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Comparative Inactivation of Poliovirus Type 3 and MS2 Coliphage in Demand-Free Phosphate Buffer by Using Ozone

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MS2 coliphage (ATCC 15597-B1) has been proposed by the U.S. Environmental Protection Agency as a surrogate for enteric viruses to determine the engineering requirements of chemical disinfection systems on the basis of previous experience with chlorine. The objective of this study was to determine whether MS2 coliphage was a suitable indicator for the inactivation of enteric viruses when ozone disinfection systems were used. Bench-scale experiments were conducted in 2-liter-batch shrinking reactors containing ozone demand-free 0.05 M phosphate buffer (pH 6.9) at 22°C. Ozone was added as a side stream from a concentrated stock solution. It was found that an ozone residual of less than 40 μg/liter at the end of 20 s inactivated >99.99% of MS2 coliphage in the demand-free buffer. When MS2 was compared directly with poliovirus type 3 in paired experiments, 1.6 log units more inactivation was observed with MS2 coliphage than with poliovirus type 3. It was concluded that the use of MS2 coliphage as a surrogate organism for studies of enteric virus with ozone disinfection systems overestimated the inactivation of enteric viruses. It is recommended that the regulatory agencies evaluate their recommendations for using MS2 coliphage as an indicator of enteric viruses.

The 1986 amendments to the Safe Drinking Water Act required the U.S. Environmental Protection Agency to promulgate primary drinking water regulations. The regulations required disinfection of all public water supplies, specified water quality criteria under which filtration of surface waters would be a requirement, and listed *Giardia lamblia* and viruses among a list of 83 parameters which would be regulated in drinking water (24). Specific treatment objectives for the inactivation or removal of *G. lamblia* and enteric viruses were 99.9 (3 log units) and 99.99% (4 log units), respectively. The guidance manual for the surface water treatment rule recommended the use of MS2 coliphage as a surrogate for enteric viruses (17). It was assumed that a 99.99% inactivation of coliphages would ensure that enteroviruses would be absent from the finished water (24).

Many ozone disinfection studies with poliovirus as the test organism have been performed (3, 5, 12, 14, 18, 19). Previous reviews of the literature suggested that polioviruses were more resistant to ozone than were other enteroviruses (13, 20). The f2 and MS2 phages have been reported to be more resistant to chlorine than are other coliphages, leading to their use as surrogates for enteric viruses in disinfection studies (10, 15, 23).

There have been fewer studies of ozone inactivation of coliphages than of inactivation of enteric viruses. An observation common to the studies to date is that coliphages 185, f2, and MS2 were significantly more sensitive to ozone than were enteric viruses, with 6- to 7-log-unit inactivation occurring very quickly and in the presence of little or no ozone residual (5, 10, 22, 25, 26). These observations cast doubt on the reliability of coliphage as an indicator for the performance of ozone disinfection of enteric viruses.

The purpose of this study was to perform paired experiments with MS2 coliphage (ATCC 15597-B1) and poliovirus type 3 to determine whether the ozone inactivation results were comparable.

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MATERIALS AND METHODS

MS2 preparation. The stock suspension of coliphage MS2 ATCC 15597-B1 was prepared from a culture grown in the host bacterium *Escherichia coli* ATCC 15597 by methods described elsewhere (17). The titer of the MS2 stock was 8.1×10^{10} PFU/ml. Samples containing MS2 were assayed by the agar overlay method by using 1.0-ml samples and 2 drops of overnight *E. coli* host culture (1). Dilutions were made in sterile 0.1% peptone.

Poliovirus type 3 preparation. Attenuated poliovirus type 3 (strain WM-3; ATCC VR-300) was obtained from the Viral Immunopathogenesis Research Unit, Department of Medical Microbiology and Infectious Diseases, University of Alberta, Edmonton, Canada. The virus was propagated in HEp-2 cells by standard methods (9, 21). Stock virus was produced by harvesting the virus from infected HEp-2 cells when the maximum cytopathic effect was apparent, usually at 24 to 48 h. The poliovirus was semipurified by centrifuging the infected cell suspension for 30 min at 5,000 × *g*, washing the pellet in cold phosphate-buffered saline (pH 7.2), recentrifuging, and then freeze-thawing the suspension three times in ethanol-dry ice to release the virus. This step was followed by another centrifugation for 20 min at 2,000 × *g* to remove cell debris, and the supernatant was then centrifuged for 45 min at 30,000 × *g*. The resulting pellet was suspended in phosphate-buffered saline (pH 7.2) and stored at −70°C.

