Ecotoxicology

New Approaches

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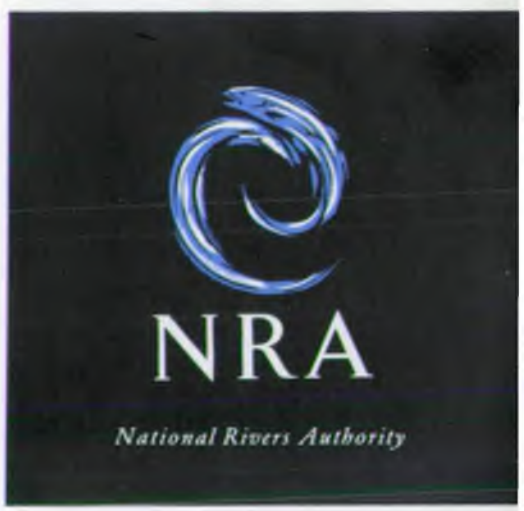

ECOTOXICOLOGY - NEW APPROACHES

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ECOTOXICOLOGY - NEW APPROACHES

M Crane, I Johnson and N Adams

SUMMARY

This report is a review for the NRA of current ecotoxicological test methods and approaches, and their role in meeting the NRA's requirements. It provides an in-depth review of available ecotoxicological techniques, from community methods, through population tests and whole organism lethal and sublethal methods to suborganism tests at the tissue, cellular, subcellular and biochemical level.

Following this in-depth review there is a discussion of how the various tests meet the current needs of the NRA and what approaches and methods it should adopt in future. The report focuses on how the NRA uses ecotoxicological methods in setting Environmental Quality Standards and monitoring environmental quality objectives for water bodies. It also considers the use of aquatic toxicity tests in setting and monitoring toxicity based consents, and their role in detecting and monitoring pollution incidents.

The report contains a review of the current levels of use of ecotoxicological techniques in these areas in the UK and other western nations. It concludes with recommendations on how to implement currently available methods into the NRA's current work practices, and in which areas it should focus research effort in the medium term.

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APPENDICES

SECTION 1 - INTRODUCTION

1.1 THE SCOPE AND STRUCTURE OF THIS REVIEW

The NRA regions currently perform toxicological tests for discharge consent setting and monitoring, receiving water quality monitoring and the investigation of pollution incidents. However, the regions use and perception of these techniques varies greatly. This report is a review for the NRA of the present status of ecotoxicological approaches both in the UK and worldwide. It will assist the NRA in making informed decisions about the use of toxicological data and the development of practical ecotoxicological tests. The aims of the project were to:

a) review established ecotoxicological methods and new approaches currently under development by surveying the literature and contacting environmental protection agencies worldwide, particularly in Europe and North America;

b) consider NRA requirements for ecotoxicological techniques, primarily through discussions with relevant NRA personnel;

c) identify any currently used ecotoxicological approaches or methods that are appropriate to the operational needs of the NRA, and advise on their introduction into their current work practices;

d) identify any new ecotoxicological approaches or methods that could be of use to the NRA in the future.

The remainder of this introductory section reviews the duties of the NRA and the techniques that are currently used to fulfil these duties. Section 2 summarises the ecotoxicological test methods currently available or under development.

Section 3 describes the ecotoxicological approaches and methods that satisfy the needs of the NRA and draws on information on the use of

ecotoxicological techniques in the UK and Worldwide contained in Appendix B. Four main categories of need have been identified:

1. The derivation of Environmental Quality Standards

2. The setting and monitoring of Environmental Quality Objectives

3. The setting and monitoring of toxicity-based consents

4. The detection and investigation of pollution incidents

Clearly, in some instances, the four main categories will have ecotoxicological techniques in common. However, this review should assist the NRA in focusing effort and attention on the most suitable methods for each situation.

The appendices contain the body of the review, a detailed examination of the use of ecotoxicology in the UK and worldwide (particularly the US) and specimen consents for industrial discharges in the UK for which there is a toxicity component.

1.2 THE ROLE OF THE NRA IN THE PROTECTION OF AQUATIC SYSTEMS

The 1989 Water Act (HMSO 1989) lists the duties and responsibilities of the National Rivers Authority. The NRA has to:

a) ensure, as far as practicable, that any Water Quality Objectives (WQOs) established by the Secretary of State for the Environment for specific types of water use are achieved;

b) monitor the extent of pollution in 'controlled' inland, estuarine and coastal waters;

c) maintain, improve and develop fisheries and

d) promote the conservation of aquatic organisms.

To achieve these objectives the NRA has both to classify and monitor the controlled waters within its jurisdiction, and derive and monitor consent conditions for effluents discharged to these waters.

The NRA has to develop Water Quality Objectives (WQOs) for all reaches of controlled waters. Three criteria have to be met for a water body to satisfy the WQO. The controlled water must:

a) meet its target classification, based on chemical and biological parameters;

b) achieve its use-related Environmental Quality Objectives (EQO). There are fourteen designated uses of a water body, of which basic amenity and general ecosystem protection are common to all reaches. The defined EQOs have supporting Environmental Quality Standards (EQSs) for a range of determinands that the water body has to satisfy to achieve its EQO.

c) satisfy standards set in any relevant EC Directives.

Waters are classified within the EQO framework by reference to the purposes for which they should be suitable. To satisfy the EQOs, specific Environmental Quality Standards (EQS) have been established for a range of dangerous substances. These chemical concentrations are derived from an evaluation of available aquatic toxicological data. An EQS for a particular water body is the concentration of a substance in receiving waters, outside a specified mixing zone, that cannot be exceeded if a defined EQO is to be maintained (NRA 1990). For general ecosystem protection the EQSs currently under development are either:

i) for List I substances, those of the relevant EC directives.

ii) for List II substances, those determined by the UK from available toxicological data with appropriate application factors (Gardiner and Mance 1984)

In cases where an EQS is not available, toxicological data can be used to derive a 'Likely Safe Environmental Concentration' (LSEC) as a surrogate EQS for consent setting. For compounds on which there is no or poor quality toxicological information, appropriate quantitative structure-activity relationships (QSARs) and biological compartment models could have a major role in deriving EQSs or developing LSECs, although the safety factors used in translating toxicity values to tentative EQSs or LSECs would have to reflect any uncertainty associated with the accuracy of the QSAR used.

EQOs are achieved by ensuring that discharges to the aquatic environment are controlled by a system of discharge consents or permits. It is an offence to cause or knowingly permit the contamination of controlled waters without a consent to discharge effluent. The consent conditions are specified at the discretion of the NRA according to the type of effluent and the character of the receiving water, and the discharger has to pay for any samples or tests required by the NRA for the establishment or monitoring of the consent.

There are approximately 139 000 'active' consents in England and Wales, of which the majority are descriptive, covering small discharges considered to be of limited environmental significance (NRA 1990). However, a large number of discharge consents contain specific numeric limits for one or several of their chemical constituents, that are designed to ensure that EQSs in the receiving water are not exceeded, and that the EQOs are maintained.

1.3 CURRENT MONITORING AND REGULATORY TOOLS USED BY THE NRA

A number of chemical, hydrological and biological techniques are currently used by the NRA when monitoring catchments, estuaries and coastal waters, and setting and monitoring discharge consents.

At present approximately 12 000 consented effluents in England and Wales are sampled regularly. Almost 5000 of these sampled effluents are from sewage treatment works (NRA 1990). Chemical techniques have

traditionally been used to analyse these samples (Metcalfe 1989), in conjunction with hydrodynamic models of transport, dilution and dispersion. Only a limited number of chemical determinands are commonly measured in water samples from consented effluents. Biological and chemical oxygen demand, suspended solids and ammonia levels are usually assessed, and further determinands are selected depending on the nature of the effluent.

The biological techniques currently used by the NRA include fish surveys, microbiological determinations and sporadic bioaccumulation studies, the latter designed to investigate the uptake and accumulation of inorganic and organic pollutants by aquatic organisms at specific sites. In freshwaters, the biological assessment of water quality has increasingly concentrated on the Biological Monitoring Working Party (BMWP) score, and its associated Average Score Per Taxon (ASPT) (Armitage *et al* 1983). The BMWP score is an index that combines into a single numerical expression both a quantitative measure of macroinvertebrate species diversity and qualitative information on the ecological sensitivities of individual taxa (Metcalfe 1989). The River Invertebrate Prediction and Classification System (RIVPACS), a multivariate technique has recently been used to identify the biological communities representative of certain physical and chemical characteristics in rivers. 'Best achievable' communities can then be identified for particular sites and comparisons made between 'observed' and 'expected' BMWP and ASPT results, with the differences possibly ascribed to particular discharges (Armitage *et al* 1983, 1987; Wright *et al* 1984, 1986, 1989; Furse *et al* 1984; Moss *et al* 1987). Considerable resources were committed by all NRA Regions during 1990 in an effort to produce observed and expected results for the majority of lotic systems in England and Wales.

In summary, the NRA uses the following techniques for meeting its statutory duty to protect aquatic organisms and ecosystems:

a) chemical analysis of several general, and certain site-specific determinands for consenting and monitoring effluent discharges and

b) fish, macro-invertebrate and microbiological surveys and limited bioconcentration studies for monitoring water quality to ensure compliance with EQOs.

In many situations the use of these techniques within a properly resourced and planned framework should be an entirely adequate approach to protecting the aquatic environment. There are, however, certain particular situations for which neither detailed chemical analysis nor biotic indices may be the most appropriate techniques or where there may be discrepancies between the conclusions drawn from these techniques.

1.4 LIMITATIONS OF CURRENT TOOLS

1.4.1 Chemical analysis

A chemical specific approach is suitable for the control of simple, well-defined and consistent effluent discharges containing substances for which EQSs have been derived. However, there are a number of disadvantages to an entirely chemical-specific approach to effluent control (OECD 1987; Hunt 1989), as

a) effluents often contain organic chemical components which cannot be readily identified or quantified by available analytical techniques;

b) there are few or no toxicological data for many organic chemicals and those that are available may not be relevant to indigenous species;

c) for highly complex effluents, particularly those of variable composition, these problems may be exacerbated by excessive costs for the accurate measurement of all the chemicals present, and problems in applying EQSs, which are derived on the basis of single substance toxicity. These do not allow for possible chemical interactions between different discharge components or synergistic or antagonistic toxicological interactions between substances.

These problems have led the OECD (1987) and Hunt (1989) to advocate the Direct Toxicity Assessment (DTA) or whole effluent approach, in which the toxicity of potentially hazardous complex, and variable, effluents is assessed directly using rapid, cost-effective toxicity tests. The results of these tests assist in establishing toxicity based consents (TBCs), with compliance being monitored by a regular programme of toxicity testing using appropriate standard methods.

The use of toxicity based consents for effluent discharge regulation has recently been recommended in the NRA report on 'Discharge Consent and Compliance Policy' (NRA 1990). Recommendation 16 of the report states that:

> "For environmentally significant discharges of complex composition where not all important constituents can be individually identified and numerically limited, consents should specify a clearly-defined toxicity limit, the appropriate form of toxicity test to be used, and the minimum frequency with which it should be applied."

1.4.2 Biotic indices

The BMWP system has proved to be a useful tool for dewatering the biological community present in British rivers. However, Metcalfe (1989) identified four major problems with the use of biotic scores for monitoring aquatic pollution;

a) the BMWP score approach was developed for assessing degradable organic pollution, such as that emanating from sewage treatment works, and may not be an appropriate indicator of industrial toxic impact;

b) organisms intolerant of organic pollution have shown tolerance to specific toxicants, and vice versa (Slooff 1983);

c) the BMWP score uses family level identification, although species belonging to the same taxon may exhibit as much variation in susceptibility to a toxicant as species from different taxa (Slooff 1983);

d) confounding factors, such as flow regime and nutrient levels, may mask the effects of toxic stress in aquatic systems (La Point *et al* 1984; Dance and Hynes 1980).

A further problem with faunal surveys is that pollutant impact cannot be detected until a change in score is registered, representing a reduction in species richness. Consequently this type of method may not be the most effective for limiting pollutant-induced damage to aquatic environments.

1.5 THE ROLE OF AQUATIC TOXICOLOGY IN WATER QUALITY REGULATION

There are limitations to both a chemical-specific approach to discharge consent setting and monitoring, and the use of biotic indices for monitoring receiving water quality. Toxicological tests are available at all levels of biological organisation and can reflect the progression of pollutant-induced effects from the initial behavioural and biochemical responses of individuals to ultimate effects at the community level. These methods can be used by water quality regulators in three main ways:

1) for setting and monitoring toxicity based consents;

2) to protect aquatic ecosystems by providing an early warning of potential problems considerably in advance of changes in community structure;

3) for monitoring receiving water and sediment quality where pollutant sources are episodic or from non-point sources, and routine chemical monitoring may not detect pollutant exposure.

In all cases, the use of ecotoxicological methods should be seen as complementary to existing techniques and a means of providing additional evidence on which to base regulatory decisions, rather than as a replacement for chemical monitoring and biological surveys.

SECTION 2 - SUMMARY OF ECOTOXICOLOGICAL APPROACHES AND TEST METHODS

2.1 INTRODUCTION

Although the use of ecotoxicology for the regulation and monitoring of aquatic pollutants has in the past mainly involved whole organism responses, techniques at all levels of biological organisation have the potential for reflecting changes in water quality. An hypothetical time-related sequence of events of pollutant impact on biological effects at various levels of organisation is shown in Figure 2.1. The effects outlined may result from the impact of pollutants present in the dissolved or particulate phases of the water column, or those associated with sediments.

Pollution may be perceived by an organism's sensory system, and this could lead to avoidance behaviour. In motile organisms this may be movement away from the region of pollution, while in sessile forms avoidance may involve reducing exposure of external body surfaces through mucous production or withdrawal into shells or burrows.

Continued exposure to pollution will generally lead to the accumulation of the toxicant and resultant biochemical responses, which may include:

a) alterations in enzyme activity and enzyme induction;

b) alterations in the rates of synthesis of nucleic acids, protein, lipid and carbohydrate and;

c) activation or suppression of metabolic pathways associated with energy production and biosynthesis.

Pollutant-induced effects on certain biochemical responses may be compensated for by changes in other processes. In addition the activation of detoxification mechanisms may restrict the effects of accumulated toxicants. However, in situations where compensatory or detoxification mechanisms cannot restrict the toxic effects of internal pollutants, physiological processes such as oxygen consumption, feeding and excretion rates, assimilation efficiency, and osmotic and ionic balance will be affected and lead to alterations in physiological status.

A continuation of chronic pollutant exposure may ultimately result in a reduced physiological status being translated into adverse effects on the growth, reproduction or survival of individuals. These changes will affect biomass and recruitment and may influence the population level. The effects of pollution on 'key' populations may then have consequences for the biological community by affecting its structure or function.

Although there is a greater understanding of response at the biochemical and physiological levels of biological organisation, there is a lower perceived ecological relevance in the results obtained from these techniques compared to those obtained from the assessment of effects at the population and community levels. These factors have to be considered in selecting an appropriate ecotoxicological technique, or suite of techniques for a particular purpose.

The conclusions from a detailed review of the literature (Appendix A) are summarised below in tables and text.

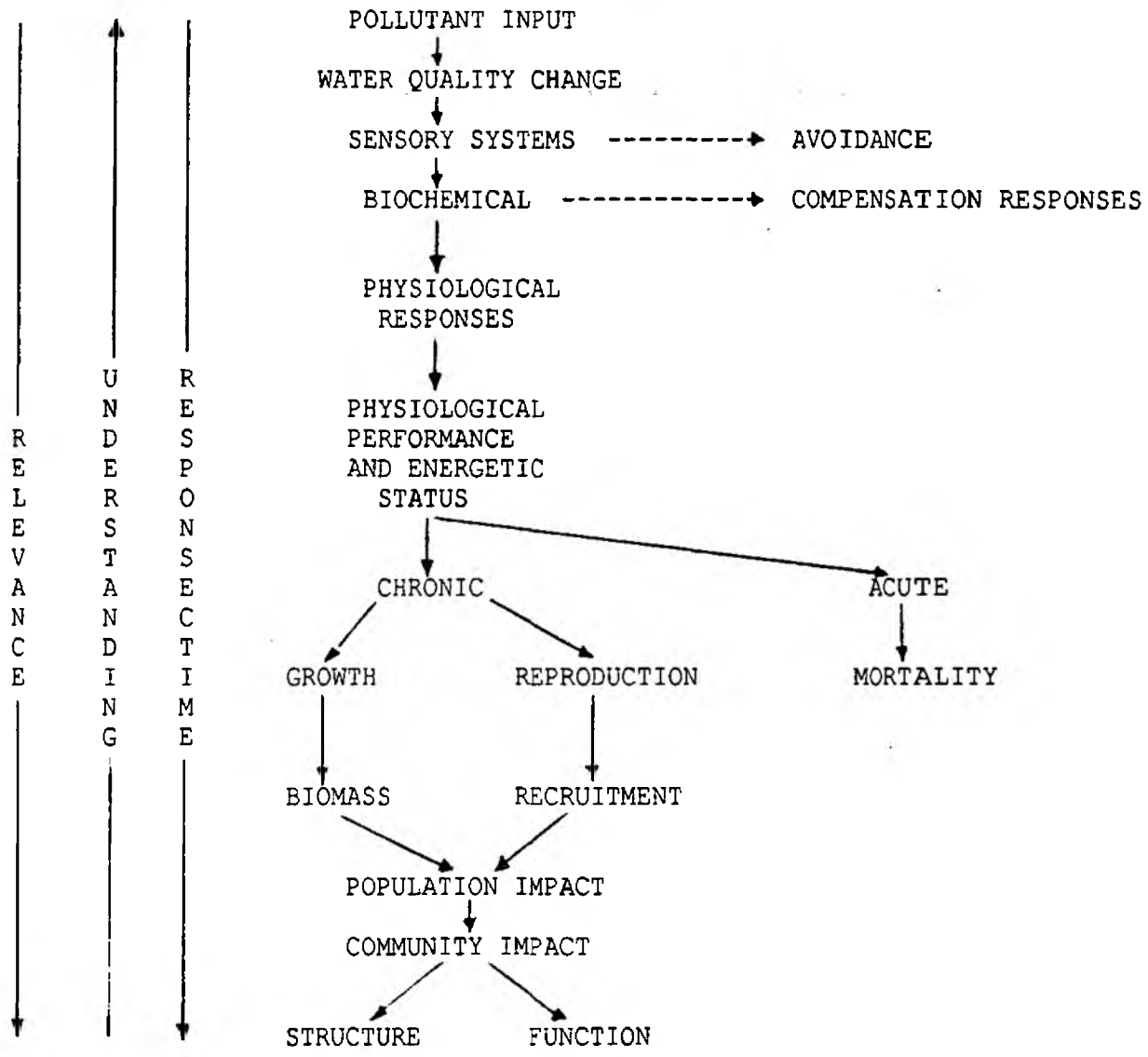

Figure 2.1 - Hypothetical sequence of events of pollutant induced effects at various levels of biological organisation and their ecological relevance and understanding

2.2 COMMUNITY TESTS

There are strong arguments in favour of increasing the use of multispecies community test systems. At present these are used at a high level in a hierarchy of effluent toxicity evaluation, after a preliminary assessment of hazard using a range of standard single-species tests. However, this situation could change if a cheap,

robust and effective multispecies test was available at the screening stage.

The selection of an appropriate community test depends upon the specific character and location of each discharge, and the organisms used should be naturally-derived and representative of the local biological community. A sufficient number of well-tested systems exist to cover the range of aquatic habitats likely to be impacted by a discharge (Tables 2.1 and 2.2). The measurements taken within these systems should determine both the fate and the biological effects of the test substance. The selected toxicological endpoints should cover the range of biological organisation, but specifically concentrate on ecosystem-level measurements such as community metabolism, diversity and richness, and the cycling of essential nutrients.

2.3 POPULATION TESTS

The intrinsic rate of population increase and age-specific survivorship and fecundity are important and ecologically relevant parameters that can be measured in the laboratory. Studies have been successfully performed with several standard toxicity test organisms. The influence of many biotic or abiotic environmental variables may, however, be critical in determining the population-level effects of a contaminant. Field or microcosm studies that investigate all or many of the key factors affecting the population of interest, such as Woltering (1985) and Stacey and Marcotte (1987), will be more ecologically realistic than those performed with single populations.

2.4 WHOLE ORGANISM LETHALITY TESTS

Toxicity tests in which death is the measured endpoint remain the most common type of test for assessing receiving water, effluent and sediment toxicity (Table 2.3). Lethality results are simple to understand and the methods used to obtain them are generally simple, standardised, cost-effective and reproducible. A wide range of organisms from different phases and habitats have been used in standard tests of this

type. Acute or chronic lethal tests will, however, fail to measure any sublethal effects caused by pollution and, as with any single-species test, population or community-level effects may be under or overestimated. In addition, large safety factors have to be applied when extrapolating from lethal effects concentrations to those which can be considered appropriate for preventing toxic impact.

2.5 WHOLE ORGANISM SUBLETHAL TESTS: PHYSIOLOGY

Several approaches are available for assessing the effect of water column and sediment contamination on sublethal physiological parameters (Tables 2.4 and 2.5). Growth and reproduction are the two parameters that can most readily provide ecologically relevant information, and should, therefore, be the measured end points of first choice in deriving information for management decisions. There are established international methods for measuring growth and reproduction in aquatic plants, algae, invertebrates and fish and the US EPA have developed short-term (7 day) tests with crustaceans and fish (Mount and Norberg 1984; Horning and Weber 1985; Weber *et al* 1988; Stewart *et al* 1990) to reduce the long testing periods (21-28 days) usually associated with other tests. The use of sub-lethal endpoints, such as growth and reproduction, obviates the need for acute to chronic (ACR) ratios in regulatory and management decisions.

Physiological techniques, such as feeding rate, may be related to growth and reproduction and can act as sensitive early warning systems. A bioluminescence-based toxicity test, Microtox, using the bacterium *Photobacterium phosphoreum* has proved to be a useful tool for assessing effluent toxicity and monitoring pollution incidents. A review of this technique has been prepared for the NRA in the 'Direct Toxicity Assessment' programme (Ref No A18.049). A further test using this response with genetically engineered *Escherichia coli* is currently under development and apparently has considerable potential (Stewart *et al* 1989).

2.6 WHOLE ORGANISM SUBLETHAL TESTS: BEHAVIOUR

The measurement of behavioural changes in aquatic organisms, such as preference-avoidance reactions, abnormal locomotor and reproductive behaviour and altered predator-prey interactions, offer considerable potential as early warning systems of pollutant impact. The methods developed vary considerably in terms of cost, complexity and required expertise (Tables 2.6 and 2.7). A number of techniques using crustaceans, molluscs and fish, and involving indices such as avoidance locomotor activity, positive rheotoxis and phototaxis, have been developed as automated continuous monitoring systems (Section 2.8).

2.7 SUB-ORGANISM TESTS

Appropriate suborganism methods should be applied either as a suite of methods, in an approach analagous to clinical testing for human health, or individually in association with methods at a higher level of biological organisation, to provide additional information on the mode of toxic action (Tables 2.8, 2.9, 2.10 and 2.11).

Tissue Damage

Pathological changes in animal tissues are the net effects of *in situ* biochemical and physiological changes, and can provide information on toxicant target sites. The use of quantitative histopathological techniques can provide sensitive and biologically meaningful methods for measuring the effects of pollutants, particularly when related to other chemical or biological monitoring techniques. Tissue changes may not always be sensitive indicators of environmental stress, particularly where obvious catastrophic damage such as destruction of the respiratory surfaces has occurred. In such cases resulting mortality may be a more easily measured and appropriate toxic endpoint.

Genotoxicity

The effect of genotoxicity on natural populations is a new area in ecotoxicology, and hence one on which there is limited information. The endogenous induction of DNA damage may be fairly common in some organisms and the ecological relevance of the effects of exogenous factors, such as pollution, could be difficult to interpret. At present, most tests for genotoxicity are probably best used for ranking substances according to hazard, identifying specific contaminants in mixtures or focusing investigations on important conventional parameters. The induction of unscheduled DNA synthesis is probably the endpoint that has most long term relevance for individual fitness.

Cytotoxicity

The effects of pollutants on specific cells are not widely used in a regulatory or monitoring role at present, although there is increasing interest in immunological assays (Anderson 1990). However, in the future, increased costs and public pressure to reduce animal testing may mean that the use of fish cell lines, and in particular, cultured fish hepatocytes, will become a valuable replacement for traditional whole organism responses.

Biochemical indices

Although considerable research activity has focused on the effects of pollution on biochemical indices, the majority of techniques have yet to be related to or calibrated with important whole organism responses such as growth and reproduction. However, the site-specific application of a suite of biochemical techniques measuring 1) the activation of enzymes associated with the detoxification of organics, such as ethoxyresorufin 0-deethylase (EROD) and glutathione-s-transferase (GST) and 2) the induction of metal-binding proteins, such as metallothioneins appears to be useful for identifying and quantifying the impact of specific pollutants.

2.8 BIOMONITORING SYSTEMS

In recent years a range of automated biosensors (in which a biological sensing element is either intimately connected to or integrated with a transducer) have been developed, primarily based on the physiological and behavioural responses of a wide range of aquatic organisms from bacteria to fish. These biomonitoring systems can be applied to water intake protection, effluent discharge assessment and environmental monitoring for pollution incidents, and can provide a rapid indication of pollutant impact. In addition to the non-specific biosensors described, which respond to a wide range of pollutants and effluents, immunoassay-based sensors specific to given compounds have been developed (Rawson *et al* 1989, Baldwin 1990).

At present, commercially available early warning systems principally involve fish, although bacteria, crustaceans and bivalve molluscs are also used (Table 2.12). Van der Schalie (1986) and Baldwin (1990) have reviewed the usefulness of available techniques and concluded that biomonitors are suitable for detecting pollutants at levels that are generally acutely toxic to aquatic organisms. The longer response times before sub-lethal effects are detected may reduce their potential. However the requirements of a continuous biomonitor, in terms of the balance between sensitivity and accuracy, will depend on the regulatory or monitoring use to which it is being applied. At this time biomonitors can complement, though not replace, existing physico-chemical water quality monitors.

2.9 BIOACCUMULATION

Standard OECD protocols are available for performing static, semi-static or flow-through bioaccumulation and bioconcentration laboratory studies with fish (OECD 1981). Numerous studies have also reported results on the bioaccumulation of organic and inorganic contaminants in many species in the field. This area of ecotoxicology will be dealt with fully in a forthcoming report by WRc on toxicity and body burdens, commissioned by the NRA (A18.1.212).

Table 2.1 - Community tests: information on methods for assessing the impact of pollutants on the aquatic environment

Method	Description of system	Taxa used	Type of endpoint	Source literature
LENTIC FRESHWATER				
Gnotobiotic Laboratory Microcosms	Several species of plants and animals are selected as 'typical' representatives of their respective functional groups.			
- Compartmentalised	Test species are exposed in separate but connected compartments. Interactions are limited to effects on inflow and outflow of medium.	Algae (producer compartment) Daphnia (herbivore compartment), Hydra or fish (carnivore compartment) bacteria (decomposer compartment).	Species numbers, bioaccumulation, functional responses	Kersting 1975, 1984 de Zwart & Langstraat 1988 Vighi 1981
- Uncompartmentalised	Test species are exposed in same container and allowed to interact.	Algae, various micro and macro crustacea, oligochaetes, mosquito larvae and occasionally fish.	Species numbers, bioaccumulation, functional responses, fate of contaminant.	Taub 1989 Huckins *et al* 1986 Metcalf *et al* 1973
NATURALLY DERIVED SYSTEMS	Natural colonisation of the water column or substrate is allowed to occur, although some additional species may be added by operator.			
- Microbial colonisation	Polyurothane blocks are colonised with pond microbes. Rate of colonisation of barren substrata then assessed in different contaminant concentrations.	Protozoans, diatoms and other microscopic taxa.	Colonisation rate, species numbers.	Cairns *et al* 1990
- Plankton (Mixed Flask Cultures)	One litre vessels are inoculated with planktonic communities.	Algae, grazers, detritivores bacteria and protozoa.	Species numbers, functional responses.	Stay *et al* 1989a, 1989b
- Multiple trophic levels	Normally sediment and water phases included in small bench top vessels.	Wide range of macrophytes, algae and invertebrates, sometimes fish.	Species numbers, bioaccumulation, functional responses, fate of contaminant.	Johnson 1986 Burnett & Liss 1990 Giddings 1986

Table 2.1 - continued

Method	Description of system	Taxa used	Type of endpoint	Source literature
- Lake enclosures	Flexible or rigid plastic enclosures, with or without sediment.	Pelagic or pelagic and benthic lake communities, sometimes with fish excluded.	Primarily species abundance, occasionally functional responses, bioaccumulation or fate of contaminant.	Gachter 1979 Sanders 1985 Brazner & Kline 1990
- Experimental ponds	Small, shallow, replicated ponds with sediment and water phases.	Naturally colonising pond flora and fauna. Fish sometimes added.	Species numbers, bioaccumulation, functional responses, fate of contaminant.	Crossland *et al* 1987 Giddings *et al* 1984 Dutka & Kwan 1984
LOTIC FRESHWATER				
	Artificial streams.	Naturally derived stream organisms.	Various (see below).	Warren & Davis 1971 Shriner & Gregory 1981
- Aufwuchs and periphyton	Usually small laboratory-based model streams, often without sediment. Test communities normally exposed on artificial substrata such as glass slides.	Bacteria, fungi, protozoa and algae.	Species numbers, bioaccumulation, functional responses, fate of contaminant.	Sanders 1982 Genter *et al* 1987 Lewis *et al* 1986
- Macroinvertebrates	Includes laboratory-scale model streams with naturally derived sediment and invertebrates, and field based flumes.	All stream flora and fauna except higher predators (eg, fish).	Species numbers and behaviour (drift), bioaccumulation, functional responses.	Clements *et al* 1989a 1989b Muirhead-Thomson 1987
- Multiple trophic levels	Usually large indoor or outdoor streams that attempt to mimic closely natural lotic systems.	All stream flora and fauna. Fish species are normally selected by the operator and may be confined to only part of the system.	Species numbers, bioaccumulation, functional responses.	Newman & Perry, 1989 Armitage 1980

Table 2.1 - continued

Method	Description of system	Taxa used	Type of endpoint	Source literature
- Artificial leaf packs	Teflon 'leaves' in mesh bag, naturally colonised by stream macroinvertebrates, are used to test contaminants in static or flow through systems.	Primarily macroinvertebrates.	Species numbers.	Livingston 1988
MARINE AND ESTUARINE				General Gearing 1989
- Enclosures	Flexible or rigid plastic enclosures, with or without sediment, normally large (>1000 litres).	Natural pelagic and/or benthic communities, but fish usually excluded.	Species numbers, bioaccumulation, functional responses, fate of contaminant.	Grice & Reeve 1982 Kuiper 1983 Topping *et al* 1982
- Land-based tanks	Rigid land-based tanks, normally with water and sediment phases. Normally large (>1000 litres).	Natural pelagic and benthic communities. Fish (eg, plaice) sometimes added.	Species numbers, bioaccumulation, functional responses, fate of contaminant.	Sullivan & Rittacco 1988 Saward *et al* 1975 Morton *et al* 1986
- Small land-based tanks	Laboratory-scale experiments with benthic colonisation from either field placement or seawater flowing through system.	Benthic invertebrates.	Species numbers.	Tagatz 1986

Table 2.2 - Critical evaluation of community methods for assessing the impact of pollutants on the aquatic environment

Method	Available Studies	Merits of the Method	Limitations of the Method
Microcosms in general	Many	Closer in character to natural systems Bioaccumulation through food chain fate and effects can all be studied Indirect effects can be investigated Systems provide large amounts of information per test	Different levels of mixing and other physico-chemical processes to natural systems. 'Edge effects' on biological parameters. Limit on number of samples that can be removed. Difficulty in choosing most sensitive end points Divergence of systems over time. Higher cost. Difficulties in interpreting effects.
LENTIC FRESHWATER			
GNOTOBIOTIC LABORATORY MICROCOSMS			
Compartmentalised	Limited	Simple to control and interpret.	Little similarity with natural systems. Interactions between species not considered. Fate of contaminants not considered.
Uncompartmentalised (Standardised Aquatic Microcosms) Taub 1989	Several with wide range of substances tested.	SAM Fully tested protocol and statistical analysis package. Generally repeatable and transferable methodology. Relatively inexpensive for a microcosm test (425 person hours. Shannon *et al* 1986).	Little similarity with natural systems. Fate of contaminants not considered.
NATURALLY DERIVED SYSTEMS			
Microbial colonisation	Several, on pure substances and complex effluents, using variations on basic techniques (eg, static or flow-through).	Simple Rapid Relatively inexpensive May be used with or without sediment	Possibly not very sensitive. Test species represent only a small albeit important section of the community. Fate of contaminants not considered.
Mixed Flask Cultures	Limited but increasing number being reported.	Simple Rapid Relatively inexpensive Low variation Repeatable in time and space Apparently sensitive to wide range of contaminants.	Divergence of replicates precludes long-term tests. Fate of contaminants not considered.
Multiple trophic levels	Many, with a wide range of contaminants.	Water and sediment phases present Closely mimic natural communities Investigation of fate of contaminants possible.	Relatively site-specific Sample sizes limited by size of microcosm Work on the systems relatively sporadic No one system has emerged as superior

Table 2.2 - continued

Method	Available Studies	Merits of the Method	Limitations of the Method
Lake enclosures	Several, including one intensive study, MELIMEX* *MELIMEX = Metal Limnological Experiment (Gachter 1979)	Fate, effects and bioaccumulation can all be studied.	Costly because of low level of replication 'Edge' effects Divergence of system from natural environment over time.
Experimental ponds	Several, especially for pesticide registration purposes in US.	Fate, effects and bioaccumulation can all be studied. Long-term studies possible.	Ponds may not be representative of the type of lenthic system normally receiving effluent. Costly
EXPERIMENTAL STREAMS			
Aufwuchs periphyton, macrophyte and multiple trophic levels	Many different functional groups in small and large streams.	Fate, effects and bioaccumulation can all be studied. Long-term studies possible. Effect of contaminants on drift can be studied.	Costly Problems in disposing of waste medium Low level of replicability in larger systems. patchy distribution of invertebrates makes sampling difficult.
Artificial leaf packs	Few	Rapid Cheap Flexible Appears to predict in-stream effects	Fate of contaminants not considered. 'Realism' of substrate low.
MARINE AND ESTUARINE			
Enclosures	Several, including one intensive study, CEPEX (Controlled Ecosystems Pollution/Populations Experiment, (Grice & Reeve 1982)	Fate, effects and bioaccumulation can all be studied.	Costly Engineering difficulties Edge effects (eg, alteration of incident light) Enclosure effects (eg, interference with mixing) Low level of replication Divergence of system from natural environment over time.
Land-based Tanks	Several, including one intensive study, (Marine Ecosystems Research Laboratory, Sullivan & Ritacco 1988)	Fate, effects & bioaccumulation can all be studied. Continuous mixing of water possible. Greater control over systems possible than for enclosures. Replication generally possible.	Costly Small size precludes natural population of top predators.
Small Land-based Tanks	Several, on a wide range of contaminants	Rapid Relatively cheap High level of replication possible.	Fewer endpoints: usually macroinvertebrate richness or diversity. Fate of contaminants not considered.

