GREAT SHIPS INITIATIVE
BENCH-SCALE TEST FINDINGS
Technical Report - Public

SeaKleen 80® (Menadione)

March 17, 2009

Compiled By: **Signed:** Matt TenEyck and Nicole Mays
Title: GSI Lead Investigator for Bench-Scale Studies and GSI Senior QAQC Officer

Reviewed and Approved By: **Signed:** Allegra Cangelosi
Title: GSI Principal Investigator

Opportunity for Final Review Given to Applicant **Date:** February 19, 2009
Publication on GSI Website: **Date:** March 17, 2009

PAGE LEFT BLANK INTENTIONALLY

ABSTRACT

The Great Ships Initiative (GSI), an innovative collaboration whose objective is to end the problem of ship-mediated invasive species in the Great Lakes-St. Lawrence Seaway System, conducts bench-scale research to aid developers of innovative technologies which could have application as ballast treatment systems. This report describes 2008 findings from bench-scale evaluations of a proposed ballast treatment produced by Vitamar, LLC called SeaKleen 80®. This treatment method is intended as a routine ballast treatment in doses up to 3 mg/L as menadione. GSI tested the formulation to assist range-finding for effective doses, determination of the rates of chemical degradation, and the potential for residual toxicity. Please see www.greatshipsinitiative.org for more information about GSI's bench-scale testing program.

GSI's bench testing of SeaKleen 80® found the compound to be highly effective on zooplankton—juvenile daphnids (*Daphnia magna*), adult rotifers (*Brachionus calyciflorus*), and rotifer resting eggs (*B. calyciflorus*)—with less than 1 % survival within 48 hours of treatment at a dose 0.4 mg/L as menadione. The substance was only minimally effective on the bacteria and the green algae species tested under the same conditions. SeaKleen 80® degraded readily under high light/high transmittance conditions. However, if either light or light transmittance of the water was attenuated, degradation was slow and incomplete.

TABLE OF CONTENTS

Introduction **5**

Background **5**

The Great Ships Initiative 5
GSI Bench Tests 6

About SeaKleen 80® **7**

Methods **8**

Bench-Scale Dose Effectiveness Test Methods for SeaKleen 80® 9
Zooplankton and Algae *9*
Bacteria *10*
Bench-Scale Chemical Degradation Test Methods for SeaKleen 80® 11
Bench-Scale Residual Toxicity Test Methods for SeaKleen 80® 12

Findings **14**

Bench-Scale Dose Effectiveness Test Findings for SeaKleen 80® 14
Zooplankton and Algae *14*
Bacteria *17*
Bench-Scale Chemical Degradation Test Findings for SeaKleen 80® 19
Bench-Scale Residual Toxicity Test Findings for SeaKleen 80® 22

Quality Assurance/Quality Control **22**

Conclusion **23**

INTRODUCTION

This technical report presents quantitative and measured findings from GSI bench-scale evaluations of SeaKleen 80®. SeaKleen 80®, produced by Vitamar, LLC, is under development as a method of routine ballast treatment in doses up to 3 mg/L metered in at the time of ballast uptake. GSI undertook these bench-scale tests during summer 2008 at the Lake Superior Research Institute's Aquatic Toxicity Laboratory (ATL) of the University of Wisconsin-Superior in Superior, Wisconsin. Tests included range-finding evaluations of dose effectiveness, chemical degradation and residual toxicity. A summary of these findings for non-scientific audiences can be accessed at http://www.nemw.org/GSI/GSI-BS-P-FS-SeaKleen.pdf. Please note that GSI's bench-scale tests do not by themselves provide adequate information to assess a prospective ballast treatment's ability to meet a particular discharge standard or to achieve environmental soundness under shipboard application. Instead these tests provide initial insights into possible strengths and weaknesses of the treatment—information that can be used by developers to better design an effective system and/or to move to the next stage of treatment evaluation.

BACKGROUND

The Great Ships Initiative

The Great Ships Initiative (GSI)[1] is a collaborative effort whose objective is to end the problem of ship-mediated invasive species in the Great Lakes-St. Lawrence Seaway System. To that end, the GSI established sophisticated independent third party ballast treatment evaluation capabilities at three scales—bench, land-based, and shipboard. Each scale is dedicated to addressing specific evaluation objectives:

- GSI Bench-Scale Tests
  - Range finding for effective doses under a range of ambient conditions;
  - Chemical degradation over time under a range of ambient conditions;
  - Detection of any residual toxicity under a range of ambient conditions; and
  - Confirmation of treatment process.

- GSI Land-Based Tests
  - Detection of scale-up, mechanical operation issues;
  - Effectiveness of a dose with respect to the full range of ambient organisms; and
  - Detection of any whole water effluent toxicity.

- GSI Shipboard Tests
  - Confirmation of biological and operational performance as expected in the ship environment; and
  - Confirmation of performance as expected under a broad range of ambient conditions.

1 www.greatshipsinitiative.org

Developers of ballast water treatment systems apply for GSI research services online, and awards are offered based on an objective review process, regardless of the state of development of the proposed treatment. GSI status testing will be performed at the scale appropriate to the treatment state of development, with the goal of helping meritorious ballast treatment systems to progress as rapidly as possible to an approval-ready and market-ready condition.

To assure relevancy of test output, GSI test protocols are as consistent with the International Maritime Organization (IMO) Convention and federal requirements as practicable. In particular, bench testing directly supports IMO G9 evaluations, and land-based testing directly supports IMO G8 evaluations.

GSI tests are third party assessments. They are completely independent of any vested interest in outcomes. The GSI tests are supported by general project funds which derive from federal and state agency grants and contributions, Great Lakes port contributions, and in-kind contributions by the local government and universities. None of these funds come to the GSI with any strings (other than public disclosure).