Poliovirus was enumerated by a standard agar overlay plaque assay method (21). When plaques appeared, at 48 to 96 h postinfection, the agar overlay was removed and the cells were fixed with 40% ethanol–10% acetic acid solution for 24 h and then stained with crystal violet. For virus dilutions producing 50 or fewer plaques per assay container, the numbers of virus particles were counted.

Ozone apparatus. The protocol for preparing the stock ozone solution and ozone demand-free phosphate buffer was identical to that used in earlier work (6, 7). Ozone concentrations in the aqueous phase were determined by UV A_{260} with a molar absorption coefficient of 3,300 M^{-1} cm^{-1}, which lies midway between the reported extremes of this

value (8). The concentration factor is 14.55 cm · mg/liter. The stock ozone solution concentration was typically 18 to 20 mg/liter at 22°C.

The ozonation experiments were conducted at room temperature (22°C) with 1.7-liter volumes of ozone demand-free 0.05 M phosphate buffer (pH 6.9) in 2-liter borosilicate glass beakers fitted with stainless steel quadrant baffles. A floating Teflon lid minimized the loss of ozone through stripping. Mixing was accomplished by using a Teflon-coated magnetic stir bar. The reactor was continuously sampled by using a peristaltic pump with a flow rate of 8 ml/min. The sample was carried through a short piece of small-diameter Teflon tubing to a 35-μl flow cell with a light path of 1 cm situated in a diode array spectrophotometer (Hewlett Packard model 8452A). Test solution A_{260} was continuously monitored with this apparatus. A slight excess of 1.0 M sodium formate was used to neutralize residual ozone, since sodium formate does not interfere with UV A_{260} measurements. The reaction of sodium formate and ozone is very fast.

Ozone disinfection procedure. Two types of experimental designs were used. The first was simply the addition of one type of virus to a reactor followed by the addition of ozone for a fixed contact time. For MS2, the contact time was fixed at 20 s and various ozone doses were added to provide a residual. This procedure proved unsuccessful for poliovirus because a high concomitant ozone demand prevented any meaningful comparisons between MS2 and poliovirus. The second experimental design was a paired design with both virus types in the same reactor. This design provides directly comparable data, since the absolute ozone doses and residuals are not important in determining the relative sensitivities of each organism to ozone.

Prior to the addition of ozone, each reactor vessel received 1.5 ml of the stock coliphage titer, providing a final MS2 concentration of approximately 10^7 PFU/ml. After the contents of each vessel were mixed, a 10-ml sample was removed to be assayed for the initial concentration of MS2 coliphage. The mean initial concentration (N_0) of MS2 was $10^{6.9}$ PFU/ml in the trials in which MS2 was the only virus.

The poliovirus stock titer was sonicated for 1 min to disrupt aggregates. Various initial concentrations (N_0) of poliovirus were added to the test solutions in the paired virus experiments (see Table 1). After the test solutions were mixed, a 10-ml sample was removed and stored at 4°C until it was assayed.

The prepared reactor vessel containing the test viruses was placed on a magnetic stirrer, and the 35-μl flow cell pump was attached to the reactor. The spectrophotometer was zeroed by using the test water containing viruses. The concentration of the stock ozone solution was determined twice: immediately before and immediately after the addition of the ozone to the test solution. If the two ozone concentration measurements differed by more than 0.5 mg/liter, the experiment was not continued. Ozone demand-free mass-calibrated pipettes were used to transfer ozone from the stock solution to the reactor vessel. For the single-contact-time experiments, a laboratory timer was used to measure the contact time. At the end of the appropriate contact time, sufficient sodium formate was added to remove any ozone residual.

