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Draft Protocol for the Derivation of Toxicity-Based Consents

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DRAFT PROTOCOL FOR THE DERIVATION OF TOXICITY-BASED CONSENTS

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EXECUTIVE SUMMARY

This report outlines a procedure for using toxicity measurement to control complex and variable discharges. The first step in the procedure is to identify and prioritise candidate effluents that may be appropriate for toxicity-based control. This involves a desk based appraisal of available data on effluent composition and variability and the dilution capacity of the receiving water. Collection of toxicity data from a battery of rapid and complementary screening tests is also usually required. For effluents considered suitable, there follows in-depth testing with the most appropriate screening test and acute higher organism (alga, invertebrate and fish) tests representative of the water that receives the discharge. The data from the most sensitive of the tests are used to assess whether a toxicity-based discharge consent should be derived or the toxicity of the effluent needs to be reduced.

For effluents appropriate for toxicity-based control, establish whether a correlation exists between the most sensitive higher organism test and the most appropriate screening test. Where a highly significant positive correlation exists, a 'calibrated' screening test consent condition can be derived. For discharges where no correlation exists the toxicity-based discharge consent should specify the most sensitive test. The toxicity-based consent can be expressed as an absolute limit or as an effective (EC_{50}) or lethal (LC_{50}) concentration. The variability in the toxicity of an effluent governs the level of testing required to establish the discharge consent and the frequency of monitoring necessary to assess compliance. Analytical quality control procedures, which need to be applied to ensure the acceptability of data to regulators, dischargers and the public, are also discussed.

KEY WORDS

Toxicity-based consents, effluents, screening tests, discharge consents, analytical quality control, control charts.

1. INTRODUCTION

This draft protocol describes the procedures for identifying complex and variable effluents which are appropriate for toxicity-based control and deriving toxicity-based consents. The philosophy underlying the use of toxicity-based consents (or direct toxicity assessment) for effluents and its overall role in the discharge consenting procedure has been given in an accompanying report (Johnson *et al* 1992). The draft protocol is intended to describe the procedures which will need to be implemented on a national basis. This will ensure there is a consistent approach which is acceptable to regulators, dischargers and the public.

2. SELECTION OF APPROPRIATE DISCHARGES (STAGE 1)

The selection of discharges which are appropriate for toxicity-based control is achieved by a desk-based appraisal and, where needed, effluent screening with a battery of appropriate toxicty tests.

2.1 Desk-based appraisal

The desk-based appraisal should collate all the available information on the effluent from the discharger and regulator, including:

- existing information on the environmental impact of the discharge from biological surveys and pollution incidents;
- the complexity of the effluent, including the range of products produced and a list of substances present in the discharge;
- toxicological data (on sub-lethal and lethal toxicity and bioaccumulation potential) for identified substances in the discharge;
- chemical monitoring data to assess the variability of the effluent;
- the volume of the effluent discharged at peak flow based on gauging data for existing discharges and projections for proposed discharges;
- the worst case flow of riverine receiving waters or information on tidal flows, dispersion and dilution for effluents discharged to estuaries and coastal waters;
- information on the toxicity of the whole effluent or of constituents of the waste stream, where available;
- current or proposed uses of the receiving water.

Most of the information required at the desk-based appraisal will probably have been obtained for existing discharges which are controlled by chemical-specific limits. In contrast, for proposed discharges most of the information will have to be determined or estimated from representative pilot-plant effluents. Table 2.1 provides a check list for use in the desk-based appraisal.

2.1.1 Calculation of the available dilution in the mixing zone and the effluent concentration at the edge of the zone

This clearly represents an area of the protocol where pollution control officers already possess considerable experience. There are also protocols available which provide guidance on the allocation of mixing zones in riverine and coastal water discharges, such as the Water Authorities Association document on "Mixing Zones" (WAA 1986).

Table 2.1 Check list of information required for the desk-based appraisal

Discharger			Regulator		
Required	Available	Obtained	Required	Available	Obtained
List of substances present in the effluent			Information on the environmental impact of the discharge (Biological survey data, pollution incidents)		
Chemical monitoring data			Chemical monitoring data		
Volume of effluent discharged at peak flow			Toxicological (toxicity and bio-accumulation) information on substances present in the discharge		
Degree of biological treatment on site			Worst case receiving water flow		
			Current or proposed uses of the receiving water		

A simple estimate of the worst case dilution factor for discharges in freshwater may be calculated from the highest effluent flow (from on-site gauges) data and a value for the worst case receiving water flow. The resulting effluent concentration at the edge of the mixing zone can be approximated from the equation:

Effluent concentration (%) at the edge of the mixing zone = 100 / Worst case dilution factor

In more complex situations, such as estuaries and coastal waters, dye studies and the use of hydrodynamic models may be needed to obtain a realistic view of effluent dispersion and dilution in the receiving water.

2.1.2 The potential impact of the effluent

The analytical data on the effluent are used to identify substances:

- having to satisfy established Environmental Quality Standards (EQSs);
- present in concentrations which, by comparison with toxicological data on levels known to impair growth and reproduction or cause death, are likely to cause toxicity in the receiving water;
- for which toxicological data indicate a propensity for bioaccumulation at the levels present in the receiving water;
- for which there are no EQSs or for which relevant toxicological data are not available.

At this time the permitted levels of chemicals currently specified in an existing discharge to satisfy EQSs or meet Likely Safe Environmental Concentrations (LSECs) should be reviewed. It may be appropriate to revise the consent if the discharge is not considered appropriate for toxicity-based control, but is considered to cause an impact in the receiving water.

In the case of proposed discharges, substances requiring chemical consent limits will have to be identified, by comparing chemical and toxicological data, and permissible concentrations for each substance defined.

2.2 Toxicity testing of the effluent with screening tests

Table 2.2 shows the available toxicity tests which are considered appropriate for screening effluents. The proposed tests have been extensively validated and all have standard operating procedures. The tests show different interspecific sensitivities to specific chemical classes (Young *et al* 1991). The use of these complementary tests should ensure that existing or proposed discharges suitable for toxicity-based control are identified and minimise the likelihood of not detecting a toxic effluent.

Table 2.2 Screening tests recommended for use in selecting appropriate effluents for toxicity-based control

Toxicity test	Receiving water	
	Freshwater	Marine waters
5-30 minute Microtox (*Photobacterium phosphoreum*) bioluminescence test (Butler *et al* 1991)	+	+
24 hr Water flea (*Daphnia magna*) immobilisation test (OECD 1984)	+	
24 hr Oyster (*Crassostrea gigas*) embyro-larval development test (ICES 1991)		+

The toxicity testing with the screening tests has to reflect the inherent variability of the effluent so that the maximum toxicity of the discharge is assessed (see Section 8 on sampling and testing regimes). Discharges where there is measurable acute toxicity in full strength effluent, that is the $EC(LC)_{50}$ for the most sensitive screening test $\leq$ 100%, should be initially considered appropriate for toxicity-based control. However, the toxicity data from the screening tests need to be considered in the light of data collected from the desk-based appraisal, particularly relating to available dilution. The data should be used to determine whether the toxicity of the effluent is essentially due to a single substance or limited number of substances, which can most effectively be controlled by a chemical-specific consent. The decisions made should be conservative, to ensure that no potentially toxic discharge is excluded unless there is clear evidence that toxicity-based control is not appropriate. An example of this would be an effluent that shows an absence of toxicity in the screening tests and a high available dilution in the receiving water.