Table 2.3 - Toxicity tests using a lethality endpoint which are currently included in OECD, EPA EC, BSI and ISO guidelines for fresh and marine waters

Trophic level	Species used	Type of test	Guidelines and toxicity indices				
			OECD	EPA	EC	BSI	ISO
Invertebrates	*Daphnia magna*	Acute	24 hr EC50	24/48 hr EC50	24/48 hr EC50	-	-
		Chronic	-	21 d EC50	-	-	-
	Daphnia pulex	Acute	-	24/48 hr EC50	24/48 hr EC50	-	-
		Chronic	-	21 d EC50	-	-	-
	Mysidopsis bahia	Acute	-	48/96 hr LC50	-	-	-
		Chronic	-	28 d LC50	-	-	-
	Penaeid shrimp	Acute	-	48/96 hr LC50	-	-	-
Fish	*Brachydanio rerio* (Zebra fish)	Acute	96 hr LC50	-	96 hr LC50	96 hr LC50	96 hr LC50
		Chronic	NOEC/LOEC	-	-	-	-
	Oncorhyncus mykiss (Rainbow trout)	Acute	96 hr LC50	96 hr LC50	96 hr LC50	-	-
		Chronic	NOEC/LOEC	-	-	-	-
		ELS	-	NOEC/LOEC	-	-	-
	Pimephales promelas (Fathead minnow)	Acute	96 hr LC50	96 hr LC50	96 hr LC50	-	-
		Chronic	NOEC/LOEC	NOEC/LOEC	-	-	-
		ELS	-	NOEC/LOEC	-	-	-
	Lepomis macrochirus (Bluegill)	Acute	96 hr LC50	96 hr LC50	96 hr LC50	-	-
		Chronic	NOEC/LOEC	-	-	-	-
	Cyprinus carpio (Carp)	Acute	96 hr LC50	-	96 hr LC50	-	-
		Chronic	NOEC/LOEC	-	-	-	-
	Poecilia reticulata (Guppy)	Acute	96 hr LC50	-	96 hr LC50	-	-
		Chronic	NOEC/LOEC	-	-	-	-
	Orzias latipes (Red killifish)	Acute	96 hr LC50	-	96 hr LC50	-	-
		Chronic	NOEC/LOEC	-	-	-	-
	Leuciscus idus (Golden orfe)	Acute	-	-	96 hr LC50	-	-
	Salvelinus fontinalis (Brook trout)	ELS	-	NOEC/LOEC	-	-	-
	Cyprinodon variegatus (Sheepshead minnow)	Acute	-	96 hr LC50	-	-	-
		Chronic		NOEC/LOEC			
		ELS		NOEC/LOEC			

The chronic fish tests are conducted over 14 days and the early life stage (ELS) tests are for 28 days after hatching for trout species and 60 days after hatching for minnows.

Table 2.4 - Physiological responses: Information on methods for assessing the impact of pollutants on the aquatic environment

Method	Measured endpoints	Species used	Types of contaminant shown to induce a response	Source literature
Growth rate	Change in biomass of individuals	Many, at different trophic levels	Inorganics and organics	OECD 1984 EPA 1982
Scope for growth	Feeding, assimilation, respiration and excretion	*Mytilus edulis* *Gammarus pulex*	Inorganics and organics	Bayne 1985 Naylor *et al* 1989
Oxygen to nitrogen ratio	Rate of oxygen consumption and ammonia excretion	Marine mussels and shrimps	Oil, metals and suspended solids	Carr *et al* 1985 Aldridge *et al* 1987
Reproduction rate	Time to first reproduction size, number and energy content of offspring. Amount of energy allocated to gamete production	Many different fish and invertebrates	Inorganics and organics	OECD 1984 EPA 1982
Whole body indices	Proportion of shell volume occupied by tissue	Molluscs	Little information	Bayne 1985
Respiration rate	Uptake of oxygen	Many, at different trophic levels	Metals and organics	Dutka *et al* 1983 Prasad 1987
Ventilation rate	Gill movement	Fish	Organics and inorganics excluding metals	Baldwin 1990
Photosynthesis	O_2 production Fate of ^{14}C tracer	Usually algae	Metals and organics	Vollenweider 1969
Osmoregulation	Tissue water content Concentration of ions in blood Sodium/potassium ATP activity	Many different fish and invertebrates	Oil and metals	Baden 1982 Oikari *et al* 1985
Bioluminescence	Light output	*Photobacterium phosphoreum* *Escherichia coli*	Organic chemicals and complex mixtures	Munkittrick *et al* 1990 Stewart *et al* 1990
Electrical activity in weakly electric fish	Rate of electrical pulse discharge	Elephant-nosed mormyrid	Metals, but possibly other toxicants	Baldwin 1990

Table 2.5 - Critical evaluation of physiological methods for assessing the impact of pollutants in the aquatic environment

Method	Available studies	Merits of the method	Limitations of the metal
Growth rate	Many in laboratory and field	- Direct ecological relevance - Standard methods available - Responds to range of pollutants	- Some standard methods insensitive (eg algae)
Scope for growth (SFG) and feeding rates	Many in laboratory and field, particularly for *Mytilus edulis*	- Direct ecological relevance - Rapid assessment of growth potential - Responds to range of pollutants	- Measurement can be arduous (especially for SFG) - Variation in individual responses often high
Oxygen to nitrogen ratio	Limited laboratory studies	- Responds to range of pollutants	- Response direction variable - Ecological relevance questionable without supporting data from other tests
Reproduction rate	Many in laboratory and field	- Direct ecological relevance - Standard methods available - Responds to range of pollutants - Generally very sensitive	- Some standard methods need to be refined
Whole body indices	Limited laboratory and field studies	- Responds to range of pollutants - Direct ecological relevance - Can be sensitive	- Only possible with one taxon - molluscs - Methodology needs refinement - Affected by reproductive condition
Respiration rate	Many in field and laboratory, especially with bacteria	- Responds to range of pollutants - May occur rapidly	- Shape of response sometimes difficult to interpret - Acclimation of response may occur - Bacterial respiration rate probably not very sensitive - Can only be linked indirectly to ecologically relevent parameters

Ventilation rate	Several field and laboratory studies	- Rapid and sensitive response
		- Responds to range of pollutants
		- Allows continuous monitoring
			- Not responsive to metals
			- Sensitivity may be affected by:
			  - species used
			  - condition and biological variability of stock
			  - interference from external stimuli other than pollutants

Photosynthesis	Many in laboratory and field	- Standard methods available - Responds to range of pollutants - Direct ecological relevance	- Generally only possible to run short term expert experiments
Osmoregulation	Many in laboratory and field	- Responds to range of pollutants	- Not always sensitive - Can only be linked indirectly to ecologically relevant parameters
Microtox	Increasing number in laboratory and field	- Inexpensive - Sensitive to a range of contaminants - Reproducible	- Insenstive to some important contaminants - Little ecological relevance
Electrical activity in weekly electric fish	Limited laboratory and field studies	- Possibly sensitive - Allows continuous monitoring	- Little ecological relevance - Expensive

Table 2.6 – Behavioural responses: Information on potential methods for assessing the impact of pollutants on the aquatic environment

Method	Functional consequence	Type of endpoint	Specificity of response	Species used	Available literature
Preference/Avoidance	Modify the extent to which organisms are exposed to aquatic pollutants	Respones of organism to increase (preference) or reduce (avoidance) pollutant exposure	Inorganics/ Organics	Fish Amphipods Molluscs	Cherry and Cairns 1982
Reproductive behaviour	Modify fecundity of organisns and effect population structure	Changes in length of time that precopula pairs are separated	Inorganics/ Organics	Amphipods	Poulton & Pascoe 1990
Activity	Reduction in fitness	Change in locomotor activity	Inorganics/ Organics	Fish Crustacean Insects	Baldwin 1990
Positive rheotaxis	Loss of habitat through drift	Response of organism to current	Inorganics/ Organics	Fish	Baldwin 1990
Phototaxis	Modify the population structure	Response of organism to light	Inorganics/ Organics	Crustaceans	Knie 1978, 1982
Predator-prey interactions	Modify the structure of the community	Changes in rate of predator (mysids) feeding on prey (copepods)	Not tested with pollutants	Invertebrates	Cooper and Goldman 1982

Table 2.7 - Critical evaluation of potential behavioural methods for assessing the impact of pollutants on the aquatic environment

Method	Available studies	Merits of the method	Limitations of the method
Behavioural responses in general		- Often extremely sensitive and easily learned techniques - Techniques offer potential for a high degree of automation	- The techniques require considerable information on the 'normal' behaviour of organisms in order that pollution effects can be infered - Although tests may be short-term, long acclimation periods to laboratory conditions may be required to establish 'normal' behavioural patterns and restrict background noise - There may be problems in applying laboratory derived behavioural responses to field situations
AVOIDANCE BEHAVIOUR:			
Simple observational methods	Laboratory only	- Sensitive easily conducted tests	- Need to be used in association with other techniques
Automated techniques (shell valve activity monitor)	Limited laboratory and field studies	- The electronic interface facilitates automated data collection and data interpretation - The small size and rigidity of the system allows its use under both laboratory and (semi) field conditions, the latter being essential for application as an Early Warning System	- Additional testing, particularly under field conditions is required Biological factors such as the effect of adaptation, seasonal variation and reproductive state have to be studied
ABNORMAL BEHAVIOUR			
Pre-copula pairs	Limited laboratory and field studies	- The techniques for measuring direct and indirect separation time of pre-copula pairs of amphipods are simple to conduct	- The interpretation of pollutant induced effects on separation times, in terms of consequences for reproduction is difficult, particularly for the induced separation response
Activity	Laboratory and field studies	- Technique can be applied to a wide range of species to assess water column and sediment toxicity	- Costly technique due to computing requirement to distinguish between background and pollutant effects
Positive rheotaxis	Laboratory and field studies	- Toxicant-induced responses have important implications for field populations	- Relationship between laboratory data and field effects has to be investigated
Phototaxis	Laboratory and field studies	- Simple, easily measured technique	- Relevance to population impact has to be established
Predator-prey interaction	Laboratory only	- The copepod-mysid technique is simple to conduct and should have ecological relevance	- Has to be tested with a wide range of pollutants

Table 2.8 – Subcellular, cellular and tissue responses: Information on potential methods for assessing the impact of pollutants on the aquatic environment

Method	Functional role in organisms	Type of endpoint	Specificity of response	Species used	Source literature
TISSUE					
Quantitative histopathology		Change in tissue (cell) structure	Heavy metals & Organics	Fish Invertebrates	Hinton & Lauren 1990
Tissue Somatic Indices (TSI)	Index of metabolic responses	Change in somatic index from normal values	Heavy metals & Organics	Fish Molluscs	Bayne *et al* 1985
CELLULAR					
In vivo cell pathology	Index of status of the feeding and reproductive processes	Quantifiable reduction in structural indices of digestive and reproductive cells	Organics	Invertebrates	Moore *et al* 1986
In vitro cell responses using cultured fish cells	Index of overall cellular condition	Quantifiable changes in cell physiology, growth morphology, detachment and survival	Heavy metals and Organics	Fish	Bols *et al* 1985
SUBCELLULAR					
Lysosomal stability	Index of cellular condition and catabolic potential	Decreased lysosomal stability/ Increased lysosomal volume	Heavy metals & Organics	Fish Invertebrates	Moore *et al* 1986
Sub-mitochondrial metabolic bioassay	Index of metabolic status in sub-mitochondrial electron transfer particles	Decrease in ratio of reduced to oxidised nicotinamide adenine dinucleotide (NADP/NAD)	Heavy metals & Organics	Isolated sub-mitochrondrial particles from rats	Blondin *et al* 1989
GENOTOXICITY					
In vitro tests					
Ames test Fluctuation test SOS-chromotest	Index of saturation rates	Revision of engineered bacteria to wild types	Mutagens	*Salmonella* *Escherichia*	Ames *et al* 1973 Van der Gaag *et al* 1990
Chromosome damage) mitotic inhibition)	Indices of genetic damage	Quantifiable effects on mitosis or chromosome structure	Genotoxins	Fish	Van der Gaag *et al* 1990
In vivo tests					
Nuclear anomaly assays	Index of genetic damage	Formation of micronuclei	Genotoxins	Amphibians Fish Mussels	Metcalfe 1988
Sister chromatid exchange	Index of genetic damage	Chromatids exchange places	Genotoxins	Fish Mussels	Metcalfe 1988
Unscheduled DNA synthesis	Index of genetic damage	Unusual DNA synthesis	Genotoxins	Fish Invertebrates	Maccubbin *et al* 1990

Table 2.9 – Critical evaluation of potential tissue, cellular and sub-cellular methods for assessing the impact of pollutants on the aquatic environment

Method	Available studies	Merits of the method	Limitations of the method
TISSUE			
Quantitative histopathology	Extensive laboratory/ field studies	– Effects can be distinguished in all life stages of small species – A number of potential target sites can be assessed rapidly in a given tissue section	– Normal tissue state has to be adequately described – Effects of seasonal and hormonal cycles, and nutrition have to be considered
Tissue Somatic Indices (TSI)	Limited laboratory/ field studies	– Measurements are simple inexpensive and should be accurate providing the problem of changing tissue water content is addressed – The technique can easily be integrated with other studies being conducted	– There is limited information on the variability associated with normal levels and the factors that affect TSIs – Research is needed to determine the situations in which TSIs could realistically be used
In vivo responses of cells	Extensive laboratory and field studies	– Easily measured	– Ecological relevance of response is not always apparent
CELLULAR			
In vitro responses of cultured fish cells	Limited laboratory and field studies	– The assays available are rapid, reproducable and sensitive – The use of cultured cell lines would reduce animal testing, which would be advantageous on 'moral' and economic grounds	– The responses of isolated cells have yet to be calibrated with whole organism responses
SUBCELLULAR			
Lysosomal stability	Extensive laboratory/ field studies	– In a range of organisms the level of lysosomal destabilisation is quantitatively related to the degree of toxicant exposure – Significant positive correlations have been established between the index and physiological parameters (SFG in *Mytilus*)	– Information on the relationship between lysosomal stability and physiological parameters in fish is limited
Sub-mitochondrial metabolic bioassay	Limited laboratory studies	– Simple rapid and reliable technique – Sensitive to a wide range of inorganic and organic pollutants	– The technique has to be tested with industrial effluents
GENOTOXICITY			
Genotoxic methods in general	Many in laboratory/ field studies	– Can detect cancer-forming saturations – May detect long-term enhancement of mutation rate	– Ecological relevance of response uncertain
In vitro tests	Many in laboratory/ field	– Rapid and cheap	– Results sometimes ambiguous – Genotoxicity may be lost during processing of sample
In vivo tests	Several in laboratory/ field	– Relevant inductors of genotoxicity at organism level	– Unknown ecological relevance

Table 2.10 - Biochemical responses: Information on methods for assessing the impact of pollutants on the aquatic environment

Method	Functional role in organisms	Type of endpoint	Specificity of response	Species used	Source literature
ENZYMIC RESPONSES					
Mixed Function Oxidases (MFO)	Detoxification of accumulated toxicants	Increased activity/ enzyme induction	Organics	Fish Molluscs	Payne *et al* 1987
Glutathione-s-transferases (GST)	Detoxification of accumulated toxicants	Increased activity	Organics	Fish Invertebrates	Lee & Keeran 1988
Plasma sorbitol dehdrogenase	Index of hepatic injury	Increased plasma concentration	Inorganics/ Organics	Fish	NRCC 1985
Cholinesterase activity	Control of the level of neurotransmitter acetylcholinesterase in the CNS	Inhibitiion of activity	Organophosphates and Carbamates	Fish	Coppage *et al* 1975
Delta Amino levulinic acid dehydratase (ALA-D)	Haeme synthesis	Inhibition of activity	Lead	Fish	Hodson *et al* 1984
NON-ENZYMIC FUNCTIONAL PROTEINS					
Metallothioneins	Detoxification of accumulated toxicants	Induction of protein	Heavy metals	Fish Molluscs	Hamilton & Mehrle 1986
Heat Shock Proteins	Binding of important proteins as a protective mechanism	Induction of proteins	Heavy metals	Molluscs Crustaceans	Anderson 1989, Schlesinger 1988
LOW MOLECULAR WEIGHT COMPOUNDS					
Adenylate Energy Charge (AEC or EC)	Representation of available energy in adenylate system	Decrease in AEC from >0.8 in unstressed organisms	Heavy metals & Organics	Molluscs Crustaceans	Ivanovici 1980
Glycogen	Representation of available data	Decrease in specific glycogen levels	Heavy metals & Organics	Fish Invertebrates	Giesy and Graney 1989
Free amino acid (FAA) pool	Protein metabolism/ Osmotic effectors for cell volume regulation	Change in free amino pool concentration	Heavy metals & Hydrocarbons	Molluscs Crustaceans	Maltby & Calow 1989 Kamminga 1989

Table 2.11 - Critical evaluation of potential biochemical methods for assessing the impact of pollutants on the aquatic environment

Method	Available studies	Merits of the method	Limitations of the method
Biochemical methods in general		- Biochemical indices can provide early warning of pollutant impact before physiological changes are evident	- In order to reliably interpret toxicant induced effects considerable backgound information on the effects of extrinsic and intrinsic factors is required
ENZYMIC RESPONSES			
Mixed Function Oxygenases (MFO)/ Glutathione-s-transferase (GST)	Extensive laboratory/ limited field studies	- Sensitive in terms of response time and pollutant levels - Response is rapid in fish (24h), though slower in invertebrates - Response to a single exposure may persist - Limited training is needed to perform the assay and costs are low	- Interactive effects of other pollutants could limit any response - Seasonal and sex-linked differences could contribute to high field sample variance and mask induction - Interpretation requires familiarity with the MFO system in species used
Plasma sorbitol dehydrogenase	Laboratory studies	- Simple rapid reproducible technique - Enzyme activity is not affected by potential modifying factors such as sex, body size, starvation and handling stress	- Appropriate field tests need to be conducted
Cholinesterase activity	Extensive laboratory and field studies	- Sensitive and rapid technique	-
Ó-Aminolevulinic Acid Dehydratase activity (ALA-D)	Extensive laboratory and field studies	- Rapid indicator of lead exposure, which is sensitive of levels (3-5 mg/l) comparable to analytical detection limits - ALA-D levels have been correlated with blood lead concentrations	- Pollutant induced changes in ALA-D activity can indicate lead exposure, though changes need to be correlated with toxic effects
NON-ENZYMIC FUNCTIONAL PROTEINS			
Metallothioneins (MT)	Extensive laboratory/ limited field studies	- Direct relationship between induction of metallothionein and tissue toxicant concentrations, though not necessarily water levels - MT's may be used as metabolic indicators of no-effect levels before resulting pathological disruption or cell death - MT's are apparently closely related to whole organism growth, development and physiological status	- There can be technical difficulties in isolating and purifying these proteins, and the method may be expensive and required extensive training - In fish the variability in tissue MT levels between individual fish, means long term exposure may be necessary to discern toxicant induced effects

Table 2.11 – continued

Method	Available studies	Merits of the method	Limitations of the method
Heat Shock Proteins (HSP)	Extensive laboratory and field studies	- HSP's should be inducible in all organisms in reponse to a wide range of pollutants - HSP's are easily detected auto-radiographically or immunologically in live samples	- The physiological function of HSP's have to be identified and available data is limited
LOW MOLECULAR WEIGHT COMPOUNDS			
Adenylate Energy Charge (AEC) $\frac{=([ATP]+[0.5ADP])}{([ATP]+[ADP]+[AMP])}$	Laboratory and field studies	- High level of precision, only small sample sizes required - Response time is rapid (<24h) - Response is applicable to a wide range of pollutants and to all organisms - AEC provides an integrated view of metabolism and is associated with physiological state, though AEC is only a measure of the energy in the adenylate system and does not include energy stored in phosphagens or macro-molecules, such as glycogen	- The highly labile nature of ATP necessitates careful handling and analytical procedures - Predictive power is limited since the effects of short and long term decreases in AEC on biological performance (ie growth, reproductive capacity and offspring viability) have not been established - As deviations from normal AEC values are strongly resisted, considerable toxicant stress may be needed to effect a steady state, rather than transient, change in AEC
Glycogen	Extensive laboratory and limited field studies	- Changes are not transitory and are non-responsive to handling stress	- Problems have been encountered in the interpretation of field data - Changes in specific tissue levels are not necessarily indicative of general decreases in whole-body energy reserves
Free Amino Acid (FAA) pool	Limited laboratory and field studies	- Significant changes in FAA pool occur in response to stressors - FAA pool is easily measured	- Sensitivity to pollutant exposure has not been established

Table 2.12 - Commercially available biological early warning systems

Monitor name	Supplier	Organism	Response	Application
WRc Mk III monitor	pHOX	Fish	Ventilation	L/F
BMI Series 6000	BMI	Fish	Ventilation	L/F
Aqua-Tox Control	Kerren	Fish	Rheotaxis	L/F
Aquatest	Quantum Science Ltd	Fish	Rheotaxis	L/F
Züge Biotest 3	Züge	Fish	Rheotaxis	L/F
Toxalert	Marine Electronics	Fish	Avoidance	L/F
Aztec FM 1000	Aztec	Fish	Electric Pulses	L/F
Truito Sem	Truito	Fish	Activity	L/F
Unirelief	Unitika	Fish	Activity	L/F
Mussel Monitor	Delta Consult	Mussels	Valve movement	L/I
Dynamische	Elektron GmbH	*Daphnia*	Activity	L/F
Microtox	Microbics Corp	Bacteria	Light emission	L/F
Lumistox	Lange	Bacteria	Light emission	L/F
Stiptox	Stip	Bacteria	Respiration	L/F
Respiration	Manotherm BV	Bacteria	Respiration	L/F
Toxiguard	Eur Control	Bacteria	Respiration	L/F

L = Laboratory, F = Field application using abstracted receiving water, I = *in situ*

SECTION 3 - ECOTOXICOLOGICAL METHODS THAT MEET THE NEEDS OF THE NRA

3.1 THE DERIVATION OF ENVIRONMENTAL QUALITY STANDARDS

An Environmental Quality Objective (EQO) can be defined as the requirement that a river should be suitable for one of fourteen identified uses (such as the protection of aquatic life, or abstraction to potable supply). An Environmental Quality Standard (EQS) is that level of a substance or substances which should not be exceeded if the objective is to be met, ie if the identified water use is not to be adversely affected.

An EQS should be based on as much information as possible. Therefore the initial stage in the derivation of an EQS is to collate all available toxicity and bioaccumulation data for the substance under consideration. These data are reviewed and critically assessed in order to determine their reliability and their relevance to the aquatic environment. Ecotoxicity tests that meet the criteria given in Table 3.1 are considered more reliable and more relevant than those that do not.

Table 3.1 - Features of ecotoxicity tests that increase their reliability and relevance

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A INCREASED RELIABILITY

1. DOSING

- Suitable control.
- Minimum of three treatments, one at a concentration predicted to cause no effects.
- Control and treatments all duplicated.

2. TEST ORGANISMS

- Organisms' age, size and stocking density.

3. CONDITIONS AND ANALYSIS

- Test concentrations analysed, at least at start, end and some point in middle.
- Dosing well regulated, eg flow-through rather than static test.
- Chemical and physical attributes of test media are measured, eg temperature, pH, salinity, hardness, type of test water.

4. INTERPRETATION OF RESULTS

- Organism's response shown to be concentration dependent.
- Endpoint is understood, eg mortality, growth or reproduction.
- All the above features are reported.

B INCREASED RELEVANCE

1. TEST ORGANISMS

- Test species is indigenous to the UK, or at least is temperate.
- Test species is likely to be sensitive, eg an alga if testing a herbicide.

2. CONDITIONS

- Chemical and physical attributes of test media are appropriate, eg temperature, pH, salinity, hardness, type of test water.
- Conditions mimic as near as possible environmental conditions.
- Exposure is long-term, preferably over at least one generation of the test species.

Once the data have been evaluated it should be possible to identify the most sensitive aquatic organisms, and to establish the lowest credible

concentration having a significant adverse effect on them. A safety factor can then be applied to arrive at a preliminary standard. The size of the safety factor depends on the data to which it is being applied, specifically:

- the duration of exposure;
- the endpoint of the test (eg lethal or sub-lethal); and
- the extent of the available data.

Typically in the UK, and elsewhere, the factors are:

- 100 for acute lethal data;
- 10 for chronic data and sublethal data; and
- (occasionally) 1 for reliable no-effects data.

These can be applied if a large database of information exists. If not then a larger safety factor may be considered necessary.

Small safety factors can be used when there is less uncertainty in extrapolating from effects concentrations in these tests to a likely no effects concentration in the environment. Therefore, when deriving an EQS, particular emphasis is placed on results from chronic studies and from those that best mimic field conditions, such as microcosms or artificial streams. There is also more emphasis on results for temperate or indigenous species, and those that are likely to be particularly sensitive.

Once a preliminary standard has been suggested, its relevance needs to be confirmed. This is done by comparison with any available field monitoring data on the concentration of the substance found in the aquatic environment and any effects that these concentrations have on the biota. Only if such monitoring data do not contradict the preliminary standard is it proposed as an EQS.

The essential point when deriving EQSs is that uncertainty must be reduced to a minimum. Uncertainties with ecotoxicological data are associated with:

- the reliability (quality) of the test; and
- the relevance of the results to the environment.

For most substances the data presently available are inadequate to enable EQSs to be derived with a reasonable degree of certainty. Future ecotoxicological testing needs to address this problem by ensuring that the data produced are both reliable and relevant. The NRA should develop a list of priority substances for which standards are required in the UK. Water Quality Objectives, of which Environmental Quality Objectives are a component, are currently only applied to river systems, although this will change to embrace all controlled waters in the future. EQSs will therefore need to be derived for EQOs applied to rivers, lakes, estuaries and coastal waters. The literature should be reviewed for each priority substance and EQSs derived from this information if at all possible. If insufficient data are available then the NRA should consider performing appropriate toxicological tests to fill gaps in the database. These tests will be chronic fish or invertebrate investigations using species typical of the habitats that may be impacted by the substance of interest. Microcosm studies should also be used to minimise uncertainty when very little is known about the fate or effects of particular substances in aquatic systems.

3.2 WATER QUALITY OBJECTIVES

The use of ecotoxicological data for setting Environmental Quality Standards was discussed above. Improved, or more extensive, EQSs allied with the appropriate use of toxicity-based consents will help protect and improve the aquatic environment and meet water quality objectives.

The monitoring of receiving water quality to ensure compliance with, or movement towards, WQOs has traditionally relied upon a combination of chemical sampling and benthic invertebrate and fish survey techniques.

Ecotoxicological approaches can be used to supplement this information in several areas:

1. For the rapid assessment of effluent impact reduction schemes. It may take weeks or months for traditional survey techniques to reveal an improvement in receiving water quality.

2. When discrepancies are noticed between observed and expected fauna, possibly in the absence of an obvious cause. There may be a quantifiable difference such as that found between a RIVPACs prediction and actual river community scores, or an intuitive feeling on the part of the field officer that a system is 'not as good as it should be'.

   In situ assays can help the regulatory officer understand the effects of effluent reduction schemes, or the cause of discrepancies between observed and expected fauna. Tests should ideally be performed with native species, and chronic mortality or impairment of growth or reproduction should be the measured endpoints. Tests in this category include *Mytilus edulis* scope for growth, *Gammarus pulex* feeding and growth rates and the death or impaired growth of caged fish and invertebrate species. Simple suites of suborganism tests will help the investigator to understand the cause of effects on survival and production, but should not be used separately from these ecologically relevant parameters. A suitable suite of suborganism tests could include EROD and GST activity, metallothionein induction and unscheduled DNA synthesis.

   Simple microcosm experiments using colonisation substrata may also be useful. The substrata can be colonised at unimpacted sites, removed to impacted sites, and assessed after a few days exposure for changes in richness, diversity and biomass caused by death or increased drift. Simple systems have been proposed for estuaries by Tagatz (1986) and for freshwaters by Livingston (1988).

3. When the receiving environment is heterogeneous. Aquatic systems can differ considerably over short distances. Changes in substratum type, salinity, turbulence and other physico-chemical parameters can mask effects caused by contamination. Controlled toxicological experiments can overcome this problem.

   Receiving waters in the United States are monitored using 7-day subchronic tests with *Ceriodaphnia* and *Pimephales* (Stewart *et al* 1990), which are often performed in site-based mobile laboratories. The NRA might consider the use of similar mobile facilities in which controlled flow-through experiments can be performed with indigenous species and site-specific water. The species selected for these tests should be the same as those selected for use in toxicity-based consents (see Section 3.3).

   Field-based laboratory tests have the advantage over *in situ* tests of controllability and randomisation, thus overcoming problems of environmental heterogeneity and pseudoreplication (Hurlbert 1984). They have the disadvantage of decreased relevance to the specific site of interest.

4. When large volumes of perhaps complex effluent enter important aquatic systems. Chemical and toxicity based consents should control these effluents, but in the case of major dischargers, continuous automatic monitoring will give an early-warning of problems in the receiving water and provide an extra incentive for the discharger to be vigilant.

   At present, there are a limited number of commercially available continuous water quality monitors, and of those available many cannot be used *in situ* and are affected by parameters other than contamination. The bivalve shell activity monitor may be an exception to this generality. Native or commercially important species can be used and further development of this technique with physico-chemical samplers could yield a very useful device for monitoring fresh, estuarine and marine waters.

3.3 THE SETTING AND MONITORING OF TOXICITY BASED CONSENTS

Although the use of a whole effluent or direct toxicity assessment (DTA) approach for regulating the potential toxic impact of effluent discharges has been limited to date, interest in this approach is growing. Attention has focused on controlling complex, and particularly variable, effluents, as highlighted by recommendation (16) of the NRA report on 'Discharge Control and Compliance Policy' (NRA 1990) and the responses obtained from NRA personnel (Appendix B2).

The widespread application of a DTA approach in the UK will require three major components;

1) A UK protocol for consistency;

2) Quality control procedures to ensure acceptability to regulatory agencies, dischargers and the public; and

3) Case studies to test the developed protocol rigorously.

These requirements are addressed in a research project currently being carried out by WRc, in collaboration with regulatory authorities throughout the UK.

Interim Protocol for Direct Toxicity Assessment

An interim DTA protocol applicable to the UK has been developed by WRc, with the NRA, and is summarised in Figure 1. The approach is based principally on that developed by the US Environmental Protection Agency (EPA), but has been adapted to circumstances in the United Kingdom. Only a brief description will be given of the proposed system of screening, prioritising, consenting and monitoring will be given, because a comprehensive description is available in Hunt (1989).

In the interim protocol, there is a three stage strategy for identifying and controlling appropriate discharges by toxicity based consents (TBC).

Stage 1

Selection and prioritisation of discharges which are appropriate for the DTA approach. The appraisal considers;

a) existing knowledge of the environmental impact of a particular discharge;

b) the presence of potentially toxic substances which are not subject to EQSs or for which toxicological data are not available;

c) the complexity of the effluent;

d) the volume of the effluent discharged in relation to the diluting capacity of the receiving water;

e) information on the toxicity of the whole effluent or of constituents of the waste stream.

At this stage no class of discharge should be excluded for further testing unless there is conclusive evidence that a chemical specific approach to their control is appropriate.

Stage 2

Application of an acute toxicity screening test to those priority discharges requiring additional investigation. Effluents are classified into four categories A-D, on the basis of their inherent toxicity.

In the United States, EPA protocols specify the use of acute toxicity tests on as many as three aquatic organisms at the screening stage. However at this time, cost and the absence of suitable facilities in many parts of the UK regulatory structure could impede adoption of DTA, and thereby delay the benefit of improved pollution control. Because of this simple, rapid and reproducible toxicological test is

specified in the protocol, which should be sensitive to a wide range of pollutants.

In the protocol, the commercially available Microtox toxicity test, based on the bioluminescence response of *Photobacterium phosphoreum* is specified for effluent characterisation. The test satisfies the required criteria and has a similar sensitivity to that of other commonly used aquatic organisms (McFeters *et al* 1983; Tarkpea *et al* 1986). However, since the Microtox system is a commercially patented test, there will inevitably be concern about the long-term availability of the system. Therefore, attention is being focused on the potential of other current or new toxicity tests which could be either more appropriate or complementary to the information obtained from Microtox.

