

# Discharge Control and Monitoring by Biological Techniques

Case Studies

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**DISCHARGE CONTROL AND MONITORING BY BIOLOGICAL TECHNIQUES - CASE-STUDIES**

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## DISCHARGE CONTROL AND MONITORING BY BIOLOGICAL TECHNIQUES - CASE-STUDIES

R Butler and N J Grandy

### SUMMARY

Currently, the control of discharges to UK surface waters is achieved principally by a chemical-specific approach. In this approach, maximum permitted concentrations of relevant polluting substances in the discharge are set and incorporated into the consent conditions. The discharge is then chemically monitored to ensure compliance with the consent standard(s). However, this approach is not well-suited to complex effluents which may be difficult, or even impossible, to characterise chemically.

In recent years there has been an increasing interest in the application of Direct Toxicity Assessment (DTA) for the evaluation and control of effluent quality, and in the establishment of discharge consent conditions expressed in terms of toxicity rather than in chemical composition alone.

In an earlier project in the Environmental Research Programme WRc produced a draft protocol for discharge control. The objective of this project (6.3.1b) is to test, by means of case-studies, the protocol for the application of biological procedures to the control and monitoring of complex discharges as an adjunct to chemical analysis. However, before any case-studies could go ahead, it has been necessary to establish quality control measures and standardised procedures, particularly for the Microtox work. This report describes the work done to standardise the Microtox methods and contains a draft standard operating procedure. It also discusses the selection process for case-study sites that cover a range of discharge types and outlines the progress on the establishment of the case-study programme outlined.

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## SECTION 1 - INTRODUCTION

Currently, the control of discharges to UK surface waters is achieved principally by a chemical-specific approach. In this approach, maximum permitted concentrations of relevant polluting substances in the discharge are set and incorporated into the consent conditions. The discharge is then chemically monitored to ensure compliance with the consent standard(s). However, there are a number of disadvantages to this approach:

- (i) analytical techniques are not readily available for the routine monitoring of the numerous organic chemicals which may occur in effluents;
- (ii) even if chemical data are obtainable, toxicological data, used to set Environmental Quality Standards (EQSs), are sparse or unavailable for many thousands of substances;
- (iii) for complex effluents, chemical analysis of all the constituent components, if possible, can be very costly;
- (iv) chemical interactions may occur in complex effluents (i.e. additive, synergistic and antagonistic effects) which will not be taken account of in the chemical-specific/EQS approach;
- (v) the variable composition of many complex effluents make adequate chemical monitoring very difficult.

In recent years there has been an increasing interest in the application of Direct Toxicity Assessment (DTA) for the evaluation and control of effluent quality and in the establishment of discharge consent conditions expressed in terms of toxicity rather than in chemical composition alone. The United States introduced toxicity-based controls, in combination with chemical controls, in 1984 (US EPA 1984).

By 1987, over 1400 of their industrial consent conditions incorporated toxicity testing requirements (Wall and Hanmer 1987). Advantages of this approach include:

- (i) DTA gives an integrated response to the effluent, i.e. it will detect the biological effects of all the compounds present in an effluent, even if they cannot be identified or measured by chemical means. Thus the complexity or variability of effluents does not affect the cost of control by DTA;
- (ii) Information on the toxicity of the individual components of the effluent is not required;

Here in the UK, there has been increasing interest in the DTA approach to the control of discharges and it has already been applied in a number of cases (NRA Welsh Region personal communication, Haig *et al* in press, Mackay *et al* in press). As a result of this interest, WRc produced a document on discharge control via DTA (Hunt 1989) under project area 6.3.1a of the Environmental Research Programme. This document discusses the DTA approach in full, compares it with existing control measures and proposes a protocol for discharge control in the UK. These proposals were presented to representatives from the NRA, the Scottish River Purification Boards and DoE Northern Ireland at a workshop held at Bisham Abbey, Marlow on 18 July 1989. The approach was welcomed and supported by the participants.

Broadly, the approach consists of an initial screening stage, when discharges suitable for control by DTA are identified and their toxicities assessed and ranked. It is proposed that the Microtox bioluminescent bacterial test be used for screening effluent toxicity since it is rapid and relatively inexpensive compared with other more conventional tests (e.g. fish, *Daphnia*). From the results of the screening tests and a knowledge of the dilution capacity of the receiving waters, the discharges will be categorised into four types, A to D, as described in Hunt (1989). Discharges in categories A and B will be those which may cause chronic toxicity in the environment and

for which further information on their toxicity is required. For these discharges, we propose that toxicity tests with more relevant organisms be performed (e.g. trout, Daphnia for freshwater, plaice, oyster embryos for seawater) and their sensitivities calibrated against that of Microtox.

The objective of this project (6.3.1b) is to test, by means of case-studies, the protocol for the application of biological procedures to the control and monitoring of complex discharges as an adjunct to chemical analysis. However, before any case-studies could go ahead, it has been necessary to establish quality control measures and standardised procedures, particularly for the Microtox work. Standardisation and quality control of methods is very important in this work since, if DTA is found to be an appropriate way to control and monitor discharges, the data will need to be defensible in a court of law when prosecutions for non-compliance with discharge consents arise.

This report describes the work done in order to standardise the Microtox methods and the progress on establishment of the case-study programme.

## SECTION 2 - TECHNICAL ASPECTS OF THE MICROTOX TOXICITY TEST

### 2.1 INTRODUCTION

The Microtox toxicity test is a technique which can be used to determine the toxicity of aquatic contaminants to the marine bacterium Photobacterium phosphoreum. It is an extremely simple and rapid test for screening potentially toxic chemicals before they are disposed of to the marine environment. It can also be used routinely to monitor the environmental impact of contaminants released into coastal, estuarine or river water bodies.

P. phosphoreum is a bioluminescent bacterium, common in marine waters. The test organisms are cultured, harvested and freeze-dried under carefully controlled conditions by Microbics Corporation.

The bioluminescence of Microtox bacteria has been shown to be a reasonably sensitive measure of toxicity broadly equivalent to acute fish and Daphnia toxicity tests. Walker (1988) compared the relative sensitivity of 149 species of test organisms to phenol. Rainbow trout had a mean 96 hour  $LC_{50}$  of 8.5 mg/l, Daphnia magna had a mean 48 hour  $LC_{50}$  of 26.2 mg/l whilst a mean 5 minute  $EC_{50}$  for Microtox was 28.9 mg/l.

The Microtox analyser utilises a photomultiplier to measure changes in the bioluminescent output of Microtox bacteria exposed to test substances compared with control solutions. The  $EC_{50}$  is defined as the concentration which results in a 50% reduction in bacterial light output after a 15 minute exposure period at 15 °C compared with the control.

The Microtox toxicity test system, supplied as a complete package by Microbics Corporation, has been examined in detail for its use in monitoring effluent toxicity. This section describes the quality control considerations, improvements and standardisation of the Microtox test, comparisons of sensitivity with other test organisms and equipment required for detailed toxicity studies in the laboratory and at remote sites.

## 2.2 THE STANDARD OPERATING PROCEDURE (SOP)

Beckman, the original manufacturer of the Microtox system, produced a comprehensive guide to conducting the Microtox test. This manual was so detailed that it could not be easily read and was superseded by several shorter versions by Microbics Corporation, an offshoot of Beckman. The majority of these operating procedures apply to conducting the test using the older, obsolete Microtox 2055 analyser. Much of the text was difficult to follow, contained vague or ambiguous statements and did not adequately describe the determination of  $EC_{50}$  values with 95% confidence intervals.

WRc have produced a draft SOP (Appendix A) which aims to simplify the Microtox test but which contains detailed sections on the principles, methodology, scope and limitations and equipment required for each test. Quality control and information required on test substances are also dealt with in detail. This draft SOP will be modified where necessary as experience with the technique grows.

It is also WRc's aim to provide NRA regions with updated software to accompany the SOP. The software will capture data directly from the analyser using a menu-driven operating procedure and analyse the data to produce  $EC_{50}$ s with 95% confidence intervals. Currently WRc is liaising with Microbics to produce an upgraded version of their software. The present software allows for three toxicity tests to be run simultaneously without replication for test concentrations. WRc's SOP recommends carrying out two tests simultaneously with duplicate cuvettes for each concentration. Duplicate concentrations allow the operator to clearly determine erroneous test results and improve the 95% confidence limits on  $EC_{50}$  calculations.

### 2.3 EQUIPMENT REQUIRED FOR MICROTOX TESTING

Microbics can supply the Microtox system as a complete package. However some items may be obtained more cheaply, locally and with better service contracts. The basic requirements are a Microtox analyser (models 500 or 2055), a chart recorder, pH meter, salinometer, non-self-defrosting fridge freezer and a centrifuge. Micropipetters, sample bottles, cuvettes, bacteria and standard test solutions are also required.

In order to conduct a testing programme efficiently, a personal computer and printer can be used to capture data directly from the Microtox analyser. Manual calculation of  $EC_{50}$ s with 95% confidence limits is difficult and time consuming. The Microbics software enables test data to be statistically analysed immediately. The software can be used for manual data input as well as direct data capture. Direct data capture reduces operator error in reading chart recordings and the menu driven prompts during testing ensure that the operator knows which cuvettes are to be examined and at which time.

The Microtox analyser is robust and portable and can easily be used at sites remote from the laboratory. A portable personal computer is therefore recommended.

