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Review and implementation study of biomonitoring for assessment of air quality outcomes

Science Report – SC030175/SR2

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Steve Killeen

Head of Science

Executive summary

This review of biomonitoring techniques should be read in conjunction with its companion report, Air Quality Outcomes in pollution regulation: strengths, limitations and potential (Bealey *et al.,* 2007, hereafter referred to as Part A), which outlines the advantages and disadvantages of an outcomes-based approach to regulating emissions to air from industrial sources, and identifies the need for biomonitoring tools to make this approach viable.

Chapter 1 of this report provides the background and context of the review of biomonitoring techniques, based on the key conclusions of its sister report.

Chapter 2 describes biomonitoring techniques currently used to measure responses of biota to air pollutants. The methods are divided into those applicable to flora, fauna and soils. The advantages and disadvantages, including guideline costs and specificity to particular pollutants, are summarised for each method along with references to their development and practice in the UK.

Chapter 3 describes methods which are currently not in use in the field, but which show promise as potential biomonitoring techniques, based on their current use under laboratory conditions. Significant development would be required before most of these could be used in the field.

Chapter 4 highlights the requirements for a sampling strategy and statistical design of any field-based sampling or biomonitoring programme. This is the most important aspect of the use of biomonitoring in support of an outcomes-based approach to regulation, yet it is often not sufficiently resourced. The requirement to provide both statistically and biologically significant data must be satisfied.

Chapter 4 provides example uses for many of the techniques described in Chapter 2, based on first-hand experience. Detailed protocols (some in the Appendix) are given, with guidance on where methods may be adapted to particular situations.

Archived samples can offer evidence of conditions prior to the start of industrial activity, or in geographical areas remote from pollution, and therefore act as a baseline against which to measure current activities. Chapter 5 provides a list of organisations that may have archived material which could be used for a retrospective analysis.

The application of a subset of biomonitoring techniques to a field study around a power station, to investigate the feasibility of an outcomes-based approach to emissions regulation, is the subject of a complementary report (Long *et al.,* 2007).

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1 Background and initial scoping

1.1 Background

The Environment Agency has a duty to protect the environment using regulatory measures. Up until now, regulation has been based on chemical and physical measurements indicating the spatial and temporal distribution of emissions from sources, for example, ambient air concentrations. Measurements of this type can also be used to model dose-response relationships, where an airborne pollutant has a known level of toxicity or critical load that can be compared with the measured concentration. However, measurements of this kind can only determine an exposure; they cannot describe the actual manifestation of an effect or impact on the biota or ecosystem. In contrast, biomonitoring can demonstrate an actual response in biota to an industrial emission, providing a first-level assessment of an ecosystem's health. Biomonitoring techniques are diverse and based on the responses of individual organisms, parts of organisms (for example, at the cellular level) or communities of organisms (ecosystem level).

The companion work to this study, Air Quality Outcomes in pollution regulation: strengths, limitations and potential (Bealey *et al.,* 2007, referred to below as 'Part A') explains the concepts underlying an outcomes-based approach to air quality regulation. It identifies the advantages and disadvantages of current (air quality) methods, and the potential for outcomes-based approaches. Part A should be read in conjunction with this review of potential biomonitoring methods, because it sets out a framework within which such techniques might be employed.

Issues associated with biomonitoring are explored further in this report, which reviews a wide range of biomonitoring techniques and their applicability to an outcomes-based approach. By their very nature, and the limited extent of our knowledge of the effects of air pollutants on biota, some methods may be more applicable for use with standardised materials imported to a site – a concept that is midway between an exposure-based and outcomes-based approach (see Part A).

The concepts of *effect*, *impact* and *outcome* are defined in Part A (see Table 1.1); the response of a biomonitoring technique can be expressed as an *effect* (such as the accumulation of a pollutant in tissue), an *impact* (an effect having a measurable consequence for the long-term behaviour, vitality or survival of an organism, community or ecosystem) or an *outcome* (defined as a significant, measurable change that leads to the loss or (re)appearance of an organism, community or ecosystem).

Table 1.1: Summary of definitions

Effect	Measurable change in properties or state, causally linked to pollutant exposure
Impact	Measurable change in function or populations, causally linked to effects
Outcome	Impact where the cost/benefit of reversing the change exceeds the value of the resource affected

This review focuses on methods that reveal *outcomes* and those that demonstrate a measurable response in the *effects*/*impacts*/*outcome* chain, which potentially leads to an *outcome*. For example, biomonitoring methods may detect *outcomes* directly, or may indicate the likelihood of future *outcomes*, such as an accumulated biological uptake or effect on some other component of the ecosystem.

The accumulation of pollutants in organisms provides an account of exposure over time, with or without visible injury symptoms. In cases with no visible symptoms, accumulation effects can be seen as an early warning system for potential *impacts* and show the transfer of pollutants within the biological chain. In other words, bioaccumulation is an *effect* which may have no immediate *impact* or *outcome* for the individual organism. However, responses at the community level, which often occur over a time span of years, can be described as constituting an *outcome*, in that the *effect* and *impacts* have already occurred and are shown by changes in species abundance. Both organism- and community-based types of biomonitoring offer a clear advantage over physical and chemical measurements in that they indicate an actual response of a community or organism, rather than predicting one based on the measured concentrations of pollutant emissions and generic expectations of response.

While physical monitoring has to consider regular temporal differences over daily, monthly and seasonal scales, biomonitoring can capture the integrated response to all these changes. Physical and chemical measurements generally require repeated monitoring over several months or years, while bioindicators and biomonitors represent the accumulated response over time. Therefore, useful information about the effects of a pollutant can be captured by measurements taken in the field on a single occasion. This makes biomonitoring an attractive technique compared to instrumental ones, provided that the response can be detected from the 'normal' (unperturbed) response of the environment at some predefined baseline of air pollution (see Part A).

1.2 Definitions

The terms *bioindicator*, *biomonitor*, *bioaccumulator*, and *biomarker* have all been used in varying ways to describe different approaches and techniques for studying biological responses to air pollution. In general, however, the field of biomonitoring can be seen as both a qualitative (bioindicator) and quantitative (biomonitor) approach to pollution control.

Biomonitors hold quantitative information on the health of an ecosystem. A biomonitor is also a bioindicator, except that it quantifies the impact or eventual outcome on an organism or ecosystem.

Bioindicators provide a pollutant measurement which can be compared with an instrument measurement. Bioindicators provide information on the quality of the environment and the actual condition of an organism or ecosystem, effectively a 'snap shot' of an ecosystem's health. However, decisions on ecosystem health are not always easy to translate from the effects on a few bioindicator species. The choice of bioindicator should be based on the pollutant, and must allow for prediction of how a species or ecosystem will respond to the pollutant stress.

Bioindication can be *active,* for example in the use of deliberately-introduced plants, or *passive,* where organisms already present in the ecosystem are examined for their reactions.

Bioaccumulators are organisms that accumulate pollutants within their tissues. They may be less sensitive to, or indeed unaffected by, air pollution but are nevertheless good indicators of the exposure of the ecosystem to a pollutant. Bioaccumulators can give invaluable information about the status of a pollutant within the environment and whether future effects or impacts are likely to occur. Bioaccumulators, as integrators of exposure over time, can be used to validate physical or chemical measurements, and can demonstrate the pathways by which pollutants move through an ecosystem.

Biomarkers are biochemical, cellular, physiological or behavioural variations in the tissue, body fluids or whole body of an organism that provide evidence of exposure to chemical pollutants, and may (or may not) also indicate a toxic effect.

1.3 Temporal and spatial scales

Any metric that assesses the effectiveness of control measures can be applied at a range of spatial and temporal scales. It is essential for biomonitoring techniques to be able to distinguish between temporal and spatial variabilities. Spatial changes in soil type, topography, aspect or climate must be accounted for in any biomonitoring design. For example, comparing exposure near to and far away from a source would have to demonstrate that any differences were not the result of some other factor unrelated to the source emissions. The temporal scale of a method will be determined by both the pollutant regime and chosen indicator. For example, community changes will require long-term studies (months to years), while standard transplant methods may involve timescales of weeks.

1.4 Advantages and disadvantages of biomonitoring

Biomonitoring is central to realising the benefits of outcomes-based monitoring. The requirements and benefits of outcomes-based monitoring identified in the Part A report are as follows:

- The outcomes-based approach includes an implicit assessment of the value of the resource affected in relation to the cost of any proposed change in emission control.
- Case studies from published literature illustrate the need for:
    - knowledge of the context of measurements used to assess an outcome;
    - explicit inclusion of any confounding factors in the sampling design;
    - biomonitoring before and after the introduction of control measures, as a means of assessing outcomes.
- Current exposure-based approaches rely on generalised agreed links between exposure to pollutants (air concentrations or deposition) and consequent effects. Compliance is assumed if exposure thresholds are not exceeded, but possible outcomes are not necessarily checked. There is little flexibility for adapting emission controls for site-specific factors.
- Outcomes-based approaches require assessment of the risk of impacts resulting in losses of a value that is at least comparable to the cost of emission controls. Criteria for demonstrating compliance have to be agreed in advance, in terms of qualitative or quantitative changes to the surrounding biota. These may need to be expressed relative to changes at unaffected sites, so that non-pollution environmental effects can be allowed for.
- An outcomes-based approach provides tangible evidence of the results of emission control, which can be given a value in terms of losses prevented.

Although biomonitoring techniques are usually simple, non-continuous and relatively inexpensive on an individual basis, the natural variability of the environment may require large numbers of samples to be taken to meet the required statistical precision. Consequently, biomonitoring should not necessarily be seen as a low-cost approach to compliance assessment. The requirement for a statistically sound method places stringent constraints on the sampling design. Knowledge of the potential confounding factors that modify direct and indirect responses of biota to air pollutants is crucial.

1.5 Biomonitoring review

The practical use of biomonitoring to estimate temporal and spatial scales of the impact of industrial emissions will be considered in this review. The review is wide-ranging, give that biological responses to pollution are manifested through a variety of traits. Among these are population characteristics, including species presence or absence, biodiversity and species abundance; morphological characteristics including necrosis, malformations and cell plasmolysis; and physiological and biochemical characteristics including enzyme activation and photosynthetic function.

Classes of response can be defined in bioindication and biomonitoring terms:

- pollutant material may accumulate in biota to produce a (semi-) permanent residue of historical exposure over a set period, with or without injury to the organism;

- exposure to a pollutant or its breakdown residue may disrupt the normal function of an organism or community, with or without a long-term detrimental effect;
- a property of the organism may respond temporarily to exposure, without necessarily leaving a long-term trace of such exposure.

Each of these responses may help to determine environmental outcomes, depending on the context in which they are employed. However, common to them all is the need to distinguish the response to the pollutant from responses to other environmental conditions, or to genetic variation within the organisms or communities being studied. Consequently, there needs to be a clear prior understanding of what is normal or unperturbed behaviour in the organism or community. This must be determined not only in terms of measurable quantities, but also in terms of the likely *variability* of the measured quantities in time and space, and their dependence on growing conditions (such as climate, soil), competition, land use and management (such as grazing). These concepts will be developed further in Chapter 4.

1.6 Structure of the review

A wide range of biomonitoring techniques and methods is described in Chapter 2. The development of each method is outlined along with examples of its use, the conditions under which it can be applied and any factors that need to be taken into account. An accompanying table summarises the resource requirements, constraints and examples of uses of these methods in the UK. The methods are broadly separated into those that can be used with plants (both higher and lower) and those that can be used for animals (vertebrates and invertebrates) and microbial populations.