It is recognised that there may be circumstances where a more detailed initial screening of the effluent is required. Acute toxicity testing using an alga, such as *Selenastrum capricornatum*, *Scenedesmus subspicatus* or *Chlorella vulgaris*, would be an appropriate adjunct to Microtox testing for an effluent from a herbicide factory. Tests with an invertebrate, such as *Daphnia magna*, could be used for insecticidal discharges.

In testing the protocol, the Microtox test is applied with an additional test. The 48 hour *Daphnia* immobilisation method is used for freshwater discharges and the oyster embyro-larval test is used for effluents released to estuarine or marine waters. Techniques currently under development which are considered to have potential in a screening role include the *Daphnia* IQ test, the biolumuninescent *E. coli* bioassay (Stewart *et al* 1989), the use of fish hepatocytes (Denizeau and Marion 1990) and the sub-mitochondrial particle (SMP) bioassay (Blondin *et al* 1989).

The acute Microtox toxicity data and other factors are then used to determine the potential chronic toxic impact on aquatic communities in the receiving waters.

Stage 3

Control of effluents for each toxicity-based category as detailed below:

Category A: Additional in-depth toxicological studies of these priority discharges estimated to cause chronic toxicity in the receiving waters, leading to toxicity and/or discharge reduction, and probable establishment of a Toxicity Based Consent (TBC).

Category B: Establishment of full TBCs for these discharges showing intermediate toxicity, until the priority discharges have been investigated.

Category C: Establishment of TBCs based on the screening toxicity test for these discharges showing low toxicity, though variability in composition.

Category D: Continued reliance on chemical specific consent conditions for these discharges showing no or limited toxicity or low toxicity with limited variability of composition.

In-depth toxicity testing

The toxicity based consents (TBC) for priority discharges and those showing intermediate toxicity, would normally be based on acute toxicity testing with three representative species and routine monitoring using a 'calibrated' Microtox system. Representative algal, invertebrate and fish species for fresh and marine waters are shown below:

Type of organism	Representative species	
	Freshwater system	Marine system
ALGAE	*Selenastrum capricornatum* *Scenedesmus subspicatus* *Chlorella vulgaris*	*Phaedactylum tricornutum*
INVERTEBRATES	*Daphnia magna* (Water flea)	Larvae of *Crassostrea gigas* (Pacific oyster) *Crangon crangon* (Brown shrimp)
FISH	*Oncorhynchus mykiss* (Rainbow trout)	Juvenile *Pleuronectes platessa* (Plaice) Juvenile *Rhombus maximus* (Turbot)

Although these species may not be the most relevant to British waters, their use is well established and standard protocols are available (OECD 1984, 1987). In the future tests could use appropriate indigenous species, such as the freshwater amphipod *Gammarus pulex* and native salmonid (brown trout *Salmo trutta*) and non-salmonid (roach and carp) fish species. However the use of these species will necessitate standard toxicity test protocols to satisfy quality control requirements.

In setting full TBCs, the absolute acute toxicity of the effluent will be assessed on no less than four occasions over a minimum period of three months. The Microtox test will be carried out in conjunction with the tests on the three other representative species. The Microtox results can then be calibrated against the most sensitive of the three test organisms so that a consented Effluent (Microtox) Acute Toxicity (EMAT) can be set, which will protect the receiving water community.

The Microtox system can then be used by the discharger and regulatory agency for routine monitoring. The discharge must be periodically retested with the most sensitive species and the consented EMAT adjusted by 're-calibration'. The frequency of recalibration will depend upon:

a) The closeness of the results of routine monitoring to the consented EMAT.

b) The expected variability of discharge composition.

c) Changes in the nature and operation of the plant.

d) The importance of the discharge to the receiving water quality.

The recalibration should be undertaken at least once every three and five years for category A and B discharges respectively.

In situations where the Microtox test proves insufficiently sensitive in the role of compliance monitoring technique, an appropriate toxicity test will be used. The test that will be used will be decided on a site-specific basis, and given the greater complexity and costs of such a test, the frequency of monitoring may need to be re-assessed.

Quality Control Procedures

In the testing of the protocol, appropriate quality control procedures are being integrated, in terms of:

1) effluent handling (collection, storage and transportation);

2) effluent and dilution water analysis; and

3) the use of reference standards during testing to ensure the health of the test species used for testing a given effluent.

An important requirement in conducting toxicity testing for DTA will be that the work is carried out according to the principles of Good Laboratory Practice to ensure the consistency and reliability of the test data.

Case studies

It is vital that the Direct Toxicity Assessment approach is thoroughly tested and evaluated before it is considered for widespread use. Consequently, the interim DTA protocol is initially being assessed in case studies of multiple discharge inputs to freshwater (River Erewash) and estuarine/marine receiving waters (Irvine Bay). After this specified important individual discharges will be assessed. These studies are being conducted in association with the NRA regions and the Clyde River Purification Board, to maximise use of available information for any discharge.

Future developments

Although standard acute toxicity tests using indigenous species are available for effluents discharged to fresh and marine waters there are at present no specific tests for monitoring discharges to estuaries. Investigations into the applicability of using indigenous estuarine zooplankton species, such as the mysid *Neomysis integer* and the copepod *Eurytemora affinis* in lethal (and sub-lethal) tests are currently being carried out at WRc under the 'Biological Method Development Programme' (Ref A18.063).

In the current protocol for particularly important discharges, chronic toxicity tests are considered to be appropriate adjuncts to acute tests to allow calibration of the Microtox data without the assumption of an acute-to-chronic ratio. Since it is important to reduce uncertainty in toxicity testing and ensure that any potential sub-lethal effects of effluents are detected it is recommended that appropriate short-term chronic toxicity tests are routinely used with acute toxicity tests in a nationally implemented protocol. This approach would ensure that the

Microtox data were calibrated against the important parameters of growth and reproduction.

For new discharges, or those which will result from changes in industrial practice, an evaluation of their potential impact with acute and chronic toxicity tests should be accompanied with an assessment using colonisation substrata, such as those described by Livingston (1988). This inexpensive, effective and simple community method can be used to assess likely receiving water impact by colonising bags upstream of the discharge and exposing these to various concentrations of pilot plant effluent. In this way the necessity for toxicity or discharge reduction could be ascertained before any impact on the actual receiving water community. In the case of discharges to particularly important receiving waters, complex microcosms, such as artificial streams, ponds and estuarine/marine tanks may be appropriate methods for assessing the consequences of effluent release.

Implementation

Toxicity testing for permit setting and compliance monitoring could be conducted either:

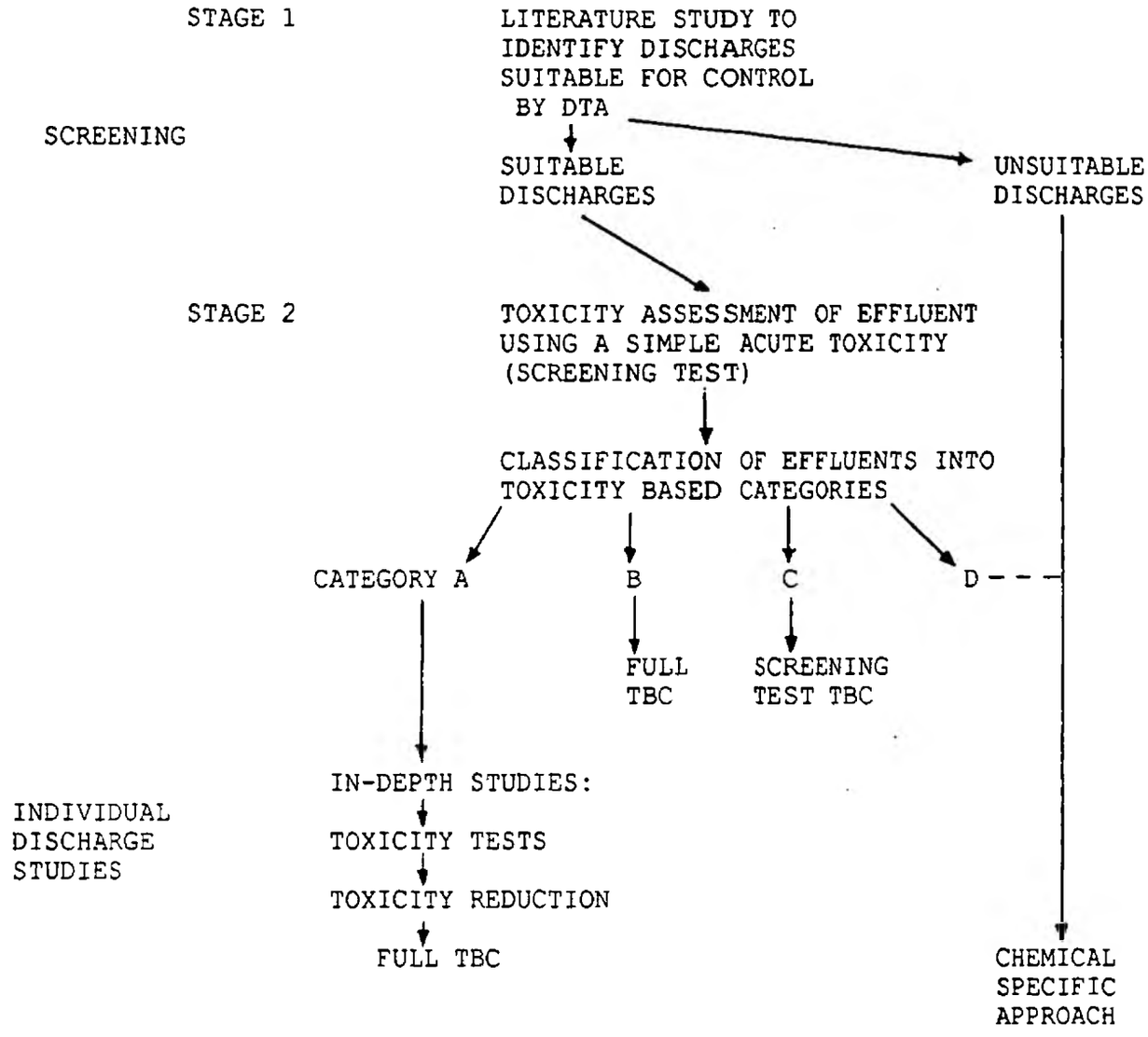
1) in-house by the NRA; or

2) by the dischargers using their own facilities, in the case of large organisations (eg ICI and Shell) or those of an independent commercial testing house.

In the UK it would seem appropriate that effluent testing for direct toxicity assessment be conducted by the discharger. In this way any effluent which failed to comply with permit limits would be an admission of failure and would avoid potential arguments. The NRA will need to establish certain suitable testing facilities in order that effluent samples taken periodically from all discharges could be analysed to ensure the accuracy and reliability of a discharger's testing procedure. This rapid, accessible biological testing capability would also be

useful in other circumstances, such as pollution incidents. In terms of effluent testing for quality control the NRA facility could be centralised rather than in each region, and the initial establishment costs would be shared.

At present, while the DTA protocol is being tested, effluents considered appropriate for control by toxicity based consents should be tested with Microtox and appropriate algal, invertebrate and fish species to ascertain the most sensitive species from which consent conditions could be derived.



CATEGORY A - PRIORITY DISCHARGES SHOWING HIGH TOXICITY
CATEGORY B - DISCHARGES SHOWING INTERMEDIATE TOXICITY
CATEGORY C - DISCHARGES SHOWING LOW TOXICITY, THOUGH VARIABLE COMPOSITION
CATEGORY D - DISCHARGES SHOWING NO OR LIMITED TOXICITY AND CONSISTENT EFFLUENT COMPOSITION

Figure 1 - The potential application of toxicity testing to effluent control

3.4 POLLUTION INCIDENTS

There are several ways in which the NRA could use ecotoxicology to help interpret and control pollution incidents, depending upon the nature of the incident.

If obvious damage has occurred to the biological community, with evidence such as fish or invertebrate kills, or a high level of migration from the impacted area, then establishment of the 'relevance' of the discharge is initially unnecessary. NRA personnel will, in this situation, have two main objectives:

1. To gain sufficient evidence of cause and effect to bring a successful prosecution against the discharger.

2. To trace the source of the incident and control any further discharges.

1. Acquisition of evidence

Speed is important when acquiring evidence for a successful prosecution. If samples of the contaminated water can be taken, with confidence, then a rapid, reproducible, off-the-shelf and standardised test can be used to assess toxicity and provide quantitative evidence. In those NRA regions without an in-house toxicological capability, Microtox is at present the only test system which fulfils these criteria. Cultured animals are not required, and the whole test facility can be left dormant until activated by an incident. The sub-mitochondrial particle bioassay may in the future complement the Microtox test, but further development and validation are required before this test can be used routinely.

The major limitations of Microtox and other similar tests are that they have a low ecological relevance and courts may therefore question whether they are reliable indicators of observed effects on aquatic systems. NRA regions with a toxicological capacity should therefore supplement the initial Microtox screening of polluted water samples with simple short-term marine or freshwater acute toxicity tests with indigenous species, preferably one fish and one macroinvertebrate. These organisms would be present in laboratory cultures for routine toxicity-based consenting purposes. Standard protocols for the culturing and testing of appropriate indigenous

species should, therefore, be developed by the NRA as soon as possible. These species can be selected objectively by reference to both the literature and the results of the 1990 biological survey which may suggest local species that are particularly sensitive to pollution. If particular NRA regions do not have a sufficiently extensive in-house toxicological capability to perform investigations of this type they should arrange for another laboratory to run the necessary tests. The NRA currently funds WRc to provide a 24-hour emergency service.

2) Tracing pollution incidents

The source and cause of a pollution incident will, in many cases, be quite apparent and ecotoxicological methods will be unnecessary except as additional evidence. If the type of contaminant is unknown, however, or if the source is unclear because of non-point, or multiple point discharges in the area, ecotoxicology may be of use.

An elevation in activity of enzyme detoxification systems, particularly EROD and GST, in sessile animals can indicate the presence of organic contamination, while metallothionein induction is indicative of metal contamination. The relative levels of EROD/GST and metallothionein activity will help locate the source of the incident. Many sedentary species can be used, including *Sphaerium*, *Pisidium* and *Chironomus* in freshwaters and *Capitella*, *Corophium*, *Nereis* and *Mytilus* in marine or estuarine systems. Ubiquitous and robust species such as these should be selected from marine, estuarine and freshwater habitats and a programme of work instituted to investigate the usefulness of this approach for pollution incident management. NRA in-house expertise need not be developed in these areas, as frozen specimens can be analysed by appropriate contractors.

The use of bioaccumulation techniques will be discussed in detail in a later report on toxicity and body burdens (Contract A18.1). It is worth noting here, however, that several regions have successfully

used mosses, seaweeds and other taxa to locate pollution 'hot-spots' caused by continuous discharges. A similar approach can be adopted when investigating pollution incidents, and should be systematised between regions.

Other uses for ecotoxicology in managing pollution incidents

Ecotoxicological techniques can also be of benefit for assessing the toxicity of discharges in incidents where few or no effects are found in the receiving water. Such situations may arise when coloured effluents or otherwise obvious pollution incidents are reported at locations where the biological community has previously been impacted by pollution. Many lowland rivers may fall into this category, and the rapid ecotoxicological test approach described above would help in deciding whether further action was necessary.

Lastly, ecotoxicological methods can be used to assess the persistent effects of a pollution incident on sediment toxicity. If contaminants have sorbed onto or into sediments, community recovery may take longer than water chemistry samples would suggest. Sediment toxicity tests used after an incident will provide insight into the likehood of persistent effects. Common native infaunal and epifaunal taxa, such as *Gammarus*, *Chironomus* and *Corophium* should be used in these tests and appropriate protocols and standard procedures developed.

Results from the 1990 River Quality Survey will, again, provide valuable information on potentially sensitive species.

3.5 CONCLUSIONS AND RECOMMENDATIONS

This review has been directed towards identifying NRA business requirements which can be satisfied by ecotoxicological approaches. To assist the NRA in making decisions on key elements within this topic area, the following guidance is given:

1) Toxicity based consents (TBCs) are well-established in other countries and have considerable potential as a means for controlling complex discharges. The NRA has to consider the development of protocols for a range of simple and rapid techniques to complement the use of Microtox for screening effluent discharges when setting and monitoring TBCs. The efficacy of Microtox could be compared with that of the *Daphnia* IQ test, the bioluminescent *Escherichia coli* and sub-mitochondrial particle bioassays, and suitable tests with fish hepatocytes. Protocols for short-term tests with indigenous algal, fish and invertebrate species should also be developed for use in TBCs (see Point 4).

2) A list of priority substances for which standards are required in British rivers, lakes, estuaries and coastal waters should be developed, and the literature for toxicological information on these substances reviewed. NRA Project A10.1 with WRc will help the NRA to select methods to acquire the appropriate data.

3) If insufficient data are available for the confident derivation of an EQS for any priority substances, then the NRA should commission, or ask the discharger to commission, toxicological studies to fill any gaps. These studies should:

   a) use at least one native fish, invertebrate and plant species in chronic tests, with mortality, growth and reproduction as the measured endpoints;

   b) examine the system-level fate and effects of the substances using relevant microcosms. These microcosms could include ponds, artificial substrata, and artificial stream, estuarine or marine systems.

4) The NRA should establish that there are standard protocols for the type of tests recommended in 2a and b, or, if there are none, the NRA should develop appropriate protocols. Standard protocols for acute toxicity tests should also be developed for indigenous species from

the sediment and water column phases of rivers, lakes, estuaries and coastal waters. These can then be used to assess short-term toxicity during pollution incidents and for the direct toxicity assessment of effluents. The same fish, invertebrate and plant species should be used in both acute and chronic tests whenever possible. Sensitive biochemical indices (such as EROD and GST activity, metallothionein induction and unscheduled DNA synthesis) should be incorporated in the acute protocols in order to assess immediate sublethal effects and increase the amount of useful data generated from each test.

Sensitive indigenous species can be identified from two main sources:

a) The results of project A18.23, Determination of Ecotoxicological Effects on Indigenous Species, in which WRc is reviewing the effects of organic and inorganic contaminants on indigenous species;

b) Discrepancies between predicted and observed biological communities and water chemistry results from the 1990 River Quality Survey.

The protocols for acute toxicity assessments should include methods for testing the selected species in fixed or mobile laboratories and *in situ*. The methods described in each protocol should be subjected to rigorous testing, publication and peer review before they are routinely used or recommended by NRA personnel.

5) The NRA should undertake field studies with continuous biological systems, such as the Mussel Monitor, in order to develop robust methods for continuously monitoring the impact of important effluent outfalls and receiving water quality.

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APPENDIX A

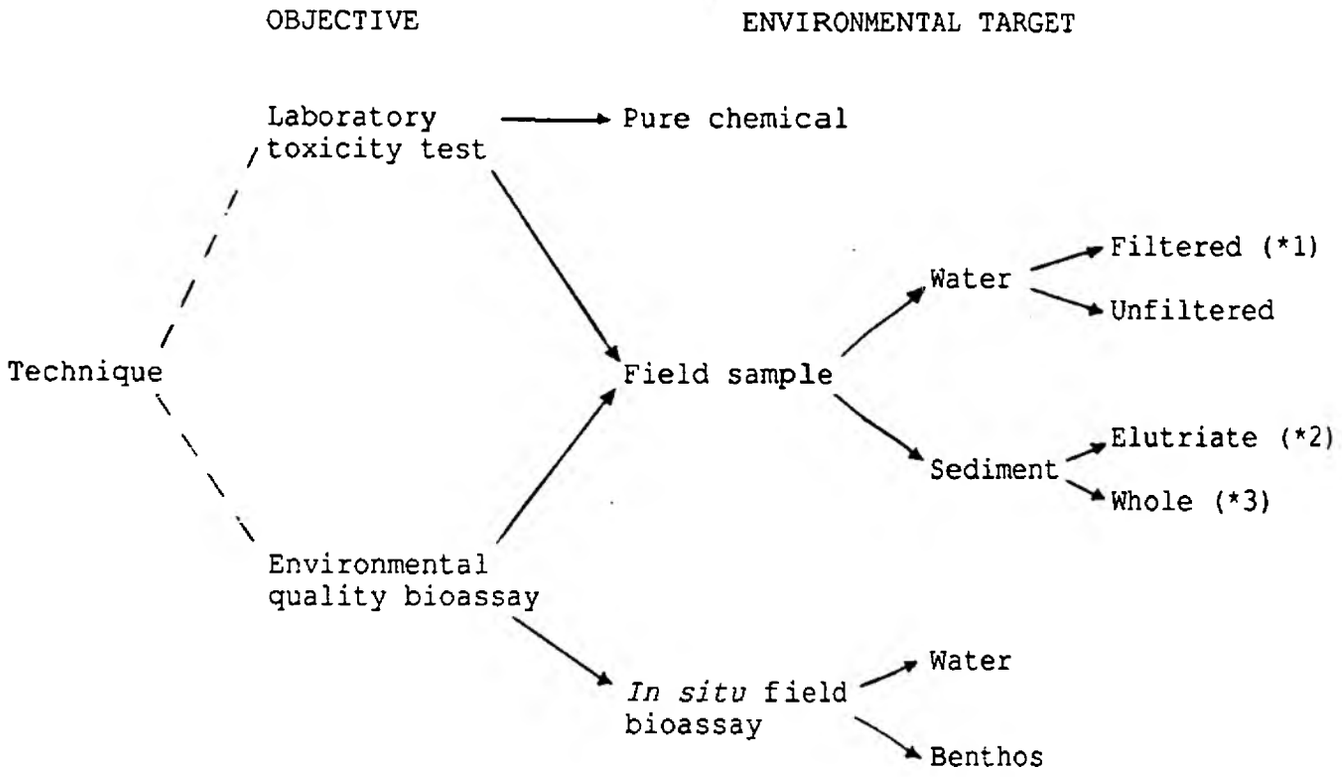
REVIEW OF ECOTOXICOLOGICAL APPROACHES AND METHODS

A1 INTRODUCTION

Fresh, estuarine and marine waters are subjected to a wide range of continuous and episodic polluting discharges. Traditionally, discharges of liquid effluents and solid wastes have been regulated and monitored on a chemical-specific basis. However, regulatory authorities have found that a purely chemistry-based approach to pollution control does not prevent damage to the aquatic environment. Because of this, biological methods providing toxicity-based assessments have become increasingly widely used and sophisticated in recent years (Cairns and Pratt 1987; OECD 1984 and 1987, MAFF 1990), and tests are available for use in both the laboratory and the field (Figure A1).

Established water quality standards at present only relate to the control of pollutants in receiving waters. However, sediments and suspended solids represent significant sinks for anthropogenic contaminants of water bodies, and methods for assessing this impact are currently being developed.

This review of methods in ecotoxicology is divided into several sections based upon different levels of biological organisation. These are community, population, whole organism and sub-organism methods. Much of the information is presented in tables in which particular methods are described and critically assessed.



*1 - Testing of water sample with suspended particulate matter removed
*2 - Testing of surface adsorbed and leachable sediment pollutants
*3 - Testing of whole sediment with the natural population removed

Figure A1 - The role of laboratory-based and *in situ* biological methods in determining the impact of polluted water and sediments on aquatic organisms

A2 COMMUNITY METHODS

A2.1 INTRODUCTION

Community, or multispecies test systems are those in which more than one species of plant or animal are exposed to a contaminant, in order to investigate the direct or indirect effects of pollution. They include simple chambers in which a limited number of selected species are exposed simultaneously or in series, and more complex 'microcosms' of the environment in which large numbers of species derived from the appropriate habitats are exposed (Tables 2.1 and 2.2 in Section 2.2).

A2.3 THE MEASUREMENT OF EFFECTS IN MULTISPECIES TEST SYSTEMS

Certain measurements taken in community studies will be similar to those obtained from other types of test. Mortality, immobility and reproduction rate may be measured directly and analysed using standard inferential statistics. Although such endpoints may be extremely valuable for determining the toxicity of a compound to particular microcosm components, it is important to integrate such single-species measurements, and take further, system-level measurements in order to extract maximum information from multispecies tests. Table A2.1, taken from a review by Sanders (1982), is an example of the types of structural and functional parameters that could be measured in an artificial stream study, although most of the endpoints could be measured in other types of microcosm.

A recent approach to effects measurement in microcosms uses pollution-induced community tolerance (PICT) as a determinant of functional change (Blanck *et al* 1988, Landner *et al* 1989). The PICT concept relies upon the assumption that contaminants exert a selective pressure upon communities, leading to the elimination of sensitive and the retention of tolerant species. Naturally-derived communities in microcosms can be exposed to a contaminant range over a suitable period of time, and then re-exposed in order to determine whether tolerance has been induced. Functional measurements can be used to measure the no observed effect concentration for the community, and preliminary work with periphyton community photosynthesis as the response parameter suggests that this approach can be robust and relatively toxicant-specific.

Table A2.1 - Experimental measurements for an artificial stream study (from Sanders 1982)

	Response to perturbation	
Within-stream functional groups and storage compartments	Structural	Functional
Microbial heterotrophs	Benthic biomass	Community respiration, processing rate of introduced leaf detritus
Periphyton	Biomass/species structure	Primary production, colonisation rate on artificial substrata
Macroinvertebrate primary consumers	Biomass/species structure	Production
Macroinvertebrate secondary consumers	Biomass/species structure	Production
Juvenile fish	Biomass	Growth, respiration, food consumption, pathology/ mortality
Benthic detritus	Standing stock in storage	Rate of accumulation

Inflow/outflow characteristics	Variables
Detritus	Inflow/outflow of particulate organic carbon
Algae	Inflow/outflow of biomass; species
Macroinvertebrates	Inflow/outflow of biomass; species, size/frequency

Abiotic conditions	Variables
Solar radiation	Daily incident radiation, attenuation in stream water

Table A2.1 - continued

Within-stream functional groups and storage compartments	Response to perturbation: Structural	Response to perturbation: Functional
General water quality	Temperature, pH, conductivity, alkalinity, dissolved oxygen	
Water flow characteristics	Depth, velocity, mixing characteristics in riffles and pools	
Sediments	Particle size-frequency	
Gross fate analysis of introduced toxicants		
Monitor toxicants in inflow and outflow, separated into water and adsorbed phases. Monitor toxicant deposition on interstitially-held detritus in riffles and pools.		

A2.2 THE USE OF MULTISPECIES SYSTEMS FOR ENVIRONMENTAL REGULATION

Many reasons have been advanced for moving from the current dependence upon the results of single-species toxicity tests to an approach that incorporates microcosms of the natural environment. Single-species testing has been criticised as an unreliable method for predicting responses to pollution at higher levels of biological organisation (Crow and Taub 1979, Draggan and Reisa 1980, Cairns 1983, Giesy and Allred 1985, Cairns and Pratt 1987). This is because the use of single 'sensitive' species may involve a number of flawed assumptions, including the beliefs (Cairns 1986) that:

1) the response of selected species will correspond to those of a larger array of organisms in a natural system;

2) the chosen endpoints are more sensitive than any other at any level of organisation;

3) the financial savings that result from using the single-species approach are not exceeded by the cost of a poor management decision, for example the imposition of an over-protective safety factor; and

4) a species shown to be sensitive to a few toxicants will be equally sensitive to a much wider range of substances.

The OECD (1981) has recognised that, 'where appreciable environmental concentrations of chemicals are likely to be involved and/or some indication of possible environmental hazard exists, it may be necessary to assess the effects in experimental systems more closely approaching something like natural conditions, especially with regard to interspecific relations and the functioning of multispecies systems'.

The European Community has also recognised the value of developing more relevant test systems, and is currently funding work on model streams at Shell Research Limited and on pond enclosures at GSF in Munich (CEC Contract EV4V-0110-UK (BA)).

Regulatory agencies in the United States have been recommended to use multispecies tests in a number of ways (Harrass and Sayre 1989) including using systems as:

1) models of ecosystems, for use at a high level in a hierarchy of tests;

2) a procedure to integrate fate and effects investigations;

3) a screening method for identifying ecosystem-level problems requiring further investigation;

4) a procedure for demonstrating post-contamination recovery;

5) a method of ranking contaminants, based upon their impact on ecosystem-level parameters.

Giddings (1980) has advocated the use of microcosms for predicting environmental fate and effects, but suggested that simpler systems may be more appropriate for the routine screening or ranking of chemicals. Slooff (1985) has also expressed the opinion that single-species tests are probably sufficient for screening chemicals, although his own research has shown that for 25-30% of test chemicals, a standard toxicity test set including an alga, a daphnid and a fish, failed to cover the toxicity of the chemical to other aquatic species. The author stated that multispecies tests are useful for 'predictive' purposes, in the development and validation of mathematical models, and for the selection of key parameters. Other authors have also suggested that the optimum role of microcosms is near the top of a hierarchy of tests (Kemp *et al* 1980, de Kock and Kuiper 1981, Hendrix *et al* 1982, Herricks and Schaeffer 1987), although Leffler (1984) and Cairns *et al* (1986) proposed that simple generic systems, like Mixed Flask Culture (MFC) or microbial colonisation microcosms, could have a role in screening.

In a lengthy review of laboratory multispecies tests available for ecotoxicological testing, Hammons (1981) concluded that naturally-derived MFCs were at that time the only test potentially efficient enough for the routine screening of chemicals. Sediment cores, periphyton communities and experimental ponds were all identified as useful methods in the intermediate or advanced stages of an hierarchical system of hazard assessment. Progress towards the production of a standardised protocol for experimental pond studies to support pesticide registrations has recently been made in the United States (Touart 1988).

Alternative views to those described above are held by some of those involved in regulation in the United States. Mount (1985) and Loewengart and Maki (1985) warn of difficulties in demonstrating that multispecies tests have 'real-world meaning', and question whether they are more sensitive than single-species tests, or can be used decisively

by regulators to answer questions and provide solutions, rather than merely identifying problems. Tebo (1985) repeats these points and identifies further problems involving the general applicability of results, interpretability, repeatability and social relevance, in terms of the meaning of responses to the public and the courts. Giesy (1985) suggests that until the relative sensitivity and efficiency of single-species and multispecies tests has been established, microcosms should only be used to study particular cases where species interactions may be affected by contaminants.

Proponents of multispecies tests have suggested these can be considerably more sensitive than tests with standard organisms (Suter 1983). Adema *et al* (1983) found effects on natural plankton communities at contaminant (pesticides and 4-chlorophenol) concentrations 1000-2000 times lower than the lowest No Observed Effect Concentration (NOEC) for *Scenedesmus quadricauda*, *Phaeodactylum tricornatum*, *Daphnia magna*, *Gammarus marinus* or *Brachydanio rerio*.

Yount and Shannon (1988) have compared the hazard rankings resulting from tests in mixed flask culture microcosms with those derived from toxicity tests with the fathead minnow, *Pimephales promelas* (Table A2.2).

Table A2.2 - Comparison of 96-hour Lowest Observed Effects Levels (LOEL) for Mixed Flask Culture (MFC) microcosms and fathead minnow

Chemical	96 hr LOEL for MFC (moles/m^3)	96 hr fathead minnow LOEL (moles/m3)
Decanol	19.0	11
Octanol	77.0	68
Hexanol	97.9	863
Hexyloxyaniline	6.7	14
Diisopropylaniline	8.5	59.8
Aniline	107.0	851
Tetrachloroaniline	1.2	0.87
Diuron	0.12	24
Salicylanilide	46.2	8.25

In this study, the 96-hour toxicity to microcosms, as Lowest Observed Effects Levels (LOEL), showed lower, similar and greater toxicity for certain chemicals relative to the 96 hr LC_{50} values obtained for *Pimephales promelas*. Giddings and Franco (1985) found significant changes in community metabolism and zooplankton populations in microcosms and ponds exposed to less than 50 µg/l of phenols, which was similar to the 28-day LOEL for *Daphnia magna*. It is likely that the ultimate resolution of the single verses multispecies sensitivity issue will be the discovery that neither single nor multispecies tests are innately superior in sensitivity in all situations (Maltby and Calow 1989).

A3 POPULATION APPROACHES

A3.1 INTRODUCTION

The community tests discussed in the previous section are designed to mimic with accuracy the various types of aquatic ecosystem that may be affected by contaminants. The virtue of such systems is their relevance to the central objective of environmental management, which is the protection of biological communities. The trade-off for this high ecological relevance is high cost, higher variation in the measured end points and, sometimes, difficulty in interpreting the results. One reason for this last problem is the general lack of knowledge about the factors responsible for structuring even unstressed communities. This is because the strength of explanatory theory seems to increase with decreasing biological organisation. The dynamics of populations is one area of ecology where theory is most fully developed, and so the population could well be the most appropriate unit of study in ecotoxicology (Moriarty 1988, van Straalen and de Goede 1987).

Vinegar (1983) makes an analogy between population studies for assessing environmental risk and epidemiology in human health investigations, and suggests that the three main roles for population studies are:

1) the field validation of laboratory experiments;

2) the design of more predictive laboratory tests; and

3) for comparing the variability of population parameters in the presence and absence of low levels of contamination, thus providing insight into the significance of contamination at the population level.

On this last point, Moriarty (1988) states that, "many field observations contradict the simplistic view that a large number of sudden deaths will permanently damage a population". Kooijman (1985) has observed that populations often appear to be less susceptible to contaminants than are the individuals of which they are composed, and Petersen and Petersen (1989) argue that aquatic toxicology must go beyond the "count-the-bodies" approach.

A3.2 POPULATION STUDIES IN THE LABORATORY

The size of animal or plant populations in any habitat is affected by only four factors: birth, death, immigration and emigration rates (Moriarty 1988). Immigration and emigration will be discussed later, in the section on behavioural tests, although it is recognised that both may have a significant impact on population dynamics and genetics.

Ignoring migration rates, population growth may be modelled using the logistic equation (Equation 1), or one of many recent variants (May 1981).

$$\frac{dN}{dt} = rN\left(\frac{1 - N}{K}\right) \qquad \text{Equation 1}$$

where: N = population size,
t = time,
K = carrying capacity of the environment and
r = intrinsic rate of population increase (birth rate minus death rate under ideal, non-limiting circumstances).

