WCMUC
Western Canada Microtox Users Committee

Standard Procedure for MICROTOX Analysis

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1.0 BACKGROUND INFORMATION

1.1 Principle

a) The Microtox test is based on monitoring changes in the level of light emission from a marine luminescent bacterium when challenged with a toxic substance or sample containing toxic materials. The test organism most closely resembles *Vibrio fischerii* (Strain NRRL B-11177).

b) The test is performed by rehydrating freeze dried cultures of the organism, supplied as the Microtox Reagent containing 10^8 bacteria/vial, and determining the initial light output of homogenized bacterial suspensions. Appropriate aliquots of osmotically adjusted sample and sample dilutions are added to the bacterial suspension and light measurements are made at specific intervals (generally at 5 or 15 minutes) after exposure to test samples. The diluent control (blank) is used to correct time-dependant change in light output.

1.2 Definition of Effective Concentration (EC) and Inhibitory Concentration (IC)

a) The test endpoint is measured as the effective concentration of a test sample (constituent) that reduces light emission by a specific amount under defined conditions of time and temperature. Normally, this is expressed as an $EC_{50}(15)$ which is the effective concentration of a sample which reduces light emission by 50% at 15 minutes at 15^oC. Alternatively, the term Inhibitory Concentration is used. This endpoint is the concentration which inhibits light emission by a specific amount under defined conditions of time and temperature. Normally, this is expressed as an $IC_{50}(15)$ which is the concentration of a sample which inhibits 50% of the light at 15 minutes at 15^oC.

b) The EC or IC is calculated by log linear plotting of Concentration (C) vs. % Light Decrease (%Δ), or more precisely by plotting Gamma (Γ) (which is the corrected ratio of the amount of light lost to the amount of light remaining) versus Concentration on a log-log graph. In practice, either a hand calculator, or various computer programs/data reduction systems are used for calculating Gamma and corresponding EC_{50} or IC_{50} values.

1.3 Limitations and Interferences

a) Colour (especially red and brown), turbidity, suspended solids, and floating or emulsified substances interfere with this test by absorbing or reflecting light and thereby affecting or causing non-specific reductions in light output from the hydrated luminescent bacteria. The absorbance (colour) correction procedure should be used to correct for the above interferences. This procedure provides a means for mathematically adjusting the light emission readings to account for light lost due to absorption, and allows the analysis of highly turbid or coloured test samples.

b) High salt concentration, in excess of 30 g/L NaCl, of test (e.g. wastewater) samples may produce hyperosmotic effects upon addition of NaCl as required in the Microtox test. No osmotic adjustment should be made if the salt concentration in the sample is between 30-50 g/L NaCl equivalent. In practice, this may be lower and should be investigated.

c) Interference of bioluminescence, not related to contaminants, may occur in samples with high organic contents/biological oxygen demand. If such interference is suspected, samples should be analyzed for appropriate parameters before conducting the Microtox test.

d) Presence of volatiles may affect the test results or reproducibility. However, unless aeration or excessive mixing is performed on these samples, volatile components should not be excessively lost.

e) Extreme sample pH (outside the ideal range of pH 6.2-6.7) may inhibit light output and affect test results. Even so, the sample pH should not be adjusted because the test relevance and sample integrity may become questionable. Altering the sample pH will usually change the solubility of both organic and inorganic constituents (e.g. precipitation of metal ions due to pH adjustment). In addition, modifying the pH can also cause chemical reactions which will change the sample matrix and integrity, and greatly alter the exhibited toxicity of the sample. Duplicate Microtox assays of both the pH-adjusted and non-adjusted samples should be performed to correct/overcome such interference and to delineate toxicity not related directly to pH. In practice, only in those samples which are below about pH 6 and above pH 8 is there toxicity only due to the effect of pH alone.

f) In complex samples matrices, synergistic and antagonistic effects may influence test results. Such samples, however, should be tested without any pre-treatment as altering the sample matrix may also cause changes in the interaction of the sample matrix with the toxicant, or in interaction of multiple toxicants. Also the speciation or change of toxicants may be modified by changing the sample matrix (e.g. pH, redox potential) causing erroneous and irreproducible results for the same level of a specific toxicant.

g) Turbidity due to high levels of suspended solids interferes with the Microtox test. The absorbance (colour) correction procedure should preferably be used to correct for this interference. Other alternatives for handling and testing highly turbid samples include: centrifugation, pressure filtration, and decanting. It should be noted, however, that although these procedures will remove this interference, levels of toxics may change through absorption, leaching and volatilization during filtration or centrifugation thus impacting the exhibited/observed toxicity.

h) Depending on the sample matrix and very much like other conventional bioassays, the Microtox test may some time provide "False Positives" (i.e. sample identified as toxic when it is actually non-toxic) and "False Negatives" (i.e. sample identified as non-toxic when it is actually toxic). Repeat assays should be performed when such results are suspected.

Alternatively, several other biological tests (the battery approach) should be conduced and used for toxicant monitoring and toxicity assessment. It should be stressed that while the use of multiple assays would eliminate or minimize "False Positives/Negatives", the battery testing approach can be very expensive, time consuming, and most likely impractical and inappropriate for screening large numbers of samples. In practice, it should be possible (particularly if prior knowledge of an area or sample type is available) to select one or two bioassays based on the specific test sensitivities relative to expected sample characteristics.

i) The procedure, as described, is specific to liquid samples only. Solid samples need to be handled differently and are discussed in a separate section.

2.0 SAMPLE COLLECTION

Collection, preservation, and storage of samples are critical to the results of microbial toxicity testing by the Microtox assay. The data are only as valid as the sample itself. In principle, collect samples that are free of secondary contamination and, when analysed in the laboratory, provide results which are representative of the source or problem under investigation. For microbial toxicity testing, as with all other bioassays and chemical and microbiological analyses, special precautions are required in sample collection, storage and handling to prevent accidental contamination by toxic substances, and loss of toxicity due to escape of volatile components, evaporation, sorption effects, biodegradation and chemical reactions. It is recommended therefore that the techniques and procedures described below are strictly followed to obtain reliable data and results.

2.1 Sample Containers - Liquids and Solids

a) Collect samples in clean and properly washed borosilicate glass containers with Teflon coated liner screw caps.

b) NOTE: Plastic containers are unsuitable due to the presence of toxic antioxidants, plasticizers, and mould-releasing agents. Also some plastic containers may adsorb certain toxicants and therefore cause erroneous test results.

c) If, for practical reasons, glass containers other than the one suggested in 2.1.a., are used, seal with Teflon sheeting or parafilm covering. Plastic lids may be placed over the seal for shipping purposes.

d) Wash sampling bottles and caps with a suitable detergent and hot water. Soak bottles and caps overnight in 1% HCl, rinse three times with distilled water, and finally rinse three times with deionized distilled water. Air dry bottles (inverted) and cap prior to storage.

e) No preservatives are required or added to the sampling bottles for microbial toxicity testing.

f) Controls for the influence of the container on the toxic response of the test should be included in the pre-screen assay when samples are submitted in containers other than the ones prescribed above in 2.1.a.

