FINAL STUDY REPORT

PROTOCOL TITLE

Sporicidal Activity of Disinfectants

Test Organisms:

Bacillus subtilis (ATCC 19659)
Clostridium sporogenes (ATCC 3584)

PRODUCT IDENTITY

Oxine,
Lot No. 0212-064, Lot No. 0305-024 and Lot No. 0305-025

DATA REQUIREMENTS

U.S. EPA 40 CFR Part 158
"Data Requirements for Registration"
Pesticide Assessment Guidelines – Subdivision G, 91-2 (a)

PROJECT NUMBER

A01464

PROTOCOL NUMBER

BCI03051203.SPOR

AUTHOR

David Rottjakob, M.T.
Study Director

STUDY COMPLETION DATE

October 20, 2003

AMENDED REPORT DATE

October 31, 2003

PERFORMING LABORATORY

ATS LABS
2540 Executive Drive
St. Paul, MN 55120

SPONSOR

Bio-Cide International, Inc.
P.O. Box 722170
Norman, OK 73070-8644

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA Section 10 (d) (1) (A), (B), or (C).

Company: Bio-Cide International, Inc.

Company Agent: Neeraj Khanna, Ph.D. (P.I.)

Director, Research & Development
Title

Neeraj Khanna
Signature

Date: 11/12/03

GOOD LABORATORY PRACTICE STATEMENT

The study referenced in this report was conducted in compliance with U.S. Environmental Protection Agency Good Laboratory Practice (GLP) regulations set forth in 40 CFR Part 160.

The studies not performed by or under the direction of ATS LABS are exempt from this Good Laboratory Practice Statement and include: characterization and stability of the compound(s).

Submitter: James P. Ringo — Date: 12 Nov 03

Sponsor: Neeraj Khanna, Ph.D. — Date: 11/12/03

Study Director: David Rottjakob, M.T. — Date: 10/31/03

QUALITY ASSURANCE UNIT SUMMARY

Study: Sporicidal Activity of Disinfectants

The objective of the Quality Assurance Unit is to monitor the conduct and reporting of nonclinical laboratory studies. These studies have been performed under Good Laboratory Practice regulations (40 CFR Part 160) and in accordance to standard operating procedures and standard protocols. The Quality Assurance Unit maintains copies of study protocols and standard operating procedures and has inspected this study on the dates listed below. Studies are inspected at time intervals to assure the integrity of the study.

Phase Inspected	Date	Study Director	Management
Critical Phase	June 27, 2003	June 27, 2003	October 20, 2003
Final Report	October 15, 2003	October 15, 2003	
Amended Report	October 30, 2003	October 30, 2003	October 31, 2003

The findings of these inspections have been reported to management and the Study Director.

Quality Assurance Auditor: Rachelle L. Ewenson Date: 10/31/03

TABLE OF CONTENTS

STUDY PERSONNEL

STUDY DIRECTOR:	David Rottjakob, M.T.

Professional personnel involved:

Douglas G. Anderson, Ph.D.	- President
Karen M. Ramm, B.A.	- Technical Director
David R. Rottjakob, M.T.	- Microbiology Program Manager
Barbara Bailey, A.A.	- Microbiology Laboratory Supervisor
Sally Nada, B.S.	- Research Scientist I
Adam W. Pitt, B.S.	- Research Assistant II
Scott R. Steinagel, B.S.	- Research Assistant I
Matthew Sathe, B.S.	- Research Assistant I
Peter Toll, B.S.	- Research Assistant I
Linda Mizeur, B.S.	- Research Assistant I

STUDY REPORT

GENERAL STUDY INFORMATION

Study Title: Sporicidal Activity of Disinfectants

Project Number: A01464

Protocol Number: BCI03051203.SPOR

Sponsor: Bio-Cide International, Inc.
P.O. Box 722170
Norman, OK 73070-8644

Test Facility: ATS LABS
2540 Executive Drive
St. Paul, MN 55120

TEST SUBSTANCE IDENTITY

Test Substance Name: Oxine

Lot/Batch(s): Lot No. 0212-064, Lot No. 0305-024 and Lot No. 0305-025

Test Substance Characterization
Test substance characterization as to content, stability, etc., (40 CFR, Part 160, Subpart F [160.105]) is the responsibility of the Sponsor.