Several laboratory-based toxicological studies over the past decade have successfully used life table or Leslie matrix methods in which the effect of pollution on r, age-specific survivorship and fecundity are calculated for cohorts or populations of aquatic species. The calculation of these parameters has yielded useful results with *Daphnia magna* (Van Leeuwen *et al* 1985 and 1987), *D. pulex* (Daniels and Allan 1981), *D. galeata mendotae* (Day and Kaushik 1987), *Moina macrocopa* (Wong and Wong 1990), *Mysidopsis bahia* (Gentile *et al* 1982, 1983; McKenney 1986) and *Eurytemora affinis* (Daniels and Allen 1981). Mathematical models have also been used to predict the effect of acute or chronic pollution on estuarine (Schaff *et al* 1987) and freshwater fish populations (Barnthouse *et al* 1987; Suter *et al* 1987) using known life-cycle information. Workers at the State University of Ghent are currently developing a chronic population toxicity test with the rotifer *Brachionus calyciflorus* on European Community contract EV4V-0110-UK (BA). Life table studies and population growth experiments are being carried out to determine the most useful and sensitive population parameters to measure in chronic toxicity tests.

Sublethal effects on individual growth, behaviour or physiology that modify survivorship or reproduction may affect populations in ways that cannot be predicted from the investigation of individuals alone (Goodyear 1983). Such sublethal effects can be caused by a reduction in food intake rate or available assimilation energy, effects on basal metabolism leading to increased maintenance energy per unit body weight or effects on reproduction leading to a longer gestation period (Kooijman and Metz 1984) or time to first reproduction (Lewontin 1965).

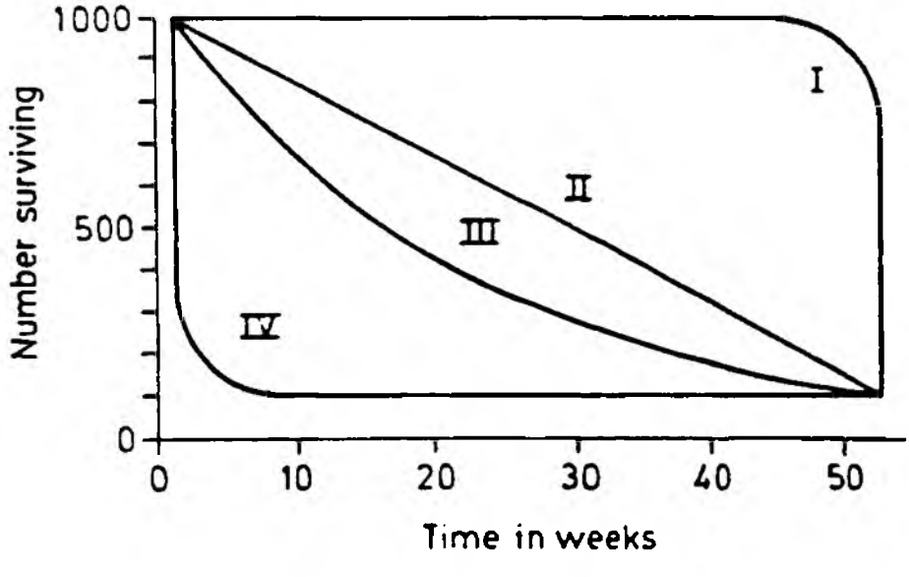
A3.3 POPULATION STUDIES IN ECOSYSTEMS

The laboratory-based, single-species population approach discussed above can be valuable for assessing the likely effects of both lethal or sublethal toxicity on optimum population size, an ecologically relevant parameter. The approach can be used to identify critical life stages and processes. However the general problem of single-species testing, as discussed in the section on microcosms, remains. This means that

there are still considerable difficulties in using the results from these tests for accurately predicting environmental effects at higher levels of biological organisation.

Immature or neonatal life stages are often the most sensitive in single-species toxicity tests (McKim 1985). However, there is a problem in applying this generality to natural populations without at least some understanding of survivorship patterns in the environment (Petersen and Petersen 1989). In Figure A2, four distinct types of survivorship curve are shown. Populations of organisms showing a Type I survivorship have a very low mortality until the end of their life cycle, a pattern commonly shown by cladocerans. Type II populations suffer a constant number of deaths through time, while Type III populations lose a constant proportion of individuals through time. The decreasing power function of Type IV populations describes a situation in which most mortality occurs during the early life stages, after which survivorship is high. Many aquatic insects and fish display a survivorship pattern somewhere between Types III and IV (Petersen and Petersen 1989; Mortensen 1977).

Figure A2



High natural mortality during early life stages is usually attributed to density-dependent biological factors such as predation, pathogens or competition for food or space. An important question for ecotoxicologists and water quality managers arising from this is whether it is possible to demonstrate the existence of any 'additive' mortality caused by a toxicant that is not compensated for by density-dependent mechanisms. Such additive effects have been demonstrated during the early life stages of caddis flies (Petersen and Petersen 1989), though it is unlikely that all populations in natural communities will always respond to contaminants in a similar way to single populations dosed in the laboratory.

A4 WHOLE ORGANISM METHODS

A4.1 LETHALITY

Assessments of the toxicity of chemicals to aquatic organisms in recent years have most frequently been carried out using tests in which death is the endpoint. In a recent review Maltby and Calow (1989) found that in the period 1979-1987 approximately 80% of studies on the effects of pollutants on single species used mortality (immobilisation or failure to respond to stimuli) as the measured response. The majority of tests are performed in the laboratory, however a few *in situ* studies have been reported (deLafontaine and Leggett 1987; Hall *et al* 1987; Johnston *et al* 1987; Ormerod *et al* 1987).

Mortality is the ultimate response to toxicant exposure and reflects an integration of effects at the biochemical and physiological levels of organisation. Consequently, though toxicity tests using lethality as an endpoint are simple, cost-effective and reproducible, the values obtained are relatively insensitive. In acute lethality tests the lethal or effective concentrations for 50% mortality (LC_{50} and EC_{50} respectively) are determined, while in longer-term chronic exposure tests, such as early life stage tests, the lowest observed effect concentration (LOEC) and no-observed effect concentration (NOEC) are derived.

There are now established international guidelines describing acute and chronic lethality tests, as summarised in Table 2.3 (Section 2.4). These standard guidelines describe the species used, the experimental protocol and data interpretation. At present the EC are evaluating a short-term early life stage test with the zebrafish, *Brachydanio rerio* as an alternative to the commonly used test with the rainbow trout *Oncorhyncus mykiss*. These standard tests are, however, restricted to toxicants present in the water column, although there is considerable interest in developing standard toxicity tests for assessing the lethal impact of polluted sediments.

The sediment tests that have been developed mainly focus on the use of in-faunal invertebrate species, such as amphipods, oligochaetes, polychaetes and insect larvae. The methods have yet to be standardised, although the US Environmental Protection Agency and the American Society for the Testing of Materials are currently considering this question. Despite the lack of standard protocols, sediment toxicity tests have been used extensively in the United States dredged material disposal permit programmes for over ten years (US EPA/Army Corps of Engineers 1977). Mearns *et al* (1986) reported an inter-laboratory comparison of the most widely-used amphipod test with *Rhepoxynius abronius* (protocol published by Swartz *et al* 1985). This study concluded that the test could reliably distinguish between toxic (<76% survival) and non-toxic (>87% survival) sediments, though not those in the intermediate range.

In the sediment toxicity test development programme currently being carried out at WRc (Roddie 1990), the lethal toxic impact of whole freshwater and marine sediments is being assessed in the midge *Chironomous riparius* and the amphipod *Corophium volutator* respectively.

A4.2 SUB-LETHAL MEASUREMENTS ON WHOLE ORGANISMS

Many techniques are currently available or being developed for investigating the sublethal effects of contaminants on whole individuals. These range from physiological responses such as growth and reproduction, to the measurement of behavioural effects. Ecological

theory (Kooijman 1985) and experimental population-level studies (see Section A3.2) suggest that contaminants may influence populations through sublethal effects on growth and reproduction, and that mortality is only one of a range of ecologically significant endpoints. The main approaches in this category are outlined below.

A4.2.1 Physiological tests

Physiological endpoints in toxicity tests may be useful indicators of pollution for a number of reasons (Widdows 1985):

1) they integrate the many cellular and biochemical processes that respond to environmental stimuli, and are complementary to specific biochemical responses;

2) they are a good general response to the totality of environmental stimuli;

3) they can reflect environmental deterioration before population or community effects are apparent.

Widdows (1985) also suggests several criteria for judging whether a physiological index is a useful measure of an organism's condition in response to pollutant exposure. In his view the index should:

1) reflect a quantitative or predictable relationship with the pollutant, and have a large range of response from optimal to lethal conditions.

2) have ecological significance.

3) reflect an integrated steady-state condition, not significantly affected by short-term environmental fluctuations such as tides or daily cycles.

4) be capable of precise measurement, with a high signal to noise ratio.

5) be applicable to laboratory or the field situations without high costs or the necessity for complicated procedures.

The types of physiological tests that are available and a critical summary of test features are described below and summarised in Tables 2.4 and 2.5 of Section 4.2.

i) Growth rate

Direct measurement of the growth rate of certain algae, crustacea and fish has been part of the standard battery of toxicity tests recommended by regulatory organisations for several years (OECD 1984; EPA 1984; EC 1984). A range of other species have also been used to assess inhibition of growth (Table A2.3). These simple protocols have become standard in toxicity testing, and the use of such tests seems to have increased in recent years. In a review of more than 2000 papers describing results from single-species tests, less than 2% of the papers from before 1979 (n=992) presented growth results. However between 1979 and 1987 this figure rose to more than 8% of the 1175 papers reviewed (Maltby and Calow 1989). Shell growth rate has sometimes been used as an indicator of stress in molluscs (Stromgren 1982), although such measurements do not provide information on the physiological factors influencing growth (Kammenga 1989).

Certain problems with standard methods have led to a number of new approaches to the measurement of growth. Rhee (1989) has suggested the use of continuous culture algal bioassays in preference to the batch cultures presently used. The advantage of continuous cultures is that a steady-state response to contamination can be obtained, which enables a more realistic prediction of environmental impact. A chronic flow-through system for algal toxicity tests is currently under development at the Fraunhofer Institut für Umweltchemie und Ökotoxikologie with funding from the European Community (CEC Contract EV4V-0110-UK (BA)). Lewis (1990) reviewed freshwater algal growth tests and concluded that they were

a necessary complement to invertebrate and fish tests, especially for detergents, textile effluents, acridine, dyes and synfuels. The combined use of several test species for contaminant evaluation was also recommended and this area of research was identified as one that should receive a higher priority from both toxicologists and regulators.

Crossland (1988) criticises fish tests for their lack of sensitivity in detecting differences in growth rate between treatment groups. He suggests that these tests can be improved by marking individuals, grading initial fish weights and providing a diet and environmental conditions that ensure a relatively fast and constant growth rate.

A 'sub-chronic' growth test with juvenile fathead and sheephead minnow is now used widely in the United States for estimating the chronic toxicity of effluents discharged to freshwater or marine receiving waters (Stewart *et al* 1990). This seven-day test is considerably more rapid than traditional fish growth tests of 28 day duration. Critics have, however, questioned whether it is as sensitive as a full fish early lifestage or growth test (Suter 1990).

Table A2.3 - Growth inhibition tests not included in OECD or EPA guidelines

Taxon	Reference
Bacteria	Bringmann and Kuhn 1980
Algae	
Anabaena cylindrica	Spencer and Greene 1981
Anabaena flos-aquae	Hughes *et al* 1988
Ankistrodesmus falvatus	Spencer and Greene 1981
Ankistrodesmus bibraianus (for sediments)	Ahlf *et al* 1989
Pediastrum tetras	Spencer and Greene 1981
Scenedesmus dimorphus	Spencer and Greene 1981
Scenedesmus quadricata	Klapwijk *et al* 1989
Mycrocystis	Lewis 1986
Dunaliella tertiolecta	Hughes *et al* 1988
Navicula pelliculosa	Hughes *et al* 1988
Macrophytes	
Duckweed (*Lemna minor*)	Health and Safety Executive 1982 Smith and Kwan 1989 Lockhart and Blouw 1980 Lockhart *et al* 1983 and 1989 Jenner and Jansson-Mommen 1989 Wang 1986 Hartman and Martin 1985 Bishop and Perry 1981 Cooley and Foy 1986 Nasu and Kugimoto 1981
(*Lemna gibba*)	Hughes *et al* 1988
Pondweed (*Potamogeton pectinatus*)	Hartman and Martin 1985
Protozoans	
Ciliates	Dive *et al* 1989
Amoebae	Bogaert *et al* 1982
Flagellates	Bringmann and Kuhn 1980
Hydroids	Stebbing 1985
Oligochaetes	
Aeolosoma headleyi	Niederlehner *et al* 1984
Nematodes	
Panagrellus silusiae	Haight *et al* 1982
Insects	
Chironomus decorus	Kosalwat and Knight 1987
Chironomus riparius	Powlesland and George 1986
Tanytarsus dissimilis	Anderson *et al* 1980

Table A2.3 - continued

Taxon	Reference
Molluscs	
Musculium partumeium	Hornbach and Childers 1987
Fish	
Lebistes reticulatus	Wroblewski 1979
Micropterus salmoides	Johansen *et al* 1987
Oncorhyncus kisutch	Miller *et al* 1979
Phoxinus phoxinus	Bengtsson 1980

A ten-day sub-chronic growth test using the midge *Chironomus tentans* has also been used in the United States (Giesy *et al* 1990), and the University of Wales are currently investigating the use of both *Chironomus riparius* and the amphipod, *Gammarus pulex* in similar growth tests in the UK.

At WRc chronic toxicity tests, using growth and development endpoints are being developed with the midge *Chironomus riparius* and the polychaete worm *Capitella capitata* to assess the toxic impact of whole freshwater and marine sediment (Roddie 1990).

ii) Scope for growth and feeding rate

The growth rate of any organism is fundamentally affected by the amount of energy available at a given time. Scope for growth (SFG) is the amount of energy absorbed from food minus the energetic costs of respiration (Warren and Davis 1967), and may be calculated from Equation 2;

$$SFG = C - D - R \qquad \text{Equation 2}$$

where C = energy consumed through feeding
D = energy lost in faeces and excreta
R = energy lost in respiration

The advantage of using SFG, rather than direct measurement of growth, as an indicator of contaminant stress is that the separate energy budget components (absorption, respiration and excretion) can be analysed after a shorter exposure period for possible causes of disturbance to growth (Bayne 1985). Toxic stress may, for example, reduce energy consumption through a depression in feeding rate and increase respiration rate, thus reducing SFG. Alternatively, C and R may remain constant, but assimilation efficiency may be reduced, leading to an increase in D and a decrease in SFG.

The SFG has been used extensively with *Mytilus edulis* to assess marine water quality around the UK and elsewhere (Bayne 1985, Widdows 1985, Widdows and Johnson 1988, Koehn and Bayne 1989, Widdows and Donkin 1989), and has demonstrated sensitivity to a wide range of organic and inorganic contaminants. Efforts are currently being made to relate *Mytilus* SFG to population-level endpoints through mathematical models currently under development at the Plymouth Marine Laboratory (PML) for the NRA (Johnson *et al* 1990).

The usefulness of the SFG technique for assessing freshwater pollution has also been demonstrated with the freshwater shrimp, *Gammarus pulex* (Naylor *et al* 1989) and a relationship has been found between reduced SFG and a reduction in female fecundity (Maltby and Naylor 1990). Studies designed to validate and simplify the *Gammarus* SFG procedure are currently in progress at WRc and Sheffield University. Feeding rate, which is simple to measure, is the largest and most sensitive parameter used in the SFG equation. Because of this, it is likely that feeding rate alone will be measured as the endpoint in future laboratory and *in situ* tests (Crane and Maltby 1991).

Several other workers have also found a reduction in feeding rate in animals exposed to contamination. Test organisms have included chironomid larvae exposed to cadmium (Heinis *et al* 1990), lugworms

(Augenfeld 1980) and marine oysters exposed to petroleum (Mahoney and Noyes 1982, Crecelius *et al* 1980), freshwater fish exposed to lindane (Bakthavathsalam and Reddy 1981) and frog tadpoles exposed to a mothproofing agent (Osborn and French 1981).

iii) Reproduction rate

A reduction in the growth rate and SFG of an organism may affect both the time to first reproduction and the size, number, or energy content of offspring (Sibly and Calow 1986, Bayne 1985). OECD and United States EPA guidelines have included tests for effects on reproduction for several years (OECD 1984, EPA 1982). The standard *Daphnia magna* reproduction test lasts for 14-21 days, with the time to production of the first brood as one endpoint (OECD 1984). The endpoint in the mysid shrimp toxicity test is the cumulative number of young per female on day 28 (EPA 1982). Several fish reproduction tests have also been developed recently and an 8-day test using the zebrafish (*Brachydanio rerio*) is currently being evaluated by the OECD. Zebrafish have been used to assess the effect of adult exposure to pulp mill wastewater on the fitness of eggs, embryos and juveniles (Landner *et al* 1985). The results from this experiment showed that reproductive success was impaired at effluent concentrations five times lower than those showing effects during the direct exposure of embryos and larvae.

A number of other species have also been used to examine the effects of pollution on reproduction in tests that have not yet been recognised by an international body. These include the cladocerans *Ceriodaphnia dubia*, *C. reticulata* and *Daphnia pulex* (Carlson *et al* 1986; Elnabarawy *et al* 1986), the copepod *Tisbe battagliai* (Hutchinson and Williams 1989), the molluscs *Helisoma trivolvis*, *Physa gyrina* and *Musculium partumeium* (Millemann *et al* 1984; Hornbach and Childers 1987), and the fish *Pimephales promelas* and *Phoxinus phoxinus* (Spehar and Fiandt 1986). However, the investigation of contaminant effects on reproduction does not seem to have increased in recent years, comprising less than 5% of the

papers reviewed by Maltby and Calow (1989) both before 1979, and during the period 1979 to 1987. This may change with the introduction in the United States of a rapid sub-chronic reproduction test using *Ceriodaphnia dubia* (Stewart *et al* 1990). This toxicity test has proved to be a useful predictor of measured toxic impact on receiving water macroinvertebrate community structure (Eagleson *et al* 1990).

Fecundity (the number, weight, or energy content of offspring) is not the only parameter of reproductive fitness that may be affected by contaminants. Reproductive effort, or the amount of energy allocated to gamete production may also be influenced (Bayne 1985; Calow 1979). Recent work with *Daphnia magna* has shown that animals may partition more or less energy in somatic growth or reproductive output, depending upon ambient concentrations of toxicant (L Maltby, Sheffield University, pers comm). Additional parameters associated with reproduction that have been shown to be affected by pollution are the onset of ovulation in fish (Tam and Payson 1986) and gametogenesis in a number of species (eg Chung and Brinkhuis 1986). Measurements of reproductive parameters other than female fitness may not, however, have any ecological relevance. Kluytmans *et al* (1988) found that although cadmium affected follicular growth in *Mytilus edulis*, no significant effects could be found on the number of gametes produced by a population.

iv) Bioluminescence

The patented Microtox (Microbics Corporation) test is an inexpensive and rapid bioassay that is increasingly used for toxicity testing. The system is supplied as a package of hardware, computer software and freeze-dried bioluminescent bacteria (*Photobacterium phosphoreum*) for the calculation of EC_{50}s, using the inhibition of bacterial bioluminescence as the test endpoint. The most recent review of the sensitivity of the test compared Microtox with daphnid, rainbow trout and fathead minnow acute lethality tests (Munkittrick *et al* 1990). Microtox was generally

as sensitive as, or more sensitive than the acute lethality tests for individual organic chemicals, but less sensitive to most inorganics and effluents with a high proportion of insecticide, herbicide, pharmaceutical, textile, or highly lipophilic components. The sensitivity and precision of the Microtox test increased with the complexity and toxicity of industrial effluents. These conclusions are consistent with those of earlier reviews of the method (Dutka and Kwan 1981; Quershi *et al* 1982; McFeters *et al* 1983; Vasseur *et al* 1984). On the basis of such comparisons, Microtox has been proposed as a useful tool for screening, and possibly monitoring, complex effluents in fresh and saline systems (Curtis *et al* 1982; Elnabarawy *et al* 1986; Hunt 1989).

A limitation of the Microtox technique is that sediment toxicity can only be assessed after solvent or acid extraction. However, a new technique in which *Photobacterium phosphoreum* is brought into direct contract with sediment has proved useful for detecting hydrophobic organic contaminants (Brouwer *et al* 1990).

An alternative toxicity test involving a bioluminescence response is currently under development by Amersham using genetically engineered *Escherichia coli* harbouring a lux plasmid (Stewart *et al* 1989). This simple and apparently sensitive technique, can establish the efficacy of biocides within 5-10 minutes through a reduction in light output. However, the test remains to be tested with a wide range of pollutants, including industrial effluents.

v) Oxygen to Nitrogen ratio

Stressed organisms tend to use nutrient reserves to meet high metabolic requirements. The oxygen to nitrogen (O:N) ratio, based on measurements of the rates of oxygen consumption and ammonia excretion, is an index of the balance between carbohydrates, lipids and proteins in an organism (Widdows 1985). An organism with a low O:N ratio is generally breaking down protein, which is normally an indication of stress, although the converse has been recorded in

some species. This technique has been shown as a sensitive measure of cadmium stress in juvenile mysid shrimps (Carr *et al* 1985), zinc and copper stress in freshwater shrimps (Correa 1987), diesel oil stress in saline shrimp species (Tedengren *et al* 1988) and suspended solid stress in freshwater mussels (Aldridge *et al* 1987).

vi) Whole body indices

Bayne (1985) describes the use of two indices of shellfish condition for assessing the effects of pollution. The Body Condition Index is the proportion of the internal shell volume which is occupied by the body tissues, and can be calculated using either wet (Equation 3) or dry (Equation 4) tissue weight.

$$\frac{\text{(Weight or volume of wet flesh)}}{\text{Shell cavity volume (ml)}} \times 100 \qquad \text{Equation 3}$$

$$\frac{\text{Dry tissue weight (g)}}{\text{Shell cavity volume (ml)}} \times 1000 \qquad \text{Equation 4}$$

The condition index depends upon the balance between food availability and feeding and catabolic rates and may be affected by a number of environmental factors. The measurement of body condition is simple, though Bayne (1985) stated that the method is generally insensitive and has a low signal-to-noise ratio. However, recent work at WRc, has demonstrated that changes in the methodology can produce a technique with low variation that in *Mytilus edulis* is highly sensitive to marine pollution (B Roddie, WRc, pers comm).

vii) Respiration rate

A number of standard methods exist for the measurement of respiratory oxygen uptake in bacteria (Yoshioka *et al* 1986), plants

(Vollenweider 1969) and animals (Grodzinski *et al* 1975). Although pollutant-induced effects on bacterial respiration have been recorded, the index may be less sensitive than other sub-lethal parameters for many species (Trevors 1982; Dutka *et al* 1983). The oxygen production of a bacterial population on a filter has been used in 'Toxiguard', a commercially available early warning system (Solyom 1977), which has proved to be both useful and sensitive. In addition the oxygen consumption of activated sludge has been applied as a parameter by Shieh and Yee (1985), while Martin (1988) has used activated sludge in an on-line respirometer to detect the bacterial biomass respiration. The potentiometric measurement of CO_2 production by *Escherichia coli* immobilised at the surface of a CO_2 sensing electrode has been developed as a rapid, inexpensive and sensitive bioassay (Dorward and Barisas 1984).

Algal respiration has been shown to be impaired by anilines and toluidines (Batterton *et al* 1978), petroleum oils (Singh and Gaur 1990) and oil refinery effluent (Reddy *et al* 1983). Caddisfly, daphnid, crab and oligochaete respiration rates were affected respectively by low pH (Correa *et al* 1986), phenol (Kolupaev *et al* 1986), chlorine-produced oxidants (Key and Scott 1986) and contaminated sediments (Chapman 1987). Fish respiration rate can be affected by many contaminants, including organic chemicals (Capuzzo *et al* 1977) and crude oil (Prasad 1987).

However the pattern of the measured response may be difficult to interpret, as respiration rate commonly increases with exposure level to a certain threshold, after which a decline is recorded until the death of the organism (eg Lee *et al* 1978). In addition, an acclimation of respiration rate to toxic concentrations has been recorded (Papathanassiou 1983).

viii) Ventilation rates

The respiration of fish is directly related to the movement of the operculum (gill cover) and this index has been used in commercially

available continuous biomonitors. Baldwin (1990) reviewed the use and limitations of fish ventilation rate as a method of monitoring for pollution events and concluded that the technique is responsive to many contaminants, although there are some exceptions, including heavy metals. The sensitivity of fish monitors such as the WRc Mk III monitor to different contaminants ranges from the detection of µg/l to mg/l levels, depending on the substance. The index is generally close to or below the 96 hr LC50 value for rainbow trout, with a response time of about 30 minutes. Sensitivity may, however, be affected by the species, condition and biological variability of the stock used, and by interference from external stimuli other than toxicants (Baldwin 1990). In addition to determining ventilation rate, important information can be obtained from the electrical signal on the strength of the movement (ventilatory depth) and coughing rate (Drummond and Carlson 1977, Diamond *et al* 1990), which can increase the sensitivity of the technique.

An 'optical-fibre light interruption biomonitoring system' has been developed by Batac-Catalan and White (1983) to detect the pollutant effects on the ventilation system of *Chironomous* sp.

ix) Photosynthesis

The effect of contaminants on the photosynthetic rate of cyanobacteria, algae and higher plants has been assessed in a number of studies. Several standard methods are available for the measurement of photosynthesis using oxygen production rates or more sophisticated ^{14}C tracer techniques (Vollenweider 1969). Algal photosynthesis can be affected by crude and fuel oils (Batterton *et al* 1978; Hutchinson *et al* 1980; Singh and Gaur 1990), metal ions (Stratton and Corke 1979), herbicides (Fritz-Sheridan 1982; Rawson *et al* 1987), insecticides (Stratton and Corke 1982), chlorobenzenes (Calamari *et al* 1983) and surfactants (Lewis and Hamm 1986).

A number of continuous monitoring devices using phototropic organisms have been developed for assessing the impact of

pollutants, particularly herbicides, on primary producers. These have been reviewed by Kammenga (1989) and toxicity responses include:

1) inhibition of fluorescence in a constant density culture of *Scenedesmus subspicatus* (Benecke *et al* 1982);

2) changes in chlorophyll fluorescence intensity and carbon assimilation in caged plankton communities as monitored by a microcomputer in the Algal Fractionation Bioassay (Munawar and Munawar 1987); and

3) inhibition of photosynthesis in the cyanobacteria *Synechococcus* sp immobilised on an electrode (Rawson *et al* 1987, 1989).

x) Osmoregulation

The effect of a contaminant on animal osmoregulation can be determined by measuring tissue water content, the overall blood osmolality, or the concentration of major ions in the blood or haemolymph. In freshwater and estuarine animals, particularly crustaceans and fish, the blood osmolality is maintained at a higher level than the external medium (ie hyperosmotic). This is primarily achieved by the active regulation of the major ions Na^+ and Cl^-. In fish, significant reductions in blood osmolality and blood Na^+ and Cl^- have been found after exposure to low pH (McDonald and Wood 1981, Harvey and Whelpdale 1986, Peres *et al* 1990), heavy metals (McCarty and Houston 1976, Stagg and Shuttleworth 1982) and paper mill effluents (Soivo *et al* 1988). Studies have also been carried out with crustaceans, and effects on osmolality found in the marine shrimp, *Palaemon adspersus*, exposed to crude oil (Baden 1982) and the estuarine amphipod *Gammarus duebeni* exposed to zinc (Johnson and Jones 1990).

Increases in tissue water content have been measured in freshwater asiatic clams exposed to zinc (Belanger *et al* 1986) and fish

exposed to cadmium (McCarty and Houston 1976). Sodium/potassium adenosine triphosphatase (Na^+-K^+ ATPase) transport activity has also been used as an indicator of effects on osmoregulation in fish (Kuhnert *et al* 1976) and crustaceans (Neufeld and Pritchard 1979; Haya *et al* 1983), although the results have been equivocal.

Leivestad and Muniz (1976) suggested that the measurement of gill ATPase activity and plasma Na^+ and Cl^- levels may be a useful index of acid water exposure, since effects on these parameters are apparently the primary cause of fish death. However, osmoregulation may not always be a sensitive indicator of contaminant stress. Oikari *et al* (1985) found that measurements of blood plasma sodium, chloride and magnesium in trout placed in cages downstream from a pulp and paper mill were less sensitive than several other blood parameters. A similar relative lack of sensitivity was found when fish were exposed to high levels of suspended solids (Redding *et al* 1988).

xi) Electrical activity

The elephant-nosed mormyrid (*Gnathonemus petersi*), a weakly electric fish has been used in a limited number of trials designed to develop a continuous water quality monitor for drinking water intake protection. The fish produces short electrical pulses for communication and orientation (Bullock and Heiligenburg 1986) and the rate of discharge changes significantly in the presence of metal and probably other toxicants (Geller 1983). A commercial system (Aztec FM1000) has been operated by the Bournemouth Water Company for over a year, and an evaluatory study is planned by Thames Water in the near future. Little information is available on the sensitivity of the system (Baldwin 1990).

A4.2.2 Behavioural responses

Behavioural toxicity may be defined as the toxicant-induced change in behaviour pattern that exceeds the normal range of variability

(Marcucella and Abramson 1978). A behavioural change may represent the initial response of an organism to environmental perturbation (Slobodkin 1968). For example, certain adaptive, defensive or avoidance responses such as shell closure prevent toxicant exposure, thereby reducing the probability of sub-lethal or lethal effects.

However, at a higher threshold, these responses may be inadequate and aberrant or abnormal behavioural responses may result, indicating that the toxicant concentration has exceeded the tolerance limit of the organism. These behavioural changes may be accompanied by biochemical or physiological damage to the sensory organs, nervous system or other parts of the body. Although many changes in normal response may indicate behavioural toxicity, only pollutant-induced changes that decrease the organism's ability to adapt and survive in the environment are ecologically significant.

In selecting a behavioural response as a toxic endpoint, Rand (1985) identified the specific requirements that:

1) the behavioural pattern has to be displayed under laboratory or controlled field investigations, in order that the behavioural responses can be isolated; and

2) General behavioural patterns that integrate or depend on diverse sensory or motor mechanisms should be evaluated

Pollutants may affect a number of behavioural responses in aquatic organisms (Table A2.4), although developed techniques have not commonly been used in the assessment of pollutant effects. This is primarily due to the requirement for a considerable body of background data, from which subtle behavioural changes can be discerned. Although the application of computer technology for data gathering and interpretation have addressed certain inherent difficulties, the high level of training and the time and expense required to generate an adequate database have restricted the general use of behavioural indices.

Table A2.4 - Behavioural responses used to monitor sub-lethal effects of chemicals

Individual responses	Inter-individual responses
LOCOMOTOR	PREDATOR-PREY INTERACTIONS
Undirected locomotion (activity): - Free (Spontaneous free-running patterns) - Forced (Swimming performance or ability)	SOCIAL INTERACTIONS Territoriality Dominance Aggregation
Directed (orientation) responses: Preference/avoidance and orientation to	
- Salinity gradient - Temperature gradient - Light - Current - Gravity - Food odour or pheremone - Chemical toxicant	
FEEDING BEHAVIOUR/MOTIVATION	
LEARNING	

The behavioural techniques which will be considered in this section can be divided into tests which measure:

i) the preference-avoidance responses of organisms to environmental stressors; and

ii) abnormal behaviour or departures from 'normal' activity patterns which may have adverse biological effects. These include changes in locomotor and reproductive behaviour, and predator-prey interactions.

A summary of the types of test available and critical evaluations of these tests have been given in Tables 2.6 and 2.7 respectively of Section 2.6.

i) Preference-avoidance behaviour

The preference-avoidance responses of motile species to environmental stressors, including industrial discharges, have been reviewed by Cherry and Cairns (1982) and can be used to determine whether:

1) fish or other motile aquatic organisms actively avoid plumes of toxic materials;

2) a thermal plume or other water discharge causing no other apparent adverse biological effects will alter the distribution of motile species in the receiving waters; and

3) the extent to which pre-exposure to elevated temperature environmental stressors alters subsequent preference-avoidance behaviour

Assessments of the extent to which motile aquatic organisms avoid toxic plumes resulting from industrial discharges are important since these behavioural responses can result in a loss of usable habitat. Furthermore, avoidance responses may be particularly important for determining pollutant impact on receiving waters used by migratory fish, as effluent plumes may restrict their passage (Smith and Bailey 1990).

Preference-avoidance studies may also be important for determining the effects of thermal effluents, which by attracting fish and other motile species to an outfall could expose the organisms to other pollutants, such as those used in the chlorination process. Indeed, Cherry and Cairns (1982) recommended that preference-avoidance

testing should be mandatory for receiving waters important for the passage of migratory species or impacted by thermal effluents.

There are no standard preference-avoidance behaviour tests and a considerable diversity of test procedures have been described in the literature ranging from simple observational techniques to elaborate methods using video recording and computer analysis. In the majority of preference-avoidance studies conducted for temperature and pollutant stressors the testing systems have been relatively sophisticated using either shallow or steep gradients between the tested temperatures or chemical concentrations (Cherry and Cairns 1982).

For chemical pollutants Hartwell *et al* (1988) have shown that although laboratory derived avoidance data are representative of field derived data, there may be inaccuracies, due to differences in the background chemical matrix and the physical setting, and large errors depending on the acclimation history of the fish. Because of these potential difficulties in extrapolating from laboratory experiments to field situations, *in situ* behavioural testing systems are apparently most appropriate for assessing pollutant impact.