Each vessel in the paired experiments had the same ozonation conditions and was sampled at 60-s intervals for a total elapsed time of 240 s. Time zero was arbitrarily defined as the time of the first addition of ozone. The initial concentration of poliovirus in trials 2 and 3 was lower than the initial concentration in trial 1, to reduce the ozone demand associated with the virus preparation. In trial 1, the ozone was applied in three aliquots (applied ozone dose of 0.6 mg/liter) for a low dose with a high poliovirus concentration. In trial 2, the stock ozone was added by using 13 10-ml aliquots (applied ozone dose of 1.3 mg/liter). The stock ozone solution was added to the reactor vessel all at once in trial 3 (applied ozone dose of 1.8 mg/liter).

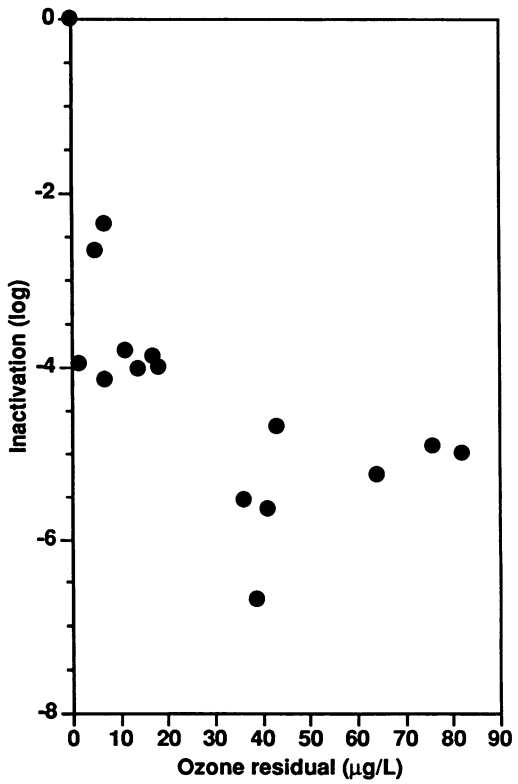

FIG. 1. Ozone inactivation of MS2 coliphage in ozone demand-free phosphate buffer at pH 6.9 and 22°C.

Samples from the reactor were transferred to sterile tubes containing a slight excess of 1.0 M sodium formate solution to neutralize any remaining ozone residual. MS2 samples of 10 ml each were transferred to a 90-ml milk dilution bottle. Poliovirus samples of 1.0 ml each were transferred to a vial. Sodium formate was also added to the controls. Plaque assays were performed with the controls and the ozone-treated samples.

RESULTS

A series of trials was performed with MS2 alone. Ozone doses were kept very low, and a contact time of 20 s was used for all trials. The data are presented in Fig. 1. Some trials were also performed with poliovirus alone but a significant amount of ozone demand was associated with the preparation of the virus titer, which prevented proper comparisons with MS2 from being made. Subsequent trials used MS2 and poliovirus in the same reactor vessel so that paired comparisons could be made.

Three trials were performed with the paired protocol. The test solution absorbance during the course of each trial is shown in Fig. 2 to 4. Ozone concentrations can be calculated from the absorbances by using the concentration factor. The plaque assay results are summarized in Table 1. The inacti-

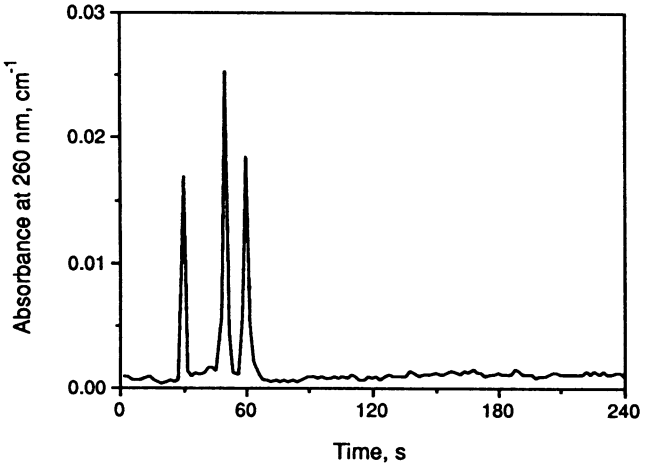

FIG. 2. Test solution absorbance during inactivation of MS2 coliphage and poliovirus type 3 in trial 1 (ozone added at 26, 44, and 54 s).

