WTP April 2014 Sample		WTP May 2014 Sample	
		Pond 2	Pond 10	Pond 2	Pond 10
COD (mg/L)	44 - 70	104	223	127	84
TOC (mg/L)	13 -16	21	11	29	9
EC (µS/cm)*	730 - 890	2150	1860	2170	1700
SS (mg/L)	13 – 24	12	4		
VSS (mg/L)	2 - 33	11	2		
Turbidity (NTU)	2.3 - 9	2.5	1.8		
pH	6.6 – 6.9	7.3	8.2		

*1000 µS/cm =~500 mg/L salt = 0.5 g/L = 0.05% w/v

The ETP water on the challenge test days had lower COD, TOC, EC and pH values. The WTP VSS values were within the range of values found in ETP water. The turbidity and SS of ETP water was generally higher than that of WTP water. These differences between ETP and WTP water, however, are not sufficient to influence the heat sensitivity of pathogens. The protective effect of salt, for example, is very small at the salt content of the ETP and WTP waters (less than 1 g/L or 0.1 % w/v). Similarly, the pH of ETP water is not sufficiently low to induce the protective effect associated with acid stress. Further discussion of the likely effect of water parameters on pasteurisation performance can be found in the Validation Protocol attached as an appendix to this report (Appendix 1).

The achieved LRVs at 75°C are plotted against the water quality parameters (Table 4.12) in Figure 4.10. The 12-month historical data record for turbidity, pH and UVT are shown in Figures 4.11 to 4.13. The turbidity on the challenge test days is shown in Figures 4.14 to 4.19. Effect of native turbidity on native *E.coli* at 60°C for ETP water is shown in Figure 4.20.

Wastewater quality parameters that can influence pasteurisation effectiveness are wastewater salt content, turbidity, pH and temperature.

From Figure 4.10, using the limited data available, it can be seen that there was generally poor correlation between water quality on the challenge test days and the LRV achieved on these days. The order of correlation coefficients (R^2) from highest to lowest was: SS (0.60) > Ca (0.42), > Turbidity (0.38) > TOC (0.33) > pH (0.31) > VSS (0.27) > COD (0.20) > UVT (0.16) > Alkalinity (0.09) > EC ($8x10^{-5}$). Only two of the parameters showed a trendline with a negative slope that would be indicative of a decrease in LRV with increase in the parameter (Alkalinity and COD). This lack of clear correlation is consistent with these parameters having no influence on inactivation of pathogens over the range of water quality variation in these experiments.

Salt content of the order of 1 to 2% w/v has been found to be protective to bacteria and viruses (Besten, 2010; Juneja, 2013; Volkin, 1997; Mazotta, 1999). The protective effect of salt is very small at the typically salt content of municipal wastewater (usually less than 1 g/L or 0.1 % w/v). The salt content of Eastern Treatment Plant (ETP) water (EC = <900 µS/cm ~= <450 mg/L or 0.045% w/v) is well below protective levels and examination of the historical record over a 12-month period reveals that the salt level has never reached protective levels.

The presence of suspended matter also has the potential to affect disinfection effectiveness of pasteurisation if the particles can decrease the rate of heat transfer to the microbes. The results of 2007 wastewater pasteurisation trials (see Figures 4.7 and 4.8) suggests that water quality plays a role in pasteurisation disinfection kinetics, particularly with regard to coliform disinfection. The water quality results presented in Table 4.9 suggest that suspended solids and organic content may play a role.

Examination of the historical record of turbidity (Figure 4.11), pH (Figure 4.12) and UV transmittance (Figure 4.13) over a 12-month period shows relatively stable water quality over this period, indicating that the heat sensitivity of the pathogen it contain would remain constant. The turbidity data had an average of 4.9 NTU with a standard deviation of 4.0. The pH data had an average of 6.5 with a standard deviation of 0.2. The UVT data had an average of 44.7 with a standard deviation of 4.7.

The baseline turbidity readings ranging from 2 to 10 NTU, with numerous spikes that on the 12-month record graph look as though they are noise in the meter readings. A closer inspection of the data, however, reveals that these are not necessarily noise. Figure 4.16, for example, shows a period of sustained elevated turbidity over a 2-hour period, while Figure 4.17 shows what might be a meter “glitch” (NB None of the challenge tests coincided with a high turbidity period in the graph, all tests were conducted between 10:30 am and 12:30 pm).

The elevated turbidity periods appear to be short in duration (in Figure 4.16 the turbidity is elevated for approximately 2 hours). The average turbidity for the data presented in Figure 4.11 is 4.9 NTU, with a standard deviation of 4.0, indicating that the turbidity is below 12 NTU (3xSD) for over 99.7% of

the time (assuming a normal distribution). The effect of 15 NTU of native ETP sediment on the survival of the cultured native *E.coli* that have been added to the ETP water is shown in Figure 4.20. The data in this figure indicates that the native sediment is not protective to the added free native *E.coli* at these levels. It does not, however, shed any light on whether *E.coli* within the sediment particles are protected. The enumeration and degree of inactivation of *E.coli* within the sediment particles is experimentally very challenging and is a topic that deserves further attention.

The growth phase that the bacteria are in can also markedly impact the heat resistance of bacteria, with late stationary phase cells being more heat resistant than log phase cells (Kaur, 1997). Similarly, bacterial cells that are in starvation conditions are more heat resistant than cells in nutrient rich environment (Casadei, 1998). It is difficult to make any definite conclusions regarding the bacterial growth conditions from the available data but it is noteworthy that the bacterial pathogens in the wastewater e.g. *Campylobacter*, *Salmonella*, and some forms of *E. coli*, are from the human gut and once outside the gut, they are in a stressed situation and they are not likely to proliferate in the sewer or WWTP.

The pH of the feedwater can also markedly influence the heat tolerance of bacteria, with bacterial resistance being higher for acid grown bacteria than for cells grown in at higher pH conditions (Shen, 2011). Examination of the available historical data (Figure 4.12) reveals that the pH of the wastewater ranges from 6.2 to 7.0. A pH value of 6.2 is not expected to impart a measurable protective effect. Tosun et al. (2005) quantified the heat resistance of *E.coli* 0157:H7 that had been acid adapted at pH 4.5, 5.0. and 5.5 and found that acid adaptation at pH 4.5.and 5.0 provided measurable protection from heat treatment, but the heat sensitivity at pH 5.5 was not statistically different from that of a non-acid-adapted control.

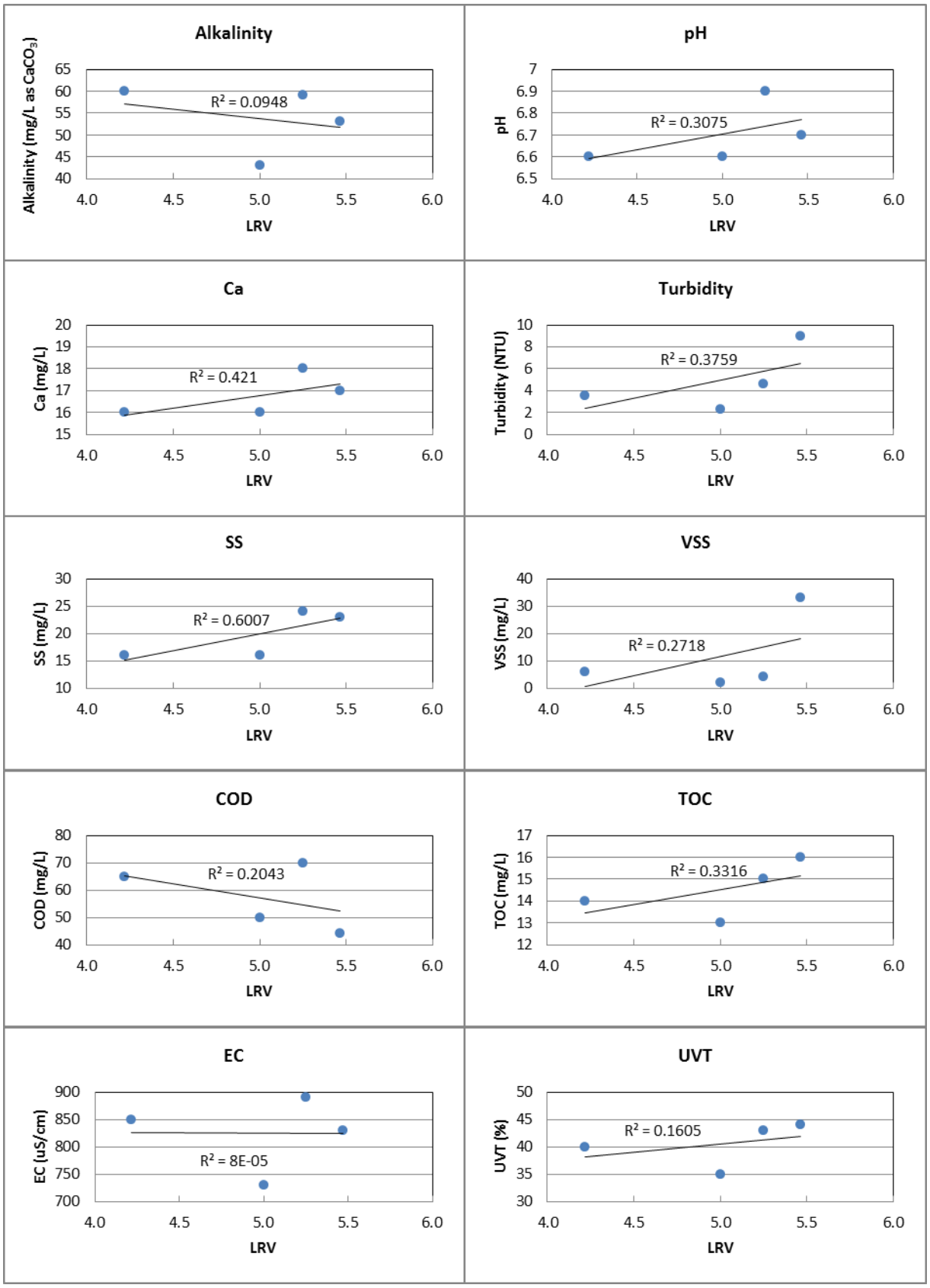


Figure 4.10: Correlation analysis, water quality vs achieved LRV at 75°C.