2.2 Sampling Procedures - Liquids and Solids

a) The sample volume will vary according to specific requirements, but the suggested minimum liquid volume for microbial toxicity testing is about 25 mL. As a general practice, however, collect 125-150 mL sample to allow for replicate/repeat analysis. For solid samples, a minimum of 50 g is required. As a general practice, however, collect 75-150 g of sample to allow for replicate/repeat analysis.

b) A "Field Data Sheet" should be completed to provide information regarding sample origin, type, source, identification code, and sampling details including the collection method, time, location and other pertinent remarks. It may also include field physical parameter measurements (pH, conductivity, dissolved oxygen, turbidity, flow rates, air/water temperature) where they apply to the study being conducted.

2.3 Sample Preservation and Transport - Liquids and Solids

a) Transport samples on ice or in a refrigeration unit.

b) Analyze samples as soon as possible after collection to prevent any unpredictable changes. If that is not possible, store samples at 4°C in a cooler with ice or a refrigerator. The recommended maximum elapsed time between sample collection and testing is 48 h for unrefrigerated samples and one week for samples stored at 4°C.

c) Samples containing highly volatile components are to be handled as little as possible to reduce losses due to stripping.

d) Samples which have high levels of biological activity (e.g. sludges, sediments) should be analyzed immediately after collection or stored frozen until required for testing. Allow sufficient air space in the container for expansion and freeze samples in slanted position to avoid breakage. These samples should be frozen due to their biological activity which may alter the toxicity of the sample. Freezing, however, should only be used as a last resort.

e) Surface water and effluent samples may be frozen for long-term transport in clean borosilicate glass sampling bottles with Teflon lined screw caps (freeze samples in slanted position to avoid breakage).

2.4 Sample Handling Precautions - Liquids and Solids

a) The Microtox test may involve the use and testing of hazardous materials and highly toxic substances and wastewaters. The handling of such samples entails potential health hazards due to exposure to chemical and biological contaminant and toxicants. Appropriate safety measures, such as the wearing of protective clothing and coverings (gloves, lab coats, face shield, safety glasses, face masks, etc.) and maintaining proper hygienic and good laboratory practices, should be utilized to minimize chance exposure and safety related problems.

b) Perform the test in a well ventilated area. Use appropriate and environmentally safe procedures (prescribed by relevant regulatory agencies) for the disposal of tested waste samples and hazardous materials.

3.0 MATERIALS

3.1 Apparatus

a) Microtox Toxicity Analyzer, Model 2055 or Model 500.

b) Micropipettors: Fixed or variable volume 10-1000 µL (0.010-1.0 mL) with plastic disposable tips.

c) Strip chart recorder (optional).

d) Computer with interface to Microtox Toxicity Analyzer (optional).

e) Refrigerator (4°C) and Non-self-defrosting Freezer (-4°C).

f) Non-self-defrosting low temperature freezer (-60°C or –20°C) (optional).

g) Thermometer and pH meter (optional).

h) Stopwatch or timer.

i) Microtox cuvettes.

j) Culture tubes (16 x 100 mm) with Teflon screw caps, especially washed and rinsed (optional).

3.2 Reagents and Media[1]

a) Microtox Reagent[2]: freeze dried (lyophilized) luminescent bacterium *Vibrio fischerii* (marine water organisms, strain NRRL B-11177), viable bacterial population of 10^8 cells/vial when reconstituted with 1 mL of reconstitution solution. According to the manufacturer, the reagent remains stable for periods of a few (3-6) months in a refrigerator at 2-8°C. Preferably the reagent should be stored in a non-self-defrosting freezer at about –20°C. The reagent stocks can also be stored for longer periods (up to 18 months) in a deep freezer at –60°C with no significant change in response to reference toxicants.

NOTE: Do not use improperly shipped or partially frozen Microtox Reagent.

b) Microtox Reconstitution Solution: specially purified distilled water free of organics/toxic material (storage 4°C, 1 year maximum).

c) Microtox Diluent: specially purified distilled water containing 2% NaCl to provide osmotic protection for the marine luminescent bacteria (storage 4°C, 1 year maximum).

d) Microtox Osmotic Adjust Solution (MOAS): specially purified water containing 22% NaCl, used to osmotically adjust samples to 2% NaCl (storage 4°C, 1 year maximum).

NOTE: Do not freeze any of these (b-d) solutions.

e) Sodium Chloride (NaCl) Reagent grade.

4.0 MODEL 2055 ANALYZER

4.1 Analyzer Preparation and Calibration (Figure 1)

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[1] All Microtox Reagents and solutions are supplied by the manufacturer, Microbics Corporation, Carlsbad, CA.

[2] Because the Microtox Reagent is perishable, it is shipped/packed with "blue ice" packs which refrigerate the reagent for three days. Ensure that the reagent is received in frozen state within three days of the original shipping date.

a) Check temperature readings of the analyzer on the digital panel meter (DPM), by depressing appropriate switches as follows:

SWITCH	DPM TEMPERATURE READING (^oC)
TURRET	± 0.2
INCUB	± 0.2
AIR	± 1.0

b) Adjust with the appropriate Temperature Set dials until the desired operating temperatures have been reached.

c) Depress the HV switch to turn on the voltage supply to the photomultiplier tube (PMT).

d) Depress HV Check Switch, voltage reading on DPM should read between -500 and -900.

e) Check PMT sensitivity by closing the turret (no cuvette) and adjusting SPAN (100% ADJ) at 4.0 turns (Clockwise) and the SENSITIVITY at 10X. Depress and hold CAL CHECK switch, the DPM reading should be 080-120 if the analyzer is functioning properly[3]. With the CAL CHECK still depressed, adjust the SPAN (100% ADJ) dial until the DPM reads 050. The recorder should agree (within ± 1% of scale) the DPM reading.

f) Depress HV, RUN and SENSITIVITY 1X switches. With the turret empty and open, and the SPAN (100% ADJ) at 10.0, the DPM should display 000 with the minus sign flashing. If necessary, unlock the ZERO % ADJ dial and readjust the instrument reading to ZERO, and relock the dial to avoid accidental changes. Maintain SENSITIVITY at 1X for conducting assays.

g) If any of the above readings fall outside the specified range, consult the manual for remedial action.

h) Place new and clean cuvettes in the pre-cooling and all 15 incubator wells (3 horizontal rows of 5 wells designated as A, B, C and 1, 2, 3, 4, 5, respectively (see Figure 1).

