Chlorine inactivation of coxsackievirus B5 in recycled water destined for non-potable reuse

Satiya Wati, Bret S. Robinson, John Mieog, Judy Blackbeard and Alexandra R. Keegan

ABSTRACT

Currently guidelines for disinfection of water with free chlorine, while primarily developed for potable water, are often used for virus disinfection of nitrified recycled water of >1 NTU (Nephelometric Turbidity Unit). More information is needed on the disinfection efficacy of free chlorine for viruses in waters of varying turbidity and pH due to significant reuse of treated wastewater of varying quality. In this study, disinfection efficacy in nitrified/denitrified activated sludge treated wastewater was investigated for coxsackievirus B5 (CB5), an enterovirus known to be highly resistant to free chlorine. The required chlorine contact times (CT) values (mg.min/L) for inactivation of CB5 were established in treated wastewater at 10 °C and of varying turbidity (0.2, 2, 5 and 20 NTU) and pH (7, 8 and 9). CTs were calculated to achieve 1 to 4 $\log_{10}$ inactivation. Robust data is presented in support of the chlorine CT values required to inactivate a chlorine-resistant virus in a range of turbidities and pHs in treated wastewaters. The testing method used a conservative approach and the data presented have been used to develop the free chlorine virus inactivation guildelines for recycled water in Victoria and South Australia, Australia.

Key words | chlorine, chlorine contact time (CT) mg.min/L, coxsackievirus B5 (CB5), free available chlorine (FAC), wastewater

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INTRODUCTION

With increasing water demands, the reuse of domestic wastewater for non-potable purposes (such as in urban, agriculture and industrial sites with uncontrolled public access in Australia) is becoming an important issue. Disinfection processes are critical for the reduction of infectious virus concentrations in wastewaters destined for reuse. Effective disinfection of human infectious viruses in wastewaters can be adversely affected by a range of factors present in wastewaters including particles, turbidity, aggregation and cell association. The United States Environmental Protection Agency's (USEPA) 'Guidance Manual for Compliance with the Filtration and Disinfection Requirements for Public Water Systems using Surface Water Sources' (USEPA 1999) provides disinfection guidance for drinking water systems using free chlorine and chloramine where the turbidity is <1 NTU (nephelometric turbidity unit). The potable water focus has been further reinforced by subsequent disinfection studies which have mainly been carried out in buffered demand free waters or source water of turbidity of <1 NTU (Liu *et al.* 1971; Jensen *et al.* 1980; Payment *et al.* 1985; Sobsey *et al.* 1988; Sobsey 1989; USEPA 1999; Thurston-Enriquez *et al.* 2003; Kahler *et al.* 2010).

Currently the USEPA (1999) free chlorine guidelines for disinfection of potable water of <1 NTU are commonly used for disinfection of low turbidity recycled water. As the source for recycled water is often predominantly domestic wastewater, the range of enteric viruses likely to be present and the potential occurrence of unidentified viruses raises

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doubts as to whether the use of drinking water guidelines is appropriate. This is exacerbated as the USEPA (1999) guidelines were developed based on disinfection of hepatitis A virus which is not the enteric virus most resistant to free chlorine. In addition, the USEPA guidelines (1999) do not provide guidance for disinfection of water of >1 NTU. USEPA's 'Guideline for Water Reuse' (2012) include disinfection requirements for chlorination based on California Title 22 with a required chlorine contact time (CT) of 450 mg.min/L for tertiary recycled water while Florida works with a sliding disinfection standard of 25 mg.min/L for faecal coliforms <1,000 MPN (most probably number)/100 mL, 40 mg.min/L for faecal coliforms 1,000 to <10,000 MPN/100 mL and 120 mg.min/L for faecal coliforms >10,000 MPN/100 mL. The work reported in this paper is based on the enteric virus most resistant to free chlorine, coxsackievirus B5 (CB5). Although CB5 is not currently considered to be responsible for a significant disease burden, it has been chosen as a conservative virus to ensure adequate inactivation of all potentially pathogenic known and unknown viruses in recycled water. By developing guidelines for waters >1 NTU, recycled water can be produced from a wider variety of treatment trains and at potentially lower cost.

An extensive literature review was carried out to identify the most chlorine resistant enteric virus which can be enumerated using culture-based methods (Keegan *et al.* 2012). Based on this review, CB5 virus was chosen as the conservative model for disinfection by free available chlorine (FAC). It is >5 times more conservative than hepatitis A virus based on reported CT values (Sobsey *et al.* 1988; Black *et al.* 2009), which has been used for developing drinking water guidelines (Sobsey *et al.* 1988; USEPA 1999) which include a safety factor to account for this lack of conservatism.

Ideally, native virus present in intact particles in wastewater would be most suitable to determine the efficacy of the current CT targets. Use of native viruses in sewage is not practical because of the variability of virus types circulating in the community and the low and variable numbers of virus present in secondary-treated water (Hejkal *et al.* 1981; Rose *et al.* 2004; Sedmak *et al.* 2005). Guidelines for health-based targets require sufficient infectious virus to be present in the recycled water prior to disinfection to demonstrate up to 4 $\log_{10}$ inactivation of virus. Surrogates such as somatic coliphage and F-specific bacteriophage are usually present in higher numbers than viruses in wastewater; however, they are far more sensitive to inactivation by free chlorine than some of the human enteric viruses (Sobsey *et al.* 1988). Therefore, use of a chlorine resistant laboratory-cultured virus was the most practical and conservative model for this type of study. Native viruses can exist free in the supernatant or attached or embedded in particles present in wastewater, thus gaining some level of protection from disinfection. Wastewater consists of a heterogeneous mixture of particles that are organic, or inorganic, with some particles being colloids. Virus binding to particles is dependent on a range of factors including particle adsorbency and the isoelectric point of each virus, as reviewed in the literature (Templeton *et al.* 2008). Within a heterogeneous particle distribution in wastewater, spiking with laboratory-cultured virus and allowing time for attachment as performed by Meschke & Sobsey (1998) would allow viruses to attach to particles. The level of attachment in a heterogeneous mixture of particles will vary depending on charge, type of individual particles and dissolved constituents present (e.g. salts).

