a) if the pH of the sample is not between 6 and 8.5, adjust the pH to 7.0 ± 0.2 with NaOH or HCl (the volume of the sample should not increase more than 5 %);

b) adjust the salinity of the sample to be equivalent to 2 % NaCl solution. With freshwater or low salinity samples add either solid NaCl to a final concentration of 2 % w/v. Alternatively, use 1/10 of the final volume **1243-559 BioTox ™ NaCl tablet dissolved in 45 ml of distilled water** solution for adjusting the salinity.

c) if the oxygen concentration of the undiluted sample is less than 3 mg/l, oxygenate the sample by aeration or stirring.

3. Reconstitute the **1243-555 BioTox™ *Vibrio fischeri* Reagent** by adding the contents of the cooled (+4 °C) **1243-551 BioTox™ Reagent Diluent** to the reagent vial. The reconstituted reagent should be equilibrated at +4°C for at least 30 minutes**.** Then stabilise the reagent at +15 °C for at least 30 min before pipeting into the cuvettes. **The reconstituted reagent must be used within the same day and it cannot be frozen.**

4. Pipette 500 µl of the bacterial suspension into the cuvettes needed for performing the tests. It is recommended to measure all dilutions and controls as duplicate samples. Stabilise the bacteria in the cuvettes for at least 15 minutes at +15 °C.

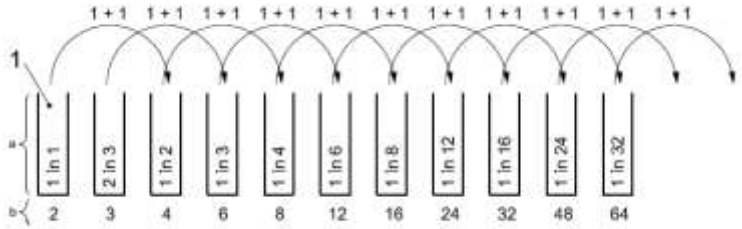
5. Dilute sufficient volume of **1243-559 BioTox™ NaCl dissolved in 45 ml of distilled water** 1:10 with distilled water to obtain 2 % NaCl solution. If solid NaCl is used for the salinity adjustment, the whole tablet of **1243-559 BioTox™ NaCl** can be dissolved in 450 ml of distilled water and stored at refrigerator. Before every measurement, check and adjust the pH of the diluted **NaCl solution** to 7.0 ± 0.2, if necessary.

6. Prepare the sample dilution series by using 2 % NaCl solution as diluent. Temperate all samples and dilutions to +15 °C for at least 15 min. Keep all samples and dilutions at +15 °C during the whole measurement. The dilution series should be in accordance with the ISO 11348 Standard. It is prepared by means of a graduated dilution and combines two geometric series (D = 2, 4, 8, 16 etc., and D = 3, 6, 12, 24 etc.) Suitable dilutions are chosen depending on the expected toxicity of the sample. The dilution series can be prepared from two stock solutions:

   – Dilution 1:1, undiluted sample (dilution factor at final assay will be 1:2). 3000 µl undiluted sample

   – Dilution 2:3, (dilution factor at final assay will be 1:3). 2000 µl sample and 1000 µl 2 % NaCl solution

   All the other dilutions can be done from the two stock dilutions by serial dilution using only the dilution factor 1:1 (for example 1.5 ml of previous dilution and 1.5 ml of diluent). The length of the dilution series must be determined separately for each sample, but with most samples about 5 -10 dilutions is sufficient for the determination of EC_{50}. The principle of the dilution procedure is shown in the picture below:



1 = sample, a = dilution of a sample, b = final dilution level D after the addition of the test suspension.

6. Measure the luminescence intensity (I_0) from the first cuvette containing bacterial suspension. Add immediately 500 µl of sample to the cuvette. Repeat for all samples using equal time intervals between each sample. Note: If it is desired to test nearly undiluted water samples, it is possible to add 800 µl of the undiluted water sample to 200 µl of a test suspension. The dilution then is 1:1,25. Prepare similar control sample.