STUDY DATES

Date Sample Received: May 20, 2003
Study Initiation Date: May 27, 2003
Experimental Start Date: June 27, 2003
Experimental End Date: September 8, 2003
Study Completion Date: October 20, 2003

TEST HISTORY

Testing started on 6/27/03 yielded growth of the test organism, *Clostridium sporogenes*, in one of the porcelain penicylinder subcultures when testing against Oxine, Lot 0305-024 and two of the porcelain penicylinders when testing against Lot 0305-025 at a one hour contact time. Per Sponsor's request, testing was repeated on 8/14/03 to evaluate for the presence of potential false positives. On the same date, each of the three lots of Oxine was also tested against *Clostridium sporogenes* on porcelain penicylinders for a 2 hour contact time. All results are valid and presented in this report.

OBJECTIVE

The purpose of this assay was to determine the presence of sporicidal activity of a germicide against specified spore-forming bacteria using the AOAC Sporicidal Activity Method.

STUDY MATERIALS

Test System/Growth Media

Test Organisms	ATCC #	Growth Medium	Incubation Parameters
Bacillus subtilis	19659	Soil Extract Nutrient Broth	35-37°C, aerobic
Clostridium sporogenes	3584	Soil Extract-Egg-Meat Medium	35-37°C, aerobic

The microorganisms used in this study were obtained from the American Type Culture Collection (ATCC), Manassas, Virginia.

Recovery Media

Neutralizing Subculture Medium: Fluid Thioglycollate Medium (test)
Modified Fluid Thioglycollate (HCl Control)
Agar Plate Medium: Tryptic Soy Agar (BAP)

Reagents

Organic Soil Load Description: 5% fetal bovine serum

Preparation of Test Organism

A stock culture was prepared for each test organism. Three tubes of growth media per twenty carriers needed for testing were inoculated. The tubes were incubated for 72±4 hours at 35-37°C. The *Clostridium sporogenes* culture was harvested from the tubes of Soil Extract Egg Meat Media and filtered through sterile gauze. The *Bacillus subtilis* culture was aspirated from the Soil Extract Nutrient Broth tubes, macerated in a tissue grinder, and filtered through sterile gauze.

Addition of Organic Soil Load

For testing on 6/27/03, a 20 mL aliquot of FBS was added to 400 mL of the test organism to yield a 5% organic soil load. For repeat testing on 8/14/03, a 16 mL aliquot of FBS was added to 304 mL of the test organism suspension to yield a 5% organic soil load.

Carriers

Porcelain penicylinders (O.D. 8mm ±1, I.D.6mm ±1, length 10mm ±1) were sterilized 2 hours in 180° C air oven. Used penicylinders were washed with 1.0% Triton X-100 and rinsed with water 4 times.

Dacron suture loop carriers were prepared by wrapping size 3 surgical dacron suture around a pencil 3 times, slipping coil off pencil, and knotting another piece of suture securely to form a 2 loop coil. The loops were extracted in a Soxhlet Extraction Apparatus using $CHCl_3$ for 24 hours and air dried in a hood for 12-18 hours at room temperature. The loops were placed in HCl for 10 minutes, liquid decanted and loops rinsed repeatedly with deionized water until the pH of the rinse water was neutral. The loops were air-dried on filter paper mats under ambient conditions.

TEST METHOD

Carrier Contamination

Carriers were immersed for 15 minutes in a 72±4 hour old broth culture at a ratio of 1 carrier per 1 mL broth culture and placed into a glass petri dish matted with 2 layers of filter paper. The contaminated carriers were transferred in a vacuum desiccator containing $CaCl_2$ and the vacuum was drawn to ≥69cm Hg for the entire drying period. The contaminated carriers were dried in the desiccator for 88.5 hours for testing on 6/27/03 and approximately 71 hours for testing on 8/14/03.