Chapter 3 describes potential biomonitoring methods that are still under development. Some may prove useful for an outcomes-based approach, but may require significant input of time, effort and resources if they are to be recognised and accepted indicators of ecosystem function. Examples are provided of how such approaches might be used.

Chapter 4 explores a selection of methods from Chapter 2 in sufficient detail to allow them to be used or developed by competent laboratories. One example of a method is worked through in detail, to illustrate the problems that may arise and the method's strengths and weaknesses. These examples are drawn from the first-hand knowledge of Centre for Ecology and Hydrology (CEH) staff. The methods and protocols are preceded by a discussion on sampling strategy and design, expanding on the general points made in Part A, with examples of the type of problems and confounding factors that can arise, again based on first-hand experience of CEH staff.

The need to establish 'background' biological status (in the absence of or at baseline or background levels of air pollution) may require access to geographically dispersed or historical samples for analysis. Chapter 5 lists the potential sources of such material in archives and collections. The assembly of a meta-database with details of available material is beyond the scope of this

review, but contact details are provided for initial investigation of possible material. The types of organisation that may hold reference collections, particularly for historical analysis, are also given.

Finally, the appendix gives examples of detailed protocols for laboratory analysis, to supplement the methods described in Chapter 4.

A third report in this series (referred to hereafter as Part C) will describe a pilot study in which a subset of the techniques described here are field-tested.

2 Biomonitoring - review of techniques and methods

2.1 Methodology Introduction

2.1.1

This section focuses on the different biomonitoring methods available, based on the main groups of techniques and their receptors, with information on the suitability of each method including confounding factors and an estimated cost. Each method or category is written up as a general description, with an indication of the extent to which the method is accepted for investigation of air pollution, and examples of its recent use, especially in the UK. The characteristics of each method are summarised in tables, whose various components are described briefly below:

- Target organism or community: What is the target organism of the biomonitoring method? There can be more than one.
- Measurement: A brief description of the type of measurement, how it is made, and what information it provides about the organism/community response.
- Field-based (in situ) or lab-based (sampling required): Tick-boxes indicate whether it is field-based, lab-based or both.
- Background knowledge: What is the method's pedigree/history?
- Level of response: At what level is the response, for example, sub-cellular, part of organism, whole organism, community?
- Mechanism of action: A brief description of what is behind the measured effect/outcome. If known, what is the evidence for a direct cause-effect relationship with the pollutant?
- Pollutant specificity: What pollutants are known to cause the measured response?
- Known interferences or uncertainties: Interactions with other pollutants, soil conditions, time of year, climatic stresses and so on.
- Timescale of response: The time period over which an effect can be measured.
- Variability in time: Seasonal or other factors to be considered.
- Variability in space: Any information on factors affecting variability at spatial scales up to 10 km, that is, factors that would affect the sampling strategy and design, for example the influence of soil type, south/north-facing slope, shading, water availability and so on.

- Sampling requirements: What are the requirements for sampling in the field, such as the level of replication and ease of sampling? Are there any temporal or spatial constraints or requirements?
- Skill requirements in the field: Is there a need for specialist skills in sampling, identification and so on?
- Skill requirements in the lab: Is there a need for specialist equipment and technicians?
- Calibration information: Are there any published data on absolute values of response to a particular combination of organism and pollutant stress?
- Indicative costs: Indicative costs are provided for each method including individual costings for fieldwork sampling, lab analysis and data interpretation.
- Existing use for monitoring: Reference to reports and papers of existing use and history, with particular emphasis on UK studies.

2.1.2 Receptor criteria

When considering biomonitoring techniques and their application to local source monitoring, the receptor organisms or communities present can often determine which methods will be most suitable. Osborn *et al.* (2000) have identified a number of receptor criteria to take into account before carrying out biomonitoring.

Receptors must be:

- readily identifiable in the field;
- widely distributed within the geographical range of predicted emissions;
- easy to sample repeatedly throughout the year, to capture temporal variability;
- affordable, where the cost of sampling, including the cost of any laboratory analyses that may follow, should be such that sufficient replicates and statisical rigour can be obtained.

In this report, methods have been classified into three receptor categories:

- higher plants, bryophytes and lichens;
- vertebrates and (above-ground) invertebrates;
- soil microbes and invertebrates.

For each category, examples of biomonitoring techniques are examined and their merits discussed. The practical aspects of each method (including an indication of cost), status of development and advantages over non-biological methods are also given, along with the method's repeatability, calibration, and the potential for such an approach to determine outcomes, impacts or effects.

2.1.3 Explanation of costs

It is difficult to give a precise cost for each method because of site-specific circumstances such as sampling area or pollutant characteristics. However, as a guide, we have separated likely costs into three broad categories of low, medium and high.

Methods with low costs generally have a high sample throughput in the field resulting in low staff costs. Analysis and data interpretation is straightforward, and a competent trained person can carry out the fieldwork. Laboratory work can be conducted using equipment normally available in a standard research laboratory such as microscopes, spectrophotometer, fluorimeter and basic electrophoresis equipment. Interpretation of the data is straightforward, and does not require specialist skills.

Medium-cost methods have a low sample throughput in the field and lab, resulting in high staff costs per sample. A competent trained person can carry out fieldwork and a specialist is not required. More complex data analysis and interpretation is required.

High-cost methods require specialist field biologists to identify relevant receptors and carry out sampling procedures. Laboratory methods require the purchase of special equipment, and consumables are expensive. There is low sample throughput, and analysis and data interpretation requires specialist skills and expertise.

These cost categories have been applied to the three aspects of biomonitoring methods – fieldwork, laboratory analyses and interpretation of results – in the following tables.

2.2 Higher plants, bryophytes and lichens

2.2.1 Measuring the accumulation of chemical elements in plant tissue

A vast number of biomonitoring methods use the whole or part of an organism to measure exposure to, and accumulation of, a pollutant. Specific responses by plants can make them good bioindicators of pollutant impacts, while tolerant species are ideally used as bioaccumulators. By analysing plant tissue, and provided that rates of clearance from the organism are known, a direct quantitative assessment of pollutant exposure can be made.

Measuring the accumulation of pollutants in plant tissue is a common method that has been used to assess the effects of many pollutants. Total tissue nitrogen (N) has been measured for many years in all types of plant tissue to assess atmospheric nitrogen deposition (Bobbink *et al.,* 1993; Pitcairn *et al.,* 1995, 1998). For example, this method has been used to assess the tissue nitrogen content of a range of plant species along a pollution gradient near four intensive livestock units (Pitcairn *et al.,* 1998). Foliar nitrogen concentrations decreased with distance from the livestock buildings and there was a close relationship between foliar N, atmospheric ammonia (NH_3) concentrations and total N deposition at every distance for each selected tree, herb and moss species.

Other studies have obtained less significant correlations (such as Kirkham, 2001).

Inconsistencies may arise from poor estimates of nitrogen deposition using low resolution data (10-20 km); 'saturation', where a maximum tissue N may be reached for certain species under certain climatic conditions; and unsuitable site selection, where disturbance factors or poor sampling protocols become important confounders. Furthermore, there can be large differences between tissue N concentrations across species groups and within species groups. Pitcairn *et al.* (1998) found the best correlation between atmospheric N and foliar tissue N in ectohydric mosses, most likely because nitrogen is accumulated by foliar uptake alone without additional root uptake, supporting the use of mosses as bioindicators of NH_3 concentrations and N deposition.

Sulphur is an essential element that plants require for growth. Sulphur is absorbed by plants roots as sulphate (SO_4^{2-}) from the soil. Sulphur dioxide (SO_2) can also be absorbed from the atmosphere through the leaves of the plant. In situations where soil S is low, atmospheric sulphur can be used as an alternative source for normal growth. However, when soil S is at an adequate level for plant growth, excess sulphur from atmospheric sources can have negative impacts on the plant. Manninen and Huttunen (1995) found very good correlations between S content in young Scots pine needles and SO_2 load. The authors concluded that needles under pollutant stress are extremely influenced by high short-term SO_2 doses, which has implications for the setting of critical levels for forest ecosystems.

Sulphur concentrations in Scots pine needles from forests of the Gulf of Finland were correlated with sulphur emissions in the region (Haapala *et al.,* 1996). Regression analysis demonstrated gradients in pine needle content with increasing distance from known pollutant sources. However, Cape *et al,* (1990) suggested that the total S content may be less important as an indicator of exposure than the ratio of S:N or of S:Mg. Separate estimation of inorganic and organic S in leaf tissue may provide additional information on the source of the S uptake. Innes (1995) found good correlation between the sulphur content of needle tissues of Sitka and Norway spruce and Scots pine, and estimated dry deposition of sulphur. Correlations were reported to be better for second-year needles than for first-year needles, particularly for the two spruce species investigated. While this method can be useful for comparing sulphur content in needles with differing exposure to atmospheric pollution, sulphur uptake could also be related to nutritional status, with good nutrition enhancing rates of uptake. Threshold sulphur contents, characteristic of crown injury to trees, do not exist. Biomonitoring of this would be difficult to translate into an outcome.

Bryophytes and lichens are both good biomonitors of metal deposition. Devoid of well-developed leaf cuticles or roots, they rely on atmospheric deposition for nutrients and growth. Because of this, they accumulate persistent atmospheric pollutants to concentrations higher than in the air. A literature review carried out by Burton (1986) reported that many surveys involving metal analysis of lichen thalli have reflected the level of metal deposition, and that lichen thalli can be

used to monitor spatial variabilities in metal deposition from localised point sources.

Accumulation in lichens is lower than in mosses, although there are many examples of the combined use of lichens and bryophytes as bioindicators of metal emissions. For example, Bargagli *et al.* (2002) compared metal concentrations in two common biomonitor species *Hypnum cupressiforme* (a moss) and *Parmelia caperata* (a lichen) around an intensive mining area in Italy. Both moss and lichen were able to indicate emissions, but the species could not be used interchangeably because of broad variation in both geochemical (natural) and anthropogenic sources of metals. *H. cupressiforme* gave higher concentrations of Al, As, B, Ba, Cr, Fe, Mn, Ni, S and Ti, while *P. caperata* accumulated elements such as Cd, Cu, Hg, Pb, V and Zn. *H. cupressiforme* was more affected by geochemical features than the lichen species, implying that in these environments epiphytic lichens may be more reliable as biomonitors of trace elements than mosses.

Differences in the uptake of different metals are commonplace. For example, Genoni *et al.* (2000) looked at metal accumulation in moss and soil near an oil-fired power plant in Northern Italy. Samples of soil and the moss, *Hypnum cupressiforme,* were taken from 12 sites in an area of 50 km^2 around the plant. Another four background sites were positioned 30 km south of the plant. Results showed that vanadium and nickel were strongly correlated in the mosses, with the highest accumulation near the stack. Levels decreased rapidly to background levels at around 4-5 km from the stack. Other metals such as arsenic and chromium showed limited changes in bioaccumulation, due to the presence of other industrial and agricultural sources in the area.

Measurements of the accumulation of trace elements using bryophytes are open to many confounding factors, including the effects of rainfall and desiccation of thalli on soluble element content, relationships between altitude and uptake rates, and the zoning of pollutants within tissue.

