AWRCoE Project: Pasteurization for the production of Class A water

Milestone 1 Report: Literature Review

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1. Scope

The primary purpose of this review of the scientific literature on pasteurization is to identify potential indicator organisms for the pasteurization of secondary effluent. This review builds upon a literature review conducted by PTG and Carollo Engineers (PTG Title 22 Report) 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 virus, protozoa (*Cryptosporidium*), helminths and bacteria in the pilot trials.

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 focussed on pasteurisation of food (primarily milk, juices, shellfish). There has been no reported application of pasteurisation to 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 $\log_{10}$ 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 paper. The PTG pasteurization plant/process is essentially a high temperature (>60°C) short time (HTST) pasteurization process, with a contact time of ~5 minutes, including ramp up and ramp down. The literature reviewed has focussed on processes with temperatures >50°C and, due to the lower number of studies that deal with HTST pasteurization, considers longer contact times to assess the *relative* heat sensitivity of the organisms of interest.

3. Pasteurization inactivation of bacteria

Only one study was found thatreported 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 done 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*, showing 3 – 4 $\log_{10}$ inactivation 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 minutes 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 higher inoculum most organisms had higher inactivation, with 3-4 $\log_{10}$ inactivation during the 3 minutes 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 for 15 and 6 $\log_{10}$ inactivation after 25 minutes at 63°C (Dumalisile et al., 2005).

Work with total coliform at the City of Ventura's Water Reclamation Plant using sand filtered secondary effluent (PTG Title Validation Report) showed that 68°C resulted in greater than 4-5 $\log_{10}$ inactivation of total and fecal 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 minutes 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 (eg 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 lab-based pasteurisation of biowaste from a biogas plant (Sahlstrom et al., 2008). The waste was a combination 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 lab 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 in raspberry puree. In the Lab & Smith study, the medium affected inactivation, with tryptone soy both 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 pasteurization for inactivation of bacteria is shown in Table 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 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 1: Summary of pasteurization log_{10} inactivation values for bacteria

Organism	Total Log_{10} inactivation	Temp (C)	Time (min)	Ramp (min)	Ramp Log_{10} inactivation	T90 (min)	Method	Matrix	Inoculum (log10)	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

Organism	Total Log_{10} inactivation	Temp (C)	Time (min)	Ramp (min)	Ramp Log_{10} inactivation	T90 (min)	Method	Matrix	Inoculum (log10)	Reference
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
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-

Organism	Total Log_{10} inactivation	Temp (C)	Time (min)	Ramp (min)	Ramp Log_{10} inactivation	T90 (min)	Method	Matrix	Inoculum (log10)	Reference
										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
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-

Organism	Total Log_{10} inactivation	Temp (C)	Time (min)	Ramp (min)	Ramp Log_{10} inactivation	T90 (min)	Method	Matrix	Inoculum (log10)	Reference
										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
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

Organism	Total Log_{10} inactivation	Temp (C)	Time (min)	Ramp (min)	Ramp Log_{10} inactivation	T90 (min)	Method	Matrix	Inoculum (log10)	Reference
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

4. Pasteurization 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 ilieal 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 s (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 s at 70°C and 4.8 $\log_{10}$ inactivation for 5 s 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 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* at 54°C. These results are

similar to those reported in an earlier study, which reported 1 – 2 $\log_{10}$ inactivation following a 10 minutes 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*, 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 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 1 with the highlighted rows in Table 2).

Table 2: Summary of pasteurization 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 (log10)	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

5. Pasteurization 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 ground water. The order was similar for complex matrices, with calicivirus > echovirus > rotavirus > FRNA > coxsackie > 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 temp 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 for 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 possess 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 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 claimed MS2 4 $\log_{10}$ inactivation = polio 5 $\log_{10}$ inactivation. This is not completely correct, 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), coxsackie virus may be a suitable surrogate for enteroviruses (eg poliovirus and HAV)

Comparison of the data in Table 3 with the *E. coli* data in Table 1 indicats 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 1) while the total $\log_{10}$ inactivation under these conditions is less for most viruses (see Table 3).

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Table 3: Summary of pasteurization 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 (log10)	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

Organism	Total Log_{10} inactivation	Temp (C)	Time (min)	Ramp (min)	Ramp Log_{10} inactivation	T90 (min)	Method	Matrix	Inoculum (log10)	Reference
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
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
murine norovirus	4	60	5	ns	ns	1.35	Thermal cycler	PBS	6 to 7	Jarke

Organism	Total Log_{10} inactivation	Temp (C)	Time (min)	Ramp (min)	Ramp Log_{10} inactivation	T90 (min)	Method	Matrix	Inoculum (log10)	Reference
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

Organism	Total Log_{10} inactivation	Temp (C)	Time (min)	Ramp (min)	Ramp Log_{10} inactivation	T90 (min)	Method	Matrix	Inoculum (log10)	Reference
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
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

6. Helminths

The PTG Title 22 report did not appear to 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 4 with the *E. coli* data in Table 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 1) while the total $\log_{10}$ inactivation under these conditions is less for *Ascaris suum* (see Table 4).

Table 4: Summary of pasteurization 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 (log10)	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

7. Conclusions

Based on reviewed scientific literature, the suggested surrogates and pathogens for inclusion in this project are listed in Table 5.

Table 5: List of pathogens and indicators suggested for lab and pilot scale testing

	Pathogens	Surrogate	Conservative surrogates
Lab-scale	• *Cryptosporidium* • *Giardia* • Adenovirus • Coxsackievirus • *Ascaris*	• *E. coli*	• FRNA • *Enterococcus*
Pilot-scale	• Adenovirus	• *E. coli*	• FRNA • *Enterococcus*

It is recommended that the lab-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 lab-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 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 6. The metadata from the Bertrand 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 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.

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