Drinking Water Decontamination with Immobilized Enzymes

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ABSTRACT

Nerve agent-degrading enzymes Organophosphorus Acid Anhydrolase (OPAA) and Organophosphorus Hydrolase (OPH) covalently-coupled to solid supports were examined as drinking water system nerve agent decontaminants. Enzymes were bound to azlactone polyacrylamide, glyoxal agarose and glyoxal-aminopropyl controlled-pore glass and tested for stability in unbuffered tap water. Kinetic analyses showed that both enzymes lost activity after covalent immobilization, but the degree of activity loss varied with immobilization method and enzyme type. Superior activity against paraoxon and *p*-nitrophenyl Soman was seen with OPH-polyacrylamide and OPAA-agarose, respectively, after 5 days tap water storage at room temperature. The azlactone-polyacrylamide coupling method gave the best activity stabilization for both enzymes. Inclusion of immobilized enzyme as a catalytic filter in a circulating, unbuffered, tap water loop system (24 hour hydraulic residence time - HRT) demonstrated >99% paraoxon catalysis over a 5 day treatment. This compares to ~6% paraoxon hydrolysis in a control column. These results indicate that immobilized OPH and OPAA can be used successfully to treat contaminated water supplies.

INTRODUCTION

Drinking water distribution systems supplying large population centers must be considered as serious potential targets for terrorists. Contamination of distribution system equipment would result from adherence of contaminants to biofilms, tubercles and other corrosion products lining the pipes, or from permeation of the pipe material itself. Because of their nontoxic, non-corrosive, and environmentally benign properties, enzymes may provide an ideal method for the treatment of agents, pesticides or other chemical contaminants in drinking water systems, as well as the decontamination of pipes and other equipment with contaminant residue. Additionally, enzymes have been demonstrated to function in foams, sprays, lotions, detergents, and other vehicles that can be used in flowing water or on material surfaces. Many special requirements need to be considered in the application of enzymes to contaminated drinking water systems. Because of the large volumes of water contained in water distribution and treatment systems, a decontaminant will need to be active for a much longer time than in military operations. Since drinking water flows very quickly in pipes, methods are needed to ensure that the enzymes maintain sufficient contact with the contaminated water or materials. The goal of this project is to identify, develop, and evaluate at least one enzyme-based method for treating flowing contaminated water.

A literature survey was initially conducted to examine the types of enzymes that could potentially be used in the decontamination of tap water as well as the methods for immobilizing and/or stabilizing them. Enzymes were identified with activity against organophosphorus nerve agents and pesticides, sulfur mustard and halogenated pesticides, carbamate pesticides, cyanide, biological agents, toxins, and biofilms. However, because of their more advanced status, the two nerve agent/pesticide degrading enzymes organophosphorus acid anhydrolase (OPAA) JD6.5 and organophosphorus hydrolase (OPH) were selected for use.

PHASE 1 PRELIMINARY STUDIES

These studies examined the effect of immobilizing OPH and OPAA on enzyme activity after exposure to tap water. This was needed to ensure that the immobilization technology chosen would result in an active enzyme system after five days, which was the examination period for the subsequent tap water bench studies. Initial studies used enzyme kinetic rate analysis as the activity benchmark. This benchmark was examined at time 0 and after 5 days storage in ECBC tap water. Kinetic rate comparisons were made between the different immobilization techniques to find the technique which resulted in the highest activity after five days storage in tap water. Paraoxon was used as an OPH substrate, as *p*-nitrophenyl Soman hydrolytic activity is a very poor substrate for OPH. Although OPAA has better catalytic activity with *p*-nitrophenyl Soman, this substrate can't be purchased commercially (unlike paraoxon), and problems encountered with *p*-nitrophenyl Soman synthesis by a local chemist precluded its use for the bench studies. As such, paraoxon was also used as the substrate for OPAA.