vations of MS2 and poliovirus for trials 1 to 3 are summarized in Fig. 5. In all trials, there was less inactivation of poliovirus type 3 than of MS2 coliphage under the same ozone conditions. Poliovirus inactivation in trial 2 was greater than anticipated, and kill is shown as greater than 3.6 log units in Table 1. The sodium formate in the ozonated samples did not affect the plaque assays as determined by the controls.

A paired t test on the inactivations [log (N/N_0)] of MS2 and poliovirus type 3 revealed an approximate (because of the imprecision of the estimated inactivation during trial 2) mean difference of 1.6 log units. There was significantly ($P \leq 0.05$) greater inactivation of MS2 coliphage than of poliovirus type 3.

DISCUSSION

Behavior of ozone during disinfection. The method of adding ozone, the ozone dose applied, and the initial concentration of viruses for each trial affected the reaction of ozone in solution. In trial 1 (Fig. 2), the ozone residual was short-lived, as shown by the rapid decrease in absorbance. This was due to the significant ozone demand of the virus

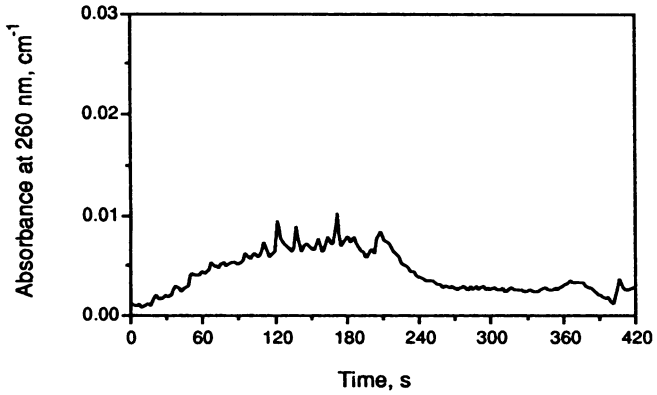

FIG. 3. Test solution absorbance during inactivation of MS2 coliphage and poliovirus type 3 in trial 2 (ozone added at 16, 32, 48, 60, 70, 80, 90, 100, 112, 130, 144, 160, and 170 s).

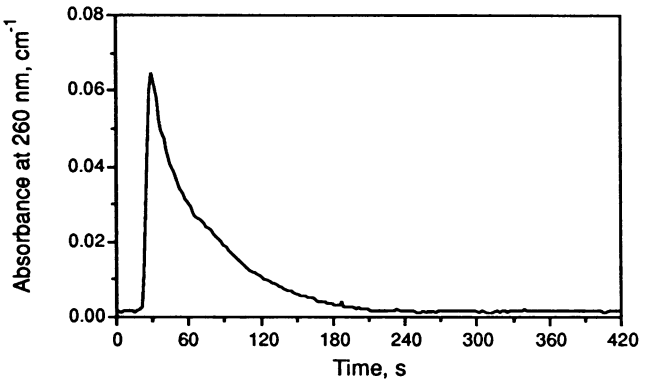

FIG. 4. Test solution absorbance during inactivation of MS2 coliphage and poliovirus type 3 in trial 3 (ozone added at 20 s).

preparation. In trial 2 (Fig. 3), in which a higher ozone dose was applied in a series of 13 10-ml aliquots, a gradual increase in absorbance in the reaction vessel occurred, with progressively slower decays of absorbance as the demand was satisfied. The stock ozone solution was added all at once to the reactor vessel in trial 3 (Fig. 4), resulting in a rapid rise of absorbance followed by a rapid decay to near zero.