Ms. Allegra Cangelosi of the Northeast-Midwest Institute is the Principal Investigator and Manager of the GSI. Researchers from the University of Wisconsin-Superior's Lake Superior Research Institute, and the University of Minnesota-Duluth's Natural Resources Research Institute, among others, provide critical scientific and technical expertise and implementation services to GSI's biological research activities, and the GSI generally. A GSI Advisory Committee comprising top-level officials of key stakeholder groups helps steer the GSI providing crucial assistance in making GSI award decisions and fund-raising. The GSI Advisory Committee includes elected leadership, environmental organizations, port directors and federal officials from the United States and Canada, and industry representatives. The American Great Lakes Ports Association advises the project, assuring that the GSI is meeting the needs of the maritime industry; and coordinating maritime industry and supply chain outreach.

The largest contributor of GSI operating funds is the United States Department of Transportation, including its Maritime Administration, and the Saint Lawrence Seaway Development Organization. GSI also receives significant funds and in-kind contributions from the National Oceanic and Atmospheric Administration, the Canadian St. Lawrence Seaway Management Corporation, the City of Superior, Wisconsin, and approximately ten U.S. and Canadian ports in the Great Lakes.

GSI Bench Tests

GSI bench-scale tests take place year-round at the Lake Superior Research Institute's Aquatic Toxicity Laboratory (ATL) of the University of Wisconsin-Superior in Superior, Wisconsin. The ATL is amply equipped with staff expertise and resources to conduct the tests, and has a long history of successfully undertaking similar tests.

The overarching goals of GSI bench testing are to explore dose-effectiveness, chemical degradation, residual toxicity, and sensitivity to challenge conditions of a proposed treatment method about which little is known. To that end, the tests are "range-finding" missions, to determine the optimal treatment dose/intensity that would maximize effectiveness and minimize residual toxicity.

Findings help treatment developers better design an effective system and/or to move to the next stage of treatment evaluation. The tests are also a form of trouble-shooting to encounter possible problems with the proposed treatment in advance of more extensive and larger scale tests.

GSI bench-scale dose effectiveness tests help estimate the range of concentrations of an active substance that is harmful to a variety of robust freshwater zooplankton and algae known to be relatively resilient to stressors. Dose effectiveness test results are expressed as percent survival, percent mortality, and percent hatch. They are also expressed in terms of a series of absolute quantifications: LC_{99}, i.e., the experimentally derived concentration of an active substance estimated to kill 99 percent of the test population following 24 or 48 hours of continuous exposure; No Observed Effect Concentration (NOEC), i.e., the highest concentration of an active substance shown to have no significantly adverse effect on the test population compared to controls; and Lowest Observed Effect Concentration (LOEC), i.e., the lowest concentration of an active substance known to have a significantly adverse effect on the test population compared to controls.

GSI bench-scale chemical degradation tests determine the effect that various water quality or environmental parameters may have on the rate of chemical degradation of a treatment system involving active substances. No organisms are used in association with these analytical assays; instead test solutions are analyzed for their concentration of active substance (or active component of the substance). Test results are expressed as the percent change in active ingredient concentration.

GSI bench-scale residual toxicity tests help estimate the effect that treated water (following a degradation period) may have on non-target organisms in the receiving system. These tests results are expressed in a manner similar to those for dose effectiveness assays. The principal difference between these tests and dose effectiveness tests is that the concentration of the active substance is adjusted to be consistent with potential discharge levels, and the tests are performed on sensitive organisms rather than robust species.

ABOUT SEAKLEEN 80®

Manufactured by Vitamar, LLC, SeaKleen 80® is described in the material safety data sheet (MSDS) as a formulation that consists of a minimum of 80 % menadione (vitamin K_3) which is the active ingredient. The other 20 % are inert ingredients and therefore not listed on the MSDS.

SeaKleen 80® is proposed for use at concentrations of up to 3 mg/L as menadione and metered into ballast water as it is pumped into the ballast tanks. No further treatment is proposed. SeaKleen 80® is supplied as a granular material and must be mixed with water prior to dosing.

SeaKleen 80® has been tested by other groups at bench-scale, dockside and at full-scale aboard ship. SeaKleen 80® is currently in the process of registration under the U.S. Environmental Protection Agency's requirements for a marine biocide. The SeaKleen 80® chemical, menadione or vitamin K_3, has already been approved for several other applications at higher doses, including as an additive in fish food for catfish farms.

METHODS

GSI bench-scale standard operating protocols (SOPs) are grounded in published standard methods by the United States Environmental Protection Agency (EPA)and ASTM, and modified to reflect ballast treatment circumstances. They are also consistent with international and domestic guidelines where they exist. All SOPs are peer-reviewed prior to acceptance and are subject to periodic review and revision to assure that the most up to date approaches are employed. Table 1 outlines the GSI bench-scale SOPs utilized for SeaKleen 80® tests. In addition, all GSI bench-scale research activities comply strictly with a detailed Quality Assurance Project Plan (QAPP) which is consistent in format, detail and stringency with EPA requirements.

Table 1. GSI Bench-Scale Standard Operating Procedures (SOPs).