Several immobilization methods were examined for both enzymes. These included covalent attachment of OPH and OPAA to solid supports such as polyacrylamide, agarose and controlled-pore glass beads. Encapsulation of the enzymes in sol-gels was also examined. The sol gel encapsulation method used in this study was the polymerization of locust bean gum (LBG) galactomannan with Tetrakis (2-hydroxyethyl) orthosilicate (THEOS) to form hybrid silica nanocomposites. The activity results of the covalently-coupled enzymes are shown in **Figures 1** and **2**. For OPH, the best activity after immobilization is seen with the azlactone-polyacrylamide coupling method. For OPAA, the best coupling method was Amino-link Plus agarose. The lowest activity was seen with the azlactone polyacrylamide method for OPAA and with controlled pore glass for OPH. Preservation of the free enzyme activity level after immobilization was much better with OPH than for OPAA with all methods. Preservation of the initial post-modification activity level after five days was best for both enzymes with the azlactone polyacrylamide coupling method.

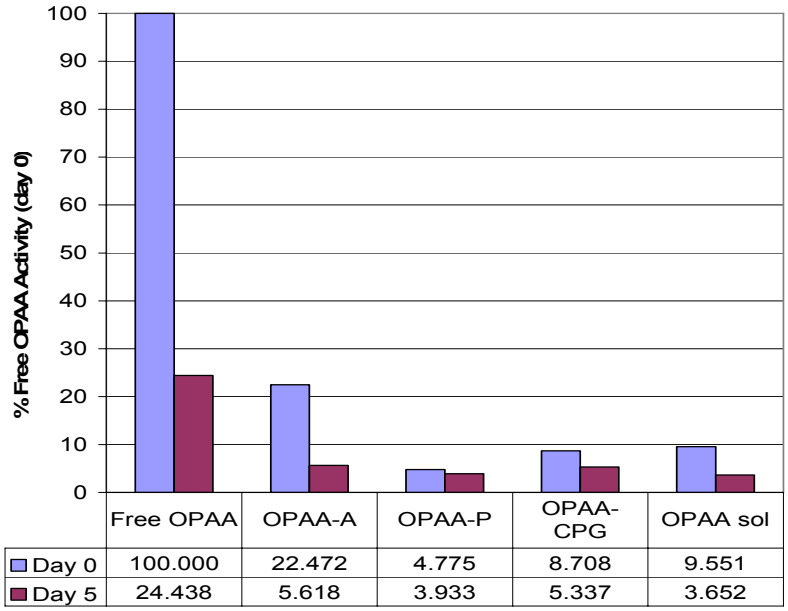
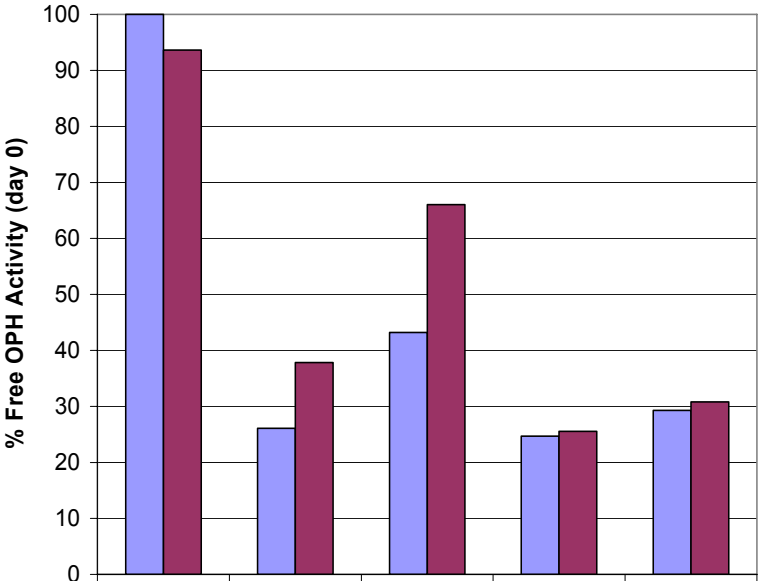


Figure 1. Specific activity of free and immobilized OPAA at 0 and 5 day in tap water. A = agarose; P = polyacrylamide; CPG= controlled pore glass; sol = THEOS-LBG sol gel.



