Microtox®

Acute Toxicity Manual

MODERN WATER
A Deepverge Company

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MATERIALS REQUIRED

- Microtox® Acute Toxicity Test Reagent
- Reconstitution solution
- Osmotic Adjusting Solution (OAS)
- Diluent
- Cuvettes
- 10 - 100 µL pipettor and tips
- 0.25 - 2.5 µL pipettor and tips
- Repeat pipettor and syringes

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Basic Test Fundamentals

Principle of Operation

The test exposes luminescent test organisms in Microtox Acute Toxicity Test Reagent to test samples, and measures the increase or decrease in light output by the test organisms.

Reagent contains living luminescent bacteria that have been grown under optimal conditions, harvested, and then lyophilized (freeze-dried). The lyophilized bacteria are rehydrated with Reconstitution Solution to provide a ready-to-use suspension of organisms.

The test system measures the light output of the luminescent bacteria after they have been challenged by a sample and compares it to the light output of a control (reagent blank) that contains no sample. A difference in light output (between the sample and the control) is attributed to the effect of the sample on the organisms.

Precision

Each test cuvette contains roughly a million individual test organisms that are challenged by the test sample. Variations among individual organisms become statistically insignificant. The system measures a single parameter, the simultaneous light output of all of the organisms.

Each batch of Reagent, containing lyophilized test organisms, is prepared under conditions within very rigidly controlled limits.

Time and Temperature

Different chemicals affect living organisms at different rates, reflecting differences in mechanism of action.

For some classes of chemicals, the effect on light output is complete in 5 minutes. For other classes of chemicals, the light output is still decreasing rapidly at 5 minutes. In these cases, 15-minute data may be more reliable. We recommend that 5-minute and 15-minute data be taken routinely when dealing with unknown samples. Some laboratories elect to collect data routinely at five, fifteen and thirty minutes. The software will automatically process data taken at any one, two, or three times you select.

The temperature during exposure to various materials will affect the response of the living organisms. For the Microtox Acute Toxicity Test procedures, the Incubator Wells and READ Well temperature are 15°C and the REAGENT Well temperature is 5.5°C.

Control (Blank)

Reagent Control is required for each test and is run concurrently with the test. Light levels normally change with time, for reasons other than the bioreactivity of the test sample.

Use of the Reagent Control allows the operator to compensate for some of these variables when reducing the data. In all variations of the Basic Test procedure, the responses of all the test cuvettes are normalized to that of the Control test response, which compensates for errors up to 20% in the 10 µL pipetting of reagent.

Supplies and Accessories

Testing requires several special items in addition to those commonly found in testing laboratories.

Reagent

The Microtox Acute Toxicity Test Reagent is specially formulated for bioreactivity testing with sensitivity to a broad range of toxicants.

The reagent is a freeze-dried preparation of a specially selected strain of the marine *bacterium Aliivibrio fischeri* (formerly known as Vibriio fischeri, NRRL number B-11177). A vial of reagent contains roughly one hundred million test organisms.

Shelf life is two years when stored at minus (-) 20°C to (-) 25°C. Do Not store the Reagent below minus (-) 25°C.

Storage at normal refrigerator temperature greatly reduces shelf-life.

Reagent should not be stored in a self-defrosting freezer, which defrosts by warming up periodically.

Use the reagent within three hours after reconstitution. The sensitivity of the reagent is essentially unchanged for 3 hours after reconstitution. Changes in sensitivity may become significant after that time for some samples.

Reconstitution Solution

Reconstitution Solution is a specially prepared nontoxic Ultra Pure Water that contains trace amounts of sodium chloride (NaCl).

Shelf life is three years when stored at room temperature.

Diluent

The Diluent is a specially prepared nontoxic 2% Sodium chloride (NaCl) solution, used for diluting the sample and the reagent.

The marine bacterium in the reagent requires osmotic protection that is provided by the 2% NaCl.

Shelf life is three years when stored at room temperature.

Osmotic Adjustment Solution

Osmotic Adjustment Solution (OAS) is a specially prepared nontoxic 22% Sodium chloride (NaCl) solution, used to adjust the osmotic pressure of the sample to approximately 2% NaCl.

To adjust a sample osmotically, add one part of OAS to ten parts of sample.

(X mL sample x 0.1 = mL OAS)

Example: 2.5 mL sample x 0.1 = 0.25 mL OAS

The sample can also be osmotically adjusted by the addition of solid AR grade NaCl to a final concentration of 2.0%.

Shelf life is three years when stored at room temperature.

DO NOT PREPARE SOLUTIONS

Do not make Diluent, Osmotic Adjustment Solution or Reference Water yourself or use substitutes. The production of uncontaminated solutions is difficult, and some laboratories have created problems using their own Diluent and Osmotic Adjustment Solution.

Cuvettes

Cuvettes are used to contain samples, controls, and Reagent during testing. They are nontoxic and disposable.

Do NOT reuse cuvettes. Used cuvettes cannot reliably be cleaned for reuse. Traces of detergent or sample contaminants interfere with later tests. The risk of interference from contamination is unacceptably high.

Pipettors and Pipettor Tips

Test protocols require repeated precise transfer of small amounts of liquid, as little as 10 µL. High precision adjustable micro-pipettors are necessary. It is highly recommended to have and use the following pipettors:

- 10 – 100 µL adjustable volume pipettor
- 0.25 – 2.5 mL adjustable volume pipettor
- repeat pipettor

Sample Collection and Preparation

Sample Collection

Use new clean, borosilicate, screw cap containers (30 to 50 mL) with Teflon® or equivalent lined caps. Fill the container completely to the top with sample, leaving NO airspace. Completely filling the container helps keep volatile material in solution.

Sample Storage

Test the sample as soon as possible after collection. If testing is delayed, store samples at normal refrigerator temperature.

The toxicity of the sample can change with time, so testing the sample within 1-2 hours after collection is best, but this is not always possible. Try to test the sample within 24-48 hours after collection.

Colored/ Turbid Sample

The Microtox LX performs automatic color correction. This feature can be turned off if color correction is not desired.

When color correction is turned on and the sample is too colored or too turbid, the software will generate a message to the user and an initial dilution may be required.

The turbidity is objectionable when the toxicity from the turbid material is not wanted. If the toxicity is desired from the material that causes turbidity, do not centrifuge the sample and ensure that "measure no turbidity" is chosen on the analyzer.

Chlorine Content

The Reagent (organism) is sensitive to chlorine as are all microorganisms.

Municipal Water Treatment (drinking water) or Waste Water Treatment Plants have a common problem with the water they are producing, bacterial contamination. Chlorination of the water is generally used to solve this problem. Chlorine is toxic: that is why it is being used for killing microorganisms.

Most of the time when either a Municipal Water Treatment or Waste Water Treatment Plant wants to check for acute toxicity, they do not want to know the effect of chlorination.

Collect the samples before chlorination, unless you are testing for the effect of chlorination. Do not collect a chlorinated sample for testing, unless you want to know the effect of chlorination or can not collect a sample that is not chlorinated. If knowing the effect of chlorination is desired, it is recommended to test the sample just before chlorination and just after chlorination. When the effect of chlorination can not be avoided, dechlorinate the sample with Sodium Thiosulfate.

Sodium Thiosulfate Dechlorination

To dechlorinate a sample, add 1 part of Sodium Thiosulfate Stock Solution to 100 parts of Sample.

Example: 100 uL Sodium Thiosulfate Stock Solution added to 10 mL Sample.
Final Sodium Thiosulfate concentration in the sample is 100 mg/L.

Sodium Thiosulfate Stock Solution Preparation

1. Weight out 1.0g Sodium Thiosulfate ($Na_2S_2O_3$).
2. Add 100.0 mL Diluent.

The Sodium Thiosulfate will last for 1-2 months when stored in the refrigerator.

Sample pH

1. Measure the pH of the sample and record the pH as part of the information in the sample description.

The bacterial reagent is sensitive to pH. There is a minimal pH effect between 6.0 and 8.0. When the pH is higher than 8.0 or lower than 6.0, and the sample has buffering capacity, the effect can be dramatic. Example: distilled water adjusted to pH 5.0 with HCl has NO pH effect because there are not enough hydrogen ions to affect the pH of the Reagent. Another sample at the same pH (5.0) may show considerable toxicity if it does have buffering capacity.

2. If the pH of the sample is below 6.0 or above 8.0 and pH adjustment is required, adjust the pH as shown below. Adjustment is required when the toxic effect of pH is not wanted.

   A. If the pH is below 6.0, adjust the pH to 6.0 with NaOH. If over-titration occurs, discard sample and start again.

   B. If the pH is above 8.0, adjust the pH to 8.0 with HCl. If over-titration occurs, discard sample and start again.

NOTE: When the sample is below pH 6.0, titrating the sample above 6.0 may precipitate the sample, thus changing the effective toxicity.

When the sample is above pH 8.0, titrating the sample below 8.0 may precipitate the sample, thus changing the effective toxicity.