For candidate effluents, the persistence of toxicity of the effluent should be assessed with a rapid screening test. This information is needed if a decision on the the most appropriate dosing regime for the fish toxicity test (that is open or closed vessels with static, semi-static or flow-through testing) is to be made. Initially, open and closed test vessels containing the effluent are prepared (Figure 2.1). Samples are taken from both at the start of the test (0 hr), after 24 hours and at the end of the test (96 hr). These samples are analysed for toxicity using the most appropriate screening test. If the measured toxicity in a test vessel after 96 hr, as an $EC(LC)_{50}$, is not significantly different from that measured initially a static, semi-static or flow-through dosing regime can be used for the fish tests. Semi-static or flow-through tests should be used where the measured $EC(LC)_{50}$ at 96 hr is signficantly different from the initial value, but there is no signficant difference in values

after 24 hr. In cases where the measured toxicity at 24 hr is significantly different from the initial value, flow-through tests should be used.

Assessment of the effluent toxicity in both open and closed vessel indicates whether changes are due to volatilisation, chemical degradation (hydrolysis or photolysis) or sorption onto the surfaces of the vessels. Changes in toxicity in the open vessels only reflect the effects of volatilisation, whereas changes in toxicity in both vessels are probably due to chemical degradation or sorption. Differences in effects between vessels may allow a more cost-effective approach to be taken using closed rather than open vessels. For example if open vessels showed a statistically significant change in toxicity after 24 hr but there was only a change in toxicity in the closed vessels after 96 hr, this would allow a semi-static system to be used rather than the flow-through system indicated by the open vessel data.

It has to be recognised that this approach has major cost implications. In addition, a closed system may be the most appropriate test system scientifically, but may not realistically reflect environmental conditions when the effluent enters the receiving water.

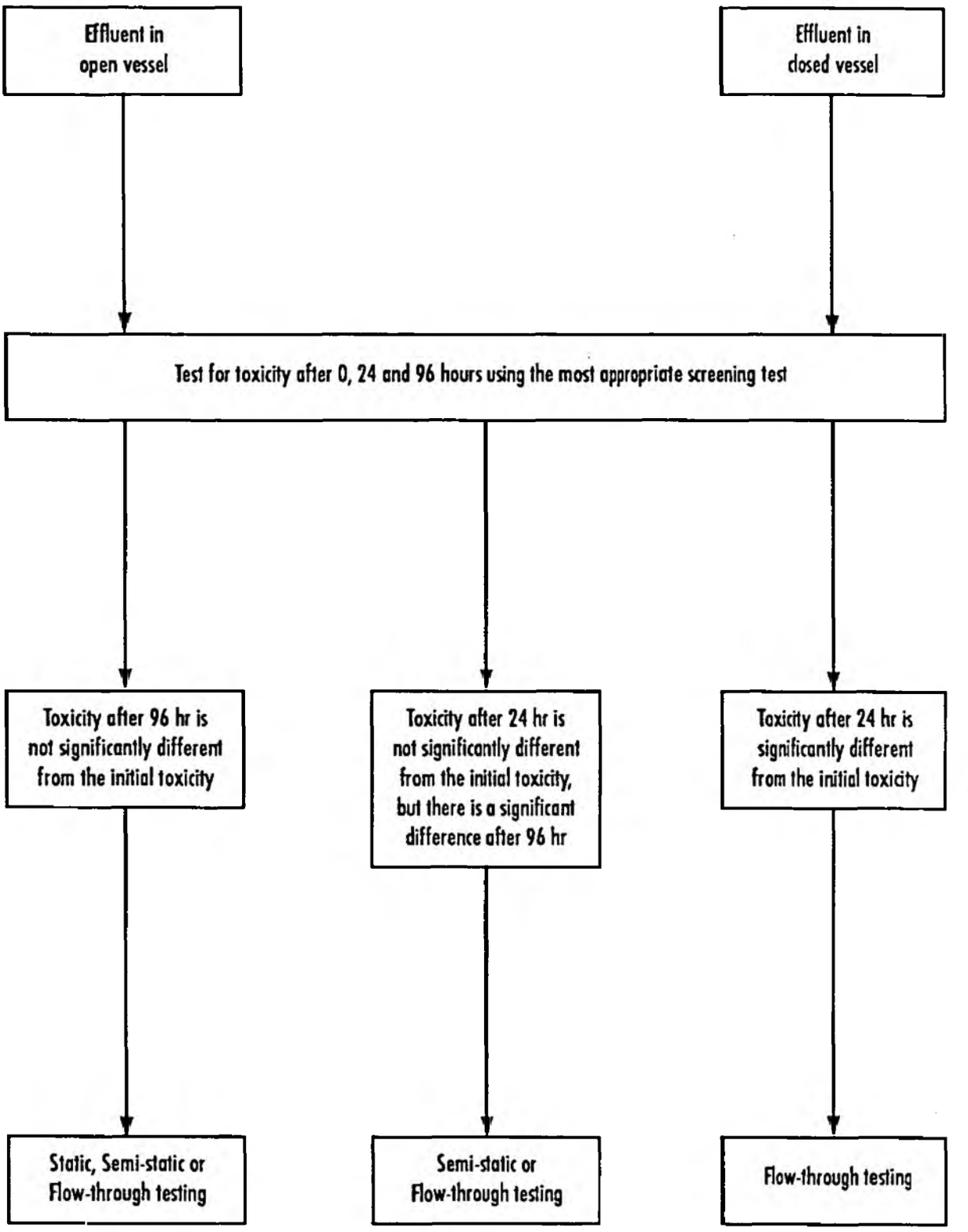

Figure 2.1 Guidance for selecting an appropriate dosing system for fish toxicity testing

3. IN-DEPTH TESTING OF THE TOXICITY OF THE EFFLUENT (STAGE 2)

3.1 Introduction

At this stage, effluents considered appropriate for toxicity-based control are tested with the most appropriate screening test from Stage 1 and higher organism (alga, invertebrate and fish) tests representative of the receiving water to which the effluent is released.

The data derived from the in-depth testing are required to:

1. identify which species of those tested is most sensitive to the effluent;

2. determine the acceptable environmental concentration (AEC) from the data for the most sensitive test;

3. compare the AEC with the likely effluent concentration at the edge of the mixing zone, that is the receiving water concentration (RWC);

4. determine whether a discharge consent can be derived or the toxicity of the effluent needs to be reduced;

5. establish which type of test should be specified in a consent, where one is deemed appropriate.

A more detailed description of each of these stages is given in the following sections.

3.2 Higher organism tests

Table 3.1 shows the algal, invertebrate and fish tests for fresh and marine waters that are proposed for use in assessing the toxicity of effluents. These represent traditional toxicity test organisms, whose use is well established and for which standard internationally recognised protocols are available (OECD 1984, ISO 1988, ICES 1991).

Standard test procedures for each method and the situations in which the tests can be applied will be described in the NRA/SNIFFER Ecotoxicology Methods Manual. The way in which the tests are carried out will often be influenced by the nature of the effluent, for example whether it is coloured, acidic or alkaline or contains high suspended solids levels. The manual will provide guidance on these areas. The procedure for each test will also specify the statistical procedures to use when calculating toxicity values.