SOP Category	Subcategory	SOP Title	SOP Code
General	Sample Custody	Procedure for Sample Custody	GSI/SOP/G/RA/SC/1
General	Sample Custody	Procedure for Labeling Bench-Scale Samples	GSI/SOP/G/RA/SC/3
Research Activities	Culturing	Procedure for Culturing the Freshwater Rotifer *Branchionus calyciflorus*	GSI/SOP/BS/RA/C/1
Research Activities	Culturing	Procedure for Culturing the Freshwater Amphipod *Hyalella azteca*	GSI/SOP/BS/RA/C/2
Research Activities	Culturing	Procedure for Culturing the Cladocerans *Daphnia magna* and *Ceriodaphnia dubia*	GSI/SOP/BS/RA/C/3
Research Activities	Chemical Degradation	Procedure for Determining Chemical Degradation of a Primary Treatment or Its By-Products	GSI/SOP/BS/RA/CD/1
Research Activities	Dose Effectiveness	Procedure for Assessing Dose-Effectiveness of a Ballast Water Treatment System Using a Copepod	GSI/SOP/BS/RA/DE/1
Research Activities	Dose Effectiveness	Procedure for Assessing Dose-Effectiveness of a Ballast Water Treatment System Using the Daphnid *Daphia magna*	GSI/SOP/BS/RA/DE/2
Research Activities	Dose Effectiveness	Procedure for Assessing Dose-Effectiveness of a Ballast Water Treatment System Using the Freshwater Rotifer *Branchionus calyciflorus*	GSI/SOP/BS/RA/DE/3
Research Activities	Dose Effectiveness	Procedure for Assessing Dose-Effectiveness of a Ballast Water Treatment System Using Resting Eggs of the Freshwater Rotifer *Branchionus calyciflorus*	GSI/SOP/BS/RA/DE/4
Research Activities	Dose Effectiveness	Procedure for Assessing Dose-Effectiveness of a Ballast Water Treatment System Using the *Selenastrum capricornutum*	GSI/SOP/BS/RA/DE/5
Research Activities	Dose Effectiveness	Procedure For Quantifying Heterotrophic Plate Counts Using IDEXX's SimPlate for HPC Method	GSI/SOP/BS/RA/MA/1
Research Activities	Dose Effectiveness	Procedure for Assessing Antimicrobial Activity Using Time-Kill Method	GSI/SOP/BS/RA/MA/2
Research Activities	Dose Effectiveness	Procedure for the Detection and Enumeration of *Enterococcus* using Enterolert™	GSI/SOP/BS/RA/MA/3
Research Activities	Dose Effectiveness	Procedure for the Detection and Enumeration of *E. coli* Using IDEXX'sColilert	GSI/SOP/BS/RA/MA/4
Research Activities	Residual Toxicity	Procedure for Measuring Residual Toxicity Using *Ceriodaphnia dubia*	GSI/SOP/BS/RA/RT/1
Research Activities	Residual Toxicity	Procedure for Measuring Residual Toxicity Using Fathead Minnows (*Pimephales promelas*)	GSI/SOP/BS/RA/RT/2

Research Activities	Residual Toxicity	Procedure for Measuring Residual Toxicity Using the Alga *Selenastrum capricornutum*	GSI/SOP/BS/RA/RT/3
Research Activities	Residual Toxicity	Procedure for Measuring Residual Toxicity Using the Freshwater Rotifer *Branchionus calyciflorus*	GSI/SOP/BS/RA/RT/5
Research Activities	Chemistry	Procedure for Measuring Organic Compounds Using High Performance Liquid Chromatography (HPLC)	GSI/SOP/BS/RA/C/5

Bench-Scale Dose Effectiveness Test Methods for SeaKleen 80®

Zooplankton and Algae

GSI's dose effectiveness tests on SeaKleen 80® examined the range of concentrations of menadione that is harmful to juvenile (less than 24 hours old) daphnids, adult copepods, newly hatched rotifers, rotifer resting eggs, and green alga. The tests evaluated five exposure concentrations and a control at a nominal temperature of 25.0 °C ± 1.0 °C. Filtered Duluth-Superior Harbor water (FHW) was used for the test water, and solutions were not renewed. All exposures with the exception of the rotifer adults and rotifer resting eggs took place over 48 hours, involved 50 mL of solution in a 300 mL beaker, and included three replicates. Tests on rotifer adults and rotifer resting eggs involved four replicates and 2.0 mL of exposure solution. Adult rotifers were only exposed for 24 hours, and instead of complete darkness, rotifer resting eggs were exposed to continuous light to stimulate hatching. GSI researchers made daily observations on mortality, and measured water quality parameters including temperature, dissolved oxygen, pH, conductivity, alkalinity and hardness. Table 2 arrays the types and numbers of organisms analyzed, the exposure concentrations used, and the number of replicates per dose effectiveness test.

Table 2. Numbers and Types of Organisms, and Average Exposure Concentrations of Menadione Analyzed in GSI Dose-Effectiveness Tests of SeaKleen 80® on Zooplankton and Alga.

Major Taxonomic Group	Type	Species	Measured Exposure Concentrations	No. of Organisms per Exposure /Control	No. of Replicates per Exposure /Control	Total Number of Organisms
Zooplankton	Juvenile daphnids	*Daphnia magna*	0.00 (control), 0.09, 0.19, 0.40, 0.84 and 1.76 mg/L	10	3	6 x 10 x 3 = 180
Zooplankton	Adult copepods	*Eucyclops* spp.	0.00 (control), 0.02, 0.04, 0.09, 0.19 and 0.39 mg/L	10	3	6 x 10 x 3 = 180
Zooplankton	Adult rotifers	*Brachionus calyciflorus*	0.00 (control), 0.06, 0.13, 0.25, 0.50 and 1.0 mg/L	5	4	6 x 5 x 4 = 120
Zooplankton	Rotifer resting eggs	*Brachionus calyciflorus*	0.00 (control), 0.28, 0.58, 1.17, 2.42 and 4.86 mg/L	20	4	6 x 20 x 4 = 480
Algae (test 1)	Green alga	*Selenastrum spp.*	0.00 (control), 0.32, 0.97, 2.53, 5.53, 11.63 mg/L	200,000 cells/mL	3	6 x 200,000 x 3 = 3,600,000
Algae (test 2)	Green alga	*Selenastrum spp.*	0.00 (control), 5.7, 11.9, 24.0, 47.7, 81.5 mg/L	200,000 cells/mL	3	6 x 200,000 x 3 = 3,600,000

Bacteria

Dose effectiveness tests were also conducted to determine the concentration of menadione most harmful to a range of ambient bacteria contained in FHW including total coliforms, *Escherichia coli*, *Enterococci* and heterotrophic bacteria. The analysis was performed with Colilert® and Enterolert ™ tests using Quanti-Tray/2000 and the Quanti-Tray sealer from IDEXX laboratories. The Colilert® test has detection limits for total coliforms and *E. coli* of 1 colony forming unit (cfu) per 100 mL, and the Enterolert ™ test can detect *Enterococci* at 1 cfu per 100 mL. Both tests use Defined Substrate Technology® (DST) in which the bacteria metabolize the enzymes in the specific media causing the sample to fluoresce. Heterotrophic plate count (HPC) bacteria in FHW were analyzed using SimPlate for HPC medium which uses IDEXX's Multiple Enzyme Technology™ (MET™). All tests were performed using three exposure concentrations (20mg/L, 40 mg/L and 80mg/L), with results expressed as Most Probable Number (MPN) per 100 mL.