Test Substance Use-Dilution
For each lot, a 2000 ppm final use concentration was prepared by adding 20 grams of citric acid activator to 100 mL of Oxine concentrate. The citric acid crystals were dissolved by gently stirring with a glass rod, then the beaker was covered with a glass petri plate for 20 minutes. The entire 100 mL of this solution was then added to 900 mL of filter sterilized tap water. Agitation was minimized to avoid gas escaping from the prepared solution.

Medication
On 6/27/03 and for repeat testing conducted on 8/14/03, five dried, contaminated carriers were placed into each of the 6 tubes containing 10 mL of the test substance at its use-dilution for a 1 hour contact period at 25°C. Additional testing conducted on 8/14/03 held the inoculated porcelain penicylinder carriers in tubes containing the test substance for 2 hours.

Subculture
Each medicated carrier was transferred by hook needle to Fluid Thioglycollate medium. After completion of subcultures, each carrier was re-transferred to a fresh tube of the same Fluid Thioglycollate medium.

Incubation and Observation
Subculture plates were incubated for 48±4 hours and subculture tubes were incubated for 21 days at 35-37°C. Tubes demonstrating growth were subcultured onto appropriate agar medium for confirmation of the test organism. Tubes demonstrating no growth of the test organisms were heat shocked for 20 minutes at 80°C and reincubated for 72±4 hours at 35-37°C. The plate subcultures from testing on 8/14/03 were stored at 2-8°C for 3 days prior to reading.

STUDY CONTROLS

Purity Control
A "streak plate for isolation" was performed on the organism culture, and following incubation, examined in order to confirm the presence of a pure culture. The acceptance criterion for this study control is a pure culture demonstrating colony morphology typical of the test organism.

Carrier Sterility Control
A representative uninoculated carrier from each carrier type was added to the subculture medium. The subculture medium containing the carrier was incubated and examined for growth. The acceptance criterion for this study control is lack of growth.

Media Sterility Control
A representative sample of uninoculated subculture medium was incubated and observed. The acceptance criterion for this study control is lack of growth.

Organic Soil Sterility Control
The serum used for soil load was cultured, incubated, and visually examined for lack of growth. The acceptance criterion for this study control is lack of growth.

Viability Control
A representative inoculated carrier from each organism and each carrier type was added to the subculture medium. The subculture medium containing the carrier was incubated and examined for growth. The acceptance criterion for this study control is growth.

Carrier Quantitation Control

Contaminated carriers were transferred to a jar of neutralizer at a ratio of one carrier to 10 mL of neutralizer and vortex mixed. This suspension was serially diluted and plated using standard microbiological techniques. For *Clostridium*, only 0.1 mL was plated to avoid pooling of the organism on the plates. Following incubation 48±4 hours at 35-37°C, and at anaerobic conditions for *Clostridium sporogenes*, the organism plates were observed to enumerate the concentration of the test organism present at the time of testing. The acceptance criterion for this study is growth of $\geq 1.0 \times 10^4$ CFU/carrier.

Neutralization Confirmation

The neutralization of the test substance was confirmed by exposing sterile carriers to the test substance for the specified exposure times and transferring them to primary subculture tubes containing 10mL of the subculture medium. Carriers were transferred from primary subculture tubes into individual secondary subculture tubes. The secondary transfer tubes were challenged with low levels of the organism (neutralization control), incubated as in test and observed for the presence of growth. The acceptance criterion for this study is growth.

HCl Control

Four contaminated carriers were placed into a tube containing 10 mL 2.5N HCl at 20±1°C. One exposed carrier was subcultured in a manner consistent with the test after 2, 5, 10 and 20 minutes using Modified Fluid Thioglycollate. These control subculture tubes were incubated with the test subculture tubes. Test spores should resist HCl for ≥ 2 minutes and may be resistant for a full 20 minutes.

STUDY ACCEPTANCE CRITERIA

Test Substance Performance Criteria

Data submitted to the EPA in support of a sterilizing claim requires that a disinfectant kill microorganisms on all carriers.

Control Acceptance Criteria

The study controls must perform according to the criteria detailed in the study controls description section.

