June 2006

Environmental Technology Verification Report

CHECKLIGHT LTD.
TOXSCREEN-II TEST KIT

Prepared by
Battelle

Battelle
The Business of Innovation

Under a cooperative agreement with

EPA **U.S. Environmental Protection Agency**

ETV ETV ETV

THE ENVIRONMENTAL TECHNOLOGY VERIFICATION PROGRAM

U.S. Environmental Protection Agency

Battelle
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ETV Joint Verification Statement

TECHNOLOGY TYPE:	**Rapid Toxicity Testing System**	
APPLICATION:	**Detecting Toxicity in Drinking Water**	
TECHNOLOGY NAME:	**ToxScreen-II**	
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The U.S. Environmental Protection Agency (EPA) has established the Environmental Technology Verification (ETV) Program to facilitate the deployment of innovative or improved environmental technologies through performance verification and dissemination of information. The goal of the ETV Program is to further environmental protection by accelerating the acceptance and use of improved and cost-effective technologies. ETV seeks to achieve this goal by providing high-quality, peer-reviewed data on technology performance to those involved in the design, distribution, financing, permitting, purchase, and use of environmental technologies. Information and ETV documents are available at www.epa.gov/etv.

ETV works in partnership with recognized standards and testing organizations, with stakeholder groups (consisting of buyers, vendor organizations, and permitters), and with individual technology developers. The program evaluates the performance of innovative technologies by developing test plans that are responsive to the needs of stakeholders, conducting field or laboratory tests (as appropriate), collecting and analyzing data, and preparing peer-reviewed reports. All evaluations are conducted in accordance with rigorous quality assurance (QA) protocols to ensure that data of known and adequate quality are generated and that the results are defensible.

The Advanced Monitoring Systems (AMS) Center, one of six technology areas under ETV, is operated by Battelle in cooperation with EPA's National Exposure Research Laboratory. The AMS Center evaluated the performance of the CheckLight Ltd. ToxScreen-II Test Kit. This verification statement provides a summary of the test results.

VERIFICATION TEST DESCRIPTION

Rapid toxicity technologies use various biological organisms and chemical reactions to indicate the presence of toxic contaminants. The toxic contaminants are indicated by a change or appearance of color or a change in intensity. As part of this verification test, ToxScreen-II was subjected to various concentrations of contaminants such as industrial chemicals, pesticides, rodenticides, pharmaceuticals, nerve agents, and biological toxins. Each contaminant was added to separate drinking water samples and analyzed. In addition to determining whether ToxScreen-II could detect the toxicity caused by each contaminant, its response to interfering compounds, such as water treatment chemicals and by-products in clean drinking water, was evaluated.

ToxScreen-II was evaluated by

- Endpoints and precision—percent inhibition for all concentration levels of contaminants and potential interfering compounds and precision of replicate analyses
- Toxicity threshold for each contaminant—contaminant level at which higher concentrations generate inhibition significantly greater than the negative control and lower concentrations do not. Note that CheckLight Ltd. recommends that a 50% inhibition is required for a conclusive indication of toxicity. During this test, a thorough evaluation of the toxicity threshold was performed. Therefore, the toxicity threshold was determined with respect to the negative control rather than the 50% inhibition threshold
- False positive responses—chlorination and chloramination by-product inhibition with respect to unspiked American Society for Testing and Materials Type II deionized water samples that exceeded 50%
- False negative responses—contaminants that were reported as producing less than 50% and/or were not significantly different from the negative control when present at lethal concentrations (the concentration at which 250 milliliters of water would probably cause the death of a 154-pound person) or negative background inhibition that caused falsely low inhibition
- Other performance factors (sample throughput, ease of use, reliability).

ToxScreen-II was verified by analyzing a dechlorinated drinking water sample from Columbus, Ohio (DDW), fortified with contaminants (at concentrations ranging from lethal levels to concentrations up to one million times less than the lethal dose) and interferences (metals possibly present as a result of the water treatment processes). Dechlorinated water was used because free chlorine kills the bacteria within the ToxScreen II reagent and can degrade the contaminants during storage. Inhibition results (endpoints) from four replicates of each contaminant at each concentration level were evaluated to assess the ability of ToxScreen-II to detect toxicity, as well as to measure the precision of ToxScreen-II results. The response of ToxScreen-II to possible interferents was evaluated by analyzing them at one-half of the concentration limit recommended by the EPA's National Secondary Drinking Water Regulations guidance. For analysis of by-products of the chlorination process, the unspiked DDW was analyzed because Columbus, Ohio, uses chlorination as its disinfectant procedure. For the analysis of by-products of the chloramination process, a separate drinking water sample was obtained from the Metropolitan Water District of Southern California (LaVerne, California), which uses chloramination as its disinfection process. The samples were analyzed after residual chlorine was removed using sodium thiosulfate. Sample throughput was measured based on the number of samples analyzed per hour. Ease of use and reliability were determined based on documented observations of the operators.

Quality control samples included method blank samples, which consisted of American Society for Testing and Materials Type II deionized water; positive control samples (fortified with sodium chloroacetate for the Pro-Organic Buffer samples and copper chloride for the Pro-Metal Buffer samples); and negative control samples, which consisted of the unspiked DDW.

QA oversight of verification testing was provided by Battelle and EPA. Battelle QA staff conducted a technical systems audit, a performance evaluation audit, and a data quality audit of 10% of the test data.

This verification statement, the full report on which it is based, and the test/QA plan for this verification test are all available at www.epa.gov/etv/centers/center1.html.

TECHNOLOGY DESCRIPTION

The following description of the ToxScreen-II Test Kit is based on information provided by the vendor. This technology description was not verified in this test.

ToxScreen-II provides on-site detection of organic and inorganic toxicants, such as heavy metals; pesticides; herbicides; chlorinated hydrocarbons; polychlorinated biphenyls; benzene, toluene, ethylbenzene, and xylenes; and phencyclidine. ToxScreen-II can be used in both field and laboratory testing. Typical applications include effluent toxicity testing; surface and ground water screening for changes in water quality; and raw drinking water monitoring for early warning of dangerous spills, accidents, and sabotage/bioterrorism.

Under proper conditions, luminous bacteria emit high and steady levels of luminescence. Chemical and biological toxicants that affect cell respiration, electron transport systems, adenosine triphosphate generation, and the rate of protein or lipid synthesis alter the level of luminescence. Similarly, agents that affect a cell's integrity and membrane function have a strong effect on luminescence. Hence, toxicants of different characteristics such as pesticides, herbicides, chlorinated hydrocarbons, and heavy metals exert a measurable effect on a bacterial luminescence system. By comparing the luminescence level obtained in a suspected toxic sample with that obtained in a clean water control sample after a short period of incubation, very low concentrations of a broad range of toxicants can be detected. To detect toxicants in water samples, ToxScreen-II uses a highly sensitive variant of *Photobacterium leiognathi* and two assay buffers: one for detecting heavy metals (Pro-Metal Buffer) and the other for organic pollutants (Pro-Organic Buffer). When used concurrently, these buffers are designed to discriminate between the presence of organic and metal toxicants at submilligram per liter concentrations.

The ToxScreen-II luminometer is 150 millimeters (mm) wide by 280 mm deep by 170 mm high and weighs approximately two kilograms. The test kit comes with stoppered vials holding freeze-dried luminous bacteria, hydration buffer, storage buffer, Pro-Metal concentrated assay buffer, Pro-Organic concentrated assay buffer, concentrated positive control solutions, and empty test tubes. The portable luminometer costs $3,950, and a starter kit including reagents for 1,000 single tests costs $550.

VERIFICATION RESULTS
Pro-Organic Buffer

Parameter	Compound	Lethal Dose (LD) Conc. (mg/L)	Average Inhibition at Concentrations Relative to the LD Concentration (%)				Range of Standard Deviations (%)	Toxicity Thresh. (mg/L)
			LD	LD/10	LD/100	LD/1,000		
Contaminants in DDW	Aldicarb	260	50	-26	0	-50	12-18	ND
	Botulinum toxin complex B	0.3	-87	14	16	-54	21-58	ND
	Colchicine	240	75	17	4	1	2-5	24
	Cyanide	250	100	100	95	72	0-2	0.25
	Dicrotophos	1,400	70	23	3	1	3-29	140
	Nicotine	2,800	83	-10	-32	-20	2-10	1,400
	Ricin	15	68[(a)]	9	1	10	2-5	ND
	Soman	1.4	-6	-202	4	15	10-68	ND
	Thallium sulfate	2,800	66	13	9	-1	3-6	28
	VX	2	-3	-5	2	-6	3-9	ND

			Average Inhibition (%)		Standard Deviation (%)	
	Interference	Conc. (mg/L)	Initial Analysis	Reanalysis[(b)]	Initial Analysis	Reanalysis[(b)]
Potential interferences in DDW	Aluminum	0.5	-4	-12	4	1
	Copper	0.6	3	5	3	15
	Iron	0.15	0	-6	1	4
	Manganese	0.25	0	3	4	3
	Zinc	2.5	-1	NR	3	NR

False positive response	None of the disinfection by-product samples produced an inhibition significantly greater than 50%, the inhibition level suggested by CheckLight Ltd. to conclusively determine toxicity.
False negative response	Aldicarb, botulinum toxin complex B, soman, and VX produced an inhibition that either did not exceed 50% or were not significantly different from the negative control at the lethal dose concentrations. For ricin in the Pro-Organic buffer, the inhibition of the lethal dose was significantly different from the negative control, but not significantly different from the inhibition generated by the preservative blank.
Ease of use	ToxScreen-II included clearly written instructions with good illustrations. The contents of the ToxScreen-II were well labeled, making it easy to follow the instructions. A minimum of three hours was required to rehydrate the bacteria, which must be stored at -14ºC prior to rehydration. After rehydration, the bacteria can be used for up to seven days; however, the vendor suggested using them within one day. Overall, the ToxScreen-II was easy to use, making it likely that a person with no formal scientific training could conduct the tests.
Field portability	ToxScreen-II was transported from a laboratory to a storage room to simulate operation in a non-laboratory location. It was tested with cyanide at the lethal dose concentration, and the results generated (>90% inhibition) were very similar to those obtained in the laboratory. No carrying case was provided with ToxScreen-II (one is available for purchase from Checklight Ltd.); however, all materials except the luminometer were transported in a small cardboard box. The box and luminometer were easily carried by one person, and setup for analysis took less than 10 minutes.
Throughput	Approximately 25 analyses were completed each hour using both buffers, and approximately 1,000 samples could be processed per kit.

ND = Significant inhibition was not detected.
NR = Not reanalyzed.
[(a)] Inhibition was not significantly different from the preservative blank.
[(b)] Potential interferences were reanalyzed due to four suspect negative inhibitions during the initial analysis with the Pro-Metal buffer.

