



EDGEWOOD

CHEMICAL BIOLOGICAL CENTER

U.S. ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND

ECBC-TR-489

CATALYTIC ENZYME-BASED METHODS FOR WATER TREATMENT AND WATER DISTRIBUTION SYSTEM DECONTAMINATION

2. EXPERIMENTAL RESULTS

Joseph J. DeFrank

RESEARCH AND TECHNOLOGY DIRECTORATE

Ilona J. Fry
Gregory J. Pellar

SCIENCE APPLICATIONS
INTERNATIONAL CORPORATION
Abingdon, MD 21009

SAIC

June 2006

Approved for public release;
distribution is unlimited.



Disclaimer

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorizing documents.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE (DD-MM-YYYY) XX-06-2006		2. REPORT TYPE Final		3. DATES COVERED (From - To) May 2004 – Sep 2005	
4. TITLE AND SUBTITLE Catalytic Enzyme-Based Methods for Water Treatment and Water Distribution System Decontamination 2. Experimental Results				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Defrank, Joseph J. (ECBC); *Fry, Ilona J.; *Pellar, Gregory J. (SAIC)				5d. PROJECT NUMBER None	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES) DIR, ECBC, ATTN: AMSRD-ECB-RT-BD, APG, MD 21010-5424 Science Applications International Corporation (SAIC) 3465A Box Hill Corporate Drive, Abingdon, MD 21009				8. PERFORMING ORGANIZATION REPORT NUMBER ECBC-TR-489	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Environmental Protection Agency, ORD/NASRC, 26 W. Martin Luther King Drive (MS-163), Cincinnati, OH 45268				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution is unlimited.					
13. SUPPLEMENTARY NOTES *When this work was conducted, the authors indicated above were employed by GEO-CENTERS, Inc., which is now part of Science Applications International Corporation (SAIC).					
14. ABSTRACT Many special considerations are needed 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 need to be developed 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, and one enzyme-based method for decontaminating drinking water pipes. A thorough literature search was previously undertaken to fully identify the potential of enzymes to treat contaminated drinking water and/or to decontaminate distribution systems equipment. Based on that literature search, methods for enzyme immobilization and stabilization were selected for evaluation.					
15. SUBJECT TERMS Enzymes Decontamination Chemical biological agents Pesticides Immobilization Carbamate Organochlorine Organophosphorus Stabilization					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON Sandra J. Johnson
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code) (410) 436-2914

Blank

PREFACE

The work described in this report was authorized under the "Catalytic Enzyme-Based Methods for Water Treatment and Water Distribution System Decontamination" project funded by the U.S. Environmental Protection Agency. This work was started in May 2004 and completed in September 2005.

The use of either trade or manufacturers names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

This report has been approved for public release. Registered users should request additional copies from the Defense Technical Information Center; unregistered users should direct such requests to the National Technical Information Service.

Blank

CONTENTS

1.	INTRODUCTION	9
2.	QUALITY ASSURANCE PROJECT PLAN	9
3.	IMMOBILIZED ENZYME DECONTAMINATION OF DRINKING WATER.....	10
3.1	Phase I Preliminary Studies	10
3.2	Systemization Process	12
3.3	Bench Scale Testing.....	15
3.4	Conclusions.....	23
4.	STABILIZED ENZYME DECONTAMINATION OF DRINKING WATER ..	25
4.1	Phase I Preliminary Studies	25
4.2	Systemization Process	27
4.3	Bench Scale Testing.....	27
4.4	Conclusions.....	37
5.	GENERAL CONCLUSIONS	38
	GLOSSARY	39

FIGURES

1.	Specific Activity of Free and Immobilized OPAA at 0 and 5 Days in Tap Water	11
2.	Specific Activity of Free and Immobilized OPH at 0 and 5 Days in Tap Water	12
3.	Comparison of Paraoxon Loss with Different Tubing	13
4.	Reverse Circulating Filter Loop System Used in the 50-and 2000 ml Systems	14
5.	Five Day Paraoxon Catalysis Treated by OPH-Agarose in the 50-ml Reverse Circulating Enzyme Filter Loop System.....	15
6.	Comparison of OPH-Agarose Treated (right) and Untreated (left) Paraoxon in tap water after 5 days in the 50 ml Pharmed™/ glass system	16
7.	Paraoxon Catalysis in Catalytic and Control 2-Liter Filter Loop Systems	16
8.	Total μ moles <i>p</i> NP Produced by the OPAA-Agarose, OPH-Agarose and Their BSA-Agarose Controls in the 2-Liter Systems	17
9.	OPAA-Agarose and BSA-Agarose 2-Liter Systems after Five Day Treatment of Paraoxon in Tap Water.....	18
10.	pH Profile of the Catalytic and Control Filter Loops during Paraoxon Hydrolysis in the 2-Liter Systems.....	19
11.	Specific Activity of PEGylated OPH at Days 0 and 5 in Tap Water.....	26
12.	Specific Activity of PEGylated OPAA at Days 0 and 5 in Tap Water	26
13.	Bench Scale System Set-Up.....	27
14.	Paraoxon Catalysis by OPH 2-kDa after 3-Day Interval.....	28
15.	OPH-2kDa Paraoxon Hydrolysis Measured at 405 nm Assayed in ECBC Tap Water	29
16.	Paraoxon Catalysis in Catalytic and Control 2-Liter Bench Scale Systems	30

17.	Total μ moles pNP Produced by the OPH-2kDa, OPAA-2kDa and Their BSA-2kDa Controls in the 2-Liter Systems	31
18.	Photograph of Bench Study on Days 0 and 5	32
19	pH Profile of Catalytic and 2-Liter Control Reactors during Paraoxon Hydrolysis	32

TABLES

1.	T Test Analysis of the OPH/BSA-Agarose Bench Scale Experimental A405 Data	20
2.	T Test Analysis of the OPAA/BSA-Agarose Bench Scale Experimental A405 Data	22
3.	T Test Analysis of the OPH/BSA 2-kDa Bench Scale Experimental A405 Data	33
4.	T Test Analysis of the OPAA/BSA 2-kDa Bench Scale Experimental A405 Data	35

Blank

CATALYTIC ENZYME-BASED METHODS FOR WATER TREATMENT AND WATER DISTRIBUTION SYSTEM DECONTAMINATION

2. EXPERIMENTAL RESULTS

1. 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 non-toxic, 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, and one enzyme-based method for decontaminating drinking water pipes.

2. QUALITY ASSURANCE PROJECT PLAN (QAPP)

The QAPP (E4) for this project was compiled jointly by the Edgewood Chemical Biological Center (ECBC) and Neptune Associates, Inc. personnel. This document provided quality assurance guidance for both phase I (baseline) and phase II (bench) studies, although it was only applicable to the phase II operations according to the work plan. This comprehensive document (EPA QAPP No: WS3.4.d.10) covered the responsibilities of the personnel involved, quality standards expected for the project, implementation of these standards, explanations of the technologies and procedures involved and statistical analysis of the results. The initial approved QAPP (5/11/2005) was revised once to reflect corrections needed in the initial document and to modify some of the experimental procedures that were updated after the original submission in March, 2005. The final corrected QAPP was approved 7/21/05 and received by ECBC personnel 8/4/05. The approved quality procedures were implemented for the Phase II bench study.

3. IMMOBILIZED ENZYME DECONTAMINATION OF DRINKING WATER

The initial part of this project, a literature survey (ECBC-TR-489a), was 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 in the Phase I and Phase II studies.

3.1 Phase I Preliminary Studies.

Immobilization/Encapsulation Process.

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 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.

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 LBG/THEOS encapsulation method retained the enzyme very well and resulted in detectable enzyme activity (Fig 1 and 2) after the encapsulation and diffusion of the excess ethylene glycol. In comparison to the other immobilization methods, the activity performance of sol-gel OPAA ranked 2nd behind OPAA-agarose and the activity of sol-gel OPH ranked 2nd behind OPH-Polyacrylamide over the 5 day tap water storage examination period. Activity retention after sol-gel encapsulation was much poorer for OPAA than for OPH, presumably because no covalent modification of the enzyme occurred during the encapsulation to protect the enzyme. OPH was far more stable as a free enzyme than OPAA, which probably accounts for its higher activity as an unmodified enzyme after sol-gel encapsulation. Unfortunately, highly concentrated preparations of enzyme were necessary for this procedure, as it was highly diluted by the addition of the THEOS and LBG. The only commercially available THEOS had a purity of 20% (v/v), with the balance of the preparation being ethylene glycol, so this decreased the volume of enzyme that could be added to the system. In addition, the aqueous solubility of LBG was low, so it was not possible to make a concentrated solution of this polymer, which further decreased the enzyme addition volume. Despite these limitations, we were able to encapsulate sufficient enzyme to compare hydrogel enzyme activity to that of the other immobilization methods.

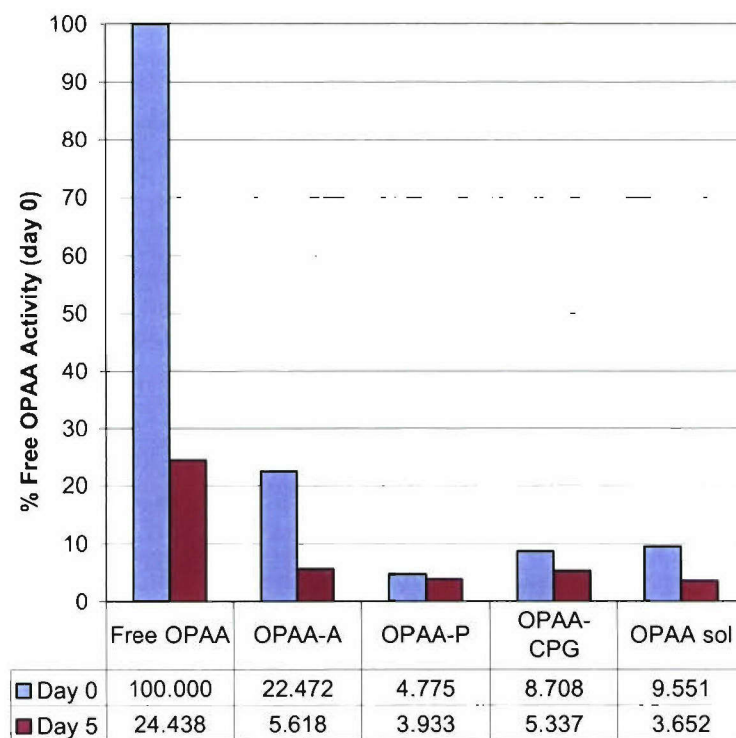


Figure 1. Specific Activity of Free and Immobilized OPAA at 0 and 5 Days in Tap Water. A = agarose; P = polyacrylamide; CPG= controlled pore glass; sol = THEOS-LBG sol gel.

