Literature reviews on ecotoxicology of chemicals with a special focus on plant protection products

Lot 6: Available protocols for testing the effects of chemicals against aquatic invertebrates other than crustacea

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Executive summary

Background

Plant protection products used within the European Union are regulated by Directive 91/414/EEC. Guidance Documents are available for notifying organisations and Member States on how to conduct particular aspects of the risk assessment. In order to facilitate the PPR's revision of the Ecotoxicology Guidance Document SANCO/3268/2001 (Aquatic Ecotoxicology), SANCO/10329/2002 (Terrestrial Ecotoxicology), literature reviews on six areas of ecotoxicology were commissioned.

EFSA's PPR Panel has reviewed the proposed methodology and approaches outlined in the Commission Working Document SANCO/10483/2006 rev.6 on the proposed data requirements for the revision of Directive 96/12/EC (ecotoxicological studies) within the framework of revisions to 91/414/EEC. In its opinion issued in 2007 (EFSA, 2007), the PPR Panel made the following main recommendations relevant to this literature review:

- Specific testing on endocrine endpoints should be included for invertebrates; endocrine endpoints from these tests should allow assessment at a population level.

- Given the high diversity among invertebrates, chronic toxicity data for another taxonomic group should be included in addition to Crustaceans, especially for compounds with an insecticidal mode of action. An insect with several generations a year that does not live in sediment would simplify the exposure situation.

Objectives

The objectives of the contract are as follows:

> To compile all available scientific information for available protocols for testing the effects of chemicals against aquatic invertebrates other than crustacean.
>
> To present the scientific information in a complete, systematic, clear and concise report written in English.

A literature search was conducted to identify whole organism toxicity tests reporting at least mortality and/or immobility as endpoints. Specific tests on endocrine endpoints are also considered. The selected literature concentrates on effects of pesticides on freshwater species although other references are considered where appropriate. Where studies have been designed to include an exposure and recovery phase only the exposure phase is considered. This is to avoid duplication of effort with Lot 5 "Evidence of potential long term effects in (aquatic and terrestrial) invertebrates after short term pulsed exposure".

In addition to conducting a search of standard methods and published literature, Contract Research Organisations (CROs) were approached. These organisations were asked to respond to a questionnaire on their experiences of conducting toxicity tests with non-standard species. The majority of the CROs canvassed provided a comprehensive response with only a small number deciding not to participate in the review.

Results

Sixteen detailed protocols were identified for testing the effects of chemicals against aquatic invertebrates other than Crustacea, that had been published by national and international standards agencies (ASTM, OECD, USEPA, APHA), comprising six acute tests and seven chronic tests (one test included acute and chronic endpoints). These included *Chironomus riparius, C. tentans, Hexagenia* sp., *Lumbriculus variegatus*, other Lumbriculidae and Tubificidae worms, *Brachionus calyciflorus* and freshwater mussels (Table 4.1, Annex 1). In addition to these protocols, guidance documents were also available on selecting appropriate additional species, test designs, endpoints, statistics and validation criteria for regulatory tests.

The chironomid Full Life Cycle tests (USEPA 2000) may address the requirement for a chronic toxicity test with an insect species that includes endpoints suitable for determining population level effects as a result of exposure to potential EDCs. However, the PPR Opinion stated a preference for a chronic insect test without sediment to simplify the exposure situation.

Seven Contract Research Organisations (CROs) responded to requests for details of any test methods they had used with non-Crustacea invertebrates. Information on the source and age of species, test parameters, test endpoints and assessment criteria were obtained. A total of 53 protocols were received employing 43 test species.

Novel protocols were identified for *Chaoborus crystallinus, Haprophlebia lauta, Serratella ignita* and *Lymnaea stagnalis.* In addition to these tests, a wide range of test species, predominantly field collected and resident in at least some areas of Europe were identified. These included Diptera, Ephemeroptera, Trichoptera, Hemiptera, Odonata, Sialidae, gastropod snails, bivalves, Oligochaeta, and rotifers. These have all been used in acute toxicity tests and in some cases, they have also been used in chronic toxicity tests.

A total of 111 protocols were identified in the literature review. Seventy-six acute protocols and 26 chronic protocols were conducted in water-only test systems. Three acute protocols and six chronic protocols specified the presence of sediment. In total there were 70 test species.

This report presents the review of protocols for testing the effects of chemicals against aquatic invertebrates other than Crustacea. Five protocols were identified as providing information additional to that provided by existing standard protocols. The species identified were *Chironomus riparius, Cloeon triangulifer, Chaoborus crystallinus, Lymnaea stagnalis and Brachionus calyciflorus*.

The insects *Chironomus riparius, Cloeon triangulifer* and *Chaoborus crystallinus* would be good candidate species for monitoring the effects of potentially endocrine disrupting insecticides. It was also demonstrated that *Chaoborus crystallinus* is suitable for long term culturing in the laboratory. *Lymnaea stagnalis, Brachionus calyciflorus* or any of the three species identified above would also be suitable for chronic toxicity tests in addition to *Daphnia*, although tests with sediment will inhibit direct comparisons between *Daphnia* endpoints and the additional test species. Summaries of the five protocols that are recommended as candidates for providing a non-crustacean chronic invertebrate test that may be sensitive to endocrine disruption are presented overleaf with more detailed descriptions listed in Appendix 4.

Summaries of the five protocols that are recommended as candidates for providing a non-crustacean chronic invertebrate test that may be sensitive to endocrine disruption

Species:	***Chironomus riparius***
Test description:	Full life cycle test including F1 viability endpoints
Source:	Laboratory culture
Test design:	Modification of OECD method with continuous exposure from 1st instar of parent through to emergence of the F1 generation
Endpoints:	Emergence ratio, development rate, no. egg ropes per female, fertility of egg ropes, viability of offspring, sex ratio of emerged adults (P and F1 generation)
Validation criteria:	Same as the OECD test guidelines
Reference:	Several similar protocols including (Taenzler et al. 2007), (USEPA 2000)
Species:	***Cloeon triangulifer***
Test description:	Two tests reported, together they cover exposure from 1st instar to hatching success of F1 generation
Source:	Laboratory culture or lab reared field collected eggs
Test design:	1st instar exposed to test item in a semi-static test system with natural water and no sediment Following emergence and egg laying the hatch success is determined.
Endpoints:	Emergence, hatch success, adult residues
Validation criteria:	Not recorded
Reference:	(Sweeney et al. 1993)
Species:	***Chaoborus crystallinus***
Test description:	Part life cycle test
Source:	Field collected and in-house stock (potential for long term culturing of laboratory stocks has been identified by the relevant CRO)
Test design:	1st instar exposed in a semi-static water only test system until emergence (30-90 d)
Endpoints:	Mortality, growth, moulting, pupation, emergence, reproduction
Validation criteria:	None specified
Reference:	CRO protocol (see Appendix 2)
Species:	***Lymnaea stagnalis***
Test description:	Reproduction in a hermaphroditic snail
Source:	Laboratory culture
Test design:	84 d semi-static test in reconstituted water with no sediment
Endpoints:	Adult mortality, fecundity, mean no. egg clutches, hatchability (in clean water)
Validation criteria:	Not specified
Reference:	(Czech et al. 2001)
Species:	***Brachionus calyciflorus*** **(rotifer)**
Test description:	Resting egg production
Source:	Laboratory culture or cysts
Test design:	4 d static test in reconstituted water initiated with neonate females. Feeding during the study, no sediment, no aeration
Endpoints:	Resting egg production
Validation criteria:	Not recorded
Reference:	(Preston et al. 2000)

1 Introduction

1.1 Background information

Plant protection products used within the European Union are regulated by Directive 91/414/EEC. Guidance Documents are available for notifying organisations and Member States on how to conduct particular aspects of the risk assessment. In order to facilitate the PPR's revision of the Ecotoxicology Guidance Document SANCO/3268/2001 (Aquatic Ecotoxicology), SANCO/10329/2002 (Terrestrial Ecotoxicology), and considering the Opinion of the PPR Panel related to the revisions of Annex II and III to Council Directive 91/414/EEC (Ecotoxicology), literature reviews on six areas of ecotoxicology were commissioned. This report is covering the available scientific information regarding aquatic invertebrates other than Crustacea.

Freshwater invertebrates are extremely diverse and include both arthropod and non-arthropod species. In freshwater systems the arthropods are dominated by Insecta and Crustacea with some Arachnida species. Non-arthropod invertebrates found in freshwater systems include Annelida (worms and leeches), Cnidaria (Hydra), Mollusca, Platyhelminthes (flatworms) and Rotifera. Protocols were identified for all of the aforementioned orders of aquatic invertebrates.

1.2 Objective

The objective of this literature review was to compile available scientific information on protocols for testing the effects of chemicals against aquatic invertebrates other than Crustacea.

The methods used in this literature review are detailed in Section 2. Section 3 provides an overview of the main recommendations derived from the Opinion of the PPR Panel related to revisions of Annex II and III to Council Directive 91/414/EEC: Ecotoxicological Studies (EFSA, 2007) and of the existing Aquatic Ecotoxicology Guidance Document and identifies reasoning for and requirements when identifying available protocols for testing the effects of chemicals against invertebrates other than Crustacea. Section 4 examines test protocols already provided by national and international standards agencies. Section 5 details those test protocols currently in use by Contract Research Organisations (CROs). Section 6 examines the peer-reviewed literature for suitable test protocols and identifies some potential methods for consideration. Finally, Section 7 provides recommendations, conclusions and a summary of potential test protocols.

2 Methods

2.1 Literature search methodology

This report considers whole organism toxicity tests reporting at least mortality and/or immobility as endpoints. Specific tests on endocrine endpoints are also considered. The selected literature concentrates on effects of pesticides on freshwater species although other references are considered where appropriate.

Studies with and without sediment present are considered separately. Studies including artificial substrates (e.g. glass beads or wire mesh) are included with water-

only test systems as the artificial substrate has usually been selected from inert materials commonly used for test vessels (i.e. glass or stainless steel).

Acute tests have been defined as tests of up to four days duration. Studies with a duration of more than four days are considered as chronic studies. Where studies have been designed to include an exposure and recovery phase, only the exposure phase is considered. This is to avoid duplication of effort with Lot 5 “Evidence of potential long term effects in (aquatic and terrestrial) invertebrates after short term pulsed exposure”.

An initial literature search was conducted using Scopus, Science Direct and British Library journal articles advanced search. Some active ingredients were identified by name in the search terms. These were taken from a list identified in previous research (Maltby et al. 2002) and the Pesticides Manual (Tomlin 2000). Only references from 1980 onwards were included in the database. In addition to the search of published peer reviewed papers, literature references were sought from OECD, ASTM, EFSA and USEPA OPPTS and APHA. The search terms used to interrogate literature databases are summarised as follows:

Search terms used*
Aquatic or freshwater or "fresh water" or stream* or pond* or river* or ditch* or lake
AND
toxic* or lethal* or risk or EC? Or EC?? or LC? Or LC?? or NOEC or LOEC or effect or exposure
AND
pesticid* or insecticid* or acaricid* or "plant protection product*" or "91/414" or carbamate* or organophosphorous or organochlori* or "chlorinated hydrocarbon" or pyrethroid or fungicide* or "plant activator*" or nematicide or bactericide or "plant host defence inducer" or "bird repellent" or neonicotinoid or triazole or phenylamide or carbamate or "aromatic hydrocarbon" or benzoylurea or "acyl-urea" or "acetylcholinesterase inhibitor" or "Azinphos?methyl" or Bendiocarb or Carbaryl or Carbofuran or Chlorpyrifos or Cyfluthrin or Cypermethrin or Deltamethrin or Diazinon or Diflubenzuron or Esfenvalerate or Fenitrothion or Fenvalerate or cyhalothrin or Lindane or Methoxychlor or Parathion or Permethrin or Phorate or Tralomethrin
AND
invertebrate or arthropod* or insect or insecta or arachnid* or annelid* or mollusc or mollusca or planar* or rotifer* or turbellari* or gastropod* or bivalve* or oligochaet* or hirudin* or odonata or coleoptera or diptera or ephemeroptera or plecoptera* or trichoptera* or hemiptera* or neuropteran* or megaloptera* or ostracoda* or hydra or larvae or midges or mussel or oligochaete or nematode or annelid or boatman or non-target or worm or snail or leech or dragonfly or damselfly or beetle or fly or mayfly or stonefly or caddis or caddisfly or bug
AND NOT
avian or bovine or human or forest or soil

The search of literature databases and additional data sources identified a total of 2394 references.

2.2 Initial selection criteria

An initial selection criteria was applied to the preliminary reference list to identify papers that addressed the literature search brief. The following papers were included:

1. Single-species laboratory studies
2. Acute and chronic duration
3. Water only and sediment:water studies
4. Lethality, immobility and other "whole organism" endpoints.
5. European and non-European species in initial assessment

References meeting the following criteria were excluded as they were either not suitable methods for standardised regulatory tests or were considered in other Lots:

1. Data for crustaceans
2. Long term effects after short term exposure
3. Only indirect effects are reported
4. Saltwater and estuarine species
5. Mesocosm studies and field studies
6. Genetic and microbiological effects

2.3 Supplementary questionnaire

In addition to conducting a search of standard methods and published literature, CROs were approached. Organisations were asked if they were willing to respond to a questionnaire. Questions included whether there was experience of conducting toxicity tests with non-standard species. Where additional species had been tested details were sought. The results of this questionnaire are presented in Section 7 and Appendix 2.

Initially, references for existing standard protocols (ASTM, OECD, USEPA) were examined to determine what methods were available and where additional protocols were required. Next, the results from the questionnaire sent to CROs were examined. Additional non-standard test species and suitable protocols already in use were identified. Finally the results of the literature search were examined. From these data additional species that may be suitable additional species in regulatory tests were identified.

3 Recommendations from the Opinion of the PPR Panel related to revisions of Annex II and III (91/414/EEC) and of the existing Aquatic Ecotoxicology Guidance Document

3.1 Opinion of the PPR Panel related to the revisions of Annex II and III to Council Directive 91/414/EEC: Ecotoxicological studies (EFSA, 2007)

EFSA's PPR Panel reviewed the Commission Working Document SANCO/10483/2006 rev.6 on the proposed data requirements for the revision of Directive 96/12/EC (ecotoxicological studies) within the framework of revisions to 91/414/EEC. In particular the PPR Panel were to review the proposed methodology and approaches outlined in the document. The PPR Panel made a number of recommendations (EFSA 2007). The main recommendations that are pertinent to this literature review included:

- "Consideration should be given to requiring more detailed information to be recorded from existing studies, such as more frequent observations on the time course of effects, to avoid any need for repeating studies"

- "Specific testing on endocrine endpoints should be included in the respective sections for both, fish and invertebrates"

- "Given the high diversity among invertebrates, toxicity data for another taxonomic group should be included in addition to Crustaceans"

Other comments in the opinion of the PPR Panel that may be relevant to the identification of protocols for testing the effects of chemicals against invertebrates other than Crustacea were:

1. Validated test protocols should be used. Where no validated method is available the notifier should justify the choice of non-standard method and provide details of their performance.

2. ECx is an alternative to NOEC and may become a preferred option therefore, this option should be available.

3. Endocrine endpoints should be included for invertebrates in the determination of effects on aquatic organisms. Endpoints from these tests should allow assessment at a population level.

4. It was considered important to include chronic toxicity data for another taxonomic group in addition to Crustaceans, especially for compounds with an insecticidal mode of action. An insect with several generations a year that does not live in sediment would simplify the exposure situation.

5. It was felt that reproduction was not appropriately addressed in the Chironomid test as young larvae development was recorded but their ability to reproduce was not considered as an endpoint.

3.2 Aquatic Ecotoxicology Guidance Document (SANCO/3268/2001)

The Ecotoxicology Guidance Document SANCO/3268/2001 (Aquatic Ecotoxicology) details a number of protocols for testing the effects of chemicals against aquatic invertebrates other than Crustacea. Data requirements relevant to this literature review (i.e. not including Crustacea) include:

1. Studies with additional invertebrate species:

    a. Studies with gastropod molluscs may be required if continued or repeated exposure is likely to occur. No accepted international guideline is currently available and gastropods are considered to be generally less sensitive than *Daphnia*. Therefore a reasoned case should be made as to why the test is not required.

    b. Mode-of-action should be considered prior to deciding whether additional species testing is required. An acute Chironomid test should be conducted for insecticides. A chronic chironomid test may also be required, for example, where the insecticide is a growth regulator.

2. Tests with sediment dwelling organisms:

    a. Annex II specifies *Chironomus* sp, as the required test organism to assess potential effects on sediment dwelling organisms. OECD 218 and 219 are available methods for testing sediment dwelling organisms. Spiked water is normally considered to be the most realistic exposure scenario. Spiked sediment tests are recommended where there is an accumulation of the pesticide in the sediment over time. However, it was noted in the guidance document that OECD 219 did not require sediment concentrations to be quantified.

    b. Toxicity to sediment dwelling invertebrates may also be addressed in a suitably designed mesocosm study.

3. Endocrine effects: At the time of publication of the Aquatic Ecotoxicology Guidance Document, it was considered premature to make firm recommendations. It was anticipated that endocrine effects will be dealt with in similar way to other expressions of effect as many of the areas of uncertainty are similar (e.g. intra and inter-species, study duration, exposure route etc.).

The use of any international guideline that is comparable with those mentioned in Annex II or III is acceptable. In principle, species mentioned in other test guidelines are acceptable, although not all are indigenous to Europe.

4 Standard methods for acute and chronic toxicity tests

Testing the effects of chemicals against aquatic invertebrates is usually carried out with a few surrogate species. This facilitates comparison between tests by regulatory agencies, minimise costs as the standard test species are easy to culture and available throughout the year and, maximise reliability by using well understood species in well characterised test systems (USEPA 1996b).

Sixteen detailed protocols were identified for testing the effects of chemicals against aquatic invertebrates other than Crustacea that are published by national and international standards agencies (ASTM, OECD, USEPA, APHA), comprising six acute tests and seven chronic tests (one test included acute and chronic endpoints). These included *Chironomus riparius, C. tentans, Hexagenia* sp., *Lumbriculus variegatus*, other Lumbriculidae and Tubificidae worms, *Brachionus calyciflorus* and freshwater mussels (Table 4.1, Annex 1). In addition to these protocols guidance documents were also available on selecting appropriate additional species, test designs, endpoints, statistics and validation criteria for regulatory tests.

Table 4.1: Summary of non-Crustacea invertebrate protocols identified from standard methods (ASTM, OECD, USEPA, APHA). See Appendix 1 for further details.

Target species	Test system	Duration	Endpoint	Effect	Reference
Chironomus riparius[1] Non-biting midge	Static	10d, 20-28 d	Immobility, growth, emergence, development rate	ECx, NOEC, LOEC	(OECD 2004b, a)
Chironomus tentans[2] Non-biting midge	Flow-through	14 d	Mortality, growth, bio-concentration	LC50, EC50, NOEC, LOEC, MATC	(USEPA 1996c)
Hexagenia sp. Mayfly	Static, recirculating or flow-through	4 d	Immobility	LC50	(Henry et al. 1986)
Lumbriculus variegatus Worm	Static	2-4 d	Mortality, fragmentation, clumping, mucus production, swelling, colour changes	LC50, EC50	(Bailey 1980)
Lumbriculus variegatus Worm	Static	28 d	Reproduction, growth, behavioural changes	ECx, NOEC, LOEC	(OECD 2007)
Tubificidae or Lumbriculidae Worms	Semi-static	10 d	Mortality	LC50, LT50	(Eaton et al. 2005)
Brachionus calyciflorus Rotifer	Static	1 d	Mortality	LC50	(ASTM 2004a)
Brachionus calyciflorus Rotifer	Static	2 d	Mortality, reproduction	LC50, EC50, NOEC, LOEC	(Eaton et al. 2005)
Hexagenia sp. Mayfly	Static or flow-through	4-7 d, 5-60 d 30-90d	Survival, Growth, Emergence	NR	(Eaton et al. 2005)
Chironomus sp. Non-biting midge	Flow-through	30 d	Mortality, growth, emergence, no. mature eggs	NR	(Eaton et al. 2005)
Freshwater mussels	Static, recirculating or flow-through	2 d	Mortality	NR	USEPA, 2006
Freshwater mussels: Glochidia	Static, renewal or flow through	1 d	Mortality	LC50, EC50, IC50, NOEC, LOEC	(ASTM 2006)
Freshwater mussels: juvenile	Static, renewal or flow through	4 d	Mortality	LC50, EC50, IC50, NOEC, LOEC	(ASTM 2006)
Freshwater mussels: juvenile	Static, renewal or flow through	10-28 d	Mortality, growth	LC50, EC50, IC50, NOEC, LOEC	(ASTM 2006)
Chironomus tentans	Flow through	50-65 d	Mortality, weight, emergence, sex ratio, adult mortality, no. egg cases laid, no. egg cases produced, no. hatched eggs		(USEPA 2000)

[1] two protocols, water spiked, and sediment spiked
[2] three exposure scenarios - aqueous, sediment:water and interstitial

4.1 Test species

In addition to the seven taxa in the 16 protocols outlined above, the test protocols frequently make reference to alternative taxa (e.g. *Chironomus riparius, C. tentans* or *C. yoshimatsui* are all identified as suitable species in the OECD guidelines). Details of the additional species for these protocols are listed in Appendix I. In addition to these protocols, less prescribed test methods are identified in more general guidance documents published by ASTM, USEPA and OECD to facilitate the choice of test species and test design. Many of the factors considered for selecting additional test species as higher tier tests are relevant to this literature review (USEPA 1996a, b, ASTM 2004b, OECD 2006, ASTM 2007a, b, 2008a).

Twenty-five species were identified as potential test species in the Standard Guide for Selection of Resident Species as Test Organisms for Aquatic and Sediment Toxicity Tests (ASTM 2004b). These included Protozoa (four), Rotifer, Coelenterata (one), Oligochaeta (four), Mollusca (one gastropod, three bivalves) and Insecta (five Ephemeroptera, three Plecoptera, one Odonata, one Trichoptera and two Diptera. APHA provide some guidance on test designs for Ephemeroptera, Plecoptera, Trichoptera, rotifers, oligochaetes and molluscs (Eaton et al. 2005).

ASTM produced a list of recommended additional test species. These are available, of commercial, recreational and ecological importance, been used in previous studies, and easy to handle in the laboratory. The list includes stoneflies, mayflies, midges, snails and flatworms. The use of species from this list is encouraged to increase comparability of results for a few species (ASTM 2007a).

When selecting species for toxicity testing it is important to consider the ease with which the species might be cultured in the laboratory or sourced from elsewhere. Test species should be taxonomically identifiable, readily available from the field or in-house cultures, easily maintained in the laboratory, have a broad geographical distribution, tolerant to broad range of sediment physiological properties (e.g. organic carbon, grain size), be compatible with exposure duration and endpoints and tolerant of water quality characteristics (ASTM 2008b).

Most non-standard species cannot be reliably cultured under laboratory conditions. However, the mayfly *Cloeon triangulifer* and the rotifer *Brachionus acuticornis* were identified as species that could be cultured in the laboratory. It should be noted that there may be changes in the sensitivities of populations that are held in laboratory cultures (ASTM 2004b). Intermittent testing against a reference toxicant can ensure that the population's sensitivity is not altered.

If the species cannot be cultured in the laboratory, they may be sourced from natural communities. Field collected organisms should be representative of species likely to be exposed to the test item in natural systems and sourced from uncontaminated areas. The species should have a wide geographical distribution to allow field collections to be made. Species of an appropriate age and condition should be easily available for most of the year. Ease of handling, collection, resistance to handling and no exposure to prior contaminants should all be considered when identifying suitable test species (ASTM 2004b). The selected taxa also need to be sensitive to the test item (ASTM 2007b).

Immature individuals are usually more sensitive and should be selected unless the endpoint of interest requires adult exposure. Some taxonomic groups are not ideally suited to acute lethality endpoints. For example, bivalves can close their valves for extended periods of time thus, limiting the potential effects of toxicants. However, reduction in shell deposition can be determined under conditions suited to rapid growth (ASTM 2007a). *Lumbriculus* were exposed to seven pesticides in a four day test (malathion, 2,4-D, sevin, chlordane, methoxychlor, treflan, DDT). The results were compared with 24 and 48h EC50 toxicity data for standard test species *Daphnia* sp. *L. variegatus* were found to be less sensitive in all instances (Bailey 1980).

It is also important to consider which life stages might be sensitive, and which will provide the required endpoints (ASTM 2004b). For example, first instar *Chironomus* larvae have been demonstrated to be more than two orders of magnitude more sensitive to some metals than later instars in sediment tests (ASTM 2008b). Consideration should also be given as to whether particular trophic levels are of interest (e.g. filter feeders, deposit feeders, algal scrapers, predators). Finally it is important that taxonomic identity is accurate (ASTM 2004b). This is particularly important for field-collected species where two or more similar species may be present, and in some instances may not be distinguished from each other until later instars (Eaton et al. 2005, ASTM 2008b).

Source and history of test organisms, including acclimation time and acclimation conditions should be reported together with full description of test design, sources of water and sediment, and full description of survival, growth and behaviour of control organisms (ASTM 2004b).

4.2 Test design

Sixteen protocols were identified; six used a static test system, one used a semi-static test system, three used a flow-through test system and six identified various test systems as appropriate. Static tests are the easiest tests to set up and perform. In static tests the water level can be topped up during the study to replace evaporated media. However, semi-static or flow-through tests are likely to be more appropriate for chronic studies or larger organisms where there is a need to maintain the exposure concentration and suitable environmental conditions. It should be noted that where sediment is present the equilibrium between sediment and water is more difficult to monitor and/or maintain (ASTM 2008b). Test item concentrations should be measured at the start and end of the test. In chronic semi-static and flow-through tests, additional measurements should be taken during the study. Measured concentrations confirm the test system has been designed appropriately and operating correctly and reflect actual concentrations to which test organism is exposed (USEPA 1996a).

Eight of the twelve protocols identified a requirement for sediment, three specified natural, two specified artificial and three allowed either natural or artificial sediments to be used. Where exposure is not via the sediment many benthic or sediment dwelling organisms may survive in water-only test system with provision of chemically inert structures to facilitate normal behaviour in natural habitat, e.g. glass tubes for sediment dwelling mayfly *Hexagenia* sp. (Henry et al. 1986, ASTM 2004b).

Artificial or dechlorinated tap water was specified for four of the protocols, seven specified either artificial or natural water and five did not record the preferred water type. Six chronic protocols identified a need to feed the test organisms. Five studies (all chronic) specified that aeration should be provided to maintain dissolved oxygen levels.

Ideally test duration should be sufficient to ensure a time-independent toxicity level can be determined. Different taxonomic groups and different life stages may require different test durations (e.g.) (ASTM 2004b). For Daphnids, chironomids and Chaoboridae, acute exposure should last for at least two days. Other invertebrate taxa should be exposed for at least four days. Organisms should not be fed during or immediately before acute toxicity tests. Uneaten food and faecal material will decrease dissolved oxygen levels and may reduce biological activity of some test items due to the additional fate pathways present in the test system (ASTM 2007a).

The physical variables of a test may influence behavioural responses of the test species. For example, factors such as temperature, light, water quality, water flow, substrate, cover and food quality (ASTM 2007b) should be considered. It is also important to ensure the loading rate (stocking density) of organisms in the test chambers is not too high. This is to ensure dissolved oxygen levels and the test item concentrations are maintained. Furthermore, stress from crowding or aggregation will be avoided. Loadings should be reduced if necessary to keep DO above 60% saturation (ASTM 2007a). Where appropriate artificial substrates can be found to simulate natural habitat benthic or sediment dwelling organisms may be tested in water only tests (ASTM 2004b).

Field collected organisms should be maintained for at least two days in dilution water at test temperatures. The transfer from field water to dilution water and adjustment to test temperature should be gradual to avoid shock. Where more than 5% of organism show signs of disease or stress the group should not be used (ASTM 2007a).

Test validity criteria were identified that should be met in most testing situations (ASTM 2007a):

- Test chambers should all be identical
- Treatments should be randomly assigned
- Dilution water or solvent control should be included
- Organisms were disease free and had not been treated for disease within ten days
- Test organisms maintained in dilution water at test temperature for at least two days prior to exposure
- Individuals randomly assigned to test chambers
- Not more than 10% organisms in control showed disease, stress, unusual behaviour or mortality during the test
- Dissolved oxygen should be measured at the start and end of the test and at least every 48 h in high, medium and low test concentrations as a minimum requirement
- Maximum and minimum temperature should be measured daily in one test chamber as a minimum requirement and maintained between prescribed parameters

- At least 60% dissolved oxygen saturation in a renewal or flow through test, at least 40% dissolved oxygen saturation in a static test
- Organic solvents should not exceed 0.5 ml/L
- Surfactants must not be used to prepare test solutions
- When calculating LC50 or EC50 one treatment should affect less than 37% and one treatment should affect more than 67% of exposed organisms.

4.3 Endpoints

When selecting species for toxicity testing, it is important to identify sensitive endpoints (e.g. survival, growth, reproduction, emergence and metabolism) and include taxa which are sensitive to specific modes of action e.g. insects for insecticides (Eaton et al. 2005, ASTM 2008b).

In the sixteen standard protocols listed in Table 4.1, fifteen listed mortality or immobility as an endpoint. Endpoints other than mortality or immobility were almost exclusively associated with chronic toxicity tests. Eight protocols identified growth parameters as an endpoint, five measured emergence, five measured reproduction and two protocols measured F1 survival. Two protocols identified bioconcentration as an endpoint. Physical impairment and behavioural changes were each identified once in the standard protocols.

Mortality and immobility are the most commonly reported endpoints. Immobility is usually defined as lack of movement except for minor spontaneous movement of appendages (ASTM 2007a). Behavioural responses can include respiration, locomotion, habitat selection (e.g. orientation, response to light), competition, feeding (feeding preferences, feeding rates, prey selection, feeding efficacy), predator avoidance and reproduction. These responses will alter by species, genetic strain, population, gender, and developmental stage of the organism (ASTM 2007b). General observations such as erratic swimming, loss of reflex, excitability, discoloration, excessive mucus production, moulting, cessation of burrowing and cannibalism should be recorded (ASTM 2007a).

4.4 Endocrine disruption

An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations (CSTEE 1999). The endocrine system in invertebrates is best described in insects due to the development of 3rd generation insecticides targeting the endocrine system of the target species. The insecticides act as juvenile hormone (ant)agonists or ecdysone (ant)agonists, interfering with various processes, including moulting, metamorphosis, vitellogenesis and reproduction (OECD 2006).

Endocrine systems in invertebrates are not analogous to vertebrate systems. Insecticides acting as hormone (ant)agonists and may have adverse effects on non-target invertebrates (Taenzler et al. 2007). Endocrine Disrupting Chemicals (EDCs) can affect moulting, morphology, behaviour, sexual maturity, time to first brood, egg development time, brood size (fecundity) and sex determination in invertebrates (OECD 2006). Development of standardized test to cover these types of effect is required (Taenzler et al. 2007).

Unfortunately knowledge of which potential EDCs affect invertebrate species, and the mode of action of any effects expressed, is incomplete. Effects associated with potential endocrine disrupters can be latent and not expressed until later in life or until reproduction occurs. In order to capture these latent effects, tests for endocrine disruption often encompass two generations. This allows effects on fertility and mating, embryonic development, sensitive neonatal growth and development, and transformation from the juvenile life state to sexual maturity to be evaluated (OECD 2006).

For species that reproduce parthenogenetically, genetic variability within the test population can be low compared to species that reproduce sexually. Reducing variability in responses allows more subtle impacts to be detected (Ingersoll et al. 1996). However, the use of parthenogenetically reproducing animals will result in important sexual reproduction processes (e.g., gametogenesis) are not being evaluated (OECD 2006)

Present regulatory ecotoxicity testing cannot detect all endocrine disrupting effects. New methods should be based on *in vivo* exposure and invertebrate tests should include endpoints to allow which cover full life-cycle effects related to endocrine disruption (CSTEE 1999).

Research on the effect of EDCs on invertebrates have identified a number of important factors that require consideration (OECD 2006):

- Chronic test may reveal impacts at much lower doses than acute tests
- EDCs may affect invertebrates differently to vertebrates
- Pesticides affecting physiological processes in the target organism may affect different processes in non-target organisms
- Responses of males and females may differ
- Endocrine effects do not necessarily correlate well with toxicity effects or octanol/water partition co-efficients (*Kow*)
- Some endpoints to potential EDCs can also be caused by non-EDCs.

4.5 Summary of standard methods and guidance

Sixteen detailed standard protocols were identified although guidance exists for many other taxa. Guidance is also provided on selecting and testing non-standard additional resident species. These documents are useful in determining the suitability of non-standard test species when additional tests are required to address effects on particular taxonomic groups.

The chironomid Full Life Cycle tests (USEPA 2000) may address the requirement for a chronic toxicity test with an insect species that includes endpoints suitable for determining population level effects as a result of exposure to potential EDCs. However, the PPR Opinion stated a preference for a chronic insect test without sediment to simplify the exposure situation.

5 Methods for acute and chronic toxicity tests in Contract and Research Organisations

CROs involved in ecotoxicity tests with invertebrate species were approached and asked for details of any test methods they had used with non-Crustacea invertebrates. Information on the source and age of species, test parameters, test endpoints and assessment criteria were obtained.

Responses were received from seven CROs. These included a total of fifty-three protocols. Thirty-eight acute protocols and six chronic protocols were reported in water only test systems. One acute and seven chronic protocols were reported in sediment:water test systems. In total, there were forty-three test species.

Twenty of the test protocols were reported to have been used in GLP compliant tests. These included one rotifer, one Chaoboridae, five Chironomidae, three Ephemeroptera (mayfly), one Trichoptera (caddisfly), one Odonata (damselfly), three Hemiptera, four molluscs and one Oligochaeta.

5.1 Test species

5.1.1 Insects

Twenty-six of the forty-three test species were insects. These included eight Diptera (*Chaoborus obscuripes*, *C. crystallinus, Dicrotendipes sp., Chironomus riparius*, *Endochironomus albipennis, Glyptotendipes sp., Macropelopia sp., Culex* sp.), six mayfly (*Cloeon dipterum*, *Caenis horaria, Serratella ignita, Haproleptoides confuse, H. lauta, Ephemera danica*), three Caddisfly (*Hydropsyche* sp., *Molanna angustata, Sericostoma* sp.), four Hemiptera (*Sigara striata, Notonecta maculate, Plea minutissima, Ranatra linearis*), three Odonata (*Erythromma viridulum*, *Anax imperator, Coenagrionidae*), one Sialidae (*Sialis lutaria*) and one Lepidoptera (*Paraponix stratiotata*).

Two species were sourced from a commercial supplier, eight species were field collected, two species were in-house stock and 16 were sourced from mesocosms or rainwater reservoirs. Where acclimation time was reported for field or mesocosm collected organisms it was for between one and five days.

5.1.2 Non-arthropod invertebrates

Seventeen of the forty-three test species were non-arthropod invertebrates. These included six gastropod (*Bithynia tentaculata, Lymnaea stagnalis, Physa fontinalis, Planorbarius corneus, Planorbis contortis , Melanoides auberculata*), one bivalve (*Sphaerium* sp.), four Oligochaeta (*Dero digitata, Stylaria lacustris, Lumbriculus variegatus*, *Tubifex* sp.), four flatworm (*Dugesia sp., D. lugubris, Polycelis nigra, P. tenuis*), one leech (*Erpobdella* sp.) and one rotifer (*Brachionus calyciflorus*).

Two species were sourced from a commercial supplier, nine species were field collected, four species were in-house stock or laboratory culture and three were sourced from mesocosms or rainwater reservoirs. Where acclimation time was reported for field or mesocosm collected organisms, it was for between one and five days.

5.2 Test design

Forty-three protocols employed a static test system and ten employed a semi-static system. Artificial sediment was used in three of the static test protocols. Sand was used as sediment in two of the semi-static protocols and stones were used in two semi-static protocols for Ephemeroptera.

Water used in these protocols included filtered natural water, Elendt M4, reconstituted water (ASTM and OECD specifications). Interestingly each CRO has selected a water source and this source is used for all species tested within that facility. With one exception (*Chironomus riparius*), test species were only fed in studies with a duration of seven or more days. There was only one chronic study were food was not specified (*Sphaerium* sp.). Aeration was supplied in all studies with a duration of ten or more days with the exception of a 30-90 d protocol for *Chaoborus crystallinus*. Aeration was only supplied in three acute protocols; these were for the Ephemeroptera; *Ephemera danica, Haproleptoides confuse, H. lauta* and *Serratella ignita*.