Pro-Metal Buffer

Parameter	Compound	Lethal Dose (LD) Conc. (mg/L)	Average Inhibition at Concentrations Relative to the LD Concentration (%)				Range of Standard Deviations (%)	Toxicity Thresh. (mg/L)
			LD	LD/10	LD/100	LD/1,000		
Contaminants in DDW	Aldicarb	260	-19	-7	33	-31	10-35	ND
	Botulinum toxin complex B	0.3	-185	-121	-91	-40	18-104	ND
	Colchicine	240	12	2	8	-9	2-6	ND
	Cyanide	250	89	64	44	19	1-7	0.25
	Dicrotophos	1,400	55	-1	-10	-3	2-4	140
	Nicotine	2,800	98	2	-10	-7	0-4	700
	Ricin	15.0	3	-2	2	2	2-4	ND
	Soman	1.4	-55	17	-66	-4	13-22	ND
	Thallium sulfate	2,800	79	53	27	4	1-4	28
	VX	2.0	5	-11	-11	-3	5-10	ND

			Average Inhibition (%)		Standard Deviation (%)	
	Interference	Conc. (mg/L)	Initial Analysis	Reanalysis[a]	Initial Analysis	Reanalysis[a]
Potential interferences in DDW	Aluminum	0.5	-395	-13	29	3
	Copper	0.6	-299	30	26	4
	Iron	0.15	-399	-8	18	3
	Manganese	0.25	-368	5	15	4
	Zinc	2.5	86	NR	0	NR

False positive response	Neither the chlorination nor chloramination samples generated an inhibition greater than 50%. However, the chloramination sample generated a result that indicated an enhancement in luminescence (i.e., a negative inhibition), which, according to Checklight Ltd., can also indicate toxicity.
False negative response	The inhibition of the chloramination by-products was -75% ± 20% with DI water as the negative control. If a contaminant causing a 75% inhibition had been present in this water and DI water was used as the negative control, the inhibition would have been close to 0%—a false negative response. This underscores the need to use negative control samples that are as similar as possible to the samples being analyzed. A second type of false negative response occurred (for aldicarb, colchicine, botulinum toxin, ricin, soman, and VX) when the inhibition was not greater than 50% in the presence of a lethal dose of contaminant.

ND = Significant inhibition was not detected.

NR = Not reanalyzed.

See the Pro-Organic Buffer table for descriptions for ease of use, field portability, and throughput.

[a] Potential interferences were reanalyzed due to four suspect negative inhibitions during the initial analysis with the Pro-Metal buffer.

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Environmental Technology Verification Report

ETV Advanced Monitoring Systems Center

CheckLight Ltd.
ToxScreen-II Test Kit

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Notice

The U.S. Environmental Protection Agency (EPA), through its Office of Research and Development, has financially supported and collaborated in the extramural program described here. This document has been peer reviewed by the Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation by the EPA for use.

Foreword

The U.S. Environmental Protection Agency (EPA) is charged by Congress with protecting the nation's air, water, and land resources. Under a mandate of national environmental laws, the Agency strives to formulate and implement actions leading to a compatible balance between human activities and the ability of natural systems to support and nurture life. To meet this mandate, the EPA's Office of Research and Development provides data and science support that can be used to solve environmental problems and to build the scientific knowledge base needed to manage our ecological resources wisely, to understand how pollutants affect our health, and to prevent or reduce environmental risks.

The Environmental Technology Verification (ETV) Program has been established by the EPA to verify the performance characteristics of innovative environmental technology across all media and to report this objective information to permitters, buyers, and users of the technology, thus substantially accelerating the entrance of new environmental technologies into the marketplace. Verification organizations oversee and report verification activities based on testing and quality assurance protocols developed with input from major stakeholders and customer groups associated with the technology area. ETV consists of six environmental technology centers. Information about each of these centers can be found on the Internet at http://www.epa.gov/etv/.

Effective verifications of monitoring technologies are needed to assess environmental quality and to supply cost and performance data to select the most appropriate technology for that assessment. Under a cooperative agreement, Battelle has received EPA funding to plan, coordinate, and conduct such verification tests for "Advanced Monitoring Systems for Air, Water, and Soil" and report the results to the community at large. Information concerning this specific environmental technology area can be found on the Internet at http://www.epa.gov/etv/centers/center1.html.

Acknowledgments

The authors wish to acknowledge the support of all those who helped plan and conduct the verification test, analyze the data, and prepare this report. We would also like to thank Karen Bradham, U.S. EPA National Exposure Research Laboratory; Steve Allgeier, U.S. EPA Office of Water; Ricardo DeLeon, Metropolitan Water District of Southern California; Yves Mikol, New York City Department of Environmental Protection; and Stanley States, Pittsburgh Water and Sewer Authority, for their careful review of the test/quality assurance plan and/or this verification report.

Contents

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Tables

List of Abbreviations

AMS	Advanced Monitoring Systems
ASTM	American Society for Testing and Materials
ATEL	Aqua Tech Environmental Laboratories
DI	deionized water
DDW	dechlorinated drinking water from Columbus, Ohio
DPD	n,n-diethyl-p-phenylenediamine
EPA	U.S. Environmental Protection Agency
ETV	Environmental Technology Verification
HDPE	high-density polyethylene
ID	identification
LD	lethal dose
mM	millimolar
µL	microliter
mg/L	milligram per liter
mL	milliliter
mm	millimeter
NSDWR	National Secondary Drinking Water Regulations
%D	percent difference
PE	performance evaluation
QA	quality assurance
QC	quality control
QMP	quality management plan
SOP	standard operating procedure
TSA	technical systems audit

Chapter 1
Background

The U.S. Environmental Protection Agency (EPA) supports the Environmental Technology Verification (ETV) Program to facilitate the deployment of innovative environmental technologies through performance verification and dissemination of information. The goal of the ETV Program is to further environmental protection by accelerating the acceptance and use of improved and cost-effective technologies. ETV seeks to achieve this goal by providing high-quality, peer-reviewed data on technology performance to those involved in the design, distribution, financing, permitting, purchase, and use of environmental technologies.

ETV works in partnership with recognized testing organizations; with stakeholder groups consisting of buyers, vendor organizations, and permitters; and with the full participation of individual technology developers. The program evaluates the performance of innovative technologies by developing test plans that are responsive to the needs of stakeholders, conducting field or laboratory tests (as appropriate), collecting and analyzing data, and preparing peer-reviewed reports. All evaluations are conducted in accordance with rigorous quality assurance (QA) protocols to ensure that data of known and adequate quality are generated and that the results are defensible.

The EPA's National Exposure Research Laboratory and its verification organization partner, Battelle, operate the Advanced Monitoring Systems (AMS) Center under ETV. The AMS Center recently evaluated the performance of the CheckLight Ltd. ToxScreen-II test kit. Rapid toxicity technologies were identified as a priority verification category through the AMS Center stakeholder process.

Chapter 2
Technology Description

The objective of the ETV AMS Center is to verify the performance characteristics of environmental monitoring technologies for air, water, and soil. This verification report provides results for the verification testing of ToxScreen-II. Following is a description of ToxScreen-II, based on information provided by the vendor. The information provided below was not verified in this test.

Figure 2-1. CheckLight Ltd. ToxScreen-II Test Kit

ToxScreen-II (Figure 2-1) provides on-site detection of a wide range of organic and inorganic toxicants, such as heavy metals; pesticides; herbicides; chlorinated hydrocarbons; polychlorinated biphenyls; benzene, toluene, ethylbenzene, and xylenes; and phencyclidine. ToxScreen-II can be used in both field and laboratory testing. Typical applications include effluent toxicity testing; surface and ground water screening for changes in water quality; and raw drinking water monitoring for early warning of dangerous spills, accidents, and sabotage/bioterrorism.

Under proper conditions, luminous bacteria emit high and steady levels of luminescence. Chemical and biological toxicants that affect cell respiration, electron transport systems, adenosine triphosphate generation, and the rate of protein or lipid synthesis alter the level of luminescence. Similarly, agents that affect a cell's integrity and membrane function have a strong effect on luminescence. Hence, toxicants of different characteristics such as pesticides, herbicides, chlorinated hydrocarbons, and heavy metals exert a dramatic and measurable effect on a bacterial luminescence system. By comparing the luminescence level obtained in a suspected toxic sample

with that obtained in a clean water control sample after a short period of incubation, very low concentrations of a broad range of toxicants can be detected.

To detect toxicants in water samples, ToxScreen-II uses a highly sensitive variant of *Photobacterium leiognathi* and two assay buffers: one for detecting heavy metals (Pro-Metal buffer) and the other for organic pollutants (Pro-Organic buffer). When used concurrently, these buffers are designed to discriminate between the presence of organic and metal toxicants at sub-milligram per liter concentrations.

The ToxScreen-II luminometer is 150 millimeters (mm) wide by 280 mm deep by 170 mm high and weighs approximately two kilograms. The test kit comes with stoppered vials holding freeze-dried luminous bacteria, hydration buffer, storage buffer, Pro-Metal concentrated assay buffer, Pro-Organic concentrated assay buffer, concentrated positive control solutions, and empty test tubes. The portable luminometer costs $3,950, and a starter kit including reagents for 1,000 single tests costs $550.

Chapter 3
Test Design

The objective of this verification test of rapid toxicity technologies was to evaluate their ability to detect certain toxins and to determine their susceptibility to interfering chemicals in a controlled experimental matrix. Rapid toxicity technologies do not identify or determine the concentration of specific contaminants, but serve as a screening tool to quickly determine whether water is potentially toxic.

As part of this verification test, ToxScreen-II was subjected to various concentrations of contaminants such as industrial chemicals, pesticides, rodenticides, pharmaceuticals, nerve agents, and biological toxins. Each contaminant was added to separate drinking water samples and analyzed. In addition to determining whether ToxScreen-II can detect the toxicity caused by each contaminant, its response to interfering compounds, such as water treatment chemicals and by-products in clean drinking water, was evaluated. Table 3-1 shows the contaminants and potential interferences that were evaluated during this verification test.

This verification test was conducted from August to December 2005 according to procedures specified in the *Test/QA Plan for Verification of Rapid Toxicity Technologies* including Amendments 1 and 2.[1] ToxScreen-II was verified by analyzing a dechlorinated drinking water sample from Columbus, Ohio (hereafter in this report, referred to as DDW), fortified with various concentrations of the contaminants and interferences shown in Table 3-1. Dechlorinated water was used because free chlorine inhibits the photosynthetic process that ToxScreen II depends on to indicate toxicity and can degrade the contaminants during storage. Where possible, the concentration of each contaminant or potential interference was confirmed independently by Aqua Tech Environmental Laboratories (ATEL), Marion, Ohio, or by Battelle, depending on the analyte.

ToxScreen-II was evaluated by

- Endpoints and precision—percent inhibition for all concentration levels of contaminants and potential interfering compounds and precision of replicate analyses

- Toxicity threshold for each contaminant—contaminant level at which higher concentrations generate inhibition significantly greater than the negative control and lower concentrations do not. Note that CheckLight Ltd. recommends that a 50% inhibition is required for a

Table 3-1. Contaminants and Potential Interferences

Category	Contaminant
Biological toxins	Botulinum toxin complex B, ricin
Botanical pesticide	Nicotine
Carbamate pesticide	Aldicarb
Industrial chemical	Cyanide
Nerve agents	Soman, VX
Organophosphate pesticide	Dicrotophos
Pharmaceutical	Colchicine
Potential interferences	Aluminum, copper, iron, manganese, zinc, chloramination by-products, and chlorination by-products
Rodenticide	Thallium sulfate

conclusive indication of toxicity. During this test, a thorough evaluation of the toxicity threshold was performed. Therefore, the toxicity threshold was determined with respect to the negative control rather than the 50% inhibition threshold

- False positive responses—chlorination and chloramination by-product inhibition with respect to unspiked American Society for Testing and Materials (ASTM) Type II deionized (DI) water samples that exceeded 50%

- False negative responses—contaminants that were reported as producing less than 50% inhibition and/or were not significantly different from the negative control when the contaminant was present at lethal concentrations or negative inhibition that could cause falsely low inhibition results.

- Other performance factors (sample throughput, ease of use, reliability).