Table 3.1 Algae, invertebrates and fish recommended for use in the testing of the toxicity of effluents discharged to fresh and marine waters

Type of organism	Freshwaters	Marine waters
ALGAE	*Selenastrum capricornutum* 72 hr Inhibition of growth (OECD 1984)	*Phaeodactylum tricornutum* *Skeletonema costatum* 96 hr Inhibition of growth (ISO 1988)
INVERTEBRATES	*Daphnia magna* (Water flea) 48 hr Immobilisation (OECD 1984)	*Crassostrea gigas* (Pacific oyster) embryos 24 hr Inhibition of development (ICES 1991)
FISH	*Salmo trutta* (Brown trout) *Oncorhynchus mykiss* (Rainbow trout) 96 hr Mortality (OECD 1984)	*Pleuronectes platessa* (Plaice) *Scopthalmus maximus* (Turbot) 96 hr Mortality

3.3 Testing regime

Representative samples of the effluent should initially be tested with an alga, invertebrate and fish and an appropriate screening test. This is to identify the most sensitive test species and establish the relationship between the toxicity of the effluent to the most sensitive test and the screening test. The algal and invertebrate tests specified are carried out as static tests. The appropriate regime for the fish tests can be determined using the procedure described in Section 2.2. The level of testing required depends on the extent of the variability in toxicity identified at the screening stage (see Section 8). Effluents with limited or defined variability should be tested on a minimum of four occasions. However, for effluents which show no definable pattern in the variability of effluent toxicity, additional testing will be required to ensure the maximum effluent toxicity has been tested.

3.4 Identifying the most sensitive test species

The most sensitive test species is determined from the mean toxicity values for each test conducted. It is considered to be the test showing the lowest mean $EC(LC)_{50}$, which takes account of instances where the most sensitive species may differ between tests.

3.5 Determination of the no effect concentration (NEC) and the acceptable environmental concentration (AEC)

The toxicity data for the most sensitive test are initially used to determine the effluent concentration causing no toxic effect (NEC). The NEC for the most sensitive test is then translated into an acceptable environmental concentration (AEC) using a safety factor.

3.5.1 Determination of the no effect concentration (NEC)

For discharges where the algal test, measuring effluent-induced inhibition of growth, or the oyster embryo-larval development test is the most sensitive test both the EC_{50} and the NEC can be determined directly from the test data. The statistical methods used will be described in the NRA/SNIFFER Ecotoxicology Methods Manual.

Where an invertebrate or fish test based on immobilisation or lethality is the the most sensitive test, an NEC can be calculated from the test data. However, this value only describes the highest predicted effluent concentration at which no mortality is expected. Exposure of the most sensitive test species to this effluent concentration may still result in effects on sub-lethal parameters such as growth and reproduction. The NEC for sub-lethal responses is therefore calculated from the $EC(LC)_{50}$ values for lethality using an appropriate acute to chronic ratio (ACR), that is:

$NEC = EC(LC)_{50} / ACR$

In comparison to pure substances, there is limited data on the ratios between $EC(LC)_{50}$ values and NECs for effluents. An ACR of 20 is recommended for use at this time since this represents the conservative end of the 10-20 range recommended by the US EPA from effluent test data (US EPA 1991).

3.5.2 Determination of the acceptable environmental concentation (AEC)

The safety factor used to translate the NEC for the most sensitive test species into an AEC accounts for the greater range of species sensitivity in the receiving water and the persistence of toxicity of the effluent. It replaces the acute to chronic ratio (ACR) and application factor (AF) specified in the initial protocol of Hunt (1989).

In cases where the algal growth inhibition test or the oyster embryo test is most sensitive and the NEC is estimated directly:

AEC = NEC/Safety factor

where: Safety factor = Factor for species sensitivity x Factor for persistence

In cases where invertebrate immobilisation and fish lethality tests are the most sensitive and the NEC is calculated from the $EC(LC)_{50}$:

$AEC = EC(LC)_{50} / ACR \times$ Safety factor

There is limited data on the range of sensitivities of receiving water organisms to toxic complex effluents. A safety factor of 10 applied to the NEC for the most sensitive test species should ensure the protection of the most sensitive species in the receiving water.

An indication of the persistence (rate of change) of effluent toxicity in the receiving water and the magnitude of the safety factor for persistence is obtained using an approach proposed by Haig *et al* (1989).

This involves measuring the changes in toxicity of the effluent to the most sensitive test species due to:

1. Sedimentation, where the effects of suspended solids are assessed by comparing the toxicity of the untreated effluent with that of the supernatent after the effluent has been allowed to settle for 2 hours;

2. Chemical degradation and volatilisation, where the toxicity of the untreated effluent is compared with that of effluent which has been aerated for 4 days prior to testing;

3. Microbial action, where the toxicity of untreated effluent is compared with that of effluent diluted with 'clean' (non-polluted) field collected river or saline water (representative of the receiving water) and aerated for 4 days prior to testing.

The data from these tests are used to ascertain the extent to which the toxicity of the effluent changes in the receiving water and determine the magnitude of the safety factor for persistence which is used to calculate the AEC. Effluents which show limited persistence will require smaller safety factors than those where these processes have little effect on toxicity.

3.6 Comparison of the acceptable environmental concentration (AEC) with the receiving water concentration (RWC)

A toxicity-based discharge consent should be derived for discharges where the effluent concentration at the edge of the mixing zone (that is the receiving water concentration, RWC) is less than or equal to the AEC. For discharges where the RWC is greater than the AEC, the effluent should be considered to be too toxic to derive a discharge consent and its toxicity should be reduced. This will involve the use of procedures to identify the constituents responsible for the toxicity and subsequent substitution or elimination of the component(s). If the toxic component(s) of a discharge cannot be identified, the effluent may need to be treated. Retesting should then be carried out to ascertain that the resultant toxicity is acceptable and a discharge consent can be derived.

3.7 Determining the type of test to be specified in the consent

A discharge consent can be derived using:

1. a 'calibrated' screening test limit, where there is a correlation between the screening test and the most sensitive test species;

2. the most sensitive test, where no correlation is evident.

An initial decision on which test should be specified is made by determining the relationship between the screening test and the most sensitive test from the in-depth testing. The extent of the correlation between the two tests is expressed by the correlation coefficient (r) and the procedure for deriving this value is described in Appendix A. A value of r is calculated from the test data and compared with statistical tables to determine whether the correlation is significant.

The use of a 'calibrated' screening test consent limit should be investigated where the in-depth testing has shown a positive correlation between the screening test and the most sensitive test. Additional testing should be carried out with both tests while the correlation coefficient increases with each test. However, testing should not be continued if the correlation coefficient between the data is not statistically significant at the 0.1 % level after 8 tests have been conducted. In these cases, a consent should be derived with the most sensitive test. The use of this stringent criterion will ensure that the likelihood of correlations occurring by chance is minimised.

If the data from the in-depth testing show either a negative correlation or an absence of a correlation between the screening test and the most sensitive test, additional testing, if needed, should only be carried out with the most sensitive test. The initial testing may be sufficient to derive a toxicity-based consent for effluents whose toxicity is consistent and those of defined variability. Effluents where the variability in toxicity shows no definable pattern will generally have to be tested more frequently to provide sufficient data to derive the consent.

In certain instances two of the higher organism tests may show comparable sensitivities to the effluent and the mean toxicity values obtained may not be statistically different. In such cases the degree of correlation between the screening test and each higher organism test may need to be explored to ascertain which shows the greatest correlation.

4. DERIVING THE CONSENT CONDITION (STAGE 3)

4.1 Introduction

A toxicity-based consent should prevent adverse ecotoxicological effects arising in the receiving water outside a defined mixing zone. In toxicological terms this means that the effluent level at the edge of the mixing zone should not cause chronic sub-lethal effects in indigenous species. This can be achieved by ensuring compliance of the effluent with the toxicity limit for a given test(s) which is specified in the consent. Regulators may consider that in certain instances a consent specifying both a 'calibrated' screening test limit and a limit for the most sensitive test is the most effective means of controlling the discharge.