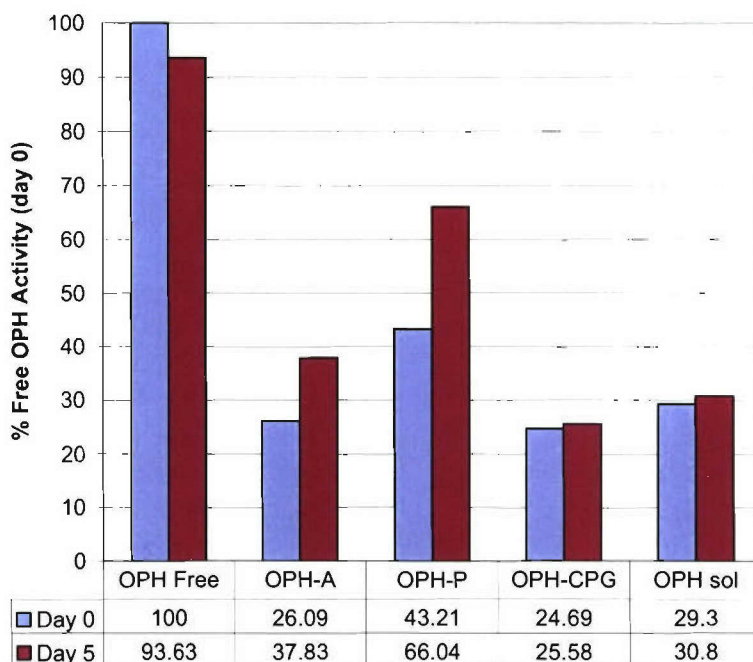


Figure 2. Specific Activity of Free and Immobilized OPH at 0 and 5 Days in Tap Water. A = agarose; P = polyacrylamide; CPG= controlled pore glass; sol = THEOS-LBG sol gel.

3.2 Systemization Process.

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 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.

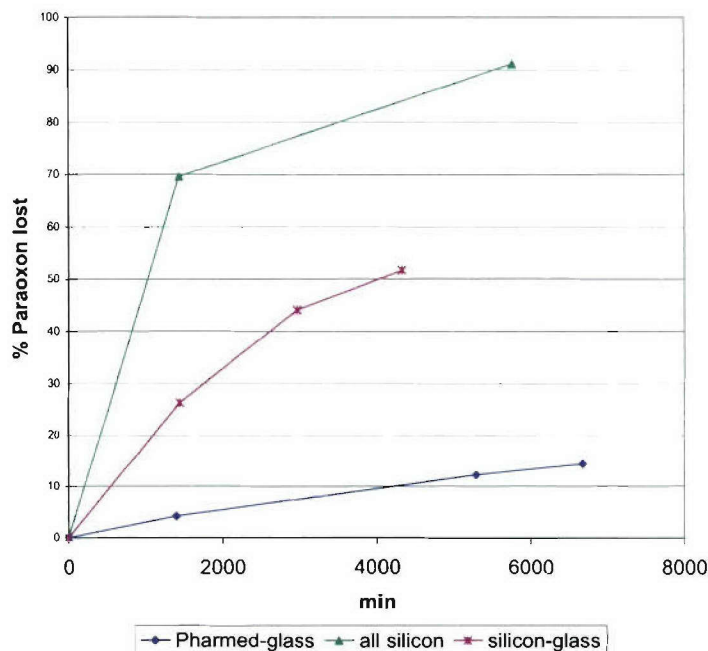


Figure 3. Comparison of Paraoxon Loss with Different Tubing. No enzyme, 50 ml, sterile circulating loop system.

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 (Figures 5 and 6).

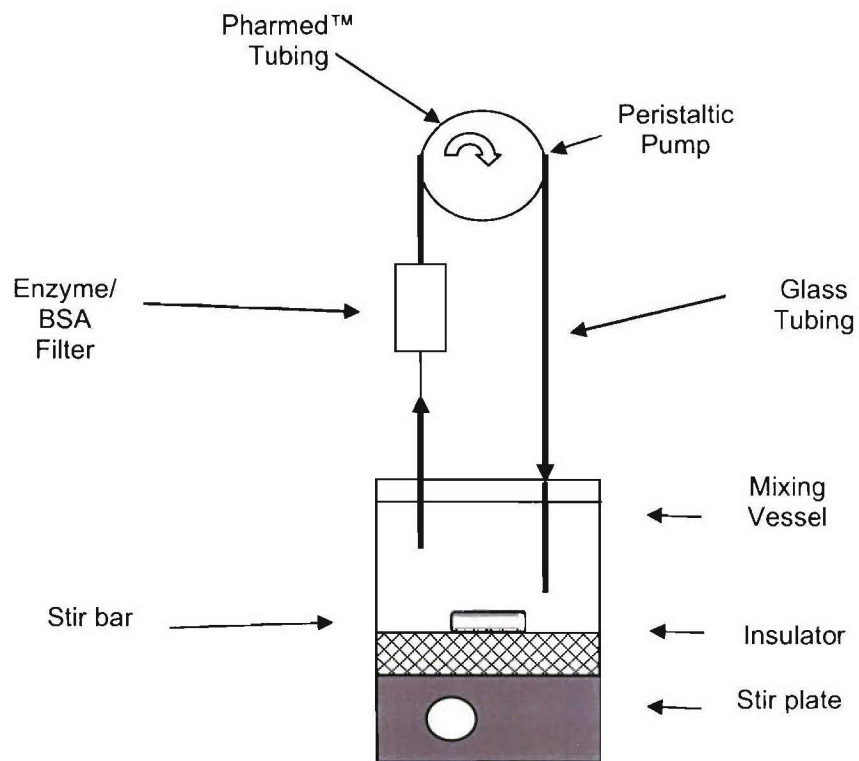


Figure 4. Reverse Circulating Filter Loop System Used in the 50- and 2000-ml Systems.

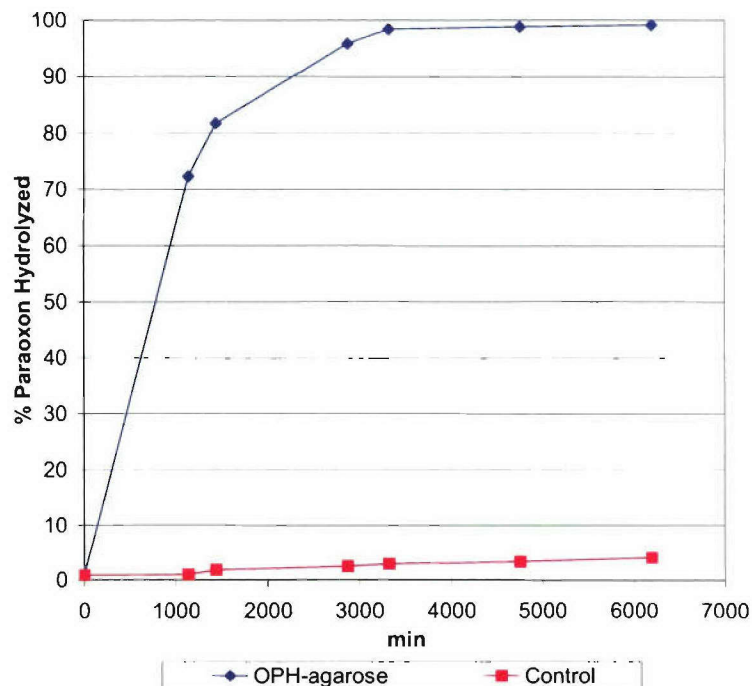


Figure 5. Five Day Paraoxon Catalysis by OPH-Agarose in the 50-ml Reverse Circulating Enzyme Filter Loop System.

3.3 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 biofilms 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 h 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 according to the schedule.

The 2 liter apparatus was an enlarged version of the 50 ml system. Larger Pharmmed™ tubing and glass capillaries were built into this system 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 observed temperature of all systems was 24°C.



Figure 6. Comparison of OPH-Agarose Treated (right) and Untreated (left) Paraoxon in Tap Water after 5 Days in the 50-ml Pharmed™/Glass System. The yellow compound is the *p*-nitrophenolate ion of *p*-nitrophenol, one of the paraoxon hydrolysis

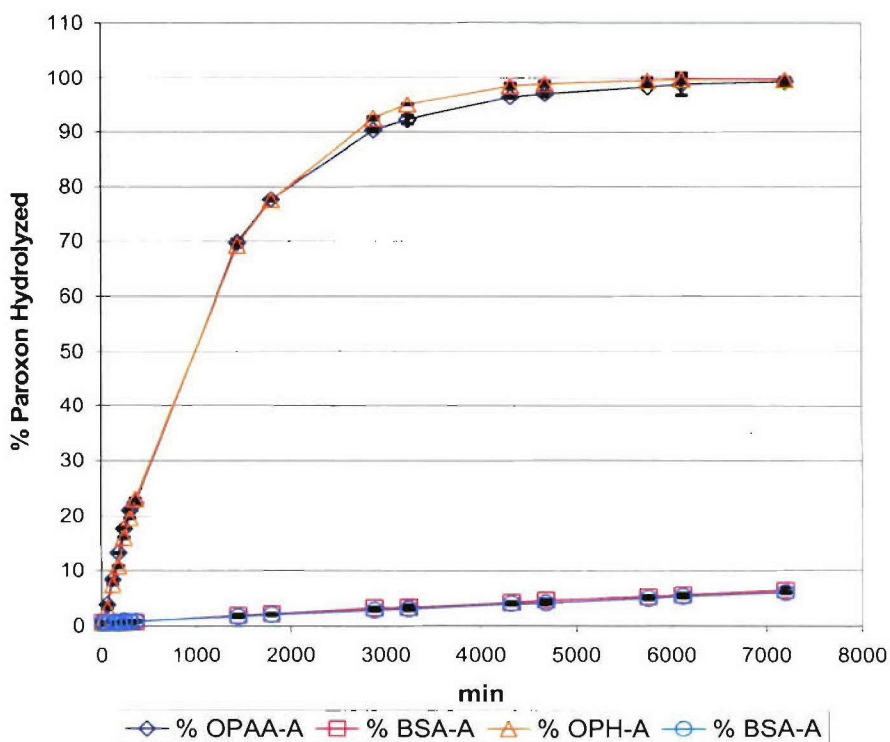


Figure 7. Paraoxon Catalysis in Catalytic and Control 2-liter Filter Loop Systems. Error bars are the +/- 95% confidence levels.