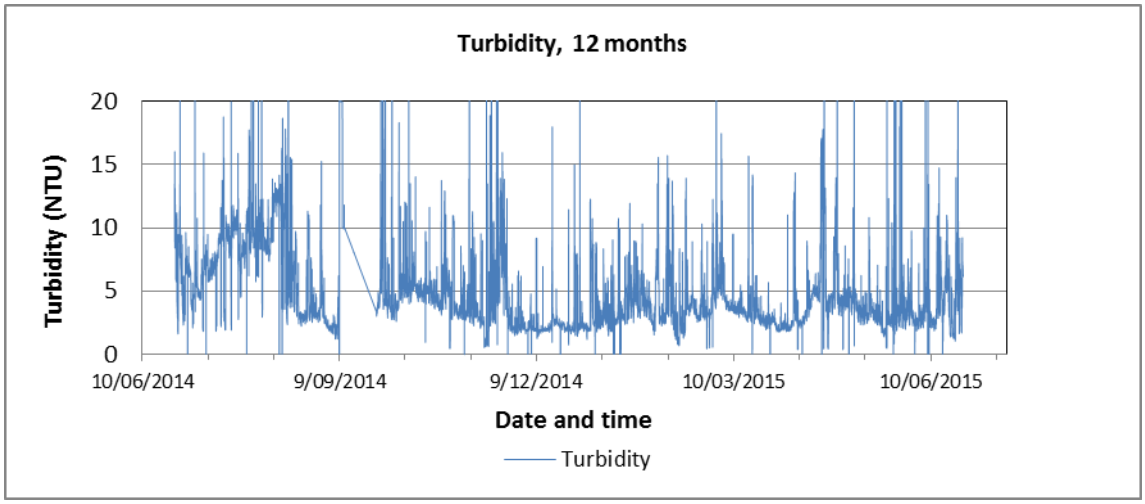


Figure 4.11: Turbidity of feed water, June 2014 to June 2015.

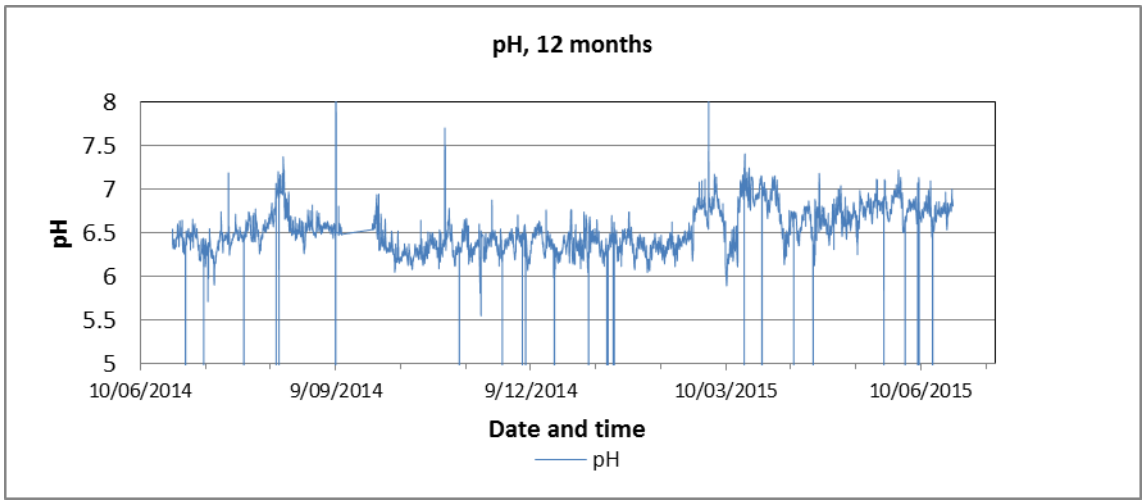


Figure 4.12: pH of feed water, June 2014 to June 2015.

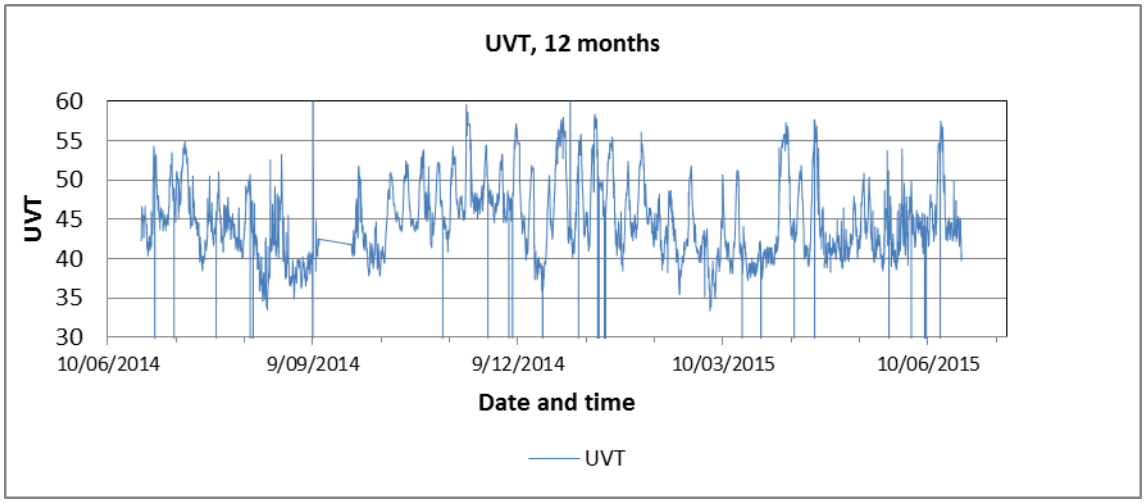


Figure 4.13: UVT of feed water, June 2014 to June 2015.

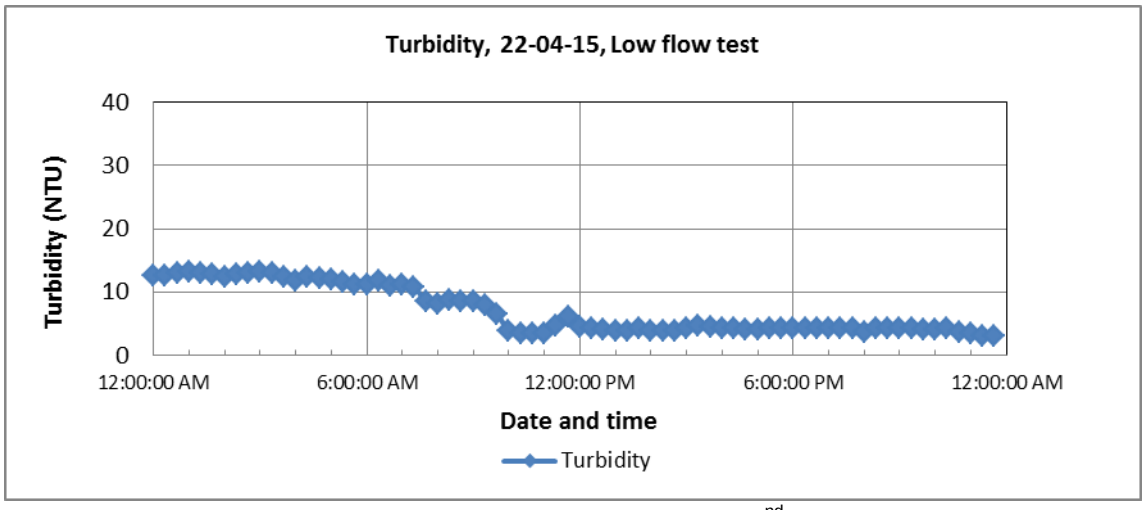


Figure 4.14: Turbidity of feed water during low flow test, April 22nd, 2015 (tests F to J, Table 4.1, results in Table 4.7).

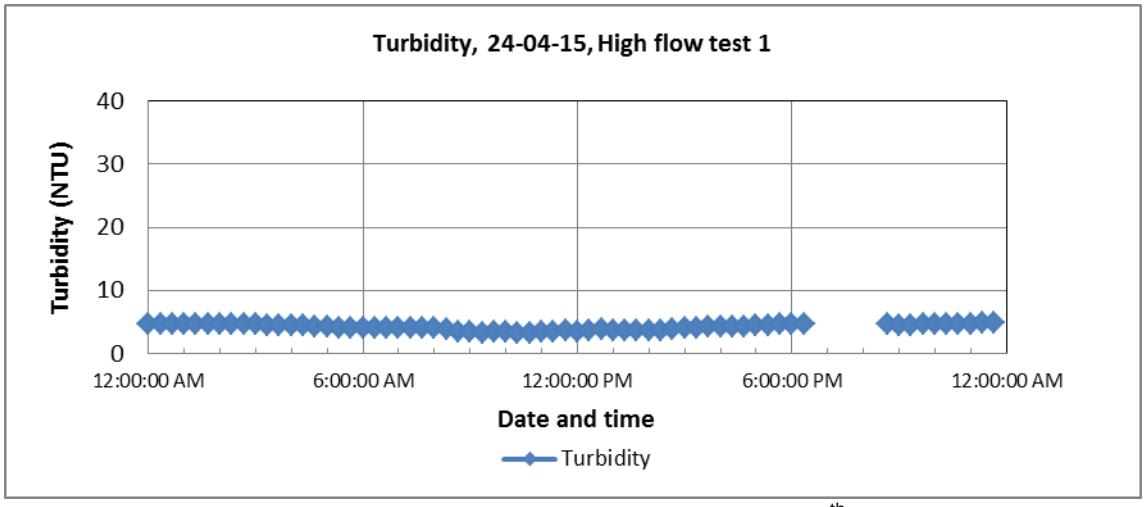


Figure 4.15: Turbidity of feed water during first high flow test, April 24th, 2015 (tests A to E, Table 4.1, results in Table 4.3).

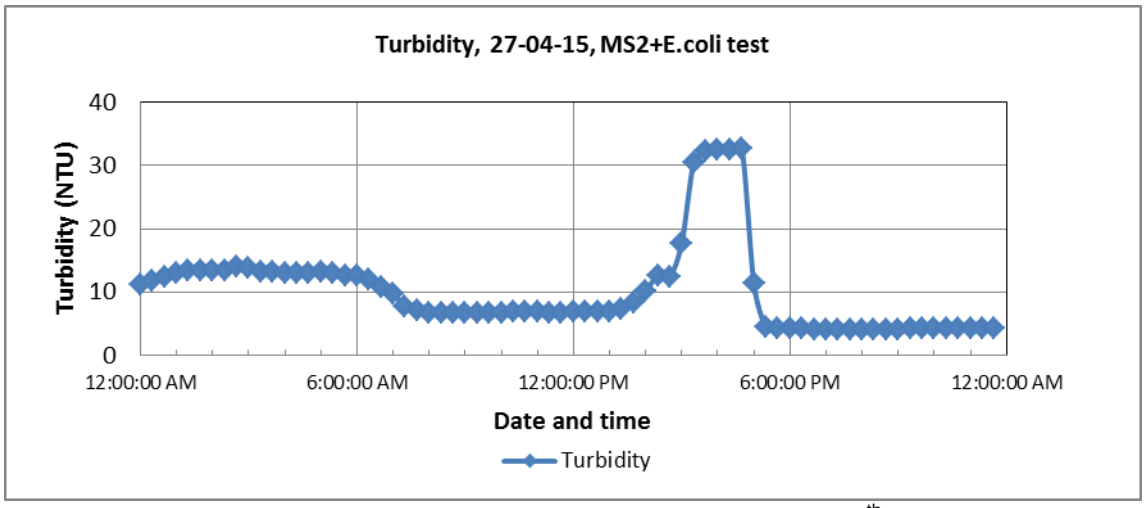


Figure 4.16: Turbidity of feed water during test of entire process, April 27th, 2015 (tests K to N, Table 4.1, results in Tables 4.10 and 4.11).