4.2 Calculation of a consent limit using the most sensitive test

In the case of effluents where the toxicity-based consent specifies the most sensitive test, an acceptable acute effluent toxicity limit must be derived for this test. This absoslute value is calculated to ensure the receiving water concentration (RWC) does not exceed the acceptable environmental concentration (AEC), using the equation:

Acceptable acute effluent toxicity (%) = AEC x Worst case dilution factor

4.3 Calculation of the 'calibrated' screening test consent limit

The case studies showed that the approach specified in the initial protocol (Hunt 1989) introduced unnecessary complexity into the calculation of 'calibrated' screening test consent limits (Butler *et al* 1992a,b). Consequently a number of changes have been made to simplify the procedure and ensure the process is consistent with that which has been used previously in the UK to derive toxicity-based consent conditions.

The absolute numeric consent limit for the 'calibrated' screening test is derived by multiplying the acceptable acute effluent toxicity for the most sensitive test by the ratio of sensitivity between the screening test and the most sensitive test derived from all the available data:

Screening test consent limit (% effluent)	=	Acceptable acute effluent toxicity	x	Ratio of sensitivity between tests

where: Ratio of sensitivity = $\dfrac{\text{Mean EC(LC)}_{50} \text{ for screening test}}{\text{Mean EC(LC)}_{50} \text{ for most sensitive test}}$

The derivation of toxicity-based consents using acute tests, based on lethality, requires the use of large safety factors and has limitations for discharges with small mixing zone dilutions. For these discharges, consent conditions should be derived from sub-lethal tests, though at present there is uncertainty about which methods are appropriate.

4.4 Additional monitoring

As the aim of the consent condition is to protect the quality of the receiving water environment there is considerable value in using *in situ* (field deployed) bioassays, such as the *Gammarus* feeding rate test or *Mytilus* scope for growth, or toxicity tests on collected water samples to assess effects around the discharge and at the edge of the mixing zone. Tests of this nature may lead to a reduction in uncertainty about the toxicity of the effluent and so ensure the discharge consent is not overly stringent. The deployment of caged mussels has been used successfully by the Clyde River Purification Board and WRc to assess the impact of pharmaceutical (Mackay *et al* 1989) and industrial (Roddie and Johnson 1988) discharges to Irvine Bay.

5. FORM OF THE CONSENT

Consents using 'calibrated' screening tests or higher organism tests can specify a percentage effect (for example 50% lethality) at a given dilution or an $EC(LC)_{50}$ value as the absolute toxicity limit which should not be breached.

The former approach has been used in toxicity-based consent conditions by NRA Anglian region and the Clyde River Purification Board. The NRA Anglian condition for a discharge from a chemical plant to tidal waters specified that:

> "When the discharge is diluted 5 times with seawater, and tested by the required procedure (see Appendix A) the cumulative mortality of brown shrimps (*Crangon crangon*), within a 96 hour test period shall not be greater than 50%".

The consent condition for a pharmaceutical discharge to marine waters issued by the Clyde River Purification Board stated that:

> "The effluent shall be conclusively deemed to comply with the terms of this consent when a sample thereof taken at the sampling point and diluted 125 times with seawater and tested according to the procedure set out in the document headed 'Toxicity Test for Effluent Discharges to Saline Waters' attached to this consent, exhibits a cumulative percentage mortality as hereinafter defined of not greater than 50 per cent".

The differences in dilution specified in the two consents reflect the differences in dilution available in the respective receiving waters.

No approach has been prescribed at this stage since there are advantages and limitations to each approach. A toxicity limit expresesed as a percentage effect at a given dilution is less ambiguous and can provide a clear indication of compliance or failure. It also reduces the level of testing associated with compliance monitoring. However, it does not allow trends in toxicity to be followed, since only one effluent concentration is tested.

The use of an EC_{50} or LC_{50} limit means the pattern of effluent toxicity can be closely followed over time. In addition, Warren-Hicks and Parkhurst (1992) stated that greater variability was associated with the use of the limit test approach and that the $EC(LC)_{50}$ approach was more statistically robust, whatever species is used. However, the use of $EC(LC)_{50}$ values allows a degree of interpretation of the results since the values are produced with confidence limits and may vary according to the statistical (probit, moving average) method used. If an $EC(LC)_{50}$ value is specified, it is important to specify the statistical procedure to be used. There would be potential problems if a measured compliance value was around the absolute limit and one method gave a value indicating compliance while another indicated failure.

6. COMPLIANCE MONITORING

6.1 Testing procedure

Where the discharge consent specifies a permitted response at a given dilution, samples should be prepared with an appropriate medium to a percentage effluent specified in the discharge consent. At this effluent concentration there should be less than the specified percentage effect on the parameter measured in the screening test or the most sensitive test. A minimum of four replicates of the effluent concentration and four controls (dilution medium only) should be tested with the screening test. In the case of a higher organism toxicity test, two replicates of the effluent concentration and two controls should represent the minimum number of samples tested to verify the consent condition.

For consents specifying an $EC(LC)_{50}$ value in the 'calibrated' screening test or the most sensitive test, assessment of compliance is carried out using the same procedures as those used in the in-depth testing (Stage 2).

6.2 Frequency of monitoring

The frequency at which discharges are monitored for consent compliance should as a necessity reflect the variability in toxicity identified in the screening test and the calibration studies. For discharges of limited or definable variability, compliance monitoring of a calibrated screening test consent should be carried out on a monthly basis as a minimum in the first year. This should coincide with the routine chemical sampling regimes of UK regulatory agencies. In cases where the most sensitive higher organism test is specified in the consent condition, quarterly monitoring for compliance should be the minimum frequency in the first year. Effluents having an unpredictable pattern of effluent toxicity will probably require a more extensive compliance monitoring programme.

6.3 Implications of compliance test data

Discharges complying with the screening test consent limit on all test occasions in the first year need not be tested so extensively in future years, providing there are no changes in processes at the plant. A reduction to quarterly monitoring would seem appropriate in the first instance, though all decisions on the frequency of monitoring will be influenced by the experiences of the relevant pollution control personnel.

Any effluent failing the 'calibrated' screening test consent limit should be subject to a retest. This should be carried out on a new formal sample since, unlike samples for chemical analysis, there are problems in retesting effluents which have been stored for greater than 24 hours as they may not reflect initial effluent quality. The rapidity with which the formal sample testing is carried out will depend to a certain extent on how the monitoring system is implemented. Compliance of the formal sample with the consent limit should result in a formal action warning being issued, but no further action. This is

consistent with the NRA approach specified for chemical-specific discharge consents (NRA 1990).

If an effluents fails on the first test but passes the retest on the formal sample, the discharge should continue to be monitored on a monthly basis until a 12 month period of compliance has been demonstrated, after which the frequency can be reduced to quarterly. Failure of the retest on the formal sample should lead to a further action which may involve:

1. a programme to identify the component(s) of the effluent responsible for the observed toxic effects. These can then be substituted or eliminated to reduce the toxicity of the effluent;

2. additional higher organism testing;

3. prosecution.

Effluents failing the consent conditions on a second occasion, having passed the retest after the first failure should also be subject to these actions.

For higher organism test consents, the procedure of retesting on a formal sample after consent failure should be used and the implications of compliance or failure should be the same as for the 'calibrated' screening test. The frequency of monitoring of these consents should not be relaxed to less than quarterly even given continued compliance.

7. REASSESSMENT OF THE DISCHARGE CONSENT

The applicability of a toxicity-based consent will need to be reviewed at regular intervals and retested with the relevant test(s). The consent condition can then be adjusted using the data generated. The frequency of reassessment will depend upon:

1. the closeness of the results of routine monitoring to the consent condition;
2. the expected variability of discharge composition;
3. changes in the nature and operation of the plant;
4. the importance of the discharge to receiving water quality.

Reassessment of the discharges should be undertaken at least once every three years.