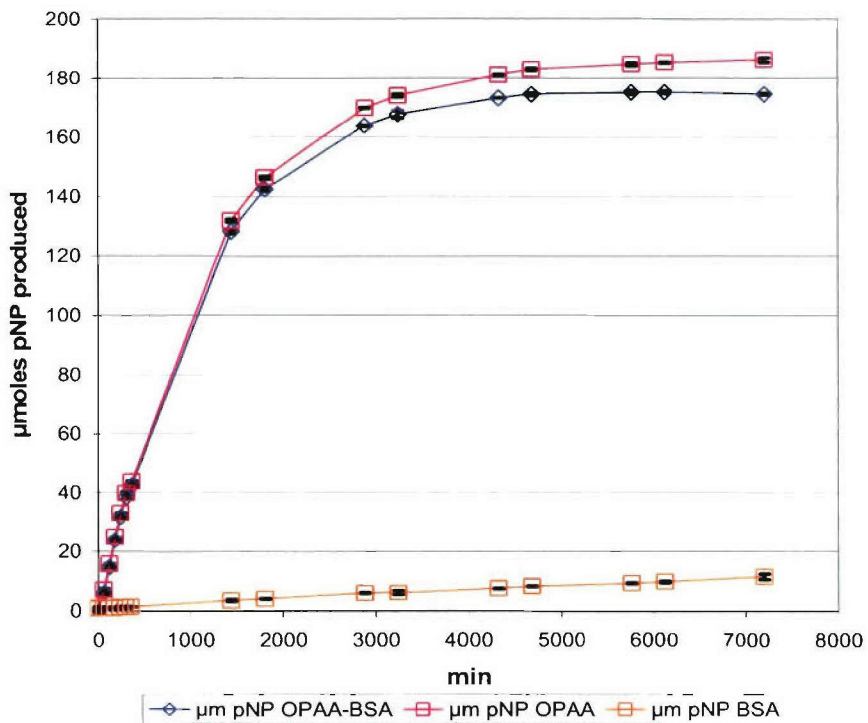
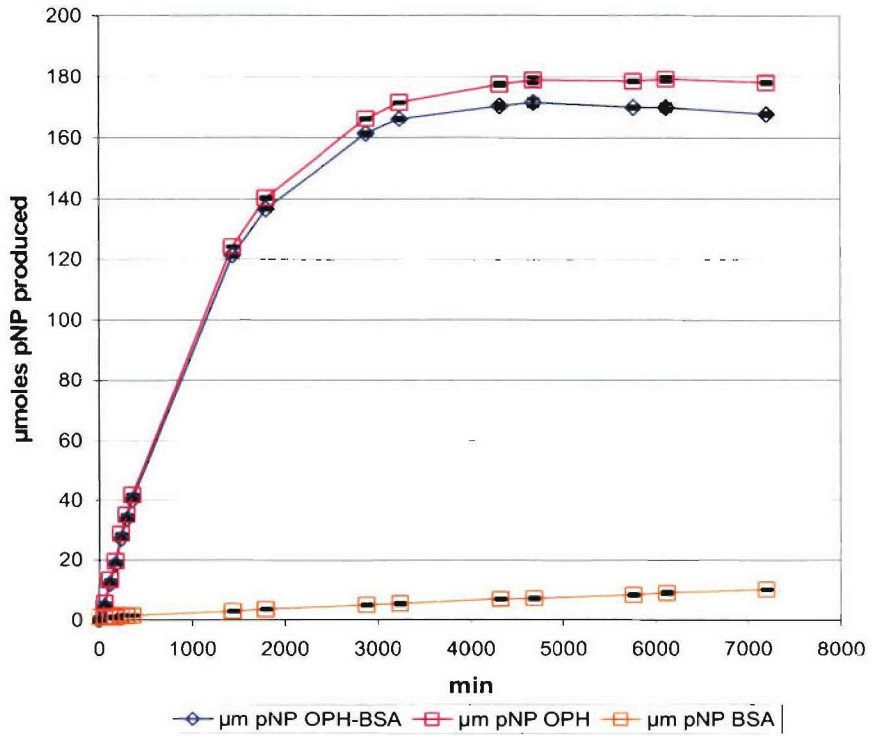


Figure 8. Total μ moles pNP Produced by the OPAA-Agarose, OPH-Agarose and Their BSA-Agarose Controls in the 2-Liter Systems. The enzyme-BSA plots show the net catalytic μ moles produced. Error bars show the \pm 95% confidence limits.

Results of the catalytic filter loop paraoxon decontamination systems showed excellent performance from both immobilized enzymes on agarose (Figure 7). 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 8). *p*-Nitrophenol production from paraoxon was quite evident in the 2 liter catalytic filter system compared with the control filter system (Figure 9).

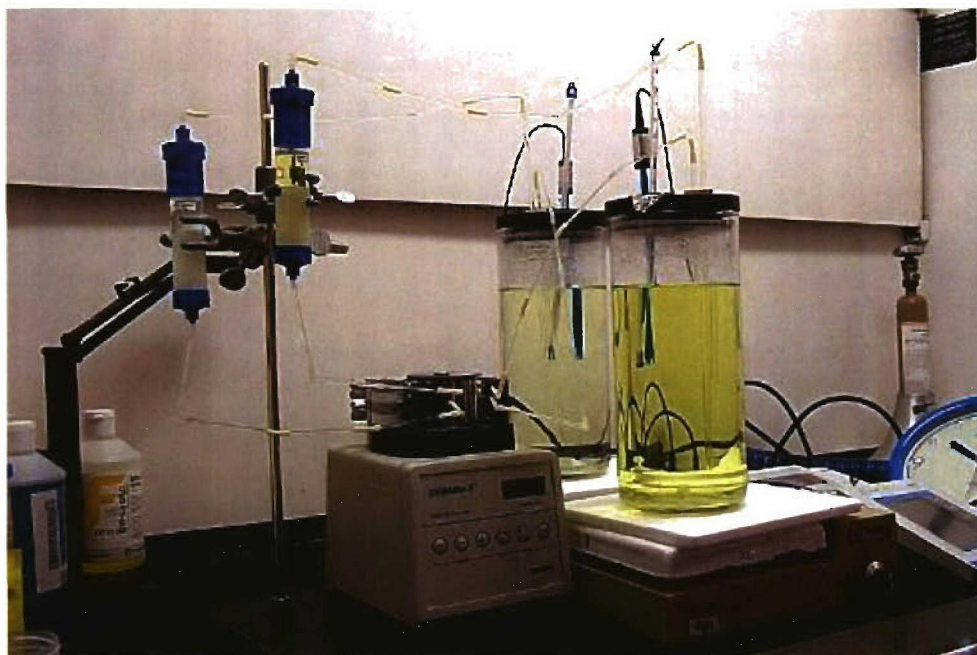


Figure 9. OPAA-Agarose (front) and BSA-Agarose (rear) 2-Liter Systems after Five Day Treatment of Paraoxon in Tap Water. The yellow compound is the *p*-nitrophenolate ion of *p*-nitrophenol, one of the paraoxon hydrolysis products.

The pH of the systems was also divergent (Figure 10). The initial mean pH of the catalytic systems was 7.57 (enzyme) and 7.60 (BSA). After the five day treatment, the final mean 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.

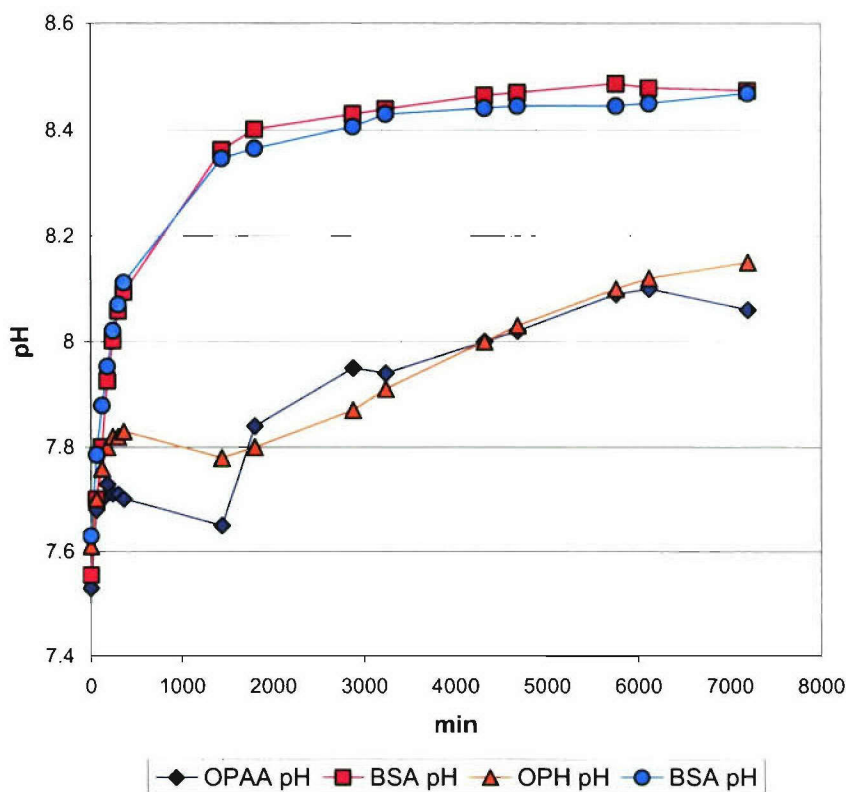


Figure 10. pH Profile of the Catalytic and Control Filter Loops during Paraoxon Hydrolysis in the 2-Liter Systems.