4.2 Chart Recorder Preparation

[3] A DPM reading of < 80 indicates that the analyzer is not operating properly most likely due to malfunction of PMT.

a) Turn the recorder on, remove cap from its pen, set chart speed to 1 cm/minute, set input dial for 1-10 mV.

b) With the analyzer DPM reading 000 (flashing minus sign), adjust the pen to the chart origin (zero) using RECORDER ZERO adjustment dial. The zero may be set above the origin in order to observe drift caused by equipment malfunction. However, if readings are taken from the recorder chart this must be accounted for.

5.0 MODEL 500 ANALYZER

The Model 500 analyzer is fully automated and temperature controlled and needs no daily adjustment or calibration. When the unit is first plugged in, it will reach the present temperatures within fifteen minutes to an hour and the Temperature Warning Indicator Light will switch off. (Figure 2) Multiple samples may be analyzed at the same time by using all of the 30 incubator wells.

6.0 MICROTOX ASSAY PROCEDURES

6.1 Reagent Reconstitution

a) Remove reagent vial from the freezer (-60°C or –20°C) to the refrigerator (4°C) and maintain for 20-30 minutes to allow temperature equilibration.

b) Pipet 1000 µL (1.0 mL) of the reconstitution solution into the precooling well cuvette and allow to stand for 10-15 minutes for temperature (3°C) stabilization.

c) Take out one reagent vial from the refrigerator, remove and discard the aluminium seal and rubber stopper. When opening, make sure that the vial was under vacuum (listen for the vacuum releasing sound), and when open, the pellet is flat at the base of the vial. DO NOT USE A VIAL WITHOUT A VACUUM FOR TESTING. A vial without a vacuum may have been improperly sealed or lost its seal and the lyophilized bacteria may not react the same as bacteria from properly sealed vials.

d) Pour the pre-cooled reconstitution solution into the reagent vial by RAPIDLY inverting the cuvette to minimize any damage during rehydration. The use of a pipet for this step is not required/recommended because what is important is not the exact volume, but rather that fact that bacteria would suddenly come in contact with water. Mix by swirling for a few seconds while holding the vial at the top (to minimize warm-up).

e) Transfer (pour back) the reconstituted reagent into the cuvette and replace the cuvette into the precooling well. Using a 500 μL micropipetor, immediately mix the reagent by aspirating and dispensing 500 μL of the solution about 5-10 times. Ensure to completely expel all liquid from the pipettor tip during the last mixing. Maintain the cuvette in the precooling well during the entire mixing step. Alternatively, the reconstituted reagent may be mixed by flicking the cuvette with a finger and mixing by swirling. Handle the cuvette at the top to minimize warming.

f) Allow the rehydrated reagent to stabilize for about 15 minutes before using it in the assay.

6.2 Sample Pre-Treatment

a) Preferably always TEST SAMPLES AS RECEIVED without any pre-treatment and alteration.

b) The Osmotic adjustment (to 2% NaCl) of samples is required (mandatory) for conducting the Microtox assay because of the use of a marine bacterium as the test organism. Such treatment may make some wastewater components less soluble, reducing concentration in solution and potentially altering exhibited toxicity profile. In practice, however, the osmotic adjustment of samples appears to have negligible impact and is critical for osmotic protection of test bacteria.

c) Uniformly disperse, by mixed agitation, any undissolved material in samples before withdrawing a measured aliquot for osmotic adjustment and subsequent testing. Avoid vigorous agitation and unnecessary exposure of the sample to the atmosphere.

d) Undissolved material (suspended solids), which will interfere with light transmission during analysis, should be removed through centrifugation (40,000 x G), decanting or filtration.

e) Samples containing highly volatile components are to be handled as little as possible to reduce losses due to stripping.

f) Any pre-treatment of samples, through the addition of any preservative or other chemical agent (including acid or base to adjust pH), will in all likelihood impact the exhibited/observed toxicity of the sample or cause chemical species changes. In most cases avoid such treatments, unless it is absolutely essential or the user is specifically testing for determining the effects of these variables and sample modifications. Normally, use 1 M HCl and 1 M NaOH for pH adjustment. Samples which are below 6 and above 8 should also be tested after pH adjustment. pH should be adjusted with the minimum amount of acid or base to bring the sample to the pH 6-8 range.

6.3 Sample Preparation

a) Osmotic Adjustment:

   i) Using MOAS Solution: Transfer 2000 µL (2.0 mL) of test sample to the cuvette in well Al. Add 200 µL (0.2 mL) of Microtox Osmotic Adjustment Solution (MOAS). Using a 1000 µL micropipetor, mix well by carefully aspirating and dispensing the solution 3-5 times (for sample containing volatiles, one pipet mixing is sufficient). If these micropipetors are unavailable, add MOAS at a rate of 10% to the sample (i.e. to 10 mL of sample add 1.0 mL of MOAS by using glass pipets or micropipetors.) Alternatively, the sample and all subsequent dilutions can be mixed by swirling but the operator must ensure complete mixing at each step of the process.

   ii) Using Dry Sodium Chloride (NaCl): To a test tube with Teflon screw cap, add 0.200 g of dry NaCl. Add 10 mL of test sample to the test tube and cap. Mix well by gently shaking the test tube.

b) Full Series 1:1 Serial Dilution:

   NOTE: The following placement of cuvettes is only a guide for easy reference. The cuvettes may be placed in any of the 15 (Model 2055) (Figure 1) or 30 (Model 500) (Figure 2) incubator wells as long as the operator is fully aware of which cuvettes are which and the placement is constant within a laboratory. Refer to Figures 1 and 2 for the letter/number designation of the wells.

   i) Add 1000 µL (1.0 mL) of Microtox diluent to A2 through A5 and B1 and B2 cuvettes. NOTE: If duplicate blanks are used, pipet 1.5 mL of diluent into B2. Cuvette B3 will then be used for the second blank.

   ii) Prepare 1:1 serial dilution of the test sample as follows: Transfer 1000 ?L (1.0 mL) of osmotically adjusted sample from A1-A2. Mix the contents of A2 by aspirating and dispensing the solution 3-5 times using a 1000 ?L micropipettor.

   iii) Transfer 1000 µL (1.0 mL) of sample from A2-A3. Mix the contents of A3 as in step <u>ii</u>.

   iv) Transfer 1000 µL (1.0 mL) of sample from A3-A4. Mix the contents of A4 as in step <u>ii</u>.