	OPH Free	OPH-A	OPH-P	OPH-CPG	OPH sol
Day 0	100	26.09	43.21	24.69	29.3
Day 5	93.63	37.83	66.04	25.58	30.8

Figure 2. Specific activity of free and immobilized OPH at 0 and 5 days in tap water. A = agarose; P = polyacrylamide; CPG= controlled pore glass; sol = THEOS-LBG sol gel.

SYSTEMIZATION

The immobilized enzymes were used to filter-decontaminate paraoxon from tap water. The benchmark for these studies was the amount of paraoxon hydrolyzed to *p*-nitrophenol (pNP) over a 5 day treatment period. A small-scale (50 ml) reservoir loop was used to transition from the initial rate studies to the 2 liter, bench scale studies. The mixing reservoir and the enzyme filter were foil-wrapped to protect the pNP from light. Many unanticipated technical challenges arose while implementing this transitional system that required resolution before the bench scale decontamination studies could be attempted.

First, paraoxon adsorbed to the Tygon and silicon tubing and *p*-nitrophenol adsorbed to the fittings. Further, paraoxon and its hydrolysis product, *p*-nitrophenol, were used as a nutritional source by the native bacteria in the tap water, resulting in formation of biofilms in the tubing. This, in turn, adversely affected the accuracy of the paraoxon and *p*-nitrophenol measurements. Sterilization of the system by autoclaving eliminated bacterial degradation of the substrate/product; however, Tygon tubing did not survive autoclaving well, so its use was discontinued. The fittings and most of the tubing were replaced with glass capillaries and polypropylene fittings. A tubing comparison showed that Pharmed™ tubing gave the least paraoxon adsorption (**Figure 3**). Silicon gave the highest paraoxon adsorption; over 90% was removed from the system in 4 days. The geometry of the system was changed (**Figure 4**) so that Pharmed™ tubing did not come into contact with the treatment water until after it had passed through the immobilized enzyme filter (reverse loop).

Using these modifications and a 24 hour residence time (time for a sample to pass through the system), >99% of the paraoxon (0.1 mM or 27.5 ppm initial) was hydrolyzed to *p*-nitrophenol during the five day treatment period with the OPH-agarose filter compared to 4% for the untreated control (**Figure 5**).