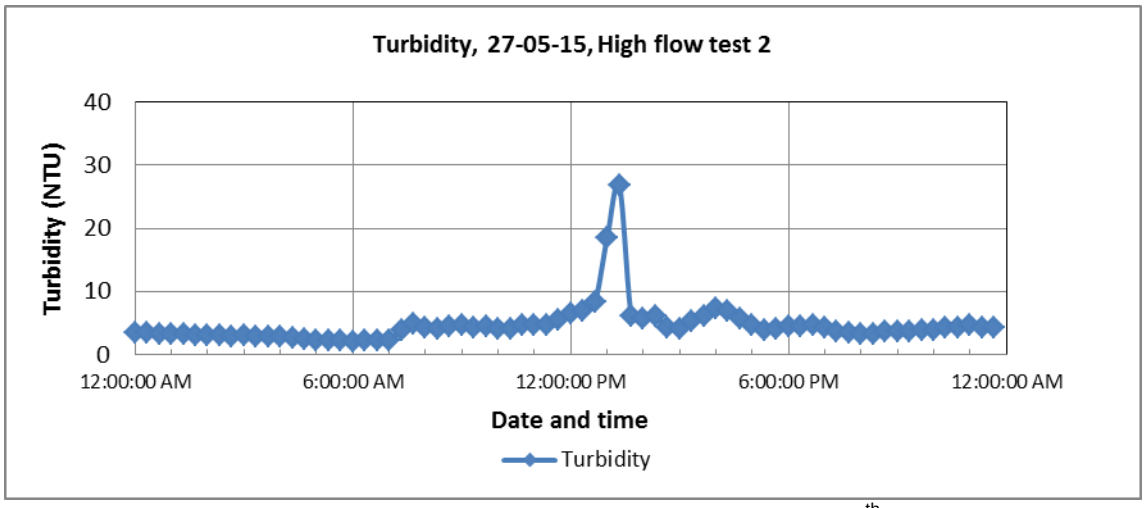


Figure 4.17: Turbidity of feed water during second high flow test, May 27th, 2015 (tests A to E, Table 4.1, results in Table 4.4).

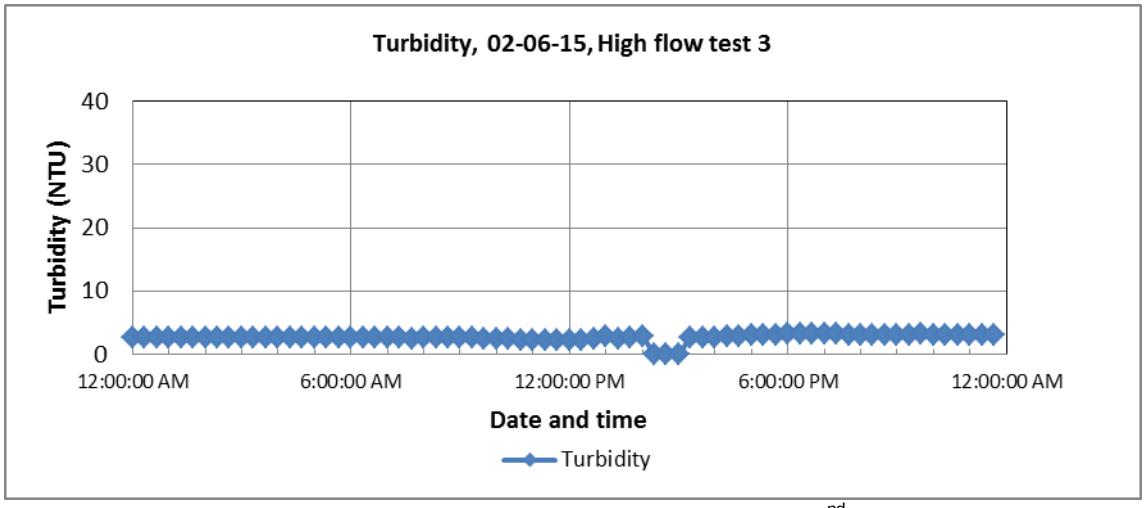


Figure 4.18: Turbidity of feed water during third high flow test, June 2nd, 2015 (tests A to E, Table 4.1, results in Table 4.5).

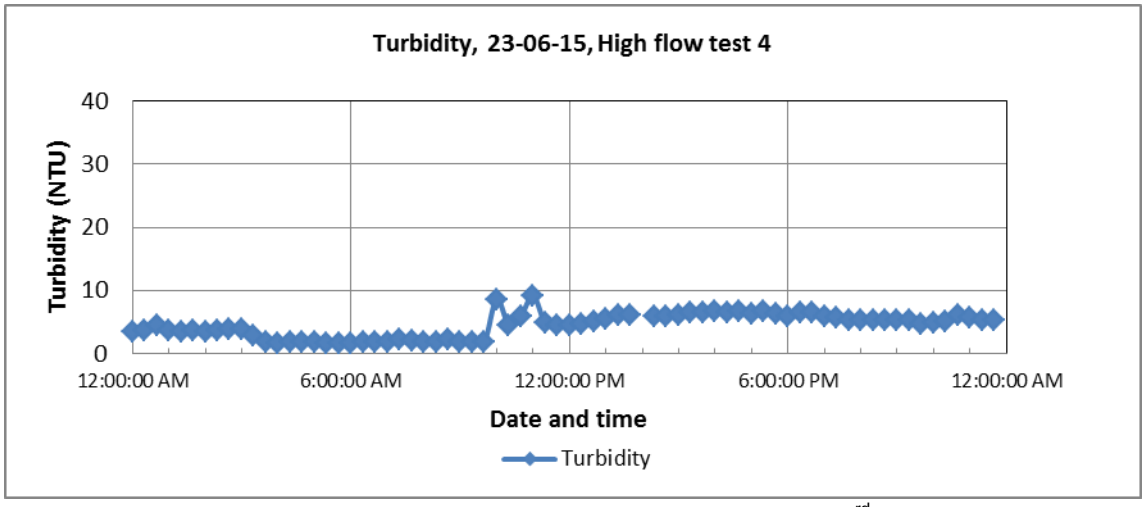


Figure 4.19: Turbidity of feed water during fourth high flow test, June 23rd, 2015 (tests A to E, Table 4.1, results in Table 4.6).

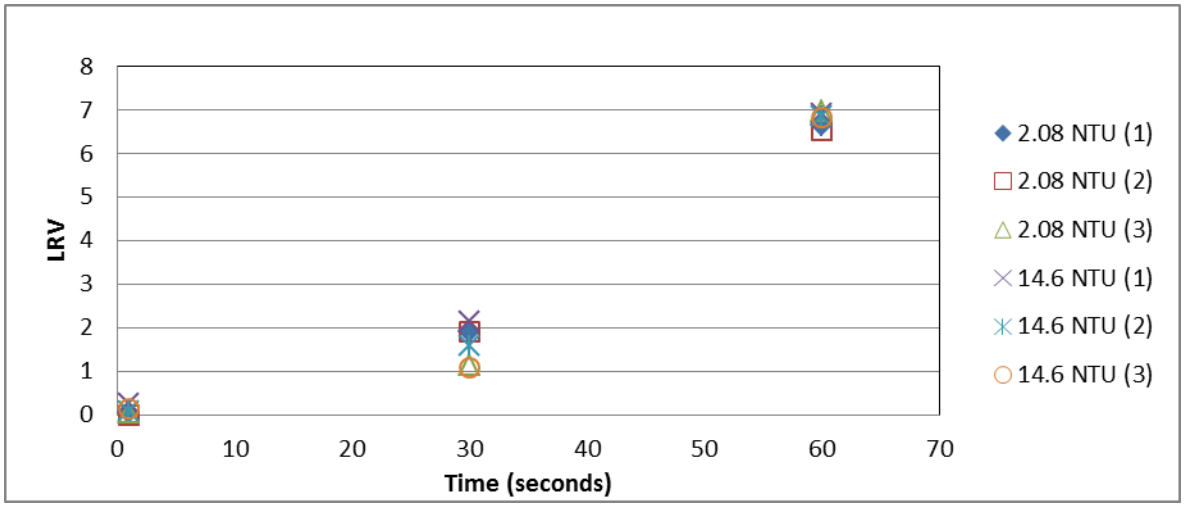


Figure 4.20: Effect of native turbidity on added cultured native *E.coli* at 60°C, ETP water, sampled 26-05-15.

References:

Ahmed, N.M., Conner, D.E. and Huffman, D.L. Heat-Resistance of Escherichia Co/i 0157:H7 in Meat and Poultry as Affected by Product Composition, Journal of Food Science,-Volume 60, No. 3, 1995.

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Tosun, H. and Gonul, S.A, The effect of acid adaptation conditions on heat resistance of *Escerichia coli O157:H7*, Polish Journal of Microbiology, 2005, vol.54, No. 4, 295-299.

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USEPA, 2012 Guidelines for water reuse, EPA/600/R-12/618.

4.3.5. Validation LRV

The Victorian Department of Health "Guidelines for validating treatment processes for pathogen reduction" sets two restrictions of the LRV that is attributed to the process:

(1) That the maximum LRV that can be attributed to the process is 4 Log; and
(2) The lower 5th percentile LRV established during challenge testing must be used. This requires at least 20 samples.

