

# Removal and Inactivation of *Cryptosporidium* and Microbial Indicators by a Quaternary Ammonium Chloride (QAC)-Treated Zeolite in Pilot Filters

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A set of pilot filters packed with Zeolite filter media treated with a quaternary ammonium chloride (QAC) were evaluated to verify the proof of concept of their combined antimicrobial capabilities. *Escherichia coli* was removed and inactivated the most (2.83 log), followed by MS-2 (2.75 log), *Klebsiella terriena* (2.21 log), PRD-1 (1.95 log), *Chlorella vulgaris* (1.92 log), and *Cryptosporidium parvum* oocysts (1.78 log). Especially, inactivation of *C. parvum* oocysts (1.19 log) was higher than physical removal (0.54 log). The data suggest that QAC-treated Zeolite in the pilot filters has microbial inactivation capabilities and may have useful applications in other types of filter media.

**Key Words:** QAC-treated Zeolite filter media; Antimicrobial capabilities.

## INTRODUCTION

It is well known that quaternary ammonium chloride (QAC) functions as cationic surface-active agent with antimicrobial activity due to cellular

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Received August 22, 2005.

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disruption of the cell membrane.<sup>[1-3]</sup> QAC displays its antimicrobial activity even after being covalently immobilized on inert supports.<sup>[4]</sup> Chemically treated Zeolite filter media, such as silver and zinc-containing Zeolite (AgION)<sup>[5]</sup> and surfactant modified Zeolite,<sup>[6]</sup> has also been shown to inactivate viruses and bacteria; however, QAC and Zeolite have not been tested together for their combined antimicrobial efficacy. Natural Zeolite (Clinoptilolite) has various sizes and shapes of pores and numerous charge sites on the surfaces available for ion exchange,<sup>[7]</sup> making it a good substrate for QAC. In this study, two commercially available QAC compounds were immobilized on natural Zeolite using a new Silane surface treatment. In addition, QAC as a cationic surfactant reduces the surface tension at interfaces and is attracted to negatively charged surfaces, including microorganisms.

The objective of this study was to evaluate microbial removal and inactivation capabilities of the pilot filters packed with Zeolite filter media treated with QAC; and to devise a strategy to calculate and differentiate between removal and inactivation in combined processes. The microorganisms used in this study included bacteriophages MS-2 and PRD-1, bacteria *Escherichia coli* and *Klebsiella terriena*, algae *Chlorella vulgaris*, and protozoan parasite *Cryptosporidium parvum* oocysts, which represent a broad spectrum of size and susceptibility to inactivation. We have demonstrated the efficacy of QAC-treated Zeolite filter media to remove microorganisms in water. Experiments have further demonstrated that algae and *C. parvum* oocysts were inactivated. The mechanism of this inactivation was elucidated by using a viability assay for algae and an infectivity assay for *C. parvum* oocysts. Also, since the pilot filters provided dual treatment processes such as filtration (physical removal) and chemical inactivation, the basic theory of probability was used to calculate the antimicrobial capability of each treatment process separately.