## **2.4 QUALITY CONTROL CONSIDERATIONS**

Several manuals have been produced, initially by Beckman and later Microbics, which outline the principles and methods used in the Microtox test. Although the manufacturer has considered quality control, several important aspects needed to be tested so that a standard operating procedure could be produced and distributed to NRA regions.

Standard reference toxicants have therefore been chosen, tested and assigned limits of acceptability. Limits on sample pH, methods of pH adjustment and acceptable test temperature ranges were tested and defined. The length of time for which a reconstituted vial of bacteria could successfully be used to give reproducible results has also been determined and standardised.

### **2.4.1 Testing zinc and phenol as standard toxicants**

Microbics recommend phenol and zinc sulphate as suitable organic and inorganic standard toxicants for the Microtox test. Stock solutions were therefore prepared, using aristar grade zinc and phenol as 10 mg Zn/l and 100 mg phenol/l in double glass-distilled water. Using the standard Microtox toxicity test (Appendix A)  $EC_{50}$  values with 95% confidence limits were generated in order to assess its reproducibility and set acceptable limits.

The results of 17 toxicity tests using phenol produced mean 5 and 15 minute  $EC_{50}$  values of 17.8 and 19.0 mg phenol/l respectively and are shown in Table 1. Microbics state that an acceptable range of 5 minute  $EC_{50}$  values is 17-28 mg/l.

An asymptotic  $EC_{50}$  is typically achieved within 5 minutes' exposure of Microtox bacteria to organic compounds. The same response to bivalent metals can take up to 30 minutes. Microbics quote a range of 2-4 mg  $ZnSO_4/l$  as the 15 minute  $EC_{50}$  for zinc sulphate. Vasseur et al (1986) found the 30 minute  $EC_{50}$  for the same compound to be 0.96 mg Zn/l. The results of 13 tests carried out at WRc produced a mean 15 minute  $EC_{50}$  of 1.92 mg Zn/l, whilst 7 tests extended to 30 minutes produced a mean 30 minute  $EC_{50}$  of 1.15 mg Zn/l (Table 2).

As a result of these studies, the standard operating procedure recommends that each vial of bacteria be tested with one or both of the standard toxicants during a series of toxicity tests and that the 15 minute  $EC_{50}$  for phenol should lie within 15-28 mg phenol/l and that the 30 minute  $EC_{50}$  for zinc sulphate should be within 0.95 to 1.5 mg Zn/l. Should the relevant  $EC_{50}$  fall outside these preset limits, the test and vial of bacteria should be rejected.

A two day Microtox workshop was held at the NRA Welsh Region laboratory at Llanelli on 14 and 15 November 1989. Five operators working in two groups carried out tests on the zinc and phenol standards using 2 Microtox machines and following a standard operating procedure.  $EC_{50}$  results were consistent between groups and machines. Different vials of Microtox bacteria gave similar results.

**Table 1 - Five and fifteen minute EC<sub>50</sub>s, with 95% confidence limits, for phenol: the standard organic toxicant.**

Test date	5 min EC <sub>50</sub> (mg/l)	95% CI	15 min EC <sub>50</sub> (mg/l)	95% CI
09.11.89	16.4	15.7-17.0	22.7	20.4-25.4
09.11.89	17.0	16.2-17.9	18.0	17.3-18.8
09.11.89	19.1	18.2-20.0	19.2	18.5-19.9
09.11.89	17.2	14.0-21.1	17.7	14.3-21.9
09.11.89	16.8	16.0-17.7	16.5	16.0-17.0
09.11.89	17.2	16.4-18.0	17.8	17.3-18.3
13.11.89	17.2	13.8-21.4	18.1	14.7-22.3
14.11.89	16.4	15.9-16.8	16.9	16.6-17.3
14.11.89	14.8	13.9-15.8	15.2	14.3-16.2
14.11.89	15.8	10.0-25.1	16.4	10.3-26.2
05.02.90	16.5	15.6-17.6	17.6	16.4-19.0
05.02.90	19.8	17.7-22.1	22.3	20.2-24.7
05.02.90	22.2	19.9-24.6	23.3	20.3-26.8
05.02.90	19.3	18.1-20.5	21.6	20.0-23.4
15.02.90	18.0	17.2-18.9	19.8	18.9-20.7
15.02.90	18.8	17.9-19.7	20.6	19.7-21.6
15.02.90	19.4	18.5-20.4	19.7	18.7-21.1
Mean EC <sub>50</sub>	17.8		19.0	

Table 2 - Five, fifteen and thirty minute  $EC_{50}$ s, with 95% confidence limits, for zinc: the standard inorganic toxicant

Test date	5 min $EC_{50}$ (mg/l)	95% CI	15 min $EC_{50}$ (mg/l)	95% CI	30 min $EC_{50}$ (mg/l)	95% CI
3.11.89	7.5	4.2-13.3	1.56	1.2-2.0	-	-
13.11.89	7.7	5.4-11.0	1.35	1.2-1.5	-	-
13.11.89	24.5	9.1-66.1	1.32	1.2-1.4	-	-
14.11.89	4.5	3.1- 6.5	1.08	1.0-1.2	-	-
14.11.89	3.2	2.6- 3.9	1.13	0.9-1.4	-	-
14.11.89	7.3	6.9- 7.7	1.44	1.3-1.6	-	-
05.02.90	21.7	14.8-31.7	3.22	3.0-3.5	1.37	1.3-1.4
05.02.90	11.5	6.0-21.8	2.42	2.1-2.8	1.13	1.1-1.2
05.02.90	10.2	5.7-18.0	2.56	2.3-2.8	1.13	1.1-1.2
05.02.90	18.8	10.7-32.9	2.20	2.1-2.3	0.96	0.9-1.0
15.02.90	8.4	6.3-11.2	2.19	2.1-2.3	1.16	1.1-1.2
15.02.90	6.9	5.9- 8.2	2.06	2.0-2.1	1.13	1.1-1.2
15.02.90	6.4	3.4-12.2	2.42	2.2-2.7	1.20	1.1-1.3
Mean $EC_{50}$	10.7		1.92		1.15	

#### 2.4.2 Determination of a suitable pH range for effluent testing

Microbics recommend that test substances should be pH-adjusted to between 6 and 8, using analytical grade hydrochloric acid and sodium hydroxide. Vasseur *et al* (1986) reported that the bacterial response was not significantly modified over a pH range of 6 to 7.5.

In order to substantiate these claims, zinc and phenol standard toxicant solutions were pH-adjusted to cover a pH range of between 4 and 10. They were then tested using the standard Microtox test. Tables 3 and 4 show that, for phenol, the 15 minute  $EC_{50}$  values were within the range

of 15-28 mg/l over the whole pH range on both occasions. The 30 minute  $EC_{50}$  value for zinc at pH 9 appeared unusually high during the first study (Table 4), whilst in the second study, even pH 10 caused no significant difference ( $p>0.05$ ) compared with pH 7 and 8. At pH 4 and 5, the 30 minute  $EC_{50}$ s for zinc were 1.66 and 1.54 mg Zn/l respectively and were significantly higher than  $EC_{50}$ s determined at higher pHs. Table 4 again illustrates the importance of continuing a test for 30 minutes in the case of zinc, in order to enhance test sensitivity and achieve asymptotic  $EC_{50}$ s.

The standard operating procedure described in this report recommends that all test substances should be pH-adjusted to between 6 and 8.

Table 3 - The sensitivity of Photobacterium phosphoreum to phenol over a pH range of 4 to 10 (at 15 °C).

	pH	5 min $EC_{50}$ (mg/l)	95% CI	15 min $EC_{50}$ (mg/l)	95% CI
(i)	4	20.2	19.5-21.0	21.2	20.6-21.9
	5	20.5	19.8-21.2	23.4	18.4-29.7
	6	19.8	17.7-22.1	22.3	20.2-24.7
	7	19.3	18.1-20.5	21.6	20.0-23.4
	8	22.2	19.9-24.6	23.3	20.3-26.8
	9	23.8	22.7-25.0	25.6	24.0-27.3
	10	23.0	21.3-24.9	22.9	21.9-23.9
(ii)	4	18.3	17.4-19.2	18.7	17.9-19.5
	5	18.0	17.1-19.0	18.1	17.3-19.0
	6	18.0	17.2-18.9	19.8	18.9-20.7
	7	18.8	17.9-19.7	20.6	19.7-21.6
	8	19.4	18.5-20.4	19.7	18.7-21.1
	9	20.6	19.3-21.9	21.2	19.8-22.8
	10	24.5	22.3-26.9	24.4	22.3-26.8