5.3 Endpoints

In protocols for acute tests, the reported endpoints were all for mortality or immobility. Twenty-six acute protocols also identified at least one behavioural endpoint but details were not provided.

In protocols for chronic tests, the reported endpoints included mortality, immobility, moulting, pupation, time to emergence, emergence success, daily and total emergence, sex ratio, reproduction and development rate (Chironomidae), survival, growth, reproduction and hatching rate of eggs (Lymnaea), emergence characteristics, sex ratio (Ephemeroptera), growth measured as total biomass (Lumbriculidae) and unspecified behavioural endpoints.

5.4 Potential test species

Three tests were identified that meet the requirement of providing chronic toxicity tests for insects. Freshly hatched *Chaoborus crystallinus* were exposed in a semi-static water-only test system for 30-90 d (until emergence). Endpoints included mortality, growth, moulting, pupation, emergence and reproduction. In addition, one CRO confirmed that long-term culturing of this species in the laboratory is possible, further suggesting its suitability as a candidate test species

Two mayfly taxa, *Haprophlebia lauta* and *Serratella ignita,* were collected from field populations and exposed in a semi-static sediment:water test system. Test durations were 56 d and 28 d respectively and endpoints from both species were mortality, emergence characteristics and sex ratio. A test was also identified for the gastropod *Lymnaea stagnalis.* Juveniles and adults were exposed in a water only test system for 28 d. Endpoints were survival, growth, reproduction, fertility and hatch rate.

5.5 Summary of literature review

Four of the 43 species tested were standard test species. Novel protocols were identified for *Chaoborus crystallinus, Haprophlebia lauta, Serratella ignita* and *Lymnaea stagnalis.* In addition to these tests a wide range of test species, predominantly field collected and resident in at least some areas of Europe were identified. These included Diptera, Ephemeroptera, Trichoptera, Hemiptera, Odonata, Sialidae, gastropod snails, bivalves, Oligochaeta, and rotifer. These have all been used in acute toxicity tests and in some cases they have also been used in chronic toxicity tests

6 Literature review of methods for acute and chronic toxicity tests

6.1 Test species

A total of 111 protocols were identified. Seventy-six acute protocols and 26 chronic protocols were conducted in water-only test systems. Three acute protocols and six chronic protocols specified the presence of sediment. In total there were seventy test species.

6.1.1 Insects

Forty-seven of the seventy test species were insects. These included one Coleoptera (*Gyrinus natator*), twelve Diptera (*Aedes aegypti, Chaoborus crystallinus, C. obscuripes, Chironomini, Chironomus riparius, C. tentans, C. thummi, Cricotopus spp., Culex pipiens, Macropelopia* sp*., Simulium latigonium, Tanytarsus dissimilis*), eleven Ephemeroptera (*Ameletus* sp*., Atalophlebia* spp*., Baetis* sp*., Caenis horaria, C. miliaria, Cinygmula reticulata, Cloeon dipterum, C. triangulifer, Epeorus longimanus, Ephoron virgo, Hexagenia bilineata*), five Hemiptera (*Anisops sardeus, Corixa punctata, Notonecta glauca, N. maculate, Sigara striata*), one Megaloptera (*Sialis lutaria*), six Odonata (*Austrolestes colensonis, Cordulia aenea, Erythromma viridulum, Lestes sponsa, Sympetrum striolatum, Xanthocnemis zealandica*), three Plecoptera (*Calineuria californica, Hesperoperla pacifica, Pteronarcys dorsata*) and eight Trichoptera (*Brachycentrus americanus, Clistoronia magnifica, Cyrnus trimaculatus, Hydropsyche angustipennis, H. siltalai, Lepidostoma unicolor, Notidobia ciliaris, Psychoglypha* sp.). Thirteen of these taxa were used in CRO tests.

6.1.2 Non-arthropod invertebrates

Twenty-three of the seventy test species were non-arthropod invertebrates. These included one Hydracarina (*Piona carnea*), two Hydrozoa (*Hydra viridissima, Hydra vulgaris*), six Oligochaeta (*Lumbriculus variegatus, Tubifex tubifex, Dero digitata, Limnodrilus hoffmeisteri, Stylaria lacustris, Stylodrilus heringianus*), nine Mollusca (*Bithynia tentaculata, Dreissena polymorpha, Juga plicifera, Lampsilis siliquoidea, Lymnaea acuminata, L. stagnalis, Physa integra, Planorbis planoris, Unio elongatulus eucirrus*), one Rotifer (*Brachionus calyciflorus*), four Turbellaria (*Dugesia lugubris, Polycelis nigra, P. tenuis, Dugesia dorotocephala*). Six of these taxa were used in CRO tests.

Insect and non-arthropod invertebrates were sourced from laboratory cultures (28%), eggs or glochidia from field populations to establish laboratory population (6%), or field collected organisms (58%). It is evident from the above species lists that a very wide range of insect species have been used in ecotoxicity tests.

6.2 Test design

In acute tests, 49 protocols reported a static test system, five reported a semi-static system and two protocols were conducted using a flow-through system. Sediment was reported in two static and one flow-through system. In chronic tests, six protocols reported a static test system, 13 tests used a semi-static test system and 8 protocols used a flow-through test system. It should be noted that reporting of test design parameters was often incomplete.

Water used in these protocols included dechlorinated or aerated (a technique employed to actively volatilise chlorine from tap water) tap water, groundwater (often dechlorinated), pond lake or river water reservoir water and reconstituted water. No attempt was made to link water source with taxa or endpoint as the responses from CROs indicated that each laboratory had a single water source that was used for most or all of their non-standard species tests. Twelve percent of acute tests reported feeding of test organisms during testing. Feeding was reported in 56% of chronic tests.

6.3 Endpoints

For many protocols, more than one endpoint was reported. Acute studies all reported mortality or immobility (59) with some including feeding inhibition (three) or emergence in late instar insects (two). One acute study with *Chaoborus* sp. reported 'ability to stay in suspension' as an acute endpoint. Growth and avoidance behaviour were also reported.

The number of endpoints measured and range of endpoints used in chronic protocols was much greater. Once again mortality and immobility were the most common endpoints (15). Endpoints relating to development and reproduction were identified in a number of chronic studies. These included growth (three), gill beats (one), moulting (three), body condition index (one), emergence (two) and reproduction (one). Endpoints relating to physical integrity were reported for a number of non-arthropod invertebrates. These included morphological abnormalities (one), head lesions (one), fissioning (one), physical integrity (one) and strength (one). Behavioural changes (five) and bioaccumulation (four) were also reported (Appendix 3).

6.4 Potential test species

Six tests were identified that meet the requirement of providing chronic toxicity tests for insects. First instar *Cloeon triangulifer,* cultured in the lab from field collected, stored eggs were exposed in a semi-static water only test system for approximately 43 d (until emergence and ovipositing). The endpoints were emergence, egg viability and adult residues. The eggs were exposed in a static or semi-static system to determine hatch success and larval mortality. Maintenance of laboratory cultures of *Ephoron virgo, Cyrnus trimaculatus* and *Hydropsyche angustipennis* were identified although the test conducted were only for acute tests. However, if the Trichoptera C. trimaculatus and H. angustipennis can be maintained in laboratory cultures then chronic and Full Life Cycle (FLC) tests may be possible. Several chronic and FLC tests are identified for *Chironomus* sp. The existence of laboratory cultures and well established chronic test protocols make this a useful test species. Unfortunately, the presence of sediment in the test system complicated the exposure profile. Finally sexually mature *Lymnaea stagnalis* were exposed for 84 d in a semi-static waster only test system. Endpoints included egg production, egg hatching success and hatching survival.

6.5 Summary of the literature review

Four of the 43 species tested were standard test species. Novel protocols that meet some or all of the PPR Panel requirements were identified for *Chaoborus crystallinus, Haprophlebia lauta, Serratella ignita* and *Lymnaea stagnalis.* There was also a chronic (two day) rotifer test for resting egg production. In addition to these tests, a wide range of test species, predominantly field collected and resident in at least some areas of Europe were identified. These included Diptera, Ephemeroptera, Trichoptera, Hemiptera, Odonata, Sialidae, gastropod snails, bivalves, Oligochaeta, and rotifer. These have all been used in acute toxicity tests and in some cases they have also been used in chronic toxicity tests. Six potential protocols and/or species were identified from the literature. These were *Cloeon triangulifer, Ephoron virgo, Cyrnus trimaculatus, Hydropsyche angustipennis, Chironomus* sp. and *Lymnaea stagnalis.*

7 Identified protocols for testing the effects of chemicals against aquatic invertebrates other than Crustacea

The aim of this literature review was to examine the existing standard test methods, identify other test species currently in use in CROs and search the peer reviewed literature to provide a short-list of species that meet some or all of the PPR Panel criteria. Protocols for a total of 75 genera from 20 taxonomic orders were identified as potential methods meeting the PPR Panel criteria (Table 7.1). Test species were dominated by arthropods, with Insecta accounting for 69% of all test species.

Table 7.1: Summary of the taxonomic composition of species considered in this review.

Phylum	Class	Order	No. of genera
Arthropoda			
	Arachnida	Hydracarina	1
	Insecta	Coleoptera	1
	Insecta	Diptera	12
	Insecta	Ephemeroptera	12
	Insecta	Hemiptera	6
	Insecta	Lepidoptera*	1
	Insecta	Megaloptera	1
	Insecta	Odonata	8
	Insecta	Plecoptera	2
	Insecta	Trichoptera	9
Non-arthropods			
Annelida	Hirudinea	Arhynchobdellida	1
Annelida	Oligochaeta	Lumbricida	2
Annelida	Oligochaeta	Tubificida	4
Cnidaria	Hydrozoa	Hydroida	1
Mollusca	Bivalvia	Unionoida	2
Mollusca	Bivalvia	Veneroida	2
Mollusca	Gastropoda	Basommatophora	4
Mollusca	Gastropoda	Neotaenioglossa	3
Platyhelminthes	Turbellaria	Tricladida	2
Rotifera	Monogonota	Plioma	1

* aquatic larval stage

7.1 Selection of test species

Test species were required for two different objectives. Firstly, to provide a species for testing potential EDCs in non-Crustacea invertebrates and provide population relevant endpoints. Secondly, to identify a non-Crustacea invertebrate from another taxonomic group to try and address the diversity among invertebrates.

An ideal test species for examining potential EDCs in a chronic test should be easily sourced, suitable for laboratory culture, not excessively stressed by handling and thrive under control conditions for most of it's lifecycle in a water-only test system (OECD 2006). It should have a short life-cycle, reproduce sexually throughout the year, be a sensitive representative of non-target species in the field and include many of the potentially endocrine sensitive processes in it's lifecycle. Some of these criteria can be met by a number of species identified in this literature review.

Many of these species may also be suited to the second objective, to identify non-Crustacea invertebrates from another taxonomic group where there is a requirement to increase the diversity in test organisms (EFSA 2007). These tests may be of a shorter duration than those used to test for EDCs. Taxa that were identified as potential test species are summarised in Table 7.2.

It was evident from the questionnaire to CROs that a wide variety of indigenous non-standard test species can be used in acute laboratory toxicity tests. A few of these species are also suited to chronic studies. Field collected organisms should be collected from a clean source and held for several days in test conditions to ensure they are not stressed by the test environment (ASTM 2004b).

In addition to existing standard test methods and the CRO questionnaire a literature review was conducted. Further potential test species were identified. Several methods for culturing Ephemeroptera and Trichoptera methods from field collected or stored eggs were also identified. Storing eggs from a field population would allow testing throughout the year. However, if such test methods were to be used it would be necessary to verify that there was no change in sensitivity with season or storage time of the eggs.

Each of the species identified in Table 7.2 has a number of advantages and limitations when it is considered as a suitable test species. These will be considered in turn. Full details of the protocols are listed in Appendix 4.

Chironomus riparius

Chironomus sp. (Diptera) has a worldwide distribution and can be found inhabiting almost every type of water body. They are an established regulatory test genus. Eggs hatch after two to six days, there are then four larval instars, followed by a short pupal stage lasting one to two days. Males emerge before females (protandry) and adults are short lived (OECD 2006).

As chironomid larvae live in the sediment a suitable substrate must be provided. (Watts et al. 2003) demonstrated that sediment type was an important factor in differences in reported responses as concentrations of some contaminants were greater in the pore waters of the artificial sediment than in those of the natural sediment, thus altering the exposure regime. (Watts et al. 2003) also found that *C. tentans* was more sensitive than *C. riparius* to the same toxicants evaluated under the same test conditions. *C. tentans* was also less physically robust resulting in greater variability in the data, especially emergence data (OECD 2006).

The chironomid protocols are two generation studies. Sexual reproduction and a pupal stage maximise the species characteristics that may be affected by EDCs. The main disadvantage of *C. riparius* is the need for a sediment:water testing system which complicates exposure routes.

Table 7.2: Potential protocols for chronic toxicity tests with non-Crustacean aquatic invertebrates (tests include endpoints that may be sensitive to endocrine disruption)

Species:	***Chironomus riparius***
Test description:	Full life cycle test including F1 viability endpoints
Source:	Laboratory culture
Test design:	Modification of OECD method with continuous exposure from 1st instar of parent through to emergence of the F1 generation
Endpoints:	Emergence ratio, development rate, no. egg ropes per female, fertility of egg ropes, viability of offspring, sex ratio of emerged adults (P and F1 generation)
Validation criteria:	Same as the OECD test guidelines
Reference:	Several similar protocols including (Taenzler et al. 2007), (USEPA 2000)
Species:	***Cloeon triangulifer***
Test description:	Two tests reported, together they cover exposure from 1st instar to hatching success of F1 generation
Source:	Laboratory culture
Test design:	1st instar exposed to test item in a semi-static test system with natural water and no sediment Following emergence and egg laying the hatch success is determined.
Endpoints:	Emergence, hatch success, adult residues
Validation criteria:	Not recorded
Reference:	(Sweeney et al. 1993)
Species:	***Chaoborus crystallinus***
Test description:	Part life cycle test
Source:	Field collected and in-house stock (potential for long term culturing of laboratory stocks has been identified by the relevant CRO)
Test design:	1st instar exposed in a semi-static water only test system until emergence (30-90 d)
Endpoints:	mortality, growth, moulting, pupation, emergence, reproduction
Validation criteria:	None specified
Reference:	CRO protocol (see Appendix 2)
Species:	***Lymnaea stagnalis***
Test description:	Reproduction in a hermaphroditic snail
Source:	Laboratory culture
Test design:	84 d semi-static test in reconstituted water with no sediment
Endpoints:	Adult mortality, fecundity, mean no. egg clutches, hatchability (in clean water)
Validation criteria:	Not specified
Reference:	(Czech et al. 2001)
Species:	***Brachionus calyciflorus*** **(rotifer)**
Test description:	Resting egg production
Source:	Laboratory culture or cysts
Test design:	4 d static test in reconstituted water initiated with neonate females. Feeding during the study, no sediment, no aeration
Endpoints:	Resting egg production
Validation criteria:	Not recorded
Reference:	(Preston et al. 2000)

Cloeon triangulifer

The test is initiated with 1st instar larvae and ends with hatch success of F1 generation. The test is conducted in a water only test system, simplifying the test design and determination of exposure concentrations. *C. triangulifer* reproduces as parthenogenetic clones, reducing intraspecific variability thus increasing the power of the test to detect subtle effects. However, parthenogenesis and no pupal stage reduce the number of species characteristics that may be affected by EDCs. There is also an absence of information on how the species is cultured in the laboratory. *Cloeon triangulifer* is a North American species. However, *Cloeon dipterum* is a widespread and abundant species in Europe and may respond well to similar treatment.

Chaoborus crystallinus

C. crystallinus (Diptera) lives in the water column of static water bodies. It is known to be extremely sensitive to some pesticide groups and, like *Chironomus* sp., undergoes full metamorphosis. Eggs are deposited on the water surface which hatch and there are four larval instars followed by a pupal stage and emergence for sexual reproduction. In natural water bodies in the UK, Chaoboridae are univoltine or bivoltine. The test is initiated with 1st instar larvae in a semi-static water only test system. The test duration is 30-90 days with mortality, growth, moulting, pupation, emergence and reproduction endpoints.

C. crystallinus has the advantages of sexual reproduction and a pupal stage maximising the species characteristics that may be affected by EDCs. It also has the added advantage of the test being conducted without sediment, simplifying the exposure regime. It is also known to be sensitive to many pesticides. In addition, *C. crystallinus* are strong candidates for a potential test species as they have been demonstrated to be suitable for long term culturing in a laboratory environment and, the use of artificial substrates is not required.

Lymnaea stagnalis

Lymnaea stagnalis is an abundant and widespread gastropod mollusc found in European fresh waters. The test is initiated with sexually mature individuals sourced from a laboratory culture. The test was conducted in a semi-static water only test system. The test duration is 84 d with endpoints of adult mortality, fecundity, mean number of egg clutches and hatch rate. The adults were exposed for up to twelve weeks. Eggs were removed and hatched in clean water and maintained under culture conditions until sexually mature.

This protocol provides a chronic exposure test for an indigenous gastropod snail that can be cultured in the laboratory. This protocol could be useful for determining the sensitivity of indigenous non-arthropod invertebrates to pesticides with a specific mode of action (e.g. molluscicides). It also presents some reproductive endpoints although eggs were not exposed to the test item. A disadvantage of this protocol is the lack of exposure of juvenile life stages. Also, the sensitivity of *L. stagnalis* relative to other gastropod snails is not known.

Brachionus calyciflorus

Brachionus calyciflorus is a rotifer species found in static water bodies. Most planktonic rotifers reproduce via cyclical parthenogenesis incorporating both asexual and sexual reproduction. Asexual reproduction predominates but sexual reproduction is important as resting eggs are produced. Seasonal resting egg production is an important population level endpoint as in temperate regions it may be only the resting eggs that survive over winter.

The test is initiated with neonate females in a static, water only test system. The test duration is four days with resting egg production as the endpoint. The test endpoint was shown to be very sensitive for pentachlorophenol (PCP) relative to other rotifer endpoints. It has the advantage of being a quick and simple test and provides information on the sensitivity of a non-arthropod invertebrate. It's main disadvantage is the relative insensitivity of rotifers to many insecticides.