Statistical Analysis of the 2-Liter Systems.

Triplicate data generated during the bench studies was subjected to the T test (in Microsoft Excel) to determine if the absorbance's at 405 nm (A405) of the enzyme filter systems were significantly different from those of the control filter systems. The T test determines the significant differences between the catalytic and the control data based upon the chance that random probability could produce the observed numbers within a predetermined confidence limit. Using the paired two sample for means analysis (two tailed), the resulting parameters of t stat, t critical and P values were examined for each time point. Our criteria was that the t stat should > the t critical value (two-tailed), and that the P value (two-tailed) should be < 0.05, using 95% confidence limits. The results are shown in Tables 1 and 2.

Table 1. T Test Analysis of the OPH/BSA-Agarose Bench Scale Experimental A405 Data

	OPH 0	BSA 0	OPH 60	BSA 60	OPH 120	BSA 120	OPH 180	BSA 180
Mean	0.008333	0.008333	0.045333	0.007667	0.108	0.01	0.158333	0.007
Variance	3.33E-07	3.33E-07	3.33E-07	3.33E-07	0	0	3.33E-07	0
Observations	3	3	3	3	3	3	3	3
Pearson Correlation	-0.5		0.5		#DIV/0!		#DIV/0!	
Hypothesized Mean Difference	0		0		0		0	
deg. freedom	2		2		2		2	
t Stat	0		113		65535		454	
P (T<=t) one-tail	0.5		3.92E-05		#NUM!		2.43E-06	
t Critical one-tail	4.302653		4.302653		4.302653		4.302653	
P(T<=t) two-tail	1		7.83E-05		#NUM!		4.85E-06	
t Critical two-tail	6.205347		6.205347		6.205347		6.205347	
	OPH 240	BSA 240	OPH 300	BSA 300	OPH 360	BSA 360	OPH 1440	BSA 1440
Mean	0.233667	0.013667	0.286333	0.011	0.340333	0.012667	1.015667	0.024333
Variance	3.33E-07	3.33E-07	3.33E-07	4.51E-36	3.33E-07	3.33E-07	3.33E-07	3.33E-07
Observations	3	3	3	3	3	3	3	3
Pearson Correlation	-0.5		3.93E-14		0.5		-1	
Hypothesized Mean Difference	0		0		0		0	
deg. freedom	2		2		2		2	
t Stat	381.0512		826		983		1487	
P(T<=t) one-tail	3.44E-06		7.33E-07		5.17E-07		2.26E-07	
t Critical one-tail	4.302653		4.302653		4.302653		4.302653	
P(T<=t) two-tail	6.89E-06		1.47E-06		1.03E-06		4.52E-07	
t Critical two-tail	6.205347		6.205347		6.205347		6.205347	

Table 1. (Continued)

	OPH 1800	BSA 1800	OPH 2880	BSA 2880	OPH 3240	BSA 3240	OPH 4320	BSA 4320
Mean	1.148333	0.029333	1.363333	0.040667	1.409667	0.044333	1.461	0.057667
Variance	1.33E-06	3.33E-07	1.33E-06	3.33E-07	3.33E-07	1.33E-06	3E-06	1.33E-06
Observations	3	3	3	3	3	3	3	3
Pearson Correlation	0.5		-0.5		-0.5		-0.5	
Hypothesized Mean Difference	0		0		0		0	
Deg. freedom	2		2		2		2	
t Stat	1938.165		1499.763		1548.142		965.8402	
	1.33E-07		2.22E-07		2.09E-07		5.36E-07	
t Critical one-tail	4.302653		4.302653		4.302653		4.302653	
P (T<=t) two-tail	2.66E-07		4.45E-07		4.17E-07		1.07E-06	
t Critical two-tail	6.205347		6.205347		6.205347		6.205347	
	OPH 4680	BSA 4680	OPH 5760	BSA 5760	OPH 6120	BSA 6120	OPH 7200	BSA 7200
Mean	1.474667	0.06	1.474	0.07	1.482	0.075667	1.474667	0.084667
Variance	1.03E-05	0.000001	0.000001	1E-06	7E-06	2.33E-06	1.33E-06	3.33E-07
Observations	3	3	3	3	3	3	3	3
Pearson Correlation	-0.77771		0.5		-0.61859		-1	
Hypothesized Mean Difference	0		0		0		0	
Deg. freedom	2		2		2		2	
t Stat	606.2857		2431.799		643.3915		1390	
P(T<=t) one-tail	1.36E-06		8.46E-08		1.21E-06		2.59E-07	
t Critical one-tail	4.302653		4.302653		4.302653		4.302653	
P (T<=t) two-tail	2.72E-06		1.69E-07		2.42E-06		5.18E-07	
t Critical two-tail	6.205347		6.205347		6.205347		6.205347	

All results meet the significance criteria, except for the time zero sample ($P = 1$, t stat = 0). This was expected, as both systems were untreated at time zero, so their absorbance values should not differ significantly. The 120' values showed no variance between replicates, so it was not possible to get a P value from this data (can't divide by zero variance). The t stat, however, was much larger than the t critical ($65535 > 6.205$), so these measurements do meet this significance criterion.

OPAA/BSA T-test analysis for the A405 data is shown in Table 2.

Table 2. T Test Analysis of the OPAA/BSA-Agarose Bench Scale Experimental A405 Data

	OPAA 0	BSA 0	OPAA 60	BSA 60	OPAA 120	BSA 120	OPAA 180	BSA 180
Mean	0.008333	0.009667	0.058333	0.008667	0.128333	0.008667	0.203	0.008333
Variance	3.33E-07	3.33E-07	2.33E-06	3.33E-07	3.33E-07	3.33E-07	1.16E-33	3.33E-07
Observations	3	3	3	3	3	3	3	3
Pearson Correlation	0.5		0.188982		0.5		0	
Hypothesized Mean Difference	0		0		0		0	
Deg. freedom	2		2		2		2	
t Stat	-4		56.31671		359		584	
P (T<=t) one-tail	0.028595		0.000158		3.88E-06		1.47E-06	
t Critical one-tail	4.302653		4.302653		4.302653		4.302653	
P (T<=t) two-tail	0.057191		0.000315		7.76E-06		2.93E-06	
t Critical two-tail	6.205347		6.205347		6.205347		6.205347	
	OPAA 240	BSA 240	OPAA 300	BSA 300	OPAA 360	BSA 360	OPAA 1440	BSA 1440
Mean	0.269333	0.010667	0.324667	0.011	0.356667	0.012	1.077667	0.029
Variance	3.33E-07	1.33E-06	2.33E-06	0.000001	3.33E-07	4.51E-36	2.33E-06	1E-06
Observations	3	3	3	3	3	3	3	3
Pearson Correlation	-0.5		-0.65465		-3.9E-14		-0.65465	
Hypothesized Mean Difference	0		0		0		0	
Deg. freedom	2		2		2		2	
t Stat	293.3004		235.25		1034		786.5	
P (T<=t) one-tail	5.81E-06		9.03E-06		4.68E-07		8.08E-07	
t Critical one-tail	4.302653		4.302653		4.302653		4.302653	
P (T<=t) two-tail	1.16E-05		1.81E-05		9.35E-07		1.62E-06	
t Critical two-tail	6.205347		6.205347		6.205347		6.205347	

Table 2. (Continued)

	OPAA 1800	BSA 1800	OPAA 2880	BSA 2880	OPAA 3240	BSA 3240	OPAA 4320	BSA 4320
Mean	1.199	0.033333	1.393667	0.049667	1.43	0.051333	1.49	0.064333
Variance	3E-06	3.33E-07	3.33E-07	3.33E-07	3E-06	6.33E-06	0.000001	3.33E-07
Observations	3	3	3	3	3	3	3	3
Pearson Correlation	-1		-0.5		-0.80296		0.866025	
Hypothesized Mean Difference	0		0		0		0	
Deg. freedom	2		2		2		2	
t Stat	874.25		2327.876		590.8571		4277	
P (T<=t) one-tail	6.54E-07		9.23E-08		1.43E-06		2.73E-08	
t Critical one-tail	4.302653		4.302653		4.302653		4.302653	
P(T<=t) two-tail	1.31E-06		1.85E-07		2.86E-06		5.47E-08	
t Critical two-tail	6.205347		6.205347		6.205347		6.205347	
	OPAA 4680	BSA 4680	OPAA 5760	BSA 5760	OPAA 6120	BSA 6120	OPAA 7200	BSA 7200
Mean	1.508667	0.070333	1.525	0.078667	1.532333	0.083	1.542333	0.096333
Variance	2.33E-06	3.33E-07	3E-06	3.33E-07	3.33E-07	0.000001	6.33E-06	9.33E-06
Observations	3	3	3	3	3	3	3	3
Pearson Correlation	0.188982		-0.5		-0.86603		0.953821	
Hypothesized Mean Difference	0		0		0		0	
Deg. freedom	2		2		2		2	
t Stat	1630.917		1203.422		1643.39		2504.545	
P (T<=t) one-tail	1.88E-07		3.45E-07		1.85E-07		7.97E-08	
t Critical one-tail	4.302653		4.302653		4.302653		4.302653	
P (T<=t) two-tail	3.76E-07		6.9E-07		3.7E-07		1.59E-07	
t Critical two-tail	6.205347		6.205347		6.205347		6.205347	

All results meet the significance criteria, except for the time zero sample ($P = 0.057$, t stat = -4). This was expected, as both systems were untreated at time zero, so their absorbance values should not differ significantly.

3.4 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.

4. STABILIZED ENZYME DECONTAMINATION OF DRINKING WATER

4.1 Phase I Preliminary Studies.

Stabilization Process.

These studies examined the effect of PEGylating OPH and OPAA on enzyme activity after drinking water storage. Initial studies used enzyme kinetic rate analysis as the activity benchmark. This benchmark was examined at 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 tap water storage. Paraoxon was used as an OPH substrate, as *p*-nitrophenyl Soman hydrolytic activity was 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 used as the substrate for OPAA as well.