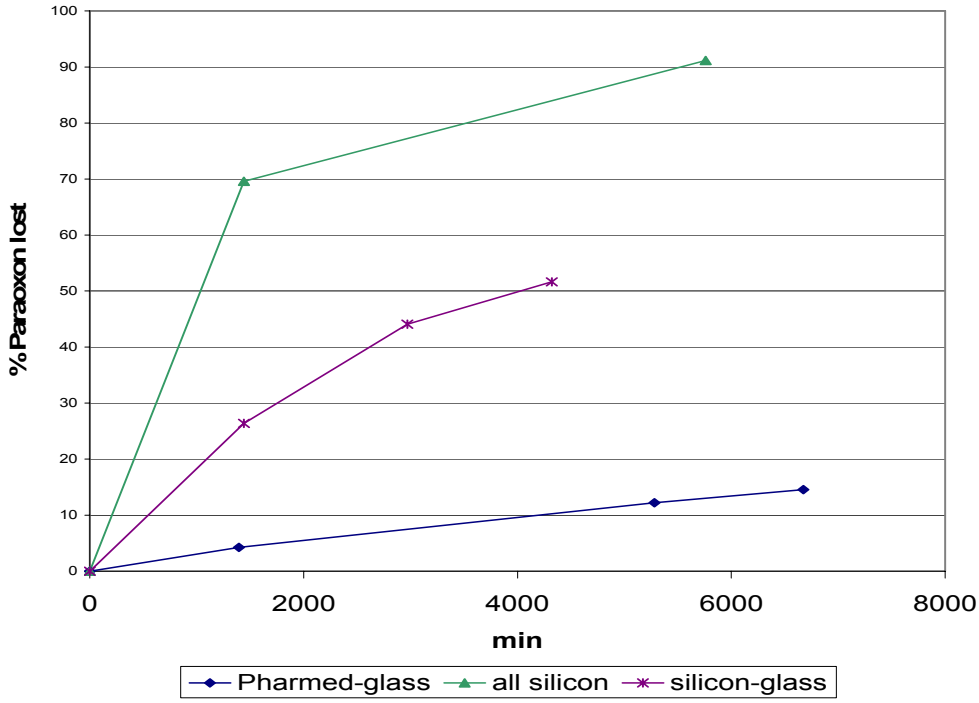


Figure 3. Comparison of paraoxon loss with different tubing (no enzyme, 50 ml, sterile circulating loop system).

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BENCH SCALE TESTING

The bench circulating loop system tested the feasibility of tap water decontamination with an immobilized enzyme filter. All systems tested were sterilized by autoclaving to prevent anomalous results from bacterial growth. Obviously, this is not feasible for large-scale application of the technology. However, in actual use it is anticipated that a disinfectant or biofilm-degrading system/enzymes will also be incorporated, thus eliminating this problem. The bench circulating loop system tested the feasibility of tap water decontamination with an immobilized enzyme filter. All systems tested were sterilized by autoclaving to prevent anomalous results from bacterial growth. Obviously, this is not feasible for large-scale application of the technology. However, in actual use it is anticipated that a disinfectant or biofilm-degrading system/enzymes will also be incorporated, thus eliminating this problem.

The enzyme filter (30-33 ml bed volume) circulated 2 liters of 0.091-0.096 mM paraoxon (actual, measured by base hydrolysis) in ECBC tap water with a hydraulic residence time of 24 hours at 24°C. The mixing reservoirs and the test filters were foil-wrapped to protect the pNP from light. Both OPH-agarose and OPAA-agarose were used in this demonstration. BSA-agarose was run in parallel with each enzyme filter as the non-enzymatic control under the same operating conditions. Temperature, pH and the absorbance (A405) were monitored during the five day demonstration period.

The 2 liter apparatus was an enlarged version of the 50 ml system. Larger Pharmed™ tubing and glass capillaries were used to handle the larger flow rates (1.39 ml/min). The 50 ml system pump (Rainin RP4) was also used in the 2 liter system. The temperature was 24ºC.