A novel *in situ* preference-avoidance technique for fish has been described by McGreer and Vigers (1983), based on pollutant-induced changes in the vertical distribution of normally surface dwelling caged juvenile coho salmon (*Oncorhynchus kela*). The method has been tested with a pulp mill effluent release into estuarine and marine waters, and response times of 30 minutes were found, even at distances of 10 km from the outfall. The cage technique appears to be a sensitive, reliable and cost-effective tool, which has modest logistical requirements and provides data which can be relatively easily interpreted. In addition, and importantly, the response realistically predicts the effects of industrial pollutants on natural fish populations, since the fish movements based on avoidance tests were consistent with those found in the field for migratory juvenile salmon.

An avoidance behaviour response for sessile organisms, currently under consideration at WRc is the shell valve activity monitor recently developed by the Dutch research organisation TNO. Pollutant-induced effects on the valve opening and closing patterns of freshwater and marine mussels are electronically measured and assessed in this method (Kramer *et al* 1989). The technique has at present been tested on the freshwater mussel *Dreissena polymorpha* and the marine mussel *Mytilus edulis*. Positive response patterns, measured as a closing response or change in activity in a proportion of tested animals, have been recorded in groups of *Dreissena* exposed to continuous sub-lethal concentrations of dispersed crude oil, tributyl-tin oxide and the heavy metals copper, cadmium and zinc. The system also detected the effects on *Mytilus* of copper pulses at ug/l levels. This method could be applied to epibenthic and infaunal bivalve species for monitoring effluents and general receiving water quality and for testing the toxicity of compounds or mixtures of interest.

ii) Abnormal behaviour

a) Reproductive behaviour

Bioassays based on direct or induced disruption of precopulatory pairing behaviour by contaminants have been described for freshwater and marine amphipods (Davis 1978; Linden 1978; Poulton and Pascoe 1990). In the direct precopula bioassay, the time taken for pairs to separate as a direct result of toxicant exposure is used as the behavioural index. The method has identified responses in the marine amphipods *Anisogammarus pugettensis* and *Gammarus oceanicus* exposed respectively to Bleached Kraft Pulp Mill Effluent (Davis 1978) and oil (Linden 1978). This method has also been used to test the response of *Gammarus pulex* to cadmium in the laboratory and to elevated ammonia and decreased dissolved oxygen in the field (Poulton and Pascoe 1990).

The behavioural index used in the induced precopula bioassay proposed by Poulton and Pascoe (1990) is the Median Induced Separation Time (IST50), which is a measure of the time taken for the male and female to resume precopulatory pairing, following induced separation, in a range of toxicant concentrations. Responses in *Gammarus pulex* were detected at 22 µg/l cadmium in this bioassay.

Although this behavioural bioassay is rapid and sensitive, it is difficult at present to ascribe ecological relevance to the technique. In contrast, the direct precopula approach has the advantage of allowing post-exposure observations of recovery and re-establishment of precopula pairs, which can be related to ecosystem recovery following a pollution incident.

b) Locomotor behaviour

Responses based on locomotory behaviour are considered in this review to include activity, positive rheotaxis and phototaxis.

Activity

Activity patterns in fish have been shown to respond to a wide range of pollutants and continuous or semi-continuous monitoring systems compare non-polluted background responses with toxicant-induced effects. A range of monitoring techniques have been used, including interruption of light beams, detection of ultrasonic echoes, monitoring of an inductive current generated by implanted magnets, detection of electric potential and designed fish fitness test systems, and these have been reviewed by Baldwin (1990).

Zooplankton species have been shown to be sensitive to a wide range of pollutants and their locomotor activity can be used as a biomonitoring tool. Buskey and Stoecker (1989) have produced a monitoring system for the swimming activity in the marine

tintinnid (*Favella* sp) using a video system with dark field infra-red illumination and subsequent automated motion analysis.

The short-term activity of chironomids to pollutants can be detected by an impedence-conversion technique (Heinis and Swain 1986, Heinis and Crommentuijn 1989, Heinis *et al* 1990). The system has been tested with heavy metals and organics under laboratory conditions, and an operational field system is under development.

Rheotaxis

Fish living in running waters generally swim upstream against the current, thereby exhibiting positive rheotaxis. Toxicant exposure can lead to a loss of positive rheotaxis and effects can be detected by the blocking of a light beam or infra-red light by photo-electric cells. The available systems have been reviewed by Van der Schalie 1986 and Kammenga 1989. These early warning systems have been tested in monitoring surface water quality in Germany (Scharf 1979) and the Netherlands (Koeman *et al* 1978) but were considered to be less sensitive than techniques that monitored ventilatory activity.

c) Predator-prey interactions

Predator-prey interactions between copepods and mysids have been developed by Cooper and Goldman (1982) as simple and environmentally relevant test methods. This technique could examine the ability of

1) contaminant exposed prey to avoid exposed predators;
2) contaminant exposed prey to avoid non-exposed predators and
3) non-contaminated prey to avoid contaminant exposed predators.

The comparison of toxicant-induced changes with background control levels may provide an indication of the consequences of observed feeding rate changes for effects on community structure.

A5 SUB-ORGANISM METHODS

A5.1 Introduction

Changes in structure and function at the biochemical, sub-cellular and cellular level should, in theory, provide rapid and highly sensitive early warning indicators of pollution in aquatic ecosystems. The observed effects can be used to identify potential problems before an integrated cellular response manifests itself at the level of whole animal physiological processes, and long before changes are evident at the population level.

At present these techniques have no established role in the regulation or monitoring of water quality and there are no standard protocols from relevant agencies, such as the OECD, EPA and ASTM. However, the considerable research effort devoted to the development of sub-organism methods and the increasing use worldwide of these techniques by regulators on a site specific basis indicates the potential of these techniques. The methods are often used in a 'clinical' or 'forensic' role and consequently would not be applied singly, but rather as a suite of techniques to enable realistic and appropriate conclusions to be drawn.

The usefulness of any sub-organism index for assessing water and sediment quality depends upon a number of response features including:

1) Ease of measurement and reproducibility
2) Sensitivity to sub-lethal stress
3) Discriminatory capacity

Furthermore, it is vital that sub-organism methods considered appropriate for assessing water and sediment quality are thoroughly

tested and validated in the field before they are routinely applied for water quality regulation. An extensive series of field tests will allow toxic effects to be separated from acclimation responses.

Several summary tables of suborganism methods have been given in Section 2.7 of the review (Tables 2.8-2.11).

A5.2 TISSUE METHODS

Histopathological changes in organs, tissues and cells are the net result of adverse biochemical and physiological changes in an organism. The quantification of these morphological alternations provides a means by which their potential effects on processes or activities, such as growth, reproduction, predator avoidance and population stability can be predicted (McKim 1985; Meyers and Hendricks 1985). Regular histopathological screening of natural populations can be used to distinguish toxicant dependent effects from environmental and non-pollutant responses. Target sites can then be identified and histopathological changes established as either acute or chronic (Hinton and Lauren 1990).

The effectiveness of quantitative histopathological techniques has increased in recent years with the development of sophisticated analytical techniques, such as high resolution light microscopy, electron microscopy and immunohistochemistry (Hinton and Lauren 1990).

Tissue pathologies, such as swelling or atrophy, are usually non-specific indicators of pollutant exposure (for review Meyers and Hendricks 1985). In fish, gills (filaments and lamellae), liver, kidney, spleen, thyroid, pituitary, gastro-intestinal tract and gonads can be affected by exposure to pesticides (Webster and Canton 1986), heavy metals (Haux and Larsson 1984) and contaminated sediments (Gardner and Yevitch 1988). A fusing of fish gill lamellae has also been found following exposure to untreated bleached kraft pulp mill effluent (Couillard *et al* 1988) and treated petroleum refinery effluent

(Onwumere and Oladimejc 1990). Fish larval morphology may be affected by contaminants such as oil (Westernhagen *et al* 1987).

In invertebrates, shell deformities in molluscs can result from exposure to heavy metals (Sunila and Lindström 1986), and the morphology of chironomid exoskeletons can be affected by oil (Cushman *et al* 1984). Algal cell shape or size has been altered by chlorine, lead and tributyl tin (Trotter *et al* 1978, Roderer 1983, Starodub *et al* 1987).

Tissue somatic indices (TSI) or body component indices (BSI), based on the ratio of the tissue weight to whole body weight, have also been used as an early indicator of pathological effects in a range of aquatic organisms. Pesticides, fungicides and hydrocarbons can affect the gonadosomal (Bayne *et al* 1978; Shukla and Pandey 1986; Ram and Sathyanesan 1986, 1987) and the hepatosomatic or digestive gland (Bayne *et al* 1978; Ram and Sathyanesan 1987) indices of fish and molluscs.

The embryos of aquatic species may also be sensitive indicators of contamination. The effect of teratogens (substances that specifically affect embryogenesis (Hubison 1980)) may be due to a variety of mechanisms. These include genetic mechanisms such as mutation, chromosome nondisjunction and altered nucleic acid function and nongenetic events such as an insufficient supply of energy sources and substrates, enzyme inhibition, altered membrane permeability and an osmolarity imbalance. However, the final effect in all cases is a morphological change in the offspring. Many classes of substances are teratogenic to aquatic species and several standard methods are available for assessing their effects. One of the measurements taken in fish life-cycle or early life stage tests is the number of abnormal embryos per treatment group (McKim 1985). In tests of this type, teratogenic effects have been observed in fish embryos exposed to mercuric compounds (Leonard *et al* 1983), dithiocarbamates (van Leeuwen *et al* 1986) and a range of other substances (McKim 1985). Frog tadpoles and other amphibians have also been used in the assessment of teratogenicity (Abbasi and Soni 1984). Invertebrate tests for teratogenicity have been developed using sea urchins (Hose *et al* 1983),

mussels and oysters (ASTM 1980, ISO 1990). The oyster embryo bioassay has been used widely in recent years and seems to be a particularly sensitive indicator of metal contamination (Martin *et al* 1981), although there is some debate about the sensitivity of the test to organic compounds (Calabrese 1984).

A5.3 CELLULAR METHODS

A5.3.1 *In vivo* cellular assay systems

i) Cell structure

Changes in the cell structure of fish and molluscs after exposure to pollution have been noted in many studies. Inflammation, atrophy or necrosis have been recorded in gill cells (Soderburg 1985), thyroid cells (Canton 1983, Katti and Sathyanesen 1987, Webster *et al* 1988), pituitary cells (Katti and Sathyanesen 1987), hepatocytes (Dixon and Leduc 1981, van Leeuwen *et al* 1986, Ram and Sathyanesan 1987) and cells in the digestive system (van Leeuwen *et al* 1986, Forlin *et al* 1986, Ram and Sathyanesen 1987). Hydrocarbon exposure in molluscs can result in the thinning of digestive tubule epithelium, reductions in the volume of mantle storage cells and ripe gametes and increased degeneration of oocytes (Lowe and Pipe 1985; Moore *et al* 1988). These cellular responses indicate direct impairment of feeding and reproductive processes and a reduction in the fitness of the animal.

Changes in the thyroid follicles of fish induced by pollution can be quantified by the ratio of epithelial height to follicle diameter (E:T). This index has been shown to increase in response to chronic sub-lethal concentrations of the insecticide malathion (Pandey and Shukla 1983), the pesticides fenitrothion and carbofuran (Saxena and Mani 1988) and DDT (Shukla and Pandey 1986).

ii) Immunological indicators

The immune response in fish can be compromised by environmental stressors, and toxicant-induced immunosuppression often increases the vulnerability of fish to disease (Anderson 1990). At present, immunological and serological assays are widely used to detect and identify disease causing agents. However, as the biological materials required for testing become more defined and increasingly available, rapid and sensitive methods will probably be more extensively used to investigated pollutants impact on the immune system of fish. Standardised responses of control fish can be compared to those of fish in polluted environments or of fish suspected of being under stress.

Immune indicator assays can be divided into three broad categories:

1) non-specific assays of general characteristics that indicate the health of the fish, without the use of antigenic stimulation. These include the physical condition of haemopoetic tissues and organs, haemocrit (fraction of blood occupied by red blood cells) and leucocrit (fraction of blood occupied by white blood cells (Section A5.3.1ii).

2) indicators that can be used with or without antigenic stimulation. These include macrophage chemotaxis and activity indices (Weeks *et al* 1987), a chemiluminescent assay for phagocytic activity against certain immunosuppressants or stimulaters (Stave *et al* 1984) and mitogenic or blastogenic response assays assessing the ability of immune system cells (eg lymphocytes) to proliferate or expand in response to a mitogenic challenge (Laudenslager *et al* 1983).

3) indicators that usually include immunisation of the fish with vaccines, bacteria or other immunogenic preparations and measurement of the specific immune responses in the form of activated cellular responses and the production of antibodies.

These range in complexity from simple agglutination assays, which can be performed by field biologists, to highly sophisticated and sensitive assays such as radioimmunoassays, enzyme linked immunosorbent assays (ELISA) and counter immunoelectrophoresis, which are costly and require highly trained personnel (Anderson 1990).

In measuring and evaluating pollutant effects by immunological indicators, the large number of environmental (temperature) and intrinsic (sex, nutritional status, reproductive and development stage) which can influence the immune response have to considered.

iii) Haematology

In fish, haematological responses to pollutant exposure can be assessed using indices such as red blood cell number, haemocrit and mean corpuscular volume (MCV), mean red blood cell haemoglobin (MCH) and haemoglobin content (MCHC), as well as white blood cell number and leucocrit.

Lehtinen *et al* (1990) found reductions in red blood cell number and haemocrit in rainbow trout exposed to a range of paper mill effluents. Haemocrit can be affected by a range of pollutants; in fish exposed to organic contaminants, levels declined and anaemia increased (Buckley *et al* 1976, Goel and Garg 1980). In contrast, inorganics, including ammonia, chlorine and heavy metals, generally cause a significant increase in haemocrit levels which may be associated with additional oxygen-carrying requirements (O'Connor and Fromm 1975, Zeitboun *et al* 1977, Sheehan and Lewis 1986).

Leucocrit provides an indication of the status or health of the immunopoietic (immune cell producing) system and resistance to disease (Wedemeyer *et al* 1983), since it specifically indicates the quantity of cells responsible for immunity. McLeay and Gordon

(1977) have identified effects of pulp mill effluents on the leucocrit in salmon and trout.

iv) Macrophages

Macrophages are cells in the immune system that are responsible for removing foreign particles and damaged cells by phagocytosis. The available data on the effects of pollution on macrophages are equivocal, although inhibitory effects have been found in fish cells exposed to chromium (Kranz and Gerken 1987) and endrin (Bennett and Wolke 1987).

A5.3.2 *In vitro* cellular assay systems

There is an increasing interest in using acute *in vitro* bioassays for assessing the toxicity of water samples. This is due to a general desire to reduce animal testing for both moral and financial reasons and a greater recognition of the importance of mechanisms at the cellular level. At present there are three main *in vitro* techniques available: organ and primary cultures and continuous cell lines. These techniques have the potential to detect all substances that are acutely toxic by mechanisms not dependent on specialised cellular functions. Although organ and primary cultures are elaborate models of specific *in vivo* situations, general biological assays using continuous cell lines are considered more applicable to assessing aquatic pollution (Hunt *et al* 1987).

There are a large number of established mammalian cell lines, and a lesser number of continuous fish and aquatic invertebrate cell lines from different organs. Commonly used endpoints in *in vitro* cytotoxicological studies are:

a) cell morphology (changes in cell shape and size, increases in cell granularity, and death and reduction in cell number);

b) inhibition of cell growth by protein measurement, vital dye uptake or electronic cell counting;

c) dye exclusion;

d) cloning efficiency (suppression of colony formation);

e) leakage of enzymes and radiolabelled metabolites;

f) adenosine triphosphate levels;

g) cell detachment and

h) inhibition of oxygen consumption.

Hunt *et al* (1987) reviewed the use of mammalian cell lines for assessing water pollution and concluded that tests measuring inhibition of cell growth (by the use of vital dyes and protein binding dyes) and cloning efficiency were the most appropriate techniques. A subsequent study of domestic sewage and industrial effluents found that cloning efficiency was more sensitive than inhibition of cell growth and detected effects after 24 hours.

In contrast to mammalian studies, the use of cultured fish cell lines to evaluate the cytotoxicity of aquatic pollutants has received limited attention. However, the effects of a range of heavy metal (Rachlin and Perlmutter 1968; Marion and Denizeau 1983a,b; Babich *et al* 1986) and organic (Bols *et al* 1985) pollutants have been studied. In the study of organic pollutants cytotoxicity was assessed as the inability of cells to attach to a growth surface after chemical exposure and the results were found to be significantly correlated with their water-borne toxicity to rainbow trout.

In recent years the use of cultured fish hepatocytes from rainbow trout (*Oncorhynchus mykiss*) has been advocated as a powerful and simple experimental tool in environmental toxicology (Moon *et al* 1985; Rauckman

and Padilla 1987). Cytotoxicological evaluations are carried out using lactate dehydrogenase (LDH) leakage into the extracellular medium as the assay endpoint. The cytotoxic potential of the heavy metals cadmium, copper and lead to cultured trout hepatocytes has been evaluated and increased LDH activity was recorded at µg/l heavy metal levels (Denizeau and Marion 1990).

A5.4 SUBCELLULAR METHODS

A5.4.1 Lysosomal stability indices

Lysosomes are membrane bound vesicles found in invertebrates (Owen 1972; Moore and Stebbing 1976; Moore 1980) and vertebrates (Dingle and Fell 1969a,b). The organelles are involved in the compartmentalisation and accumulation of a wide range of organic chemicals and heavy metals, and contain catalytic enzymes which function in the breakdown of these contaminants and endogenous cellular components. The nature of pollutant-induced changes on lysosomal structure have been discussed by Kammenga (1989).

In laboratory and field studies, changes in the latency of lysosomal enzymes (as measured by a lysosomal enzyme release assay) and alterations in lysosomal volume (as measured by stereology of tissue sections) have been used as sensitive general indices of cellular condition when assessing the impact of aquatic pollutants (Lowe *et al* 1981; Harrison and Berger 1982; Moore 1985; Versteeg and Giesy 1985). Destabilisation of the lysosomal membrane by pollution is quantitatively related to tissue body burdens (Widdows *et al* 1979) and physiological stress responses (Bayne *et al* 1979). In fish blood plasma, levels of the lysosomal enzyme leucine amino naphthylamidase (LAN) can be used as an indicator of lysosomal stability (Dixon *et al* 1985).

A5.4.2 Sub-mitochondrial metabolic bioassay

Blondin *et al* (1989) have described a simple, rapid and reliable *in vitro* sub-mitochondrial bioassay for toxicity assessment. This

spectrophotometric method measures pollutant-induced metabolic changes in easily isolated sub-mitochondrial electron transfer particles (SMP). These are apparent as alterations in the ratio of reduced to oxidised nicotinamide adenine dinucleotide (NADP/NAD), due to the inhibition of NADH production. Greater correlations were found in this study between fathead minnow LC_{50}s and SMP derived EC_{50} toxicity values for a wide range of industrial chemicals than was evident for fish and comparable Microtox EC_{50} data. The SMP technique was considerably more sensitive than the Microtox test to heavy metals and organochlorine compounds.

The authors advocated the technique as a versatile and sensitive technique which may be capable of quantifying a broad spectrum of toxicants and could be used as a pre-screening method for environmental samples or industrial effluents. Since additional tests for specific groups of toxicants, such as lipophilic substances, are being developed, the SMP may be able to provide a toxicity 'fingerprint' of hazardous chemicals to impart a diagnostic feature to the test system.

A5.4.3 Genotoxicity

i) Introduction

Substances capable of damaging DNA are termed 'genotoxic' and are generally electrophilically reactive towards DNA (Lohman *et al* 1990). Mutagens and clastogens are genotoxic substances that cause gene mutations and chromosomal breaks respectively (Thilly and Liber 1980).

De Raat *et al* (1990) discussed the ecotoxicologically relevant effects of the presence of mutagens in the aquatic environment and concluded that these effects can be divided into two types. Effects on survival, reproduction and fitness can be detected by appropriate 'conventional' tests, although the underlying mechanisms of toxic action will not be obvious. However, some effects such as cancer-forming mutations (eg West *et al* 1988) and the long-term enhancement of mutation rates will not be detected in

conventional tests, and require assays that are specific for genotoxicity. Chemical analysis alone will often not be sufficient for determining genotoxicity (eg Donnelly *et al* 1985).

Much of the work in this field has been performed on terrestrial organisms for human risk assessment and is reviewed in detail in Brusick (1982), but the application of these techniques to aquatic systems is common. Recently, attention has increasingly focused on aquatic ecotoxicological effects with relevant aquatic species (De Raat *et al* 1990) and the NRA is involved in a European initiative called ECOGENTOX which is designed to investigate several genotoxicological techniques and assess their usefulness for monitoring water quality (J Fawell, WRc, pers comm).

The testing of substances for human risk normally follows a tiered approach, such as that recommended by the Department of Health (1981) in which prokaryotic and eukaryotic cells are tested *in vitro*, and both mutagenesis and clastogenesis are tested *in vivo*. Parallel tests with greater relevance to aquatic systems are also increasingly available.

ii) Prokaryotic and eukaryotic *in vitro* tests

The Ames test (Ames *et al* 1973), otherwise known as the Salmonella/microsome test, is widely used in the assessment of genotoxicity at an early stage in tiered testing. The technique uses engineered strains of the bacterium *Salmonella typhimurium* mixed with small amounts of histidine and microsomes from rat or fish extract. This mixture is exposed to the test substance and the proportion of bacteria reverting to the wild type is used as a measure of the mutagenic properties of the substance. There are several variations on the basic Ames test, such as the fluctuation test, which uses similar constituents, but a different methodology. The SOS-chromotest is a newer bacterial assay that uses engineered strains of *Escherichia coli*, and may be more sensitive than the Ames test to some complex mixtures (Dutka *et al* 1987, Van der Gaag

et al 1990). The Microbics corporation is currently developing a luminescent bacterial system, called Mutatox, in which light production from a dark strain of *Photobacterium phosphoreum* is assessed in the presence of genotoxins (R Butler, pers comm).

Chromosome damage and mitotic inhibition can also be measured *in vitro* in aquatic species and methods include the use of rainbow trout gonads and bluegill fry tissues (Kocan *et al* 1985). There are some general problems with short-term *in vitro* tests, including ambiguous results and the loss of genotoxicity that occurs when a sample is filtered for sterilisation (Van der Gaag *et al* 1990). Samples may also have to be concentrated prior to testing, a process that can affect genotoxicity. These problems have led to increased reliance on *in vivo* techniques.

iii) *In vivo* tests

In vivo tests are performed to assess chromosome damage or the induction of dominant lethals. Rodents receiving oral doses are the most widely used organisms for the assessment of the *in vivo* effects of genotoxic agents, although aquatic species are increasingly used (Choroulinkoff and Jaylet 1989, Jaylet and Zoll 1989). Three approaches: nuclear anomaly assays (NAA), the induction of sister chromatid exchanges (SCEs) and the measurement of unscheduled DNA synthesis are commonly performed.

Nuclear anomaly assays include tests for the formation of micronuclei in the blood cells of larval newts and other amphibians (Jaylet *et al* 1986, Jaylet *et al* 1990), fish (Hooftman and De Raat 1982, Das and Nanda 1986) and mussels (Majone *et al* 1987). Sister chromatid exchange, in which the two chromatids comprising a chromosome exchange places, has been measured in fish (van der Kerkhoff and van der Gaag 1985), and adult mussels (Dixon and Clarke 1982) and larvae (Harrison and Jones 1982, Dixon 1985). The induction of micronuclei and SCEs were of a similar level of sensitivity in experiments in which fish species were dosed with

two organic chemicals (Metcalfe 1988). The genotoxic response of mussels tended to be less sensitive than other species when exposed to an organic wastewater, possibly because valve closure reduced the exposure of the organism (Van der Gaag *et al* 1990).

Unscheduled DNA synthesis has been measured in fish hepatocytes (Kelly and Maddock 1985) and chromosomal aberrations found in fish adults (Maddock *et al* 1986), eggs and larvae (Means *et al* 1988) and marine worms (Dixon 1985). The formation of DNA adducts may also be used as an indication of genotoxicity and has been initiated in mussels by low concentrations of benz(a)pyrene and 2-aminofluorene (Kurelec *et al* 1988) and in fish by exposure to sediments contaminated by hydrocarbons (Maccubbin *et al* 1990). The inhibition of mitosis in sea urchin embryos was also a sensitive indicator of benzo(a)pyrene concentrations above 1 ng/l. DNA strand breaks have been measured directly or indirectly in fish, although such breaks may be repaired rapidly and hence their significance is uncertain (Shugart 1988).

A5.5 BIOCHEMICAL INDICES

Biochemical indices of pollution are based on the measurement of molecular components of the cells, tissues or extracellular fluids such as blood. The indices used may respond to a wide range of environmental stressors or to a specific stressor or class of stressors. Since the initial point of toxic action of accumulated toxicants is generally at the biochemical level, appropriate biochemical indices can provide fundamental information on the impact of pollutants on aquatic organisms. Furthermore biochemical indices can be used for pollutants dissolved in the water column, associated with suspended solids or present in sediments.

Livingstone (1985) has stated that for a biochemical response to be acceptable as an index of biological effect, two important criteria have to be satisfied:

a) the measurable change in the biochemical process has to result from, or be a response to, a change in environmental water quality. The relationship between the environmental toxicant level and the biochemical response may be either quantitative, semi-quantitative or a qualitative presence or absence of response.

b) a pollutant induced change in the biochemical index should be linked to detrimental effects on an organism's growth, reproduction or survival. In certain instances a change in a given biochemical process may be compensated for by a change in another process. However a biochemical index which cannot be directly related to growth, reproduction or survival may be useful if it is characteristic of a physiological condition which does have this affect and sufficient background information can be obtained.

There are numerous types of biochemical indices which respond to pollutant-induced stress and which could theoretically be used in the regulation and monitoring of water quality. However, the majority are apparently of limited diagnostic value as sensitive indicators of environmental impact. In this review only those techniques which apparently satisfy the criteria of Livingstone (1985) described previously have been considered in detail. These have been assigned to the following classes for consideration in the review:

1) Enzymes

2) Non-enzyme functional proteins

3) Low molecular weight components

A5.5.1 Enzymes

In recent years the effects of aquatic pollutants on a wide range of enzymes have been investigated, although this diversity of study has meant that, with the exception of the mixed-function oxygenase system, only limited information is available on pollutant-induced effects for

any given enzyme. It is clear, however, that pollution may alter enzyme properties other than activity, leading to effects at the cellular level (Gould *et al* 1976, Gould 1979).

Mixed function oxidase (MFO) enzyme system

In the metabolism of endogenous organic substrates and accumulated organic pollutants, Phase I biotransformation reactions of organic pollutant metabolism by the mixed function oxidase enzyme system convert lipid soluble aromatic hydrocarbons into water soluble products, which can be excreted or conjugated with glutathione or other small molecules in the Phase II conjugation reactions (Gelboin 1980).

The cytochrome P-450 monooxygenase, or mixed function oxidase (MFO) system in the hepatopancreas or liver of aquatic organisms is a multi-component enzyme system. It plays an important role in the metabolism of accumulated liposoluble foreign compounds, such as pesticides and polyaromatic hydrocarbons (PAH), in addition to natural substrates, such as steroid hormones, vitamins and bile acids (Gelboin 1980).

It is now well established that there are multiple forms of cytochrome P-450 and that the monooxygenase activity measured depends on the relative amounts of different cytochrome P-450 isozymes present (Nebert and Gonzales 1987).

The commonly employed MFO assay is based on the conversion of the model poly-cyclic aromatic hydrocarbon substrate benzo(a)pyrene to 3-hydroxy derivative. The measurement of this intensely fluorescent derivative has been shown to be a good overall indicator of benzo(a)pyrene hydroxylase activity (Gelboin 1980). The observed MFO responses of fish and invertebrates to organic pollutants have been reviewed by Kammenga (1989).

However the activities of monooxygenases can be measured with several different substrates and considerable recent interest has focused on

assays for the enzymes ethoxycoumarin O-deethylase (ECOD) and ethoxyresorufin O-deethylase (EROD). The EROD assay is particularly sensitive and reproducible (Burke *et al* 1985) and recent investigations have shown EROD activities in fish are elevated in response to bleached kraft mill effluents (Andersson *et al* 1987, 1988). An effluent concentration-EROD response relationship was established in the laboratory (Andersson *et al* 1987) and in field studies an inverse relationship was found between EROD activity and distance from the discharge point (Andersson *et al* 1988).

Glutathione-s-transferase (GST) enzyme system

The conjugation of organic toxicants in the MFO system, for subsequent excretion, may be achieved by the activity of glutathione-s-transferases (GST). These are ubiquitous within aquatic invertebrates and vertebrates and the use of this enzyme system as a biochemical index of aquatic pollution is currently being evaluated by WRc in an NRA funded research and development programme (Ref No A18.063). Preliminary research using a rapid and reproducible GST assay has shown that elevations in enzyme activity occur after exposure of the freshwater mussel *Sphaerium corneum* to organochlorine pesticides such as lindane and dieldrin.

The enzyme systems described above function as detoxification mechanisms, so toxicant-induced effects at higher levels of organisation in the organism will not be evident until the protective mechanisms have been saturated and there is metabolically active toxicant in the animal.

A5.5.2 Non enzymic functional proteins - Metallothioneins

In unpolluted conditions, metallothioneins and other proteins within cells function as specific metal binding proteins (MBP) and are involved in the storage and transport of the essential metals copper and zinc. Metallothioneins (MT) are a widely distributed class of low molecular weight (6800-7000) metallo-proteins, which have a high binding affinity for certain metal cations. Elevated rates of metallothionein synthesis

and production have been recorded in response to sub-lethal concentrations of copper and zinc and the non-essential metals cadmium and mercury. Cellular toxicity possibly only occurs after metal body burdens have exceeded the binding and detoxifying capacity of the proteins. After this there is a spill-over of free metals which are then able to cause pathological effects at critical target sites by binding to components of the high molecular weight (HMW) protein pool. Analysis of the ratio of a given heavy metal associated with metallothioneins and high molecular weight proteins in a specific tissue, such as the hepatopancreas, may be used as a sensitive index of toxicological status.

Viarengo (1985) reviewed the role of metallothioneins in the uptake, homoeostasis and toxic effects of trace metals and the available data on the 'spill-over' hypothesis is equivocal. The use of metallothionein induction as a specific biochemical index of pollutant stress (Lee *et al* 1980) has been demonstrated in invertebrates (Bayne *et al* 1985) and vertebrates (Klaverkamp *et al* 1984).

A5.5.3 Low molecular weight components

Low molecular weight compounds, such as the substrates and products of intermediary metabolism, have been studied as potential biochemical indicators of aquatic pollution. However, the general nature, rapid turnover leading to changeable concentrations during analysis, and low concentrations in the tissues of these molecules is considered to limit their usefulness. In addition with indices based on tissue and blood carbohydrates, lipids and protein levels there may be difficulties in distinguishing toxicant-induced responses from background variability (Giesy and Graney 1989). However certain indices, such as adenosine phosphates, free amino acids and certain blood metabolites appear to have potential in the assessment of water quality (Versteeg *et al* 1988).

A5.5.4 Other biochemical indices

Other biochemical indices considered useful for assessing the effects of pollution have been reviewed by Kammenga (1989). These include:

a) Specific acetylcholinesterase (AchE) and non-specific cholinesterase enzyme activities. These are selectively depressed by organophosphorus insecticides and non-selectively inhibited at high metal concentrations.

b) Delta amimolevulinic acid dehydratase enzyme activity (ALA-D) in fish. This is inhibited *in vitro* by a number of metals, although it is highly specific to lead *in vivo*.

c) Heat shock proteins (HSP). These are induced to varying degrees in the cells of many species, depending on the nature and magnitude of pollutant stress (Andersen 1989).

d) Adenylate energy charge (AEC). This index of energy production and utilisation is based upon the relationship between adenosine triphosphate, diphosphate and monophosphate and has been used to assess pollution in water (Sylvestre and Le Gal 1987, Picado and Le Gal 1990) and sediments (Zaroogian and Johnson 1989). In a recent study, Picardo and Le Gal (1990) recorded large decreases in whole body AEC levels in *Cardium edule* following 24-hour immersion in a range of paper mill effluents.

APPENDIX B - THE USE OF ECOTOXICOLOGICAL TECHNIQUES IN THE UK AND WORLDWIDE

B1 THE USE OF ECOTOXICOLOGICAL TECHNIQUES IN THE UK BY ORGANISATIONS OTHER THAN THE NRA

Questionnaires were sent to senior individuals in academic, industrial and regulatory organisations in which toxicological investigations are regularly performed (Table B1.1). The following organisations were contacted:

1. Academia: Universities of Stirling, Sheffield, Reading and Wales, Queen Mary's College, Kings College and Plymouth Marine Laboratory.

2. Regulators: The Clyde and Forth River Purification Boards, the Ministry of Agriculture, Food and Fisheries at Burnham (The Scottish Office Agriculture and Fisheries Department, and DoE Northern Ireland.

3. Industry: All ten water utilities, Powergen, ICI Brixham, Shell Research Limited, Unilever Research, Schering Agrochemicals Ltd, Huntingdon Research Centre Limited, Inveresk Research International Limited, Life Science Research and WRc Medmenham.

