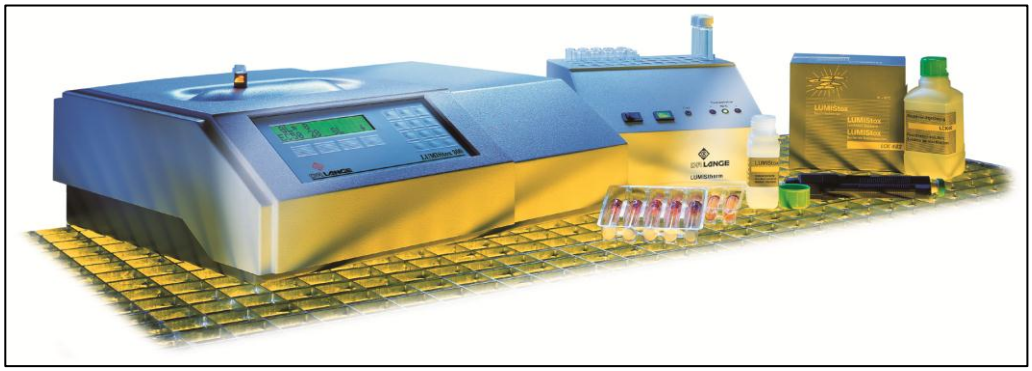
LUMIStox 300 Bench Top Luminometer
ECLOX Handheld Luminometer

Joint verification report

Luminescent bacteria test for use in wastewater

Handheld ECLOX



LUMIStox 300

May 2011

Final

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ECLOX Handheld Luminometer

Joint verification report

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TABLE OF CONTENTS

1 INTRODUCTION

Environmental technology verification (ETV) is an independent (third party) assessment of the performance of a technology or a product for a specified application, under defined conditions and quality assurance.

This verification is a joint verification between Danish Centre for Verification of Climate and Environmental Technologies (DANETV), the U.S. Environmental Technology Verification (U.S. EPA ETV) Advanced Monitoring Systems (AMS) Center and the Canadian ETV Program (ETV Canada). The objective of the verification was to evaluate the performance of a wastewater rapid toxicity technology that can be used to monitor industrial or domestic wastewater.

1.1 Name of products

The verification report covers two products from the same vendor; both are acute toxicity tests with luminescent bacteria. The target products were LUMIStox 300 bench top luminometer and ECLOX handheld luminometer. Both can operate in connection with a LUMIStherm thermostat and the PC software LUMISsoft4, version 2.0.2.56 (December 2009).

1.2 Name and contact of vendor

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Contact: Dr. Elmar Grabert, email: elmar.grabert@hach-lange.de, phone +49 211 5288 241.

Web site: www.hach-lange.de

1.3 Name of center/verification responsible

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Verification responsible: Mette Tjener Andersson, email mta@dhigroup.com, phone +45 16 91 48.

U.S. EPA ETV Advanced Monitoring Systems Center (Battelle), Battelle Memorial Institute, 505 King Avenue, Columbus, Ohio 43201-2693, U.S.A.

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Verification responsible: Mona El Hallak, email melhallak@etvcanada.ca, phone +1 905 822 4133 extension 239.

1.4 Verification test organization

The verification was conducted as a joint verification between the DANETV, the U.S. EPA ETV program and ETV Canada. The verification was planned and conducted to satisfy the requirements of the ETV scheme currently being established by the European Union (EU ETV) as well as the U.S. and Canadian ETV programs. Verification and tests were performed by DHI as DANETV Water Technology ETV Center (DHI DANETV Water Centre) under contract with the Danish Agency for Science, Technology and Innovation. Battelle participated as the manager of the ETV AMS Center through a cooperative agreement with the U.S. Environmental Protection Agency (EPA). ETV Canada participated as manager of the Canadian ETV Program.

The day-to-day operations of the verification and tests were coordinated and supervised by DHI personnel, with participation of the vendor, HACH-LANGE. The testing was conducted in DHI laboratories, Hørsholm, Denmark. DHI personnel operated the luminometers during the verification. HACH-LANGE provided luminometers, thermostats, bacteria, software, user manuals and operation instructions. HACH-LANGE furthermore participated in development of protocol and plans by providing input to DHI. Battelle and ETV Canada ensured that the verification and tests were planned, conducted and reported to satisfy the requirements of the U.S. and Canadian ETV programs, including input and concurrence from their stakeholder groups, as described in the process document /1/. Battelle and ETV Canada also participated in the development of the verification protocol, test plan, verification report, and verification statement and they performed quality assurance (QA) of the verification and tests. The verification protocol, test plan, test report, verification report, and verification statements were reviewed and approved by ETV Canada, while U.S. EPA ETV AMS Center and Environment Canada reviewed and approved all listed documents except the test report.

Three technical experts provided independent expert reviews of the planning documents. Four experts provided reviews of the verification report. The test report is solely a DANETV report; DANETV requires review by one external expert. The test report was therefore reviewed by only one of the external experts.

The chart in Fig 1.1 identifies the relationships of the organizations associated with this verification and test.

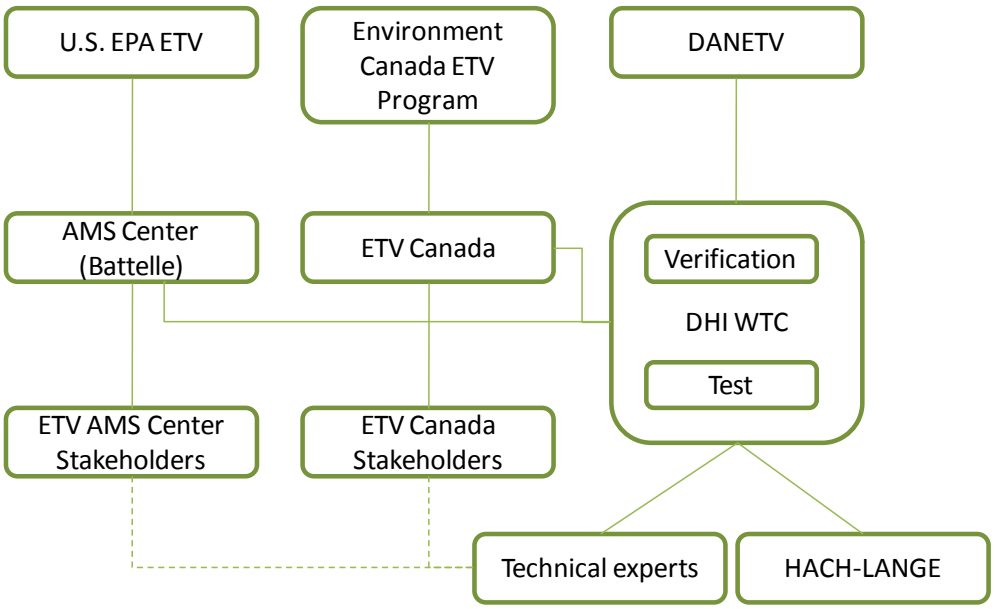

Fig 1.1 Organization of the verification and tests.

1.5 Technical experts

The technical experts are:

Dr. Joel Allen, email: allen.joel@epa.gov, phone +1 513 487 2806. U.S.EPA, Office of Research and Development/National Risk Management Research Laboratory/Water Supply and Water Resources Division/Water Quality Management Branch.

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Dr. Max Lee, email: mmlee@dow.com, phone +1 979 238 7726. Environmental Tech Center, Dow Chemical Company.

1.6 Verification process

The principles of operation with the role of the verification and test documents and the different sub-bodies responsible are given in Fig 1.2.

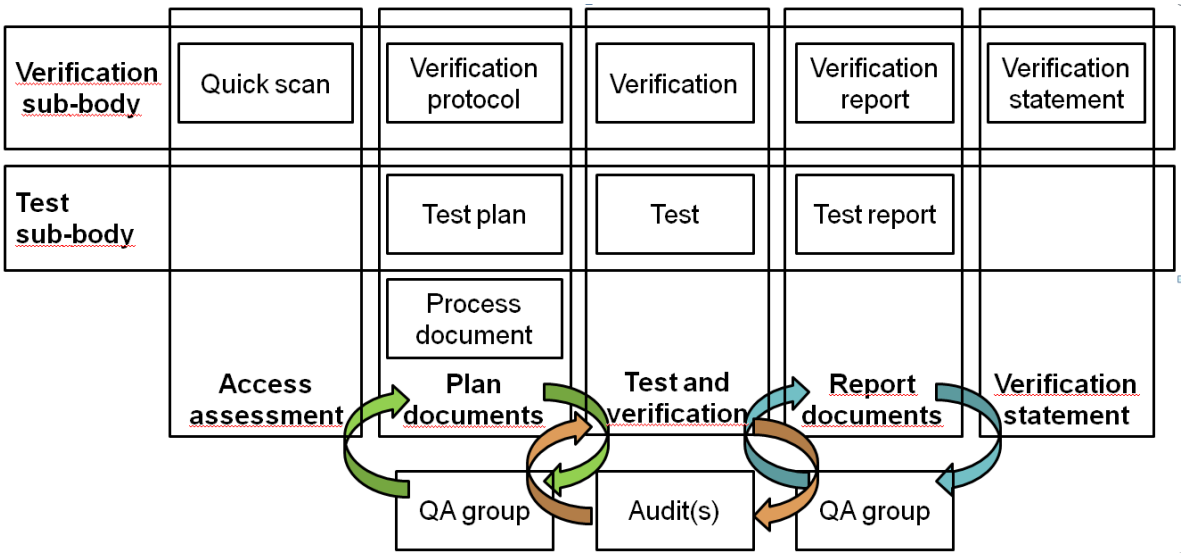

Fig 1.2 Principles of operation of the DANETV verification scheme for joint verification.

The QA group covers the expert group, Battelle, U.S. EPA ETV and ETV Canada. Audits were performed internally by DHI and Battelle for U.S. EPA ETV.

References for the verification process were the Quality Management Plan from Battelle /2/, the General Verification Protocol from ETV Canada /3/ and the Quality Manual for the ETV operations at DHI following the DANETV Quality Manual Template /4/.

The final verification protocol, the test plan, and the above mentioned process document were the planning documents for this verification test.

Two separate joint verification statements, one for each product, were issued after completion of the verification. The results of verification and testing were described in one verification report and one test report covering both the LUMIStox 300 Bench Top Luminometer and the ECLOX Handheld Luminometer.

2 DESCRIPTION OF THE TECHNOLOGY

Luminometers such as LUMIStox and ECLOX are *in vitro* testing systems that use bioluminescent bacteria to detect toxic compounds in water. Luminometers can directly determine toxicity of water soluble chemicals, and from a number of compatible water matrices such as river, lake and wastewater, and leachates from soil, waste or rubble. Bioluminescence tests are metabolic inhibition tests that provide acute toxicity analyses. For the LUMIStox and ECLOX technologies, a strain of naturally occurring luminescent bacteria, *Vibrio fischeri*, is used. *Vibrio fischeri* is a non-pathogenic, marine, luminescent bacterium which is sensitive to a wide range of toxicants and is commonly used in rapid toxicity tests. When properly grown, luminescent bacteria produce light as a by-product of their cellular respiration. Any inhibition of cellular activity results in a decreased rate of respiration and a corresponding decrease in the rate of luminescence. For this verification, the light emission/luminescence was measured with a LUMIStox or ECLOX luminometer.

Inhibition of the light emission in the presence of a sample is determined relative (as percent inhibition) to a non-toxic control. The luminescence is measured after a contact time of five (optional), 15 and 30 minutes at 15 °C, taking into account a correction factor, which is a measure of the control sample's intensity change during the exposure time.

3 DESCRIPTION OF THE PRODUCTS

3.1 LUMIStox 300

The LUMIStox 300 (referred to as "LUMIStox" throughout this report) is a bench top luminometer that has been developed as a measuring unit for the luminescent bacteria test. In combination with the LUMIStherm incubation block, it conforms to the technical requirements of ISO 11348. This ISO standard describes determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri.* The ISO standard contains three parts, using freshly prepared bacteria, liquid-dried bacteria and freeze-dried bacteria, respectively. For the LUMIStox (and ECLOX) freeze-dried bacteria are used. Therefore ISO 11348-3 /23/ applies.

The LUMIStox 300 has a built-in photometer function and an automatic measuring and evaluation routine, which enables it to recognize color effects in the luminescent bacteria test, and to take these into account in the test results.

The photometer function also allows the color effect to be estimated in advance, and can be used to determine the extinction (as OD - optical density) of bacteria suspensions for the purpose of assessing light extinction.

The LUMIStox 300 can be connected to a personal computer running the LUMISsoft4 that enables the operator performing and recording luminescent bacteria tests to conduct all of the ISO 11348-3 requirements. The results from the measurements are percent inhibition, but with use of the software LUMISsoft4 either Lowest Ineffective Dilution (LID) or Effective Concentration (EC) values, representing concentrations causing less than 20%[1] inhibition can be determined. EC values can be extrapolated to concentration values causing 50% inhibition (EC_{50}) using a model not validated in this verification. EC_{50} values are the commonly used results from toxicity tests internationally, while the LID is used as a standard practice in Germany.

3.2 ECLOX

The ECLOX is a portable instrument designed to provide data appropriate for risk assessments in the event of environmental releases, emergency situations, preventive security measures, and regulatory monitoring.

The ECLOX is designed in particular to be used for the luminescent bacteria toxicity test and to be used with a chemiluminescence toxicity test. Both tests will give quick results in the field or in the laboratory. The ECLOX used in the field provides values of percent inhibition.

Additionally, the ECLOX can be used in the laboratory in the same way as the LUMIStox. When the ECLOX is used with the thermal block LUMIStherm and connected to a PC with the software LUMISsoft4, the principles of the luminescent bacteria test according to ISO 11348 can be followed (however, tests performed on the ECLOX are not ISO 11348-3 compatible). For the LUMIStox, the percent inhibition results can be used to calculate LID and EC_{50} values.

[1] LID of 20% inhibition is stated in ISO 11348-3, Annex B, Section B.5.

4 APPLICATION AND PERFORMANCE PARAMETER DEFINITIONS

The application has been defined in detail in Appendix 3 for matrices for use, targets of monitoring, and effects. The application and performance parameters are summarized in this section.

4.1 Application definition

An overview of matrix, effect, targets and technologies is given in Table 4.1.

Table 4.1 Description of matrix, effect, targets and technologies.