   v) Transfer 1000 µL (1.0 mL) of sample from A4-A5. Mix the contents of A5 as in step <u>ii</u>.

   vi) Transfer 1000 µL (1.0 mL) of sample from A5-B1. Mix the contents of B1 as in step <u>ii</u>.

   vii) Discard 1000 µL (1.0 mL) of sample from B1 in order to maintain a constant volume in all cuvettes for temperature equilibration.

   viii) Do not add any sample to B2 which will be used as non-toxic control (Reagent Blank).

   ix) Allow 15 minutes for 15°C temperature equilibration. The sample concentrations at this point are:

SAMPLE CONCENTRATION (%)

Cuvette	MOAS Adjusted	NaCl Adjusted
A1	90.9	100
A2	45.5	50
A3	22.7	25
A4	11.4	12.5
A5	5.7	6.2
B1	2.8	3.1
B2 (Control)	00.0 (Reagent Blank)	00.0 (Reagent Blank)

x) If desired, fewer dilutions can be done.

c) Other Dilution Series: Other dilution series may be done, however, the dilution procedure and sample concentrations should be checked before use.

6.4 Standard Assay Procedure

NOTE: The following placement of cuvettes is only a guide for easy reference. The cuvettes may be placed in any of the 15 (Model 2055) or 30 (Model 500) incubator wells as long as the operator is fully aware of which cuvettes are which and the placement is constant within a laboratory.

a) Pipet 500 µL (0.5 mL) of Microtox diluent into cuvettes B3 through B5 and C1 through C5 (a total of 8 cuvettes). Allow 15 minutes for 15°C temperature equilibration.

b) Transfer 10 µL (0.01 mL) of the reconstituted bacterial reagent (from the precooling well) to cuvettes B3 through B5 and C1 through C5 (the same tip may be used for all transfers). Mix (3-5 times) by aspirating and dispensing with a 500 µL micropipettor.

c) Allow approximately 15 minutes for 15°C temperature equilibration. This can be done by using recorder set at a Chart Speed of 1 cm/minute and measuring the distance required for the 15 minutes time period or using a stopwatch.

d) MODEL 2055: After the equilibration period, check to ensure that HV, RUN and 1X SENSITIVITY switches are in depressed position. Transfer the cuvette B3 into the turret (opening handle in the READ position) and turn on the recorder. Adjust the recorder to read approximately 70-80% on the chart paper by adjusting the SPAN (100% ADJ) dial. Plot light level until a suitable slope (stable light output) is obtained. If no recorder is available,

take light readings every 20-30 seconds for 3 to 4 minutes. If these light readings do not drop appreciably (<5 point drop) the assay may begin. Adjust the SPAN (100% ADJ) dial for a DPM reading of approximately 090 (90% on chart recorder). Open the turret and remove cuvette B3 and return it to its incubator well.

e) MODEL 500: After the equilibration period, transfer the cuvette B3 into the turret. Press the CALIBRATE button to set the digital reading to 90. Take light readings by pressing the READ button every 20-30 seconds for 3 to 4 minutes. If these light readings do not drop appreciably, (<5 point drop) begin the assay. Remove cuvette B3 and return it to its incubator well.

f) Cycle the cuvettes through turret in the order B3, B4, B5, C5, C4, C3, C2, C1 to check for variability in light levels. Discard the most deviant cuvette (an acceptable light level is 080-100 on the DPM) leaving the B3 well vacant. DO NOT CHANGE THE ORDER OF CUVETTES.

NOTE: If duplicate blanks are used, do not discard blank B3 and treat this cuvette exactly as cuvette B4. Cycling of cuvettes begin with B3, B4, B5, etc. as follows.

g) Cycle the cuvettes through the turret in the following order to obtain initial light (I_0) levels for each cuvette: B4, B5, C5, C4, C3, C2, C1. Allow 10-20 sec between each reading.

h) Immediately, and without removing the cuvettes from the incubator wells, transfer 500 µL (0.5 mL) of reagent blank (cuvette B2) and sample dilutions (cuvettes B1 and A5-A1) into cuvettes B4 and B5 and C5-C1 as follows:

Pipet 500 µL from B2-B4 (start the stopwatch at this point)
Pipet 500 µL from B1-B5,
Pipet 500 µL from A5-C5,
Pipet 500 µL from A4-C4,
Pipet 500 µL from A3-C3,
Pipet 500 µL from A2-C2,
Pipet 500 µL from A1-C1.

Ensure that transfers are made by pipetting up the concentration gradient (i.e. order of increasing sample concentration) as specified above. Since the same sample has been serially diluted, use the same tip to transfer all sample dilutions. Mix each transfer thoroughly by aspirating and dispensing 500 µL volumes 3-5 times.

Also ensure that this challenge step is completed in the same amount of time in which the initial light measurements were made. A 10-20 sec interval between each transfer is usually sufficient.

i) Five minutes after the first challenge, cycle the cuvettes through the turret in the following order (of increasing concentrations) to obtain (I_5) light levels: B4, B5, C5, C4, C3, C2, C1.

j) Repeat the cycling of cuvettes (as in step i) for light readings at 10(I_{10}) (if required), 15(I_{15}) minutes and, if required, for 20(I_{20}) and 30 (I_{30}) minutes. The longer time periods are generally used for observation of slow-acting toxicants such as heavy metals.

k) In the above procedure 500 µL (0.5 mL) of the diluted sample is mixed with 510 µL (0.51 mL) of diluted reagent, the final (test) concentrations of sample assayed are one-half of the prepared dilutions. Therefore, the approximate (since the actual dilution factor is 500/1010 and not 50%) final concentrations (of sample) in the test cuvettes for the designated serial dilution scheme and test procedure outlined above are:

SAMPLE (FINAL) CONCENTRATION (%)		
Cuvette	MOAS Adjusted	NaCl Adjusted
B4	0.0 (Reagent Blank)	0.0 (Reagent Blank)
B5	1.4	1.5
C5	2.8	3.1
C4	5.6	6.2
C3	11.2	12.4
C2	22.5	24.8
C1	45.0	49.5

NOTE: The final assay concentration for each cuvette and the light output readings I_0, I_5, I_{10}, I_{15}, I_{20} and I_{30} are required for data reduction and EC_{50} calculations.

l) Perform data reduction and all calculations as outlined in Section 9.0.