(i) refers to the initial test carried out on 05.02.90

(ii) refers to the second test carried out on 13.02.90

**Table 4 - The sensitivity of *Photobacterium phosphoreum* to zinc over a pH range of 4 to 10 (at 15 °C)**

	pH	5 min EC <sub>50</sub> (mg/l)	95% CI	15 min EC <sub>50</sub> (mg/l)	95% CI	30 min EC <sub>50</sub> (mg/l)	95% CI
(i)	4	21.6	7.5-62.5	3.1	2.8-3.3	1.19	1.1-1.3
	5	4.0	1.0-15.6	2.4	1.8-3.1	1.37	1.2-1.6
	6	11.5	6.0-21.8	2.4	2.1-2.8	1.13	1.1-1.2
	7	10.2	5.7-18.0	2.6	2.3-2.8	1.13	1.1-1.2
	8	18.8	10.7-32.9	2.2	2.1-2.3	0.96	0.9-1.0
	9	43.1	4.2-44.4	6.7	4.7-9.6	2.98	2.6-3.4
(ii)	4	4.5	2.6- 7.7	3.1	2.9-3.3	1.66	1.5-1.8
	5	8.5	6.4-11.4	2.7	2.5-2.9	1.54	1.5-1.6
	6	8.4	6.3-11.2	2.2	2.1-2.3	1.16	1.1-1.2
	7	6.9	5.9- 8.2	2.1	2.0-2.2	1.13	1.1-1.2
	8	6.4	3.4-12.2	2.4	2.2-2.7	1.20	1.1-1.3
	9	6.1	3.8- 9.6	2.3	2.0-2.7	1.22	1.1-1.3
	10	11.1	6.7-18.4	2.1	1.9-2.2	1.14	1.1-1.2

(i) refers to the initial test carried out on 05.02.90

(ii) refers to the second test carried out on 15.02.90

#### **2.4.3 Determination of the effects of different temperatures on the response of Microtox bacteria to standard toxicants**

Vasseur et al (1986) suggest that by increasing the Microtox test temperature to 20 °C, the bacterial sensitivity is significantly enhanced for some test substances. However, Vasseur also states that in the case of volatile organic compounds such as benzene, volatilisation effects outweigh the benefits of increased sensitivity.

According to Microbics, the standard Microtox toxicity test should be performed at 15 °C. The model 500 analyser is preset to 15 °C and cannot easily be altered. It was therefore decided to standardise the test temperature at 15 °C. Microbics claim that the model 500 analyser can maintain the test temperature at 15 °C  $\pm$  0.25 °C at an ambient laboratory temperature range of between 15 and 28 °C.

Possible fluctuations in test temperature needed to be assessed in order to determine whether differences of  $\pm$  1 °C would significantly effect the test sensitivity. Using a model 2055 Microtox analyser, phenol and zinc standard toxicants were tested at 14, 15 and 16 °C with a single vial of bacteria. Tables 5 and 6 show that the EC<sub>50</sub>s at 15 minutes for phenol and 30 minutes for zinc are within the pre-defined acceptable quality control limits described in section 2.4.1.

**Table 5 - The sensitivity of Photobacterium phosphoreum to phenol at 14, 15 and 16 °C**

Test Temperature (°C)	5 min EC <sub>50</sub> (mg/l)	95% CI	15 min EC <sub>50</sub> (mg/l)	95% CI
14	16.4	11.8-22.7	20.0	18.9-21.1
15	16.5	15.6-17.6	17.6	16.4-19.0
16	22.7	21.5-24.0	24.5	23.0-26.0

**Table 6 - The sensitivity of Photobacterium phosphoreum to zinc at 14, 15 and 16 °C**

Test Temperature (°C)	5 min EC <sub>50</sub> (mg/l)	95% CI	15 min EC <sub>50</sub> (mg/l)	95% CI	30 min EC <sub>50</sub> (mg/l)	95% CI
14	33.7	14.5-78.9	3.0	2.9-3.1	1.37	1.3-1.4
15	21.7	14.8-31.7	3.2	3.0-3.5	1.37	1.3-1.4
16	13.9	7.9-24.5	2.1	2.0-2.2	1.00	0.9-1.1

#### 2.4.4 Determination of the change in bacterial sensitivity with time after reconstitution

The freeze-dried Microtox bacteria is reconstituted with distilled water in a standard cuvette and placed in a pre-cooled well, set at 5 °C. The reconstituted bacteria are maintained at 5 °C until required for testing. A single vial is sufficient for up to 8 standard tests. Microbics recommend that the bacteria should not be used for more than three hours after reconstitution. They claim that "aged" bacteria loses its test reproducibility and sensitivity. In practice, a single vial of bacteria can be completely used within three hours when a series of tests are conducted. However, an experiment was carried out to monitor bacterial sensitivity to phenol with increasing time.

Table 7 shows that five and a half hours after reconstitution, the Microtox bacteria produced results consistent with the previously identified quality control standard limits. However there did appear to be a slight trend towards increased  $EC_{50}$  with time.

Reconstituted bacteria left in the pre-cooling well overnight lose their light output almost completely.

The standard operating procedure (Appendix A) recommends that hydrated bacteria should be used within 3 hours of reconstitution for standard toxicity tests. Further work needs to be carried out over longer periods of time and with different test substances in order to confirm these initial findings and the acceptable time limit for vial use.

Table 7 - The sensitivity of Photobacterium phosphoreum to phenol with time after reconstitution

Time after reconstitution (hours)	5 min EC <sub>50</sub> (mg/l)	95% CI	15 min EC <sub>50</sub> (mg/l)	95% CI
0.5	16.6	15.5-17.8	21.1	19.5-22.8
1.5	18.5	17.3-19.7	22.8	20.9-24.9
3.0	18.5	17.5-19.6	24.3	22.7-26.0
3.5	19.1	18.1-20.1	22.8	21.6-24.1
4.5	20.9	19.6-22.3	24.9	22.1-28.1
5.5	22.4	20.3-24.7	26.9	23.6-30.7

#### 2.4.5 Colour or absorbance correction

Highly coloured aqueous test samples, particularly red or brown samples, may cause non-specific reductions in light level when analysed by the Microtox technique. These light level reductions cannot be distinguished from those caused by toxicants in the standard Microtox procedure. An absorbance correction cuvette (ACC) can be used to measure the colour interference. The results derived from the standard Microtox test can then be corrected accordingly.

Microbics state that very few effluent samples need correcting for colour. The point at which a sample needs correcting for colour appears to be very subjective and dependent on the operators' experience. Microbics do not, at present, supply a coloured standard toxicant but were considering the idea. WRc is currently testing phenol coloured with food dyes as a possible standard coloured toxicant.

## 2.5 A COMPARISON OF MICROTOX SENSITIVITY WITH SOME OTHER TOXICITY TESTS

A large data base exists in the scientific literature which compares the sensitivity of the Microtox test with other toxicity tests, such as the acute rainbow trout and Daphnia tests. The literature is currently being reviewed in order to gain a better appreciation of the comparative sensitivity of the test for a range of test substances. In using the Microtox test as a tool for ranking effluents in terms of their toxicity and for consent compliance monitoring, it will be important to have an appreciation of those chemicals for which Microtox is not particularly sensitive.

To add to the database on comparative sensitivity we are attempting to use Microtox alongside other tests being done on other research projects. So far the Microtox test has been carried out in parallel with the oyster embryo-larval, turbot and brown shrimp tests. The inter-test comparisons are shown in Table 8. From this limited number of tests it can be seen that the Microtox test was consistently less sensitive than the oyster embryo-larval test by approximately one order of magnitude. Both the shrimp and turbot acute toxicity tests produced similar results to the Microtox test for the gas plant effluent.

**Table 8 - A comparison of the Microtox test with oyster embryo-larval, shrimp and turbot toxicity tests**

Test substance	BOD (mg/l)	Microtox (bacteria) 15 min EC <sub>50</sub>	Oyster (embryo) 24 hour EC <sub>50</sub>	Shrimp (adult) 96 hour LC <sub>50</sub>	Turbot (juveniles) 96 hour LC <sub>50</sub>
Gas plant effluent - (filtered)		79	>20	-	-
Gas plant effluent - (unfiltered)		26	-	21	15
Domestic sewage	510	4	0.5	-	-
Mixed domestic/ industrial sewage	26	57	5.5	-	-
Mixed domestic/ industrial sewage	112	111	5.7	-	-

EC<sub>50</sub> values shown in Table 8 are expressed as % effluent in the test diluent (seawater or sodium chloride solution).

### SECTION 3 - CASE-STUDY PROGRAMME

#### 3.1 INTRODUCTION

As stated earlier in Section 1, the objective of this area of the Environmental Research Programme is to test, by means of case-studies, the protocol for discharge control by DTA proposed by WRc (Hunt 1989). It is intended that this protocol be addressed in three ways:

- (i) By screening exercises, in order to evaluate the role of Microtox in identifying discharges suitable for control by DTA;
- (ii) By more detailed case-studies on the toxicity of individual discharges in order to test the protocol relating to the establishment of full Toxicity-Based Consents (TBCs);

(iii) By assessments of the efficacy of the TBCs

- do they help regulatory authorities detect failure to comply with discharge consents?
- do they help pollution control officers do their job better?
- does the receiving environment improve when TBCs are applied?

It has been agreed, during discussions with the relevant Research Programme Project Leaders, that parts (i) and (ii) would be co-ordinated, and largely performed by WRc, whilst part (iii) would be performed by NRA Welsh Region, who already have a programme of including a Microtox component in discharge consents.