Matrix	**Effect**	**Targets**	**Technologies**
LUMIStox and ECLOX are applied for wastewater; river and lake water; leachate from soil, waste, rubble, etc.; or directly in fluent chemicals. Verification testing was conducted on domestic and industrial wastewater effluents.	Measurement of toxicity as indicated by inhibition of luminescent bacteria by a variety of compounds including metal ions, organic pesticides, inorganic and organic pollutants and surfactants. **Additional parameters:** User manual quality, product cost, environmental health and safety.	The target for the application is measurement of toxicity, specifying criterion of detection (CD), range of application, precision (repeatability and reproducibility), agreement with accepted values and robustness.	ECLOX and LUMIStox analyses for inhibition of light emitting luminescent bacterium *Vibrio fischeri.*

4.2 Performance parameters for verification

The performance parameters relevant for the application, as derived in Appendix 3, are presented in Table 4.2. The ranges presented for these parameters were used for planning the verification and testing only and will not be compared to actual performance.

Table 4.2 Relevant ranges of performance parameters in effluent industrial and domestic wastewater.

	Criterion of detection	**Range of application**	**Precision (RSD) %**		**Agreement with accepted values**	**Robust-ness**
			Repeatability	**Reproducibility**		
	% inhibition	Dilution L/L			%	%
LUMIStox	< 10	> 1/2 - < 1/32	< 20	< 30	100±50	100±50
ECLOX	< 10	> 1/2 - < 1/32	< 20	< 30	100±50	100±50

For toxicity testing, it is not possible to determine the limit of detection (LoD). Instead, a criterion of detection (CD) was chosen, above which inhibition is seen as significant, based on the standard deviation of blanks (2% NaCl solution and bacteria suspension, no toxic compound added).

The range of application for a chemical analysis is usually the range of analyte concentration from the limit of detection to the highest concentration with linear response. This concept is not meaningful for a toxicity test of a water sample, because the test does not measure a concentration but an inhibitory effect as a function of the dilution of the sample. The range of application for determining EC_{50} therefore has to be considered in terms of dilution. According to the HACH-LANGE manual, estimation of EC_{50} for a water sample requires a minimum of three measurements where the inhibition is between 10% and 90%. In addition one of the three measurements must be above 50%. If the standard dilution row is considered as described in the LUMIStox 300 operation manual and in Annex B of the ISO 11348-3:2007 with nine dilutions (2, 3, 4, 6, 8, 12, 16, 24, 32 times dilution in the test suspension), then EC_{50} should be in the range of dilutions greater than two and less than 32 times. This assumes three measurements with inhibition between 10 and 90%. Based on test results, ranges of concentrations of the compounds tested should give inhibition within the range of application. The range of application will be given in mg compound/L and is valid for undiluted samples. If samples are more toxic than the maximum value in the range of application, additional dilution shall take place prior to testing. If samples are less toxic, a minimum value in the range of application (EC_{50} values) cannot be determined.

Precision can be evaluated under repeatability and reproducibility conditions. Repeatability is defined as the relative standard deviation of measurements done with the same measurement procedure, operators, measuring system, operating conditions, and location with replicate measurements on the same or similar objects over a short period of time. Reproducibility is defined as the relative standard deviation (RSD) of measurements under different conditions such as locations, operators, and measuring systems with replicate measurements on the same or similar objects. In laboratory terminology, repeatability is the within-series precision and the reproducibility the between-series precision. For reproducibility of luminescent toxicity testing, the difference in bacteria batches is considered to be the greatest source of deviation and is one of the variables which were evaluated in this verification. The other variables were different days and different technicians. Precision has been determined as the RSD of the EC_{20} and EC_{50} results generated during testing.

“Trueness” is the closeness of agreement between the (mean) concentrations found in measurements, and the true or accepted concentration. According to ISO 11348-3 the true or accepted EC_{50} value of a substance is obtained, as long as the criteria in the ISO method are met. For this verification it was chosen to determine trueness as “agreement with accepted values.” This agreement is the inhibition results (EC_{50} values) obtained in the tests compared to robust literature values for EC_{50} values, with clear reference to tests performed according to the ISO 11348-3 method for the same compound. The agreement with accepted values was only determined for test substances where robust literature values were available.

The verified parameters for “robustness” included pH change, temperature change, presence of color or turbid material in the sample, difference in initial concentration

(i.e., lowest dilution of the sample), matrix variation, and type of cuvette. Robustness was the trueness for each of the verified parameters.

Samples were tested with different concentration of color and turbid material, since the ISO standard specified this would cause interference. Available color correction methods were used for both the LUMIStox and ECLOX during the verification.

The ISO 11348-3 recommends testing be performed at a pH range of 7.0±0.20, but stated that pH values of 6.0-8.5 are acceptable. Tests were performed comparing three pH values (6.0, 7.0, and 8.5).

The ISO 11348-3 specifies that a thermostat should be used to cool the test vials to 15±1 °C. A monitored thermostat was used during the verification testing. Tests were performed comparing temperatures of 14.0 °C, 15.4 °C and 16.1 °C.

When testing wastewater samples, it is not always possible to cover the ideal range from 10 to 90% inhibition. Tests were therefore performed with maximum concentrations of approximately 30% and 60% inhibition (EC_{30} and EC_{60}), to see how that affected the determination of EC_{20} and EC_{50}. Initial concentrations causing approximately 30% and 60% inhibition were used to determine $EC_{20.}$ Initial concentrations causing 60% inhibition were used to determine EC_{50}.

Testing of industrial and domestic effluent wastewater samples was included. This included testing of these wastewaters as they were received. To show they were non-inhibitory, these water samples were tested with and without spiking using inhibitory chemicals. These tests were performed to evaluate the effect of the wastewater matrix on the luminescent test.

Typically glass cuvettes are used in the LUMIStox, and plastic cuvettes are used in the ECLOX. HACH-LANGE has stated that plastic cuvettes can also be used in LUMIStox. To be consistent, all tests were performed with plastic cuvettes except for test L, where the LUMIStox was tested for robustness using both types of cuvettes (glass and plastic).

4.3 Additional parameters

Besides the performance parameters obtained by testing, a compilation of parameters describing the ease of understanding the user manual, product costs, and occupational health and safety issues of the product were included in the verification.

5 EXISTING DATA

5.1 Summary of existing data

The vendor recently performed tests with the LUMIStox and ECLOX instruments for determination of precision expressed by the relative standard deviation (RSD). Table 5.1 provides results from HACH-LANGE at a contact time of 15 minutes.

Table 5.1 Results from testing performed by HACH-LANGE of LUMIStox and ECLOX.

Compound	Range 10-90% inhibition	LUMIStox			ECLOX		
		No. of bacteria batches/no. of replicates	EC_{50}	RSD	No. of bacteria batches/no. of replicates	EC_{50}	RSD
	mg/L		mg/L	%		mg/l	%
Cr^{6+}	1.7-27	3/5	6.6	38	1/3	8.6	26
Zn^{2+}	1.5-9.0	2/4	4.3	25	1/3	4.2	15
Pb^{2+}	0.21-2.5	2/4	0.49	8.0	1/3	0.48	8.7
SDS[1]	0.14-2.3	3/6	0.66	16	1/3	0.55	2.8
CTAB[2]	0.33-6.0	2/4	0.84	5.8	1/3	1.1	16
Formaldehyde	4.4-35	2/4	15	9.5	1/3	14	5.1
Hydroquinone	0.03-0.20	2/7	0.09	46	Not tested		
p-Cresol	0.38-6.0	2/4	1.5	33	1/3	1.6	6.6
CN^-	0.51-8.1	2/6	2.7	74	Not tested		

1: Sodium Lauryl Sulphate.

2: Cetyl Trimethyl Ammonium Bromide.

The range 10% to 90% inhibition was the measurement interval used for calculating the EC_{50} values. Ten percent inhibition equals EC_{10}, while 90% inhibition equals EC_{90}. This range for compounds was used as guidance for the test range included in the verification.

It should be mentioned that the RSD was calculated by the vendor as a general RSD including all results, and with no reference to number of samples tested in each bacteria batch. Note that the test of LUMIStox was performed on two to three different bacteria batches, while the test of ECLOX was performed on one bacterial batch only. This resulted in higher RSDs for LUMIStox as compared to ECLOX.

The vendor made a note on results regarding cyanide being difficult to work with in the laboratory at a pH =7.

At pH = 7, almost all cyanide is in the volatile and toxic hydrogen cyanide (HCN) form, and evaporation of HCN can occur.

5.2 Quality of existing data

The tests were performed by the vendor, and not by an independent body. Furthermore, the analyses were not conducted by a laboratory with ISO 17025 accreditation.

5.3 Accepted existing data

No existing data were accepted for use as part of the verification test. However, these data did provide useful background for planning the test.

6 TEST PLAN REQUIREMENTS

Based upon the application and performance parameters identified in Section 4, the requirements for test design were established in the test plan. The detailed test plan was prepared separately, based upon the test requirements summarized below.

6.1 Test design

The outline of the required tests is shown in Table 6.1. More details of the test design can be found in the test report /30/. The principle behind the design was that three test set-ups were used:

- LUMIStox 300 bench top luminometer with LUMIStherm thermostat and LUMISsoft4 PC software. According to ISO 11348-3.
- ECLOX handheld luminometer with LUMIStherm thermostat and LUMISsoft4 PC software. Conditions similar to ISO 11348-3.
- ECLOX handheld luminometer with use of firmware.

Three matrices were used in the testing: spiked 2% sodium chloride (NaCl) MilliQ water, domestic effluent wastewater, and industrial effluent wastewater. Salinity of the wastewaters was increased to 2% by addition of solid NaCl.

Tests were performed with specific compounds in 2% NaCl MilliQ water to determine their EC_{20}- and EC_{50} values. The tests showed the range of responses towards these specific toxic compounds (zinc (Zn^{2+}), chromium (Cr_2O72-), triclosan, cyanide (CN-), sodium lauryl sulphate (SDS) and cetyl trimethyl ammonium bromide (CTAB)). Secondly, tests were performed on effluent wastewater with and without spiking with a toxic compound. This showed the robustness of the luminescent tests towards the wastewater matrix. The last test evaluated the effect on results between use of glass cuvettes and plastic cuvettes in the LUMIStox Benchtop.

Table 6.1 Test design and associated performance parameters.

Test no.	Performance parameters	Equipment			Matrix	
		LUMIStox	ECLOX incl. thermostat and software	ECLOX incl. firmware	2% NaCl MilliQ	Wastewater
A	Range, Repeatability, Agreement with accepted values	x	x		x	
B	Criterion of detection	x	x		x	
C	Robustness, effect of start conc. on repeatability	x	x		x	
D	Reproducibility	x	x		x	
E	Robustness, sample temperature at field use			x	x	
F	Robustness, sample temperature at laboratory use	x	x		x	
G	Robustness, pH	x	x		x	
H	Robustness, color	x	x		x	
I	Robustness, turbidity	x	x		x	
J+K	Robustness, matrix	x	x			x
L	Robustness, cuvettes	x			x	

ISO 11348-3 requires that each batch of bacteria is tested by determining the inhibition by three reference substances. These tests were performed solely on the LUMIStox, since the operation of the ECLOX is not in compliance with the ISO 11348-3.

6.2 Comparable tests and chemical analysis

Reference tests conducted by an independent laboratory using *Vibrio fischeri* following ISO 11348-3 were originally planned. However, the limited utility of the planned reference tests was noted in the Joint Verification Protocol /33/ and in the Joint Test Plan /34/.

The reference tests were intended to be done under ISO 17025 accreditation, using the ISO 11348-3 luminescent bacteria test method with Microtox® equipment. ALcontrol was selected as the independent laboratory to conduct the ISO 11348-3 accredited testing. The results obtained by ALcontrol for one of the reference compounds were lower than anticipated, that is the control compound appeared more toxic than anticipated (see section 7.3.3). The systems audit in section 7.4 identified that ALcontrol was accredited to conduct ISO 11348-3, but used a modified method. After scrutinizing the first set of results from the laboratory, and after subsequent discussions with them, the data impact of the modified ISO 11348-3 method were realized. Hence, it was determined that the comparison of ALcontrol data to the HACH-LANGE results would be of lower value, since the two methods were not directly comparable. It was investigated if the tests could be performed elsewhere fulfilling the ISO 11348-3 and the accreditation requirement as well as operating different equipment than the LUMIStox (or ECLOX). A laboratory meeting these criteria could not be found in Germany, where the HACH-LANGE equipment is widely used, or in Norway. Laboratories were found that conducted a modified version of the ISO 11348-3 method, but none were found that would conduct the ISO method as written. Therefore, it was decided to exclude further reference tests rather than include measurements from a laboratory that was performing a modified method, since the

results would likely be different. This change to the test plan was documented in Deviation 9B (see Appendix 4). Precedence for not using a reference test for water toxicity verification testing followed the U.S. ETV Test/QA Plan for Verification of Rapid Toxicity Technologies /35/. DHI believes the impact of not including these analyses was minimal, since these data were not intended to be used as true reference measurements but rather to present results that would have been obtained by a comparable technology. Additionally, data from peer-reviewed scientific literature based on ISO 11348-3 had been planned for comparison to the testing data, and are available (see section 7.2.4).

Reference chemical analyses of stock solutions were done under ISO 17025 accreditation /17/ with appropriate methods by an independent laboratory.

6.3 Data management

Data storage, transfer and control were done in accordance with the requirements of ISO 9001 /18/ enabling full control and retrieval of documents and records. The filing and archiving requirements of the DHI Quality Manual were followed (10 years archival).

6.4 Quality assurance

The quality assurance (QA) of the tests included audits of the test system at DHI DANETV Water Centre and the external laboratory performing reference tests, as well as performance evaluations of the laboratory providing stock solution confirmations. Data quality audits were performed on data generated during testing to ensure data quality and integrity.

This verification report was subjected to review by the QA group indicated in Fig 1.2.

Since this verification was a joint verification with the U.S. EPA ETV and ETV Canada, an on-site technical systems audit (TSA) by the Battelle AMS Center was included as part of the quality assurance. An audit debrief occurred at the conclusion of the TSA, and issues identified during the audit were brought to DHI's attention. This included issues which were ultimately identified as one finding, four observations, and one recommendation. The finding raised during the TSA debrief was that the external laboratory was performing a modified ISO 11348-3. At the time of the TSA, the impact of the modifications on data quality was not known. However, DHI further investigated the external laboratory upon receipt of the first batch of data, and determined that the modifications had impact on usability of the data. As a result, the use of modified ISO 11348-3 was discontinued and documented as a finding in the final audit report.

The Battelle Quality Manager and the ETV Canada Quality Manager also performed an audit of data quality. This was a review of data acquisition and handling procedures and an audit of at least 10% of the data acquired in the test and verification. The Quality Managers traced the data from initial acquisition, through reduction and statistical comparisons, to final reporting. All calculations performed on the data undergoing the audit were checked.