ToxScreen-II was used to analyze the DDW samples fortified with contaminants at concentrations ranging from lethal levels to concentrations up to one million times less than the lethal dose. The lethal dose of each contaminant was determined by calculating the concentration at which 250 milliliters (mL) of water would probably cause the death of a 154-pound person. These calculations were based on toxicological data available for each contaminant that are presented in Amendment 2 of the test/QA plan.[1] Inhibition results (endpoints) from four replicates of each contaminant at each concentration level were evaluated to assess the ability of ToxScreen-II to detect toxicity at various concentrations of contaminants, as well as to measure the precision of ToxScreen-II results.

The response of ToxScreen-II to compounds used during the water treatment process (identified as potential interferences in Table 3-1) was evaluated by analyzing separate aliquots of DDW fortified with each potential interference at one-half of the concentration limit recommended by the EPA's National Secondary Drinking Water Regulations (NSDWR)[2] guidance. For analysis of by-products of the chlorination process, the unspiked DDW was analyzed because Columbus, Ohio, uses chlorination as its disinfectant procedure. For the analysis of by-products of the

chloramination process, a separate drinking water sample was obtained from the Metropolitan Water District of Southern California (LaVerne, California), which uses chloramination as its disinfection process. The samples were analyzed after residual chlorine was removed using sodium thiosulfate. Sample throughput was measured based on the number of samples analyzed per hour. Ease of use and reliability were determined based on documented observations of the operators.

3.1 Test Samples

Test samples used in the verification test included drinking water and quality control (QC) samples. Table 3-2 shows the number and type of samples analyzed. QC samples included method blanks and positive and negative control samples. The fortified drinking water samples were prepared from a single drinking water sample collected from the Columbus, Ohio, system. The water was dechlorinated using sodium thiosulfate and then fortified with various concentrations of contaminants and interferences. The DDW containing the potential interferences was analyzed at a single concentration level, while at least four dilutions were analyzed for each contaminant using ToxScreen-II. Solutions were analyzed using both the Pro-Organic and Pro-Metal buffers. Mixtures of contaminants and possible interfering compounds were not analyzed.

3.1.1 Quality Control Samples

QC samples included method blanks, positive controls, negative controls, and preservative blanks. The method blank samples consisted of ASTM Type II DI water and were used to ensure that no sources of contamination were introduced in the sample handling and analysis procedures. Positive control samples consisted of ASTM Type II DI water fortified with a vendor-specified contaminant at a vendor-specified concentration level. Sodium chloroacetate (Pro-Organic) and copper chloride (Pro-Metal) were used as positive control samples throughout the verification test with their respective buffer solutions. While performance limits were not placed on the results, an inhibition of approximately 50% for these positive control samples indicated to the operator that ToxScreen-II was functioning properly. The negative control samples consisted of unspiked DDW and were used to set a background inhibition of the DDW, the matrix in which each test sample was prepared. To ensure that the preservatives in the contaminant solutions did not have an inhibitory effect, preservative blank samples were prepared. These preservative blanks consisted of DDW fortified with a concentration of preservative equivalent to that in the test solutions of botulinum toxin complex B, ricin, soman, and VX.

3.1.2 Drinking Water Fortified with Contaminants

Approximately 50 liters of Columbus, Ohio, tap water were collected in a low-density polyethylene container. The water was dechlorinated with sodium thiosulfate. Dechlorination was confirmed by adding an n,n-diethyl-p-phenylenediamine (DPD) tablet to a 10-mL aliquot of the water. Lack of color development in the presence of DPD indicated that the water was dechlorinated. All subsequent test samples were prepared from this DDW.

A stock solution of each contaminant was prepared in DDW at concentrations at or above the lethal dose level. The stock solution was further diluted to obtain one sample containing the

lethal dose concentration for each contaminant and three additional samples with concentrations 10, 100, and 1,000 times less than the lethal dose. Additional concentrations of some contaminants were prepared and analyzed for two reasons: one was because of the large difference in response between two concentration levels. For example, if only one dilution level was almost completely inhibitory and the next dilution level was non-inhibitory, several intermediate concentrations were analyzed to better determine the toxicity threshold of that contaminant. The other reason was because sometimes the lowest concentration analyzed was mostly inhibitory, thus, not providing even an estimate of the toxicity threshold. For these contaminants, additional tenfold dilutions were analyzed to more accurately determine the toxicity threshold. Table 3-2 lists each concentration level and the number of samples analyzed at each level.

3.1.3 Drinking Water Fortified with Potential Interferences

Individual aliquots of the DDW were fortified with one-half the concentration specified by the EPA's NSDWR for each potential interference. Table 3-2 lists the interferences, along with the concentrations at which they were tested. Four replicates of each of these samples were analyzed. To test the sensitivity of ToxScreen-II to by-products of the chlorination process as potential interferences, the unspiked DDW (same as the negative control) was used since the water sample originated from a utility that uses chlorination as its disinfectant procedure. In a similar manner, by-products of the chloramination process were evaluated using a water sample from the Metropolitan Water District of Southern California. The residual chlorine in both of these samples was removed using sodium thiosulfate, and then the samples were analyzed in replicate with no additional fortification of contaminants.

3.2 Test Procedure

The procedures for preparing, storing, and analyzing test samples and confirming stock solutions are provided below.

3.2.1 Test Sample Preparation and Storage

A drinking water sample was collected as described in Section 3.1.2 and, because free chlorine kills the bacteria within the ToxScreen-II reagent and can degrade the contaminants during storage, was immediately dechlorinated with sodium thiosulfate. Dechlorination of the water sample was qualitatively confirmed by adding a DPD tablet to a 10-mL aliquot of the DDW. All the contaminant samples, potential interference samples, preservative blanks, and negative control QC samples were made from this water sample, while the method blank sample was prepared from ASTM Type II DI water. The positive control samples were made by adding the vendor-specified positive control solution to ASTM Type II DI water using calibrated auto-pipettes. All QC samples were prepared prior to the start of the testing and stored at room temperature. The stability of each contaminant for which analytical methods are available was confirmed by analyzing it three times over a two-week period. Throughout this time, each contaminant maintained its original concentration to within approximately 25%. Therefore, the aliquots of DDW containing the contaminants were prepared within two weeks of testing and were stored at room temperature without chemical preservation. The contaminants without analytical methods were analyzed within 48 hours of their preparation. To maintain the integrity of the test, test samples were labeled only with sample identification numbers so that the operators did not know their content.

Table 3-2. Summary of Quality Control and Contaminant Test Samples

Type of Sample	Sample Characteristics	Concentration Levels	No. of Sample Analyses
Quality control	Method blank (ASTM Type II water)	NA	16
	Positive control: Pro-Organic	1:100 dilution of sodium chloroacetate stock provided in kit	16 (Pro-Organic test only)
	Positive control: Pro-Metal	1:100 dilution of copper chloride stock provided in kit	15 (Pro-Metal test only)
	Negative control (unspiked DDW)	NA	56
	Preservative blank: botulinum toxin complex B	0.015 millimolar (mM) sodium citrate	4
	Preservative blank: VX and soman	0.21% isopropyl alcohol	4 with VX, 4 with soman
	Preservative blank: ricin	0.00024% NaN_3, 0.00045 molar NaCl, 0.03mM phosphate	4 each at concentration: lethal dose (LD) (conc. at left), LD/10, LD/100, and LD/1,000
DDW fortified with contaminants	Aldicarb	260; 26; 2.6; 0.26 milligrams/liter (mg/L)	4 per concentration level
	Botulinum toxin complex B	0.30; 0.030; 0.0030; 0.00030 mg/L	4 per concentration level
	Colchicine	240; 24; 2.4; 0.24 mg/L	4 per concentration level
	Cyanide	250; 25; 2.5; 0.25; 0.025; 0.0025; 0.00025 mg/L	4 per concentration level
	Dicrotophos	1,400; 1,000; 500; 140; 14; 1.4; mg/L	4 per concentration level
	Nicotine	2,800; 2,100; 1,400; 700; 280; 28; 2.8 mg/L	4 per concentration level
	Ricin	15; 1.5; 0.15; 0.015 mg/L	4 per concentration level
	Soman	1.4; 0.14; 0.014; 0.0014 mg/L	4 per concentration level
	Thallium sulfate	2,800; 280; 28; 2.8 mg/L	4 per concentration level
	VX	2.0; 0.2; 0.02; 0.002 mg/L	4 per concentration level
DDW fortified with potential interferences	Aluminum	0.5 mg/L	4
	Copper	0.6 mg/L	4
	Iron	0.15 mg/L	4
	Manganese	0.25 mg/L	4
	Zinc	2.5 mg/L	4
Disinfectant by-products	Chloramination by-products	NA	4
	Chlorination by-products	NA	56

NA = not applicable, samples not fortified with any preservative, contaminant, or potential interference.

3.2.2 Test Sample Analysis Procedure

To analyze the test samples, the luminescent marine bacteria *Photobacterium leiognathi* (strain SB) were reconstituted with hydration buffer, incubated at ambient temperature for approximately 5 minutes, then transferred into storage buffer and mixed well. The rehydrated bacteria were stored at 4°C until use. The bacteria were prepared the afternoon before their use for all tests, with the exception of the field portability test. For the field portability test, two sets of bacteria were prepared. One set was prepared approximately 3 hours before use and the second set approximately 24 hours before use to assess the performance of the minimum incubation time (3 hours) against the more standard time (24 hours) used during this testing program. Once the bacteria were properly rehydrated and incubated, 800 microliters (µL) of the test sample were added to a sample cuvette along with 200 µL of either the Pro-Metal or Pro-Organic buffer, and this combination was mixed. Then, 10 µL of rehydrated bacteria were added to each water/buffer solution, mixed well, and incubated at ambient temperature for 60 minutes. After 60 minutes, luminescence was measured. The luminescence of the test sample was compared with that of the negative control to determine percent inhibition.

For each contaminant, a minimum of the lethal dose concentration and three additional concentration levels were analyzed four times using ToxScreen-II. Only one concentration of each potential interference was analyzed four times. The luminescence was recorded, and the percent inhibition was calculated for each sample. Two operators performed all the analyses using ToxScreen-II. One operator performed testing with contaminants that did not require special chemical and biological agent training and one performed testing with those that did. Both held bachelor's degrees in the sciences and were trained by the vendor to operate ToxScreen-II.

3.2.3 Stock Solution Confirmation Analysis

The concentrations of the contaminant and interfering compound stock solutions were verified with standard analytical methods, with the exception of colchicine, ricin, and botulinum toxin complex B—contaminants without standard analytical methods. Aliquots to be analyzed by standard methods were preserved as prescribed by the method. In addition, the same standard methods were used to measure the concentration of each contaminant/potential interference in the unspiked DDW so that background concentrations of contaminants or potential interferences were accounted for within the displayed concentration of each contaminant/potential interference sample. Table 3-3 lists the standard methods used to measure each analyte; the results from the stock solution confirmation analyses (obtained by analyzing the lethal dose concentration for the contaminants and the single concentration that was analyzed for the potential interferences); and the background levels of the contaminants and potential interferences measured in the DDW sample, which were all non-detect or negligible.

Standard methods were also used to characterize several water quality parameters such as alkalinity; dissolved organic carbon content; specific conductivity; hardness; pH; concentration of haloacetic acids, total organic carbon, total organic halides, and trihalomethanes; and turbidity. Table 3-4 lists these measured water quality parameters for both the water sample collected in Columbus, Ohio, representing a water system using chlorination as the disinfecting process, and the water sample collected at the Metropolitan Water District of Southern California, representing a water system using chloramination for disinfection.