Although the guideline states that the LRV must be calculated by subtracting the log of the concentration of challenge surrogate after treatment from the concentration of challenge surrogate before treatment, i.e.,

LRV = LV (before treatment) – LV (after-treatment),

the guideline does not restrict the manner in which the representative LRV is calculated. Consequently, there are numerous methods that could be used to calculate the representative LRV for a test process. As stated in the USEPA Membrane Guidance Manual:

(1) If multiple feed/filtrate sample pairs are collected, a LRV can be calculated for each set of paired data, and the LRV could be selected as the lowest LRV (more conservative) or the average of the LRVs (less conservative) can be quoted.
(2) Another approach is to average all the respective feed and filtrate concentrations from among the various samples collected and calculate a single LRV for the process.
(3) A more conservative approach would be to use the average feed concentration but with the maximum post-treatment concentration sampled, which would result in a lower representative LRV.
(4) Likewise, a still more conservative approach would be to use the minimum feed and maximum after treatment concentrations.

The Membrane guidance manual notes that these methods simply represent potential options and other approaches may be used for calculating a representative LRV.

Yet another option for calculating the LRV is that used by Carollo Engineers in their previous validation studies at Ventura (Carollo Engineers, 2012). This method involves using the average of the influent samples for a given test condition and the use of each of the individual effluent samples to calculate one LRV for each after-treatment sample. This is the preferred option for calculating the LRV.

The low fluctuations in the before-treatment counts seen in the data is in agreement with Carollo Engineers justification for calculation of the LRV in the Ventura validation trials by using the average

of the influent samples and each of the individual effluent samples. In his Sat 9/05/2015 11:16 AM email to the project team wrote:

> "*During any test run, we have the MS2 dosing pump set to a single pump rate, pumping from one large container of MS2. We also have one set flow and one set temperature, which our tests show as consistent with very little variation. What this means to me is that all six samples of the seeded MS2 should be equal for any given test, and variation is more the result of analytical precision than other factors. This is especially true for the "no dose" MS2 numbers, also called "influent", as we know that the influent temperatures have no impact on MS2 concentrations. These numbers should all be the same. So, when we do our (California) analysis of this kind of work, we average the influent samples to decrease the analytical noise (average of 6 points), and then use each of the individual effluent samples to create 6 log reduction values (LRVs) for each test*."

This method of calculating the LRV at each temperature, allows the generation of as many LRV values as there are samples, and also allows direct determination of the bottom 5th percentile LRV required for the validation from 20 or more samples.

Department of Health was asked in May and June 2014 if there was a preferred method of calculation the LRV for the process, but an answer had not been received at the time of writing this report. In the absence of guidance from the Department of Health, analysis of the data will adopt the method used by Carollo Engineers in the Ventura studies (hereafter referred to as the Ventura method).

The before treatment and after-treatment concentrations of MS2 surrogates on the four test days used to accumulate the required number of test samples (>20) for determination of the bottom 5th percentile LRV are shown in Tables 4.14 and 4.15.

Table 4.14: Before-treatment (Feed) MS2 concentrations.

	Test Date											
	24/04/2015			27/05/2015			2/06/2015			23/06/2015		
Temp. (°C)	MS2 count (pfu/mL)	LV	Average LV	MS2 count (pfu/mL)	LV	Average LV	MS2 count (pfu/mL)	LV	Average LV	MS2 count (pfu/mL)	LV	Average LV
Amb.	7,300	3.86	4.0	253,000	5.40	5.4	246,000	5.39	5.4	298,000	5.47	5.5
	10,900	4.04		266,000	5.42		272,000	5.43		260,000	5.41	
	11,133	4.05		264,000	5.42		274,000	5.44		292,333	5.47	
	9,400	3.97		270,000	5.43		248,000	5.39		288,000	5.46	
	10,900	4.04		269,000	5.43		272,000	5.43		284,000	5.45	
	14,700	4.17		311,000	5.49		277,000	5.44		294,000	5.47	

Table 4.15: After treatment MS2 concentrations and calculated LRVs using the average feed LVs from Table 4.14.

Test Date												
	24/04/2015			27/05/2015			2/06/2015			23/06/2015		
Temp. (°C)	MS2 count (pfu/mL)	LV	LRV	MS2 count (pfu/mL)	LV	LRV	MS2 count (pfu/mL)	log (count)	LRV	MS2 count (pfu/mL)	log (count)	LRV
75.0	0.3	-0.52	4.5	1.7	0.23	5.2	5.0	0.70	4.7	1.2	0.08	5.4
	1.0	0.00	4.0	1.3	0.11	5.3	1.8	0.26	5.2	3.0	0.48	5.0
	1.0	0.00	4.0	2.0	0.30	5.1	7.0	0.85	4.6	1.0	0.00	5.4
	0.2	-0.70	4.7	1.0	0.00	5.4	2.0	0.30	5.1	0.7	-0.15	5.6
	2.0	0.30	3.7	1.0	0.00	5.4	1.5	0.18	5.3	0.3	-0.52	6.0
	0.4	-0.35	4.4	2.0	0.30	5.1	2.0	0.30	5.1	1.0	0.00	5.4
72.0	79	1.90	2.1	1,370	3.14	2.3	1,110	3.05	2.4	920	2.96	2.5
	80	1.90	2.1	820	2.91	2.5	1,210	3.08	2.4	1,150	3.06	2.4
	65	1.81	2.2	1,240	3.09	2.3	1,190	3.08	2.4	950	2.98	2.5
	50	1.70	2.3	960	2.98	2.5	1,380	3.14	2.3	1,030	3.01	2.4
	79	1.90	2.1	480	2.68	2.8	1,300	3.11	2.3	1,020	3.01	2.4
	47	1.67	2.3	950	2.98	2.5	1,530	3.18	2.2	1,170	3.07	2.4
69.0	2,030	3.31	0.7	38,000	4.58	0.9	23,500	4.37	1.1	29,700	4.47	1.0
	1,610	3.21	0.8	51,000	4.71	0.7	26,700	4.43	1.0	27,600	4.44	1.0
	1,930	3.29	0.7	53,000	4.72	0.7	23,500	4.37	1.1	26,500	4.42	1.0
	1,920	3.28	0.7	38,000	4.58	0.9	27,200	4.43	1.0	26,100	4.42	1.0
	1,980	3.30	0.7	43,000	4.63	0.8	24,500	4.39	1.0	28,900	4.46	1.0
	1,950	3.29	0.7	21,000	4.32	1.1	26,800	4.43	1.0	31,300	4.50	0.9

The bottom 5th percentiles of the LRVs calculated using the method adopted by Carollo Engineers in previous validation trials at Ventura, i.e., using:

LRV = Average LV (ambient) – LV (sample),

are shown in Table 4.16.

Table 4.16: Bottom 5th percentile LRV using the Ventura method.

Temperature	Average LRV	SD	Bottom 5th Percentile LRV
75	5.0	0.5	4.0
72	2.4	0.1	2.1
69	0.9	0.1	0.7

References:

USEPA. 2005. Membrane filtration guidance manual, edited by U. S. EPA: U.S. EPA Office of Water

Department of Health, Guidelines for validating treatment processes for pathogen reduction Supporting Class A recycled water schemes in Victoria February 2013

Carollo Engineers, Testing Results of the Pasteurization Demonstration Unit at the Ventura WRF, December 2012.

4.3.6. Plant process control

The major factors that affect the efficacy of the treatment process in reducing the target pathogen(s) are temperature and time, and strict control of these factors is required in the pasteurisation process. The validation process, therefore, requires that the level of control of these two parameters be quantified.

This project experienced major delays in the installation of the pilot plant, leaving only 8 weeks for the challenge testing and continuous operation. This period was further reduced by delays in the installation of a safety feedwater gate valve without which continuous operation was not deemed to be safe (3 weeks), a plant shutdown due to the malfunction of an important plant component (burner fan motor, 2 weeks), and a PLC related issue that shut down the plant during continuous operation. This prevented the planned collection of process control related data during long periods of continuous operation. The longest continuous period of operation was ~36 hours. The data presented here is data from this 36-hour period.

The temperature and flow readings recorded over a 24-hour period of continuous operation at a set temperature of 75°C, and a constant medium pump frequency of 30 Hz (max. = 60 Hz) is shown in Figures 4.21 and 4.22 respectively. The average and standard deviation are shown in Table 4.17.

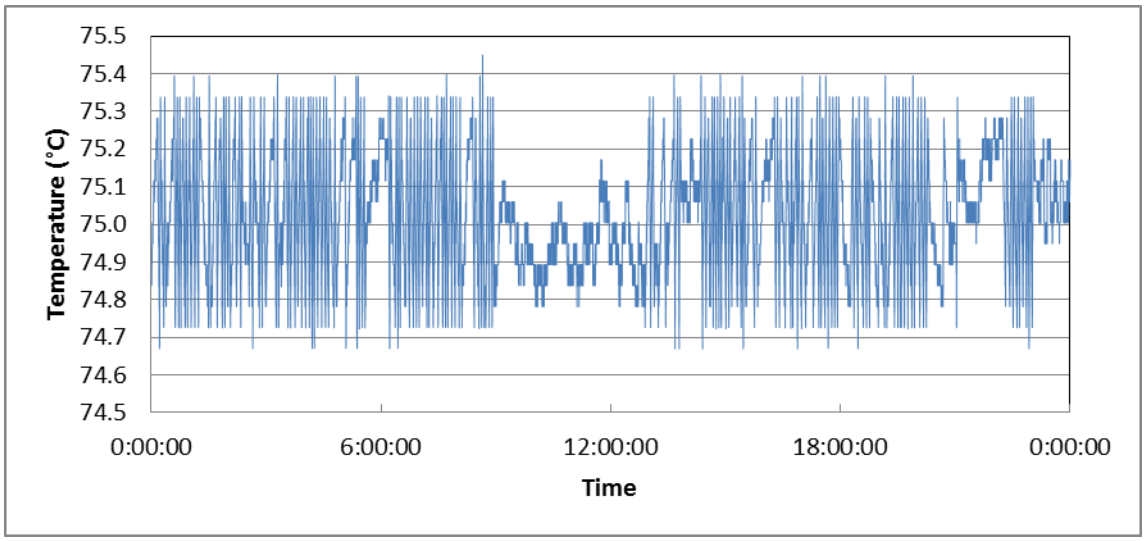

Figure 4.21: Fluctuations in temperature over a 24-hour period, June 4, 2015.