6.5 Test report

The test report /30/ followed the principles of the template of the DHI DANETV verification center quality manual template /4/ with data and records from the tests presented.

The test report was not reviewed by the U.S. ETV program or the Battelle AMS Center, since the purpose of the test report was a specific requirement for DANETV.

One test report was prepared for both verified technologies (LUMIStox and ECLOX).

7 EVALUATION

The evaluation included calculation of the performance parameters from Section 4.2, evaluation of the data quality based upon the test quality assurance from Section 6.4, and compilation of the additional parameters from Section 4.3.

The calculations involved in the EC_{20} and EC_{50} determination by the LUMISsoft4 software were not independently verified as part of this test. However, results generated by the software were spot-checked by comparison to calculations derived independently, e.g. when performing manual color correction of results from the ECLOX and calculating EC values.

7.1 Calculation of performance parameters

By testing a dilution series with inhibitions in the range from 10%-90%, $EC_{20}$and EC_{50} values can be calculated according to principles in ISO 11348-3. This is performed by the software LUMISsoft4 connected to the HACH-LANGE instruments. To estimate EC_{50} values, a minimum of three measurements have to be in the range from 10%-90% inhibition. Furthermore, one concentration has to give response above 50% inhibition of a valid EC_{50} value.

For use of the ECLOX without connection to a computer, the results were recorded as percent inhibition and, as such EC values could not be determined directly.

Calculations of parameters and EC values (and in the case of ECLOX using firmware, percent inhibition) were performed according to accepted statistical principles (Table 7.1 and /9/). Table 8.1 includes updates to the calculations originally listed in the verification protocol /33/ that were added to improve the quality of the evaluation, and are described in Deviation 8 (see Appendix 4).

Table 7.1 Calculations used for the test results

Parameter	Calculation	Explanations
Criterion of detection	$CD = t_{0.95}(f) s_k (1 + \frac{1}{n})^{½}$	CD is criterion of detection; $t_{0.95}$(f) is the Student's t factor for f where f= n-1 degrees of freedom; n is number of measurements; s_k is a pooled estimate for standard deviation of luminescent in control glasses
Range of application	Minimum: just above 2*EC_{50} Maximum: just less than 32*EC_{50}	EC_{50}: Concentration causing 50% inhibition
Precision (repeatability), as relative standard deviation, RSD	$D_i = \lvert x_{i\max} - x_{i\min} \rvert$ $\bar{x}_i = \frac{\sum x_i}{n}$ $d_i = \frac{D_i}{\bar{x}_i}$ $\bar{d} = \frac{\Sigma d_i}{m}$ $RSD = \frac{\bar{d} * 100}{Divisor}\%$	D_i is the range at level i; x_{imin} and x_{imax} are the lowest and highest measurements at level i; $\bar{x}_i$ is the average of n measurements; m is the number of levels; d_i is the relative range at level i; $\bar{d}$ $\bar{d}$ is the mean relative range for all m levels Divisor is for i=3 equal to 1.693 and for i=4 equal to 2.059
Precision (reproducibility), as relative standard deviation, RSD	$\bar{x}_i = \frac{\sum x_i}{n}$ $\bar{\bar{x}}_t = \frac{\Sigma \bar{x}_i}{m}$ $s_{Between\ groups} = \sqrt{MS_{Between\ groups}}$ $RSD = \frac{s_{Between\ groups}}{\bar{\bar{x}}_t} * 100\ \%$	$\bar{x}_i$ is the average of n measurements in group; m is the number of levels; s is standard deviation $\bar{\bar{x}}_t$ is average of average in groups; s is standard deviation $MS_{Between\ groups}$ is variance between groups obtained by single factor ANOVA in Excel
Agreement with accepted values, A. Based on robust literature values (obtained by use of ISO 11348-3)	$\bar{x}_i = \frac{\sum x_i}{n}$ $\bar{y}_i = \frac{\sum y_i}{n}$ $A_i = \frac{\bar{x}_i}{\bar{y}_i} \times 100\ \%$ $A = \frac{\sum A_i}{m}$	$\bar{x}_i$ is the mean of measurements at level i, x_i; $\bar{y}_i$ is the literature value at level i, y_i; Ai is the agreement at level i; A is the mean agreement for all levels
Robustness, R	$R = \frac{\bar{x}_{ro}}{\bar{x}_{re}} \times 100\ \%$	$\bar{x}_{ro}$ is the average of measurement under conditions of robustness test; $\bar{x}_{re}$ is the average of measurements under reference conditions
Test of significant deviation from reference. Used for robustness results	$s_{xy} = \sqrt{\frac{(f_x \times s_y^2 + f_y \times s_y^2)}{f_x + f_y}}$ $f_{xy} = f_x + f_y$ $\frac{\lvert \bar{x} - \bar{y} \rvert}{s_{xy}} \times \sqrt{\frac{n_x \times n_y}{n_x + n_y}} > t_{0.975\,(f_{xy})}$	s_x is standard deviation on dataset x s_y is standard deviation on dataset y f_x is degree of freedom for dataset x f_y is degree of freedom for dataset y $\bar{x}$ is the average of measurements of dataset x $\bar{y}$ is the average of measurements of dataset y s_{xy} is average deviation n_x is number of measurement in dataset x n_y is number of measurement in dataset y $t_{0.975}$ is student t-factor for two-sided test

Calculations of performance parameters were conducted in Excel 2007.

7.2 Performance parameter summary

Results in the test report /30/ are given for a test time of both 15 and 30 minutes. When EC values are calculated both EC_{20} and EC_{50} values are listed. In the verification report only EC_{50} values are listed, since they are most widely used. In the verification statement, only results for EC_{50} with a test time of 30 minutes are listed, since this test time is used in reporting more frequently than the 15-minute test times.

7.2.1 Criterion of detection

The criterion of detection, the level above which inhibition is significant (95%), was calculated based on series of nine 2% MilliQ water samples including bacteria, but no toxic compounds. The criteria of detection for LUMIStox and ECLOX after 15 and 30-minute exposures, respectively, are given in Table 7.2.

Table 7.2 Criterion on detection (% inhibition). Number of replicates (n) is 3.

Test time (min)	**LUMIStox** (% inhibition)	**ECLOX** (% inhibition)
15	6.7	7.5
30	5.8	5.5

7.2.2 Range of application

Range of application in this context means the concentration range where (pure water) samples can be tested without dilution or pre-concentration.

Table 7.3 LUMIStox range of application in 2% NaCl MilliQ water for target compounds (mg/L). Number of replicates (n) is 3 and 4 for cyanide.

LUMIStox	**15 min**			**30 min**		
Compound	**Average EC_{50}** (mg/L)	**Range of application** (mg/L)		**Average EC_{50}** (mg/L)	**Range of application** (mg/L)	
		Minimum	**Maximum**		**Minimum**	**Maximum**
Zn^{2+}	8.5	>17	<270	4.1	>8.3	<130
$Cr_2O_7^{2-}$	n.c. [1]	-	-	17	>35	<560
Triclosan [3]	0.40	>0.79	<13	0.53	>1.1	<17
Cyanide	24 [2]	>48	<770	24	>48	<780
SDS [3]	1.4	>2.8	<44	1.0	>2.0	<32
CTAB [3]	1.3	>2.7	<43	0.97	>1.9	<31

n.c.: Not calculated.

[1] EC_{50} for $Cr_2O_7^{2-}$ was not possible to calculate after 15 minutes. The requirement of one measurement above 50% inhibition was not fulfilled.

[2] EC_{50} for cyanide was only possible to calculate after 15 minutes for two out of four replicates. The requirement of one measurement above 50% inhibition was not fulfilled.

[3] The recovery of these compounds in mixed solutions was not near 100%. The listed EC values are based on the added amount of compound. See details on recovery later in section 7.3.3.

The range of application was based on EC_{50} values determined for six target compounds. Note that originally the verification protocol /33/ called for using nine target compounds; however, three of these compounds: $CuSO_4$ (heavy metal), Flutriafol (organic pesticide), and 4-NPE (surfactant) were not sufficiently toxic at concentrations without precipitation to be used for testing and were therefore excluded.

Removal of these three target compounds is described in Deviations 1, 2 and 4, respectively (see Appendix 4). The six remaining target compounds represent the compound categories of heavy metals, organic compounds, industrial pollutants, and surfactants.

Table 7.4 ECLOX range of application in 2% NaCl MilliQ water for target compounds (mg/L). Number of replicates (n) is 3 and 4 for cyanide.

ECLOX	**15 min**			**30 min**		
Compound	**Average EC_{50}** (mg/L)	**Range of application** (mg/L)		**Average EC_{50}** (mg/L)	**Range of application** (mg/L)	
		Minimum	**Maximum**		**Minimum**	**Maximum**
Zn^{2+}	8.4	>17	<270	4.1	>8.2	<130
$Cr_2O_7^{2-}$	n.c. [1]	-	-	18	>37	<590
Triclosan [4]	0.39	>0.77	<12	0.53	>1.1	<17
Cyanide	23 [3]	>45	<730	18	>35	<570
SDS [4]	1.4	>2.8	<45	0.99	>2.0	<32
CTAB [4]	1.4	>2.9	<46	0.96	>1.9	<31

n.c.: Not calculated.

[1] EC_{50} for $Cr_2O_7^{2-}$ was not possible to calculate after 15 minutes. The requirement of one measurement above 50% inhibition was not fulfilled.

[2] EC_{50} for cyanide was only possible to calculate after 15 minutes for three out of four replicates. The requirement of one measurement above 50% inhibition was not fulfilled.

[3] EC_{50} for cyanide was only possible to calculate after 30 minutes for three out of four replicates. The requirement of one measurement above 50% inhibition was not fulfilled.

[4] The recovery of these compounds in mixed solutions was only 2-7%. The listed EC values are based on the addition of compound. See details on recovery in section 7.3.3.

To be able to determine the EC_{50} value, an initial concentration greater than twice the EC_{50} is needed, since the standard procedure is to dilute the sample to half the initial concentration before testing. Without extraordinary dilution of the sample, the EC_{50} value has to be detected within the regular dilution series containing nine dilutions (limitation by the thermoblock). The maximum concentration in the sample can therefore be less than 32 times the EC_{50}. The compound specific ranges of application are listed in Table 7.3 and Table 7.4 together with the average EC_{50} values.

The tested concentrations of chromium were not inhibiting at levels necessary to calculate EC_{50} values after 15 minutes.

7.2.3 Precision

The precision in terms of repeatability is presented in Table 7.5 and Table 7.6. The repeatability is calculated for the six target compounds based on the results from Test A.

Generally it was noticed that the repeatability was improved for EC_{50} values compared to EC_{20} values. For example, 30 minute LUMIStox EC_{20} values have RSDs for Zn^{2+}, $Cr_2O_7^{2-}$ etc. as follows: 12, 55, 13, 73, 44 and 6.3. EC_{20} results are provided in the test report /30/.

The log-log linearity, used by the model for EC calculation, was relatively low for cyanide, causing high relative standard deviations.

The precision in terms of reproducibility is presented in Table 7.7. Reproducibility is based on the results from Test D, which was performed with Zn^{2+} as the target compound. EC_{50} values are closely related to the activity of the bacteria, as explained in Section 7.2.4, 8.3.3, and in further details in the test report /30/.

Table 7.5 LUMIStox repeatability as relative standard deviation (RSD) in percent. For target compounds in 2% NaCl MilliQ water. Number of replicates (n) is 3 but 4 for cyanide.

LUMIStox	**15 min**	**30 min**
	EC_{50} RSD (%)	**EC_{50} RSD** (%)
Zn^{2+}	4.5	5.0
$Cr_2O_7^{2-}$	n.a.	29
Triclosan	7.4	5.5
Cyanide	18	24
SDS	29	33
CTAB	3.6	2.4

n.a.: Not applicable. EC_{50} could not be determined.

Table 7.6 ECLOX repeatability as relative standard deviation (RSD) in percent. For target compounds in 2% NaCl MilliQ water. Number of replicates (n) is 3 but 4 for cyanide.

ECLOX	**15 min**	**30 min**
	EC_{50} RSD (%)	**EC_{50} RSD** (%)
Zn^{2+}	2.7	4.9
$Cr_2O_7^{2-}$	n.a.	24
Triclosan	4.6	2.2
Cyanide	15	16
SDS	34	38
CTAB	6.3	1.2

n.a.: Not applicable. EC_{50} could not be determined.

Table 7.7 LUMIStox and ECLOX reproducibility as relative standard deviation (RSD) in percent. For Zn^{2+} in 2% NaCl MilliQ water. Test was performed on three bacteria batches on three different days. Number of replicates (n) is 3, except for ECLOX batch 02099 where 4 replicates were tested.

Zn^{2+}	**15 min**	**30 min**
	EC_{50} RSD (%)	**EC_{50} RSD** (%)
LUMIStox	28	30
ECLOX	63	51

7.2.4 Agreement with accepted values

The agreement with accepted values was calculated for each target compound and the average agreement was also determined (from Test A). The sources of accepted literature values obtained with the ISO 11348-3 are listed in Appendix 3. The average agreement was determined for all compounds which had literature values and where it is known that the test was performed according to the ISO 11348-3.

Table 7.8 LUMIStox EC_{50} agreement with accepted values (A) in percent.

Compound	Accepted values			LUMIStox	
	EC_{50} ± 1 RSD (mg/L)	**Test time** (min)	**According to ISO 11348-3**	**EC_{50} ± 1 RSD** (mg/L)	**A_i** (%)
Zn^{2+} ($ZnSO_4 \cdot 7H_2O$)	2.2 ± 23%	30	Yes	4.1 ± 4.4%	186
$Cr_2O_7^{2-}$ ($K_2Cr_2O_7$)	19 ±11%	30	Yes	17 ± 27%	91
Triclosan	0.28	15	Yes	0.40 ± 6.3%	143
	0.28	30	Yes	0.53 ± 4.7%	189
CTAB	0.97	30	Yes	0.97 ± 2.2%	100

Table 7.9 ECLOX EC_{50} agreement with accepted values (A) in percent.