7. Incubate sample dilutions at +15 °C for the chosen contact time (either 5, 15 or 30 minutes). Determine the luminescence intensity (I_t) from the first sample (cuvette number 1). Repeat for all samples using exactly the same time interval as during the first measurement. When the **BioTox™ Software** is used, the computer will prompt the operator when each sample should be measured. For further information about the usage of the **BioTox™ Software, refer to the software manual.**

NOTES:

– Temperature changes during the measurement may affect the results. Ensure that all reagents have reached the same temperature (+15 °C).

– The contact time should be exactly the same for all the samples and the controls. The most commonly used contact times are 5, 15 and 30 minutes.

CALCULATION OF RESULTS

You can calculate the results using a spreadsheet calculation or The **BioTox™ Software** which performs automatically all calculations needed. If the software is not used, the inhibition percentage (INH%) is calculated as follows (in this example the contact time is 15 min.):

$$KF = IC_{15}/IC_{0}$$

$$INH\% = 100 - 100 \times (IT_{15} / KF \times IT_{0})$$

Where:

KF	=	Correction factor.
IC_{15}	=	Luminescence intensity of control after contact time (15 min) in RLU.
IC_0	=	Initial luminescence intensity of control sample in RLU.
IT_{15}	=	Luminescence intensity of test sample after contact time (15 min) in RLU.
IT_0	=	Initial luminescence intensity of the test sample in RLU.

The EC_{50}-value is determined by using standard linear regression analysis. If the range of value pairs cannot be linearized, the EC_{50}-value can be determined graphically using a double logarithmic co-ordinate system. The INH% is plotted on the y-axis and the concentration (in mg/l, mol/l or % of original sample) on the x-axis.

When the **BioTox™ Software** is used for controlling the measurement, all calculations and graphic presentations of the results are performed automatically.

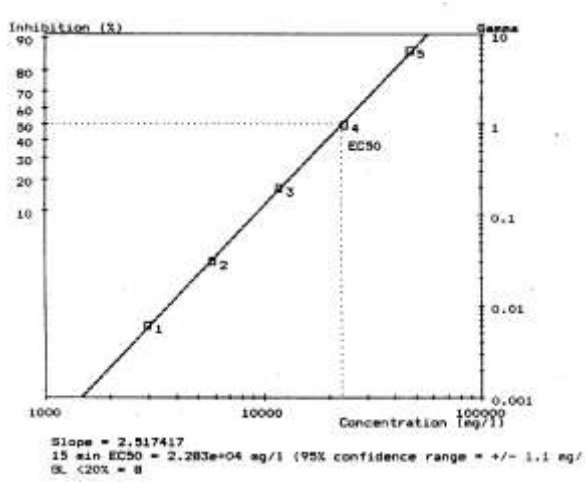
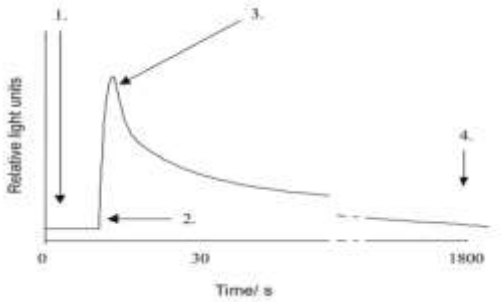


Figure 1: The toxicity of ethanol calculated with the **BioTox™ Software**.

TURBID AND COLOURFULL SAMPLES

It is possible to use the ISO standard protocol 21338 with the 1243-500 BioTox™ Kit (Kinetic luminescent bacteria test). This standard protocol should be used if sample colour or turbidity is an issue. Contact Aboatox for detailed information.



Schematic protocol for the kinetic test. The photobacteria reagent is dispensed on top of a sample in the measurement chamber of the luminometer. 1. Start measurement. 2. Inject bacteria. 3. Record peak value from 0-2 s. 4. Mix sample and record signal at 30 min.