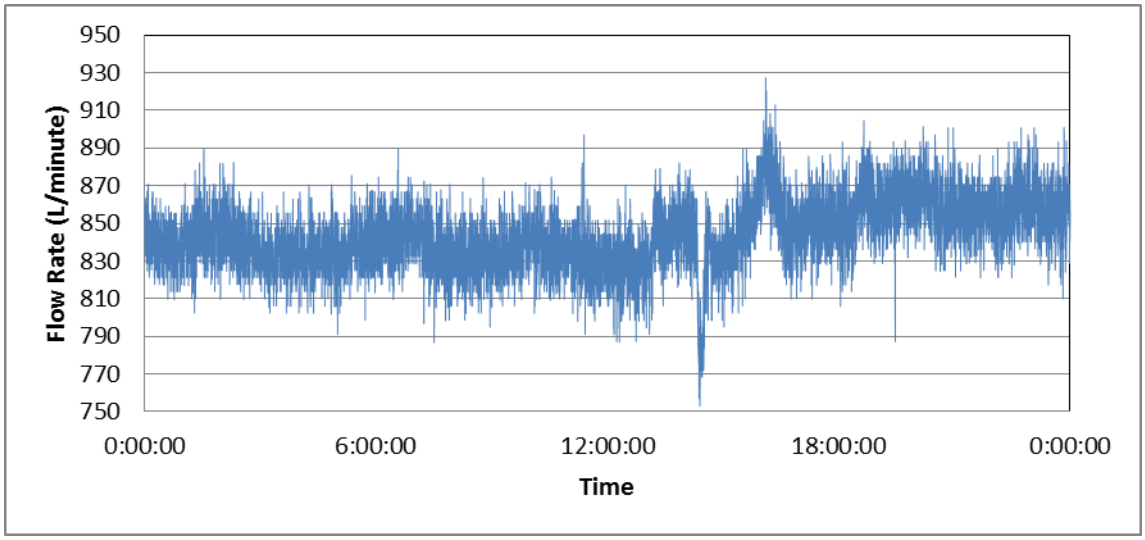

Figure 4.22: Fluctuations in flow rate over a 24-hour period, June 4, 2015.

Table 4.17: Average and standard deviations in temperature and flow

	Temperature (°C)	Flow (L/min)
Average	75.0	844
SD	0.2	18

The observed fluctuations over the 24-hour period were the same as over the much shorter challenge test sample collection period, 0.2% for temperature and 2% for flow.

4.3.7. Gas usage and running costs

The brevity of the available trial period (8 weeks), delays in the installation of a safety feedwater gate valve without which continuous operation was not deemed to be safe (3 weeks), a plant shutdown due to the malfunction of an important plant component (burner fan motor, 2 weeks), and an unresolved PLC related issue that shut down the plant during continuous operation have prevented the planned collection of gas usage and scale formation data required to evaluate the economic viability of the process. The predicted performance summary in Figure 4.23 is for a pasteurisation system which generates 30 kW of power from gas and uses the waste heat to run the pasteurisation plant. It predicts the heat usage for the pasteurisation of 0.2 MGD (0.76 ML/d) of wastewater to be 0.2 MMBtu/hr (0.2 million BTU/h, 58.6 kW).

Predicted Performance Summary for Bob Rawson, Graton WWTP

Prepared by Greg Ryan on September 22, 2015

Pasteurization System Demand and Design

			Metric Conversion
Pasteurization System Design Parameters			
Specified Effluent Flow	0.25	MGD	0.95 ML/d
Ambient Temperature for Design Basis	70	°F	21 °C
Cogeneration System Design Parameters			
1 x Capstone CR 30 (30kW)	30	kW Total	

Estimated System Performance

Cogeneration System			
Power Output	30	kW	
Prime Mover Fuel Input (HHV)	0.4	MMBtu/hr	117 kW
Heat Used for Pasteurization	0.2	MMBtu/hr	58.6 kW
Effluent Treated	0.2	MGD	0.76 ML/d
System Efficiency (HHV)	72.4	%	
Fuel Chargeable to Power (HHV)	0.2	MMBtu/hr	58.6 kW
Equivalent Power Generation Heat Rate	6,460	Btu/kWhr	6,816 kJ/kWhr
Equivalent Power Generation Efficiency	52.8	%	
System Performance Summary			
Effluent Treated	0.2	MGD	0.76 ML/d
Fuel Consumed (HHV)	0.4	MMBtu/hr	117 kW
Overall System Efficiency (HHV)	72.4	%	

Contact information: 510-357-0562, gryan@pastechgroup.com, or www.ptgwaterandenergy.com

Figure 4.23: Predicted pasteurisation system performance summary, Graton Waste Water Treatment Plant.

4.4 Conclusions and recommendations

Pilot testing of the PTG pasteurisation demonstration unit contact chamber using Eastern Treatment Plant water showed that, at temperatures between 75°C and 69°C and a contact chamber contact time of 30 seconds (at ~1,100 L/min), average log reductions values (LRVs) between 5.0 ± 0.5 and 0.9 ± 0.1 respectively for the chosen heat resistant surrogate (MS2) can be achieved. The bottom 5th percentile LRVs under these treatment conditions were found to range between 4.0 and 0.7. The trial also showed that doubling the contact time by halving the flow rate can increase the contact chamber LRV at 72°C from 2.4 ± 0.1 to 4.0 ± 0.3. The results of this testing are expected to be a very conservative estimate of the capability of the entire treatment unit since the time the water spends in the heat exchangers at elevated temperatures also contributes to the disinfection performance of the test unit (see Figure 4.1). The total log reduction achieved during the Ventura trial at 165°F (74°C) was 22 logs (5 logs in the heat exchanger on the way in, 5 logs in the stack heater, 7 logs in the contact chamber and 5 logs in the heat exchanger on the way out) (Carollo Engineers, 2012).

Testing of the entire pasteurisation process, including the heat exchangers, with sampling of influent and effluent to/from the plant showed that an *E.coli* LRV of 2.9 ± 0.3 can be achieved at 68°C and a contact time of 254 seconds (at ~1,100 L/min). The MS2 inactivation at this temperature was found to be 0.8 ± 0.2. The trend in *E.coli* LRVs at varying temperatures was found to indicate that a minimum temperature of approximately 72°C is required for complete inactivation of the native *E.coli* in this water, that were present at ~ 5 LV.

The LRV results achieved during the current work (0.9, 2.4 and 5.0 at 69°C, 72°C and 75°C respectively) are considerably different to those achieved for the contact chamber in the Ventura study (Carollo, 2012) where LRVs of 5.5, 7.0 and 7.2 were reported at 72°C, 73°C and 79°C respectively, using the same strain of MS2 (ATCC 15597-B1) and similar contact times. There was, however, considerable agreement between the current results and those in other US, California, studies at Graton and Santa Rosa (LRVs of 4.5 to 5.3 at approximately 75°C, at contact times between 15 and 40 seconds). The observed difference between the Ventura trial data and the other trial data may possibly be attributable to the use of high seed doses in the Ventura trial, which can cause artificially high LRVs (USEPA, 2005). Also, the sampling protocol used at Ventura was not fully documented, so it is unknown if the samples were adequately cooled to avoid further pathogen inactivation after sampling. Another possible cause of the differences observed is a difference in water quality. The results of a 2007 wastewater pasteurisation trial suggests that water quality plays a role in pasteurisation disinfection kinetics, particularly with regard to coliform disinfection. There was no indication that the water quality influenced the achieved LRV during challenge test days. It was found that there was generally poor correlation between the tested water quality parameters on the challenge test days and the LRV achieved on these days. The order of correlation coefficients (R^2) from highest to lowest was: SS (0.60) > Ca (0.42), > Turbidity (0.38) > TOC (0.33) > pH (0.31) > VSS (0.27) > COD (0.20) > UVT (0.16) > Alkalinity (0.09) > EC ($8x10^{-5}$). Only two of the parameters

showed a trendline with a negative slope that would be indicative of a decrease in LRV with increase in the parameter (Alkalinity and COD). More data is required to establish whether or not there is a correlation between LRV and the tested water quality parameters, but the lack of clear correlation is consistent with the range of water quality parameters that occurred over the trials having no influence on LRV.

The observed fluctuations over a 24-hour period of continuous operation at 75°C and medium to high flow (845 L/min) were the same as over the much shorter challenge test sample collection periods at temperatures between 66°C and 75°C, and flows between 570 and 1,100 L/min – 0.2% for temperature and 2% for flow (and contact time).

Major project delays, however, have reduced the pilot plant trial period such that pasteurisation performance over extended periods of continuous operation could not be evaluated. This has prevented evaluation of the reliability of the pilot plant and the cost of operation (gas use, power use and cleaning requirements). Further testing is required to assess the economic viability of the technology in Australia.

5. REPORT CONCLUSIONS AND RECOMMENDATIONS

This study has shown that pasteurisation can be used to decrease the levels of pathogens in wastewater to safe levels. Laboratory tests using treatment plant water showed that FRNA (MS2) is a good surrogate for a wide range of organisms of concern due its higher resistance to heat. Tests showed that FRNA were the most heat resistant, followed by enterococci and *E. coli. Ascaris* and adenovirus 2 showed some survival at 55°C. Coxsackie virus B5 and *Cryptosporidium* were highly temperature sensitive, being rapidly inactivated even after brief time exposures to 55°C. The results are summarised in Table 5.1.

Table 5.1: Laboratory trial results summary.

Organism	Temperature (°C)								
	55			65			75		
	Contact time (seconds)			Contact time (seconds)			Contact time (seconds)		
	5	30	60	5	30	60	5	30	60
MS2	0		0.1			0.8	1	6	**>7****
Enterococci					0.6	2	2	**>6**	**>6**
E. coli				1.0	2	**>6**	**>6**	**>6**	**>6**
Coxsackie virus		5	6	**>7**	**>7**	**>7**	**>7**	**>7**	**>7**
Ascaris	~0	~0	0.9	**>2**	**>2**	**>2**	**>2**	**>2**	**>2**
Adenovirus	~0	2	**>8**	**>8**	**>8**	**>8**	**>8**	**>8**	**>8**
Cryptosporidium		**>3**			**>3**		**>3**		

* LRV = $\log_{10}$ (organism number before treatment) - $\log_{10}$ (organism number after treatment)
** when the organism number after treatment is zero, a one is substituted for the organism number and the LRV is expressed as “greater than” the calculated value (>).

The effect of different water quality was evaluated using MS2 phage. There was no evidence for any difference in temperature inactivation for phage spiked into Pond 2 water (turbidity 8.5 NTU, TOC 21.2 mg/L) or Pond 10 water (turbidity 1.8 NTU, TOC 10.7 mg/L). The lack of effect of turbidity was confirmed in later tests with ETP water where heat inactivation of native *E.coli* at 2 NTU was found to be indistinguishable from their heat inactivation at 16 NTU. It was found that there was generally poor correlation between the tested water quality parameters on the challenge test days and the LRV achieved on these days (SS, Ca, Turbidity, TOC, pH, VSS, COD, UVT, Alkalinity and EC).