8. ASSESSMENT OF EFFLUENT VARIABILITY

The accurate assessment of the variability of the effluent requires an appropriate sampling regime and testing systems. The approach adopted depends largely on the inherent variability of the effluent over time and the persistence of toxicity, but will also be influenced by available resources.

8.1 Sampling regimes

There are three types of sampling which can be used in the course of toxicity testing:

1. Spot (snap), in which discrete samples are collected over a short period of time, for example 15 minutes;

2. Composite, in which discrete samples are collected over a specified period of time, such as the daily operation cycle of a plant. Composite samples may be collected as a single sample or a number of samples which are then pooled;

3. Continuous flow, in which the effluent is sampled and tested directly.

It is important to use a sampling regime which ensures that the maximum toxicity of the effluent is accurately assessed. Although each effluent should be considered on its own merits, the Organisation for Economic Development and Cooperation (OECD 1987) and the United States Environmental Protection Agency (US EPA 1991) have provided guidance on the sampling of effluents for toxicity testing. The merits and limitations of each approach are given in Table 8.1, along with situations where each is appropriate. A brief discussion of appropriate sampling schedules for different types of effluents is given in Sections 8.3 - 8.5.

The Pollution Inspectorate of the Clyde River Purification Board has suggested that only spot samples are likely to be accepted as formal (tri-partite) samples in a court of law. Clearly this is an area which, given the potential implications, needs to be clarified.

8.2 Testing systems

The algal and invertebrate tests specified in the protocol have static exposure regimes. However, there are three types of testing system which can be used to measure effluent toxicity to fish:

1. Static, in which the test solutions are prepared manually by adding the effluent and the dilution media to the tanks at the beginning of the test;

2. Semi-static, which are similar to static tests, but with renewal of the test solutions on a predetermined schedule, for example every 24 hours. Fresh effluent samples should, ideally, be collected to renew the test solutions;

Table 8.1 Sampling regimes which can be used to assess variability in effluent toxicity

Sampling regime	Advantages	Disadvantages	Recommended use
Spot (snap)	Can assess the peak toxicity of processes Realistically reflects the toxicity of effluents containing volatile compounds or other materials that are likely to change rapidly after collection	May miss peak toxicity if the pattern of effluent variability is not identified	EPA (1980) recommends the use of spot samples where the hydraulic retention time of the treatment system is 14 d or longer and where there is little dispersion or mixing in the receiving water and the peak of toxicity persists. Static or semi-static tests should be used for these samples.
Composite	Ties in with monitoring of daily average of specific pollutants	Averages peaks of toxicity Deterioration of volatile substances may result in differences in toxicity between the initial and final portions of the sample	Static or semi-static testing of effluents of consistent composition or predictable variability where the peaks are of long duration (hours) over weekly or longer cycles
Continuous	Accurate simulation of time varying concentrations in the receiving water by dilution of the effluent	Difficult to interpret whether the measured responses are caused by an average or varying quality of effluent	On site flow-through testing for highly variable effluents which are continuously discharged or are intermittent

3. Flow-through, which use a diluter system and a continuous supply of effluent and dilution water. Flow through systems may be more resource intensive than static or semi-static systems, require complex delivery systems and large volumes of effluent and dilution water.

The testing system used should be determined based on the extent of effluent variability and the persistence of toxicity of the effluent.

Guidance on the selection of an appropriate testing regime based on the persistence of effluent toxicity is given in Section 2.2. Table 8.2 shows the minimum volumes of effluent required for each test system. The standard test protocols for Microtox, the algal growth inhibition test, the *Daphnia* immobilisation test and the oyster embryo-larval test all require comparatively small volumes of effluent. All these tests could be carried out for a proposed discharge where the availability of effluent may be a problem. However, for the fish tests, larger volumes of effluent are needed, particularly for flow-through testing, and this may present a problem in the case of a proposed discharge.

8.3 Sampling and testing schedule for consistent (non-variable) effluents

For discharges where there is limited variation in effluent toxicity, random spot samples or composite (24 hr) samples can be used to conduct static or semi-static acute toxicity tests. Grab samples are most appropriate for discharges containing volatile substances, which may be lost over the longer periods required to collect composite samples.

8.4 Sampling and testing schedule for effluents of definable variability

For discharges varying on a regular and predictable basis, spot or composite samples which are considered to be representative of the maximum effluent toxicity should be used to conduct static or semi-static toxicity tests. Spot samples will be appropriate where the peaks of toxicity are short lived and occur each day, whereas spot or composite samples can be used where the peaks of toxicity last for hours and occur over a longer period, for example weekly.

8.5 Sampling schedule for effluents of undefinable variability

For highly variable discharges where the fluctuations are not apparently predictable, it may be necessary to initially sample intensively over an operationally appropriate period to attempt to determine the pattern of toxicity and identify at which stage peak effluent toxicity is apparent. On-line flow-through testing with rapid toxicity tests is a useful means of characterising effluent toxicity.

At present there are no validated on-line toxicity test devices, but methods under development should be incorporated when they are considered reliable and deliver repeatable results.

On-line flow-through testing also provides the most appropriate means of assessing the toxicity to higher organisms of effluents with no definable pattern of variation. However, test organisms will only be exposed to peak toxicity for periods proportional to the flow through rate, the duration of the peak in toxicity and the length of the test.

Table 8.2 Volumes of effluent required to conduct each type of test specified in the protocol

Type of test	Test procedure	Number and range of effluent concentrations	Test solution volume	Volume of effluent required Per day	During test
Microtox	Static	4 (5.7,11.4,22.8,45.5%)	1 ml	-	2.5 ml
Algal growth inhibition	Static	5 (1.0,3.2,10.0,32,100%)	1 litre	-	1.46 litres
Daphnia immobilization	Static	5 (1,0,3.2,10,32,100)	100 ml	-	146 ml
Oyster embryo-larval development	Static	7 (0.032,0.1,0.32,1.0,3.2,10,20)	30 ml	-	10.4 ml
Fish lethality	Static	6 (0.32,1.0,3.2,10,32,100)	10 litres	-	14.7 litres
	Semi-static	"	"	14.7 litres	58.8 litres
	Flow-through:				
	50 ml min^{-1}	"	"	105 litres	420 litres
	100 ml min^{-1}	"	"	210 litres	840 litres

9. MULTIPLE INPUTS TO A RECEIVING WATER

The description of the toxicity-based assessment approach given to date has focussed on controlling single effluents discharged to a receiving water. In situations where two or more discharges are exerting an effect, the acceptable toxicity from each has to be controlled to ensure that there is an absence of sub-lethal toxicity outside the relevant mixing zones. The absolute toxicity data derived from effluent testing and the estimated dilution factors for all the discharges concerned have to be used to determine the toxicity each contributes to the receiving water. The diluting capacity can then be apportioned between the discharges.

Table 9.1 shows the testing programme which will be needed to determine the toxicity of an individual discharge in a multiple source situation.

The procedure involves determining:

1. the absolute toxicity of the effluent using toxicity tests with non-toxic dilution water;

2. the toxicity of the receiving water upstream of the discharge in rivers or in an area outside of a mixing zone for estuaries and coastal waters;

3. the relative toxicity of the effluent in toxicity tests using receiving water as the dilution water.

The purpose of the relative toxicity test procedure is to determine the toxic impact of the effluent after it is mixed at the point of discharge. Analysis of toxicity trends resulting from the relative toxicity tests are used to assess effluent toxicity in relation to other sources and receiving water conditions.