Bench-Scale Chemical Degradation Test Methods for SeaKleen 80®

The GSI chemical degradation tests assessed the effect of various water quality or environmental parameters on the rate of SeaKleen 80® degradation. The parameters included four types of water quality:

1. Laboratory water (LW) - City of Superior tap water that has been passed through an activated carbon column to remove chlorine;
2. Salt water (SW) - Prepared using deionized water and a commercial salt mix such that the salinity was 32.0 ppt,
3. Duluth-Superior Harbor water (HW); and
4. FHW.

Two different ambient temperatures (15 °C and 25 °C), and two different light regimes (laboratory light, i.e. 255 lumens/ m^2, 1.6 µW/cm^2 UVA, and 0.08 µW/cm^2 UVB on a 16:8 light:dark cycle, and complete darkness) were also tested.

The tests were performed using three menadione exposure concentrations (0.5 mg/L, 5 mg/L and 50 mg/L), and consisted of 50 mL of solution in a 300 mL beaker. There were three replicates per exposure concentration and all beakers were covered with a glass plate to prevent evaporation before being introduced to the exposure conditions. Following 96 hours of exposure, the solutions were analyzed for the concentration of menadione using High Performance Liquid Chromatography (HPLC). Test results are expressed as the percent change in menadione concentration.

Following this set of tests, a second set of chemical degradation experiments on SeaKleen 80® were performed. These tests involved addition of a third light regime to better simulate natural conditions. The light level selected was based on mid-depth (approximately 3.0 meters) in Ashland Harbor, Wisconsin. Measurements integrated the effects of weather (sunny and cloudy days) and time of day the sample was collected during one day in each month of June and August, 2005. The level is described as follows: 730 lumens/m^2, 89 µW/cm^2 UVA and 4.8 µW/cm^2 UVB. Exposures were only carried out using FHW, though the rate of degradation was measured on five different nominal concentrations: 3.0 mg/L, 5.0 mg/L, 10.0 mg/L, 20.0 mg/L, and 50.0 mg/L of menadione. The solutions were analyzed for the concentration of menadione using HPLC at 0, 2, 4, 6, 8, 10, 12, 24, 34, 48, 72 and 96 hours.

Table 3 arrays the test conditions and exposure concentrations used for both sets of chemical degradation tests.

Table 3. Test Conditions and Exposure Concentrations (as Menadione) used for SeaKleen 80® Chemical Degradation Tests.

Test Number	Water Type	Ambient Temperature	Light Regime	Nominal Exposure Concentrations	No. of Replicates per Exposure /Control
Test 1	LW	15 and 25 °C	Laboratory light on a 16:8 light:dark cycle and complete darkness	0.5, 5, and 50 mg/L)	3
Test 1	SW	15 and 25 °C	Laboratory light on a 16:8 light:dark cycle and complete darkness	0.5, 5, and 50 mg/L	3
Test 1	HW	15 and 25 °C	Laboratory light on a 16:8 light:dark cycle and complete darkness	0.5, 5, and 50 mg/L	3
Test 1	FHW	15 and 25 °C	Laboratory light on a 16:8 light:dark cycle and complete darkness	0.5, 5, and 50 mg/L	3
Test 2	FHW	25 °C	730 lumens/m2, 89 µW/cm2 UVA and 4.8 µW/cm2 UVB continuous light	3.0, 5.0,10.0, 20.0, and 50.0 mg/L	3

Bench-Scale Residual Toxicity Test Methods for SeaKleen 80®

The proposed treatment is designed to degrade primarily through photodegradation, a process which will not commence until after the treated ballast water is discharged to a receiving system. As a result potential residual toxicity issues include:

- Residual menadione toxicity in the discharge, post dark-retention in the ballast tank;
- Residual menadione toxicity post-discharge due to incomplete degradation in the receiving system; and
- Residual toxicity post discharge due to toxic menadione degradation products in the receiving system.

It was not possible to fully investigate all of these scenarios as residual toxicity tests. Fortunately, the levels of toxicity that may be associated with undegraded menadione in the discharge to a receiving system, and the toxicity of menadione which may remain in the receiving system post

discharge if photodegradation is inhibited by low transmittance properties of the receiving water, such as in HW, were explored relative to robust organisms in the dose effectiveness tests described above.

Residual toxicity tests were therefore performed to explore toxicity that may be associated specifically with degradation by-products of menadione. In these tests, identical sets of organisms were exposed to 1) water freshly treated with menadione at a given concentration, and 2) water treated with a much higher level of menadione that was allowed to degrade under high light to the same level over a defined retention period. In the first case, few degradation products can be expected to be present, while in the second case, degradation products would be present. The relative toxicity of the two treatments were compared to see if water containing SeaKleen 80® and its degradation products was more toxic than water containing SeaKleen 80® at the same concentration and relatively little in the way of degradation products.

Sensitive fresh water surrogate organisms including the daphnid (*Ceriodaphnia dubia)* that were <24 hours old, fathead minnows (*Pimephales promelas*) <24 hours old, and newly hatched rotifers (*Brachionus calyciflorus*) were added to both types of treated water and their survival compared. Table 4 arrays the test conditions and number of organisms used in the residual toxicity experiments.

