# Ecotoxicological Methods: Improving the Assessments of Complex Effluents – Assessment of Sub-Lethal Fish Toxicity Test Methods

**R&D Technical Report P2-202/TR1**

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**Statement of use**

This report is one of a series that provides recommendations for appropriate ecotoxicity test methods that may be used to improve the assessment and control of whole effluents. The series is intended to advise the future of the DTA approach following implementation, in response to the need to incorporate long-term, sub-lethal effects on organisms in addition to acute toxicity. The series comprises reports that recommend new, adapted or refined methods and the reasoning behind the recommendations for algae, invertebrates and fish. This individual report deals with the selection of test methods for determining sub-lethal effects of effluents in marine and freshwater fish. This work will be implemented via a further piece of R&D designed to trial the two recommended ecotoxicity tests for their suitability for use in effluent control.

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# EXECUTIVE SUMMARY

Following the conduct of a joint regulator/industry funded Direct Toxicity Assessment (DTA) Demonstration Programme at three case study locations Technical Guidance has been developed for its application to scenarios where there is evidence of biological damage in the receiving water which is perceived to be due to acute toxicity (UKWIR 2000). The Technical Guidance document describes the approaches to be adopted and the battery of acute (algal, invertebrate and fish) toxicity test procedures which should be used when applying a DTA approach at locations, sub-catchments or catchments where the problems are due to the presence of chemicals at concentrations sufficient to cause short-term largely lethal effects on resident populations and communities. However, further guidance is currently being developed for the application of DTA to other scenarios. This will require the use of a battery of test methods from different trophic levels that are able to address more subtle water quality issues through the measurement of general sub-lethal toxic endpoints (such as growth and reproduction) and specific mechanisms of toxicity (such as endocrine disruption).

The overall objective of Sub-Project 2 of Project P2-202 described in this report is to select and develop long-term (chronic) sub-lethal freshwater and marine fish tests for effluent control and receiving water monitoring. The specific objectives of **Phase 1** of Sub-Project 2 are to:

- critically and concisely review available freshwater and marine fish tests (with a preference for those with existing guidelines) for assessing long-term (chronic) sub-lethal toxicity of effluents and receiving waters to fish. The review needs to consider alternative approaches for assessing fish ecotoxicity in order to reduce the number of fish used or to replace whole organism tests.

- select the most appropriate developed tests for immediate use within the Environment Agency DTA effluent control initiative.

- produce a) supplementary notes to any existing internationally recognised guideline for application of the guidelines to effluent testing and receiving water assessment or b) a full method guideline where none exists.

- make recommendations for improvement of the tests in relation to key parameters (such as practicality, cost, suitability for the operational role and ethical considerations)

- identify tests which have the potential to be developed in the medium term into methods which will be more appropriate for the operational role in terms of their assessment against the key parameters.

The key question that needed to be addressed in the review is "*what are the most appropriate currently available chronic fish toxicity test methods for assessing effluent and receiving water toxicity?*". This includes both whole organism assays and also alternative approaches involving the use of fish cell lines or biosensors. Alternative validated approaches are being considered in the review because the Home Office is continually seeking approaches which will allow the use of fish testing under the Animals (Scientific Procedures) Act to be reduced.

The review has also addressed another important question, namely: "*what type of information is required from chronic fish tests?*". In this context, any potential method should ideally be able to provide information directly (or indirectly) on parameters which are important in defining population effects including growth, reproduction and lethality. Single or multi-generational life cycle tests can be considered to be the most robust mean of assessing the long-term toxicity of individual substances or complex mixtures to fish and providing data to establish long-term "safe" concentrations for fish populations. However, the logistical difficulties and cost constraints of conducting such tests for effluents, particularly species with long live cycles) mean that the practical reality is that tests assessing sub-lethal effects focus on particular elements of the life cycle which are deemed to be most sensitive to pollutants and thereby have the greatest bearing on the maintenance of populations. Therefore, methods are needed which can deliver ecologically relevant results indicative of potential life cycle effects, particularly those related to critical windows of sensitivity, in a cost-effective manner. Such methods include those using:

- early life stages of the animals (from fertilised eggs through to juveniles)
- adults during the reproductive period

These include a range of internationally recognised standardised sub-chronic and chronic fish toxicity test methods which have been promulgated by bodies such as the Organisation for Economic Cooperation and Development (OECD), International Standards Organisation (ISO) and the United States Environmental Protection Agency (US EPA). These methods vary in duration from 7 days to potentially 95 days depending on the organism used and its life history

If comparisons between different types of methods are to be unbiased and objective, rather than subjective, then it is necessary to judge each potential method against a series of criteria which are considered important if the method is to be 'fit for purpose'. The criteria which have been used to assess the appropriateness of methods for use in assessing effluent toxicity (and receiving water quality) were developed by the Steering Group and are divided into five categories:

1. relevance of test data
2. test method variability
3. previous application to effluent assessment
4. methodology
5. ethical and legal issues

The following conclusions have been drawn from the review of methods for assessing the chronic toxicity of effluents and receiving waters to fish:

- On the basis of the evaluation procedure used in this review the routine assessment of the toxicities of effluents and receiving waters could *currently* be carried out using either:
  - the 7 day embryo test using rainbow trout promulgated by Environment Canada as Method EPS1/RM/28 (Environment Canada 1998) for freshwaters. No comparable test

is available using turbot (the preferred species for acute toxicity testing in the DTA Methods Guidelines);

- the 7 day larval survival and growth test using freshwater (*Pimephales promelas*) or estuarine/marine (*Cyprinodon variegatus* or *Menidae beryllina*) species promulgated by the United States Environment Agency as Methods 1000.0, 1004.0 and 1006.0 (US EPA 1995a,b);

In the absence of comparative sensitivity and performance data the most appropriate method should ideally be determined by a conducting a limited evaluation exercise using a range of effluents types.

Reproduction tests should not be used for the routine assessment of effluent or receiving water toxicity but need to be reserved for situations where an evaluation of effects on reproduction is key to determining potential effects on fish populations, for example discharges from pharmaceutical plants which may release human or veterinary products.

In the *medium term* there are areas of research that could be pursued to address ethical concerns over the use of fish for testing. These include developing a combined 11 day embryo-larval survival, growth and teratogenicity test (based on the procedures promulgated by the United States Environmental Protection Agency as Methods 1000, 1001, 1004, 1005 and 1006) so that additional information is gained from each test. In this revised procedure it is recommended that fish are provided with appropriate food once the test organism reaches a life history stage where it is capable of free feeding.

In the *medium to long term* it is recommended that work is carried out to evaluate whether *in vitro* assays could be appropriate candidates for replacing acute fish toxicity tests in a screening role so that *in vivo* tests were used in a confirmatory role where necessary. However, further work is needed to develop a battery of appropriate systems covering different modes of toxic action that have the required level of sensitivity. At this time there is no immediate prospect of *in vitro* systems being used as surrogates for chronic tests with sub-lethal endpoints.

# KEY WORDS

Fish, embryos, larvae, juveniles, adults, toxicity tests, chronic, sub-chronic, sub-lethal, effluents, receiving waters

# 1. INTRODUCTION

## 1.1 Current regulatory approaches for effluent discharges

In England and Wales current regulatory practice for the control of effluent discharges to riverine, estuarine and marine receiving waters (under the Water Resources Act 1991[1] or the Pollution Prevention and Control (England and Wales) Regulations 2000, SI2000/1973 and other statutory legislation[2]) essentially relies on a substance-specific approach. Under this approach the levels (and loads) of indivdual substances which may be discharged from industrial plants (under IPPC authorisations) or sewage treatment works (under WRA discharge consents) are generally derived to satisfy Environmental Quality Standards (EQS) at defined points in the receiving water body and, thereby, protect the resident populations and communities.

Although this approach has led to improvements of water quality over the past decades there are a number of limitations which have to be addressed if these improvements are to continue: namely:

- the chemical-specific approach can only be applied to those substances which can be identified and quantified analytically and for which EQSs are available. At present there are only sound standards for approximately 100 (0.1%) of the 100000+ industrial substances which are listed on the European Inventory of New and Existing Chemical Substances (EINECS);

- the chemical-specific approach cannot account for interactive effects (such as antagonism or synergism) of the complex mixtures of substances (or breakdown products) frequently found in effluents .

As a result of these issues there has been increasing interest in the use of a biological-effects based approach for controlling and monitoring effluents and assessing receiving water quality as part of an integrated approach alongside analytical chemistry and biological survey techniques.

[1] Under Section 85 of the Water Resources Act (1991) it is an offence to cause or knowingly permit poisonous, noxious or polluting matter, or any solid waste matter, to enter controlled waters.

[2] The Integrated Pollution Prevention and Control (IPPC) Directive (EC/61/96) has been implemented into English and Welsh legislation under the Pollution Prevention and Control (England and Wales) Regulations 2000, SI2000/1973 and other statutory legislation and lays down measures designed to prevent, or where that is not practicable, to reduce emissions to air, land and water from these activities, including measures concerning waste. This approach is being taken in order to achieve a high level of protection of the environment taken as a whole. These conditions are based on the use of the Best Available Technology (BAT) which balances the costs to the operator against the benefits to the environment.

## 1.2 Application of a Direct Toxicity Assessment (DTA) approach to effluent control and monitoring

Following the conduct of a joint regulator/industry funded Direct Toxicity Assessment (DTA) Demonstration Programme at three case study locations[3] Technical Guidance has been developed for its application to scenarios where there is evidence of biological damage in the receiving water which is perceived to be due to acute toxicity (UKWIR 2000). The Technical Guidance document describes the approaches to be adopted and the battery of acute (algal, invertebrate and fish) toxicity test procedures which should be used when applying a DTA approach at locations, sub-catchments or catchments where the problems are due to the presence of chemicals at concentrations sufficient to cause short-term largely lethal effects on resident populations and communities. The document provides information on how to deal with the different requirements of a DTA approach; namely

- screening of effluent toxicity;
- characterisation of effluent toxicity;
- identification of the causes and sources of effluent toxicity using toxicity identification evaluations (TIEs) and toxicity source evaluations (TSEs);
- monitoring of effluent quality against a toxicity reduction target;
- assessment of receiving water quality.

A key requirement for the application of the DTA approach to control short-term, largely lethal effluent toxicity was a series of robust test method guidelines to detect acutely toxic effects. These procedures describe both internationally recognised standardised tests and modified high throughput versions of these tests using algae, invertebrates and fish (see Table 1.1) which can be applied to a range of operational roles The guidelines also indicate the level of quality assurance/quality control that has to be applied to the tests in different operational roles to ensure that the data generated is “fit for purpose”.

[3] The three case study locations used in the Direct Toxicity Assessment (DTA) Demonstration Programmes were the Rivers Aire and Esk and the Lower Tees Estuary. The studies involved testing a draft protocol that addressed the issues of prioritisation of locations based on chemical and biological survey information, screening of discharges for further action, characterisation of discharges of interest and development of a toxicity reduction plan for problem effluents including, where necessary, the identification of causes and sources of toxicity and monitoring against a toxicity reduction target as remedial action was taken.

**Table 1.1 Short-term largely lethal toxicity tests specified for use in a DTA approach**

| Freshwater | Estuarine/marine waters |
|---|---|
| 72h Algal (*Pseudokirchnereilla subcapitata*) growth inhibition test (based on OECD Guideline 201) | 72h Algal (*Skeletonema costatum*) growth inhibition test (based on ISO Standard 10253) |
| 48h *Daphnia magna* immobilisation test (based on OECD Guideline 202) | 24h Oyster (*Crassostrea gigas*) embryo-larval development test (based on ICES Standard No 11)<br>48h Marine copepod (*Tisbe battagliai*) lethality test (based on ISO Standard 14669) |
| 96h Rainbow trout (*Oncorhynchus mykiss*) lethality test (based on OECD Guideline 203) | 96h Turbot (*Scophthalmus maximus*) lethality test (based on ISO Standard ISO/WD 15990) |

Although Technical Guidance has been developed for scenarios where there is evidence of biological changes in the receiving water due to acute toxicity (UKWIR 2000) further guidance is currently being developed for the application of DTA to other scenarios. This will require the use of a battery of test methods from different trophic levels that are able to address more subtle water quality issues through the measurement of general sub-lethal toxic endpoints (such as growth and reproduction) and specific mechanisms of toxicity (such as endocrine disruption).

It should also be recognised that in the United States and Canada toxicity-based criteria using acute and (sub) chronic measures have been included in regulatory permits for effluent discharges for more than ten years and the approach is gaining wider acceptance in a number of European countries such as Germany, the Netherlands, Denmark and Sweden. Furthermore, as part of the activities of the OSPAR Point Group a workshop was recently held in the Netherlands to investigate the use of Whole Effluent Assessment (WEA) as a tool to improve the quality of Best Available Technology (BAT) decision making within IPPC. The discussions resulted in the general conclusion that "the objective of the OSPAR strategy with regard to hazardous substances will be served by operationalisation of Whole Effluent Assessment".

## 1.3 Objectives

### 1.3.1 Overall objectives

The overall objective of Project P2-202 is to identify and make available a battery of long-term (chronic) sub-lethal aquatic ecotoxicity tests suitable for application to the hazard assessment of complex effluents and receiving water samples for both freshwater and marine environments.

The overall objective of Sub-Project 2 of Project P2-202 is to select and develop long-term (chronic) sub-lethal freshwater and marine fish tests for effluent control and receiving water monitoring.

### 1.3.2 Specific objectives

The specific objectives of **Phase 1** of Sub-Project 2 are to:

- critically and concisely review available freshwater and marine fish tests (with a preference for those with existing guidelines) for assessing long-term (chronic) sub-lethal toxicity of effluents and receiving waters to fish. The review needs to consider alternative approaches for assessing fish ecotoxicity in order to reduce the number of fish used or to replace whole organism tests.

- select the most appropriate developed tests for immediate use within the Environment Agency DTA effluent control initiative.

- produce a) supplementary notes to any existing internationally recognised guideline for application of the guidelines to effluent testing and receiving water assessment or b) a full method guideline where none exists.

- make recommendations for improvement of the tests in relation to key parameters (such as practicality, cost, suitability for the operational role and ethical considerations)

- identify tests which have the potential to be developed in the medium term into methods which will be more appropriate for the operational role in terms of their assessment against the key parameters.

The specific objectives of **Phase 2** of Sub-Project 2 are to:

- develop and comparatively evaluate the improved test designs/methods identified in Phase 1 with the standard methods already in existence in order to validate the improvements in relation to the identified key parameters.

- produce new methods guidelines and/or supplementary notes to existing guidelines to incorporate these modifications.

The consideration of which test procedures are most appropriate for assessing the potential chronic effects to fish raises a number of issues which need to be addressed, namely:

- can existing standardised chronic methods (which were developed for testing of individual substances or formulations) be used directly or in a modified form to assess the long-term, sub-lethal toxicity of effluents?

- can the requirements for data on the long-term sub-lethal toxicity of effluents and receiving waters be integrated with existing guidelines for assessing short-term lethal toxicity?

- are there alternative (*in vitro*) methods available to assess the long-term, sub-lethal toxicity of effluents which do not use whole organisms, but which are predictive of *in vivo* responses?

- how is the potential instability of effluent samples be addressed by the procedures detailed in existing or modified chronic whole organism or alternative methods?

These issues are considered in the following sections of the report and their resolution in the project will be key for the identification of appropriate tests for assessing effluent toxicity to fish.

# 2. REVIEW OF SUB-CHRONIC AND CHRONIC FISH TOXICITY TEST METHODS AND ALTERNATIVE APPROACHES

## 2.1 Scope of the review

The key question that needs to be addressed in the review is "*what are the most appropriate currently available chronic fish toxicity test methods for assessing effluent and receiving water toxicity?*" based on a consideration of data against defined criteria. This includes both whole organism assays and also alternative approaches involving the use of fish cell lines or biosensors. The tests identified clearly need to be fit for purpose but alternative validated[4] approaches are being considered in the review because the Home Office is continually seeking approaches which will allow the use of fish testing under the Animals (Scientific Procedures) Act to be reduced. This approach is consistent with the Organisation for Economic Development (OECD) strategy for reduction, replacement and refinement concerning the use of fish (and mammals) in toxicological studies as initially defined by Russell and Birch in 1959 and re-iterated in a number of international fora. It should also be remembered that the Direct Toxicity Assessment (DTA) Methods Working group recommended that within a framework to assess and control the toxicity of effluents, fish should only be used where they are likely to be the most sensitive species given the composition of the discharge.

However before addressing this issue it is necessary to consider another important question, namely: "*what type of information is required from chronic fish tests?*".

Ideally any potential method should be able to provide information directly (or indirectly) on parameters which are important in defining potential population level effects including growth, reproduction and lethality. Single or multi-generational life cycle tests can be considered to be the most robust mean of assessing the long-term toxicity of individual substances or complex mixtures to fish and providing data to establish long-term "safe" concentrations for fish populations.

A life cycle test demands a minimum laboratory exposure of the animal from 'embryo to embryo' which for many animals especially fish requires a minimum of 6-12 months of concentrated effort. To ensure that all life stages and life processes are exposed, the test typically begins with embryos or newly hatched young fish less than 8 h old, continues through maturation and reproduction, and ends not less than 28 days (or 60 days for salmonids) after hatching of the next generation. For the fathead minnow (*Pimephales promelas*) 12 months are required for a life cycle test while for brook trout (*Salvelinus fontinalis*) a life cycle test has a duration of thirty months.

[4] The validation of the techniques should be based on the principles of the OECD Workshop on Harmonisation of Validation and Acceptance Criteria for Alternative Toxicological Test methods held in Solna, Sweden in January 1996 (OECD 1996)

However, the logistical difficulties and cost constraints of conducting such tests for effluents, particularly species with long life cycles, mean that the practical reality is that tests assessing sub-lethal effects focus on particular elements of the life cycle which are deemed to be most sensitive to pollutants and thereby have the greatest bearing on the maintenance of populations. Therefore, methods are needed which can deliver ecologically relevant results indicative of potential life cycle effects, particularly those related to critical windows of sensitivity, in a cost-effective manner. Such methods include those using early life stages of the animals (from fertilised eggs through to juveniles) and adults during the reproductive period

In the following sections currently available standardised methods or those under development (using different life stages) are reviewed. It needs to be recognised that different endpoints will be of greater or lesser importance depending on the life stage of the organism. For example whilst lethality may be a sensitive endpoint of exposure during the early life stages of a fish associated with hatchlings and yolk-sac fry stage, this may not be the case during the juvenile life stage.

## 2.2 Internationally accepted and standardised sub-chronic and chronic fish toxicity test procedures

At present there are a range of internationally recognised standardised sub-chronic and chronic fish toxicity test methods which have been promulgated by bodies such as the Organisation for Economic Cooperation and Development (OECD), International Standards Organisation (ISO) and the United States Environmental Protection Agency (US EPA). These methods (see Table 2.1) vary in duration from 7 days to potentially 95 days depending on the organism used and its life history (particularly the water temperatures that the organism tolerates and the resulting growth rate).

### 2.2.1 Tests with fish early life stages

In early life stage (ELS) tests, fish of a given species are exposed for $\leq$95 days from ova fertilisation through embryonic, larval and early juvenile development. The major toxic effects typically measured are those for growth and mortality. Early life stages are sensitive parts of the life cycle of a fish because of the many critical events that take place in this short span of the organism. For example in the fathead minnow in a period of 7-8 days from fertilisation through hatching to the free feeding stage, the fish goes from two cells to a swimming, feeding and functional organism with well developed organ systems. If the organism is exposed to pollutants during this period, changes in the timing of these developmental events can have marked consequences for their immediate to longer-term fitness and survival. Indeed it is the rapidity of growth and morphological changes during early fish development that is the key factor that make early life stages valuable for toxicity testing. Different early life stages can vary in their sensitivity to different toxicants (Mayer *et al.*, 1986; Kristensen, 1990). Therefore, it is preferable to monitor effects of continuous toxicant exposure on several early life stages (and a variety of endpoints), and during the transition from one stage to the next, to obtain a good estimate of a sub-lethally safe concentration

**Table 2.1 Standardised chronic fish toxicity test procedures using early life stages of fish**

| Body | Guideline number | Description (Date) | Recommended test species (Life stage at the start of the test) | Duration (days) | Endpoint |
|---|---|---|---|---|---|
| OECD | 210 | Fish, Early-life Stage Toxicity Test (1992) | *Danio rerio*, *Oncorhynchus mykiss*, *Oryzias latipes*, *Pimephales promelas* and *Cyprinodon variegatus* (Newly fertilised eggs) | ≤ 95 | Hatching success, Juvenile lethality and growth |
| | 212 | Fish, Short-term Toxicity Test on embryo and sac-fry stages - Draft (1997) | *Cyprinus carpio*, *Danio rerio, Oncorhynchus mykiss*, *Oryzias latipes* and *Pimephales promelas* (Embryos) | ≤ 55 | Hatching success, juvenile lethality |
| ISO | 12890 | Water quality - Determination of embryo-larval toxicity to freshwater fish - Semi-static method (1997) | *Danio rerio* (Embryos) | 10 | Hatching success, Juvenile lethality |
| US EPA | 1000.0 | Fathead minnow larval survival and growth effluent toxicity test (1995) | *Pimephales promelas* (Larvae) | 7 | Larval survival and growth |
| | 1001.0 | Fathead minnow embryo-larval survival and teratogenicity effluent toxicity test (1995) | *Pimephales promelas* (Embryos) | 7-9 | Hatching success and abnormalities Larval survival |
| | 1004.0 | Sheepshead minnow larval survival and growth effluent toxicity test (1995) | *Cyprinodon variegatus* (Larvae) | 7 | Larval survival and growth |
| | 1005.0 | Sheepshead minnow embryo-larval survival and teratogenicity effluent toxicity test (1995) | *Cyprinodon variegatus* (Embryos) | 7-9 | Hatching success and abnormalities Larval survival |
| | 1006.0 | Inland silverside larval survival and growth effluent toxicity test (1995) | *Menidia beryllina* (Larvae) | 7 | Larval survival and growth |
| Environment Canada | EPS1/RM/22 | Fathead minnow larval growth and survival test (1992) | *Pimephales promelas* (Larvae) | 7 | Larval survival and growth |
| | EPS1/RM/28 | Salmonid (rainbow trout) early life stage test (1998) | *Oncorhynchus mykiss* (Newly fertilised eggs) | 7 - ~70 | Larval development and survival |

A number of freshwater fish species and several saltwater species have been used in ELS toxicity tests (McKim 1984 for review) and currently available standardised ELS procedures for salmonid and cyprinid species are described in the following sections.