Multiple inputs also represent a case where *in situ* (field deployed) tests using suitable indigenous species (such as caged *Gammarus* in rivers or caged *Mytilus* around sea outfalls) should be carried out to:

1. determine whether or not the effluent exerts measurable toxicity in the receiving water body;

2. measure the persistence of toxicity from all sources contributing to receiving water toxicity;

3. determine the combined toxicity resulting from the mixing of multiple point and non-point sources of toxicity.

The *in situ* bioassays should be conducted during low flow or worst case dilution periods. These tests should be conducted simultaneously where possible for each discharge. Where this is not possible the tests should be conducted concurrently within a short time period (1-2 days). Repeated *in situ* bioassays will be necessary where variable effluents are discharged to a receiving water. The data from the effluent toxicity testing programme can be used to indicate an acceptable frequency of sampling. An assessment of the impact

of multiple inputs to rivers, estuaries and coastal waters usually requires dye studies and possibly the use of hydrodynamic models. This allows analysis of the effluent concentration at selected sampling stations around the discharge points.

Table 9.1 Effluent and receiving water toxicity tests for multiple inputs to a water body

Aim	Test methodology		Interpretation of results
	Test group medium	Control group medium	
Assess the specific toxicity of a particular effluent (that is the toxicity attributable to a specific effluent)	Effluent plus non-toxic dilution water	Non-toxic dilution water	If the test group shows a toxic effect compared with the control, **the effluent is considered to be toxic**. No toxic effect in the test group indicates the effluent is probably not toxic
Assess the specific toxicity of the receiving water in the absence of a particular effluent	Receiving water (collected above the discharge of the particular effluent)	Non-toxic dilution water	If the test group shows a toxic effect compared with the control, **the receiving water is considered to be toxic**. No toxic effect in the test group indicates the receiving water is probably not toxic.
Assess relative toxicity (that is the toxicity of particular effluent/ water mix relative to the toxicity of the receiving water alone	Effluent plus receiving water as diluent	Receiving water	If the test group shows a greater toxic effect than the control, the contribution of the particular effluent to toxicity may be additive or synergistic. An effect greater than the sum of the specific effects of the receiving water and the effluent may indicate **synergism**. Test group toxicity equal to the sum of effluent and receiving water toxicity may indicate **additivity**. If the test group has a lower toxicity than the sum of effluent and receiving water toxicity then the effluent may be **antagonistic** to the other effluents in the multiple source situation

If the relative toxicity assessment only is being carried out an additional control using non-toxic water should also be used to ensure the validity of the test.

10. QUALITY ASSURANCE AND CONTROL PROCEDURES

Quality assurance and control procedures are an integral component of the effluent testing programme and have to be rigorously observed to ensure the data generated will be accepted in a court of law should this be required. Effluent test data should be accompanied by a QA/QC report describing any problems which could affect the validity of the results.

There are a number of areas of the effluent testing programme for which defined procedures are necessary and these are considered in the following sections.

10.1 Effluent sampling and handling

The time between sampling and testing should in all cases be minimised and should not exceed 24 hours. Ideally, effluents should be transported from the point of collection to the test facility and stored at 4 ± 2 °C at all times before they are tested. The fate of a sample from the point of collection to testing should be recorded on sample custody forms, such as that shown in Table 10.1. Effluent samples should be collected in appropriate containers which will minimise losses due to adsorption to the walls of the vessel. Glass containers should be used for effluents which contain largely organic chemicals, whereas polypropylene or polyethylene containers should be used for essentially metalliferous discharges.

10.2 Analysis of dilution water

Fresh and saline waters, which are used as dilution media in the toxicity tests, have to be analysed routinely for the presence of inorganic and organic contaminants which could modify the toxicity of the effluent. Table 10.2 shows the determinands which should be analysed for in fresh and saline dilution waters, the methods which should be used and the acceptable levels. Total organic carbon (TOC) and halogenated organics (AOX) are used to provide an indication of contamination of water sources by organics such as pesticides. Parameters such as pH and hardness in freshwater should be monitored weekly, while the other determinands should be measured monthly. The dissolved oxygen level in the dilution medium should always exceed the level required for the controls in each test.

Table 10.1 Effluent sample collection and custody record

1. SAMPLE COLLECTION

Sample description & method of collection	Sample code(s)	Date and time collected	Collector's signature	Special treatment * (see Study Plan)

2. CHAIN OF CUSTODY

Task performed	Date and time	Collector's signature	Special treatment * (see Study Plan)

* Examples of 'Special Treatment' include cooling to 4 ^{o}C, returning to laboratory within a certain time period, etc. These will be specified in the Study Plan. The sample collector must record the particular 'Special treatment' and sign and date this record to confirm that it has been undertaken.

Table 10.2 Determinands in fresh and saline dilution waters which need to be routinely measured, methods of measurement and acceptable levels

Determinand	Freshwater		Saline water	
	Method of measurement	Acceptable level (mg l^{-1})	Method of measurement	Acceptable level (mg l^{-1})
pH	pH meter	7.4-8.5		
Cadmium (Cd)	ICP-AES	0.005[3]	FAAS	0.005[1]
Chromium (Cr)	"	0.050[2]	-	-
Copper (Cu)	"	0.028[2]	FAAS	0.010[1]
Lead (Pb)	"	0.050[1]	-	-
Nickel (Ni)	"	0.100[2]	-	-
Zinc	"	0.100[2]	FAAS	0.040[2]
Ammonia (NH_3)	Flow injection analysis	0.50	Flow injection analysis	0.50
Chloride (Cl)	Ion chromatography	250	-	-
Nitrate (NO_3)	"	1.00[4]	Ion chromatography	1.00[4]
Nitrite (NO_2)	"	0.06	"	0.003
Phosphate (PO_4)	"	0.03[4]	"	0.03[4]
Sulphate (SO^4)	"	400	-	-
Total organic carbon (TOC)	Oxidation and IR detection	1.00[1]	Oxidation and IR detection	1.00[1]
Halogenated organics (AOX)	Gas chromatography	0.01[5]	Gas chromatography	0.001[5]

ICP-AES = Inductively Coupled Plasma Atomic Emission Spectrometry, FAAS = Flame Atomic Absorption Spectrophotometry, IR = Infra red

1 Limit of detection, 2 DoE (1989), 3 CEC (1983), 4 Cartwright and Painter (1991), 5 EQS for permethrin (Zabel *et al.* 1988)

10.3 Assessing the precision of toxicity test methods

10.3.1 Intralaboratory precision

Introduction

The precision of the toxicity test methods used at each facility to generate data to derive consents or assess compliance should be assessed. Precision can be described using the coefficient of variation which is calculated from means and relative standard deviations using the equation:

$$CV\ (\%) = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

The procedure recommended for assessing toxicity test precision in each laboratory involves:

1. conducting a series of toxicity tests for each test procedure with appropriate inorganic and organic reference toxicants to assess the variability in test results;

2. Developing control charts for each reference toxicant/protocol combination to identify an acceptable range of test variability. The acceptable limits for the control charts have to be defined before the facility conducts tests to measure effluent toxicity;

3. compare reference toxicity data obtained during effluent testing with the control chart to determine the validity of the test results.

Intralaboratory precision for a specific test method should ideally be assessed using two reference toxicants (one inorganic and one organic chemical), which is the approach adopted by Environment Canada (Environment Canada 1990) and the US EPA (US EPA 1991). Table 10.3 shows substances considered to be appropriate as reference toxicants by Environment Canada, which have been ranked using the criteria described (Environment Canada 1990). Table 10.4 shows an assessment of the suitability of the reference substances in Table 10.3 for each of the type of tests specified in this protocol. This is based on the data in Table 10.5 from the UK case studies, which assessed the usefulness of cadmium as a reference toxicant for higher organism tests, and other published data (Environment Canada 1990, US EPA 1991). Although each laboratory involved in effluent testing could select different reference toxicants, it would be preferable for consistency to have a standardised approach. From the available evidence it is recommended that zinc and phenol are used as the respective inorganic and organic reference toxicants for each test procedure.