One set of organisms, with the exception of the rotifer adults, was added to beakers containing FHW (50.0 mL of solution in a 300.0 mL beaker) immediately after the addition of menadione, at five different concentrations and to a control. The samples were subjected to 16:8 light:dark cycle (the same light composition and intensity as the higher light scenario as in the chemical degradation methods above) for 48.0 hours, The rotifer adult exposure consisted of four replicates, with 2.0 mL of solution, and was 24.0 hours in duration.

The second set of organisms was added to beakers containing a stock solution of filtered FHW containing partially degraded SeaKleen. A nominal concentration of 3.0 mg/L menadione was added to FHW and retained in complete darkness for 24.0 hours. After dark retention, the stock solution was then placed in the higher light condition as in the chemical degradation experiments. This solution was allowed to degrade 24 hours at which time organisms were added. The tests were performed at temperatures of 25.0 °C ± 1.0 °C and the test water was not renewed. All exposures were conducted in a 16:8 light:dark cycle, were 48.0 hours in length, consisted of 50.0 mL of solution in a 300.0 mL beaker, and consisted of three replicates with the exception of the rotifer adults. The rotifer adult exposure consisted of four replicates, with 2.0 mL of solution, and was 24.0 hours in duration.

Table 4. Numbers and Types of Organisms, used in GSI Residual Toxicity Tests of SeaKleen 80®.

Major Taxonomic Group	Type	Species	No. of Organisms per Exposure /Control	No. of Replicates per Exposure /Control	Total Number of Organisms
Zooplankton	Adult daphnids	*Ceriodaphnia dubia*	10	3	3 x 10 x 4 = 90
Fish	Juvenile fathead minnows	*Pimephales promelas*	15	3	3 x 15 x 3 = 135
Zooplankton	Newly hatched rotifers	*Brachionus calyciflorus*	5	4	3 x 5 x 4 = 60

FINDINGS

Bench-Scale Dose Effectiveness Test Findings for SeaKleen 80®

Zooplankton and Algae

All zooplankton tested, including rotifer resting eggs, succumbed at a rate of 97 % or greater when exposed to menadione concentrations within the proposed dose range (i.e. up to 3 mg/L as menadione). Adult daphnia and adult rotifers were the most vulnerable to treatment using SeaKleen 80®, while the green algae species tested was the most resistant. Table 5 indicates those species that had less than 1 % survival within 24 hours and 48 hours, respectively, of treatment. Table 6 details LC_{99}, NOEC and LOEC concentrations of menadione associated with the test organisms as determined by Dunnett's Test or Steel's Many-One Rank Test (both at $p<0.05$) using ToxCalc 5.0 software. Tables 7-11 provide more detailed test results.

Table 5. Species With Less Than 1% Survival (noted as ✓) Within 24 and 48 Hours of Treatment with SeaKleen 80® at up to 3 mg/L as Menadione.

Major Taxonomic Group	Species	Less Than 1 % Survival	
		24 Hours	48 Hours
Algae§	*Selenastrum sp.*		
Zooplankton	*Daphnia magna*	✓	**Not applicable**
Zooplankton	*Eucyclops sp.*		
Zooplankton	*Branchionus calyciflorus*	✓	**Not applicable**
Zooplankton	*B. calyciflorus cysts**	**Not measured**	✓

*Resting egg survival is scored as number hatched.
§ Highest algae test concentration was 81.5 mg/L

Table 6. LC_{99}, NOEC and LOEC Concentrations of SeaKleen 80® (as Menadione) Associated with Test Organisms Used To Estimate Dose Effectiveness Following 24 or 48 Hour Exposures.

Test Organism	LC_{99}	NOEC	LOEC
Juvenile daphnids (*Daphnia magna*)	0.46	0.09	0.19
Adult Copepods (*Eucyclops spp*.)	0.43	0.02	0.04
Adult Rotifers (*Brachionus calyciflorus*)	0.45	0.09	0.20
Algae (*Selenastrum spp*.)	*NC	*NC	*NC

***** NC = (Not calculable) no significant mortality was observed in any treatments

Table 7. Average (n=3) Percent Survival (+/- Standard Error) of *Daphnia magna* Juveniles Exposed to SeaKleen 80® (as Menadione) in Filtered Duluth-Superior Harbor Water (FHW)

Initial Measured Concentration (mg/L)	Measured Concentration at 48 Hours (mg/L)	Percent Survival Following 48 Hours Exposure (%)
0.00	0.00	100 +/- 0
0.11	0.08	100 +/- 0
0.22	0.16	73 +/- 12
0.45	0.35	3 +/- 6
0.93	0.75	0 +/- 0
1.90	1.62	0 +/- 0

Table 8. Average (n=3) Percent Survival (+/- Standard Error) of Adult Copepods *(Eucyclops spp.)*

Exposed to SeaKleen 80® (as Menadione) in Filtered Duluth-Superior Harbor Water (FHW)

Initial Measured Concentration (mg/L)	Measured Concentration at 48 Hours (mg/L)	Percent Survival Following 48 Hours Exposure (%)
0.00	0.00	96 +/- 3
0.03	0.01	83 +/- 9
0.06	0.03	73 +/- 9
0.11	0.07	20 +/- 12
0.22	0.16	3 +/- 3
0.45	0.33	3 +/- 3

Table 9. Average (n=4) Percent Survival (+/- Standard Error) of Adult Rotifers *(B. calyciflorus)* Exposed to SeaKleen 80® (as Menadione) in Filtered Duluth-Superior Harbor Water (FHW)

Initial Measured Concentration (mg/L)	Measured Concentration at 24 Hours (mg/L)	Percent Survival Following 24 Hours Exposure (%)
0.0	0.00	100 +/- 0
0.06	0.04	100 +/- 0
0.13	0.08	85 +/- 5
0.20	0.18	40 +/- 8
0.45	0.34	0 +/- 0
0.93	0.72	0 +/- 0

Table 10. Average (n=4) Percent Hatch (+/- Standard Error) of *B. calyciflorus* Resting Eggs Exposed to SeaKleen 80® (as Menadione) in Filtered Duluth-Superior Harbor Water (FHW)