### 3.2 IDENTIFICATION OF SUITABLE CASE-STUDY SITES

Discussions at the Bisham Abbey workshop on the selection of suitable case-study sites led to agreement on the following:

- (i) Screening studies - that this procedure should be tested on a number of fairly small, well defined and characterised catchments (both rivers and estuaries) having a number of industrial inputs. Complex catchments should be avoided.
- (ii) Individual discharge studies - that a number of case-studies be established to investigate the DTA approach for discharges to both freshwater and marine environments.
- (iii) Discharge types - that the programme should incorporate as wide a range of effluent types as possible, preferably including representatives from the following industries:

- petrochemical
- chemical manufacturers
- pharmaceutical
- pesticide manufacturers/formulators
- paper
- textile/dye
- steel works
- sewage treatment works with a significant industrial component

(iv) Chemical analysis - that this should be performed on the effluents involved in order that the DTA approach to discharge control could be compared and contrasted with the existing chemical-specific approach.

Following the workshop, representatives from each of the NRA regions, the Scottish RPBs and DoE Northern Ireland were requested to nominate suitable case-study sites for both the screening and individual discharge studies. The nominations received are shown in Tables 9 and 10 for screening and individual discharge studies respectively. A short-list was then drawn-up and further information requested, in the form of questionnaires, from the regions concerned in order that final site selection could occur. The information requested will also be of use for the actual case-studies themselves. The questionnaires were compiled in collaboration with NRA Welsh Region and are given in Appendix B.

At present two case-studies have been identified:

(i) Irvine Bay, proposed by Clyde RPB for a marine screening study.

There are 15 direct discharges into the Bay, including industrial inputs (chemical and pharmaceutical), domestic sewage and sewage discharges with significant trade components, and two major estuaries. This therefore represents a manageable-sized screening case-study. As CRPB have already screened most of the

discharges for their acute toxicity using selected marine species (e.g. brown shrimp, scallops and turbot), a good opportunity exists for the comparison of sensitivity with Microtox. The chemical composition of the effluents are also well documented.

- (ii) Paper Mill discharging into the Svale Estuary, proposed by NRA Southern Region for an individual discharge study.

The mill continuously produces paper for corrugating and packaging and also produces paper pulp from wood chips using a semi-chemical process. The paper manufacturing effluent is treated by screening, flow balancing, chemical flocculation and settlement. The pulp waste is treated by solids removal using fine screens and by desludging in a holding tank. Thus there are two process effluents being discharged both of which have failed to comply with consent limits for BOD and suspended solids (SS) in the past. Substantial improvements to treatment facilities are being commissioned and effluent quality is apparently improving. The environmental impact of the discharges has been investigated previously by Southern Water. This will provide useful information for the case-study which will involve assessments of the effluents' toxicities using a range of relevant marine species, their sensitivities being calibrated against that of Microtox. The high BOD and SS loadings of the discharges will provide valuable experience in testing effluents of this kind.

Work on the Irvine Bay study is scheduled to begin in the first week of March. Investigations on the paper mill will follow. Discussions for the selection of further case-studies, particularly for freshwater environments, are in progress.

TABLE 9. TOXICITY BASED CONSENTS - PROPOSED CASE-STUDY SITES - SCREENING

REGULATOR	DISCHARGE TYPE											
	petro-chemical	chemical	pharmaceutical	pesticide	paper mill	textile /dye	steelworks	mine	stw	tannery	airport drainage	refuse tip
SOUTH-WEST:												
R Cula					+				+			
Red River								+	+			
Taw		●							●			
YORKSHIRE												
R Aire									+			
R Calder								+				
R Rother		+										
R Wharfe					+							
SEVERN-TRENT												
R Erewash						+	+	+	+			
WELSH												
Dee Estuary												
NORTHUMBRIAN												
Tees Estuary	●	●	●				●			●		
SOUTHERN												
Southampton Water	●	●							●			
NORTH EAST RPB												
R Don					+						+	
DOE (NI)												
R Lagan		●					●		●			●

stw - sewage treatment works

+ - discharge to freshwater

RPB - River Purification Board

● - discharge to estuary

TABLE 10. TOXICITY BASED CONSENTS - PROPOSED CASE-STUDY SITES - INDIVIDUAL DISCHARGES

REGULATOR	DISCHARGE TYPE						
	petro-chemical	chemical	pharma-ceutical	pesticide	paper mill	textile /dye	stw
SOUTH-WEST		●		⊕	⊕	⊕	●
YORKSHIRE		⊕			⊕		⊕
SEVERN-TRENT						⊕	
WELSH		●					
NORTHUMBRIAN	●	●	●			●	●
SOUTHERN	●				●		
WESSEX		●	●				
THAMES							●⊕
TAY RPB			●				
NORTH EAST RPB	⊕				⊕		
FORTH RPB		●					
HIGHLAND RPB		●					●
TWEED RPB							⊕
DOE (NI)						⊕	

stw - sewage treatment works

RPB - River Purification Board

⊕ - discharge to freshwater

● - discharge to estuary

## REFERENCES

HAIG A J N, CURRAN J C, REDSHAW C J and KERR R Use of mixing zone to derive a toxicity test condition. Journal of the Institution of Water and Environmental Management (Accepted for publication, 1989).

HUNT D T E H (1989) Discharge control by Direct Toxicity Assessment (DTA) - Interim Protocol. Water Research Centre Report No. PRS 2160-M/1.

MACKAY D W, HOLMES P J and REDSHAW C J The application of bioassay techniques to water pollution problems - the United Kingdom experience. Hydrobiologia, (Accepted for publication, 1989).

US ENVIRONMENTAL PROTECTION AGENCY (1984) Development of Water Quality-Based Permit Limitations for Toxic Pollutants: National Policy. Federal Register, 49(48), 9016-9019.

VASSEUR P, BOIS F, FERARD J F AND RAST C. (1986) Influence of physicochemical parameters on the microtox test response. Toxicity assessment: An International quarterly vol. 1, 283-300. John Wiley and Sons, Inc.

WALKER J D (1988) Relative sensitivity of algae, bacteria, invertebrates and fish to phenol: analysis of 234 tests conducted for more than 149 species. Toxicity assessment: An International Journal vol. 3, 415-447 (1988). John Wiley and Sons, Inc.

WALL T M and HANMER R W (1987) Biological testing to control toxic water pollutants. Journal of the Water Pollution Control Federation, 59(1), 7-12.

**APPENDIX A - A DRAFT STANDARD OPERATING PROCEDURE**

DETERMINATION OF THE  $EC_{50}$  OF TEST SUBSTANCES TO THE

MICROTOX REAGENT PHOTOBACTERIUM PHOSPHOREUM

R BUTLER

FEBRUARY 1990

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## SECTION 1 - INTRODUCTION

The Microtox toxicity test is a technique which can be used to determine the toxicity of aquatic contaminants to the marine bacterium Photobacterium phosphoreum. It is an extremely simple, cost effective and rapid test for screening potentially toxic chemicals before they are disposed of to the aquatic environment. It can also be used routinely to monitor the environmental impact of contaminants released into coastal, estuarine or river water bodies.

P.phosphoreum is a bioluminescent bacterium common in marine waters. The test organisms are cultured, harvested and freeze-dried under carefully controlled conditions by Microbics Corporation. The Microtox system determines the mean toxic response of approximately one million bioluminescent organisms. The use of such a large population gives the Microtox results very high resolution and statistical significance, compared with those obtained from relatively small populations, such as those used in fish or invertebrate studies.

The bioluminescence of Microtox bacteria has been shown to be a reasonably sensitive measure of toxicity broadly equivalent to acute fish and Daphnia toxicity tests (Walker 1988).

The Microtox Analyser utilises a photomultiplier to measure the light output of the luminescent bacteria before and after exposure to toxicants.

## SECTION 2 - PRINCIPLE

By determining changes in the bioluminescent output of the Microtox bacteria in duplicate test concentrations compared with control solutions, the 15 minute median effect concentration ( $EC_{50}$ ) can be calculated using appropriate statistical techniques.

The  $EC_{50}$  is defined as the concentration which results in a 50% reduction in Microtox bacterial light output after a 15 minute exposure at 15 °C compared with the control.

### SECTION 3 - SCOPE AND LIMITATIONS

#### 3.1 SCOPE OF THE MICROTOX TOXICITY TECHNIQUE

- i) The Microtox system can be used to monitor pollution events, on site, as and when they occur. Being an extremely rapid test, data can be generated for a pollution event within hours as opposed to days or weeks for the majority of other biological toxicity tests.
- ii) The toxicity and environmental impact of complex industrial effluents can be monitored.
- iii) The potential hazard of new chemicals can be determined.
- iv) The synergism and antagonism of toxicant mixtures can be studied.
- v) The exposure time of test substances to an organism is a critical toxicity test parameter. When the toxic response is increasingly significant with time, the Microtox test may easily be extended in time whereas other tests may not, and therefore may achieve enhanced sensitivity. This is because light output rate is the test endpoint, rather than death as in other acute toxicity tests. Some toxic responses are virtually instantaneous, some are essentially complete after ten to thirty minutes, but a few, notably bivalent metals, show increasing toxicity beyond thirty minutes.

- vi) Since Microtox bacteria can be held in the laboratory in a freeze-dried state, the test can be performed at any time of the year and at short notice. Other toxicity tests may require expensive holding facilities, be possible only at certain times of the year and require several weeks notice before testing.