## MATERIALS AND METHODS

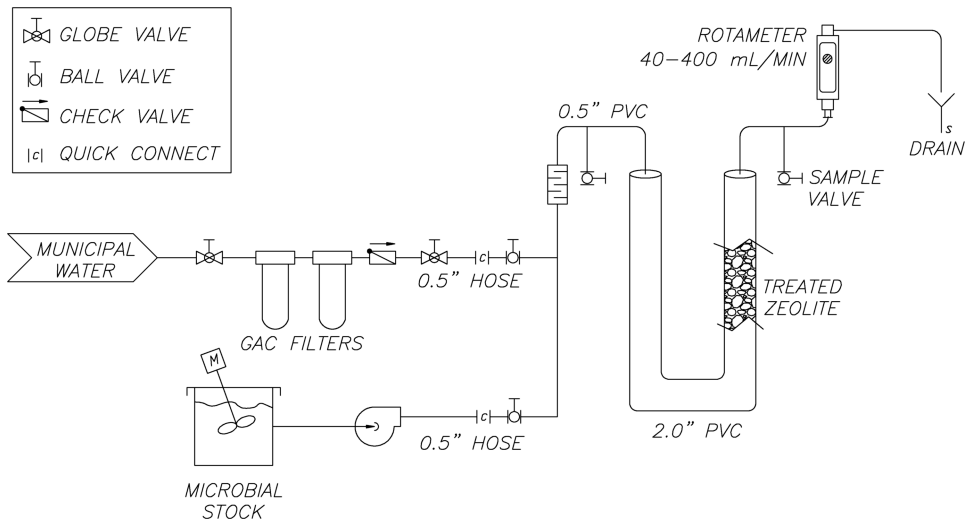
### Microorganisms

MS-2 coliphage (ATCC #15597-B1) and PRD-1 coliphage were propagated as described by Gerba et al.<sup>[8]</sup> Briefly, 1-liter flasks containing 200 mL of tryptic soy broth (TSB) were inoculated with 2 mL of host bacteria *E. coli* (ATCC #15597) and *Salmonella typhimurium* (LT2), respectively. The culture flasks were placed in a shaking incubator maintained at 37°C until the bacterial density reached approximately  $1 \times 10^8$  colony forming units (cfu)/mL (previously determined), followed by addition of an aliquot of each virus stock. The culture was shaken continuously for 4 h, and 0.02 g of lysozyme and 6 mL of sterile 0.2 M ethylenediaminetetraacetic acid (EDTA) were added to the

culture to lyse any intact host cells and incubated for an additional 30 min. The suspension was centrifuged at  $3,300 \times g$  for 20 min and then passed through a  $0.45 \mu\text{m}$  pore size sterile filter to remove cellular debris. The stock was titered by the agar overlay method and stored at  $4^\circ\text{C}$  until needed.<sup>[9]</sup> Bacteria hosts *E. coli* (ATCC #15597) and *Salmonella typhimurium* (LT2) were used to detect MS-2 coliphages and PRD-1 coliphages, respectively. One ml samples were added to molten top agar with the appropriate host and plated on bottom agar plates in duplicate. The plates were incubated at  $37^\circ\text{C}$  overnight, and plaques were counted after 12 h. Positive and negative controls were included in each set of assays and for each coliphage group.

*E. coli* (ATCC #25922) and *Klebsiella terriena* (ATCC #33257) were prepared the day of the experimental runs by inoculating 100 mL of TSB and incubating overnight at  $37^\circ\text{C}$ . The membrane filter technique using mEndo LES agar (Difco, Sparks, MD) was used to enumerate these bacteria by Standard Method 9222.<sup>[10]</sup> Samples in triplicate were filtered using 47-mm diameter cellulose acetate membranes with  $0.45 \mu\text{m}$  pore size (Pall Gelman Laboratory, Ann Arbor, MI). The plates were counted after 24-hour incubation at  $35^\circ\text{C}$ .

Algal cells of *Chlorella vulgaris* (ATCC #16487) were obtained from ATCC, Manasses, VA. The algal cells were grown in a medium containing 0.33 g/l yeast extract, 0.33 g/l of beef extract, 3.33 g/l glucose, 0.67 g/l of tryptose, and 0.6 mg/l of ferrous sulfate per liter of nanopure water at pH 7.2. Algal stock was maintained by cell passages every 4 to 5 days in a  $75 \text{ cm}^2$  tissue culture flask with caps equipped with  $0.2 \mu\text{m}$  pore size filter, which allows the exchange of oxygen and carbon dioxide. The flasks were incubated at room temperature under fluorescent lights. The samples were collected and concentrated by centrifugation. The final volume of the sample after concentration was 1.7 ml. The cells in the concentrates were digested and stained with Fluorescein Diacetate (FDA) described as follows. A stock solution of 2% trypsin (mass/vol) (Sigma Chemical Co., St. Louis) in Hanks Balanced Salt Solution (THBSS) was prepared, and a total of  $200 \mu\text{l}$  of THBSS per ml of algal cell suspension was added to digest the cell walls. These were incubated at  $37^\circ\text{C}$  for 30 minutes and subsequently quenched with  $200 \mu\text{l}$  of trypsin inhibitor (5.8 mg/ml in HBSS, Sigma Chemical T-6522). Finally, a stock solution containing  $50 \mu\text{l}$  of 1% fluorescein diacetate was added. The cells were incubated at room temperature for 10 minutes in the dark. Samples were placed on ice in the dark during the counting process. FDA is a non-polar ester that passes through cell membranes. Once inside the cell, FDA is hydrolyzed by esterase (an enzyme present in viable cells) to produce fluorescein, which accumulates inside viable cell walls and fluoresces under UV light. Live and dead algal cells were enumerated by counting with a hemacytometer in a  $400 \times$  magnification microscope equipped with UV light and white light, respectively (Olympus America Inc., Melville, NY).