### A) Organisation for Economic Cooperation and Development (OECD) procedures

The OECD has promulgated three procedures using early life stages of fish (see Table 2.1) which use salmonids and/or cyprinid species.

In ***OECD Test Guideline 210*** (Fish, Early-life stage toxicity test) early life stages of fish are exposed to the test substance preferably under flow-through conditions or, for less degradable or volatile materials, semi-static conditions (OECD 1992). The test is initiated by placing fertilised eggs (preferably before cleavage of the blastodisc commences) in the test chambers and is continued at least until all the control fish are free feeding (see Table B1 for full details of the method including test validity criteria). In the test the effects of chemicals are measured using the following parameters: cumulative mortality, numbers of healthy larvae at the end of the test, the time to the start and end of hatching, numbers of larvae hatching each day, the length and weight of surviving animals at the end of the test and the numbers of larvae that are deformed, are of abnormal appearance or exhibit abnormal behaviour.

The test can be carried out with a range of freshwater and saltwater species, those recommended being rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), fathead minnow (*Pimephales promelas*), japanese medaka (*Oryzias latipes*), zebrafish (*Danio rerio*) and the sheepshead minnow (*Cyprinodon variegatus*). However, the procedure can also be used with other species can such as brown trout, lake trout atlantic salmon, coho salmon, chinnok salmon, northern pike, white sucker, bluegill sunfish, channel catfish, flagfish, three spined stickleback, common carp, atlantic silverside and tidewater silverside. The test duration depends on the species and Table 2.2 provides information on durations for recommended species and ranges from 30 – 60 days post hatch.

**Table 2.2 Summary of the duration of early life stage tests with different species**

| Species | Test temperature ($^{o}$C) | Typical duration of test |
|---|---|---|
| Rainbow trout | 10 $\pm$ 2 for embryos<br>12 $\pm$ 2 for larvae and juveniles | 2 weeks after controls are free-feeding (or 60 days post-hatch) |
| Fathead minnow | 25 $\pm$ 2 | 32 days from start of test (or 28 days post-hatch) |
| Japanese medaka | 24 $\pm$ 1 for embryos<br>23 $\pm$ 2 for larvae and juveniles | 30 days post-hatch |
| Zebrafish | 25 $\pm$ 2 | 30 days post –hatch |
| Sheepshead minnow | 25 $\pm$ 2 | 32 days from start of test (or 28 days post-hatch) |

In the ***proposed OECD Test Guideline 212*** (Fish, Short-term toxicity test on Embryo and Sac-fry stages) life stages from the newly fertilised egg to the end of the sac-fry stage are exposed (OECD 1996). No feeding is provided in the egg and sac-fry test and studies are terminated while the sac fry are still nourished from the yolk sac on the basis that mortalities due to starvation do not occur in the controls (see Table B2 for full details of the method including test validity criteria). In the test the effects of chemicals are measured using the

following parameters: cumulative mortality, numbers of healthy larvae at the end of the test, the time to the start and end of hatching, numbers of larvae hatching each day, the length and weight of surviving animals at the end of the test and the numbers of larvae that are deformed, are of abnormal appearance or exhibit abnormal behaviour.

The recommended test species are rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), fathead minnow (*Pimephales promelas*), japanese medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*), though a number of other freshwater fish (bluegill sunfish and goldfish) and saltwater fish (cod, herring, inland silverside and sheepshead minnow) could also be used. The duration of the test for the different recommended species is summarised in Table 2.3 and ranges from 8-9 days to 50-55 days.

**Table 2.3 Summary of the duration of embryo and sac-fry tests with different species**

| Species | Test temperature ($^{o}$C) | Typical duration of test |
|---|---|---|
| Rainbow trout | 10 $\pm$ 1 for embryos<br>12 $\pm$ 1 for larvae and juveniles | As soon as possible after fertilisation (early gastrula stage) to 20 days post-hatch (50-55 days) |
| Common carp | 21 - 25 | As soon as possible after fertilisation (early gastrula stage) to 4 days post-hatch (8-9 days) |
| Fathead minnow | 25 $\pm$ 2 | As soon as possible after fertilisation (early gastrula stage) to 4 days post-hatch (8-9 days) |
| Japanese medaka | 24 $\pm$ 1 for embryos<br>23 $\pm$ 2 for larvae and juveniles | As soon as possible after fertilisation (early gastrula stage) to 5 days post-hatch (13-16 days) |
| Zebrafish | 25 $\pm$ 2 | As soon as possible after fertilisation (early gastrula stage) to 5 days post-hatch (8-10 days) |

It is recognised that the embryo and sac-fry test would be less sensitive than the full Early-life stage test, particularly with respect to chemicals with high lipophilicity (log Kow > 4) and chemicals with a specific mode of action (for example acetylcholinesterase inhibitors or respiratory inhibitors). However, smaller differences between the two tests would be expected for chemicals with non-specific, narcotic modes of action (Kristensen 1990). Clearly for effluents, the relative sensitivity of the two methods would depend on the constituents present.

**B) International Standards Organisation (ISO) Procedures**

***Draft International Standards Organisation Method 12890*** (Water quality – determination of embryo-larval toxicity to freshwater fish, semi-static method) is designed to assess the toxicity of chemicals, waters and wastewaters (under specified conditions) for embryos and early larval development stages of the freshwater zebrafish (*Danio rerio*) (ISO 1997). Newly fertilised eggs are exposed to the sample under a semi-static renewal procedure and hatched eggs or larvae are recorded daily. The standard period of exposure for the test is 10 days, but the test duration may be prolonged up to 14 days in order to increase test sensitivity. ISO Draft Standard 12890 is consistent with the procedures for zebrafish in OECD Test Guideline 212 with only minor modifications (see Table B3 for full details of the method including test validity criteria).

During 1984-1985 the INSTA (Internordic Standardization Commission) conducted a preliminary inter-calibration of this method whereby two substances, potassium dichromate ($K_2Cr_2O_7$) and zinc sulphate ($ZnSO_4.2H_2O$) were tested on two occasions at five laboratories in the Nordic countries. The results of the inter-calibration were evaluated according to ISO 5725 (Dave *et al* 1987).

The variation in the logarithmic no effect concentration (NEC) for ***survival*** expressed as coefficient of variation was <10% for intra-laboratory variation (repeatability) for both potassium dichromate and zinc. Inter-laboratory variability (reproducibility) ranged from < 15% (for zinc sulphate) to < 25% (for potassium dichromate).

For the ***hatching*** endpoint only zinc sulphate caused effects at the concentrations tested and hatching was inhibited at lower concentrations than those resulting in shorter times of survival. The variation in the logarithmic no effect concentration (NEC) for hatching expressed as coefficient of variation for zinc sulphate data was approximately 75% for intra-laboratory variation (repeatability) and approximately 100% for inter-laboratory variability (reproducibility).

**C) United States Environmental Protection Agency (US EPA) Procedures**

The United States Environmental Protection Agency have been able to develop a series of short-term sub-chronic toxicity methods of 7-9 days duration utilising freshwater and marine fish (and algal, macrophyte and invertebrate) species to assess effluent and receiving water toxicity as part of the National Pollutant Discharge Elimination System (US EPA 1991). The fish species used are the fathead minnow (*Pimephales promelas*), the sheepshead minnow (*Cyprinodon variegatus*) and the inland silverside (*Menidia beryllina*) and the measured endpoints include embryo-larval survival, larval hatching success, abnormalities and growth (see Table 2.1 and Tables B4 – B8 for full details of the methods including test validity criteria). Statistical analysis of the power of the endpoints in the fathead minnow and inland silverside larval survival and growth tests has been carried out by US EPA Region 9 to determine the minimum detectable differences for the tests. It was concluded from the analyses that the test designs commonly adopted detect differences from the controls of > 15% using hypothesis testing or point estimate methods.

In 1998, as part of a settlement agreement (US EPA vs Edison Electric Institute *et al*, Settlement Agreement, July 24, 1998) to resolve a judicial challenge to the use of these tests in the NPDES programme, the US EPA conducted an inter-laboratory variability study of 12 US EPA short-term acute and chronic whole effluent toxicity test methods (the WET Variability Study). The purpose of the WET Variability study (US EPA 2001) was to characterise:

- inter-laboratory variability
- the rate of successful test completion
- the rate of false positive incidence

The tests of relevance to this review were:

1. the fathead minnow (*Pimephales promelas*) larval survival and growth test (Table B4 )

2. the sheepshead minnow (*Cyprinodon variegatus*) larval survival and growth test (Table B6)

3. the inland silverside (*Menidia beryllina*) larval survival and growth test (Table B8 )

For these tests the US EPA selected a minimum of 7 and a maximum of 20 participant laboratories to constitute a "base" study design. Additional volunteer or externally sponsored laboratories participated on a more limited basis as part of an extended study design. Each participant laboratory was required to prequalify for the study by documenting that their capacity and capabilities, experience and proficiency, and quality assurance and quality control systems met the needs of the study.

Table 2.4 summarises the results from the relevant studies from the US EPA WET Variability Study. It is evident that for the three sub-chronic fish tests of interest there was and extremely high completion rate ($\geq$ 98%) and a low false positive rate ($\leq$ 4.4%).

**Table 2.4 Summary of the results from the US EPA WET Variability Study**

| Test method | Number of tests (and laboratories) | Successful completion rate (%) | False positive rate (%)[a] |
|---|---|---|---|
| Fathead minnow larval survival and growth rate | 101 (27) | 98 | 4.4 |
| Sheepshead minnow larval survival and growth rate | 28 (7) | 100 | 0 |
| Inland silverside larval survival and growth rate | 36 (9) | 100 | 0 |

**Key:** a - False positive rates reported for each method represent the higher of false positive rates observed for hypothesie testing results or point estimates

Table 2.5 summarises the coefficients of variation for intra- (within) and inter (between)-laboratory variability measured in the study for the tests of interest. Intra-laboratory variability was < 15% for all the tests where data was available. However, while inter-laboratory variability was low (< 15%) for the fathead minnow larval survival and growth endpoints it was higher (40 – 45%) for these endpoints in tests with inland silverside.

**Table 2.5 Summary of the coefficient of variation data for the fathead minnow, sheepshead minnow and inland silverside larval survival and growth tests**

| Test type | Sample type | Coefficient of variation (CV) % | | | | | |
|---|---|---|---|---|---|---|---|
| | | Survival $LC_{50}$ values | | | Growth $IC_{25}$ values | | |
| | | Within laboratory | Between laboratory | Total | Within laboratory | Between laboratory | Total |
| Fathead minnow larval survival and growth test | Effluent | 9.2 | 12.0 | 15.1 | 19.1 | 12.9 | 23.1 |
| | Receiving water | - | - | 12.6 | - | - | 19.8 |
| | Reference toxicant | 6.6 | 10.6 | 12.5 | 10.0 | 17.2 | 19.9 |
| | **Average** | **7.9** | **11.3** | **13.4** | **14.6** | **15.0** | **20.9** |
| Sheepshead minnow larval survival and growth test | Effluent | - | - | 2.3 | - | - | 6.1 |
| | Receiving water | - | - | 16 | - | - | 7.2 |
| | Reference toxicant | - | - | 22.2 | - | - | 18.4 |
| | **Average** | **-** | **-** | **8.7** | **-** | **-** | **10.5** |
| Inland silverside larval survival and growth test | Effluent | 12.2 | 46.8 | 48.4 | 7.2 | 55.5 | 56.0 |
| | Receiving water | - | - | 40.0 | - | - | 39.1 |
| | Reference toxicant | 9.2 | 32.2 | 33.5 | 22.0 | 29.1 | 36.4 |
| | **Average** | **10.7** | **39.5** | **40.6** | **14.6** | **42.3** | **43.8** |

**Notes:**

Within and between laboratory components of variability were not calculated for this method since no within laboratory replication was provided

For test methods and sample types that included within-laboratory replication (that is multiple tests on the same sample type from a given laboratory) the PROC MIXED procedure in SAS Version 8 (SAS Institute 2000) estimated the within laboratory, between laboratory and total variance components

## D) Environment Canada Procedures

The US EPA ***Fathead minnow larval survival and growth test (1000.0)*** has also been adopted by Environment Canada (EPS1/RM/22) for use in the Environmental Effects Monitoring Programs for pulp and paper mill and mine effluents (Scroggins *et al* 2002a,b). Sub-lethal fish toxicity tests have been used to measure the quality of pulp and paper mill effluents since 1992. The Aquatic Effects Technology Evaluation for mine effluents is a joint government-industry programme to evaluate the cost-effectiveness of technologies for the assessment of mining related impacts in the aquatic environment (Natural Resources Canada, 1997)(see Table B9 for full details of the method including test validity criteria).

Environment Canada (1998) have promulgated a toxicity test using ***early life stages of rainbow trout*** (*Oncorhynchus mykiss*) which starts at the onset of embryo development and measures the development and survival of early life stages ***(EPS1/RM/28)***. Three test options using a semi-static or flow-through procedure are described (see Table B10 for full details of the methods and Table 2.6 for a summary of the duration of the different tests and the endpoints measured):

- an embryo (E) test for frequent or routine monitoring, which ends seven days after fertilisation. This test is based on an early embryo test with rainbow trout (Birge *et al* 1985, Birge and Black 1990);

- an embryo/alevin (EA) test for measuring effects on multiple phases of development which is terminated seven days after half the alevins are seen to have hatched in the control;

- an embryo/alevin/fry (EAF) test for more definitive investigations which ends 30 days after half the surviving fry in the control show swim-up behaviour.

**Table 2.6 Summary of the duration of the different tests and the endpoints measured**

| Type of test | Termination of test after fertilisation (days) | Observations |
|---|---|---|
| Embryo test | 7 | Proportion on non-viable embryos at the end of the test |
| Embryo/alevin test | 27 - 29 | Proportion on non-viable embryos at the end of the test<br>Narrative statements on delayed hatching and deformed alevins |
| Embryo/alevin/fry test | ~70 | Proportion on non-viable individuals at swim-up<br>Mortality of fry during final 30 days<br>Average dry weight of surviving fry at end of test<br>Narrative statements on delayed hatching, deformed alevins, delayed swim-up and abnormal behaviour of fry |

Selection of the most suitable test option will depend on the objectives of the test and on the physico-chemical characteristics of the substance being tested. The procedures can be used to

assess the toxicity of chemicals, effluents, elutriates, leachates or receiving waters. The 7 day embryo test has been used in Canada to assess the toxicity of pulp and paper mill and mine effluents as part of Environmental Effects Monitoring Programmes (Scroggins *et al* 2002a,b)

**E) Comparison of data from early life stage tests with full life cycle tests**

Evaluation of the usefulness of ELS toxicity tests with fish has been made possible by the large database from life cycle toxicity tests generated in the 1970s. At that time several investigators showed that the ELS portions of life cycles were the most sensitive to single toxicants. However, acceptance of the ELS toxicity test approach and its widespread use only came after comprehensive reviews of all existing data from freshwater life cycle toxicity tests (Macek and Sleight 1977, McKim 1977). Table 2.7 provides a summary of the data presented in McKim (1984) and these confirm that for species such as brook trout and fathead minnow exposed to a range of substances (with differing modes of toxic action) and sewage effluents the embryo-larval, early juvenile portion of the life cycle is the most, or one of the most sensitive in the life cycle of these species. This is evident from the fact that the MATC values for the embryo-larval tests are the same as for the whole life cycle test. These data are typical of those for a range of other freshwater and saltwater species.

Birge *et al.* (1985) showed that, for the substances evaluated, short-term embryo-larval tests with rainbow trout were more sensitive than similar tests using fathead minnows or bluegill sunfish.

### 2.2.2 Tests with juvenile fish

The ***proposed OECD Test Guideline 215*** (Fish, Juvenile Growth Test) assesses the effects of prolonged exposure ($\geq$ 28 days) to chemicals on the growth of juvenile rainbow trout (*Oncorhynchus mykiss*), though japanese medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) may be used (OECD 1997)(see Table 2.8 and Table B11 for full details of the method including test validity criteria). The weights of fish maintained under flow through or, for less degradable or volatile materials, semi-static conditions, are measured at the beginning ($t_1$ and end $t_2$) of the test and the specific growth rate is calculated in the interval between $t_1$ and $t_2$.

The proposed OECD Test Guideline is similar to ***International Standards Organisation (ISO) Method 10229***: Water quality – Determination of the prolonged toxicity to substances to freshwater fish – Method for evaluating the effects of substances on the growth rate of rainbow trout [*Oncorhynchus mykiss* Walbaum (Teleostei, Salmonidae)](ISO 1994). There are only minor procedural differences between the OECD method and the ISO method (see Table 2.8 and Table B 12 for full details of the method), but importantly the ISO method specifies testing of wastewaters as an option.

The results of a European ring test using two test substances (3,4-dichloroaniline and linear alkylbenzenesulphonate) yielded estimates of inter-laboratory variability (as coefficients of variation) of 29% and 31% respectively (Ashley *et al* 1990).

Work at AstraZeneca has been carried out on developing a short-term growth test using the turbot (*Scophthalmus maximus*)(see Table B13 for full details of the method). The 14 day test is initiated with animals <1g wet weight and the endpoints measured are growth (as standard length and wet weight) and survival.