Comparing results within and between species is problematic, due to differences in ecophysiological properties among species. For example, Reimann *et al.* (2001) compared two moss species and found that although interspecies variability increased for a number of elements, it was not possible to use results from co-located pairs of moss species to calibrate responses across species; single species biomonitoring is more advisable. The majority of elements analysed showed big variations within catchments, and hence composite sampling over a large area would be necessary.

2.2.1.1 Summary

Measuring pollutants in tissue requires both field sampling and laboratory analyses, but is relatively inexpensive compared to other types of physical monitoring. Bioindicators and bioaccumulators do not necessarily indicate the outcome for the ecosystem, but the signals they give can provide an early warning system for pollutant responses. Any pollutant gradient detected may serve as a useful pointer to the source, and will thus be of value for pollutant mapping and source apportionment.

While bioindicators can show a biochemical effect within the plant tissue that may eventually be expressed visibly (active biomonitors), bioaccumulators only indicate the level of pollutant in tissue (passive biomonitors). It is important to determine how measured tissue content relates to atmospheric gaseous concentrations or deposition of pollutants. Separating the signals from various sources can be tricky, as seen in the work carried out by Genoni *et al.* (2000) cited above. However, knowledge of the form and nature of likely emitted pollutants can help to interpret the bioaccumulants present in plant tissue, and unravel the pollutant 'noise' from multiple sources.

Table 2.1: Characteristics of methods measuring pollutant levels in tissue

Target organism or community	Higher and lower plants including trees, shrubs, herbs, mosses and lichens.
Measurement	Concentration of pollutant in tissue.
Field-based (*in situ)* or lab-based (sampling required)	Field: ☒ Lab: ☒
Background knowledge	Measurement of the foliar content is routinely undertaken to investigate accumulations of pollutants. Evidence has come from transplant experiments and field studies. Historically, measurement of pollutant in tissue to study atmospheric pollutants is well-established and examples of this method go back to the 1960s/70s. Hill (1971) studied the effect of sulphite on lichens with reference to atmospheric pollution. McCune and Hitchcock (1970) looked at fluoride accumulation in forage. Goodman and Roberts (1971) researched plants and soils as indicators of metals in air.
Level of response	Cellular and sub-cellular responses.
Mechanism of action	Uptake of pollutant is through the roots or foliage cuticle and stomata.
Pollutant specificity	Nitrogen (NH_x and NO_x), SO_2, heavy metals, hydrogen fluoride.
Known interferences or uncertainties	Interactions with other pollutants, soil conditions, seasonal changes, climatic stresses, grazing management (land management).
Timescale of response	This method can be used to assess long-term or short-term changes in concentrations or deposition. However, care must be taken in selecting species suitable for the type of change. Tree species can be used for long-term change, especially conifers which have potentially up to five years of needle age class. Mosses may be more suitable for response to short-term changes. For fluorides, grass species give integration over days to one week. Tree foliage accumulation is over growing season.
Variability in time	Seasonal factors need to be considered. Variation in emissions and subsequent concentration levels.
Variability in space	Factors such as wind direction and speed can affect the episodicity of pollutant effects. Habitat structure/composition can influence the rates of pollutant deposition.
Sampling requirements	Sampling constraints include not sampling where human or livestock interference is evident (disturbances or near roads), and avoiding sampling under canopy where throughfall can influence tissue concentrations. Sampling during meteorological extremes (very dry or wet periods) should be avoided.
Skill requirements - field	Sampling requires training in species identification, particularly of bryophytes or lichens. When age or growth phase of the plant or

	foliage is important, these must be identifiable by staff. Ideally, samples should be collected, stored in cold conditions, cleaned (removal of litter, other species and so on) and oven-dried before being transported to an analytical laboratory by post. When suitable ovens are not available, samples can be air-dried before being transported to the laboratory.
Skill requirements - lab	Analysis using specialist, but well-proven laboratory techniques.
Calibration information	None.
Indicative costs	Fieldwork – medium, although some expertise required to sample bryophytes and lichens. Laboratory costs – medium.. Interpretation – low.
Existing use for monitoring	EuroBionet (European network for the assessment of air quality by the use of bioindicator plants) has used many types of plant to monitor pollutants in tissue, including Italian rye grass (*Lolium multiflorum*) to measure the accumulation of sulphur and heavy metals, and curly kale (*Brassica oleraceato*) to measure the accumulation of PAHs. Cape *et al*. (1990) looked at the nutritional status of *Picea abies* (L) Karst across Europe, and the implications for forest decline. Nitrogen has become of interest as an important air pollutant with studies by Pitcairn *et al.* (1998) on nitrogen deposition in the vicinity of a livestock farm, and Hicks *et al.* (2000), who looked at the foliar concentration of upland vegetation to predict atmospheric nitrogen deposition.

2.2.2 Community-level changes

Changes in species composition or diversity provide a useful method for monitoring the status of an ecosystem and the impacts of emissions, where changes in community structure are a clear 'outcome' of pollutant impacts. By comparing species lists at the polluted site with a 'clean' reference site having the same physical characteristics, the status of a community can be assessed. Examples of community structure change methods are well-established in the aquatic environment, for example RIVPACS (Wright *et al.,* 1993), while the impacts of air pollution on lichen communities have been studied for many years (see review by Wolterbeek *et al.,* 2003). Changes in species composition are an actual measure of the biological response within the impacted community.

This type of biomonitoring is relatively cheap compared to chemical analyses of plant material. Furthermore, community changes offer a more quantitative approach to biomonitoring than single species bioindicators, by showing the full integration of biotic and abiotic factors. However, this approach leads to more uncertainties and problems of identifying confounding factors.

Methods using the presence or absence of species as a biomonitor for air pollution have to date been used mainly to determine the impacts of atmospheric nitrogen deposition. Of these methods, Ellenberg indicator values are the best known. Ellenberg (1979) devised a comprehensive indicator system for vascular plants in central Europe and described the response of individual species to a range of ecological conditions including light, temperature, 'continentality', moisture, pH and nitrogen. The Ellenberg N index consists of allocating a N-score to each plant species, so that the overall community has a total score based on a scale ranging from nutrient poor (1) to nutrient rich (10).

Ellenberg values by themselves have no capacity for detecting the unique effects of nitrogen deposition; the approach needs to be applied jointly with local estimates of atmospheric N deposition from physical or other biomonitoring methods (Sutton *et al.,* 2004). A number of confounding factors can influence the spatial and temporal changes in Ellenberg indices, including succession, disturbance, weather and light. Despite this, their use has been demonstrated recently in the UK with data from the UK-wide countryside surveys between 1978 and 1998. Results suggest that N deposition across the UK has led to widespread changes in plant communities (Smart *et al.,* 2005). Such index methods may also be used more widely to study the effects of sulphur dioxide (as an acidifying pollutant) and N (for example, see Brakenhielm and Liu, 1995).

Many lichen species are sensitive to sulphur dioxide and other air pollutants. The presence or absence of different species growing on similar substrates (for example, the bark of a given tree species) can be used to infer the spatial limits of air pollution. During the 1970s, it was assumed that sulphur dioxide was the most important toxic pollutant for lichens. Classic work on this subject was published by Hawksworth and Rose (1976). The method may also be used to show temporal changes in air pollution, such as recovery following reductions in SO_2 emissions (van Dobben and de Bakker, 1996; Poikolainen *et al.,* 1998), or to show whether a potential pollution source has changed air quality (Bartholmess and Wuerzner, 1993).

Nowadays, nitrogen is far more dominant than sulphur as a major pollutant in the UK, with potential effects of NO_x from urban sources and NH_x from intensive farming. In the vicinity of point sources, lichen bioindicators can provide a valuable context for impacts of N deposition. The most useful results are obtained where complementary methods are used, including estimated N deposition, lichen species occurrence, lichen N chemistry and pH of substrate (Sutton *et al.,* 2004). Results from a lichen survey around a poultry farm in southern Scotland (Pitcairn *et al.,* 2004) showed that twig acidophytes were found to be more sensitive to NH_3 concentrations than trunk acidophytes. Furthermore, nitrophyte species only increased at air concentrations larger than 3-8 µg m^{-3}, but acidophytes decreased at concentrations below 2 µg m^{-3}. Effects on lichens in this study were observed at air concentrations considerably lower than the currently accepted critical levels for ammonia effects on plants.

2.2.2.1 Summary

Community changes represent a definitive outcome within an ecosystem as species are either lost/gained or reduced/increased. Exploiter species can increase because of a lack of competition from eliminated species, or because of reduced competition with greater nutrient availability. Changes in an ecosystem can be limited to only a small number of sensitive species or species on a specific substrate. For example in nitrogen monitoring, twig lichens offer a better early warning system than trunk lichens. Sensitive species may show a measurable environmental change in a number of ways, for example through mortality, age class or community structure. Since changes in community structures can be attributed to a wide range of factors, including atmospheric pollution, it is important to monitor a variety of species within the community to

help discriminate between pollutant effects and other environmental changes. To obtain a representative sampling of all species present, sampling may need to be carried out on a number of occasions throughout the year.

Table 2.2: Characteristics of methods measuring community-level changes

Target organism or community	Higher plants, bryophytes and lichens
Measurement	The presence or absence of different species growing on similar substrates (for example, the bark of a given tree species) can be used to infer the spatial limits of air pollution. Methods rely on simply measuring changes in species composition, diversity or frequency.
Field-based (*in situ)* or lab-based (sampling required)	Field: ☒ Lab: ☐
Background knowledge	Extensive published literature on the application, for example, the Ellenberg indicator values. Hawksworth and Rose (1970) developed a ten zone scheme for epiphytic lichens to study SO_2 gradients across England and Wales, while other scales are being developed for nitrogenous pollutants.
Level of response	Community level.
Mechanism of action	Plants favouring pollutant conditions out-compete sensitive species for nutrients and light. Some species are vulnerable to direct toxic damage. Secondary factors can be influenced by pollutant levels, such as drought, frost, pest and diseases.
Pollutant specificity	Nitrogen, SO_2 (acidification).
Known interferences or uncertainties	A number of other factors can potentially influence the spatial and temporal changes in plant communities including nutrient limitation, pH, disturbance and land management practices, bioclimatic conditions, and light levels.
Timescale of response	The method reflects the time constant of colonisation and species change. While differences as small as three years have been examined, in general the method reflects responses over several decades. Some direct toxic effects can occur over shorter periods (of less than a year) depending on the pollutant source and strength.
Variability in time	Biomonitoring should be carried out when plants are physiologically active (during the growing season).
Variability in space	Community structure is important as well as the length of the sampling transect, in order to capture the full range of pollutant emissions.
Sampling requirements	The simplicity depends on species diversity and/or survey of cover versus presence/absence. There is no requirement for plant sample collection and transport. However, a solid botanical knowledge is required. The method has potential for fine spatial resolution, depending on the averaging scale of the plant community considered. For nitrogen and forest ground flora, some approaches have been applied across transects of smaller than 100 m. The uniformity of habitat structure over the desired transect length is essential. Species sensitivities to the target pollutant need to be known, as some species/communities will be more susceptible than others.
Skill requirements - field	The method can be applied by staff skilled in botanical identification.
Skill requirements - lab	No lab work is required.
Calibration information	Established indices such as Ellenberg's for vascular plants and Van Herk's for lichen species.