In this study, we investigated the required chlorine CTs for inactivating CB5, an enterovirus known to be highly resistant to chlorine (Liu *et al.* 1971; Payment *et al.* 1985; Black *et al.* 2009) in wastewater. Disinfection efficiency was determined at 10 °C for cultured virus spiked into secondary-treated wastewater, of varying turbidity (0.2, 2, 5 and 20 NTU) and pH (7, 8 and 9) reflecting normal and extreme water turbidity that could be produced by a variety of wastewater treatment plants. Disinfection concentration contact time (CT = concentration of free chlorine ($C_{mg/L}$) × contact time ($T_{minutes}$) was determined for each condition tested to aid in establishing guidelines for free chlorine disinfection of viruses in treated wastewater for non-potable reuse.

METHODS AND MATERIALS

Viruses and cell lines

ATCC VR-185^{TM} CB5 was cultured in Buffalo green monkey kidney (BGM) cell line. The cells were propagated in

complete Eagle's minimum essential medium (MEM; Gibco) supplemented with 10% foetal bovine serum (FBS; Gibco), 2 mM L-glutamine and 10 mM non-essential amino acid (NEAA; Gibco). Three freeze-thaw cycles were performed to release virus from the infected cells. The CB5 was partially purified by centrifuging at 10,000 g for 10 minutes at 4 °C to remove cell debris, and filtering through a 0.2 μm Acrodisc syringe filters (PALL Corporation, Port Washington, NY, USA). Working solution (supernatant) titre was determined by a plaque assay (as described below) and was determined to be 1×10^9 PFU/mL.

Plaque-forming units assay

Filtered cell culture supernatants from infected cells and water samples were assayed for infectious virus by plaque assay. Viruses were enumerated by the plaque-forming unit (PFU) method in six-well tissue culture plates as described previously (Kahler *et al.* 2011), with some modifications. Briefly, overnight confluent cell monolayers were washed and infected with 150 μl of serially diluted (10^{-1}–10^{-6}) supernatant or water sample. Ten-fold dilutions of the supernatant were prepared in MEM without FBS and inoculated onto monolayers. After a 90 minute adsorption period, the monolayers were washed and overlayed with 1% Sea Plaque agarose (Lonza Rockland, Inc., USA) containing 2× MEM and 5% FBS. Following a 2-day incubation (at 37 °C, 5% CO_2), a second agarose overlay containing 2% neutral red was added to visualise plaques for counting within 8–24 hours.

Glassware and water preparation

All glassware was made chlorine demand-free by acid washing with 10% nitric acid as per Australian/New Zealand Standards (AS/NZS 1998). Secondary-treated wastewater was used from Wastewater Treatment Plant A (WWTP A) (South Australia). The water collected had undergone primary sedimentation, activated sludge treatment (ASP) including clarification and was sampled prior to entry to the lagoon (sample point 4,004). Wastewater was stored at 4 °C for up to 7 days with negligible change in water chemistry (see below). Total dissolved solids (TDS) of WWTP A was normally around 1,000 mg/L and therefore the lagoon influent was collected and diluted with ultrapure water to obtain TDS of approximately 600 mg/L to match Victorian WWTP conditions (this was routinely a 60% dilution). This was done to overcome the effects of potentially improved disinfection efficacy. Higher TDS has been previously shown to improve inactivation rates through the presence of increased ionic strength in the form of NaCl or KCl (Jensen *et al.* 1980). Wastewater treatment processes vary from plant to plant, hence to ensure coverage of the range of water qualities produced at different plants, the range of turbidities 0.2–20 NTU were tested. The turbidity was adjusted by either filtering water through a Dialyser – Hemoflow-HF80s (Fresenius, Homburg, Germany) to remove most of the suspended particulate matter (0.2 NTU), diluting water (2 NTU) or concentrating suspended particulates by filtering through a 0.45 μm filter from this water type and resuspending in wastewater to obtain turbidities of 5 and 20 NTU.

Basic chemistry tests performed on all waters included pH, TDS, volatile suspended solids (VSS), dissolved organic carbon (DOC), total suspended solids (TSS), ammonia, total Kjeldahl nitrogen (TKN), biochemical oxygen demand (BOD_5), chemical oxygen demand (COD) and total phosphorous using Australian Standard Methods at the Australian Water Quality Centre (AWQC). Particle profiling measured particle size and distribution using a laser optical counter (Liquilaz, SO5 model), as turbidity does not provide complete information about the size and number of particles.

Buffered demand-free (BDF) water was also used to reproduce published results and was prepared as per Black *et al.* (2009).

Determining chlorine demand of wastewater and adjustment of pH

The chlorine demand of the virus and water was obtained to determine the amount of chlorine required to achieve a measurable FAC residual at the 30 minute time point post chlorination (preferably 0.5 mg/L) in the test flasks. This was obtained by adding known concentrations of chlorine to flasks containing 200 mL of wastewater spiked with CB5 and incubating in a shaking water bath (approximately

60–70 oscillation per minute) at 10 °C for 30 minutes. The FAC residuals were measured at 30 minutes post chlorination and the chlorine amount chosen to test each water type was based on the lowest amount of FAC initially added that gave a measurable FAC at 30 minutes for each of the water types tested. Comparison of wastewater with and without virus showed the virus used in these experiments had a chlorine demand of approximately 0.5 mg/L.

These experiments involved use of an aqueous chlorine stock solution (described below) as the source of chlorine and large amounts (6.5–9 mg/L) were required to achieve a measurable FAC residual (approximately 0.5 mg/L at the end of each experiment) after satisfying the wastewater and virus demand. It was necessary to adjust the pH prior to addition of chlorine to allow for the instantaneous decrease in pH observed on addition of the chlorine stock solution. This observation has not been reported in published literature as the amounts (1–3 mg/L) used in those studies were significantly lower and used demand free buffered water. The pH was adjusted with 1 N sodium hydroxide to obtain the desired pH ±0.5 being tested within the 30 minute time frame for each experiment. The pH was determined at the start and end of each experiment to ensure that the disinfection experiments were carried out at the desired pH ±0.5.