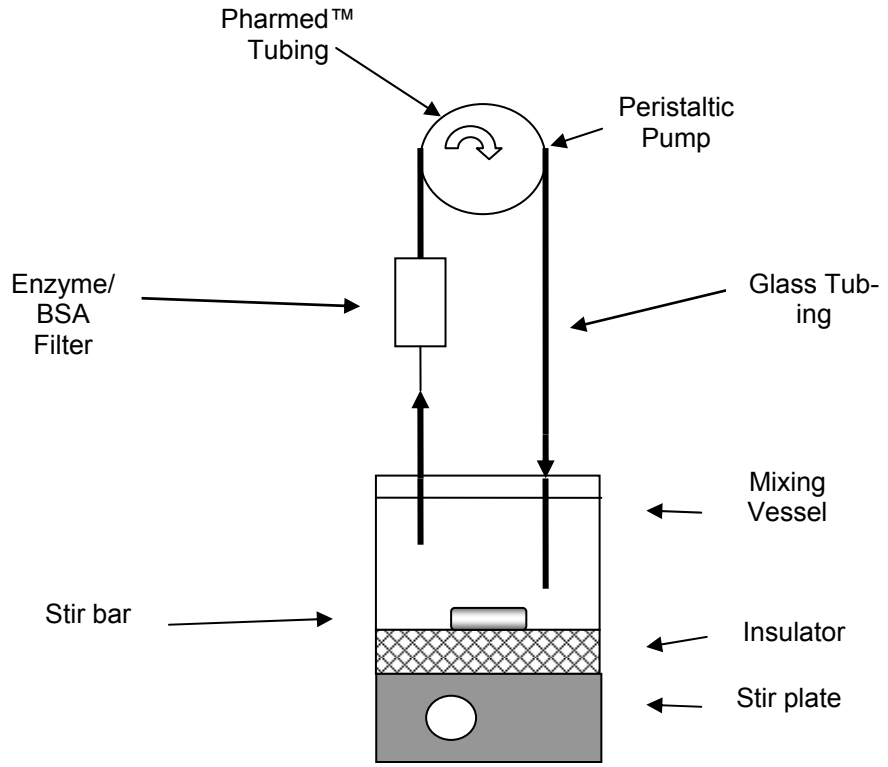

Figure 4. Reverse circulating filter loop system used in the 50 and 2000 ml systems.

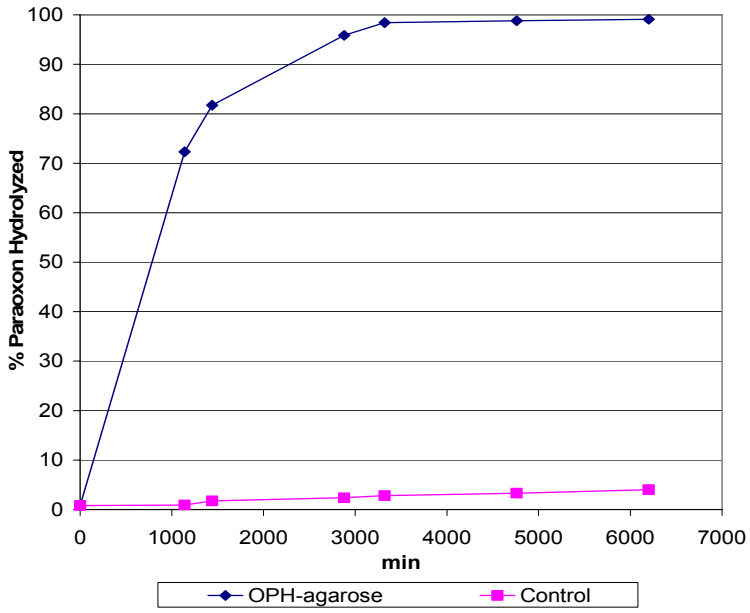

Figure 5. 5 day paraoxon catalysis by OPH-agarose in the 50 ml reverse circulating enzyme filter loop system.

Results of the catalytic filter loop paraoxon decontamination systems showed excellent

