Comparison of bacteriophages for use in iodine inactivation: batch and continuous flow studies

Gail M. Brion, N. Brennan O'Banion and George L. Marchin

ABSTRACT

Inactivation rates in batch studies for four commonly used surrogate bacteriophages were measured in stable aqueous iodine solutions for the purpose of determining which was the most suited to evaluate iodine disinfection efficacy in batch and continuous flow conditions. Two types of group Leviviridae bacteriophages were used, Type I (MS2) and Type II (GA), along with group Microviridae, Phi-X174, and group Tectiviridae, PRD1. Inactivation was compared at iodine doses of 1.0–1.5 mg I_2/l. MS2 was the most susceptible to iodine inactivation of the four phages tested. Inactivation of naked, icosahedral bacteriophages, MS2 and Phi-X174 demonstrated removals to below detection limits (>99.99%) in less than 10 min. Lipid-containing PRD1 and F+ssRNA GA bacteriophages demonstrated the greatest iodine resistance in batch experiments with an average of 1.82 logs of inactivation (98.5%) after 60 min and 1.05 logs of inactivation (91.1%) after 30 min respectively. Similarly, in continuous flow studies through pentaiodide quaternary ammonium strong base resin, MS2, GA and Phi-X174 were more strongly inactivated than PRD1. The lipid component of PRD1 is thought to enhance resistance to iodine over non-lipid-containing bacteriophages by protecting easily oxidized groups on the protein capsid, but further research is needed before proving this hypothesis. The results from this research may provide a surrogate standard for more rigorous and developed research into the mode of iodine disinfection and its inactivation kinetics.

Key words | bacteriophages, disinfection, iodine, inactivation, pentaiodide resin, viral surrogates

Gail M. Brion
N. Brennan O'Banion
Department of Civil Engineering,
University of Kentucky,
Lexington,
KY 40506-0281,
USA

George L. Marchin (corresponding author)
Division of Biology,
Kansas State University,
Manhattan,
KS 66506-4901,
USA
E-mail: *gmarchin@ksu.edu*

INTRODUCTION

Extensive viral inactivation research has been conducted on potable water disinfectants such as chlorine, ozone and UV, resulting in the publication of an array of defined surrogate bacteriophages and methods that can be used to model the response of pathogenic enteroviruses (Cramer *et al*. 1976; Alvarez & O'Brien 1982; Battigelli *et al*. 1993). However, limited research has been conducted utilizing iodine; and an adequate bacteriophage surrogate for iodine inactivation of pathogenic enteroviruses is as yet undefined. Indeed, little is known about the disinfection properties of iodine such as target sites and mode(s) of inactivation. With the emergence of point-of-use water disinfection devices containing iodinated materials (Marchin & Fina 1989), such data for critical testing strategies are needed. Published inactivation studies have utilized iodine concentrations well above that considered palatable for human consumption and have yielded limited kinetic information on bacteriophages due to experimental concentrations that reduce phage numbers quickly below detectable ranges. This study presents an analysis of different bacteriophage groups to determine which among these groups are most suited to evaluate iodine disinfection as indicated by superior persistence in the presence of low doses of free iodine.

MATERIAL AND METHODS

Hosts and bacteriophages stock preparation

Crude stocks ($\approx 10^{10}$ PFU/ml) of the F+ssRNA group Leviviridae bacteriophage Type I (MS2) and Type II (GA)

Downloaded from https://iwaponline.com/jwh/article-pdf/2/4/261/396230/261.pdf
by guest

obtained from the laboratories of M. D. Sobsey (University of North Carolina at Chapel Hill) were obtained by propagation in soft agar with host *E. coli* C-3000 (ATCC 15597). The multiplicity of infection was 0.001. After incubation, Petri dishes showing confluent lysis were covered with 15 ml of sterile phosphate buffered saline (PBS). After 30 min, PBS was aspirated, placed into sterile polypropylene tubes, and stored at −70°C until needed. Crude stocks of ssDNA bacteriophage group Microviridae (Phi-X174), originally obtained from the laboratories of M. D. Sobsey (University of North Carolina at Chapel Hill) and enveloped bacteriophage group Tectiviridae (PRD1) originally obtained from the laboratories of C. P. Gerba (University of Arizona) were produced in the same manner as MS2 and GA with the exception of the hosts used for growth: C-13 (ATCC 13706) and a strain of *Salmonella typhimurium* obtained from the laboratories of C. P. Gerba (University of Arizona), respectively. To prepare cleaner stocks for disinfection experiments, crude stocks of MS2, GA and Phi-X174 were pelleted by ultra-centrifugation at 90,000 *g* for 4 h (Battigelli *et al*. 1993). The pellet was then resuspended in sterile borate buffer (pH 7) up to 1 ml and placed at 4°C for no longer than 1 day before usage in disinfection experiments. PRD1 crude stock was pelleted at 90,000 *g* for 2 h in a Beckman SW41Ti rotor and then resuspended in borate buffer and used the same day.