Table B1.1 - Organisations replying to a request for information on their use of ecotoxicological test methods

Organisation	Name	Job title
ACADEMIA		
NERC unit of aquatic biochemistry, University of Stirling	Stephen George	Head of Environmental Toxicology
Biological Sciences, Queen Mary and Westfield College, University of London	P S Rainbow	Reader in Marine Biology
Department of Animal and Plant Sciences University of Sheffield	L Maltby E Cox C Naylor	Academic staff
School of Pure and Applied Biology, University of Wales, College of Cardiff	David Pascoe	Senior Lecturer
REGULATORS		
Forth River Purification Board (FRPB)	Mike Elliott	Senior Marine Biologist
Clyde River Purification Board (CRPB)	John Redshaw	Water Pollution Control Scientist
Industrial Science Centre, Dept. of Economic Development, DoE Northern Ireland	Imelda O'Neill	Scientific Officer
INDUSTRY		
Water utilities:		
Wessex Water	Fiona Bowles	Senior Biologist
Severn Trent Water	M S Farrimond	Technology Development Manager
Yorkshire Water Services Ltd	Peter Hiley	
North West Water	Keith Wilson	Quality Assessment and Technical Information Manager

Table B1.1 - continued

Organisation	Name	Job title
Testing facilities:		
Life Science Research	Richard Jenkins	Chief Scientist, Aquatic Studies
Huntingdon Research	M T Douglas	Head of Fish Toxicology Centre
WRc Medmenham	All ecotoxicological staff	
Other		
ICI, Brixham	Barrie Williams	Group Manager, Ecology/Ecotoxicology
Powergen	R J Aston	Research Officer

The selected individuals were asked:

1. What toxicological approaches are presently, or have in the past, been used by you or your colleagues?

2. Are you currently developing or assessing any new ecotoxicological method(s) for use in the testing of substances, or the monitoring of water quality?

3. What, in your opinion, are the most useful techniques, either in use or under development, for biomonitoring or toxicity testing?

The answers given by those individuals that responded are summarised in Table B1.2. The majority of respondents performed algal, invertebrate or fish tests, with lethality, growth or reproduction as the measured endpoints. The Microtox test was also used by several organisations. Suborganism, behavioural, population and community tests were performed by only one or two of the respondents. The techniques considered useful naturally matched those used by the respective organisations, but there

Table B1.2 - Ecotoxicological tests used or under development by non-NRA organisations in the UK

Type of method	Used at present	No longer used	Considered useful	Under development/assessment
SUB-ORGANISM METHODS				
Mixed function oxidase activity	NERC, FRPB		FRPB	FRPB, WRc
Other enzyme activity	NERC		NWW	
Metallothionein induction in				
crabs				QM
fish	NERC, FRPB		FRPB	FRPB
Shock proteins	NERC			
Fish cell lines	NERC			
Cellular viability				
Algae	FRPB			
Immunoassays in fish			ICI	ICI
WHOLE ORGANISM METHODS				
Lethal				
LC/EC50:			CRPB	
Unspecified	YWS			
Trout	LSR, HRC, ICI, CRPB, PG, UWCC, WRc		LSR	
Other fish	NERC, LSR, HRC, ICI, CRPB, PG, WRc		WW	
Fish early life stage	LSR, YWS, UWCC, PG, WRc		HRC, CRPB, WRc	
Daphnia	LSR, HRC, ICI, S, DoENI, WRc		FRPB, LSR, YWS, S, NWW	S
Other invertebrates	NERC, FRPB, LSR, ICI, S, CRPB, PG, WRc		FRPB, WW, LSR, YWS	S
Corophium in sediment	QM, WRc		CRPB	ICI
Other sediment assays	S, WRc		S, WRc	S, WRc
Trout rapid bioassay	ICI			
Sublethal				
Growth				
Unspecified	YWS			
Algae	LSR, HRC, ICI, S, WRc		S	S
Invertebrates	S, UWCC, WRc			
Fish	NERC, FRPB, LSR, ICI, CRPB, PG, WRc	PG	ICI	
Scope for Growth			PG	
Mussels	CRPB, FRPB, WRc		CRPB	
Gammarus	S, WRc		S	WRc
O/N ratio	FRPB			

Table B1.2 – continued

Type of method	Used at present	No longer used	Considered useful	Under development/assessment
Reproduction				
Fish	LSR, HRC, ICI, WRc		WRc	WRc
Daphnia	LSR, HRC, ICI, S,	HRC, WRc	WRc	WRc
Short-term *Ceriodaphnia*			ICI	ICI
Mysids	ICI, WRc			WRc
Tisbe	ICI			ICI
Other invertebrates	SUWCC, WRc			WRc
Oyster embryos	CRPB, WRc	CRPB, FRPB, WRc		
Microtox	FRPB, CRPB, WW, STW, ICI, YWS, DoENI, WRc	HRC	WW, YWS, CRPB	
Automated bacterial systems			ICI	YWS, WRc
Respiration rate				
Bacterial	STW, LSR, HRC, WRc		WW, YWS	WW
Invertebrates	S, UWCC	WRc		
Bivalve ventilation rate				WRc
Bioaccumulation				
Fish	LSR, CRPB, WRc			
Invertebrates	QM, WRc			
Behaviour				
Swimming behaviour				
Fish		ICI		
Invertebrates	UWCC, CRPB, YWS			
Preference/avoidance		ICI	CRPB	
Fish monitors	WRc	ICI, WW	CRPB, NWW	
Gammarus precopula separation	UWCC		ICI	
POPULATION METHODS				
P/B ratios	FRPB			
Colonisation substrata	WW, CRPB			
Life tables	UWCC			
COMMUNITY METHODS				
Estuarine microcosms	FRPB			
Model streams	PG, UWCC		PG	
Artificial ponds	UWCC, WRc			

Notes

CRPB = Clyde River Purification Board
DoENI = Department of Economic Development, DoE Northern Ireland
FRPB = Forth River Purification Board
HRC = Huntingdon Research Centre

Notes to Table B1.2 continued

ICI = ICI Brixham
LSR = Life Science Research
NERC = NERC Unit of Aquatic Biochemistry, University of Stirling
NWW = North West Water plc
PG = Powergen
S = Sheffield University
STW = Severn Trent Water plc
QM = Queen May and Westfield College, London
UWCC = University of Wales, College of Cardiff
WRc = WRc Medmenham
WW = Wessex Water plc
YWS = Yorkshire Water Services

was general agreement that single species chronic lethality, growth and reproduction tests were most cost-effective. Only a few organisations were involved in the active development of new techniques, and these techniques ranged across the levels of biological organisation.

The regulatory organisations that responded to the questionnaire used ecotoxicology for several purposes. The Clyde River Purification Board (CRPB) have some simple toxicity based consents with rainbow trout, brown shrimp, turbot or plaice using protocols developed by the Ministry of Housing and Local Government (HMSO 1969). They also use *in situ* caged trout and shellfish, colonisation bags and 96-hour laboratory tests with trout, *Gammarus*, scallops, turbot, mussels and *Artemia* to assess water quality at specific locations. The CRPB uses rapid techniques such as Microtox and the oyster embryo larval test for the general monitoring of effluents, waters and sediments.

The Forth River Purification Board have used many tests, covering the range of biological organisation. These, mostly *in situ* or field-based tests, are used to assess water quality with a variety of fish, macroinvertebrate and planktonic species.

The Industrial Science Division of the Department of Environment in Northern Ireland use a limited number of simple tests to assess effluents on a site-specific basis. Microtox and *Daphnia magna* have been used to examine the toxicity of car washes, gas works wastes and solubilised tar waste.

B2 USE AND PERCEPTIONS OF ECOTOXICOLOGY BY THE NRA

A number of NRA personnel directly involved in the acquisition or interpretation of biological data were approached for information on the use of ecotoxicological techniques in the NRA regions (Table B2.1). NRA biologists were contacted in the first instance, and interviewed at their home base.

They were asked the following questions about the use of ecotoxicology in their region:

1. Do you use ecotoxicological methods for monitoring or regulating water quality ?

2. If ecotoxicological methods are used, for what length of time they been applied and are they used routinely or occasionally? Are they an integrated part of your current working practices? Have the methods been validated in your laboratory?

3. Have you been involved in setting any consents with an ecotoxicological component and if so, has the efficacy of these consents been established in a legal context?

4. If you do not use ecotoxicological methods, have you encountered any instances in the past year when a knowledge of the toxic effects of a particular chemical or effluent on aquatic organisms, populations or communities would have assisted in decision making?

5. Do you envisage increasing your use of ecotoxicological methods in the future and are you engaged in developing or assessing any new techniques for this purpose?

A synopsis of their views was written and sent to NRA pollution, fisheries and water quality managers. These individuals were asked to complete a questionnaire based upon the questions above and to comment upon the views expressed by the biologists.

Table B2.1 - List of NRA personnel directly contacted or responding to questionnaire

Region	Name	Job title
BIOLOGISTS		
ANGLIAN	Alastair Ferguson	Biological Scientist
	Tessa Crawshaw	Assistant Scientist (Toxicology)
NORTHUMBRIAN	John Orr	Principal Biologist
NORTH WEST	Dick Chambers	Senior Biologist
	David Holland	Area Biologist
SEVERN-TRENT	Shelley Howard	Area Biologist
SOUTHERN	Bob Dines	Senior Scientist
	Dave Lowthion	Principal Biologist
SOUTH WEST	John Murray-Bligh	Assistant Scientist (Biologist)
	Jane Driver	Tidal Waters Scientist
	Tom Mercer	Special Investigations Biologist
	Simon Culling	Tidal Waters Assistant Scientist (Biologist)
	Richard Smith	Freshwater Assistant Scientist
THAMES	Derek Tinsley	Area Biologist (East)
WELSH	Neil Reynolds	Environmental Appraisal Officer
	Roger Milne	Toxicity Scientist
WESSEX	Alan Frake	Senior Biologist
	George Green	Senior Biologist
YORKSHIRE	Brian Hemsley-Flint	Senior Biologist (South)
	Liz Chalk	Senior Biologist (North)
FISHERIES SCIENTISTS		
ANGLIAN	Nigel Tomlinson	Fisheries Laboratory Manager
	Tony Owen	Fish Health Scientist
SEVERN-TRENT	Roy Sedgwick	Fisheries Scientist
YORKSHIRE	Stephen Axford	Senior Fisheries Scientist
WATER QUALITY PERSONNEL		
ANGLIAN	W J Forbes	Environmental Manager (Northern)
	D J Tester	Environmental Manager (Central)

Table B2.1 - continued

Region	Name	Job title
NORTH-WEST	A W Wither	Marine and Special Projects Manager
	Leslie Hughes	Area Pollution Control Manager
SEVERN-TRENT	R I Harvey	Principal, Pollution Control
SOUTHERN	R B Edmunds	Principal, Water Quality Officer (Planning)
	K M Jury	Principal Pollution Prevention Officer
	John Foster	Water Quality Office, Pollution Prevention
SOUTH-WEST	G R Bateman	Pollution Controller
	Adam Davies	Water Quality Regulation
WELSH	Andrew Dixon	Divisional Scientist
WESSEX	Ian Nutter	Senior Scientist
	R J Huggins	Area Catchment Control Officer
YORKSHIRE	R Armitage	Pollution Control Officer (Special Projects)
	John Herring	Pollution Control (Southern)
	G Tane	Area Pollution Control Manager (Northern)
	Geoff Firth	Laboratory Services Manager
	Gerrard Morris	Environmental Scientist

B2.1 USE AND PERCEPTION OF ECOTOXICOLOGY BY NRA BIOLOGISTS

A range of ecotoxicological tests are, or have recently been, used in the regions to supplement biological survey or chemical data (Table B2.2). Fish and invertebrate acute lethal tests are most commonly used, with the work performed either in-house, or by a sub-contractor. These standard short term toxicity tests are generally used to assess the toxicity of effluents. NRA, Anglian Region, in

Table B2.2 - Ecotoxicological tests currently used by the NRA

Ecotoxicological approach	Type of method (Region)
A) SUB-ORGANISM METHODS	
Enzyme responses (eg MFO activity)	
Protein induction	
Adenylate energy charge	
Other tests	
B) WHOLE ORGANISM METHODS	
1) Acute/Chronic lethal:	
LC50 tests	*Daphnia* LC50 (N-W) *Corophium* on sediment (S,S-W) Trout (T,A,Wel) Flounder on sediment (W)
Early life stage tests	Algae LC50 (Y) Gammarus (T,N-W,Wel) Brown shrimp (A)
Other tests	Oyster embryo-larval (S)
	Asellus/chironomids/oligochaetes & other simple acute tests with relevant organisms (S-T,Wel,A)
2) Acute/Chronic sublethal:	
Growth tests	Oyster shell thickening (S) Imposex in dog whelks (S)
Reproduction tests	Fish egg viability (S-W)
Scope for Growth	*Gammarus* feeding *in situ* (Y) *Gammarus* feeding in lab (S)
Microtox	(N, S-W, Wel)
Other tests	
3) Behavioural:	
Preference/avoidance response	
Swimming behaviour	
Respiratory responses (fish)	
Other tests	
C) POPULATION METHODS	
Biomass/Recruitment	
Other tests	
D) COMMUNITY METHODS	
Microcosms	
Gnotobiotic systems	
E) BIOACCUMULATION METHODS	
Marine and estuarine	Seaweeds (S-T,Y,N-W,S,A,W) Nereis (Y,A) *Mytilus* (N-W,N,S) Oysters (N)
Freshwater	Limpets (S,W) Shrimps (A) Fish (S-W,A) Mosses (Y,N-W,S-W) Eels (W)

KEY TO NRA REGIONS: Y = Yorkshire, N-W = North-West, S = Southern, S-W = South-West, T = Thames, A= Anglian, Wel = Welsh, W = Wessex, S-T = Severn-Trent, N = Northumbrian

particular, have adopted a structured approach to this use of ecotoxicology, and field staff have a clear route of access to the toxicological testing service. At present, only the Anglian and Welsh Regions of the NRA use toxicity tests for consenting effluent discharges. Table B2.3 shows the nature of the effluent discharges to which toxicity based consent conditions have been applied in these regions, and specimen consents are contained in Appendix C.

Acute, 96-hour lethality tests are specified by NRA Anglian Region for effluents discharged to fresh, estuarine and marine waters. Fish tests, usually with the rainbow trout, are conducted to OECD guidelines for freshwater discharges. The consent conditions for discharges to estuarine or marine waters specify a simple protocol for use with brown shrimp. Microtox is the test used to control the only discharge with a toxicity based component in NRA Welsh Region. An additional eight discharges are currently being reviewed and may in the future contain a toxicity test component.

The remaining eight regions in the NRA have used ecotoxicology and bioaccumulation methods outside a legal "discharge consent" setting as a tool for effluent hazard ranking, investigations of pollution incidents and the location of pollution "hot spots". Techniques used in this way include the oyster embryo-larval test and various ad-hoc lethal and sublethal whole organism tests with plants, fish and invertebrates. Bioaccumulation studies are widely, although not extensively, used to locate the spatial source of metals and pesticides in the environment. Naturally occurring seaweeds, molluscs, ragworms and fish have been used regularly for monitoring contaminants in the marine and estuarine environment, and moss implants, mussels, eels and other fish have been used for the same purpose in fresh waters.

Several priority areas were identified in which it was thought that ecotoxicological tests could provide useful information. The most important of these were the investigation of the effects of complex mixed effluents from the agrochemical, pharmaceutical, petrochemical and piscicultural industries, sewage and farm waste pollution episodes, and

Table B2.3 - The application of toxicity-based consents to effluent discharges by regulatory agencies in the United Kingdom

Regulatory agency	Freshwater receiving waters		Estuarine/Marine receiving waters	
	Number controlled	Test protocol	Number controlled	Test protocol
NRA Anglian region	3	Acute (96 hr) lethality fish tests (Rainbow trout - *Oncorhynchus mykiss*) OECD guidelines: Semi-static	6	Acute (96 hr) lethality tests with brown shrimp (*Crangon crangon*) Specified methodology: Semi-static
NRA Welsh Region	0	-	1	Microtox
Clyde River Purification Board (CRPB)	4-5	Acute (48 hr) lethality test with rainbow trout (*Oncorhynchus mykiss*) MHLG based methodology - Static	2	Acute (96 hr) lethality test with brown shrimp (*Crangon crangon*), turbot (*Scopthalmus maximus*) and/or plaice (*Pleuronectes platessa*) MHLG/MAFF hybrid methodology: Semi-static

the resolution of any discrepancies between data from biological surveys, RIVPAC's predictions and water chemistry. Other potential uses for ecotoxicology proposed by some regions were site-specific estuarine and coastal monitoring and the examination of the effects of sewage disinfection, blue-green algal toxicity, industrial cooling waters and non-point pollution.

A number of test features were considered desirable in any technique used directly by NRA personnel. Most regions agreed that tests for use in setting and monitoring discharge consents had to be standard, rapid, sensitive, simple, widely recognised, cheap and mostly available "off the shelf". Tests that were considered to meet most of these criteria were Microtox and OECD-approved single species acute tests. It was generally agreed that a less rigid approach was acceptable for problem-solving exercises and other objectives without a specified legal end. Sensitive tests involving the use of common indigenous species, general and pollutant-specific sub-organism tests, or fish avoidance and invertebrate drift behavioural tests were considered appropriate in this context. Opinion was divided over the use of multispecies test systems. Some biologists felt that the ecosystem end points measured in these tests were highly relevant to the needs of the NRA and that this type of test should be used regularly, whilst others believed that such techniques were too expensive and complicated for regular use by NRA personnel and that the results would overlap with those obtained from routine biological surveys.

Several regions are currently expanding their use of ecotoxicological methods (Table B2.4). Most of the tests under consideration are single species toxicity or bioaccumulation studies, but North West Region are considering the use of metallothionein induction in *Littorina*.

All of the biologists that were interviewed felt that there was a rôle for ecotoxicology in the work of the NRA. It was generally felt that the biological and chemical tools at present available to the NRA were insufficient for meeting several important objectives, such as the

Table B2.4 - Ecotoxicological tests under consideration by the NRA

Ecotoxicological approach	Type of method (region)
A) SUB-ORGANISM METHODS	
Enzyme responses (eg MFO activity)	
Protein induction	
Adenylate energy charge	
Other tests	Metallothionein induction in Littorina (N-W)
B) WHOLE ORGANISM METHODS	
1) Acute/Chronic lethal:	Guppies (A)
LC50 tests	
Early life stage tests	
Other tests	
2) Acute/Chronic sublethal:	
Growth tests	
Reproduction tests	Oyster embryo-larval (N-W,A,W)
Scope for Growth	Mussels (N)
Microtox	All regions
Other tests	Imposex in dogwhelks (Y)
3) Behavioural:	
Preference/avoidance response	
Swimming behaviour	
Respiratory responses (fish)	
Other tests	
C) POPULATION METHODS	
Biomass/Recruitment	
Other tests	
D) COMMUNITY METHODS	
Microcosms	
Gnotobiotic systems	
E) BIOACCUMULATION METHODS	
Marine and estuarine	Barnacles (N-W) Fish (N)
Freshwater	Fish (N) Eels (S-W)

KEY TO NRA REGIONS: Y = Yorkshire, N-W = North-West, S = Southern, S-W = South-West, T = Thames, A= Anglian, Wel = Welsh, W = Wessex, S-T = Severn-Trent, N = Northumbrian

consenting of complex effluents and the monitoring of episodic pollution. Most interviewees felt that standard tests would become widely used for setting and monitoring discharge consents and that imaginative, less formal tests would be valuable supplements to routine biological and chemical monitoring.

B2.2 THE USE AND PERCEPTION OF ECOTOXICOLOGY BY FISHERIES SCIENTISTS

The fisheries scientists who replied to the questionnaire largely agreed with the views of the NRA biologists as outlined above. The respondents used fish toxicity and bioaccumulation tests either in the field or the laboratory and these tests seem to have been useful for monitoring water quality downstream from important discharges. Their usefulness in determining the cause of fish kills in other situations was, however, difficult to determine.

Several supplementary objectives were stated for the development of toxicological methods useful to the NRA. These were:

1. the use of a range of indigenous fish and higher invertebrate species;

2. the elimination of 'application factors' by standardising methods for determining no effect concentrations;

3. the investigation of pollutant depuration rates in dead organisms for evidential purposes;

4. the development of continuous monitors;

5. the derivation of data on synergistic and antagonistic effects between both contaminants and contaminants and different water chemistries.

B2.3 THE USE AND PERCEPTION OF ECOTOXICOLOGY BY WATER QUALITY PERSONNEL

The responses of water quality personnel are summarised in Table B2.5. Every region that replied to questionnaires wished to increase their use of ecotoxicological test methods and data, especially for consenting discharges, although the preferred methods varied between regions. Respondents were also asked to estimate the number of complex effluents in their region, and the responses suggest that more than 300 discharges fell into this category across the country. This figure should, however, be treated with caution, as several of the respondents based in the same regions provided estimates that differed greatly.

Several criteria were considered important when developing or using toxicity tests:

1. Setting and monitoring consents. Tests used for this purpose should have a standardised protocol for use with relevant indigenous organisms. They should be cheap, simple, repeatable and accurate. Monitoring should, ideally, be continuous and provide both acute and chronic information. There should be good correlations between field and laboratory investigations.

2. Setting Environmental Quality Standards. Most of the respondents felt that standardised chronic sublethal or multispecies studies with indigenous and possibly local species would be most appropriate for refining quality standards.

3. For providing general information on the toxicity of substances. Test systems used for this purpose should provide repeatable and easily interpretable results from standardised, perhaps nationally organised, tests on a wide range of organisms and life stages. There should be more information on synergistic effects. Any resulting database should be compatible with other sources of data used by the NRA.

Table B2.5 - The use and perception of ecotoxicology by NRA water quality personnel

NRA region	Type of test currently used	Situation currently used	Effectiveness	Number of complex effluents	Type of test under consideration for future use	Situation in which new tests likely be used
ANGLIAN	Rainbow trout, brown shrimp and plant bioassay.	Consenting discharges	Good	>30	Oyster embryo larval test. Fish toxicity.	Consenting discharges.
	Bioaccumulation of metals in seaweeds	Routine monitoring, effect of inputs,	Fair to very good		Bioaccumulation	River quality evaln. Assessment of effluents.
	Bioaccumulation in fish and invertebrates	abatement improvements. Monitoring and impact assessment.	Limited or uncertain			
	Acute 48-hr toxicity	Toxic pollution incidents	Dependent upon species used			
NORTH-UMBRIAN	No reply	-	-	-	-	-
NORTH-WEST	Daphnia toxicity test	Fish kill investigation	Very effective	100	Microtox	Consenting discharges
	Moss bioaccumulation	Copper discharges	Very effective		Systematic use of currently available tests	
	Microtox	Effluent toxicity	Average, perhaps due to lack of familiarity with technique			
SEVERN-TRENT	Fish toxicity tests	New effluent discharge	Good	>10	Bioaccumulation methods	Pesticide uptake
SOUTHERN	Bioaccumulation in mussels	Effect of sea outfall	Very effective for identifying toxic sources	74	Microtox	Consenting discharges pollution incidents
SOUTH-WEST	None	-	-		Whole organism methods	Tracing pollution and identifying biological significance
					Bioaccumulation methods	Protection of intakes/public health
WELSH	Microtox bioaccumulation	Consenting discharges	Very effective	>12	Microtox	More widely used for consents
WESSEX	None	-	-	50	Microtox	Effluent monitoring
					Single species tests	Effluent monitoring
					Fish monitors	River water quality
YORKSHIRE	Fish toxicity	Consenting discharges	Good	40	Daphnia and fish acute & chronic toxicity tests	Consenting discharges and investigating
	Microtox	Effluent assessment	Very good		Phytotoxicity tests, Microtox and respirometers	pollution incidents

4. For investigating pollution incidents and performing routine quality monitoring. These tests should be cheap, fast, repeatable, relevant portable, valid in a court of law and consistent with laboratory-based tests.

5. For monitoring general water quality. These tests should have ecological relevance for the water being monitored and it should be possible to relate the results to chemical data. Tests should be accurate, reliable, repeatable, cost-effective and, where possible, automated. Methods should have widespread acceptance in the water industry and the data should be relevant over the long term.

B3 THE APPLICATION OF ECOTOXICOLOGICAL METHODS IN OECD COUNTRIES

B3.1 INTRODUCTION

This section of the report describes the findings from a literature review, questionnaires and personal visits to organisations in N America and Europe directly involved in using ecotoxicological methods to control and regulate point discharges and monitor receiving water quality. An overview of the extent to which test methods at different biological levels of organisation are used in these management roles in various OECD countries is given using information provided by both regulatory and industrial organisations.

B3.2 TOXICITY-BASED CONSENT SETTING AND COMPLIANCE MONITORING

United States

Introduction

In the United States the use of toxicity based consents for controlling effluent discharges is now widely established, and usually follows the Environmental Protection Agency (EPA) guidelines, although the precise

method of implementation varies between individual states. The implementation of whole effluent based control to point source discharges is through the National Pollution Elimination Discharge Scheme (NPDES). Legislation for this process was provided in the Federal Water Pollution Control Act of 1972 and subsequent amendments of the legislation in 1977 and 1987 (Table B2.6). The effectiveness of whole effluent toxicity testing in regulating the toxic impact of surface water discharge has been demonstrated by the EPA in studies at industrial facilities and publicly owned treatment works (POTW). Correlations between whole effluent toxicity test results and in-stream response as determined by benthic survey data have been shown at these facilities (Mount *et al* 1984 and 1985, Mount and Norberg-King 1986, Norberg-King and Mount 1986, Eagleson *et al* 1990).

The US EPA has issued a national policy statement (US EPA 1984) which describes the use of toxicity data to assess and control the discharge of toxic substances to receiving waters, through the NPDES permits programme. All US states now have water quality standards which include statements prohibiting the discharge of toxic materials in toxic amounts.

In current state standards the criteria for toxicity range from narrative prohibition, such as "no discharge of toxic chemicals in toxic amounts", to detailed requirements specifying test species and the allowable toxicity level. The EPA have established national water quality criteria, which are recommended to limit effluent toxicity and ensure protection of aquatic organisms in receiving waters against acute (short-term) and chronic (long-term) toxic effects. These receiving water, or 'instream', standards can be considered to have three components: magnitude, duration and frequency, and specifications for these components are required for both acute and chronic levels.

The recommended instream acute toxicity standard is called the criterion maximum concentration (CMC) and represents the highest instream effluent (or toxicant) concentration to which organisms can be exposed for a restricted time period without causing mortality. In the proximity of a

Table B2.6 - Legislation in the United States pertaining to the introduction of toxicity-based control of effluent discharges

Year	Legislation	Details
Pre 1972		Few legal tools available in the US to control surface water pollution
1972	Federal Water Pollution Control Act Amendment (Clean Water Act)	Designed to " restore and maintain the chemical, physical and biological integrity of the nations waters". The Act required that the discharge of any pollutant from a fixed location be regulated by a National Pollution Discharge Elimination Scheme (NPDES) permit system, driven by the EPA. Specified industrial discharges had to satisfy effluent limitations by applying Best Practicable Control Technology (BPT) and Best Available Technology (BAT) economically achievable.
1972-1977		The EPA failed to satisfy deadlines specified in the 1972 Act for developing and promoting regulations, and resultant pressure and legal action by environmental groups, such as the National Resource Defense Council (NRDC) highlighted the need for additional regulation. The resultant settlement between the EPA and environmental groups required the Agency to develop a programme for promoting Best Available Technology (BAT) effluent limitation guidelines and pre-treatment and new source performance standards for 65 chemicals and classes of chemicals (129 priority pollutants).
1977	Clean Water Act reauthorisation	Stated that " it is the national goal that the national discharge of toxic pollutants in toxic amounts is prohibited". The reauthoriation lists 129 (now 126) priority pollutants, from a NRCD list, for which industries have to derive BAT based discharges limit values. The Act permitted industry to use methods other than BAT prescribed by the EPA, provided the technology is at least comparable or better.
1984		The EPA issued a national policy statement 'Policy for the Development of Water Quality-Based Permit Limitations for Toxic Pollutants' (US EPA 1984). The use of toxicity data to assess and control the discharge of toxic substances to receiving waters, through the NPDES permits programme, was proposed. The policy stated that "biological testing of effluents is an important aspect of the water quality-based approach for controlling toxic pollutants". Acute and short-term chronic effluent toxicity tests were then increasingly used to establish control priorities, assess compliance with state water quality standards and establish discharge permit limitations to achieve those standards.
1987	Clean Water Act reauthorisation	Water quality- based toxics control, including toxicity-based regulation was introduced.

discharge or within a mixing zone the acute toxicity should not exceed 0.3 acute toxic units (TUa), based on effluent toxicity test data from the most sensitive of at least three test species from ecologically diverse taxa. The toxic unit TUa value for a measured effluent is derived from the equation,

$TUa = 100/LC_{50}$

where the LC_{50} is the effluent concentration causing 50% mortality in the most sensitive test species (eg alga, invertebrate or fish) after a specified exposure time.

A factor of 0.3 is used to adjust the typical acute toxicity test LC_{50} value to an LC_1 value (effluent concentration causing 1% mortality in the test species after the specified time). This value has been derived on the basis of a large number of effluent toxicity tests conducted by the EPA, which have shown that the value includes 91% of observed LC_{50} to LC_1 ratio's.

For the protection of aquatic organisms against chronic (long-term) toxic effects, the national water quality standard recommended is the criterion continuous concentration (CCC). This represents the highest instream effluent concentration to which organisms can be exposed indefinitely without causing adverse effects. The value should not exceed 1.0 chronic toxic units (TUc) to the most sensitive of three test species. The TUc value is derived from the equation:

$TUc = 100/NOEL$

where the no observed effect effluent level (NOEL) is the highest effluent concentration at which no observed effect will occur in the most sensitive test species during continuous chronic toxicity testing.

In situations where only two species have been tested, the acute and chronic standards to be maintained should be divided by 10 (ie acute =

0.03TUa and chronic = 0.1 TUc) to account for uncertainty in species sensitivity.

Toxic impact in aquatic communities is a function of both the magnitude (concentration) and duration (time) of exposure. Organisms in the receiving water often experience fluctuating, rather than continuous steady exposure, and short exposure periods to high toxicant levels can have adverse effects. Therefore in addition to limiting the concentration of an effluent, regulatory agencies have to also specify the length of time receiving water biota are exposed to effluent standards. For acute effects the duration of exposure has to be restricted to as short a period as practicable and the CMC may not be exceeded by a 1 hr average instream exposure concentration.

Studies have shown that to avoid long-term sub-lethal toxic impact the maximum period during which the receiving water exposure concentration may be continuously higher than the CCC should be four days. The EPA therefore recommends that the average exposure concentration over a 4 day period has to be equal to or less than the CCC.

The frequency with which the criteria concentrations can be exceeded should depend on site-specific factors, although the EPA recommends that the CMC and CCC are only exceeded once in three years for both whole effluent and chemical specific approaches.

The implementation of the NPDES system involves the appropriate regulatory agency issuing discharge permits to protect designated water uses, such as the survival and propagation of aquatic life. These list:

a) analytical determinands and their limits, including the 126 priority pollutants;
b) monitoring methods that should be used; and
c) any toxicity tests required for monitoring the effluent relative to the discharge consent.

The USA has a system of major and minor permittees (permit holders), with major permittees defined as those considered likely to discharge significant quantities of potentially toxic substances. In practice, however, recent EPA surveys have shown that some minor permittees are in fact discharging toxic effluents. All major permittees are subject to 'water quality based toxics control', with each permit lasting five years. As these expire or require modifications for other reasons (such as changes in plant processes), regulators introduce a toxicity based assessment component into the dischargers consent.

Toxicity based discharge permits are issued by the relevant organisation in each state having 'primacy' (ie legal responsibility under State law). In states which have insufficient staff or expertise to effectively carry out the permit programme, the regional EPA office issues discharge permits. At present there are about 10 states in this category, and the EPA Dallas office is responsible for issuing permits for New Mexico, Arkansas, Oklahoma and Nevada. However the EPA has granted primacy to most states, which means the relevant state agencies can establish permits within their state, based on guidelines issued by the EPA. Although states have to follow the guidelines, they may exhibit a degree of freedom in the interpretation of the EPA protocols. In this way the principle is followed consistently, although the actual detail may vary between individual states.

a) Environmental Protection Agency (EPA) guidelines for a toxicity based discharge consent

The EPA's recommendations for controlling the discharge of toxic pollutants to receiving waters are detailed in the Technical Support Document (TSD) for Water Quality-Based Toxics Control, (US EPA 1985a) which provides guidance on the water quality-based toxics control process from screening to compliance monitoring. These specify an integrated whole effluent (or toxicity) and chemical specific approach, using either or both of these approaches as appropriate. In this section an overview of the permit setting procedure and compliance

monitoring is given indicating the type of toxicity testing which is recommended by the EPA at different stages of the process.

The mechanisms by which permit limits are derived are also determined on a site specific basis and may involve setting limits directly or carrying out effluent characterisation. Indeed, the principle established by the EPA that it is not always necessary to generate any effluent toxicity data to establish toxicity based permit limits is significant. However, there are advantages and limitations to both this approach and effluent characterisation (Table B2.7).

Table B2.7 Advantages and limitations of setting permit limits directly or carrying out effluent characterisation

Approach	Advantages	Limitations
Setting limits directly:		
	Costs of data generation prior to permit issuance is eliminated.	There is uncertainty as to whether there is a toxicity problem, as no data is available, and the permittee may object.
	Permit limits are quickly derived and permit issuance is not delayed.	Exposure has to be simplified so that only steady state exposure conditions can be used. This may over or under protective depending on the design flow used.
Effluent characterisation:		
	Indicates whether there is a real toxicity problem.	The cost of testing can be high.
	Can generate the data needed to assess exposure in a sophisticated manner with a sensitive test species being identified and effluent variability being assessed.	The testing and analysis could delay permit issuance.
	There is reduced likelihood of objection to the permit limits due to the sound basis on which they were derived.	

In the case of single point sources, toxicity based limits (in toxic units) can be derived without effluent characterisation from the following relationship:

allowable effluent concentration < criteria concentration x dilution factor

using criteria concentrations and data on the dilution of the effluent. Although these values can be derived without effluent toxicity testing, a biological monitoring programme would be required to ensure compliance with the permit limit.

In the case of multiple sources, toxicity additivity is assumed and there is summation of flows, with allowable toxicity allocated to individual sources using an option for establishing toxicity wasteload allocations. The relationship for multiple sources is given by:

$$\frac{\text{(Effluent flow x Effluent toxicity)}}{\text{Stream flow at the reference point}} < \text{criteria concentration}$$

However in numerous instances effluent characterisation may be needed to provide data needed to establish priorities for control, whether by toxicity and/or chemical specific analysis.

a) Effluent characterisation

The EPA consider a tiered testing approach to be the most cost-effective method of generating data to assess potential toxic impact, and an approach combining a first screening tier with a second definitive data generation tier is recommended (Figure B1).