Chlorine stock, chlorine analysis and CT calculation

FAC stock solution was prepared by bubbling gaseous chlorine through ultrapure water to give a stock concentration of 1,000 mg/L of FAC. Concentration of FAC in the stock solution and sample during the course of the experiments were measured by the standard N, N, diethyl-P-phenylenediamine-ferrous ammonium sulphate (DPD-FAS) titration method (APHA 2005). For virus inactivation purposes, the important parameters were the FAC concentration and the time over which the virus was exposed to chlorine. The appropriate degree of inactivation was obtained by determining the CT where concentration is multiplied by time of chlorine exposure. Therefore CT for 1 to 4 $\log_{10}$ inactivation value of CB5 in these experiments was calculated by determining the area under a curve of chlorine concentration vs time (Ho *et al.* 2006). Combined chlorine residuals were also measured but were not used for CT calculation due to the presence of low levels of other species and the short 30 minute exposure time of these experiments where FAC was present.

Experimental protocol

Two parameters, pH and turbidity, were varied to determine CT values for inactivation of CB5 in secondary-treated wastewater. This included four different turbidities 0.2, 2.5, 5 and 20 NTU and pH 7, 8 and 9 (to represent operational bounds for disinfecting recycled water) at a constant temperature of 10 °C (the minimum temperature a number of WWTP plants operating in South Eastern Australia with improved inactivation observed at higher temperatures). The pH values and turbidities tested were carefully chosen to represent different treated wastewater types that are currently being disinfected at WWTPs in South East Australia (<2 NTU from membrane process, 2 NTU from membrane bioreactors, 5 NTU from media filters with elevated turbidity and 20 NTU from a lagoon system experiencing elevated turbidity). Chlorine disinfection experiments were performed in a bench-scale batch system using chlorine demand-free glassware in a 10 °C shaking water-bath. Each experimental condition was tested in triplicate with controls alongside each test flask to determine the initial virus concentration in the reaction flask and to evaluate whether virus inactivation occurred under the tested pH, NTU, temperature and wastewater condition in the absence of chlorine.

Determination of virus inactivation by chlorine

Six flasks containing 200 mL of wastewater with pre-adjusted pH and turbidity were inoculated with CB5 ($4–5 \times 10^5$ PFU/mL) to give a concentration that would allow detection of a 4 $\log_{10}$ inactivation of CB5. Flasks were incubated in a 10 °C shaking water-bath (approximately 60–70 oscillations per minute) for at least 2 hours prior to the experiment to allow equilibration and mixing of the virus with the particulate matter in the test wastewater. The pre-determined concentration of chlorine stock solution was added to the three test flasks (in succession). In order to determine viral inactivation by free chlorine, 1 mL samples were taken at pre-determined time points (0.5, 1, 1.5, 2.5, 5, 10, 20 and 30 minutes) and neutralised with 2% sodium

thiosulphate in 1 mL of 2 × MEM. Virus samples were stored at 4 °C for 1 to 2 hours before being assayed.

Determination of chlorine decay kinetics

To determine chlorine decay during the experiment, 20 mL samples from the same test flask were taken at time points 0.5, 2.5, 5, 10, 20 and 30 minutes and the residual free chlorine and other chlorine species was measured immediately.

Determination of virus persistence in the absence of disinfection (control)

Sampling from the control flask (no chlorine added) was done at time points 0 and 30 minutes and consistently showed negligible variation in virus numbers during its exposure to the different experimental water types across the 30 minute time period of the study.

RESULTS

Clumping of CB5 virus

The objective of this study was to determine FAC CT values for CB5 in different types of treated wastewater destined for non-potable reuse. An extensive literature review (Keegan *et al.* 2012) identified CB5 as the enteric virus most resistant to FAC disinfection. It was thus chosen as a conservative model for establishing FAC CT values for disinfection of all known pathogenic enteric viruses. The increased resistance to FAC of CB5 is partially attributed to clumping, and previously it has been shown that that purified CB5 aggregated rapidly at pH between 3 and 10 (Jensen *et al.* 1980) when purified using PBS (without calcium and magnesium) and addition of Freon 113 followed by sucrose gradient and dilution in phosphate buffer (Sharp *et al.* 1976). In this study, filtered virus in freeze/thawed cell culture supernatant was used without any further purification. The virus was filtered through a 0.2 μm filter to ensure continued sterility and remove larger particles of cell debris and so reduce the FAC demand. As CB5 is reported to be 30 nm in size (Rossman 2002), it was hypothesised that clumps of no more than six virus particles would be able to pass through the filter. Viruses present in aggregates in the same plane may pass through the filter but do not harbour protected viruses such as those seen internally in clumps. To determine if the virus stock clumped further at different pH values, known concentrations of viruses were incubated for 2 hours in water types of differing turbidity (4 and 20 NTU) and three different pH values (pH 7, 8 and 9). No difference in virus PFU numbers was observed at the three different pH values and two turbidities tested (results not shown). An additional 1 minute sonication step using a low power sonic bath sonicator (Ney Ultrasonik) was also carried out to determine if PFU numbers increased due to breaking up of potential virus clumps. No increase in virus numbers were seen with the additional sonication step (data not shown). CB5 appeared to be present in the stock solution in single virion units at pH values of 7, 8 and 9 and turbidities of 4 and 20 NTU.