When the sample has been over-titrated, back titrating may not resolubilize the sample, if precipitation occurred. Use a strong concentration of acid or base (5 N) for coarse pH adjustment of a sample with a high/strong buffering capacity to minimize sample dilution.

Use a weak concentration of acid or base (0.5 N) when the sample has a low/weak buffering capacity.

Sample Collection and Preparation

The table below shows the combinations of numbers of tests, controls and dilutions that can be performed at one time. The Basic Test protocol with slight modifications can take readings on one to three controls, plus three to fourteen serial dilutions of the same sample (a combined maximum of fifteen cuvettes).

TEST VERSATILITY

No. Tests	No. Controls	No. Dilutions
1	1	4–14
1	2	4–13
1	3	4–12
2	1	4
3	1	4
ID	1	4–9
ID	2	4–8
ID	3	4–7
2D	1	4

D = Test in duplicate

Applications & Methods

Wastewater treatment

*Influent (inc trucked waste): Basic, 2% Basic, 2% Screening, In process: Basic

*Effluent: 81.9% Basic, Comparison, WET, ISO, DIN

*TRE: Basic, Comparison

Drinking water

Source: 81.9% Basic

*Finished - Drinking water monitoring: 81.9% screening, Confirmation, Comparison

Stormwater

*TRE: Basic, Comparison

Recreational water

81.9% Basic, Comparison

Ecotoxicology

Basic, 2% Basic, 81.9% Basic, ISO, DIN

Emergency response

45% screening, 2% screening, 81.9% screening

Personal care/ Household chemicals

(products, formulations and raw materials along with typical WWTP apps)

Basic, 45% Screening, 2% Basic, 2% Screening

Food and beverage:

*Water security monitoring program: 81.9% Screening, Confirmation, Comparison

Medical device

Basic

Drilling muds/ sediments

Solid Phase, Basic Solid Phase

Mining

Basic, 2% Basic, 2% Screening

* Applications available

Microtox Methods

There are 19 methods on the Microtox LX software and 17 standard protocols:

- 2% Basic
- 2% Screening
- 45% Screening
- 81.9% Basic
- 81.9% Screening
- ASTM Basic
- Basic Solid-Phase
- Basic
- Comparison
- Confirmation
- DIN
- International Standard ISO 11348-3
- Solid-Phase
- SOLO 2% Screening
- SOLO 45% Screening
- SOLO 81.9% Screening
- Whole Effluent Toxicity (WET)

2 Quality Assurance:

- Phenol Standard
- Zinc Sulfate Standard

Methods in detail:

Basic:
A 45% concentration sample test, used for samples on unknown toxiocity. This test provides the most flexibility along with the highest confidence level and test precision. The test measures relative toxicity; producing data for calculating EC_{50} or IC_{50} values or other EC_{xx} or IC_{xx} values. The test is recommended for: pure compounds, septage, wastewater influent, wastewater treatment digester.

81.9% Basic:
Designed for use with samples of low to medium toxicity, such as post secondary treatment, wastewater effluent, stormwater runoff, and pore water, can also be used for toxicity baseline studies. Recommended for samples with expected EC_{50}'s between 30% and 100%.

2% Basic:
Designed for use with samples of high toxicity (e.g. industrial waste streams) where an EC_{50} is required. Recommended for wastewater influent streams and pure compounds.

ASTM Basic:
This method is currently an ASTM Standard Method (D5660). The "Standard Test Method for Assessing the Microbial Detoxification of Chemically Contaminated Water and Soil Using a Toxicity Test with a Luminescent Marine Bacterium," describes the effective use of Microtox to evaluate the 'toxicity of wastewaters and aqueous extracts from contaminated soils and sediments prior to and following biological treatment.'

Basic Solid Phase:
Designed for use with soil, sediment, and other solid samples. The initial concentration is 99,000mg/L. The Basic Test should also be used when the toxicity of the sample is unknown.

Solid Phase:
This procedure allows the testing of a solid sample using serial dilutions of the sample. The procedure tests a sample at nine (9) dilutions with 9.9% (99.01 mg/L) being the highest concentration.

The procedure described is for freshwater samples, but it could also be used for marine and estuarine samples except for using the appropriate diluent for sample preparation. The SPT procedure allows the test organisms to come in direct contact with the solid sample in an aqueous suspension of the test sample. Thus it is possible to detect toxicity which is due to the insoluable solids that are not in solution. Use for whole sediment or soil (sediment or soil from which the liquid phase has been removed).

Screening:
For testing at a single concentration. No dose response curve is generated and no relative toxicity is determined.