### 3.2 LIMITATIONS

- i) The sensitivity of the Microtox toxicity test is broadly equivalent to the sensitivity of other acute toxicity tests such as 96 hour fish and Daphnia studies. However, lower levels of toxicity, harmful to sensitive aquatic organisms such as early life history stages of bivalve molluscs may not be detected by the Microtox system.
- ii) In order to fully define the hazard of an aquatic contaminant, a suite of toxicity tests should be performed. The Microtox results cannot be used in isolation to predict environmental impact except in the crudest terms.
- iii) The Microtox bacteria are of marine origin and therefore freshwater test samples require salinity adjustment. The addition of sodium chloride may affect the sample toxicity or form precipitates. Precipitate formation may result in sample turbidity and hence the need for centrifugation or absorption correction.
- iv) Hormesis, the stimulation of increased light output at low levels of toxicants, and nutrient enhanced light output cannot be distinguished.

## SECTION 4 - INFORMATION REQUIRED ON TEST SUBSTANCES

The Microtox toxicity test can be used to determine the toxicity of a wide range of substances from pure compounds (where stock solutions may need to be prepared) to complex effluents and sediment extracts. This section details the information required for the three major categories of test substances analysed using the Microtox toxicity test.

### 4.1 PURE TEST SUBSTANCES

#### 4.1.1 Essential information required

- i) Solubility of the test substance in water, or if insoluble, the name of any appropriate solvents.
- ii) Stability under test conditions.
- iii) Any hazards associated with the test substance.
- iv) Relevant analytical techniques.
- v) If the test substance is a formulated product, then the other components should be listed and the percentage activities given.
- vi) The pH and salinity of resulting test solutions.

#### 4.1.2 Useful information required

- i) Name.
- ii) Molecular weight.
- iii) Structural/Empirical formula.
- iv) Percentage purity and nature of impurities.

## **4.2 ENVIRONMENTAL SAMPLES**

This group includes influents, effluents and water samples.

### **4.2.1 Essential information required**

- i) pH and salinity.

### **4.2.2 Useful information required**

- i) The type and quantity of contaminants likely to be found in the sample.
- ii) Analytical techniques and hazards associated with any identified contaminants.

## **4.3 SEDIMENT EXTRACTS**

Sediment toxicity may be determined by conducting the Microtox test on aqueous or solvent extracts of the sediment sample.

### **4.3.1 Essential information required**

- i) pH and salinity of the sediment extract.

### **4.3.2 Useful information required**

- i) The organic carbon content of the sediment.
- ii) The particle size distribution of the sediment.
- iii) The water content of the sediment.

## SECTION 5 - APPARATUS

### 5.1 GLASSWARE

- i) Microtox standard disposable cuvettes as supplied by Microbics Corporation. These are supplied free from contamination and should be handled as little as possible and without touching the lower surface, where light transmission is important. They should be covered at all times during storage to avoid dust and bacterial contamination.
- ii) Microtox Absorbance Correction Cuvettes (ACC) are special double chamber cuvettes required only when testing highly coloured samples (Ref 7.3).
- iii) Volumetric flasks for preparing toxicant stock solutions.
- iv) Graduated cylinders for performing initial dilutions (10 ml).
- v) Disposable vessels (30 ml) for sample storage after initial dilution.
- vi) Sample collection bottles. Borosilicate glass containers (250-500 ml) with Teflon inserts in the caps, are required for sample collection and storage.

### 5.2 CHEMICALS, REAGENTS AND SOLUTIONS

- i) Microtox Reagent. Each vacuum-sealed vial of reagent contains approximately one hundred million freeze-dried bacteria. It should be stored at -20 °C in a freezer (not self-defrosting, which defrosts by warming up periodically). The bacteria is a common marine species,

non-pathogenic to mammals and can be disposed of by dilution with tap water (causing the cells to lyse) and flushing it down a normal laboratory sink. Dates of receipt and opening should be recorded on each batch of reagent. The vial and lot numbers should also be recorded.

(For information of the optimal use of the Microtox Reagent refer to Section 8.)

- ii) Microtox Reconstitution Solution. This is distilled water, supplied by Microbics Corporation with each batch of Microtox Reagent, and should be used to reconstitute the Microtox Reagent. It should be stored in a refrigerator at  $4 \pm 2$  °C (and not frozen). It is stable for one year. Date of receipt and opening should be recorded on each bottle.
- iii) Microtox Osmotic Adjustment Solution (MOAS). This is a solution of 22% sodium chloride in distilled water and is used to adjust the salinity of a sample. It should be stored in a borosilicate glass container in a refrigerator at  $4 \pm 2$  °C. This solution can either be obtained from Microbics Corporation or prepared as 22 g NaCl per 100 mls of double distilled water.
- iv) Standard Microtox Diluent. This is a solution of 2% sodium chloride in distilled water. The diluent is used to prepare dilutions of the test sample and of the reconstituted Microtox Reagent before testing. It should also be stored in a glass borosilicate container in a refrigerator at  $4 \pm 2$  °C. Marine test samples require the preparation of  $3.4 \pm 0.1\%$  NaCl in distilled water (Ref 7.2.2) although salinities of up 4.0 % NaCl could be used if necessary.
- v) Analytical grade (Normal) hydrochloric acid for acid cleaning sample bottles and reducing the pH of alkaline test samples.

- vi) Analytical grade (Normal) sodium hydroxide for increasing the pH of acidic test samples.
- vii) Double distilled or Reverse Osmosis (Polished) water for preparing Microtox diluents, MOAS and stock solutions of some test substances.
- viii) Aristar grade sodium chloride for preparing Microtox diluents and MOAS.
- ix) Detergent for preliminary washing of sample bottles (DECON 90).
- x) Deionised water for rinsing sample bottles following acid cleaning.
- xi) Aristar grade phenol and zinc sulphate for use as standard toxicants (Ref 8).

### 5.3 EQUIPMENT

- i) Microtox analyser (Model 500).
- ii) Chart recorder with a paper speed of 1 cm per minute and a sensitivity of 1 volt / cm.
- iii) Micropipetters capable of accurately dispensing 10, 250, 500 and 1000 microlitre volumes.
- iv) Non-toxic disposable tips for each micropipetter.
- v) pH meter with a robust small volume electrode.
- vi) Salinometer, refractometer or conductivity meter.
- vii) Non-self defrosting freezer at -20 °C.

- viii) Refrigerator set at  $4 \pm 2$  °C.
- ix) Centrifuge capable of 10,000 G.
- x) IBM compatible personal computer, preferably portable so that work can be carried out at remote sites.
- xi) Printer to connect to the personal computer.

## SECTION 6 - COLLECTION OF SAMPLES

### 6.1 SAMPLE BOTTLES

250-500 ml borosilicate glass containers with Teflon inserts in caps. (e.g. Red top, Schott bottles).

### 6.2 SAMPLE COLLECTION

Sample bottles should be filled to minimise volatilisation and evaporation. All samples are collected in duplicate in case of breakage or leakage. All samples must be labelled with site (name, number or code), date and time of collection.

### 6.3 SAMPLE STORAGE

Purified compounds as well as complex effluents may either gain or lose toxic components during storage due to evaporation, sorption effects, biodegradation and/or chemical reactions. Therefore, it is recommended that samples be tested as soon as possible after collection. To minimise losses during storage, samples should be maintained in a refrigerator at  $4 \pm 2$  °C.

### 6.4 CLEANING SAMPLE BOTTLES

- i) Soak all sample bottles and tops in a 2% solution of Decon 90 for at least 30 minutes.

- ii) Hand brush and rinse in tap water.
- iii) Rinse once in deionised water.
- iv) Soak in a solution of 10% hydrochloric acid for 24 hours.
- v) Rinse twice in deionised water.
- vi) Oven dry.

## SECTION 7 - EXPERIMENTAL PROCEDURES

### 7.1 ROUTINE SCREENING TECHNIQUE

A screening test to determine approximate toxicity of test substances is carried out prior to a standard Microtox test, using concentrations of 9.1 and 45.5%. By testing two concentrations an approximate  $EC_{50}$  can be calculated, providing information on the dilution series required for a standard test.

#### 7.1.1 Instrument and sample preparation

- i) Add 1.0 ml reconstitution solution to a cuvette in the reagent well (G).
- ii) Add 2 ml Microtox diluent to cuvettes A1 and A2.
- iii) Add 0.5 ml Microtox diluent to cuvettes B1, B2, B3, C1, C2 and C3.
- iv) Add 2.5 ml of test substance to cuvette A3 and 250  $\mu$ l of MOAS in order to adjust the salinity (see section 7.2.2).
- v) Allow 5 minutes for the solutions to achieve thermal equilibrium.

### 7.1.2 Reagent preparation

- i) Reconstitute a vial of Microtox reagent (see section 7.2.3).
- ii) Transfer 10  $\mu$ l of reconstituted reagent to B1, B2, B3, C1, C2 and C3 and mix by aspirating and dispensing 250  $\mu$ l, 5 times.

### 7.1.3 Screening procedure

- i) Transfer cuvette B1 into read well H.
- ii) Press the SET button and wait for the green ready light.
- iii) Press the READ button and record the initial light level (I0) for cuvette B1.
- iv) READ light levels for the remaining test cuvettes in the following order: C1, B2, C2, B3 and C3.
- v) READ I5 and I15 light levels, by cycling the cuvettes in the previous order, five minutes after taking the first initial light reading.
- vi) Calculate gamma values for each sample (see section 9.2.2).