Method validation and calculation of CT values for FAC in BDF

A difference between this study and the work of Black *et al.* (2009) was the preparation of the CB5 stock solution method which did not include the secondary purification step of polyethylene glycol precipitation and Vertrel treatment. As a result, the use of partially purified virus increased the demand of FAC requiring 1.5 mg/L more FAC than used in the Black *et al.* (2009) study. FAC CTs have not previously been determined for recycled waters having a higher FAC demand than BDF water. As a result, validation of the experimental methods in this study could only be done using published CTs for disinfection of CB5 performed using spiked BDF water. To determine if the test techniques used in this study gave similar results to published work, CTs for CB5 were determined at 5 °C in BDF for pH 7.5 and 9 using the method described by Black *et al.* (2009). In this study, the CT values were calculated by determining the area under the curve of chlorine concentration vs time (Ho *et al.* 2006), whereas Black *et al.* (2009) calculated CT using the 'Efficiency Factor Hom' (EFH). In the EFH approach, the rate constant of chlorine decay is used to calculate the integral. However, with treated wastewater, the decay kinetics of chlorine is more complex, being at least biphasic. The fast initial decay rate is considered to

represent the rapid interaction of HOCl with ammonia, and the second slower decay rate the slower interaction with organic amines and other compounds present in the water matrix. As a result there is no constant rate of chlorine decay in wastewater making the use of the EFH approach impractical. The empirical approach used in Ho *et al.* (2006) uses Microsoft Excel 2007, to calculate the CT by determining the integral between time 0 and time taken for virus inactivation of a respective $\log_{10}$, directly from chlorine decay graphs and is not dependent on a constant decay rate. Hence for wastewater CT studies the Ho *et al.*. (2006) method of CT calculation was considered more accurate. CT values from triplicate experiments carried out using conditions from Black *et al.* (2009) and calculated using the method of Ho *et al.* (2006) were not statistically different to published results (Table 1). Passing and Bablock regression analysis show a small constant bias (0.69), no proportional bias and a *P*-value of >0.1 indicating agreement between the two results with no significant deviation from linearity. This demonstrated that the laboratory experimental method and the calculation method used for calculation of CT could produce results similar to the previously published work of Black *et al.* (2009).

Determination of CT for CB5 in wastewater

The CTs were established in secondary-treated wastewater adjusted to reflect a range of recycled water qualities including 0.2, 2, 5 and 20 NTU, pHs 7, 8 and 9 and TDS was kept relatively constant at 600 mg/L. The chlorine concentration added to ensure a measurable residual at the end of the experiment in wastewater was 6.5 to 9 mg/L depending on the turbidity of water. When FAC was added to wastewater, the concentration of residual chlorine decreased over time in two phases following a log-logistic model (Figure 1). In the first phase, the concentration decreased rapidly. This was followed by a much slower but steady decrease in concentration over time. A significant amount of chlorine demand was observed in secondary-treated wastewater due to the reaction of chlorine with ammonia and nitrogenous compounds which reduces the availability of free chlorine for disinfection. Similar decay curves were observed for all combinations tested (pH 7–9 and turbidity 0.2–20 NTU). Use of partially purified virus (that has only gone through 0.2 μm filtration and is present in MEM with FBS) also increased the chlorine demand by 0.5 mg/L.

Table 1 | Inactivation of CB5 using chlorine at 5 °C, pH 7.5 and 9 in BDF water

Log $_{10}$ inactivation	pH 7.5 CT values[a]	Published CT values for pH 7.5 (Black *et al.* 2009)	pH 9 CT values[a]	Published CT values for pH 9 (Black *et al.* 2009)
2	6.55	5.4	13.57	14.00
3	8.93	8.4	18.79	18.70
4	11.03	11.5	23.56	22.90

[a]CTs were obtained using 2.5 mg/L of FAC and a virus concentration of 2×10^5 PFU/mL at 5 °C. The CTs presented are average of triplicate experiments. Black *et al.* (2009) used 1 mg/L of FAC and 3.6×10^5 PFU/mL at 5 °C.

For each of the wastewater types containing spiked CB5, $\log_{10}$ reduction values shown in Tables 2–5 and graphs showing the survival curves are presented in Figure 2. The survival curves show mainly linear inactivation of CB5 for nearly all pHs at the lower turbidities (0.2–5 NTU) and a linear regression line was used to determine time points for 1, 2, 3 and 4 $\log_{10}$ inactivation of viruses to calculate CTs. The only exception in this group was 5 NTU, pH 8 where the graph departed from linear indicating initially slower virus inactivation; however, it was processed using linear regression lines, shown in Figure 2, as there was no apparent reason for the observed non-linearity of the graph.

At all pH values tested for 20 NTU wastewaters, the relationships were shown to be non-linear (curved). Predominant non-linear virus inactivation curves in experiments with 20 NTU showed accelerated virus inactivation in the early stages (but slower when compared to lower turbidity results) and may be due to the presence of particulates in these higher turbidity wastewaters shielding virus from disinfection at earlier time points.

Mean CT values for disinfection of CB5 in recycled waters of turbidities 0.2–20 NTU and pH 7, 8 and 9 are shown in Table 6. The lower turbidities particularly 0.2 and 2 NTU showed no significant difference in CTs (*P*-value ≤0.05) (Table 6). CT data for 0.2 and 2 NTU have hence been combined to represent wastewater of up to 2 NTU in Victorian Department of Health (Australia) guidelines for validating treatment processes for pathogen reduction in Class A recycled waters that include urban (non-potable) water use with uncontrolled public access, in agriculture, e.g. where human food crops are consumed

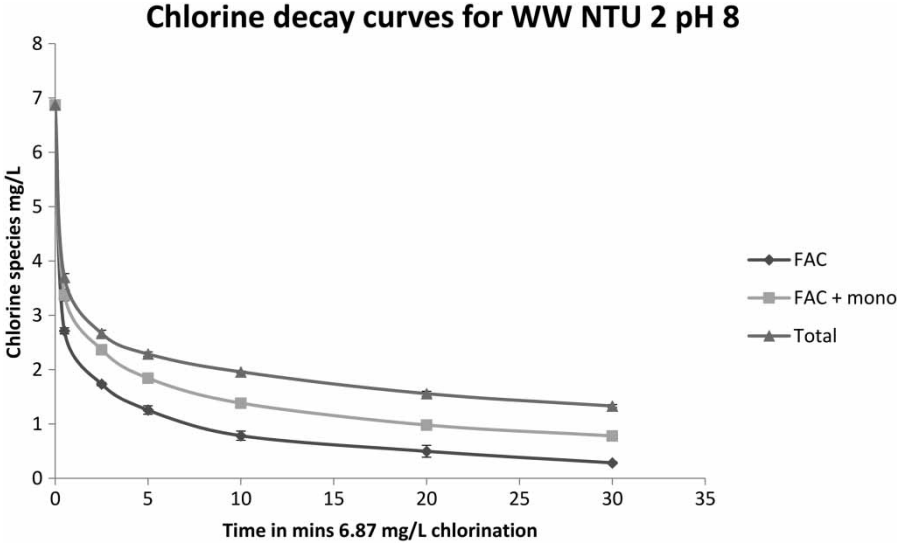

Figure 1 | Chlorine decay curve for CT experiments in wastewater of turbidity 2 NTU, pH 8 and 10 °C. FAC represents free available chlorine, FAC + mono represents free available chlorine and monochloramine, and 'Total' represents a cumulative value of all chlorine species.

Table 2 | Inactivation of CB5 using 6.50 mg/L of chlorine at 10 °C pH 7, 8 and 9 in wastewater of 0.2 NTU

pH	Time (min)	Residual[a]	Titre (PFU/mL)[a]	SD for triplicate titre	Log $_{10}$ reduction
7	0.5	3.07	8.44×10^{3}	2.14×10^{3}	1.35
	1	ND	2.01×10^{3}	2.85×10^{2}	1.97
	1.5	ND	1.7×10	7.50	4.03
	2.5	2.00	0	0	>5
	5	1.49	0	0	>5
	10	0.99	0	0	>5
	20	0.53	0	0	>5
	30	0.37	0	0	>5
8	0.5	2.86	1.67×10^{5}	2.37×10^{4}	0.07
	1	ND	7.55×10^{4}	1.5×10^{4}	0.41
	1.5	ND	2.30×10^{4}	4.66×10^{3}	0.93
	2.5	2.06	5.09×10^{3}	1.14×10^{3}	1.59
	5	1.62	2.53×10^{2}	9.80×10	2.89
	10	1.18	0		>5
	20	0.75	0		>5
	30	0.49	0		>5
9	0.5	3.42	1.36×10^{5}	1.96×10^{4}	0.16
	1	ND	1.31×10^{5}	2.31×10^{4}	0.17
	1.5	ND	8.20×10^{4}	2.76×04	0.38
	2.5	2.61	1.36×10^{4}	2.14×10^{3}	1.16
	5	2.18	8.22×10^{5}	2.77×10^{2}	2.38
	10	1.74	1.80×10	4	4.04
	20	1.22	0		>5
	30	0.9	0		>5

[a]Average of triplicate results, ND = not determined.

Table 3 | Inactivation of CB5 using 6.87 mg/L of chlorine at 10 °C pH 7, 8 and 9 in wastewater of 2 NTU

pH	Time (min)	Residual[a]	Titre (PFU/mL)[a]	SD for triplicate titre	Log $_{10}$ reduction
7	0.5	2.87	7.55×10^{3}	1.02×10^{3}	1.42
	1	ND	9.78×10^{2}	3.90×10	2.31
	1.5	ND	5.4×10	11.50×10	3.57
	2.5	1.57	0	0	>
	5	1.07	0	0	>
	10	0.63	0	0	>
	20	0.32	0	0	>
	30	0.22	0	0	>
8	0.5	2.71	1.00×10^{5}	0	0.30
	1	ND	9.33×10^{4}	0	0.33
	1.5	ND	2.75×10^{4}	3.85×10^{2}	0.86
	2.5	1.73	1.00×10^{4}	0	1.30
	5	1.26	1.00×10^{3}	6.93×10^{2}	2.30
	10	0.78	1.80×10	8	4.04
	20	0.50	0	0	>
	30	0.28	0	0	>
9	0.5	3.75	1.29×10^{5}	3.46×10^{3}	0.24
	1	ND	1.09×10^{5}	1.03×10^{4}	0.31
	1.5	ND	7.57×10^{4}	1.02×10^{4}	0.47
	2.5	2.83	1.51×10^{4}	4.23×10^{2}	1.17
	5	2.39	1.64×10^{3}	1.01×10^{2}	2.1
	10	1.72	2.20×10	7.50	4.03
	20	1.12	0	0	>
	30	0.82	0	0	>

[a]Average of triplicate results; ND = not determined.

Table 4 | Inactivation of CB5 using 6.87 mg/L of chlorine at 10 °C pH 7, 8 and 9 in wastewater of 5 NTU

pH	Time (min)	Residual[a]	Titre (PFU/mL)[a]	SD for triplicate titre	Log $_{10}$ reduction
7	0.5	3.39	7.11×10^3	3.73×10^3	1.39
	1	2.82	3.22×10^3	1.62×10^3	1.74
	1.5	2.50	2.70×10	1.70×10	3.82
	2.5	2.06	0	0	>
	5	1.63	0	0	>
	10	1.16	0	0	>
	20	0.63	0	0	>
	30	0.43	0	0	>
8	0.5	3.75	1.66×10^5	2.41×10^4	0.07
	1	3.32	9.09×10^4	7.89×10^3	0.33
	1.5	3.05	2.51×10^4	2.87×10^3	0.89
	2.5	2.68	3.64×10^3	1.24×10^3	1.73
	5	2.22	2.09×10^2	4.30×10	2.97
	10	1.71	9.00	8.00	4.34
	20	1.12	0	0	>
	30	0.80	0	0	>
9	0.5	4.16	1.15×10^5	2.97×10^4	0.22
	1	3.79	9.76×10^4	9.88×10^3	0.30
	1.5	3.56	7.32×10^4	1.78×10^4	0.42
	2.5	3.23	1.09×10^4	1.96×10^3	1.25
	5	2.73	1.06×10^3	4.84×10^2	2.26
	10	2.17	9.00×10^2	7.50×10^2	4.34
	20	1.50	0	0	>
	30	1.05	0	0	>

[a]Average of triplicate results; ND = not determined.