6.5 Increased Sensitivity Assay Procedure (90% Assay) (AEC Modification)

NOTE: The following placement of cuvettes is only a guide for easy reference. The cuvettes may be placed in any of the 15 (Model 2055) or 30 (Model 500) incubator wells as

long as the operator is fully aware of which cuvettes are which and the placement is constant within a laboratory.

a) Reconstitute the bacterial reagent as described in the SAP (Standard Operating Procedure) and place in the precooling well.

b) Prepare the sample and use the Microtox Osmotic Adjust Solution (MOAS) to adjust the sample to 2% NaCl (see Section 6.3a, Sample Preparation). Alternatively, the sample may be osmotically adjusted using dry analytical grade NaCl (e.g. 0.2 g for 10 mL of sample). Perform 1:1 serial dilutions of the test sample, as described in SAP. Add 1.0 mL of diluent (Blank) to the cuvette in B2.

c) Pipet 0.9 mL of Microtox diluent into a cuvette and place in the B3, allow 15 minutes for temperature equilibration.

d) Pipet 0.1 mL of reconstituted bacterial reagent into the cuvette in B3 and mix using a 1.0 mL micropipet. Immediately after mixing, dispense 0.1 mL of the diluted reagent from B3 to the empty cuvettes in test positions B4, B5 and C1-C5. Discard the cuvette from B3.

e) Allow 15 minutes for bacterial reagent equilibration. Adjust the gain control (100% ADJ) to give DPM readings in the 080-085 range.

f) Cycle the cuvettes B4, B5, C1-C5 through the turret to obtain initial light levels (I_o) for each cuvette.

g) Challenge bacteria (Start Timer) by removing 0.9 mL of each sample dilution and pipetting to the cuvettes containing 0.1 mL (diluent/bacterial reagent) as follows:

   Pipet 0.9 mL dilution blank from B2 - B4
   Pipet 0.9 mL sample dilution from B1 - B5
   Pipet 0.9 mL sample dilution from A5 - C5
   Pipet 0.9 mL sample dilution from A4 - C4
   Pipet 0.9 mL sample dilution from A3 - C3
   Pipet 0.9 mL sample dilution from A2 - C2
   Pipet 0.9 mL sample dilution from A1 - C1

h) Five minutes after the first challenge, cycle the cuvettes through the turret in the order of increasing concentrations (B4, B5, C5, C4, C3, C2, C1) to obtain (I5) light levels. Repeat the cycling of cuvettes for light readings after 10(I10), 15(I15) minutes exposure. If required, also obtain 20(I20) and 30(I30) minutes light readings.

i) Perform data reduction and calculations as outlined in Section 9.0.

j) For calculations in this 90% assay, the sample concentrations (% original sample) are as follows:

SAMPLE (FINAL) CONCENTRATION (%)		
Cuvette	MOAS Adjusted	NaCl Adjusted
B4	0.0 (Reagent Blank)	0.0 (Reagent Blank)
B5	2.6	2.8
C5	5.1	5.6
C4	10.2	11.2
C3	20.4	22.5
C2	40.9	45
C1	81.8	90

NOTE: The final assay concentration for each cuvette and the light output readings I_0, I_5, I_{10}, I_{15}, I_{20} and I_{30} are required for data reduction and EC_{50} calculations. In the 90% assay, the bacterial reagent is "pre-diluted" (steps c and d above) and therefore the bacterial reagent does not add an additional 10 mL (0.01 mL) to each final volume (1.01 mL) as in the SAP (50% assay).

k) It is also important to note that inherent to the Increased Sensitivity (90%) Assay, a slight stimulation (about 5-10%) is observed in all light levels directly after challenge. However, as this effect is general and common to the control and test samples, it does not change the EC50 values obtained for reference toxicants as compared to EC50 values obtained using SAP in the 50% assay. Other increased sensitivity schemes (e.g. 100% assay) may be useful for certain applications, but are less precise than the 90% assay due to the fact that individual initial light (I_0) readings are not determined.

6.6 ERCB Procedure for the Analysis of Drilling Wastes

NOTE: See Appendix I for the complete ERCB document on Microtox analysis of drilling wastes.

a) Osmotic Adjustment: Using MOAS Solution: Transfer 2000 µL (2.0 mL) of test sample to the cuvette in well A5. Add 200 µL (0.2 mL) of Microtox Osmotic Adjustment Solution (MOAS). Using a 1000 µL micropipettor, mix well by carefully aspirating and dispensing the solution 3-5 times (for sample containing volatiles, one pipet mixing is sufficient).

b) Full Series 1:1 Serial Dilution:

NOTE: The following placement of cuvettes is only a guide for easy reference. The cuvettes may be placed in any of the 15 (Model 2055) (Figure 1) or 30 (Model 500) (Figure 2) incubator wells as long as the operator is fully aware of which cuvettes are which and the placement is constant within a laboratory. Refer to Figures 1 and 2 for the letter/number designation of the wells.

i) Add 1000 µL (1.0 mL) of Microtox diluent to A1 through A4 and B1 cuvettes.
ii) Prepare 1:1 serial dilution of the test sample as follows: Transfer 1000 µL (1.0 mL) of osmotically adjusted sample from A5-A4. Mix the contents of A4 by aspirating and dispensing the solution 3-5 times using a 1000 µL micropipettor.
iii) Transfer 1000 µL (1.0 mL) of sample from A4-A3. Mix the contents of A3 as in step ii.
iv) Transfer 1000 µL (1.0 mL) of sample from A3-A2. Mix the contents of A2 as in step ii.
v) Transfer 1000 µL (1.0 mL) of sample from A2-A1. Mix the contents of A1 as in step ii.
vi) Discard 1000 µL (1.0 mL) of sample from A1 in order to maintain a constant volume in all cuvettes for temperature equilibration.
vii) Do not add any sample to B1 which will be used as non-toxic control (Reagent Blank).
viii) Allow 5 minutes for 15°C temperature equilibration. The sample concentrations at this point are:

SAMPLE CONCENTRATION (%)		
Cuvette	MOAS Adjusted	NaCl Adjusted
A5	90.9	100
A4	45.5	50
A3	22.7	25
A2	11.4	12.5
A1	5.7	6.2

NOTE: The following placement of cuvettes is only a guide for easy reference. The cuvettes may be placed in any of the 15 (Model 2055) or 30 (Model 500) incubator wells as long as the operator is fully aware of which cuvettes are which and the placement is constant within a laboratory.

c) Pipet 500 µL (0.5 mL) of Microtox diluent into cuvettes B2 through B5 and C1 through C5 (a total of 9 cuvettes). Allow 5 minutes for 15°C temperature equilibration.

d) Transfer 10 µL (0.01 mL) of the reconstituted bacterial reagent (from the precooling well) to cuvettes B2 through B5 and C1 through C5 (the same tip may be used for all transfers). Mix (3-5 times) by aspirating and dispensing with a 500 µL micropipettor.