Indicative costs	Fieldwork – medium. The method is difficult to cost because it depends on the biodiversity of the site. Species identification and recording can be very time-consuming on species-rich sites such as typical mixed woodland ground flora. Laboratory costs – low. Interpretation – low.
Existing use for monitoring	Türk (1982) monitored air pollutants by lichen mapping, while van Dobben (1996) mapped epiphytic lichen biodiversity in the Netherlands while studying the effects of decreasing SO_2 and increasing NH_3. Pitcairn *et al*. (2002) defined the spatial impacts of poultry farm ammonia emissions on species composition of adjacent woodland ground flora using Ellenberg indicators.

2.2.3 Visible injury impact

Symptoms of visible injury can be seen in plants and animals. Typical plant symptoms include leaf chlorosis (loss of chlorophyll), necrosis (tissue death) or abscission (leaf loss) (Taylor *et al.,* 1986), while in mammals symptoms have included dental lesions in voles (Boulton *et al.,* 1994). In each case, a picture of zones of exposure can be built around emissions from point sources. Visible injuries may be acute, when organisms are exposed to high concentrations of a pollutant over short periods, or symptoms may be chronic where an organism undergoes low but prolonged exposure.

An important issue to consider when using visible injury methods is that effects from pollutants often mimic effects from other stress, such as drought and frost. Symptoms from different pollutants quite often resemble each other, and where more than one source is present it may be difficult to link the source to its corresponding effects. Visible injury alone does not necessarily imply reduced vitality of the affected plant species or community (Mills *et al.,* 2003). Visible injury is widely used in the ICP (International Cooperative Programme) of the UNECE to indicate ozone effects on plants. Ozone, however, is not likely to be part of an assessment of air quality outcomes from controlled emission sources. Ozone is a regional pollutant formed through photochemical reactions in the atmosphere, reaching the highest concentrations hundreds of kilometres downwind of its precursor emission sources.

High concentrations of sulphur dioxide can have harmful effects on vegetation. However, the threshold of damage varies according to the plant species, growing location, altitude, and the combined influences of various environmental factors. There is also evidence to suggest that the presence of other pollutants (such as ozone and NO_2) can, under some circumstances, produce markedly increased adverse effects of SO_2 on vegetation.

For example, Rautio *et al.*(1998) examined the effect on Scots pine needles along the heavily polluted Kola Peninsula. Foliage samples were collected from 12 sampling plots located on a 400-km transect. Of the needle damage variables studied, necrotic needle tips and fewer needle age classes were explained best by high total foliar concentrations of S, Ni and Cu and low total concentrations of Mn and Zn. The best predictors for stomatal chlorosis and other discolorations were increased total foliar concentrations of Ca, Fe, Si and Cl. However, stomatal chlorosis and discoloration also occurred in areas where SO_2 was low, indicating that factors other than SO_2 can cause needle damage. The author suggests that multi-stress symptoms can be caused not only by SO_2 and heavy metals, but also by ozone, acid deposition and frost.

Visible injury using bioindicator plants is the classic method for assessing the spatial impact of fluoride emissions (Posthumus, 1983). Effects on plants are caused by direct uptake of hydrogen fluoride from the air, not by deposition on soil or uptake by roots. Fluoride, unlike sulphur, nitrogen and even chlorine, is not an essential element for plants. Hydrogen fluoride (HF) is the main concern as it is up to 1,000 times more toxic than ozone, SO_2 or NO_2 (Weinstein *et al.*, 1998). Because of the high toxicity of HF, relatively small releases into the atmosphere

can result in damage to vegetation. Visible injury is seen at the tips and margins of leaves, where HF has accumulated over time. Injury to the most sensitive species begins at concentrations below 0.8 µg m^{-3} for a one- to three-day exposure period, with a long-term threshold of 0.25-0.30 µg m^{-3} (Weinstein and Davison, 2003). However, there is a large variation in tolerance to HF between species, and species that are sensitive in one region may not be in another, due to differences in environmental and genetic factors.

2.2.3.1 Summary

Visible impacts indicate the possibility of a potentially negative outcome on the plant species or ecosystem. Symptoms of loss of growth or crown deformation in trees can have implications for timber yield and quality of wood products, and therefore could be seen as an outcome. However, visible symptoms may be only temporary, with the plant recovering at a later stage. Many other biotic and abiotic factors can mimic visible injury symptoms (chlorosis, necrosis or abscission of leaves), including drought, cold, heat, sun scald, nutrient deficiencies or excesses, other toxic chemicals (such as pesticides and herbicides), and pests and pathogens. It is therefore important to have some knowledge of the pollutants being emitted and the impacts expected. Occurance of symptoms will, in general, approximate the dispersal pattern of the pollutant, being greatest downwind from source and decreasing with distance away from the source. What makes any biomonitoring scheme difficult is having enough plant species covering the sampling domain to capture not only the pollutant dispersion, but also the environmental variability arising from factors such as microclimate, slope or shading.

Table 2.3: Characteristics of methods measuring visible injury impact

Target organism or community	Higher plants (including agricultural crops), bryophytes and lichens
Measurement	Visible symptoms of injury including defoliation, crown degeneration, needle and leaf discolouration.
Field-based (*in situ)* or lab-based (sampling required)	Field: ☒ Lab: ☒
Background knowledge	Surveys have been used in most developed countries. Methods for assessing fluoride emissions, particularly by the aluminium industry, have been used for at least 40 years as a convenient way of assessing the impact of these emissions.
Level of response	Acute exposure can lead to chlorosis, necrosis, abscission of leaves, inhibition or promotion of flowering, and growth reduction.
Mechanism of action	Pollutant enters the leaf through the stomata and dissolves in the water permeating the cell walls. The natural flow of water in a leaf is towards the sites of greatest evaporation, which are the margins and tip. Carried by the water, the pollutant concentrates in the margins and tip, so it is these areas that are generally the first to show visible injury.
Pollutant specificity	Hydrogen fluoride, SO_2, NO_x, (ozone).
Known interferences or uncertainties	Water stress closes stomata, reducing uptake and therefore decreasing the effects. Exposure during humid periods increases uptake, but may delay the onset of symptoms. Several other environmental factors may cause similar symptoms - drought, frost, insects and herbicides.
Timescale of response	The period over which the leaf is expanding. In some species, this

	may be as little as two to three weeks, but in others it may be two months or more. Symptoms are often similar to premature ageing of leaves.
Variability in time	Emerging leaves are more susceptible, indicating that the early season's growth in plants is more prone to damage than later in the year.
Variability in space	Dependent on canopy structure and leaf age.
Sampling requirements	The main limitation is the availability of sensitive species around the source. If these are absent, then consider using the transplant method.
Skill requirements - field	It is essential that staff have a basic knowledge of plant pathology and are able to recognise the main causes of lesions and discolourations shown by leaves, even if they do not know the exact individual causes. It is also essential that they have seen authentic injury in other locations and have had some field training by an experienced operator. If these requirements are not met, the result is usually false-positive identification of injury.
Skill requirements - lab	Some lab examination of samples may be required to separate the contribution of other factors such as pests and pathogens.
Calibration information	Standardised photographic publications are available for tree species in the ICP forest network, and for some crop and natural species for characteristic ozone injury.
Indicative costs	Fieldwork – medium. The cost is for the time of a trained person to visit and walk around the area. For an operation as large as an aluminium smelter, this may take two days in the first instance but probably less on repeat visits. Large industrial complexes take longer. Lab – low. Interpretation – medium. The cost will also include time to produce a report that should include a list of species at each site visited, symptoms, a map of injury and photographs.
Existing use for monitoring	Although many studies have looked at the visible symptoms of ozone (such as EuroBionet), this method is mainly used to assess impacts from fluorides (Weinstein *et al*., 1998). Visible changes are also a key component of the ICP forest monitoring network across Europe.

2.2.4 Transplants – native and standardised

There is a long history of using standardised and native transplants as active bioindicators and bioaccumulators. The sensitive BelW3 cultivar of the tobacco plant (*Nicotiana tabacum*), has been used for decades as a bioindicator for exposure to ground-level ozone (Ashmore *et al.,* 1978). Recently, a range of standardised plants has been used in the EuroBionet programme covering 11 European cities (Klumpp *et al.,* 2004b). The suite of standardised bioindicators includes: BelW3 tobacco and poplar (*Populus nigra*) as bioindicators of ozone; Italian rye grass (*Lolium multiflorum* Lam) as a bioaccumulator of sulphur compounds and heavy metals/trace elements; curly kale (*Brassic oleracea acephala*) to accumulate PAHs (polycyclic aromatic hydrocarbons), and *Tradescantia* to indicate mutagenic substances.

Transplant experiments are not conducted under controlled conditions, and therefore other factors may influence the results. Native transplant experiments involve moving a group of individuals from relatively unpolluted sites to sites receiving high levels of pollution or *vice versa,* and can involve a range of

methods (see Brodo, 1961; Ferry and Coppins, 1979; Kauppi, 1976). Ideally, all environmental and physical variables between the two sites should be the same, except the pollution level. This allows an assessment of the impact of the pollutant on a given species to be made.

Bryophytes and lichens may hold a memory of their exposure history, which fades in time. However, clear indications of responses to different pollution levels can be measured if transplant experiments are designed rigorously (Mitchell *et al.,* 2004).

An example of the use of standardised plant material to measure the impacts of industrial pollutants on a previously unknown environment is given in recent studies around industrial complexes in Brazil (Klumpp *et al.,* 1994) that included fertiliser plants, steel works, refineries and chemical and petrochemical plants. On the basis of previous studies in Europe, a number of plant species were used to monitor hydrogen fluoride, including *Gladiolus* hybr. (cv. White Friendship) and *Hemerocallis* hybr. (cv. Red Moon), a native bioindicator. *Lolium mulitflorum italicum* (cv Lema) was used to monitor accumulating toxic elements, including fluoride, sulphur and metals. After exposing plants for 28 days at 12 sites around the valley, plants were brought to the lab for evaluation of visible injury symptoms and chemical analysis. Typical fluoride-induced injury was found on plants across most sites, with the most severe effects being seen near the fluoride-emitting fertiliser industries. Visible symptoms on native *Hemerocallis* leaves were similar to those of *Gladiolus*. Exposure of the standard *Lolium* culture resulted in very high leaf fluoride concentrations of up to 700 μg g^{-1} dry weight. These by far exceeded the recommended European values for fluoride concentration in forage (75-125 μg g^{-1} dry weight; VDI, 1989).

Standardised grass cultures have been used as far back as the end of the 1960s (Scholl, 1971), when studies were carried out on fluoride and lead pollution in North Rhine-Westphalia. By 1978, grass cultures became the first standardised bioindicators to be established as a VDI-Guideline (VDI, 1978) (VDI is a German association of engineers and natural scientists). Grass cultures are capable of accumulating a large number of inorganic and organic air pollutants without incurring visible damage. Under the EuroBionet programme (Klumpp *et al.,* 2002), grass cultures were exposed for 28 days and were analysed for SO_2 and heavy metal concentrations. Data from air monitoring stations in the immediate vicinity of bioindicator stations were used to compare accumulated pollutants in the grass cultures. There was no correlation between measured SO_2 and amounts of S in the plant tissues. The authors concluded that the relatively low concentrations of SO_2 measured at the monitoring stations had only very minor negative effects on higher plants. The accumulation of heavy metals and trace elements was especially high where stations had been set up close to roadsides. Close correlations were found between iron and vanadium, both of which are released during the combustion of heavy fuel oil in power generation.