Table 5 | Inactivation of CB5 using 9 mg/L of chlorine at 10 °C pH 7, 8 and 9 in wastewater of 20 NTU

pH	Time (min)	Residual[a]	Titre (PFU/mL)[a]	SD for triplicate titre	Log $_{10}$ reduction
7	0.5	5.08	5.56×10^3	3.85×10^2	1.62
	1	ND	2.24×10^3	3.80×10^2	2.01
	1.5	ND	1.02×10^3	7.7×10^1	2.36
	2.5	3.31	5.55×10^2	3.9×10^1	2.62
	5	2.39	1.29×10^2	8.0×10^0	3.26
	10	1.52	9.00	8.0×10^0	4.43
	20	0.74	0		>
	30	0.42	0		>
8	0.5	4.62	7.11×10^4	7.69×10^3	0.51
	1	ND	3.78×10^4	3.85×10^3	0.79
	1.5	ND	2.60×10^4	1.16×10^3	0.95
	2.5	2.983	8.44×10^3	7.70×10^2	1.44
	5	2.113	1.07×10^3	6.70×10^1	2.34
	10	1.297	1.62×10^2	3.0×10^0	3.15
	20	0.87	1.30×10^1		4.25
	30	0.487	0		>
9	0.5	4.84	9.78×10^4	7.70×10^3	0.38
	1	ND	8.44×10^4	7.70×10^3	0.43
	1.5	ND	5.78×10^4	3.85×10^3	0.60
	2.5	3.63	3.24×10^4	2.04×10^3	0.85
	5	2.80	9.26×10^3	3.85×10^2	1.38
	10	1.96	1.20×10^3	3.34×10^2	2.28
	20	1.07	6.20×10^1	8.00×10^0	3.57
	30	0.63	9.00×10^0	8.00×10^0	4.42

[a]Average of triplicate results; ND = not determined.

raw, and industrial sites with open systems where workers are likely to be exposed (Victorian DoH 2013).

Water characteristics and their effect on disinfection

Nitrogenous compounds present in wastewater, particularly ammonia, react with chlorine quickly (within a few seconds) forming monochloroamine, dichloramine and nitrogen trichloride. The combined chlorine residuals were also measured using the DPD method but were not used for CT calculations in this study due to the presence of low levels of these species and short exposure time. CT required to achieve 2 $\log_{10}$ inactivation of virus are set at 643 mg.min/L (USEPA 1999) and levels reached in this study were less than 90 mg.min/L. To achieve a 2 $\log_{10}$ inactivation of coxsackievirus with FAC CT 5 mg/L.min was required while using dichloramine required a CT 300 mg/L.min (Hendricks 2016). The results presented here were carried out in water with low ammonia levels (<0.5 mg/L) (Table 7). Wastewaters that do not go through nitrifying/denitrifying activated sludge can generally have high ammonia levels but these studies used wastewater that had been treated by activated sludge which reduced the ammonia level to <0.5 mg/L. Where water has a high ammonia concentration, monochloramine would be the disinfectant choice.

Water quality characteristics (of water used to generate CTs in Table 6) are provided in Table 7. The quality of water did not vary greatly between the collection days and was reasonably stable during storage at 4 °C for up to 7 days prior to use. Turbidity adjustment was performed either by dilution or addition of concentrated turbidity isolated from wastewater, to achieve the required higher turbidity levels. Characterisation of turbidity was also done by analysing the organic and inorganic nature of modified and unmodified waters (modified shown in Table 7). Turbidity itself does not give adequate information about size and numbers of particles. Particle sizing, using a laser liquid

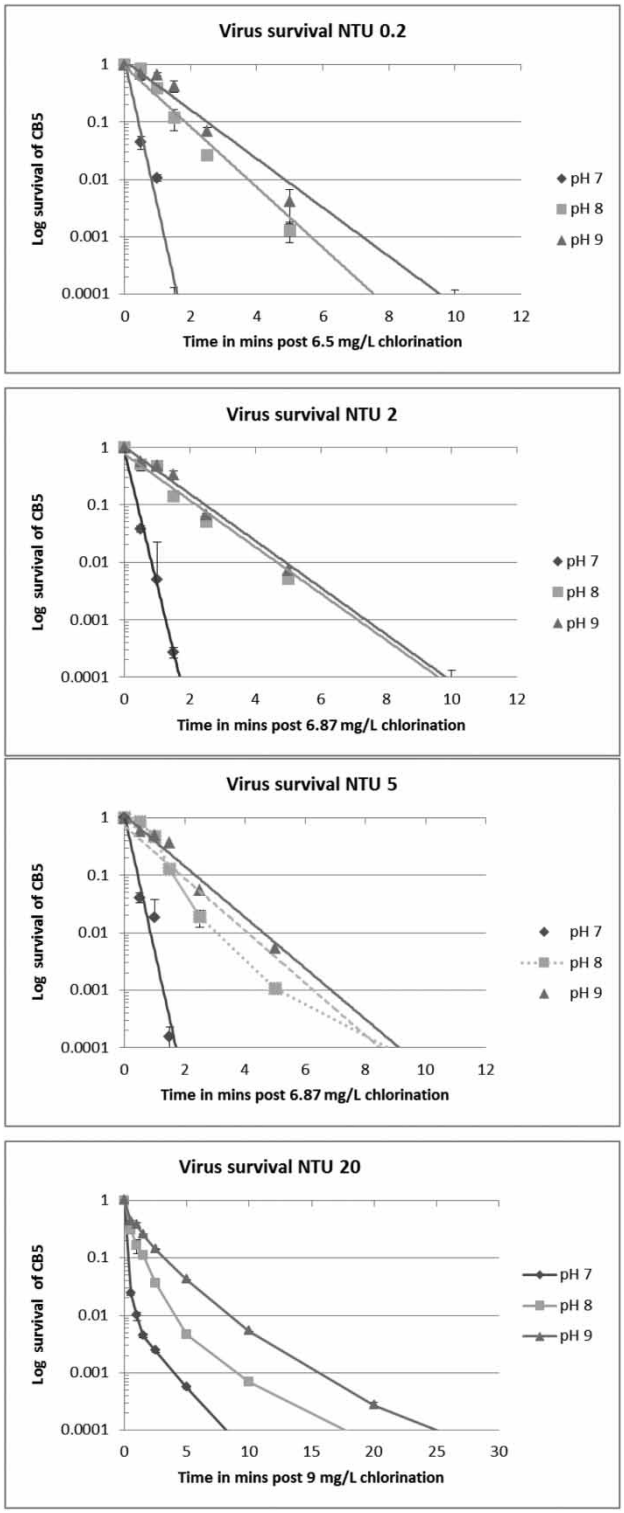

Figure 2 | Inactivation of spiked CB5, in wastewater of varying turbidity (0.2–20 NTU) were determined at 10 °C. 6.5–9 mg/L of FAC was used to test various wastewaters at three different pHs (7, 8 and 9) to determine time taken for (1 to 4) $\log_{10}$ inactivation of CB5.