2% Screening:
Designed for use with samples of high toxicity. This protocol is appropriate for samples such as untreated industrial effluents, trucked waste from industrial sources, and any other sample types of high toxicity. Microtox Acute Reagent is used with this protocol. For the same test using Microtox SOLO reagent, choose the SOLO 2% Screening Test.

45% Screening:
Designed for use with samples of medium toxicity. For the same test using Microtox SOLO reagent, choose the SOLO 45% Screening Test.

81.9% Screening:
Designed for use with samples of low toxicity. This protocol is appropriate for the Microtox Drinking Water Program. Microtox Acute Reagent is used with this protocol. For the same test using Microtox SOLO reagent, choose the 81.9% SOLO Screening.

Comparison:
Measures the relative acute toxicity of an unknown sample against a control sample. The control sample can be either another sample of known toxicity or prepared from Microtox Reconstitution Solution. All samples, control or unknown, must be osmotically adjusted. The Comparison Test is the best protocol for testing low toxicity samples when an EC_{xx} value cannot be determined using a basic test.

The Comparison Test uses multiple replicates of the sample at a single concentration. The results are reported as mean percent difference as compared to a reference sample.

Recommended sample types are: Wastewater treatment plant effluent, Storm water runoff, Drinking water (high confidence testing), Pore Water, Eluate.

Confirmation:
The best protocol for confirming the toxicity of a sample. The sample is tested against itself (with fresh preparation) to establish if the first test result was accurate. This test is primarily designed for use with the Microtox Water Quality Monitoring Program.

Deutsches Insitut für Normung (DIN) 38412 Teil Test:
Germany granted Microtox a DIN Standard (DIN 38412 part 34) for the testing of water samples as indicated in the ISO Standard 11348-3, allowing for fresh water, marine and estuarine sample reporting.

International Standard Organisation (ISO) 11348-3:
Microtox is an ISO Standard 11348-3: 1998. The ISO Standard states that this method is applicable to wastewater, fresh water (surface and ground water), sea and brackish water as well as elutes of sediment, pore water, aqueous extracts and leachates.

Whole Effluent Toxicity (WET) Test:
This is not an EPA approved test.
This test is designed for use on wastewater treatment effluent.

Phenol Standard:
Reference standard best chosen when sample testing involves samples that contain organics.

Zinc Sulfate Standard:
Reference standard best chosen when sample testing involves samples that contain metals and other inorganics.

Purpose of Standard

Testing a standard whose test results are well characterized, confirms your understanding of a test protocol and checks the performance of the complete test system (e.g. analyzer, Reagent, Diluent and Reconstitution Solution).

The Basic Test Protocol (with two controls, eight sample dilutions in duplicate) is the best procedure for testing "Standards," and provides a high level of precision and flexibility.

When testing "Standards" never accept an EC_{50} derived from extrapolated data, always retest using the appropriate "Standard" dilutions.

Phenol Standard

Phenol EC_{50} 5 minutes = 13 to 26 mg/L

1. Weigh out ~50 mg (~0.050 g) of crystalline phenol, add it to a 500 milliliter (mL) amber volumetric flask.

Do not try to weigh out the exact amount of sample, calculate sample concentration for the amount weighed out.

If an amber volumetric flask is not available, cover the entire flask with aluminum foil to protect the Phenol Standard from light.

2. Add Diluent to the 500 mL mark on the volumetric flask.
3. Seal the flask, and mix well by inverting the volumetric flask.
4. Label the flask and store at normal refrigerator temperature (2-8°C).

The Phenol standard will last for 3-4 months when stored in this manner.

Zinc Sulfate Standard

EC_{50} 15 minutes

$ZnSO_4.7H_2O$ = 3 to 10 mg/L

Zn^{++} = 0.6 to 2.2 mg/L

5. Weigh out ~ 50 mg (~ 0.050 g) of $ZnSO_4.7H_2O$, add it to a 500 milliliter (mL) volumetric flask.
   Do not try to weigh out the exact amount of sample; calculate sample concentration for the amount weighed out.
6. Add Diluent to the 500 mL mark on the volumetric flask.
7. Seal the flask and mix well by inverting the volumetric flask.
8. Label the flask and store at normal refrigerator temperature (2-8°C).

The Zinc sulfate standard will last for 3-4 months when stored in this manner.

Data Reduction Formulas

Gamma for the Microtox Acute Toxicity Basic Test Protocols

Correction Factor

The Correction Factor (R_t) is the fraction obtained when the light output of the Control (Blank) remaining after time t is divided by the initial light output of the untreated Control (Blank):

$$R_t = I_t \div I_o$$

Gamma

The gamma value (Γ) is the ratio of the light lost at time t to the light remaining at time t for a given sample concentration:

$$\Gamma_t = (R_t I_o - I_t) / I_t = [(R_t \times I_o) \div I_t] - 1$$

The second form of this equation stresses the fact that gamma is the ratio of light expected for a nontoxic sample to that observed, minus (-) 1.