Iodine analysis

The Hach DPD colorimetric method for iodine analysis was used to determine iodine residuals as mg I_2/l by spectrophotometry (Hach, Loveland, CO).

Iodine inactivation experiments

All batch inactivation experiments were conducted at 10°C in 0.05 molar borate buffer (pH 5.6) made with halogen demand free water (HDFW). The selected pH ensured that the most stable iodine residual, I_2, would be predominant. The borate buffer is known to slow the rate of active iodine residual decomposition (Brion & Silverstein 1999). Ultracentrifuged stocks containing a mixture of phages were diluted in borate buffer to the desired concentrations and combined as either pooled Stock A (MS2 + PRD1) or pooled Stock B (Phi-X174 + PRD1) for use in replicate inactivation experiments. A stock iodine solution (resublimed I_2 crystals and halogen demand free water (HDFW)) was diluted with borate buffer to 1.4 mg I_2/l and dispensed in 27 ml amounts into four 50 ml polypropylene centrifuge tubes. This initial iodine concentration would decrease to near 1.0 mg/l upon the addition of the phage-containing stock. Three tubes containing iodinated buffer had 3 ml of combined phage stock added and were then used to measure phage concentration (1), pH (2) and iodine residual (3) throughout the experiment. Aliquots from tube 1 were taken at 1, 5, 10, 30 and 60 min and immediately diluted in PBS containing 1% Na_2SO_3 to quench the reaction. Solution pH and residual concentrations were measured at 0, 30 and 60 min. In the fourth tube (4), containing 27 ml of iodinated buffer and 3 ml of non-iodinated buffer, measurements were taken at 0 and 60 min to check for halogen demand of the buffer and containers. Phage controls were taken at the beginning and end of the experiments from a fifth tube (5) that contained non-iodinated borate buffer and phage spike solution. The GA inactivation experiments were done in a similar manner, with two major differences: only GA phage stock was used instead of a stock containing a combination of bacteriophages and the initial iodine titer was increased to 2.1 mg/l iodine which resulted in an initial iodine concentration of 1.5 mg/l after the addition of the GA phage stock.

Iodinated resin studies

A commercial grade of pentaiodide quaternary ammonium strong base resin was obtained commercially (PentaPure, Eagan, MN) to see if the behavior of the selected phage groups would be similar in a kinetic study that used both bound and free forms of iodine. The resin (2.5 cm^3) was packed in a 3.0 cm^3 plastic syringe barrel (Promega, Madison, WI) fitted with a fiber glass plug. Suspensions of phages in HDFW at 25°C were pumped through the resin bed at a flow rate of 10 ml/min using a

Downloaded from https://iwaponline.com/jwh/article-pdf/2/4/261/396230/261.pdf
by guest

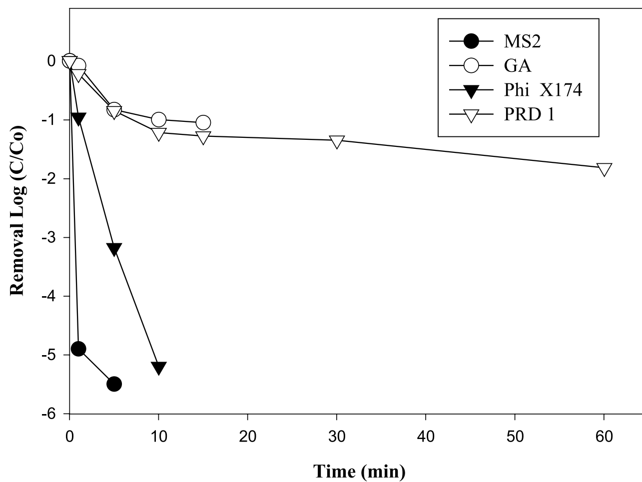

Figure 1 | Kinetics of temporal inactivation of phages by iodine.

peristaltic pump (Masterflex, Cole-Palmer). An initial sample without phages was collected to determine free iodine concentration as described above. Phage samples were collected in 1% Na_2SO_3 to quickly neutralize the iodine residual upon passage through the resin column. Surviving phages were titered as described below.