e) Allow approximately 15 minutes for 15°C temperature equilibration. This can be done by using recorder set at a Chart Speed of 1 cm/minute and measuring the distance required for the 15 minutes time period or using a stopwatch.

f) MODEL 2055: After the equilibration period, check to ensure that HV, RUN and 1X SENSITIVITY switches are in depressed position. Transfer the cuvette B2 into the turret (opening handle in the READ position) and turn on the recorder. Adjust the recorder to read approximately 70-80% on the chart paper by adjusting the SPAN (100% ADJ) dial. Plot light level until a suitable slope (stable light output) is obtained. If no recorder is available, take light readings every 20-30 seconds for 3 to 4 minutes. If these light readings do not drop appreciably (<5 point drop) the assay may begin. Adjust the SPAN (100% ADJ) dial for a DPM reading of approximately 090 (90% on chart recorder). Open the turret and remove cuvette B2 and return it to its incubator well.

g) MODEL 500: After the equilibration period, transfer the cuvette B2 into the turret. Press the CALIBRATE button to set the digital reading to 90. Take light readings by pressing the READ button every 20-30 seconds for 3 to 4 minutes. If these light readings do not drop appreciably, (<5 point drop) begin the assay. Remove cuvette B2 and return it to its incubator well.

h) Cycle the cuvettes through turret in the order B2, B3, B4, B5, C5, C4, C3, C2, C1 to check for variability in light levels. Place the most deviant cuvette (an acceptable light level is 080-100 on the DPM) in the B2, B3, and B4 wells. These 3 cuvettes will not be used in the remainder of the analysis. DO NOT CHANGE THE ORDER OF CUVETTES.

i) Cycle the cuvettes through the turret in the following order to obtain initial light (I_0) levels for each cuvette: B5, C1, C2, C3, C4, C5. Allow 10-20 sec between each reading.

j) Immediately, and without removing the cuvettes from the incubator wells, transfer 500 µL (0.5 mL) of reagent blank (cuvette B1) and sample dilutions (cuvettes A5-A1) into cuvettes B5 and C1-C5 as follows:

   Pipet 500 µL from B1-B5 (start the stopwatch at this point)
   Pipet 500 µL from A1-C1,
   Pipet 500 µL from A2-C2,
   Pipet 500 µL from A3-C3,
   Pipet 500 µL from A4-C4,
   Pipet 500 µL from A5-C5.

   Ensure that transfers are made by pipetting up the concentration gradient (i.e. order of increasing sample concentration) as specified above. Since the same sample has been

serially diluted, use the same tip to transfer all sample dilutions. Mix each transfer thoroughly by aspirating and dispensing 500 μL volumes 3-5 times.

Also ensure that this challenge step is completed in the same amount of time in which the initial light measurements were made. A 10-20 sec interval between each transfer is usually sufficient.

k) Five minutes after the first challenge, cycle the cuvettes through the turret in the following order (of increasing concentrations) to obtain (I_5) light levels: B5, C1, C2, C3, C4, C5.

l) Repeat the cycling of cuvettes (as in step i) for light readings at 15(I_{15}) minutes.

m) In the above procedure 500 μL (0.5 mL) of the diluted sample is mixed with 510 μL (0.51 mL) of diluted reagent, the final (test) concentrations of sample assayed are one-half of the prepared dilutions. Therefore, the approximate (since the actual dilution factor is 500/1010 and not 50%) final concentrations (of sample) in the test cuvettes for the designated serial dilution scheme and test procedure outlined above are:

SAMPLE (FINAL) CONCENTRATION (%)	
Cuvette	MOAS Adjusted
B5	0.0 (Reagent Blank)
C1	2.8
C2	5.6
C3	11.2
C4	22.5
C5	45.0

NOTE: The final assay concentration for each cuvette and the light output readings I_0, I_5, and I_{15} are required for data reduction and EC_{50} calculations.

n) Perform data reduction and all calculations as outlined in Section 9.0.

7.0 ABSORBANCE (COLOR) CORRECTION PROCEDURE

Highly coloured (especially red or brown) aqueous samples may cause non-specific reductions in light levels, which cannot be distinguished from those caused by toxicants, when analyzed using the Standard Assay Procedure (SAP). The following procedure, utilizing a specially designed double-walled absorbance correction cuvette (ACC), allows indirect measurement of the amount of light loss caused by sample colour, cloudiness (turbidity) or both. The measurements are then used to mathematically normalize the results obtained in the SAP to correct for colour or turbidity interferences.

a) Perform the normal assay using the SAP described in Section 6.0. Pipet 1.5 mL of Microtox diluent into the outer chamber of a clean ACC and place it into the turret well. Pipet 1.0 mL of diluent into a clean standard cuvette and place it in well A1. Also pipet approximately 2 mL of the highest final test sample concentration (i.e. 500 µL osmotically adjusted sample and 510 µL of Diluent) into each of two clean standard cuvettes and place them in incubator wells C1 and C2. Allow all solutions to temperature equilibrate for about 5 minutes.

b) Pipet 50 µL (0.05 mL) of reconstituted bacterial reagent to the diluent in cuvette A1. Mix well with a 500 µL pipet by aspirating and dispensing five times. Lift the ACC out of the turret and transfer, using a Pasteur pipet, the cell suspension from A1 into the inner chamber of the ACC to a level equal to that of the diluent in the outer chamber. Be sure that there are no bubbles in the cuvette by filling starting with the pipet tip at the bottom and slowly raising it as the cuvette fills. Return ACC to the turret to minimize any effect of warming. DO NOT move the ACC again.

c) MODEL 2055: Close the turret. Set the Span (100% ADJ) dial for a reading of approximately 90% (090 on the DPM) and then record the light level output for about 10-20 minutes until a stable light reading is obtained. (Note that the last four minutes recorded light output will be used for data reduction). Reset the Span to 90% if the output drops below 70 and record the light output for additional 5 minutes. Open the turret but do not remove the ACC. Using a plastic aspirator or Pasteur pipet, remove the diluent from the outer chamber of the ACC and discard. Rinse the outer chamber of the ACC by pipetting 1.5 mL of test sample from cuvette C1 into the outer chamber of the ACC. Remove as much of the sample as possible with the aspirator and discard. Pipet 1.5 mL of test sample from cuvette C2 into the outer chamber of the ACC. Close the turret and record the light level for an additional ten minutes.

d) MODEL 500: Press the CALIBRATE button. Take light readings every 30 seconds by pressing the READ button until a stable light reading is obtained. Record light levels for an additional 4 minutes and plot on linear 10 cycle graph paper. Recalibrate the turret if the output drops below 70 and record the light output for an additional 5 minutes. Using a plastic aspirator or Pasteur pipet, remove the diluent from the outer chamber of the ACC and discard. Pipet 1.5 mL of test sample from cuvette C1 into the outer chamber of the ACC. Remove as much of the sample as possible with the aspirator and discard. Pipet 1.5 mL of

test sample from cuvette C2 into the outer chamber of the ACC. Take light readings every 30 seconds for an additional 10 minutes.