**Table 2.7 Results of partial and complete life cycle toxicity tests with fish including the MATC established by each life cycle test and an MATC estimated by embryo, larval and early juvenile exposures conducted as part of each life cycle test**

| Toxicant | Species | Type of life cycle test | Critical life stage endpoints | MATC for full life cycle tests ($\mu g\ l^{-1}$) | Estimated MATC for embryo-larval tests ($\mu g\ l^{-1}$) | Duration of embryo- larval test | Reference |
|---|---|---|---|---|---|---|---|
| Cadmium | Brook trout | C | ELEJ-G | 1.7 – 3.4 | 1.7 – 3.4 | 90 d | Benoit *et al* (1976) |
| | Fathead minnow | P | ELEJ-J | 37 - 57 | 37 - 57 | 90 d | Pickering and Gast (1972) |
| Copper | Brook trout | C | ELEJ-G | 9.5 – 17.4 | 9.5 – 17.4 | 90 d | McKim and Benoit (1974) |
| | Fathead minnow | P | ELEJ-M, A-S | 10.6 – 18.4 | 10.6 – 18.4 | 120 d | Mount and Stephan (1969) |
| Chromium | Brook trout | C | ELEJ-M | 200 - 350 | 200 - 350 | 90 d | Benoit (1976) |
| | Fathead minnow | P | ELEJ-M, G | 1000 - 3950 | 1000 - 3950 | 30 d | Pickering (Pers comm) |
| Zinc | Brook trout | P | ELEJ-F | 532 - 1368 | 532 - 1368 | 90 d | Holcombe *et al* (1979) |
| | Fathead minnow | C | ELEJ-F | 78 - 145 | 78 - 145 | 90 d | Benoit and Holcombe (1978) |
| Methyl-mercury | Brook trout | C | ELEJ-M, G | 0.29 – 0.93 | 0.29 – 0.93 | 90 d | McKim *et al* (1976) |
| | Fathead minnow | C | A-M, S | 0.07 – 0.13 | 0.07 – 0.13 | 60 d | Mount and Olson (Pers comm) |
| Diazinon | Brook trout | P | ELEJ-G | <0.8 | <0.8 | 120 d | Allison and Hermanutz (1977) |
| | Fathead minnow | P | ELEJ-M, G | 6.8 – 13.5 | 6.8 – 13.5 | 60 d | Allison and Hermanutz (1977) |
| Toxaphene | Brook trout | P | ELEJ-M, G | <0.039 | <0.039 | 60 d | Mayer *et al* (1975) |
| | Fathead minnow | C | ELEJ-G | 0.025 – 0.054 | 0.025 – 0.054 | 30 d | Mayer *et al* (1975) |
| Sewages | | | | | | | |
| Nondisinfected | Fathead minnow | C | ELEJ-M, G | 25 – 50 % | 25 – 50 % | 30 d | Ward *et al* (1976) |
| Chlorinated | Fathead minnow | C | ELEJ-G | 7 – 14 % | 7 – 14 % | 30 d | Ward *et al* (1976) |
| Dechlorinated | Fathead minnow | C | ELEJ-M | 50 – 100 % | 50 – 100 % | 30 d | Ward *et al* (1976) |

Key: A – Adult, M – Mortality, G – Growth, F – egg fragility, H – Hatchability, D – Deformities, S - Spawning

### 2.2.3 Tests with adult fish

Existing fish toxicity tests, with the exception of the full life-cycle test are not adequate for assessing the reproductive effects of chemicals. In response to the requirement to assess the effects of endocrine disrupting chemicals on fish reproduction, Harries *et al* (2000) described the development of a short-term (6 week) paired breeding test using fathead minnows (*Pimephales promelas*) (see Table 2.9 and Table B14 for full details of the method). In the test, reproductive performance in paired fish is assessed over two 3 week periods, one with exposure to the test substance and one with exposure to 'clean water'. Four breeding pairs (two tanks of fish, each containing two breeding pairs that were separated by a perforate stainless) for each treatment. Only data from pairs of fish that continued to spawn throughout the full 3 week pre-exposure are subsequently used for analysis of reproductive performance.

The test is considered to be highly integrative and measures effects on fecundity (and other factors). The measurements of reproductive performance include number of spawnings, fecundity as egg number and egg size. Each day, spawning events are recorded and the eggs collected. The eggs are counted and the diameter of 20 eggs are measured from each spawning under a low power dissecting microscope fitted with an eyepiece graticule. However, the test requires large volumes of test material and, therefore, it is questionable whether the test could easily be routinely conducted for assessing effluent or receiving water toxicity.

**Comparison of adult reproduction data with full life cycle data**

Suter *et al.* (1987) pointed out that fecundity of adults (i.e., the number of viable eggs produced per female surviving to the initiation of reproduction) is usually the most sensitive effect in a full life-cycle test, with larval growth and survival less sensitive and about equal in sensitivity to the mortality of adults.

**Table 2.8 Standardised chronic fish toxicity test procedures using juvenile fish**

| Body | Guideline number | Description (Date) | Recommended test species (Life stage at the start of the test) | Duration (days) | Endpoint |
|---|---|---|---|---|---|
| OECD | 215 | Fish, Juvenile Growth Test - Draft (1998) | *Oncorhynchus mykiss* (juveniles) | 28 | Growth, lethality |
| ISO | 10229 | Water quality, Determination of the prolonged toxicity of substances to freshwater fish – Method for evaluating the effects of substances on the growth of rainbow trout (1994) | *Oncorhynchus mykiss* (juveniles) | 28 | Growth, lethality |
| AstraZeneca protocol | - | | *Scopthalmus maximus* (juveniles) | 14 | Growth, lethality |

**Table 2.9 Standardised chronic fish toxicity test procedures using adult fish**

| Body | Guideline number | Description (Date) | Recommended test species (Life stage at the start of the test) | Duration (days) | Endpoint |
|---|---|---|---|---|---|
| Harries *et al* (2000) Ankley *et al* (2001) | - | Short-term paired or group breeding reproduction test | *Pimephales promelas* (Adults) | 42 | Reproductive performance |

## *2.3 Ethical considerations in the assessment of effluents*

### 2.3.1 Introduction

When evaluating which methods should be used to assess the toxicity of effluents and receiving waters a key consideration has been the ethical issues associated with such tests and the potential for using approaches by which:

- Numbers of animals used could be reduced by the adoption of a more focussed *in vivo* testing strategy
- Alternative methods not involving whole organisms could be used to replace *in vivo* methods

A more focussed *in vivo* testing strategy could involve initially conducting single concentration limit tests (whether acute or chronic) at the lowest threshold effluent concentration for effects in algae or invertebrates. In this way the effluent tests with fish would be used to establish whether full concentration tests were needed on the basis that fish represent the most sensitive taxonomic group.

Alternative approaches that could be used as replacements for whole organism tests include *in vitro* assays using fish cells and tissues and biosensors (such as the Fishsense system) which utilise fish cells.

### 2.3.2 *In vitro* test systems

There are merits and limitations to the use of *in vitro* systems and these are summarised in Table 2.10. Interactions of an anthropogenic chemicals with biota initially occur at the cellular level, making cellular responses not only the initial manifestation of toxicity but also suitable tools for the early, sensitive and cost-effective detection of chemical exposure (and potentially effects). However, *in vitro* systems do not account for all the biochemical and physiological processes that occur in whole organisms meaning the results of *in vitro* tests will not necessarily be realistic surrogates for *in vivo* responses.

In aquatic toxicology, fish cell lines have been used mainly to study the mechanisms of action of xenobiotics including assessments of :

- biochemical systems and responses including the regulation of xenobiotic metabolizing enzymes, the biotransformation of xenobiotics, the induction of DNA damage and repair, cell membrane transport system function, ionic homeostatis perturbation, expression of ionic mimicry, induction of oxidative stress and expression of metallothioneins and stress proteins;
- morphological changes in cytotoxic responses;
- the action of xenobiotics on specialised cell functions such as immune cell function and responses to oestrogenic compounds.

**Table 2.10 Merits and limitations of *in vitro* systems**

| Merits of *in vitro* systems | Limitations of *in vitro* systems |
|---|---|
| • *In vitro* tests are fundamental in identifying/ characterising mechanisms of action, and therefore, may help to elucidate potential endpoints which should be measured *in vivo*<br><br>• Metabolites, when known and available, can be tested individually in vitro. This may characterise the role of metabolism and its contribution to toxicity, in terms of the relative potency and amount of the metabolites, *in vivo* and may provide important information if the capacity for the production of a specific metabolite differs across species.<br><br>• *In vitro* tests can be run using small amounts of test material or environmental samples and can be used to ascertain the types of activity and/or the predominant activity present. This information may be relevant to on-going field surveys.<br><br>• *In vitro* tests can be low cost, rapid and high throughput with the possibility of automation | • *In vitro* systems possess only a portion of the metabolic system present in whole organisms<br><br>• *In vitro* systems often only consider one site of action of a test substance whereas many toxicants exhibit multiple sites of action *in vivo*<br><br>• *In vitro* systems do no accurately model pharmacodynamic/pharmacokinetic considerations<br><br>• *In vitro* systems do not model the effects of bioconcentration and bioaccumulation which can be important factors in *in vivo* effects<br><br>• *In vitro* systems cannot identify critical windows within the development or lifecycle of an organism which may be sensitive to particular types of effect (for example endocrine disruption) |

Table 2.11 summarises the types of *in vitro* systems which could be applied to the assessment of the toxicity of effluents and receiving waters. The endpoints used in the *in vitro* systems include measurements of disruption of membrane integrity (using neutral red or tetrazolium salt reduction) and cell death

**Table 2.11 Fish cells used in toxicity testing**

| Cell name | Type of cell | Origin |
|---|---|---|
| R1 | Fibroblastic cell line | Liver of rainbow trout |
| Hepatocytes | Primary culture | Liver of rainbow trout |
| RTG-2 | Fibroblastic cell line | Gonads of rainbow trout |
| FHM | Epitheloid cell line | Tissue posterior to the anus from fathead mnnow |
| PLHC-1 | Hepatoma cell line | From top minnow |
| BB | | Posterior trunk tissue from brown bullhead |
| CCB | Fibroblastic cell line | Brain cells from carp |
| BG/F | Epitheloid cell line | Fin tissue from bluegill sunfish |
| OLF-136 | Fibroblastic cell line | Fin tissue from japanese medaka |

In terms of the validation of fish cell lines the emphasis to date has been on developing *in vitro*/*in vivo* comparisons by investigating different groups of compounds, groups of reference chemicals and environmental samples.

In the large number of cases for specific chemical groups statistically significant *in vitro*/*in vivo* correlations have been obtained based on comparisons of *in vitro* effect concentrations (concentrations that inhibit the response by 50% as determined *in vitro*) with *in vivo* lethality data (concentrations that cause 50% lethality in whole organisms). However, a number of exceptions have been reported in particular for lipophilic solvents based on a comparison of $EC_{50}$ data for reductions in protein content in FHM cells with published $LC_{50}$ data for golden orfe (Dierickx 1993)

Studies of the comparability between *in vitro* and *in vivo* data for environmental samples have also shown statistically significant correlations. Rusche and Kohlpoth (1993) found a correlation coefficient of 0.868 between in vitro data for R1 cells and those obtained in parallel with golden orfe fish tests for a range of wastewater samples. However, it also has to be remembered that for *in vitro* tests to act as a surrogate for *in vivo* tests there needs to be comparable sensitivity between the methods (see Table 2.11).

Gagné and Blaise (1995) have developed a particularly novel strategy for the validation of the rainbow trout hepatocyte model for testing industrial wastewaters. It was found that artificial neural network modelling of *in vitro* data allowed toxic effluents to be classified in a way that was predictive of the toxicity observed *in vivo* with 96h rainbow trout lethality tests. This approach was considered to be potentially useful for screening large numbers of waste water samples and identifying which samples required *in vivo* evaluations.

In another approach a three year project has been completed to develop and evaluate an *in vitro* assay using cultured fish cells, modified to allow the health of the cells to be monitored by their bioluminescence (Fishsense)(see Section D). The work was carried out by AstraZeneca in collaboration with researchers at the University of Luton and with Seraph Technologies, specialists in the development of biosensor instrumentation. The project was successful in gaining matching DTI funding under the LINK Cell Engineering programme. The work arose out of previous studies with Luton, employing fish cells on electrochemical (amperometric) biosensors using the CellSense™ system. Although successful (Polak *et al* 1996) the chemical mediators employed exhibit certain toxicity to the cells, suggesting the need for alternative methods of cell interrogation. The conclusions from the study were that effects of chemicals on fish populations can only be predicted accurately by the use of whole fish experiments. However, luminescent fish cells provide a rapid estimate of the acute effect level, generally within an order of magnitude of the *in vivo* value, with the potential for improvement by extending the exposure period. Thus, such screening tests could help to minimising the number of fish used in experiments.

### 2.3.3 Summary

Overall there exists the potential for *in vitro* systems to be used in a screening role and to limit the use of acute fish toxicity tests to situations where there is clear need to confirm the screening test results. This will require further development of the in vitro systems to address the limitations highlighted in Table 2.10. However, at present, there is no immediate prospect of *in vitro* assays being used as replacements for chronic *in vivo* tests with sub-lethal endpoints.

## 2.4 Criteria for the evaluation of potential methods

If comparisons between different types of methods are to be unbiased and objective, rather than subjective, then it is necessary to judge each potential method against a series of criteria which are considered important if the method is to be 'fit for purpose'.

The criteria that have been used to assess the appropriateness of methods for use in assessing effluent toxicity (and receiving water quality) were developed by the Steering Group and are shown in Table 2.13.

The criteria are divided into five categories:

1. relevance of test data
2. test method variability
3. previous application to effluent assessment
4. methodology
5. ethical and legal issues

The relevance of test data and test method variability categories were selected to be consistent with the two key OECD criteria for the development of toxicity tests procedures, namely:

- Does the test provide ecologically relevant data?
- Does the test generate reproducible results?

The criteria in the suitability for effluent assessment category are designed to assess whether the test is 'fit for purpose' and provide any evidence of its current successful application. The methodology category incorporates criteria related to practical test issues while the criteria in the ethical and legal category are designed to ensure that any methods considered satisfy current ethical and legal requirements. A more detailed description of the criteria is given in Appendix A.

## 2.5 Evaluation procedure for methods

There are alternative approaches to achieving this aim including:

- Assigning scores for the criteria using a series of guidelines and then summing these to obtain a total score. A method is considered fit for purpose if it achieves a certain threshold score or more pragmatically if it is the highest score among the available methods for a particular role. In the procedure key criteria can be weighted so that they exert a greater influence on the total score;
- Assigning threshold requirements for the criteria and considering a method fit for purpose if they satisfy these requirements for all the criteria or all the *key* criteria.

Versions of each of the above approaches have been used previously in the selection of methods for different roles including those described previously by the Marine Pollution Monitoring Groups Co-ordinating Group on methods suitable for monitoring at United Kingdom sewage sludge disposal sites (MAFF 1990) and Hill *et al* 1993 for the selection of methods for assessing sediment toxicity. In the selection of methods for monitoring toxicity at UK sewage sludge disposal grounds tests were scored on a scale of 1-5 against a series of criteria. Additionally the criteria were weighted (also on a scale of 1-5) according to their perceived importance. A total score was then obtained by summing the weighted score for each criterion.

In developing the selection procedure for the Environment Agency both options were considered and evaluated before it was decided to opt for the latter option of assessing methods against threshold requirements for the criteria. This approach was advocated to ensure that tests were not selected which were deficient in one or more key areas but which still scored highly overall.

Table 2.14 summarises the criteria which are used to identify the most appropriate currently available methods for assessing effluent toxicity. The assessment is carried out using a combination of information based evaluations and expert judgement with many of the criteria scored on a Yes/No basis. Tests which show a large number of Yes answers are considered to be more appropriate for assessing effluent and receiving water toxicity.

**Table 2.12 Summary of the criteria to be used to select currently available methods which are appropriate for assessing effluent toxicity**

| Category and criterion | Description of criteria |
|---|---|
| ***1.Relevance of test data*** | |
| 1.1 Use of ecologically relevant test species | Assessment of whether the test species used is representative of the receiving water at a location |
| 1.2 Importance of test species | Assessment of whether the test species used is of economic or ecological importance |
| 1.3 Diversity of endpoints | Assessment of whether multiple (sub-lethal and lethal) endpoints can be measured in the method |
| 1.4 Ecological relevance of test endpoint(s) | Assessment of whether the test endpoint(s) are surrogates for parameters important to fish population dynamics (for example growth, development and reproduction) |
| 1.5 Extrapolation to population effects | Assessment of whether the test data is predictive of longer-term population effects as measured in long-term laboratory tests based on information on the *sensitivity* and *spectrum of response* of the test. |
| ***2. Test method variability*** | |
| 2.1 Test method repeatability | Assessment of whether the intra-laboratory variability for a method (as measured using the results of repeat tests in a laboratory with reference toxicant) satisfies a threshold coefficient of variation of 30% |
| 2.2 Test method reproducibility | Assessment of whether the inter-laboratory variability for a method (as measured using the results of tests at different laboratories with reference toxicants) satisfies a threshold of coefficient of variation of 50% |
| 2.3 Availability of a validated method describing the procedure | Assessment of whether a validated method describing the conduct of the test procedure (for example to regulatory guidelines) is available |
| 2.4 Test organism variability | Assessment of whether the quality of test organisms will be markedly affected by factors such as their source and holding conditions (for example in house culturing or field collection) |
| 2.5 Training effort | Assessment of the time taken to achieve consistent results with a method |
| ***3.Previous application to effluent assessment*** | |
| 3.1 Current or proposed use in national effluent control strategies | Assessment of whether the method is currently used successfully or is proposed for use in effluent control programmes in different countries |
| 3.2 Discrimination between samples | Assessment of whether the method provides graded responses for effluent samples and can discriminate between effluents |
| 3.3 Influence of exposure conditions | Assessment of whether data is available on the influence of key physico-chemical parameters (such as temperature, dissolved oxygen, pH hardness or salinity, suspended solids) on the test response |
| ***4. Methodology*** | |
| 4.1. Application to DTA | Assessment of whether the test method design is practical for effluent assessment in terms of factors such as sample volumes and the frequency of test solution replacement |
| 4.2 Timescale of the method | Assessment of the time taken from initiating a test to obtaining the test data |
| 4.3 Cost of method | Assessment of the cost of the method |
| *4.3.1 Cost of implementing the method* | *Assessment of the cost of staff time and equipment to establish a method at a location* |
| *4.3.2 Cost of conducting the method* | *Assessment of the cost per unit test including staff time and consumables and the cost of culturing or collecting the test organisms (where this is appropriate)* |
| 4.4 Availability of test organisms | Assessment of whether the test organisms are available throughout the year |
| 4.5 Suitability of the method for abbreviated testing | Assessment of whether the test can be miniaturised into an abbreviated version for particular operational roles |
| ***5. Ethical and legal issues*** | |
| 5.1 Exemption from the Animal Scientific Procedures Act | Assessment of whether the Animal Scientific Procedures Act applies to the test |
| 5.2 Potential for reduction of animal numbers | Assessment of whether the method can be adapted to minimise the number of animals used without compromising the statistical power of the method to detect effects |

**Table 2.13 Summary of the criteria used to select appropriate currently available methods for assessing effluent toxicity**

| Category and criterion | Type of criteria | Assessment against criteria | Means of assessment |
|---|---|---|---|
| ***1.Relevance of test data*** | | | |
| 1.1 Use of ecologically relevant test species | ***High priority*** | Is the test species used in the method representative of the receiving water at a location (Y/N)? | Expert judgement |
| 1.2 Importance of test species | Low priority | Is the test species used in the method of economic or ecological importance (Y/N)? | Expert judgement |
| 1.3 Diversity of endpoints | **Key** | Are multiple (sub-lethal and lethal) endpoints be measured in the test (Y/N)? | Information based |
| 1.4 Ecological relevance of test endpoint(s) | **Key** | Are the test endpoint(s) surrogates for parameters which are important to fish population dynamics (Y/N)? | Expert judgement |
| 1.5 Extrapolation to population effects | **Key** | Is the test data is predictive of longer-term population effects as measured in long-term laboratory tests based on information on the *sensitivity* and *spectrum of response* of the test (Y/N)? | Information based |
| ***2. Test method variability*** | | | |
| 2.1 Test method repeatability | **Key** | Is the intra-laboratory variability for the method (as assessed using the results of repeat tests in a laboratory with reference toxicants) below a threshold coefficient of variation of 30% (Y/N) | Information based |
| 2.2 Test method reproducibility | **Key** | Is the inter-laboratory variability for the method (as assessed using the results of tests at different laboratories with reference toxicants) below a threshold coefficient of variation of 50% (Y/N)? | Information based |
| 2.3 Availability of a validated method describing the procedure | **Key** | Is a validated method describing the conduct of the test procedure (for example to regulatory guidelines) available (Y/N)? | Information based |
| 2.4 Test organism variability | ***High priority*** | Are the effects of the source and holding conditions on the quality of test organisms minimised (Y/N)? | Expert judgement |
| 2.5 Training effort | Low priority | How long a training period is required to achieve consistent results with a method? | Information based |

**Table 2.13 Continued**

| Category and criterion | Type of criteria | Assessment against criteria | Means of assessment |
|---|---|---|---|
| ***3.Previous application to effluent assessment*** | | | |
| 3.1 Current or proposed use in national effluent control strategies | ***High priority*** | Is the method currently used successfully or proposed for use in effluent control programmes (Y/N)? | Information based |
| 3.2 Discrimination between samples | **Key** | Does the method provide graded responses for effluent samples and can it discriminate between effluents (Y/N)? | Information based |
| 3.3 Influence of exposure conditions | ***High priority*** | Are data available on the influence of physico-chemical parameters on the test response (Y/N)? | Information based |
| ***4. Methodology*** | | | |
| 4.1 Application to DTA | **Key** | Is the test method design practical for effluent assessment in terms of factors such as sample volumes and the frequency of test solution replacement (Y/N)? | Expert judgement |
| 4.2 Timescale of the method | ***High priority*** | What is the time taken from initiating a test to obtaining the test data? | Information based |
| 4.3 Cost of method | | | |
| *4.3.1 Cost of implementing the method* | ***High priority*** | What is the cost of staff time and equipment to establish a method at a location)? | Information based |
| *4.3.2 Cost of conducting the method* | ***High priority*** | What is the cost per unit test including staff time and consumables and the cost of culturing or collecting the test organisms (where this is appropriate)? | Information based |
| 4.4 Availability of test organisms | ***High priority*** | Are the test organisms available throughout the year (Y/N)? | Information based |
| 4.5 Suitability of the method for abbreviated testing | Low priority | Can the test can be modified into a miniaturised version for particular operational roles (Y/N)? | Information based |
| ***5. Ethical and legal issues*** | | | |
| 5.1 Exemption from the Animal Scientific Procedures Act | **Key** | Is the method outside scope of the Animal Scientific Procedures Act (Y/N)? | Information based |
| 5.2 Potential for reduction of animal numbers | **Key** | Can the method can be adapted to satisfy the requirements for minimising the number of animals used without compromising the statistical power of the method to detect effects (Y/N)? | Expert judgement |

# 3. IDENTIFICATION OF THE MOST APPROPRIATE CURRENTLY AVAILABLE METHODS FOR ASSESSING EFFLUENT TOXICITY

## 3.1 Introduction

On the basis of the evaluation of currently available methods which could be applied to assessing effluent and receiving water toxicity it is evident that a number of the methods promulgated by international (Organisation for Economic Cooperation and Development or International Standards Organisation) or national bodies (Environment Canada or United States Environmental Protection Agency) are similar. As a result the actual number of test methods available (which can be used with different species) is more limited. Table 3.1 summarises the types of procedures available and the species used which comprise:

- the early-life stage test (OECD TG 210) and the embryo, embryo/alevin and embryo/alevin/fry tests (Environment Canada Method EPS1/RM/28)
- the short-term toxicity test using embryo larval and sac-fry stages (Proposed OECD TG 212 and ISO Standard 12890)
- the short-term larval growth and survival test (US EPA Methods 1000, 1004 and 1006 and Environment Canada Method EPS1/RM/22)
- the short-term larval survival and teratogenicity test (US EPA Methods 1001 and 1005)
- the juvenile growth test (Proposed OECD 215, Draft ISO Standard 10229, AstraZenenca in-house methodology)
- the adult paired reproduction test (Harries *et al* 2000, Ankley *et al* 2001)

## 3.2 Evaluation of currently available methods for assessing effluent toxicity

### 3.2.1 Early life stage tests

There are a number of early life stage tests which in their present form (or with minor changes) would be suitable for routinely assessing the chronic toxicity of effluents and receiving waters including:

- The 7 day larval survival and growth methods for freshwater and estuarine/marine species promulgated by the United States Environmental Protection Agency (Methods 1000, 1004 and 1006) and Environment Canada Method EPS1/RM/22.

