November 2003

Environmental Technology Verification Report

STRATEGIC DIAGNOSTICS INC.

MICROTOX®

RAPID TOXICITY TESTING SYSTEM

Prepared by
Battelle

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EPA U.S. Environmental Protection Agency

ETV ETV ETV

November 2003

Environmental Technology Verification Report

ETV Advanced Monitoring Systems Center

Strategic Diagnostics Inc.
Microtox®
Rapid Toxicity Testing System

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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 seven 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.

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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
EC_{50}	effective concentration causing 50% inhibition
EPA	U.S. Environmental Protection Agency
ETV	Environmental Technology Verification
HDPE	high-density polyethylene
ID	identification
LD	lethal dose
µL	microliter
mL	milliliter
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 Strategic Diagnostics Inc. Microtox® rapid toxicity testing system. Rapid toxicity testing systems were identified as a priority technology 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 Microtox®. Following is a description of Microtox®, based on information provided by the vendor. The information provided below was not subjected to verification in this test.

Microtox® is an *in vitro* testing system that uses bioluminescent bacteria to detect toxins in air, water, soil, and sediment. Microtox® is a metabolic inhibition test that provides both acute toxicity and genotoxic analyses. Microtox® uses a strain of naturally occurring luminescent bacteria,*Vibrio fischeri*. *Vibrio fischeri* are non-pathogenic, marine, luminescent bacteria that are sensitive to a wide range of toxicants. When properly grown, luminescent bacteria produce light as a by-product of their cellular respiration. Cell respiration is fundamental to cellular metabolism and all associated life processes. Bacterial bioluminescence is tied directly to cell respiration, and any inhibition of cellular activity (toxicity) results in a decreased rate of respiration and a corresponding decrease in the rate of luminescence.

Microtox® Model 500 Analyzer was tested as a stand-alone instrument along with the Microtox® reagent. The *Vibrio fischeri* are supplied in a standard freeze-dried (lyophilized) state and, to analyze water samples, are reconstituted in a salt solution, 2.5 milliliters (mL) of the water sample are diluted with 250 microliters (µL) of a Microtox® reagent, then approximately 1 mL of water sample is added to 100 µL of the reconstituted bacteria. Luminescence readings are taken prior to adding the drinking water and then at 5 and 15 minutes after the addition. When analyzing unknown samples, it is recommended that inhibition data be collected at both time intervals to determine the most appropriate data collection time since the rates can vary depending on how the toxicant affects the bacteria. Results are displayed as absolute light units.

Figure 2-1. Microtox® Rapid Toxicity Testing System

The temperature-controlled Microtox® maintains the test organisms and samples at a standard temperature of 15ºC. As such, the Microtox® must be operated in a laboratory setting at ambient temperatures of between 15 and 30ºC. It detects light intensity at 490 nanometers, the wavelength emitted by the bacteria. Microtox® can be used with Microtox®Omni™ software and a personal computer to collect, analyze, track, and store test data. Microtox® weighs 21 pounds, measures 7-1/8 inches x 15-3/8 inches x 16-1/8 inches, and runs on 120/240 volts alternating current. The Microtox® Model 500 costs $17,895, and the reagents cost $360 for approximately 200 samples.

Chapter 3
Test Design and Procedures

3.1 Introduction

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. Rapid toxicity technologies use bacteria (e.g., *Vibrio fischeri*), enzymes (e.g., luciferase), or small crustaceans (e.g., *Daphnia magna*) that either directly, or in combination with reagents, produce a background level of light or use dissolved oxygen at a steady rate in the absence of toxic contaminants. Toxic contaminants in water are indicated by a change in the color or intensity of light produced or by a decrease in the dissolved oxygen uptake rate in the presence of the contaminants.

As part of this verification test, Microtox® 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 Microtox® can detect the toxicity caused by each contaminant, its response to interfering compounds in clean drinking water, such as water treatment chemicals and by-products, was evaluated. Table 3-1 shows the contaminants and potential interferences that were evaluated during this verification test.

This verification test was conducted according to procedures specified in the *Test/QA Plan for Verification of Rapid Toxicity Technologies*.[1] Microtox® was verified by analyzing a dechlorinated drinking water (DDW) sample from Columbus, Ohio, fortified with various concentrations of the contaminants and interferences shown in Table 3-1. Hereafter in this report, DDW will refer to dechlorinated drinking water from Columbus, Ohio. 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.