Other species

Culturing methods also exist for the European species *Ephoron virgo, Hydropsyche angustipennis* and *Cyrnus trimaculatus*. The protocols detailed in the literature for these taxa are all acute tests. However, *H angustipennis* and *C. trimaculatus* were both cultured in the laboratory and could therefore be candidate taxa for whole life cycle or chronic tests. *Cloeon dipterum* or *Ephoron virgo* were both reared in the laboratory from field collected eggs.

In addition to these species, a wide range of field-collected European species could be used as additional test species. However, with field collected organisms their history is unknown. For higher tier testing this level of variability may be acceptable as long as the source of the organisms is identified and described. However, it is less suitable for lower tier tests where results from different facilities and different chemicals need to be compared. For this reason, it is recommended that additional test species are limited to those species that can be either reared in the laboratory or will tolerate an acclimation period of several days.

7.2 Recommendations and conclusions

The objectives of this report were to "compile all available scientific information for available protocols for testing the effects of chemicals against aquatic invertebrates other than crustacean". This literature review considered whole organisms tests and focused on effects of pesticides in freshwater species. A supplementary questionnaire was sent out to CRO's to identify taxa and protocols in use by these facilities.

Five protocols were identified as providing information additional to that provided by existing standard protocols. The species identified were *Chironomus riparius, Cloeon triangulifer, Chaoborus crystallinus, Lymnaea stagnalis and Brachionus calyciflorus*. An overview of the reasons for identifying each method are given below. Test methods are presented in Section 7.2:

- *Chironomus riparius* (Chironomid) was considered a suitable test species because the protocol is for a full life cycle test including F1 viability endpoints. The test method is an extension of the existing OECD test guidelines and the taxa is easily cultured in the laboratory. The method includes additional individual and population relevant endpoints for two generations making the protocol a good candidate for monitoring the effects of potentially endocrine disrupting insecticides. The main disadvantage of *C. riparius* is the need for a sediment:water testing system which complicates exposure routes.

- *Cloeon triangulifer* (Mayfly) was considered a suitable test species because the protocol covers exposure from early instar parent to F1 hatching success and may therefore be a good candidate for monitoring the effects of potentially endocrine disrupting insecticides. However, *C. triangulifer* reproduces as parthenogenetic clones and does not have a pupal stage, reducing the number of species characteristics that may be affected by EDCs. The test duration also means this protocol would be a suitable candidate for chronic toxicity tests in addition to *Daphnia.*

- *Chaoborus crystallinus* (Phantom midge or non-biting midge) was considered a suitable test species as the protocol provides a chronic toxicity test in addition to *Daphnia.* It may also be a good candidate for monitoring the effects of potentially endocrine disrupting insecticides as a number of potentially sensitive endpoints (e.g. moulting, pupation, emergence) are included. *C. crystallinus* was also identified as being suitable for long term culturing in the laboratory and does not require sediment in the test system.

- *Lymnaea stagnalis* (Pond snail) could be useful for determining the sensitivity of indigenous non-arthropod invertebrates to pesticides with a specific mode of action (e.g. molluscicides). A disadvantage of this protocol is the lack of exposure of potentially sensitive juvenile life stages.

- *Brachionus calyciflorus* (Rotifer) was considered a suitable test species because the protocol provides a chronic toxicity test in addition to *Daphnia.* It's main disadvantage is the relative insensitivity of rotifers to many insecticides.

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Appendix 1: Existing standard methodologies

A1.1: Acute toxicity tests in water only test systems

ASTM Standard Guide for Acute Toxicity Test with the Rotifer Brachionus	
Reference:	(ASTM 2004a)
Test species	
Species tested:	*Brachionus calyciflorus*
Source of organisms:	Hatched from cysts
Age of organisms:	0-2 hours
Acclimation time:	16 – 22 hours to hatch
Acclimation conditions:	Hatched at 25 ºC in standard dilution water
Test design	
Test type:	Static
Test duration (days):	1 d
Endpoints:	Mortality
Effects:	LC50
No. treatments:	5
Replicates per treatment:	4
Organisms per replicate:	10
Feeding :	None
Aeration or additional substrate:	None
Test acceptability criteria:	Dissolved oxygen >90%, <10% control mortality, <37% mortality in at least one test concentration, >67% mortality in at least one test concentration.
Test conditions	
Test chamber size (ml):	2.5
Test chamber material:	Tissue culture plates
Water source:	Reconstituted water
Water volume(ml):	1
Water quality measurements:	Dissolved oxygen, temperature, pH, hardness
Temperature (ºC):	25 ± 1ºC
Illuminance (lux):	-
Photoperiod:	0L:24D

Role of artificial burrows in Hexagenia toxicity tests: Recommendations for protocol developments.	
Reference:	(Henry et al. 1986)
Test species	
Species tested:	*Hexagenia* sp.
Source of organisms:	Eggs collected from gravid females in the field, *H. limbata* eggs have been stored for 120 d, *H. bilineata* eggs stored for 380 d but with declining viability
Age of organisms:	
Acclimation time:	
Acclimation conditions:	Laboratory or pond culture from collected eggs.
Test design	
Test type:	Static, recirculating or flow-through
Test duration (days):	4 d
Endpoints:	Immobility
Effects:	LC50
No. treatments:	-
Replicates per treatment:	-
Organisms per replicate:	-
Feeding :	-
Aeration or additional substrate:	Synthetic substrate or sediment. For chronic studies lightly dust each replicate with *Cerophyl* powdered grass.
Test acceptability criteria:	-
Test conditions	
Test chamber size (ml):	-
Test chamber material:	-
Water source:	-
Water volume(ml):	-
Water quality measurements:	-
Temperature (ºC):	-
Illuminance (lux):	Low level yellow light
Photoperiod:	24L:0D

Standard guide for conducting laboratory toxicity tests with freshwater mussels.	
Reference:	(USEPA 2006)
Test species	
Species tested:	Not specified
Source of organisms:	Flush gills with syringe to obtain glochidia
Age of organisms:	<1 d
Acclimation time:	-
Acclimation conditions:	-
Test design	
Test type:	Static, renewal or flow through
Test duration (days):	Up to 2 d
Endpoints:	Survival
Effects:	-
No. treatments:	-
Replicates per treatment:	3
Organisms per replicate:	500
Feeding :	None
Aeration or additional substrate:	None if dissolved oxygen maintained
Test acceptability criteria:	>90% control survival
Test conditions	
Test chamber size (ml):	100
Test chamber material:	Glass
Water source:	Study specific
Water volume(ml):	75
Water quality measurements:	DO, pH, ammonia, hardness, alkalinity, conductivity,
Temperature (ºC):	20
Illuminance (lux):	100-1000
Photoperiod:	16L:8D

ASTM Standard guide for conducting laboratory toxicity tests with freshwater mussels: glochidia acute test	
Reference:	(ASTM 2006)
Test species	
Species tested:	Not specified
Source of organisms:	Not specified
Age of organisms:	<2 h after glochidia isolated from female mussels
Acclimation time:	-
Acclimation conditions:	-
Test design	
Test type:	Static, semi-static or flow through
Test duration (days):	1 d, longer for species where glochidia remain viable for several days
Endpoints:	Mortality (valve closing in response to salt solution)
Effects:	LC50, EC50, IC50, NOEC, LOEC
No. treatments:	-
Replicates per treatment:	3
Organisms per replicate:	500
Feeding :	None
Aeration or additional substrate:	-
Test acceptability criteria:	Control survival >90% at end of study
Test conditions	
Test chamber size (ml):	100
Test chamber material:	Glass
Water source:	Reconstituted or natural water
Water volume(ml):	75
Water quality measurements:	Dissolved oxygen, pH, ammonia, hardness, alkalinity, conductivity
Temperature (ºC):	20
Illuminance (lux):	100-1000
Photoperiod:	16L:8D

ASTM Standard guide for conducting laboratory toxicity tests with freshwater mussels: acute juvenile freshwater mussels	
Reference:	(ASTM 2006)
Test species	
Species tested:	Not specified
Source of organisms:	Not specified
Age of organisms:	<5 d after release from host
Acclimation time:	-
Acclimation conditions:	-
Test design	
Test type:	Static, semi-static or flow through
Test duration (days):	4 d
Endpoints:	Mortality
Effects:	LC50, EC50, IC50, NOEC, LOEC
No. treatments:	
Replicates per treatment:	Minimum 4
Organisms per replicate:	Minimum 5
Feeding :	None
Aeration or additional substrate:	None
Test acceptability criteria:	>90 % control survival additional requirements listed in reference
Test conditions	
Test chamber size (ml):	50
Test chamber material:	Glass
Water source:	Reconstituted or natural
Water volume(ml):	30
Water quality measurements:	Dissolved oxygen, pH, ammonia, hardness, alkalinity, conductivity
Temperature (ºC):	20
Illuminance (lux):	100-1000
Photoperiod:	16L:8D

ASTM Standard guide for conducting laboratory toxicity tests with freshwater mussels: chronic juvenile freshwater mussels	
Reference:	(ASTM 2006)
Test species	
Species tested:	Not specified
Source of organisms:	Not specified
Age of organisms:	2-4 months old
Acclimation time:	-
Acclimation conditions:	-
Test design	
Test type:	Static, semi-static or flow through
Test duration (days):	10-28 d
Endpoints:	Mortality, growth (shell length)
Effects:	LC50, EC50, IC50, NOEC, LOEC
No. treatments:	-
Replicates per treatment:	3
Organisms per replicate:	10
Feeding :	Yes, algae
Aeration or additional substrate:	None
Test acceptability criteria:	>80 % control survival additional requirements listed in reference
Test conditions	
Test chamber size (ml):	300
Test chamber material:	Glass
Water source:	Reconstituted or natural
Water volume(ml):	200
Water quality measurements:	Dissolved oxygen, pH, ammonia, hardness, alkalinity, conductivity
Temperature (ºC):	20
Illuminance (lux):	100-1000
Photoperiod:	16L:8D

Lumbriculus variegatus, a Benthic Oligochaete, as a Bioassay Organism	
Reference:	(Bailey 1980)
Test species	
Species tested:	*Lumbriculus variegatus*
Source of organisms:	Lab culture
Age of organisms:	-
Acclimation time:	-
Acclimation conditions:	19 litre glass aquaria, flow-through conditions, 100g *L. variegatus* per tank, 5cm sand substrate, no aeration, fed trout food, 16L:8D
Test design	
Test type:	Static
Test duration (days):	2-4 d
Endpoints:	Mortality, fragmentation, clumping, localised swelling, mucus production, overall swelling, colour changes
Effects:	LC50
No. treatments:	
Replicates per treatment:	10
Organisms per replicate:	5-10
Feeding :	-
Aeration or additional substrate:	-
Test acceptability criteria:	-
Test conditions	
Test chamber size (ml):	250
Test chamber material:	Glass
Water source:	Dechlorinated tap water
Water volume(ml):	200
Water quality measurements:	Dissolved oxygen, temperature, pH,
Temperature (ºC):	20ºC
Illuminance (lux):	-
Photoperiod:	12L:12D

A1.2: Chronic toxicity tests in sediment:water test systems

OECD Sediment-water chironomid test using spiked water	
Reference:	(OECD 2004a)
Test species	
Species tested:	*Chironomus riparius.* Also *C. tentans, C. yoshimatsui*
Source of organisms:	Laboratory culture
Age of organisms:	1st instar (2-3 d, 1-4 d for *C tentans*)
Acclimation time:	1 d in test vessels
Acclimation conditions:	Test conditions
Test design	
Test type:	Static without renewal
Test duration (days):	20-28 d (28-65 d for *C. tentans*); also 10 d
Exposure scenario:	Spiked water
Endpoints:	28 d - emergence, development rate; 10d – immobility, growth (AFDW[1])
Effects:	ECx; NOEC/LOEC
No. treatments:	5
Replicates per treatment:	Minimum 3 (ECx); 4 (NOEC/LOEC)
Organisms per replicate:	20
Feeding :	Fish food at least 3 times per week
Aeration or additional substrate:	Aeration from 7 days prior to test to test end
Test acceptability criteria:	70% control emergence in 12-23 days (20-65 d for *C. tentans*); Oxygen > 60% ASV[2] ;pH 6-9; temperature ± 1.0 ºC
Test conditions	
Test chamber size:	600 ml, 8cm diameter
Test chamber material:	Glass with glass cover
Water source:	Any water conforming to prescribed chemical characteristics
Water volume:	6cm depth
Water quality measurements:	-
Sediment source:	Artificial (2% TOC[3]) or conditioned natural sediment
Sediment volume:	1.5-3.0 cm deep; 2-3 cm^2 sediment per larvae; 1:4 sediment:water depth ratio
Temperature (ºC):	20 (*C. riparius*); 23 (*C. tentans*); 25 (*C. yoshimatsui*)
Illuminance (lux):	500-1000
Photoperiod:	16L:8D

[1]Ash Free Dry Weight
[2]ASV Air Saturation Value
[3]Total Organic Carbon

OECD Sediment-water chironomid test using spiked sediment	
Reference:	(OECD 2004b)
Test species	
Species tested:	*Chironomus riparius.* Also *C. tentans, C. yoshimatsui*
Source of organisms:	Laboratory culture
Age of organisms:	1st instar (2-3 d, 1-4 d for *C tentans*)
Acclimation time:	1 d in test vessels
Acclimation conditions:	Test conditions
Test design	
Test type:	Static without renewal
Test duration (days):	20-28 d (28-65 d for *C. tentans*); also 10 d
Exposure scenario:	Spiked sediment (OECD 1984)
Endpoints:	28 d - emergence, development rate; 10d – immobility, growth (AFDW[1])
Effects:	ECx; NOEC/LOEC
No. treatments:	5
Replicates per treatment:	Minimum 3 (ECx); 4 (NOEC/LOEC)
Organisms per replicate:	20
Feeding :	Fish food at least 3 times per week
Aeration or additional substrate:	Aeration from 7 days prior to test to test end
Test acceptability criteria:	70% control emergence in 12-23 days (20-65 d for *C. tentans*); Oxygen > 60% ASV[2] ;pH 6-9; temperature ± 1.0 ºC
Test conditions	
Test chamber size:	600 ml, 8cm diameter
Test chamber material:	Glass with glass cover
Water source:	Any water conforming to prescribed chemical characteristics
Water volume:	6cm depth
Water quality measurements:	-
Sediment source:	Artificial (2% TOC[3]) or conditioned natural sediment
Sediment volume:	1.5-3.0 cm deep; 2-3 cm^2 sediment per larvae; 1:4 sediment:water depth ratio
Temperature (ºC):	20 (*C. riparius*); 23 (*C. tentans*); 25 (*C. yoshimatsui*)
Illuminance (lux):	500-1000
Photoperiod:	16L:8D

[1]Ash Free Dry Weight
[2]ASV Air Saturation Value
[3]Total Organic Carbon

OECD Sediment-water Lumbriculus toxicity test using spiked sediment	
Reference:	(OECD 2007)
Test species	
Species tested:	*Lumbriculus variegatus*
Source of organisms:	Laboratory culture
Age of organisms:	Synchronised adults of similar size
Acclimation time:	1 day in test vessels
Acclimation conditions:	Test conditions
Test design	
Test type:	Static without renewal
Test duration (days):	28 d
Exposure scenario:	Spiked sediment followed by equilibrium period of 2-7 d
Endpoints:	Total number of worms, reproduction (increase of worm numbers), growth (increase of dry biomass), behavioural observations
Effects:	ECx; NOEC/LOEC as mg/kg sediment dry weight based on nominal or initial measured concentrations
No. treatments:	5
Replicates per treatment:	Minimum 3 (ECx); 4 (NOEC/LOEC); 6 for control
Organisms per replicate:	10
Feeding :	Powdered *Urtica* sp. in sediment
Aeration or additional substrate:	Gentle aeration
Test acceptability criteria:	Average no. individuals/replicate in controls increase by at least a factor of 1.8; Oxygen >30% ASV[1]; pH 6-9
Test conditions	
Test chamber size:	250 ml, 6cm diameter
Test chamber material:	Glass
Water source:	Reconstituted water
Water volume:	Approx. 6 cm depth
Water quality measurements:	Temperature, dissolved oxygen, air supply, pH, total water hardness, total ammonia
Sediment source:	Artificial or natural; food added prior to dosing; 2% TOC[2]
Sediment volume:	1.5-3.0 cm deep; 1:4 sediment:water depth ratio; 43g sediment (dry weight) per 10 worms
Temperature (ºC):	20 ± 2
Illuminance (lux):	100-500
Photoperiod:	16L:8D

[1]ASV Air Saturation Value
[2]Total Organic Carbon

USEPA Ecological Effects Test Guidelines: OPPTS 850.1790 Chironomid sediment toxicity test.	
Reference:	(USEPA 1996c)
Test species	
Species tested:	*Chironomus tentans, C. riparius*
Source of organisms:	Laboratory culture
Age of organisms:	Second instar(< 10 days)
Acclimation time:	4 days
Acclimation conditions:	100% dilution water
Test design	
Test type:	Flow through
Test duration (days):	14 d, longer tests may be required for high log K_{ow} chemicals
Exposure scenario:	1. aqueous exposure test, minimal sediment, water spiked 2. sediment-water test, sediment present, sediment spiked 3. interstitial exposure test, sediment present, water spiked
Endpoints:	Mortality, growth (wet weight), bioconcentration factors
Effects:	LC50, EC50, concentration response curves, MATC, NOEC, LOEC
No. treatments:	5
Replicates per treatment:	2
Organisms per replicate:	15
Feeding :	yes
Aeration or additional substrate:	Aeration if required
Test acceptability criteria:	<20% control mortality, dissolved oxygen >60% in test solutions,
Test conditions	
Test chamber size:	1-5.7 litre
Test chamber material:	Glass or borosilicate glass
Water source:	Any water conforming to prescribed chemical characteristics
Water volume:	<30 chironomids per litre per day in the flow-through test system
Water quality measurements:	Dissolved oxygen, pH, temperature, test substance concentrations,
Sediment source:	Natural sediments with 1-15% organic carbon; sieved to remove large particles; described by particle size and chemical characteristics
Sediment volume:	1. <2 mm 2. 4-6 cm with varying amounts of organic carbon
Temperature (ºC):	20 ± 1ºC for *C. tentans*; 22 ± 1ºC for *C. riparius*
Illuminance (lux):	-
Photoperiod:	16L:8D

APHA Standard methods for the examination of water and wastewater. Section 8700: Aquatic insects – *Hexagenia* sp.	
Reference:	(Eaton et al. 2005)
Test species	
Species tested:	*Hexagenia bilineata, H. limbata, H. rigida* (Mayfly) Alternative test species: Stoneflies (Plecoptera): *Pteronarcys dorsata, P. californica, Hesperoperla lycorias, H. pacifica*; Mayflies (Ephemeroptera): *Ephemerella subvaria*; Caddisflies (Trichoptera): *Brachycentrus americanus, B. occidentalis, Clistoronia magnifica*
Source of organisms:	Cultures where possible, otherwise collected from clean natural waters
Age of organisms:	Early instars for lethality and growth, late instars for emergence
Acclimation time:	> 7 d
Acclimation conditions:	Flowing water, test temperature, stone substrate, 3-5 cm layer of unsterilised mud, material for larval and pupal cases. *Pteronarcys* sp, *Ephemerella* sp. coarse chopped maple, birch or aspen leaves; *Hexagenia* sp. finely ground leaves and fish food;
Test design	
Test type:	Flow through or static with airstones to provide movement.
Test duration (days):	4-7 d survival, 5-60 d growth and survival, 30-90 d emergence tests or full life-cycle,
Endpoints:	Mortality, growth (length, weight, head capsule width), emergence (emergence, incomplete emergence, sex ratio), no. of mature eggs
Effects:	-
No. treatments:	-
Replicates per treatment:	-
Organisms per replicate:	50 larvae, 200 eggs
Feeding :	-
Aeration or additional substrate:	Fine mesh stainless steel screens formed into cylinders or cubes, 10-15 cm^2 per insect. Sticks or stones protruding from water for emergence tests.
Test acceptability criteria:	-
Test conditions	
Test chamber size:	8 or 20 litre aquariums for quiet-water species (inc. *Hexagenia* sp.), 90cm long troughs for riffle species.
Test chamber material:	Glass, stainless steel, epoxy painted troughs
Water source:	
Water volume:	8-20 cm deep
Water quality measurements:	-
Sediment source:	Organic ooze with similar characteristics to source site
Sediment volume:	4-5 cm
Temperature (ºC):	10-20
Illuminance (lux):	-
Photoperiod:	Natural photoperiod for locality. Increase day length 0.5 hours every 2 weeks in emergence tests. Most species are univoltine, emergence tests should start not later than March 1st.

APHA Standard methods for the examination of water and wastewater. Section 8700: Aquatic insects – chironomid	
Reference:	(Eaton et al. 2005)
Test species	
Species tested:	*Chironomus* sp.
Source of organisms:	Laboratory cultures
Age of organisms:	1st instar, <24 h old
Acclimation time:	-
Acclimation conditions:	-
Test design	
Test type:	Flow through, 2 L/h
Test duration (days):	Short-term survival, 30 d emergence tests
Endpoints:	Mortality, emergence (emergence, pupal cases, sex ratio), hatchability, F1 survival reared to adulthood.
Effects:	-
No. treatments:	-
Replicates per treatment:	-
Organisms per replicate:	-
Feeding :	Yes, during 30 d emergence test
Aeration or additional substrate:	Sticks or stones protruding from water for emergence tests.
Test acceptability criteria:	-
Test conditions	-
Test chamber size:	20 litre aquariums
Test chamber material:	Glass, stainless steel
Water source:	-
Water volume:	-
Water quality measurements:	-
Sediment source:	Mud or powdered dry cereal grass
Sediment volume:	-
Temperature (ºC):	25 ± 1
Illuminance (lux):	-
Photoperiod:	Natural photoperiod for locality. Increase day length 0.5 hours every 2 weeks in emergence tests. Most species are univoltine, emergence tests should start not later than March 1st.

USEPA Ecological Effects Test Guidelines: OPPTS 850.1735 Whole sediment acute toxicity invertebrates, freshwater	
Reference:	(USEPA 2000)
Test species	
Species tested:	*Chironomus tentans*
Source of organisms:	Laboratory culture
Age of organisms:	1 d (<24 h)
Acclimation time:	-
Acclimation conditions:	-
Test design	
Test type:	Flow-through or intermittent flow, application to sediment
Test duration (days):	50-65 d
Exposure scenario:	
Endpoints:	20 d survival and weight, emergence, sex ratio, adult mortality, no. egg cases laid, no. eggs produced, no. hatched eggs
Effects:	-
No. treatments:	-
Replicates per treatment:	16
Organisms per replicate:	12
Feeding :	Yes
Aeration or additional substrate:	Dissolved oxygen maintained at >2.5 mg/L
Test acceptability criteria:	*C. tentans* in control at 20 d >0.6 mg/surviving organism dry weight, emergence ≥ 50%, mean number eggs/egg case ≥800, percent hatch ≥ 80%
Test conditions	
Test chamber size:	300 ml
Test chamber material:	-
Water source:	-
Water volume:	175 ml
Water quality measurements:	Hardness, alkalinity, conductivity, ammonia, temperature, dissolved oxygen, pH,
Sediment source:	-
Sediment volume:	100 ml
Temperature (ºC):	23 ± 1
Illuminance (lux):	100-1000
Photoperiod:	16L:8D

APHA Standard methods for the examination of water and wastewater. Section 8420: Rotifers	
Reference:	(Eaton et al. 2005)
Test species	
Species tested:	*Brachionus calyciflorus* (Rotifer). Alternative test species are *Brachionusrubens; Brachionuspatulus; Asplancha brightwelli; Philodina roseola; Philodina acutiocornis*
Source of organisms:	Hatched from cysts
Age of organisms:	<2 hours
Acclimation time:	-
Acclimation conditions:	-
Test design	
Test type:	Static
Test duration (days):	2 d (2 generations)
Endpoints:	Mortality, reproduction
Effects:	LC50, EC50, NOEC, LOEC
No. treatments:	5
Replicates per treatment:	5
Organisms per replicate:	6
Feeding :	*Nannochloris oculata* monoculture or *Selenastrum capricornutum*/*Chlorella vulgaris* mix.
Aeration or additional substrate:	None
Test acceptability criteria:	Control *r* at least 0.7 (minimum accepted population growth rate)
Test conditions	
Test chamber size:	Borosilicate glass test tubes
Test chamber material:	
Water source:	Artificial water
Water volume:	12 ml
Water quality measurements:	-
Temperature (ºC):	25ºC
Illuminance (lux):	Dark
Photoperiod:	0L:24D

APHA Standard methods for the examination of water and wastewater. Section 8510E: Sediment test procedures using freshwater and marine oligochaetes *Pritina leidyi, Tuifex tubifex,* and *Lumbriculus variegatus*	
Reference:	(Eaton et al. 2005)
Test species	
Species tested:	Tubificidae (*Limnodrilus hoffmeisteri; Tubifex tubifex; Branchiura sowerbyi*), Lumbriculidae (*Stylodrilus heringianus; Lumbriculus variegatus*)
Source of organisms:	Laboratory culture from population at uncontaminated site
Age of organisms:	Mixed age
Acclimation time:	-
Acclimation conditions:	-
Test design	
Test type:	Static renewal
Test duration (days):	10 d
Endpoints:	Survival
Effects:	LC50, LT50
No. treatments:	-
Replicates per treatment:	-
Organisms per replicate:	5 *T. Tubifex*, 10 *L. variegatus,* density below 0.5 g/L
Feeding :	None
Aeration or additional substrate:	-
Test acceptability criteria:	-
Test conditions	
Test chamber size:	250 ml
Test chamber material:	-
Water source:	Synthetic or natural water
Water volume:	100 ml
Water quality measurements:	-
Sediment source:	Natural sediments
Sediment volume:	-
Temperature (ºC):	20 – 25 ºC
Illuminance (lux):	550 – 1100
Photoperiod:	16L:8D

Appendix 2: Methods in use in contract and research laboratories

A2.1: Acute toxicity tests in water only test systems in contract research organisations

Parameter	**Response**	**Response**	**Response**
Species	*Chaoborus obscuripes*	*Chironomus riparius*	*Chironomus riparius*
Family	Chaoboridae (Diptera)	Chironomidae (Diptera)	Chironomidae (Diptera)
Common name	phantom midge	Bloodworm	Freshwater midge
Source of organisms	Mesocosm	In-house stock	In-house stock
Life stage	Larvae	Larvae	1st instar
Size of organisms	-	13 d	<1 cm
Acclimation period	3-4 d	13 d	egg ropes transferred in test water
Exposure regime	static	Semi-static (daily renewal)	Static
Test medium	filtered natural water	Purified drinking water (OECD-Guideline 202).	Reconstituted water according to OECD 211 (M7-Medium)
Include sediment	no	No	No
Organisms fed	no	No	Yes, *Scenedesmus subspicatus*, Tetra Min fish food in 48 h test
Aeration	no	No	No
Temperature range (ºC)	18-21	20.0 ± 2°C	18-22
Light intensity (Lux)	-	250 - 300	520-690
pH range	6.6-8.5	8.1-8.3	7.6-7.9
Dissolved 0_2 range	7-10.2	7.6 mg/L - 8.5 mg/L	7.9-8.4
Other environmental parameters	-	-	-
Photoperiod	14-16L:8-10D	16L:8D	16L:8D
Range-finding		Yes	Yes
No. concentrations	6	5	5
Replicates/level	2-3	4	4
No. controls	2-4	4	5
No. organisms/replicate	-	5	5
Test duration (days)	4	2	1 or 2
No. GLP compliant tests conducted		1-3	>10
No. non-GLP compliant tests conducted	1-3	None	>10
Measured endpoints	behaviour/mortality	Immobility	Immobility
Statistical output	-	ECx, LOEC, NOEC	EC50, NOEC, EC100
Suggested reference substance	-	-	-
Validity criteria	80% survival in controls	control immobility <10 %, no pathological symptoms, no abnormal behaviour, oxygen content >3 mg/L for whole test	Immobility < 10% in the control
Additional comments	no dose-response observed in range finder	-	Based on OECD 202, and 92/69/EEC

A2.1: Acute toxicity tests in water only test systems in contract and research laboratories contd.

Parameter	Response	Response	Response
Species	*Endochironomus albipennis*	*Glyptotendipes sp.*	*Macropelopia sp.*
Family	Chironomidae (Diptera)	Chironomidae (Diptera)	Chironomidae (Diptera)
Common name	phantom midge	phantom midge	phantom midge
Source of organisms	Mesocosm	Mesocosm	Mesocosm
Life stage	Larvae	Larvae	Larvae
Size of organisms	<1 cm	1-3 cm	-
Acclimation period	-	-	-
Exposure regime	static	static	static
Test medium	filtered natural water	filtered natural water	filtered natural water
Include sediment	no	no	no
Organisms fed	no	no	no
Aeration	no	no	no
Temperature range (ºC)	20.6-21.1	20.6-21.1	20 ± 0.9
Light intensity (Lux)	-	-	-
pH range	7.2-7.5	7.2-7.5	7.4-7.8
Dissolved 0_2 range	8.-8.7	8.-8.7	7.0-7.8
Other environmental parameters	-	-	-
Photoperiod	14L:10D	14L:10D	14L:10D
Range-finding	-	-	-
No. concentrations	6	6	6
Replicates/level	2	2	2
No. controls	4	4	2
No. organisms/replicate	-	-	-
Test duration (days)	4 d	4 d	4 d
No. GLP compliant tests conducted	-	-	-
No. non-GLP compliant tests conducted	1	1	1
Measured endpoints	behaviour/mortality	behaviour/mortality	behaviour/mortality
Statistical output	-	-	-
Suggested reference substance	-	-	-
Validity criteria	80% survival in controls	80% survival in controls	80% survival in controls
Additional comments	-	-	-

A2.1: Acute toxicity tests in water only test systems in contract and research laboratories contd.

Parameter	Response	Response	Response
Species	*Culex* sp.	*Cloeon dipterum*	*Caenis horaria*
Family	Culicidae (Diptera)	Baetidae (Ephemeroptera)	Caenidae (Ephemeroptera)
Common name	-	mayfly	mayfly
Source of organisms	Rainwater collector	Mesocosm	Mesocosm
Life stage	Larvae	Larvae	Larvae
Size of organisms	-	<1 cm	<1 cm
Acclimation period	3 d	3-4 d	-
Exposure regime	static	static	static
Test medium	filtered natural water	filtered natural water	filtered natural water
Include sediment	no	no	no
Organisms fed	no	no	no
Aeration	no	no	no
Temperature range (ºC)	-	18.5-20.9	21-23.4
Light intensity (Lux)	-	-	-
pH range	6.6-8.5	6.5-8.5	6.9-8.0
Dissolved 0_2 range	7-9.0	7.0-9.4	6.4-8.9
Other environmental parameters	-	-	-
Photoperiod	14-16L:8-10D	14-16L:8-10D	14L:10D
Range-finding	-	-	-
No. concentrations	6	6	6
Replicates/level	3	2-3	2
No. controls	3	2-4	2
No. organisms/replicate	-	-	-
Test duration (days)	4 d	4 d	4 d
No. GLP compliant tests conducted	-	1-3	-
No. non-GLP compliant tests conducted	1	4-6	1-3
Measured endpoints	behaviour/mortality	behaviour/mortality	behaviour/mortality
Statistical output	-	-	-
Suggested reference substance	-	-	-
Validity criteria	-	80% survival in controls	80% survival in controls
Additional comments	Limit test (7 d) also conducted with uptake/elimination as endpoints	Limit test (7 d) also conducted with uptake/elimination as endpoints	-

A2.1: Acute toxicity tests in water only test systems in contract and research laboratories contd.

Parameter	Response	Response	Response
Species	*Serratella ignita*	*Haproleptoides confusa/lauta*	*Hydropsyche* sp.
Family	Ephemerellidae (Ephemeroptera)	Leptophlebiidae (Ephemeroptera)	Hydropsychidae (Trichoptera)
Common name	-	-	Caddisfly
Source of organisms	Field collected	Field collected	Field collected
Life stage	Larvae	Larvae	Juvenile
Size of organisms	3-6 cm	3-6 cm	11.1-12.1 mm
Acclimation period	1-3 d	1-3 d	5 d
Exposure regime	Static	Static	Semi-static (daily renewal)
Test medium	Synthetic (M4 Elendt)	Synthetic (M4 Elendt)	Purified drinking water (OECD-Guideline 202).
Include sediment	No	No	No
Organisms fed	No	No	No
Aeration	Yes	Yes	No
Temperature range (ºC)	7-15	7-14	12.5 ± 2°C
Light intensity (Lux)	1000-4000	1000-4000	250 - 300
pH range	7-8	7-8	7.3 - 8.3
Dissolved 0_2 range	>8 mg/L	> 8 mg/L	7.6 mg/L - 10.7 mg/L
Other environmental parameters	-	-	-
Photoperiod	16L:8D	16L:8D	16L:8D
Range-finding	Yes	Yes	Yes
No. concentrations	5	5	5
Replicates/level	4	4	4
No. controls	4	4	4
No. organisms/replicate	5	5	4
Test duration (days)	4 d	4 d	4 d
No. GLP compliant tests conducted	-	-	1-3
No. non-GLP compliant tests conducted	1-3	1-3	None
Measured endpoints	Mortality	Mortality	Immobility
Statistical output	EC50	EC50	ECx, LOEC, NOEC
Suggested reference substance	-	-	-
Validity criteria	-	-	control immobility <10 %, no pathological symptoms, no abnormal behaviour, oxygen content >3 mg/L for whole test
Additional comments	-	-	-

A2.1: Acute toxicity tests in water only test systems in contract and research laboratories contd.

Parameter	Response	Response	Response
Species	*Molanna angustata*	*Sigara striata*	*Paraponix stratiotata*
Family	Molannidae (Trichoptera)	Corixidae (Hemiptera)	Pyralidae (Lepidoptera)
Common name		water boatman	
Source of organisms	Field collected	Mesocosm	Mesocosm
Life stage	Larvae	Adult	Larvae
Size of organisms	-	-	-
Acclimation period	3 d	-	3 d
Exposure regime	static	static	static
Test medium	filtered natural water	filtered natural water	filtered natural water
Include sediment	no	no	no
Organisms fed	no	no	no
Aeration	no	no	no
Temperature range (ºC)		20 ± 0.7	18.3-19.5
Light intensity (Lux)	-	-	-
pH range	6.6-8.5	6.8-8.1	6.6-8.5
Dissolved 0_2 range	7-9.0	7.2-9.8	7-9.0
Other environmental parameters	-	-	-
Photoperiod	14-16L:8-10D	14L:10D	14-16L:8-10D
Range-finding	-	-	-
No. concentrations	6	6	6
Replicates/level	3	2	2
No. controls	3	2-4	2-4
No. organisms/replicate	-	-	-
Test duration (days)	4 d	4 d	4 d
No. GLP compliant tests conducted	-	1 (Corixa punctata)	-
No. non-GLP compliant tests conducted	1	1	1-3
Measured endpoints	behaviour/mortality	behaviour/mortality	behaviour/mortality
Statistical output	-	-	-
Suggested reference substance	-	-	-
Validity criteria	-	80% survival in controls	80% survival in controls
Additional comments	-	-	Limit test (7 d) also conducted with uptake/elimination as endpoints

A2.1: Acute toxicity tests in water only test systems in contract and research laboratories contd.

Parameter	Response	Response	Response
Species	*Sialis lutaria*	*Erythromma viridulum*	*Anax imperator*
Family	Sialidae (Megaloptera)	Coenagrionidae (Odonata)	Aeshnidae (Odonata)
Common name	alderfly	damselfly	
Source of organisms	Mesocosm	Mesocosm	Mesocosm
Life stage	Larvae	Larvae	Larvae
Size of organisms	-	-	-
Acclimation period	3 d		3 d
Exposure regime	static	static	static
Test medium	filtered natural water	filtered natural water	filtered natural water
Include sediment	no	no	no
Organisms fed	no	no	no
Aeration	no	no	no
Temperature range (ºC)	20 ± 0.5	22 ± 1.0	-
Light intensity (Lux)	-	-	-
pH range	6.6-8.5	7.1-7.7	6.6-8.5
Dissolved 0_2 range	6.7-9.0	5.6-7.8	7-9.0
Other environmental parameters	-	-	-
Photoperiod	14-16L:8-10D	14L:10D	14-16L:8-10D
Range-finding	-	-	-
No. concentrations	6	6	6
Replicates/level	2-10	2	3
No. controls	2-20	2	3
No. organisms/replicate	-	-	-
Test duration (days)	4 d	4 d	4 d
No. GLP compliant tests conducted	-	-	-
No. non-GLP compliant tests conducted	1-3	1	1
Measured endpoints	behaviour/mortality	behaviour/mortality	behaviour/mortality
Statistical output	-	-	-
Suggested reference substance	-	-	-
Validity criteria	80% survival in controls	80% survival in controls	-
Additional comments	Limit test (7 d) also conducted with uptake/elimination as endpoints	-	Limit test (7 d) also conducted with uptake/elimination as endpoints

A2.1: Acute toxicity tests in water only test systems in contract and research laboratories contd.

Parameter	Response	Response	Response
Species	*Notonecta maculata*	*Notonecta maculata*	*Notonecta maculata*
Family	Notonectidae (Hemiptera)	Notonectidae (Hemiptera)	Notonectidae (Hemiptera)
Common name	Backswimmer	Backswimmer	
Source of organisms	In-house stock	Mesocosm	Mesocosm
Life stage	Larvae	Juvenile	Adult
Size of organisms	Larval development in 5 instars, 3-15 mm	5th instar, 8.1 - 11.7 mm	-
Acclimation period	None	2 d	3 d
Exposure regime	Static	Semi-static (daily renewal)	static
Test medium	Synthetic (M4 Elendt)	Purified drinking water (OECD-Guideline 202).	filtered natural water
Include sediment	No	No	no
Organisms fed	No	No	no
Aeration	No	No	no
Temperature range (ºC)	20	18.0 ± 2°C	-
Light intensity (Lux)	500-700	250 - 300	
pH range	7-8	7.6 - 8.3	6.6-8.5
Dissolved 0_2 range	> 8 mg/L	7.7 mg/L - 11.4 mg/L	7-9.0
Other environmental parameters	-	-	-
Photoperiod	16L:8D	16L:8D	14-16L:8-10D
Range-finding	Yes	Yes	-
No. concentrations	5	5	6
Replicates/level	>5	>5	4
No. controls	>5	>5	4
No. organisms/replicate	1	1	
Test duration (days)	2 d	2 d	4 d
No. GLP compliant tests conducted	none, up to now used in ecological studies	1-3	1-3
No. non-GLP compliant tests conducted	none, up to now used in ecological studies	None	1-3
Measured endpoints	Mortality	Immobility	behaviour/mortality
Statistical output	EC50	ECx, LOEC, NOEC	
Suggested reference substance	-	-	-
Validity criteria	-	Low control immobility (max. 2 specimens), no pathological symptoms and abnormal behaviour, oxygen content >3 mg/L for whole test	-
Additional comments	no hatching during experiment	-	Limit test (7 d) also conducted with uptake/elimination as endpoints

A2.1: Acute toxicity tests in water only test systems in contract and research laboratories contd.

Parameter	Response	Response	Response
Species	*Plea minutissima*	*Ranatra linearis*	*Bithynia tentaculata*
Family	Pleidae (Hemiptera)	Nepidae (Hemiptera)	Bithyniidae (Gastropoda)
Common name	-	-	freshwater snail
Source of organisms	Mesocosm	Mesocosm	Field collected
Life stage	Adult	Adult	Adult
Size of organisms	-	-	-
Acclimation period	3-4 d	3 d	-
Exposure regime	static	static	static
Test medium	filtered natural water	filtered natural water	filtered natural water
Include sediment	no	no	no
Organisms fed	no	no	no
Aeration	no	no	no
Temperature range (ºC)	-	-	18.3-20.4
Light intensity (Lux)	-	-	-
pH range	6.6-8.5	6.6-8.5	-
Dissolved 0_2 range	7-9.0	7-9.0	6.3-11.0
Other environmental parameters	-	-	-
Photoperiod	14-16L:8-10D	14-16L:8-10D	14L:10D
Range-finding	no		
No. concentrations	6	6	6
Replicates/level	3	2	2
No. controls	3	2	2
No. organisms/replicate	-	-	-
Test duration (days)	4 d	4 d	4 d
No. GLP compliant tests conducted	-	-	-
No. non-GLP compliant tests conducted	1-3	1	1-3
Measured endpoints	behaviour/mortality	behaviour/mortality	behaviour/mortality
Statistical output	-	-	-
Suggested reference substance	-	-	-
Validity criteria	-	-	80% survival in controls
Additional comments	Limit test (7 d) also conducted with uptake/elimination as endpoints	Limit test (7 d) also conducted with uptake/elimination as endpoints	Not good test due to closing of operculum

A2.1: Acute toxicity tests in water only test systems in contract and research laboratories contd.

Parameter	Response	Response	Response
Species	*Lymnaea stagnalis*	*Lymnaea stagnalis*	*Physa fontinalis*
Family	Lymnaeidae (Gastropoda)	Lymnaeidae (Gastropoda)	Physidae (Gastropoda)
Common name	Freshwater snail	freshwater snail	freshwater snail
Source of organisms	In-house stock	Mesocosm	Field collected
Life stage	Juvenile	Adult	Adult
Size of organisms	1-3 cm	1-3 cm	<1 cm
Acclimation period	1-3 d	-	-
Exposure regime	Static	static	static
Test medium	Reconstituted test water according to OECD 202 (ISO - Medium)	filtered natural water	filtered natural water
Include sediment	No	no	no
Organisms fed	No	no	no
Aeration	No	no	no
Temperature range (ºC)	23	18.6-21.1	20.3 ± 1.2
Light intensity (Lux)	320	-	-
pH range	6.6-8.0	6.7-8.0	7.7-8.1
Dissolved 0_2 range	5.0-8.6	0.2-9.1	6.1-8.9
Other environmental parameters	-	-	-
Photoperiod	16L:8D	14L:10D	14L:10D
Range-finding	Yes	-	-
No. concentrations	1 (Limit test)	4-6	6
Replicates/level	4	2	2
No. controls	4	2-4	2-4
No. organisms/replicate	5	-	-
Test duration (days)	2 d	4 d	4 d
No. GLP compliant tests conducted	1-3	-	-
No. non-GLP compliant tests conducted	1-3	1-3	1
Measured endpoints	Mortality, immobility	behaviour/mortality	behaviour/mortality
Statistical output	EC50, NOEC, EC100	-	-
Suggested reference substance	-	-	-
Validity criteria	Immobility <10% in the control; dissolved 0_2 >60% at end of test	80% survival in controls	80% survival in controls
Additional comments	Test is based on OECD 202, and Commission Directive 92/69/EEC	Low DO levels due to faeces in medium, no apparent effect on test animals	-

A2.1: Acute toxicity tests in water only test systems in contract and research laboratories contd.

Parameter	Response	Response	Response
Species	*Planorbarius corneus*	*Planorbis contortis*	*Melanoides auberculata*
Family	Planorbidae (Gastropoda)	Planorbidae (Gastropoda)	Thiaridae (Gastropoda)
Common name	great ramshorn	freshwater snail	Freshwater snail
Source of organisms	In-house stock	Field collected	Commercial supplier
Life stage	Juvenile	Adult	Juvenile
Size of organisms	<1 cm	<1 cm	1-3 cm
Acclimation period	>21 d	3 d	1-3 d
Exposure regime	Semi-static (daily renewal)	static	Static
Test medium	Purified drinking water (OECD-Guideline 202).	filtered natural water	Reconstituted test water according to OECD 202 (ISO - Medium)
Include sediment	No	no	No
Organisms fed	No	no	No
Aeration	No	no	No
Temperature range (ºC)	20.0 ± 2°C	18.8-21.4	20-21
Light intensity (Lux)	250 - 300		570-740
pH range	7.8 - 8.4	7.82-8.09	7.7 - 8.2
Dissolved 0_2 range	4.9 mg/L - 8.2 mg/L	8.0-8.8	7.1 - 9.2
Other environmental parameters	-	-	-
Photoperiod	16L:8D	14L:10D	16L:8D
Range-finding	Yes		Yes
No. concentrations	5	6	5
Replicates/level	4	2	4
No. controls	4	4	4
No. organisms/replicate	4	-	5
Test duration (days)	4 d	4 d	2 d
No. GLP compliant tests conducted	1-3		1-3
No. non-GLP compliant tests conducted	None	1	1-3
Measured endpoints	Immobility for 24 h post-exposure.	behaviour/mortality	Mortality, immobility
Statistical output	ECx, LOEC, NOEC	-	EC50, NOEC, EC100
Suggested reference substance			
Validity criteria	control immobility <10 %, no pathological symptoms, no abnormal behavior, oxygen content >3 mg/L for whole test	80% survival in controls	Immobility <10% in controls, dissolved O_2 > 3 mg/L
Additional comments	-	-	Test is based on OECD 202, and Commission Directive 92/69/EEC

A2.1: Acute toxicity tests in water only test systems in contract and research laboratories contd.

Parameter	Response	Response	Response
Species	*Sphaerium* sp.	*Dero digitata, Stylaria lacustris*	*Lumbriculus variegatus*
Family	Sphaeridae (Bivalvia)	Oligochaeta	Oligochaeta
Common name	-	-	-
Source of organisms	Field collected	Mesocosm	Field collected
Life stage	Adult	Adult	Adult
Size of organisms	<1 cm	-	3-6 cm
Acclimation period	-	-	-
Exposure regime	static	static	static
Test medium	filtered natural water	filtered natural water	filtered natural water
Include sediment	no	no	no
Organisms fed	no	no	no
Aeration	no	no	no
Temperature range (ºC)	20.3 ± 0.8	-	20.0 ± 1.0
Light intensity (Lux)	-	-	-
pH range	7.4-8.1	-	7.8-7.9
Dissolved 0_2 range	7.7-8.8	-	8.3-8.3
Other environmental parameters	-	-	-
Photoperiod	14L:10D	-	14L:10D
Range-finding	-	-	-
No. concentrations	6	-	6
Replicates/level	2	-	2
No. controls	2	-	2-4
No. organisms/replicate	-	-	-
Test duration (days)	4 d	-	4 d
No. GLP compliant tests conducted	-	-	-
No. non-GLP compliant tests conducted	1-3	1	1-3
Measured endpoints	behaviour/mortality	behaviour/mortality	behaviour/mortality
Statistical output	-	-	-
Suggested reference substance	-	-	-
Validity criteria	80% survival in controls	-	80% survival in controls
Additional comments	-	-	-

A2.1: Acute toxicity tests in water only test systems in contract and research laboratories contd.

Parameter	Response	Response	Response
Species	*Tubifex* sp.	*Dugesia sp./lugubris, Polycelis nigra/tenuis*	*Erpobdella* sp.
Family	Oligochaeta	Turbellaria	Hirudinea
Common name	-	Flatworm	leech
Source of organisms	Commercial supplier	Field collected	Mesocosm
Life stage	Adult	Adult	Juvenile
Size of organisms	<1 cm	-	1-3 cm
Acclimation period	-	-	-
Exposure regime	static	static	static
Test medium	filtered natural water	filtered natural water	filtered natural water
Include sediment	no	no	no
Organisms fed	no	no	no
Aeration	no	no	no
Temperature range (ºC)	20.6-21.1	18.7-21.1	20.0 ± 0.7
Light intensity (Lux)	-	-	-
pH range	7.7-7.8	7.4-8.2	7.7-8.2
Dissolved 0_2 range	8.4-8.8	8.4-11.2	8.3-9.0
Other environmental parameters	-	-	-
Photoperiod	14L:10D	14L:10D	14L:10D
Range-finding	-	-	-
No. concentrations	6	4-6	6
Replicates/level	2	2	2
No. controls	2-4	2-4	2-4
No. organisms/replicate	-	-	-
Test duration (days)	4 d	4 d	4 d
No. GLP compliant tests conducted	-	-	-
No. non-GLP compliant tests conducted	1-3	1-3	1-3
Measured endpoints	behaviour/mortality	behaviour/mortality	behaviour/mortality
Statistical output	-	-	-
Suggested reference substance	-	-	-
Validity criteria	80% survival in controls	80% survival in controls	80% survival in controls
Additional comments	-	-	-

A2.1: Acute toxicity tests in water only test systems in contract and research laboratories contd.

Parameter	Response	Response
Species	*Brachionus calyciflorus*	*Brachionus calyciflorus*
Family	Rotifer	Rotifer
Common name	-	-
Source of organisms	Laboratory culture	raised from resting eggs of a test kit
Life stage	Egg hatching	Newly hatched cysts, 2 h old
Size of organisms	-	<1cm
Acclimation period	-	<24 h
Exposure regime	static	Static
Test medium	filtered natural water	Reconstituted water according to OECD 202 (ISO - Medium)
Include sediment	no	No
Organisms fed	no	No
Aeration	no	No
Temperature range (ºC)	-	25
Light intensity (Lux)	-	Dark
pH range	-	7.9-8.1
Dissolved 0_2 range	-	8.7 - 9.0
Other environmental parameters	-	-
Photoperiod	14L:10D	0L:24D
Range-finding		Yes
No. concentrations	6	5
Replicates/level	2	5
No. controls	2	5
No. organisms/replicate	-	5
Test duration (days)	4 d	1 d
No. GLP compliant tests conducted	-	1-3
No. non-GLP compliant tests conducted	1	1-3
Measured endpoints	behaviour/mortality	Immobility
Statistical output	-	EC50, NOEC, EC100
Suggested reference substance	-	-
Validity criteria	80% survival in controls	Immobility < 10% in the control
Additional comments	-	Based on OECD 202, 92/69/EECand commercial test kit

A2.2: Acute toxicity tests in sediment:water test systems in contract and research laboratories

Parameter	Response
Species	*Ephemera danica*
Family	Insecta, Ephemeroptera
Common name	Mayfly
Source of organisms	Field collected
Life stage	Larvae
Size of organisms	<1cm
Acclimation period	5 d
Exposure regime	Semi-static (daily renewal)
Test medium	Purified drinking water (OECD-Guideline 202).
Include sediment	Yes, quartz sand layer of 2 mm
Organisms fed	No
Aeration	Yes
Temperature range (ºC)	12.5 ± 2°C
Light intensity (Lux)	250 – 300
pH range	7.8 – 8.7
Dissolved 0_2 range	7.5 mg/L - 11.3 mg/L.
Other environmental parameters	-
Photoperiod	16L:8D
Range-finding	Yes
No. concentrations	5
Replicates/level	4
No. controls	4
No. organisms/replicate	4
Test duration (days)	4 d
No. GLP compliant tests conducted	1-3
No. non-GLP compliant tests conducted	None
Measured endpoints	Immobility
Statistical output	ECx, LOEC, NOEC
Suggested reference substance	-
Validity criteria	control immobility <10 %, no pathological symptoms, no abnormal behavior, oxygen content >3 mg/L for whole test
Additional comments	-

A2.3: Chronic toxicity tests in water only test systems in contract and research laboratories

Parameter	Response	Response	Response
Species	*Chaoborus crystallinus*	*Chaoborus obscuripes*	*Dicrotendipes sp.*
Family	Chaoboridae	Chaoboridae	Chironomidae
Common name	Phantom midge	phantom midge	phantom midge
Source of organisms	Field collected and In-house stock	Mesocosm	Commercial supplier
Life stage	1st instar	Larvae	Larvae
Size of organisms	-	-	-
Acclimation period	Freshly hatched	3-4 d	4 d
Exposure regime	Semi-static (4-10 d renewal)	static	static
Test medium	Synthetic (M4 Elendt)	filtered natural water	filtered natural water
Include sediment	No	no	no
Organisms fed	Yes (Rotifer, Bosmina and juvenile daphnids)	yes,in GLP test	yes
Aeration	No	no	no
Temperature range (ºC)	20	18-21	18.6-19.4
Light intensity (Lux)	300-500	-	-
pH range	7-8.5	6.6-8.5	7.3-8.0
Dissolved 0_2 range	> 6 mg/L	8	8.9-9.1
Other environmental parameters	-	-	-
Photoperiod	16L:8D	-	-
Range-finding	No	yes, in GLP test	yes
No. concentrations	4	1-8	8
Replicates/level	10	1-5	1
No. controls	20	2-5	2
No. organisms/replicate	1	-	-
Test duration (days)	30-90 d (until emergence or death)	7 d	7 d
No. GLP compliant tests conducted	-	1-3	1
No. non-GLP compliant tests conducted	1-3	1-3	-
Measured endpoints	mortality, growth, moulting, pupation, emergence, reproduction	behaviour/mortality	behaviour/mortality
Statistical output	NOEC, EC50	-	-
Suggested reference substance	-	-	-
Validity criteria	-	-	-
Additional comments	-	Limit test (7 d) also conducted with uptake/elimination as endpoints	-

A2.3: Chronic toxicity tests in water only test systems in contract and research laboratories contd.

Parameter	Response	Response	Response
Species	*Cloeon dipterum*	*Coenagrionidae*	*Plea minutissima*
Family	Ephemeroptera	Odonata	Pleidae (Hemiptera)
Common name	mayfly	-	-
Source of organisms	Mesocosm	Mesocosm	Mesocosm
Life stage	Larvae	Larvae	Adult
Size of organisms	-	-	-
Acclimation period	3-4 d	4 d	3-4 d
Exposure regime	static	static	static
Test medium	filtered natural water	filtered natural water	filtered natural water
Include sediment	no	no	no
Organisms fed	yes, in GLP study	yes	yes in GLP test
Aeration	no	no	no
Temperature range (ºC)	19.6-20.5	18.6-20.1	19.5-20.8
Light intensity (Lux)	-	-	-
pH range	7.5-8.5	7.5-8.0	7.1-8.0
Dissolved 0_2 range	7.9-9.0	8.9-9.0	8.8-9.1
Other environmental parameters	-	-	-
Photoperiod	-	-	-
Range-finding	yes	yes	yes
No. concentrations	8	8	8
Replicates/level	1	1	1
No. controls	2	2	2
No. organisms/replicate	-	-	-
Test duration (days)	7 d	7 d	7 d
No. GLP compliant tests conducted	1-3	1	1-3
No. non-GLP compliant tests conducted	-	-	-
Measured endpoints	-	behaviour/mortality	-
Statistical output	-	-	-
Suggested reference substance	-	-	-
Validity criteria	80% survival in controls	-	-
Additional comments	Limit test (7 d) also conducted with uptake/elimination as endpoints	-	Limit test (7 d) also conducted with uptake/elimination as endpoints

A2.3: Chronic toxicity tests in water only test systems in contract and research laboratories contd.