This study has shown that pasteurisation can be used to reduce the levels of pathogens in wastewater to achieve Class A water quality standards. Challenge tests of the contact chamber conducted over short periods of operation showed that operation of the pilot plant at 1,100 L/min (contact time of 30 seconds) and a temperature of 75°C can achieve an average log reduction value of 5.0. The bottom 5th percentile LRV was found to be 4.0 under these conditions.

The results of this testing are expected to be a very conservative estimate of the capability of the entire treatment unit since the time the water spends in the heat exchangers at elevated temperatures also contributes to the disinfection performance of the test unit. The total log reduction achieved during the Ventura trial at 165°F (74°C) was 22 logs (i.e. 5 logs in the heat exchanger on the way in, 5 logs in the stack heater, 7 logs in the contact chamber and 5 logs in the heat exchanger on the way

out, Carollo Engineers, 2012). Other results are summarised in Table 5.2.

It was found that there was generally poor correlation between the tested water quality parameters on the challenge test days and the LRV achieved on these days (SS, Ca, Turbidity, TOC, pH, VSS, COD, UVT, Alkalinity and EC).

Table 5.2: Pilot trial results summary.

Process Stage	Challenge test assay	Contact time (seconds)	Flow (L/min)	Temperature (°C)	Average LRV	SD	Bottom 5th Percentile LRV
Contact chamber	MS2	30	1,100	75	5.0	0.5	4.0
				72	2.4	0.1	2.1
				69	0.9	0.1	0.7
				66	0.8	0.3	-
Contact chamber	MS2	58	570	75	5.3.	0.4	-
				72	4.1	0.3	-
				69	1.6	0.2	-
				66	0.6	0.3	-
Entire process	MS2	254	1,100	68	0.8	0.2	-
				64	0.0	0.1	-
				60	0.0	0.1	-
				57	0.0	0.1	-
Entire process	Native *E.coli*	254	1,100	68	2.9	0.3	-
				64	1.5	0.2	-
				60	0.8	0.2	-
				57	0.4	0.3	-

Major project delays were experienced. These have reduced the pilot plant trial period such that pasteurisation performance over extended periods of continuous operation could not be evaluated. This has prevented evaluation of the reliability of the pilot plant and the cost of operation (gas use, power use and cleaning requirements).

This technology has been found to be economically viable in the US state of California when using waste heat and this needs to be confirmed with further testing in Australia. This testing should include consideration of the fouling potential of the feedwater and the influence of fouling on the effectiveness of the process and its energy efficiency.

Further research is also required to confirm the poor correlation between the tested water quality parameters and the effectiveness of the pasteurisation process for municipal wastewater over long periods to confirm that there are indeed no matrix effects that can render the pasteurisation process less effective for municipal wastewater.

Although the current study deals with the pasteurisation treatment of municipal wastewater, this technology can also be applied to other wastewaters such as stormwater or wastewater from food processing. This would, however, require careful consideration of the microbial and chemical composition of each wastewater and the potential pathogen protective effects that may arise as a result of the chemical composition. Furthermore, the economic feasibility for the treatment of different wastewater may vary considerably. The process is expected to be most economical when it utilises a source of waste heat such as the waste heat from on-site electricity generation.

Appendix A

Protocol for the Validation of Pasteurisation

for

Municipal Wastewater

Draft Protocol for the Validation of Pasteurisation

for

Municipal Wastewater

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1. Introduction

This protocol aims to provide guidance on the validation of pasteurisation equipment or plants to treat municipal wastewater for the production of class A recycled water. It outlines the requirements for demonstrating that pasteurisation can produce recycled water of Class A standard under a defined range of operating conditions. It also outlines the real time monitoring to provide assurance that the water quality objectives are being continuously met. The protocol involves consideration of the microbial and chemical characteristics of the municipal wastewater, the mechanism of microbial inactivation, as well as the municipal water quality factors that can influence this inactivation. Adaptation of this protocol to other wastewaters and other product waters objectives requires major revision as this requires careful consideration of the pathogen and chemical composition of the feed wastewater, and the possible effect of the chemical components on the heat sensitivity of the pathogens to be inactivated.

2. Mechanisms of pathogen removal/inactivation by the treatment process unit

The mechanism of pathogen inactivation via heat treatment involves melting, denaturation and disruption of cell and virus macromolecules and structures that are required for microbial function. Heat affects non-virus pathogens by the melting of cell wall lipids, denaturing the proteins in the cell membranes and the proteins in the cytoplasm, and possibly melting portions of the DNA and RNA strands (Mackey et al 1991). Some of these effects, such as the melting of lipids, are reversible, but others such as the unfolding of the most thermally labile regions of the ribosome or the denaturation of RNA polymerase enzyme cause cell death (Nguyen et al 2006). Similarly, the mechanism for inactivation of viruses by heat includes denaturation of viral proteins, as well as disassembly of virus particles into non-infectious viral subunits and single proteins (Song et al 2010).

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3. Target pathogens, or appropriate surrogates, that are the subject of the validation study.

Municipal wastewater can contain a wide array of microbial pathogens. Some of these have been identified as ***organisms of concern*** in raw sewage in Table 3.1 of the Australian Guidelines for Water Recycling. Validation of the pasteurisation process to treat wastewater requires the selection of at least one ***reference pathogen*** that conservatively represents each of the major groups (i.e., bacteria, viruses, protozoa and helminths), and then the selection of one or more ***surrogate organism(s)*** that

will be monitored during the pasteurisation validation process to assess whether pasteurisation has been effective in the inactivation of the surrogate organism(s). ***The surrogate organism must be demonstrated to be more heat resistant than the reference pathogens under the water quality and test conditions used in the validation.*** Evidence of the greater heat resistance of the surrogate can be literature based, **but confirmation of the heat resistance of the surrogate relative to the reference pathogens in the test wastewater is required**. This confirmation can be performed in small scale laboratory tests.

E.coli is a suitable reference organism for bacterial pathogens for wastewater from sewage treatment. *E.coli* has been found to be more resistant to heat inactivation than other bacterial pathogens such as *Bacillus coagulans, Staphylococcus aureus, Pseudomonas putida, Chryseobacter meningosepticum* [Dumulisile, 2005]. A more conservative representative of bacteria is *Enterococcus* as these organisms have been found to be more heat resistant than Faecal coliforms [Bonjoch, 2009].

Giardia and *cryptosporidium* are two suitable reference organisms for protozoa. Their heat resistance [Fayer, 1994 and Ongerth, 1989] is less than that of *E.coli* and Enterococci [Dumulisa, Bonjorch], and these two bacterial organisms are hence good indicator organisms for these protozoa.

Ascaris Suum is a suitable reference organism for helminths, and has been found to have a heat sensitivity close to that of *E.coli*.

MS2 coliphage has been found to be more resistant to heat than poliovirus type one and various other enteroviruses (Moce-Llivina et al, 2003), making it a good reference organism for viruses.

MS2 is also more heat resistant than the above reference organisms - *E.coli, Giardia, Cryptosporidium* and *Ascaris* – making it a good surrogate for these organisms when assessing the ability of pasteurisation process to disinfect wastewater.

Unless the feedwater is substantially the same as that used in previous pasteurisation trials, it is a requirement of the validation that laboratory or demonstration scale testing be performed prior to the testing of the test unit to compare the relative heat sensitivity of the MS2, E.coli, Cryptosporidium and Ascaris in the selected test water (or waters) at temperatures and times relevant to the full-scale system. MS2 or a more heat resistant surrogate must be used as a surrogate in the validation testing of the test unit. Furthermore, if using laboratory grown bacteria, these should not be in rapid growth phase as they are likely to be more heat resistant than cultures that are rapidly growing (see Section 3).

References:

Australian Guidelines for Water Recycling: Managing Health and Environmental Risks (Phase 1). 2006

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Ongerth, J.E., Johnson, R.L., Macdonald, S.C., Frost, F. and Stibbs, H.H. (1989) Back-country water treatment to prevent giardiasis. Am J Public Health 79(12), 1633-1637.

4. Influencing factors that affect the efficacy of the treatment process unit to reduce the target pathogen

The major factors that affect the efficacy of the treatment process in reducing the target pathogen(s) are temperature and time, and strict control of these factors is required in the pasteurisation process. ***The validation process requires that the level of control of these two parameters be quantified using accurately calibrated instruments.***

Another factor that can influence the effectiveness of the pasteurisation process is salt content. Salt content of the order of 1 to 2% w/v has been found to be protective to bacteria and viruses (Besten 2010, Juneja 2013, Volkin 1997, Mazotta 1999). The protective effect of salt is very small at the salt content typical of municipal wastewater (usually less than 1 g/L or 0.1 % w/v), but evidence is required from historical data or monitoring of the salt content via the use of conductivity measurements and/or TDS analysis of grab samples to give an indication of the level of microbial protection being offered by the salt content of the feedwater. ***Analysis of the expected fluctuation or variability in feedwater salt content that may render the process less effective is required for the validation process.***

The presence of suspended matter (turbidity) also has the potential to reduce the effectiveness of pasteurisation if the particles decrease the rate of heat transfer to the microbes. The results of recent wastewater pasteurisation trials (Appendix D, page D55, USEPA 2012) suggest that the protective effect of particulates manifests itself at higher temperatures for bacteria. ***It is, therefore, required that the effect of the native turbidity on pasteurisation inactivation be quantified at turbidity values normally encountered in the wastewater.***

The growth phase of the bacteria can also markedly impact the heat resistance of bacteria, with late stationary phase cells being more heat resistant than log phase cells (Kaur 1997). Similarly, bacterial cells that are in starvation conditions are more heat resistant than cells in nutrient rich environment (Casadei 1998). ***It is, therefore, required that validation be performed under conditions that are not conducive to rapid growth as the cells are likely to be less heat resistant under these conditions.*** It is important to emphasise that the bacterial pathogens in municipal wastewater e.g. *Campylobacter, Salmonella*, and some forms of *E. coli*, are from the human gut and once outside the gut, they are in a stressed situation and they are not likely to proliferate in the sewer or large well managed wastewater treatment plants. The conditions for rapid growth may, however, occur in small municipal wastewater treatment plants with inputs from food processing (e.g. dairy or meat processing). Growth phase conditions may be ones where a sudden influx of nutrients enters the wastewater treatment plant, and/or at times of high water temperatures. The expected fluctuations

in nutrient content of the wastewater needs to be determined through consultation of historical data or the measurement of available carbon, nitrogen and phosphorus content of the wastewater. The expected fluctuation in wastewater temperature also needs to be determined to assess whether a rise in temperature alone or in conjunction with increased nutrient content can alter the growth phase of the pathogens.