- The 7 day salmonid embryo test method promulgated by Environment Canada (Method EPS1/RM/28) with a corresponding method for turbot if practicable.

Both these methods are of a realistic timescale for use as the initial assessment tool and should provide robust data on the potential risks posed by discharges at sensitive life stages of the organisms. The data in Table 2.7 for brook trout and fathead minnows exposed to a range of toxicants (cadmium, copper, chromium, zinc, methylmercury, diazinon and toxaphene) in embryo-larval tests indicates that there is no consistent trend in terms of the relative sensitivities of the different species.

The routine use of other methods using early life stages of fish for the assessment of effluent or receiving water toxicity is problematical due to the duration and resulting time/cost implications. Early-life stage methods to which this applies are:

- the 95 day early life stage test using rainbow trout and the 32 day early life stage test using fathead minnows which forms part of OECD TG 210;

- the 27-29 day embryo/alevin and 70 day embryo/alevin/fry (EAF) test using rainbow trout which forms part of Environment Canada Method EPS1/RM/28.

Furthermore since it is important that methods used avoid any additional suffering outside that resulting directly from toxicant exposure it is not considered appropriate to use tests where animals are not fed during the procedure (for example OECD 212, ISO Standard 12890 and short-term US EPA embryo-larval survival and teratogenicity Method (1001 and 1005). It is recommended that if such methods are used they should be modified by the provision of appropriate food once the test organism reaches a life history stage where it is capable of free feeding.

In the following sections the information for the candidate early life stage tests for assessing effluent and receiving water toxicity are considered.

***Methods using species recommended for acute toxicity testing in the DTA Method Guidelines***

The use of the short-term (7 day) embryo test with rainbow trout would have the advantage that it uses the test species currently specified in the DTA Method Guidelines for assessing acute toxicity. This procedure is one recommended for use in certain parts of Canada as part of the Environmental Effects Monitoring Programmes for pulp and paper mill and mine effluents (Scroggins *et al* 2002a,b). In the assessment of pulp and paper mill effluents 7 day embryo tests, using an effective concentration for 25% lethality of embryos as the endpoint, identified 33.3% of the 54 samples tested in 1992-1996 as having an $EC_{25}$ of $\leq$ 100% and 54.3% of the 70 samples tested in the period 1997-2000 as having an $EC_{25}$ of $\leq$ 100%. In the periods 1992-1996 and 1997-2000 the test measured $EC_{25}$ values from 6.3% to >100 % for 11 mills tested. In the assessment of mine effluents the 7 day embryo test is used west of the Rocky Mountains where fathead minnows are not native and the 7 day larval survival and growth procedure is not applied.

There is no corresponding early embryo test procedure for the turbot (the marine fish test species recommended for acute toxicity testing), though this could be investigated. However, it needs to be recognised that recently an issue has arisen with the routine availability of

juvenile turbot for toxicity testing which clearly compromises the use of acute and/or chronic tests using this species. It may be possible to use an alternative indigenous estuarine/marine species in acute and/or chronic tests but such a species would need to satisfy a number of criteria, namely being:

1. readily available in sufficient numbers for testing from a number of suppliers throughout the year at an acceptable cost.

2. of comparable sensitivity to turbot for substances with a range of modes of toxic action

3. providing robust data for the test endpoints

***United States Environmental Protection Agency Procedures***

The 7 day larval survival and growth (and the 8 day embryo-larval survival and teratogenecity) procedures promulgated by the United States Environment Agency (and using fathead minnows, sheepshead minnow and inland silverside) have been widely used in the United States as part of the National Pollutant Discharge Elimination System operated under the Clean Water Act. It is estimated that at present in the region of 20% of discharge permits may contain a requirement for toxicity testing with sub-lethal fish tests.

This procedure is also one recommended for use in certain parts of Canada as part of the Environmental Effects Monitoring Programmes for pulp and paper mill and mine effluents (Scroggins *et al* 2002a,b). In the assessment of pulp and paper mill effluents 7 day fathead minnow larval survival and growth tests, using an inhibiting concentration for 25% effect (growth or survival) as the endpoint, identified 66.3% of the 306 samples tested in 1992-1996 as having an $IC_{25}$ of $\leq$ 100% and 24.2% of the 451 samples tested in 1997-2000 as having an $IC_{25}$ of $\leq$ 100%. In the period 1992-1996 the test measured $IC_{25}$ values from 0.36% to >100 % and in 1997-2000 the test measured $IC_{25}$ values from 2.8% to >100 % for the mills tested. The test was also able to detect changes in effluent toxicity resulting from the implementation of secondary treatment at 12 mills tested. Tests using the inland silverside were also conducted as part of the programme and these showed 42.7% of the 103 samples tested in 1992-1996 as having an $IC_{25}$ of $\leq$ 100% and 2.5% of the 122 samples tested in 1997-2000 as having an $IC_{25}$ of $\leq$ 100%.

In the assessment of mine effluents the 7 day embryo test is used east of the Rocky Mountains where fathead minnows are native. The 7 day $IC_{25}$ values measured in mine effluent samples taken in the period 1996-1997 ranged from 46.8% to >100% (Scroggins *et al* 2002b).

At the Pellston workshop in 1995 on Whole Effluent Toxicity the available data on comparisons between fathead minnow larval survival and growth data for tests on effluents and receiving waters and effects on resident biological communities was evaluated (Grothe *et al* 1996). It was stated that in the 10 validation studies using receiving water samples (Birge *et al* 1989, US EPA 1991, Dickson *et al* 1992) laboratory measurements of toxicity provided good indications of higher level community effects under some circumstances (most notable when there was clear receiving water toxicity). This finding is not unexpected, though it must be remembered that evidence of toxicity in receiving waters could be found before effects on resident communities are evident. Furthermore changes in the resident community could be due to factors others than water column toxicity (for example habitat changes or historical sediment contamination). In the AETE Programme in Canada (Scroggins *et al* 2002b) the

tests with fathead minnows proved the least effective at 'predicting' in stream effects, which is consistent with the fact that the test was demonstrated to be of lower sensitivity to mine effluents than the algal (*Selenastrum capricornutum*) growth inhibition test, the *Lemna minor* growth inhibition test and the 7 day *Ceriodaphnia dubia* reproduction test. However, at the Heath Steele mine fish toxicity in the fathead minnows was observed at concentrations in the receiving water where fish catch per unit effort (CPUE) and biomass per unit effort (BPUE) were affected. Furthermore the threshold for growth impairment in fathead minnow exposed to the Dome mine effluents occurred at concentrations greater than those found downstream under conditions of effluent discharge. This is consistent with the observation that growth is not impaired in yellow perch or pearl dace downstream of the Dome mine .

Assessments of the intra- and inter-laboratory variability of the larval survival and growth procedure have indicated that the methods can provide repeatable and reproducible data (US EPA 2001).

Finally from a logistical viewpoint another advantage of using the fathead minnows for assessing effluent toxicity is that methods are available for detecting effects on larval survival growth and teratogenicity and adult reproduction (accepting the limitations described) which removes issues associated with interspecies comparisons. However, from an ethical viewpoint all these tests use fathead minnows at a relatively developed (larval) stage, whereas if possible it would be appropriate to use earlier, less developed (early embryonic) stages which are currently unprotected by the Animals (Scientific Procedures) Act 1986, such as the salmonid embryo test described above. This would be more in line with the use of the invertebrate species recommended in the DTA Method Guidelines, which are also unprotected by the Act.

### 3.2.2 Tests with juvenile fish

The available juvenile growth tests with rainbow trout and turbot are not the most appropriate for routinely assessing effluent and receiving water toxicity due to the lower sensitivity of this life stage compared to embryo or larval tests and the greater durations (28 days for rainbow trout and 14 days for turbot) and costs associated with the juvenile growth tests. Furthermore there remains the issue of the current limited availability of turbot (see Section 3.2.1)

### 3.2.3 Tests with adult fish

It is recognised that effects of effluents on reproduction would not be considered by the use of an embryo-larval development and growth tests. However, the costs and resource implications of conducting reproduction tests (such as the paired or group breeding assays) with adults mean that they should not be used for effluent assessment on a routine basis. Instead there use should be restricted to situations where assessment of effects on reproduction is key to evaluating potential effects on fish species, for example in the case of discharges from pharmaceutical plants which may release human or veterinary products. The option for using a reproduction test should be based on chemical specific drivers following analysis of the effluent for substances of concern.

## 3.3 Summary

Table 3.2 provides a summary of the evaluation of the methods against the criteria given in Table 2.13. Full details of the evaluation of each method are given in Appendix C. In the evaluation where Yes/No answers are required the most appropriate methods will give Yes answers to these questions. On the basis of the evaluation procedure (see Table 3.2) the routine assessment of effluent toxicity of discharges to freshwater could currently be carried out using either:

- the 7 day embryo test using rainbow trout promulgated by Environment Canada as Method EPS1/RM/28 (Environment Canada 1998)

- the 7 day larval survival and growth test using fathead minnows promulgated by the United States Environment Agency as Method 1000.0 (US EPA 1995)

In the absence of comparative sensitivity and performance data the most appropriate method should ideally be determined by a conducting a limited evaluation exercise using a range of effluents types.

Reproduction tests should not be used for the routine assessment of effluent or receiving water toxicity but need to be reserved for situations where an evaluation of effects on reproduction is key to determining potential effects on fish populations, for example discharges from pharmaceutical plants which may release human or veterinary products.

In the *medium term* there are areas of research that could be pursued to address ethical concerns over the use of fish for testing. These include developing a combined 11 day embryo-larval survival, growth and teratogenicity test (based on the procedures promulgated by the United States Environmental Protection Agency as Methods 1000, 1001, 1004, 1005 and 1006) so that additional information is gained from each test. In this revised procedure fish would need to be provided with food.

**Table 3.1 Summary of the species used in the currently available methods which could be used to assess effluent toxicity**

| Type of test | Procedure | Recommended species | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Rainbow trout | Common carp | Fathead minnows | Japanese medake | Zebrafish | Sheepshead minnow | Inland silverside |
| Early-life stage toxicity test | OECD 210 | Yes | Yes | Yes | Yes | Yes | No | No |
| Toxicity test with embryos and sac-fry stages | Proposed OECD 212<br>ISO 12890 | Yes<br>No | Yes<br>No | Yes<br>No | Yes<br>No | Yes<br>Yes | No<br>No | No<br>No |
| Larval survival and growth toxicity test | Environment Canada EPS1/RM/22<br>US EPA 1000, 1004 and 1006 | No<br>No | No<br>No | Yes<br>Yes | No<br>No | No<br>No | No<br>Yes | No<br>Yes |
| Embryo-larval survival and teratogenicity toxicity test | US EPA 1001 and 1005 | No | No | Yes | No | No | Yes | No |
| Embryo toxicity test<br>Embryo/alevin toxicity test<br>Embryo/alevin/fry toxicity test | Environment Canada EPS1/RM/28<br>“<br>“ | Yes<br>Yes<br>Yes | No<br>No<br>No | No<br>No<br>No | No<br>No<br>No | No<br>No<br>No | No<br>No<br>No | No<br>No<br>No |
| Juvenile growth test | Proposed OECD 215<br>ISO 10229 | Yes<br>Yes | No<br>No | No<br>No | No<br>No | No<br>No | No<br>No | No<br>No |
| Adult reproduction test | Harries *et al* (2000), Ankley *et al* (2001) | No | No | Yes | No | No | No | No |

**Table 3.2 Assessment of currently available chronic methods for evaluating effluent toxicity (ND – no data, P – potentially)**

| Category and criterion | Type of criteria: priority | Summary of scoring against criteria for different methods (Table number in Appendix C) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Early life stage test (1) | Test with embryos and sac-fry (2) | Larval survival and growth test (3) | Embryo-arval survival and teratogenicity (4) | Embryo test (5) | Embryo/alavin test (6) | Embryo/alevin/ fry test (7) | Juvenile growth test (FW)(8) | Juvenile growth test (SW) (9) | Adult reproduction (10) |
| ***1.Relevance of test data*** | | | | | | | | | | | |
| 1.1 Use of ecologically relevant test species | *High* | *Yes* | *Yes* | *Yes* | *Yes* | *Yes* | *Yes* | *Yes* | *Yes* | *Yes* | *Yes* |
| 1.2 Importance of test species | Low | Yes | Yes | No | No | Yes | Yes | Yes | Yes | Yes | Yes |
| 1.3 Diversity of endpoints | **Key** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** |
| 1.4 Ecological relevance of test endpoint(s) | **Key** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** |
| 1.5 Extrapolation to population effects | **Key** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **P** | **P** | **Yes** |
| ***2. Test method variability*** | | | | | | | | | | | |
| 2.1 Test method repeatability | **Key** | **ND** | **No** | **Yes** | **Yes** | **ND** | **ND** | **ND** | **ND** | **ND** | **ND** |
| 2.2 Test method reproducibility | **Key** | **ND** | **No** | **Yes** | **ND** | **ND** | **ND** | **ND** | **ND** | **ND** | **ND** |
| 2.3 Availability of a validated method describing the procedure | **Key** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **No** | **Yes** |
| 2.4 Test organism variability | *High* | *Yes* | *Yes* | *Yes* | *Yes* | *Yes* | *Yes* | *Yes* | *Yes* | *Yes* | *Yes* |
| 2.5 Training effort | Low | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| *3.Previous application to effluent assessment* | | | | | | | | | | | |
| 3.1 Current or proposed use in national effluent control | *High* | *No* | *No* | *Yes* | *Yes* | *No* | *No* | *No* | *No* | *No* | *No* |
| 3.2 Discrimination between samples | **Key** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** |
| 3.3 Influence of exposure conditions | *High* | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| *4. Methodology* | | | | | | | | | | | |
| 4.1 Application to DTA | **Key** | **P** | **P** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **No** |
| 4.2 Timescale of the method | *High* | *≤ 95 d* | *≤ 55 d* | *7 d* | *7-9 d* | *7d* | *27-29d* | *~70d* | *28d* | *14d* | *42d* |
| 4.3.1 Cost of implementing the method | *High* | *ND* | *ND* | *ND* | *ND* | *ND* | *ND* | *ND* | *ND* | *ND* | *ND* |
| 4.3.2 Cost of conducting the method | *High* | *ND* | *ND* | *ND* | *ND* | *ND* | *ND* | *ND* | *ND* | *£4K* | *>£25K* |
| 4.4 Availability of test organisms | *High* | *Yes* | *Yes* | *Yes* | *Yes* | *Yes* | *Yes* | *Yes* | *Yes* | *No* | *Yes* |
| 4.5 Suitability of the method for abbreviated testing | Low | P | Yes | Yes | Yes | Yes | Yes | Yes | No | No | No |
| *5. Ethical and legal issues* | | | | | | | | | | | |
| 5.1 Exemption from the Animal Scientific Procedures Act | **Key** | **No** | **Yes** | **No** | **Yes** | **Yes** | **No** | **No** | **No** | **No** | **No** |
| 5.2 Potential for reduction of animal numbers | **Key** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** |

# 4. CONCLUSIONS AND RECOMMENDATIONS

## 4.1 Conclusions

The following conclusions have been drawn from the review of methods for assessing the chronic toxicity of effluents and receiving waters to fish:

- On the basis of the evaluation procedure used in this review the routine assessment of the toxicities of effluents and receiving waters could currently be carried out using either:
  - the 7 day embryo test using rainbow trout promulgated by Environment Canada as Method EPS1/RM/28 (Environment Canada 1998) for freshwaters. No comparable test is available using turbot (the preferred species for acute toxicity testing in the DTA Methods Guidelines);
  - the 7 day larval survival and growth test using freshwater (*Pimephales promelas*) or esturaine/marine (*Cyprinodon variegatus* or *Menidae beryllina*) species promulgated by the United States Environment Agency as Methods 1000.0, 1004.0 and 1006.0 (US EPA 1995a,b);

In the absence of comparative sensitivity and performance data the most appropriate method should ideally be determined by a conducting a limited evaluation exercise using a range of effluents types.

Reproduction tests should not be used for the routine assessment of effluent or receiving water toxicity but need to be reserved for situations where an evaluation of effects on reproduction is key to determining potential effects on fish populations, for example discharges from pharmaceutical plants which may release human or veterinary products.

## 4.2 Recommendations

In the *medium term* there are areas of research that could be pursued to address ethical concerns over the use of fish for testing. These include developing a combined 11 day embryo-larval survival, growth and teratogenicity test (based on the procedures promulgated by the United States Environmental Protection Agency as Methods 1000, 1001, 1004, 1005 and 1006) so that additional information is gained from each test. In this revised procedure it is recommended that fish are provided with appropriate food once the test organism reaches a life history stage where it is capable of free feeding.

In the *medium to long term* it is recommended that work is carried out to evaluate whether *in vitro* assays could be appropriate candidates for replacing acute fish toxicity tests in a screening role so that *in vivo* tests were used in a confirmatory role where necessary. However, further work is needed to develop a battery of appropriate systems covering different modes of toxic action that have the required level of sensitivity. At this time there is no immediate prospect of *in vitro* systems being used as surrogates for chronic tests with sub-lethal endpoints.

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# APPENDIX A DESCRIPTION OF THE CRITERIA USED TO EVALUATE METHODS

The criteria which can be used to assess the appropriateness of methods for use in effluent assessment (and receiving water monitoring) are divided into five categories:

1. relevance of test data
2. test method variability
3. previous application to effluent assessment
4. methodology
5. ethical and legal issues

The criteria specified under each category are described in greater detail in the following sections.

## A1. Relevance of test data

### *A1.1 Use of ecologically relevant test species*

In order to ensure that the data from laboratory tests on effluent toxicity can be considered relevant to receiving water communities the test species used should be representative of indigenous species even if it is not actually present in the water body of interest.

### *A1.2 Importance of test species*

The ecological or economic relevance of fish toxicity tests can be enhanced when species which are a key component of their relevant ecosystem and/or are economically important nationally are used.

### *A1.3 Diversity of endpoints*

There is considerable practical and economic benefits in utilising test methods which enable both sub-lethal and lethal endpoints to be measured and (if required) specific biomarkers of toxic effect can be incorporated into the test design.

### *A1.4 Ecological relevance of the test endpoint*

Since one of the key aims of environmental regulation for water bodies is to protect resident communities it is important to ascertain whether the results from toxicity tests are indicative of likely effects on the resident organisms. Therefore, where possible, relationships between toxicity test data and field measurements need to be considered.

### *A1.5 Extrapolation to population effects*

In order to assess the extent to which test data is predictive of longer-term populations effects information from the test for a range of test substances with different modes of toxic action should be compared with laboratory life cycle test measurments for the same substances.