Table 3-1. Contaminants and Potential Interferences

Category	Contaminant
Carbamate pesticide	aldicarb
Pharmaceutical	colchicine
Industrial chemical	cyanide
Organophosphate pesticide	dicrotophos
Rodenticide	thallium sulfate
Biological toxins	botulinum toxin, ricin
Nerve agents	soman, VX
Potential interferences	aluminum, copper, iron, manganese, zinc, chloramination by-products, and chlorination by-products

Microtox® was evaluated by

- Endpoint and precision—percent inhibition for all concentration levels of contaminants and potential interfering compounds and precision of replicate analyses
- Toxicity threshold for each contaminant
- False negative responses—contaminants that were reported as producing inhibition results similar to the negative control when the contaminant was present at lethal concentrations
- False positive responses—occurrence of inhibition significantly greater than the inhibition reported for unspiked American Society for Testing and Materials (ASTM) Type II deionized (DI) water samples (zero inhibition)
- Ease of use
- Throughput.

3.2 Test Design

Microtox® was used to analyze the DDW sample fortified with contaminants at concentrations ranging from lethal levels to concentrations 1,000 times less than the lethal dose. The lethal dose of each contaminant was determined by calculating the concentration at which 250 mL of water would probably cause the death of a 154-pound person. These calculations were based on toxicological data available for each contaminant. For soman, the stock solution confirmation showed degradation in the water; therefore, the concentrations analyzed were less than anticipated. Whether the concentration is still a lethal dose, as is the case for all contaminants, depends on the characteristics of the individual person and the amount of contaminant ingested.

Inhibition results (endpoints) from four replicates of each contaminant at each concentration level were evaluated to assess the ability of Microtox® to detect toxicity at various concentrations of contaminants, as well as to measure the precision of Microtox® results.

The response of Microtox® 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 approximately 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 from St. Petersburg, Florida, which uses chloramination as its disinfection process, was obtained. 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 and the verification test coordinator.

3.3 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. Using this DDW (Columbus, Ohio, dechlorinated drinking water), individual solutions containing each contaminant and potential interference were prepared and analyzed. The DDW containing the potential interferences was analyzed at a single concentration level, while four dilutions (made using the DDW) were analyzed for each contaminant using Microtox®. Mixtures of contaminants and interfering compounds were not analyzed.

3.3.1 Quality Control Samples

QC samples included method blank samples, which consisted of ASTM Type II DI water; positive control samples, which consisted of ASTM Type II DI water or DDW (depending on vendor preference) fortified with a contaminant and concentration selected by the vendor; and negative control samples, which consisted of the unspiked DDW. The method blank samples were used to help ensure that no sources of contamination were introduced in the sample handling and analysis procedures. Either zinc sulfate or phenol were suggested by the vendor for use as positive control samples, and both were used at times throughout the verification test. While performance limits were not placed on the results, significant inhibition for either of these contaminants indicated to the operator that Microtox® was functioning properly. The negative control sample was used to set a background inhibition of the DDW, the matrix in which each test sample was prepared.

3.3.2 Drinking Water Fortified with Contaminants

Approximately 150 liters of Columbus, Ohio, tap water were collected in a high-density polyethylene (HDPE) container. The sample was dechlorinated with 0.5 mL of 0.4 M sodium thiosulfate for every liter of water. All subsequent test samples were prepared from this DDW and stored in glass containers to avoid chlorine leaching from HDPE containers.

A stock solution of each contaminant was prepared in ASTM Type II DI water at concentrations above the lethal dose level. The stock solution was diluted in DDW 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. Table 3-2 lists each concentration level and the number of samples analyzed at each level.

3.3.3 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 Microtox® 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 test involving the by-products of the chloramination process, an additional water sample was obtained from St. Petersburg, Florida, a city that uses chloramination as its disinfectant procedure. 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.

3.4 Test Procedure

3.4.1 Test Sample Preparation and Storage

A drinking water sample was collected as described in Section 3.3.2 and, because free chlorine kills the bacteria within the Microtox® reagent and can degrade the contaminants during storage, was immediately dechlorinated with sodium thiosulfate. Prior to preparing each stock solution, dechlorination of the water sample was qualitatively confirmed by adding an n,n-diethyl-p-phenylenediamine tablet to a 25-mL aliquot of the DDW. Once dechlorination was confirmed, all the contaminant samples, potential interference samples, and negative control QC samples were made from this DDW, while the method blank sample was prepared from ASTM Type II DI water. The positive control samples were made using ASTM Type II DI water in Class A volumetric glassware. All QC samples were prepared prior to the start of the testing and stored at room temperature for a maximum of 60 days. The aliquots of DDW containing the contaminants were prepared within seven days of testing and stored in the dark at room temperature without chemical preservation. Aliquots to be analyzed by each technology were placed in uniquely labeled sample containers. The sample containers were assigned an identification (ID) number. A master log of the samples and sample ID numbers for each technology was kept by Battelle.