The pH of the feedwater can also markedly influence the heat tolerance of bacteria, with bacterial resistance being higher for acid grown bacteria than for cells grown at higher pH values (Shen, 2011). Tosun et al (2005) quantified the heat resistance of *E.coli* 0157:H7 that had been acid adapted at pH 4.5, 5.0. and 5.5 and found that acid adaptation at pH 4.5.and 5.0 provided measurable protection from heat treatment, but the heat sensitivity at pH 5.5 was not statistically different from that of a non-acid-adapted control. Again, this is unlikely to influence the heat sensitivity of bacteria in large, well managed, municipal treatment plants as they are not likely to experience large fluctuations in wastewater pH. The bacteria in these plants is also unlikely to be actively growing, so higher heat tolerance due to acid stress is unlikely for these municipal plants. Higher heat resistance due to acid stress may, however, be evident in small, less stable, low flow, municipal treatment plant with industrial inputs. ***It is, therefore, required that pH be monitored if the historical water quality data shows pH fluctuations to below 5.5. Validation must not be performed at pH >7.5 if the pH data in the historical record fluctuates to below 5.5 as the cells may have a lower heat tolerance at these higher pH values.***

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Casadei, M.A., de Matos, R.E, Harrison, S.T. and Gaze, J.E., Heat resistance of Listeria monocytogenes in dairy products as affected by the growth medium, Journal of Applied Microbiology 1998, 84, 234–239.

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5. Operational monitoring parameters that can be measured continually (ideally) and that will relate to the reduction of the target pathogen

Operational on-line monitoring of temperature and flow rate is required. Monitoring of feedwater conductivity, turbidity and pH is required if the levels of salt, particulates and acidity over a 12 month historical record approach the levels that may influence heat sensitivity of the organisms as outlined in Section 3 and Table 1. Operational monitoring of nutrient content has not been included here because active growth of bacteria does not adversely influence pasteurisation performance. It increases the effectiveness of pasteurisation by increasing the heat sensitivity of bacteria. Furthermore, active growth of bacteria may possibly occur for short periods due to sudden influx of nutrients (for small WWTP's) but is not the prevalent bacterial growth phase in municipal wastewater. It is, however, important to ensure that the bacteria are not actively growing during validation as this will yield higher LRVs that are unrepresentative of the pasteurisation performance when bacteria are not actively growing. This would require microbial testing with concurrent nutrient level testing in the period before, during and after challenge testing. ***Table 1: Influencing factors and monitoring requirements for validation process***

Factor	Monitoring	Influence	Comments
Temperature	Temperature	Denaturation and disruption of microbial proteins and lipids that are required for microbial function	Pathogen specific and time dependent. Monitoring and control of temperature is required because temperature and time are the two key influencing factors in the pasteurisation process.
Contact time	Flow rate	The faster the flow rate, the shorter the time the pathogen is exposed to the denaturing and disrupting effect of temperature	Pathogen specific and temperature dependent Monitoring and control of flow rate is required because temperature and time are the two key influencing factors in the pasteurisation process.
Salt content	Conductivity	Salt content of the order of 1 to 2% w/v has been found to be protective to bacteria and viruses	Variability of salt content of the wastewater must be assessed Weekly monitoring of conductivity is required to ensure that the protective effect of salt (if any) during the validation period is the same as or similar to that during process operation. An increase of 150% is considered acceptable provided the salt concentration is less than 0.1% w/v.
Turbidity	Turbidity	Can be protective if particles can decrease the rate of heat transfer to the microbes	Variability of wastewater turbidity must be assessed The ability of the wastewater sediment material to affect heat sensitivity of pathogens and surrogates must be assessed and quantified. Turbidity monitoring is required to ensure that the protective effect of turbidity (if any) during the validation period is the same as or similar to that during process operation.
Acidity	pH	Low pH can be protective	Variability of wastewater pH must be assessed Monitoring of pH is required to ensure that the

	protective effect of acid stress (if any) during validation is the same or more conservative as that during process operation

6. Validation method to demonstrate the capability of the treatment process unit

The steps required to demonstrate the capability of the treatment process unit are outlined in Figure 1.

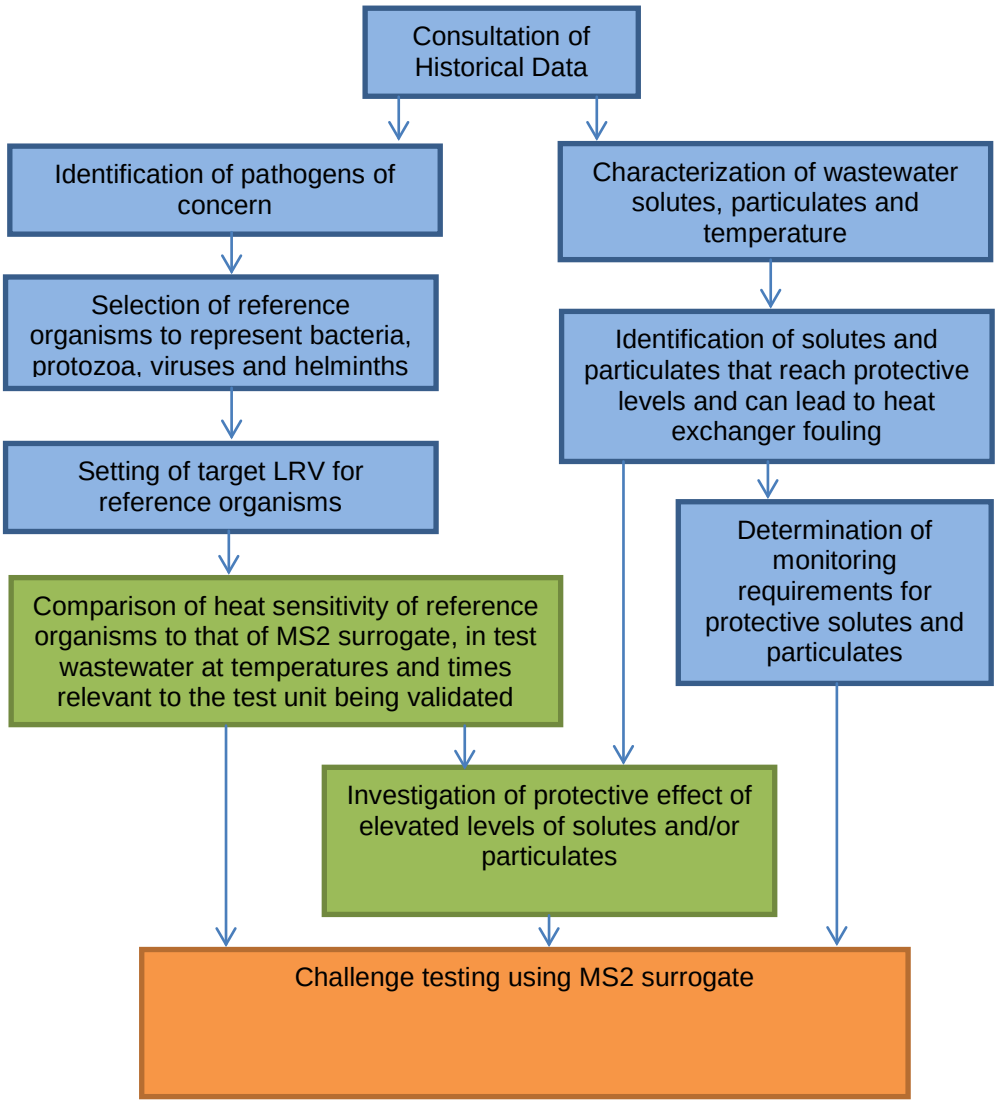

Figure 1: Wastewater characterization (blue boxes), laboratory testing (green boxes) and challenge testing of process unit (orange box).

Wastewater chemical composition and microbial content are site-specific and a number of chemical and microbial factors can influence the effectiveness of the pasteurisation process. Consultation of historical data for the wastewater is required to determine the expected variability with regards to the microbial content of the wastewater and the major influencing factors that can alter the sensitivity of the organisms to the pasteurisation process conditions (see Section 3). This allows

selection of representative organisms and the disinfection performance targets for each of these organisms, and the identification of matrix factors that can influence the sensitivity of these organisms to the pasteurisation process. If historical data does not exist, monitoring is required to establish the level and variability of these factors in the wastewater. The effect of these influencing factors needs to be investigated if their concentration and variability at any time in the historical record is such that there is potential for the constituent to significantly affect the pasteurisation process (see Section 4).

It is required that the sensitivity to heat inactivation of the key pathogens of concern in the test wastewater be compared to that of the surrogate organisms that will be monitored in the test wastewater (MS2 or a more heat resistant surrogate). The temperature and exposure time conditions at which this comparison is made need to be within the range of temperatures and times that the key pathogens and surrogates will be exposed to in the validation testing of the pasteurisation treatment unit. If the historical record indicates that any of the matrix factors is likely to be present at protective levels, it is a requirement of the validation process to quantify the pasteurisation disinfection at these protective levels.

Validation challenge testing of the pasteurisation unit shall take place using the surrogate organism (MS2 or a more heat resistant surrogate, see Section 2) at a time when the level of protection (if any) offered to the pathogens and the surrogate by matrix influencing factors is the same as that during normal operation. If the historical records indicate that the matrix influencing factors can reach levels that may be protective, monitoring of the key influencing factors is required:

- ***During validation to demonstrate that the matrix influencing factors have not changed to levels that would increase the heat sensitivity of the pathogens and surrogate during the validation period***
- ***During non-validation process operation to allow action to be taken to prevent the production of recycled wastewater that does not meet Class A standard at times of high microbial protection from heat.***

Challenge tests can be performed via batch seeding or in-line injection. In-line injection involves continuous introduction of the challenge surrogate into the feed stream and requires a dosing pump, a suitable injection port and in-line mixing to evenly disperse the challenge particulates in the test medium. Batch seeding requires an upstream reservoir large enough to supply feed throughout the challenge test and efficient mixing in the upstream reservoir.

The two key parameters that are controlled by the pasteurisation process are the temperature and the contact time to which the microbes are exposed. It is a requirement of the validation process that the effect of these two parameters on the inactivation of pathogens of health concern be tested for each wastewater. This can be done entirely at the pilot or full scale, or partially in laboratory testing prior to pilot testing.

The temperature and time settings used in the validation of the treatment unit define the conditions for future use of this treatment unit. Operation outside these conditions requires revalidation. It is, therefore, important to validate under a range of time and temperature conditions to accommodate potential future needs (e.g. lower or higher flow and temperature, or lower or higher LRV

requirement). The contact time at each flow rate needs to be determined via tracer studies according to accepted methods outlined in Appendix D of the USEPA Guidance Manual for Disinfection Profiling and Benchmarking (1999).