Table 3-3. Stock Solution Confirmation Results

	Method	Average Concentration ± Standard Deviation N = 4 (mg/L)[(b)]	Background in DDW (mg/L)
Contaminant			
Aldicarb	Battelle method	260 ± 7	<0.005
Botulinum toxin complex B	[(a)]	NA	NA
Colchicine	[(a)]	NA	NA
Cyanide	EPA 335.3[(3)]	249 ± 4 296 ± 26 (field portability)	0.006
Dicrotophos	Battelle method	1,168 ± 18	<3.0
Nicotine	Battelle method	2,837 ± 27	<0.01
Ricin	[(a)]	NA	NA
Soman	[(a)]	1.3 ± 0.1 (10/18/05) 1.16 ± 0.06 (10/21/05)	<0.025
Thallium sulfate	EPA 200.8[(4)]	2,469 ± 31	<0.001
VX	Battelle method	1.89 ± 0.08 (10/17/05) 1.77 ± 0.03 (10/20/05)	<0.0005
Potential Interference			
Aluminum	EPA 200.7[(5)]	0.50 ± 0.02	<0.2
Copper	EPA 200.7[(5)]	0.60 ± 0.03	<0.02
Iron	EPA 200.7[(5)]	0.155 ± 0.006	<0.04
Manganese	EPA 200.7[(5)]	0.281 ± 0.008	<0.01
Zinc	EPA 200.7[(5)]	2.63 ± 0.05	0.27

NA = Not applicable.
[(a)] No standard method available. QA audits and balance calibration assured accurately prepared solutions.
[(b)] Target concentration was highest concentration for each contaminant or interference on Table 3-2.

Table 3-4. Water Quality Parameters

Parameter	Method	Dechlorinated Columbus, Ohio, Tap Water (disinfected by chlorination)	Dechlorinated Southern California Tap Water (disinfected by chloramination)
Alkalinity (mg/L)	SM 2320 B[6]	40	71
Specific conductivity (μmho)	SM 2510 B[6]	572	807
Hardness (mg/L)	EPA 130.2[7]	118	192
pH	EPA 150.1[7]	7.6	8.0
Total haloacetic acids (μg/L)	EPA 552.2[8]	32.8	17.4
Dissolved organic carbon (mg/L)	SM 5310 B[6]	2.1	2.9
Total organic carbon (mg/L)	SM 5310 B[6]	2.1	2.5
Total organic halides (μg/L)	SM 5320B[6]	220	170
Total trihalomethanes (μg/L)	EPA 524.2[9]	74.9	39.2
Turbidity (NTU)	SM 2130[10]	0.1	0.1

NTU = nephelometric turbidity unit.

Chapter 4
Quality Assurance/Quality Control

QA/QC procedures were performed in accordance with the quality management plan (QMP) for the AMS Center[11] and the test/QA plan for this verification test.[1]

4.1 Quality Control of Stock Solution Confirmation Methods

The stock solutions for the contaminants cyanide and thallium sulfate and for the potential interferences aluminum, magnesium, zinc, iron, and copper were analyzed at ATEL using standard reference methods. As part of ATEL's standard operating procedures (SOPs), various QC samples were analyzed with each sample set. These included matrix spike, laboratory control spike, and method blank samples. According to the standard methods used for the analyses, recoveries of the QC spike samples analyzed with samples from this verification test were within acceptable limits of 75% to 125%, and the method blank samples were below the detectable levels for each analyte. For VX, soman, aldicarb, nicotine, and dicrotophos, the confirmation analyses were performed at Battelle using a Battelle SOP or method. Calibration standard recoveries of VX and soman were always between 62% and 141%, and most of the time were between 90% and 120%. Dicrotophos standard recoveries ranged from 89% to 122%. Aldicarb standard recoveries ranged from 95% to120%. Nicotine standard recoveries ranged from 96% to 99%. Standard analytical methods for colchicine, ricin, and botulinum toxin complex B were not available and, therefore, not performed. QA audits and balance calibrations assured that solutions for these compounds were accurately prepared.

4.2 Quality Control of Drinking Water Samples

A method blank sample consisting of ASTM Type II DI water was analyzed once by ToxScreen-II for approximately every 20 drinking water samples that were analyzed. Because an inhibition has to be calculated with respect to a control sample, none were calculated for the method blank samples. The method blanks were used as the control for calculating the inhibition of the DDW for the disinfecting by-product evaluation. A positive control sample also was analyzed once for approximately every 20 drinking water samples. While performance limits were not placed on the results of the positive control sample, the vendor informed Battelle that, if the positive control samples did not cause inhibition that was significantly greater than DI water, it would indicate to the operator that ToxScreen-II was not functioning properly. For 16 sodium chloroacetate (Pro-Organic) positive control samples and 15 copper chloride (Pro-Metal) positive

control samples, inhibition results of 50% ± 14% and 75% ± 18%, respectively, were measured after a 60-minute incubation. These inhibition values indicated the proper functioning of ToxScreen-II. A negative control sample (unspiked DDW) was analyzed with approximately every four samples. The percent inhibition calculation for each sample incorporated the average inhibition of the negative control samples analyzed with that particular sample set; therefore, by definition, the average inhibition of four negative control samples was 0%.

4.3 Audits

A performance evaluation (PE) audit, a technical systems audit (TSA), and an audit of data quality were performed for this verification test.

4.3.1 Performance Evaluation Audit

The accuracy of the reference method used to confirm the concentrations of the stock solutions of the contaminants and potential interferences was confirmed by analyzing solutions of each analyte from two separate commercial vendors. The standards from one source were used to prepare the stock solutions during the verification test, while the standards from a second source were analyzed as the PE sample. The percent difference (%D) between the measured concentration of the PE sample, and the nominal concentration of that sample was calculated using the following equation:

$$\%D = \frac{M}{A} \times 100\% \quad (1)$$

where M is the absolute value of the difference between the measured and the nominal concentration, and A is the nominal concentration. The %D between the measured concentration of the PE standard and the nominal concentration had to be less than 25% for the measurements to be considered acceptable. Table 4-1 shows the results of the PE audit for each compound. All %D values were less than 25.

PE audits were performed when more than one source of the contaminant or potential interference was commercially available and when methods were available to perform the confirmation; therefore, PE audits were not performed for all of the contaminants. To assure the purity of the other standards, documentation, such as certificates of analysis, was obtained for colchicine, botulinum toxin complex B, and ricin. In the cases of VX and soman, which were obtained from the U.S. Army, the reputation of the source, combined with the confirmation analysis data, provided assurance of the concentration analyzed.

4.3.2 Technical Systems Audit

The Battelle Quality Manager conducted a TSA to ensure that the verification test was performed in accordance with the test/QA plan[1] and the AMS Center QMP.[11] As part of the audit, the Battelle Quality Manager reviewed the contaminant standard and stock solution confirmation methods, compared actual test procedures with those specified in the test/QA plan, and reviewed data acquisition and handling procedures. Observations and findings from this audit were documented and submitted to the Battelle Verification Test Coordinator for response. No findings were documented that required any significant action. The records concerning the TSA are permanently stored with the Battelle Quality Manager.

Table 4-1. Summary of Performance Evaluation Audit

		Measured Concentration (mg/L)	Nominal Concentration (mg/L)	%D
Contaminant	Aldicarb	0.057	0.050	14
	Cyanide	1,025	1,000	3
	Dicrotophos	1.10	1.00	10
	Nicotine	0.120	0.100	20
	Thallium	1,010	1,000	1
Potential interference	Aluminum	960	1,000	4
	Copper	1,000	1,000	0
	Iron	960	1,000	4
	Manganese	922	1,000	8
	Zinc	1,100	1,000	10

4.3.3 Audit of Data Quality

At least 10% of the data acquired during the verification test were audited. Battelle's Quality Manager traced the data from the initial acquisition, through reduction and statistical analysis, to final reporting, to ensure the integrity of the reported results. All calculations performed on the data undergoing the audit were checked.

4.4 QA/QC Reporting

Each internal assessment and audit was documented in accordance with Sections 3.3.4 and 3.3.5 of the QMP for the ETV AMS Center.[11] Once the assessment report was prepared, the Battelle Verification Test Coordinator ensured that a response was provided for each adverse finding or potential problem and implemented any necessary follow-up corrective action. The Battelle Quality Manager ensured that follow-up corrective action was taken. The results of the TSA were sent to the EPA.

4.5 Data Review

Records generated in the verification test were reviewed before they were used to calculate, evaluate, or report verification results. Table 4-2 summarizes the types of data recorded. The review was performed by a technical staff member involved in the verification test, but not the staff member who originally generated the record. The person performing the review added his/her signature or initials and the date to a hard copy of the record being reviewed.

Table 4-2. Summary of Data Recording Process

Data to be Recorded	Responsible Party	Where Recorded	How Often Recorded	Disposition of Data[(a)]
Dates, times of test events	Battelle	Laboratory record books	Start/end of test, and at each change of a test parameter	Used to organize/check test results; manually incorporated in data spreadsheets as necessary
Sample preparation (dates, procedures, concentrations)	Battelle	Laboratory record books	When each sample was prepared	Used to confirm the concentration and integrity of the samples analyzed; procedures entered into laboratory record books
Test parameters (contaminant concentrations, location, etc.)	Battelle	Laboratory record books	When set or changed	Used to organize/check test results, manually incorporated in data spreadsheets as necessary
Stock solution confirmation analysis, sample analysis, chain of custody, and results	Battelle or contracted laboratory	Laboratory record books, data sheets, or data acquisition system, as appropriate	Throughout sample handling and analysis process	Transferred to spreadsheets/agreed upon report

[(a)] All activities subsequent to data recording were carried out by Battelle.

Chapter 5
Statistical Methods and Reported Parameters

The statistical methods presented in this chapter were used to verify the performance parameters listed in Section 3.

5.1 Endpoints and Precision

The luminometer provided with the ToxScreen-II reported the absolute light units for each sample analyzed. Each DDW sample was compared with a negative control sample that, for this verification test, was unspiked DDW. This comparison was made by accounting for the inhibition of the negative control in the calculation of the percent inhibition. Therefore, the percent inhibition of the four negative control samples within each sample set always averaged zero. The percent inhibition for each sample was calculated using the following equation:

$$\%\ \text{inhibition} = \left(1 - \frac{L_{\text{sample}}}{\bar{L}_{\text{negative control}}}\right) \times 100\% \qquad (2)$$

Where L is the absolute light units produced for each test sample and $\bar{L}_{\text{negative control}}$ is the average negative control of the four negative control samples analyzed in the same sample set as the subject test sample. The negative control sample was always DDW, except when the inhibition of the disinfectant by-products was being determined, in that case, ASTM Type II DI water served as the control sample.

The standard deviation (*SD*) of the results for the replicate samples was calculated, as follows, and used as a measure of technology precision at each concentration. The standard deviation around the average negative control results represented the variability of the inhibition caused by the negative control water. Similarly, the standard deviation of the rest of the contaminant concentrations represented the precision of the inhibition caused by the background water combined with the contaminant.

$$SD = \left[\frac{1}{n-1}\sum_{k=1}^{n}\left(I_k - \bar{I}\right)^2\right]^{1/2} \qquad (3)$$

where n is the number of replicate samples, I_k is the percent inhibition measured for the k^{th} sample, and $\bar{I}$ is the average percent inhibition of the replicate samples. Because the average inhibition was frequently near zero for this data set, relative standard deviations often would have greatly exceeded 100%, making the results difficult to interpret. Therefore, the precision results were left in the form of standard deviations of the percent inhibition so the reader could easily view the uncertainty around the average percent inhibition for results that were both near zero and significantly larger than zero.