Methods used in effluent testing (and receiving water monitoring) should respond to a wide range of substances with different modes of toxic action which may be present in complex effluents to minimize incidences of false negatives. Ideally if the spectrum of response of the method to individual substances is to be realistically assessed data should be available for substances representative of chemical classes with different modes of toxic action. Investigators at the United States Environmental Protection Agency have described eight modes of toxic action for fish exposed to contaminants (for review McCarty and Mackay 1993): non-polar narcotics, polar narcotics, uncouplers of oxidative phosphorylation, cholinesterase inhibitors, membrane irritants, central nervous system convulsants, respiratory blockers and dioxin-like substances. It is also important to consider the sensitivity of test methods to herbicides (whose modes of toxic action are specific to algae and aquatic macrophytes. include cell division inhibition and photosynthesis inhibition) and heavy metals such as cadmium, chromium, mercury and zinc which are not considered representative of any of the specified modes of toxic action but are obviously important pollutants found in indutsrial effluents.

## A2. Test method variability

### *A2.1 Test method repeatability*

Test method precision (repeatability) is assessed based on the mean coefficient of variation for repeat intra-laboratory tests on the representative substances for which test data are available. There are no internationally accepted ranges for intra-laboratory variability. However, Environment Canada (1990) have suggested that coefficients of variation of 20-30 % should be achievable for intra-laboratory variability.

### *A2.2 Test method reproducibility*

Test method (reproducibility) is assessed based on the mean coefficient of variation for an inter-laboratory ring test with a representative substance involving at least three laboratories. There are no internationally accepted ranges for inter-laboratory variability. However ..

### *A2.3 Availability of a validated method describing the procedure*

A threshold requirement for test methods is the availability of a draft procedure describing the methodology which, as a minimum, has received peer review.

### *A2.4 Test organism variability*

The variability associated with tests will depend to a degree on the quality of the sources of test organisms or fish cell lines, but such variability should be accounted for and minimised in the test methodology.

### *A2.5 Training effort*

For both effluent screening or monitoring the methods employed should be easy to use (and robust) since those requiring considerable time and skill to learn will probably not be widely used. In the selection procedure the ease of use of a method is assessed using the time taken for staff to become proficient with a test. Proficiency is considered to be achieved when testing with an appropriate reference substance on a number of occasions (usually 5-6) leads to similar test results ($IC_{50}$, $EC_{50}$ and $LC_{50}$) and a coefficient of variation below a an intra-laboratory threshold level of 30%.

## A3. Previous application to effluent assessment

### *A3.1 Current or proposed use in national effluent control programmes*

The capability of a chronic fish test for assessing effluent toxicity will be proven if it is currently being used successfully in a national effluent control programme or is proposed for use on the basis of the results of a pilot study.

### *A3.2 Discrimination between samples*

In any operational role methods should be able to successfully discriminate between samples such that they are grouped into a number of toxicity categories (based on the measured toxicity endpoints as toxic units) rather than a single category. For effluents (and receiving waters) it is important that methods do not either identify all samples of varying chemical contaminant concentrations as either non-toxic or highly toxic but rather show a clear discrimination range.

### *A3.3 Influence of exposure conditions*

In the testing of effluents (and receiving water monitoring) it is vital to have information on the effects of physico-chemical parameters of the sample (such as pH, dissolved oxygen, hardness, salinity, suspended solids, colour) on test responses so that the influence can be separated from those of residual chemical contaminants.

### *A3.4 Volume of sample needed*

From a practical viewpoint there is benefit in utilising test methods which require the smallest volumes of effluent.

## A4. Methodology

### *A4.1 Application to DTA*

Practicality of the method assesses the extent to which the time needed for a method to respond to toxicant exposure matches the practicalities of assessing effluents. This is important given that the degradation of samples requires that methods be of shorter duration than methods used for assessing the toxicity of pure substances.

### *A4.2 Timescale of the method*

There are clear benefits in terms of resources and costs from using methods of short duration (in terms of the time taken from initiating a test to obtaining the test data) providing that the test provides robust and relevant data.

### *A4.3 Cost of the method*

The cost of the method is assessed based on two criteria: *cost of conducting the method* and *cost of implementing the method.*

The *cost of implementing the method* needs to include the purchase of specialised dedicated equipment and the staff costs for setting up the method. A standard figure of £40 per hour should be used for calculating staff costs to ensure consistency of application between users even though local rates may vary.

The *cost of conducting the method* comprises both the staff costs and materials. For methods where specific materials (including test organisms) may need to be bought in the determination of the materials cost is straight-forward. However, for methods using cultured organisms the exact costs allocated to each test may be less obvious. This should be determined by the frequency of use of the method, with the cost per test being the annual costs divided by the number of tests which are expected to be conducted each year. The culturing costs comprise staff costs and materials, and staff costs have again been calculated using the figure of £40 per hour.

### *A4.3 Availability of test organisms*

The availability of material from commercial suppliers depends largely on the number of organisations capable of supplying test substrate or organisms, and reliance should not be placed on fewer than two suppliers.

### *A4.4 Suitability of the method for abbreviated testing*

Abbreviated testing may be required in certain circumstances and consequently it is important that test methods can be used in a modified format.

## A5 Ethical and legal issues

### *A5.1 Exemption from the Animal Scientific Procedures Act*

There are practical and legal requirements to be complied with if the fish toxicity test method is covered by the Animal Scientific Procedures Act (HMSO 1986). The Act currently relates to tests on free feeding fish.

### *A5.2 Potential for reduction, replacement and refinement*

The Organisation for Economic Cooperation and Development (OECD) has a strategy for reduction, replacement and refinement of fish (and mammalian) toxicity tests. The issues associated with these requirements are:

- the potential for minimising the number of animals used without compromising the statistical power of the method to detect effects (reduction);

- the existence of a reliable, currently available and validated *in vitro* method which can replace the whole organism method (replacement);

- the use of sub-lethal endpoints in the method rather than lethal endpoints (refinement).

For currently available *in vivo* methods the key question is whether there are opportunities for addressing the reduction issue.

# APPENDIX B SUMMARIES OF TEST METHODS

**Table B1 Summary of Recommended Test Conditions for Organisation for Economic Co-operation and Development (OECD) Proposed Guideline 210: Fish, Early life stage Toxicity Test**

| Parameter | Recommendation |
|---|---|
| Reference (regulatory body and number) or author | OECD TG 210 |
| Test Species | Rainbow trout (*Oncorhynchus mykiss*)<br>Fathead minnow (*Pimephales promelas*)<br>Zebrafish (*Danio rerio*)<br>Japanese medaka/ricefish (*Oryzinas latipes*)<br>Sheepshead minnow (*Cyprinodon variegatus*)<br>Also potentially<br>Coho salmon (*Oncorhynchus kisutch*)<br>Chinook salmon (*Oncorhynchus tschawytscha*)<br>Brown trout (*Salmo trutta*)<br>Atlantic salmon (*Salmo salar*)<br>Brook trout (*Salvelinus fontinalis*)<br>Lake trout (*Salvelinus namaycush*)<br>Northern pike (*Esox lucius*)<br>White sucker (*Catostomus commersoni*)<br>Bluegill (*Lepomis macrochirus*)<br>Channel catfish (*Ictalurus punctatus*)<br>Flagfish (*Jordanella floridae*)<br>Three-spined stickleback (*Gasterosteus aculeatus*)<br>Common carp (*Cyprinus carpio*)<br>Atlantic silverside (*Menidia menidia*)<br>Tidewater silverside (*Menidia peninsulae*) |
| Test type | Flow-through (preferably) or semi-static |
| Test duration | As soon as possible after fertilisation (early gastrula stage):-<br>• 28 days three-spined stickle back, atlantic silverside and tidewater silverside<br>• 28 days post-hatch common-carp, fathead minnow (32 days total) and sheepshead minnow (32 days total)<br>• 30 days post-hatch zebra fish and ricefish<br>• 32 days total for northern pike, white sucker, bluegill and channel catfish<br>• 60 days post-hatch for rainbow trout (or 2 weeks after controls free feeding), coho salmon, chinook salmon, brown trout, atlantic salmon, brook trout and lake trout |
| Age of test organisms at start of the test | Fertilised eggs |
| Temperature | • Rainbow tout 10 $\pm$ 2°C (embryo)<br>12 $\pm$ 2 °C (larvae & juvenile)<br>• Fathead minnow 25 $\pm$ 2 °C<br>• Zebra fish 25 $\pm$ 2 °C<br>• Ricefish 24$\pm$ 1 °C (embryo)<br>23 $\pm$ 2 °C (larvae & juvenile)<br>• Sheepshead minnow 25 $\pm$ 2 °C<br>• Coho salmon 10 °C (embryo) |

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| | 12 °C (larvae & juvenile)<br>• Chinook salmon 10 °C (embryo)<br>12 °C (larvae & juvenile)<br>• Brown trout 10 °C<br>• Atlantic salmon 10 °C<br>• Brook trout 10 °C<br>• Lake trout 12-18 °C<br>• Northern pike 7 °C<br>• White sucker 15 °C<br>• Bluegill 28 °C<br>• Channel catfish 26 °C<br>• Flagfish 24-26 °C<br>• Three-spined stickleback 18-20 °C<br>• Common carp 21-25 °C<br>• Atlantic silverside 22-25 °C<br>• Tidewater silverside 22-25 °C |
| Light quality and intensity | Not stated |
| Photoperiod | • For rainbow trout, coho salmon, chinook salmon, brown trout, atlantic salmon, brook trout and northern pike darkness for larvae until one week after hatching then subdued lighting throughout test.<br>• 16h light: 8h dark for fathead minnow, lake trout, bluegill, channel catfish and flagfish.<br>• 13h light: 11h dark for atlantic silverside and tidewater silverside.<br>• 12-16h light: 8-12h dark for all other species |
| Test vessel size and test solution volume | Sufficient for a dissolved oxygen volume of 60% ASV to be maintained without aeration. For flow-through tests a loading rate not exceeding 0.5g/l per 24hrs and not exceeding 5g/l of solution at any time has been recommended |
| Renewal of test concentrations | For flow-through tests a flow rate equivalent to at least five test chamber volumes per 24h has been found suitable. Flow rates of stock solutions and dilution waters should not vary by more than 10% throughout the test. For semi-static techniques, two different procedures may be followed. Either new solutions are prepared in clean vessels and surviving eggs and larvae are gently transferred into the new vessels, or the test organisms are retained in the test vessels whilst a proportion 9at least two thirds) of the test water is changed |
| Number of organisms per test vessel | Minimum 30 |
| Replicate test vessels per concentration | Minimum of 2 |
| Number of organisms per concentration | Minimum of 60 |
| Number of concentrations | Normally 5 concentrations and a dilution-water control and a control containing solubility agent (if relevant) |
| Concentration spacing | $\leq$3.2 between concentrations |
| Dilution water | Any clean water in which the test species shows control survival. For marine species the following salinities are required: |

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| | Sheepshead minnow 15-30 $^{o}/_{oo}$ |
| Feeding regime | Food and feeding critical for each stage. Essential correct food is supplied from appropriate time to support normal growth.<br>• Trout food[1] and trout starter[3]; for rainbow trout, coho salmon, chinook salmon, brown trout, atlantic salmon, brook trout and lake trout. No food required for newly-hatched larvae<br>• FBS[1]; BSN[2] and BSN48[3]; for fathead minnows<br>• BSN48, flake food[1]; protozoa, protein[2] and BSN48[3] for zebra fish<br>• Flake food[1]; BSN, flake food (or protozoa or rotifers)[2] and BSN48, flake food (or rotifers)[3] for ricefish<br>• FBS or flake food[1]; BSN[2] and BSN48[3] for sheepshead minnow<br>• Live minnows[1]; BSN48[2] and larval fish[3] for northern pike<br>• FBS[1] and BSN48[3] for white sucker. No food required for newly-hatched larvae<br>• FBS, trout food[1]; BSN[2] and BSN48[3] for bluegill<br>• Catfish food[1] and modified oregon[2,3] for channel catfish<br>• Tetramin FBS[1]; *Brachionus rubens* (rotifer)[2] and BSN48, tetramin[3] for three-spined stickleback<br>• Proprietary carp food, freeze dried tubifex or carp food[1]; BSN[2] and BSN48, ground, trout starter or flake food[3] for common carp<br>• BSN48, flake food[1] and BSN48 and rotifers (*Brachionus plicatilis*)[2,3] for atlantic silverside and tidewater silverside |
| Aeration | Not required |
| Cleaning | Surplus food and faeces should be removed as necessary to avoid accumulation of waste |
| Water quality measurements | During the test, dissolved oxygen, pH, total hardness and salinity (if relevant) should be measured in all test vessels. As a minimum, dissolved oxygen, salinity (if relevant) and temperature should be measured weekly, and pH and hardness at the beginning and end of the test. Temperature should preferably be monitored continuously in at least one test vessel |
| Effects measured | • Time to start of hatching and end of hatching<br>• Numbers of larvae hatching each day<br>• Numbers of deformed larvae<br>• Numbers of healthy fish at end of test<br>• Numbers of fish exhibiting abnormal behaviour<br>• Length and weight of surviving animals<br>• Cumulative mortality |
| Endpoints | LOEC and NOEC (embryonic development and hatching success and mortality) |
| Test validity criteria | • The dissolved oxygen concentration must be between 60 and 100 per cent of the air saturation |

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| | value throughout the test<br>• The water temperature must not differ by more than ±1.5°C between test chambers or between successive days at any time during the test, and should be within the temperature range specified for the test species<br>• Evidence must be available to demonstrate that the concentrations of the test substance in solution have been satisfactorily maintained within ±20% of the mean measured values<br>• Overall survival of fertilised eggs in the controls and, where relevant, in the solvent-only controls must be greater than or equal to the limits defined for the test species<br>• When a solubilising agent is used it must have no significant effect on survival nor produce any other adverse effects on the early-life stages as revealed by a solvent-only control |
| Reference toxicant testing | No requirement stated |
| Requirements for effluents and receiving waters | No reference made to testing effluents and receiving waters |
| Additional comments | - |
| Key reference(s) for further information | OECD (1992) |

**Key:**

[1] Brood fish
[2] Newly-hatched
[3] Juveniles

**Table B2 Summary of Recommended Test Conditions for Organisation for Economic Development and Co-operation (OECD) Proposed Guideline 212: Fish, Short-term Toxicity Test on Embryo and Sac-fry Stages**

| Parameter | Recommendation |
|---|---|
| Reference (Regulatory body and number) or author | Proposed OECD TG 212 |
| Test species | Rainbow trout (*Oncorhynchus mykiss*)<br>Fathead minnow (*Pimephales promelas*)<br>Zebrafish (*Danio rerio*)<br>Common carp (*Cyprinus carpio*)<br>Japanese medaka/ricefish (*Oryzias latipes*)<br>Also potentially<br>Bluegill sunfish (*Lepomis macrochirus*)<br>Goldfish (*Carassus auratus*)<br>Sheepshead minnow (*Cyprinodon variegahs*)<br>Cod (*Gadus morhua*)<br>Herring (*Clupea harengus*)<br>Tidewater silverside (*Menidia peninsulae*) |
| Test type | Static renewal or flow-through |
| Test duration | As soon as possible after fertilisation (early gastrula stage) to:<br>• 3 days post hatch for cod (18 days total) and herring (23-27 days total)<br>• 4 days post hatch for fathead minnow (8-9 days total), common carp (8-9 days total), bluegill sunfish (7 days total) and goldfish (7 days total)<br>• 5 days post hatch for zebrafish (8-10 days total), Japanese medaka (13-16 days total) and tidewater silverside (6-7 days total)<br>• 4-7 days post hatch for sheepshead minnow (28 days total)<br>• 20 days post hatch for rainbow trout (50-55 days total) |
| Age of test organisms at start of the test | Fertilised eggs (30 min after fertilisation) |
| Temperature | • Rainbow trout 10±1°C (embryos), 12±1°C (larvae)<br>• Fathead minnow 25±2°C<br>• Zebrafish 25±1°C<br>• Common carp 21-25°C<br>• Japanese medaka 24±1°C (embryos), 23±2°C (larvae)<br>• Bluegill sunfish 21±1°C<br>• Goldfish 24±1 °C<br>• Sheepshead minnow 25±2°C<br>• Cod 5±1°C<br>• Herring 10±1°C<br>• Tidewater silverside 22-25°C |
| Light quality and intensity | Not stated |
| Photoperiod | For rainbow trout darkness for embryos and larvae until one week after hatching then subdivided lighting 12-16h light: 8-12h dark for all other species |

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| Test vessel size and test solution volume | In semi-static, sufficient for a dissolved oxygen level of 60% ASV to be maintained without aeration. For flow-through, a loading rate not exceeding 0.5g/l per 24 hours and not exceeding 5g/l of solution at any time has been recommended |
| Renewal of test concentrations | Daily as a minimum |
| Number of organisms per test vessel | 10 fertilised eggs |
| Replicate test vessels per concentration | Minimum of 3 |
| Number of organisms per concentration | Minimum of 30 |
| Number of concentrations | Normally five test concentrations and a control |
| Concentration spacing | $\leq$3.2 between concentrations |
| Dilution water | Any clean water in which the test species shows suitable long-term survival and growth. For marine species the following salinities are required:<br>Sheepshead minnow: 15-30 $^{o}/_{oo}$<br>Cod: 5-30 $^{o}/_{oo}$<br>Herring: 8-15 $^{o}/_{oo}$<br>Tidewater silverside: 15-22 $^{o}/_{oo}$ |
| Feeding regime | Feeding not provided |
| Aeration | Not external aeration to be used |
| Cleaning | Not stated |
| Water quality measurements | During the test, dissolved oxygen, pH and temperature should be measured in all test vessels. Total hardness and salinity (if relevant) should be measured in the controls and one vessel at the highest concentration. As a minimum, dissolved oxygen and salinity (if relevant) should be measured three times – at the beginning, middle and end of the test. In semi-static tests, it is recommended that dissolved oxygen be measured more frequently, preferably before and after each water renewal or at least once a week. pH should be measured at the beginning and end of each water renewal in semi-static test and at least weekly in flow-through tests. Hardness should be measured once each test. Temperature should be measured daily and it should preferably be monitored continuously in at least one test vessel |
| Effects measured | • Time to start of hatching and end of hatching<br>• Numbers of larvae hatching each day<br>• Number of healthy larvae at end of test<br>• Numbers of larvae that are deformed or of abnormal appearance<br>• Numbers of larvae exhibiting abnormal behaviour<br>• Length (and weight) of surviving animals at end of test<br>• Cumulative mortality |
| Endpoints | NOEC, LOEC and $LC_{50}$ (hatching success and mortality);<br>NOEC, LOEC and $EC_{50}$ (morphological abnormalities and growth as length and weight) |
| Test validity criteria | • Overall survival of fertilised eggs in the controls and, where relevant, in the solvent-only vessels must be greater than or equal to the limits defined |

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| | in Annexes 3 and 4;<br>• The dissolved oxygen concentration must be between 60 and 100 per cent of the air saturation value (ASV) throughout the test;<br>• The water temperature must not differ by more than $\pm 1.5^{o}C$ between the test chambers or between successive days at any time during the test, and should be within the temperature ranges specified for the test species |
| Reference toxicant testing | No requirement stated |
| Requirements for effluents and receiving waters | No reference made to testing effluents and receiving waters |
| Additional comments | - |
| Key reference(s) for further information | OECD (1997a) |