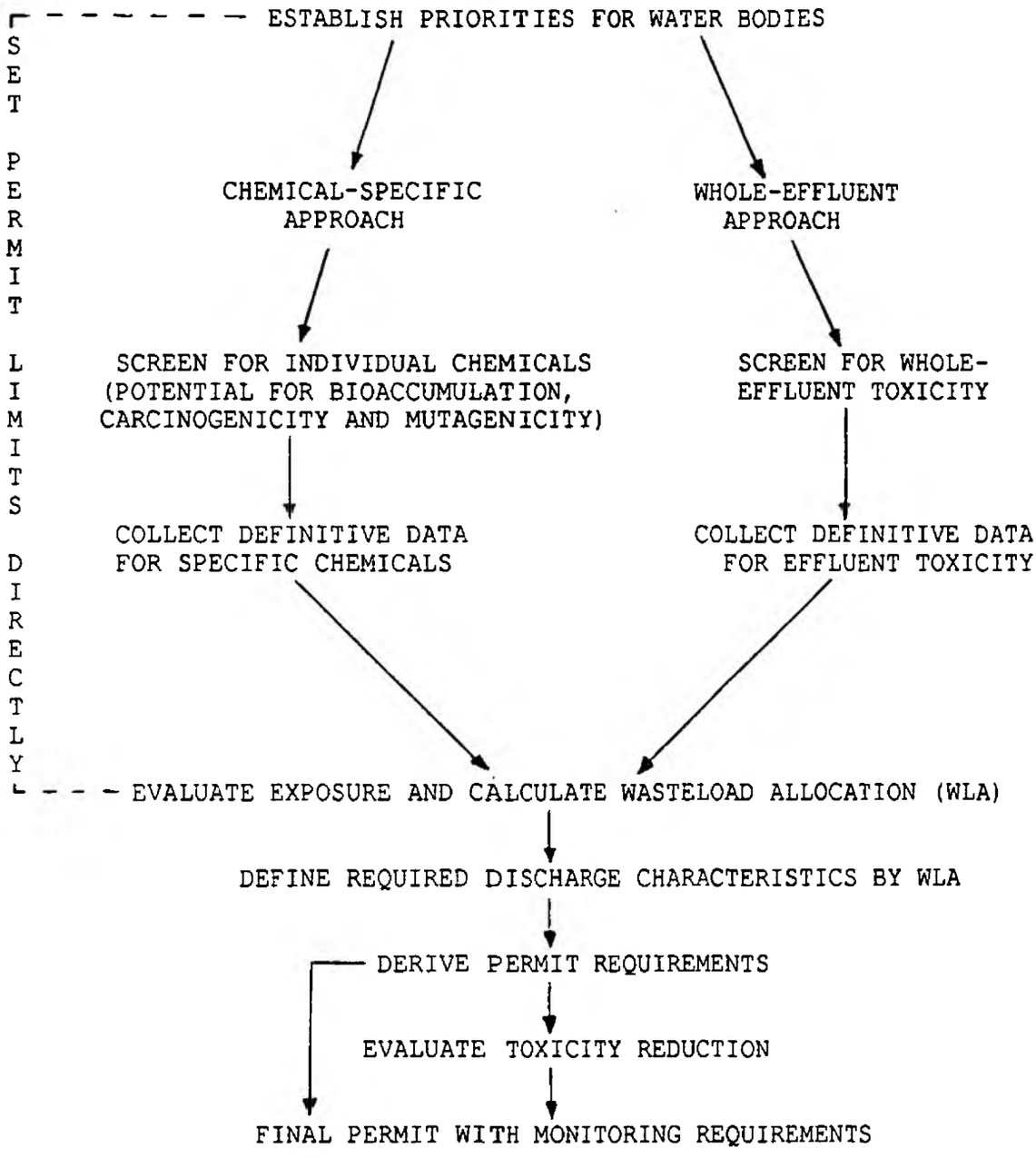


Figure B1 - Overview of the Water Quality-Based Toxics Control Process

Tier 1 - Screening

The screening approach is used to identify potential water quality problems from priority discharges, with the most cost-effective methods available, whilst avoiding data collection in situations where the

effluent will cause minimal toxic impact. The decisions as to which NPDES effluents are candidates for water quality-based toxics control will be made on a site-specific basis and depend on a number of pertinent factors including;

a) the available dilution capacity;
b) the type of discharge;
c) the complexity of the effluent and the type and character of discharged pollutants;
d) the characteristics of the receiving water; and
e) the history of the discharge, in terms of previous toxic impact and compliance problems.

Priorities for toxicity analysis are established on the basis of an evaluation of the available data. In cases where water quality violations are documented it may be appropriate to move to the next testing tier. This may be more applicable to the chemical specific rather than the whole effluent approach, since effluent toxicity data are usually unavailable for NPDES discharges. Consequently, for complex effluents or those for which there is limited toxicological information, data generation for whole effluent screening will probably be needed. The EPA has a database of whole effluent toxicity testing data for industrial and municipal wastewaters, the Complex Effluent Toxicity Information System (CETIS). The data, from EPA Regional and State NPDES permitting programs and published literature can be used to assess a discharge in a specific industrial category for potential toxicity by comparison with a similar discharge for which toxicity data are available.

The recommendations for whole effluent toxicity screening involve comparing the receiving water flow (in terms of the designated low flow specified by the regulatory agency) to the average effluent flow rate. In the initial dilution study the consequences for given dilutions are:

1) dilution > 10000:1 and a reasonably rapid mixing of the effluent outside of an initial dilution area in the receiving water - LOW PRIORITY FOR FURTHER ATTENTION;

2) dilution 1000 - 10000:1 or mixing is not rapid and toxicity within a plume is in a large receiving body (dilution >10000:1) - SHORT-TERM ACUTE TOXICITY TESTS (24 hr);

   Four to six grab or short-term composite samples collected on one day are tested individually at 100% effluent concentration using a daphnid and a fish:

   A low priority for additional testing is given if 50% or greater mortality is found in two or less samples, whereas additional testing is needed if this level of mortality is recorded in three samples.

3) dilution 100 - 1000:1 - CHRONIC TOXICITY TESTS (Short-term tests are recommended);

   Four to six effluent (24 hr composite) samples collected on four to six successive days are used to conduct 7 day static screening tests at 100% effluent concentration using a cladoceran and a fish:

   A low priority for additional testing is given if less than a 50% effect on growth or reproductive output is observed between controls and test organisms, whereas additional testing is needed if a greater than 50% effect is observed.

   Acute tests can be used for these dilutions, although there will be cases where no acute toxicity is measured even though the effluent is chronically toxic.

4) dilution < 100:1 - TOXICITY-TESTING BASED SCREENING IS NOT RECOMMENDED, (Initiate Definitive data generation)

In the screening tier, receiving water toxicity analysis can also be used to identify areas of instream toxicity associated with specific discharges.

Tier 2 - Definitive Data Generation

The definitive data generation tier is used to generate sufficient data on high priority effluents to support regulatory decisions, whether relating to technical measures or additional testing. On the basis of the information obtained, the environmental impact of a toxic discharge can be quantified to design appropriate discharge limits and necessary controls.

The EPA recommendations for testing are presented in terms of eliminating uncertainty levels associated with initial or 'baseline' screening toxicity tests, such as effluent variability, species sensitivity and the use of an acute to chronic (ACR) ratio. The values specified by the EPA for these factors are 10-100 for effluent variability, 10 for species sensitivity and 10 for the ACR ratio.

A simple relationship relating the acute (LC50) or chronic (NOEL) effluent concentration divided by the Instream Water Concentration (IWC) to the level of uncertainty is used to determine whether additional data are required, to end testing and initiate the setting of permit conditions, or to cease analysis due to a wide margin between toxic response and the IWC. If:

1. (LC50 or NOEL)/IWC > level of uncertainty ---> no further testing,

2. (LC50 or NOEL)/IWC < level of uncertainty ---> additional testing required,

where the level of uncertainty equals the combined uncertainty factors associated with a toxicity test. The EPA stress that this equation should be used to evaluate the need for additional analysis and the relationship should not form the basis for the development of permit

limits or conditions. The Instream Waste Concentration (IWC) is the receiving water effluent concentration after mixing, where the most appropriate or allowable mixing is determined by the regulatory authority. The IWC can be calculated in two ways depending on the source of the effluent:

$$IWC = \frac{\text{average daily effluent flow}}{\text{receiving water flow}}$$

where the effluent source is a receiving stream;

$$\text{or } IWC = \frac{\text{average daily effluent flow}}{\text{average daily effluent flow + receiving water flow}}$$

where the effluent source is discrete and not a receiving stream

In order to reduce the uncertainty associated with previously conducted toxicity tests, an increased number of tests using more advanced or extensive biological (and chemical) tests techniques are used. The EPA recommends that the following toxicity test procedures are used to address given uncertainty factors:

- Effluent variability

Acute toxicity tests should be conducted on a least two species (an invertebrate and a fish) at a frequency appropriate to the suspected variability of the discharge.

For effluents exhibiting short-term (12-48 hr) variability, the highest toxicity during a short duration period, such as one discharge cycle or a 24 hr period), should be tested using 4-6 grab or short-term composite samples. The tests should be conducted monthly for at least one year. An alternative is to require a continuous flow-through acute test on each species, which will integrate the effects of variable toxic concentrations but will not quantify effluent variability.

For effluents exhibiting suspected long-term variability, testing frequencies have to be correlated with the nature of expected changes (eg weekly, monthly, seasonal or process changes). A year long monitoring program may be required to determine long-term variability.

- Species sensitivity

Acute toxicity tests should be conducted on a range of species representative of several taxonomic groups, including plants, invertebrates and fish, to identify the most sensitive species (US EPA 1985b).

- Acute to chronic (ACR) factor

Short-term chronic toxicity tests on three species are conducted using tests such as invertebrate reproduction and fish growth tests (US EPA 1985c, 1988).

- Additional considerations

At this stage the use of more advanced hydrological and fate models to evaluate exposure and persistence respectively and chemical specific analytical techniques for identifying potentially toxic effluent characteristics is strongly recommended.

In multiple source discharge situations, where more than one discharge may be contributing to toxic impact, additional data may be required. A detailed testing procedure may be needed to measure important factors such as, additivity, antagonism and persistence. The assessment of toxic impact can be made either by considering each source separately, using the procedures described previously, or by treating each discharge as an interactive component of the whole system, using the following procedures.

For effluent dominated receiving waters, where the effluent(s) account for >1% of the total measured flow, chronic toxicity tests following

prescribed procedures should be conducted. In steam dominated receiving waters, where the effluent(s) account for <1% of the total measured flow, acute toxicity tests using prescribed protocols should be conducted.

An additional data requirement where there are multiple discharge sources is the assessment of relative and absolute toxicity of each source in order that appropriate permit conditions can be established for individual discharges. The following procedure has been suggested:

- absolute toxicity measurements of the effluent can be obtained by conducting toxicity tests on the effluents using a control dilution water (either from an upstream, uncontaminated receiving water station of similar chemical composition or reconstituted water);

- relative effluent toxicity measurements can be derived by conducting parallel toxicity tests using a dilution water taken directly upstream from the point of discharge. This dilution water may be contaminated with upstream effluents or other toxicant sources and the purpose of these tests is to establish toxicity measurements of the effluent, where it is mixed at the point of discharge.

  For toxic receiving water, due to an upstream toxic source, the dilution water for the relative toxicity test may cause significant toxic effects (mortality, growth or reproduction) at the lower effluent concentrations. However this mortality does not invalidate the test, and analysis of toxicity trends resulting from relative toxicity tests can be used to assess the effluents toxicity in relation to other sources and receiving water conditions.

- receiving water toxicity tests should be carried out during a low flow period to determine:

  a) whether or not the effluent has a measurable toxicity after mixing;

b) in-stream persistence of toxicity from all sources contributing to total in-stream toxicity; and

c) combined in-stream toxicity resulting resulting from the mixing of multiple, point and non-point sources of toxicity.

The receiving water monitoring, usually with short-term chronic toxicity tests, can be conducted by the regulatory agency or can be required of each discharger, who would then be responsible for the upstream and downstream areas around their outfall(s).

The tests should, if possible, be conducted simultaneously by all dischargers and at a minimum the tests should be conducted currently starting within a short time period (1-2 days). An appropriate frequency of testing should be applied with repeated effluent and receiving water testing required where effluent variability may be important.

In assessing the effect of various discharges in a multiple source situations, dye studies of effluent dispersion for rivers, lakes reservoirs and estuaries are strongly recommended. This method allows analysis of effluent concentration at the selected sampling stations above and below the discharge points.

Exposure and Wasteload allocation

In determining an effluent composition that will protect aquatic organisms, there has to be an assessment of the extent to which the relevant community are exposed to the effluent. This assessment should reflect the amount of a waterbody affected by the discharge, and the duration and frequency of effects so that an appropriate wasteload allocation of pollutant load can be derived. The subsequent wasteload allocation will depend on an assessment of whether complete rapid mixing occurs or there is a defined mixing zone.

In cases of complete rapid mixing, the initial step in the WLA process is the calculation of the allowable acute and chronic effluent toxicity levels which satisfy the CMC and CCC in the receiving water at the established duration and frequency. The allowable acute and chronic toxicity levels are compared and the more stringent is used to calculate the permit limits.

In situations where mixing is not rapid or complete the wasteload allocation has to be based on mixing zone analysis and modelling (where state standards allow a mixing zone. The derived WLA has to ensure that, under chronic conditions, effluent concentrations at the mixing zone boundary satisfy the CCC, which for whole effluent toxicity based control is the NOEL that will prevent lethality, and impairment of growth and reproduction in tested species. In situations where a mixing zone is prohibited,concentrations in the pipe itself cannot exceed the CCC.

In situations of complete, rapid mixing and mixing zone analysis, exposure can be assessed using several models, ranging from the steady-state model, using a critical low flow condition, to dynamic computer and statistical models to derive effluent control values. The EPA considers dynamic models to have greater realism which may often result in the establishment of less stringent effluent limits, with a high degree of assurance that the receiving water will be protected from adverse effects. The EPA have recommended models for each type of receiving water, such as rivers, lakes, estuaries and marine waters (EPA 1985).

Permit requirements

The requirements of a wasteload allocation (WLA) have to be incorporated into the wastewater discharge permit, though in many cases permit limits will be different than the WLA to reflect different assumptions and means of expressing effluent quality. Permit limits are designed to require a particular level of effluent quality and, since effluent quality is generally variable, the limits are established at a level

where the probability of exceedence will be low (<0.05) if the plant maintains the desired level of performance. The requirements are usually expressed using two types of permit limits::

a) the daily maximum permit limit, which is the maximum allowable value for any single observation; and

b) the 'average daily' or monthly permit limit, which is the maximum allowable value for the average of all observations obtained during one month.

For toxicity based control the permit will detail the effluent toxicity levels to be achieved and the test organisms and methods to be used in acute and chronic toxicity tests for compliance monitoring.

Compliance monitoring

The frequency of monitoring is considered to be the most important factor in determining compliance with permit limitations, since sampling and analysis have to be sufficiently frequent to detect violations of permit limits. In issuing a typical permit, the EPA Permits Division would incorporate the following aquatic toxicity test regime. On a monthly basis:

- 48 hr acute tests on the effluent to determine the LC_{50} (effluent concentration for 50% mortality) of the most sensitive of an invertebrate and fish species (US EPA 1985b).

- 7 day sub-chronic tests for growth and reproductive output to determine the no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) with an invertebrate and fish species (US EPA 1985c, 1988).

The discharger, or an appointed testing house, should use the appropriate freshwater or marine invertebrate and fish tests specified in the EPA test protocols.

On the basis that the test results indicate no or limited effluent toxicity after a year of monitoring, routine testing can be adjusted, usually to a quarterly regime.

In situations where the tests show significant effluent toxicity the discharger may have to conduct a Toxicity Reduction Evaluation (TRE), in which the toxic fraction of the effluent is isolated and either substituted with a component of lower toxicity or eliminated from the effluent.

Ecotoxicity Test Methods

The EPA has published detailed guidelines for toxicity tests that should be used in discharge permit setting and compliance monitoring (US EPA 1985b,c, 1988). Although the guidelines have no statutory role at present, the EPA are currently attempting to make the methods mandatory. The majority of permits issued specify a combination of the following studies:

Acute (48 hr) static invertebrate tests using species such as *Daphnia pulex* (freshwater) and *Mysidopsis bahia* (marine);

Acute (48 hr) static fish tests using species such as *Pimephales promelas* and *Poecilia reticulata* (freshwater) and *Cyprinodon variegatus* (marine);

7 day invertebrate reproduction tests with *Ceriodaphnia* (freshwater) or *Mysidopsis bahia* (marine);

7 day fish larval growth tests with *Pimephales* and *Cyprinodon*.

Indigenous species may be more appropriate than the standard species specified in the guidelines in certain instances, and trout are generally tested in all designated trout waters.

Quality Assurance and Control

Organisations (whether permittees or their contractors) are not required at present to conduct toxicity tests to the requirements of Good Laboratory Practice, as defined by the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) and Toxic Substances Control Act (TSCA). However these tests have to satisfy the guidelines stated in the EPA's Technical Support Document for Water Quality Based Toxics Control (EPA 1985a).

In order to evaluate the acceptability and validity of submitted toxicity test results regulatory authorities require information on:

1) effluent sampling, including the sampling dates, collection methods and storage and transportation procedures;

2) toxicity test data;

3) chemical analysis of the effluent and dilution water; and

4) appropriate quality control procedures adopted during testing.

In acute and chronic toxicity tests the raw data of the number of test organisms used and their responses (survival, growth, reproduction) at the end of the test period in each effluent concentration should be reported, in addition to the derived LC_{50} and NOEC/LOEC values. The use of a reference toxicant to validate biological responses and organism health is strongly recommended by regulatory authorities.

For each effluent sample collected for analysis, the toxicity test data should be accompanied by chemical analysis for appropriate parameters, such as pH, hardness, conductivity, total residual and free chlorine, surfactants and ammonia, as well as parameters listed under 'Monitoring' in the NPDES permit. Chemical analysis should be conducted using EPA approved methods.

The EPA regional offices (in states without primacy) and relevant state regulatory organisations will inspect laboratories and ring tests with reference standards may be used to establish the competence of laboratories conducting toxicity tests.

Toxicity reduction Evaluation (TRE)

On the basis of an effluent failing to comply with the conditions prescribed in a permit, the discharger is required to conduct a Toxicity Reduction Evaluation (TRE) of the problem effluent within a specified number of days. A TRE is a study conducted to determine which control options for a toxic effluent are effective for ensuring compliance with toxicity or chemical concentration requirements. A TRE can be required prior to permit issuance, during the permit term in response to a monitoring trigger or to limits being exceeded, or in response to an administrative order.

In the TRE details are given of the actions which will be carried out to enable the effluent to satisfy the permit conditions within a specified time. The TRE may include:

a) chemical analysis;

b) effluent characterisation tests (physical/chemical properties);

c) toxicity tests on the effluent prior to and after characterisation treatment;

d) toxicity tests on the effluent after physical/chemical separation;

e) in-stream toxicity tests;

f) chemical identification after physical/chemical separation of the toxic phase;

g) assessment of treatment technology available to remove the toxic substance(s) from the effluent; and

h) an implementation schedule.

The EPA has published manuals on procedures for identifying toxic components of effluents, with specific reports for problems arising from the discharges of sewage treatment works and industrial operations.

b) Guidelines adopted by states with primacy

States with the legal responsibility to issue discharge permits have adopted various protocols, which often reflect the diversity of water quality problems encountered in a state and the extent of available resources. It is apparent from the information obtained that there is considerable variation between states in discharge consent requirements, in terms of the type and frequency of both initial studies and subsequent monitoring regimes. Therefore, two examples, from the Commonwealth of Virginia and Connecticut, are given to illustrate these different approaches.

Commonwealth of Virginia

In Virginia the control of discharges of toxic pollutants to surface waters from facilities holding VPDES (NPDES) permits is controlled by the Office of Water Resource Management (OWRM). The OWRM assesses the requirements for toxics management (chemical and biological), whenever VPDES permits for discharges to surface waters are issued, re-issued or modified. The selection of sites requiring a toxics management programme involves all major facility permits, non-major permits for facilities which fall into one of the Standard Industrial Classification (SIC) codes listed in the Toxic Management Regulations, and any other sites the regional offices consider have the potential for toxic discharge.

The initial step of toxics management is a programme of biological and chemical monitoring for toxic pollutants to develop data to aid in establishing water quality based effluent limitations and assessing the extent of effluent toxicity. Following the initial data generation, biological monitoring may be required of certain categories to ensure continued compliance or to ascertain the extent of toxicity and any need for a toxicity reduction evaluation (TRE).

In this regulatory process Toxics Management Programmes (TMP) for effluent discharges with no prior information (Appendix B1) generally require the following toxicity tests in the first year of the permit;

Quarterly within 3 months of the effective date of the permit;

- Acute (48 hr) static tests for lethality with an invertebrate and fish appropriate to the receiving water.

- Chronic (7 day) static renewal invertebrate reproduction and fish growth tests with species appropriate to the receiving water.

The tests are carried out, wherever possible, using 24 hr composite samples of effluents from outfalls. Grab samples are used for intermittent, storm water and batch type discharges. These acute and chronic tests are conducted in such a manner, and at sufficient dilutions, for the calculation of valid LC_{50} and No Observed Effect Concentrations (NOEC) respectively.

The results of the tests over the first year are then compared with the Instream Waste Concentration (IWC), of a given % effluent, to ascertain whether effluents comply with or fail permit conditions. Chronic testing is not required when the IWC is less than 1%.

Permit compliance

Compliance with the ecotoxicological component of the permit results if acute LC_{50} values $\geq$ 100% effluent and chronic NOEC levels for

reproduction and growth ≥ Instream Waste Concentration (IWC) in 6 or more of the total of 8 acute toxicity tests, or 75% of the total tests conducted (if the total is greater than 8). Acute and chronic toxicity testing of the effluent continues on an annual basis with the first annual tests being conducted within 3 months of the last quarterly tests.

Permit failure

There is failure to comply with the permit if:

a) acute LC_{50} values < 100% effluent or NOEC values < predicted IWC in 3 or more of the total of 8 acute toxicity tests or more than 25% of the total tests conducted (if the total is greater than 8). In this case a toxicity reduction evaluation will be required or the discharger can conduct benthic surveys to establish that the effluent, though failing the permit conditions, has not adversely affected the receiving water communities. The benthic survey approach is a part of a TRE, which has to be implemented if a pollutant-induced impact on benthic communities is established.

b) any annual acute or chronic toxicity tests yield LC_{50} values < 100% effluent or NOEC values < the predicted IWC respectively. On this basis the test(s) shall be repeated within 3 months with;

   - resumption of the original annual compliance schedule if the retest contradicts the initial result;

   - resumption of quarterly toxicity testing, within 3 months, if the retest confirms the initial test.

Although the effluent may satisfy the biological monitoring requirements, no instream exceedence of water quality standards (for any of the 126 priority pollutants) or criteria for the protection of aquatic life or human health is permissible, pursuant to appropriate Virginia Water Quality standards, otherwise a TRE will be required.

In cases where a TRE is required the permittee shall submit:

a) a toxicity reduction plan; or at the permittees option
b) a receiving water impact study plan and accompanying implementation schedule

within 120 days of the notification of such a requirement by the State Water Control Board. On completion of the review of the plan, the permit may be modified or alternatively revoked and reissued in order to reflect appropriate permit conditions and a compliance schedule.

In certain instances, though not in cases of acute toxicity, permittees may request an exemption from the requirement for toxicity reduction. These exemptions may be for technological, socio-economic or other reasons as outlined in the State and/or Federal regulations governing the adoption and maintenance of water quality standards. The demonstration that a designated use of a water body is improper or unattainable for some reason beyond the control of the discharge are potential reasons for exemption from toxicity reduction.

Virginia Power Permit

A VPDES discharge permit reissued to the Virginia Electric and Power Company in the Toxics Management Programme (TMP), specified biological monitoring of site outfalls 1, 2, 3 and 12, the effluents of which have the following composition:

Outfall 1: Condenser cooling water, demineraliser wastes and reverse osmosis water;

2: Wastewater from an ashpond receiving ash sluice water, ash landfill runoff, and effluents from the metals treatment basin, the sewage treatment plant and the oily waste treatment basin. The ashpond effluent is subject to sedimentation prior to discharge;

3: Coal pile drainage, which is subject to sedimentation prior to discharge;

12: Storm-water runoff, which is discharged without treatment.

The following toxicity testing regime for the multiple effluent discharges on the site is specified in the discharge permit.

Outfall	Starting time after permit effective date	Year				
		1	2	3	4	5
1 & 2	3 Months	Acute annual tests with *Mysidopsis bahia*	----------	----------	----------	----------
		Quarterly chronic tests with *M. bahia* and *Cyprinodon variegatus*				
3	6 Months	Acute annual tests with *M. bahia*	----------	----------	----------	----------
12	6 Months	Semi-annual acute tests with *M. bahia* and *C. variegatus*	-----			

A flow chart detailing the consequences of permit compliance or failure over the initial two years of the permit are shown in Figure B2.

b) Connecticut

The strategy adopted by the Connecticut Department of Environmental Protection for water quality based permitting of toxic pollutant discharges to surface waters has been extensively described by Dunbar (1987). The strategy initially involves screening of NPDES major industrial permits to identify toxic discharges and establish priorities

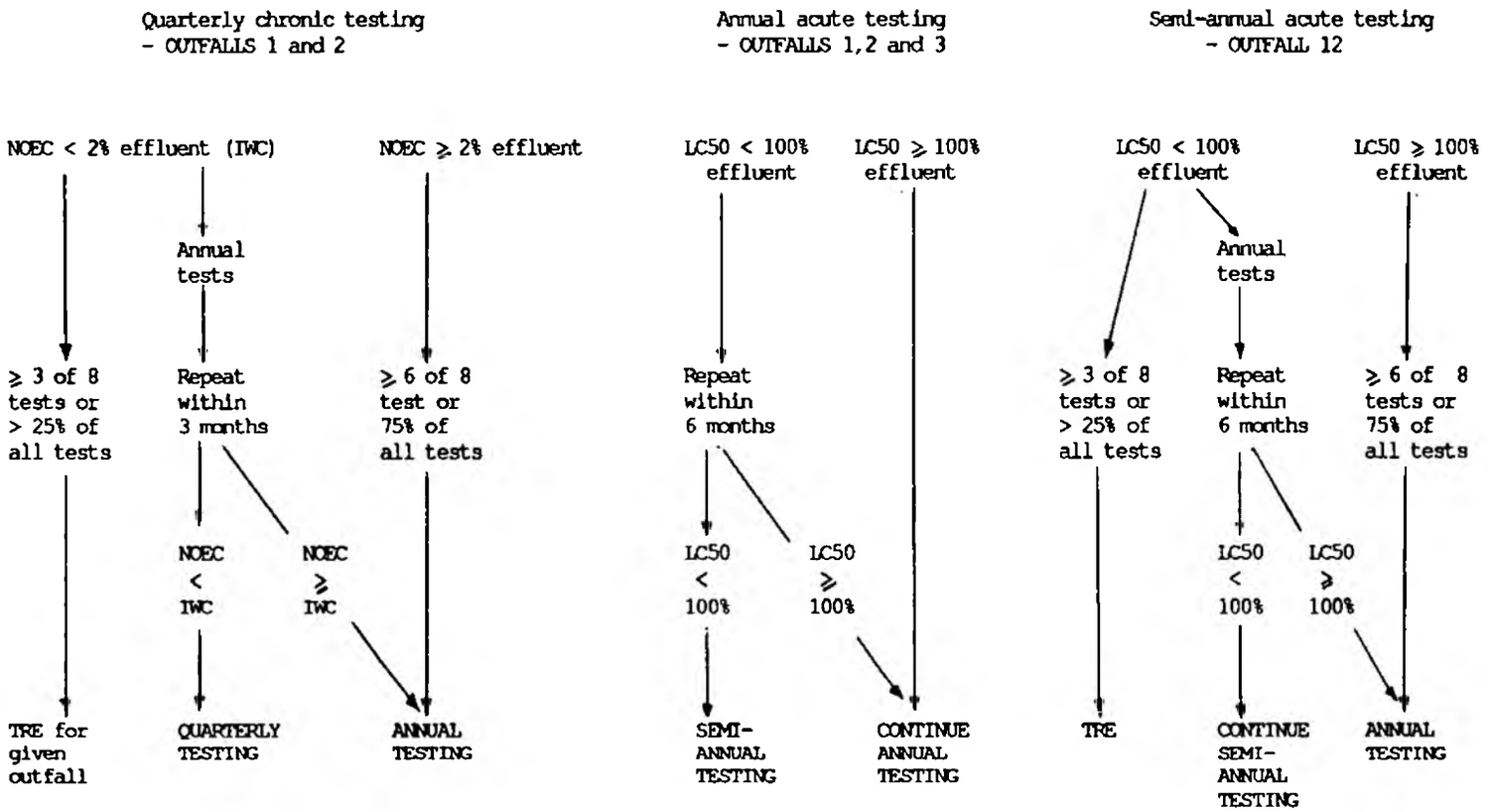


Figure B2 - Implications of permit compliance or failure for the four outfalls regulated by toxicity-based control at the Virginia Electric and Power Company facility

for permit revision. The permitting scheme is also applied to state permitted discharges to publicly operated treatment works (POTW) and discharges of treated effluent from a POTW, where in-stream toxicity of receiving waters may result.

In the screening analysis, data from the existing permit is used to establish in-stream pollutant concentrations for each effluent parameter, based on simple dilution calculations of daily average limits for critical low stream flow periods (7Q10). Acute and chronic screening toxic units (TU) for each effluent parameter are then calculated as the ratio of estimated in-stream concentrations to the relevant acute and chronic numeric criteria for the protection of aquatic life. These values are obtained from the water quality standards published by the EPA, based on receiving water hardness.

On the basis of toxicant additivity for effluents containing more than one toxic component, acute and chronic screening toxic units were summed to obtain a total toxic unit (TTU) value. Effluents which exhibited a chronic TTU > 1.0 under 7Q10 low flow conditions are considered to present a potential toxic problem and therefore require water quality based permits. In the case of discharges showing in-stream toxicity < 1.0 TU further toxicity assessment was not generally considered necessary unless other evidence existed indicating effluent toxicity. The TTU approach to ranking permits was considered to provide a means of prioritising the workload for efficient use of DEP resources. In the state, 124 of the 800 issued discharge permits in 1987 were classified as NDPES major permits and screening analysis indicated 105 (78%) exhibited in-stream values > 1.0 TTU.

In the case of effluents shown to have potential in-stream toxic impact, dischargers are required to prepare a Discharge Toxicity Evaluation (DTE). In nearly all cases the discharger is required to obtain biological test data on effluent toxicity and the ability of the receiving water to accumulate the toxicity, based on available dilution capacity. Hydrological investigations of effluent dilution and mixing characteristics may also be required. In order to ensure that the data

generated by the DTE will be acceptable to the regulators, dischargers are required to submit a proposed scope of studies for review and approval before initiation of data collection. The toxicity test results are reviewed to ensure minimum performance standards are satisfied regarding quality control and data reliability, and if appropriate are used to derive toxicological effluent limits.

In the DTE the in-stream waste concentration (IWC) is calculated as the % effluent concentration which will be present at a 7Q10 low stream flow. The results of a minimum of 3 static acute toxicity tests, several weeks apart and employing an invertebrate and fish species representative of the diluting medium, are used to calculate LC_{50} values. The LC_{50} value for the most sensitive species is used to derive:

1) the acute no observable effect level (ANOEL) = $LC_{50}/3$;

2) the maximum allowable toxicant concentration (MATC) = $LC_{50}/20$

The chronic toxicity value is derived on the basis of an acute-chronic ratio (ACR) of 20, though values can be measured directly and the MATC calculated as the geometric mean of the chronic no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC). The IWC and MATC values are compared and if the IWC is less than or equal to the MATC a permit will be issued. In situations where IWC is greater than the MATC toxicity reduction may be required before a permit is issued.

A discharge is evaluated on its own merits, with consideration of the interactions between neighbouring outfalls restricted to the process of influence zone allocation. The influence zone is allocated by the DEP following an evaluation of a number of factors including:

a) the volume, strength and persistence of toxic substances present in the effluent;

b) the potential for bioaccumulation of toxic substances in the effluent;

c) allowance for a zone of passage of aquatic organisms in the receiving water;

d) the location of the zone relative to sensitive areas, such as spawning grounds;

e) the location of other discharges in the receiving water; and

f) the use(s) of the receiving water.

In compliance with Conneticut Water Quality standards, chronic toxicity has to be limited to the designated zone of influence during 7Q10 conditions for rivers and streams. For discharges to large rivers, estuaries and marine waters, a dye dilution study will frequently be required to evaluate the zone of influence.