Similar measurements of heavy metals have been carried out for many years using moss bags. Fernandez *et al.* (2000) examined the compatibility of using both native and standard moss transplants around an industrial facility releasing mercury. In both cases, there was a clear relationship with distance from the

source, irrespective of the time of year. However, native mosses appeared to adapt to low concentrations of Hg because, at increasing distances from the source, native mosses were classified as uncontaminated while the transplanted mosses maintained high levels of contaminant.

There have been many transplant experiments involving lower plants and SO_2, but few involving N pollution. Because bryophytes and lichens are closely coupled to the atmosphere, most experiments use these groups of plants. However some experiments have used vascular plants (Spink and Parsons, 1995; Hicks, 1996). While turfs can be moved, epiphytic species attached to bark, twigs or stones are more easily moved without disturbing the plants. Several confounding factors must be taken into account.

Transplant experiments to investigate the effects of nitrogen pollution have mainly been used to assess the impact on a given species, rather than to provide a direct measure of N pollution *per se*. Responses of epiphytic bryophytes to changes in atmospheric N can vary according to how they obtain their nutrients, the chemistry and roughness of the bark and how effectively the bark substrate traps dust particles (Brown, 1982). Species that grow on acid and basic-barked tree species may respond differently to the same N levels. Climatic differences between sites may also confound results, especially since they influence growth and nutrient dilution.

Table 2.4: Characteristics of transplant methods, both native and standardised

Target organism or community	Higher plants, bryophytes and lichens
Measurement	Biomass accumulation, biochemical changes or physical changes (visible injury). The basis behind native reciprocal transplants is to take a native species from one location and transplant it at another.
Field-based (*in situ)* or lab-based (sampling required)	Field: ☒ Lab: ☒
Background knowledge	Used extensively in EuroBionet in 2000-2001 (www.eurobionet.de). Background levels (less than 3 mg per g dry weight) served as a reference for the enhancement of S concentrations in rye grass in cities, where concentrations of S in leaves could be double the background. Recent application of the EuroBionet growth system for *Lolium perenne* for N accumulation downwind of a poultry farm showed a very close relationship to NH_3 concentrations in each of dry matter production, tissue %N content and total above-ground N (Pitcairn *et al*., 2003) Surveys have been used in most developed countries, particularly by the aluminium industry, for at least 40 years as a convenient way of assessing the impact of fluoride emissions. Tradescantia micronucleus mutagenicity test (Trad-MCN) was also used widely in EuroBionet project. Initial results showed greater mutagenesis at sites within cities with significant exposure to traffic-related pollution.
Level of response	Sub-cellular, part of organism, whole organism.
Mechanism of action	Direct and indirect toxic effects, biochemical effects and influences on growth.
Pollutant specificity	Nitrogen, SO_2, heavy metals, hydrogen fluoride.

Known interferences or uncertainties	Transplant experiments are conducted under field conditions and therefore several other factors may influence the results, such as local variability and a range of biotic and other abiotic factors. Bryophytes and lichens may hold a memory of their exposure history, which fades in time. Allowance (calibration) must be made for such an effect.
Timescale of response	Potentially can be hours, days, weeks, years depending on species and pollutant.
Variability in time	Standard growing conditions may be required for indicator plants, restricting their use to certain times of the year or climatic zones
Variability in space	The use of standardised transplants allows for a better spatial coverage at any biomonitoring site. Specimens can be carefully placed to avoid areas where cross-contamination or disturbance may occur. Native transplants are susceptible to all other spatial factors including altitude and coverage.
Sampling requirements	Identification of suitable pollutant-specific plant material, and preparation in standardised form. Comparability of transplant sites is important to avoid confounding factors such as altitude, aspect, temperature, precipitation levels and soil type.
Skill requirements - field	Staff need to be trained in plant identification, particularly of bryophytes and lichens, and in transplantation methods. They also need training to monitor the responses of transplanted material (such as growth and sampling for chemical analysis). Skills are also needed for preparing suitable standardised plant material, and sampling native material for transplanting.
Skill requirements - lab	Specimens need to be grown to standardised methods, while basic laboratory techniques are required for analytical testing.
Calibration information	Published data on absolute values of response to a particular organism/pollutant stress.
Indicative costs	Fieldwork – medium. Lab – variable. Interpretation – medium.
Existing use for monitoring	Methods of measuring the accumulation of sulphur in standardized grass cultures have been developed in Germany over many years, leading to the publication of standard methods (VDI 3792, Part 1, 1978; VDI 3957, Part 2, 2001). The same grass cultures can be used to simultaneously measure accumulation of heavy metals and organic micropollutants (such as PAHs). Sommer and colleagues have used standard transplants to measure ammonia deposition around farms (Sommer 1988 and Sommer and Jensen 1991). Hitchcock *et al*. (1962) spent 10 years studying the effects of fluoride on gladiolus transplants.

2.2.5 Stable isotopes

Stable isotopes are forms of an element with different masses but the same chemical characteristics and similar behaviour. Stable isotopes are a valuable tool for investigating the origins of compounds within the biogeochemical cycle. Elements like sulphur and nitrogen display natural variation in the composition of their stable isotopes due to fractionation that occurs during biogeochemical processes (Wadleigh, 2003). These variations provide an isotopic signature that can be used to trace the origins of compounds and separate out these origins in multi-source situations.

Wadleigh (2003) looked at three studies that assessed the apportionment of sulphur isotopes with the biomonitoring capabilities of lichens. The studies were carried out in Newfoundland around two main anthropogenic sources: a thermal generating station and an oil refinery. Other smaller sources included pulp and paper mills. In the first study, stable isotope $\delta^{34}S$ of atmospheric sulphur was measured using the lichen *Alectoria sarmentosa* to assess the importance of long-range versus local sources of sulphur. The second study attempted to measure the response of *A. sarmentosa* to a change in atmospheric sulphur, by transplanting lichens from a clean coastal area to an industrial area. The final study looked at the heterogeneity of sulphur distribution within the lichen. Results showed that isotopic composition demonstrated the relative contributions of sulphur from different sources, providing a good indication of anthropogenic impact when combined with concentration measurements. Transplanted lichens experienced a change in isotope composition within a period of 18 months, although the author noted that this was not a linear process. This method can offer a useful early warning system when other impacts are hard to find.

Stable isotopes of nitrogen ($\delta^{15}N$) have also been studied widely in connection with plant processes and crop cycling (Stevens *et al.,* 2005). There is evidence to suggest that the two species of nitrogen (NO_y and NH_x) have different $\delta^{15}N$ signatures, with NO_y having a positive signal while NH_x is negative (Garten, 1992). This is useful for attributing nitrogen to a mixture of source emitters.

Pearson *et al.* (2000) sampled eight species of moss growing next to roads at different traffic densities in the UK. Shoots were sampled for both total N content and tissue $\delta^{15}N$. While there was a poor correlation between tissue N and traffic exposure, there was a good correlation between $\delta^{15}N$ in tissue and traffic exposure. $\delta^{15}N$ values ranged between +6 and -1 ‰, while in rural areas with low traffic densities $\delta^{15}N$ values ranged between -2 and -12 ‰. A separate survey of mosses compared the average $\delta^{15}N$ on busy roadsides in London (NO_y) with samples collected from farm buildings near poultry and cattle pens (NH_x). $\delta^{15}N$ around the busy road networks averaged +3.66 ‰, while around the farm it was -7.8 ‰. The authors concluded that there is strong evidence to support the use of this method to distinguish between the two types of nitrogen, but further work would be needed to determine the mechanism of fractionation between dry, wet and particulate $\delta^{15}N$.

2.2.5.1 Summary

Stable isotopes can be used to detect changes in sources and amounts of pollutant over time. Isotope fractionation can be used to determine the components of both local and background sources, making it a useful method for attributing pollutants to a source.

Table 2.5: Characteristics of stable isotope methods

Target organism or community	Particularly bryophytes and lichens, but also higher plants.
Measurement	Molecular weight of isotopic compounds of nitrogen $\delta^{15}N$, sulphur $\delta^{34}S$, and PAHs using carbon isotopes ($\delta^{12}C$, $\delta^{13}C$).
Field-based (*in situ)* or lab-based (sampling required)	Field: ☒ Lab: ☒
Background knowledge	The method has been used for measuring isotopes of carbon, oxygen, nitrogen and sulphur. Sulphur, with its distinctive isotopic composition, has been used to attribute pollution sources and as a tracer in hydrological studies. Stable isotopes of nitrogen have been used to explore variations in the natural abundance of $\delta^{15}N$ in plants and waters, including nitrogen cycling in the soil.
Level of response	Sub-cellular.
Mechanism of action	Uptake of pollutant is through the roots, or foliage cuticle and stomata. Most work has been done on lichen thalli and bryophyte shoots.
Pollutant specificity	Nitrogen, SO_2, PAHs.
Known interferences or uncertainties	Many factors affect the fractionation of isotopes in tissue. Wet and dry deposition processes may also influence fractionation.
Timescale of response	Normally one year or longer. Some studies have sampled transplanted material every month to measure the response time of the species.
Variability in time	For transplanted material, monthly variations in accumulation may occur. Transplanted material reaches equilibrium after around 12-18 months of exposure.
Variability in space	This method is good for determining the spatial extent of an impact from a single source point, since isotopic composition can be distinguished between natural sources.
Sampling requirements	It is important to select each site based on similar ecological states. Three samples (for example, thalli) per site are often recommended.
Skill requirements - field	Sampling requires some training in identification techniques for bryophytes and lichens.
Skill requirements - lab	Samples will need to be cleaned by hand to remove detritus. Specialist equipment is required to analyse samples.
Calibration information	Extracts of sampled material are measured against a calibrated standard laboratory gas of sulphide, sulphate or ammonium.
Indicative costs	Fieldwork – low. Lab – high. Interpretation – medium.
Existing use for monitoring	Stable isotopes have been used in the aquatic environment as tracers to study the chemistry in catchment hydrology and effects of nitrates from agriculture (Kendall and McDonnell, 1998). Stable isotopes have been used in Germany to investigate their use as biomonitors of air quality (Jung *et al*., 1997).

2.2.6 Plant emergence and growth tests

As well as supporting the existence of all other organisms, plants have a high inherent conservation value of their own. Many of the sites covered by Part IIA of the Environmental Protection Act 1990 have a wide variety and diversity of plants. A diverse plant fauna helps to maintain the functional integrity of terrestrial ecosystems.

Many species of plants are protected by law in the UK. The recording activity of both amateur and professional botanists means that changes in the occurrence of different species can be used to monitor the effects of large-scale and small-scale environmental changes in the UK (Thomas *et al.,* 2004). The protection of plants at contaminated sites is therefore a key concern within the Ecological Risk Assessment (ERA) Framework. Tests to measure the phytotoxicity of potentially contaminated soils to both monocotyledonous and dicotyledonous plant species are recommended for inclusion in the suite of biological assays.

Plants have inherent properties that make them good candidates for toxicity testing:

- they interact intimately with the soil and its constituents;
- they have a large root (and mycorrhizal) surface area, with which they actively and passively absorb mobile contaminants within the soil;
- they are static.

These methods monitor the effects of pollutants within the soil on various parameters of terrestrial plant growth, such as survival and shoot and root growth. The plant species chosen (Environment Canada recommend 12 species) should be able to survive in the field soil; for example, carrot tolerates a wide pH range (4.2-8.7) but grows best at pH 6.5-7.8. A plant species other than carrot should therefore be chosen if the field soil has a pH below six. Seedlings from terrestrial plants are placed in the soil, which should then be hydrated to a specific water holding capacity and placed in a constant temperature room. After a designated time period (usually seven days), the numbers of seedlings which have emerged are recorded. At the end of the exposure period (either 14 or 21 days, depending on the species chosen), seedling emergence and shoot length are recorded and the dry weight of the shoots and roots recorded 48 hours after oven drying.