Initial Measured Concentration (mg/L)	Measured Concentration at 48 Hours (mg/L)	Percent Hatched Following 48 Hours Exposure (%)
0.0	0.0	30.0 +/- 5
0.28	0.06	17.5 +/- 5
0.58	0.10	7.5 +/- 3
1.17	0.30	0 +/- 0
2.42	0.57	0 +/- 0
4.86	0.97	0 +/- 0

Table 11. Average (n=3) Percent Survival (+/- Standard Error) of *Selenastrum spp.* Exposed to SeaKleen 80® (as Menadione) in Laboratory Water (LW)

Initial Measured Concentration (mg/L)	Measured Concentration at 48 Hours (mg/L)	Percent Survival Following 48 Hours Exposure (%)
0.0 (control – test 1)	0.0	100 +/- 0.3
0.0 (control – test 2)	0.0	100 +/- 0.3
0.48 (test 1)	0.32	99 +/- 0.6
1.33 (test 1)	0.97	100 +/- 0.4
2.68 (test 1)	2.53	99 +/- 0.1
5.79 (test 1)	5.53	99 +/- 0.7
5.89 (test 2)	5.42	99 +/- 0.2
11.70 (test 1)	11.63	91 +/- 1.5
12.20 (test 2)	11.63	99 +/- 0.5
24.5 (test 2)	23.47	91 +/- 1.1
49.2 (test 2)	46.13	99 +/- 0.5
80.5 (test 2)	80.0	99 +/- 0.1

Bacteria

Menadione concentrations measured in the exposure solutions at the start and the end of the test period for the bacteria tests are provided in table 16. The Colilert® and Enterolert ™ tests showed a decrease in the number of total coliforms, *E. coli*, and *Enterococci* in the control, due to low starting densities, and all exposure concentrations during the 48 hour exposure period (tables 12-14). Higher concentrations of menadione resulted in lower numbers of the specific indicator bacteria relative to controls. For example, following 48 hours exposure, total coliforms decreased by 58 % in the control, by 86 % in the 20 mg/L concentration, and by 98 % in the 40 mg/L concentration. Both total coliforms and *E. coli* decreased by 100 % in the 80 mg/L concentration within 24 hours and enterococcus decreased by 100 % within 48 hours. In contrast, heterotrophic bacteria counts increased in all samples, and menadione, even at the highest concentration, had no effect (table 15).

Table 12. Most Probable Number (MPN) of Total Coliforms per 100 mL (+/- Standard Deviation) in Control and Three Concentrations of SeaKleen 80® (as Menadione) Following 24 and 48 Hours of Exposure Time.

Sample	Initial MPN of Total Coliforms per 100 mL	MPN of Total Coliforms per 100 mL at 24 Hours	MPN of Total Coliforms per 100 mL at 48 Hours
Control	431 +/- 75	301 +/- 39	179 +/- 22
20 mg/L		82 +/- 12	60 +/- 9
40 mg/L		3.8 +/- 3.1	8.3 +/- 2.4
80 mg/L		0 +/- 0	0 +/- 0

Table 13. Most Probable Number (MPN) of *E. coli* per 100 mL (+/- Standard Deviation) in Control and Three Concentrations of SeaKleen 80® (as Menadione) Following 24 and 48 Hours of Exposure Time.

Sample	Initial MPN of Total *E. coli* per100 mL	MPN of Total *E. coli* per 100 mL at 24 Hours	MPN of Total *E. coli* per 100 mL at 48 Hours
Control	31 +/- 5.7	17 +/- 2.1	2.7 +/- 2.2
20 mg/L		8.2 +/- 2.5	3.4 +/- 1.2
40 mg/L		1.7 +/- 1.2	1.2 +/- 0.6
80 mg/L		0 +/- 0	0 +/- 0

Table 14. Most Probable Number (MPN) of *Enterococci* per 100 mL (+/- Standard Deviation) in Control and Three Concentrations of SeaKleen 80® (as Menadione) Following 24 and 48 Hours of Exposure Time.

Sample	Initial MPN of Total *Enterococci* per 100 mL	MPN of Total *Enterococci* per 100 mL at 24 Hours	MPN of Total *Enterococci* per 100 mL at 48 Hours
Control	16.3 +/- 2.9	13 +/- 2.7	5.2 +/- 1.1
20 mg/L		0.67 +/- 0.58	0.67 +/- 1.2
40 mg/L		6.7 +/- 5.8	5 +/- 0
80 mg/L		10 +/- 0	0 +/- 0

Table 15. Most Probable Number (MPN) of Heterotrophic bacteria per 1 mL (+/- Standard Deviation) in Control and Three Concentrations of SeaKleen 80® (as Menadione) Following 24 and 48 Hours of Exposure Time.

Sample	Initial MPN of Total Heterotrophs per 100 mL	MPN of Total Heterotrophs per 100 mL at 24 Hours	MPN of Total Heterotrophs per 100 mL at 48 Hours
Control	1.8E+03 +/- 6.3E+02	>4.1E+03* +/- 2.9E+03	>7.3E+03 +/- 2.8E+03
20 mg/L		>7.4E+03 +/- NC	>7.4E+03 +/- NC
40 mg/L		>7.4E+03 +/- NC	>7.4E+03 +/- NC
80 mg/L		>7.4E+03 +/- NC	>7.4E+03 +/- NC

*One of the sample replicates was > 7380. This value was used to calculate average to get a minimum average value.
NC = Not calculable. All replicates were greater than range of the plate.

Table 16. Concentration of Menadione at the Beginning and End of the Exposure Tests for Bacteria.