**Figure 1:** Schematic of experimental setup.

*C. parvum* oocysts (Iowa isolate) were obtained from the Sterling parasitology laboratory, the University of Arizona, Tucson, AZ. The oocysts were stored in antibiotic solution (100  $\mu\text{g}/\text{mL}$  penicillin and 100  $\mu\text{g}/\text{mL}$  gentamicin) containing 0.01% Tween 20. The number of oocysts on concentrated samples was determined by direct count using a hemacytometer. The focus detection method-most probable number (FDM-MPN) assay was used to detect infectious oocysts as described by Slifko et al.<sup>[11,12]</sup>

### Assembly of Pilot Filters

Pilot filters packed with QAC-treated Zeolite filter media were provided by Coating Systems Laboratories, Inc., Chandler, Arizona, and Northern Filter Media International, Muscatine, Iowa. Two commercially available materials, 3-(trimethoxysilyl) propyldimethyloctadecyl ammonium chloride and 3-(trimethoxysilyl)propyldidecylmethyl ammonium chloride were utilized to prepare the filter media. The experimental apparatus consisted of a set of three filters attached to a manifold, which included fittings for hose connections and sample ports at the inlet and outlet for each filter (Fig. 1). An inline mixer was included in the pipe assembly before the inlet port to maximize microbial mono-dispersivity in test water.

### Experimental Operation

Municipal tap water (Tempe, AZ) was dechlorinated by granular activated carbon (GAC) filters and used for the spiked experiment and also for flushing

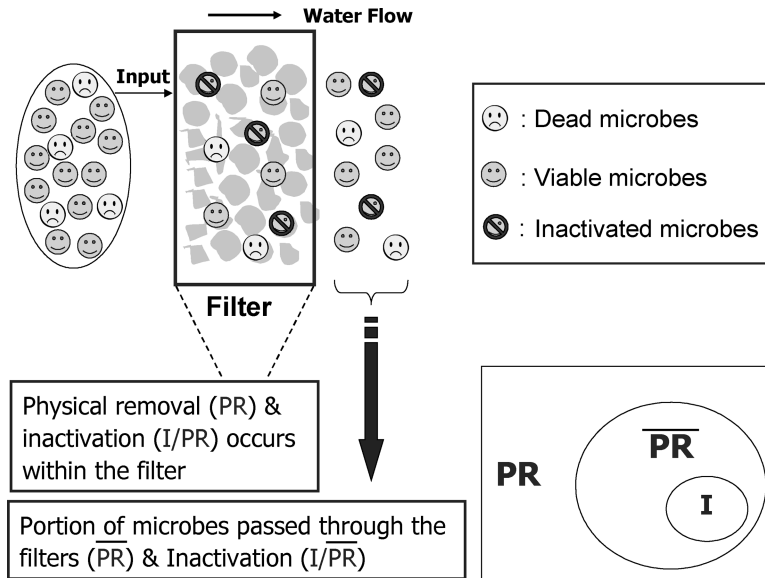
the system before and after the challenge study. The chlorine residual was measured before and after the dechlorination using Hach Method 8167 (Hach, Loveland, CO). Prior to each microbial challenge, the filters were flushed for 25 min with dechlorinated tap water.

The challenge test water was prepared by adding a known number of microorganisms to 20 L of dechlorinated tap water in a polypropylene container (Nalgene, Rochester, NY). The microorganisms were washed with  $1\times$  phosphate buffered saline just before spiking in the container. The water was continuously mixed with a Teflon-coated stir bar to provide homogenous distribution of microorganisms in the influent water. The water was pumped into each filter inlet using a thermally protected pump (Little Giant Potent Pump, Oklahoma City, OK). The flow rate was adjusted to 330 ml/min. Empty bed volume of the pilot filters is about 2.5 liters (2" diameter and 36" length), and working volume is about 1.25 L assuming 50% media porosity. Based on the hydraulic parameters of the system, hydraulic residence time (HRT) was estimated to be 4 min assuming plug flow. For steady state, each filter was run for 12 min (3 times HRT). Effluent samples were taken from each filter after 12 min, and an influent sample was collected from the container after 8 min.

## Determination of Log Removal and Inactivation of Microorganisms

To accurately assess microbial removal during filtration processes, both physically removed and inactivated microbes should be measured. Physical removal can be easily exaggerated if dead microbes in the filtrate are not measured. In many circumstances, microbes can be inactivated during filtration and still go through the filter media. The culture based technique only measures viable cells and dead cells are not enumerated. Therefore, the dead microorganisms which passed through the filter are not measured as inactive and are assumed physically removed, resulting in lack of accuracy when only the physical removal is calculated.