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

Type of Sample	Sample Characteristics	Concentration Levels (mg/L)	No. of Sample Analyses
Quality control	Method blank	NS[a]	9
	Positive control	115 (Phenol) 25 (Zinc sulfate)	9 17
	Negative control (unspiked DDW)	NS	51
DDW fortified with contaminants	Aldicarb	280; 28; 2.8; 0.28	4 per concentration level
	Colchicine	240; 24; 2.4; 0.24	4 per concentration level
	Cyanide	250; 25; 2.5; 0.25	4 per concentration level
	Dicrotophos	1,400; 140; 14; 1.4	4 per concentration level
	Thallium sulfate	2,400; 240; 24; 2.4	4 per concentration level
	Botulinum toxin[b]	0.30; 0.030; 0.0030; 0.00030	4 per concentration level
	Ricin[c]	15; 1.5; 0.15; 0.015	4 per concentration level
	Soman[d]	0.068; 0.0068; 0.00068; 0.000068	4 per concentration level
	VX	0.22; 0.022; 0.0022; 0.00022	4 per concentration level
DDW fortified with potential interferences	Aluminum	0.36	4
	Copper	0.65	4
	Iron	0.069	4
	Manganese	0.26	4
	Zinc	3.5	4
Disinfectant by-products	Chloramination by-products	NS	4
	Chlorination by-products	NS	4

[a] NS = Samples not fortified with any contaminant or potential interference.
[b] Lethal dose solution also contained 3 mg/L phosphate and 1 mg/L sodium chloride.
[c] Lethal dose solution also contained 3 mg/L phosphate, 26 mg/L sodium chloride, and 2 mg/L sodium azide.
[d] Due to the degradation of soman in water, the stock solution confirmation analysis confirmed that the concentration of the lethal dose was 23% of the expected concentration of 0.30 mg/L.

3.4.2 Test Sample Analysis Procedure

To analyze the test samples, the *Vibrio fischeri* were reconstituted in a salt solution, and an aliquot of drinking water was added to a small amount of the reconstituted bacteria. Luminescence readings were taken prior to adding the drinking water and then at 5 and 15 minutes after the addition. When analyzing unknown samples, the vendor recommended collecting inhibition data at both time intervals to determine the most appropriate data collection

time since the rates can vary depending on how the toxicant affects the bacteria. Since these samples were all treated as unknowns, both data collection times were used.

For each contaminant, Microtox® analyzed the lethal dose concentration and three additional concentration levels four times. Only one concentration of potential interference was analyzed. The absolute light units were recorded and the percent inhibition was calculated for each sample. When Microtox® produced percent inhibitions greater than 50% for a contaminant, EC_{50} (effective concentration causing 50% inhibition) values were also calculated and reported. Two operators performed all the analyses using Microtox®. Both held bachelor's degrees in the sciences and spent approximately four hours with the vendor to become familiar with using Microtox®.

3.4.3 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—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 concentrations 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 reporting 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 the concentration of trihalomethanes, haloacetic acids, and total organic halides; turbidity; dissolved organic carbon content; pH; alkalinity; specific conductivity; and hardness. 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 in St. Petersburg, Florida, representing a water system using chloramination as the disinfecting process.

Table 3-3. Dose Confirmation Results

	Method	Average Concentration ± Standard Deviation N = 4 (mg/L)	Background in DDW (mg/L)
Contaminant			
Aldicarb	EPA 531.1[3]	280 ± 28	<0.0007
Colchicine	[a]	NA[b]	NA
Cyanide	EPA 335.1[4]	250 ± 15	0.008
Dicrotophos	EPA SW846 (8141A)[5]	1,400 ± 140	<0.002
Thallium sulfate	EPA 200.8[6]	2,400 ± 24	<0.001
Botulinum toxin	[a]	NA	NA
Ricin	[a]	NA	NA
Soman	[c]	0.068[d] ± 0.001	<0.05
VX	[c]	0.20 ± 0.02	<0.05
Potential Interference			
Aluminum	EPA 200.8	0.36 ± 0.01	<0.10
Copper	EPA 200.8	0.65 ± 0.01	0.011
Iron	EPA 200.8	0.069 ± 0.008	<0.04
Manganese	EPA 200.8	0.26 ± 0.01	<0.01
Zinc	EPA 200.8	3.5 ± 0.35	0.3

[a] No standard method available. QA audits and balance calibration assured accurately prepared solutions.
[b] NA = Not applicable.
[c] Purity analyses performed on chemical and biological agent materials using Battelle standard operating procedures.
[d] The result of the dose confirmation analysis for soman was 23% of the expected concentration of 0.30 mg/L.