Each of these figures illustrates that ozone is rapidly consumed by the poliovirus preparation, resulting in no detectable ozone residual at the end of the 2-min contact time. However, there was detectable ozone residual for varying periods in all of the trials. Thus, if the competing reaction rates were higher (but not much higher) than the reactions with the virus, these short-lived residuals would be sufficient for a detectable inactivation of MS2 and poliovirus. The inactivation curves for MS2 and poliovirus under these conditions are provided in Fig. 5. These curves are not directly comparable in terms of absolute inactivation of poliovirus, since the ozone doses ranged from 0.6 to 1.76 mg/liter. However, it is of interest to note that the results of trials 2 and 3 appear to be somewhat similar. This was likely a result of the different methods of adding ozone to the

TABLE 1. Ozone inactivation in paired experiments with MS2 coliphage and poliovirus type 3

Trial	Concn of ozone applied (mg/liter)[a]	Contact time(s)	Inactivation [log (N/N_0)][b] of:	
			MS2	Poliovirus type 3
1	0.60	0	0	0
		60	2.96	1.63
		120	2.72	1.64
		240	2.66	1.98
2	1.29	0	0	0
		60	5.68	>3.6
		120	5.14	>3.6
		240	4.81	>3.6
3	1.76	0	0	0
		60	7.00	3.52
		120	6.15	3.52
		240	5.46	3.52

[a] Calculated mass of ozone added to the reactor divided by reactor volume.
[b] N_0, $10^{6.9}$ PFU/ml for all MS2 trials and $10^{5.3}$, $10^{3.9}$, and $10^{4.0}$ PFU/ml for poliovirus trials 1, 2, and 3, respectively.

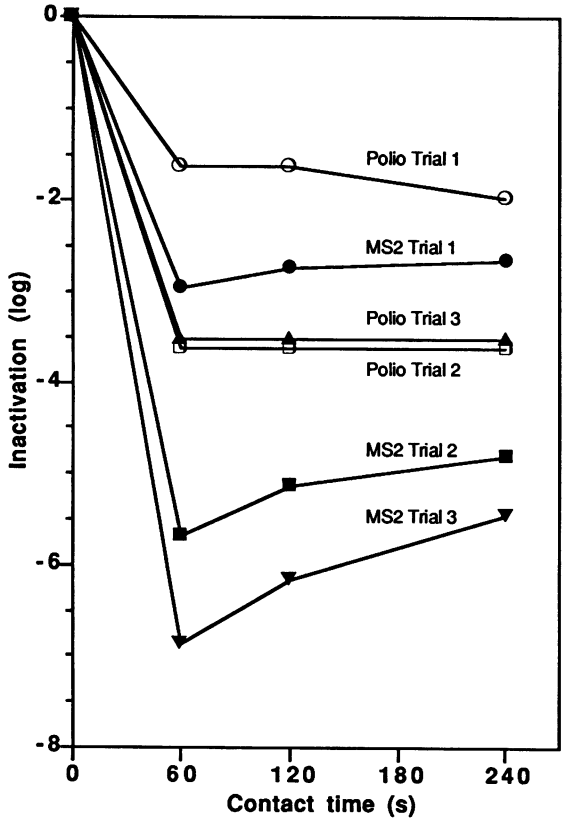

FIG. 5. Comparison of MS2 coliphage and poliovirus type 3 inactivation by using ozone in the same reactor vessel. Ozone doses applied were 0.60 (trial 1), 1.29 (trial 2), and 1.76 (trial 3) mg/liter.

reactors. The effect of the method of addition of ozone could be determined through further well-designed experiments. This was beyond the scope of the present study.

MS2 coliphage in ozone demand-free buffer. The extraordinary sensitivity of MS2 coliphage to ozone in demand-free buffer is illustrated in Fig. 1. For 20-s contact time and <40 μg of residual ozone per liter, there was a 4-log-unit inactivation of MS2. Increasing the ozone residual to 80 μg/liter provided another log unit of inactivation, bringing the overall inactivation to 5 log units. These data corroborate those reported for other studies (5, 10, 25). Evison (5) had to add ozone demand to her studies with coliphage so that her ozone apparatus and methods of measuring residual could be used to determine the inactivation of coliphage. The importance of this observation by Evison is that competing reactions for ozone must be very fast to successfully compete with the ozone required for inactivation of coliphage. Also, if one is contemplating an engineering design criterion, the ozone dose is somewhat meaningless, since the competing reactions will vary from water to water. Therefore, considering ozone residual as a design criterion may provide a means of comparing waters of different quality. Wolfe and his coworkers (25) performed all of their work at pilot scale using two types of surface waters from California with similar ozone demands. The inactivation of MS2 was observed to be independent of the contact times, which ranged from 3 to 12 min, used in the pilot plant.