Gamma for the Microtox Acute Toxicity Solid- Phase Test Protocols

The gamma value (Γ_t) is the ratio of the light output of the Control (Blank) at time t to the light output for a given sample concentration at the same test time, minus (-) 1:

$$\Gamma_t = (R_t c / I_t) - 1$$

Note that this is equivalent to making the correction factor (R_t) equal to one in the above equation for the Basic Test, which means that no correction for reagent pipetting errors is made.

Regression Statistics

Rate theory for biological inhibition predicts a simple mathematical relationship between the concentration of a toxic material and the response of a susceptible organism when the response is measured in terms of gamma values:

$$\Gamma = kCP$$

where C is concentration, p is the number of toxic molecules per target site, and k is a composite factor relating to free energy and volume changes of the reaction. This relationship may be restated in the form of a linear equation for prediction of concentration from gamma values:

$$\log C = b \log \Gamma + \log a$$

This equation describes a line with a slope of b and an intercept of log a, in which C represents concentration and Γ the corresponding gamma. Logs of any base may be used, as long as they are used consistently.

The equations below utilise LOG to denote logs (base 10)

Regression statistics of logC on log Γ are used to estimate the concentration which would give a nominal effect, for example an EC_{50}. In this example EC_{50} represents a gamma of 1. Since log 1 is zero, canceling the effect of the slope, the log C estimate is identical to log a. Therefore, the EC_{50} is equal to the antilog of the intercept (log a). Concentrations which cause other percent effects (%E) can be estimated by substituting the log of the equivalent gamma value in the regression equation.

To convert %E to gamma, divide %E by 100 minus %E. Thus for an EC_{20}:

Γ = 20/(100-20) = 20/80 = .250 which is the equivalent Γ. Percent E can be derived from gamma by:

$$\%E = [\Gamma \div (1 + \Gamma)] \times 100\%$$

Standard statistics are used to calculate confidence intervals for this estimate. The specific equation for a 95% confidence factor in this case is:

$$CF=\sqrt{[S^2(1 \div N)+((\log \Gamma_o - \log \Gamma_a)^2 \div \Sigma \gamma^2)]}$$

In this equation Γo is the gamma corresponding to the EC_{XX} of interest, log Γa is the mean of log (gamma) values, and N is the number of data pairs used for regression. The residual variance, S2, is calculated from the regression statistics as:

$$S^2 = [\Sigma c^2 - (\Sigma c\gamma)^2 \div (\Sigma \gamma^2)] \div (N - 2)$$

The product of CF and t.05 (from a Student's t table) is added to and subtracted from log Co to give the logs of the higher and lower 95% confidence limits. The antilogs are then taken of log Co and its limits.

Comparison Test Data Reduction

The Comparison Test compares a single dilution of a sample with a control, such as diluent, or with another sample to determine if there is a significant difference between the two samples. Five replications of each control and sample are used. The statistical approach is conventional, and is referenced in the bibliography.

This document discusses the approach in the specific terms of the test protocol. Note that an equal number of replicates, fixed at 5 each for sample and control, simplifies the math involved over that of a general case.

This approach assumes that the data are normally distributed, but is robust for moderate departures from normality.

The fundamentals of the statistical calculations are outlined below:

1. Means, estimated variances, and corresponding standard deviations are calculated for the corrected readings for the control and the sample for each exposure time.
2. Variances are used to calculate an F statistic, which is compared with the critical F distribution value for a two-tailed, 95% confidence level. For 4x4 degrees of freedom (df) this value is 9.6.
3. If the calculated F statistic is less than 9.6, then a standard student's t test is used to test for significant differences of the means. If the F value is 9.6 or greater, indicating that the variances are not homogeneous, then a modified t test is used to test for significance.
4. Percent difference, 95% confidence limits, and a "t" value are calculated. The calculated t statistic is compared with look-up table values of t for 8 df (homogeneous) or 4 df (not homogeneous) at different levels of significance from 60% and above.
5. The results are given as percent difference ± the 95% confidence limits. The confidence level of the difference is also stated (60% - 99.9%). If the confidence level of the difference is less than 60%, no confidence level is stated (indicated by "-"). If the difference is stated as a negative number, it indicates that the sample was less toxic than the control (or the sample with which it was being compared.)