Table 3-4. Water Quality Parameters

Parameter	Method	Dechlorinated Columbus, Ohio, Tap Water (disinfected by chlorination)	Dechlorinated St. Petersburg, Florida, Tap Water (disinfected by chloramination)
Turbidity	EPA 180.1[7]	0.1 NTU[a]	0.3 NTU
Organic carbon	SM 5310[8]	2.5 mg/L	2.9 mg/L
Specific conductivity	SM 2510[8]	364 μmho	460 μmho
Alkalinity	SM 2320[8]	42 mg/L	97 mg/L
pH	EPA 150.1[9]	7.65	7.95
Hardness	EPA 130.2[9]	112 mg/L	160 mg/L
Total organic halides	SM 5320B[8]	190 μg/L	83 μg/L
Total trihalomethanes	EPA 524.2[10]	52.8 μg/L	2.4 μg/L
Total haloacetic acids	EPA 552.2[11]	75.7 μg/L	13.5 μg/L

[a]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[12] and the test/QA plan for this verification test.[1]

4.1 Quality Control of Stock Solution Confirmation Methods

The stock solutions for aldicarb, cyanide, dicrotophos, and thallium sulfate were analyzed using a standard reference method at ATEL. 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 and soman, the confirmation analyses were performed at Battelle using a Battelle SOP. Calibration standard recoveries of VX and soman were always between 69% and 130%, and most of the time were between 90% and 100%. Standard analytical methods for colchicine, ricin, and botulinum toxin 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 Microtox® for approximately every 20 drinking water samples that were analyzed. No detectable inhibition was observed in any of these samples. 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 greater than approximately 50% inhibition, it would indicate to the operator that Microtox® was operating incorrectly. For nine positive control samples of phenol, inhibitions of 86% ± 1% and 87% ± 2% were measured at 5 and 15 minutes, respectively. For 17 positive control samples of zinc sulfate, inhibitions of 77% ± 1% and 96% ± 2% were measured at 5 and 15 minutes. These inhibition values indicated the proper functioning of Microtox®. A negative control sample (unspiked DDW) was analyzed with approximately every four samples. The percent inhibition calculation incorporated the inhibition of the negative control; therefore, by definition, the negative control samples had 0% inhibition.

4.3 Audits

4.3.1 Performance Evaluation Audit

The concentration of the standards used to prepare stock solutions of the contaminants and potential interferences was confirmed by analyzing solutions of each analyte prepared in ASTM Type II DI water from two separate commercial vendors using the confirmation methods. The standards from one source were used to prepare the stock solutions during the verification test, while the standards from a second source were used exclusively to confirm the accuracy of the measured concentration of the first source. The percent difference (%D) between the measured concentration of the performance evaluation (PE) sample, and the prepared 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 prepared concentration, and A is the prepared concentration. The %D between the measured concentration of the PE standard and the prepared 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.

Table 4-1. Summary of Performance Evaluation Audit

		Average Measured Concentration ± Standard Deviation (mg/L)	Actual Concentration (mg/L)	Percent Difference
Contaminant	Aldicarb	0.00448 ± 0.000320	0.00500	11
	Cyanide	0.207 ± 0.026	0.200	4
	Dicrotophos	0.00728 ± 0.000699	0.00748	3
	Thallium sulfate	0.090 ± 0.004	0.100	10
Potential interference	Aluminum	0.512 ± 0.013	0.500	2
	Copper	0.106 ± 0.002	0.100	6
	Iron	0.399 ± 0.004	0.400	0.30
	Manganese	0.079 ± 0.003	0.100	21
	Zinc	0.106 ± 0.016	0.100	6

Given the lack of confirmation methodology for some of the contaminants in this verification test, PE audits were not performed for all of the contaminants. 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. To assure the purity of the other standards, documentation, such as certificates of analysis, was obtained for colchicine, botulinum toxin, 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 technical systems audit (TSA) to ensure that the verification test was performed in accordance with the test/QA plan[1] and the AMS Center QMP.[12] 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.

The EPA Quality Manager also conducted a TSA to ensure that the verification test was performed in accordance with the test/QA plan[1] and the AMS Center QMP.[12] As part of the audit, the EPA Quality Manager compared actual test procedures with those specified in the test/QA plan and reviewed data acquisition and sample preparation records and procedures. No significant findings were observed during the EPA TSA. The records concerning the TSA are permanently stored with the EPA Quality Manager.

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.[12] 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 these records 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 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 3.1.

5.1 Endpoints and Precision

Microtox® reports absolute light units as a measure of light intensity (*I*). Each sample was compared with a reference sample that, for this verification test, was unspiked DDW. This comparison was made by calculating gamma (G_t), the ratio of the light lost at time *t* to the light remaining at time *t*, using the two following equations:

$$R_t = \frac{I_{ct}}{I_{ci}} \tag{2}$$

$$G_t = \frac{R_t \cdot I_{si}}{I_{st}} - 1 \tag{3}$$

where R_t corrects for any inhibition induced by the negative control sample and I_{ct} and I_{ci} are the absolute light intensities produced by the negative control at time *t* and at the initial time, *i*, respectively. In addition, I_{si} and I_{st} are the light intensities produced by the water sample at the initial time, *i*, and time *t*, respectively. G_t is converted to percent inhibition using the following equation:

$$\%\ inhibition = \frac{G_t}{1+G_t} \times 100 \tag{4}$$

Percent inhibition data were calculated and are presented with respect to each test sample analyzed as a part of this verification test. For contaminants that induced inhibition of greater than 50%, the concentration of contaminant that affects 50% of the bacteria in the Microtox® reagent (EC_{50}) was estimated from the linear regression of the log of each concentration level of the contaminant versus the percent inhibition. For contaminants that did not induce inhibition of greater than 50%, this calculation was not appropriate.