e) Initial (I_o) and final (I_f) light levels are determined by drawing best-fit lines through recorder tracing for the diluent blank (I_o) and sample (I_f) using the last four minutes of tracing. The lines are then extended forward and backward on the time scale to a point on the chart that would correspond to removal of the diluent and replacement with sample. (Figure 3)

f) Use I_o and I_f to calculate absorbance due to colour (A_c) by using the following formulae

i) Absorbance due to colour (A_c) of the sample:

$$A_c = 3.1 \text{ Ln } \frac{I_o}{I_f} \quad OR \quad A_c = 7.14 \text{ log } \frac{I_o}{I_f}$$

The 3.1 and 7.14 (= log to the base 10 and 3.1 x 2.303, respectively) are constants which correct for geometric difference between ACC and the standard cuvette.

ii) Calculate contribution to colour absorbance (A_x) for each test concentration:

$$A_x = \frac{C}{Co} A_x$$

Where C is the concentration of interest (used in the assay) and Co is the concentration used to determine A_c.

iii) Calculate transmittance (T_x) for each test sample concentration C.

$$T_X = \frac{1 - e}{A_x} A_x$$

iv) Calculate the colour corrected Gamma (Γ_c) for each test sample concentration:

$$\Gamma_c = T_x (1 + \Gamma) - 1$$

Where Γ is the observed effect, including both colour and toxicity effects.

8.0 SOLIDS HANDLING - AQUEOUS EXTRACTION PROCEDURE

8.1 Aqueous Extraction of Solid Samples

a) Perform a 1:1 extraction as follows. To 25 g of solid, add 25 mL of distilled deionized water in a glass container which has been cleaned as described previously. (See Section 2.1 Sample Containers). Close container with a Teflon-lined cap.

b) Place the container on a wrist-action shaker at the maximum mixing arc for 1 hour. If a wrist-action shaker is unavailable, a suitable mechanical shaker should be used, providing there is complete mixing between the solid and liquid phases. Ensure that the maximum surface contact is obtained. Record the type of shaker used.

c) Transfer the aqueous extract solution to a glass centrifuge tube using a glass pipet. If glass centrifuge tubes are unavailable, non-toxic plastic may be used. Record the type of centrifuge tube used.

d) Clarify the extract solution by centrifuging at 16,000 x g for 15 minutes in a refrigerated centrifuge at approximately 10 to 15 ^{o} C. If a refrigerated centrifuge is unavailable, cool the extract solution to 10 ^{o} C prior to centrifuging. Record the speed, g force, and temperature used for centrifugation. If the aqueous extract does not appear optically clear after centrifugation, re-centrifuge in a clean centrifuge tube. Relative centrifugal force (RCF) may be calculated by the formula:

   $RCF = 11.17\ r\ (w/1000)^2$

   r = radius in cm of the centre of the rotor head to the centrifuge tube and
   w = angular speed in revolutions per minute.

e) Immediately, transfer the aqueous supernatant to a clean glass container which has been cleaned as described previously. (See Section 2.1 Sample Containers). Close the container with a Teflon-lined cap. Avoid transferring any oily solvent layer which may have been extracted. This will be the aqueous extract sample to be used for Microtox analysis.

8.2 Microtox Analysis of Aqueous Extracts of Solid Samples

a) Record the aqueous extract pH. It should be within the range of 6.0 to 8.0 described previously. If it is not, adjust pH using 1.0 M HCl or NaOH as required. Record the volume of acid or base used to adjust pH. Use the minimum required amount to bring the sample pH to the test range.

b) Perform Microtox analysis on the aqueous extract using normal procedure (see either Section 6.4 Standard Assay Procedure or 6.5 Increased Sensitivity Assay Procedure (90% Assay)).

c) Results are reported as the percentage of the aqueous extract which causes a toxic response. Results are **not** back-calculated to the amount of solid extracted.

9.0 DATA HANDLING AND CALCULATIONS

9.1 Data Recording

a) Record all details of sampling and sample preparation and treatment on Sample Preparation/Collection Sheets.

b) Record the initial (I_0) and subsequent (final) I_5, I_{10}, I_{15}, I_{20} and I_{30} (if required) light output readings for the reagent blank and each assay sample dilution, on a Raw Data Sheet. Preferably record light readings directly from the DPM during the assay. Alternatively, readings may be transcribed from the chart recorder after the assay is completed. If data is entered directly onto a computer through an interface, the digital readings should still be manually recorded in case of computer failure or non-communication. In addition, the sample pH should be determined and recorded on the data sheet.

9.2 Data Reduction

NOTE: For basic data reduction and all calculations, use the equations, formulations and procedures as described in the Microtox System Operating Manual (#015-555879).

a) Calculate and determine the assay endpoint (toxic responses) as the effective concentration of a test sample (constituent/toxicant) causing 50% reduction in light output (EC_{50}) or 50% inhibition of light (IC_{50}).

b) Use Gamma (Γ) for all calculations and data reduction in place of the percent light decrease ($\%\Delta$). Gamma (Γ) is the ratio of the amount of light lost to the amount of light remaining.

c) Calculate and use the Blank Ratio (BR) to correct for the normal (time dependent) light output variation by the bacteria in the absence of a toxicant (in the blank cuvettes). Determine BR by dividing the final time light intensity readings of the reagent blank (B_t) by the initial light intensity of the blank (B_o) using the following formula.

$$BR = \frac{B_t}{B_o}$$

a)

a) A Microtox test would be unacceptable if the difference between two blank ratios exceeds 0.02. (See Quality Control 10.2(b))

b) Calculate the normalized light loss (Gamma, Γ effect) for each test cuvette using the following equation.

$$\Gamma_t = \frac{Corrected\ Light\ Lost}{Light\ Remaining} = B\,R_t \frac{I_o}{I_t} - 1$$

Where:
Γ_t = Γ effect calculated for exposure time t.
BR_t= blank ratio for time t.
I_0 = the initial light reading for any given test cuvette at "zero" time, just before challenging the bacteria.
I_t = the final light reading for the corresponding test cuvette at time t.