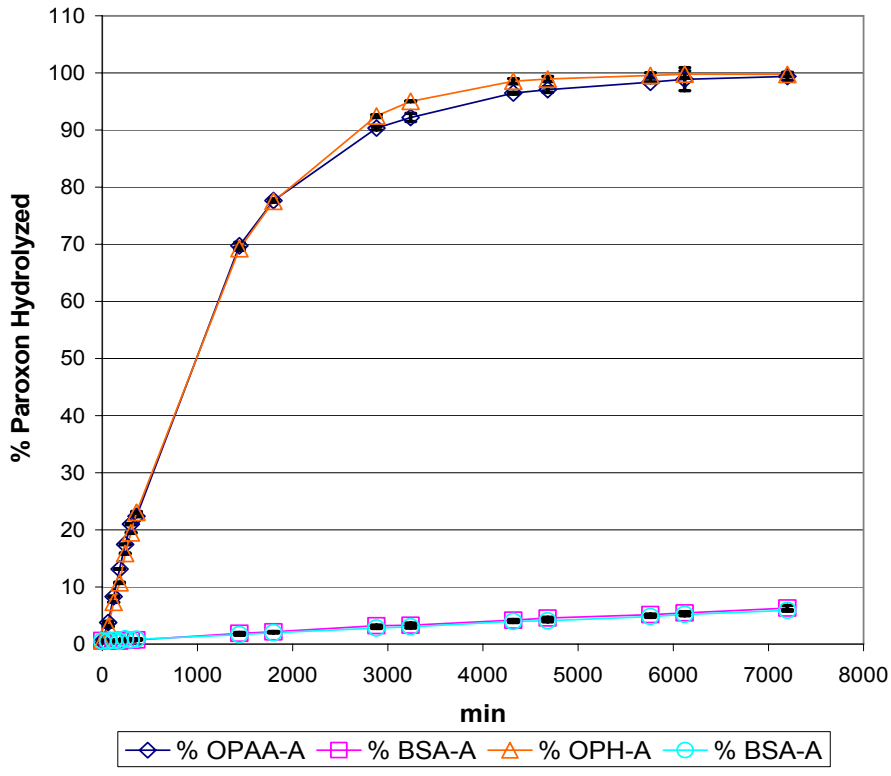

Figure 6. Paraoxon catalysis in catalytic and control 2 liter filter loop systems. Error bars are the +/- 95% confidence levels.

performance from both immobilized enzymes on agarose (**Figure 6**). After the five day treatment, the catalytic filters hydrolyzed 99.4 – 99.8% of the paraoxon. This is in contrast to the control filter loop, which showed only 5.9-6.3% paraoxon hydrolysis during the same examination period. The net catalytic paraoxon hydrolysis from OPAA-agarose and OPH-agarose was 92.8 and 93.9%, respectively (**Figure 7**). *p*-Nitrophenol production from paraoxon was quite evident in the 2 liter catalytic filter system compared with the control filter system (**Figure 8**).