The standard deviation (*S*) of the results for the replicate samples was calculated, as follows, and used as a measure of technology precision at each concentration.

$$S = \left[\frac{1}{n-1} \sum_{k=1}^{n} \left(I_k - \bar{I} \right)^2 \right]^{1/2} \quad (5)$$

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 inhibitions were frequently near zero for this data set, relative standard deviations would often have greatly exceeded 100%, making the results difficult to interpret. Therefore, the precision results were left in the form of standard deviations so the reader could easily view the uncertainty around the average 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 a percent inhibition significantly greater than the negative control. Also, the inhibition of the toxicity threshold had to be significantly different than the inhibition of the other concentrations analyzed. 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. An inhibition was significantly greater than the negative control if the average inhibition plus or minus the standard deviation did not include zero.

5.3 False Positive/Negative Responses

A response would be considered false positive if an unspiked drinking water sample produced an inhibition significantly greater than zero when determined with respect to ASTM Type II DI water. Depending on the degree of inhibition in the sample, toxicity due to subsequent contamination of that sample may not be detectable or could be exaggerated as a result of the baseline inhibition. To test for this possibility, the percent inhibition of the unspiked drinking water was determined with respect to ASTM Type II DI water. Drinking water samples collected from water systems using chlorination and chloramination as the disinfecting process were analyzed in this manner. An inhibition was considered significantly different from zero if the average inhibition, plus or minus the standard deviation, did not include zero.

A response was considered false negative when Microtox® was subjected to a lethal concentration of some contaminant in the DDW and did not indicate inhibition significantly greater than the negative control (zero inhibition) and the other concentration levels analyzed. Requiring the inhibition of the lethal dose sample to be significantly greater than zero and the other concentration levels more thoroughly incorporated the uncertainty of all the measurements made by

Microtox® in determining a false negative result. 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.

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 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-i present the percent inhibition data for nine contaminants, and Table 6-2 presents data for five potential interferences and the drinking water samples disinfected by both chlorination and chloramination. 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. Results are given for the samples analyzed at five minutes and then again at 15 minutes. For the most part, the results at both time intervals were consistent, but according to the vendor protocol for unknown samples, both data sets were collected and reported. EC_{50} values also are given when applicable. 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 Microtox® during this verification test produced one of two trends apparent from Tables 6-1a-i. Contaminants caused percent inhibitions that, starting from the lowest concentration that produced inhibitions near zero, either increased in proportion to the concentration in the sample so the two highest concentrations had significantly higher inhibition, or did not change considerably, regardless of what concentration was analyzed. Aldicarb, dicrotophos, and thallium sulfate (15 minutes only) fall into the former category, while botulinum toxin, ricin, VX, and soman fall into the latter category. The only exceptions to these trends were colchicine, for which the lethal dose (highest concentration) exhibited a higher percent inhibition than for the rest of the concentration levels, and cyanide, for which the two highest concentrations exhibited a higher percent inhibition than for the other two concentration levels at 5 minutes. At 15 minutes, all four concentration levels of cyanide had distinct inhibitions. For aldicarb, cyanide, and dicrotophos, whose inhibitions increased with concentration and spanned the range from approximately no inhibition to greater than 50% inhibition, EC_{50} values were calculated and reported in Tables 6-1a, 6-1c, and 6-1d. Because inhibitions did not reach 50% for the other contaminants, EC_{50} values could not be calculated.

Table 6-1a. Aldicarb Percent Inhibition Results

Concentration (mg/L)	Results after 5 minutes				Results after 15 minutes			
	Inhibition (%)	Average (%)	Standard Deviation (%)	EC_{50} (mg/L)	Inhibition (%)	Average (%)	Standard Deviation (%)	EC_{50} (mg/L)
0.28	-3 6 2 6	3	4	45	-2 2 2 10	3	5	41
2.8	5 5 7 2	5	2		6 2 8 3	4	3	
28	29 30 31 30	30	1		30 29 33 32	31	2	
280 (Lethal Dose)	78 77 82 79	79	2		79 80 83 80	81	2	

Table 6-1b. Colchicine Percent Inhibition Results

Concentration (mg/L)	Results after 5 minutes				Results after 15 minutes			
	Inhibition (%)	Average (%)	Standard Deviation (%)	EC_{50} (mg/L)	Inhibition (%)	Average (%)	Standard Deviation (%)	EC_{50} (mg/L)
0.24	8 4 2 6	5	3	NA[(a)]	5 2 1 3	3	2	NA[(a)]
2.4	7 6 6 6	6	1		3 4 6 6	5	1	
24	7 5 5 2	5	2		5 3 1 -1	2	3	
240 (Lethal Dose)	12 10 10 11	11	1		11 11 13 13	12	1	

[(a)] NA = Not applicable.