Table 10.3 Ranking of potential reference toxicants according to primary selection criteria

Criteria	Organic				Inorganic						
	4-CP	SDS	Phenol	NaPCP	Cd	Cr	Cu	KCl	Ag	NaCl	Zn
Detection of abnormal organisms	L	No	Yes	Yes	L	E	No	L	L	E	Yes
Established toxicity database	No	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
Readily available in pure form	Yes	Yes[a]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Soluble	Yes	Yes[b]	Yes	Yes	Yes	Yes	Yes[b]	Yes	Yes	Yes	Yes
Stable in solution	Yes	No	No	Yes	Yes	Yes	Yes[b]	Yes	E	Yes	Yes
Stable shelf life	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Limited intra-laboratory water quality effects	Yes	Yes	Yes	E[c]	L[d]	Yes[cd]	Yes[d]	Yes	No	Yes	Yes[d]
Easily analysed	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
TOTAL SCORE	5	4	6	7	6	7	6	5	4	7	8

4-CP = 4 Chlorophenol, SDS = Sodium Dodecyl Sulphate, NaPCP = Sodium pentachlorophenol, Cd = Cadmium as Cadmium chloride ($CdCl_2$), Cr = Chromium as Potassium chromate ($KCr0_4$) or Potassium dichromate ($K_2Cr_20_7$), Cu = Copper as Copper sulphate ($CuSO_4$), KCl = Potassium chloride, Ag = Silver as Silver Nitrate ($AgNO_3$), NaCl = Sodium chloride, Zn = Zinc as Zinc chloride ($ZnCl_2$).
The score was calculated by adding 'yes' items and subtracting 'no' items, other symbols have no score (E = equivocal data, L = limited data) a = batches may vary in toxicity, b = not in some waters, c = pH effects, d = hardness effects

Table 10.4 Suitability of various reference toxicants for specific toxicity tests

Type of test	Organic			Inorganic				
	4-CP	Phenol	NaPCP	Cd	Cr	Cu	NaCl	Zn
Microtox	ND	Yes	Yes	No	ND	No	No	Yes
Algal growth inhibition	ND	Yes	ND	E	Yes	Yes	No	Yes
Daphnia immobility	Yes	Yes	Yes	No	Yes	Yes	ND	Yes
Oyster embryo larval development	ND	ND	ND	E	ND	Yes	-	Yes
Fish lethality	Yes	Yes	Yes	No	Yes	Yes	No	Yes

Abbrevations for substances are given in Table 10.3. ND = No data, E = Equivical data
The conclusions are drawn from the results of the case studies and an Environment Canada document (Environment Canada 1990)

Table 10.5 Data on the coefficients of variation of test organism responses to different reference substances

Reference toxicant	Test species	Toxicity index	Coefficient of variation (%)	Number of samples	Reference
Organics					
Phenol	Microtox	15 min EC_{50}	17	35	Butler *et al* (1992b)
Sodium dodecyl sulphate (SDS)	*Daphnia magna*	48 hr EC_{50}	29	8	US EPA (1991)
Sodium pentachloro-phenol	*Daphnia magna*	48 hr EC_{50}	10	13	US EPA (1991)
Inorganics					
Cadmium	*Daphnia magna*	48 hr EC_{50}	72	8	US EPA (1991)
	Phaeodactylum tricornutum	96 hr EC_{50}	32	6	Butler *et al* (1992b)
	Crassostrea gigas larvae	24 hr EC_{50}	64	18	"
	Scopthalmus maximus	96 hr LC_{50}	29	9	"
Copper	*Oncorhynchus mykiss*	96 hr LC_{50}	3[a]	5	US EPA (1980a)
	"	"	40[b]	8	"
	"	"	62[c]	10	"
Zinc	Microtox	15 min EC_{50}	10	7	Butler *et al* (1992b)
	Oncorhynchus mykiss	96 hr LC_{50}	45[d]	5	US EPA (1980b)
	"	"	36[c]	3	"

a = Copper as Sulphate, Hardness 30-32 mg $CaC0_3$ l^{-1}
b = Copper as Sulphate, Hardness 98-102 mg $CaCO_3$ l^{-1}
c = Copper as Chloride, Hardness 194 mg $CaCO_3$ mg l^{-1}
d = Zinc as Sulphate, Hardness 30 mg $CaCO_3$ mg l^{-1}

Preparation of control charts

A control chart of the response of a test species to a specific reference toxicant is prepared by plotting the results of a successive series of tests on a chart where the x axis represents the test date or test number and the y axis indicates the endpoint of the acute toxicity test (that is EC_{50} or LC_{50}s). The mean and standard deviation of the reference toxicity test data are then used to define a range of 'normal' or 'acceptable' variation for that test. The US EPA requires that a minimum of five tests are conducted before 95 per cent limits are established (Weber *et al* 1989). Tests should be carried out until the limits do not change markedly with the addition of each new point, thereby reflecting the minimum variability for that method. A total of 15-20 tests may be necessary to obtain a representative range (Dux 1986). Figure 10.1 shows the control chart derived for Microtox using the reference toxicant phenol.

In all reference toxicant tests, samples of the stock solution and a representative selection of samples from the exposure concentrations should be taken and analysed for the substance using an appropriate method. In accordance with protocols for the tests the measured exposure concentrations should not be less than 80% of the nominal concentrations for the tests to be considered valid (OECD 1984, ISO 1988, ICES 1991).

Warning limits on the control chart are defined as the values two times the standard deviation above and below the mean. For a large data set these represent the upper and lower 95 per cent confidence limits. Action limits are derived as values three times the standard deviation above and below the mean, which represent the 99 per cent confidence limits.

The mean $\bar{X}$ is calculated as the sum of the individual values (X_i) from toxicity tests divided by the number of tests (n), that is

$$\bar{X} = \sum_{i=1}^{n} X_i / n$$

The standard deviation (S) is calculated from the equation:

$$S = \sqrt{\frac{\sum_{i=1}^{n} X_i^2 - \left(\sum_{i=1}^{n} X_i\right)^2 / n}{n - 1}}$$