PROTOCOL CHANGES

Protocol Amendments:

1. Per Sponsor's request the protocol was amended to change the concentration to be tested from 1500 ppm to 2000 ppm. This change is reflected in the revised test substance activation procedure supplied by the Sponsor.

2a. Per Sponsor's request the protocol is amended to allow for the repeat testing of Lot 0305-024 and 0305-025 against *Clostridium sporogenes* on porcelain penicylinders for the protocol specified 1 hour exposure time. The testing is being conducted for the evaluation of false positives.

2b. Per Sponsor's request the protocol is amended to allow for the additional testing of all three lots of test substance against *Clostridium sporogenes* on porcelain penicylinders. The additional testing will be performed utilizing a 2 hour exposure time.

Protocol Deviations:

No protocol deviations occurred during this study.

DATA ANALYSIS

Calculations

Carrier Population Control Calculation:

CFU/carrier: $\frac{\text{(avg. \# colonies found/plate @ dilution used) (dilution factor) (volume neutralizer)}}{\text{(\# of carriers tested) (volume plated)}}$

The carrier population was calculated and reported using data from the most appropriate dilutions.

Statistical Analysis

None used.

STUDY RETENTION

Record Retention

All of the original raw data developed exclusively for this study shall be archived at ATS LABS, 2540 Executive Drive, St. Paul, MN 55120. These original data include, but are not limited to the following:

1. All handwritten raw data for control and test substances including, but not limited to notebooks, data forms and calculations.
2. Any protocol amendments/deviation notifications.
3. All measured data used in formulating the final report.
4. Memoranda, specifications, and other study specific correspondence relating to interpretation, and evaluation of data, other than those documents contained in the final study report.
5. Original signed protocol
6. Certified copy of final study report
7. Study-specific SOP deviations made during the study.

Test Substance Retention

The test substance will be discarded following study completion per Sponsor approved protocol. It is the responsibility of the Sponsor to retain a sample of the test material.

REFERENCES

1. AOAC Official Methods of Analysis, Sporicidal Activity of Disinfectants, Sixteenth Edition, 1995, 966.04.
2. U.S. Environmental Protection Agency Pesticide Assessment Guidelines, Subdivision G 91-2 (a): Product Performance, November, 1982.
3. U.S. Environmental Protection Agency, Registration Division, Office of Pesticide Programs, DIS/TSS-1, January 22, 1982.

RESULTS

Control and Neutralization Results (Tables 1-4)

All data measurements/controls for the culture purity, viability, organic soil sterility, neutralizing subculture media sterility, carrier sterility, neutralization confirmation, carrier quantitation and HCl controls were within acceptance criteria.

Test Results (Table 5)

ANALYSIS

On 6/27/03, Oxine (Lot No. 0212-064, Lot No. 0305-024 and Lot No. 0305-025), diluted at 2000 ppm in filter sterilized tap water, demonstrated no growth of *Bacillus subtilis* in any of the 60 primary subcultures and no growth in any of the 60 secondary subcultures after a 1 hour exposure time when tested using Dacron suture and porcelain penicylinder carriers.

On 6/27/03, Oxine (Lot No. 0212-064, Lot No. 0305-024 and Lot No. 0305-025), diluted at 2000 ppm in filter sterilized tap water, demonstrated no growth of *Clostridium sporogenes* in any of the 60 primary subcultures and no growth in any of the 60 secondary subcultures after a 1 hour exposure time when tested using Dacron suture carriers.

On 6/27/03, Oxine (Lot No. 0305-024 and Lot No. 0305-025), diluted at 2000 ppm in filter sterilized tap water, demonstrated no growth of *Clostridium sporogenes* in any of the 60 primary subcultures and growth in 1 (Lot No. 0305-024) and 2 (Lot No. 0305-025) respectively, of the 60 secondary subcultures after a 1 hour exposure time when tested using porcelain penicylinder carriers. On 6/27/03, under the same conditions, Oxine (Lot No. 0212-064) demonstrated no growth of *Clostridium sporogenes* in any of the 60 primary or secondary subcultures when testing using porcelain penicylinder carriers.