optical particle counter, showed a similar distribution of the particle sizes in modified water compared to non-modified water (Table 8), although while the ratio between particle bins was similar, as expected the total numbers of particles increased with an increase in turbidity (data not shown). The wastewater TSS and VSS showed that the samples had fairly constant proportions of organic solids although some variability was observed in the 5 NTU wastewater.

The pH adjustment varied in experiments and proved difficult for wastewater experiments. Addition of chlorine (6.5–9 mg/L to satisfy demand and provide a FAC residual) to wastewater initially decreased pH and during incubation at 10 °C for 30 minutes, the pH levels tended to increase (Table 7). Chlorine was applied in the form of a chlorine water stock solution, based on gaseous chlorine and ultra pure water, which explains the generally observed decrease in the pH of wastewater due to formation of HCl. As chlorine gas is dissolved in water, it hydrolyses rapidly according to this equation: $Cl_2 + H_2O \rightarrow H^+ + Cl^- + HOCl$. In these experiments, pH was maintained ±0.5 of the required pH. Initial pH was read within 2 minutes post chlorination in a sample flask. The delayed pH reading time depended on how long it took for the pH meter to give a stabilised reading due to the presence of relatively high concentrations of ionic solutes typical of these water types. Final pH readings were taken from the three test flasks, in succession, 30 minutes after chlorination. The 20 NTU waters were the most difficult to obtain readings from, as the pH meter took a lot longer to stabilise, i.e. 3–4 minutes. It is important to note pH changes observed in these experiments were only observed in wastewater that was diluted to lower TDS and was not observed in undiluted wastewater or at treatment plants. Dilution of wastewater, as expected, diluted its buffering capacity and, to avoid any further manipulation of wastewater, additional buffering was not considered.

DISCUSSION

Current USEPA chlorine disinfection guidelines for enteric virus were developed for drinking water of low turbidity (<1 NTU). In the absence of guidelines specific to recycled water, these USEPA guidelines have frequently been applied

Table 6 | Calculated CT values by determining the integral of residual chlorine vs time of CB5 in wastewater of various turbidities and pH at 10 °C

pH	Log_{10} inactivation	CT (mg.min/L) 0.2 NTU using 6.5 mg/L chlorine	CT (mg.min/L) 2 NTU using 6.87 mg/L chlorine	CT (mg.min/L) 5 NTU using 6.87 mg/L chlorine	CT (mg.min/L) 20 NTU using 9 mg/L chlorine	[a]USEPA free chlorine CT (mg.min/L) pH 6.0–9.0 at 10 °C
7	1	2.05	2.13	2.24	2.55	
	2	3.29	3.37	3.71	5.95	3.0
	3	4.41	4.75	4.88	16.47	4.0
	4	5.44	5.46	5.99	25.81	6.0
8	1	5.72	6.67	7.78	7.99	
	2	9.60	10.32	13.16	15.09	3.0
	3	12.80	12.90	17.79	24.81	4.0
	4	15.49	15.68	21.94	34.52	6.0
9	1	8.25	8.94	9.66	13.70	
	2	14.06	15.50	16.33	28.73	3.0
	3	19.10	20.88	22.03	41.32	4.0
	4	23.97	26.00	27.93	51.89	6.0

[a]Source modified by linear interpolation between 5 °C increments. US EPA (1999).

to low turbidity recycled waters. This project has attempted to establish a wider reaching set of guidelines specific for recycled water produced from a variety of wastewater treatment processes. By including higher turbidities typical of less complex treatment processes such as ponds, guidelines have been developed that may allow the use of recycled water from a wider range of processes while protecting human health.

Disinfection of human infectious viruses in wastewater is affected by a number of factors including pH, turbidity, ionic strength and temperature. In this study, data was developed using a conservative approach, a range of pHs and turbidities that represent a variety of secondary-treated wastewaters in South Eastern Australia and at a temperature of 10 °C, which is typical of treated wastewater in coastal areas in winter. It is a well-established fact that increasing temperature increases the efficiency of chlorine disinfection hence the CT values established will be conservative as peak summer temperatures of treated wastewater can reach more than 22 °C (USEPA 1999).

Ideally, a native enteric virus present in secondary-treated wastewater should be used to account for particle association and any differences between laboratory grown vs indigenous strains. In the absence of adequate numbers of indigenous virus, a laboratory strain was used. To allow for virus attachment to particles, virus was added and incubated for at least 2 hours prior to the disinfection experiments. This allowed time for virus particles to interact and associate with wastewater particulate matter within the test water, and despite acknowledged limitations, this is currently one of the better models available to imitate native virus particle association. The concentration of virus chosen was dictated by the need to meet health-based targets which required demonstration of 4 log_{10} inactivation of virus.

There are a number of methods reviewed in Templeton *et al.* (2008) where various eluents and physical treatments have been previously used to determine levels of viruses adsorbed to wastewater sludge, aquatic sediments and soils; however, these methods would not yield information on whether the viruses were completely embedded within the particle or attached to the surface. Determining the total number of viruses that are particle associated does not give an indication of whether the virus is embedded in the particle, and hence effectively shielded from disinfection, or surface attached and hence more vulnerable to disinfection. As a result, the measure of particle association was not investigated in this study.