## 7.2 STANDARD MICROTOX TOXICITY TEST

### 7.2.1 Analyser preparation

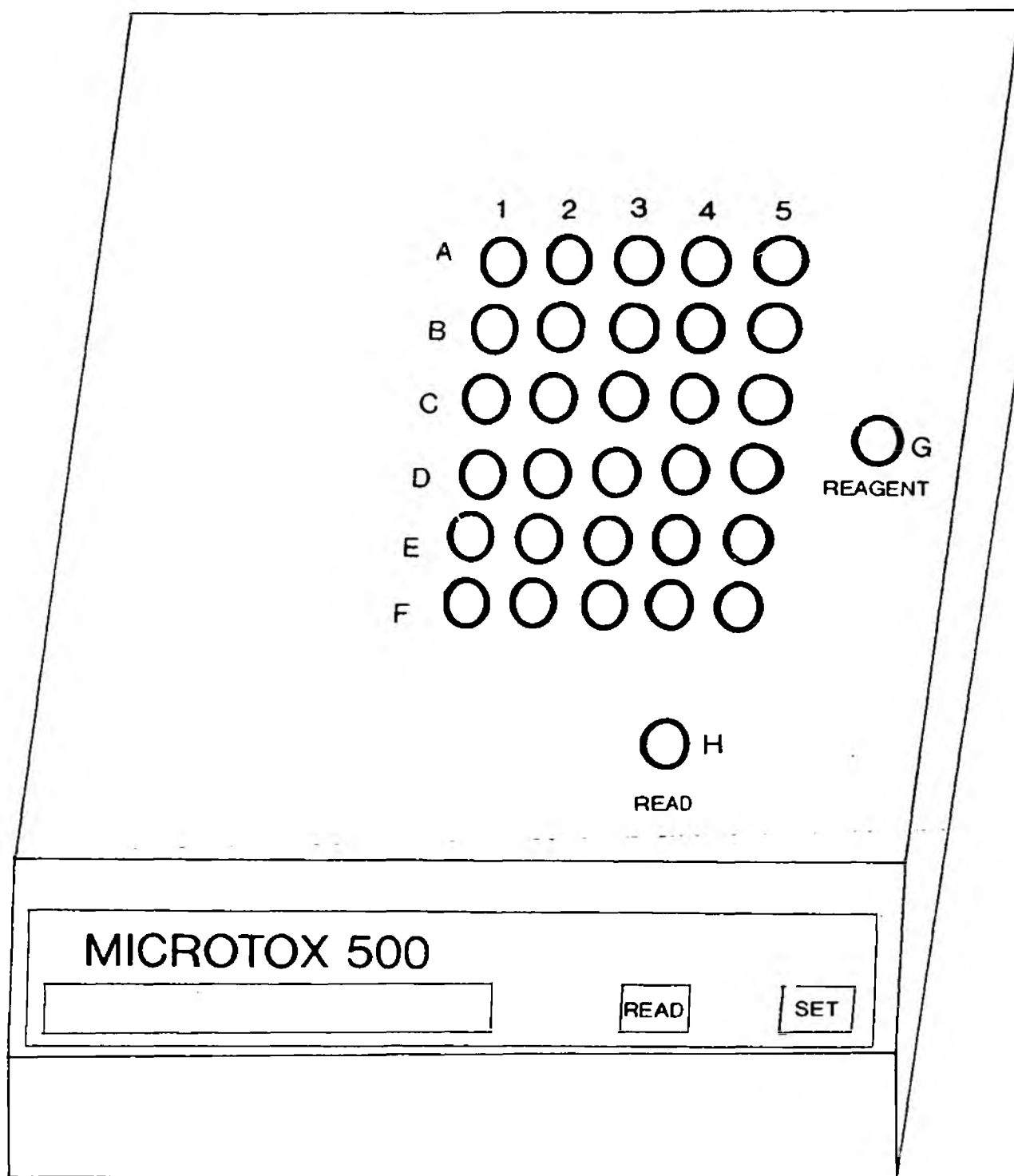
- i) Verify analyser readiness. When switched on, the analyser brings the reagent, incubator and turret wells to preset temperatures. The "temperature warning indicator light" switches off once the wells have reached the desired temperatures. No adjustment or calibration is required.

- ii) Fill all wells (shown in figure 1), except read well (H), with clean empty cuvettes. New unused cuvettes should be used for the standard Microtox test since the risk of cuvette contamination and cost to clean them outweighs the cost of using new cuvettes for each test.
- iii) Add 1.0 ml Reconstitution Solution to the cuvette in precooling well G.
- iv) Add 1.5 ml Microtox Diluent to: A1, A2, A3, A4.
- v) Add 500  $\mu$ l Microtox Diluent to: B1, B2, B3, B4, B5, C1, C2, C3, C4, C5.

#### 7.2.2 Sample preparation

- i) Sample turbidity interferes with the Microtox toxicity determination. To avoid absorbance correction, all samples should be centrifuged at between 8000 and 10000 G for 15 minutes or until a clear supernatant is achieved.
- ii) Visually inspect the test sample to determine if absorbance correction (colour correction) procedure is necessary (see section 7.3).
- iii) Measure the salinity of the sample. Ideally a good quality bench top salinometer should be used, although a refractometer or conductivity meter can be used to determine sample salinity. The salinity of freshwater samples is adjusted by adding 250  $\mu$ l MOAS to 2.5 mls sample in cuvette A5. Mix by aspirating and dispensing 500  $\mu$ l of the sample, five times. The sample salinity is now 20‰ (or 2% NaCl) and Standard Microtox Diluent should be used,

Figure 1. The well positions of the Model 500 Microtox analyser



prepared as a 2% NaCl solution in distilled water. If the sample is saline, the Microtox diluent should be prepared as  $3.4 \pm 1\%$  NaCl (i.e. 3.4 g NaCl in 100 ml distilled water).

When a sample has been osmotically adjusted with MOAS a dilution of 10/11 occurs. The original concentration of the sample must be multiplied by 0.9091 in order to obtain the actual sample concentration after osmotic adjustment.

- iv) Measure the sample pH. If the pH value lies outside the range 6 to 8, then it should be adjusted to  $7 \pm 1$  using either sodium hydroxide for acidic samples or hydrochloric acid for alkaline samples. Providing that the volumetric change after adjustment is  $< 1\%$  of sample no correction need be made to the assumed dilution series concentrations.
- v) Following the prescreening test (Ref 7.1), primary sample dilution may be required (i.e. toxic samples will need diluting before addition to the test cuvettes).
- vi) Transfer 1.5 ml of the osmotically adjusted sample in A5 to A4.
- vii) Mix A4 by aspirating and dispensing 500  $\mu$ l, 5 times.
- viii) Transfer 1.5 ml from A4 to A3.
- ix) Mix A3 by aspirating and dispensing 500  $\mu$ l, 5 times.
- x) Transfer 1.5 ml from A3 to A2.
- xi) Mix A2 by aspirating and dispensing 500  $\mu$ l, 5 times.

These serial dilutions result in the following concentrations of the original sample (providing no primary dilution has been made):

prepared as a 2% NaCl solution in distilled water. If the sample is saline, the Microtox diluent should be prepared as  $3.4 \pm 1\%$  NaCl (i.e. 3.4 g NaCl is 100 ml distilled water).

When a sample has been osmotically adjusted with MOAS a dilution of 10/11 occurs. The original concentration of the sample must be multiplied by 0.9091 in order to obtain the actual sample concentration after osmotic adjustment.

- iv) Measure the sample pH. If the pH value lies outside the range 6 to 8, then it should be adjusted to  $7 \pm 1$  using either sodium hydroxide for acidic samples or hydrochloric acid for alkaline samples. Providing that the volumetric change after adjustment is  $< 1\%$  of sample no correction need be made to the assumed dilution series concentrations.
- v) Following the prescreening test (Ref 7.1), primary sample dilution may be required (i.e. toxic samples will need diluting before addition to the test cuvettes).
- vi) Transfer 1.5 ml of the osmotically adjusted sample in A5 to A4.
- vii) Mix A4 by aspirating and dispensing 500  $\mu$ l, 5 times.
- viii) Transfer 1.5 ml from A4 to A3.
- ix) Mix A3 by aspirating and dispensing 500  $\mu$ l, 5 times.
- x) Transfer 1.5 ml from A3 to A2.
- xi) Mix A2 by aspirating and dispensing 500  $\mu$ l, 5 times.

These serial dilutions result in the following concentrations of the original sample (providing no primary dilution has been made):

A5	=	91%	Sample in diluent
A4	=	45.5%	" " "
A3	=	22.75%	" " "
A2	=	11.4%	" " "

xii) Allow 5 minutes for temperature equilibration.

### 7.2.3 Reagent preparation

#### (a) Reconstitute the Microtox Reagent (bacteria)

- i) Remove a single vial of reagent from the freezer.
- ii) Open the vial, without too much handling, to minimise warming of the vial.
- iii) The vials are vacuum sealed and when opened a distinct hiss should be heard as the air rushes into the vial. If no sound is heard the reconstituted reagent should be immediately checked for light output since lost vacuum implies that the reagent is unfit for testing.
- iv) Shake and tap the vial gently to ensure the pellet of reagent is seated on the bottom of the vial.
- v) Take the precooled cuvette of reconstitution solution from well G and quickly pour the solution into the opened vial.
- vi) Swirl the vial 3 or 4 times quickly, pour the mixture back into the cuvette and return it to well G.
- vii) Mix the reagent thoroughly with a 500  $\mu$ l pipette by aspirating and dispensing, 20 times. Reconstituted reagent should not be used for longer than 3 hours. Further tests after this period require freshly reconstituted reagent.
- viii) Transfer 10  $\mu$ l reconstituted reagent to:

B1, B2, B3, B4, B5

C1, C2, C3, C4, C5

- ix) Mix the contents of each cuvette with a 250  $\mu$ l pipette by aspirating and dispensing 250  $\mu$ l, 5 times.
- x) Allow 15 minutes for reagent stabilisation.