**Table B3 Summary of Recommended Test Conditions for International Standards Organisation (ISO) Method 12890: Water quality – Determination of embryo-larval toxicity to freshwater fish – Semi – static method**

| Parameter | Recommendation |
|---|---|
| Reference (Regulatory body and number) or author | ISO 12890 |
| Test species | Zebrafish (*Danio rerio*)<br>Method can be used for other freshwater fish if appropriate modifications are made to test conditions |
| Test type | Semi-static |
| Test duration | 10 days (can be prolonged to 14 days in order to increase sensitivity of test, when at least 90% of larvae in all test solutions have died) |
| Age of test organisms at start of the test | Newly fertilised eggs (2-4 hours after spawning) |
| Temperature | $26 \pm 2^{o}C$ |
| Light quality and intensity | Normal laboratory illumination |
| Photoperiod | Normal laboratory illumination with 12h light: 12h dark; 14h light: 10h dark or 16h light: 8h dark |
| Test vessel size and test solution volume | 100ml shallow petri dish, inner diameter 100mm, with cover; containing 25ml test solution |
| Renewal of test concentrations | Daily |
| Number of organisms per test vessel | 15<br>Day 0 transfer 15 eggs to each dish; after 24h record number of dead embryos in each dish and reduce number of viable eggs per dish to a maximum of 10 when transferring to new solutions. Determination of median times for hatching and survival shall only be based on these remaining 10 individuals, which are 1 day old. |
| Replicate test vessels per concentration | Minimum 2 (exposure concentration); minimum 4 (controls) |
| Number of organisms per concentration | Minimum 30 (exposure concentrations); minimum 60 (controls) |
| Number of concentrations | Control and minimum of 6, where at least the two highest concentrations give a significant effect on hatching or survival and the lowest concentration produces no significant effect |
| Concentration spacing | Geometric spacing<br>eg. 2, 1, 1/2, 1/4, 1/8 and 1/16 x 96h $LC_{50}$ for test according to ISO 7346-1, 2, or 3 – 1996<br>If suspected the tested sample contains substances for which the toxic effect is delayed the number of concentrations shall be increased by several lower concentrations (1/32, 1/64, 1/128, 1/256, 1/512 x 96h $LC_{50}$)<br>Lower concentrations must be tested if the lowest concentration produces any kind of effect relative to the controls |
| Dilution Water | Prepared 1-7 days before use<br>pH 7.5 $\pm$ 0.2 and hardness corresponding to (100 $\pm$ 10) |

| | |
|---|---|
| | mg $CaCO_3$ $l^{-1}$, prepared as follows:<br>• Dissolve 11.76g $CaCl_2 2H_2O$ in water, make up to 1l with water<br>• Dissolve 4.93g $MgSO_4 7H_2O$ in water, make up to 1l with water<br>• Dissolve 2.59g $NaHCO_3$ in water, make up to 1l with water<br>• Dissolve 0.23g KCl in water, make up to 1l with water<br>Add 100ml of each solution to 5l of water and dilute to a total volume of 10l. Aerate the dilution water through a glass tube until concentration of dissolved oxygen reaches 90-100% of air saturation at 26°C. Stabilise pH, if required, using appropriate dilutions of hydrochloric acid or sodium hydroxide. No further aeration is required before use |
| Feeding Regime | Not required |
| Aeration | Not required |
| Cleaning | Not stated |
| Water quality measurements | At start of test (day 0) measure the oxygen concentration, pH and temperature in all the samples and control solutions. Levels obtained should be within those for the test validity criteria. Measure the oxygen content, pH and temperature in both the new and old solutions on subsequent days. Check controls and samples with highest and lowest concentrations first. If the difference in values are large between these solutions then all solutions shall be measured |
| Effects measured | • Number of dead and living eggs and larvae when transferring to new solutions daily<br>• Numbers of eggs and hatched larvae every morning and afternoon (record exact time) on second, third and fourth days<br>• Numbers of surviving larvae after 10 days |
| Endpoints | NEC (determined from concentration effect relationships for hatching and survival)<br>$EC_{xs}$ can be determined and NOEC and LOEC evaluated in relation to controls |
| Test validity criteria | • Concentration of dissolved oxygen has been maintained between 70 and 110% of the air saturation value for dilution water at 26°C<br>• pH in all fresh solutions has been $7.5 \pm 0.2$<br>• temperature in the test solutions has been maintained at $(26 \pm 2)$ °C<br>• more than 70% of the embryos (eggs) in the controls were alive after 24h<br>• the median for hatching was 2-4 days in the controls<br>• the proportion of surviving larvae in the controls after 10 days was >90%<br>• if the test is prolonged the median time for survival in the controls shall be 12-16 days |
| Reference toxicant testing | No requirement stated. Data available for potassium |

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| | dichromate and zinc |
| Requirements for effluents and receiving waters | Reference made to testing effluents and receiving waters |
| Additional comments | - |
| Key reference(s) for further information | ISO (1997) |

**Table B4 Summary of Recommended Test Conditions for United States Environmental Protection Agency Method 1000.0: Fathead minnow (*Pimephales promelas)* Larval Survival and Growth Test**

| Parameter | Recommendation |
|---|---|
| Reference (Regulatory body and number) or author | US EPA 1000.0 |
| Test species | Fathead minnow (*Pimephales promelas*) |
| Test type | Static renewal |
| Test duration | 7 days |
| Age of test organisms at start of the test | Newly hatched larvae (preferably <24 h old) |
| Temperature | 25 $\pm$ 2°C |
| Light quality and intensity | Ambient laboratory illumination - 10-20uE/$m^2$/s (50-100 ft-c) |
| Photoperiod | 16 h light, 8 h darkness |
| Test vessel size | 1-litre containers |
| Test solution volume | 500ml per replicate |
| Renewal of test concentrations | Daily |
| Number of organisms per test vessel | 10 larvae |
| Replicate test vessels per concentration | Minimum of two |
| Number of organisms per concentration | Minimum of 20 larvae per concentration |
| Number of concentrations | At least five test concentrations and a control |
| Concentration spacing | Approximately 3.2 (1, 3, 10, 30, 100%) or 2 (0.8, 1.6, 3.2, 6.2, 12.5, 25, 50, 100%) |
| Dilution Water | Moderately hard standard water, receiving water, other surface water, ground water, or synthetic water similar to receiving water |
| Feeding Regime | 0.1ml of newly hatched brine shrimp nauplii fed 3 times daily, 4 h between feeding (at the beginning, midway and end of work day) |
| Aeration | None, unless dissolved oxygen concentration falls below 40% saturation. Rate should be less than 100 bubbles per minute |
| Cleaning | Siphon daily, immediately before test solution renewal |
| Water quality measurements | |
| Effects measured | ▪ Growth (as increase in weight)<br>▪ Survival |
| Endpoints | NOEC and LOEC for growth<br>$LC_1$, NOEC and LOEC for survival |
| Test validity criteria | Survival in the controls $\geq$ 80%, except where survival in any test concentration is 80% or better |
| Reference toxicant testing | Potassium chloride |
| Requirements for effluents and receiving waters | Reference made to testing effluents and receiving waters |
| Additional comments | - |
| Key reference(s) for further information | US EPA (1995b) |

**Table B5 Summary of Recommended Test Conditions for United States Environmental Protection Agency Method 1001.0: Fathead minnow (*Pimephales promelas)* Embryo-Larval Survival and Teratogenicity Test**

| Parameters | Recommendations |
|---|---|
| Reference (Regulatory body and number) or author | US EPA 1001.0 |
| Test species | Fathead minnow (*Pimephales promelas*) |
| Test type | Static renewal |
| Test duration | 8 days |
| Age of test organisms at the start of the test | 2 – 24 h old embryos; preferably less than 12 h old |
| Temperature | 25 $\pm$ 2°C |
| Light quality and intensity | Ambient laboratory illumination - 10-20 uE/$m^2$/s, or 50-100 ft-c |
| Photoperiod | 16 h light, 8 h darkness |
| Test vessel size | 500ml |
| Test solution volume | 400ml |
| Renewal of Test Concentrations | Daily |
| Number of organisms per test vessel | 20 to 50 |
| Replicate test vessels per concentration | Two |
| Number of organisms per concentration | 40 to 100 |
| Number of concentrations | Five test concentrations and a control |
| Concentration spacing | Approximately 3.2 (1, 3, 10, 30, 100%) or 2 (0.8, 1.6, 3.2, 6.2, 12.5, 25, 50, 100%) |
| Dilution Water | Hardness greater than 25 mg/l ($CaCO_3$); receiving water or other surface water, ground water, or synthetic water if similar to receiving water |
| Feeding Regime | Feeding not provided |
| Aeration | None, unless dissolved oxygen falls below 40% saturation. |
| Cleaning | Siphon daily, immediately before test solution renewal |
| Water Quality measurements | |
| Effects measured | • Percent hatch<br>• Percent larvae with terata,<br>• Percent of normal larvae surviving 4 days post-hatch;<br>• Surviving normal larvae from original embryos |
| Endpoints | NOEC and LOEC for growth<br>$LC_1$, NOEC and LOEC for survival |
| Test validity criteria | Survival in the controls $\geq$ 80%, except where survival in any test concentration is 80% or better |
| Reference toxicant testing | |
| Requirements for effluents and receiving waters | Reference made to testing effluents and receiving waters |
| Additional comments | - |
| Key reference(s) for further information | US EPA (1995b) |

**Table B6 Summary of Recommended Test Conditions for United States Environmental Protection Agency Method 1004.0: Sheepshead minnow (*Cyprinodon variegatus)* Larval Survival and Growth Test**

| Parameter | Recommendation |
|---|---|
| Reference (Regulatory body and number) or author | US EPA 1004.0 |
| Test species | Sheepshead minnow (*Cyprinodon variegatus)* |
| Test type | Static renewal |
| Test duration | 7 days |
| Age of test organisms at start of the test | Newly hatched larvae (preferably <24 h old) |
| Temperature | 25 $\pm$ 2°C |
| Light quality and intensity | Ambient laboratory illumination - 10-20uE/$m^2$/s (50-100 ft-c) |
| Photoperiod | 16 h light, 8 h darkness |
| Test vessel size | 1-litre containers |
| Test solution volume | 500ml per replicate |
| Renewal of test concentrations | Daily |
| Number of organisms per test vessel | 10 larvae |
| Replicate test vessels per concentration | Minimum of two |
| Number of organisms per concentration | Minimum of 20 larvae per concentration |
| Number of concentrations | At least five test concentrations and a control |
| Concentration spacing | Approximately 3.2 (1, 3, 10, 30, 100%) or 2 (0.8, 1.6, 3.2, 6.2, 12.5, 25, 50, 100%) |
| Dilution Water | |
| Feeding Regime | 0.1ml of newly hatched brine shrimp nauplii fed 3 times daily, 4 h between feeding (at the beginning, midway and end of work day) |
| Aeration | None, unless dissolved oxygen concentration falls below 40% saturation. Rate should be less than 100 bubbles per minute |
| Cleaning | Siphon daily, immediately before test solution renewal |
| Water quality measurements | |
| Effects measured | ▪ Growth (as increase in weight)<br>▪ Survival |
| Endpoints | NOEC and LOEC for growth<br>$LC_1$, NOEC and LOEC for survival |
| Test validity criteria | Survival in the controls $\geq$ 80%, except where survival in any test concentration is 80% or better |
| Reference toxicant testing | |
| Requirements for effluents and receiving waters | Reference made to testing effluents and receiving waters |
| Additional comments | - |
| Key reference(s) for further information | US EPA (1995a) |

**Table B7 Summary of Recommended Test Conditions for United States Environmental Protection Agency Method 1005.0: Sheepshead minnow (*Cyprinodon variegatus)* Embryo-Larval Survival and Teratogenicity Test**

| Parameters | Recommendations |
|---|---|
| Reference (Regulatory body and number) or author | US EPA 1005.0 |
| Test species | Sheepshead minnow (*Cyprinodon variegatus)* |
| Test type | Static renewal |
| Test duration | 8 days |
| Age of test organisms at the start of the test | 2 – 24 h old embryos; preferably less than 12 h old |
| Temperature | 25 $\pm$ 2°C |
| Light quality and intensity | Ambient laboratory illumination - 10-20 uE/$m^2$/s, or 50-100 ft-c |
| Photoperiod | 16 h light, 8 h darkness |
| Test vessel size | 500ml |
| Test solution volume | 400ml |
| Renewal of Test Concentrations | Daily |
| Number of organisms per test vessel | 20 to 50 |
| Replicate test vessels per concentration | Two |
| Number of organisms per concentration | 40 to 100 |
| Number of concentrations | Five test concentrations and a control |
| Concentration spacing | Approximately 3.2 (1, 3, 10, 30, 100%) or 2 (0.8, 1.6, 3.2, 6.2, 12.5, 25, 50, 100%) |
| Dilution Water | |
| Feeding Regime | Feeding not provided |
| Aeration | None, unless dissolved oxygen falls below 40% saturation. |
| Cleaning | Siphon daily, immediately before test solution renewal |
| Water Quality measurements | |
| Effects measured | • Percent hatch<br>• Percent larvae with terata,<br>• Percent of normal larvae surviving 4 days post-hatch;<br>• Surviving normal larvae from original embryos |
| Endpoints | NOEC and LOEC for growth<br>$LC_1$, NOEC and LOEC for survival |
| Test validity criteria | Survival in the controls $\geq$ 80%, except where survival in any test concentration is 80% or better |
| Reference toxicant testing | |
| Requirements for effluents and receiving waters | Reference made to testing effluents and receiving waters |
| Additional comments | - |
| Key reference(s) for further information | US EPA (1995a) |

**Table B8 Summary of Recommended Test Conditions for United States Environmental Protection Agency Method 1006.0: Inland silverside (*Menidia beryllina)* Larval Survival and Growth Test**

| Parameters | Recommendations |
|---|---|
| Reference (Regulatory body and number) or author | US EPA 1006.0 |
| Test species | Inland silverside (*Menidia beryllina)* |
| Test type | Static renewal |
| Test duration | 8 days |
| Age of test organisms at the start of the test | 2 – 24 h old embryos; preferably less than 12 h old |
| Temperature | 25 $\pm$ 2$^{o}$C |
| Light quality and intensity | Ambient laboratory illumination - 10-20 uE/m$^2$/s, or 50-100 ft-c |
| Photoperiod | 16 h light, 8 h darkness |
| Test vessel size | 500ml |
| Test solution volume | 400ml |
| Renewal of Test Concentrations | Daily |
| Number of organisms per test vessel | 20 to 50 |
| Replicate test vessels per concentration | Two |
| Number of organisms per concentration | 40 to 100 |
| Number of concentrations | Five test concentrations and a control |
| Concentration spacing | Approximately 3.2 (1, 3, 10, 30, 100%) or 2 (0.8, 1.6, 3.2, 6.2, 12.5, 25, 50, 100%) |
| Dilution Water | |
| Feeding Regime | Feeding not provided |
| Aeration | None, unless dissolved oxygen falls below 40% saturation. |
| Cleaning | Siphon daily, immediately before test solution renewal |
| Water Quality measurements | |
| Effects measured | ▪ Growth (as increase in weight)<br>▪ Survival |
| Endpoints | NOEC and LOEC for growth<br>LC1, NOEC and LOEC for survival |
| Test validity criteria | Survival in the controls $\geq$ 80%, except where survival in any test concentration is 80% or better |
| Reference toxicant testing | |
| Requirements for effluents and receiving waters | Reference made to testing effluents and receiving waters |
| Additional comments | - |
| Key reference(s) for further information | US EPA (1995a) |

**Table B9 Summary of Recommended Test Conditions for Environment Canada Fathead Minnow (*Pimephales promelas*) Larval Growth and Survival Test**

| Parameter | Recommendations |
|---|---|
| Reference (Regulatory body and number) or author | Environment Canada EPS1/RM/22 |
| Test species | Fathead minnow (*Pimephales promelas*) |
| Test type | Static, renewal (volatile/unstable chemicals may require use of flowthrough tests) |
| Test duration | 7 days |
| Age of test organisms at start of test | Newly hatched larvae (preferably <24 h old) |
| Temperature | 25 $\pm$ 1°C, extreme fluctuations within range 23 to 27 °C |
| Light quality and intensity | At water surface, $\leq$500 lux |
| Photoperiod | 16 $\pm$ 1 h light : 8 $\pm$ 1 h dark, preferably with gradual transition & preferably supplied by full-spectrum fluorescent lights |
| Test vessel size | Depth $\geq$3 cm, $\approx$ diameter |
| Test solution volume | $\geq$250 ml, preferably 500 ml |
| Number of organisms per test vessel | Minimum 10 larvae |
| Replicate test vessels per concentration | Minimum of 3 replicate test vessels per test concentration, 4 replicates recommended |
| Number of organisms per concentration | Miniumum of 30 larvae |
| Number of concentrations | |
| Concentration spacing | |
| Dilution water | Ground, surface, or if necessary, dechlorinated municipal water; "upstream" water to assess toxic impact at specific location*; reconstituted water if requiring high degree of standardisation; DO to 100% saturation at time of use. |
| Feeding Regime | 2 or 3 times/day with newly-hatched brine shrimp nauplii; feed at the start of the test but do not feed during the final 12 h |
| Aeration | No pre-aeration unless a test solution has DO <40% or >100% saturation upon preparation, in which case aerate all test solutions for $\leq$20 mins at minimal rate before starting test or renewing solution; DO 40 to 100% saturation throughout test, with more frequent renewal if required to maintain DO; if necessary to meet objectives of test, gentle aeration of all vessels |
| Water quality measurements | Temperature, pH & DO at start & end of 24 h periods, representative concentrations; conductivity at least at start of 24 h periods; hardness of control/ dilution water & highest concentration at start of test |
| Effects measured | • Mortality,<br>• Swimming behaviour<br>• Mean dry weight after 7 days |
| Endpoints | NOEC/LOEC and/or ICp for growth, mortality; if appropriate, $LC_{50}$ at selected time(s) |
| Test Validity | Invalid if >20% of control fish die or exhibit clearly |

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| | atypical swimming behaviour, or if average weight of control fish is not ≥250µg; validity & usefulness of test is questionable if the Minimum Significant Difference of Weight is >20% of the mean control dry weight |
| Reference Toxicant | Sodium chloride, phenol and/or zinc; test for NOEC/LOEC and/or ICp, monthly |
| Requirements for effluents and receiving waters | Reference made to testing effluents and receiving waters |
| Additional comments | - |
| Key reference(s) for further information | Environment Canada (1992) |

**Table B10 Summary of Recommended Test Conditions for Environment Canada Rainbow Trout (*Oncorhynchus mykiss*) Early Life Stage Test**

| Parameter | Recommendations |
|---|---|
| Reference (Regulatory body and number) or author | Environment Canada EPS1/RM/28 |
| Test species | Rainbow trout *(Oncorhynchus mykiss)* |
| Test type | Static-renewal or flow-through. |
| Test options | Embryo test (*E test)* for frequent or periodic testing,<br>Embryo/alevin test *(EA test)* for measuring effects on multiple developmental stages,<br>Embryo/alevin/swim-up fry test *(EAF test)* for definitive investigations |
| Test duration | *For E test:* 7 days after fertilization<br>*For EA test:* 7 days after half of eggs in control are seen to have hatched,<br>*For EAF test:* 30 days after half of the surviving fish in control show swim-up behaviour |
| Age of organisms at start of test | Within 30 minutes immediately following period of 5 to 20 minutes for dry fertilization of eggs |
| Temperature | Daily mean of 14 $\pm$ 1°C throughout test, for E, EA, or EAF test |
| Light quality and intensity | Controlled at water surface, 100 to 500 lux |
| Photoperiod | Dark until one week after hatching is completed, with dim or red light during solution renewals; 16 $\pm$ 1 h light : 8 $\pm$ 1 h dark, preferably with gradual transition & preferably using full-spectrum fluorescent lights or equivalent |
| Test vessel size | For embryos & alevins, an 800ml plastic beaker with solid bottom & slits in side, suspended in plastic pail or glass aquarium (the test chamber),<br>For swim-up fry, a plastic pail or glass aquarium |
| Renewal of test concentrations | For embryos & alevins with static- renewal or flow-through replacement of test solutions at $\geq$0.5 L/g.d,<br>For swim-up fry with either static-renewal or flow-through replacement of solutions at $\geq$0.5 L/g.d |
| Number of organisms per test vessel | For *E test*, $\geq$ 120 embryos per concentration including the control,<br>For *EA test* or *EAF test*, 120 to 320 embryos/concentration; $\geq$3 replicates for standard point-estimation techniques (i.e. at least 40 embryos in each of 3 replicates in the E test); if hypotheses testing is to be done, $\geq$4 replicates/ concentration needed if parametric analysis proved to be invalid & non-parametric analysis required (i.e. $\geq$30 embryos in each of 4 replicates); $\geq$1 incubation unit/test chamber, the chamber being a replicate |
| Replicate test vessels per concentration | |
| Number of organisms per concentration | |
| Number of concentrations | At least 5 test concentrations and a control |
| Dilution factor | |
| Control/Dilution Water | Ground, surface, reconstituted, or if necessary, dechlorinated municipal water; "upstream" water to |

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| | assess toxic effect at a specific location. |
| Feeding Regime | *For E & EA tests:* no feeding<br>*For EAF test:* feed fry 4% body wt/d with commercial starter feed, ≥4 times/d, starting when half of surviving control fish show swim-up behaviour, continuing for a 30-d exposure, but without feed in final 24 h of exposure |
| Aeration | Control/dilution water 90 to 100% DO saturation before use; normally no pre-aeration unless sample or test solution has do<60% or >100% upon preparation, in which case pre-aerate sample or all solutions for 30 minutes & if necessary for additional period of ≤90 minutes, at 6.5 ± 1 ml/min.L; if static-renewal test, gentle aeration; if flow-through test, aerate if necessary or desired to maintain DO at 60% to 100% saturation, and/or increase rate of exchange |
| Cleaning | |
| Water quality measurements | Temperature, pH & DO in representative concentrations, at start & end of 24 h periods in static-renewal, or daily in flow-through tests; optionally, conductivity of each new test solution before dispensing |
| Effects measured | ▪ *For E test:* percent nonviable embryos at test end<br>▪ *For EA test:* percent nonviable alevins, & narrative statements on delayed hatching & deformed alevins;<br>▪ *For EAF test:* percent nonviable individuals at swim-up, mortality of fry during final 30 days, average dry weight of surviving fry at test end, & narrative statements on delayed hatching, deformed alevins, delayed swim-up, & abnormal behaviour of fry |
| Endpoints | *For E test:* $EC_{50}$ and/or $EC_{25}$ for nonviable embryos,<br>*For EA test:* $EC_{50}$ and/or $EC_{25}$ for nonviable alevins (failure to reach alevin stage); narrative statements on delayed hatching & deformed alevins,<br>*For EAF test:* $EC_{50}$ and/or $EC_{25}$ for nonviable individuals at swim-up (failure to survive at any stage up to time of early swim-up); $LC_{50}$ for swim-up; $IC_{25}$ for average dry weight of surviving swim-up fry at test end; narrative statements on deformed alevins, delayed swim-up, & abnormal behaviour of fry |
| Test Validity | Invalid if any of following occurs:<br>*For E test:* >30% of controls nonviable at end of test<br>*For EA test:* >35% of controls nonviable at end of test<br>*For EAF test:* >40% of controls nonviable at time of 50% swim-up of survivors |
| Reference Toxicant | Phenol and/or zinc; perform as an E test at time that each E, EA, or EAF test is initiated, using a portion of the same batch of fertilized eggs used to start the definitive test; use procedures described herein for performing an E test with a chemical; determine EC50 |
| Requirements for effluents and receiving waters | Reference made to testing effluents and receiving waters |
| Additional comments | - |
| Key reference(s) for further information | Environment Canada (1998) |

**Table B11 Summary of Recommended Test Conditions for Organisation for Economic Co-operation and Development (OECD) Proposed Guideline 215: Fish, Juvenile Growth Test.**

| Parameter | Recommendation |
|---|---|
| Reference (Regulatory body and number) or author | Proposed OECD 215 |
| Test species | Rainbow trout (*Oncorhynchis mykiss*), zebrafish (*Danio rerio*) or Japanese medaka/ricefish (*Oryzias latipes*) |
| Test type | Static, renewal or preferably flow-through |
| Test duration | 28 days for rainbow trout, ≥28 days for zebrafish or Japanese medaka |
| Age of test organisms at start of the test | 1-5g for rainbow trout and 0.05-0.1g for zebrafish and Japanese medaka |
| Temperature | 12.5-16$^{o}$C for rainbow trout, 21-25 $^{o}$C for zebrafish and Japanese medaka |
| Light quality and intensity | Not stated |
| Photoperiod | 12-16h light : 8-12h dark |
| Test vessel size and test solution volume | Sufficient for a loading rate of 1.2-2.0 g/l rainbow trout and 0.5-2.0 g/l zebrafish or Japanese medaka |
| Renewal of test concentrations | Daily as a minimum |
| Number of organisms per test vessel | 10 |
| Replicate test vessels per concentration | Minimum of 1 |
| Number of organisms per concentration | Minimum of 10 |
| Number of concentrations | At least five test concentrations and a control |
| Concentration spacing | An appropriate gas metre or preferably logarithmic series should be used |
| Dilution water | Any clean water in which the test species shows suitable long-term survival and growth |
| Feeding regime | Daily (Minimum ratios 2% body weight per day, preferably 4% body weight per day) |
| Aeration | Dissolved oxygen >60% ASV throughout the test |
| Cleaning | Daily, siphon uneaten food and faecal material |
| Water quality measurements | During the test, dissolved oxygen, pH and temperature should be measured in all test vessels. Total hardness, alkalinity and salinity (if relevant) should be measured in the controls and one vessel at the highest concentration. As a minimum, dissolved oxygen and salinity (if relevant) should be measured three times – at the beginning, middle and end of the test. In semi-static tests, it is recommended that dissolved oxygen be measured more frequently, preferably before and after each water renewal or at least once a week. pH should be measured at the beginning and end of each water renewal in static renewal test and at least weekly in flow-through tests. Hardness and alkalinity should be measured once each test. Temperature should be preferably be monitored continuously in at least one test vessel |
| Effects measured | ▪ Growth (as increase in weight) and. Fish weighed |

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| | at day 0 and 28 and possibly 14 days<br>▪ Survival |
| Endpoints | NOEC and LOEC and ECp for growth |
| Test validity criteria | • Mortality in the control(s) must not exceed 10% at the end of the test;<br>• The mean weight of fish in the control(s) must have increased enough to permit the detection of the minimum variation of growth rate considered as significant. A ring-test (2) has shown that for rainbow trout the mean weight of fish in the controls must have increased by at least the half (i.e. 50%) of their mean initial weight over 28 days; e.g. initial weight: 1g/fish (=100%), final weight after 28 days: $\geq$ 1.5g/fish ($\geq$ 150%);<br>• The dissolved oxygen concentration must have been at least 60% of the air saturation value (ASV) throughout the test;<br>• The water temperature must not differ by more than $\pm 1^{o}C$ between test chambers at any one time during the test and should be maintained within a range of $2^{o}C$ within the temperature ranges specified for the test species |
| Reference toxicant testing | None specified. |
| Requirements for effluents and receiving waters | No reference made to testing effluents and receiving waters |
| Additional comments | - |
| Key reference(s) for further information | OECD (1997b) |

**Table B12 Summary of Recommended Test Conditions for International Standards Organisation (ISO) Method 10229: Water quality – Section 5.17 Determination of prolonged toxicity of substances to freshwater fish – Method for evaluating the effects of substances on the growth rate of rainbow trout**

| Parameter | Recommendation |
|---|---|
| Reference (Regulatory body and number) or author | ISO 10229 |
| Test species | Rainbow trout (*Oncorhynchus mykiss*)<br>Method can be adapted for use with other freshwater fish and marine and brackish water fish with appropriate modification of test conditions |
| Test type | Semi-static and flow-through |
| Test duration | 28 days total |
| Age of test organisms at start of the test | Mass of not less than 3g and not more than 5g.<br>For the whole batch of fish used in the test, the range in individual masses at the start of the test shall lie within ±10% of the arithmetic mean of the masses |
| Temperature | 12.5 – 17.5°C ± 1 °C |
| Light quality and intensity | Normal laboratory illumination |
| Photoperiod | Normal laboratory illumination with photoperiod of 12h light: 16h dark |
| Test vessel size and test solution volume | Vessels with capacity of at least 45l containing 40l of test solution or control |
| Renewal of test concentrations | Minimum rate of 200 l/day continuously or by additions at short intervals. If fish of initial mass >5g are used, test shall not be invalidated but the rate of replacement increased so that, in all cases, the dissolved oxygen concentration remains >70% ASV and conditions concerning concentrations of test substances are met. Concentrations of stock solution in test vessels are to maintained within 10% of the mean |
| Number of organisms per test vessel | 16<br>Mark fish individually using freeze branding or other methods, provided they do not interfere with the test |
| Replicate test vessels per concentration | 1 |
| Number of organisms per concentration | 16 |
| Number of concentrations | Minimum of 5 test concentrations and a control. Prepare a second control if an organic solvent has been used to dissolve or disperse a substance, with the dilution water containing sufficient of the organic solvent to give the maximum concentration at which this solvent is present in the test solutions |
| Concentration Spacing | Not exceed √10(3.162) |
| Dilution Water | Suitable for long-term survival and growth of the test fish. Average pH shall be within the range 6.7-8.5, but not vary by more than ±0.2 pH units from the mean value during a given test |
| Feeding Regime | Fish shall be a fed minimum rate of 1% wet body mass per day during pre-acclimation and acclimation, and 4% of their mass per day during the test. Food shall be |

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| | a dry proprietary salmonid fry food and shall be divided into two equal portions and given to the fish in two feeds per day, separated by a minimum of 5h. After 14 days, when fish are re-weighed, rations are recalculated for each test vessel |
| Aeration | Continuous aeration to prevent DO falling below 70% ASV |
| Cleaning | Faecal material and uneaten food removed daily |
| Water quality measurements | DO concentration, pH and temperature of the solution from each of the test vessels at least once daily and at the beginning and end of the test. Measure the concentrations of the test substances in the solutions leaving the test vessels at least at the beginning, middle and end of the test |
| Effects measured | • Individual growth rate of fish (mass and length) or mean growth of each group of fish at 14d and 28d<br>• Cumulative mortalities<br>• Abnormal behaviour monitored daily |
| Endpoints | LOEC and NOEC (if individual growth rates have not been measured the mean specific growth rate can be used to calculate the IC10 which can be taken as an approximation of the LOEC) |
| Test validity criteria | • The DO concentration in the test solutions during the test was at least 70% ASV<br>• The temperature was in the range 12.5-17.5$^{o}$C and did not vary by more than 2 $^{o}$C<br>• The concentration of the test substance were known to have remained within $\pm$20% of the median value throughout the test<br>• The mortality of the control fish did not exceed 10% |
| Reference toxicant testing | No requirement stated |
| Requirements for effluents and receiving waters | Reference made to testing effluents and receiving waters |
| Additional comments | - |
| Key reference(s) for further information | ISO (1994) |

**Table B13 Summary of Recommended Test Conditions for Turbot (*Scophthalmus maximus*) juvenile growth test**

| Parameter | Recommendation |
|---|---|
| Reference (Regulatory body and number or author) | Based on US EPA larval growth study - AstraZeneca protocol |
| Test species | Turbot (*Scophthalmus maximus)* |
| Test type | Short-term growth assay |
| Test duration | 14 days |
| Age of test organisms at start of the test | Juvenille Turbot < 1g |
| Temperature | 15 ± 1 Cº |
| Light quality and intensity | Standard laboratory lighting approximately 500 lux |
| Photoperiod | 16h light 8 h dark with dawn dusk transition period |
| Test vessel size | 2 l beaker |
| Test solution volume | 2000 ml |
| Renewal of test concentrations | Semi static minimum change every 48 h |
| Number of organisms per test vessel | 10 |
| Replicate test vessels per concentration | 4 |
| Number of organisms per concentration | 40 |
| Number of concentrations | Dependant on resources available |
| Concentration spacing | Logarithmic scale |
| Dilution water | Sea water filtered to 5 µm |
| Feeding regime | 2 feeds daily: live brine shrimp and micro encapsulated food. Operator judgement of quantities *ad libitum* |
| Aeration | None |
| Cleaning | Daily siphoning and test solution renewal (could be reduced to 48 h renewal) |
| Water quality measurements | pH, DO and temperature daily. Daily salinity measurement of seawater supply |
| Effects measured | Growth and survival |
| Endpoints | Standard length and wet weight |
| Test validity criteria | <10% mortality in control |
| Reference toxicant testing | None (used for in house effluent assessment) |
| Requirements for effluents and receiving waters | Reference made to testing effluents and receiving waters |
| Additional comments | - |
| Key reference(s) for further information | US EPA (1995a) Short-term methods for estimating the chronic toxicity of effluents and receiving water to marine and estuarine organisms |

**Table B14 Summary of Recommended Test Conditions for Fathead minnow (*Pimephales promelas*) reproduction test**

| Parameter | Recommendation |
|---|---|
| Reference (Regulatory body and number or author) | Harries *et al* (2000), Ankley *et al* (2001) |
| Test species | Fathead minnow (*Pimephales promelas*) |
| Test type | Short-term reproduction test |
| Test duration | 42 days (Pre-exposure period 21 d, exposure period 21 d) |
| Age of test organisms at start of the test | Sexually mature adults >90 days post hatch |
| Temperature | 25 ± 1 Cº |
| Light quality and intensity | Standard laboratory lighting approximately 500 lux |
| Photoperiod | 16h light 8 h dark with dawn dusk transition period |
| Test vessel size | 12 l (approximate dimensions: 20cm x 20cm x 30cm) |
| Test solution volume | 9.5 l (under consideration, likely to increase) |
| Renewal of test concentrations | Continuous flow through at least 7 tank replacements per day |
| Number of organisms per test vessel | 2 (paired) or group breeding |
| Replicate test vessels per concentration | 6 |
| Number of organisms per concentration | 12 |
| Number of concentrations | Dependant on resources available |
| Concentration spacing | Halving steps or logarithmic scale |
| Dilution water | Dechlorinated water filtered to 5 µm |
| Feeding regime | 3 feeds daily: frozen brine shrimp and pellets. Operator judgement of quantities *ad libitum* |
| Aeration | None |
| Cleaning | Twice weekly scrapping and siphoning |
| Water quality measurements | Temperature biweekly; pH, DO, Hardness, conductivity, alkalinity and chlorine weekly. (Continuous temperature monitoring used at AstraZeneca) |
| Effects measured | Reproduction, hatch success and VTG. |
| Endpoints | Reproductive performance: number of spawnings/total egg number and fecundity. $F_1$ hatchability. Assessment of behaviour and secondary sexual characteristics eg fat pads. Optional measurement of gonadal somatic index and plasma vitellogenin level |
| Test validity criteria | Statistically robust results |
| Reference toxicant testing | Ethynyloestradiaol, Oestradiol |
| Requirements for effluents and receiving waters | Reference made to testing effluents and receiving waters |
| Additional comments | - |
| Key reference(s) for further information | Harries *et al* (2000) Development of a reproductive performance test for endocrine disrupting chemicals using pair-breeding fathead minnows (*Pimephales promelas*). *Environmental Science and Technology*, 34, 3003-3011)<br>Ankley *et al* (2001) Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*) *Environmental Toxicology and Chemistry*, **20 (6)**, 1276-1290. |

# APPENDIX C DATASHEETS PROVIDING SUMMARY INFORMATION AGAINST SELECTION CRITERIA

**Table C1 Summary data for fish, early-life stage toxicity test (OECD TG 210)**

| Category and criterion | | Type of criteria | Assessment against criteria (see Table 2.13) | Data summary (where relevant) |
|---|---|---|---|---|
| No. | Description | | | |
| ***1*** | ***Relevance of test data*** | | | |
| 1.1 | Use of ecologically relevant test species | *High* | *Yes* | - |
| 1.2 | Importance of test species | Low | Yes (for some test species) | - |
| 1.3 | Diversity of endpoints | **Key** | **Yes** | - |
| 1.4 | Ecological relevance of test endpoint(s) | **Key** | **Yes** | - |
| 1.5 | Extrapolation to population effects | **Key** | **Yes** | - |
| ***2*** | ***Test method variability*** | | | |
| 2.1 | Test method repeatability | **Key** | **No data** | - |
| 2.2 | Test method reproducibility | **Key** | **No data** | - |
| 2.3 | Availability of a validated method describing the procedure | **Key** | **Yes** | - |
| 2.4 | Test organism variability | *High* | *Yes* | - |
| 2.5 | Training effort | Low | No data | - |
| ***3*** | ***Previous application to effluent assessment*** | | | |
| 3.1 | Current or proposed use in national effluent control strategies | *High* | *No* | |
| 3.2 | Discrimination between samples | **Key** | **Yes** | |
| 3.3 | Influence of exposure conditions | *High* | *Yes* | |
| ***4*** | ***Methodology*** | | | |
| 4.1 | Application to DTA | **Key** | Potentially (for some test species) | - |
| 4.2 | Timescale of the method | *High* | *≤ 95 days (28 – 60 days post hatch)* | - |
| 4.3 | Cost of method | | | |
| 4.3.1 | Cost of implementing the method | *High* | *No data* | - |
| 4.3.2 | Cost of conducting the method | *High* | *No data* | - |
| 4.4 | Availability of test organisms | *High* | Yes | From in house cultures or hatcheries |
| 4.5 | Suitability of the method for abbreviated testing | Low | Potentially | - |
| ***5*** | ***Ethical and legal issues*** | | | |
| 5.1 | Exemption from the Animal Scientific Procedures Act | **Key** | **No** | - |
| 5.2 | Potential for reduction of animal numbers | **Key** | **Yes** | - |

**Table C2 Summary data for short-term toxicity test on embryo and sac fry stages (Proposed OECD TG 212 and ISO 12890)**

| Category and criterion | | Type of criteria | Assessment against criteria (see Table 2.13) | Data summary (where relevant) |
|---|---|---|---|---|
| No. | Description | | | |
| ***1*** | ***Relevance of test data*** | | | |
| 1.1 | Use of ecologically relevant test species | *High* | *Yes* | - |
| 1.2 | Importance of test species | Low | Yes (for some test species) | - |
| 1.3 | Diversity of endpoints | **Key** | **Yes** | - |
| 1.4 | Ecological relevance of test endpoint(s) | **Key** | **Yes** | - |
| 1.5 | Extrapolation to population effects | **Key** | **Yes** | - |
| ***2*** | ***Test method variability*** | | | |
| 2.1 | Test method repeatability | **Key** | **No** | <10% for survival, ~75% for hatching |
| 2.2 | Test method reproducibility | **Key** | **No** | <25% for survival, ~100% for hatching |
| 2.3 | Availability of a validated method describing the procedure | **Key** | **Yes** | - |
| 2.4 | Test organism variability | *High* | *Yes* | - |
| 2.5 | Training effort | Low | No data | - |
| ***3*** | ***Previous application to effluent assessment*** | | | |
| 3.1 | Current or proposed use in national effluent control strategies | *High* | *No* | - |
| 3.2 | Discrimination between samples | **Key** | **Yes** | - |
| 3.3 | Influence of exposure conditions | *High* | *Yes* | - |
| ***4*** | ***Methodology*** | | | |
| 4.1 | Application to DTA | **Key** | **Potentially (for some test species)** | - |
| 4.2 | Timescale of the method | *High* | *8-9 to 50-55 days* | - |
| 4.3 | Cost of method | | | |
| 4.3.1 | Cost of implementing the method | *High* | *No data* | - |
| 4.3.2 | Cost of conducting the method | *High* | *No data* | - |
| 4.4 | Availability of test organisms | *High* | *Yes* | - |
| 4.5 | Suitability of the method for abbreviated testing | Low | Yes | - |
| ***5*** | ***Ethical and legal issues*** | | | |
| 5.1 | Exemption from the Animal Scientific Procedures Act | **Key** | **Yes** | - |
| 5.2 | Potential for reduction of animal numbers | **Key** | **Yes** | - |

**Table C3 Summary data for larval survival and growth effluent toxicity test using freshwater species (Environment Canada EPS1/RM/22 and US EPA 1000.0) and estuarine marine species (US EPA 1004.0 and 1006.0)**

| Category and criterion | | Type of criteria | Assessment against criteria (see Table 2.13) | Data summary (where relevant) |
|---|---|---|---|---|
| No. | Description | | | |
| ***1*** | ***Relevance of test data*** | | | |
| 1.1 | Use of ecologically relevant test species | *High* | *Yes* | - |
| 1.2 | Importance of test species | Low | No | - |
| 1.3 | Diversity of endpoints | **Key** | **Yes** | - |
| 1.4 | Ecological relevance of test endpoint(s) | **Key** | **Yes** | - |
| 1.5 | Extrapolation to population effects | **Key** | **Yes** | Grothe *et al* (1996) for review |
| ***2*** | ***Test method variability*** | | | |
| 2.1 | Test method repeatability | **Key** | **Yes** | 7.8% (FW) – 11.0% (SW) for survival, 14.5% (FW) – 14.7% (SW) for growth |
| 2.2 | Test method reproducibility | **Key** | **Yes** | 11.2% (FW) – 8.3 – 40.3 % (SW) - for survival, 15.0% (FW) – 10.5 – 41.7% (SW) for growth |
| 2.3 | Availability of a validated method describing the procedure | **Key** | **Yes** | - |
| 2.4 | Test organism variability | *High* | *Yes* | - |
| 2.5 | Training effort | Low | No data | |
| ***3*** | ***Previous application to effluent assessment*** | | | |
| 3.1 | Current or proposed use in national effluent control strategies | *High* | *Yes* | Canadian AETE Programme (FW) and US NPDES (FW and SW) |
| 3.2 | Discrimination between samples | **Key** | **Yes** | - |
| 3.3 | Influence of exposure conditions | *High* | *Yes* | - |
| ***4*** | ***Methodology*** | | | |
| 4.1 | Application to DTA | **Key** | **Yes** | - |
| 4.2 | Timescale of the method | *High* | *7 days* | |
| 4.3 | Cost of method | | | |
| 4.3.1 | Cost of implementing the method | *High* | *No data* | - |
| 4.3.2 | Cost of conducting the method | *High* | *No data* | - |
| 4.4 | Availability of test organisms | *High* | *Yes* | From in house cultures of from hatcheries |
| 4.5 | Suitability of the method for abbreviated testing | Low | Yes | - |
| ***5*** | ***Ethical and legal issues*** | | | |
| 5.1 | Exemption from the Animal Scientific Procedures Act | **Key** | **No** | - |
| 5.2 | Potential for reduction of animal numbers | **Key** | **Yes** | - |