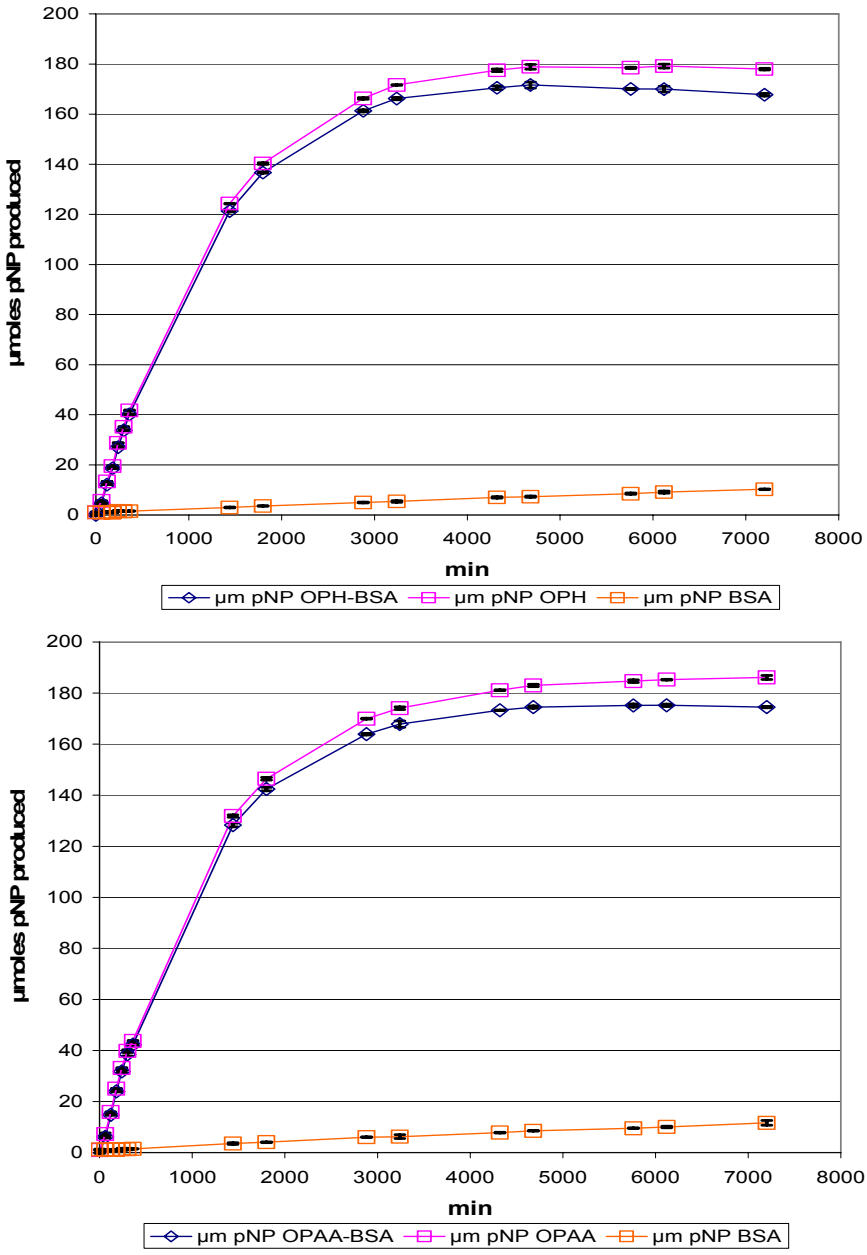

Figure 7. Total µmoles *p*NP produced by the OPAA-Agarose, OPH-Agarose and their BSA-Agarose controls in the 2 liter systems. The enzyme-BSA plots show the net catalytic µmoles produced. Error bars show the +/- 95% confidence limits.

The pH of the systems was also divergent (**Figure 9**). The initial mean pH of the catalytic systems was 7.57 (enzyme) and 7.60 (BSA). After the five day treatment, the final mean

Figure 8. OPAA-Agarose (front) and BSA-Agarose (rear) 2 liter systems after the five day treatment of paraoxon in tap water. The yellow compound is the *p*-nitrophenolate ion of *p*-nitrophenol, one of the paraoxon hydrolysis products.

pH was 8.11 (enzyme) and 8.47 (BSA). The lower pH of the catalytic filter systems is from the production of the acidic products of paraoxon hydrolysis. The rapid accumulation of these products during the first day accounts for the observed drop in pH during this period for both enzyme filter systems.

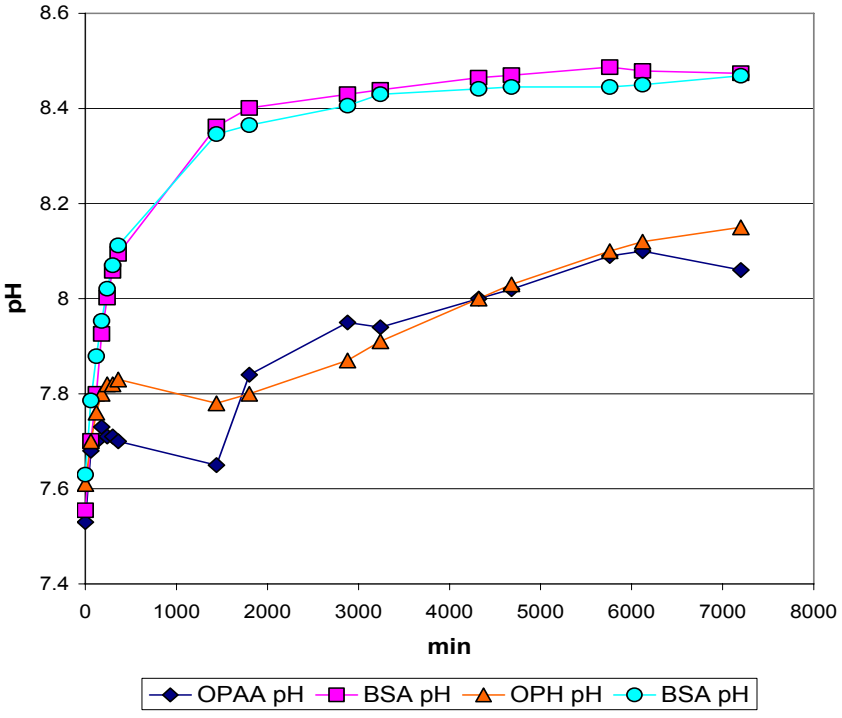

Figure 9. pH profile of the catalytic and control filter loops during paraoxon hydrolysis in the 2 liter systems.