In the permits issued, effluent limits are derived to prevent acute and chronic toxicity outside of any zone of influence and are expressed as the % effluent dilution at which no toxicity is allowed. For the protection of aquatic life in the receiving water from acute toxicity the ANOEL ($LC_{50}/3$) cannot be exceeded, whereas for to restrict chronic toxicity and maintain water quality the MATC cannot be exceeded. Compliance is achieved whenever the IWC is less than the concentration of effluent which results in acute ($LC_{50}/3$) or chronic ($LC_{50}/20$) toxicity using toxicity tests and test species specified in the permit.

c) Industrial Organisations

The ecotoxicological methods used by British Petroleum and Texaco Inc in monitoring effluent discharges from their industrial plants to fresh, estuarine and marine receiving waters are shown in Table B2.8. The BP discharges are at most monitored monthly for acute effects and quarterly for chronic effects, while biomonitoring is usually conducted

Table B2.8 - Ecotoxicological techniques used for toxicity-based effluent discharge regulation by industrial organisations in the USA

Type of method	Organisation	
	British Petroleum	Texaco Inc
SUB-ORGANISM METHODS	N/A	N/A
WHOLE ORGANISM METHODS		
Lethal (LC/EC50) endpoints:		
Invertebrates:		
Daphnia magna (F)	* (L)	*
Ceriodaphnia sp (F)	* (C)	-
Hyalella azteca (E)	* (C)	-
Crassostrea sp (M)	-	*
Mysidopsis bahia (M)	* (C)	*
Fish:		
Pimephales promelas (Fathead minnow) (F)	*	*
Oncorhynchus mykiss (Rainbow trout) (F/E)	*	-
Cyprinodon variegatus (Sheepshead minnow) (M)	-	*
Sub-lethal endpoints:		
Fish larval growth tests (7d)		
Pimephales promelas (F)	*	*
Oncorhynchus mykiss (F/E)	-	*
Cyprinodon variegatus (M)	-	*
Invertebrate reproduction tests (7d)		
Ceriodaphnia sp (F)	*	*
Mysidopsis bahia (M)		*
Others	Sediment toxicity tests with the estuarine/marine amphipod *Rhepoxyinius abronius*	Sea urchin or sand-dollar fertility/Oyster and mussel embryo larval development/ *Mytilus* hydrocarbon accumulation
POPULATION METHODS	N/A	N/A
COMMUNITY METHODS	Benthic macro-invertebrate abundance study (Community structure analysis)	Benthic sampling for population composition

F, E and M are fresh, estuarine and marine receiving waters; C = common use, L = limited use; * = applied test, N/A = not applied

on a quarterly basis for Texaco's effluent discharges. At a given discharge not all the biological methods described may be specified in a permit, though the discharger may carry these out to provide additional information on the impact of the effluent on the receiving water.

Canada

An acute lethal toxicity test using fish has been used in Canada since 1971 to regulate pulp and paper mill effluents (Environment Canada 1971). A 24 hr limit test or an acute (96 hr) lethality test is commonly carried out with the rainbow trout *Oncorhnychus mykiss*. The Ontario Ministry of the Environment is also using the 48 hr static lethality test with *Daphnia magna* as an additional regulatory tool for discharge control.

A strong programme for using toxicity based control in controlling and monitoring all industrial sector effluents and water treatment wastes (municipal) is currently under development. The use of growth, reproduction and survival tests for estimating chronic toxicity levels in non-acutely toxic effluents once every 6 months has been proposed for industrial monitoring.

Republic of Ireland

In Ireland the guidelines for restrictions on the discharge of toxic effluents, expressed in terms of toxicity, are industry specific (Table B2.9) and are incorporated on a case by case basis in individual discharge permits. The values given below for various industries represent the toxic unit limits which should not be exceeded, and these values have been derived from the 96 hr effluent LC_{50} values (% effluent) by TU = 100/96 hr LC_{50}.

Table B2.9 - Effluent toxicity limits for specific industries under Republic of Ireland guidelines

Priority group	Category	96 hr LC_{50} (% effluent)	Toxic Units (TU)
A	Chemical or pharmaceutical manufacturing	4	25
B	Metal extractions, plating or finishing	10	10
C	Textiles, tanning, paper and glass making	20	5
D	Agricultural and food industries untreated municipal sewage	70	1.4
E	Treated municipal sewage	100	1

In order to ensure sufficient mixing of the discharges in receiving waters the guidelines stipulate that at least 20 dilutions be available, in the vicinity of an outfall, for each toxic effluent unit discharged. Although the values given represent values which should not be exceeded, in certain instances the limit may be suspended. This would be with the proviso that adequate dilution capacity existed throughout the year to satisfy the dilution requirements dictated by measured toxic unit values.

The compliance of selected industrial discharges with established toxicity limits is ascertained by annual or biannual tests on representative effluent samples. The rainbow trout is commonly used and the results obtained from 96 hr exposure tests are expressed as toxic units. The national guidelines recommend the use of an abbreviated 'limit test' for compliance monitoring (ie one control and a test concentration equal to the toxicity limit), though this procedure is not widely used and expanded tests with a range of effluent concentrations are used. Flow measurements, mixing and dispersion studies are a necessary adjunct to monitoring toxicity limits, to ensure the minimum requirement of 20 dilutions per toxic unit discharged in the immediate

vicinity of the outfall is satisfied. The efficacy of toxicity limits in protecting receiving waters is confirmed through biological surveys at least once every three years, particularly in area's of special biological importance or sensitivity.

Denmark/Finland/Germany/Sweden

In Finland, Germany and Sweden a wide range of ecotoxicological techniques have been used in deriving permit limits and monitoring compliance on a site-specific basis (Table B2.10). Established acute and chronic whole organism methods using survival, growth and reproductive output in algae, invertebrates and fish are most commonly used. However, sub-organism methods (principally MFO activity), population (recruitment) and community (mesocosms) methods have also been used in all these countries. A general approach for controlling Danish marine industrial discharges, employing acute and chronic whole organism methods, has been described by Nyholm *et al* (1990).

In Germany toxicity testing is also used under the Wastewater Act to levy charges on industrial operators discharging effluents to fresh, estuarine and marine waters. A toxicity test with the golden orfe *Leuciscus idus* is one of four methods which are used to determine an industrial facilities 'Unit of Pollution' which determine the extent of the charges. In the toxicity test the dilution required to render the effluent non-acutely toxic to fish is measured.

France/The Netherlands

The use of ecotoxicological methods for establishing permit limits and compliance monitoring in France and the Netherlands is at present restricted to established acute and chronic lethal and sub-lethal whole organism responses (Table B2.10).

Table B2.10 - Ecotoxicological techniques used for toxicity-based effluent discharge regulation in European countries

Type of method	FRANCE	FINLAND	GERMANY	NETHERLANDS	SWEDEN	DENMARK
SUB-ORGANISM METHODS						
Tissue	N/A	N/A	N/A	N/A	Somatic indices (LSI, GSI)	Histological effects on gills (17 days)
Cellular	N/A	N/A	Fish cell culture	N/A	Haematology	N/A
Sub-cellular	N/A	N/A	N/A	N/A	N/A	N/A
Biochemical						
MFO activity in fish	N/A	* Cholinesterase inhibition	*	N/A	* Glycogen/Lactate/ Amino acids	N/A
WHOLE ORGANISM METHODS						
Lethal (LC/EC50) endpoints:						
Invertebrates:	*Daphnia magna*	N/A	*Daphnia magna*	*Daphnia magna*	*Ceriodaphnia*	*Nitocra spinipes* *Crangon crangon*
Fish:	Zebrafish Rainbow trout	Not specified	Zebrafish Rainbow trout Golden orfe	Zebrafish	Zebrafish Rainbow trout	Zebrafish Goby Turbot
Early life stage tests	N/A	Fish species	N/A	Zebrafish	Fathead minnow	Plaice
Sub-lethal endpoints:						
Growth tests	N/A	Stickleback (6 months)	Rainbow trout (14 days)	Zebrafish	Fathead minnow (7 days)	Eelgrass growth and development ^{14}C assimilation by eelgrass and phytoplankton Phytoplankton (3d) Turbot (17d)
Reproduction tests	N/A	N/A	Zebrafish	*Daphnia magna*	*Ceriodaphnia* (7d) *Nitocra spinipes*	*Nitocra spinipes* (12d)
Microtox	N/A	*	*	N/A	*	*
POPULATION METHODS	N/A	Sticklebacks	N/A	N/A	*Lemna minor*	N/A
COMMUNITY METHODS	N/A	Fresh/brackish water mesocosms	Freshwater mesocosms	N/A	Brackish water mesocosms	N/A

* = applied test, N/A = not applied

B3.3 RECEIVING WATER QUALITY MONITORING

United States

In the United States, regulatory agencies at both state and federal level use a variety of approaches to monitor surface water quality, including sediment and aquatic organism analysis. The principal approaches (US EPA 1987) are:

- Networks of fixed stations at which there is repeated sampling;

- Intensive surveys at a specific site or in well-defined geographical areas, such as river basins, lakes and estuaries;

- Statistically designed special studies with a specific focus.

In all these approaches rapid, reliable and cost-effective ecotoxicological methods are widely used by regulatory agencies, in association with chemical analysis and biological surveys.

In the assessment of the impact of effluent discharges on the receiving water communities, short-term chronic toxicity tests are used to measure the toxicity of receiving water above, below and at the site of outfalls. Since the actual instream toxicity is measured, significant toxic impacts should be detected and this is particularly useful for multiple source discharge situations where toxicity may not be apparent from individual screening analysis. The analysis has to be conducted concurrently with discharge-specific testing and should be carried out at low flow conditions. The analysis may be conducted either by the relevant regulatory agency or by the discharger in conjunction with effluent tests.

Canada

In Canada, state regulatory offices use a variety of biological methods (Table B2.11) in association with chemical analysis and biological

Table B2.11 - Ecotoxicological techniques used for monitoring receiving water quality by State and Federal regulatory agencies in Canada

Type of method	British Columbia Ministry of Environment: Environmental Protection Division	Canadian Centre for Toxicology	Newfoundland Dept. of Environment and Lands	Saskatchewan Research Council
SUB-ORGANISM METHODS	Metallothionein synthesis in invetebrates	N/A	N/A	N/A
WHOLE ORGANISM METHODS				
Lethal endpoints:				
Algal EC50	-	*	-	-
Daphnia sp EC50 (48 hr)	-	*	*	-
Rainbow trout EC50 (24/96 hr)	*	-	*	-
Early life stage tests	-	-	-	Fish
Sub-lethal endpoints:				
Growth tests	-	Algae	-	-
Reproduction tests	2 generation *Daphnia* test	-	-	*Ceriodaphnia* (7d)
Other tests	Microtox	-	-	-
POPULATION METHODS	N/A	N/A	N/A	N/A
COMMUNITY METHODS	N/A	Limnocoral mesocosms	N/A	N/A

* = applied test, N/A = not applied

surveys to monitor receiving water quality. Established acute and chronic whole organism methods are routinely used by regulatory agencies in all the states which responded. In contrast sub-organism (MFO activity and metallothionein synthesis), population and community methods are only occasionally used by given states.

The responses of the various branches of the Ontario Ministry of the Environment are indicative of the way ecotoxicological methods can be applied to provide data required to answer specific problems (Table B2.12).

Europe

The ecotoxicological methods principally used to complement chemical analysis and biological surveys in France, Finland, Germany, the Netherlands and Sweden are those at the sub- and whole organism levels of organisation (Table B2.13). The whole organism methods used are generally acute and chronic invertebrate and fish toxicity tests using survival, growth and reproductive output as the endpoints. The Microtox test and MFO activity are also used on a site-specific basis in all countries, except France. Population and community methods are not generally used, although Finnish and German regulatory agencies have used mesocosms.

B3.4 SEDIMENT QUALITY CRITERIA

In many OECD countries, including the United Kingdom, there is considerable interest and research activity being directed towards the development of sediment quality criteria to complement national receiving water quality criteria. As sediments integrate the effects of surface water contamination, they can present a hazard to aquatic communities (both water column and benthic), which may not be directly predictable from water quality contaminant concentrations (Shea 1988). The sediment quality criteria will:

Table B2.12 – Ecotoxicological techniques used for monitoring receiving water quality by sections of the Ontario Ministry of the Environment

Type of method	Aquatic Toxicology Unit	Water Resource Branch	
		Biohazards	Sediments
SUB-ORGANISM METHODS	N/A	MFO activity (EROD/GST) AMES mutagenicity tests (effluents, receiving waters, sediments) *Tradescantia* mutagenicity/ genotoxicity (effluents, receiving waters Fish health evaluations (pathology for cancers)	N/A
WHOLE ORGANISM METHODS			
Lethality	48 hr LC50 *Daphnia magna* 96 hr LC50 Rainbow trout	N/A	21 day Mortality and bio-accumulation with Fathead minnow Early life stage tests: 10 day test with 10 day old midge larvae *Chironomus tentans;* 21 day test with 3-4 month old burrowing mayfly nymphs *Hexagenia* sp
Sub-lethal growth tests	7 day Fathead minnow	N/A	10 day tests with midge larvae; 21 day tests with mayfly nymphs
POPULATION METHODS	N/A	Condition factors/growth rates/ reproductive performance in wild fish populations	N/A
COMMUNITY METHODS	N/A	N/A	N/A

N/A = not applied

Table B2.13 - Ecotoxicological techniques used for monitoring receiving water quality in European countries

Type of method	FRANCE	FINLAND	GERMANY	NETHERLANDS	SWEDEN	DENMARK
SUB-ORGANISM METHODS						
Tissue	N/A	N/A	N/A	Somatic indices	Somatic indices (LSI/GSI)	Histopathological effects on gills
Cellular	N/A	N/A	Fish cell culture/ Phagocytosis of mussel blood cells	N/A	Haematology	N/A
Sub-cellular	N/A	N/A	*	N/A	N/A	N/A
Biochemical						
MFO activity in fish	N/A	* Cholinesterase inhibition	*	N/A	* ALA-D activity Glycogen/Lactate/ Amino acids	*
WHOLE ORGANISM METHODS						
Lethal (LC/EC50) endpoints:						
Invertebrates: *Daphnia magna*	*Daphnia magna*	*Anodonta/Unio*	*Daphnia magna*	*Daphnia magna*	*Ceriodaphnia*	*Daphnia magna* *Nitocra spinipes* *Crangon crangon*
Fish:	Zebrafish Rainbow trout	Not specified	Zebrafish Rainbow trout Golden orfe	Zebrafish	Zebrafish Rainbow trout	Zebrafish Goby
Early life stage tests	Zebrafish	Fish species	Zebrafish Rainbow trout Carp	Zebrafish Oysters	Fathead minnow Mussels	Plaice
Sub-lethal endpoints:						
Growth tests	Zebrafish	Stickleback (6 months)	Rainbow trout (14 days)	Zebrafish *Mytilus edulis*	Fathead minnow (7 days)	Eelgrass growth and development Phytoplankton (3d) Turbot (17d) ^{14}C assimilation by eelgrass and phytoplankton
Reproduction tests	*Ceriodaphnia*	N/A	*Daphnia magna* Zebrafish	*Daphnia magna* Bathyporia	*Ceriodapdhia* (7d) *Nitocra spinipes*	*Nitocra spinipes*
Scope for Growth	N/A	N/A	N/A	*Mytilus edulis*	*Mytilus edulis*	
Microtox	N/A	*	*	*	*	

Table B2.13 - continued

Type of method	FRANCE	FINLAND	GERMANY	NETHERLANDS	SWEDEN	DENMARK
Other tests	N/A	*Anodonta/Unio* bioaccumulation	On-line cyano-bacteria test Fish monitor	Fish monitor	Skeletal composition	N/A
Behavioural endpoints:	N/A	N/A	*Daphnia* monitor	Shell-valve activity monitor *Daphnia* monitor	N/A	N/A
POPULATION METHODS	N/A	Sticklebacks	N/A	N/A	N/A	
COMMUNITY METHODS	N/A	Fresh/brackish water mesocosms	Freshwater mesocosms	Freshwater mesocosms	N/A	N/A

* = applied test, N/A = not applied

1) protect habitats and communities from the effects of sediment contamination; and

2) provide a basis for the long-term management of contaminated sediments.

A number of approaches to classifying and evaluating sediment toxicity have been proposed and these have been described extensively by Roddie (1989). These approaches range from chemical concentration-based screening to multidisplinary evaluation schemes in which biological criteria are employed to different extents. These multidisciplinary schemes include:

a) the Apparent Effects Threshold (AET) approach, which combines field data on sediment contaminant concentrations with at least one biological effects measure (eg whole sediment toxicity tests, infaunal community structure or bioaccumulation) to identify sediment contaminant levels above which biological effects are always observed (Chapman 1989); and

b) the Sediment Quality Triad (SQT) in which information is required on sediment chemistry and toxicity and *in situ* biological effects, such as community structure, (Chapman 1989), and chemical concentrations associated with minimal and severe limits of biological effect.

None of the approaches developed have been incorporated into legislation and all have recognised limitations.

An ecotoxicological assessment of sediment toxicity can be obtained from the responses of epibenthic and infaunal species to sediment interstitial (pore) water, sediment elutriates or whole (bulk) sediments. The toxicity test endpoints employed include lethality, growth, development and physiological and behavioural indices. At present whole sediment toxicity tests using infaunal species provide the most robust basis for assessment and these have been widely used in a management programmes in the United States. These tests can be used with field collected or laboratory-defined sediments, and can be applied to assessing the effects of single contaminants and complex mixtures.

Although standardised test methods are being agreed, the major limitations of sediment toxicity tests relate to the development of standard sampling, storage and handling methods to minimise changes in contaminant partitioning and speciation.

APPENDIX C

SPECIMEN DISCHARGE CONSENTS CONTAINING TOXICITY-BASED COMPONENTS FROM THE UNITED KINGDOM AND THE UNITED STATES

Consent No. PR/C/T/S/1592

NATIONAL RIVERS AUTHORITY

ANGLIAN REGION

WATER ACT 1989 SCHEDULE 12

NOTICE OF VARIATION OF CONSENT

To: Dow Chemical Company Limited
Stana Place
Fairfield Avenue
Staines
Middlesex
TW18 4SX

In pursuance of Schedule 12 Paragraph 6 of the above Act, Notice is hereby given that the prescribed conditions attached to.Consent No. PR/1/T/S/1399 issued to Dow Chemical Company Ltd, Riverside Industrial Estate, King's Lynn, Norfolk by Anglian Water Authority under the Rivers (Prevention of Pollution) Acts 1951 to 1961 for a discharge of trade effluent from factory premises at North Lynn, King's Lynn into the tidal River Ouse at National Grid Reference Point TF 6097 2169 and dated 12th August 1983 are VARIED with effect from the date of this Notice.

The conditions stated in Consent No. PR/1/T/S/1399 are revoked and replaced by the following conditions :-

1. The discharge shall consist solely of trade effluent derived solely from the manufacture of Dursban and Latex, AND/OR surface water from the production plant areas at the above premises.

2. The discharge shall be made to the tidal River Great Ouse via an outlet at National Grid Reference TF 6100 2161.

3. The volume discharged shall not exceed 2045 cubic metres in any period of 24 hours.

4. The rate of discharge shall not exceed 220 cubic metres per hour.

5. The discharge shall only be made during the ebb period in any tidal cycle commencing one half hour after high water and ending five and a half hours after high water.

6. For the purposes of this Consent the times of high water on any particular day shall be determined by reference to the Tide Tables published by the King's Lynn Conservancy Board.

7. The discharge shall not contain more than 500 milligrams per litre of suspended solids (measured after drying at 105°C).

8. The discharge shall not contain more than 350 milligrams per litre of biochemical oxygen demand (determined in the presence of 0.5 milligrams per litre of allyl-thiourea after 5 days at 20°C.)

9. The discharge shall not contain more than 500 milligrams per litre of total organic carbon.

10. The discharge shall not contain more than 1 milligram per litre of free chlorine.

11. The discharge shall not contain more than 2 micrograms per litre of γ hexachlorocyclohexane (lindane) nor shall the total amount of lindane discharged in any one calendar year exceed 1 kilogram.

12. The discharge shall not contain more than 3 micrograms per litre of chlorpyrifos or chlorpyrifos methyl either singly or in combination.

13. The discharge shall have a pH value of not less than 6.0 nor greater than 10.0 .

14. 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%.

15. A sampling point with facilities enabling fully representative samples of the discharge to be readily taken at all times shall be installed by the discharger at the valve on the pumping main at National Grid Reference point TF 6104 2160 before the main passes through the river bank. The discharger shall ensure that all constituents of the discharge pass through the said sampling point at all times and in any legal proceedings it shall, for the purposes of Section 10(2) of the Rivers (Prevention of Pollution) Act 1961, be presumed until the contrary is proved that any sample of the discharge taken by the Authority at the said sampling point is a sample of what was passing into relevant waters from the said premises.

16. A continuous recorder, to enable the volume and the instantaneous rate of the discharge to be measured, shall be provided within 12 months of the date of this consent and thereafter maintained in full working order.

17. Records as to the volume of the discharge shall be maintained by the Company and these records shall be kept conveniently available to representatives of the Authority for inspection at all reasonable times.

Consent No. PR/1/T/S/1399 as varied to No. PR/C/T/S/1592 with effect from the date of this Notice will not be revoked nor will the conditions thereof be modified without the written agreement of the person making the discharge before the period ending with the last day of November 1991.

National Rivers Authority, Anglian Region
Aqua House
London Road
Peterborough, PE2 8AG

Dated 15th November 1989

J.A. Tetlow
Regional Manager
Environment and Fisheries

The procedure for appealing against the Authority's decision is set out in the Control of Pollution (Consents for Discharges etc.) (Secretary of State Functions) Regulations 1989 S.I. 1989 No.1151.

TOXICITY TEST PROCEDURES

1. STANDARD ACUTE TOXICITY TEST (STATIC)

014. Cleaning equipment

1. Wear a lab coat and rubber gloves for all washing up.

2. If any of the containers waiting to be washed up contain an unidentified liquid or solid, check with whoever is thought to have placed them there before disposing of the liquid.

3. Use a solution of hot water and 'Pyroneg' to wash all glassware, then rinse well with first hot, and then cold, water; if anything has been in contact with biological sludge use 'Diversol' sanitizer instead of 'Pyroneg'.

4. Place pipettes in a pipette washer with a small amount of 'Pyroneg' (or 'Diversol') and allow at least two washing cycles with hot water followed by two rinsing cycles with cold water.

5. Allow the glassware, etc to drain.

6. Dry all glassware with a clean tea towel and put away immediately in a clean cupboard.

7. Rinse the tanks with cold water to remove most of the test material.

8. Use a solution of hot water and 'Pyroneg' to wash the tanks, lids, and aerator pipettes and end pieces; if they have been in contact with biological sludge use 'Diversol' sanitizer instead of 'Pyroneg'. If the green mesh tap guards are badly soiled remove them from the tanks and wash separately.

9. Rinse everything well, using first hot, and then cold water.

10. Allow the tanks to drain.

11. Dry everything with a clean tea towel.

12. Store the tanks upside down on the test racks when they are not in use.

a) (i) The sample shall be tested by the Authority as soon as is reasonably practicable after the receipt thereof but in any event not later than five days after receipt thereof.

(ii) If the sample cannot for any reason be tested immediately by the Authority it shall be securely stored by the Authority pending the carrying out of such test such storage to be at a constant temperature of 4°C.

b) (i) Prior to commencement of the test a bulk sample of 110 litres of sea water of a salinity of between 30 and 35‰ shall be taken for use in the test procedure and in the control tank by which the test procedure shall be regulated and references to 'sea water' in this appendix shall be taken to be references to the sea water comprised in the said bulk sample.

(ii) In this Consent references to dilution water shall mean sea water filtered to remove all matter contained therein of a dimension greater than 10 um and aerated to maintain a dissolved oxygen air saturated value (ASV) of 80-100%.

c) The test shall be carried out at a temperature of 15 °C ± 1 °C.

d) Two test tanks shall be used each containing ten litres of sample diluted as to the proportion of two litres of the sample mixed with ten litres of dilution water, and one control tank shall also be used containing ten litres of dilution water only.

e) The contents of the two test tanks and of the control tank shall be constantly aerated during the carrying out of the test to maintain at all times a dissolved oxygen concentration of not less than 80% saturation therein.

f) The pH of the dilution water in the control tank shall be measured before the test commences and the test shall not commence until the pH reading thereof measures between pH 7.5 and pH 8.0. If the pH reading thereof shall either measure below pH 7.5 or above pH 8.0 either a sufficient quantity of hydrochloric acid or as the case may be of sodium hydroxide shall be added to the tank until the pH reading shall measure between pH 7.5 and pH 8.0.

g) The test creatures shall be brown shrimps (Crangon crangon) of between 40 and 60 mm in length (which measurement shall exclude the length of any antenna).

h) The creatures shall be gradually acclimatised to the test conditions for between two and four days prior to the commencement of the test by being placed in (a) tank(s) of sea water which shall be constantly aerated to maintain at all times a dissolved oxygen concentration of not less than 80% saturation therein and which sea water shall at all times during the final two days of the said period of acclimatisation be kept at a temperature of 15 °C ± 1 °C. During the said period of acclimatisation any ovigerous female, dead, damaged or newly moulted creature(s) shall be removed. The creatures should not be fed for 2 days prior to the start of the test or during the test.

i) At the commencement of the test the creatures shall be hand netted from the acclimatisation tank(s) referred to in Condition h) hereof to be distributed randomly as to twenty to each of the two test tanks and as to a further twenty to the one control tank.

j) (i) The test shall continue for a continous period of ninety six hours.

(ii) Each test and the control tank shall be emptied every twenty four hours and the respective contents thereof replaced with a fresh solution of dilute sample as to the two test tanks and of dilution water only as to the control tank in each case. Care should be taken to avoid physical damage to the shrimps when the tanks are drained.

The test tank and the control tank shall from time to time and in any event at intervals no less frequent than once every twenty four hours for which the test continues be visually inspected to ascertain the condition of the creatures therein.

l) (i) 'Dead animals' shall be taken to mean (a) those creatures in the test and control tanks which shall fail to respond by leg pleopod or antenna movement to gentle prodding and (b) those creatures which are seen to be moulting and dying and any such dead animals as aforesaid shall be removed from the control tank or from the test tanks as the case may be and any moult skin of such dead animal shall also be removed from the said tanks provided that creatures which have moulted and which are found partially or wholly eaten in the said tanks shall not be regarded as dead animals but any remains and moult skins of such creatures shall also be removed.

(ii) On each occasion on which a periodic inspection under the provisions of Condition k) hereof is carried out a count shall be taken of the number of dead animals in each of the two test tanks and in the control tank and in addition on each such occasion a count shall also be taken of the number of living creatures in each such tank as a check on the number of creatures which may have been wholly eaten and of which no trace shall remain.

m) The temperature in degrees centigrade pH value and dissolved oxygen concentration shall be measured and be recorded one hour after the commencement of the test and, in addition, at least once in each period of twenty four hours during which the test continues.

n) At the end of the test period the number of live creatures remaining in each test tank and in the control tank shall be counted and separately recorded.

o) The cumulative percentage mortality for the test shall be calculated for each test tank and for the control tank and shall be calculated in manner following that is to say it shall be taken to be in each case and by reference to each tank separately the number of dead animals divided by the sum of the number of dead animals and the number of creatures counted as living at the end of the test period which said total shall then be multiplied by one hundred provided that no account shall be taken in calculating the cumulative percentage mortality of any creatures which are counted under the provisions hereof as either partially or wholly eaten during the period of the test.

p) The test shall be deemed to be invalid and no account shall be taken thereof if the cumulative percentage mortality calculated as aforesaid in the control tank is either equal to or in excess of 20%.

q) The sample of effluent shall be conclusively deemed to fail to satisfy the conditions of this Consent if in a case in which the test shall be deemed to be valid under the provisions of Condition p) hereof the cumulative percentage mortality calculated as aforesaid in both of the said test tanks shall exceed 50%.

NATIONAL RIVERS AUTHORITY

(ANGLIAN REGION)

WATER ACT 1989
SCHEDULE 12

NOTICE OF CONSENT

To: Schering Agrochemicals Limited ("The Company")
Hauxton
Cambridge
CB2 5HU

The NATIONAL RIVERS AUTHORITY ("The Authority") in pursuance of its powers under the above Act HEREBY CONSENTS to the making of a discharge of TRADE EFFLUENT and/or SEWAGE EFFLUENT and/or MATTER OTHER THAN TRADE OR SEWAGE EFFLUENT from premises at Chesterford Park Research Station, Little Chesterford, Saffron Walden, Essex, in accordance with the application received on 10 April 1989, subject to the following conditions :-

1. The discharge shall consist of treated trade effluent, derived from laboratory wastes, and/or treated sewage effluent and/or surface water from Chesterford Park Research Station, Little Chesterford.

2. The discharge shall be made through an outlet at National Grid Reference TL 5314 4237 into a tributary of the River Cam.

3. The volume discharged under dry weather conditions shall not exceed 450 cubic metres in any period of 24 hours .

4. The rate of discharge shall not exceed 30 cubic metres per hour.

5. The discharge shall not contain more than 30 milligrams per litre of suspended solids (measured after drying at 105°C).

6. The discharge shall not contain more than 15 milligrams per litre of biochemical oxygen demand (determined in the presence of 0.5 milligrams per litre of allyl-thiourea after 5 days at 20°C).

7. The discharge shall not contain more than 5 milligrams per litre of ammonia (expressed as N).

8. The discharge shall not contain more than 15 micrograms per litre of 2,3,6-trichlorobenzoic acid.

9. The discharge shall not contain more than 30 micrograms per litre of total pesticides.

10. The discharge shall contain no visible oil or grease.

11. The discharge shall at no time contain any matter which will cause the receiving waters to be poisonous or injurious to fish, the spawn of fish or the food of fish.

12. When tested in accordance with the current OECD Guidelines for Testing of Chemicals 203 "Fish, Acute Toxicity Test" the effluent shall have a median lethal concentration in 96 hours (96 hour LC_{50}) of not less than 50%.

13. The effluent shall not contain any matter such that in a solution when tested in a bioassay test as provided in the Annex attached hereto and titled "Plant Bioassay Test for Unknown Herbicide" causes any deleterious effect as described in the said Annex.

14. A continuous recorder shall be provided and maintained to enable the volume and rate of the trade effluent and/or sewage effluent discharge to be measured.

15. Records of the volume and rate of the trade effluent and/or sewage effluent discharge shall be maintained by the Company and these records shall be kept conveniently available to representatives of the Authority for inspection at all reasonable times.

16 The discharge shall be made through an outlet to the watercourse constructed and maintained so that a direct sample of the discharge may be readily obtained.

This consent will not be revoked nor will the conditions thereof be modified without the written agreement of the person making the discharge before the expiration of the period ending with the last day of November 1992.

National Rivers Authority
Anglian Region
Kingfisher House
Goldhay Way
Orton Goldhay
Peterborough
PE2 0ZR

Dated: 3rd December 1990

J. A. Tetlow
Regional Manager
Environment and Fisheries

Attention is drawn to the notes overleaf.

STANDARD CONSENT CONDITIONS
OTHER CONDITIONS FOR DISCHARGES TO WATERCOURSES

W3 The discharge shall consist solely of treated trade effluent from **********.

W5 The volume discharged shall not exceed <$$$> cubic metres in any period of 24 hours.

W6 The rate of discharge shall not exceed <$$$> cubic metres per hour.

W11 The discharge shall not contain more than <$$$> milligrams per litre of <$$$>

W12 The discharge shall not contain more than <$$$> micrograms per litre of <$$$>

W13 The discharge shall not contain <$$$>

W14 The effluent from the treatment plant shall be discharged to an inspection chamber, fitted with a removable cover, before any surface water connection.

W20 An alarm system shall be provided and maintained to provide a warning of pumping station failure or breakdown.

W21 A continuous recorder shall be provided and maintained at the sample point to enable the volume and rate of the discharge to be measured.

W22 On every day that an effluent has been discharged (including at weekends and public holidays) a sample of that effluent shall be taken by the Company and kept available by them for collection by the Authority during the following day but if not collected in that time it may (subject to Condition ** below) be discarded.

W23 The company shall, in addition to any analysis which may be carried out by the Authority, analyse a weekly flow related sample of the effluent on a monthly basis in respect of pH and all the substances in Condition ** of this Consent, and shall determine the toxicity of a weekly flow related composite sample as described in Condition ** of this Consent, on a quarterly basis.

W24 Records of the volume, nature and composition of the discharge shall be maintained by the Company and these records shall be kept conveniently available to representatives of the Authority for inspection at all reasonable times.

W25 The Company shall supply the Authority with copies of the aforementioned records upon request and shall notify the Authority in writing within 14 days of the end of every quarter of each calendar year whether any of the limits specified in this Consent have been exceeded and if so of the steps which have been taken or are proposed to be taken in consequence.

W26 The toxicity of the effluent discharged (as measured by procedures conforming to a protocol of the Ministry of Agriculture, Fisheries and Food No. AEP2) to the brown shrimp, *Crangon crangon* as expressed by its 24 hour LC50 value shall not exceed 10 per cent effluent.

W27 The Company shall forthwith notify the Authority in writing if any change in the manufacturing processes and /or raw materials occurs which might increase or introduce into the effluent any substance specified in the EEC Dangerous Substances Directive List I or List II (EEC Directive 76/464).

NRA WELSH REGION

WATER ACT 1989

Continuation sheet

Conditions of Consent

Conditions prescribed for the discharge of treated trade effluent from X into the River Y Estuary from a new 250 mm diameter outlet at National Grid Reference Z.

1. The treatment plant shall be maintained in optimum operational condition at all times.

2. The maximum rate of effluent discharge shall not exceed 135 litres/sec.

3. The maximum volume of effluent discharge in any consecutive period of 24 hours shall not exceed 8000 cubic metres.

4. The quality of the effluent at all flow rates up to the maximum quoted in condition 2 shall not exceed the following limits:

   i) Total suspended solids shall not exceed 250 milligrams per litre.

   ii) The five day Biochemical Oxygen Demand (BOD ATU) determined after the suppression of nitrification using allyl thiourea at 20 °C shall not exceed 200 milligrams of oxygen per litre of sample.

   iii) The pH value of the treated effluent shall not be less than 6 nor greater than 10 pH units.

5. The MICROTOX test EC50 (15 minute) value shall be equal to or greater than 10% for effluent flows up to 4840 cubic metres per day and equal to or greater than 20% for flows from 4840 to 8000 cubic metres per day.

6. Facilities satisfactory to the Authority shall be provided to enable representative samples of the treated effluent passing to the estuary to be conveniently collected by any authorised officer of the Authority, at any time.