5.2 Toxicity Threshold

The toxicity threshold was defined as the lowest concentration of contaminant to exhibit an average inhibition significantly greater than the negative control. Also, each concentration level higher than the toxicity threshold had to be significantly greater than the negative control, and the inhibition produced by each lower concentration analyzed had to be significantly less than that produced by the toxicity threshold concentration. Since the inhibition of the test samples was calculated with respect to the inhibition of each negative control sample, the percent inhibition of the negative control was always zero. A significant difference in the average inhibition at two concentration levels required that the average inhibition at each concentration level, plus or minus its respective standard deviation, did not overlap.

CheckLight Ltd. suggests that a 50% inhibition be attained for a conclusive indication of toxicity; however, for this test, a more thorough evaluation of sensitivity was performed. Therefore, the toxicity threshold was determined as described here, and the 50% inhibition threshold was used for the false negative/false positive evaluation.

5.3 False Positive/Negative Responses

A response was considered false positive if an unspiked drinking water sample produced an inhibition greater than 50% when determined with respect to DI water. Depending on the degree of background inhibition in a sample, toxicity from subsequent contamination of that sample may not be detectable or could be exaggerated as a result of the baseline inhibition. Drinking water samples collected from water systems using chlorination and chloramination as the disinfecting process were analyzed in this manner.

A response was considered false negative if, when a lethal concentration of some contaminant was analyzed, the average inhibition did not exceed 50%, was not significantly different from the negative control, or was not significantly different from the other concentration levels analyzed (for lethal dose inhibition less than 100%). The inhibition of the lethal dose sample was required to be significantly greater than the other concentration levels because it more thoroughly incorporated the uncertainty of all the measurements made by the ToxScreen-II in determining false negative results. A difference was considered significant if the average inhibition plus or minus the standard deviation did not encompass the value or range of values that were being compared. In addition, background water samples that increased the light production of the ToxScreen-II organisms (i.e., negative inhibition) were considered false negative because such samples could cancel out the effect of a contaminant that inhibits light production, making it seem that the contaminant had no toxic effect.

5.4 Other Performance Factors

Ease of use (including clarity of the instruction manual, user-friendliness of software, and overall convenience) was qualitatively assessed throughout the verification test through documented observations of the operators and Verification Test Coordinator. Sample throughput was evaluated quantitatively based on the number of samples that could be analyzed per hour.

Chapter 6
Test Results

6.1 Endpoints and Precision

Tables 6-1a-m present the percent inhibition data for 10 contaminants; and Table 6-2 gives the percent inhibition data for preservatives with concentrations similar to what would be contained in a lethal dose of botulinum toxin complex B, ricin, soman, and VX. Given in each table are the concentrations analyzed, the percent inhibition results for each replicate at each concentration, and the average and standard deviation of the inhibition of the four replicates at each concentration. Contaminant test samples that produced negative percent inhibition values indicated an increase in light production by the bacteria and were considered non-toxic.

6.1.1 Contaminants

The contaminants that were analyzed by ToxScreen-II during this verification test produced results that differed depending on whether the Pro-Organic or Pro-Metal buffer was used (Tables 6-1a-m). The inhibition in both buffers was determined for each contaminant at the concentration levels indicated in the tables. Since the buffers were developed to enhance the sensitivity of specific classes of compounds (metal or organic pollutants), the results were expected to show this difference.

In the Pro-Organic buffer, all the contaminants except aldicarb, botulinum toxin complex B, soman, and VX exhibited an inhibition that was significantly larger than the negative control. Aldicarb generated a positive average inhibition at only the lethal dose concentration. However, the uncertainties around all of the aldicarb measurements, including the negative control, were rather large; therefore, not even the lethal dose sample was significantly different from the negative control. Thallium sulfate produced a detectable inhibition at the top three concentration levels, colchicine and dicrotophos generated a detectable inhibition for the lethal dose and the first tenfold dilution concentration level, while nicotine generated a detectable inhibition at the lethal dose concentration. Additional dilutions for cyanide, dicrotophos, and nicotine were performed to more closely determine the toxicity threshold (Tables 6-1e, g, and i). ToxScreen-II was especially sensitive to cyanide. The first four concentrations that were analyzed (250, 25, 2.5, 0.25 mg/L) produced an inhibition that was significantly larger than the negative control;

Table 6-1a. Aldicarb Percent Inhibition Results

Concentration (mg/L)	Pro-Organic Buffer			Pro-Metal Buffer		
	Inhibition (%)	Average (%)	Standard Deviation (%)	Inhibition (%)	Average (%)	Standard Deviation (%)
Negative Control	54	0	45	31	0	38
	-6			-13		
	7			-48		
	-55			30		
0.26	-62	-50	12	-28	-31	24
	-55			-55		
	-33			-42		
	-50			1		
2.6	-10	0	12	53	33	35
	-9			44		
	3			-19		
	14			55		
26	-48	-26	18	-8	-7	13
	-29			7		
	-22			-24		
	-5			-5		
260 (Lethal Dose)	34	50	15	-22	-19	10
	42			-30		
	60			-10		
	65			-11		

Table 6-1b. Botulinum Toxin Complex B Percent Inhibition Results

Concentration (mg/L)	Pro-Organic Buffer			Pro-Metal Buffer		
	Inhibition (%)	Average (%)	Standard Deviation (%)	Inhibition (%)	Average (%)	Standard Deviation (%)
Negative Control	-42	0	46	7	0	52
	-21			12		
	-3			53		
	65			-73		
0.0003	-77	-54	58	-70	-40	26
	-92			-9		
	-79			-33		
	32			-51		
0.003	-16	16	21	-102	-91	60
	31			-10		
	23			-96		
	25			-154		
0.03	45	14	25	-104	-121	18
	5			-107		
	20			-138		
	-15			-136		
0.3 (Lethal Dose)	-29	-87	44	-217	-185	104
	-104			-274		
	-133			-215		
	83			-35		
Lethal Dose Preservative Blank	52	-6	46	-256	-212	57
	-51			-129		
	7			-220		
	-31			-243		
0.3 (Lethal Dose)	-21	-77	42	-2	9	33
	-93			-20		
	-120			-1		
	-73			57		
Lethal Dose Preservative Blank	55	0	43	-14	0	18
	-43			27		
	12			-3		
	-24			10		

Shading indicates inhibition calculated with respect to the preservative blank.

Table 6-1c. Colchicine Percent Inhibition Results

Concentration (mg/L)	Pro-Organic Buffer			Pro-Metal Buffer		
	Inhibition (%)	Average (%)	Standard Deviation (%)	Inhibition (%)	Average (%)	Standard Deviation (%)
Negative Control	-2	0	3	3	0	2
	3			-1		
	-2			-1		
	1			-1		
0.24	4	1	3	-17	-9	6
	0			-6		
	2			-6		
	-2			-6		
2.4	1	4	3	1	8	4
	[a]			11		
	7			9		
	3			10		
24	16	17	5	-1	2	4
	11			3		
	23			-1		
	19			7		
240 (Lethal Dose)	74	75	2	13	12	2
	78			14		
	73			9		
	73			12		

[a] Deleted -98% because it was an outlier.

Table 6-1d. Cyanide Percent Inhibition Results

Concentration (mg/L)	Pro-Organic Buffer			Pro-Metal Buffer		
	Inhibition (%)	Average (%)	Standard Deviation (%)	Inhibition (%)	Average (%)	Standard Deviation (%)
Negative Control	-3	0	4	-1	0	8
	0			-3		
	-3			11		
	6			-7		
0.25	72	72	2	17	19	2
	69			18		
	71			19		
	74			20		
2.5	95	95	0	46	44	2
	95			43		
	95			42		
	96			44		
25	100	100	0	75	64	7
	100			62		
	100			59		
	100			62		
250 (Lethal Dose)	100	100	0	89	89	1
	100			88		
	100			88		
	100			89		
Negative Control (3-hour incubation	-9	0	9	-22	0	17
	-3			-1		
	12			4		
	1			19		
250 (3-hour incubation)	98	99	1	90	91	1
	99			91		
	100			92		
	99			91		
Negative Control (24-hour incubation)	10	0	11	0	0	4
	-7			3		
	-11			3		
	8			-6		
250 (24-hour incubation)	98	98	0	95	96	1
	98			97		
	97			98		
	98			95		

Shading indicates results from field portability testing.

Table 6-1e. Cyanide Percent Inhibition Results—Additional Dilutions

Concentration (mg/L)	Pro-Organic Buffer		
	Inhibition (%)	Average (%)	Standard Deviation (%)
Negative Control	-4	0	11
	12		
	-14		
	6		
0.00025	-15	-11	3
	-9		
	-10		
	-11		
0.0025	-16	-13	3
	-12		
	-9		
	-14		
0.025	-10	-14	3
	-17		
	-15		
	-15		
0.25	32	31	2
	29		
	31		
	34		

Table 6-1f. Dicrotophos Percent Inhibition Results

Concentration (mg/L)	Pro-Organic Buffer			Pro-Metal Buffer		
	Inhibition (%)	Average (%)	Standard Deviation (%)	Inhibition (%)	Average (%)	Standard Deviation (%)
Negative Control	-1	0	2	1	0	2
	1			0		
	-2			-2		
	2			2		
1.4	4	1	5	-6	-3	4
	6			-3		
	0			2		
	-5			-5		
14	2	3	3	-11	-10	3
	1			-8		
	2			-8		
	8			-15		
140	12	23	7	-1	-1	2
	27			2		
	26			-3		
	29			-1		
1,400 (Lethal Dose)	84	70	29	57	55	2
	25			54		
	84			54		
	84			54		

Table 6-1g. Dicrotophos Percent Inhibition Results—Additional Dilutions

Concentration (mg/L)	Pro-Organic Buffer			Pro-Metal Buffer		
	Inhibition (%)	Average (%)	Standard Deviation (%)	Inhibition (%)	Average (%)	Standard Deviation (%)
Negative Control	2	0	2	1	0	2
	-1			-2		
	2			2		
	-3			-1		
140	4	2	2	8	8	1
	-1			8		
	3			10		
	1			8		
500	7	8	1	13	14	1
	7			15		
	9			14		
	9			14		
1,000	15	19	4	21	21	2
	20			20		
	20			25		
	23			20		
1,400 (Lethal Dose)	27	26	3	21	19	2
	22			18		
	29			18		
	25			21		

Table 6-1h. Nicotine Percent Inhibition Results

Concentration (mg/L)	Pro-Organic Buffer			Pro-Metal Buffer		
	Inhibition (%)	Average (%)	Standard Deviation (%)	Inhibition (%)	Average (%)	Standard Deviation (%)
Negative Control	19	0	13	-2	0	4
	-9			-4		
	-2			6		
	-8			0		
2.8	-17	-20	5	-5	-7	2
	-16			-5		
	-22			-10		
	-27			-8		
28	-41	-32	9	-16	-10	4
	-20			-7		
	-36			-10		
	-31			-9		
280	-9	-10	10	3	2	2
	-18			-1		
	-16			1		
	3			3		
2,800 (Lethal Dose)	82	83	2	98	98	0
	84			98		
	84			99		
	81			98		

Table 6-1i. Nicotine Percent Inhibition Results—Additional Dilutions

Concentration (mg/L)	Pro-Organic Buffer			Pro-Metal Buffer		
	Inhibition (%)	Average (%)	Standard Deviation (%)	Inhibition (%)	Average (%)	Standard Deviation (%)
Negative Control	2	0	19	-6	0	4
	25			3		
	-6			0		
	-20			3		
700	14	22	7	20	16	3
	21			14		
	32			12		
	20			16		
1,400	79	79	2	75	72	3
	77			75		
	79			71		
	80			68		
2,100	55	57	3	85	85	1
	58			86		
	61			84		
	55			84		
2,800 (Lethal Dose)	64	64	3	96	96	0
	67			96		
	60			96		
	66			96		