A number of studies have used these methods (Marwood *et al.,* 1998; Rutherford *et al.,* 2005) and there are Environment Canada guidelines for conducting these tests (Environment Canada, 2005). Responses are not pollutant-specific but are usually carried out alongside chemical analysis of the soil. The responses provide evidence of the effects of complex mixtures of pollutants, but cannot determine which pollutant is responsible for the changes observed. Season, water moisture and temperature will affect responses; however, these should be controlled under laboratory conditions. The timescale of response is days to weeks. These bioassays may be useful to determine the effectiveness of remediation techniques and control measures.

Table 2.6: Characteristics of plant growth methods

Target organism or community	Plants.
Measurement	Measures the emergence of seedlings and the root and shoot biomass after a fixed period (usually 14 or 21 days, depending on the plant species chosen).
Field-based (*in situ)* or lab-based (sampling required)	Field-collected soil used in laboratory (controlled temperature) environment.
Background knowledge	In use in regulatory and academic sectors to monitor the effects of chemicals on seed emergence and growth. Currently being trialled for use in the tiered framework for environmental risk assessments in the UK.
Level of response	Individual.
Mechanism of action	These tests measure soil toxicity using terrestrial plants.
Sampling requirements	Four replicates should be collected from each site and stored at 4^{o}C in the dark.
Skill requirements - field	No specialist skills are needed, as the only activity in the field is to collect soil.
Skill requirements - lab	No specialist skills are needed.
Calibration information	There are guidelines and criteria for the number of control soils (positive and negative) that should survive for the test to be valid; these are given in Environment Canada guidelines.
Indicative costs	Low.
Existing use for monitoring	Plant growth tests are being trialled by the Environment Agency as a screening tool for assessing the risk of contaminated land (Fountain *et al*., 2005), while Environment Canada offers procedural guidelines.

2.3 Vertebrates and invertebrates

Several academically established tests have been published in the open literature, but have not necessarily undergone international standardisation. Some of these are recognised as useful for biomonitoring schemes and have been used by regulatory bodies around the world. These biomonitoring tools are measures of effects of chemicals upon organisms; some may not reflect the impact on the ecosystem *per se,* but act as an early warning system of higher-level effects/impacts/outcomes.

2.3.1 Cytochrome P450 enzyme induction

Cytochrome P450 (CYP) enzyme induction is caused by exposure to chemicals such as dioxins, PCBs (polychlorinated biphenyls), polycyclic aromatic hydrocarbons (PAHs) and numerous insecticides including organophosphates (OPs), carbamates and fungicides. Induction of cytochrome P450 enzymes is a biomarker of exposure, in that elevated levels in organisms (compared to levels in organisms from reference/clean areas) demonstrate exposure to organic compounds. There are many enzymes in the CYP family; the enzymes predominantly measured in biomonitoring studies are CYP1, 2 and 3, as these are the enzymes that are mainly induced following exposure to chemicals. CYP enzymes are involved in phase I of the detoxification of lipophilic organic compounds. The resulting metabolites are in a chemical state (with an extra

oxygen added to the structure) that can then be conjugated by Phase II enzymes (including glutathione S-transferase, discussed below) to make them more water soluble so that they can be excreted from the body.

Because vertebrates can metabolise certain organic compounds (predominantly PAHs) easily, P450 enzymes provide the best evidence of exposure. Compared to the chemical itself (which may have been metabolised), these enzymes stay elevated for a number of days after exposure. This is important if exposure occurs in a one-off release. Continuous exposure will result in continued elevation of CYP enzymes. Once exposure has stopped, the level of induction declines to control levels. The remainder of this section will concentrate on CYP1A enzymes, as these are the enzymes that are predominantly used in biomonitoring studies.

CYP1A enzymes are induced following exposure to dioxins, dioxin-like PCBs (those with planar structure) and PAHs. The response is well characterised in vertebrates, including mammals, birds and fish. Many biomonitoring studies have investigated exposure to environmental pollutants and have successfully used induction of CYP1A in aquatic and terrestrial environments to show exposure (including Bowyer *et al.,* 2003; Fisk *et al.,* 2005; Lochmiller *et al.,* 1999; Sanderson *et al.,* 1994a and 1994b; Schmitt *et al.,* 1999; Schmitt and Dethloff, 2000; Schmitt, 2002; Van den Brink *et al.,* 2005). However, the CYP1A response is less well characterised in invertebrates (Brown *et al.,* 2004; Livingstone, 1993), and de Knecht *et al.* (2001) failed to measure CYP1A in isopods exposed to pyrene. Measurement of the '418 peak'of the carbon-monoxide difference spectrum of reduced microsomes can be used in invertebrate biomonitoring (Sole and Livingstone, 2005) to determine exposure of these organisms.

There are a several confounding factors (other pollutants/naturally occurring chemicals as well as biotic and abiotic factors) which should be taken into account when using cytochrome P450 measurements in biomonitoring studies. Certain heavy metals inhibit P450 induction while some plant toxins may elevate the response; these must be accounted for in residue analysis to ensure they have not affected CYP levels. Other confounding factors include:

- developmental stage, where new-born vertebrates do not have a well-developed P450 system − studies have shown that it can take up to 21 days for the P450 system to develop fully;
- sex, where Van den Brink *et al.* (2005) observed gender-specific CYP enzyme induction in shrews collected from PCB-polluted sites;
- species differences;
- season.

The test can be used to determine whether remediation activities have been successful.

Table 2.3: Characteristics of cytochrome P450 enzyme induction method

Target organism or community	Vertebrates; the response is not well characterised in some invertebrates.
Measurement	Increase in cytochrome P450 mixed function oxidase (MFO) enzyme activity or expression (CYP1, 2, 3, 4), such as CYP1A - ethoxyresorufin-O-deethylase (EROD) activity, protein or mRNA. Cytochrome P450 enzymes biotransform organic lipophilic compounds. Also total P450 and P418 concentration. Samples from the liver, hepatopancreas, digestive gland or whole body should be taken for CYP determinations.
Field-based (*in situ)* or lab-based (sampling required)	Field and lab-based. The assays cannot be carried out in the field. Need to collect samples and then return to laboratory for analysis.
Background knowledge	In use in academic and regulatory sectors. Successfully used to monitor dioxin-like chemicals and PAHs in wildlife. Recommended biomarker by ICES (International Council for the Exploration of the Sea) and OSPAR (Convention for the Protection of the Marine Environment of the North-East Atlantic) for biomonitoring of marine environment.
Level of response	Biochemical.
Sampling requirements	Sampling can be conducted in the field; however, tissues must be snap-frozen in liquid nitrogen and stored at -80^{o}C immediately after sampling. Requires at least three replicates per sampling and time point. Need to sample food-digesting tissues, that is, liver, hepatopancreas or digestive gland, depending on taxa; in certain organisms whole body is analysed. Destructive biomarker.
Skill requirements - field	Specialist skills required in the field are identification of species and ability to dissect.
Skill requirements - lab	The molecular biological methods require specialist equipment (such as PCR machines); however, an increasing number of laboratories have this equipment. Enzyme assay is inexpensive and relatively simple without any need for specialist equipment (requires centrifuge, homogeniser, fluorimeter/microplate reader). Results are easy to interpret.
Calibration information	There is not a general level of CYP induction which can be used to demonstrate exposure to organic compounds; therefore, it is important to have a field control site (no/low induction) to compare responses to and also a positive control (samples with a known level of CYP induction) to ensure that the laboratory assay is working correctly (where a negative result is due to low/no induction rather that poor laboratory technique).
Indicative costs	Fieldwork – medium for both labour and expertise as need to trap and identify small mammals or dig up and identify earthworms/invertebrates. Laboratory costs – low. Equipment required for EROD/total P450 analysis is generally found in laboratories (see above skill requirements). Interpretation – low, once the user is experienced in the analysis.
Existing use for monitoring	The majority of examples of using cytochrome P450 induction in biomonitoring studies come from the US and Canada. Used in the BEST program by USGS (Schmitt *et al*., 1999; Schmitt and Dethloff, 2000; Schmitt, 2002) to monitor effects in aquatic ecosystems, and used in Canada to monitor effects of halogenated aromatic hydrocarbons on birds (Sanderson *et al*., 1994a&b). Used for biomonitoring petrochemical-polluted sites in US (Lochmiller *et al*., 1999), where levels of cytochrome P450 (EROD) were measured in the livers of small mammals collected from polluted and unpolluted sites. Cytochrome P450 enzymes were induced in animals from the polluted sites.

2.3.2 Metallothionein/phytochelatin induction

Metallothionein and phytochelatin proteins are induced following exposure to metals such as cadmium, zinc and mercury in vertebrates and invertebrates (Elliott *et al.,* 1999; Ikemoto *et al.,* 2004; Lukkari *et al.,* 2004; Spurgeon *et al.,* 2004, 2005a and b; Weeks *et al.,* 2004). Metallothioneins bind the metal, rendering it biologically inactive in the organism; thus, induction of

metallothionein is a detoxification mechanism for organisms exposed to toxic metals. Phytochelatins are metal-binding peptides which are involed in detoxification of heavy metals in plants (Pomponi *et al.,* 2006). Zhang *et al.* (2005) measured phytochelatins and metallothionein (through measuring the protein itself and by gene expression) in garlic seedlings exposed to cadmium and found that there was an induction in both proteins following exposure. However, the laboratory study showed that other stresses, including heat shock, also resulted in increased phytochelatin gene expressison and phytochelation content in the roots.

A number of confounding factors (chemical, biotic and abiotic) must be taken into account when using metallothionein and phytochelatin in biomonitoring studies. Chemicals that cause changes include free radicals, endogenous compounds such as peptide hormones and essential metals. Levels may also be affected by previous exposure as the response is irreversible, so knowledge of previous exposure is important during interpretation of the results. Season, moulting and tissue regeneration (important for insects and invertebrates) and nutritional status may also influence the metallothionein and phytochelatin response. Because the liver, kidneys, hepatopancreas digestive gland or whole body/plant are analysed, this biomarker method is destructive. Because the response is not reversible, metallothionein is not a good biomarker to determine if remediation or other exposure control techniques have been successful unless transplanted organisms are used, because previous exposure will have elevated metallothionein levels.