#### 7.2.4 Test procedure

- i) If a computer is part of the Microtox system, follow the start up procedure specified for the software.
- ii) Start the chart recorder; set at 1 cm per minute.
- iii) Place cuvette B1 into Read Well H. Press the SET button and wait for the green ready light to come on. The model 500 analyser automatically sets the light level to 90.
- iv) Press the READ button in order to obtain the initial light level IO for cuvette B1.
- v) Cycle the remaining cuvettes in the following order to obtain IO light levels:

C1, B2, C2, B3, C3, B4, C5, B5 C5.

- vi) If any of the cuvettes read over 100 reset the light reading to 90 by pressing the SET button again and waiting for the green ready light to come on. The initial light levels must be measured again, from B1, if the light level has been reset.
- vii) Immediately following the IO measurements:

Transfer 500 µl from A1 to each of B1 and C1 (and mix five times)											
"	"	"	"	A2	"	"	"	B2 and C2	"	"	"
"	"	"	"	A3	"	"	"	B3 and C3	"	"	"
"	"	"	"	A4	"	"	"	B4 and C4	"	"	"
"	"	"	"	A5	"	"	"	B5 and C5	"	"	"

viii) Five minutes after the first solution transfer (A1 to B1), cycle the cuvettes in the following order to obtain I5 light levels:

B1, C1, B2, C2, B3, C3, B4, C4, B5, C5.

ix) Repeat this procedure at 15 minutes to obtain I15 light levels.

x) Tabulate the data and calculate gamma and EC<sub>50</sub> values (see sections 9.1 and 9.2).

### 7.3 ABSORBANCE CORRECTION MEASUREMENT

Highly coloured aqueous samples, particularly those that are red or brown, may cause non-specific reductions in light level when analysed according to the standard Microtox technique. These light level reductions cannot be distinguished from those caused by toxicants in the standard Microtox procedure. An Absorbance Correction Cuvette (ACC) can be used to measure a samples colour interference and the results derived from the standard Microtox test can then be corrected accordingly.

Immediately after performing the standard toxicity test, one or all of the sample dilutions can be colour-corrected using the following procedure. It is most appropriate to colour-correct the concentration closest to the 15 minute EC<sub>50</sub> value.

- i) Pipette 1.5 ml of Microtox Diluent into the outer chamber of a clean ACC and place it in Read well H.
- ii) Pipette 1 ml of Microtox Diluent into a standard cuvette and place it in incubator well A1.
- iii) Pipette 2 ml of the chosen sample, normally at the  $EC_{50}$ , into each of 2 standard cuvettes and place them in incubator wells C1 and C2.
- iv) Wait five minutes for all solutions to achieve thermal equilibrium.
- v) Pipette 50  $\mu$ l of reconstituted reagent into cuvette A1. Mix by aspirating and dispensing 500  $\mu$ l five times.
- vi) Lift the ACC from the Read well and transfer sufficient cell suspension from cuvette A1 into the inner chamber of the ACC to provide a level equivalent to that of diluent in the outer chamber.

\* Avoid air bubbles in the reagent in the ACC chamber.

- vii) Immediately return the ACC to the turret well to minimise warming.
- viii) Press the SET button and wait for the green ready light.
- ix) Press the READ button and record the first blank light level ( $B_0$ ).

\* Do not move or rotate the ACC until the entire colour correction procedure has been completed.

- x) Use a disposable plastic pipette to remove as much Microtox Diluent as possible from the outer chamber.

- xi) Transfer 0.5-1.5 ml of test sample from cuvette C1 into the outer chamber of the ACC. Either a pipette or an aspirator may be used for this transfer.

\* It is important not to contaminate the reagent in the inner chamber with sample as this would cause a toxic response and invalidate the colour correction.

- xii) Five minutes after taking the initial light reading, press the READ button and record I5.
- xiii) Remove as much sample as possible from the outer chamber.
- xiv) Pipette 1.5 ml of Microtox Diluent into the outer chamber of the ACC.
- xv) Five minutes after the sample reading, press the READ button and record the second blank light level.
- xvi) The sample concentration, C0, and the three light readings are required in order to calculate the colour correction (see section 9.2).

#### SECTION 8 - QUALITY CONTROL CONSIDERATIONS

- i) The sensitivity of the Microtox Reagent to standard toxicants needs to be monitored at regular periods to ensure consistency and reproducibility of the Microtox technique. Microbics (1988) recommend phenol and zinc sulphate as acceptable positive control substances. The approximate  $EC_{50}$  concentrations for these compounds are (for a temperature of 15 °C):

Phenol	15-28 mg phenol/l 5 min
Zinc sulphate	0.95-1.5 mg Zn/l 30 min

These are nominal values, and the  $EC_{50}$  will also depend upon chemical source, purity, age, stability in solution and storage conditions. It is therefore recommended to use aristar grade test substances, stored at ambient laboratory temperature. Stock solutions should be prepared freshly for each test as 1000 mg/l of test substance in distilled water and diluted down with distilled water to give initial upper test concentrations of 45.5 mg Phenol/l and 9.1 mgZn/l.

ii) For optimum reagent sensitivity and precision the following guidelines should be observed:

- a) store unopened bacterial reagent at  $-20^{\circ}\text{C}$  in a non-cycling freezer.
- b) The reagent should be used within one year of the manufacturing date printed on the vial.
- c) The hydrated reagent should be used within 3 hours following reconstitution.
- d) New, unused cuvettes should be used once for a standard Microtox test and then discarded.
- e) Carefully monitor the test temperature, which should be  $15 \pm 0.25^{\circ}\text{C}$  as standard.
- f) Carefully monitor the temperature achieved in the precooling well G which should be  $5 \pm 1^{\circ}\text{C}$ .

## SECTION 9 - DATA HANDLING

### 9.1 RECORDING RAW DATA

Hard copies of data generated by the Microtox test must be produced and filed as chart recordings and or computer print-outs. It is difficult to record values of light output during a standard Microtox test, by

hand and this is not recommended. At the end of the test, data should be transferred to the data sheets (Tables A1 and A2).

## 9.2 TREATMENT OF RESULTS

Computer software is available from Microbics which enables direct data capture from the Microtox analyser. The menu driven software can be used to calculate  $EC_{50}$  values with 95% confidence limits. This section describes the manual calculation of blank ratios, gammas and  $EC_{50}$  values.

### 9.2.1 Calculating the blank ratio ( $R_t$ ) and normalising light loss

The light output of the reagent diminishes with time, even in the absence of toxic contamination. A control or blank is therefore included in every Microtox test in order to determine this light loss.

The blank ratio at time  $t$  ( $R_t$ ), is defined by the formula:

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

where  $B_t$  = the sum of the final light readings ( $t=5$  or  $15$ ), for the duplicate blank cuvettes.

$B_o$  = the sum of the initial light readings ( $t=0$ ), for the duplicate blank cuvettes.

The response observed for each test substance cuvette is normalised against the blank response by multiplying the initial light output of each cuvette by the blank ratio for time  $t$ ,  $R_t$ . This product ( $R_t I_o$ ) is the light output expected at time  $t$  for a particular cuvette if it had been used as a blank.

### 9.2.2 Calculating gamma, the ratio between light lost due to toxicity and remaining light at a particular time

The toxic response of a test substance to the Microtox reagent is calculated using the actual final light reading and the light reading expected for a non-toxic sample ( $RtIo$ ), both at time  $t$ . This method corrects for the effects of both light drift and the offset in light output due to the dilution which occurs when the organisms are exposed to the test substance dilution after the initial light levels have been recorded.

The normalised gamma effect is calculated for each cuvette using the equation:

$$Gt = (Rt.Io/It)-1$$

where  $Io$  = the initial light reading for any given cuvette at time zero, before the addition of any test substance.

$It$  = the final light reading for the corresponding test cuvette at time( $t$ ).

$Rt$  = the blank ratio for time ( $t$ ).

$Gt$  = the normalised gamma effect for exposure time ( $t$ ).

### 9.2.3 Determination of the $EC_{50}$

The  $EC_{50}$  can be determined graphically by plotting gamma values against the associated concentrations on log:log graph paper. A dose-response curve can then be fitted to the data points. The concentration at which the line crosses 1.0 on the vertical scale is the  $EC_{50}$ . A gamma of 1 is the concentration of a sample which causes the bacterial light output to be reduced by 50%. Since the dose-response is typically linear, the line may be extrapolated to a gamma of 1 should all the data points be above or below this value.

The most appropriate method of calculating the 95% confidence intervals associated with the  $EC_{50}$  is currently being considered by statisticians at WRc.