Terms:

X_c, X_s = Individual readings at time t for control & sample

$\sum X_c$, $\sum X_s$ = Sums of the readings

$\bar{X}_c$, $\bar{X}_s$ = Means of the readings, $= \sum X/n$,

where n = 5 = number of replications

$1 + (1 - \bar{X}_s / \bar{X}_c)$ for zero time means = correction factor for timed sample data

$\sum D_c^2$, $\sum D_s^2$ = Sums of squares of differences from the mean, $= \sum X^2 - (\sum X)^2/n$

S_c^2, S_s^2 Estimated Variances, $= \sum D^2/n-1$

S_c, S_s Standard deviations, $= \sqrt{S^2}$

$F = S_c^2 / S_s^2$ or $F = S_s^2 / S_c^2$
(larger variance is placed in numerator)

$$t_{obs} = (\bar{X}_s - \bar{X}_c / \sqrt{ (D_c^2 + D_s^2)}) (\sqrt{20}).$$

[Note: $\sqrt{(n_c n_s / n_c + n_s)}\ \sqrt{(n_c + n_s - 2)} = \sqrt{20}$ for n=5]

Comparison Test Details:

1. Means are calculated for the zero time readings for control and sample. The ratio of these means is used to calculate a correction factor for the purpose of normalizing the timed sample data. (See example below.)
2. Means, variances, standard deviations, and the F factor are calculated for each set of timed data. The result of the F test for homogeneity will determine the method for testing for significance and for calculating the 95% confidence limits.
3. The t_{obs} is calculated from the above given formula. The standard deviation used for calculating confidence limits is the average of the two standard deviations, $S_{cs} = S_c + S_s / 2$
4. Case of homogeneous (equal) variances. The degrees of freedom are $2(n -1) = 2 \times 4 = 8$.

The t_{05} for 8 df from Table 1 is 2.306, thus the difference with limits is the difference between the two means ± 2.306 Scs. The confidence level that the difference is real can be computed by comparing the observed t, t_{obs}, with the table values of t for each probability level. If t_{obs} is greater than the table value of t, then the difference is significant at the table probability.

Case of heterogeneous (unequal) variances. In this case the treatment is the same as the above case except that the degrees of freedom are 5 -1 = 4, and the t_{05} for 4 df is 2.776. The table 1 values for t correspond to 4 df.

Comparison Test Calculation Examples

The following raw data were obtained comparing a water sample against reference water in a 5 minute test:

	IO	I5	I5 corrected
Control	97.12	103.91	
Control	91.88	102.76	
Control	94.75	102.93	
Control	94.62	104.17	
Control	93.88	103.24	
Mean	94.45	103.40	
Sample	90.88	93.78	95.32
Sample	90.16	94.52	96.07
Sample	95.05	94.66	96.21
Sample	94.83	94.86	96.42
Sample	93.58	95.21	96.77
Mean	92.90	94.61	96.16

Comparison Test Details:

Correction factor, CF = 1 + (1 - 92.90/94.45) = 1.01641. [indicates 1.01641% difference between the zero time means] Corrected sample I5 mean = CF x 94.61 = 96.16; individual readings are corrected in the same way. The corrected values are used in subsequent calculations. For the five minute data $\sum D_c^2$ & $\sum D_s^2$ are 1.5091 and 1.158 respectively as calculated by the formula in Terms above. The variances are thus these numbers divided by 4 (df), or 0.3773 for the controls and 0.2895 for the samples. F is the larger of these variances divided by the smaller; 0.3773 / 0.2895 = 1.303. The calculated F is smaller than the critical F value of 9.6, so these variances may be considered equal and the standard t test may be used.

From the formula for t:

t_{obs}
$(X_s - X_c / \sqrt{ (D_c^2 + D_s^2)}) (\sqrt{20})$
$((103.4 - 96.16) / \sqrt{(1.5091 + 1.158)}) (\sqrt{20})$
$(7.24 / \sqrt{2.6671})(\sqrt{20})$
19.83

From the look-up table, Table 1, it can be seen that the calculated t is greater than the 99.9% look-up value for 8 df of 5.041 indicating that the difference between means is significant with a probability greater than 99.9%.