Based upon the literature survey, the best method for stabilizing an enzyme was through the covalent attachment of polyethylene glycol (PEG) groups, also known as PEGylation. PEG groups were attached to the proteins via primary and secondary amines employing succinimide activated PEG's, the oldest and best tested coupling chemistry, to yield a stable amide linkage. Activated PEG's consisted of either a succinimidyl α -methypropionate or succinimidyl α -methybutanoate group attached to the PEG polymer. The optimal size of the polyethylene glycol group for enzyme stabilization varies from one enzyme to another and must be determined empirically. Several different PEG sizes of 2, 5, 20 and 30 kDa, as well as a 40 kDa branched chain polymer were chosen for testing.

Phase 1 testing of modified OPH indicated that the 2-kDa PEG was optimal for enzyme stability with 114% activity of day-5 control (Figure 11). Due to difficulties in obtaining *p*-nitrophenyl Soman, paraoxon was used as the substrate for both enzymes. OPAA results were similar to OPH with the 2-kDa PEG retaining 99% of the day 5 control activity (Figure 12). Out of the five polymer sizes tested the 2-kDa PEG polymer yielded the best activity for both OPH and OPAA after five days in tap water.

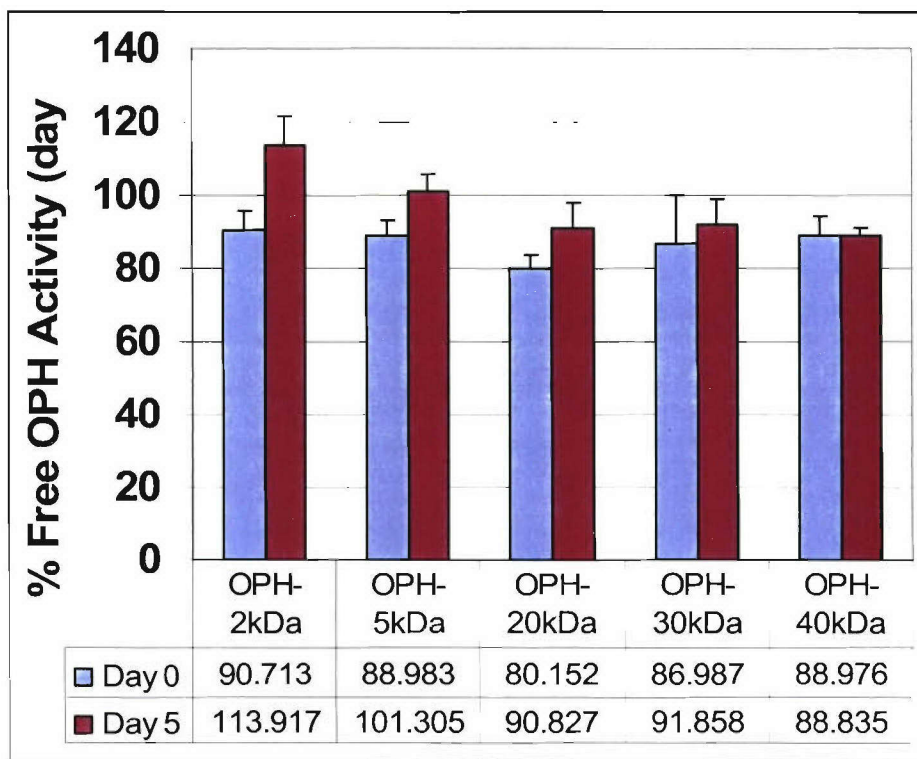


Figure 11. Specific Activity of PEGylated OPH at Days 0 and 5 in Tap Water.

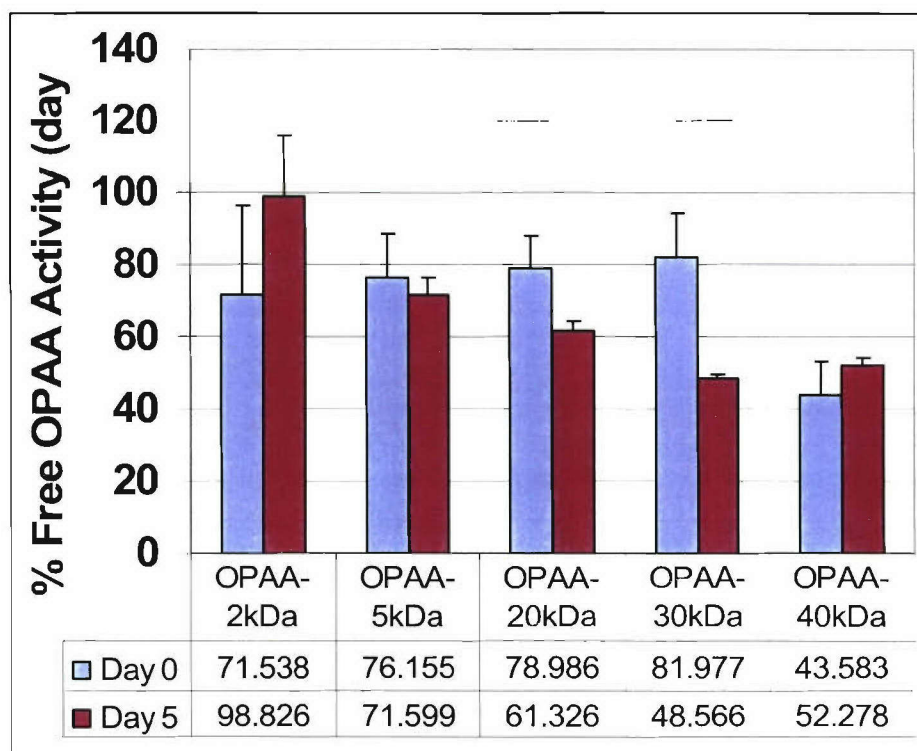


Figure 12. Specific Activity of PEGylated OPAA at Days 0 and 5 in Tap Water. PEG molecular weights indicated. Error bars are the +/- 95% confidence levels.

4.2 Systemization Process.

The stabilized enzymes were used to decontaminate paraoxon from tap water. The benchmark for these studies was the amount of paraoxon hydrolyzed to *p*-nitrophenol over a 5 day treatment period. A small-scale (50 ml) reservoir was employed for transition from the initial rate studies to the 2 liter, bench scale studies. The same modifications required for the investigation into the immobilized enzymes (Section 3.2) were employed in this study.

4.3 Bench Scale Testing.

The bench system tested the feasibility of drinking water decontamination with a stabilized enzyme. Based upon the phase 1 studies the 2-kDa PEG polymer was chosen for the bench scale experiments. The reactor set-up consisted of a sealed 2-L vessel with a pH probe and thermometer passing through the lid. An insulated stir plate was used to drive a stir bar in the bottom of the vessel (Figure 13). Samples were taken via an access port in the top. During the experiment the vessel was protected from light with aluminum foil due to possible photosensitivity of *p*-nitrophenol (It was done for all full scale runs). The stabilized enzyme circulated with 2 liters of 0.091-0.103 mM paraoxon (actual, measured by base hydrolysis) in ECBC tap water at 24°C. Both OPH-2kDa and OPAA-2-kDa were used in this demonstration. BSA-2-kDa was run in parallel with each enzyme as the non-enzymatic control under the same operating conditions. Temperature, pH and the absorbance ($A_{405_{nm}}$) were monitored during the five day demonstration period according to the schedule.

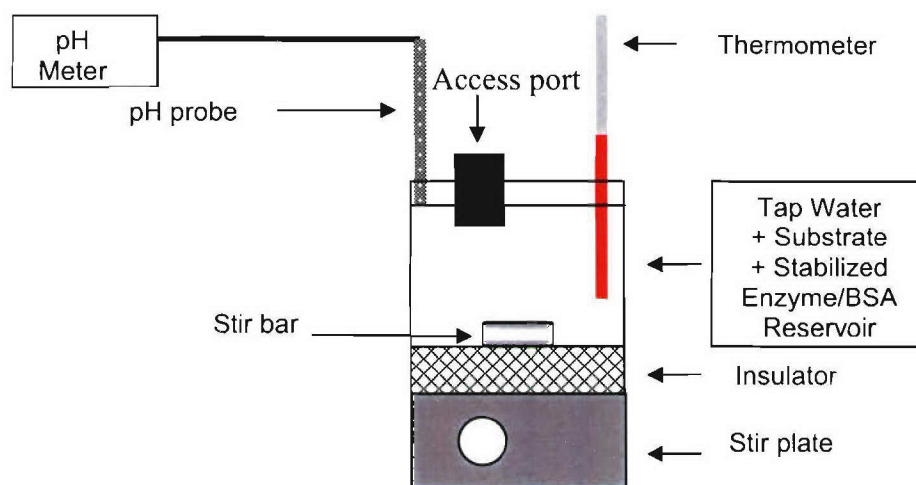


Figure 13. Bench Scale System Set-Up

In the first bench scale experiment OPH failed to hydrolyze any paraoxon after 3-days. Stabilized OPH-2kDa equal to that used on day 0 was added directly to the reactor on day-3. The enzyme behaved as was initially expected with greater than

90% of the paraoxon hydrolyzed in 24-hours (Figure 14). It was suspected that the filtering process had removed or damaged the initial addition of stabilized enzyme.

The experiment was performed again without filtering the stabilized enzyme. There was no paraoxon hydrolyzed by OPH even after five days incubation. The addition of OPH-2kDa after day 5, equivalent to that used in day 0, again resulted in complete hydrolysis of paraoxon in less than 72 hours.