An example of Gamma calculations is as follows.
Light Readings: Initial Reagent Blank B_0 = 84, Final (5 minutes) Reagent Blank B_5 = 81, Initial Sample I_0 = 80, Final (5 minute) sample I_5 = 50.

$$BR = \frac{81}{84} = 0.96$$

$$\Gamma(5) = \frac{0.96(80)}{50} - 1 = 0.54$$

f) Determine the effective concentration values (EC's)or inhibitory concentrations (IC's) by either log linear plotting of concentration vs. percent light decrease (% Δ) or more precisely by plotting Gamma (Γ) values as a function of sample concentration on log-log graph paper. The relationship (linear regression) between Gamma and the toxicant (sample) concentration is expressed as: Log Γ = S log C + log B. The plotting of Γ and C on log-log paper converts the usually S-shaped dose-response curve to a straight line and allows the easy determination of EC_{50} (or IC_{50}) intercept (log Γ = 0). This approach results in greater precision for the EC_{50} (or IC_{50}) value, as well as allowing estimation of other test endpoints (e.g. EC_{20}, EC_{80}, etc.)(e.g. IC_{20}, IC_{80}, etc.). The slope of the regression line, S, relates to the mode of action of the toxicant. The intercept, Log B, is a composite factor which relates to

free energy and volume changes at the test temperature, as well it also reflects the mechanism of toxicant action.

9.3 EC Calculations

a) Following completion of the plot (manual or computerized), the determination of the EC (or IC) values is made by drawing a best fit line through the points when they scatter about a line, or by connecting points with several line segments when significant deviation from linearity of the log-log plot is observed. In order to ensure that the results are as accurate as possible, the line drawn should have at least one point above and one point below the EC (or IC) value. Since extrapolated data leads to variation in results, highly toxic samples should be diluted to within the concentration range tested.

b) See Table 1 for the determination of various EC (or IC) values.

9.4 Results Reporting

a) Report the Microtox assay results as the "Effective Concentration of a test sample causing a 50% decrease in light production (EC_{50}) at 15°C after 15 minutes exposure". Alternatively results may be reported as the "Concentration of a test sample which inhibits 50% of light production (IC_{50}) at 15°C after 15 minutes exposure.

b) Results based on other test endpoints (e.g. EC_{20}, EC_{80}), (e.g. IC_{20}, IC_{80}, etc.) after 15 minutes exposure, may be provided if required for specific purposes. Likewise, EC_{50} (IC_{50}) values for other time points (e.g. 5, 10, 20 or 30 minutes exposure) may also be provided if required for specific purposes.

c) For aqueous samples, results are given in percent original sample or in mg/L (ppm) where toxicant concentration is known.

d) For aqueous extracts of solid samples, results are given in percent of extract of a 1:1 aqueous extract of the solid sample.

e) Extrapolated results should be reported as such, since extrapolation of an EC leads to increased variability.

10.0 MICROTOX QUALITY CONTROL

10.1 General Considerations

a) In general, quality data are produced when test procedures are followed as stated or when a Standard Assay Procedure is routinely used for all analyses. In conducting the Microtox assay, the greatest source of error will likely be due to an operator error. Errors are most likely to occur during sample preparation, osmotic adjustment, sample and reagent dilutions, sample transfer and mixing steps, reagent re-constitution, sample filtration, colour correction (if required), data reduction and resulting calculations, and data interpretation. Use of the properly calibrated equipment (analyzer) and development of the appropriate skills for sample/reagent preparation and data handling/calculations is imperative in producing quality data for the Microtox assay. As well, the Microtox data quality could be improved and maintained by conducting internal quality control testing producing control charts using selected Reference Toxicants.

10.2 Specific Considerations

a) A Microtox test would be unacceptable if initial light readings of diluted reconstituted reagent are < 80 at 1x Sensitivity. This may be due to malfunction of the PMT or loss of reagent viability.

b) A Microtox test would be unacceptable if the difference between the two blank ratios exceeds 0.02 indicating a spurious blank. This can be caused by poor sample dilution and transfer techniques, contaminated glassware and outdated or improperly stored reagent.

c) A Microtox test is unacceptable if the coefficient of variation (CV) of duplicate or comparative testing exceeds 20%.

d) The raw data (sample dilutions, initial and final light readings) generated by the assay procedure will determine whether EC_{50} values can be calculated with reasonable accuracy.

e) The determination of 95% confidence interval and the correlation coefficient values (acceptable range 0.85-0.99) will assist the investigator in validating the quality of generated EC_{50} (IC_{50}) values and test results.

10.3 Precision and Inter/Intra-Laboratory Testing

Precision is a measure of the degree of agreement (closeness) among replicate analyses of a given sample (standard), under the same conditions; a measure of random error generally expressed quantitatively as the Standard Deviation (SD).

a) Results of both the inter- and intra-laboratory studies/trials have demonstrated excellent reproducibility (between lab precision) and repeatability (within lab precision) of the Microtox assay. Control (MEANS) Charts are also useful in developing and establishing precision of the Microtox assay procedure.

b) Recommended compounds for intra-laboratory testing are Phenol (5 minute EC_{50} approximately 20 mg/L Phenol) or Zinc Sulphate (15 minute EC_{50} approximately 1.5 mg/L Zn^{+2}). A long term Quality Control Chart should be prepared and maintained for one or both of these compounds.
   i) For the preparation of Zinc Sulphate (10mg/L Zn^{2+} stock). Using an analytical balance, weigh out 43.98 mg of $ZnSO_4 \cdot 7H_2O$. Transfer to a 1L volumetric flask and make to volume using distilled, deionized (ASTM Type 1) water. Store for up to 1 year in a borosilicate glass bottle with a Teflon liner.
   ii) For the preparation of Phenol (100 mg/L stock). Using an analytical balance, weigh out 100 mg of Phenol. Transfer to a 1 L volumetric flask and make to volume using distilled, deionized (ASTM Type 1) water. Make fresh for each day's use.

TABLE 1

Relationship Between GAMMA, Percent Light Decrease, Percent Effect and EC Values

Gamma (Γ)	Percent Light Decrease (%Δ)	Percent Effect (%)	EC Values (IC Values)[1]
<0.00[2]			
0.00	0	0	
0.01	1	1	
0.05	5	5	
0.10	10	10	EC10
0.25	20	20	EC20
0.50	35	35	
0.70	40	40	
1.00	50	50	EC50
1.50	60	60	
2.00	68	68	
3.00	75	75	
4.00	80	80	EC80
9.00	90	90	EC90
?	100	100	

[1] The 10%, 20%, 50%, and 80% effects (light decrease) corresponds to Gamma of 0.1, 0.25, 1.00, and 4.0, respectively. The EC (IC) values corresponding to these effects are determined by the intersection of the Log Gamma VS Log Concentration plot for each time of exposure to the toxicant.

[2] Negative gamma values generally indicate stimulation of bacterial light output.

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