Sample assay

Phage concentrations at sample times were determined by plaque formation on double-layer agar assay as previously described (Brion & Silverstein 1999) with triplicate plating of each dilution. Detection limit was calculated to be 30 PFU/ml in the iodine inactivation tube 1.

RESULTS AND DISCUSSION

The difference in susceptibility of each bacteriophage group to iodine residuals is clearly illustrated by the rate at which they are inactivated in static batch experiments over time (Figure 1). MS2 is the most susceptible to low doses of iodine, showing almost 5 logs of removal (99.999%) at 5 min. Phi-X174 was the next most susceptible with greater than 99.999% removal at 10 min. PRD1 exhibited the next greatest amount of resistance to the biocidal effects of iodine with a log reduction of 96.0 to >99.4% at 60 min (Table 1). The slight difference in inactivation for PRD1 between experiments using Stock A and B is thought to be due to slight differences in the overall halogen demand of the pooled stocks. It is of note that, in subsequent single phage stock experiments with

Table 1 | Phage inactivation in batch experiments

				Log (C/C_0) at times (min)				
Phage	***n***	**Control (PFU/ml)**	**Initial iodine (mg/l)**	**1**	**5**	**10**	**30**	**60**
MS2	2	1.0×10^7	1.01	– 4.90	– 5.50[a]	BDL	BDL	BDL
Phi-X 174	2	5.2×10^6	1.02	– 0.97	– 3.19	– 5.20[a]	BDL	BDL
PRD1	2	4.2×10^5	1.01	– 0.11	– 0.63	– 0.91	– 1.10	– 1.39
Stock A PRD1	2	3.1×10^5	1.02	– 0.32	– 1.07	– 1.54	– 1.59	– 2.25
Stock B PRD1	4	3.6×10^5	—	– 0.22	– 0.85	– 1.22	– 1.35	– 1.82
Average GA[b]	2	2.2×10^4	1.5	– 0.08	– 0.83	– 1.00	– 1.05	—

[a]Lowest measurable concentration.
[b]Single phage stock experiment.
BDL = below detection limit.

Downloaded from https://iwaponline.com/jwh/article-pdf/2/4/261/396230/261.pdf
by guest

GA at higher iodine residuals (1.5 versus 1.0), that GA phage type showed removal similar to PRD1. As can be seen in Table 1, the average inactivation of PRD1 and GA at 10 and 30 min is quite close, even though the GA experiment had higher iodine residual. In Figure 1, the two-run average for GA inactivation falls within the error bars for the four-run PRD1, making a statistical determination of the most resistant phage group impossible.

It is thought that the enhanced survival of PRD1 in iodine containing water can be attributed to the bilipid component surrounding the protein coat. The mechanism of iodine inactivation is thought to involve oxidation of the protein coat (Hsu 1964; Cramer *et al*. 1976; Alvarez & O'Brien 1982). The bilipid component of PRD1 may act as a barrier that prevents iodine from reaching sulfhydryl groups, tyrosine and histidine rings, and oxidizing tryptophan, a morphological advantage that neither MS2, GA, nor Phi-X174 possesses. Although the MS2 phage group is a recommended model phage for chlorine, ozone and other disinfectant inactivation studies where destruction of the genetic material takes place as well as protein coat oxidation, it is clearly not the best choice for study of the kinetics of iodine inactivation in batch experiments as it does not survive long enough to obtain kinetic information even at low doses of iodine. MS2 is not even clearly superior with respect to survival in iodinated water to other types of the group Leviviridae such as GA in the experiments summarized in Figure 1 and Table 1. Other studies have shown GA to exhibit superior survival to inactivation in surface waters when compared to MS2 (Brion *et al*. 2002) and our batch study with iodine is in agreement with these previous results.

From the results above, MS2 is not the hardiest of the phage groups tested, and when reviewing other studies, it would not appear to be as resistant against iodine inactivation at pH values below 7 as enteric viruses such as poliovirus, echovirus and hepatitis A virus. A previous study by Sobsey *et al*. (1991) has shown animal viruses to be resistant to larger doses (8 mg/l versus 1.0–1.5 mg/l) of iodine than were used in this study with MS2. They found poliovirus type 1 and echovirus type 1 to have inactivation times of <3.9 and <4.9 min to drop 99.99% of the initial concentration ($\log C/C_0 = -4$) at pH 7.0, iodine dose = 8 mg/l and temperature = 25°C (Sobsey *et al*. 1991). As pH decreased to 4.5 (temperature and iodine dose remained the same), the times for 99.99% inactivation increased to 7.2, 54 and <13 min for hepatitis A virus, poliovirus type 1 and echovirus type, respectively (Sobsey *et al*. 1991). In our study, MS2 declined more than 99.99% within 1 min, exhibiting less survival than all of these enteric viruses at doses of iodine only 1/8 as great as that used in the comparison study. Although care must be taken when comparing results between studies due to differences in disinfectant residual, temperature, buffer and virus stock preparation, MS2 survival in iodine disinfectant experiments conducted similarly is not as great as that reported for other enteric viruses at higher iodine doses and higher temperatures.