CRITERIA OF VALIDITY

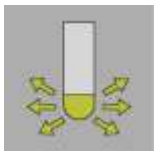
1. The three reference substances, solutions not neutralized, cause 20 % to 80 % inhibition after 30 min contact time at the following concentrations **in the final test suspension:**

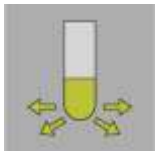
   3,4 mg/l 3,5-dichlorophenol
   2,2 mg/l Zn(II), equivalent to 9,67 mg/l Zinc sulfate heptahydrate
   18,7 mg/l Cr(VI), equivalent to 52,9 mg/l potassium dichromate

2. Correction factor for 30 min incubation ranges between 0,6 – 1,8.

REFERENCES:

1. ISO 11348-3, 1999, Determination of the Inhibitory Effect of Water Samples on the Light Emission of Vibrio fischeri (Luminescent bacteria test).
2. Kahru, Anne, In vitro Toxicity testing Using Marine Luminescent Bacteria (*Photobacterium Phosphoreum*): the BioTox™ test; ATLA 21, 210-215, 1993
3. Kahru, Anne and Borchardt Barbara, Toxicity of MEIC Chemicals to Bioluminescent Photobacteria (the BioTox™ test): Correlation with other test systems.
4. Lappalainen, J., Juvonen, R., Nurmi, J. and Karp, M. (2000). Automated colour correction method for *Vibrio fischeri* toxicity test. Comparison of standard and kinetic assays. *Chemosphere* 45 (2001) 635-641.



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1243-500 BioTox™ Kit

Instructions for use

INTENDED USE

The **BioTox™ Kit** is used for the determination of toxicity of water soluble samples. The inhibitory effect of the sample on the light emission of luminescent bacteria, A*liivibrio fischeri* (formerly *Vibrio fischeri*), is measured with a luminometer. The **BioTox™ Kit** provides a rapid, easy to use method for measuring toxicity of aqueous samples such as WASTE WATER, AQUEOUS EXTRACTS AND LEACHATES, FRESH WATER, SEA AND BRACKISH WATER, ELUATES OF SEDIMENT, PORE WATER AND SINGLE SUBSTANCES. SOIL AND SEDIMENT samples can also be tested with the kinetic modification of the test.

ASSAY PRINCIPLE

The inhibition of the luminescence is determined by combining different dilutions of the test sample with luminescent bacteria. The decrease of light intensity is measured after a contact time of 5 - 30 minutes. The inhibitory effect of dilutions is compared to a toxin free control to give the percentage inhibition (INH%). The value is plotted against the dilution factor and the resultant curve is used to calculate the EC_{50} (Effective Concentration causing 50% inhibition of light output) of the sample.

KIT CONTENTS AND STORAGE

1. **1243-555 BioTox™ *Vibrio fischeri* Reagent**, 6 vials. Lyophilised *Aliivibrio fischeri* NRRL B-11177 together with stabilisers.
2. **1243-551 BioTox™ Reagent Diluent**, 6 vials, 12.5 ml.
3. **1243-559 BioTox™ NaCl** tablet 9,0 g.

Reagent storage

The stability of the reagents is guaranteed until the best before date if stored at -18 °C. The Kit should be stored at -18 °C. **1243-551 BioTox™ Reagent Diluent** and **1243-559 BioTox™ NaCl tablet** may be stored at room temperature.

The test kit does not contain any equine, ruminant, swine, or avian species or materials.
For laboratory use only. Not for drug, household or other use.

Liability: In case of problems, the user is requested to return this specification sheet to the distributor, with a detailed description of the problem(s) encountered. The claim will be analysed and the outcome communicated to the claimer. The liability is restricted to the replacement of the Biotox materials.

REAGENTS AND INSTRUMENTS

- **BioTox™ Kit** (prod. no. 1243-500)
- **Luminometer**
- +15 °C dry block incubator
- Luminometer cuvettes
- Solid NaCl (pro analysis grade)
- Adjustable 200 µl, 1 ml and 5 ml pipettes and pipette tips
- pH-meter (accuracy 0,1 pH units)
- Oxygen probe
- 1257-130 BioTox™ Software (optional) or Microsoft Excel Spreadsheet for calculations
- 0,1 M and/or 0,01 M NaOH
- 0,1 M and/or 0,01 M HCl

ASSAY PROCEDURE (according to International Standard, ISO 11348-3)

1. Dissolve the 1243-559 Biotox™ NaCl tablet to 45 ml of distilled water to obtain 20 % NaCl solution.

2. Prepare the original sample for the toxicity assay: