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Guidance on the use of bioassays in ecological risk assessment

Science report SC070009/SR2c

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This report is the result of research commissioned and funded by the Environment Agency's Science Programme.

Published by:
Environment Agency, Rio House, Waterside Drive, Aztec West, Almondsbury, Bristol, BS32 4UD
Tel: 01454 624400 Fax: 01454 624409
www.environment-agency.gov.uk

ISBN: 978-1-84432-948 -9
Front cover image: RSPB images, taken by Nigel Blake

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Dissemination Status:
Publicly available / released to all regions

Keywords:
Bioassay, Biological test, Operating Window, Ecological Risk Assessment, Part 2A, Contaminated Land, Environmental Protection Act, Soils, Contamination

Environment Agency's Project Manager:
Stephen Roast, Science Department

Science Project Number:
SC070009

Product Code:
SCHO1008BORU-E-P

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- **Delivering information, advice, tools and techniques**, by making appropriate products available to our policy and operations staff.

Steve Killeen

Head of Science

Executive summary

The Environment Agency has developed an Ecological Risk Assessment (ERA) Framework for soil contamination which can be used to support determinations under Part 2A of the Environmental Protection Act 1990.

Tier 2 of the framework seeks to establish whether or not significant harm or the significant possibility of significant harm is occurring. A risk characterisation is conducted using ecological surveys and biological tests identified as relevant to the site and the receptors potentially at risk. This document considers the biological tests that can be used to assess the impacts of contaminants on organisms representative of the Receptors of Potential Concern.

The Environment Agency has developed biological tests for use in Part 2A assessments and has documented the process in the following research reports:

- *Review of sub-lethal ecotoxicological tests for measuring harm in terrestrial ecosystems* (R&D Technical Report P5-063/TR1);
- *Assessing risks to ecosystems from land contamination* (R&D Technical Report P299);
- *Application of sub-lethal ecotoxicological tests for measuring harm in terrestrial ecosystems* (R&D Technical Report P5-063/TR2);
- *Biological test methods for assessing contaminated land. Stage 2: a demonstration of the use of a framework for the ecological risk assessment of land contamination* (R&D Technical Report P5-069/TR1).

This document provides a summary of the biological tests currently recommended at Tier 2 of the ERA Framework, when they are applicable and how to apply them. It does not contain detailed information on the tests; rather, this can be found in the Standard Operating Procedures for Bioassay, a separate document intended for the use of testing laboratories (ERA 3).

This document details:

- strengths and weaknesses of the biological tests currently recommended for use in the ERA Framework;
- guidance notes and considerations for deciding which biological tests to use and when (operating windows);
- restrictions as to when certain biological tests should **not** be used;
- criteria for ‘new’ or additional biological tests (i.e. tests not currently recommended) proposed for use in Tier 2;
- considerations and recommendations when commissioning laboratories to conduct biological tests.

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1.1 The purpose of this document

This document provides a summary of the biological tests currently recommended at Tier 2 of the Ecological Risk Assessment (ERA) Framework, when they are applicable and how to apply them. It gives the risk assessor an overview of the strengths and weaknesses of the various tests, provides guidance on their selection, sets out the criteria for the adoption of new or additional tests and highlights factors that the risk assessor should be aware of when commissioning laboratories to undertake tests.

1.2 How this document fits into the ERA framework

This document is one of six guidance documents that support the ERA framework.

The purpose of this guidance is to support Tier 2 of an ERA.

The position of this document (shown in red) within the overall ERA framework is summarised in the flow chart shown in Figure 1.1.

This report and the guidance documents in the series refer to each other in the following manner (full details can also be found in the reference list):

- This report is referred to as ERA 2c (Guidance on the use of bioassays).
- The overarching Ecological risk assessment framework for contaminants in soil is referred to as ERA 1 (Framework document).
- The Guidance on desk studies and Conceptual Site Models in Ecological Risk Assessment is referred to as ERA 2a (Guidance on desk studies and CSM).
- The Guidance on the use of Soil Screening Values in Ecological Risk Assessment is referred to as ERA 2b (Guidance on the use of SSV).
- The Guidance on the use of Ecological Surveys in Ecological Risk Assessment is referred to as ERA 2d (Guidance on the use of ecological surveys).
- The Guidance on the Attribution of Cause and Effect in Ecological Risk Assessment is referred to as ERA 2e (Guidance on the attribution of cause and effect).
- The Standard Operating Procedures for Bioassays is referred to as ERA 3 (SOPs for bioassays).

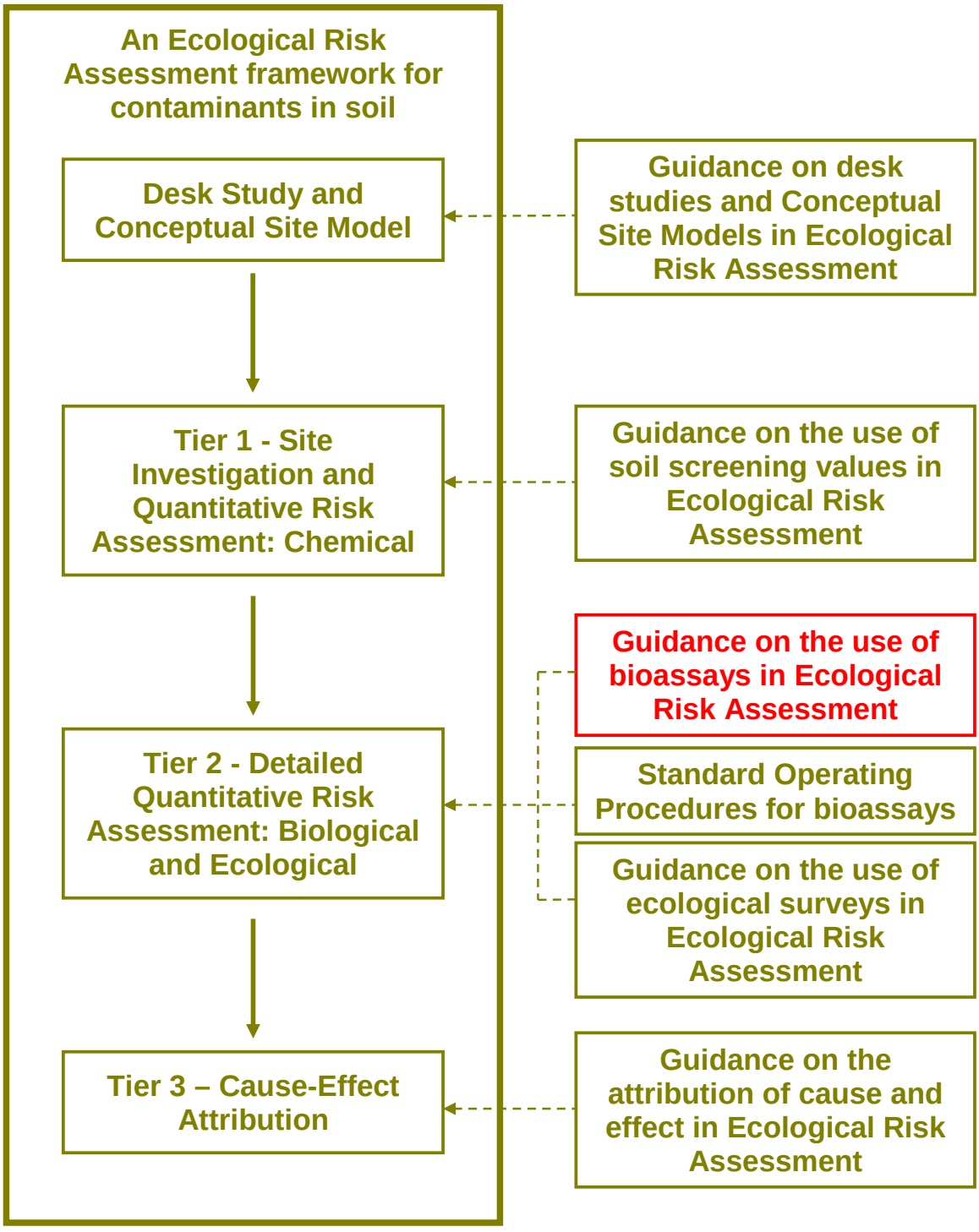

Figure 1.1 Position of this guidance document within the overall ERA framework

1.3 Potential regulatory drivers for ERA

The primary driver is Part 2A of the Environmental Protection Act 1990. Other potential regulatory drivers include the Habitats Directive and the planning regime.

1.3.1 Part 2A of the Environmental Protection Act

Section 57 of Part 2A of the Environmental Protection Act 1990 (EPA 1990) introduced a new statutory regime for the identification and control of contaminated land in England and Wales (DEFRA 2006, WAG 2006 and Scottish Executive 2006). The Act states that:

> *'Contaminated land' is any land which appears to the local authority in whose area it is situated to be in such a condition, by reason of substances in, on or under the land, that –*
>
> *significant harm is being caused or there is a significant possibility of such harm being caused; or pollution of controlled waters is being, or is likely to be, caused…*

where 'harm' is defined as:

> *harm to the health of living organisms or other interference with the ecological systems of which they form a part, and in the case of man includes harm to his property.*

'Ecological harm' within Part 2A is confined to specified receptors as set out in Table A of the Statutory Guidance (DEFRA 2006, WAG 2006 and SE 2006). In summary, these are:

- any ecological system, or living organism forming part of such a system, within a location which is:
  - a site of special scientific interest (SSSI) notified under section 28 of the Wildlife and Countryside Act 1981;
  - a national nature reserve (declared under section 35 of the above act);
  - a marine nature reserve (designated under section 36 of the above act);
  - an area of special protection for birds (under section 3 of the above act);
  - any habitat or site afforded policy protection under paragraph 6 of Planning Policy Statement (PPS 9) on nature conservation;
  - any nature reserve established under section 21 of the National Parks and Access to the Countryside Act 1949;
  - any European site within the meaning of regulation 10 of the Conservation (Natural habitats etc) Regulations 1994;
  - any candidate Special Areas of Conservation or potential Special Areas of Conservation given equivalent protection.

1.3.2 Habitats Directive

Regulation 3 of the Conservation Regulations 1994 (commonly known as the Habitats Regulations) implements the requirements of the European Habitats Directive 92/43/EEC in Great Britain. It also secures the protection of areas classified under the Wild Birds Directive 79/409/EEC.

The Environment Agency is the competent authority (in England and Wales) for these regulations. As such, it applies the regulations when considering all applications for authorisations, permissions, permits, consents and environmental licences and for all relevant Environment Agency policy and operational activities.

A risk assessment process is initiated in situations where an application under the UK system of land use planning or a review of permits, licences, etc. is likely to impact on sites protected under the regulations. There are four stages to the risk assessment:

- identifying relevance;
- likely significant effect;
- identifying adverse impacts;
- implementing any changes.

The ERA framework will be a useful aid in this process.

1.3.3 Planning

Planning Policy Statement (PPS) 23: *Planning and Pollution Control* states that:

> *Land contamination, or the possibility of it, is a material planning consideration in the preparation of development plan documents and in taking decisions on individual planning applications* (ODPM 2004).

The remediation of contaminated land through the planning process should secure the removal of unacceptable risk and make the site suitable for its new use. Following redevelopment, the land should not be capable, as a minimum, of being determined as contaminated land under Part 2A of the Environmental Protection Act 1990.

Development plans and decisions on individual planning applications should take into account the potential sensitivity of the area to adverse effects from pollution, including nature conservation interests such as:

- SSSIs;
- National Parks;
- Areas of Outstanding Natural Beauty (AONBs);
- Special Areas of Conservation (SACs);
- Special Protection Areas (SPAs);
- wetlands of international importance (RAMSAR sites).

Where appropriate, soil screening values and the wider ERA framework can be used to assess the possible risks to nature conservation interests when potentially polluting activities are proposed. Where necessary, they can also be applied to the assessment and remediation of historic contamination.

1.4 Report structure

Section 2 provides guidance on the bioassays recommended for use as part of the ERA framework along with the strengths and weaknesses of each test. Section 3 provides guidance on the application of bioassays, including how to select tests. Section 4 details operating windows for bioassays, section 5 details the criteria for the acceptance of new bioassays into the ERA framework and section 6 provides guidance on implementing bioassays in commercial laboratories.

2 Bioassays for use in the ERA framework

The Environment Agency has developed an Ecological Risk Assessment (ERA) Framework for Contaminants in Soils (ERA1) which can be used to support determinations under Part 2A of the Environmental Protection Act 1990. Tier 2 of this framework seeks to establish whether or not significant harm or the significant possibility of significant harm is occurring. A risk characterisation is conducted using ecological surveys and biological tests identified as relevant to the site and receptors potentially at risk (see ERA 2a, Guidance on desk studies and CSM).

Biological tests can be used to identify impacts of contaminants on organisms representative of the Receptors of Potential Concern (RoPC). The Environment Agency has developed biological tests for use in Part 2A assessments and has documented the process in the following research reports:

- *Review of sub-lethal ecotoxicological tests for measuring harm in terrestrial ecosystems* (Environment Agency 2002);
- *Assessing risks to ecosystems from land contamination* (Environment Agency and SNIFFER 2002);
- *Application of sub-lethal ecotoxicological tests for measuring harm in terrestrial ecosystems* (Environment Agency 2004a);
- *Biological test methods for assessing contaminated land. Stage 2: a demonstration of the use of a framework for the ecological risk assessment of land contamination* (Environment Agency 2004b).

This document provides a summary of the biological tests currently recommended, when they are applicable and how to apply them within Tier 2 of the ERA Framework. Standard Operating Procedures for Bioassays (SOPs) intended for use in laboratories are provided in a separate document (ERA3).

2.1 Introduction

A bioassay is:

> *'a laboratory test in which the toxicity of a contaminant or environmental sample is measured by exposing a specific organism and measuring a life-cycle parameter (for example, survival, reproduction, development, growth). In general, bioassays are conducted under controlled conditions so that the effects of environmental factors that could confound interpretation of results are avoided'* (Environment Agency 2002).

Bioassays are null hypothesis type tests. This means that they are based on a null (or negative) hypothesis stating that, when an organism is exposed to a contaminant, the contaminant has no significant effect on that organism. The null hypothesis is not supported if the test provides a statistically significant result. If there is a significant difference at the 95 per cent confidence level ($P < 0.05$) then, in most instances, this is deemed to be an 'effect' worthy of further consideration.

Appropriately selected bioassays can be used to help determine whether soil contamination may cause harm to identified (RoPCs). Bioassays chosen as

measurement endpoints must be relevant to the assessment endpoint, i.e. the organisms selected for testing are appropriate models for the receptors. Within the ERA Framework, a bioassay that indicates an effect on such an organism at the agreed level of confidence can be considered as supporting evidence that harm is or could occur at the site from which the samples were taken, provided the testing has been conducted properly according to the appropriate SOP (see ERA 3, SOPs for bioassays).

A variety of bioassays have been reviewed, tested and recommended for use in the ERA Framework (Environment Agency 2002a, 2004a, 2004b). These are:

- solid-phase Microtox®;
- bait lamina;
- nitrogen mineralisation;
- earthworm survival and reproduction test;
- earthworm lysosomal stability test – also known as neutral red retention time (NRRT);
- Collembolan survival and reproduction test;
- plant seedling emergence and vegetative vigour test.

The process by which these bioassays were selected for inclusion in the ERA framework is described in an earlier report (Environment Agency 2002). The recommendations were made on the basis of literature reviews and performance in a series of trials (Environment Agency 2004a, 2004b).

This chapter presents the comparative strengths and weaknesses of each bioassay. It is intended to provide guidance to risk assessors as to when a specific bioassay might be suitable. Equally it can be used to help identify when bioassays are not appropriate, i.e. if the soil type, contaminants present, receptor of interest or pollutant pathway is such that none of the recommended bioassays can provide relevant, interpretable results.

Details are provided on how well each bioassay performed in two field trials performed when testing the ERA framework in the following documents:

1. Environment Agency (2004b) describes a study where the ERA process was applied to two contaminated sites. Environment Agency (2004a) describes the same bioassay trials in isolation.
2. An evaluation of how well the bioassays worked on samples of contaminated soils was performed in collaboration with industrial partners. The bioassays were used to assess the contaminant effects from industrial sites chosen to represent a variety of different soil types and contaminants.

Finally, a tabulated summary is given at the end of the chapter (Table 2.1). The information in this chapter is intended to be used in combination with guidance on:

- the application of the bioassays (see Chapter 3);
- operating windows (see Chapter 4);
- SOPs for bioassays (ERA 3).

2.2 Microtox® with solid-phase extracts

Microtox® is a commercially available ecotoxicological test based on the bioluminescent bacterium, *Vibrio fischeri*, which produces light as a by-product of its cellular respiration. This test measures the amount of light emitted by the exposed bacteria in relation to that of the control organisms as a measure of the degree of suppression of cellular respiration as a surrogate expression of contaminant effects.

The test is recognised by many international regulators. American Society for Testing and Materials (ASTM) and United States Environmental Protection Agency (US EPA) protocols are available as well as a draft International Standards Organization (ISO) protocol. A revised protocol for using solid-phase soil samples is recommended for use in the ERA Framework (see ERA 3 - SOPs for bioassays).

2.2.1 Strengths

Speed

The Microtox® test itself is simple and can be performed rapidly (in less than 30 minutes per sample) using commercially available kits. Software for interpreting the results is also commercially available.

Affected by a range of contaminant types

There is a considerable amount of literature on the use of Microtox® as a toxicity test and there is good evidence of its sensitivity to a range of contaminant types including metals, organics and pesticides. Furthermore, the test is demonstrably sensitive to contaminant concentrations likely to occur in soils.

Small sample size

The Microtox® test requires only a small sample of soil (7 g), making it highly practicable in terms of both sampling and laboratory procedures.

Highly standardised

Protocols for using Microtox® are described by a number of significant international regulatory organisations including ASTM, ISO and US EPA. The commercial availability of the testing kits and interpretation software adds to the level of standardisation.

2.2.2 Weaknesses

Unrepresentative

Vibrio fischeri is a marine bacterium and therefore not specifically representative of a soil organism. Extrapolating effects from a marine bacterium to a soil invertebrate

necessarily incurs uncertainty. The ecological relevance of this test to soil invertebrates is therefore questionable. But although not suitable for detailed soil assessment, Microtox® is still considered suitable for initial screening assessments (Environment Agency 2002).

Solid-phase equivalents have recently been developed for testing soil toxicity directly. However, this version of the test requires genetic manipulation of more representative soil invertebrates to carry the *V. fischeri* gene, and is therefore subject to greater control (e.g. by the Health and Safety Executive).

Low sensitivity to some chemicals

Microtox® is reported to have low sensitivity to certain substances, including some metals, pesticides with certain modes of action and polyaromatic hydrocarbons (PAHs) (Environment Agency 2004a). However, the solid phase test that uses an aqueous suspension of soil is generally more sensitive than tests on aqueous leachates.

Sample treatment

Microtox® works only in the aqueous phase (i.e. only aqueous solutions/suspensions can be applied to the bacteria). So for evaluating contaminant toxicity in soils, the contaminants must first be extracted from the soil into solution. A number of protocols exist for extracting contaminants from soils, but there is uncertainty as to their efficiency at removing the bioavailable fraction.

The aim when using Microtox® on soils is to assess the effect of soil contaminants on the soil fauna. However, this is only possible if the soluble extract is representative of the exposure experienced by the soil infauna. Therefore the extracted solution should contain:

- only contaminants that the soil organisms can take up (i.e. the bioavailable fraction); and
- all of the contaminants that are bioavailable.

Salt must then be added the extract so that the marine bacterium is not osmotically challenged. However, changing the salinity may also affect the bioavailability of contaminants. This can introduce further uncertainty into the analysis.

2.2.3 Performance

Environment Agency (2004a) reported that at a site contaminated with metals the test performed well and demonstrated a clear concentration response. However, sensitivity at the site with hydrocarbon contamination was low, with the light response even being stimulated in some cases.

The industrial partners reported a similarly low sensitivity to hydrocarbons and a more variable response to metals.

Microtox® with solid phase extractions is not recommended for use at Tier 2 of the ERA Framework, but may have application at Tier 1 when assessing the extent of contamination and identifying pollutant ‘hotspots’

2.3 Bait lamina

The bait lamina test assesses the feeding activity of soil organisms by measuring the breakdown of organic material set into plastic strips that have been inserted into the soil.

Bait lamina is an academically recognised method for measuring soil function, but has no international standard or agreed protocol. However, the bait lamina equipment is available commercially and is low cost. Earlier reviews recommended it as a screening assay in ERA assessments (Environment Agency 2002).

2.3.1 Strengths

Used in situ

Bait lamina strips are deployed *in situ*, being inserted directly into the soil on the study site. Therefore the test is representative of the site under natural conditions.

This is advantageous in that none of the other bioassays recommended for use in the ERA Framework are performed *in situ*. Although several of the other tests could be performed *in situ,* the requirement for controlled environmental conditions makes *in situ* deployment impractical.

Process focussed

Bait lamina gives a direct measure of the functioning of the whole soil as determined by the breakdown of organic matter. This process is considered important in the functioning of soil ecosystems.

Ecologically relevant

Bait lamina integrates the functioning of soil invertebrates and micro-organisms and gives a measure of total soil infauna feeding activity. This is particularly important as the two groups are thought to be interdependent. It can be considered as an assessment of impact at a community level which has relevance for ecosystem functioning as a whole.

2.3.2 Weaknesses

Low interpretive power

Although one of the strengths of the bait lamina test is that it measures ecosystem functioning at a community level, this factor can also reduce the power of the test because it cannot discriminate changes in the community.

Bait lamina measures the feeding activity of the total soil infauna and does not identify the phyla or species involved. Therefore it cannot indicate when changes in soil community structure have occurred. For example, overall feeding activity might remain similar in a contaminated and uncontaminated soil – so the bait lamina test will produce

the same result. However, the contaminated soil might have an entirely different community structure, brought about by the presence of the contamination. Therefore the bait lamina test does not establish that a 'natural' soil community is functional.

Interference

The results of the bait lamina test can be influenced by a number of factors including soil condition (moisture, matrix, etc.) and season. Although this does not preclude the use of the test, modifying factors must be considered when interpreting the results.

Spatially limited

Bait lamina can only be deployed at the soil surface and can therefore only be used to measure impacts of contaminants in top 15cm of the soil. The test is unable to demonstrate effects of contaminants deeper in the soil strata.

2.3.3 Performance

The bait lamina test clearly identified the effects of metal contamination on the feeding activity of the soil community but it failed to show effects of hydrocarbon exposure (Environment Agency 2004a). It was suggested that where the hydrocarbon contamination was very localised at the soil surface, the strips were not properly exposed.

The industrial partners similarly reported variability in their results and also that it was not possible to deploy the strips in some types of made ground.

Overall, the ecological relevance of bait lamina and the low cost and ease of use make it a recommended test for use at Tier 2 of the ERA Framework, but results should be interpreted with care according to the season and soil conditions.

2.4 Nitrogen mineralization

Nitrogen is essential to plant growth but its availability is often reduced in contaminated soils. This test estimates the amount of mineralisable nitrogen (i.e. the bioavailable fraction) in soils by measuring nitrate production over time. The test has considerable international recognition with full protocols available from OECD and ISO.

2.4.1 Strengths

Representative

Nitrogen is essential to plant growth and the nitrogen cycle is a critical process of soil functioning. The measurement of nitrogen mineralisation gives a representative indication of the health of the soil.

Indicative of long-term effects

Nutrient cycling within soils occurs over comparatively long timescales. Impacts on this cycling may only manifest themselves over similar timescales. As a measure of nutrient cycling within soils, this test gives an indication of long-term effects of contaminants on soil functioning.

Highly standardised

ISO (1997) and OECD (2000a) have both published protocols for this test as an assessment of soil health.

2.4.2 Weaknesses

Sensitive to physico-chemical parameters

The nature of this test is that the results depend on the physical and chemical characteristics of the soil under investigation. The nitrogen cycle interacts closely with other nutrient cycling in the soil (e.g. carbon cycling) and is therefore strongly influenced by soil physico-chemistry. When interpreting the results, attention must be given to the soil properties and how these affect the results.

Low interpretive power

The test is non-specific and measures soil health as a function of a chemical cycle. It cannot be used to identify impacts on particular organisms or communities.

Not concentration dependant

Unlike many toxicity tests where degree of impact often depends on the concentration of the contaminant, soil nitrogen processes do not react in a manner relative to contaminant levels. The test can only show that nitrogen cycling processes are significantly affected or not affected by a contaminant but this is not related to the amount of contaminant present.

2.4.3 Performance

Both Environment Agency (2004a) and the industrial partners reported that the nitrogen mineralisation test produced highly variable results with no correlation to contaminant levels. However, it is recommended for use at Tier 2 of the ERA Framework as an indication of soil functioning and health.

2.5 Earthworm reproduction

The most pertinent end-point of the earthworm test for soil samples from contaminated sites is reproduction (the production of cocoons). It is also possible to assess the growth of the juvenile worms following hatching. However where gross contamination is

present, it may be necessary to collect information on the survival of the exposed adults as an end-point.

Earthworms are universally accepted as representative 'sentinel' organisms for soil and have a long history of use (e.g. OECD 1984a). Two common genera are used (*Eisenia fetida* or *E. andrei*, and *Lumbricus rubellus*). These are cultured and available commercially.

Bioassay protocols using earthworms have been published by the ISO (1998a) and OECD (2000b). The tests are comparatively easy and are low cost, although they are usually run over a period of weeks and may require a degree of operator skill. They were recommended for use in the ERA Framework in earlier reviews (Environment Agency 2002).

2.5.1 Strengths

Representative

Earthworms are common inhabitants of the soil ecosystem and they are also known to be important to the functioning of soils. Earthworms (particularly the three species used in these tests) are accepted by regulators and academics as suitable test species for identifying contaminant impacts in soils.

Unlike *Eisenia andrei*, the compost-dwelling *E. fetida* will not be present in the soils of interest of the ERA framework, but it is still a suitable alternative as it is a close relative. The deeper dwelling *Lumbricus rubellus* can be used if a slower-growing, longer-lived organism is more relevant to the objective of the testing.

Sensitive to a range of contaminant types

There is considerable literature on the use of earthworms in soil toxicity testing. It contains good evidence that earthworms are impacted by a range of contaminant types including metals, organics and pesticides.

Sensitive to 'realistic' concentrations

Sub-lethal effects of contamination are usually manifest at much lower concentrations than those that cause mortality. Effects on reproduction in earthworms can be expected at concentrations of contaminants that are commonly encountered in contaminated soils.

Indicative of higher level impacts

Sub-lethal tests such as reproductive output can be indicative of impacts on higher levels of biological functioning (e.g. Heimbach 1998). Reproductive output can be extrapolated to consider whether the population is in decline or is likely to become extinct. Population level impacts can affect higher species for which worms are a significant part of their diet.

Indicative of long-term impacts

Effects on reproduction can demonstrate longer term impacts, especially if multi-generational studies are considered (either experimentally or via extrapolation models).

Highly standardised

Methodologies for performing reproduction tests with *Eisenia* sp. are available as draft protocols from ISO (1998b) and OECD (2000a).

2.5.2 Weaknesses

pH sensitive

Earthworms are sensitive to low pH (<3) and reproduction is reduced (Spurgeon and Hopkin 1996, Spurgeon and Weeks 1998). Earthworm tests are therefore unsuitable for use in very acidic soils where the low pH may affect the test.

Soil matrix sensitive

Earthworms are soft bodied and therefore susceptible to damage from coarse or sharp soil matrices. The soil matrix type will not necessarily affect the bioavailability of the contaminants, but the worms may become physically damaged in very coarse or sharp matrices. This may affect the viability of the earthworms used. Therefore earthworm tests may not be suitable in all soil types.

Food effects

For all tests, horse manure is added to the soil substrate as a food source for the worms. It is possible that the manure might bind contaminants, reducing their availability to the earthworms. Alternatively, it may increase availability if the earthworms select and efficiently metabolize the manure. The dynamics of contaminants in tests where food is provided is not well understood.

2.5.3 Performance

The earthworm reproduction test was found to be sensitive to metals and hydrocarbons (Environment Agency 2004b). The industrial partners described the results of this test as promising, with some samples giving good correlation with contaminant levels, but others being more variable.

Overall, the earthworm reproduction test is recommended for use at Tier 2 of the ERA Framework where effects on invertebrates are suspected, and also where earthworms are a common food source for vertebrates.

2.6 Earthworm lysosomal stability test

The lysosomal stability test, also known as neutral red retention time (NRRT) assay, is a measure of the structural integrity of the lysosomes in the epithelial cells of the digestive gland of earthworms.

The test is based on exposure of earthworms to a toxicant causes the lysosomal membranes to become damaged and therefore allow a dye (neutral red) to leach from the lysosomes. A reduction in the amount of dye retained in the lysosome indicates that the membrane has been damaged, which in turn indicates an impact on the individual exposed to the toxicant.

This test can be performed following removal of the surviving adult worms from the end of the survival and reproduction tests (see section 2.5).

The test is academically recognised (Moore 1988, Lowe and Pipe 1994, Lowe et al. 1995, Svendsen et al 1996), but has no ISO or OECD protocol.

2.6.1 Strengths

Concentration responsive

The degree to which lysosomal stability is reduced depends on the concentration of the toxicant. The results from the test are therefore indicative of the concentration of toxicant to which the worms have been exposed.

Insensitive to physico-chemical factors

Compared with several of the other assays recommended for use in the ERA Framework, the lysosomal stability assay is comparatively insensitive to physico-chemical factors. Because the test is non-specific (i.e. it is a measure of 'general health' of the worm), the power of the test would be significantly reduced if lysosomal stability was affected by factors other than toxicant exposure. Although research to date implies that reduced lysosomal stability occurs only following exposure to toxicants, this does not negate the requirement for robust experimental design using representative controls.

Affected at 'realistic' concentrations

Lysosomal stability is affected at concentrations lower than those required to affect reproduction (and other sub-lethal end-points) in earthworms. This has been demonstrated for a range of toxicant types including metals and a variety of organic compounds.

Affected by a range of contaminant types

The lysosomal stability assay responds to a range of toxicant types including metals, organics and mixtures of compounds. However, this is not universal and the test has been shown to be completely insensitive to the insecticide methiocarb and only sensitive to the fungicide iprodione at approaching lethal concentrations.

Used in-situ

Although the test is more usually applied following laboratory exposure of commercially purchased worms to contaminated soils, the lysosomal stability assay can be used to test worms collected from the site of interest. Furthermore, Hankard et al. (1998) released earthworms to sites where a natural worm population did not exist, prior to testing them with this assay.

2.6.2 Weaknesses

Labour-intensive methodology

Although the lysosomal stability assay is comparatively simple and requires no expensive equipment, it is labour-intensive as the results are recorded by making a visual count using a light microscope. Although this is a relatively unskilled operation, it is time consuming and laboratory staff need appropriate training to ensure the results are recorded correctly.

Potential for inter-laboratory differences

As stated above, results are subjectively interpreted by visual observation using a light microscope. There is therefore the potential for differences between operators and between samples recorded by the same operator.

Subjectivity can be greatly reduced by ensuring that operators are trained properly, disciplined and unbiased – but it cannot be completely removed. An automated system for reading and recording results would remove such subjectivity, but this has yet to be developed.

Non-specific

The lysosomal stability assay provides a measure of the general health of an organism; it is non-specific and does not necessarily indicate that the individual is adversely affected to the degree of causing detrimental effects on reproduction or at any higher population level.

Food effects

In laboratory tests, horse manure is added to the soil substrate as a food source for the worms. The effect that the manure might have on the dynamics of the contaminants in the test system is unknown. This does not apply to earthworms released into or collected from a field site.

2.6.3 Performance

Environment Agency (2004a) found the lysosomal stability assay performed well. However, the industrial partners found the test to produce highly variable results. Overall, the lysosomal stability assay is recommended for use at Tier 2 of the ERA Framework due to its adaptability between laboratory and field situations.

2.7 Collembolan reproduction

The most pertinent end-point of the collembolan test for soil samples from contaminated sites is reproduction (the number of juveniles produced). However where gross contamination is present, it is possible to collect information on the survival of the exposed adults as an end-point.

Collembolans, or springtails as they are more commonly known, are a diverse and ubiquitous group that are recognised as important components of the soil infauna. For this reason, and due to their ease of culture, they are suitable for use in soil bioassays. An ISO protocol (1998c) for a standard soil toxicity test using springtails has been published; the species recommended as a test organism is *Folsomia candida*.

2.7.1 Strengths

Representative

Springtails are ubiquitous inhabitants of the soil ecosystem. They are usually present in large populations and have an important role in soil functioning (Environment Agency 2002). They inhabit all types of soil and are therefore useful surrogates for assessing impacts on soil fauna and functioning.

Affected by a range of contaminant types

The standard laboratory based assay is demonstrably sensitive to a range of organic compounds including TNT, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and phenols (see Markwiese et al. 2001, Environment Agency, 2002). The bioassay has also been shown to be sensitive to metals and mineral oils (Environment Agency 2002) when appropriately modified for testing these contaminants in soils (see ERA 3, SOPs for bioassays).

Sensitive to 'realistic' concentrations

Sub-lethal effects of contamination are usually manifest at much lower concentrations than those that cause mortality and effects on reproduction in springtails can be expected at concentrations of compounds that are commonly encountered in contaminated soil samples.

Indicative of higher level impacts

Sub-lethal tests in springtails can be indicative of impacts on higher levels of biological functioning (e.g. Heimbach 1998). Reproductive output can be extrapolated to consider whether the population is in decline. The absence of a species with a significant role in soil functioning has the potential to alter soil condition and therefore the survival of higher organisms.

Indicative of long-term impacts

Effects on reproduction can demonstrate longer term impacts, especially if multi-generational studies are considered (either experimentally or via extrapolation models).

Speed

Springtail cultures are comparatively fast growing and so tests can usually be completed within a month. In addition, image recognition software is now available.

Flexibility

A number of modifications can be made to the test making it flexible and able to measure a number of endpoints. For example, a rapid assessment of population growth rate can be made, which is an ecologically relevant indication of soil health.

2.7.2 Weaknesses

Sensitive to soil physico-chemistry

The reproductive rate of springtails is significantly influenced by soil physico-chemistry including soil temperature, pH and organic matter content. These effects can be taken into account by performing the test with suitable control soils. This is important to ensure that the proper interpretation of the results is made.

Monitoring difficulties during test

It is not possible to monitor the progress of the test as the assessment method is destructive (flooding the soil sample with water to flush out the springtails). Therefore, in the standard test, it is not possible to continuously assess mortality or juvenile production (Scott-Fordsmand et al. 2000; Environment Agency 2002). However, modification of the test can allow periodic measures to be made (Fountain and Hopkin 2001).

2.7.3 Performance

A number of difficulties were reported with the springtail test, including failure of the test due to fungal growth in the test chambers (Environment Agency 2004) and the industrial partners reported an unsuitability for use in ‘made ground’ and some variability in their test results.

However there were some good correlations between contaminant levels and effects on springtail reproduction, and so the test is recommended for use at Tier 2 of the ERA Framework where the test can be performed by experienced operators and the test conditions can be suitably controlled.

2.8 Plant seedling emergence and growth

Plants depend on the soil for moisture and nutrients via their root system, which provides an interface between the plant and the soil. Degradation of soil quality is therefore likely to be reflected in impacted plant health, either physically or physiologically. Measurements of impacted functions such as germination, growth, etc are therefore valuable tools for assessing the quality of soils.

Plant tests have been used for the commercial testing of herbicides (for weed inhibition) and pesticides (for crop protection). However, the published literature does not reflect this as many studies remain confidential.

Although OECD and ISO protocols for performing plant tests have long been available (OECD 1984b, ISO 1993, ISO 1995), they have received considerable criticism and have since been revised. The tests developed here are based on the revised OECD seedling emergence and growth tests (OECD 2000c).

The Environment Agency (2002, 2004b) recommends three species of plant for use, namely:

- wheat (variety ZBB 065, a monocotyledon that represents other grasses);
- tomato (variety 'Garden Pearl', a dicotyledon to represent wildflowers etc.);
- Chinese cabbage (a dicotyledon to represent more foliate species).

2.8.1 Strengths

Sensitive to a range of contaminant types

Although the plant tests described were originally designed for commercial purposes (the development of herbicides and pesticides), the tests are sensitive to a range of other soil contaminant types including metals and a variety of organic compounds, e.g. PAHs, petroleum fuels and chlorinated solvents (Environment Agency 2002).

Indicative of higher ecological effects

Seedling emergence and growth are indicative of general soil health and functioning and are, therefore, indicative of higher ecological effects and ecosystem functioning. Different species can be used as surrogates for different plant communities, e.g. wheat being a monocotyledon to represent grass assemblages. Also, potential impacts on dependant herbivorous animals or those using plants for cover can also be considered.

Highly standardised

The plant tests have a comparatively long history of use and their protocols have undergone several refinements. Current protocols are published by ISO (1993), OECD (2000c), US EPA (1994) and Canadian Council of Ministers for the Environment (CCME 1996).

2.8.2 Weaknesses

Sensitive to soil physico-chemical parameters

The success of seedling emergence and growth is closely associated with the condition of the supporting soil. While this is an advantage in terms of identifying toxicant impacts, it can make interpretation of the results complex as other soil conditions (pH, moisture, organic content, conductivity, etc.) must also be carefully controlled. Representative control trials must be performed so that the effects of toxicants on emergence or growth can be isolated from effects of other soil conditions.

Potential for plant specific differences

Although studies have shown these plant tests are sensitive to a range of chemicals, most studies to date have concentrated on pesticides. The effects of the types of industrial contaminants likely to be present at Part 2A sites are not widely tested.

It is also possible that different plant species may have different sensitivities to certain toxicants. Therefore careful selection of plant species to represent the RoPC is important.

2.8.3 Performance

Seedling growth showed a very good correlation between effects and contaminant concentration for metals, however the results for hydrocarbon contaminated soils were more variable (Environment Agency 2004b). Seedling emergence was reported to be unaffected by contaminant concentrations.

The industrial partners reported that overall the plant tests were the most promising bioassays trialled, and they are therefore recommended for use at Tier 2 of the ERA Framework.

2.9 Concluding remarks

This chapter has discussed the strengths and weaknesses of each of the biological tests currently recommended for use at Tier 2 of the ERA Framework and a summary is provided in Table 2.1. It is clear that no single bioassay is perfect and that no one bioassay would be suitable for use in all risk assessments. But by careful consideration of the various attributes of the bioassays developed here,[1] together with the further information presented in Chapters 3 (application of the bioassays) and 4 (‘operating windows, i.e. when the bioassays can be used) of this guidance, practitioners should be able to make an informed choice. Such decisions should be made in consultation with the relevant conservation agencies, and the mode of interpretation of test outcomes should be agreed with the regulator prior to any tests being commissioned.

[1] Described in more detail in Environment Agency 2002, 2004a.

Table 2.1 Summary of the strengths and weaknesses of the bioassays recommended for use in the ERA framework

Test	Strengths	Weaknesses
Microtox® solid phase	Fast Sensitive to a range of contaminants Requires small sample size Highly standardised	Unrepresentative (uses marine bacterium) Variability of sensitivity Requires treatment of sample
Bait lamina	Is used *in situ* Process focussed Ecologically relevant	Low interpretive power Sensitive to physico-chemical parameters Spatially limited Unsuitable for all soil types (including some types of made ground)
Nitrogen mineralisation	Representative Indicative of higher level effects Highly standardised	Sensitive to physico-chemical parameters Low interpretative power Not concentration-dependent
Earthworm reproduction	Representative Sensitive to a range of contaminants Sensitive to 'realistic' concentrations Indicative of higher level impacts Indicative of long-term impacts Highly standardised	pH sensitive Soil matrix sensitive Potential of food to alter bioavailability of contaminants
Earthworm neutral red retention time	Concentration-dependent response Not affected by physico-chemical parameters Sensitive to a range of contaminants Sensitive to 'realistic' concentrations[1] Can be used *in situ*	Laborious methodology Potential for subjectivity giving inter-laboratory differences Non-specific Potential of food to alter bioavailability of contaminants

Notes: [1] Even lower than those impacting earthworm reproduction

Table 2.1 Summary of the strengths and weaknesses of the bioassays recommended for use in the ERA framework (cont'd)

Test	Strengths	Weaknesses
Collembolan reproduction	Representative Sensitive to a range of contaminants Sensitive to 'realistic' concentrations Indicative of higher level impacts Indicative of long-term impacts Relatively fast and flexible	Sensitive to physico-chemical parameters Cannot monitor during test
Plant seedling emergence, growth and vegetative vigour	Sensitive to a range of contaminants Indicative of higher level effects Highly standardised	Sensitive to physico-chemical parameters Potential for plant specific differences

3 Guidance on application of bioassays

3.1 Introduction

The reasoning behind the recommendation of the bioassays selected for use in the ERA framework is provided in Environment Agency (2002). Information on how the tests performed in a series of trials is supplied in two further reports (Environment Agency 2004a, 2004b). The methodology of how each bioassay should be performed is out in Standard Operating Procedures (SOPs) for bioassays that can be found in a separate guidance document (ERA 3).

The guidance provided here is intended to be general advice on the choice and application of biological tests within the ERA Framework.

3.2 Choice of appropriate biological tests

Seven biological tests are recommended for use in the ERA Framework (see Chapter 2). These tests are diverse, covering a variety of:

- biological systems – microbial, plant and animal;
- types of response – survival, growth and reproduction;
- levels of organisation – cellular and organism;
- types of result – deterministic (discrete) and stochastic (allowing for natural variation);
- methodologies – *in situ* and laboratory.

The recommended bioassays, combined with any additional tests that meet the criteria (see Chapter 5 for acceptance of new tests for use in the ERA Framework), presents practitioners with a wide selection of tests to assess the potential for impacts at their sites. However, it is essential the correct tests are chosen in order to generate information relevant to the RoPCs.

Throughout the ERA process, the risk assessor should refer to the Conceptual Site Model developed prior to Tier 1 (see ERA 2a, Guidance on desk studies and CSM). This has identified the source–pathway–receptor linkages and is fundamental when proposing/agreeing suitable tests with the regulator and conservation agencies. The tests should provide measurement end-points that can be related to potential harm to the receptor, and the level of test response that constitutes harm should also be agreed beforehand.

None of the biological tests is compulsory for risk assessments; indeed, some of them are likely to be unsuitable at some sites. The intention is that only those tests that are appropriate for a site and for a given pathway and/or receptor should be used. If none of the recommended tests is considered suitable for use then bioassays need not be used at all. An ecological survey where the assessments are compared with historical data or a reference site may be considered more appropriate (see ERA 2d, Guidance on the use of ecological surveys).

It is accepted that the range of biological tests recommended for use in the ERA Framework is comparatively small and that, for most scenarios, assays are not available that specifically refer to the pathway or RoPC. Bioassays use species that are well understood and amenable to laboratory culture, because receptor organisms are not usually available for testing. It is intended that the **most** appropriate bioassay should be chosen **but** only when the outcome is likely to provide information that is relevant to the receptor species concerned and the objectives of the test

The following examples demonstrate some of the considerations that can be made when deciding which biological tests to use for certain scenarios and species of special interest. The list is neither prescriptive nor exhaustive. It serves only to aid in an understanding of the issues a risk assessor should address when deciding which, if any, biological tests should be used.

These decisions will be made during the desk study and development of the Conceptual Site Model (CSM), and will be agreed with the regulator before any samples are taken. If a site is being considered under Part 2A, then the testing strategy should link directly back to the statutory guidance definitions of harm. Guidance on the development of the CSM is provided in ERA 2a (Guidance on desk studies and CSM).

3.2.1 Species of special interest is a plant

If the species of special interest at a site is a plant, a risk assessor might consider two types of biological test:

(a) a test that assesses general soil health or functioning:

- nitrogen mineralization; and/or
- bait lamina.

(b) a test that assesses contaminant impacts on plants specifically:

- seedling emergence; and/or
- plant growth.

For (a), plants are dependant on the soil for obtaining fixed nitrogen so a test based on nitrogen cycling may be of benefit for assessing the soil conditions as suitable for plant growth. Similarly, a functioning population of microbes is important for releasing other micro-nutrients to plant roots.

For (b), site assessors have further choices as there are several different plant species recommended for use. A test species that is similar in seedling structure (monocotyledon or dicotyledon) to the species being protected is most relevant. Combining these tests with an ecological survey to assess the extent of the species of special interest both on the contaminated site and on a reference site is likely to be beneficial.

3.2.2 Species of special interest is an invertebrate

It may be that the species of special interest is an invertebrate, e.g. a butterfly species. Potential impacts to a butterfly include effects on the larval (caterpillar) stage as well as the adult. An exposure pathway for a caterpillar is via the food chain and so a plant assay might be a suitable test. If plant emergence or growth is affected the food source for the caterpillar may be reduced leading to reductions in the butterfly populations. In this case, a test using a foliate plant species such as Chinese cabbage may be

appropriate. However, where a butterfly feeds on the nectar of specific flowers, it may be of greater benefit to propose an ecological survey where plant numbers are assessed (see ERA 2d, Guidance on the use of ecological surveys).

Where other soil dwelling invertebrates are a RoPC, e.g. a spider or beetle that feeds on other smaller invertebrates, then the most appropriate test may be a collembolan reproduction assay to assess the health of the soil for supporting micro-invertebrates.

3.2.3 Species of special interest is an amphibian

The species of special interest might be an amphibian, e.g. a great-crested newt. The pathway identified in the Conceptual Site Model might be the food chain where earthworms are a major food source for newts. Therefore an earthworm test might give an indication of contaminant effects on the newts' food supply. Also, a bait lamina test could give a general indication of soil functioning, health and ability to support the invertebrate populations that the newts predate.

An ecological survey where newt numbers are assessed at the contaminated site and in similar uncontaminated areas, or where there are data on newt numbers prior to contamination, would also be of benefit (see ERA 2d, Guidance on the use of ecological surveys).

3.2.4 Species of special interest is a bird

Generally, biological tests are unlikely to provide sufficient evidence of contaminant impacts on birds and ecological surveys will be required. Risk assessors should consult the guidance document on performing ecological surveys in this series (see ERA 2d, Guidance on the use of ecological surveys).

Notwithstanding this, biological assays **might** provide useful additional information under some circumstances. Whereas direct exposure from contaminant to bird is unlikely, there may be extended links via the food chain, with the contaminant being taken up from the soil by soil invertebrates such as earthworms. For a thrush feeding on earthworms, for example, this would be a complete pathway and so an earthworm test might provide some useful additional information. A similar case might be made for plant tests where seed-eating finches are the receptor.

Alternatively, there might be one or more links in the food chain with intermediate species (e.g. small birds or mammals) feeding on earthworms or other large soil invertebrates before being eaten by a species of raptor. In this instance, an earthworm test and/or a lysosomal stability assay might provide useful additional information on the health of the lowest organisms in the food chain that supports a diverse and extended predation network.

An alternative scenario to that described above is where a raptor feeds on herbivorous prey items such as rabbits. For similar reasons to those described above, a plant test might provide some useful information here as it demonstrates an impact on a link in the pathway (i.e. the food chain – either by reduction in prey availability or possible bioaccumulation).

Where bioaccumulation is suspected due to the nature of the contaminant (e.g. it has log k_{OW} >3), body burden analysis of the earthworms might be considered as supporting evidence.

In all cases, however, biological tests are only likely to provide additional, supporting information. Ecological surveys are more appropriate for assessing impacts on birds.

3.2.5 Assessing risks to the functioning of the ecological system

Where the RoPC is the 'functioning of the ecological system', ecological surveys are more appropriate for assessing potential impacts. Risk assessors should consult ERA 2d, Guidance on the use of ecological surveys. Bioassays may be able to provide additional, supporting information, for example, in a chalk grassland where the ecological functioning depends on the status of grasses, a monocotyledon plant test may be appropriate. Also, more generally, nitrogen mineralisation, bait lamina and collembolan reproduction assays can provide information on soil health and condition.

3.2.6 Where biological tests are not appropriate

It is accepted that the range of biological tests recommended for use in the ERA framework is comparatively small and that, for most scenarios, assays are not available that specifically refer to the pathway or RoPC. Bioassays use species that are well understood and amenable to laboratory culture, because receptor organisms are not usually available for testing. However, from the examples described it can be seen that a suitable surrogate test may be available to provide relevant information on the identified pathway.

There may be scenarios where none of the recommended tests appears suitable. In such cases, it is likely that the detailed ecological survey also recommended for use at Tier 2 is more appropriate (see ERA 2d, Guidance on the use of ecological surveys). Cases where bioassays are clearly not suitable include where the soil substrate is 'made ground' or contains a high proportion of gravel and rubble making it unsuitable for laboratory testing. Bioassays are also less relevant where the receptor is dependant on a very particular set of circumstances, e.g. highly salt tolerance plants growing on mudflats, because the surrogate model species are not similarly adapted.

3.3 Use of appropriate controls

Most of the tests recommended for use in the ERA Framework are sensitive to the physico-chemical qualities of the soil being investigated. Temperature, moisture, pH, and organic matter can affect the results of the tests (e.g. seedling growth, nitrogen mineralisation and earthworm survival/reproduction) to varying degrees. The heterogeneous nature of soils means that the results reported from the majority of tests will differ according to the specific soil(s) being investigated. In general, this variability according to soil type will not affect the interpretation of the test result because the effects of physico-chemical parameters on the measurement endpoints are, on the whole, known. However, it does mean that it is important to run suitable controls when using these tests (see Environment Agency 2002 and ERA 3 – SOPs for bioassays).

For all tests, the results from the soil being assessed should be compared with results of the same test on two reference soils:

- a 'clean' soil – Kettering loam or an artificial soil prepared according to OECD Guideline 207 (OECD 1984a) as specified in the various SOPs;
- an 'unimpacted' soil of similar physico-chemical parameters – ideally collected from a contaminant-free part of the site being investigated (see ERA 3, SOPs for bioassays).

A positive control is also performed by 'spiking' a clean soil with a reference toxicant (e.g. cadmium) to demonstrate that the test organism is affected to a predictable

degree by exposure to contaminants (i.e. to demonstrate that the test itself is sensitive and performing within expected parameters).

3.4 Biological test data and population modelling

Biological tests are primarily used in the ERA Framework as tools to assess the effects of contaminants on the biota at a site. For example, the main purpose of the earthworm test is to identify whether soil contaminants are sufficiently toxic to inhibit the reproduction of the earthworms inhabiting the soil. However, the data collected during the biological testing can potentially be used in other applications at Tier 2 of the ERA Framework. The use of ecological models for identifying impacts of contaminated land on higher levels of biological organisation (e.g. population, community or ecosystem impacts) has been reviewed in a previous report (Environment Agency, 2007).

The choice of biological test used at Tier 2 (if any) should be made on the relevance of the test to the source–pathway–receptor linkage and the ecological function or species of special interest. Certain tests, e.g. the collembolan reproduction assay and the associated rate of population increase test, lend themselves to use in population/ecological models. However, models should only be employed where a clear benefit can be seen and their use and the proper interpretation of their outcomes have been agreed by all the stakeholders.

3.5 Concluding remarks

Biological tests can be useful within the ERA process, providing information as to how toxicants in the soil impact biota. It is important only to use those tests that are relevant to the source–pathway–receptor linkage. Use of non-relevant or inappropriate biological tests can be misleading and a waste of resources.

Close regard of the Conceptual Site Model and consideration of the information in this document, the SOPs for bioassays (ERA 3) and the other supporting report for Tier 2 assessments (ERA 2d, Guidance on the use of ecological surveys) should ensure an appropriate selection of measurement end-points for the risk assessment.

4 Operating windows

4.1 Introduction

A number of bioassays have been assessed and trialled for use in the ERA Framework. The Standard Operating Procedures (SOPs) for bioassays (ERA 3) should be adhered to when performing the tests. The SOPs provide the quality criteria required for performing the tests as well as any confounding factors that may affect the results. However, the SOPs do not give full account of the circumstances of when a certain bioassay should or should not be performed.

This guidance draws together information on each bioassay including required quality criteria, generic 'good practice' guidelines and practical experience to describe when each bioassay can and, more importantly, should not, be used.

Essentially any parameter that can affect the result of a bioassay is relevant, i.e. all parameters that impact on the health or survival of the test organism or the biological activity being measured (e.g. feeding rate). Consequently 'operating windows' have been compiled with reference to the measurement end-point for each test so that risk assessors have a guide to whether a certain bioassay is suitable for use as part of their assessment.

Factors affecting each test are described and also summarised in Table 4.1 at the end of the chapter. Where an operating window prevents the use of a bioassay, Table 4.1 gives alternative bioassays that **might be** suitable for the conditions (where available). Each operating window is intended to be used in conjunction with the respective SOP for the bioassay (see ERA 3) to ensure its appropriate and informative use.

4.2 Performance

During the development of the recommended bioassays, the Environment Agency performed trials at 2 contaminated sites (2004a; 2004b)

1. an area contaminated by aerial deposits of metal from a primary cadmium/lead/zinc smelter;
2. a former (demolished) tank farm area where crude oil and refined petroleum products were stored.

Additionally, a further evaluation was performed in collaboration with industrial partners where all the bioassays were trialled at several sites chosen to represent a variety of soil and contamination types. The owner of each site reported on the performance of the bioassays and their suitability for use in different situations. The results and lessons learnt from all of the evaluation exercises have been incorporated into the following operating windows.

4.3 Microtox® with solid-phase extracts

Solid-phase Microtox® tests follow a series of specific assay procedures using a suspension of the test soil (refer to ERA 3, SOPs for bioassays). It is run under

controlled laboratory conditions and has no restrictive operating window. The chemical parameters of the sample (such as pH) are controlled by the operator. Microtox® can be performed on soil of any physical or chemical nature.

4.4 Bait lamina

Temperature

The temperature of the soil must be ≥5°C and ≤15°C for the duration of the test. This is the temperature range for normal biological activity of temperate soil organisms.

Soil moisture content

A moisture content of 12–15 per cent is *preferred* for a bait lamina test. The test can still be deployed in soils with moisture outside of this range, though the impacts of moisture on test results should be carefully considered.

Low moisture greatly reduces feeding rates whereas high moisture increases feeding rates. In very dry soils, the test is unlikely to provide a result due to cessation of feeding activity. In very damp soils, the test is likely to show very high feeding rates in a short test duration.

Soil matrix

The physical structure of the soil matrix must allow successful deployment of the bait lamina feeding strips. For the test to succeed there must be close contact between the soil and the feeding strip. If the soil is not in contact with the feeding strip, the soil organisms might not be able to traverse the gap between the soil and the strip, resulting in no feeding activity on the strip. Soils with a matrix of large particles may leave gaps between the feeding strip and the soil particles, and may not therefore be suitable for bait lamina tests. This is often the case for ‘made ground’ at many industrial sites.

Contamination depth

The bait lamina strips are inserted into the soil to a depth of 16 cm. Consequently they can only be used to assess soil contamination in the top 16 cm of the soil (i.e. the surface or top soil). They cannot be used to assess the effects of contaminants occurring deeper in the soil. Also, as the bait delivery section of the strip must be buried, bait lamina tests can not be used to assess the effects of toxicants lying on the surface of the soil.

4.5 Nitrogen mineralisation

The nitrogen mineralisation test follows a specific laboratory procedure (refer to ERA 3, SOPs for bioassays). The test is performed in laboratory conditions and chemical parameters of the samples (such as pH and moisture content) are controlled by the operator. Therefore the test has no restrictive operating window.

4.6 Earthworm tests

All of the tests that use earthworms (including the lysosomal stability assay) follow the same protocol of exposing the earthworms. The operating windows for these assays are therefore the same.

Soil matrix

The physical structure of the soil matrix must not be so course as to damage the soft body of the earthworms. Soils composed of a matrix containing sharp fragments (shales, flints, broken glass, etc.) will cause damage to the earthworms. Physical damage to the earthworms will adversely affect the test result.

Soil pH

The soil being tested must not be highly acidic. Earthworms are sensitive to low pH and soils with a pH <3 will cause mortality in the test population. Earthworm tests should therefore only be conducted where soil pH is >3.

Contaminant type

Earthworm tests are conducted over a relatively long period and the earthworms continually perturb the test soils. Therefore volatile compounds will evaporate over time and the earthworms will be exposed to decreasing concentrations of the contaminants. This factor should be a consideration when proposing or interpreting tests where the contaminants may be volatile.

4.7 Collembolan tests

Previous reports show that the springtail species used in these tests (*F. candida*) is comparatively robust and is therefore a suitable test organism for most soils with no restrictive operating windows. However, the soil moisture content is a particular consideration because collembolans are very sensitive to humidity and soils that are too dry will cause mortality of the test population. Therefore soil moisture levels must be carefully controlled by the operator (refer to ERA 3, SOPs for bioassays). Where soils are too moist, fungal growth can impede air moving through the soil matrix, which will also cause mortality of the test population. The SOP describes sterilization techniques to reduce fungal growth.

4.8 Plant seedling emergence and plant growth

Plant tests are generally straight-forward to perform and the species selected as test organisms are comparatively robust. However, the soil moisture content is a particular consideration because plants are dependant on soil moisture for evapo-transpiration during the test. Different soil types will absorb and hold different amounts of water and therefore soil moisture levels must be carefully controlled by the operator (refer to ERA 3, SOPs for bioassays).

Contaminant type

Plant tests are conducted over a relatively long period and root growth continually perturbs the test soils. Therefore volatile compounds will evaporate and the earthworms will be exposed to decreasing concentrations of the contaminants. This factor should be a consideration when proposing or interpreting tests where the contaminants may be volatile.

4.9 Concluding remarks

This chapter describes the operational restrictions that apply to some of the biological tests recommended for use at Tier 2 of the ERA framework. The information is also summarised in Table 4.1. When deciding which, if any, biological test to use, practitioners should ensure that tests are not used outside of their operating window as this could invalidate the test results.

Table 4.1 Operating windows for bioassays recommended for use at Tier 2 of the ERA

Test	Measure	Temperature	pH	Soil matrix	Soil moisture	Site considerations	Contaminant type	Possible alternative test
Microtox® solid phase	Cellular activity of a test bacterium	–	–	–	n/a, but should be measured if volatiles suspected	–	–	None
Bait lamina	Soil biological activity	5–15°C inclusive	–	Small particles	Preferably 12–15%	Only measures top 16 cm of soil. Unsuitable for contaminants on soil surface.	–	None
Nitrogen mineralisation	Rate of mineralisation of nitrogen in the test soil	–	–	–	n/a; made up to 75% in laboratory	–	–	None

Notes: '–' indicates that the tests has no operational restriction for the described parameter or that the parameter is controlled by the operator during the test (e.g. pH is adjusted to 6 ±0·5 for Microtox®).

Table 4.1 Operating windows for bioassays recommended for use at Tier 2 of the ERA (cont'd)

Test	Measure	Temperature	pH	Soil matrix	Soil moisture	Site considerations	Contaminant type	Possible alternative test
Earthworm reproduction	Reproductive output of earthworms	–	≥ 3	Particles not sharp	–	–	Careful consideration is required when volatilization of test compounds is likely	Collembolan reproduction
Earthworm NRRT	Integrity of lysosomal cells	–	≥3	Particles not sharp	–	–	Careful consideration is required when volatilization of test compounds is likely	None
Collembolan reproduction	Reproductive output of springtails exposed to test soil	–	–	–	Must be carefully controlled by operator	–	–	Earthworm reproduction
Seedling emergence and growth	Successful germination and growth of a range of mono- and dicotyledonous plants	–	–	–	Must be carefully controlled by operator	–	Careful consideration is required when volatilization of test compounds is likely	None

Notes: '–' indicates that the test has no operational restriction for the described parameter or that the parameter is controlled by the operator during the test (e.g. pH is adjusted to 6±0·5 for Microtox®).

5 Criteria for acceptance of new bioassays into the ERA framework

5.1 Introduction

At present the ERA framework recommends a range of bioassays for use at Tier 2 (Environment Agency 2002, 2004a, 2004b). These tests have undergone considerable evaluation using contaminated soils and the results have been compared against desirable criteria to assess their suitability and applicability.

However, the ERA Framework should to be flexible and allow new techniques to be added to improve it. A large range of bioassays are currently described in the literature covering all media and many species, but comparatively few are suitable for use in an ERA (see Environment Agency 2002, 2004b and Environment Agency and SNIFFER 2002).

This guidance sets out the attributes of bioassays that make them suitable for use in the ERA Framework and defines criteria that should be met by any new bioassay being proposed for use at Tier 2.

5.2 Criteria

In the initial report reviewing bioassay techniques for their potential inclusion in the ERA framework (Environment Agency 2002), the '5R' criteria described by Hopkin (1993) were adopted (Environment Agency 2002). The '5Rs' state that the bioassay should be reproducible, representative, responsive, robust and relevant. A sixth criterion, 'practicality', was proposed to include a measure of the ease of use of a bioassay in routine assessments. Full definitions of these six criteria, as reported by Environment Agency (2002), are presented in Table 5.1.

Table 5.1 Criteria and definitions used to judge initial bioassays included in the ERA framework (after Environment Agency 2002)

Criterion	Definition
Reproducible	The assay should produce similar responses to the same level of pollution after repetition of the assay.
Representative	It should be possible to use the assay at a range of potentially contaminated sites to facilitate comparisons between separate locations. In this respect the ecological community or species used for the test should be present at each site.
Responsive	The biological response should be measurable after exposure to pollutants, when compared to the results of assays conducted in uncontaminated soils.
Robust	The assay should be suitable for use with naturally contaminated field soils and should not respond to environmental factors unrelated to pollution or environmental degradation.
Relevant	The assay should provide data that is ecologically meaningful or can be related directly, preferably in a mechanistic way, to effects at higher levels of organisation (population, community, ecosystem).
Practical	The assay should not be overly technical or require extensive technical expertise. It should be comparatively quick and the results easy to interpret. Costs should not be excessive.[1]

Notes [1] Whereas the 5R definitions are quoted directly from Environment Agency (2002), this definition is derived from the measures of practicality described in Environment Agency (2002).

Because these criteria were used to select the initial list of bioassays, it is appropriate that they are also used to assess any new bioassays proposed for inclusion in the ERA Framework. Table 5.2 presents an example of how these criteria were applied to the earthworm reproduction test (taken from Environment Agency 2002).

A number of bioassays have internationally agreed protocols (e.g. via OECD or ISO). This means that they have been thoroughly evaluated (e.g. ring-testing at different laboratories to compare reproducibility) and should already meet at least four of the 5R criteria. However, this does not necessarily mean that the test has been trialled on contaminated soils and consideration given to its suitability for use within the ERA Framework (i.e. the fifth criterion, relevant). There should therefore be an assessment as to whether a bioassay gives meaningful results for these soil samples even if it has been internationally accepted for use in chemical screening. The assessment should include trials at several laboratories and feedback collated from all operators as to the practicality of the bioassay in routine use.

Table 5.2 Example evaluation of a bioassay (earthworm reproduction) against the criteria used to assess the initial bioassays included in the ERA framework (after Environment Agency 2002)

Criterion	Definition
Reproducible	As an OECD draft guideline, the reproducibility of the test has been satisfactorily demonstrated during ring-testing.
Representative	As well as being used for single substance testing with a wide range of potential contaminants, the test has also been used to assess sites contaminated with a range of pollutants. The results of this work have indicated the suitability of the test for ecological risk assessment.
Responsive	Earthworms, particularly those recommended in the test guidelines, are widely accepted as representative of higher invertebrates. As *Eisenia fetida* is not naturally present within soils of interest, use of a deep-dwelling species such as *Lumbricus rubellus* may be recommended in some cases.
Robust	Though originally developed for the testing of individual substances, the ISO/OECD draft test has been successfully developed for mixtures of contaminants in field soils. Selection of reference soils and species, and pre-treatment of field soils should be carefully controlled as soil conditions such as pH and organic matter content can all influence results.
Relevant	The test has been accepted as relevant for ecological risk assessment. The measurement of demographically important sub-lethal effects is an advantage over the acute test.
Practical	The test is relatively easy to carry out and requires simple equipment. In adapting the test to field soils, a number of practical issues need to be addressed such as the maintenance of soil conditions and choice of reference soils. While limitations of the test are the time needed for completion (eight weeks) and the relatively large amount of effort required, these are comparable to many of the internationally accepted tests. A further advantage of this test is that it can be carried out *in situ* if required. For *in situ* testing, practical considerations include the need to avoid human and animal interference with the test containers and the difficulty of wet sieving for cocoons in 'dirty' soils.

Risk assessors proposing the use of a bioassay not currently recommended for use in the ERA Framework must be able to:

- demonstrate that it fulfils the criteria outlined in Table 5.1;
- demonstrate that it is likely to provide useful data for the site under study.

The case for the inclusion of other tests at Tier 2 should be made in consultation with the regulator and other relevant stakeholders, and the level of effect that is indicative of unacceptable risk agreed.

In general, the performance of bioassays that are accepted by international organisations or government agencies will have been closely evaluated and are likely to be suitable for use in the ERA Framework once the relevance and practicality of the

tests have been established. However, it should be noted that recognised accreditation schemes exist (e.g. United Kingdom Accreditation Service; UKAS) that laboratories can employ to demonstrate the quality of the bioassays they conduct. This does not necessarily indicate that it is suitable for the ERA Framework, because any measurement made can be accredited for quality. In practice, laboratories usually seek to offer tests that meet the 5Rs and accreditation is a desirable, but not always available, additional option.

Commercial bioassay developers are also mindful of the criteria required so commercially developed assays often meet the 5Rs. However, the availability of a test (e.g. whether the protocol is freely available or whether it can only be used under licence) should be considered. Commercially developed tests are intended to make a profit and may require a costly licence or special equipment available only from the developer. This may pose a barrier to the practical use of the test on a routine basis.

5.3 Concluding remarks

Criteria that should be met by a biological test used at Tier 2 of the ERA Framework are described. This improves the reliability and interpretive power of the risk assessment because the bioassays are:

- **relevant** to ecological processes and ERAs for soil;
- **reproducible** with minimal experimental or natural variance;
- **representative** of organisms or processes likely to be found at most sites;
- **responsive** to the types and concentrations of toxicants likely to be encountered;
- **robust** in that its success is well-tested at a number of sites and scenarios.

Another criterion that practitioners should consider is how **practical** it is to perform the test. For example:

- Is any specialist equipment required?
- How long will it take to perform the assessment?
- How easy it is to analyse and interpret the results?

Endorsement by an international organisation for testing or standardisation such as ISO, OECD or ASTM demonstrates that the performance of the bioassay has been properly evaluated. However, it does not necessarily mean the test is **relevant** or **practical** for the assessment of contaminated land.

6 Implementing bioassays in commercial laboratories

A set of biological tests have been selected and trialled for use within the ERA Framework. Risk Assessors procuring biological testing of soil need to ensure that the testing is conducted to appropriate standards. Demanding that laboratories are accredited to conduct biological tests is beyond the scope of the ERA framework. Steps have been taken to constrain variability and potential bias by selecting tests that meet certain criteria (see Chapter 5) and by referring to the detailed SOPs for bioassays (ERA 3).

However, there are a number of considerations to be made when selecting a laboratory to conduct the biological testing:

- The laboratory must be able to demonstrate that it is proficient in carrying out the method(s) and that appropriate internal validation of the method(s) has been undertaken. The laboratory would usually hold (and be able to supply) an internal SOP that complies with the relevant Environment Agency SOP for the bioassay (see ERA 3). If this is not available, details of the methodology should be available in a study plan for review by the risk assessor.
- The laboratory must be able to provide full records for each sample that must be clearly traceable and include (as a minimum):
  - a unique sample code, reference or other identifier;
  - the sample location (site and position);
  - date and time the sample was taken;
  - the duration and condition of sample transport and storage.
- Full details of the sample characteristics (e.g. particle size, geological composition, visual appearance, total organic carbon content, moisture content, etc.) and any sample pre-treatment procedures (e.g. removal of stones, crushing or grinding, sieving or preservation) must be recorded and reported.
- Internal laboratory quality control (QC) must include:
  - **Equipment**. All equipment used within the testing process must be subject to appropriate analytical quality control (AQC) and calibration procedures. Records should be kept and supplied on request.
  - **Test process and test organisms**. The sensitivity of batches of test organisms and potential differences in application of test procedures (e.g. by different operators) should be assessed by using suitable reference testing to support the testing of soil samples. Reference tests should be performed at intervals commensurate with the numbers of samples tested and different batches of test organisms used.
  - **Supplies and services**. Reagents, apparatus and other laboratory supplies (including test organisms where appropriate) must be suitable for the purpose for which they are employed in the testing process. Any other services that are employed (either internally or externally) in the

derivation of the test results (e.g. chemical analyses) must also be subject to the same level of control as the testing process itself.

- **Staff training**. The laboratory must be able to demonstrate that staff involved in the testing process are adequately trained and are proficient in the procedures to be carried out.

- **Internal laboratory quality assurance (QA)**. It is not necessary for laboratories to be formally accredited or to involve third party auditing/ inspection. However, given the regulatory process within which these results may be used, an appropriate level of QA for the testing process, particularly with regard to the recording of data, contract review and dealing with complaints/queries regarding test results is encouraged.

- Full document control and traceability of all analytical records and test reports must be maintained throughout. Primary (raw) data and all subsequent calculations must be available in a project file and retained for a minimum of six years.

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List of abbreviations

ASTM	American Society for Testing and Materials
CCME	Canadian Council of Ministers for the Environment
CSM	conceptual site model
Defra	Department for Environment, Food and Rural Affairs [UK]
ERA	Ecological Risk Assessment
ISO	International Standards Organization
NRRT	neutral red retention time
OECD	Organisation for Economic Co-operation and Development
PAHs	polycyclic aromatic hydrocarbons
QA	quality assurance
RoPC	Receptor of Potential Concern
SE	Scottish Executive
SNIFFER	Scottish and Northern Ireland Forum for Environmental Research
SOP	Standard Operating Procedure
US EPA	United States Environmental Protection Agency
WAG	Welsh Assembly Government

Glossary

Adverse effect	An impairment of biological functions or description of ecological processes that results in unfavourable changes in an ecological system.
Assessment endpoint	An explicit expression of the environmental resource that is to be protected. It is defined operationally in structural terms (e.g. a population of a particular species) or functionally (e.g. supporting processes that are typical of a particular habitat).
Bioavailability	The degree to which a chemical can be taken into the tissues of an exposed organism.
Bioassay	A laboratory test in which the toxicity of a contaminant or environmental sample is measured by exposing a specific organism and measuring a life-cycle parameter (e.g. survival, reproduction, development, growth).
Community	Interacting populations of species (plants or animals) living in the same habitat.
Concentration	The amount of a chemical substance expressed relative to the amount of environmental medium, e.g. µg/g (micrograms of chemical per gram of soil).
Conservation	The preservation, management, and care of natural and cultural resources.
Contaminant	In general terms, a substance that is in, on or under the land and that has the potential to cause harm or to cause pollution of controlled waters. Within ecological risk assessment the specific emphasis will be on contaminants that have the potential to cause harm to ecological receptors.
Contaminant of Potential Concern (CoPC)	A contaminant identified as being present or likely to be present at the study site, included in the CSM and agreed to be of concern by all the stakeholders.
Conceptual Site Model (CSM)	A representation of the characteristics of the site in diagrammatic or written form that shows the possible relationships between contaminants, pathways and receptors.
Dicotyledon	A plant that germinates with two cotyledons (first 'leaves').
Dose	The amount of chemical taken into an organism per unit of time.
Dose-response relationship	The relationship between the dose of a contaminant administered or received and the incidence of adverse effects in the exposed population. From the quantitative dose-response relationship, values are derived that are used to estimate the likelihood of adverse effects occurring at different exposure levels.
Ecological survey (ecosurvey)	Surveys for habitats and species; a method of gathering spatial and/or temporal ecological data on a site.
Ecosystem	An ecological community of plants and animals together with its physical environment or habitat, regarded as a unit.

Effect	A change in the state of an organism or other ecological component resulting from exposure to a chemical or other stressor.
Endpoint	The biological or ecological entity or variable being measured or assessed (see measurement endpoint and assessment endpoint).
Exposure	The amount of a chemical available for intake by a target population at a particular site. Exposure is quantified as the concentration of the chemical in the medium (e.g. air, water, food) integrated over the duration of exposure. It is expressed in terms of mass of substance per kg of soil, unit volume of air or litre of water (e.g. mg/kg, mg/m^{-3} or mg/l).
Foliate plant	A plant bearing many broad leaves.
Food chain	A series of organisms each dependent on the next as a source of food.
Food web	Interconnected food chains that describe the pathways of energy and matter flow in nature.
Function	A variable quantity regarded as depending on another variable; a consequence.
Germination	The process of a plant emerging from a seed.
Habitat	A place in which a particular plant or animal lives. Often used in the wider sense referring to major assemblages of plants and animals found together.
In-situ bioassay	A bioassay that can be performed at the study site without the need to remove samples of soil to a laboratory.
Lethal concentration (LCx)	The concentration of a substance at which a lethal effect of magnitude x occurs. The x is usually 50 per cent of the exposed population, in which case LC50 is known as the median lethal concentration.
LOEC	Lowest Observed Effect Concentration. The lowest concentration of a material used in a bioassay or toxicity test that has a statistically significant adverse effect on the exposed population of test organisms compared with the controls.
Medium (plural; media)	The substance in which a chemical may exist such as air, soil, sediments and water.
Measurement endpoints	Quantifiable indicators that relate directly to assessment endpoints, for example, viable offspring per female bird.
Monocotyledon	A plant that germinates with one cotyledon (first 'leaf').
NOEC	No Observed Effect Concentration. In test organisms, the highest concentration at which no significant adverse effects, such as growth or reproduction, were observed.
Organism	An individual plant or animal.
Pathway	A route or means by which a receptor could be, or is exposed to, or affected by a contaminant.
Pollutant	Any substance that contaminates one other substance, causing harm or not.

Pollutant linkage	The relationship between a contaminant, pathway and receptor.
Population	A group of individuals of the same species interacting within a given habitat.
Receptor of Potential Concern (RoPC)	An ecological receptor identified as present or likely to be present at the study site, included in the CSM and agreed to be of concern by all the stakeholders.
Remediation	Action taken to prevent or minimise, or remedy or mitigate the effects of any identified unacceptable risks.
Sentinel organism	An organism the health of which is used to indicate the overall health of the ecosystem in which it lives.
Species of Special Interest	A species within a protected location that, through discussion with relevant conservation organisations, has been established as being of special interest.
Sub-lethal	Effects at concentrations below those that cause death. A sublethal test focuses on endpoints other than mortality.
Stressor	A physical, chemical or biological agent that can induce an adverse response in organisms or other compartments of ecosystems.
Terrestrial	Living or growing on land.
Trophic level	Broad class of organisms within an ecosystem characterised by mode of food supply

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Published by:

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Rio House
Waterside Drive, Aztec West
Almondsbury, Bristol BS32 4UD
Tel: 0870 8506506
Email: enquiries@environment-agency.gov.uk
www.environment-agency.gov.uk