The average of the two variances is:

S_{cs}^2
(0.3773 + 0.2895) / 2
0.6668 / 2
0.3334

The standard deviation of the average is:

S_{cs}
$\sqrt{S_{cs}^2}$
$\sqrt{0.3334}$
0.5774

This value is used to calculate the 95% confidence limits. The difference between means is:

103.4 - 96.16 = 7.24

The limits are ± 2.306(.5774), yielding 7.24 ± 1.33. These are converted to percent differences by:

7.24 / 103.4 x 100 = 7.00% and
1.33 / 103.4 x 100 = 1.29%

Thus the results for 5 minute data are reported as:

% Difference	7.0 ± 1.3
Significance level	99.9%

Table 1: Look-up table for standard and modified t tests*

Significance Level %	t for 8 df (standard)	t for 4 df (modified)
60	0.889	0.941
80	1.397	1.533
90	1.860	2.123
95**	2.306**	2.776**
97.5	2.752	3.495
99	3.355	4.604
99.5	3.832	5.598
99.9	5.041	8.610

** values used for the 95% confidence limits calculations

Special Sample Handling

Difficult Samples Made Easy

In several situations, samples may require special handling.

1. **Samples that have known special requirements**
   Some samples are already saline, requiring omission of the osmotic adjustment step; others are colored, others have unusual physical characteristics. Such samples are characteristic of certain industries and activities and various manufacturing situations.
   This manual contains a number of variations on the fundamental Microtox Acute Test protocols designed specifically for dealing with samples known to require special handling. Examine these carefully, and select one that serves your particular need.

2. **Samples that are exceptionally toxic**
   Some samples are so bioreactive that the tests you normally run show complete light loss at all concentrations. Retest the sample, using the Basic Test Protocol and make a "primary dilution" of the sample. With adequate dilution, the EC_{XX} of any highly toxic sample can be found.
   It is desirable to use the Basic Test Protocol when testing a sample of completely unknown toxicity.

3. **Samples that are of exceptionally low toxicity**
   The toxicity of some samples is extremely low, but important to know. When the toxicity levels are very low, they may be confused with the background "noise" produced by irregularities in pipetting.
   Run an 81.9% Basic Test protocol, in duplicate, using two controls in each set. When the 81.9% Basic Test protocol does not provide EC_{xx}, run a Comparison Test.

4. **Samples that have an extremely steep dose-response curve**
   Some test samples show a dose-response curve so steep that the light level drops from very high to very low from one sample concentration to the next, providing too few useful data points for calculation of ECXX.
   Complete light loss may occur at all concentrations in an Abbreviated Basic Test Protocol. After a second test with a primary dilution, all concentrations may show no light loss at all. A trial-and-error search for the narrow range in which the change occurs can be time-consuming. This sort of data has a dose-response curve so steep at times that it cannot be plotted by the system.
   Use narrower (1:1.5) serial dilutions.

5. **Samples that have a negative slope**
   Some test samples show a negative slope (reverse dose- response curve). The light level appears to increase with increase in sample concentration. This condition is caused by one of three mistakes:

   1. The sample dilutions were made in reverse sequence.
   2. The It light readings were read in reverse sequence.
   3. The most common cause is background noise (pipetting error) which by chance forms a negative slope.

   Background noise can be more noticeable with low toxicity samples and the 81.9% Basic Test protocol.
   Run the test protocol used, in duplicate, using two controls with higher sample concentration if possible.
   During the test, be very careful in pipetting, making the sample dilutions and reading the light levels.

6. **Samples that have a flat dose-response curve**
   Some samples show dose-response curves so flat (at 1:2 dilution ratio) that the data points may be confused with background (pipetting) noise, and calculation of EC_{XX} may require extrapolation to a distant point, widening the confidence range beyond cceptable range limits.
   Rerun the test, using the wide (1:10) serial dilutions.

7. **The ECXX is determined by extrapolation**
   Some samples are so bioreactive, or so slightly bioreactive, at the range of concentrations tested that all of the data points fall on one side of the EC_{XX}, which can only be determined by extrapolation. This is always less desirable than an EC_{XX} bracketed by data points.

   If the gammas are all larger than that corresponding to the EC_{XX} of interest, all gammas greater than 1.0 for EC_{50}, retest the sample using a primary dilution testing lower concentrations. The problem with extrapolation to determine EC_{XX} is that the sample may be nonlinear at lower or higher concentrations.

   If the gammas are all smaller than that corresponding to EC_{XX} (gammas less than 1.0 for EC_{50}) retest using a more concentrated sample.

FAQs

These common Questions & Answers provide insight into the decisions and practices that produce high quality data.

Q. How do I know which test to run?

A. The Applications and methods section of the manual has detailed descriptions of each protocol, choose the test that is appropriate for your application.

Q. Which test should be used for samples that show no toxicity at concentrations of 45% and lower.

A. Use the 81.9% Basic Test protocol or the Comparison Test.

Q. Duplicate tests. What are they, and when would they be used?

A. The use of duplicates provides additional data to check the quality of the test (operator pipetting), and provides a larger context in which to evaluate the data.