Mechanisms of microbial removal and inactivation by the filter are presented in Figure 2. Culturable assays were used to calculate total removal of microorganisms including physical removal and inactivation, assuming that physical removal rates for both dead and viable microorganisms are identical (Eq. (1));  $C_I$  and  $C_O$  are the number of viable/infectious microorganisms for input and output, respectively. Total numbers of microorganisms, including dead and viable ones determined by direct count using a hemacytometer, were used to calculate physical removal rates (Eq. (3));  $T_I$  and  $T_O$  are the number of total microorganisms for input and output, respectively.



**Figure 2:** Schematic mechanisms and Venn diagram of filtration including physical removal (PR) and inactivation (I) processes.

Total removal of bacteria and bacteriophages were computed by Eq. (1), and log removals of microorganisms (L) are defined by Eq. 2;

$$\text{Total Removal: } TR = \frac{C_I - C_o}{C_I} \tag{1}$$

$$\text{Log Removal (L)} = -\text{Log}_{10}(1 - TR) \quad \text{for } 0 = TR < 1 \tag{2}$$

where,  $TR$  is total removal as a fraction of one. Note:  $TR = 0$  for no removal and  $TR = 1$  for total removal. If there is total removal, the computed log removal would be the same as the original number of microorganisms spiked.

For algae *Chlorella vulgaris* and *C. parvum*, a physical removal rate and an inactivation rate were computed separately, assuming that the mechanisms are independent (Figure 2).

$$\text{Physical Removal: } PR = \frac{T_I - T_o}{T_I}, \overline{PR} = 1 - PR \tag{3}$$

$$\text{Inactivation: } (I/\overline{PR}) = \frac{(C_I/T_I) - (C_o/T_o)}{(C_I/T_I)} \tag{4}$$

where,  $PR$  and  $(I/\overline{PR})$  are both fractions of one,  $\overline{PR}$  is the complement of  $PR$ , which means a portion of microorganisms passed through filters, and  $(I/\overline{PR})$  is a portion of inactivated microorganisms among microorganisms passed

through filters. In this study, the portion of microorganisms physically removed and subsequently inactivated, ( $I/PR$ ), was not determined. Total log removal credits were calculated using summation of log physical removal and log inactivation, and the total probability theorem<sup>[13]</sup> was used to confirm this calculation.

The union of sets  $PR$  and  $I$  is indicated as  $\Pr(PR \cup I)$ ; the resulting set includes all members of  $PR$  and  $I$ . The intersection of sets  $PR$  and  $I$  is indicated as  $\Pr(PR \cap I)$ ; the resulting set includes only members that belong to both  $PR$  and  $I$ . The relationship between union and intersection is given by Eq. (5);

$$\Pr(PR \cup I) = \Pr(PR) + \Pr(I) - \Pr(PR \cap I) \quad (5)$$

If  $PR$  and  $I$  are mutually exclusive,  $\Pr(PR \cap I)$  is zero. According the total probability theorem, probability of inactivation,  $\Pr(I)$ , can be estimated by Eq. (6). The conditional probability of an event  $A$  given an event  $B$  is denoted as  $\Pr(A/B)$  for  $\Pr(B) > 0$ ;

$$\Pr(I) = \Pr(I/PR) \Pr(PR) + \Pr(I/\overline{PR}) \Pr(\overline{PR}) \quad (6)$$

$$\Pr(A/B) = \frac{\Pr(A \cap B)}{\Pr(B)} \quad (7)$$

As mentioned previously,  $\Pr(I/PR)$  is zero. Therefore, the following Equation for probability of total removal of microorganisms can be generated from combining Eqs. (5), (6), and (7);

$$\Pr(PR \cup I) = \Pr(PR) + \Pr(I/\overline{PR}) \Pr(\overline{PR}) \quad (8)$$

## RESULTS AND DISCUSSION

The average log removals of MS-2 and PRD-1 by the three filters for the three experiments were  $2.75 \pm 0.48$  [geometric mean  $\pm$  standard deviation] and  $1.95 \pm 0.56$ , respectively. Our experiments showed less removal and inactivation of PRD-1 than MS-2, suggesting PRD-1's resistance to removal or greater survival in water.<sup>[14]</sup> Many factors could potentially affect removal and inactivation of viruses by the filters. These factors include the size and isoelectric points of each virus, protein coat structure or its hydrophobicity, and antimicrobial activity of QAC immobilized on Zeolite. Virus removal was reported to be poor using direct filtration without prior destabilization of particles (Table 1). Gerba et al.<sup>[8]</sup> reported that the average log removals of MS-2 and PRD-1 by pilot-scale multimedia filtration were 0.68 and 1.07, respectively. The pilot filters tested in this study resulted in an average of more