Table 6-1c. Cyanide Percent Inhibition Results

Concentration (mg/L)	Results after 5 minutes				Results after 15 minutes			
	Inhibition (%)	Average (%)	Standard Deviation (%)	EC_{50} (mg/L)	Inhibition (%)	Average (%)	Standard Deviation (%)	EC_{50} (mg/L)
0.25	7 5 5 11	7	3	8	10 5 7 11	8	3	4
2.5	12 5 7 12	9	4		50 41 44 48	46	4	
25	82 83 83 85	83	1		97 96 96 95	96	1	
250 (Lethal Dose)	100 100 100 100	100	0		100 100 100 100	100	0	

Table 6-1d. Dicrotophos Percent Inhibition Results

Concentration (mg/L)	Results after 5 minutes				Results after 15 minutes			
	Inhibition (%)	Average (%)	Standard Deviation (%)	EC_{50} (mg/L)	Inhibition (%)	Average (%)	Standard Deviation (%)	EC_{50} (mg/L)
1.4	1 13 5 9	7	5	160	0 4 0 6	2	3	200
14	11 8 4 8	8	3		10 7 2 4	6	4	
140	41 39 35 34	37	3		39 35 32 33	34	3	
1,400 (Lethal Dose)	82 84 81 81	82	1		80 82 78 79	80	2	

Table 6-1e. Thallium Sulfate Percent Inhibition Results

Concentration (mg/L)	Results after 5 minutes				Results after 15 minutes			
	Inhibition (%)	Average (%)	Standard Deviation (%)	EC_{50} (mg/L)	Inhibition (%)	Average (%)	Standard Deviation (%)	EC_{50} (mg/L)
2.4	3 14 5 4	7	5	NA[a]	2 8 3 2	4	3	NA
24	3 5 6 3	4	2		2 6 5 9	6	3	
240	6 8 5 4	6	1		16 18 16 17	17	1	
2,400 (Lethal Dose)	27 16 14 11	17	7		41 30 27 28	32	6	

[a] NA = Not applicable.

Table 6-1f. Botulinum Toxin Percent Inhibition Results

Concentration (mg/L)	Results after 5 minutes				Results after 15 minutes			
	Inhibition (%)	Average (%)	Standard Deviation (%)	EC_{50} (mg/L)	Inhibition (%)	Average (%)	Standard Deviation (%)	EC_{50} (mg/L)
0.00030	-6 -4 -4 -2	-4	2	NA[a]	-2 -2 -3 0	-2	1	NA
0.0030	3 -2 -1 -3	-1	3		3 -1 -3 -2	-1	2	
0.030	-1 2 -6 -4	-2	3		7 -2 -3 -1	0	5	
0.30 (Lethal Dose)	4 -1 -2 1	0	3		-2 -3 -5 -5	-4	1	

[a] NA = Not applicable.

Table 6-1g. Ricin Percent Inhibition Results

Concentration (mg/L)	Results after 5 minutes				Results after 15 minutes			
	Inhibition (%)	Average (%)	Standard Deviation (%)	EC_{50} (mg/L)	Inhibition (%)	Average (%)	Standard Deviation (%)	EC_{50} (mg/L)
0.015	-2 -1 2 1	0	2	NA[a]	-5 -2 -1 0	-2	2	NA
0.15	3 -3 2 -1	0	3		3 -7 1 1	0	4	
1.5	2 -2 0 -1	-1	2		-1 -4 -2 -7	-4	3	
15 (Lethal Dose)	-1 3 -1 2	0	2		-2 1 -2 0	-1	2	

[a] NA = Not applicable.

Table 6-1h. Soman Percent Inhibition Results

Concentration (mg/L)	Results after 5 minutes				Results after 15 minutes			
	Inhibition (%)	Average (%)	Standard Deviation (%)	EC_{50} (mg/L)	Inhibition (%)	Average (%)	Standard Deviation (%)	EC_{50} (mg/L)
0.000068	1 -6 -1 -9	-4	5	NA[a]	1 -5 -2 -9	-4	4	NA
0.00068	5 1 -4 1	1	3		5 0 -4 -2	0	4	
0.0068	-2 0 -4 0	-2	2		-1 0 -3 -3	-2	2	
0.068[b] (Lethal Dose)	3 2 -3 -4	-1	4		2 3 -2 -2	0	3	

[a] NA = Not applicable.