Q. How many controls should be used?

A. The number of controls can range from one to three. The highest confidence in results would come from running three controls. Multiple controls provide another measure of pipetting consistency.

Q. How many sample dilutions should be used?

A. When dealing with well-characterized samples, four sample dilutions that have at least one dilution on either side of the EC_{XX} are enough. In that case, you will typically be testing samples from the same source on a regular basis, looking for significant changes in toxicity levels.

Tests using more dilutions are important if you are testing samples with which you have no previous experience, and you don't know what to expect.

For instance, if you run a test with just a few dilutions of a very toxic sample, every concentration may cause complete loss of light output from the reagent. This indicates that the sample has detectable toxicity, but does not allow calculation of EC_{XX}, because the few sample dilutions used were in the wrong range.

For unknown, or highly variable samples, the Basic Test Protocol is appropriate. This protocol is much more likely to bracket the EC_{XX} of interest.

Q. Examples of tests in this manual use 1:2 serial dilutions to prepare the serial dilutions of the sample.
Wouldn't a higher ratio provide a greater range of concentrations?

A. Yes, but it reduces the resolution of the test. The 1:2 dilutions are a good standard compromise between range and resolution, and work well for most samples.

Q. How long can the frozen Microtox Acute Test Reagent be stored before it is used?

A. The shelf life of the reagent, from date of manufacture, is at least two years conditions. See the discussion of Microtox Acute Test Reagent in the FUNDAMENTALS section of this manual. The expiration date of the reagent is printed on the label on the reagent vial. Make a point of using up the reagent with earlier expiration dates before opening new boxes of reagent with later expiration dates. First in/first out.

Q. How can I tell if the fresh reagent always produces the same amount of light?

A. The reagent does not always produce the same amount of light, and the question is of little practical importance. The system measures the percent of light lost in response to change in toxicity, which is consistent, no matter what the original light level. Light levels may be different for several reasons:

a) The light output of different batches of reagent varies.

b) Poor reconstitution technique reduces the potential light output of the reagent.

c) The light output will change with the age (shelf life) of the reagent.

These variations are unimportant because the gradual light loss or gain during a test is measured, and accounted for in data reduction calculations of the Test Procedures.

In general, light output is not the primary consideration in evaluating the quality of the reagent.

More important is the response of the reagent to various toxic materials.

Q. How long can the reagent be used after reconstitution?

A. For at least three hours after reconstitution, the organisms respond consistently to a wide spectrum of toxic materials. After that time, the response may begin to change. The reagent does not just become generally "less sensitive." Its response to different chemicals may either increase or decrease, changing the response spectrum.

The useful life of the reagent after reconstitution is nominally three hours. Many laboratories reconstitute a vial of reagent for use all morning or afternoon. The conditions under which you use Microtox Acute Test Reagent beyond the initial three hours will depend on your need for precision and verifiability of results.

For instance, if you intend to use the data for routine monitoring, and data quality requirements may not be critical, the use of reagent over several hours may be perfectly acceptable. If unusual or suspicious results are seen, these results could be confirmed with tests using a "fresh" bottle of reagent.

Well-characterized samples can be tested repeatedly with reagent of increasing age, so that the "normal" response of the reagent at any age is well known. The Phenol Standard is useful for this. It is also helpful to run several tests on one or more well characterized stable samples of known toxicity.

To establish baseline information, you may also elect to test each new lot of Microtox Acute Test Reagent with a standard such as Phenol or Zinc sulfate over a period of hours after reconstitution, so you know what the normal response curve is over time.

Once again, the decision on how long the reconstituted reagent may be used is based on what information the test is intended to produce.

Q. Some of the protocols call for transfer of only 10 µl of reagent mixture to the test cuvettes. Can't we increase that to 20 µl or 30 µl, as long as we do it every time?

A. No, don't increase the amount of reagent.

Q. How can we measure and improve pipetting skills?

A. The Microtox Acute Test requires transfer of small amounts of liquid, as little as 10 µL at a time, with an assortment of icropipettors. Pipetting is an ordinary laboratory skill and usually improves with repetition.

To check your reagent pipetting consistency:

Using an analytical balance, transfer 10 µL of water into a tared cuvette and weigh the amount of water pipetted. Repeat this 10 times and calculate the mean.

Q. How can we calibrate a pipettor?

A. Using an analytical balance, weigh the amount of water transfered into a tared cuvette. Repeat this 10 times and calculate the mean.

Q. Sometimes several or all of the sample cuvettes produce negative gammas. Does that always indicate a stimulatory effect of the sample?

A. Not always. The effect may also be produced by pipetting error.

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