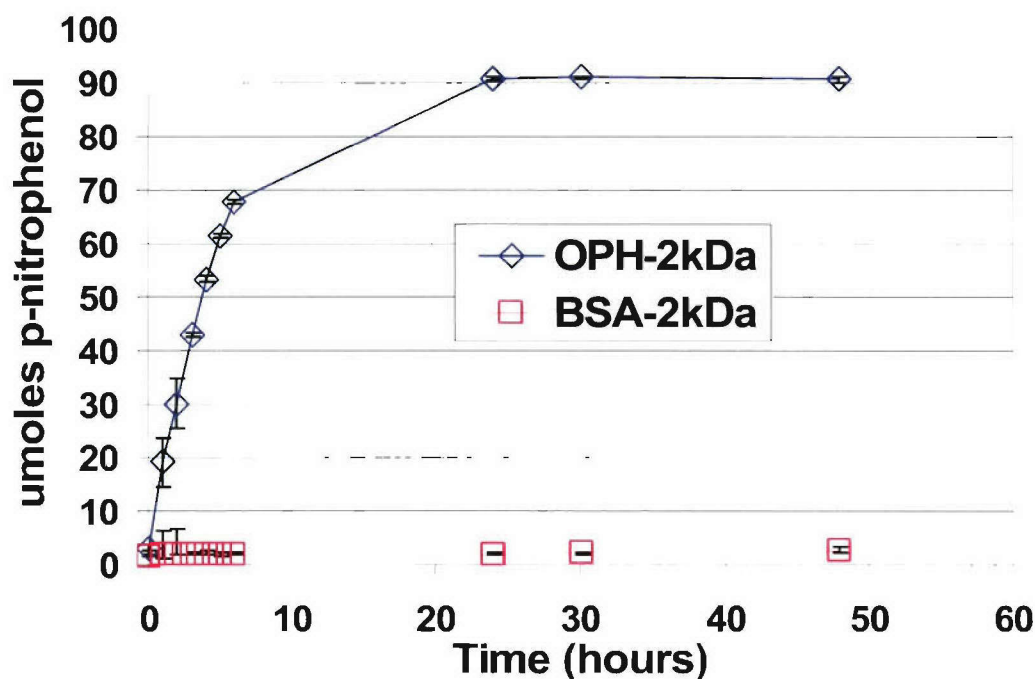


Figure 14. Paraoxon Catalysis by OPH 2-kDa after 3-Day Interval. Error bars are the +/- 95% confidence levels.

A series of troubleshooting experiments were performed to try and clarify why the enzyme initially failed to hydrolyze the substrate in the bench scale study. As part of the preparation process the stabilized enzyme was dialyzed into cold aged tap water (48-72hr) for final storage and testing (phase-1). Dialyzing into tap water did not affect the activity when assayed in BTP buffer. When assayed directly in fresh, filtered or 1-day old unbuffered tap water the enzyme exhibited a rapid loss in activity greater than 98% in the first 30 seconds (Figure 15).

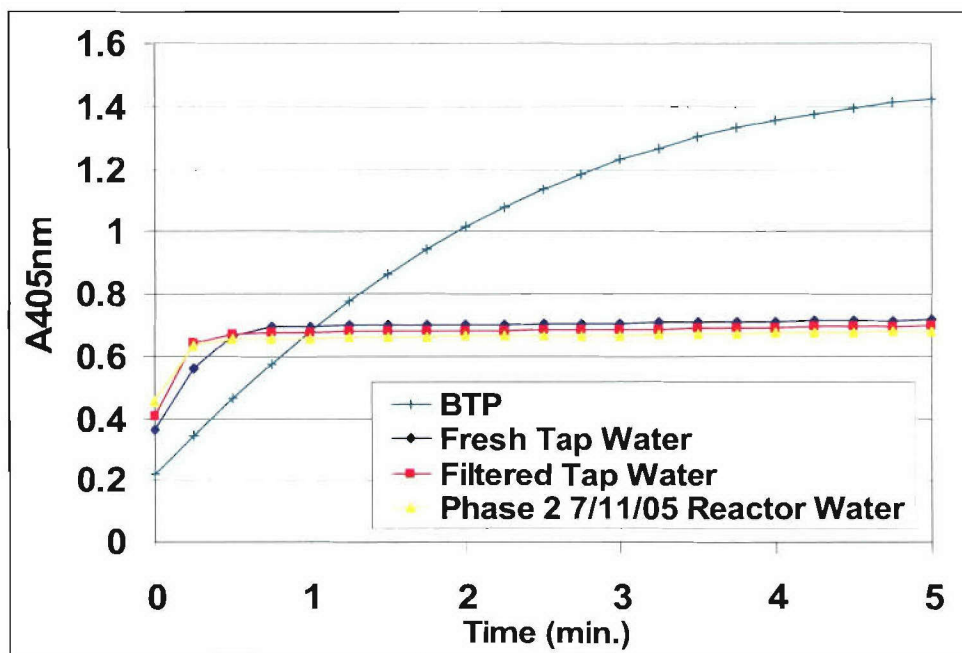


Figure 15. OPH-2kDa Paraoxon Hydrolysis Measured at 405_{nm} Assayed in ECBC Tap Water.

The enzyme was diluted 1:50 and incubated in fresh tap water, 1-day old tap water and 1-month old tap water (sterile filtered) for 15 minutes at room temperature. After incubation enzyme activity was assayed in 50mM BTP pH 8.5, 100mM paraoxon. All three samples retained 100% of their pre-incubation paraoxon activity. The OPH bench study was repeated for a third and final time following some recommendations made during a quality audit of the second study. Additionally, a much larger amount of stabilized enzyme was used for this study. The amount of stabilized enzyme used should have hydrolyzed all the paraoxon in the reactor in less than ten minutes based on the measured activity immediately prior to initiation.

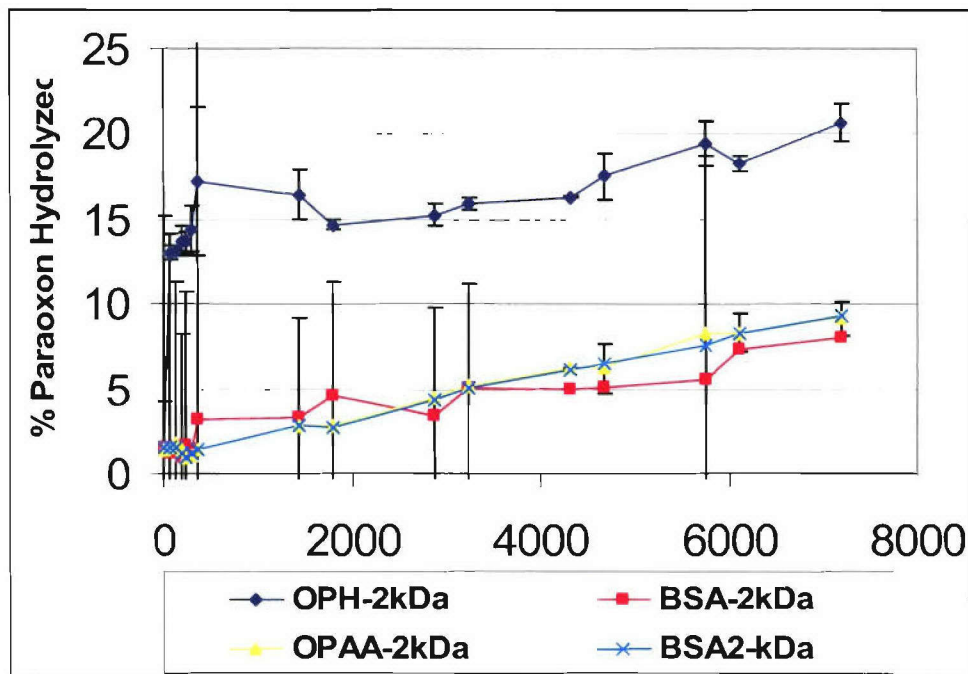


Figure 16. Paraoxon Catalysis in Catalytic and Control 2-Liter Bench Scale Systems. Error bars are the +/- 95% confidence levels.

Results of the catalytic paraoxon decontamination systems showed little performance from both stabilized enzymes (Figure 16). After the five day treatment, the stabilized OPH hydrolyzed 19.4% \pm 0.2% of the paraoxon compared to 6.8% \pm 2.7% in the BSA control. OPAA failed to hydrolyze any paraoxon and was not statistically different from the BSA control. The net catalytic paraoxon hydrolysis from OPH-2kDa and OPAA-2kDa was 11.5% and 0%, respectively (Figure 17). *p*-Nitrophenol production from paraoxon was barely discernable in the 2-liter catalytic system compared with the control filter system for OPH and undetectable in the OPAA system (Figure 18).