Compound	Accepted values			ECLOX	
	EC_{50} ± 1 RSD (mg/L)	**Test time** (min)	**According to ISO 11348-3**	**EC_{50} ± 1 RSD** (mg/L)	**A_i** (%)
Zn^{2+} ($ZnSO_4 \cdot 7H_2O$)	2.2 ± 23%	30	Yes	4.1± 4.3%	186
$Cr_2O_7^{2-}$ ($K_2Cr_2O_7$)	19 ± 11%	30	Yes	18± 22%	96
Triclosan	0.28	15	Yes	0.39± 3.8%	139
	0.28	30	Yes	0.53± 2.3%	190
CTAB	0.97	30	Yes	0.96± 1.0%	99

When evaluating the agreement with accepted values, it should be taken into account that bacterial activity for some compounds affects the EC_{50} values. It has been shown that a low bacterial sensitivity, indicated by a low inhibition by the Zn^{2+} standard, results in a higher EC_{50}. For Test A, the activity of the bacteria caused an inhibition of approximately 25% for the Zn^{2+} standard in a concentration that should equal EC_{50} according to the ISO 11348-3 method. The inhibition was therefore half of what could be expected from the EC_{50} value, but still within the accepted range from 20%-80% inhibition, the acceptable range in the ISO 11348-3 method. The concentration needed in Test A to obtain 50% inhibition was, due to the low bacteria activity, a factor of two higher than the EC_{50} value listed in the ISO 11348-3, and resulted in an agreement with accepted value (A_{Zn}^{2+}) of 186%. Further details on this can be seen in the test report /30/. The result for zinc is therefore seen as in general agreement with accepted values, since the difference is explained by the bacteria activity, and the bacteria activity met the requirements of the ISO 11348-3 method.

7.2.5 Robustness

Initial concentration, temperature, pH, color, turbidity and type of cuvettes

The robustness of the LUMIStox and ECLOX measurements was tested against differences in initial concentration, temperature, pH, color, turbidity and type of cuvettes. The robustness was calculated as the average inhibition under conditions of the robustness test divided by average inhibition under reference conditions, and reported as a percent.

The results of the robustness test are both EC values (initial concentration) and percent inhibition (all other robustness tests). The robustness under different test conditions is listed in Table 7.10 to Table 7.13. Three different concentrations of dye were used for color tests and three concentrations of $BaSO_4$ were used for turbidity tests.

Difference in initial concentration, temperature in laboratory, pH within requirements listed in ISO 11348-3, and the use of plastic cuvettes in the LUMIStox caused insignificant effects. The measurements and results show good robustness of the methods and equipment for these parameters.

The use of ECLOX under field temperatures (5 °C and 23 °C) gave significantly different results from the reference test conducted at 16 °C. The bacterial activity at 5 °C was generally low, resulting in high variation in the results. The robustness for the two tested target compounds differed, showing that the robustness against field temperature is compound specific.

Table 7.10 LUMIStox robustness (R) in percent. Test results are presented as EC values. R values significantly different (95% confidence level, two-sided t-test) from 100% indicated in bold.

LUMIStox	**Target compound**	**Condition**	**15 min**	**30 min**
			EC_{50}	**EC_{50}**
			R (%)	**R** (%)
Initial concentration Ref. ~EC_{90}	SDS	Initial concentration ~EC_{60}	93	96

Table 7.11 LUMIStox robustness (R) in percent. Test results are presented as % inhibition. R values significantly different (95% confidence level, two-sided t-test) from 100% indicated in bold.

LUMIStox	**Target compound**	**Condition**	**15 min R** (%)	**30 min R** (%)
Temperature, lab Ref.15.4 °C	SDS	14.0 °C	99	105
		16.1 °C	69	71
pH Ref. 7.0	SDS	6.0	96	110
		8.5	101	107
Color Ref. no color	SDS	0.2% dye, with c.c.	94	102
		0.2% dye, without c.c.	98	105
		6.25% dye, with c.c.	108	107
		6.25% dye, without c.c.	**170**	**156**
		12.5% dye, with c.c.	117	114
		12.5% dye, without c.c.	**220**	**197**
Turbidity Ref. no turbidity	SDS	0.05 g $BaSO_4$/L, with c.c.	55	70
		0.05 g $BaSO_4$/L, without c.c.	112	106
		0.10 g $BaSO_4$/L, with c.c.	**8**	41
		0.10 g $BaSO_4$/L, without c.c.	105	97
		0.20 g $BaSO_4$/L, with c.c.	**-90**[2]	**-20**[2]
		0.20 g $BaSO_4$/L, without c.c.	97	88
Cuvette material[1]	Zn^{2+}	Plastic	101 (99-160)	107 (106-117)
Ref. glass	SDS	Plastic	108 (93-108)	99 (90-101)

c.c.: Color correction.

[1] Test performed in triplicates (with 3 replicates in each test). Median and interval are given as result.

[2] Negative values occur when there inhibition is negative. Negative inhibition means that the solution tested gives better growth conditions for the bacteria than the control.

Table 7.12 ECLOX robustness (R) in percent. Test results are presented as EC values. R values significantly different (95% confidence level, two-sided t-test) from 100% indicated in bold.

ECLOX	**Target compound**	**Condition**	**15 min**	**30 min**
			EC_{50}	**EC_{50}**
			R (%)	**R** (%)
Initial concentration Ref. ~EC_{90}	SDS	Initial concentration ~EC_{60}	94	97

n.a.: Not applicable.

Table 7.13 ECLOX robustness (R) in percent. Test results are presented as % inhibition. R values significantly different (95% confidence level, two-sided t-test) from 100% indicated in bold. Number of replicates are 3, except for Test I on turbidity where the number of replicates is 4.

ECLOX	**Target compound**	**Condition**	**15 min R** (%)	**30 min R** (%)
Temperature, field[1] Ref. 16 °C	Zn^{2+}	5 °C	**27 (11-35)**	n.d.
		23 °C	**116 (108-171)**	n.d.
	SDS	5 °C	100 (93-105)	n.d.
		23 °C	**75 (73-75)**	n.d.
Temperature, lab Ref.15.4 °C	SDS	14.0 °C	88	100
		16.1 °C	91	85
pH Ref. 7.0	SDS	6.0	111	113
		8.5	101	105
Color Ref. no color	SDS	0.2% dye, with c.c.	124	124
		0.2% dye, without c.c.	105	110
		6.25% dye, with c.c.	107	112
		6.25% dye, without c.c.	**155**	**148**
		12.5% dye, with c.c.	128	115
		12.5% dye, without c.c.	**214**	**180**
Turbidity Ref. no turbidity	SDS	0.05 g $BaSO_4$/L, with c.c.	**135**	111
		0.05 g $BaSO_4$/L, without c.c.	109	93
		0.10 g $BaSO_4$/L, with c.c.	**154**	130
		0.10 g $BaSO_4$/L, without c.c.	118	107
		0.20 g $BaSO_4$/L, with c.c.	115	101
		0.20 g $BaSO_4$/L, without c.c.	92	86

n.d.: Not determined.

c.c.: Color correction.

[1] Performed at three different concentrations. Median and interval are given as result.

The results showed that the use of color correction is essential when testing colored samples, while the results for turbid $BaSO_4$ samples showed that the color correction function is not applicable. However, this could differ for other types of turbid samples. Additional testing is needed for verification.

According to ISO 11348-3 strongly turbid samples should be allowed to settle for 1 h, centrifuged, or be filtered. The robustness test performed was done on turbid samples (no settling, centrifugation, or filtration) to see the effect if these guidelines were not followed. The HACH-LANGE manuals are not clear in this recommendation from the ISO standard. It is suggested that the vendor revise the manuals to ensure the best test results for turbid samples.

Wastewater matrix

Wastewater contains ions, organic compounds and particles which may potentially alter the detected toxicity of substances in the wastewater by processes such as complexation and adsorption. Two non-toxic wastewater types (industrial and domestic) were therefore used as the matrix and compared to 2% NaCl MilliQ water. The wastewaters were evaluated for toxicity as part of Test K using the dilution series to determine EC_{20} and EC_{50}. EC_{20} or EC_{50} values generated were not calculated when the inhibition was lower than 10%. Originally, triplicate measurements were to be made; however, during testing an error resulted in the loss of one replicate for industrial wastewater. It was decided that two replicate measurements were sufficient to document that both the domestic wastewater and the domestic wastewater were non-toxic. This is documented in Deviations 5 and 6 (see Appendix 4). Individual inhibition measurements of the wastewaters that were based on triplicate measurements as part of Test J are included in Table 8.14 below.

The baseline luminescence of the non-toxic wastewater differed slightly from the baseline of the 2% NaCl MilliQ water, illustrated in Table 7.14. The domestic wastewater appears to enhance the luminescence, causing negative inhibition.

Table 7.14 Wastewater baseline luminescence given as % inhibition. Number of replicates is 3.

Wastewater	**LUMIStox**		**ECLOX**	
	15 min % inhibition	**30 min** % inhibition	**15 min** % inhibition	**30 min** % inhibition
Industrial	1.2	1.5	-2.9	-3.3
Domestic	-8.1	-5.7	-6.6	-5.3

Table 7.15 and Table 7.16 show the results of robustness towards wastewater. The domestic wastewater is reported both with and without an adjustment to the baseline to account for the wastewater's negative inhibition (positive growth effect) on the bacterial luminescence (see Table 7.14).

Table 7.15 LUMIStox robustness (R) towards wastewater given in percent. R values significantly different (95% confidence level, two-sided t-test) from 100% indicated in bold.

LUMIStox	Target compound and concentration	Wastewater			Adjusted baseline	
			15 min Inhibition R (%)	30 min Inhibition R (%)	15 min Inhibition R (%)	30 min Inhibition R (%)
Matrix Ref. 2% NaCl	Zn^{2+} 4.0 mg/L	Industrial	77	43		
		Domestic	**31**	84	127	**123**
MilliQ water	$Cr_2O_7^{2-}$ 2.8 mg/L	Industrial	31	**0**		
		Domestic	**-50**[1]	**-10** [1]	15	**22**
	Triclosan 0.60 mg/L	Industrial	114	141		
		Domestic	84	57	105	96
	SDS 0.80 mg/L	Industrial	68	**28**		
		Domestic	66	**64**	107	96
	CTAB 1.2 mg/L	Industrial	102	68		
		Domestic	75	**52**	118	78

1 Negative value occurs when inhibition is negative. Negative inhibition means that the solution tested gives better growth conditions for the bacteria than the control.

Table 7.16 ECLOX robustness (R) towards wastewater given in percent. R values significantly different (95% confidence level, two-sided t-test) from 100% indicated in bold.

ECLOX	Target compound	Wastewater			Adjusted baseline	
			15 min Inhibition R (%)	30 min Inhibition R (%)	15 min Inhibition R (%)	30 min Inhibition R (%)
Matrix Ref. 2% NaCl MilliQ water	Zn^{2+} 4.0 mg/L	Industrial	**56**	**22**		
		Domestic	37	85	132	**125**
	$Cr_2O_7^{2-}$ 2.8 mg/L	Industrial	**12**	**-20** [1]		
		Domestic	**-60** [1]	**-10** [1]	**14**	**13**
	Triclosan 0.60 mg/L	Industrial	116	141		
		Domestic	89	62	110	101
	SDS 0.80 mg/L	Industrial	68	**35**		
		Domestic	**71**	**67**	111	101
	CTAB 1.2 mg/L	Industrial	99	61		
		Domestic	**64**	**49**	101	**73**

[1] Negative value occurs when inhibition is negative. Negative inhibition means that the solution tested gives better growth conditions for the bacteria than the control.

Chromium showed a change in toxicity when added to the wastewater, but effects are also seen in some cases for zinc, SDS and CTAB.

7.3 Evaluation of test data quality

7.3.1 Reference chemical analysis performance data

Control data for the reference chemical analysis obtained from Eurofins are summarized in Table 7.17.

Table 7.17 Performance parameters for reference chemical analysis control data.

Target compound	Limit of detection µg/L	Precision (RSD) %	Trueness %
Zn^{2+}	0.50	15	98-99
$Cr_2O_7^{2-}$	0.50	15	103
Triclosan	0.10	Not specified	103
Cyanide (CN^-)	1.0	10	99
SDS (anionic surfactants[1])	25	15	101
CTAB (cationic surfactants[2])	100	20	95

[1] Reference compound is SDS.

[2] Reference compound is benzyl di-methyl tetradecyl ammonium chloride-dihydrate, molar weight 404,00 g/mol.

Table 7.19, in section 7.3.3, lists analyses of blank samples, performed to test the Eurofins detection limits. Eurofins participates in proficiency tests for most of the tested compounds. The results of their most recent proficiency tests are shown in Table 7.18.

Table 7.18 Results of Eurofins proficiency tests.

Parameter	Nominal value	Zeta-score	Supplier
Zinc	614 µg/L	0.316	APG, November 2009 WS, 1. round
Chromium	83.1 µg/L	0.157	FAPAS (LEAP), Wastewater, G20+G21
Triclosan	Eurofins has not participated in proficiency testing, since triclosan is a new parameter for them and is not covered by their accreditation		
Cyanide	7.00-11.3 µg/L	0.377	KIWA, drinking water, 09-03
Anionic surfactants	50.0-120 µg/L	-0.464	KIWA, drinking water, 09-03
Cationic surfactants	Eurofins is not aware of supplier of proficiency tests for cationic surfactants within the measuring area		

7.3.2 Comparable test performance data

ALcontrol uses zinc sulfate and phenol as reference compounds. The results of the data were within the specification of the bacteria supplier, though the control chart for zinc shows that over the period the references have been at a low level, around 70% of the expected average.

ALcontrol participates in an annual proficiency test with the Microtox®. The results were audited by Battelle as part of the technical systems audit (TSA) at ALcontrol and found to be within the acceptance criteria.

7.3.3 Test system control data

Blank samples

The 2% NaCl MilliQ water used to prepare stock solutions of test compounds was tested for background levels of the target compounds. The results are shown in Table 7.19.

The results showed that the 2% NaCl MilliQ water did not contain any of the target compounds in significant concentrations. These results also showed that the water purifier was operating within normal parameters, and the NaCl was free of contaminants.

Table 7.19 Concentrations of target compounds in 2% NaCl MilliQ water (blank) samples.

Target compound	**Concentration** µg/L	
	Replicate 1	**Replicate 2**
Zn^{2+}	<0.50	<0.50
$Cr_2O_7^{2-}$	0.50	0.60
Triclosan	<0.10	0.19
Cyanide (CN^-)	<1.0	<1.0
SDS (anionic surfactants[1])	<25	<25
CTAB (cationic surfactants[2])	<100	<100

[1] Reference compound for anionic surfactant is SDS.

[2] Reference compound for cationic surfactant is benzyl di-methyl tetradecyl ammonium chloride-dihydrate.

The 2% NaCl MilliQ water was tested for toxicity at ALcontrol.

The results are shown in Table 7.20.

Table 7.20 Toxicity in percentage of sample volume of 2% NaCl MilliQ water (blank) samples.