[b] Due to the degradation of soman in water, the stock solution confirmation analysis confirmed that the concentration of the lethal dose was 23% of the expected concentration of 0.30 mg/L.

Table 6-1i. VX Percent Inhibition Results

Concentration (mg/L)	Results after 5 minutes				Results after 15 minutes			
	Inhibition (%)	Average (%)	Standard Deviation (%)	EC_{50} (mg/L)	Inhibition (%)	Average (%)	Standard Deviation (%)	EC_{50} (mg/L)
0.00022	-1 5 2 1	2	2	NA[(a)]	4 4 2 1	3	2	NA
0.0022	3 11 20 20	14	8		-2 6 16 18	9	9	
0.022	-2 0 1 12	3	7		-3 -3 1 -1	-2	2	
0.22 (Lethal Dose)	32 -4 -2 0	7	17		33 -5 -4 -1	6	18	

[(a)] NA = Not applicable.

6.1.2 Potential Interferences

Table 6-2 presents the results from the samples that were analyzed to test the effect of potential interferences on Microtox®. Aluminum and iron exhibited percent inhibitions near zero, indicating little or no response to those compounds, while manganese exhibited inhibitions of about 9% and zinc at 5 minutes had an inhibition at 6%. Exhibiting higher inhibitions were copper, 40% and 61% at 5 and 15 minutes, respectively, and zinc, 28% at 15 minutes only.

All of the contaminant and potential interference samples were prepared in the DDW and compared with unspiked DDW. Therefore, any background inhibition in the DDW was mathematically corrected. To investigate whether Microtox® is sensitive to by-products of disinfecting processes, DDW from water systems that use chlorination and chloramination were analyzed and compared with ASTM Type II DI water as the control sample. This determination is crucial because the ability of Microtox® to detect toxicity is dependent on the light production of the reagents in a clean drinking water matrix. If clean drinking water produces 100% inhibition of light, the detection of subsequently added contaminants would not be possible. On average, the chlorinated sample exhibited no detectable inhibition, indicating no toxicity, while the chloraminated sample exhibited nearly complete inhibition (87% and 98% at 5 and 15 minutes, respectively. This suggests that samples that have been disinfected by using a chloramination process are likely to interfere with the Microtox® results because the background water sample would completely inhibit the Microtox® reagent.

Table 6-2. Potential Interferences Results

Potential Interferences	Concentration (mg/L)	Results after 5 minutes			Results after 15 minutes		
		Inhibition (%)	Average (%)	Standard Deviation (%)	Inhibition (%)	Average (%)	Standard Deviation (%)
Aluminum	0.36	3 2 0 2	2	1	8 -3 0 0	1	5
Copper	0.65	37 45 41 38	40	3	62 62 60 59	61	1
Iron	0.069	-2 -1 -2 -1	-1	0	-4 -4 -8 -4	-5	2
Manganese	0.26	8 10 7 11	9	2	9 8 5 13	9	3
Zinc	3.5	5 6 7 6	6	1	29 28 29 26	28	1
Chlorination by-products	NA[(a)]	-11 -11 2 1	-5	8	-15 -14 -8 -10	-14	11
Chloramination by-products	NA	86 87 87 88	87	1	98 98 98 98	98	0

[(a)] NA = Not applicable.

6.1.3 Precision

Across all the contaminants and potential interferences, the standard deviation was measured and reported for each set of four replicates to evaluate the Microtox® precision. The standard deviation of the four replicate measurements was greater than 10% for only one sample and, in most cases, it was less than 5%.

6.2 Toxicity Threshold

Table 6-3 gives the toxicity thresholds, as defined in Section 5.2, for each contaminant. The lowest toxicity threshold concentration was for cyanide at 0.25 mg/L after the 15-minute reaction time, indicating that Microtox® was most sensitive to cyanide. For botulinum toxin, ricin, soman, and VX, no inhibition greater than the negative control was detected, regardless of the concentration level, indicating that the technology was not highly responsive to these contaminants.

Table 6-3. Toxicity Thresholds

Contaminant	Concentration (mg/L)[a]
Aldicarb	28
Colchicine	240
Cyanide	25 (5 minutes) 0.25 (15 minutes)
Dicrotophos	140
Thallium sulfate	ND[b] (5 minutes) 240
Botulinum toxin	ND
Ricin	ND
Soman	ND
VX	ND

[a] Unless otherwise noted, toxicity thresholds were the same at 5 and 15 minutes.
[b] ND = Significant inhibition was not detected.