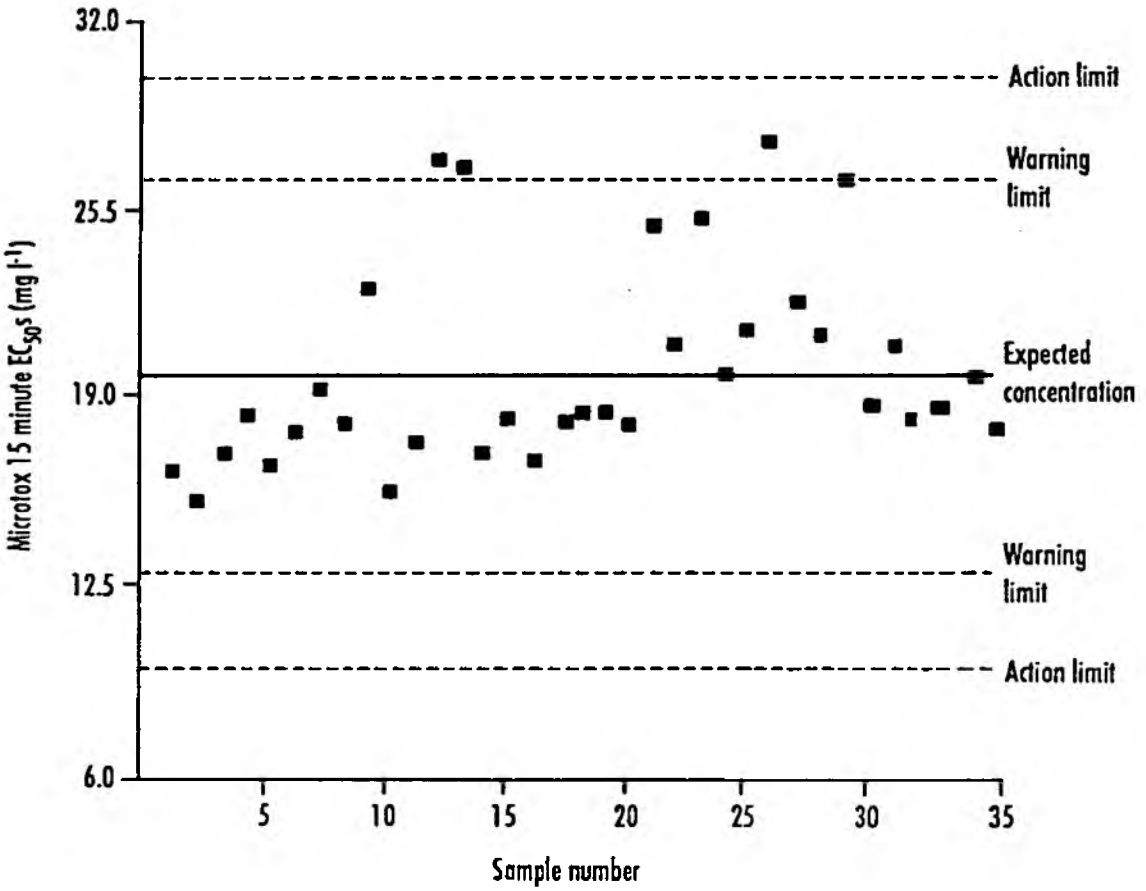

Figure 10.1 Shewart control chart of acceptable limits for Microtox 15 minute EC_{50}s for phenol with warning and action limits based on two and three standard deviations from the mean.

Acceptable ranges

There are no accepted ranges for the width of 95 per cent confidence limits among regulatory agencies (such as the US EPA and Environment Canada) that have implemented reference toxicant testing. However, Environment Canada have suggested that coefficients of variation (CV) of 20-30 % for test methods should represent an achievable goal (Environment Canada 1990). Where tests show higher CVs, potential causes of variation should be identified and restricted. This may be achieved by defining environmental holding conditions more closely and rigorously ensuring they are achieved. It may be necessary to culture organisms in-house rather than obtaining them from a supplier to reduce phenotypic variability.

Interpretation of the control charts

At the 95 per cent confidence level, five per cent of the tests would be expected to fall outside the warning limits by chance. Reference toxicant tests which are outside these limits should prompt a review of the test to identify the cause, which could be due to an error in preparing either the stock solution or the exposure concentrations or problems with the health of the test organisms. Analysis of stock solutions and exposure concentrations in reference tests should be carried out to allow technical errors to be identified. Where an outlying reference toxicant value can be attributed to technical problems, concurrent effluent toxicity tests should be accepted.

In contrast, where an outlying reference toxicant value can be attributed to the health of the organism, effluent toxicity data should not be used in deriving consents or accepted for compliance monitoring.

If no cause for the outlier can be detected or outliers are due to technical problems, the data should be accepted and a note detailing this interpretation should be made in the QA/QC report.

An outlier from the 99 per cent confidence limits is unlikely to occur by chance and the test system should be reviewed. Data on corresponding effluent tests should only be accepted where the cause of the reference toxicant outlier can be traced to technical problems. Toxicity values from acceptable reference toxicant tests should be added to the control chart to obtain a more accurate assessment of test precision.

Data trends

Control charts should not only be used to monitor where each data point falls with respect to the warning limits, but also to monitor trends and patterns that develop in the data. Analysis of the data for trends using a simple probabalistic approach can detect problems at an early stage.

Probability theory dictates that there is a 50% or 1/2 probability (assuming random sources of variation) of a single data point falling above or below the line. The probability of two consecutive points being on the same side of the line is 25% or 1/4 and the

probability of 'n' points being on the same side of the line is $1/(2^n)$. Since the chance of five consecutive points being on the same side of the line is approximately 3% this should represent a threshold at which action should be taken to detect the source of bias (Dux 1986).

10.3.2 Interlaboratory precision

It is important that ring testing with appropriate reference toxicants is carried out between the laboratories of regulatory agencies for each test in the protocol. Standardisation of procedures between laboratories will be required. Subsequent interlaboratory testing of a method by regulatory agencies will show the current level of variability, and allow causes of differences to be assessed and rectified to reduce variability to a minimum level. This level of variability can then be assessed against that for commercial testing facilities to assess whether the precision of the test house is acceptable.

The procedure for conducting an assessment of interlaboratory variability has been discussed in Butler *et al* (1992b) and involves distributing samples of reference toxicants of verified concentrations to all laboratories. These are tested using the standard test protocol and the results analysed to derive an $EC(LC)_{50}$. The values from each laboratory are then compared to ascertain the CV for the tests. The distribution of the values should also be analysed to identify obvious outliers. Each laboratory will be able to judge the validity of their results from the QA/QC report which will assist in identifying reasons for outliers. In situations where the variability is considerable, in-depth studies may be necessary, along with additional ring tests.

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APPENDIX A - CALCULATION OF CORRELATION COEFFICIENT (R) BETWEEN TOXICITY TESTS

The general formula used to calculate the correlation between data from two toxicity tests is:

$$\text{Correlation coefficient } r = \frac{\Sigma xy}{\sqrt{(\Sigma x^2)(\Sigma y^2)}}$$

In the calculation it does not matter which data set is designated as X or Y, since the indices are assummed to be independent of each other.

The calculation of the correlation coefficient between Microtox EC_{50}s and oyster embryo larval EC_{50}s for a pharmaceutical discharge is shown below:

	Microtox EC_{50} (% effluent)	Oyster embryo larval EC_{50} (% effluent)
	X	Y
	1.0	0.27
	30.8	1.29
	9.1	0.59
	7.9	0.31
	3.3	0.005
Mean (SD)	10.42 (11.46)	0.49 (0.49)

$\Sigma X = 52.1$ $\quad \Sigma Y = 2.47$

$\Sigma X^2 = 1105.8$ $\quad \Sigma Y^2 = 2.18$ $\quad \Sigma XY = 47.8$

$$\Sigma x^2 = \Sigma X^2 - \frac{(\Sigma X)^2}{n} \qquad \Sigma Y^2 = \Sigma Y^2 - \frac{(\Sigma Y)^2}{n} \qquad \Sigma XY = \Sigma XY - \frac{(\Sigma X.\Sigma Y)}{n}$$

$$= 1105.8 - \frac{(52.1)^2}{5} \qquad = 2.18 - \frac{(2.47)^2}{5} \qquad = 47.8 - \frac{(52.1 \times 2.47)}{5}$$

$= 1105.8 - 542.9$ $\quad = 2.18 - 1.22$ $\quad = 47.8 - 25.7$

$= 562.9$ $\quad = 0.96$ $\quad = 22.1$

$$r = \frac{22.10}{\sqrt{562.9 \times 0.96}}$$

$$r = 0.950$$

The correlation coefficient is compared with the appropriate value in statistical tables to determine whether the calculated value is significant. The value which r must exceed for significance at the 5% level for n-2 = 3 degrees of freedom is $r_{0.05(2)3} = 0.878$.

Therefore the calculated value of 0.950 is significant and there is a correlation between the Microtox and oyster embryo EC_{50}s for this effluent.