Time (min)	**EC value**	**Concentration** %
5	EC_{20}	78
	EC_{50}	>82
15	EC_{20}	>82
	EC_{50}	>82
30	EC_{20}	>82
	EC_{50}	>82

The results showed no detectable toxicity of the 2% NaCl MilliQ water after 15 and 30 minutes.

Control, stock solutions

The concentrations and the stability of the stock solutions were evaluated by sending subsamples of the solutions to Eurofins laboratory for chemical analysis. Table 7.21 shows the results of this analysis and the recovery of the concentrations in the stock solutions.

The surfactants SDS and CTAB were expected to adhere to the cuvettes. In addition, CTAB was difficult to dissolve. The stock solutions were therefore treated as the test samples (added to cuvettes and left for 30 minutes) before sending to Eurofins.

Table 7.21 Concentrations (average and relevant range (high/low value divided by average)) of target compounds in spiked 2% NaCl MilliQ water stock solutions.

Target compound	Measured concentration		Prepared concentration	Recovery
	Average µg/L	Relevant range %	µg/L	%
Zn^{2+}	17,500	± 5.7	22,000	80
$Cr_2O_7^{2-}$	52,000	± 7.7	56,100	93
Triclosan	355	± 2.8	1,600	22
Cyanide (CN^-)	31,500	± 9.5	32,885	96
SDS (anionic surfactants[1])	2,550	± 12	35,950	7.1
CTAB (cationic surfactants[2])	725	± 32		
CTAB[3]	560	± 32	30,000	1.9

[1] Reference compound is SDS.

[2] Reference compound is benzyl di-methyl tetradecyl ammonium chloride-dihydrate ($(C_6H_5CH_2)(CH_3)_2N(C_{12}$-$C_{14}Alkyl)^+Cl^-$), molar weight 404.00 g/mol. Molar weight of reference compound cation 368.5 g/mol.

[3] Concentration of CTAB, molar weight 364.45 g/mol has been calculated based on CTAB (cationic surfactants) results. Molar weight of CTAB cation 284.5 g/mol.

The concentration of SDS and CTAB in the cuvettes were found to be low compared to the expected (7% and 2% recovery). The triclosan stock solution also showed a significant loss, with a recovery of only 22%. These losses were not taken into account when calculating the EC values. This was noted in Table 7.3 and Table 7.4. Despite the low recoveries of triclosan and CTAB we found EC_{50} values similar to the accepted literature values (Table 7.8 and Table 7.9). The reason for the low recovery has not been determined in this verification. Therefore, a conclusion regarding the risk of reduced inhibition due to losses during handling of the samples cannot be drawn from these results. It is noted that chemical analysis of the sample is not required in the ISO 11348-3.

The concentration of cyanide in the dilutions was determined using a test kit. An artificial cyanide sample was carried through the test procedure. Instead of adding bacteria solution, 2% NaCl was added. No measurements of luminescence were performed. Instead, the cyanide concentration was measured using a HACH-LANGE test (LCK 315). Test row B was analyzed at time 0 and test row C was analyzed after 30 minutes. The results showed that cyanide was stable during the test, i.e. the concentration after 30 minutes was within the acceptable range of 80-120% of the initial concentrations. Cyanide was therefore included in the test program.

Test of inhibition by reference substances according to ISO 11348-3

The bacterial batches used in the tests were tested for compliance with the requirements in the ISO 11348-3, section 11. For all reference standard compounds the criteria is 20%-80% inhibition. The results of the reference tests are shown in Table 7.22.

Table 7.22 Mean %-inhibition and standard deviation (st. dev.) from reference tests of bacteria batches performed in accordance with ISO 11348-3. Tests are performed on LUMIStox, number of replicates are two except for 02099 where the reference standards were only tested once.

Batch	**Zn^{2+} (2.2 mg/L)** %	**$Cr_2O_7^{2-}$ (18.7 mg/L)** %	**3,5-dichlorophenol (3.4 mg/L)** %
10129	22 ± 0.03	60 ± 0.12	20 ± 2.3
11169	36 ± 0.90	53 ± 1.4	28 ± 0.62
02099	15	96	39
ISO requirement	20-80		

For batches 10129 and 11169, both Zn^{2+} and 3,5-dichlorophenol were close to the lower limit of 20%. One tested sample was below 20% for 3,5-dichlorophenol. The reference standard, Zn^{2+} was included in all tests to be able to follow the bacteria activity.

The bacteria batch 02099 was only used in one test (Test D). The results for this bacteria batch did not meet the replicates required on the percent inhibition to fulfill the requirement of the ISO standard. Use of this bacteria batch is documented in Deviation 7 (see Appendix 4). This resulted in slightly higher standard deviations and higher relative standard deviations reported in Table 8.7. As noted in Deviation 3, this batch was used even though it did not fulfill the ISO requirement, because at least three batches were needed for the reproducibility evaluation and no other bacteria batches were immediately available from the vendor.

Since the references were close to the requirements in the ISO, one reference (Zn^{2+}) was included in all test runs. This is more stringent than what is stated in the ISO standard. Few of the Zn-reference standard test results (approximately 10% of those measured in the LUMIStox) did not fulfill the ISO requirement. However, all results have been included in the evaluation since the check with the original reconstitution (reported in Table 7.22) fulfilled the ISO requirements.

The ISO standard also sets limits for the variation (i.e., deviation) between the duplicate control sample measurements. Duplicate measurements should not deviate from their mean by more than 3%. HACH-LANGE informed DHI that for the ECLOX, this can be difficult to fulfill. In some cases the deviation of EC_{50} values between triplicate samples was as low as 1.2%, even though the deviation between the replicate control measurements were above the required 3%. No data has therefore been excluded because of the deviation between duplicate control measurements.

Test of samples by the external laboratory

A solution of 96.7 mg/L of zinc sulfate heptahydrate (22 mg/L Zn^{2+}) in 2% NaCl MilliQ water was tested for toxicity at ALcontrol. The results are shown in Table 7.23. $EC_{50,30\ min}$ was considerably lower than the accepted value (see Table 7.8).

Table 7.23 Toxicity in percentage of sample volume of 96.7 mg/L zinc sulfate heptahydrate (22 mg/L Zn^{2+}) in 2% NaCl MilliQ water.

Time (min)	**EC value**	**Concentration** %	***Concentration** (mg Zn^{2+}/L)
5	EC_{20}	19	4.2
	EC_{50}	>82	> 18
15	EC_{20}	2.5	0.60
	EC_{50}	12	2.6
30	EC_{20}	0.20	0.05
	EC_{50}	1	0.20

** Calculated nominal concentration based on added amount. See also Table 7.21.*

A solution containing 7.2 mg/L SDS in 2% NaCl MilliQ water was tested for toxicity at ALcontrol. The results are shown in Table 7.24. These results are similar to the results obtained with the both LUMIStox (Table 7.3) and ECLOX (Table 7.4).

Table 7.24 Toxicity in percentage of sample volume of 2% NaCl MilliQ water added 7.2 mg/l of SDS.

Time (min)	**EC value**	**Concentration** %	***Concentration** (mg SDS/L)
5	EC_{20}	9	0.60
	EC_{50}	18	1.3
15	EC_{20}	6	0.40
	EC_{50}	11	0.90
30	EC_{20}	4	0.30
	EC_{50}	9	0.60

**Calculated nominal concentration based on added amount. There was a low recovery of SDS as determined by the chemical analyses. See Table 7.21.*

7.3.4 Audits

Two onsite audits were performed during the testing. An internal audit performed by Bodil Mose Pedersen from DHI /31/ resulted in one deviation in the internal protocol (Appendix 4 in the test plan /25/). The deviation was resolved directly after the audit.

An on-site audit was performed by Battelle AMS Center for U.S. EPA /32/. One finding, four observations and one recommendation were noted. The final audit report is permanently stored with the Battelle AMS Center Quality Manager.

7.3.5 Deviations

There were no amendments to the verification protocol or the test plan.

Four deviations were made to the verification protocol.

There have been 10 deviations to the test plan, all deviations have been approved. The test report reflects these deviations.

All deviations to the verification protocol and test plan are included in Appendix 4.

7.4 Additional parameters summary

7.4.1 User manual

The assessment for the user manual evaluated if the manual describes the use of the equipment adequately. The evaluation considered whether the manual was

understandable for a typical laboratory technician. This evaluation was based on a number of specific points of importance; see Table 7.25 and Table 7.26 for the parameters included and the assessment outcomes.

Table 7.25 Assessment of the user manual for LUMIStox.

Parameter	**Complete description**	**Summary description**	**No description**	**Not relevant**
Product				
Principle of operation	√			
Intended use	√			
Performance expected	√			
Limitations	√			
Preparations				
Unpacking			√	
Transport				√
Assembly	√			
Installation	√			
Function test	√			
Operation				
Steps of operation	√			
Points of caution	√			
Accessories	√			
Maintenance	√			
Trouble shooting		√		
Safety				
Chemicals	√			
Power				√

Table 7.26 Assessment of the user manual for ECLOX.

Parameter	Complete description	Summary description	No description	Not relevant
Product				
Principle of operation	√			
Intended use	√			
Performance expected	√			
Limitations	√			
Preparations				
Unpacking	√			
Transport			√	
Assembly	√			
Installation	√			
Function test	√			
Operation				
Steps of operation	√			
Points of caution	√			
Accessories	√			
Maintenance	√			
Trouble shooting		√		
Safety				
Chemicals	√			
Power	√			

A description was considered complete if all essential steps were described, if they were illustrated with a figure or a photo, where relevant, and if the descriptions were understandable without reference to other guidance.

7.4.2 Product costs

The capital investment costs and the operation and maintenance cost – components of product sustainability – were itemized based upon a determined design basis /28/; see Table 7.27 for the items that were included.

The design basis was determined based on one laboratory day. According to HACH-LANGE the shelf life of the dried reagent is one year, the lifetime of the rehydrated bacteria suspension is 4 hours. Within that time it was possible to perform an EC_{50} test according to the ISO 11348-3 on three samples plus associated controls and standards.

Table 7.27 List of capital cost items and operation and maintenance cost items per product unit.

Item type	Item	Number/duration
Capital		
Buildings and land	Laboratory facility	1
Equipment	LUMIStox or ECLOX LUMIStherm PC with LUMISsoft4	1 1 1
Utility connections	Power supply	3
Installation	Can be done by operator/laboratory technician	1 day
Start up/training	Training of laboratory technician	1 day
Operation and maintenance		
Materials, including chemicals	Bacteria Cuvettes Reconstitution solution 2% NaCl solution Solid NaCl	1 batch 20 pr. sample (=60 per day) 1 bottle 1 bottle 1 bottle
Utilities, including water and energy	Power	PC and screen ~6 kWh ECLOX 4 AA batteries LUMIStox ~0.4 kWh LUMIStherm ~0.4 kWh
Labor	One laboratory technician	1 day

Costs associated with the equipment at the time of testing were:

- LUMIStox, LUMIStherm, the software LUMISoft4: 13,000 Euro (17,800 $U.S.),
- ECLOX, LUMIStherm, the software LUMISoft4: 6,500 Euro (9,600 $U.S.).

Additional equipment such as cuvettes, bacteria and chemicals on a cost-per-sample basis as used for testing for EC_{50} according to the ISO 11348-3: 18 Euro (23 $U.S.).

7.4.3 Occupational health and environment

The risks for occupational health and safety and for the environment associated with the use of the products were compiled. The compilation emphasized chemicals classified as hazardous used during product operation /29/. No application of hazardous chemicals was identified during testing.

No risk from installation, operating and maintaining the product were identified, based on an assessment of risks for human health, power supply, and danger of infections. No additional risks compared to conventional effluent wastewater testing or analyses were identified.

7.5 Operational parameters

The effluent wastewater parameters covered in the test are summarized in Table 7.28. The wastewater parameters were measured by Eurofins.

Table 7.28 Results of analytical parameters analyzed in wastewater.

Parameters	Unit	Industrial wastewater	Domestic wastewater
Turbidity	FTU	15	2.4
TOC	mg/L	39	10
Conductivity	mS/m	4300	140
Alkalinity	mmol/L	6.9	5.5
pH	-	7.7	7.5
COD	mg/L	110	28
Suspended solids (SS)	mg/L	83	4.9
Nitrogen (total)	mg/L	6.3	6.9
Phosphorus (total)	mg/L	4.2	0.23
BOD_5	mg/L	3.4	5.2

The operational parameters tested in the DHI laboratory are summarized in Table 7.29.

Table 7.29 Operational parameters evaluated during testing.

Temperature of thermal block	pH in sample	Color correction	Temperature at field use (ECLOX)	Cuvette material (LUMIStox)
14.0 - 16.1 °C	6.0 - 8.5	Colored samples Turbid samples	5 - 23 °C	Glass Plastic

7.6 Recommendation for verification statement

The verification statement is a summary of the results described in the verification report. The results included in the verification statement are listed in this section.

Table 7.30 Description of matrix and effect for LUMIStox and ECLOX.

Matrix	Effect
LUMIStox and ECLOX are applied for wastewater; river and lake water; leachate from soil, waste, rubble, etc.; or directly in fluent chemicals. Verification testing was conducted on domestic and industrial wastewater effluents	Measurement of toxicity as indicated by inhibition of luminescent bacteria by a variety of compounds including metal ions, organic pesticides, inorganic and organic pollutants and surfactants **Additional parameters included:** User manual quality, product cost, environmental health and safety

The primary results include short description of the matrix and effect as given in Table 7.30, the performance parameters verified for LUMIStox and given in Table 7.31 and for ECLOX, given in Table 7.32. Listed are results for EC_{50} values or percent inhibition after 30 minutes.

Table 7.31 LUMIStox performance parameter summary.

LUMIStox	**Criterion of detection**	**Range of application**	**Precision**		**Agree-ment with accepted values**	**Robustness**		
Compound	% inhibition	mg/L	**Repeat-ability** %	**Reproduc-ibility** %	%	**pH, color, turbidity, laboratory temperature**[1)] %	**Cuvette material** %	**Waste-water matrix**[1)] %
General	5.8							
Zn^{2+}		>8.3-<130	5.0	30	186		106-117	43-123
$Cr_2O_7^{2-}$		>35-<560	29		91			0-22
Triclosan		>1.1-<17	5.5		189			96-141
Cyanide		>48->780	24					
SDS		>2.0-<32	33			71-114	90-101	28-96
CTAB		>1.9-<31	2.4		100			68-78

[1)] For colored samples are given robustness after use of color correction. For $BaSO_4$-turbide samples is given robustness without use of color correction. For domestic wastewater adjustment was made to account for the negative inhibition from the wastewater, if color correction was used the robustness was -20% to 70%. The values given are therefore the best achievable robustness.