Nominal Concentration (mg/L)	Measured Concentration (mg/L)		
	0 Hours	48 Hours	Mean
0	<0.01	<0.01	<0.01
20	19.7	19.4	19.6
40	40.0	39.8	39.9
80	71.9	78.1	75.0

Bench-Scale Chemical Degradation Test Findings for SeaKleen 80®

Results of the first set of chemical degradation tests are described in table 17 and indicate that light influenced SeaKleen 80® degradation, while temperature did not have a major influence. Average percent change in the 5.0 mg/L concentrations of SeaKleen 80® following 96 hours of exposure to light were -55 % for LW, -26 % for HW, and -28 % for FHW respectively. The lower reductions in HW and FHW compared to LW are likely the result of the water containing high concentrations of transmittance-limiting dissolved organic carbon (DOC). Only slight reductions (0.6 – 6 %) were noticeable in the same water types when exposed to complete darkness. In addition, SW had an average change of 22 % when exposed to light and 12 % when exposed to dark, possibly resulting from delayed solubility or concentration due to evaporation.

Table 17. Average Percent Change of Menadione Following 96 Hours Exposure to Various Water Quality Parameters, Including Water Quality, Light[2] and Temperature.

Initial Concentration (mg/L)	Parameters	Water Type			
		LW	SW	HW	FHW
0.5	Light at 15 °C	-33	11	-23	-14
	Light at 25 °C	-58	7	-29	-30
	Dark at 15 °C	8	13	-7	-2
	Dark at 25 °C	14	12	-14	-10
5	Light at 15 °C	-54	31	-26	-21
	Light at 25 °C	-55	22	-26	-28
	Dark at 15 °C	-2	18	-2	-0.9
	Dark at 25 °C	0.7	11	-6	-5
50	Light at 15 °C	-50	16	26	-29
	Light at 25 °C	-45	12	-24	-29
	Dark at 15 °C	-4	5	-0.5	-2
	Dark at 25 °C	-5	10	-2	-4

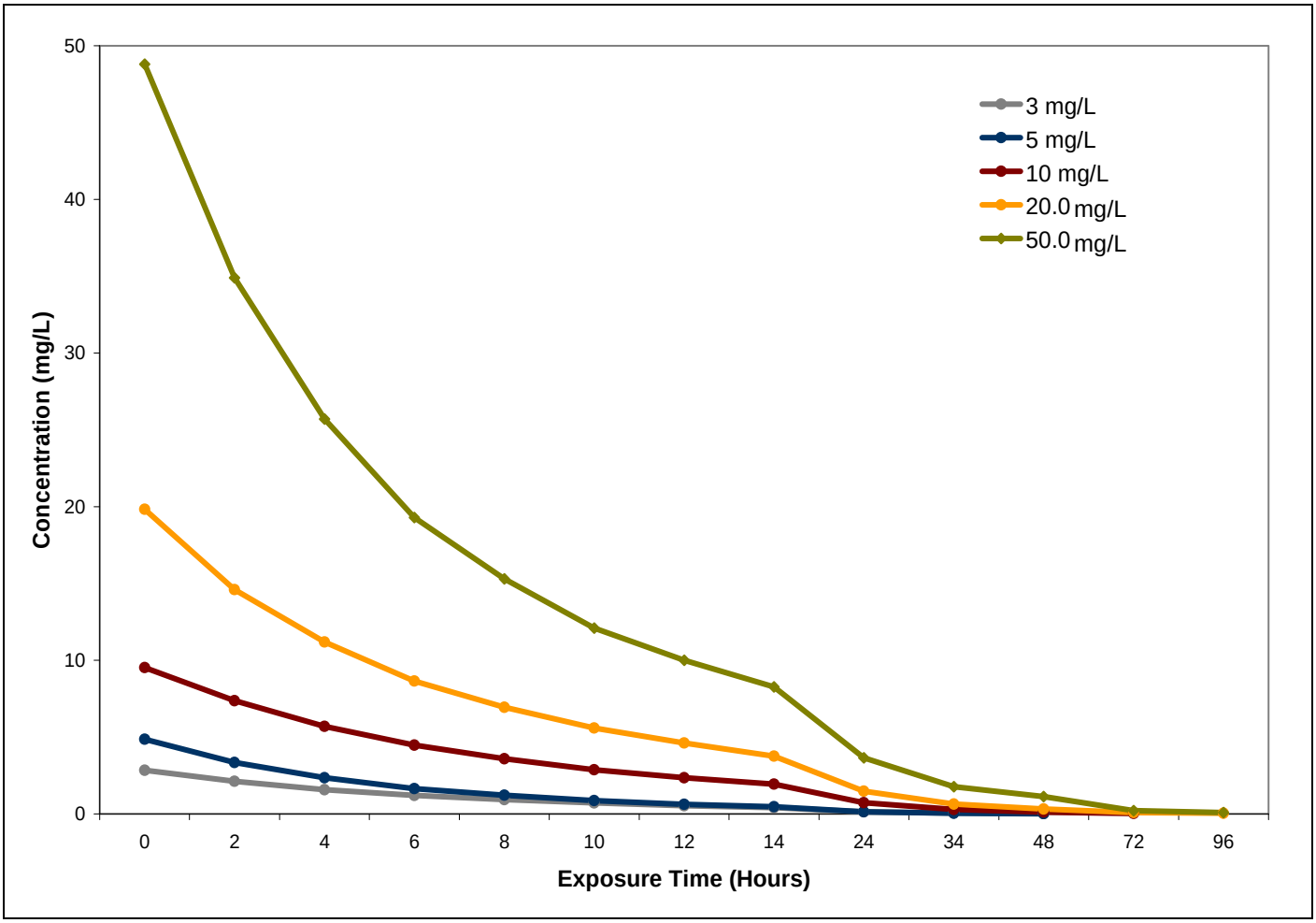
Results of the second set of GSI chemical degradation tests on SeaKleen 80® are described in table 18 and indicate that the lower concentrations (i.e., 3.0 and 5.0 mg/L of SeaKleen 80® as menadione degraded to below detection following 48 hours exposure to the higher light regime, while the higher concentrations (i.e., 20 and 50 mg/L) had some residual concentration still remaining following 96 hours exposure. All concentrations however, appeared to degrade exponentially with a rapid rate of degradation within the first six hours of exposure (see figure 1).