**Table 1:** Overview on size and susceptibility to removal and inactivation of challenge microorganisms.

Organisms	Size	2-log Inactivation by Chlorine CT values [mg/l – min]	Log removal by media filtration	References
MS-2	24 nm	12	0.5 to 1.0	(8, 15, 16)
PRD-1	63 nm			
<i>E. coli</i>	1 $\mu\text{m}$	0.5 to 1	0.8 to 2.1	(8, 17, 18)
<i>K. terrigena</i>	1 $\mu\text{m}$			
<i>Chlorella vulgaris</i>	9 $\mu\text{m}$	0.7	1	(19)
<i>C. parvum</i>	6 $\mu\text{m}$	>7200	0.4 to 1	(20, 21)

than 2 logs removal and inactivation of coliphages, suggesting that the viruses probably had a higher rate of inactivation than physical removal. However, mechanisms of removal and inactivation of coliphages by the pilot filters were not clear.

The log removals of *E. coli* and *Klebsiella* averaged  $2.83 \pm 1.02$  and  $2.21 \pm 0.73$ , respectively. During media filtration in conventional treatment plants, bacteria can be removed physically from water by straining it through small pores, by bacteria-bacteria settling, and by bacteria-media adhesion. In general, bacterial removal by media filtration during water treatment processes is greater than virus removal (Table 1). Schulze-Makuch et al.<sup>[6]</sup> reported that the iron-oxide-coated sand filter pack failed to remove viruses and bacteria to a significant degree, but the surfactant modified Zeolite filter pack removed more than 2 logs of the viruses and 4 logs of *E. coli* from ground water. The silver-Zeolite mixture proved to be able to reduce various types of bacteria from 0.6 to 5 logs after 4 hours exposure and from 5 to 7 logs after 24 hours in all cases.<sup>[5]</sup> The challenged pilot filters in this study demonstrated a greater degree of bacterial removal than sand filtration, possibly due to antimicrobial activity of QAC immobilized on the filter media.

**Table 2:** Average log removal and inactivation of test microorganisms by the pilot filters.

Organisms	Physical removal	Inactivation	Total
<i>Chlorella vulgaris</i>	$1.80 \pm 0.75$	$0.11 \pm 0.17$	$1.92 \pm 0.72$
<i>C. parvum</i>	$0.54 \pm 0.08$	$1.19 \pm 0.50$	$1.78 \pm 0.43$

Note: These values represent a total of three experiments run with three pilot filters. The differentiation between removal and inactivation were calculated based on the basic theory of probability as described in Materials and Methods. Log removals were estimated using the following equation;  $-\log_{10}(1 - \text{probability of physical removal or inactivation})$ .



The log removal of *Chlorella vulgaris* by filtration (physical removal) and inactivation averaged  $1.80 \pm 0.75$  and  $0.11 \pm 0.17$ , respectively, resulting in cumulative log credit of  $1.92 \pm 0.72$ . Physical removal seemed to be the main mechanism of algae removal. The average log credits of *C. parvum* oocysts were  $0.54 \pm 0.08$  for physical removal and  $1.19 \pm 0.50$  for inactivation, resulting in cumulative log credit of  $1.78 \pm 0.43$ . *Cryptosporidium* oocysts are very resistant to chlorination, and a range of 0.4 to 1.0 log removal by dual-media filter has been reported (Table 1). The results of algae and oocysts showed that cumulative log credits obtained from two removal mechanisms were similar, whereas the removal efficiency ratios of physical removal to inactivation were different. However, direct comparison of inactivation efficiency of algae and oocysts may be not feasible due to different characteristics of detection methods. The FDA method is designed for testing the viability of algae, whereas FDM is used to detect infectious oocysts. In general, infectivity assays of oocysts are more sensitive than viability assays. Therefore, if a viability assay is used for oocyst inactivation study, treatability efficiency may be underestimated.

The data imply that QAC-treated Zeolite in the pilot filters has inactivation and removal capabilities for test microorganisms. Moreover, due to the inactivation properties, the treated Zeolite may be useful in other types of filter media for water and wastewater treatment.

## ACKNOWLEDGMENTS

This research was supported by Northern Filter Media International, Muscatine, Iowa, Coating Systems Laboratories, Inc., Chandler, Arizona, and the National Science Foundation Water Quality Center at Arizona State University.

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