Table 7.32 ECLOX performance parameter summary.

ECLOX	**Criterion of detection**	**Range of application**	**Precision**		**Agree-ment with accepted values**	**Robustness**		
Compound	% inhibition	mg/L	**Repeat-ability** %	**Reproduc-ibility** %	%	**pH, color, turbidity, laboratory temperature**[1)] %	**Field temperature (15 minutes)** %	**Waste-water matrix**[1)] %
General	5.5							
Zn^{2+}		>8.2-<130	4.9	51	186		11-171	22-125
$Cr_2O_7^{2-}$		>37-<590	24		96			-20-13
Triclosan		>1.1-<17	2.2		190			101-141
Cyanide		>35->570	16					
SDS		>2.0-<32	38			85-115	73-105	35-101
CTAB		>1.9-<31	1.2		99			61-73

1) For colored samples are given robustness after use of color correction. For $BaSO_4$-turbide samples is given robustness without use of color correction. For domestic wastewater adjustment was made to account for the negative inhibition from the wastewater, if color correction was used the robustness was 101% to 130%. The values given are therefore the best achievable robustness.

The user manual and other instructions were described as complete. The manual described that color correction shall be used for colored as well as turbid samples. The robustness test with $BaSO_4$-turbid samples showed that application of color correction was not appropriate.

The product costs based on a scenario for one laboratory day are as listed:

Table 7.33 List of capital cost items and operation and maintenance cost items per product unit.

Item type	Item	Number/duration
Capital		
Buildings and land	Laboratory facility	1
Equipment	LUMIStox or ECLOX LUMIStherm PC with LUMISsoft4	1 1 1
Utility connections	Power supply	3
Installation	Can be done by operator/laboratory technician	1 day
Start up/training	Training of laboratory technician	1 day
Operation and maintenance		
Materials, including chemicals	Bacteria Cuvettes Reconstitution solution 2% NaCl solution Solid NaCl	1 batch 20 pr. sample (=60 per day) 1 bottle 1 bottle 1 bottle
Utilities, including water and energy	Power	PC and screen ~6 kWh ECLOX 4 AA batteries LUMIStox ~0.4 kWh LUMIStherm ~0.4 kWh
Labor	One laboratory technician	1 day

Costs associated with the equipment at the time of testing were:

- LUMIStox, LUMIStherm, the software LUMISoft4: 13,000 Euro (17,800 $U.S.),
- ECLOX, LUMIStherm, the software LUMISoft4: 6,500 Euro (9,600 $U.S.).

Additional equipment such as cuvettes, bacteria and chemicals on a cost-per-sample basis as used for testing for EC_{50} according to the ISO 11348-3: 18 Euro (23 $U.S.).

Application of the test systems does not give rise to any special risk or contact to hazardous substances other than what occur doing conventional testing of wastewater effluents.

The operational parameters are shown in Table 7.34 and the wastewater chemistry is listed in Table 7.35 as range of concentration or parameter measured.

Table 7.34 Operational parameters evaluated during testing.

Temperature of thermal block (° C)	pH in sample	Color correction	Temperature at field use (ECLOX)	Cuvette material (LUMIStox)
14.0 - 16.1	6.0 - 8.5	Colored samples Turbid samples	5 - 23 °C	Glass Plastic

Table 7.35 Range of analytical parameters analyzed in wastewater.

Parameters	**Unit**	**Range**
Turbidity	FTU	2.4 - 15
TOC	mg/L	10 - 39
Conductivity	mS/m	140 - 4300
Alkalinity	mmol/L	5.5 - 6.9
pH	-	7.5 - 7.7
COD	mg/L	28 - 110
Suspended solids (SS)	mg/L	4.9 - 83
Nitrogen (total)	mg/L	6.3 - 6.9
Phosphorus (total)	mg/L	0.23 - 4.2
BOD_5	mg/L	3.4 - 5.2

8 VERIFICATION SCHEDULE

The verification was planned and performed from October 2009 through April 2010. The overall schedule is provided in Table 8.1.

Table 8.1 Verification schedule.

Task	**Timing**
Quick scan	October 2009
Verification protocol and test plan	October to December 2009
Test	January to April 2010
Test reporting	February to April 2010
Verification	April 2010
Verification report	April 2010
Report and verification statement preparation and review	April 2010 to February 2011

9 QUALITY ASSURANCE

The quality assurance of the verification is described in Table 9.1 and Fig 1.2. The quality assurance of the tests was described in the test plan as well as in the process document prepared by Battelle /1/, and is summarized in Table 10.1 below.

Table 9.1 QA summary for the verification.

	DHI		Battelle AMS Center	U.S. EPA ETV	ETV Canada	Environ-ment Canada	Expert Group
Initials	MWN	BOP	ZW	JMK, MH	MEH	BD	KOK, JA, AA, ML (verification report only)
Tasks							
Plan document with verification protocol and test plan	Review	-	Review	Review	Review	-	Review
Test system	-	Audit	Audit	-	-	-	-
Test report	Review	-	-	-	Review	-	Review by KOK
Verification report	Review	-	Audit/Review	Review	Review	Review	Review

An internal review of plan and report documents was conducted by the Head of Innovation, Margrethe Winther-Nielsen (MWN). A test system audit (see test plan) was conducted on 22 January 2010 following GLP audit procedures by a trained auditor: Senior Chemical Engineer, Bodil Mose Pedersen (BOP).

The Battelle Quality Manager, Zachary Willenberg (ZW) performed a technical systems audit (TSA) during this verification and test on 26-29 January 2010. An audit of data quality was conducted 13 May through 4 June 2010.

U.S. EPA staff, John McKernan (JMK) and Michelle Henderson (MH) and Mona El-Hallak (MEH) from ETV Canada reviewed all plan and report documents, except the test report. In addition, Mona El-Hallak (MEH) from ETV Canada reviewed the test report, and Benoit Desforges (BD) from Environment Canada reviewed the verification report.

The expert group, Kresten Ole Kusk (KOK), Dr. Joel Allen (JA) and Dr. Ali Amiri (AA) reviewed the plan and report documents, though only Kresten Ole Kusk (KOK) reviewed the test report. Dr. Max Lee also reviewed the verification report.

A P P E N D I X 1

Terms and definitions used in the verification protocol

The abbreviations and definitions used in the verification protocol are summarized below.

Where discrepancies exist between DANETV and U.S. EPA ETV terminology, definitions from both schemes are given.

Word	DANETV	U.S. EPA ETV
Agreement with accepted values	Here defined as the % agreement between literature values and test results	
AMS Center	Advanced Monitoring Systems Center at Battelle	
Analytical laboratory	Independent analytical laboratory used to analyze reference samples	
Application	The use of a product specified with respect to matrix, target, effect and limitations	
CD	Criterion of detection	
CTAB	Cetyl trimethyl ammonium bromide	
DANETV ETV	The Danish Centre for Verification of Climate and Environmental Technologies	
EC	Effect concentration, e.g. causing 50% inhibition (EC_{50})	
ECLOX	ECLOX handheld luminometer from HACH-LANGE	
Effect	The way the target is affected	
EN	European standard	
ETV	Environmental technology verification (ETV) is an independent (third party) assessment of the performance of a technology or a product for a specified application, under defined conditions and adequate quality assurance	EPA program that develops generic verification protocols and verifies the performance of innovative environmental technologies that have the potential to improve protection of human health and the environment
EU	European Union	
Evaluation	Evaluation of test data for a technology product for performance and data quality	An examination of the efficiency of a technology
Experts	Independent persons qualified on a technology in verification or on verification as a process	Peer reviewers selected for a verification
GLP	Good Laboratory Practice	
ISO	International Standardization Organization	
LID	Lowest ineffective dilution. Often seen as the dilution in a dilution series causing less than 20% inhibition	
Limit of detection LoD	Calculated from the standard deviation of replicate measurements at less than 5 times the detection limit evaluated. Corresponding to less than 5% risk of false blanks	
LUMISsoft4	PC software from HACH-LANGE, produced for LUMIStox	
LUMIStherm	Thermostat from HACH-LANGE, produced for LUMIStox	
LUMIStox	LUMIStox 300 bench top luminometer from HACH-LANGE	
Matrix	The type of material that the product is intended for	

Word	DANETV	U.S. EPA ETV
Method	Generic document that provides rules, guidelines or characteristics for tests or analysis	
OD	Optical density	
PC	Personal computer	
Performance claim	The effects foreseen by the vendor on the target (s) in the matrix of intended use	
Performance parameters	Parameters that can be documented quantitatively in tests and that provide the relevant information on the performance of an environmental technology product	
Precision	The relative standard deviation obtained from replicate measurements, here measured under repeatability or reproducibility conditions	
(Environmen-tal) product	Ready to market or prototype stage product, process, system or service based upon an environmental technology	(Environmental) technology
QA	Quality assurance	
Range of application	Generally: the range from the LoD to the highest concentration with linear response. For this verification the range is based on range of dilution of a test sample	
Reference analyses	Analysis by a specified reference method in an accredited (ISO 17025) laboratory	
Repeatability	The precision obtained under repeatability conditions, that is with the same measurement procedure, same operators, same measuring system, same operating conditions and same location, and replicate measurements on the same or similar objects over a short period of time	
Reproducibility	The precision obtained under reproducibility conditions. Measurement performed at different locations, operators, measuring systems, and replicate measurements on the same or similar objects	
Robustness	% variation in measurements resulting from defined changes in matrix properties	
RSD	Relative standard deviation in %	
SDS	Sodium lauryl sulphate	
Stakeholder		Buyers and users of technology, technology developers/vendors, the consulting engineers, the finance and export communities, government permitters, regulators, first responders, emergency response, disaster planners, public interest groups, and other groups interested in the performance of innovative environmental technologies
Standard	Generic document established by consensus and approved by a recognized standardization body that provides rules, guidelines or characteristics for tests or	

Word	DANETV	U.S. EPA ETV
	analysis	
Target	The measurable property that is affected by the product	
(Environmen-tal) technology	The practical application of knowledge in the environmental area	An all-inclusive term used to describe pollution control devices, controls, monitoring systems, waste treatment processes and storage facilities, and site remediation technologies and their components that may be utilized to remove pollutants or contaminants from, or to prevent them from entering the environment
Test/testing	Determination of the performance of a product by parameters defined for the application	
Trueness	The % recovery of true value obtained either from knowledge on the preparation of test solutions or from measurements with reference methods	
TSA	Technical system audit	
U.S. EPA	United States Environmental Protection Agency	
Vendor	The party delivering the product or service to the customer	The technology developer, owner, or licensee seeking verification
Verification	Evaluation of product performance parameters for a specified application under defined conditions and adequate quality assurance	Establishing or proving facts of the performance of a technology under specific, predetermined criteria, test plans and adequate data QA procedures
Vibrio fischeri	Light producing bacteria used in luminescent bacteria test	

A P P E N D I X 2

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A P P E N D I X 3

Application and performance parameter definitions

This appendix defines the applications and the relevant performance parameters used to verify the performance of an environmental technology following the DANETV Program. The appendix was prepared as part of the verification protocol /33/.

1 Applications

The intended application of the product for verification is defined in terms of the matrix, the targets and the effects of the product.

The LUMIStox and ECLOX are luminometers which measure light from the light producing bacteria *Vibrio fischeri*, as indicator of acute toxicity.

1.1 Matrix/matrices

The luminometers are sold for testing of wastewater; river and lake water; leachates from soil, waste, rubble, etc.; or directly in fluent chemicals. The matrix in which the application is being verified is wastewater effluent from both domestic and industrial sources.

1.2 Effect

The luminometers can measure any acute toxicity that causes an effect on the light emission from *Vibrio fischeri*. In the ISO 11348-3 /23/ standard, which the LUMIStox is being tested according to, three compounds are listed as reference substances to be included in validity testing. These are 3,5-dichlorophenol, zinc (II) as zinc sulphate heptahydrate and chromium (VI) as potassium dichromate.

The verification will include these reference substances as well as selected metal ions, organic pesticides, organic toxic compounds, industrial chemicals and surfactants.

1.2.1 Compounds to be tested

The vendor has suggested a list of compound to be included in the verification; these are listed in Appendix Table 1.

Appendix Table 1 List of compounds suggested by vendor.

Group	Compound
Heavy metals	Hg-complexes as $HgCl_2$ Pb^{2+} as $Pb(NO_3)_2$ Zn^{2+} as $ZnSO_4$+$7H_2O$ $Cr_2O_7^{2-}$ as $K_2Cr_2O_7$
Organic pesticide	2,4,5 Trichloroanilin
Organic pollutants	Formaldehyde p-Crecol Hydroquinone (benzene-1,4-diol)
Industrial pollutant	Cyanide (CN-) as KCN
Surfactants	SDS (sodium lauryl sulphate) CTAB (cetyl trimethyl ammonium bromide)

The vendor has performed tests on all suggested compounds except $HgCl_2$ and 2,4,5-trichloroanilin.

Each of the target groups and vendor suggested compounds was evaluated as follows:

Hg is banned in the EU; it is therefore not likely to be found in European domestic wastewater today. Hg is difficult to work with in the laboratory. For these reasons Hg is excluded.

Copper is included since it is a good representative for heavy metals in both domestic and industrial wastewater, and since it is found in wastewater as many different ions.

The ISO 11348-3 uses 3,5-dichlorophenol, Zn^{2+} (as $ZnSO_4+7H_2O$) and Cr^{6+} (as $K_2Cr_2O_7$, in water resulting in $Cr_2O_7^{2-}$) as reference substances for testing the quality of delivered bacteria batches. $Cr_2O_7^{2-}$ will be included giving the possibility to do some reference to the standard and the precision test which is described in Appendix Table 7. Zn^{2+} will be included since good literature values exist.

Having two positive metals ions (Cu^{2+} and Zn^{2+}), seems sufficient and Pb^{2+} has therefore been excluded from the test program.

2,4,5-trichloranilin is not a regularly used pesticide. Instead a pesticide produced by the Danish company Cheminova and included in their standard effluent wastewater analyses is included. The specific pesticide, flutriafol, has been chosen in cooperation with Cheminova.

Hydroquinone is not seen as a compound with special relevance for effluent wastewater and is therefore excluded.

Formaldehyde and p-cresol are easily degradable and relatively volatile. It is therefore unlikely that they will remain in the wastewater effluent after treatment in the plant. Instead, triclosan, which is widely used in household products and found in domestic wastewater, is included. Triclosan is toxic to bacteria.