**Key:** FW = Freshwater species, SW – Saltwater species

**Table C4 Summary data for embryo-larval survival and teratogenicity effluent toxicity test using freshwater and estuarine/marine species (US EPA 1001.0 and 1005.0)**

| Category and criterion | | Type of criteria | Assessment against criteria (see Table 2.13) | Data summary (where relevant) |
|---|---|---|---|---|
| No. | Description | | | |
| ***1*** | ***Relevance of test data*** | | | |
| 1.1 | Use of ecologically relevant test species | *High* | *Yes* | - |
| 1.2 | Importance of test species | Low | No | - |
| 1.3 | Diversity of endpoints | **Key** | **Yes** | - |
| 1.4 | Ecological relevance of test endpoint(s) | **Key** | **Yes** | - |
| 1.5 | Extrapolation to population effects | **Key** | **Yes** | - |
| ***2*** | ***Test method variability*** | | | |
| 2.1 | Test method repeatability | **Key** | **Yes** | 2.9% (SW) – 62.0% (FW) for survival, ≤35 % (SW) for teratogenecity |
| 2.2 | Test method reproducibility | **Key** | **No data** | |
| 2.3 | Availability of a validated method describing the procedure | **Key** | **Yes** | - |
| 2.4 | Test organism variability | *High* | *Yes* | - |
| 2.5 | Training effort | Low | | |
| ***3*** | ***Previous application to effluent assessment*** | | | |
| 3.1 | Current or proposed use in national effluent control strategies | *High* | *Yes* | US NPDES (FW and SW) |
| 3.2 | Discrimination between samples | **Key** | **Yes** | |
| 3.3 | Influence of exposure conditions | *High* | *Yes* | |
| ***4*** | ***Methodology*** | | | |
| 4.1 | Application to DTA | **Key** | Yes | - |
| 4.2 | Timescale of the method | *High* | *7-9 days* | |
| 4.3 | Cost of method | | | |
| 4.3.1 | Cost of implementing the method | *High* | *No data* | |
| 4.3.2 | Cost of conducting the method | *High* | *No data* | |
| 4.4 | Availability of test organisms | *High* | *Yes* | From in house cultures of from hatcheries |
| 4.5 | Suitability of the method for abbreviated testing | Low | Yes | - |
| ***5*** | ***Ethical and legal issues*** | | | |
| 5.1 | Exemption from the Animal Scientific Procedures Act | **Key** | **Yes** | - |
| 5.2 | Potential for reduction of animal numbers | **Key** | **Yes** | - |

Key: FW – Freshwater species, SW – Saltwater species

**Table C5 Summary data for salmonid embryo toxicity test (Environment Canada EPS1/RM/28)**

| Category and criterion | | Type of criteria | Assessment against criteria (see Table 2.13) | Data summary (where relevant) |
|---|---|---|---|---|
| No. | Description | | | |
| ***1*** | ***Relevance of test data*** | | | |
| 1.1 | Use of ecologically relevant test species | *High* | *Yes* | - |
| 1.2 | Importance of test species | Low | Yes | - |
| 1.3 | Diversity of endpoints | **Key** | **Yes** | - |
| 1.4 | Ecological relevance of test endpoint(s) | **Key** | **Yes** | - |
| 1.5 | Extrapolation to population effects | **Key** | **Yes** | - |
| ***2*** | ***Test method variability*** | | | |
| 2.1 | Test method repeatability | **Key** | **No data** | - |
| 2.2 | Test method reproducibility | **Key** | **No data** | - |
| 2.3 | Availability of a validated method describing the procedure | **Key** | **Yes** | - |
| 2.4 | Test organism variability | *High* | *Yes* | - |
| 2.5 | Training effort | Low | No data | |
| ***3*** | ***Previous application to effluent assessment*** | | | |
| 3.1 | Current or proposed use in national effluent control strategies | *High* | *No* | - |
| 3.2 | Discrimination between samples | **Key** | **Yes** | |
| 3.3 | Influence of exposure conditions | *High* | *Yes* | |
| ***4*** | ***Methodology*** | | | |
| 4.1 | Application to DTA | **Key** | **Yes** | - |
| 4.2 | Timescale of the method | *High* | *7 days* | |
| 4.3 | Cost of method | | | |
| 4.3.1 | Cost of implementing the method | *High* | *No data* | - |
| 4.3.2 | Cost of conducting the method | *High* | *No data* | - |
| 4.4 | Availability of test organisms | *High* | *Yes* | From hatcheries |
| 4.5 | Suitability of the method for abbreviated testing | Low | Yes | - |
| ***5*** | ***Ethical and legal issues*** | | | |
| 5.1 | Exemption from the Animal Scientific Procedures Act | **Key** | **Yes** | - |
| 5.2 | Potential for reduction of animal numbers | **Key** | **Yes** | - |

**Table C6 Summary data for salmonid embryo/alevin toxicity test (Environment Canada EPS1/RM/28)**

| Category and criterion | | Type of criteria | Assessment against criteria (see Table 2.13) | Data summary (where relevant) |
|---|---|---|---|---|
| No. | Description | | | |
| ***1*** | ***Relevance of test data*** | | | |
| 1.1 | Use of ecologically relevant test species | *High* | *Yes* | - |
| 1.2 | Importance of test species | Low | Yes | - |
| 1.3 | Diversity of endpoints | **Key** | **Yes** | - |
| 1.4 | Ecological relevance of test endpoint(s) | **Key** | **Yes** | - |
| 1.5 | Extrapolation to population effects | **Key** | **Yes** | - |
| ***2*** | ***Test method variability*** | | | |
| 2.1 | Test method repeatability | **Key** | **No data** | - |
| 2.2 | Test method reproducibility | **Key** | **No data** | - |
| 2.3 | Availability of a validated method describing the procedure | **Key** | **Yes** | - |
| 2.4 | Test organism variability | *High* | *Yes* | - |
| 2.5 | Training effort | Low | No data | |
| ***3*** | ***Previous application to effluent assessment*** | | | |
| 3.1 | Current or proposed use in national effluent control strategies | *High* | *No* | - |
| 3.2 | Discrimination between samples | **Key** | **Yes** | |
| 3.3 | Influence of exposure conditions | *High* | *Yes* | |
| ***4*** | ***Methodology*** | | | |
| 4.1 | Application to DTA | **Key** | **Yes** | - |
| 4.2 | Timescale of the method | *High* | *27-29 days* | |
| 4.3 | Cost of method | | | |
| 4.3.1 | Cost of implementing the method | *High* | *No data* | - |
| 4.3.2 | Cost of conducting the method | *High* | *No data* | - |
| 4.4 | Availability of test organisms | *High* | *Yes* | From hatcheries |
| 4.5 | Suitability of the method for abbreviated testing | Low | Yes | - |
| ***5*** | ***Ethical and legal issues*** | | | |
| 5.1 | Exemption from the Animal Scientific Procedures Act | **Key** | **Yes** | - |
| 5.2 | Potential for reduction of animal numbers | **Key** | **Yes** | - |

**Table C7 Summary data for salmonid embryo/alevin/fry toxicity test (Environment Canada EPS1/RM/28)**

| Category and criterion | | Type of criteria | Assessment against criteria (see Table 2.13) | Data summary (where relevant) |
|---|---|---|---|---|
| No. | Description | | | |
| ***1*** | ***Relevance of test data*** | | | |
| 1.1 | Use of ecologically relevant test species | *High* | *Yes* | - |
| 1.2 | Importance of test species | Low | Yes | - |
| 1.3 | Diversity of endpoints | **Key** | **Yes** | - |
| 1.4 | Ecological relevance of test endpoint(s) | **Key** | **Yes** | - |
| 1.5 | Extrapolation to population effects | **Key** | **Yes** | - |
| ***2*** | ***Test method variability*** | | | |
| 2.1 | Test method repeatability | **Key** | **No data** | - |
| 2.2 | Test method reproducibility | **Key** | **No data** | - |
| 2.3 | Availability of a validated method describing the procedure | **Key** | **Yes** | - |
| 2.4 | Test organism variability | *High* | *Yes* | - |
| 2.5 | Training effort | Low | No data | |
| ***3*** | ***Previous application to effluent assessment*** | | | |
| 3.1 | Current or proposed use in national effluent control strategies | *High* | *No* | - |
| 3.2 | Discrimination between samples | **Key** | **Yes** | |
| 3.3 | Influence of exposure conditions | *High* | *Yes* | |
| ***4*** | ***Methodology*** | | | |
| 4.1 | Application to DTA | **Key** | **Yes** | - |
| 4.2 | Timescale of the method | *High* | *~70 days* | |
| 4.3 | Cost of method | | | |
| 4.3.1 | Cost of implementing the method | *High* | *No data* | - |
| 4.3.2 | Cost of conducting the method | *High* | *No data* | - |
| 4.4 | Availability of test organisms | *High* | *Yes* | From hatcheries |
| 4.5 | Suitability of the method for abbreviated testing | Low | Yes | - |
| ***5*** | ***Ethical and legal issues*** | | | |
| 5.1 | Exemption from the Animal Scientific Procedures Act | **Key** | **Yes** | - |
| 5.2 | Potential for reduction of animal numbers | **Key** | **Yes** | |

**Table C8 Summary data for juvenile fish growth test (Proposed OECD TG 215, ISO 10229)**

| Category and criterion | | Type of criteria | Assessment against criteria (see Table 2.13) | Data summary (where relevant) |
|---|---|---|---|---|
| No. | Description | | | |
| ***1*** | ***Relevance of test data*** | | | |
| 1.1 | Use of ecologically relevant test species | *High* | **Yes** | - |
| 1.2 | Importance of test species | Low | **Yes** | - |
| 1.3 | Diversity of endpoints | **Key** | **Yes** | - |
| 1.4 | Ecological relevance of test endpoint(s) | **Key** | **Yes** | - |
| 1.5 | Extrapolation to population effects | **Key** | **Yes** | - |
| ***2*** | ***Test method variability*** | | | |
| 2.1 | Test method repeatability | **Key** | **No data** | - |
| 2.2 | Test method reproducibility | **Key** | **No data** | - |
| 2.3 | Availability of a validated method describing the procedure | **Key** | **Yes** | - |
| 2.4 | Test organism variability | *High* | *Yes* | - |
| 2.5 | Training effort | Low | No data | - |
| ***3*** | ***Previous application to effluent assessment*** | | | |
| 3.1 | Current or proposed use in national effluent control strategies | *High* | *No* | - |
| 3.2 | Discrimination between samples | **Key** | **Yes** | - |
| 3.3 | Influence of exposure conditions | *High* | *Yes* | - |
| ***4*** | ***Methodology*** | | | |
| 4.1 | Application to DTA | **Key** | **Yes** | - |
| 4.2 | Timescale of the method | *High* | 28 days | - |
| 4.3 | Cost of method | | | |
| 4.3.1 | Cost of implementing the method | *High* | *No data* | - |
| 4.3.2 | Cost of conducting the method | *High* | *No data* | - |
| 4.4 | Availability of test organisms | *High* | *Yes* | From hatcheries |
| 4.5 | Suitability of the method for abbreviated testing | Low | No | |
| ***5*** | ***Ethical and legal issues*** | | | |
| 5.1 | Exemption from the Animal Scientific Procedures Act | **Key** | **Yes** | |
| 5.2 | Potential for reduction of animal numbers | **Key** | **Yes** | |

**Table C9 Summary data for juvenile turbot (*Scopthalmus maximus*) growth test**

| Category and criterion | | Type of criteria | Assessment against criteria (see Table 2.13) | Data summary (where relevant) |
|---|---|---|---|---|
| No. | Description | | | |
| ***1*** | ***Relevance of test data*** | | | |
| 1.1 | Use of ecologically relevant test species | *High* | *Yes* | - |
| 1.2 | Importance of test species | Low | Yes | - |
| 1.3 | Diversity of endpoints | **Key** | **Yes** | - |
| 1.4 | Ecological relevance of test endpoint(s) | **Key** | **Yes** | - |
| 1.5 | Extrapolation to population effects | **Key** | **Potentially** | - |
| ***2*** | ***Test method variability*** | | | |
| 2.1 | Test method repeatability | **Key** | **No data** | - |
| 2.2 | Test method reproducibility | **Key** | **No data** | - |
| 2.3 | Availability of a validated method describing the procedure | **Key** | **No** | - |
| 2.4 | Test organism variability | *High* | *Yes* | - |
| 2.5 | Training effort | Low | No data | - |
| ***3*** | ***Previous application to effluent assessment*** | | | |
| 3.1 | Current or proposed use in national effluent control strategies | *High* | No | - |
| 3.2 | Discrimination between samples | **Key** | **Yes** | - |
| 3.3 | Influence of exposure conditions | *High* | *Yes* | - |
| ***4*** | ***Methodology*** | | | |
| 4.1 | Application to DTA | **Key** | **Yes** | - |
| 4.2 | Timescale of the method | *High* | *14 days* | - |
| 4.3 | Cost of method | | | |
| 4.3.1 | Cost of implementing the method | *High* | *No data* | - |
| 4.3.2 | Cost of conducting the method | *High* | *£4000* | - |
| 4.4 | Availability of test organisms | *High* | *No* | From hatcheries normally |
| 4.5 | Suitability of the method for abbreviated testing | Low | No | - |
| ***5*** | ***Ethical and legal issues*** | | | |
| 5.1 | Exemption from the Animal Scientific Procedures Act | **Key** | **No** | - |
| 5.2 | Potential for reduction of animal numbers | **Key** | **Yes** | - |

**Table C10 Summary data for adult fathead minnow (*Pimephales promelas*) reproduction test**

| Category and criterion | | Type of criteria | Assessment against criteria (see Table 2.13) | Data summary (where relevant) |
|---|---|---|---|---|
| No. | Description | | | |
| ***1*** | ***Relevance of test data*** | | | |
| 1.1 | Use of ecologically relevant test species | *High* | *Yes* | - |
| 1.2 | Importance of test species | Low | Yes | - |
| 1.3 | Diversity of endpoints | **Key** | **Yes** | - |
| 1.4 | Ecological relevance of test endpoint(s) | **Key** | **Yes** | - |
| 1.5 | Extrapolation to population effects | **Key** | **Yes** | - |
| ***2*** | ***Test method variability*** | | | |
| 2.1 | Test method repeatability | **Key** | **No data** | - |
| 2.2 | Test method reproducibility | **Key** | **No data** | - |
| 2.3 | Availability of a validated method describing the procedure | **Key** | **Yes** | - |
| 2.4 | Test organism variability | *High* | *Yes* | - |
| 2.5 | Training effort | Low | No data | - |
| ***3*** | ***Previous application to effluent assessment*** | | | |
| 3.1 | Current or proposed use in national effluent control strategies | *High* | *No* | - |
| 3.2 | Discrimination between samples | **Key** | **Yes** | - |
| 3.3 | Influence of exposure conditions | *High* | *Yes* | - |
| ***4*** | ***Methodology*** | | | |
| 4.1 | Application to DTA | **Key** | **No** | - |
| 4.2 | Timescale of the method | *High* | *42 Days* | - |
| 4.3 | Cost of method | | | |
| 4.3.1 | Cost of implementing the method | *High* | *No data* | - |
| 4.3.2 | Cost of conducting the method | *High* | *>£25000* | - |
| 4.4 | Availability of test organisms | *High* | *Yes* | From in-house cultures or hatcheries |
| 4.5 | Suitability of the method for abbreviated testing | Low | No | - |
| ***5*** | ***Ethical and legal issues*** | | | |
| 5.1 | Exemption from the Animal Scientific Procedures Act | **Key** | **No** | - |
| 5.2 | Potential for reduction of animal numbers | **Key** | **Yes** | - |

# APPENDIX D INFORMATION ON THE FISHSENSE SYSTEMS

The Fishsense approach involved the development and evaluation of an *in vitro* assay using cultured fish cells, modified to allow the health of the cells to be monitored by their bioluminescence. The work was carried out by AstraZeneca in collaboration with researchers at the University of Luton and with Seraph Technologies, specialists in the development of biosensor instrumentation. The project was successful in gaining matching DTI funding under the LINK Cell Engineering programme. The work arose out of previous studies with Luton, employing fish cells on electrochemical (amperometric) biosensors using the CellSense™ system. Although successful (Polak *et al* 1996) the chemical mediators employed exhibit certain toxicity to the cells, suggesting the need for alternative methods of cell interrogation.

The objective was to obtain stable insertion of the firefly luciferase (*Luc*) gene into laboratory cultured Bluegill Sunfish fibroblast (BF-2) cells, to allow the metabolic status of the cells to be monitored as luminescence when provided with luciferin substrate. The reaction relies on energy (ATP) being supplied by actively metabolising cells; therefore, the degree of luminecsence is a function of the cell's energy supply, which would be expected to be depleted or damaged by toxicants. A recombinant bacterial clone (pCIneoLuc) was constructed containing the *Luc* gene and the G418 (neomycin) resistance marker. Expression of the luciferase gene in *Escherichia coli* transformed with pCIneoLuc was confirmed. Plasmid DNA was used to transform BF-2 fish cells and transfected cells were cultured in the presence of high levels of G418 for several weeks. Surviving cells were re-plated in order to establish stable cell lines showing significant levels of luciferase activity, confirming expression of the *Luc* gene in BF-2 cells. The transfection of BF-2 cells was reproducible; three independent transfections each gave multiple resistant colonies. Cultures of the transformed fish cells (BF-2/luc) were harvested and resuspended at a standard cell density. Aliquots of the cells were incubated (typically 30 minutes to 6 hours) in a range of concentrations of the toxicant. Bioluminescence was assayed using Brightglo™ reagent, which lysed the cells and provided the substrate, luciferin. Initially, luciferase activity was measured using a commercial luminometer but subsequently a sensitive, low-cost instrument based on a photo-multiplier tube, with customised software, was developed by Seraph. Inhibition relative to control cells was used to calculate EC50 values (the concentration causing 50% inhibition of luminescence). Lysis of the cells was necessary to achieve maximum luminescence, because luciferin does not cross the cell membrane readily at normal pH levels. Cryopreservation protocols have been defined to allow long-term storage of clones.

The BF-2/luc clone has been used to test a range of toxicants and $EC_{50}$ values are shown in Table D1, where they are compared with results from earlier work using the amperometric method (Polak *et al* 1996), and literature data for fish cells (neutral red assay) and for *in vivo* (whole fish) tests. It is evident that the relative sensitivity of the luminescent cells varies with the toxicant. It is probable that the shorter exposure period, compared with 24 hours for the neutral red and *in vivo* tests, accounts for the apparent low sensitivity to certain toxicants, since these tend to be the more hydrophobic substances that would be expected to take longer to reach equilibrium between the cells and the medium. This was demonstrated when using an exposure periods of 6 hours, which gave $EC_{50}$ values up to 2 orders of magnitude lower

than the 30-minute tests. Additionally, toxicants with specific modes of action might not necessarily be so active in isolated, non-specialised cells.

**Table D1 Sensitivity of luminescent BF-2 fish cells and comparative *in vitro* and *in vivo* data**

| Toxicant | BF-2/luc1 Luminescence[a] $EC_{50}$ (mM) | Neutral red (24-hour) $EC_{50}$ (mM) | BF-2 Biosensor[e] (Amperometric) $EC_{50}$ (mM) | *In vivo* $LC_{50}$[f] (mM) |
|---|---|---|---|---|
| Catechol | 6.9 | 0.17[b] | No data | 0.084 |
| 3-Methylcatechol | 1.4 | 0.052[b] | No data | - |
| 4-Methylcatechol | 3.8 | 0.057[b] | No data | >1.6 |
| Benzaldehyde | 0.30 | 6.2[b] | No data | 0.33 |
| Methylbenzoate | 0.43 | 11[b] | No data | - |
| o-Cresol | 1.6 | 2.2[b] | No data | 0.19 |
| m-Cresol | 2.3 | 2.9[b] | No data | 0.23 |
| p-Cresol | 3.8 | 3.0[b] | No data | 0.24 |
| Diuron | 0.68 | 1.8[e] | 1.1 | 0.11 |
| Mercuric chloride | 0.006 | 0.15[c] | 0.09 | 0.0014 |
| Potassium dichromate | 4.8 | 1.05[c] | 5.2 | 2.1 |
| Dichlorophenol | 0.32 | 1.32[e] | 0.95 | 0.029 |
| Zinc sulphate | 0.046 | 0.14[c] | No data | 0.076 |
| Linuron | 0.21 | No data | No data | 0.12 |
| Hydrogen peroxide | 1.1 (6 h) | 0.9[d] | No data | 1.1 |
| Paraquat | 73 (6 h) | 5.0[d] | No data | 2.65 |
| Ethanol | 140 (6 h) | No data | No data | 240 |
| 2-propanol | 170 (6 h) | No data | No data | 190 |
| 1-butanol | 18 (6 h) | No data | No data | 26 |
| 1-pentanol | 9.1 (6 h) | No data | No data | 5 |

**Notes**

[a] 30 mins exposure unless indicated. [b] BF-2 cells (Shen *et al* 2000) [c] Rainbow trout cells (Segner *et al* 1994)

[d] BF-2 cells (Babich *et al* 1993) [e] Polak *et al* (1996). [f] Various fish species and authors, 24 h where available