Parameter	Response	Response
Species	*Lymnea stagnalis*	*Sphaerium* sp.
Family	Lymnaeidae	Sphaeridae (Bivalvia)
Common name	-	-
Source of organisms	In-house stock	Field collected
Life stage	Juvenile and adults	Adult
Size of organisms	juveiles 1.5-2.0 cm, adults 3.5-4.0 cm	<1 cm
Acclimation period	3-7 d	-
Exposure regime	Semi-static, renewal every 3-4 d	static
Test medium	Synthetic	filtered natural water
Include sediment	No	no
Organisms fed	Yes, fresh salad leaves and Tetraphyll	no
Aeration	Yes	no
Temperature range (ºC)	20-21	18.4-19.7
Light intensity (Lux)	180-500	
pH range	7.1-7.9	7.73-8.28
Dissolved 0_2 range	>6 mg/L	6.9-8.7
Other environmental parameters	-	-
Photoperiod	16L:8D	14L:10D
Range-finding	Yes	-
No. concentrations	>5	6
Replicates/level	4	2
No. controls	4	4
No. organisms/replicate	5	-
Test duration (days)	28 d	7 d
No. GLP compliant tests conducted	1-3	-
No. non-GLP compliant tests conducted	1-3	1-3
Measured endpoints	survival, growth, reproduction, fertility, hatching rate of eggs	-
Statistical output	NOEC	-
Suggested reference substance	-	-
Validity criteria	-	80% survival in controls
Additional comments	-	Limit test (7 d) also conducted with uptake/elimination as endpoints

A2.3: Chronic toxicity tests in sediment:water test systems in contract and research laboratories

Parameter	**Response**	**Response**	**Response**
Species	*Haprophlebia lauta*	*Serratella ignita*	*Sericostoma* sp.
Family	Leptophlebiidae (Ephemeroptera)	Ephemerelllidae (Ephemeroptera)	Sericostomatidae
Common name	-	-	-
Source of organisms	Field collected	Field collected	Field collected
Life stage	Larvae	Larvae	Larvae
Size of organisms	3-6 cm	3-6 cm	3-6 cm
Acclimation period	1-3 d	1-3 d	1-3 d
Exposure regime	Semi-static	Semi-static	Semi-static
Test medium	Synthetic (M4 Elendt)	Synthetic (M4 Elendt)	Synthetic (M4 Elendt)
Include sediment	Yes, stones	Yes, stones	Yes, sand
Organisms fed	Yes, stones with periphyton	Yes, stones with periphyton	Yes, leaves
Aeration	Yes	Yes	Yes
Temperature range (ºC)	7-15	7-15	15
Light intensity (Lux)	4000	4000	1000-4000
pH range	7-8	7-8	7-8
Dissolved 0_2 range	> 8 mg/L	>8 mg/L	> 8 mg/L
Other environmental parameters	-	-	-
Photoperiod	16L:8D	16L:8D	16L:8D
Range-finding	Yes	Yes	Yes
No. concentrations	>5	>5	>5
Replicates/level	1	1	1
No. controls	4	4	4
No. organisms/replicate	10	10	5
Test duration (days)	56 d (until emergence)	28 d (until emergence)	Until emergence
No. GLP compliant tests conducted	-	-	-
No. non-GLP compliant tests conducted	1-3	1-3	1-3
Measured endpoints	Mortality, emergence characteristics, sex ratio	mortality, emergence characteristics, sex ratio	Mortality
Statistical output	EC50	EC50	EC50
Suggested reference substance	-	-	-
Validity criteria	-	-	-
Additional comments	-	-	-

A2.3: Chronic toxicity tests in sediment:water test systems in contract and research laboratories contd.

Parameter	Response	Response	Response
Species	*Chironomus riparius*	*Chironomus riparius*	*Lumbriculus variegatus*
Family	Chironomidae	Chironomidae	Lumbriculidae
Common name	Midge	non biting midge	Blackworm
Source of organisms	Commercial supplier	In-house stock	In-house stock
Life stage	1st instar	Larvae	Adult
Size of organisms	<1 cm	<1 cm	1-3 cm
Acclimation period	7-14 d in sediment	>21 d	14- 21 d
Exposure regime	Static	Static	Static
Test medium	Synthetic sediment reconstituted water	Synthetic medium (ASTM)	Synthetic sediment
Include sediment	Mixture of course sand, kaolin clay and peat	OECD 218 and 219 sediment	Peat, calcium carbonate plus sand, kaolin clay and peat
Organisms fed	Yes, powdered nettle leaf incorporated into sediment	Yes, Suspension of flaked fish food. 0.5 mg per larva per	Yes, urtica powder incorporated into sediment
Aeration	Yes	Yes	Yes
Temperature range (ºC)	18 – 22	18-22	18 – 22
Light intensity (Lux)	500-1000	500-1000	Artificial daylight
pH range	5-6	6-9	7.5-8.5
Dissolved 0_2 range	> 60% ASV	>60% ASV	62 – 96%
Other environmental parameters	-	-	-
Photoperiod	16L:8D	16L:8D	16L:8D
Range-finding	Yes	Yes	Yes
No. concentrations	>5	5	5
Replicates/level	4	4	4
No. controls	2	4	1
No. organisms/replicate	20	20	10
Test duration (days)	28 d	28 d	28 d
No. GLP compliant tests conducted	4-6	>10	1-3
No. non-GLP compliant tests conducted	None	1-3	None
Measured endpoints	daily and total emergence, sex of organisms and development rate	Emergence success, time to emergence and sex ratio	survival, total biomass (growth)
Statistical output	EC50, NOEC	EC50	ECx, NOEC
Suggested reference substance	-	-	-
Validity criteria	70% control emergence	as per OECD 218 and 219	-
Additional comments	Radiolabelled test item	-	Radiolabelled test item

Appendix 3: Methods in peer-reviewed references (first draft)

A3.1: Acute toxicity tests in water only test systems

Test species	Source	Age	Feeding	Measured endpoint	Effect	Test design	Duration	Water	Reference
Ephemeroptera (Mayfly)									
Ameletus sp.	Field pop.	12 mm	No	Mortality	LC50, LC1	Static	4 d	Lake water, stainless steel mesh, quartz rock	(Peterson et al. 2001)
Atalophlebia spp.[1]	Field pop.	<10 mm length				Static with renewal	1-2 d	River	(Hose et al. 2003)
Caenis horaria		Larvae	None	Mortality, immobility	ECx, LCx	Static	4 d	Pond with stainless steel guaze	(Schroer et al. 2004)
Caenis miliaria	Field pop.			Mortality	LC50		4 d	Pond	(Beketov 2004)
Cinygmula sp.	Field pop.	Mean length 8.8 mm	-	Mortality	LC50		4 d	Lake water, stainless steel mesh, quartz rock	(Peterson et al. 2001)
Cloeon dipterum	Field pop.			Mortality	LC50		4 d	Pond	(Beketov 2004)
Cloeon dipterum	Field pop.		None		ECx, LCx		4 d	Tap water	(Van Wijngaarden 1993)
Cloeon dipterum	Field pop.	Larvae	None	Mortality, immobility	EC50, LC50	Static	1 d	Dechlorinated tap	(Stephenson 1982)
Cloeon dipterum	Field pop.	Nymph	None	Mortality, immobility	EC50, LC50	Static	4 d	Reservoir	(Van Wijngaarden et al. 2009)
Cloeon dipterum		Larvae	None	Mortality, immobility	ECx, LCx	Static	4 d	Pond with stainless steel guaze	(Schroer et al. 2004)
Epeorus longimanus	Field pop.	Early and late instar	-	Mortality	LC50	Static	1-4 d	Ground water	(Alexander et al. 2007)

[1]Mixture of two species used

A3.1: Acute toxicity tests in water only test systems contd.

Test species	Source	Age	Feeding	Measured endpoint	Effect	Test design	Duration	Water	Reference
Ephemeroptera (Mayfly)									
Epeorus longimanus	Field pop.	Early and late instar	Yes	Mortality, feeding inhibition	LC50, EC50	Static	1d	Ground water	(Alexander et al. 2007)
Epheron virgo	Eggs from field pop.	2 d	Yes	Mortality	LC50	Static	4 d	Artificial	(Van der Geest 2000), (Van Der Geest et al. 2000), (Greve et al. 1999)
Epheron virgo	Eggs from field pop.	2 d	Yes	Mortality	LC50	Static	4 d	Artificial	
Hexagenia sp.	Eggs from field pop.			Immobility		Static or recirculating	<4 d	Synthetic substrates	(Fremling & Mauck 1980)
Plecoptera (Stonefly)									
Calineuria californica	Field pop.	8.4 mm	No	Mortality	LC50, LT50, LC1	Static	4 d	Lake water, stainless steel mesh, quartz rock	(Peterson et al. 2001)
Pteronarcys dorsata	Field pop.	2 or 3 years (>0.2 g)	None	Mortality	LC50	Flow-through	1 h to 4 d	Lake water	(Anderson & Shubat 1984)
Trichoptera (Caddisfly)									
Brachycentrus americanus	Field pop.	8.3 mm	No	Mortality	LC50, LT50, LC1	Static	4 d	Lake water, stainless steel mesh, quartz rock	(Peterson et al. 2001)
Cyrnus trimaculatus	Lab culture	2nd instar	Yes	Mortality	LC50	Static	4 d	Artificial	(Van Der Geest et al. 2000)

[1] This review only considers the methods for the exposure phase (1 d) and not the recovery phase.

A3.1: Acute toxicity tests in water only test systems contd.

Test species	Source	Age	Feeding	Measured endpoint	Effect	Test design	Duration	Water	Reference
Hydropsyche angustipennis	Lab culture	1st instar	Yes	Mortality, immobility	EC50, LC50	Static	4 d	Artificial	(Stuijfzand et al. 2000), (Greve et al. 1998), (Van Der Geest et al. 1999)
Hydropsyche angustipennis	Lab culture	5th instar	Yes	Mortality, immobility	EC50, LC50	Static	4 d	Artificial with glass beads	(Stuijfzand et al. 2000)
Lepidostoma unicolor	Field pop.	8.7 mm	No	Mortality	LC50, LC1	Static	4 d	Lake water, stainless steel mesh, quartz rock	(Peterson et al. 2001)
Notidobia ciliaris	Field pop.	Approx. 3rd instar	None	Mortality	LC50		1 d[1]	Mesocosm water	(Beketov & Liess 2008)
Psychoglypha sp.	Field pop.	8.3 mm	No	Mortality	LC50, LC1	Static	4 d	Lake water, stainless steel mesh, quartz rock	(Peterson et al. 2001)
Diptera (True flies)									
Aedes aegypti	Lab culture	Larvae	None	Mortality, immobility	EC50, LC50	Static	1 d	Dechlorinated tap	(Stephenson 1982)
Chaoborus crystallinus	Field pop.	Larvae	None	Mortality, immobility	EC50, LC50	Static	1 d	Dechlorinated tap	(Stephenson 1982)
Chaoborus obscuripes	Field pop.	Larvae	None	Ability to stay in suspension	ECx, LCx	Static	4 d	Tap or pond	(Van Wijngaarden et al. 1998)
Chaoborus obscuripes	Field pop.	Larvae	None	Mortality, immobility	EC50, LC50	Static	4 d	Reservoir	(Van Wijngaarden et al. 2009)
Chaoborus obscuripes		Larvae	None	Mortality, immobility	ECx, LCx	Static	4 d	Pond with stainless steel guaze	(Schroer et al. 2004)

[1]This review only considers the methods for the exposure phase (1 d) and not the recovery phase.

A3.1: Acute toxicity tests in water only test systems contd.

Test species	Source	Age	Feeding	Measured endpoint	Effect	Test design	Duration	Water	Reference
Diptera (True flies)									
Chironomini	Field pop.	Larvae	None	Mortality, immobility	EC50, LC50	Static	4 d	Reservoir	(Van Wijngaarden et al. 2009)
Chironomus riparius	Lab culture	1st instars	Yes	Mortality, immobility, growth	EC50, LC50	Static	4 d	Artificial	(Stuijfzand et al. 2000)
Chironomus riparius	Lab culture	4th instar	Yes	Mortality, immobility	EC50, LC50	Static	4 d	Artificial and glass beads	(Stuijfzand et al. 2000)
Chironomus thummi	Field pop.	Larvae	None	Mortality, immobility	EC50, LC50	Static	1 d	Dechlorinated tap	(Stephenson 1982)
Culex pipiens	Lab culture	1st instar (<24 hours)		Mortality	LC50	Static	1 d[1]	Artificial	(Beketov & Liess 2008)
Macropelopia sp.		Larvae	None	Mortality, immobility	ECx, LCx	Static	4 d	Pond with stainless steel guaze	(Schroer et al. 2004)
Simulium latigonium	Field pop.	Approx. last instar	None	Mortality	LC50		1 d[1]	Mesocosm water	(Beketov & Liess 2008)

[1]This review only considers the methods for the exposure phase (1 d) and not the recovery phase.

A3.1: Acute toxicity tests in water only test systems contd.

Test species	Source	Age	Feeding	Measured endpoint	Effect	Test design	Duration	Water	Reference
Odonata (Damselfly and Dragonfly)									
Austrolestes colensonis	Field pop.	12th instar	None	Mortality	LC50	Static	2 d	Aerated tap	(Hardersen 1996)
Coenagrionidae	Field pop.	Larvae	None	Mortality, immobility	EC50, LC50	Static	4 d	Reservoir	(Van Wijngaarden et al. 2009)
Cordulia aenea	Field pop.			Mortality	LC50		4 d	Pond	(Beketov 2004)
Erythromma viridulum	Field pop.	Larvae	None	Mortality, immobility	ECx, LCx	Static	4 d	Pond with stainless steel guaze	(Schroer et al. 2004)
Lestes sponsa	Field pop.			Mortality	LC50		4 d	Pond	(Beketov 2004)
Sympetrum striolatum	Lab reared[2]	2nd instar (< 2 days)	None	Mortality	LC50		1 d[1]	Artificial	(Beketov & Liess 2008)
Xanthocnemis zealandica	Field pop. or lab reared[2]	Various instars		Mortality	LC50		2 d	Aerated tap	(Hardersen & Wratten 2000)
Xanthocnemis zealandica	Field pop.	12th instar	None	Mortality	LC50	Static	2 d	Aerated tap	(Hardersen 1996)

[1]This review only considers the methods for the exposure and not the recovery phase.
[2]Field collected eggs reared in laboratory cultures

A3.1: Acute toxicity tests in water only test systems contd.

Test species	Source	Age	Feeding	Measured endpoint	Effect	Test design	Duration	Water	Reference
Hemiptera (Backswimmer)									
Anisops sardeus	Field pop.	Adult females		Mortality	LC50	Static	2 d	Well water	(Lahr et al. 2001)
Corixa punctata	Field pop.	Adult	None	Mortality, immobility	EC50, LC50	Static	1 d	Dechlorinated tap	(Stephenson 1982)
Corixa punctata	Field pop.	Adult	None	Mortality, immobility	EC50, LC50	Static	4 d	Reservoir	(Van Wijngaarden et al. 2009)
Notonecta maculate	Field pop.	Adult	None	Mortality, immobility	EC50, LC50	Static	4 d	Reservoir	(Van Wijngaarden et al. 2009)
Notonecta glauca	Field pop.	Adult	None	Mortality, immobility	ECx, LCx	Static	4 d	Pond with stainless steel guaze	(Schroer et al. 2004)
Sigara striata		Adult	None	Mortality, immobility	ECx, LCx	Static	4 d	Pond with stainless steel guaze	(Schroer et al. 2004)
Megloptera (Alderflies)									
Sialis lutaria	Field pop.	Larvae	None	Mortality, immobility	ECx, LCx	Static	4 d	Pond with stainless steel guaze	(Schroer et al. 2004)
Hydracarina (Water mites)									
Piona carnea	Field pop.	Adult	None	Mortality, immobility	EC50, LC50	Static	1 d	Dechlorinated tap	(Stephenson 1982)
Coleoptera (Beetle)									
Gyrinus natator	Field pop.	Adult	None	Mortality, immobility	EC50, LC50	Static	1 d	Dechlorinated tap	(Stephenson 1982)

A3.1: Acute toxicity tests in water only test systems contd.

Test species	Source	Age	Feeding	Measured endpoint	Effect	Test design	Duration	Water	Reference
Oligochaeta (Worm)									
Dero digitata	Lab culture	Fully grown	None	-	ECx, LCx	Static	2 d	Tap or pond water	(Van Wijngaarden et al. 1998)
Limnodrilus hoffmeisteri	Field pop.			Mortality	LC50	Static	4 d	Dechlorinated tap	(Chapman et al. 1982)
Lumbriculus variegatus	Lab culture	Similar size		Mortality	LC50	Static	4 d	Dechlorinated groundwater	(Alexander et al. 2007)
Lumbriculus variegatus	Lab culture	Mixed age		Mortality	LC50	Static	4 d	Lake water	(Ankley & Collyard 1995)
Stylodrilus heringianus	Field pop.			Mortality	LC50	Static	4 d	Dechlorinated tap	(Chapman et al. 1982)
Tubifex tubifex	Field pop.			Mortality	LC50	Static	4 d	Dechlorinated tap	(Chapman et al. 1982)
Turbellaria (Flatworms)									
Dugesia lugubris	Field pop.	Half to fully grown	-	-	LCx	Static	2 d	Tap or pond	(Van Wijngaarden et al. 1998)
Polycelis nigra		Adult	None	Mobility behaviour	ECx, LCx	Static	4 d	Pond with stainless steel guaze	(Schroer et al. 2004)
Polycelis tenuis		Adult	None	Mobility behaviour	ECx, LCx	Static	4 d	Pond with stainless steel guaze	(Schroer et al. 2004)
Hydrozoa									
Hydra vulgaris	Lab culture	Non-budding	No	Tentacle and body contraction	EC50	Static	4 d	Dechlorinated tap water	(Pollino & Holdway 1999)
Hydra viridissima	Lab culture	Non-budding	No	Tentacle and body contraction	EC50	Static	4 d	Dechlorinated tap water	(Pollino & Holdway 1999)

A3.1: Acute toxicity tests in water only test systems contd.