U.S. EPA ETV has performed verification of similar equipment, but to be used on a chlorinated drinking water matrix. The selection of compounds for those tests was made with a different focus than in this verification. However, the U.S. EPA ETV verification included cyanide, which also is included in the list of compounds suggested by vendor. The vendor has found cyanide to be difficult to work with at pH 7. Cyanide will be included as target compound, but special actions will be taken to ensure and monitor loss of cyanide from test solutions.

In addition to the listed surfactants, nonylphenol ethoxylate will be included in the test since it is a well know surfactant that is very toxic to aquatic organisms and is unwanted in the water environment. By including nonylphenol ethoxylate the three surfactants will represent anionic, cationic and nonionic detergents.

The final list of compounds to be included in the verification is listed in Appendix Table 2.

Appendix Table 2 List of compounds to be included in test with notification on whether compound is typical for domestic or industrial wastewater.

Group	Compounds suggested by vendor	Chosen compounds	Domestic	Industrial
Heavy metals	Hg-complexes as $HgCl_2$ Pb^{2+} as $Pb(NO_3)_2$ Zn^{2+} as $ZnSO_4+7H_2O$ $Cr_2O_7^{2-}$ as $K_2Cr_2O_7$	Cu^{2+} as $Cu(NO_3)_2$ CrO_7^{2-} as K_2CrO_7 Zn^{2+} as $ZnSO_4+7H_2O$	X X X	X X X
Organic pesticides	2,4,5 Trichloroanilin	Flutriafol		X
Organic pollutants	Formaldehyde p-Crecol Hydroquinone (benzene-1,4-diol)	Triclosan	X	X
Industrial pollutant	Cyanide (CN-) as KCN	Cyanide (CN^-) as KCN		X
Surfactants	SDS (sodium lauryl sulphate) CTAB (cetyl trimethyl ammonium bromide)	SDS (sodium lauryl sulphate) CTAB (cetyl trimethyl ammonium bromide) Nonylphenol ethoxylate	X X X X	X X X X

Appendix Table *3* is a list of EC_{50}-values for the selected compound found in the literature.

Appendix Table 3 EC_{50}-values from literature for the selected compounds.

Group	CAS no.	Compound	EC_{50} (*Vibrio fischeri)* mg/L	According to ISO 11348-3	Reference
Heavy metals	7758-98-7	Cu^{2+} (cupper sulfate)	7.1 (0.35 – 19.5, n=3)	to be determined	/26/
	7778-50-9	$Cr_2O_7^{2-}$ (potassium dichromate)	18.7 mg/L ±11%	Yes	/23/
	7733-02-0	Zn^{2+} (zinc sulphate heptahydrate)	2.2 mg/l ± 23%	Yes	/23/
Organic pesticides	7667-21-0	Flutriafol	no data found		
Organic pollutants	3380-34-5	Triclosan	0.28	Yes	/21/
Inorganic pollutant	57-12-5	Cyanide (CN^-)	4	No	/7/
Surfactants	151-21-3	SDS	2.09	unknown	/22/
	57-09-0	CTAB	0.97[2]	Yes	/27/
	104-35-8	Nonylphenol ethoxytale	no data found		

1.3 Target(s)

The targets for the application are generally reported in terms of limit of detection (LoD), precision (repeatability and reproducibility), trueness, range of application and robustness. For toxicity testing the limit of detection is not possible to determine. Instead it is chosen to determine the criterion of detection (CD) based on the standard

[2] 30 minutes incubation time.

deviation of blanks. The trueness of the inhibition is difficult to measure, and therefore the verification of trueness will be replaced a verification of agreement with accepted values, which will be evaluated by comparing the measured value to available robust literature values obtained by use of the ISO 11348-3 method, for same compound. The range of the application cannot be determined directly by identification of linear range as for regular measurements. For this verification range is based on the inhibitions needed to determine EC_{50}-values, see description in Section 4 Performance parameter definitions.

The values of the targets claimed by the vendor are given in Appendix Table 4 for the products.

The vendor has incorporated equipment in the LUMIStox for color correction of inhibition. With the use of the color correction on colored samples a robustness of 95-113% was shown. Without color correction, the robustness was 109-148% /45/.

The robustness is the relative results (relative to standard) due to defined variations in e.g. concentration level, temperature, pH, color, turbidity, cuvette types, matrix (pure water versus wastewater). The ISO 11348-3 standard includes the possibility of testing (marine) saltwater samples; however, saltwater samples are not included in robustness testing of the products.

Appendix Table 4 Vendor claim of performance /5/.

	Criterion on detection[3]	**Precision (RSD)** %		**Range of application (linear screening range)**	**Agreement with accepted values**	**Robustness**
		Precision of instrument	**Precision of test**[4]			
	% inhibition			% inhibition	%	%
LUMIStox	(10)	0.7	< 20	10-90	Not specified	Not specified
ECLOX	(10)	2	< 20	10-90	Not specified	Not specified

The vendor has recently tested selected compounds. The results can be found in Table 5.1, in Section 5.1 Summary of existing data.

In the ECLOX manual the vendor states the following:

> *Due to nature of the simplified procedure and that the test is carried out at ambient temperatures the results may differ if compared directly with results [derived] for the same sample using the ISO 11348 method.*

1.4 Exclusions

The verification is to be performed on one effluent domestic wastewater and one industrial wastewater, other media are excluded. However, individual test substances are tested in 2% NaCl MilliQ-water.

[3] Given as part of linear range.

[4] Is not clearly stated from vendor as repeatability or reproducibility.

According to the vendor, samples containing chlorine as a result of drinking water chlorination will interfere with the test results by affecting the viability of the bacterial agents. Chlorine containing samples are excluded from the test.

2 General performance requirements

No formal performance requirements for the application have been identified in the European Union or the U.S. and Canada.

The conventional performance parameters of analytical and monitoring methods and equipment are limit of detection (LoD), precision (repeatability and reproducibility), trueness, specificity, linearity and matrix sensitivity. The uncertainty of measurements may be used to summarize the performance. Parameters may be added to characterize variations of equipment, e.g. on-line or on-site monitoring instruments.

2.1 Regulatory requirements

No regulatory requirements exist for measurement of luminescent toxicity. The new Water Framework Directive 2009/90/EC of 31 July 2009 contains a minimum performance criteria of 25% RSD, applicable for all methods of analysis.

In Germany, wastewater regulations include results from luminescent bacteria tests (LID, lowest ineffective dilution) as quality criteria for several industries including the chemical industry, the rubber industry, cooling towers and waste treatment plants /24/. For the chemical industry a LID = 32 times is accepted, meaning that the wastewater has to be diluted a maximum of 32 times to obtain a toxicity below 20% inhibition towards the luminescent bacteria.

For a few of the compounds, environmental quality standards for surface waters are given by the EU /14/. These are listed in Appendix Table 5.

Appendix Table 5 Environmental quality standards stated by EU /14/ and Denmark. For Denmark values in normal writing are effective /15/, while values in italic are planned to come in force within 2010 /16/.

Group	**Compound**	**EU**		**Denmark**	
		Inland surface water µg/L	**Other surface water** µg/L	**Fresh water** µg/L	**Marine water** µg/L
Heavy metals	Cr(VI)			*4.9 (dissolved)*	*3.4 (dissolved)*
	Cu			*1.0* (dissolved) max. 12	*1.0* (dissolved) max. 2.9
	Zn			*7.8 (dissolved) Soft water: (H<24 mg $CaCO_3$/L) 3.1 (dissolved)*	*7.8 (dissolved)*
Organic pesticides	Flutriafol			*31*	*3.1*

2.2 Application based needs

A validity check is required according to ISO 11348-3. The validity check involves analysis of three reference standards which should cause 20 to 80% inhibition after 30 minutes of contact time. The results from the validity check are shown in Appendix Table 6, as reported for the LUMIStox by vendor.

Appendix Table 6 Vendor quality data for LUMIStox according to ISO 11348-3 /6/.

	3,5 dichlorophenol	**Zn^{2+}**	**$Cr_2O_7^{2-}$**
Standard concentration	3.4 mg/L	2.2 mg/l (zinc sulphate heptahydrate)	19 mg/L (potassium dichromate)
No. of data set	70	60	70
Range of inhibition	22-64%	21-49%	48-79%
Mean inhibition	44%	31%	63%
RSD	27%	23%	11%

In ISO 11348-3, results from an interlaboratory trial with the three reference standards are listed for information. The results are shown in Appendix Table 7.

Appendix Table 7 Interlaboratory trial, Annex C, ISO 11348-3.

	3,5 dichlorophenol		**Zn^{2+}**		**$Cr_2O_7^{2-}$**	
	EC_{20}	EC_{50}	EC_{20}	EC_{50}	EC_{20}	EC_{50}
No. of laboratories	14	13	15	14	15	14
Average conc.	2.32 mg/L	3.36 mg/L	1.08 mg/L	2.17 mg/L	3.60 mg/L	18.71 mg/L
RSD	18.6%	9.6%	43.6%	33.6%	52.4%	32.9%

3 State of the art performance

Other similar luminometers exist on the market. Some selected luminometers are listed in Appendix Table 8. Information as to whether they have been verified is included.

Appendix Table 8 Luminometers and verification of these.

Name	**Verification**	**Reference**
Portable		
BioFix Lumi-10	None known	/11/
Triathler	None known	/12/
ToxScreen-II	U.S. EPA ETV	/9/
Deltatox	U.S. EPA ETV	/8/
Laboratory		
Microtox	U.S. EPA ETV	/7/
Field installation		
TOXcontrol BioMonitor	TESTNET	/10/

The three U.S. EPA ETV verifications have all been performed using drinking water with a focus on chemical compounds toxic to humans. One compound, cyanide, is also relevant with regards to wastewater. Performance on cyanide measurements for the three products is listed in Appendix Table 9. The toxicity threshold is the lowest

concentration of the tested dilutions where toxic effects were significant. For ToxScreen-II a special set-up was used and EC_{50} could therefore not be retrieved.

Appendix Table 9 Results from U.S.EPA ETV verification on cyanide.

Luminometer	**Microtox**	**Deltatox**	**ToxScreen-II**
Cyanide EC_{50} at 5 minutes	8.0 mg/L	7.6 mg/L	Not measured
Cyanide EC_{50} at 15 minutes	4.0 mg/L	Not measured	Not measured
Repeatability. Range of relative standard deviation	0-4.0%	1.0-4.0%	0-29%
Toxicity threshold	0.25 mg/L	0.25 mg/L	0.25 mg/L

For the TOXcontrol BioMonitor the LoD, RSD, repeatability etc. were tested and reported for several test set-ups. The compounds used were Zn^{2+} and 3,5 dichlorophenol. Some of the results are summarized in Appendix Table 10.

Appendix Table 10 Results from TESTNET verification of TOXcontrol BioMonitor.

	Range	**Comment**
Lowest detectable change	7.2-17% inhibition	Calculated based on solution of approximately 20%, 50% and 80% inhibition
RSD	5.7-39%	
Repeatability	2.4-5.8% inhibition	
Day-to-day repeatability	2.5-31% inhibition	Calculated based on solution of approximately 20% and 80% inhibition
Memory effect	Not relevant	No significant effect
Interference (Tropaeolin-color)	Not relevant	Increased inhibition was significant at concentrations from 0.25 mg/L

Vendors of *Vibrio fischeri* test the bacteria lots and state an interval for EC_{50} for selected standard parameters. They also test each lot before shipment. An example of such a test from an anonymous vendor including user laboratory reference testing is shown in Appendix Table *11*.

Appendix Table 11 EC_{50} performance of Vibrio fischeri *on standard parameters stated by vendor and tested by vendor and user laboratory.*

Standard parameter	**Phenol**	**Zinc sulfate**	**Zinc^{2+} (ion)**
Specification from vendor			
EC_{50} interval at specification	13-26 mg/L	3.0-10 mg/L	0.60-2.2 mg/L
Test time	5 minutes	15 minutes	15 minutes
Vendor test result			
No. of LOTs	9	9	9
Mean	18 mg/L	4.9 mg/L	1.0 mg/L
RSD	19%	27%	25%
User laboratory test result			
No. of LOTS	9	9	Not tested
No. of tests	14	15	-
Mean	18 mg/L	5.5 mg/l	-
RSD	10%	20%	-

4 Performance parameter definitions

Based on the above-mentioned performance requirements, a set of relevant ranges of performance parameters for activated sludge tanks (and treated wastewater) have been set up and are listed in Appendix Table 12.

Appendix Table 12 Relevant ranges of performance parameters in effluent wastewater.

	Criterion of detection	Range of application	Precision (RSD) %		Agreement with accepted values	Robustness
			Repeat-ability	Reproduce-ability		
	% inhibition	L/L			%	%
LUMIStox	< 10	>1/2 - < 1/32	< 20	< 30	100 ±50	100±50
ECLOX	< 10	>1/2 - < 1/32	< 20	< 30	100 ±50	100±50

The limit of quantification is set to 10% because this is equal to the vendor claim for linear range and because EC_{10}-values often are used for reporting ecotoxicological results.

The range of application for a chemical analysis is usually the range of analyte concentration from the limit of detection to the highest concentration with linear response. This concept is not meaningful for a toxicity test of a water sample, because the test does not measure a concentration but an inhibitory effect as a function of the dilution of the sample. The range of application for determining EC_{50} therefore has to be considered in terms of dilution. According to the HACH-LANGE manual estimation of an EC_{50} of a water sample requires a minimum of three measurements where the inhibition is between 10% and 90%. In addition one of the three measurements must be above 50%. If the standard dilution row is considered as described in the LUMIStox 300 Operation manual and in Annex B of the ISO 11248-3:2007 with 9 dilutions (2, 3, 4, 6, 8, 12, 16, 24, 32 times dilution in the test suspension) then EC_{50} should be in the range of dilutions > 2 and < 32 times dilution assuming three measurements with inhibition between 10 and 90%. Based on test results will be given ranges of concentrations of the compounds tested in this study, which will give an inhibition within the range of application.

Repeatability in Appendix Table 9 and Appendix Table 10 is less than 6% in all cases, except for the ToxScreen-II, where a repeatability of 0-29% is seen. The vendor claims a precision for the products of < 20%, see Appendix Table 4. A repeatability of less than 20% is chosen, since the vendor claims to fulfill this.

The day-to-day repeatability for TOXcontrol BioMonitor, as shown in Appendix Table 10, lists RSD values up to 31.2%. The vendor states, as mentioned, a test precision of < 20%, while the quality check of LUMIStox in Appendix Table 6 shows a reproducibility of up to 27%. Here a reproducibility of 30% is chosen.