During repeat testing on 8/14/03, Oxine (Lot No. 0305-024 and Lot No. 0305-025), diluted at 2000 ppm in filter sterilized tap water, demonstrated no growth of *Clostridium sporogenes* in any of the 60 primary subcultures and growth in 1 (Lot No. 0305-024) and 1 (Lot No. 0305-025) respectively, of the 60 secondary subcultures after a 1 hour exposure time when tested using porcelain penicylinder carriers.

On 8/14/03, Oxine (Lot No. 0212-064, Lot No. 0305-024 and Lot No. 0305-025), diluted at 2000 ppm in filter sterilized tap water, demonstrated no growth of *Clostridium sporogenes* in any of the 60 primary subcultures and no growth in any of the 60 secondary subcultures after a 2 hour exposure time when tested using porcelain penicylinder carriers.

STUDY CONCLUSION

Under the conditions of this investigation, Oxine (Lot No. 0212-064, Lot No. 0305-024 and Lot No. 0305-025), diluted at 2000 ppm in filter sterilized tap water, was SPORICIDAL when tested against *Bacillus subtilis* following a 1 hour exposure period.

Under the conditions of this investigation, Oxine (Lot No. 0212-064, Lot No. 0305-024 and Lot No. 0305-025), diluted at 2000 ppm in filter sterilized tap water, was NOT SPORICIDAL when tested against *Clostridium sporogenes* on porcelain penicylinder carriers following a 1 hour exposure period.

Under the conditions of this investigation, Oxine (Lot No. 0212-064, Lot No. 0305-024 and Lot No. 0305-025), diluted at 2000 ppm in filter sterilized tap water, was SPORICIDAL when tested against *Clostridium sporogenes* on Dacron suture carriers following a 1 hour exposure period.

Under the conditions of this investigation, Oxine (Lot No. 0212-064, Lot No. 0305-024 and Lot No. 0305-025), diluted at 2000 ppm in filter sterilized tap water, was SPORICIDAL when tested against *Clostridium sporogenes* on porcelain penicylinder carriers following a 2 hour exposure period.

In the opinion of the Study Director, there were no circumstances that may have adversely affected the quality or integrity of the data.

TABLE 1: CONTROL RESULTS

The following results from controls confirmed study validity:

Type of Control		Results		
		Bacillus subtilis	***Clostridium sporogenes***	***Clostridium sporogenes***
Date Performed		**6/27/03**		**8/14/03**
Purity Control		Pure	Pure	Pure
Viability Control – Dacron Suture in Fluid Thioglycollate		Growth	Growth	NA
Viability Control – Porcelain Penicylinder in Fluid Thioglycollate		Growth	Growth	Growth
Viability Control – Dacron Suture in Modified Fluid Thioglycollate		Growth	Growth	NA
Viability Control – Porcelain Penicylinder in Modified Fluid Thioglycollate		Growth	Growth	Growth
Media Sterility Control – Fluid Thioglycollate	FTM061103-12	No Growth		NA
	FTM062603-11	No Growth		NA
	FTM062703-11	No Growth		NA
	FTM072903-11	NA		No Growth
Media Sterility Control – Modified Fluid Thioglycollate		No Growth		No Growth
Organic Soil Sterility Control	Vial # 3	No Growth		NA
	Vial # 4	No Growth		NA
	Vial # 5	No Growth		NA
	Vial # 17	NA		No Growth
	Vial # 25	NA		No Growth
Carrier Sterility Control – Dacron Suture		No Growth		NA
Carrier Sterility Control – Porcelain Penicylinder	PCS041403	No Growth		NA
	PCS012303	NA		No Growth
	PCS021303	NA		No Growth
	PCS070303	NA		No Growth

NA= Not applicable

TABLE 2: 2.5N HCl VIABILITY TEST

Test Organism	Date Performed	Carrier Type	2 MINUTES		5 MINUTES		10 MINUTES		20 MINUTES	
			1°	2°	1°	2°	1°	2°	1°	2°
Bacillus subtilis	6/27/03	Dacron Sutures	+	+	0	0	0	0	0	0
		Porcelain	0	+	0	+	0	0	0	0
Clostridium sporogenes	6/27/03	Dacron Sutures	+	+	0	+	0	0	0	0
		Porcelain	0	+	0	+	0	0	0	0
	8/14/03	Porcelain	0	+	0	+	0	0	0	0