2 Light = 255 lumens/ m^2, 1.6 µW/cm^2 UVA, and 0.08 µW/cm^2 UVB on a 16:8 light:dark cycle.
Dark =complete darkness.

Table 18. Average Concentration of Menadione in Filtered Duluth-Superior Harbor Water (FHW) During 96 Hours Exposure to a Continuous Light Regime of 730 lumens/m^2, 89 µW/cm^2 UVA and 4.8 µW/cm^2 UVB Light Regime.

Concentration (mg/L)	Exposure Time (Hours)												
	0	2	4	6	8	10	12	14	24	34	48	72	96
3.0	2.84	2.12	1.58	1.21	0.94	0.72	0.54	0.42	0.14	0.06	0.023		
5.0	4.87	3.35	2.36	1.65	1.22	0.87	0.63	0.47	0.13	0.05	0.018		
10.0	9.53	7.37	5.70	4.48	3.59	2.88	2.35	1.94	0.74	0.29	0.126	0.03	
20.0	19.8	14.6	11.2	8.65	6.94	5.6	4.62	3.76	1.48	0.64	0.319	0.095	0.042
50.0	48.8	34.9	25.7	19.3	15.3	12.1	10.0	8.27	3.65	1.77	1.13	0.21	0.096

Figure 1. Degradation of Menadione in Filtered Duluth-Superior Harbor Water (FHW) During 96 Hours Exposure to a Continuous Light Regime of 730 lumens/m^2, 89 µW/cm^2 UVA and 4.8 µW/cm^2 UVB Light Regime.



Bench-Scale Residual Toxicity Test Findings for SeaKleen 80®

Table 19 describes the results of the degradation experiment in which the organisms were exposed to menadione concentrations in which the menadione solution was not degraded and to 3.0 mg/L of menadione which was allowed to degrade as described in the above residual toxicity methods section. There was no statistical significant ($p > 0.05$) difference when comparing the survival of the organisms exposed to the degraded menadione solutions to those of similar concentrations of the undegraded menadione solutions.

Table 19. Average (n=3) Percent Survival (+/- Standard Error) of Adult Daphnids (*Ceriodaphnia dubia)*, Juvenile Fathead Minnows (*Pimephales promelas)* and Newly Hatched Rotifers (*Brachionus calyciflorus*) Following 0 and 24 Hours Degradation of 3.0 mg/L Menadione in Filtered Harbor Water (FHW) Under a Light Regime of 16 hour/8 hour Light/Dark Cycle at 730 Lumens/m^2, 89 µW/cm^2 UVA and 4.8 µW/cm^2 UVB.

Organism	Degradation Time (Hours)	Average Percent Survival (%)	Average Concentration of Menadione (mg/L)
Daphnids*	0	20	0.02
Daphnids*	24	20	0.08
Fathead Minnows*	0	75	0.06
Fathead Minnows*	24	76	0.10
Rotifers**	0	40	014
Rotifers**	24	35	0.11

*Daphnids and fathead minnows were exposed to the degraded solution for 48 hours;
**Rotifers were exposed to the degraded solution for 24 hours.

Quality Assurance/Quality Control

Quality control (QC) sample analysis for menadione determination consisted of analyzing duplicate samples and samples spiked with known amounts of menadione. Approximately 10 % of samples analyzed were analyzed in duplicate and for spike recovery. Table 23 provides the results of the QC sample analysis.

Table 23. Menadione Duplicate Agreement and Spike Recovery Results.

	Duplicate Agreement (%)	Spike Recovery (%)
Mean	98.75	95.41
Standard Deviation	2.37	4.78
Maximum Value	100.00	116.05
Minimum Value	85.71	83.53
Number of QC Samples	95	98

Toxicity tests were initiated with healthy, vigorous animals. Reference toxicant tests were performed with all test species prior to the start of the definitive test. Control charts are available upon request. Test conditions were monitored daily for parameters that might affect the outcome of the test (i.e., temperature, and dissolved oxygen). Daily and weekly calibration of test meters ensured optimal performance. The GSI Interim Quality Assurance and Quality Control Officer performed inspections of logbooks, recorded measurements, and instrumentation used during the tests. Any deviations were discussed with the principal investigator and documented in the study logbook.

CONCLUSION

GSI's bench testing of SeaKleen 80® found the compound to be highly effective at inactivating zooplankton—juvenile daphnids (*Daphnia magna*), adult rotifers (*Brachionus calyciflorus*), and rotifer resting eggs (*B. calyciflorus*)—with less than 1 % survival within 48 hours of treatment at a dose 0.4 mg/L as menadione. The substance was only minimally effective on the green algae species *Selenastrum* spp. No dose tested achieved 99 % mortality for this algal species, though the concentrations tested (20, 40, and 80 mg/L as menadione) were substantially greater than the proposed dose of 3.0 mg/L.

Light had the most influence on treatment degradation. SeaKleen 80® degraded readily under high light/high transmittance conditions. However, if either the light or transmittance of the water was attenuated, degradation was slow and incomplete. Some receiving systems are quite transparent, and degradation will likely be rapid. Others have little or no light penetration at the depths at which ballast will be discharged, so little or no degradation by light will take place. In our tests, thirty six hours were needed to degrade the dose required to kill 99 % of zooplankton tested, approximately 0.5 mg/L, to below detection under light conditions. At 96 hours, the end of the study, 0.5 mg/L did not degrade appreciably in dark conditions. Accordingly, water treated with SeaKleen 80® was still toxic to the species tested after a 24 hour degradation period in HW under light conditions. There is no mechanism within the treatment process to assure degradation, so discharge to harbors which have ample transmittance properties will be critical to detoxification.

A by-product peak was observed on the chromatograph accompanying the degradation of the proposed dose of menadione (3 mg/L). The by-product appeared to increase in concentration as the parent compound, menadione, decreased over time. Our tests did not include toxicity testing of this unidentified compound.