The maximum log reduction value (LRV) that can be attributed to any one treatment process, regardless of its capability, is 4 $\log_{10}$. If the aim of the validation is to demonstrate this level of reduction of pathogens, the initial (before treatment) concentration must be greater than 4 $\log_{10}$ since calculation of an LRV requires that the after treatment surrogate concentration be greater than zero. The initial concentration also needs to be high enough to not result in non-detects since the occurrence of non-detects yields a LRV that depends on the starting concentration as well as the ability of the treatment process to inactivate the target pathogen or surrogate. The maximum surrogate LRV that can be used in validation testing is 6.5 log because over-seeding can result in artificially high LRVs (see Section 3.10.3 of USEPA Membrane Filtration Guidance Manual, 1999).

References:

USEPA Guidance Manual Disinfection Profiling and Benchmarking, 1999, EPA 815-R-99-013

USEPA, Membrane Filtration Guidance Manual, EPA 815-R-06-009, November 2005

7. Method to collect and analyse data to formulate evidence-based conclusions

The sampling methods and techniques used during validation must be consistent with the Standard Methods for the Examination of Water and Wastewater (American Public Health Association et al. 2012). National Association of Testing Authorities (NATA) accredited methods must be used where available. Where NATA accredited methods are not available, the laboratory must demonstrate that the method employed is consistent with a standard method (where this is available) and document the method used to perform the analysis. The raw data and its analysis must be appended to the validation report. If data is excluded from the analysis the rationale must be clearly provided. All procedures must be performed by suitably qualified personnel and be subject to quality assurance/quality control procedures.

The removal efficiency of a treatment process unit demonstrated by the challenge test results is determined according to the following equation:

$$LRV = \log_{10}(\text{feed concentration}) - \log_{10}(\text{product water concentration})$$

The challenge tests and the collection of these samples must be performed over at least 3 different days, at least one week apart, over the available test period to account for day-to day variability in feedwater quality and plant operating conditions.

For paired samples, this formula will be applied to individual feed and product water surrogate concentrations. For unpaired samples, this formula will be applied to averages of the feed surrogate concentrations, and to individual product water concentrations. This latter method of calculation was used by Carollo Engineers in validation testing of pasteurisation for treatment of water from a water reclamation plant (p.D-55, USEPA, 2012). Regardless of which of these methods is used, regulatory authorities generally take a conservative approach to how validation data are analysed to

establish the challenge test LRV for a process. The Victorian regulatory authority, for example, specifies that the lower 5th percentile LRV established during challenge testing must be used. This requires the collection and analysis of at least 20 before and 20 after treatment samples. All calculations and statistical analysis performed on the raw data must be detailed.

References:

USEPA, 2012 Guidelines for water reuse, EPA/600/R-12/618.

8. Critical limits and operational monitoring and control

The temperature and time settings used in the validation of the treatment unit define the operating conditions for future use of this treatment unit. Operation outside these conditions requires revalidation. Furthermore, the LRV requirement from the treatment process and the achieved LRV as a function of temperature and flow rate defines the acceptable temperature range for operation at each flow rate tested. *For each flow rate tested, there will be a critical temperature below which the achieved surrogate LRV is likely to be less than the required surrogate LRV and the plant may fail to deliver fit-for-purpose recycled wastewater. If this occurs, action must be taken, such as plant shut down or diversion of the treated water back to storage, to avoid a risk to public health. Consideration of the error involved in the measurement of the LRV is required to ensure the achieved LRV has a high likelihood of being above the required LRV.*

Failure to deliver safe recycled water can also occur if the feedwater characteristics change in a manner that can make the pathogens more heat resistant than during validation:

(a) Turbidity increases to more protective values. The ability of the wastewater sediment material to affect heat sensitivity of pathogens and surrogates must be assessed and quantified for each wastewater

(b) Salt content (as measured by conductivity) increases to more protective levels: Salt levels of more than 1% w/v salt have been shown to be protective. Higher salt levels are likely to be further protective.

(c) pH decreases to more protective levels: i.e. to levels where the organisms are more acid stressed than during validation. Due to the nature of the pH scale, the lower the pH during validation, the smaller the pH decrease required for additional acid stress and protection during normal operation.

If the historical record demonstrates that the wastewater characteristics do not change to values that may be protective, and future changes to the treatment process are not likely to lead to values that may be protective, these parameters need not be monitored and controlled. See Section 3 for discussion of these influencing factors.

9. Method to determine the LRV for each pathogen group (protozoa, virus, bacteria, helminths) in each specific treatment process unit performing within defined critical limits

Wastewater can contain a wide array of microbial pathogens. Some of these are of particular concern as they may pose a greater health risk because of their concentration, infectivity or adverse health effects/impacts. These may be too many to test for in the validation, and ***reference***

pathogens are chosen from the organisms of concern to represent the major pathogen groups – bacteria, virus, protozoa and helminths. These are readily tested for at the laboratory scale but it is often too expensive or impractical to test for these during routine monitoring of the performance of pilot or full scale units. For this monitoring, a ***surrogate*** organism is chosen. Due to its high resistance to heat, MS2 is the recommended surrogate. Other surrogates may be used, but these must be demonstrated to be more heat resistant than MS2 and the reference pathogens under the water quality and test conditions used in the validation. Evidence of the greater heat resistance of the surrogate can be literature based, but confirmation of the heat resistance of surrogate relative to the reference pathogens in the test wastewater is more often required. This confirmation can be performed in small scale laboratory tests.

Laboratory scale confirmation of the relative heat sensitivity of the reference pathogens and the surrogate must be performed under temperature and contact time conditions that are relevant to those that will be use in the pasteurisation unit to be validated and in water representative of that being treated. An illustration of the expected trends in LRV at constant time for various pathogens and a surrogate for these pathogens is shown in Figure 2. This figure shows three pathogens, A, B, and C, with various levels of heat resistance. Pathogen A is the least heat resistant, showing high log reduction values at lower temperatures that the other two pathogens and the surrogate. Very low inactivation of Pathogen B can be seen at temperatures that result in high LRV for Pathogen A. Similarly, Pathogen B is more heat resistant than Pathogen A, but less heat resistant than Pathogen C and the surrogate. High LRVs for Pathogen A and Pathogen B are expected at temperatures where high LRV are seen for Pathogen C. The surrogate is ***and must be*** the most heat resistant such that the LRV achieved at any particular temperature/contact time combination for the surrogate is always lower than the LRV achieved for the reference pathogens at that temperature/time combination. In the example shown in Figure 2, at the critical temperature Tc, the surrogate LRV is lower than that of all the pathogens.

Figure 2 also shows the target LRV for each pathogen. These can be determined from the historical data for the wastewater. Alternatively, the default values in the Australian Guidelines for Water Recycling (2006) may be used. The target LRV for the surrogate is the lowest acceptable LRV for process unit.

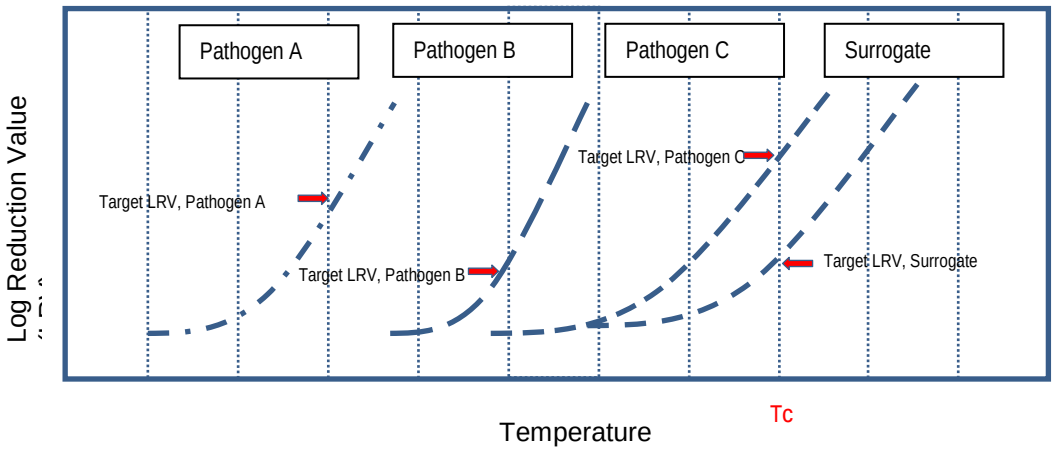

Figure 2: Expected trends in relative heat resistance of reference pathogens and surrogate at constant contact time.

It is a requirement of the validation process that laboratory scale or larger scale LRV data be obtained for each reference pathogen (E.coli, Cryptosporidium and Ascaris) and for the surrogate (MS2 or a more heat resistant surrogate) at:

(1) the expected contact times of the process unit,

(2) temperatures where the LRVs are greater than the target LRV for the pathogen or surrogate, using a wastewater that is similar with respect to the influencing factors outlined in Section 3 to the pasteurisation process unit feedwater during validation.

If the pasteurisation unit used heat exchangers, the temperature used must be such that a total kill of organisms that can colonise the heat exchangers takes place in the contact chamber. Biofouling on the effluent side of the heat exchangers can also lead apparently poor disinfection performance despite good disinfection in the contact chamber. It has been found that faecal coliforms can colonise the effluent side of heat exchangers if a total kill of the colonising microbes is not achieved in the upstream contact chamber (Carollo, 2012). So, apart from the health risk mitigation requirements, there is also a need to achieve total kill of faecal coliforms to prevent biofouling of the heat exchangers.

References:

Carollo Engineers, Testing Results of the Pasteurization Demonstration Unit at the Ventura WRF, December 2012.

10. Re-validation or additional onsite validation where proposed modifications are inconsistent with the previous validation test conditions

A validation study applies to the treatment process unit that is specified during the study. The temperature and time settings used in the validation of the treatment unit define the conditions that may be used in future use of this treatment unit, and operation outside these conditions requires revalidation if the change in operating conditions has the potential to decrease the effectiveness of the pasteurisation process. It is, therefore, important to validate under a range of time and temperature conditions to accommodate potential future needs. Lower flow within the tested temperature envelope does not require revalidation as it will increase contact time and increases the killing effect at each temperature tested. Higher flow, however, requires revalidation.

Re-validation is also required if there is a change in the design of the plant such that the water quality related protective effects outlined in Sections 3 and 4 are enhanced beyond the natural variability in the historical records.

Re-validation or additional onsite validation testing may also be required if there are design modifications to the validated treatment process unit, control philosophy and operational monitoring parameters (including critical limits) that are different to the documented validation test conditions.