Table 2.4: Characteristics of metallothionein/phytochelatin induction method

Target organism or community	Vertebrates, invertebrates and plants.
Measurement	Induction of metallothionein/phytochelatin proteins, measured by molecular biology techniques, enzyme-linked immunosorbent assay (ELISA) or atomic absorption spectroscopy (AAS) substitution assay.
Field-based (*in situ)* or lab-based (sampling required)	Laboratory-based assay. Need to collect samples from the field and return to laboratory for analysis.
Background knowledge	In use in academic and regulatory sectors. Successfully used in US to monitor metal contamination in wildlife species. Recommended by ICES as a biomarker.
Level of response	Sub-cellular.
Mechanism of action	Metallothionein and phytochelatin are proteins that bind essential metals during normal metabolic function and non-essential metals as a protective mechanism.
Sampling requirements	Sampling can be conducted in the field; however, tissues must be snap-frozen in liquid nitrogen and stored at -80°C immediately after sampling. Requires at least three replicates per sampling and time point. MT is present in all tissues but high in liver and kidney (vertebrates), digestive gland/hepatopancreas (invertebrates) or whole body.
Skill requirements - field	Specialist skills required in the field are identification of species and ability to dissect.
Skill requirements - lab	The molecular biological methods require specialist equipment (such as PCR machines); however, an increasing number of

	laboratories have this equipment. Protein can be determined through electrophoresis or ELISA (using a microplate reader). Substitution assay conducted using atomic absorption spectroscopy. Results are easy to interpret.
Calibration information	Site controls should be included in the analysis to ensure that a comparison can be made between induced and non-induced organisms.
Indicative costs	Fieldwork – medium costs (similar to those for cytochrome P450). Laboratory costs – low, as general laboratory equipment required for the analysis (ELISA and substitution assay) is low cost. However, if molecular biololgy methods are used this will increase costs to medium, as will need equipment and reagents that are relatively expensive. Data interpretation – low, once the user is familiar with the data analysis.
Existing use for monitoring	Invertebrate metallothioneins were measured in a UK study to investigate techniques to measure exposure and effect of toxicants on soil invertebrates in the field (Weeks *et al.,* 2004). Six sites were selected at a range of distances from a zinc smelter. Invertebrates were collected and metallothionein measurements made. Results showed that invertebrates from sites with high metal concentrations had high levels of the protein.

2.3.3 Induction of heat shock proteins (HSPs)/stress proteins

These proteins are involved in the normal functioning of the cell, by ensuring nascent (novel) polypeptides produced by the cell fold in the correct formation to ensure the resulting proteins are biologically active. HSPs are non-specific biomarkers of exposure as they can be induced by exposure to many different stressors including PAHs, metals and also natural stressors such as temperature, salinity, anoxia, light. As such they can be used as a general measure of stress.

If the concentration of pollutant reaches toxic levels within the organism and the organism is close to death, HSP production is reduced in favour of more energy-preserving biochemical responses, so low HSP response may lead to false negative results. Induction of HSPs by cells is a protective mechanism, as stress may result in the production of incomplete/nonsense polypeptides; HSP bind to these molecules and promote their elimination from the cell. A number of studies have used HSP in laboratory and field exposures to pollutants (including Arts *et al.,* 2004 and La Porte, 2005) and natural stressors (including Piano *et al.,* 2005; Barua and Heckathorn, 2006). Most studies concluded that, because of their non-specific nature, induction of HSP could not be linked to a specific pollutant.

The response is reversible and the time to induction is relatively small, depending on how sensitive the method of detection is. For example, with ELISA and Western blotting (measuring protein synthesis), induction could be measured after eight hours exposure, but more sensitive methods (gene expression) could detect induction within a couple of hours. As the response is transient, a negative response may not necessarily mean that the organism had not been exposed to pollutants/stressors. HSP could be used to determine if remediation/control has been successful. However, the confounding factors listed above must be considered if a negative result is observed.

Table 2.5: Characteristics of heat shock protein (HSP)/stress protein method

Target organism or community	Vertebrates, invertebrates and plants.
Measurement	ELISA, gene expression through mRNA analysis and one-dimensional electrophoresis and Western blotting techniques.
Field-based (*in situ)* or lab-based (sampling required)	Lab-based assay. Need to collect samples from the field and return to laboratory for analysis.
Background knowledge	In use in academic sector as a general indicator of environmental health.
Level of response	Biochemical.
Mechanism of action	Eliminates denatured proteins from cells and promotes correct conformation of polypeptide chains.
Sampling requirements	All tissues manufacture HSPs, therefore any tissue may be collected. Tissues must be snap-frozen in liquid nitrogen and stored at -80°C immediately after sampling.
Skill requirements - field	The only specialist skills required in the field are identification of species and ability to dissect.
Skill requirements - lab	Requires a spectrophotometer for the assay, and electrophoresis equipment which should be available in most research laboratories. Results are fairly easy to interpret.
Calibration information	A positive control should always be run to ensure the assay was conducted correctly. There are no definite levels of HSP induction which can be used to determine whether exposure has occurred; for that reason, a site control should be included for comparison between polluted and non-polluted areas.
Indicative costs	Fieldwork – medium. As for P450 and metallothionein, expertise in species identification is necessary. Laboratory work – low (for ELISA and one-dimensional electrophoresis), medium for gene expression (due to the purchasing of equipment and sample preparation). Interpretation – low costs.
Existing use for monitoring	HSPs are not routinely used in biomonitoring projects because of the many confounding factors. They could be used as a measure of general stress to organisms at a particular site, but would not be able to directly link high HSP induction with a specific pollutant.

2.3.4 Increase of phase II detoxification enzymes (including glutathione S-transferase (GST) activity)

These enzymes are involved in Phase II of the detoxification process (the stage after biotransformation by CYP enzymes); they make the metabolites from Phase I more water soluble and promote elimination from the body. The enzymes are elevated following exposure to many different types of chemical including those inducing CYP enzymes, such as dioxins, PCBs and PAHs (De Luca-Abbott *et al.,* 2005). Compounds that induce Phase II (but not CYP) enzymes include metals (Casalino *et al.,* 2006; Lopes *et al.,* 2002; Lukkari *et al.,* 2004), herbicides (Xiao *et al.,* 2006) and free radicals. Phase II enzymes are also measured in pollutant-exposed organisms that do not use CYP enzymes, including invertebrates (de Knecht *et al.,* 2001). The response can be measured within 24 hours of exposure, but is transient in nature in some species and may decline to control levels during exposure. Data should therefore be interpreted with caution. Other

confounding factors include temperature, season (Pavlovic *et al.,* 2004), soil moisture (Chen *et al.,* 2004) and soil type (terrestrial invertebrates). These biomarkers can be used to determine if remediation techniques and controls have been successful, as the response is reversible.

Table 2.6: Characteristics of phase II detoxification enzymes method

Target organism or community	Vertebrates, invertebrates and plants.
Measurement	Enzyme activity, through spectrophotometric and immunoassay techniques.
Field-based (*in situ)* or lab-based (sampling required)	Lab-based assay. Need to collect samples from the field and return to laboratory for analysis.
Background knowledge	In use in academic sectors. Further research into mechanism and variation of response in earthworms required. Successfully used in vertebrate studies following organic contaminant exposure.
Level of response	Biochemical.
Mechanism of action	Increases water solubility of products (biotransformed pollutants) from cytochrome P450 reactions to promote elimination from the body.
Sampling requirements	Food-digesting organs must be sampled for analysis (same organs as for cytochrome P450 analyses). Tissues must be snap-frozen in liquid nitrogen and stored at -80^{o}C immediately after sampling.
Skill requirements - field	The only specialist skills required in the field are identification of species and ability to dissect.
Skill requirements - lab	Requires a spectrophotometer or electrophoresis/ELISA equipment for the assays, which should be available in most research laboratories. Results are fairly easy to interpret.
Calibration information	A field control sample should be included in the analysis to compare against samples from the polluted area, as there is no official level of enzyme activity which is considered to prove that exposure has taken place.
Indicative costs	Fieldwork – medium costs (as above). Laboratory work – low, general lab equipment used and method is straightforward. Data interpretation – low.
Existing use for monitoring	There are no examples of use in UK biomonitoring studies, mainly used in laboratory and field studies from academic institutions. Used in preliminary studies to look at the effects of organic pollutants on aquatic plants and shown to be induced compared to controls (Lindstrom-Seppa *et al*., 2001)

2.3.5 Increase in antioxidant enzymes

These enzymes protect cells from oxygen free radicals which damage the cells and produce toxic effects. Some biochemical/biotransformation reactions triggered by exposure to contaminants also result in the production of free radicals as a byproduct of the reactions, so these enzymes are usually measured in addition to phase II detoxification enzymes, using the same or similar sample preparations. Antioxidant enzymes include superoxide dismutase (SOD), catalase, peroxidase and glutathione reductase. Antioxidant enzymes are induced by many different chemicals (including PCBs, PAHs, dioxins, heavy metals, pesticides and ozone) and are therefore non-specific. Elevation of these

enzymes provides evidence of exposure to free radical-forming compounds, which may lead to cell damage and ultimately tumour formation. A number of studies have measured antioxidant enzymes relative to exposure to pollutants in the field (Lopes *et al.,* 2002; De Luca-Abbott *et al.,* 2005; Manduzio *et al.,* 2004; Reid and MacFarlane, 2003; Rosety *et al.,* 2005) and the laboratory (Brown *et al.,* 2004; Reid and MacFarlane, 2003). The majority of studies investigated the effects of pollution in the marine environment, which is well characterised. The timescale of response is short, where enzyme activity begins to rise within hours to days following exposure. However, the response is reversible, so knowledge of exposure pattern is important to ensure correct interpretation of the results. Other confounding factors include changes in abiotic or endogenous conditions, seasonal and climatic processes. Elevation of antioxidant enzymes could be used in remediation studies because the response is reversible. Gene chip technology offers the potential to speed up such assays, and is reviewed later in this report.

Table 2.7: Characteristics of antioxidant enzymes method

Target organism or community	Vertebrates, invertebrates and plants.
Measurement	Enzyme activity by spectrophotometric assays.
Field-based (*in situ)* or lab-based (sampling required)	Lab-based assay. Need to collect samples from the field and return to laboratory for analysis.
Background knowledge	In use in academic sector. Method well developed to measure plant and vertebrate enzymes; needs further development in terrestrial invertebrates. Recommended by ICES as a biomarker.
Level of response	Biochemical.
Mechanism of action	Scavenges free radicals and reactive oxygen species to reduce their toxic effects.
Sampling requirements	Same tissues sampled as for cytochrome P450 and GST, that is, food digesting tissues - liver, digestive gland, hepatopancreas, whole body for certain organisms. Other tissues including gills can also be analysed in certain organisms (gills are the first site of exposure in aquatic organisms and so are a useful tissue to measure biochemical responses). Tissues must be snap-frozen in liquid nitrogen and stored at -80^{o}C immediately after sampling.
Skill requirements - field	Specialist skills required in the field are identification of species and ability to dissect.
Skill requirements - lab	Requires a spectrophotometer for the assay, which should be available in most research laboratories. Results are easy to interpret.
Calibration information	A field control sample should be included in the analysis to compare against samples from the polluted area, as there is no official level of enzyme activity which proves that exposure has taken place.
Indicative costs	Fieldwork – medium, as above. Laboratory costs – low, spectrophotometers are usually in most research labs, methods are straightforward to carry out. Interpretation – low, results are easy to determine.
Existing use for monitoring	Used in preliminary studies to look at effects of organic pollutants on aquatic plants and shown to be induced compared to controls (MacFarlane, 2002). Research into plant biomarkers to assess metal stress in the field (Hartley-Whitaker and Meharg, 2001). Used in BEEP (Biological Effects of Environmental Pollution in marine coastal ecosytems) programme to monitor pollution in coastal ecosystems around Europe.

2.3.6 Increase in DNA adduct formation

DNA adducts form following exposure to organic pollutants, which bind to DNA resulting in genotoxicity to the cells. DNA adducts detected in organisms following exposure may lead to genetic changes in these organisms in future. While the pollutants in question may not be acutely toxic, they may result in delayed effects. Measuring DNA adducts can provide an early warning of possible long-term effects. However, this method is still in the early stages of research and the link to subsequent effects needs to be much better documented. The technique involves radioactive compounds and so requires laboratories with radiological procedures and licences.