Table 6-1j. Ricin Percent Inhibition Results

Concentration (mg/L)	Pro-Organic Buffer			Pro-Metal Buffer		
	Inhibition (%)	Average (%)	Standard Deviation (%)	Inhibition (%)	Average (%)	Standard Deviation (%)
Negative Control	-1	0	6	0	0	4
	-6			-4		
	-1			-2		
	9			6		
0.015 (Lethal Dose/ 1,000)	12	10	5	0	2	2
	16			2		
	4			2		
	7			4		
Lethal Dose/1,000 Preservative Blank	12	14	6	1	1	1
	12			3		
	9			-1		
	22			1		
0.15 (Lethal Dose/ 100)	-2	1	3	3	2	2
	3			4		
	4			-1		
	0			0		
Lethal Dose/100 Preservative Blank	-1	3	3	-2	-2	1
	3			0		
	6			-3		
	5			-1		
1.5 (Lethal Dose/10)	7	9	2	3	-2	4
	11			-5		
	9			-6		
	7			1		
Lethal Dose/10 Preservative Blank	22	18	6	3	2	2
	13			5		
	14			3		
	26			-1		
15 (Lethal Dose)	67	68	3	7	3	3
	66			-1		
	68			3		
	73			1		
Lethal Dose Preservative Blank	67	66	2	3	1	6
	63			9		
	66			-4		
	69			-5		

Table 6-1k. Soman Percent Inhibition Results

Concentration (mg/L)	Pro-Organic Buffer			Pro-Metal Buffer		
	Inhibition (%)	Average (%)	Standard Deviation (%)	Inhibition (%)	Average (%)	Standard Deviation (%)
Negative Control	3	0	25	-25	0	21
	-18			27		
	34			2		
	-20			-4		
0.0014	-8	15	23	-14	-4	13
	25			14		
	43			-13		
	0			-3		
0.014	33	4	33	-80	-66	17
	-30			-62		
	-18			-44		
	33			-78		
0.14	-304	-202	68	-14	17	22
	-160			24		
	-175			40		
	-170			17		
1.4 (Lethal Dose)	-2	-6	10	-40	-55	17
	-5			-55		
	-21			-80		
	2			-47		
Lethal Dose Preservative Blank	13	-18	35	41	27	26
	-27			31		
	4			46		
	-64			-10		

Table 6-1l. Thallium Sulfate Percent Inhibition Results

Concentration (mg/L)	Pro-Organic Buffer			Pro-Metal Buffer		
	Inhibition (%)	Average (%)	Standard Deviation (%)	Inhibition (%)	Average (%)	Standard Deviation (%)
Negative Control	0	0	2	2	0	1
	0			0		
	-2			0		
	2			-1		
2.8	3	-1	3	7	4	4
	-4			0		
	0			7		
	-3			2		
28	2	9	6	32	27	4
	11			27		
	8			23		
	16			25		
280	21	13	5	53	53	2
	9			51		
	12			55		
	11			54		
2,800 (Lethal Dose)	68	66	3	79	79	1
	68			81		
	66			78		
	63			79		

Table 6-1m. VX Percent Inhibition Results

Concentration (mg/L)	Pro-Organic Buffer			Pro-Metal Buffer		
	Inhibition (%)	Average (%)	Standard Deviation (%)	Inhibition (%)	Average (%)	Standard Deviation (%)
Negative Control	1	0	2	1	0	5
	-2			5		
	3			-8		
	-2			2		
0.002	-6	-6	3	-6	-3	5
	-4			3		
	-4			-1		
	-10			-9		
0.02	1	2	5	-24	-11	10
	-3			-11		
	10			-11		
	2			1		
0.2	-10	-5	5	-18	-11	7
	-8			-5		
	-4			-4		
	1			-18		
2.0 (Lethal Dose)	3	-3	9	8	5	6
	-8			5		
	-13			9		
	6			-3		
Lethal Dose Preservative Blank	-12	-9	3	-3	-10	12
	-12			-20		
	-9			-21		
	-5			2		

therefore, additional dilutions had to be performed to reach a concentration that did not produce detectable inhibition. These additional dilutions confirmed that the lowest concentration that generated detectable inhibition was 0.25 mg/L. Interestingly, upon reanalysis, the 0.25 mg/L sample produced less than half of the inhibition that it did upon the initial analysis. There wasn't a clear reason for this.

In the Pro-Metal buffer, colchicine, cyanide, dicrotophos, nicotine, and thallium sulfate exhibited an inhibition that was significantly greater than the negative control. Colchicine produced an inhibition that was significantly greater than the negative control at the 240- and 2.4-mg/L concentration levels. However, samples at 24 and 0.24 mg/L were not significantly greater than the negative control. All four cyanide concentrations generated an inhibition significantly different from the negative control. Inhibition produced by dicrotophos were significantly different from the negative control only at the lethal dose level, so additional dilutions were performed to elucidate the toxicity threshold of dicrotophos (Table 6-1g); and, during the additional analyses, all four concentrations that were analyzed (between 1,400 mg/L and 140 mg/L) generated an inhibition significantly larger than the negative control. During the analysis of the additional dilutions, the lethal dose sample produced an inhibition of 19% ± 2% compared to an inhibition of 55% ± 2% during the first analysis. There was no clear reason for this discrepancy. Similarly, upon initial analysis of nicotine, only the lethal dose generated an inhibition that was significantly greater than zero. Upon analysis of several concentrations

between the lethal dose and the first tenfold dilution (Table 6-1i), it was shown that nicotine concentrations between the lethal dose and 700 mg/L generated an inhibition significantly greater than zero. As in the Pro-Organic buffer, thallium sulfate produced a detectable inhibition at the top three concentration levels analyzed.

It is important to note that the botulinum toxin complex B, ricin, soman, and VX stock solutions used to prepare the test samples for this verification test were stored in various preservatives that included sodium azide, sodium chloride, and sodium phosphate for ricin; sodium citrate only for botulinum toxin complex B; and isopropyl alcohol for soman and VX. During the previous ETV test of this technology category, the preservatives were not accounted for in the negative control; therefore, the results from each test should be interpreted accordingly. The results for this test are more thorough because they show the sensitivity (or lack thereof) to both the preservative and the contaminant. In the in the earlier verification test, toxicity could have been the result of either. Table 3-2 details the concentrations of preservatives in the lethal dose samples of each contaminant. These data could be evaluated in two ways to determine the sensitivity of ToxScreen-II to contaminants stored in preservatives. The first approach would be to determine the inhibition of the test samples containing preservatives with respect to the background negative control, as was the case with the contaminants that were not stored in preservatives. This technique, however, could indicate that ToxScreen-II was sensitive to the contaminant when, in fact, it was sensitive to one of the preservatives. Since these contaminants are only available (either commercially or from the government) in aqueous formulations with the preservatives, this may be appropriate. The second approach would be to fortify negative control samples with the same concentrations of preservative contained in all the samples so that the inhibition resulting from the preservatives could be subtracted from the inhibition caused by the contaminant. This approach would greatly increase the number of samples required for analysis. Therefore, for this test, aspects of both approaches were incorporated without substantially increasing the number of samples. Negative control samples fortified with a concentration of each preservative equivalent to the concentration in the lethal dose test samples (preservative blanks) were analyzed prior to and with every set of test samples. For those sets of test samples for which it was especially difficult to determine whether inhibitory effects were from the contaminant or the preservative, the preservative blank was diluted identically to all the contaminant samples and analyzed so a background subtraction could take place if necessary.

During the initial analysis of the preservative blanks (Table 6-2), the only sample that generated an inhibition significantly different from the unfortified negative controls was the sample representing the ricin preservative, with an inhibition of 61% ± 3% in the Pro-Organic buffer.

Therefore, for the ricin test samples, all of the preservative blanks were diluted with the same concentration of preservatives as the test samples containing ricin. For the other contaminant test samples, only the samples containing preservatives equivalent to those of the lethal dose were analyzed with the contaminant samples.

Using the Pro-Organic buffer, the inhibition of botulinum toxin complex B was not significantly different from the DDW negative control or the preservative blank. Note that the lethal dose concentration sample did generate an average inhibition that was considerably more negative than the lethal dose preservative blank. According to Checklight Ltd., it is possible that this increase in luminescence could indicate possible toxicity. However, during this evaluation, the large uncertainty surrounding these average results make it difficult to be confident that the

average inhibition is different from the controls. Checklight Ltd. also noted that it is possible that botulinum toxin complex B inhibition is linear over a smaller concentration range than was evaluated during this verification test. While that is true, the higher concentrations would be expected to generate some level of toxicity if the lower concentrations did. In the case of botulinum toxin complex B, the large relative variability at each concentration level made any distinctions from the negative control difficult to determine. In the Pro-Metal buffer, the negative average inhibition of the botulinum toxin complex B samples increased as the sample concentration increased. Unexpectedly, the preservative blank also generated a very large negative inhibition (-212% ± 57%), while previously it had generated an inhibition of -10% ± 2%. The uncertainties surrounding this inhibition data were rather large. As a result there was no clear trend of distinctly increasing or decreasing inhibition with concentration, and the inhibition was all negative. However, because the preservative blank analyzed with this set of test samples contained the same preservative concentration as the lethal dose sample, the inhibition of the 0.3-mg/L sample was calculated with respect to the preservative blank. When performing the calculation in this

Table 6-2. Lethal Dose Level Preservative Blank Percent Inhibition Results

Preservative Blank	Pro-Organic Buffer			Pro-Metal Buffer		
	Inhibition (%)	Average (%)	Standard Deviation (%)	Inhibition (%)	Average (%)	Standard Deviation (%)
Negative Control	-5	0	4	-4	0	4
	0			-1		
	0			-1		
	5			6		
Ricin	59	61	3	14	3	8
	58			0		
	62			-1		
	66			-3		
Soman/VX[(a)]	0	-2	4	2	-2	3
	-6			-5		
	-4			-4		
	3			0		
Botulinum Toxin Complex B	-7	0	8	-11	-10	2
	-6			-8		
	5			-9		
	8			-12		

[(a)] Soman and VX use the same preservative.

way, the inhibition of the preservative blank was 0% ± 18%, and the inhibition of the lethal dose of botulinum toxin complex B was 9% ± 33%, indicating the lack of a toxic effect from botulinum toxin complex B. The large negative inhibition when calculated with respect to the DDW negative control did indicate that, during this set of analyses, the preservative in the botulinum toxin complex B samples was causing enhanced luminescence in both the preservative blank sample and the botulinum toxin complex B samples. Further, even though no additional preservative blank samples were analyzed, results from the less concentrated botulinum toxin complex B samples generated progressively fewer negative results as the concentration decreased. Direct comparison of the lethal dose botulinum toxin complex B sample and the preservative blank showed that the botulinum toxin complex B exhibited no toxicity, strongly

suggesting that the decreasing enhanced luminescence shown by the increased dilutions of the botulinum toxin complex B really indicates the increased dilution of the preservative.