Test species	Source	Age	Feeding	Measured endpoint	Effect	Test design	Duration	Water	Reference
Mollusca (Snails)									
Bithynia tentaculata		(Sub)adult	None	Mortality, immobility, avoidance behaviour[1]	ECx, LCx	Static	4 d	Pond with stainless steel guaze	(Schroer et al. 2004)
Dreissena polymorpha	Field pop.	1.5-2.0 cm	Yes	Filtration rate	EC50	Semi-static	2 d	Lake water	(Kraak et al. 1997)
Dreissena polymorpha	Field pop.	1.6-2.3 cm	No	Mortality (shell closing reflex)	LC50	Recirculating with renewal	4 d	Dechlorinated tap water	(Dauberschmidt et al. 1996)
Lampsilis siliquoidea[2]	Mature glochidia from field pop.	Glochidia	No	Immobility	EC50	Static	2 d	NR	(Bringolf et al. 2007a), (Bringolf et al. 2007b)
Lampsilis siliquoidea[2]	Lab reared from glochidia	Juvenile (1-2 months)	No	Immobility	EC50	Semi-static	4 d	NR	(Bringolf et al. 2007a), (Bringolf et al. 2007b)
Lymnaea stagnalis		(Sub)adult	None	Mobility	ECx, LCx	Static	4 d	Pond with stainless steel guaze	(Schroer et al. 2004)
Unio elongatulus eucirrus[3]	Field pop.	25-27 g	No	Mortality	LC10-LC90	Semi-static	4 d	Dechlorinated tap water	(Köprücü & Seker 2008)

[1] Closing operculum
[2] based on ASTM test guidelines
[3] based on APHA test guidelines

A3.2: Acute toxicity tests in sediment:water test systems

Test species	Source	Age	Feeding	Measured endpoint	Effect	Test design	Duration	Water	Sediment	Reference
Lumbricidae (Worm)										
Lumbriculus variegatus	Lab culture	Individuals of similar mass and length	Yes	Immobility, feeding inhibition[1].	LC50, EC50 (foodstuffs egested)	Static	1 d	Dechlorinated groundwater	Lake sediment (16% OC[2])	(Alexander et al. 2007)
Diptera (True fly)										
Chironomus tentans	Lab culture	3rd instar	-	Mortality	LC50	Static	4 d	Lake water	Sand	(Ankley & Collyard 1995)
Ephemeroptera (Mayfly)										
Hexagenia sp.	Field collected eggs			Immobility		Flow-through (recirculating)	<4 d		Yes	ASTM (Fremling & Mauck 1980)

[1]This review only considers the methods for the exposure phase (1 d) and not the recovery phase.
[2]Organc Content.

A3.3: Chronic toxicity tests in water only test systems

Test species	Source	Age	Feeding	Measured endpoint	Effect	Test design	Duration	Water	Reference
Ephemeroptera (Mayfly)									
Hexagenia bilineata	Culture pond	3 months	Yes	Mortality, molting, gill beats, growth	LC50	Flow-through, with or without burrows	14 d	Well water	(Henry et al. 1986)
Cloeon triangulifer	Lab culture	Eggs	Yes	Hatch success, larval mortality	EC50, LC50	Static/sem-static	Until hatching	Natural water	(Sweeney et al. 1993)
Cloeon triangulifer	Lab culture	1st instar	Yes	emergence, egg viability, adult residues	EC50, LC50	Static/sem-static	Approx. 43 d	Natural water	(Sweeney et al. 1993)
Trichoptera (Caddisfly)									
Brachycentrus americanus	Field pop.	-	Yes	Mortality, behavioural changes, bioaccumulation	LC50, EC50	Intermittent flow-through	28 d	Unfiltered lake water	(Anderson & DeFoe 1980)
Clistoronia magnifica	Field pop.	4th and 5th instars	None	Mortality, emergence	LC50, EC50	Flow-through	28 d		(Nebeker et al. 1983)
Hydropsyche sp.	Field pop.	-	-	Mortality, behavioural changes, bioaccumulation	LC50, EC50	Intermittent flow-through	28 d	Unfiltered lake water	(Anderson & DeFoe 1980)
Hydropsyche siltalai	Field pop.	5th instar	Yes	Net building anomolies	-	Static with renewal	8 d	Artificial with glass slides as substrate	(Wendt-Rasch 1998)
Plecoptera (Stonefly)									
Pteronarcys dorsata	Field pop.	-	Yes	Mortality, behavioural changes, bioaccumulation	LC50, EC50	Intermittent flow-through	28 d	Lake	(Anderson & DeFoe 1980)

A3.3: Chronic toxicity tests in water only test systems contd.

Test species	Source	Age	Feeding	Measured endpoint	Effect	Test design	Duration	Water	Reference
Diptera (True flies)									
Chironomus tentans	Lab culture		Yes	Mortality	LC50	Flow-through	10 d	Lake water	(Phipps et al. 1995)
Cricotopus spp.	Lab culture	4th instar	None	Moulting success, adult emergence		Static and Flow-through	7 d		(Nebeker et al. 1983)
Tanytarsus dissimilis	Lab culture	2nd instar	None	Moulting success		Static	5 d		(Nebeker et al. 1983)
Rotifer									
Brachinus calyciflorus	Lab culture	Neonate females	Yes	Resting egg production	EC50, NOEC, LOEC	Static	4 d	Synthetic	(Preston et al. 2000, Preston & Snell 2001)
Turbellaria (Flatworm)									
Dugesia dorotocephala	Lab culture	-	No	Mortality, head lesions, fissioning	LC50, EC50	Static renewal	13 d	Aged tap	(Best et al. 1981)
Dugesia dorotocephala	Lab culture	20-25 mg, intact and decapitated	None	Mortality, immobility, morphological abnormalities,	LC50		7 d		(Villar 1993)
Dugesia lugubris	Field pop.	Half to fully grown	-	Integrity, immobility	ECx, LCx	Static with renewal	30 d	Tap or pond	(Van Wijngaarden et al. 1998)

A3.3: Chronic toxicity tests in water only test systems contd.

Test species	Source	Age	Feeding	Measured endpoint	Effect	Test design	Duration	Water	Reference
Mollusca (Snail)									
Bithynia tentaculata	Field pop.	(sub)adult	Yes	Immobility, strength	LCx, ECx	Static with renewal	28 d	Tap or pond	(Van Wijngaarden et al. 1998)
Dreissena polymorpha	Field pop.	1.5-2.0 cm	Yes	Mortality, filtration rate	EC50	Semi-static	10 weeks	Lake water	(Kraak et al. 1997)
Juga plicifera	Field pop.	3-6 mm shell length	None	Mortality	LC50	Flow-through	7 d		(Nebeker et al. 1983)
Lampsilis siliquoidea	Lab reared from glochidia	Juvenile (1-2 months)	Yes	Immobility	EC50	Semi-static	21 d	NR	(Bringolf et al. 2007a)
Lymnaea acuminata	Field pop.	Adult	None	egg production, hatching success and hatchling survival	% change from control	NR	50 d	Dechlorinated tap water	(Tripathi & Singh 2004b), (Tripathi & Singh 2004a)
Lymnaea stagnalis	Lab culture	Sexually mature	Yes	Adult mortality, fecundity, mean no. egg clutches, hatchability (in clean water),	NOEC, LOEC	Semi-static	84 d	Synthetic	(Czech et al. 2001)
Physa integra	Field pop.	-	Yes	Mortality, behavioural changes, bioaccumulation	LC50, EC50	Intermittent flow-through		Lake	(Anderson & DeFoe 1980)
Physa sp.	Field pop.	12-20 mm shell length	None	Mortality, growth, reproduction	LC50, EC50	Flow-through	21 d		(Nebeker et al. 1983)
Planorbis planoris	Field pop.	(sub)adult	Yes	Immobility	LCx, ECx	Static with renewal	28 d	Tap or pond	(Van Wijngaarden et al. 1998)
Oligochaeta (Worm)									
Lumbriculus variegatus	Lab culture		No	Mortality	LC50	Flow-through	10 d	Lake water	(Phipps et al. 1995)
Stylaria lacustris	Lab culture	Fully grown	None	Immobility	ECx, LCx	Static with renewal	21 d	Tap or pond water	(Van Wijngaarden et al. 1998)

A3.4: Chronic toxicity tests in sediment:water test systems

Test species	Source	Age	Feeding	Measured endpoint	Effect	Test design	Duration	Water	Sediment	Reference
Diptera										
Chironomus tentans	Lab culture	3rd and 4th instar	Not reported	Immobility, IGR[1], body condition index, growth	LC50, EC50, NOEC, LOEC	Static renewal	10 d	Artificial	Sieved soil	(Maul et al. 2008)
Chironomus riparius	Lab culture	1st instar	Yes	Emergence, sex ratio, egg depostion	EC10	Static	28 d	1st instar	Synthetic	(Bettinetti & Provini 2002)
Chironomus riparius	Lab culture	1st instar	Yes	Pupation, emergence, emergence accidents, sex ratio	LC50, NOEC, LOEC	Static	24 d	Tapwater	3 mm quartz sand	(Hahn et al. 2001)
Chironomus riparius	Lab culture	4th instar	Yes	Pupation, emergence, emergence accidents, sex ratio	LC50, NOEC, LOEC	Semi-static	to emergence	Tapwater	3 mm quartz sand	(Hahn et al. 2001)
Chironomus riparius	Lab culture	1st instar	Yes	Emergence, development, sex ratio, fertility, fecundity	NOEC, LOEC	Static	Full Life Cycle (44 d)	Reconstituted	Artificial	(Taenzler et al. 2007), (Tassou & Schulz 2009)
Tubifex tubifex	Lab culture	Sexually mature	Yes	Mortality, no. cocoons, no. young worms	EC10	Static	28 d	Mineral water	Artificial	(Bettinetti & Provini 2002)

[1]Instantaneous Growth Rate

Appendix 4: Protocols identified for testing the effects of chemical against invertebrates other than Crustacea

Higher tier Full Life Cycle test for *Chironomus riparius*	
Reference:	(Taenzler et al. 2007)
Test species	
Species tested:	*Chironomus riparius*
Source of organisms:	Lab culture
Age of organisms:	1st instar
Acclimation time:	-
Acclimation conditions:	-
Test design	
Test type:	Static
Test duration (days):	Full Life Cycle (44 d)
Endpoints:	Emergence, development, sex ratio, (no. of egg ropes), fertility of egg ropes
Effects:	NOEC, LOEC
No. treatments:	-
Replicates per treatment:	-
Organisms per replicate:	20
Feeding :	Yes
Aeration or additional substrate:	-
Test acceptability criteria:	>70% control emergence
Test conditions	-
Test chamber size:	-
Test chamber material:	Glass exposure vessels and breeding cages
Water source:	Reconstituted
Water volume:	380 ml
Water quality measurements:	-
Sediment source:	Artificial
Sediment volume:	140 g
Temperature (ºC):	20
Illuminance (lux):	500-1000
Photoperiod:	16L:8D

Two-generation test with *Chrionomus riparius*	
Reference:	(Tassou & Schulz 2009)
Test species	
Species tested:	*Chironomus riparius*
Source of organisms:	Lab culture
Age of organisms:	1st instar
Acclimation time:	-
Acclimation conditions:	-
Test design	
Test type:	Static
Test duration (days):	Until F1 emergence
Endpoints:	Development time, no. fully emerged adults, sex ratio, fecundity, fertility, F1 emergence ratio, F1 development rate
Effects:	NOEC, LOEC
No. treatments:	6
Replicates per treatment:	8
Organisms per replicate:	20
Feeding :	Yes
Aeration or additional substrate:	Aeration
Test acceptability criteria:	>70% control emergence
Test conditions	
Test chamber size:	600 ml
Test chamber material:	Glass
Water source:	Artificial (M7-medium)
Water volume:	400 ml
Water quality measurements:	-
Sediment source:	Artificial
Sediment volume:	100 g
Temperature (ºC):	20 ± 2
Illuminance (lux):	-
Photoperiod:	16L:8D

USEPA Ecological Effects Test Guidelines: OPPTS 850.1735 Whole sediment acute toxicity invertebrates, freshwater	
Reference:	(USEPA 2000)
Test species	
Species tested:	*Chironomus tentans*
Source of organisms:	Laboratory culture
Age of organisms:	1 d (<24 h)
Acclimation time:	-
Acclimation conditions:	-
Test design	
Test type:	Flow-through or intermittent flow, application to sediment
Test duration (days):	50-65 d
Exposure scenario:	-
Endpoints:	20 d survival and weight, emergence, sex ratio, adult mortality, no. egg cases laid, no. eggs produced, no. hatched eggs
Effects:	-
No. treatments:	-
Replicates per treatment:	16
Organisms per replicate:	12
Feeding :	Yes
Aeration or additional substrate:	Dissolved oxygen maintained at >2.5 mg/L
Test acceptability criteria:	*C. tentans* in control at 20 d >0.6 mg/surviving organism dry weight, emergence ≥ 50%, mean number eggs/egg case ≥800, percent hatch ≥ 80%
Test conditions	
Test chamber size:	300 ml
Test chamber material:	-
Water source:	-
Water volume:	175 ml
Water quality measurements:	Hardness, alkalinity, conductivity, ammonia, temperature, dissolved oxygen, pH,
Sediment source:	-
Sediment volume:	100 ml
Temperature (ºC):	23 ± 1
Illuminance (lux):	100-1000
Photoperiod:	16L:8D

***Cloeon triangulifer* in a chronic water only test system.**	
Reference:	(Sweeney et al. 1993)
Test species	
Species tested:	*Cloeon triangulifer*
Source of organisms:	Laboratory culture
Age of organisms:	1st instar larvae and F1 eggs
Acclimation time:	Not applicable
Acclimation conditions:	-
Test design	
Test type:	Static or semi-static
Test duration (days):	Until hatching
Endpoints:	Larval survival, time to emergence, adult dry weight, egg hatch success
Effects:	% effect reported
No. treatments:	7
Replicates per treatment:	6
Organisms per replicate:	30 1st instar, 1000-2000 eggs (hatch success)
Feeding :	Periphyton cultures
Aeration or additional substrate:	Aeration, netting over jar to capture emerging adults
Test acceptability criteria:	Not reported
Test conditions	
Test chamber size:	6.5 cm deep, 5.5 cm tall
Test chamber material:	Glass
Water source:	Stream water (filtered for egg exposure)
Water volume:	30 ml
Water quality measurements:	Not recorded during test
Sediment source:	None
Sediment volume:	-
Temperature (ºC):	20 ± 1
Illuminance (lux):	Fluorescent lights
Photoperiod:	13.5L:10.5D

***Chaoborus crystallinus* in a chronic water only test system**	
Reference:	CRO protocol
Test species	
Species tested:	*Chaoborus crystallinus*
Source of organisms:	Field collected and In-house stock
Age of organisms:	1st instar
Acclimation time:	Freshly hatched
Acclimation conditions:	-
Test design	
Test type:	Semi-static
Test duration (days):	30-90 d
Endpoints:	Mortality, growth, moulting, pupation, emergence, reproduction
Effects:	EC50, NOEC
No. treatments:	4
Replicates per treatment:	10
Organisms per replicate:	1
Feeding :	Yes (Rotifer, Bosmina and juvenile daphnids)
Aeration or additional substrate:	No
Test acceptability criteria:	Not specified
Test conditions	
Test chamber size:	Not specified
Test chamber material:	Not specified
Water source:	Synthetic (M4 Elendt)
Water volume:	-
Water quality measurements:	Not specified
Sediment source:	None
Sediment volume:	-
Temperature (ºC):	20
Illuminance (lux):	300-500
Photoperiod:	16L:8D

***Lymnaea stagnalis* in a chronic water only test system**	
Reference:	(Czech et al. 2001)
Test species	
Species tested:	*Lymnaea stagnalis*
Source of organisms:	Lab culture
Age of organisms:	Sexually mature
Acclimation time:	-
Acclimation conditions:	F1 generation maintained under normal culture conditions after hatching
Test design	
Test type:	Semi-static
Test duration (days):	84 d
Endpoints:	Adult mortality, fecundity, mean no. egg clutches, hatchability (in clean water)
Effects:	NOEC, LOEC
No. treatments:	-
Replicates per treatment:	-
Organisms per replicate:	15-20
Feeding :	Yes
Aeration or additional substrate:	-
Test acceptability criteria:	-
Test conditions	
Test chamber size:	20 L
Test chamber material:	Glass
Water source:	Synthetic
Water volume:	-
Water quality measurements:	-
Sediment source:	None
Sediment volume:	-
Temperature (ºC):	22
Illuminance (lux):	-
Photoperiod:	16L:8D

***Brachionus calyciflorus* in a chronic water only test system**	
Reference:	(Preston et al. 2000, Preston & Snell 2001)
Test species	
Species tested:	*Brachionus calyciflorus*
Source of organisms:	Laboratory culture
Age of organisms:	Neonate females
Acclimation time:	-
Acclimation conditions:	-
Test design	
Test type:	Static
Test duration (days):	4 d
Endpoints:	Resting egg production
Effects:	EC50, NOEC, LOEC
No. treatments:	-
Replicates per treatment:	5
Organisms per replicate:	6
Feeding :	Yes
Aeration or additional substrate:	No
Test acceptability criteria:	Not reported
Test conditions	
Test chamber size:	16x150 mm
Test chamber material:	Glass
Water source:	Synthetic
Water volume:	12 ml
Water quality measurements:	Not recorded
Sediment source:	None
Sediment volume:	-
Temperature (ºC):	25
Illuminance (lux):	Darkness
Photoperiod:	0L:24D

Appendix 5: Abbreviations

µg	Microgram
APHA	American Public Health Association
ASTM	American Society for Testing and Materials
CRO	Contract Research Organisation
CSTEE	Comité Scientifique de Toxicologie, Ecotoxicologie et l'Environnement (European Scientific Committee on Toxicity, Ecotoxicity and Environment)
EC50	Effective concentration for 50% effect
ECx	Effect concentration for x%
EDC	Endocrine Disrupting Chemical
EEC	European Economic Community
IC50	Inhibition Concentration for 50% effect
L	Litre
LC50	Lethal concentration for 50% effect
LOEC	Lowest Observed Effect Concentration
LT50	Lethal time for 50% effect
MATC	Maximum Acceptable Toxicant Concentration
NOEC	No Observed Effect Concentration
NR	Not Reported
OECD	Organisation for Economic Co-operation and Development
OPPTS	Office of Prevention, Pesticides & Toxic Substances
PPR	Plant Protection Products and their Residues
USEPA	United States Environmental Protection Agency

Appendix 5: Acknowledgements