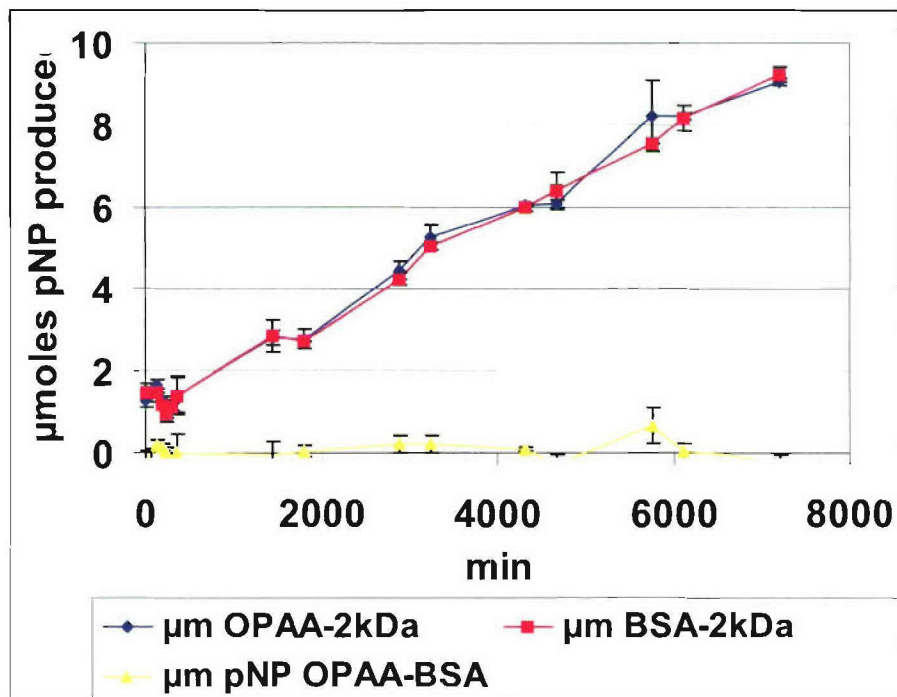
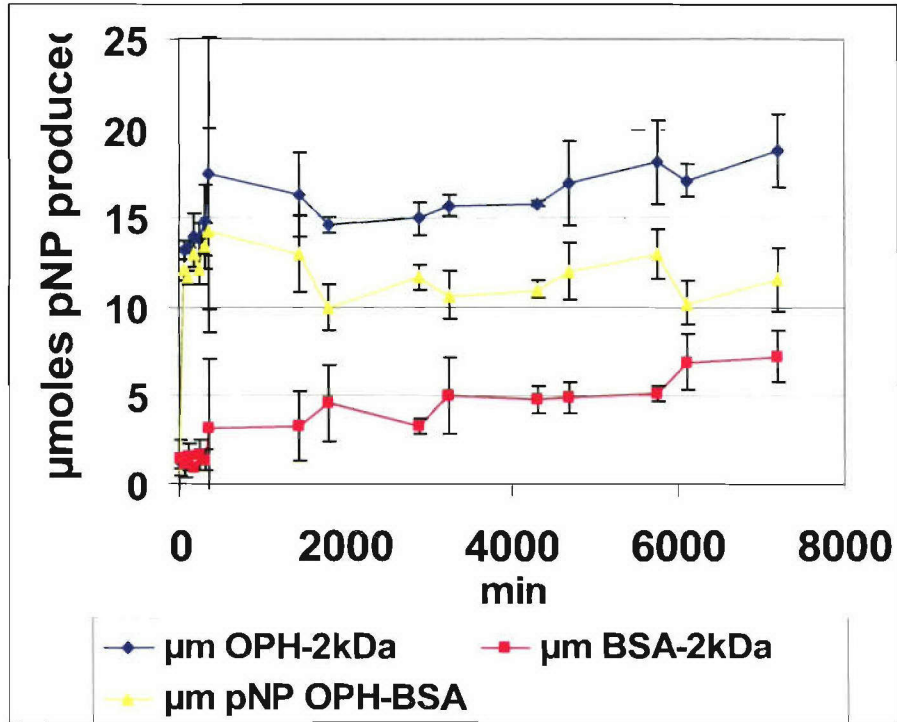


Figure 17. Total $\mu\text{moles pNP}$ produced by the OPH-2kDa, OPAA-2kDa and Their BSA-2kDa Controls in the 2-Liter Systems. The enzyme-BSA plots show the net catalytic μmoles produced. Error bars show the $\pm 95\%$ confidence limits.

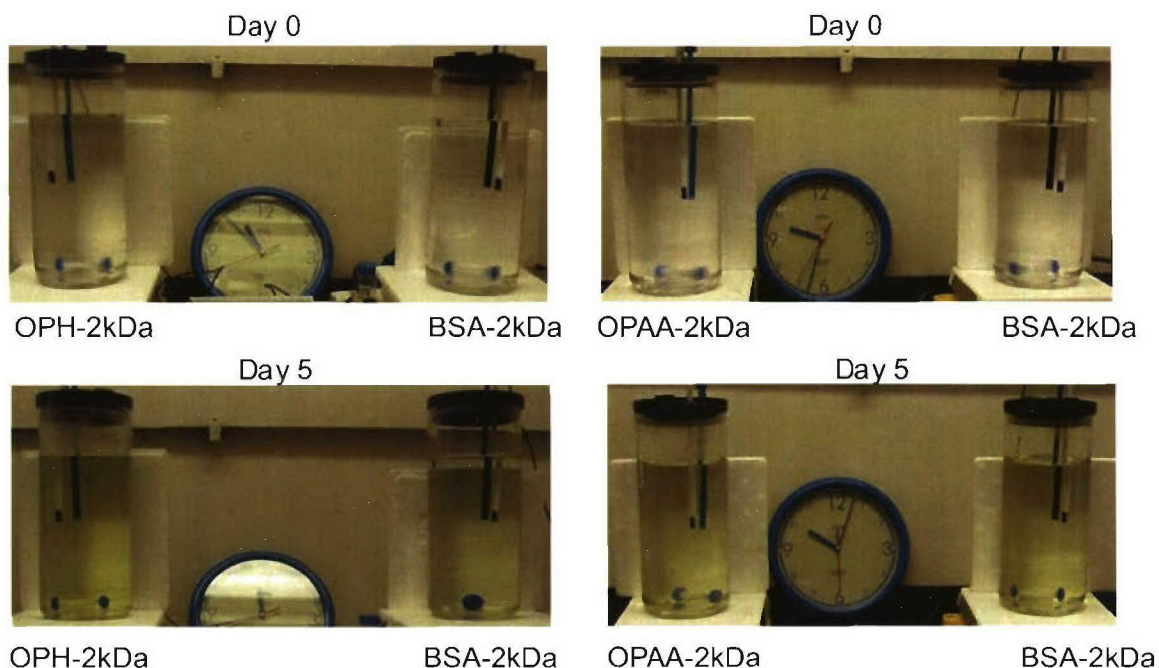


Figure 18. Photograph of Bench Study on Days 0 and 5.

Other parameters measured during the bench scale studies included temperature and pH. The temperature during the study was relatively stable ($23^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$). The initial mean pH of the catalytic systems was 7.93 (enzyme) and 7.85 (BSA). After the five day treatment, the final mean pH was 8.30 (enzyme) and 8.25 (BSA) (Figure 19).

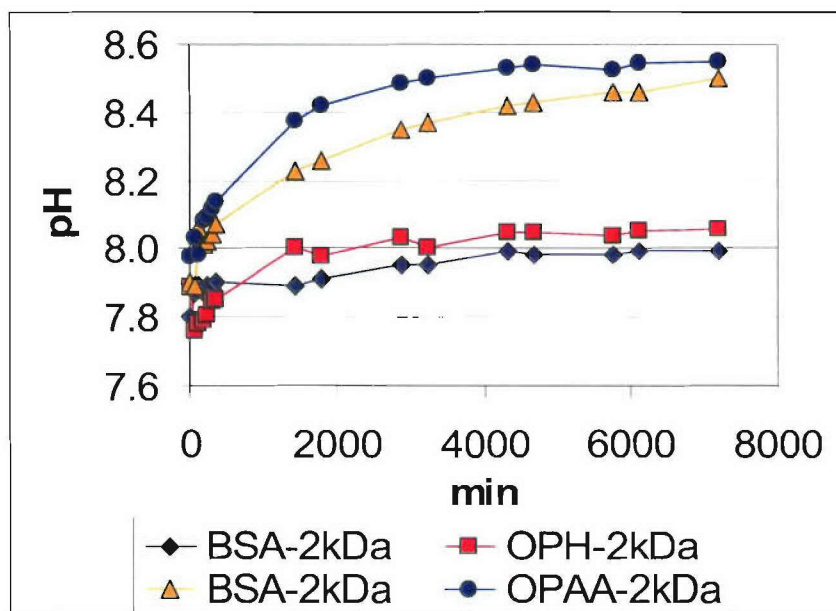


Figure 19. pH profile of Catalytic and Control 2-Liter reactors during Paraoxon Hydrolysis.

Statistical Analysis of the 2-Liter Systems.

Triplicate data generated during the bench studies was subjected to the T test (in Microsoft Excel) to determine if the absorbance at 405 nm (A405) of the enzyme systems were significantly different from those of the control systems. The T test determines the significant differences between the catalytic and the control data based upon the chance that random probability could produce the observed numbers within a predetermined confidence limit. Using the paired two samples for means analysis (two tailed), the resulting parameters of t stat, t critical and P values were examined for each time point. Our criteria was that the t stat should > the t critical value (two-tailed), and that the P value (two-tailed) should be < 0.05, using 95% confidence limits. The results are shown in Tables 3 and 4.

Table 3. T Test Analysis of the OPH/BSA-2kDa Bench Scale Experimental A405 data

	BSA 0	OPH 0	BSA 60	OPH 60	BSA 120	OPH 120	BSA 180	OPH 180
Mean	0.012	0.01067	0.009	0.10433	0.01267	0.105	0.008	0.11033
Variance	4.3E-05	6.3E-06	2.1E-05	1E-05	2.1E-05	0	1E-06	6.5E-05
Observations	3	3	3	3	3	3	3	3
Pearson Correlation	-0.69686		0.8825		#DIV/0!		-0.37115	
Hypothesized Mean Difference	0		0		0		0	
deg. freedom	2		2		2		2	
t Stat	0.27154		71.5		34.625		20.8405	
P (T<=t) one-tail	0.40572		9.8E-05		0.00042		0.00115	
t Critical one-tail	4.30265		4.30265		4.30265		4.30265	
P (T<=t) two-tail	0.81144		0.0002		0.00083		0.00229	
t Critical two-tail	6.20535		6.20535		6.20535		6.20535	
	BSA 1800	OPH 1800	BSA 2880	OPH 2880	BSA 3240	OPH 3240	BSA 4320	OPH 4320
Mean	0.03633	0.115	0.02667	0.12	0.04033	0.12567	0.03867	0.127
Variance	0.00019	7E-06	9.3E-06	3.7E-05	0.00019	1.4E-05	2.5E-05	1E-06
Observations	3	3	3	3	3	3	3	3
Pearson Correlation	-0.50683		-0.26906		-0.51923		0.39736	
Hypothesized Mean Difference	0		0		0		0	
deg. freedom	2		2		2		2	
t Stat	8.90091		21.5385		9.26782		32.3749	
P (T<=t) one-tail	0.00619		0.00107		0.00572		0.00048	
t Critical one-tail	4.30265		4.30265		4.30265		4.30265	
P (T<=t) two-tail	0.01239		0.00215		0.01144		0.00095	
t Critical two-tail	6.20535		6.20535		6.20535		6.20535	

Table 3. (Continued)