The types of batch inactivation studies presented in this study often result in first order models where the logarithm of the concentration is plotted against the logarithm of time, yielding a constant value that is related to concentration × time (*Ct*). This constant is used to determine the size of clearwells in water treatment plants and other holding structures for disinfection to obtain a desired degree of inactivation for a pathogen. If one used MS2 as a surrogate for enteric viruses, the *Ct* value for MS2 would be lower than that for the enteric viruses it is supposed to model, underestimating the amount of time at a set residual required to inactivate 99.99% of the viruses of public health concern. Clearly, another one of the phage groups should be considered a better surrogate in the light of published research and the findings of this study.

However, there are more factors to consider when selecting a good phage surrogate for enteric viruses to test disinfection processes than similar disinfection kinetics; one should consider similar structure as well. PRD1, although a hardier phage than MS2, is not similar enough in structure to the naked human enteric viruses and therefore will not behave similarly due to capsid protection provided by its lipid component. However, the more similarly structured F + ssRNA coliphage GA may be more appropriate to use based on its similar structural and iodine survival characteristics similar to those reported for poliovirus type 1. Our results show that GA coliphages drop 90% in titer at iodine levels of 1.5 mg/l as I_2 at

Downloaded from https://iwaponline.com/jwh/article-pdf/2/4/261/396230/261.pdf
by guest

Table 2 | Phage inactivation in continuous flow experiments

Phage	Flow rate (ml/min)	Iodine[a] (mg/ml)	Temperature (°C)	Input phage (PFU/ml)	Log(C/C_0)
MS2	10.0	12.8	25	1.23×10^7	– 2.66
GA	10.0	12.3	25	3.29×10^6	– 2.81
PRD1	10.0	11.7	25	4.60×10^6	– 0.22
Phi-X174	10.0	12.2	25	1.16×10^7	– 2.57

[a]Free iodine residual in samples not collected in 1% $Na_2S_2O_3$.

10 min. This is close to the 90% reduction shown by Alvarez & O'Brien (1982) for poliovirus at 2 min, pH = 6.0, temperature = 25°C, iodine dose = 2.5 mg/l as I_2. It would appear that, of the F + ssRNA phages, which are similar in structure to enteric viruses of public health concern, the GA type would be a better surrogate of animal viruses than MS2 in batch experiments.

Continuous flow experiments through iodinated resin columns were conducted to determine if the survival trends would be similar to those noted in the previously discussed batch experiments. In continuous flow experiments (Table 2) the resistance of PRD1 to iodine inactivation to high concentrations of residual iodine was again evident, but a similar degree of inactivation of phage groups MS2, GA and Phi-X174 was observed. With a relatively short residence time in the column of 15 s (bed volume/flow rate) and quick neutralization of free iodine with 1% $Na_2S_2O_3$ the relatively strong inactivation of MS2, GA and Phi-X174 may be explained by a combination of relatively high iodine concentrations in the resin bed and electrostatic properties of demand-release disinfectants (Marchin & Fina 1989). The 15 s residence time is similar to that in some commercial devices with the kind of pentaiodide employed in these studies. Other polyiodide resins may have different residence times depending on their composition and application (Marchin & Fina 1989).

When conducting disinfectant studies it is important to understand the behavior of the halogen in the buffered water matrix with and without viruses (Table 3). In this way, the experimental system can be modified to maintain the most stable disinfectant residual for the length of time required with minimal residual losses. As noted in prior studies by Brion & Silverstein (1999) borate buffers maintain a slower rate of hypoiodous acid (HOI) decomposition to iodate and iodide, while pH selection to

Table 3 | Residual iodine measurements in batch experiments

			Iodine residual (mg I_2/l) (tube 3)			
Pooled stock experiment	*n*	Initial iodine residual (mg I_2/l) (tube 4)	0[a]	30[a]	60[a]	Final iodine residual (mg I_2/l) (tube 4)
Stock A (PRD1 + MS2)	2	1.01	0.88	0.84	0.82	1.01
Stock B (PRD1 + Phi-X174)	2	1.02	0.99	0.92	0.83	1.02
GA	2	1.5	1.49	1.48	—	1.5

[a]Minutes.