\+ = Growth of Organism
0 = No Growth of Organism

TABLE 3: CARRIER POPULATION CONTROL RESULTS

Test Organism	Carrier Type	Date Performed	CFU/carrier
Bacillus subtilis	Dacron Sutures	6/27/03	1.04×10^6
	Porcelain		1.60×10^6
Clostridium sporogenes	Dacron Sutures	6/27/03	4.7×10^5
	Porcelain		4.5×10^5
	Porcelain	8/14/03	8.0×10^5

CFU = Colony Forming Unit

TABLE 4: NEUTRALIZATION CONFIRMATION RESULTS

Test Substance	Test Organism	Carrier Type	Date	Exposure Time (hours)	Inoculum (CFU)	Number of Tubes Tested	Number of Tubes Positive
Oxine, Lot No. 0212-064	*B. subtilis*	Dacron Sutures	6/27/03	1	104	6	6
		Porcelain		1	160	6	6
	C. sporogenes	Dacron Sutures	6/27/03	1	47	6	6
		Porcelain	6/27/03	1	45	6	6
			8/14/03	1	80	6	6
				2		6	6
Oxine, Lot No. 0305-024	*B. subtilis*	Dacron Sutures	6/27/03	1	104	6	6
		Porcelain		1	160	6	6
	C. sporogenes	Dacron Sutures	6/27/03	1	47	6	6
		Porcelain	6/27/03	1	45	6	6
			8/14/03	1	80	6	6
				2		6	6
Oxine, Lot No. 0305-025	*B. subtilis*	Dacron Sutures	6/27/03	1	104	6	6
		Porcelain		1	160	6	6
	C. sporogenes	Dacron Sutures	6/27/03	1	47	6	6
		Porcelain	6/27/03	1	45	6	6
			8/14/03	1	80	6	6
				2		6	6

CFU = Colony forming units

TABLE 5: TEST RESULTS

Test Substance	Test Organism	Date Performed (Exposure)	Carrier Type	Total Carriers Tested	Number of Carriers Showing Growth of The Test Organism			
					1°	2°	1° HS	2° HS
Oxine, Lot No. 0212-064	*B. subtilis*	6/27/03 (1 hour)	Dacron Sutures	60	0	0	0	0
			Porcelain	60	0	0	0	0
	C. sporogenes	6/27/03 (1 hour)	Dacron Sutures	60	0	0	0	0
			Porcelain	60	0	0	0	0
		8/14/03 (2 hour)	Porcelain	60	0	0	0	0
Oxine, Lot No. 0305-024	*B. subtilis*	6/27/03 (1 hour)	Dacron Sutures	60	0	0	0	0
			Porcelain	60	0	0	0	0
	C. sporogenes	6/27/03 (1 hour)	Dacron Sutures	60	0	0	0	0
			Porcelain	60	0	1	0	1
		8/14/03 (1 hour)	Porcelain	60	0	1	0	1
		8/14/03 (2 hour)	Porcelain	60	0	0	0	0
Oxine, Lot No. 0305-025	*B. subtilis*	6/27/03 (1 hour)	Dacron Sutures	60	0	0	0	0
			Porcelain	60	0	0	0	0
	C. sporogenes	6/27/03 (1 hour)	Dacron Sutures	60	0	0	0	0
			Porcelain	60	0	2	0	2
		8/14/03 (1 hour)	Porcelain	60	0	1	0	1
		8/14/03 (2 hour)	Porcelain	60	0	0	0	0

1° = Primary subculture 2° = Secondary subculture HS = After Heat Shock Treatment

The subcultures of positive broths (tubes showing growth) demonstrated pure cultures of the test organism.

AMENDMENT TO GLP FINAL REPORT

Modification(s) to Report:

Per Sponsor's request, the final report was amended to elaborate on the study conclusions for *Clostridium sporogenes* testing at the 1 hour exposure time. The porcelain penicylinder and Dacron suture conclusions will be listed separately.

Study Director

10/31/03
Date