#### 9.2.4 Determination of absorbance correction for coloured samples

The colour correction procedure determines the difference between the bacterial light ( $I_f$ ) penetrating a selected concentration ( $C_o$ ) of a coloured sample and a control (or blank) without colour ( $I_o$ ). The absorbance ( $A$ ) of a sample is calculated using:

$$A = (EC_{50}/C_o) (3.1 \ln(I_o/I_f))$$

The constant 3.1 has been derived by Microbics and corrects for the geometry of the cuvettes and photodetector. The contribution to colour absorbance ( $A_x$ ) can be calculated for each test concentration ( $C$ ) of interest using:

$$A_x = (C/C_o).A$$

Transmittance ( $T_x$ ) is calculated for each test concentration using:

$$T_x = \frac{1 - e^{-A_x}}{A_x}$$

The corrected gamma ( $G_c$ ) is calculated using:

$$G_c = T_x(1+G) - 1$$

where  $G$  is the observed effect, including both colour and toxicity effects. The corrected gamma values can then be used to recalculate  $EC_{50}$  values as described in section 9.2.3.

## REFERENCES

CASLER L (1982) Microtox System Operating Manual. Beckman Instruments Inc, Microbics Operations, Carlsbad, CA 92008, USA.

MICROBICS CORPORATION (1988) How to run a standard microtox test.  
Appendix Table A1. Microtox Data Sheet.

WALKER J D (1988) Relative sensitivity of algae, bacteria, invertebrates and fish to phenol: analysis of 234 tests conducted for more than 149 species.  
Toxicity assessment: An International Journal 3, 415-447 (1988) John Wiley and Sons, Inc.

Table A1 - Standard Microtox test data sheet 1

Sample name/reference: \_\_\_\_\_

Sample Collection ( or preparation) Date/Time: \_\_\_\_\_/\_\_\_\_\_

Set\_\_ Visual Colour: Yes\_\_\_\_,No\_\_\_\_      Colour Corrected: Yes\_\_\_\_,No\_\_\_\_

Set\_\_ Visual Colour: Yes\_\_\_\_,No\_\_\_\_      Colour Corrected: Yes\_\_\_\_,No\_\_\_\_

Set\_\_ Visual Turbidity: Yes\_\_\_\_,No\_\_\_\_      Centrifuged: Yes\_\_\_\_,No\_\_\_\_

Set\_\_ Visual Turbidity: Yes\_\_\_\_,No\_\_\_\_      Centrifuged: Yes\_\_\_\_,No\_\_\_\_

Set\_\_ Osmotic Adjustment By: Dry NaCl\_\_\_\_,Moas\_\_\_\_,None\_\_\_\_

Set\_\_ Osmotic Adjustment By: Dry NaCl\_\_\_\_,Moas\_\_\_\_,None\_\_\_\_

Set\_\_ pH\_\_\_\_\_ Adjusted pH\_\_\_\_\_

Set\_\_ pH\_\_\_\_\_ Adjusted pH\_\_\_\_\_

Additional Sample Information:

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Table A2 - Standard Microtox test data sheet 2

Microtox Reagent Lot No.: \_\_\_\_\_ Vial No.: \_\_\_\_\_

Bacterial reconstitution time: \_\_\_\_\_

Sample Assay Date/Time: \_\_\_\_\_ / \_\_\_\_\_

Sample Concentration	Light Level				Gamma				Blank Ratio			
	I0	I5	I15	I30	I5	I15	I30	I0	I5	I15	I30	
Blanks	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
B2	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
C2	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
B3	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
C3	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
B4	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
C4	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
B5	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
C5	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
E2	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
F2	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
E3	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
F3	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
E4	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
F4	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
E5	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
F5	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
Set (t) EC <sub>50</sub>	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
Min. EC <sub>50</sub>	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
Max. EC <sub>50</sub>	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
Set (t) EC <sub>50</sub>	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
Min. EC <sub>50</sub>	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
Max. EC <sub>50</sub>	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
Set Colour Correction Data: C0	_____	I0	_____	It	_____	_____	_____	_____	_____	_____	_____	
Set Colour Correction Data: C0	_____	I0	_____	It	_____	_____	_____	_____	_____	_____	_____	
Set (t) cEC <sub>50</sub>	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
Min. cEC <sub>50</sub>	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
Max. cEC <sub>50</sub>	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
Set (t) cEC <sub>50</sub>	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
Min. cEC <sub>50</sub>	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	
Max. cEC <sub>50</sub>	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	

## **APPENDIX B - CASE-STUDY QUESTIONNAIRES**

APPLICATION OF DIRECT TOXICITY ASSESSMENT TO  
DISCHARGE CONTROL - CASE STUDIES

**Questionnaire on discharge details for NRA regions:**

These are questions that would be asked if the impact of multiple discharges on a body of water was being assessed, or if a COPA2/Water Act Consent was being established or reviewed. Please provide answers to as many of the questions as possible. Where there are gaps in knowledge, provision for appropriate extra investigations may be needed.

**Multiple input screening survey**

The intention of a multiple input screening survey is to:

- (a) identify all potential polluting inputs to the defined area;
- (b) to assess their significance in terms of polluting impact.

To do this we require: quantitative hydraulic information,  
quantitative and qualitative chemical  
information and  
qualitative biological information.

As the case study is designed to assess the usefulness of a direct toxicity measure on polluting discharges, this chemical information is necessary to establish the hypothesis to be tested. The hydraulic information is needed to interpret potential impact and target limited resources.

Specific requirements are as follows:

1. Define catchment area and important sub catchments (by area). List hydraulic inputs (to account for approximately 95% of total) with ADF for each input. - Estimate if possible 95% exceedence flow at point of discharge for all consented discharges (see 3).
2. Describe general water quality of receiving waters.
3. List consented discharges (Actual and deemed) with consented determinands and conditions (including flow, timing) by category [(a) sewage only, (b) sewage with trade, (c) industrial]. Include broad description of type of trade or industry involved in each discharge. Frequency of chemical monitoring and size of database (i.e. number of samples/dates) would be helpful.
4. List possible diffuse inputs (agrochemicals?) in chosen catchment.

5. List parts of catchment where impoverishment of biological community is evident or suspected (Species absent, BMWP score if any, or your own assessment scheme score, with explanation). Describe briefly the type of biological community, including fish species, expected in the area.
6. Have any inputs been the subject of toxicological assessment?

Note: A practical limit of around 50 polluting discharges should be adopted to make the project area manageable. Not all these would be expected to be acutely toxic.

APPLICATION OF DIRECT TOXICITY ASSESSMENT TO  
DISCHARGE CONTROL - CASE STUDIES

Questionnaire on discharge details for NRA regions:

These are questions that would be asked if the impact of multiple discharges on a body of water was being assessed, or if a COPA2/Water Act Consent was being established or reviewed. Please provide answers to as many of the questions as possible. Where there are gaps in knowledge, provision for appropriate extra investigations may be needed.

Individual (Type) Discharges

(Inland)

We require the following information where available:

1. Hydraulic information: Hydrograph for receiving water course, with 95% exceedence flow.
2. General water quality of receiving water, with indication of impact from any upstream discharges.
3.
  - 3.1 COPA consent conditions (determinands, quality, quantity, timing (if any), special limitations).
  - 3.2 Number and description of all discharges from site.
  - 3.3 Description of on-site activities: Products, processes, materials stored, nature of processes (e.g. continuous, batch), treatment if any, with type.
  - 3.4 Flow measurement facilities (continuous recording, measurements).
  - 3.5 Sampling provisions (how is sample collected?) Autosampling facility, if so of what type).
4. Pollution control managers description of site and operation including comments on relationship with site owner/operator, and impact of site on receiving environment.
5. Compliance chemical data set (and summary) and special surveys, if any, for last two years (or before if relevant).
6. Evidence of impact on biota in receiving watercourse (if any).

**APPLICATION OF DIRECT TOXICITY ASSESSMENT TO  
DISCHARGE CONTROL - CASE STUDIES**

**Questionnaire on discharge details for NRA regions:**

These are questions that would be asked if the impact of multiple discharges on a body of water was being assessed, or if a COPA2/Water Act Consent was being established or reviewed. Please provide answers to as many of the questions as possible. Where there are gaps in knowledge, provision for appropriate extra investigations may be needed.

**Individual (Type) Discharges**

**(To tidal waters)**

We require the following information where available:

1. Hydraulic information: Details of any dilution dispersion studies carried out on the discharge/receiving water body.
2. General water quality of receiving water (salinity, suspended solids, diss. Oxygen), with indication of impact from any other discharges to the same water body.
3.
  - 3.1 COPA consent conditions (determinands, quality, quantity, timing of discharge in relation to tidal cycle, any special limitations.
  - 3.2 Number and description of all discharges from site.
  - 3.3 Description of on-site activities: Products, processes, materials stored, nature of processes (e.g. continuous or batch). If batch processes are used, give timing and duration if possible. Also describe effluent treatment if any.
  - 3.4 Flow measurement facilities (continuous recording, measurements).
  - 3.5 Sampling provisions (how is sample collected?) Autosampling facility, if so of what type).
4. Pollution control manager's description of site and operation including comments on relationship with site owner/operator, and impact of site on receiving environment.
5. Compliance chemical data set (with summary) and special surveys, if any, for last two years (or before if relevant).
6. Evidence of impact on biota in receiving waterbody or adjacent shoreline, if any.

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