As explained previously, the original analysis of the ricin preservative blank test samples resulted in the preservative blank producing a 61% ± 3% inhibition with respect to the DDW negative control shown in Table 6-2 in the Pro-Organic buffer. Therefore, equivalent dilutions of the preservative blank samples were analyzed with the ricin test samples for both the Pro-Organic and the Pro-Metal buffers. With respect to the negative control without preservative, the 15 mg/L sample (68% ± 3%) and the 1.5 mg/L sample (9% ± 2%) generated detectable inhibition. The preservative blank samples corresponding to these contaminant concentrations generated very similar inhibition data. The 15-mg/L preservative blank generated an inhibition of 66% ± 2%, and the 1.5 mg/L preservative blank generated an inhibition of 18% ± 6%; showing that the ricin preservatives, rather than the ricin, probably contribute to a toxic effect on the ToxScreen-II organisms. For ricin in the Pro-Metal buffer, neither the ricin test samples nor the preservative blanks showed an inhibition significantly different from the negative control, indicating that neither ricin nor the ricin preservatives inhibit the ToxScreen-II organisms in the Pro-Metal buffer.

For soman in the Pro-Organic buffer, only the samples at the 0.14-mg/L concentration generated an inhibition significantly different from the negative control. The average inhibition at that concentration was -202% ± 68%. At the other concentrations, as well as for the preservative blank, inhibition was not detectable. As for botulinum toxin complex B, Checklight Ltd. indicated that this enhancement of luminescence could indicate a toxic effect at this concentration; but, again, during this evaluation, the fact that none of the other three concentration levels generated a change from the negative control in either direction didn't seem to support this. Checklight Ltd. also stated that it is possible that the indication of soman's toxicity is linear over a smaller range than was analyzed during this test. While true, if that is the case, it seems unlikely that a higher concentration would not exhibit any inhibition at all. The large negative inhibition for the 0.14 mg/L concentration level was not easily explained; however, the variability in the ToxScreen-II results seemed somewhat higher during the soman analyses than it had been throughout the rest of the verification test. In the Pro-Metal buffer, the 0.014- and the 1.4-mg/L samples both generated an inhibition significantly different from the negative control; however, both were negative. Also, there was no clear trend of positive (or negative) inhibition with concentration; that is, some of the concentrations exhibited an average inhibition that was negative and some that was positive, making an evaluation of the toxic effect of soman difficult to determine. The inhibition for the preservative blank was not significantly different from the negative control, so there did not seem to be a toxic effect from the preservative.

For VX in the Pro-Organic buffer, the average inhibition at each concentration was within 10% of that of the negative control, and none of the concentrations (including the preservative blank) generated a positive inhibition that was significantly different from the negative control. Samples at two concentrations generated an inhibition that was negative and significantly different from the negative control; but again, no matter what the concentration, all of the average inhibition results were within 10% of the negative control, which indicates a minimal toxic effect. In the Pro-Metal buffer, none of the samples (including the preservative blank) exhibited an inhibition that was significantly different from the negative control, indicating a lack of toxic effect for all samples.

6.1.2 Potential Interferences

All of the potential interference samples were prepared in DDW and compared with the negative control to determine the level of inhibition. This determination is crucial because the ability of ToxScreen-II to detect toxicity is dependent on the bacteria's background light production in whatever drinking water matrix is being used. If the background drinking water sample produces 100% inhibition of light, inhibition caused by contaminants could not be detected. However, even if a drinking water sample generated some degree of inhibition, it can still be used as the background sample provided there is adequate background light available to indicate the presence of subsequent contamination.

Table 6-3 presents the results from the samples that were analyzed to test the effect of potential interferences on ToxScreen-II. In the Pro-Organic buffer, none of the potential interferences exhibited an inhibition significantly different from the negative control. In the Pro-Metal buffer, four out of the five metal solutions exhibited a large negative inhibition, while the zinc solution exhibited an 86% inhibition. There was no obvious explanation for the negative inhibition because the positive control analyzed with that sample set (a copper solution) exhibited positive (62%) inhibition as was expected, and the background luminescence from the negative control was rather typical. A negative inhibition, which indicates an increase in light production by the ToxScreen-II bacteria, caused by these solutions does not necessarily mean that these compounds will interfere with the analysis. A direct interference would cause all of the background luminescence to be inhibited. In this case, a DDW sample with similar concentrations of these metals would likely be amenable to the ToxScreen-II because there would actually be an increase in background light that could potentially be inhibited by contaminants. However, for zinc, the background luminescence was 86% depleted, leaving not much available luminescence for inhibition due to contamination. Because of the large, negative inhibition, these four possible interferences were reanalyzed using freshly prepared samples (Table 6-4). During this second analysis, only the copper test sample generated an inhibition that was significantly larger than the negative control. In the Pro-Metal buffer, iron and aluminum produced a slightly negative inhibition, while the average inhibition of manganese was not different from the negative control. In the Pro-Organic buffer, these possible interferences generated an inhibition that was either just slightly negative or not significantly different from the negative control, very similar to the results during the initial analyses of these samples. It is not clear why the results for the Pro-Metal buffer were so different during the initial analysis.

Table 6-3. Potential Interferences Results

Potential Interferences	Concen-tration (mg/L)	Pro-Organic Buffer			Pro-Metal Buffer		
		Inhibition (%)	Average (%)	Standard Deviation (%)	Inhibition (%)	Average (%)	Standard Deviation (%)
Negative control (Metals)	NA	1	0	1	4	0	9
		-2			9		
		-1			-11		
		1			-3		
Aluminum	0.5	-5	-4	4	-353	-395	29
		-6			-400		
		-7			-405		
		2			-420		
Copper	0.6	7	3	3	-277	-299	26
		1			-288		
		4			-296		
		0			-336		
Iron	0.15	1	0	1	-401	-399	18
		0			-373		
		1			-406		
		-1			-414		
Manganese	0.25	6	0	4	-358	-368	15
		-2			-381		
		-2			-351		
		-4			-381		
Zinc	2.5	-2	-1	3	86	86	0
		-4			86		
		-4			86		
		3			86		
Negative control (By-products)	NA	-2	0	3	-1	0	5
		4			4		
		1			-6		
		-2			3		
Chlorination by-products	NA	[a]	18	26	[a]	13	34
Chloramination by-products	NA	-2	-3	3	-89	-75	20
		-5			-94		
		0			-63		
		-6			-54		

NA = Not applicable.
[a] Average inhibition across all DDW negative control samples (N=56).

Table 6-4. Potential Interference Results—Reanalysis

Potential Interferences	Concentration (mg/L)	Pro-Organic Buffer			Pro-Metal Buffer		
		Inhibition (%)	Average (%)	Standard Deviation (%)	Inhibition (%)	Average (%)	Standard Deviation (%)
Negative control	NA	4	0	11	2	0	2
		12			-1		
		-14			2		
		6			0		
Aluminum	0.5	-12	-12	1	-13	-13	3
		-13			-17		
		-13			-10		
		-10			-11		
Copper	0.6	17	5	15	30	30	4
		17			25		
		1			31		
		-16			33		
Iron	0.15	-11	-6	4	-12	-8	3
		-5			-6		
		-6			-9		
		-3			-6		
Manganese	0.25	1	3	3	10	5	4
		1			8		
		3			2		
		6			2		

NA = Not applicable.

To investigate whether the ToxScreen-II is sensitive to by-products of disinfecting processes, DDW samples from water systems that use chlorination and chloramination were analyzed and compared with ASTM Type II DI water as the control sample (these results are presented in Table 6-3). In the absence of a background water sample, it seems likely that DI water may be used as a "clean water" control; therefore, it would be helpful to know what the results would be if this is done. In the Pro-Organic buffer, the sample from the water supply disinfected with chlorination exhibited an inhibition of 18% ± 26% (N=56), while the sample from the water supply disinfected by chloramination exhibited an inhibition of -3% ± 3% on four replicates. The difference in the number of replicates is because the dechlorinated water was used as the negative control with each sample set; therefore, much more data were collected on that water. This suggests that samples that have been disinfected using either process are not likely to interfere with ToxScreen-II results because the inhibition caused by the "clean" drinking water matrix left most of the light to potentially be inhibited by contamination. For the Pro-Metal buffer, the inhibition of the sample from the water supply disinfected by chlorination was 13% ± 34%, and the inhibition of the sample from the water supply disinfected by chloramination was -75% ± 20%. In the former case, interference is unlikely because of the weak inhibition caused by the background; while, in the latter case, the inhibition could be underestimated unless the negative control sample is very similar to the background water sample. For example, if a contaminant that exhibited approximately 75% inhibition was placed in water from a

chloraminated system, and ASTM Type II DI water was used as the reference sample, the percent inhibition would be approximately zero. However, if the chloraminated water was used as the negative control, an appropriate inhibition would be determined. Overall, as long as a similar negative control sample is used for the Pro-Metal buffer, water disinfected using either process is not likely to interfere with the ToxScreen-II results.

6.1.3 Precision

Across all the contaminants and potential interferences, the standard deviation (not relative standard deviation) was measured for each set of four replicates to evaluate ToxScreen-II precision. Out of 170 opportunities, the standard deviation of the four replicate measurements was less than 10% 122 times (72%), between 10% and 20% 24 times (14%), and greater than 20% 24 times (14%). Overall the standard deviation was less than 10% more than twice as often as not. As described in Section 3.2.2, the analysis procedure required that each replicate undergo the entire analysis process; therefore, the measurement of precision represents the precision of the analysis method performed on a single water sample on a given day. The precision does not reflect the repeatability of the method across more than one day or more than one preparation of reagents or more than one operator.

6.2 Toxicity Threshold

Table 6-5 gives the toxicity thresholds, as defined in Section 5.2, for each contaminant. Note the difference between detectability with respect to the negative control and the toxicity threshold with respect to the other concentration levels analyzed. A contaminant concentration level can have an inhibition significantly different from the negative control (thus detectable), but if its inhibition is not significantly different from the concentration levels below it, it would not be considered the toxicity threshold because in the context of this test, its inhibition would not be distinguishable from that of the lower concentrations. The lowest toxicity threshold concentration was for cyanide at 0.25 mg/L when both the Pro-Metal Pro-Organic buffers were used. Note that a concentration level was not determined to be detectable unless all the concentrations below it generated a significantly smaller inhibition.

6.3 False Positive/Negative Responses

The chlorination and chloramination by-product samples at times generated results that were significantly different from the negative control; but, on average, the inhibition of both types of water was less than 50%, therefore not exceeding the inhibition level suggested by CheckLight Ltd. as a minimum for determining toxicity without being considered a false positive result. Since the background inhibition is not complete, it can be accounted for by using negative control samples that are very similar to the water being analyzed. If samples are analyzed daily, a good practice would be to archive a negative control sample each day in case of contamination the next day.

Table 6-5. Toxicity Thresholds

Contaminant	Concentration (mg/L)	
	Pro-Organic	Pro-Metal
Aldicarb	ND	ND
Botulinum toxin complex B	ND	ND
Colchicine	24	ND
Cyanide	0.25	0.25
Dicrotophos	140	140
Nicotine	1,400	700
Ricin	ND	ND
Soman	ND	ND
Thallium sulfate	28	28
VX	ND	ND

ND = Significant inhibition was not detected.