The data of Evison (5), Wolfe and coworkers (25), and this study suggest that adequate coliphage inactivation can be achieved when a small ozone residual is detectable and within some short contact time. However, the question of whether coliphage results are comparable to enteric virus results remains.

Comparison of MS2 coliphage and poliovirus type 3. A paired comparison of coliphage and poliovirus was desirable to eliminate variations in water quality which could adversely affect the ozone demand and the comparability of independent trials. It was noted in these experiments that the animal virus preparation had a significant amount of ozone demand. This had been reported in an earlier study (5).

Examination of Fig. 5 reveals two interesting phenomena. The first was the consistent "tailing" appearance of the kinetic plot, which was very similar to that reported by others who maintained a relatively constant ozone residual during the course of the experiments (14). After an initial sharp drop, no significant inactivation was observed after about 60 s at any of the doses used. This has been observed by other researchers using both bacteria and viruses (4, 6, 14, 18). The reason for this feature of ozone disinfection is not clear. Finch et al. (6) explained the phenomenon in terms of competing ozone reaction kinetics. It may also be an artifact of the experimental protocol used. The practical significance of this phenomenon is that inadequacies in ozone residual concentration cannot be compensated for by increasing the contact time. In full-scale ozone reactors, prevention of hydraulic short-circuiting is very important to ensure that all water containing microorganisms comes into contact with the designed ozone residual concentration for some minimum contact time.

The second phenomenon was an apparent rebound of MS2 coliphage in trials 2 and 3 after an apparent initial inactivation in excess of 5 log units. The rebound in trial 2 was approximately 0.9 log units from 60 to 240 s. The rebound in trial 3 was higher, approximately 1.5 log units over the same period. The cause of this phenomenon is not known but perhaps could be attributed to unclumping and clumping of the viruses due to the action of ozone, since the MS2 preparation did not include an ultrasonic treatment as did the poliovirus type 3 preparation. Katzenelson and coworkers (14) demonstrated the beneficial effects of ultrasonic treatment with ozone-treated poliovirus.

The significant difference between the susceptibilities of MS2 and poliovirus to ozone observed in this study has been reported in other comparative studies (5, 10, 14). Whereas the results here suggest a mean difference of approximately 1.6 log units, Harakeh and Butler (10) reported a 3.5-log-unit difference between poliovirus type 1 and f2 coliphage at the end of 15 min of contact with 0.26 mg of ozone residual per liter. Katzenelson and coworkers (14) investigated poliovirus type 1 and T2 coliphage using a protocol very similar to the one in the present study. They reported a 2.3-log-unit difference in kill between poliovirus and coliphage at an approximate ozone residual of 0.3 mg/liter and 2 min of contact. Evison (5) reported an approximately 3-log-unit difference between poliovirus type 3 and the coliphages 185 and MS2 at the end of 10 min and a constant ozone residual of approximately 0.08 mg/liter. She also reported a 2-log-unit difference between the coliphages and poliovirus type 1 under the same conditions. Katzenelson et al. (14) observed a similar difference between coliphage and poliovirus type 1 after 2 min and an ozone residual of approximately 0.3 mg/liter.