	BSA 240	OPH 240	BSA 300	OPH 300	BSA 360	OPH 360	BSA 1440	OPH 1440
Mean	0.01367	0.10967	0.01133	0.11767	0.02533	0.13867	0.026	0.12867
Variance	3E-05	3.2E-05	1.4E-05	0.00016	0.00062	0.00236	0.00015	0.00023
Observations	3	3	3	3	3	3	3	3
Pearson Correlation	-0.73974		0.99124		-0.42464		0.99874	
Hypothesized Mean Difference	0		0		0		0	
deg. freedom	2		2		2		2	
t Stat	15.9264		20.5486		3.10053		58.2065	
P (T<=t) one-tail	0.00196		0.00118		0.04509		0.00015	
t Critical one-tail	4.30265		4.30265		4.30265		4.30265	
P (T<=t) two-tail	0.00392		0.00236		0.09017		0.0003	
t Critical two-tail	6.20535		6.20535		6.20535		6.20535	
	BSA 4680	OPH 4680	BSA 5760	OPH 5760	BSA 6120	OPH 6120	BSA 7200	OPH 7200
Mean	0.03933	0.13633	0.15033	0.04267	0.057	0.14167	0.06233	0.16167
Variance	3E-05	0.00023	0.00024	9.3E-06	0.00011	3.4E-05	0.0001	0.00021
Observations	3	3	3	3	3	3	3	3
Pearson Correlation	0.60294		0.98533		-0.25337		0.8345	
Hypothesized Mean Difference	0		0		0		0	
deg. freedom	2		2		2		2	
t Stat	13.1595		14.9148		11.1066		21.1247	
P (T<=t) one-tail	0.00286		0.00223		0.004		0.00112	
t Critical one-tail	4.30265		4.30265		4.30265		4.30265	
P (T<=t) two-tail	0.00573		0.00447		0.00801		0.00223	
t Critical two-tail	6.20535		6.20535		6.20535		6.20535	

All results meet the significance criteria, except time zero and hour six (Table 3).

Table 4. T Test Analysis of the OPAA/BSA-2kDa Bench Scale Experimental A405 data

	BSA 0	OPAA 0	BSA 60	OPAA 60	BSA 120	OPAA 120	BSA 180	OPAA 180
Mean	0.01167	0.01033	0.01167	0.01133	0.01167	0.013	0.00933	0.01033
Variance	2.3E-06	1.3E-06	3.3E-07	1.3E-06	3.3E-07	1E-06	3.3E-07	3.3E-07
Observations	3	3	3	3	3	3	3	3
Pearson Correlation	0.94491		-0.5		0.86603		-0.5	
Hypothesized Mean Difference	0		0		0		0	
deg. freedom	2		2		2		2	
t Stat	4		0.37796		4		1.73205	
P (T<=t) one-tail	0.0286		0.3709		0.0286		0.1127	
t Critical one-tail	4.30265		4.30265		4.30265		4.30265	
P (T<=t) two-tail	0.05719		0.7418		0.05719		0.2254	
t Critical two-tail	6.20535		6.20535		6.20535		6.20535	
	BSA 1800	OPAA 1800	BSA 2880	OPAA 2880	BSA 3240	OPAA 3240	BSA 4320	OPAA 4320
Mean	0.022	0.02233	0.034	0.03567	0.04033	0.042	0.04833	0.049
Variance	1.8E-35	2.3E-06	1E-06	2.3E-06	3.3E-07	4E-06	3.3E-07	7.2E-35
Observations	3	3	3	3	3	3	3	3
Pearson Correlation	0		-0.98198		0.86603		9.8E-15	
Hypothesized Mean Difference	0		0		0		0	
deg. freedom	2		2		2		2	
t Stat	0.37796		1.14708		1.88982		2	
P (T<=t) one-tail	0.3709		0.18503		0.09968		0.09175	
t Critical one-tail	4.30265		4.30265		4.30265		4.30265	
P (T<=t) two-tail	0.7418		0.37006		0.19936		0.1835	
t Critical two-tail	6.20535		6.20535		6.20535		6.20535	

Table 4. (Continued)

	BSA 240	OPAA 240	BSA 300	OPAA 300	BSA 360	OPAA 360	BSA 1440	OPAA 1440
Mean	0.00733	0.00733	0.00867	0.008	0.011	0.011	0.023	0.02267
Variance	1.3E-06	3.3E-07	3.3E-07	0	7E-06	9E-06	7E-06	1.3E-06
Observations	3	3	3	3	3	3	3	3
Pearson Correlation	0.5		#DIV/0!		-0.75593		-0.65465	
Hypothesized Mean Difference	0		0		0		0	
deg. freedom	2		2		2		2	
t Stat	0		2		0		0.1644	
P (T<=t) one-tail	0.5		0.09175		0.5		0.44226	
t Critical one-tail	4.30265		4.30265		4.30265		4.30265	
P (T<=t) two-tail	1		0.1835		1		0.88453	
t Critical two-tail	6.20535		6.20535		6.20535		6.20535	
	BSA 4680	OPAA 4680	BSA 5760	OPAA 5760	BSA 6120	OPAA 6120	BSA 7200	OPAA 7200
Mean	0.05167	0.04933	0.06	0.06533	0.065	0.06533	0.07467	0.07333
Variance	8.3E-06	3.3E-07	0	3E-05	4E-06	3.3E-07	1.3E-06	3.3E-07
Observations	3	3	3	3	3	3	3	3
Pearson Correlation	-0.5		#DIV/0!		-0.86603		-0.5	
Hypothesized Mean Difference	0		0		0		0	
deg. freedom	2		2		2		2	
t Stat	1.25724		1.67726		0.22942		1.51186	
P (T<=t) one-tail	0.16779		0.11775		0.41994		0.13485	
t Critical one-tail	4.30265		4.30265		4.30265		4.30265	
P (T<=t) two-tail	0.33559		0.23549		0.83987		0.2697	
t Critical two-tail	6.20535		6.20535		6.20535		6.20535	

None of the time points measured for OPAA were statistically different from the BSA control according to our criteria.

4.4 Conclusions.

Initial studies demonstrated that the paraoxon (and nerve agent) hydrolyzing enzymes OPH and OPAA could be PEGylated with a variety polymer sizes (2-40 kilodaltons). The stabilized enzymes were tested for activity stability before and after 5 days storage in tap water. Preliminary studies identified the smallest polyethylene polymer as the optimal size for activity stabilization of both OPH and OPAA enzymes.

Bench-scale experiments with the catalytic reactors were conducted with paraoxon in 2 liters ECBC tap water. The 2kDa succinimide activated PEG polymer was used to stabilize the enzymes OPH and OPAA. Stabilized OPH hydrolyzed a statistically significant amount of paraoxon in the bench study, but the vast majority of this hydrolysis (94.3%) occurred during the first hour. The statistical difference appears to be the result of a brief initial activity between time zero and hour one. The hydrolysis rate after the first hour does not appear to be statistically different from the BSA control, indicating the stabilized enzyme was inactivated during the first hour of incubation, likely within the first minute due to the limited amount of paraoxon hydrolyzed relative to the amount of catalytic activity added.

Stabilized OPAA did not hydrolyze a statistically significant amount of paraoxon in the bench study. OPAA is far slower at hydrolyzing paraoxon than OPH. Therefore, all available stabilized OPAA was employed for the experiment. The amount of enzyme added should have hydrolyzed all the paraoxon in the reactor in approximately 3.2 days based on hydrolysis rates measured at pH 8.5, immediately prior to initiation of the experiment. The stabilized OPAA was likely inactivated shortly after addition to the reactor in a manner similar to that of stabilized OPH. Stabilized OPAA (less than 1/20 of the amount used the initial bench study) was added post-experiment in order to determine if the inactivating material had dissipated in a manner similar to that of the OPH bench studies. After five additional days 6.6% of the remaining paraoxon had been hydrolyzed, compared to an additional 2.9% for the BSA indicating that the stabilized enzyme was indeed active.

Bench scale troubleshooting experiments indicated that the enzyme was not directly inactivated by simple exposure to tap water. However, exposure to tap water in the presence of substrate apparently caused irreversible inactivation. The ability of the tap water to inactivate enzyme was lost after three or more days indicating the transitory nature of the agent(s) responsible. Further research into the mechanism of enzyme inactivation was outside of the scope of this work.

After the five day treatment, the stabilized OPH hydrolyzed 19.4% \pm 0.2% of the paraoxon compared to 6.8% \pm 2.7% in the BSA control. OPAA failed to hydrolyze any paraoxon in the bench study. pH values for the system ranged from an initial average of 7.93 (enzyme) and 7.85 (BSA) to a final average of 8.30 (enzyme) and 8.25 (BSA).

5. GENERAL CONCLUSIONS

Both preliminary and bench scale studies resulted in the successful immobilization of OPAA and OPH enzymes. The activity appeared to be stable for at least 5 days and potentially much longer. As with many other techniques, the support that gives the best activity and/or stability will be dependent on the particular enzyme system being utilized. It appears that enzyme immobilization onto solid supports (enzyme filters) for the decontamination of flowing tap water is a viable technology for use in civilian or military water distribution systems.

For enzyme stabilization, modification of the enzymes using polyethylene glycol polymers (pegylation) was successfully demonstrated in the preliminary studies. However, when scaled up for the bench scale studies, both of the modified OPAA and OPH enzymes showed significant inhibition. Since all other conditions were the same as in the preliminary studies except for the volume of the systems, the cause of this inhibition is currently unknown. This will require additional research and development to overcome this limitation. It is also unknown whether other methods of stabilization may have the same effect.

GLOSSARY

Cyclosarin	GF, O-Cyclohexyl methylphosphonofluoridate
LBG	Locust bean gum
OPAA	Organophosphorus acid anhydrolase
OPH	Organophosphorus hydrolase
Paraoxon	O,O-Diisopropyl <i>p</i> -nitrophenylphosphate
PEG	Polyethylene glycol
<i>p</i> NP	<i>para</i> -Nitrophenol
Sarin	GB, O-Isopropyl methylphosphonofluoridate
Soman	GD, O-Pinacolyl methylphosphonofluoridate
Tabun	GA,
THEOS	Tetrakis (2-hydroxyethyl) orthosilicate
VX	S-Diisopropylaminoethyl methylphosphonothiolate

DEPARTMENT OF THE ARMY
CDR USARDECOM
ATTN AMSRD CII
5183 BLACKHAWK ROAD
APG MD 21010-5424
OFFICIAL BUSINESS

FIRST CLASS