The inhibition of the sample from the water system disinfected by chloramination was -75% ± 20% when using the Pro-Metal buffer. According to Checklight Ltd., a negative inhibition can also indicate toxicity in a sample. If this is the case, this should be considered a false positive result. Ironically, it seems that this phenomenon also introduces the possibility of a false negative response if the reference sample is not similar to the water sample. If ASTM Type II DI water was used as the reference sample, and a contaminant in a chloraminated water sample caused a 75% inhibition, the inhibition would be approximately zero—a false result. In this case, using a reference sample similar to the water sample would solve the problem, but the possibility of false negative results must be considered if ASTM Type II water is used as the reference. A second type of false negative result could occur when a lethal dose of contaminant is present in the water sample and the inhibition is not at least 50%—the lower limit for a positive response according to Checklight Ltd—and significantly different from the negative control. Table 6-6 gives these results. The lethal dose concentration of aldicarb, botulinum toxin complex B, colchicine, ricin, soman, and VX produced an inhibition that either did not exceed 50% or that was not significantly different from the negative control in at least one of the two buffers used by ToxScreen-II. For ricin in the Pro-Organic buffer, the inhibition of the lethal dose was significantly different from the negative control, but not significantly different from the inhibition generated by the preservative blank

Table 6-6. False Negative Responses

Contaminant	Lethal Dose Concentration (mg/L)	False Negative Response[a]	
		Pro-Organic Buffer	Pro-Metal Buffer
Aldicarb	280	yes	yes
Botulinum toxin complex B	0.30	yes	yes
Colchicine	240	no	yes
Cyanide	250	no	no
Dicrotophos	1,400	no	no
Nicotine	2,800	no	no
Ricin	15	[b]	yes
Soman	1.4	yes	yes
Thallium sulfate	2,800	no	no
VX	2.0	yes	yes

[a] Defined as the lethal dose sample having <50% inhibition or not exhibiting an inhibition significantly different from the control.

[b] When compared with the negative control, the ricin was detectable at the lethal dose, however, when that result was compared with the preservative blank, the results was falsely negative.

6.4 Other Performance Factors

6.4.1 Ease of Use

The ToxScreen-II contained instructions with clearly written information and illustrations. Contents of ToxScreen-II were well identified with labels on the vials with the exception of the bacteria. The bacteria vial was labeled on the outer box, but not on the vial itself. Storage requirements were marked on all outer packages. Storage conditions were also marked on the buffer solution vials, but were not on the bacteria or positive control stock vials. Overall, the packaging was easy to open, with the exception of a wax seal on the bacteria vial. All procedures could be carried out at room temperature and were not sensitive to light. ToxScreen-II requires that all samples be analyzed twice, once with the Pro-Organic buffer and once with the Pro-Metal buffer; however, these two tests could be run in parallel.

Prior to rehydration, the bacteria need to be stored at -14° C while all of the other reagents required storage at 2 to 4°C. The procedure required a three-hour wait between bacteria rehydration and testing; however, the vendor recommended that, for optimal performance, the bacteria should be rehydrated the day before use. After preparation, the hydrated bacteria can be used for up to seven days. The freeze-dried bacteria have a shelf-life of one year, while the shelf life of the buffer reagents is eight months when refrigerated.

All equipment was supplied with ToxScreen-II except for pipettes with tips and the ASTM Type II water used to prepare reagents. The luminometer was easy to use and required no calibration before use. The digital display was easy to read, and only one number needed to be recorded. On occasion, consecutive readings of the same sample resulted in a wide range of relative light units. The luminometer was easily wiped clean and did not require any routine maintenance.

No formal scientific education would be required for operation. However, good laboratory skills, especially pipetting, would be beneficial. Verification testing staff were able to operate ToxScreen-II after a brief training session. Approximately 2 mL of liquid waste were generated per sample, along with leftover bacteria and positive control dilutions. In addition, two tubes per sample, bacteria and positive control vials, and pipette tips were generated as solid waste. Information on whether the bacteria, buffers, or positive controls should be considered hazardous waste was available on material safety data sheets available from Checklight Ltd.

6.4.2 Field Portability

ToxScreen-II was transported from a laboratory to a storage room to simulate a situation in which the ToxScreen-II would be operated in a non-laboratory location. The storage room contained several tables and light and power sources, but no other laboratory facilities. During this evaluation of field portability, ToxScreen-II was tested with cyanide at the lethal dose concentration. The inhibition results from this portion of the test are given in Table 6-1d. Two sets of the lethal dose of cyanide were analyzed. The first set was analyzed after the bacteria were incubated for 3 hours, which is the minimum suggested by the vendor, and after a 24-hour incubation, which is what was suggested by the vendor for this verification test. For both incubation times and for both buffers, the inhibition was greater than 90%, nearly complete inhibition. This is similar to the results obtained during the laboratory portion of the test. ToxScreen-II produced an inhibition of 100% and 89% for the Pro-Organic and Pro-Metal buffer respectively.

No carrying case was provided with ToxScreen-II (there is one available for purchase from Checklight Ltd.); however, all materials except the luminometer were transported in a small cardboard box. The box and luminometer were easily carried by one person. ToxScreen-II was easily set up in less than 10 minutes. The luminometer operated on battery power. While the neat bacteria must be kept in a freezer, if the bacteria were reconstituted prior to leaving for the field, they could be stored at refrigerator temperatures until use. ToxScreen-II instructions indicate that the reconstituted bacteria are good for seven days if refrigerated; therefore, they could be reconstituted ahead of time for easier transport to the field. A refrigerator or cooler would be needed to transport the reconstituted bacteria and could also be used to transport the assay buffers; however, the buffers could be kept at ambient temperatures for short field tests (i.e., less than 10 hours). The following items not provided in ToxScreen-II were needed for field use: a cooler to transport and store reagents, high-purity water to prepare positive control dilutions, a timer or watch, and a waste container. Unless reconstituted bacteria are constantly kept available (in a cooler), ToxScreen II would require a minimum lead time of three hours because of the time required for bacteria rehydration. Overall ToxScreen-II was easy to transport to the field and, with the reagents prepared ahead of time, was deployed in a matter of minutes. Analysis of samples was performed as in the laboratory and results were within 60 minutes.

6.4.3 Throughput

Approximately 25 analyses were completed using both the Pro-Organic and Pro-Metal buffers in one hour. The 25 analyses included method blanks and positive controls, as well as test samples. Approximately 1,000 samples could be processed per kit.

Chapter 7
Performance Summary

Pro-Organic Buffer Performance Verification Results

Parameter	Compound	Lethal Dose (LD) Conc. (mg/L)	Average Inhibition at Concentrations Relative to the LD Concentration (%)				Range of Standard Deviations (%)	Toxicity Thresh. (mg/L)
			LD	LD/10	LD/100	LD/1,000		
Contaminants in DDW	Aldicarb	260	50	-26	0	-50	12-18	ND
	Botulinum toxin complex B	0.3	-87	14	16	-54	21-58	ND
	Colchicine	240	75	17	4	1	2-5	24
	Cyanide	250	100	100	95	72	0-2	0.25
	Dicrotophos	1,400	70	23	3	1	3-29	140
	Nicotine	2,800	83	-10	-32	-20	2-10	1,400
	Ricin	15	68[a]	9	1	10	2-5	ND
	Soman	1.4	-6	-202	4	15	10-68	ND
	Thallium sulfate	2,800	66	13	9	-1	3-6	28
	VX	2	-3	-5	2	-6	3-9	ND

			Average Inhibition (%)		Standard Deviation (%)	
	Interference	Conc. (mg/L)	Initial Analysis	Reanalysis[b]	Initial Analysis	Reanalysis[b]
Potential interferences in DDW	Aluminum	0.5	-4	-12	4	1
	Copper	0.6	3	5	3	15
	Iron	0.15	0	-6	1	4
	Manganese	0.25	0	3	4	3
	Zinc	2.5	-1	NR	3	NR

False positive response	None of the potential interferences or disinfection by-product samples produced an inhibition significantly greater than 50%, the inhibition level suggested by CheckLight Ltd. to conclusively determine toxicity.
False negative response	Aldicarb, botulinum toxin complex B, soman, and VX produced an inhibition that either did not exceed 50% or were not significantly different from the negative control at the lethal dose concentrations. For ricin in the Pro-Organic buffer, the inhibition of the lethal dose was significantly different from the negative control, but not significantly different from the inhibition generated by the preservative blank.
Ease of use	ToxScreen-II included clearly written instructions with good illustrations. The contents of the ToxScreen-II were well labeled, making it easy to follow the instructions. A minimum of three hours was required to rehydrate the bacteria; however, for optimal performance the vendor suggests preparing the bacteria the day before use. The bacteria must be stored at -14ºC prior to rehydration. After rehydration, the bacteria can be used for up to seven days. Overall, the ToxScreen-II was easy to use, making it likely that a person with no formal scientific training could conduct the tests.

Field portability	ToxScreen-II was transported from a laboratory to a storage room to simulate operation in a non-laboratory location. It was tested with cyanide at the lethal dose concentration, and the results generated (>90% inhibition) were very similar to those obtained in the laboratory. No carrying case was provided with ToxScreen-II (one is available for purchase from Checkligiht Ltd.); however, all materials except the luminometer were transported in a small cardboard box. The box and luminometer were easily carried by one person, and setup for analysis took less than 10 minutes.
Throughput	Approximately 25 analyses were completed each hour using both buffers, and approximately 1,000 samples could be processed per kit.

ND = Significant inhibition was not detected.
NR = Not reanalyzed.
(a) Inhibition was not significantly different from the preservative blank.
(b) Potential interferences were reanalyzed due to four suspect negative inhibitions during the initial analysis with the Pro-Metal buffer.

Pro-Metal Buffer Performance Verification Results

Parameter	Compound	Lethal Dose (LD) Conc. (mg/L)	Average Inhibition at Concentrations Relative to the LD Concentration (%)				Range of Standard Deviations (%)	Toxicity Thresh. (mg/L)
			LD	LD/10	LD/100	LD/1,000		
Contaminants in DDW	Aldicarb	260	-19	-7	33	-31	10-35	ND
	Botulinum toxin complex B	0.3	-185	-121	-91	-40	18-104	ND
	Colchicine	240	12	2	8	-9	2-6	ND
	Cyanide	250	89	64	44	19	1-7	0.25
	Dicrotophos	1,400	55	-1	-10	-3	2-4	140
	Nicotine	2,800	98	2	-10	-7	0-4	700
	Ricin	15.0	3	-2	2	2	2-4	ND
	Soman	1.4	-55	17	-66	-4	13-22	ND
	Thallium sulfate	2,800	79	53	27	4	1-4	28
	VX	2.0	5	-11	-11	-3	5-10	ND

			Average Inhibition (%)		Standard Deviation (%)	
	Interference	Conc. (mg/L)	Initial Analysis	Reanalysis[a]	Initial Analysis	Reanalysis[a]
Potential interferences in DDW	Aluminum	0.5	-395	-13	29	3
	Copper	0.6	-299	30	26	4
	Iron	0.15	-399	-8	18	3
	Manganese	0.25	-368	5	15	4
	Zinc	2.5	86	NR	0	NR

False positive response	Neither the chlorination nor chloramination samples generated an inhibition greater than 50%. However, the chloramination sample generated a result that indicated an enhancement in luminescence (i.e., a negative inhibition), which, according to Checklight Ltd., can also indicate toxicity.
False negative response	The inhibition of the chloramination by-products was -75% ± 20% with DI water as the negative control. If a contaminant causing a 75% inhibition had been present in this water and DI water was used as the negative control, the inhibition would have been close to 0%—a false negative response. This underscores the need to use negative control samples that are as similar as possible to the samples being analyzed. A second type of false negative response occurred (for aldicarb, colchicine, botulinum toxin complex B, ricin, soman, and VX) when the inhibition was not greater than 50% in the presence of a lethal dose of contaminant.

ND = Significant inhibition was not detected.
NR = Not reanalyzed.
[a] Potential interferences were reanalyzed due to four suspect negative inhibitions during the initial analysis with the Pro-Metal buffer.
See the Pro-Organic Buffer table for descriptions for ease of use, field portability, and throughput.

Chapter 8
References

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