CONCLUSIONS

Preliminary studies showed that the paraoxon (and nerve agent)-hydrolyzing enzymes OPAA and OPH could be successfully immobilized with four different methods. Three of these were covalent immobilization on solid supports (agarose, polyacrylamide and controlled pore glass) and one encapsulated the enzymes in a hybrid silica nanocomposite (sol-gel). All immobilization reactions resulted in loss of enzyme activity, but this loss varied with the enzyme type and the immobilization method. The immobilized enzymes were tested for activity stability before and after 5 days tap water storage. The best immobilization method for activity was with azlactone-polyacrylamide for OPH (paraoxon) and with Amino-link Plus agarose for OPAA (*p*-nitrophenyl Soman). The best stability after 5 days tap water storage was with azlactone-polyacrylamide for both enzymes. Although *p*-nitrophenyl Soman was the OPAA substrate for these preliminary studies, it was substituted with paraoxon in the bench studies. This change was prompted by purity problems associated with the *p*-nitrophenyl Soman synthesis needed for the 2 liter experiments. High purity paraoxon (99%) was purchased commercially for the bench studies.

Systemization experiments with a small 50 ml loop filter system and paraoxon in tap water showed that there were several problems with the initial apparatus. First, native tap water bacteria used the paraoxon and *p*-nitrophenol as nutritional sources, causing growth (turbidity) in the treatment water, lowered *p*-nitrophenol levels and biofilm formation in the pump tubing. After sterilizing the system, problems were encountered with paraoxon adsorption to the pump tubing. A study of paraoxon adsorption in non-filter loops showed that a combination of glass capillaries and Pharmed™ tubing gave the least paraoxon adsorption. To further reduce the adsorption of paraoxon to the tubing, the geometry of the system was changed so that the pump tubing encountered the treatment water after it exited the filter, not before. If most of the paraoxon is degraded in the filter to *p*-nitrophenol, then less paraoxon is available to adsorb to the tubing after the treatment water exits the filter. A 5 day study using this new system geometry and apparatus gave excellent paraoxon hydrolysis over 5 days (99.1%) vs. the control (4%). Paraoxon loss from the filter system was negligible.

Bench-scale experiments with the catalytic filter loops were conducted with paraoxon in 2 liters ECBC tap water. The Aminolink Plus agarose coupling method was used for both enzymes, due to the discontinuation of the Azlactone-polyacrylamide by the manufacturer. This situation caused a delay in the OPH coupling (backorder followed by re-ordering different material), putting the bench demonstration behind schedule by several weeks. The catalytic filter loop systems used a 30-33 ml coupled enzyme or BSA filter with a 2 liter total tap water volume system.

OPAA-Agarose and OPH-Agarose catalytic filter loop systems gave very similar results in the bench study. Absorbance measurements revealed that both catalytic systems hydrolyzed >99% of the paraoxon (99.4% for OPAA; 99.8% for OPH), vs. 5.9-6.3% hydrolysis for the BSA-Agarose control systems. pH values for the filter loop systems ranged from an initial average of 7.57 (enzyme) and 7.60 (BSA) to a final average of 8.11 (enzyme) and 8.47 (BSA). Statistical T test analysis confirmed that all but the time zero absorbance measurements for the enzyme filter-treated water were significantly different from the BSA-filter treated water for both the OPAA-agarose and OPH-agarose bench studies and could not have arisen by random chance within 95% confidence limits.