The agreement with accepted values is evaluated by looking at the EC_{50}-values specified by a vendor of *Vibrio fischeri* LOTs in Appendix Table *11*. The largest relative interval is given for zinc^{2+}; the "mean" here is 1.4 mg/L with an acceptable range of ± 57%. The ISO standard 11348-3 requires inhibition of 20-80% of specified concentrations. These numbers cover both reproducibility and repeatability. The agreement with accepted values is set to ± 50%.

Robustness has been tested directly for the TOXcontrol BioMonitor, where the dye chemical tropaeolin was added. The results showed a significant interference at 0.25 mg tropaeolin/L, where an increased inhibition was seen. Color correction is part of the LUMIStox product; see section 1.3 Target(s). The robustness can be interfered by other parameters. The general robustness is set to the level seen without color correction; here values of 148% of true value were seen.

A P P E N D I X 4

Deviation report for verification and testing

Deviation reports

The test plan version approved must be followed. If (or rather when) deviations are needed during testing, the deviations are noted and justified in the format:

Dev. No.	Experiment label Test Plan	Deviation	Cause	Impact assessment	Corrective action, if any	Date	Signature test responsible	Date	Signature verification responsible	Date	Signature Battelle AMS QM	Date	Signature ETV Canada
1	Test A	CuSO4 testing was not completed.	Precipitation was observed. Not toxic at concentration with no precipitation. Frozen samples very different from fresh samples. Inaccurate pH adjustment gave different toxicity even when final pH was identical.	The Test A evaluation for the heavy metal category will be based on one fewer compound. However, results for two other heavy metals (Zn and Cr) will still be performed and should adequately represent performance for the heavy metal category.	None	6/5-10		6/5 2010				10/5/2010	
2	Test A	Flutriafol testing was not completed.	Flutriafol was not sufficiently toxic at concentrations with no precipitation.	The Test A evaluations for the organic pesticide category will not be completed. Performance will still be evaluated for organic compounds by testing with Triclosan and the detergents.	None	6/5-10		6/5 2010				10/5/2010	

3	Test D	Will be performed with 3 instead of 4 bacteria batches.	Only 3 different batches could be delivered from Hach Lange.	Test on reproducibility is reduced from 4 to 3 bacteria batches. By switching to three bacteria batch means for the calculation of relative standard deviation (RSD), the 95 percent confidence interval range of values for RSD is likely to be approximately 15% larger than it would be with 4 batches. This was determined by a simulation study assuming that the data are normally distributed.	None			6/5 2016				

Deviation reports

The test plan version approved must be followed. If (or rather when) deviations are needed during testing, the deviations are noted and justified in the format:

Dev. No.	Experiment label Test Plan	Deviation	Cause	Impact assessment	Corrective action, if any	Date	Signature test responsible	Date	Signature verification responsible	Date	Signature Battelle AMS QM	Date	Signature ETV Canada
4	Test A	Repeatability for 4-NPE was not completed. The sample was not sent for chemical analyses or Microtox testing.	Found solubility in literature around 1-3 mg/l. Dissolved 3 mg in one liter. Pre-screening showed that this concentration was non-toxic.	The evaluation of repeatability will be based on fewer compounds. 4-NPE and a detergent 2 other detergents are present in the results for evaluation.	None	25/3-10		25/3-2010		4-28-10		29/4/2010	

① Spelling error BW 4-28-10

Deviation reports

The test plan version approved must be followed. If (or rather when) deviations are needed during testing, the deviations are noted and justified in the format:

Dev. No.	Experiment label Test Plan	Deviation	Cause	Impact assessment	Corrective action, if any	Date	Signature test responsible	Date	Signature verification responsible	Date	Signature Battelle AMS QM	Date	Signature ETV Canada
5	K	The test on industrial waste water was only performed as replicate, not triplicate as specified	One tube was accidentally measured twice. When trying to correct the error the test was aborted.	The waste water is non toxic. Therefore duplicate measurements are considered sufficient	None	23/4-10		24/5-10		4-29-10		30-4-10	
6	K	The test of the municipal waste water will be run in duplicate in case of non toxicity.	To be consistent with the test on industrial waste water.	If the waste water is non toxic, a duplicate measurement will be sufficient and consistent with the industrial water test. If toxic, there is no deviation and test will be run in triplicate per the original test plan.	None	23/4-10		24/5-10		4-29-10		30-4-10	

Deviation reports

The test plan version approved must be followed. If (or rather when) deviations are needed during testing, the deviations are noted and justified in the format:

Dev. No	Experiment label: Test Plan	Deviation	Cause	Impact assessment	Corrective action, if any	Date	Signature test responsible	Date	Signature verification responsible	Date	Signature Battelle AMS QM	Date	Signature ETV Canada
7	Test D, Test Plan section 9.4.4, Appendix 4	The ISO requests test of standards and acceptance of control for bacteria batches. Each delivered batch shall be checked with three reference substances. That has been done; however, batch 02099 did not meet the criteria of being within 20-80% inhibition for all three reference substances, but this batch has been used in Test D to evaluate batch to batch variability	It is not possible to change the activity of the bacteria. We have asked Hach Lange for additional batches; however, Hach Lange could only provide three and 02099 did not meet all of the ISO bacteria quality control. Since the purpose of Test D is to evaluate reproducibility with different bacteria batches it is important to have at least three batches included, and the three batches represent real-world availability from the vendor; therefore, we have left batch 02099 in the evaluation.	When the bacteria batch does not pass the criteria for all three reference standards, it will cause slightly higher standard deviations on the calculated results and slightly higher relative standard deviation.	It will be noted in the report, that results for Test D have been calculated using one bacteria batch which did not meet the ISO reference standard criteria and the impact on the results will be noted.	25-10		22/5 2010		5-27-10		4-6-10	

Deviation reports

The verification protocol version approved must be followed. If deviations are needed during testing, the deviations are noted and justified in the format:

Deviation number	Verification protocol Chapter	Deviation	Cause	Impact assessment	Corrective action, if any	Date	Signature verification responsible	Date	Signature internal auditor	Date	Signature Battelle AMS QM	Date	Signature ETV Canada
8	8.1 Table 8.1	The equation for repeatability and reproducibility was changed from that listed in Verification Protocol Table 8.1 to those in the attached Table.	1) The number of replicates for repeatability was actually 3 and 4, not only 3 as originally specified. The table has been updated to include appropriate information for when there were 4 replicates. 2) Reproducibility required a different set of equations than repeatability. The listed equation was for variance within groups, but has to be for variance between groups. 3) Test of significance between	The updated equations will provide a better assessment of repeatability and reproducibility and will improve the quality of the evaluation.	Two changes in equations used for data evaluation 1) Adjustment of equation for repeatability 2) New equation for reproducibility 3) Equations added	30 Nov 2010		3/6/2010		6-7-10		10/6/2010	

Deviation number	Verification protocol Chapter	Deviation	Cause	Impact assessment	Corrective action, if any	Date	Signature verification responsible	Date	Signature internal auditor	Date	Signature Battelle AMS QM	Date	Signature ETV Canada
			difference in conditions in robustness test included										

Appendix to deviation no. 8

Precision (repeatability), as relative standard deviation, RSD	$D_i = \lvert x_{i\max} - x_{i\min} \rvert$ $\bar{x}_i = \frac{\sum x_i}{n}$ $d_i = \frac{D_i}{\bar{x}_i}$ $\bar{d} = \frac{\sum d_i}{m}$ $RSD = \frac{\bar{d} * 100}{Divisor}$ %	D_i is the range at level i; $x_{i\min}$ and $x_{i\max}$ are the lowest and highest measurements at level i; $\bar{x}_i$ is the average of n measurements; m is the number of levels; d_i is the relative range at level i; $\bar{d}$ is the mean relative range for all m levels Divisor is for i=3 equal to 1.693 and for i=4 equal to 2.059
Precision (reproducibility), as relative standard deviation, RSD	$\bar{x}_i = \frac{\sum x_i}{n}$ $\bar{\bar{x}}_i = \frac{\sum \bar{x}_i}{m}$ $s_{Between\ groups} = \sqrt{MS_{Between\ groups}}$	$\bar{x}_i$ is the average of n measurements in group; m is the number of levels; s is standard deviation $\bar{\bar{x}}_i$ is average of average in groups; s is standard deviation $MS_{Between\ groups}$ is variance between groups obtained by single factor ANOVA in Excel

	$RSD = \frac{s_{between\ groups}}{\bar{x}_i} \cdot 100\ \%$	
Test of significant deviation from reference. Used for robustness results	$s_{x,y} = \sqrt{\frac{(f_x \cdot s_x^2 + f_y \cdot s_y^2)}{f_x + f_y}}$ $f_{x,y} = f_x + f_y$ $\frac{\lvert\bar{x} - \bar{y}\rvert}{s_{x,y}} \times \sqrt{\frac{n_x \cdot n_y}{n_x + n_y}} > t_{(1-\alpha/2,f_{x,y})}$	s_x is standard deviation on dataset x s_y is standard deviation on dataset y f_x is degree of freedom for dataset x f_y is degree of freedom for dataset y $\bar{x}$ is the average of measurements of dataset x $\bar{y}$ is the average of measurements of dataset y s_{xy} is average deviation n_x is number of measurement in dataset x n_y is number of measurement in dataset y $t_{(1-\alpha/2)}$ is student t-factor for two-sided test

Deviation reports

The verification protocol version approved must be followed. If deviations are needed during testing, the deviations are noted and justified in the format:

Deviation number	Verification protocol Chapter	Deviation	Cause	Impact assessment	Corrective action, if any	Date	Signature verification responsible	Date	Signature internal auditor	Date	Signature Battelle AMS QM	Date	Signature ETV Canada
9 part A	7.2	Reference luminescent bacteria tests were not performed	The tests should be performed according to ISO 11348-3 and under ISO 17025 accreditation and with different equipment than tested in the verification. The selected laboratory AL-control (using Microtox equipment) was after test of 3 samples found not to fulfill the requirements.	The results were only intended to be used to give indication of toxicity level and e.g. as false positive or negative. The results were not intended to be used as true values. The value of the tests were from the beginning limited.	It was investigated if any other laboratory could fulfill the requirements, but none could be found (expect for laboratories using the HACH-LANGE LUMIStox). In the verification report text will be included that describes the planned reference tests and why they were not performed.	2/5 2010		7/5-2010		5-7-10		9-6-2010	

Deviation reports

The test plan version approved must be followed. If (or rather when) deviations are needed during testing, the deviations are noted and justified in the format:

Dev. No.	Experiment label Test Plan	Deviation	Cause	Impact assessment	Corrective action, if any	Date	Signature test responsible	Date	Signature verification responsible	Date	Signature Battelle AMS QM	Date	Signature ETV Canada
9 part B	4 4.3	Reference luminescent bacteria tests were not performed	The tests should be performed according to ISO 11348-3 and under ISO 17025 accreditation and with different equipment than tested in the verification. The selected laboratory ALcontrol (using Microtox equipment) was after test of 3 samples found not to fulfill the requirements.	The results were only intended to be used to give indication of toxicity level and e.g. as false positive or negative. The results were not intended to be used as true values. The value of the tests were from the beginning limited.	It was investigated if any other laboratory could fulfill the requirements, but none could be found (expect for laboratories using the HACH-LANGE LUMIStox). In the test report, text will be included that describes the planned reference tests and why they were not performed.	9/6 - 2010		9/6-2010		6-7-10		8-6-2010	

Dev. No.	Experiment label Test Plan	Deviation	Cause	Impact assessment	Corrective action, if any	Date	Signature test responsible	Date	Signature verification responsible	Date	Signature Battelle AMS QM	Date	Signature ETV Centaur
9 part C	6.3	Reference luminescent bacteria test of blanks was performed on one instead of two samples	The tests should be performed according to ISO 11348-3 and under ISO 17025 accreditation and with different equipment than tested in the verification. The selected laboratory AL control (using Microtox equipment) was after test of 3 samples found not to fulfill the requirements.	2 blanks of DHI MilliQ water should be tested as part of test system control. One result will be listed.	One of the tested 3 samples was a blank. The results will be listed with a comment related to the reference laboratory not fulfilling the ISO 11348-3 requirement and a reference to the associated deviation in the test method.								

Deviation reports

The test plan version approved must be followed. If (or rather when) deviations are needed during testing, the deviations are noted and justified in the format:

Dev No.	Experiment label Test Plan	Deviation	Cause	Impact assessment	Corrective action, if any	Date	Signature test responsible	Date	Signature verification responsible	Date	Signature Battelle AMS QM	Date	Signature ETV Canada
10	Section 4.3, Tabel 4.2	Method for SDS analyses changed from MK8230-LC-MS to DS 237	Eurofins had mixed up SDS with LAS. MK8230-LC-MS is for LAS while DS 237 is for anionic surfacts (as SDS)	Correct method used, therefore no impact	No	25/5 - 10		25/5 2010		5-26-10		4.6. 2010	
11 part A	Section 2.4	Review of test report will only be performed by external expert Kresten Ole Kusk	US EPA and ETV Canada do not want their experts to review test report, they find review of verification report sufficient	Since one external expert is reviewing report requirements in DANETV QA manual are still fulfilled.	No	25/5 - 10		2/5 2010		5-26-10		4-6 2010	

Deviation reports

The verification protocol version approved must be followed. If deviations are needed during testing, the deviations are noted and justified in the format:

Deviation number	Verification protocol Chapter	Deviation	Cause	Impact assessment	Corrective action, if any	Date	Signature verification responsible	Date	Signature internal auditor	Date	Signature Battelle AMS QM	Date	Signature ETV Canada
1st part B	Section 2.4 and 10	Review of test report will only be performed by external expert Kresten Ole Kusk	US EPA and ETV Canada do not want their experts to review test report, they find review of verification report sufficient.	Since one external expert is reviewing report requirements in CANETV QA manual are still fulfilled.	No	3/6 2010	Claire Anderson	3/6 2010	Rachel Fuller	6-7-10	Barb Williams	16-6-2010	Kresten Kusk
		Verification report and statement will be reviewed by Environment Canada, Benoit Derforges.	Environment Canada wants to review verification report and statement.	No impact	No								
		Dr. Max Lee, Environmental Tech Center, Dow Chemical is added as external expert and will review verification report	US EPA requested review by their water stakeholder committee	No impact	No								

Deviation reports

The verification protocol version approved must be followed. If deviations are needed during testing, the deviations are noted and justified in the format:

Deviation number	Verification protocol Chapter	Deviation	Cause	Impact assessment	Corrective action, if any	Date	Signature verification responsible	Date	Signature internal auditor	Date	Signature Battelle AMS QM	Date	Signature ETV Canada
12	2.6	Two verification statements will be produced	Vendor wants a verification statement for each of the two products tested	No impact to the verification	None	2/3 2010		2/3 2010		2-6-10		2/17/2010	