Downloaded from https://iwaponline.com/jwh/article-pdf/2/4/261/396230/261.pdf
by guest

below 6.0 keeps the bulk of iodine residual in the most stable form of I_2. Initial and final iodine controls from the unspiked iodine residual control tube 4 clearly show stable iodine residuals for the duration of the experiments with only minimal residual losses in the spiked control tubes (Table 2). This ability to maintain stable iodine concentrations throughout the time period of experimentation is due to the selection of borate buffer in the suspending matrix, pH selection below 6.0 for the stable I_2 as the predominant iodine disinfectant species and low halogen demand of the ultrapurified bacteriophage stocks prepared. Another study (Sobsey *et al*. 1991) looking at virus inactivation by iodine-releasing, globaline tablets in unspiked control tubes of 0.1 M phosphate buffered halogen demand free water noted residual losses of 18–22% (1.7 mg/l average) of 8 mg I_2/l initial iodine doses at pH = 4.5. In our study using borate buffered water instead of phosphate, only measurements from the inactivation reactors (tube 3) show any residual loss (0.19 mg I_2/l) from an average initial free iodine residual of 1.01 over 60 min. None of the controls (tube 4) show any residual loss. The minimal loss of halogen residual in the inactivation reactors is thought to be the result of the demand added by the 3 ml of pooled phage stock solution.

In conclusion, when comparing two types of group Leviviridae bacteriophage (Type I (MS2) and Type II (GA)) in batch inactivation experiments, it is clear that the more commonly used phage group MS2 is not an appropriate surrogate choice for testing iodine disinfection processes based upon its limited resistance to iodine inactivation. The GA phage demonstrated the best survival in the batch iodine inactivation experiments, followed by the enveloped PRD1 and then the naked DNA-containing Phi-X174. In continuous flow experiments, the enveloped PRD1 phage group demonstrated the best survival upon passage through high concentrations of iodine residuals generated by commercially available resins, while the MS2, GA and Phi-X174 phage groups all showed similar, but considerably reduced, survival. Since the focus of this study was the comparison of commonly used surrogate phage groups against each other, and animal virus assay was not in the funded scope of research, more studies are needed before definitively establishing the best types of phage surrogates under all the types of conditions when iodine is applied. More low-level iodine inactivation research conducted in borate and carbonate buffers should be funded that includes side-by-side comparisons of likely surrogate phage candidates and enteric viruses to provide a definitive basis for establishing guidelines for the industry.

ACKNOWLEDGEMENTS

This research was sponsored by the NSF POWRE program, ID no 9805904. Some of the continuous flow experiments were funded by a NASA-Bioserve grant to Kansas State University. The authors would like to thank Dr Mark Sobsey and Dr Charles Gerba for provision of bacteriophage stocks, bacterial hosts and technical assistance.

REFERENCES

Alvarez, M. E & O'Brien, R. T. 1982 Mechanisms of inactivation of poliovirus by chlorine dioxide and iodine. *Appl. Environ. Microbiol.* **44**, 1064–1071.

Battigelli, D. A., Sobsey, M. D. & Lobe, D. C. 1993 The inactivation of hepatitis A virus and other model viruses by UV irradiation. *Wat. Sci. Technol.* **27**(3–4), 339–342.

Brion, G. M. & Silverstein, J. 1999 Iodine disinfection of a model bacteriophage, MS2, demonstrating apparent rebound. *Wat. Res.* **33**, 169–179.

Brion, G. M., Meschke, J. S. & Sobsey, M. D. 2002 Male-specific coliphage: prevalence, types, and survival in natural waters. *Wat. Res.* **36**(9), 2419–2425.

Cramer, W. N., Kawata, K. & Kruse, W. K. 1976 Chlorination and iodination of poliovirus and f2. *J. Wat. Pollut. Con. Fed.* **48**, 61–76.

Hsu, Y. 1964 Resistance of infectious RNA and transforming DNA to iodine which inactivates *f2* phage and cells. *Nature* **203**, 152–153.

Marchin, G. L. & Fina, L. R. 1989 Contact and demand-release disinfectants. *CRC Crit. Rev. Environ. Cont.* **19**, 277–290.

Sobsey, M. D., Oldham, C. E. & McCall, D. E. 1991 Comparative inactivation of hepatitis A and other enteroviruses in water by iodine. *Wat. Sci. Technol.* **24**(2), 331–337.

Downloaded from https://iwaponline.com/jwh/article-pdf/2/4/261/396230/261.pdf
by guest