6.3 False Positive/Negative Responses

False positive responses were observed for unspiked chloraminated tap water. As described in Section 6.1.2, for a clean tap water sample that had been disinfected using a chloramination process, Microtox® reported almost complete inhibition (87% and 98%). By-products of the chloramination process apparently inhibited the Microtox® reagent. The water sample treated by chlorination and then subsequently dechlorinated caused no detectable inhibition. Similarly, the method blank samples caused no significant decrease of absolute light units and, therefore, were not the cause for any false positive responses.

A false negative response is when a lethal dose of contaminant is present in the water sample and no inhibition is detected. Table 6-4 gives each contaminant's lethal dose concentration and shows whether or not each contaminant exhibited a false negative response at that concentration level, Microtox® detected an inhibition induced by lethal doses of aldicarb, colchicine, cyanide, dicrotophos, and thallium sulfate (15 minutes only), while botulinum toxin, ricin, soman, and VX inhibitions were not detected at the lethal dose, indicating false negative responses.

Table 6-4. False Negative Responses

Contaminant	Lethal Dose Concentration (mg/L)	False Negative Response
Aldicarb	280	no
Colchicine	240	no
Cyanide	250	no
Dicrotophos	1,400	no
Thallium sulfate	2,400	yes (5 minutes)
		no (15 minutes)
Botulinum toxin	0.30	yes
Ricin	15	yes
Soman	0.068[(a)]	yes
VX	0.22	yes

[(a)] Due to the degradation of soman in water, the stock solution confirmation analysis confirmed that the concentration of the lethal dose was 23% of the expected concentration of 0.30 mg/L.

6.4 Other Performance Factors

The step-by-step pictorial instruction manual for Microtox® was easy to understand, which enabled operators to become quickly adept at analyzing multiple sample sets. The necessity to read the initial light level of the bacteria prior to adding the sample was helpful in confirming the operation of the instrument and the health of the Microtox® reagent prior to the analysis of samples. Microtox®, with only two buttons, was very straightforward to operate. Although the operators had scientific backgrounds, based on observations of the verification test coordinator, an operator with little technical training would probably be able to follow the instructions to analyze samples successfully. The operators analyzed 15 samples per hour. Microtox® was not tested in a non-laboratory setting because it is designed to be only a laboratory benchtop instrument.

Chapter 7
Performance Summary

Parameter	Compound	Lethal Dose (LD) Conc. (mg/L)	Average Inhibitions at Concentrations Relative to the LD Concentration (% after 15 minutes) %				Range of Standard Deviations (%)	Toxicity Thresh. (mg/L)[a]
			LD	LD/10	LD/100	LD/1,000		
Contaminants in DDW	Aldicarb	280	81	31	4	3	2–5	28
	Colchicine	240	12	2	5	3	1–3	240
	Cyanide	250	100	96	46	8	0–4	0.25
	Dicrotophos	1,400	80	34	6	2	2–4	140
	Thallium sulfate	2,400	32	17	6	4	1–6	240
	Botulinum toxin[b]	0.30	-4	0	-1	-2	1–5	ND[c]
	Ricin[d]	15.0	-1	-4	0	-2	2–4	ND
	Soman	0.068[e]	0	-2	0	-4	2–4	ND
	VX	0.22	6	-2	9	3	2–18	ND
Potential interferences in DDW	**Interference**	**Conc. (mg/L)**	**Average Inhibitions at a Single Concentration (% after 15 minutes)**				**Standard Deviation (%)**	
	Aluminum	0.36	1				5	
	Copper	0.65	61				1	
	Iron	0.069	-5				2	
	Manganese	0.26	9				3	
	Zinc	3.5	28				1	
False positive response	Nearly complete (87% and 98%) inhibition in water from a system disinfected by chloramination. Inhibition due to contamination may not be detectable due to background inhibition. Inhibition from water disinfected by chlorination was not significantly greater than zero.							
False negative response	No inhibition was detected for lethal doses of botulinum toxin, ricin, soman, and VX.							
Other performance factors	The pictorial manual was useful, initial light measurements served as a good check of bacterial health and instrument operation, sample handling was easy, and sample throughput was 15 samples per hour. Although the operators had scientific backgrounds, operators with little technical training would probably be able to analyze samples successfully using only the instructions as a guide. Microtox® was not tested in a non-laboratory setting because it is designed to be only a laboratory benchtop instrument.							

[a] See Tables 6-1a-i in the report for the precision around each individual inhibition result.

[b] Lethal dose solution also contained 3 mg/L phosphate and 1 mg/L sodium chloride.

[c] ND = Not detectable.

[d] Lethal dose solution also contained 3 mg/L phosphate, 26 mg/L sodium chloride, and 2 mg/L sodium azide.

[e] Due to the degradation of soman in water, the stock solution confirmation analysis confirmed that the concentration of the lethal dose was 23% of the expected concentration of 0.30 mg/L.

Chapter 8
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