Although the previous studies yielded similar inactivation spreads for poliovirus and coliphage, these occurred under very different ozonation conditions. Only Evison's (5) and

TABLE 2. Summary of selected poliovirus inactivations from various ozone inactivation studies

Poliovirus type	Water type (pH, temp [°C])	Protocol of residual	Contact time	Ozone dose (mg/liter)	Ozone residual (mg/liter)	Inactivation [log (N/N_0)]	Reference
1	Wastewater (7.2, 15)	Constant	15 min	NR[a]	0.26	−0.5	10
1	Phosphate buffered (7.2, 20)	Constant	0.1 s	0.17[b]	0.10	−4	12
1	Phosphate buffered (7.2, 20)	Constant	2 min	NR	0.21	−3	18
1	Distilled water (N/R, 20)	Declining	4 min	0.6[b]	NR	−1	16
1	Phosphate buffered (7.0, 25)	Constant	10 min	NR	0.13	−2.5	5
2	Phosphate buffered (7.0, 25)	Constant	10 min	NR	0.13	−4.9	5
3	Phosphate buffered (7.0, 25)	Constant	10 min	NR	0.13	−2.7	5
1	Phosphate buffered (7.2, 5)	Declining	2 min	0.3	>0.24	−3.6	14
1	Distilled	Declining	4 min	4–5	0.23	−2.7	3

[a] NR, not reported.
[b] Recorded after instantaneous ozone demand was satisfied.

Katzenelson's (14) experiments are comparable to the one reported here. Evison's data indicated much greater differences between poliovirus type 3 and coliphage than were observed in the present study. In addition, her data suggest that poliovirus type 3 is much more difficult to inactivate with ozone than poliovirus type 1 is. Considering the fact that many poliovirus inactivation studies have used poliovirus type 1 as the test organism, this may be cause for concern when the regulatory requirements for ozone inactivation of animal viruses are being defined (17, 24).

The significant variations in the disinfection performances for poliovirus reported in the literature are possibly the result of the different ozone disinfection protocols used in the various studies. Many ozone disinfection studies have continuously added ozone to the reaction system with the goal of achieving a steady-state ozone residual concentration (11, 12, 19). This protocol removes competing ozone demands, but the actual amount of applied ozone is not usually reported. Since the virus inactivation reaction is fast, significant amounts of inactivation could occur during the initial, ozone demand satisfaction stage, resulting in an overestimation of the residual ozone required to achieve the desired inactivation. Other studies have followed a protocol similar to the one used in this study but have not compared enteric viruses with MS2 coliphage (3, 14).

It is informative to summarize some of these studies (Table 2). What is clear from Table 2 is that there is little consistency in the reported inactivations of poliovirus when ozone is used. Harakeh and Butler (11) reported only a 0.5-log-unit inactivation for the reported conditions of 0.26 mg of ozone residual per liter for 15 min in wastewater. This can be contrasted with the report of Herbold and coworkers (12), who calculated that 0.10 mg of ozone residual per liter provided a 4-log-unit inactivation within 0.1 s. Coin and coworkers (2, 3) provided some of the earliest data on ozone inactivation of poliovirus. They found that a >4-log-unit inactivation of poliovirus type 1 in filtered river water was achieved when an ozone residual of 0.3 mg/liter was reached at the end of 4 min (2, 3). Their work eventually led to the standard disinfection condition at ozone treatment plants in France, where 0.4 mg of ozone residual per liter at the end of 4 min is the design goal for sufficient inactivation of enteric viruses.

The conclusions which can be drawn from the review of the literature in comparison with the results of the present study are that current understanding of ozone inactivation of enteric viruses in water is poor and that well-defined ozone experimental protocol in combination with improved virology methods will be necessary before reliable comparable data are obtained.

The present study examined ozone inactivation of MS2 coliphage and poliovirus type 3 in ozone demand-free phosphate buffer. It was observed that MS2 coliphage was very sensitive to ozone and that poliovirus type 3 was apparently more resistant to ozone than MS2 coliphage was. Consequently, MS2 coliphage may not be a good surrogate for enteric viruses in ozone disinfection studies. It was also observed that contact time was not as important as ozone concentration for the inactivation of enteric viruses, although the response of viruses to ozone may be influenced by the ozonation protocol which is used. After the literature was reviewed, it was apparent that a well-defined experimental protocol for ozone disinfection studies of animal viruses is required before comparable data can be used to define the engineering design criteria for ozone inactivation of enteric viruses.

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