Use of particles from wastewater was considered the best model for these experiments. Turbidity was increased by concentration of the particles present in the treated wastewater. These particles consisted of a heterogeneous population with organic, inorganic and colloid particles. Application of the CT values derived from this work to

Table 7 | Modified wastewater water quality chemistry used for CT studies

NTU	0.2	0.2	0.2	2	2	2	5	5	5	20	20	20
pH to be tested	7	8	9	7	8	9	7	8	9	7	8	9
pH adjusted pre-chlorination	7.8	8.9	9.6	7.7	9.2	9.8	7.6	8.9	9.7	7.7	9.2	9.8
pH few mins post-chlorination	7.17	7.74	9.18	6.87	7.6	9.2	6.9	7.76	8.85	6.78	7.6	8.87
pH 30 mins after chlorination 3 flasks	7.3 to 7.4	8.1 to 8.3	8.9 to 9.0	7.4 to 7.5	7.8 to 7.9	8.9 to 9	7.2 to 7.4	7.89	8.5 to 8.6	7.1	7.6 to 7.9	8.6 To 8.7
Nitrate + Nitrite as N mg/L	ND	ND	ND	12.4	12.2	11.8	ND	ND	ND	15.9	15.9	15.9
Ammonia mg/L	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Phosphorous mg/L	0.221	0.221	0.248	1.78	1.73	1.87	0.724	0.718	0.705	4.09	4.09	4.09
TKN mg/L	1.85	1.85	1.67	2.51	2.43	2.34	2.28	2.16	2.09	6.18	6.18	6.18
DOC mg/L	9.4	9.4	9.7	10.6	10.9	10.8	8	7.7	7.8	9.1	9.1	9.1
TOC mg/L	9.8	9.8	10	12.1	12	11.8	9.6	8.9	9	13.4	13.4	13.4
TDS mg/L	570	570	580	610	630	620	560	600	570	560	560	560
BOD mg/L	<2	<2	<2	5	4	4	6	8	4	16	16	16
COD mg/L	27	27	ND	24	40	78	42	ND	72	79	79	79
SS mg/L	<1	<1	<1	10	5	6	16	10	8	44	44	44
VSS mg/L	<1	<1	<1	8	4	6	9	9	6	43	43	43

ND = not determined. For each experimental condition tested in triplicate, wastewater used was adjusted accordingly and tested for a number of chemical and biological properties listed in this table.

recycled water from wastewater treatment processes using such metal salts as part of the treatment process is not recommended without further experimental work.

CT values were established for pH values 7, 8 and 9 likely to typify treated wastewater. These results demonstrated reduced disinfection which is typical of increasing pH. This is attributed to the known effect of pH on dissociation of HOCl to OCl^-. HOCl is the more powerful oxidant and decreases in favour of the less powerful oxidant OCl^- with increasing pH.

Increases in turbidity from 0.2 to 5 NTU demonstrated that only slightly increased CTs were required to achieve the required $\log_{10}$ inactivation. However, the increase in turbidity from 5 to 20 NTU lead to a greater than 2-fold increase in CT values for all pH values as seen in Table 6. Turbidity and ionic strength (the latter kept constant between 560 and 630 mg/L) have been previously implicated in playing a role in the differential inactivation rates of viruses. However the effect of increasing turbidity in reducing disinfection is not observed between 0.17 and 0.6 NTU as shown by Kahler *et al.* (2010). As these values are considerably lower than the turbidities tested in this study, this observation was not considered. Ionic strength was chosen to represent typical recycled water around Australia and was kept constant throughout this study, hence its effect on disinfection cannot be determined based on the data presented here.

Black *et al.* (2009) investigated free chlorine disinfection of a range of viruses in BDF water at 5 °C at pH values of 7.5 and 9. CTs for CB5 inactivation in BDF water at pH 9 and 5 °C were determined to be 14.00 mg.min/L for 2 $\log_{10}$ inactivation, 18.70 mg.min/L for 3 $\log_{10}$ and 22.90 mg.min/L for 4 $\log_{10}$ inactivation (Table 6). In this study, very similar CTs of 14.06, 19.10 and 23.97 mg.min/L are reported for 2 $\log_{10}$, 3 $\log_{10}$, 4 $\log_{10}$ inactivation of CB5 respectively (Table 6) tested at 0.2 NTU, pH 9 and 10 °C in wastewater. This demonstrates that at lower NTUs of $\leq$0.2, the CT changes are not significant.

This study demonstrates that CB5, the currently known enteric virus most resistant to FAC can be effectively disinfected in waters destined for recycling at varying turbidities up to 20 NTU and at a temperature of 10 °C. At pH 8 and above, the increase in turbidity above 2 and up to 20 NTU required a much higher increase in CT compared to similar

Table 8 | Particle size distribution (expressed in percentage) in modified and unmodified WWTP A secondary-treated wastewater used for disinfection experiments

Particle size (μm)	0.2 NTU filtered wastewater (%)	2 NTU diluted wastewater (%)	5 NTU wastewater concentrate added (%)	20 NTU wastewater concentrate added (%)	Unmodified wastewater (%)
≥0.5	90	60	64	65	65
1	9	26	18	19	21
2	1	6	8	5	6
3	0	3	5	3	3
4–20	0	5	5	8	5

turbidity increases at pH 7. Use of a conservative approach, such as use of the most resistant enteric virus, low temperature, a range of turbidities up to 20 NTU and a range of pH values allowed development of a robust data set to establish guidelines for disinfection of similar types of secondary treated-wastewater. The USEPA guidelines have a safety factor of 3× applied to ensure that they are sufficiently protective of public health. The CT values developed in this study and used in the Australian Victorian Department of Health guidelines (DoH 2013) have not included a safety factor due to the layers of conservatism already present in the development of the data together with the wide ranges of pH and turbidity and have been deemed adequate for protection of public health. However, some conservatism may be required as mixing and controlling CT in practice is often imperfect, turbidity structure and composition may vary between WWTPs and environmental viruses may vary in their resistance to disinfection (Payment *et al.* 1985) and this may have an effect on actual treatment efficiency.

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