Some studies have measured DNA adducts following exposure to PAHs (through crude oil) in marine environments. Harvey *et al.* (1999) measured adduct formation in invertebrates and vertebrates following the *Sea Empress* oil spill in 1996. They found DNA adducts in fish, but not invertebrates. Lemiere *et al.* (2004) measured DNA adducts in rats exposed to mussels taken from the environment close to the *Erika* oil spill, and found that DNA adducts were present in rat liver following exposure. Confounding factors that must be taken into account when interpreting DNA adduct measurements include exposure to pesticides and endogenous/exogenous carcinogens. Season also has an effect. The existence of DNA repair mechanisms means that the response is reversible which may, over time, mask the formation of DNA adducts. DNA adducts could, however, be used to assess the success of remediation schemes.

Table 2.8: Characteristics of DNA adduct formation method

Target organism or community	Vertebrates, invertebrates and plants.
Measurement	Covalent binding of environmental pollutants to DNA.
Field-based (*in situ)* or lab-based (sampling required)	Lab-based assay. Need to collect samples from the field and return to laboratory for analysis.
Background knowledge	In use in both academic and regulatory sectors. Used successfully for monitoring aflatoxin exposure in humans, and used to measure PAH effects in benthic fish in US.
Level of response	Molecular.
Mechanism of action	Covalent binding of environmental pollutants to DNA .
Sampling requirements	All tissues with a nucleus can be sampled to measure DNA adducts. Tissues must be snap-frozen in liquid nitrogen and stored at -80°C immediately after sampling.
Skill requirements - field	The only specialist skills required in the field are identification of species and ability to dissect.
Skill requirements - lab	Requires specialist equipment and the use of radiolabelled compounds.
Calibration information	Published data on absolute values of response to a particular organism/pollutant stress.
Indicative costs	Fieldwork – medium costs. Laboratory work – high. Specialist equipment is needed and there are health and safety costs of working with radioactive compounds. Interpretation – medium to high, data analysis may be difficult as

	the method is still relatively new.
Existing use for monitoring	There are no biomonitoring studies currently ongoing in the UK where DNA adducts are being used. Used to monitor PAH contamination in coastal areas of US and also to monitor aflatoxin exposure in humans (Gompertz *et al*., 1996). DNA adducts were monitored in herring gulls collected from the Great Lakes *(for review see Lagadic et al 2000)*..

2.3.7 Histopathological lesions

Measuring histopathological lesions involves measuring changes in cellular morphology in the tissues of internal or external organs following exposure to chemicals, for example changes/enlargement of cells, proliferation of white blood cells, changes in epithelial cells and lung alveolar cells. Changes in morphology can be early warning signals (similar to DNA adducts) for diseases that may come to light after exposure has ceased. Measuring histopathological changes in the tissues of organisms has been carried out in a number of studies. For example, a study by Ghosh *et al.* (2006) looked at the impact of diesel oil effluent on various structures in the alimentary canal of fish. They found changes in epithelial cells of the intestine which could hamper the absorption of nutrients through the alimentary canal and impair growth in the fish. There are a number of ways in which histopathology can be used; for example, different staining techniques (such as heamotoxylin and eosin (general stain) or PAS (for acidic areas such as the stomach)) can highlight different changes in cell chemistry using the same tissue/organ and may be used for the diagnosis of diseases or conditions which may occur in the future.

These biomarkers are not specific and can be induced following exposure to PCBs, PAHs, dioxins, metals (Mateo *et al.,* 2006) and pesticides (Pereia *et al.,* 2006; Rosety *et al.,* 2005). Other confounding factors include disease, age, sex and nutritional state. The time to response is usually longer than for cellular and molecular biomarkers but may be as fast as within 48 hours, depending on the tissue, or weeks. The response tends to be irreversible but the confounding factors need to be taken into account, for example if the organism is showing signs of age. These biomarkers may be used to determine the success of remediation techniques/control measures; however, some lesions may not be reversible so knowledge of the lesion type and its reversibility should be taken in to account when interpreting the results.

Table 2.9: Characteristics of histopathological lesions method

Target organism or community	Vertebrates, invertebrates and plants.
Measurement	Histological measurement of tissues after staining.
Field-based (*in situ)* or lab-based (sampling required)	Lab-based assay. Need to collect samples from the field and return to laboratory for analysis.
Background knowledge	In use in both academic and regulatory sectors. Used to monitor extent of PAH contamination in sediment in coastal areas of US. Recommended by ICES as a biomarker (liver) or potential biomarker (kidneys).
Level of response	Physiological.
Mechanism of action	Measures lesions or abnormalities on a cellular level.
Sampling requirements	All tissues can be sampled for histopathological analyses. Tissues must be preserved in fixative solution as soon as possible post mortem.
Skill requirements - field	The only specialist skills required in the field are identification of species and ability to dissect.
Skill requirements - lab	Histological staining techniques using haematoxylin and eosin (H&E) for general histological analyses or periodic acid schiff (PAS) depending on the type of lesions measured. Light microscopy. Interpretation of changes requires specialist training.
Calibration information	Need information on the baseline responses of organisms under control/natural conditions to be able to compare with exposed organisms.
Indicative costs	Fieldwork – medium. Laboratory costs – high, requires specialist equipment to prepare samples on slides, although staining techniques are relatively simple. Low throughput of samples increases the cost. Interpretation – medium to high, requires specialist expertise to interpret cellular changes.
Existing use for monitoring	Mainly used in aquatic biomonitoring programmes; however, technique could be used to measure changes in terrestrial organisms. Used to monitor PAH contamination in fish in coastal areas of US (Hahn, 1999). Used to monitor health of fish as part of the BEST programme (Biomonitoring Environmental Status and Trends) (Schmitt *et al*., 1999; Schmitt and Dethloff, 2000). Used as part of a survey of UK coastal waters in the National Marine Monitoring Programme (NMMP) (Marine Pollution Monitoring Management Group, 1998).

2.3.8 Lysosomal membrane stability

Lysosomes are intracellular organelles involved in the breakdown of unnecessary cellular material and so are important components of the cell. If these organelles begin to lose their integrity (for example, if their membrane becomes more permeable), then the cell will not function efficiently. Lysosomal membrane stability can show whether the membrane is intact and whether there is a problem with intracellular functioning. A number of studies have shown that lysosomal membrane stability is reduced following exposure to pollutants in terrestrial (Spurgeon *et al.,* 2002, 2004) and aquatic organisms (Domouhtsidou *et al.,* 2004; Marigomez *et al.,* 2005; Martins *et al.,* 2005). However, the response is not particularly sensitive to low levels of pollution. This biomarker is not pollutant-specific and so can only offer a general measure of the health of an organism.

Confounding factors which must be taken in to account when interpreting the data include hypoxia, hypothermia, osmotic shock and nutritional status; season also has an effect. Lysosomal stability could be used to determine whether remediation measures have been successful, as the response is reversible.

Table 2.10: Characteristics of lysosomal membrane stability method

Target organism or community	Vertebrates and invertebrates.
Measurement	Disruption to intracellular membrane stability, which is measured by staining the cells with a dye and measuring the time taken for the dye to accumulate in the cells.
Field-based (*in situ)* or lab-based (sampling required)	Lab-based assay. Need to collect samples from the field and return to laboratory for analysis.
Background knowledge	In use in both academic and regulatory sectors. Successfully being used to assess health of aquatic and terrestrial ecosystems. Recommended by ICES as a biomarker. Being trialled in UK as a biomarker for contaminated land risk assessments.
Level of response	Cellular.
Mechanism of action	Increased fragility of lysosomal membrane results in reduction of subcellular function.
Sampling requirements	All tissues can be sampled to measure lysosomal stability. Samples must be taken from organisms before death and analysed immediately if using neutral red retention time (NRRT), but other techniques allow the use of fixed tissue samples.
Skill requirements - field	The only specialist skills required in the field are identification of species and ability to dissect.
Skill requirements - lab	Requires light microscope.
Calibration information	There are no absolute values known to determine whether the response is due to high levels of pollutants. Negative controls are necessary to compare organisms from clean and polluted sites. Good baseline information is also required to determine the natural variation in response due to season, sex, age and so on.
Indicative costs	Fieldwork − medium, as above. Laboratory costs − high staff costs; the technique is time-consuming and so there is low throughput of samples; low equipment costs, which just require a light microscope. Interpretation – medium.
Existing use for monitoring	Decreased lysosomal integrity observed in earthworms collected from polluted accident sites in UK (Spurgeon *et al*., 2002, 2004). Used in Italy to assess residual biological effects in molluscs (transplanted and wild) in biomonitoring programme following an oil spill (Viarengo *et al*., 2002) and as part of an ERA in freshwater ecosystems (Capri *et al*., 2002).

2.3.9 Increase in porphyrins

These biochemicals are produced during the synthesis of haemoglobin (animals) and chlorophyll (plants). An increase in porphyrins is evidence of disruption in the haem or chlorophyll biosynthesis cascade, which may lead to detrimental effects. A family of porphyrins can be measured in biomonitoring studies including coporphyrin III. The response is reversible and there is a short time to onset of response, in hours to days. Porphyrin levels are elevated throughout exposure and decline once exposure has ceased. They are a useful biomarker in vertebrates as they are non-destructive, being sampled from faeces. This is

important if only small numbers of organisms are present, or if the organism of interest is a protected species or in decline. Porphyrins are elevated by halogenated aromatic hydrocarbons (PCBs, dioxins) and PAHs.

Confounding factors may influence porphyrin concentrations in organisms, including inherited disorders and iron. Nevertheless, these biomarkers can be used to determine the success of remediation or control measures, as the response is reversible. A study by Bowyer *et al.* (2003) used porphyrins as biomarkers to investigate whether otters were still at risk from PAHs in Prince William Sound in the years following the Exxon Valdez oil spill. Results showed that after a number of years, porphyrin levels declined in otters at the oiled sites to levels similar to those in otters at other sites.

Table 2.11: Characteristics of porphyrin method

Target organism or community	Vertebrates, invertebrates and plants.
Measurement	Involved in synthesis of haemoglobin and chlorophyll. Porphyrins are measured using high performance liquid chromatography.
Field-based (*in situ)* or lab-based (sampling required)	Lab-based assay. Need to collect samples from the field and return to laboratory for analysis.
Background knowledge	In use in both academic and regulatory sectors.
Level of response	Molecular.
Mechanism of action	Involved in synthesis of haemoglobin and chlorophyll.
Sampling requirements	Measure porphyrin concentration in liver, digestive gland, hepatopancreas and faeces (vertebrates and invertebrates) and leaves (plants). Tissues must be snap-frozen in liquid nitrogen and stored at -80^{o}C immediately after sampling.
Skill requirements - field	The only specialist skills required in the field are identification of species and ability to dissect.
Skill requirements - lab	The analysis uses high performance liquid chromatography (HPLC); therefore, it requires an HPLC machine.
Calibration information	It is important to have samples from clean reference sites to ensure that porphyrin concentrations in the unknown samples are elevated compared to these sites.
Indicative costs	Fieldwork – medium; collecting samples may be time-consuming. Laboratory costs – low (high throughput of samples), chemistry laboratories should have HPLC machines with which to do the analysis. Method is relatively easy. Interpretation – low, results are easy to interpret.
Existing use for monitoring	Environment Canada measured hepatic porphyrin in fish-eating birds in the Great Lakes to monitor PAH toxicity (Fox, 1993). Elevated levels of porphyria were detected a year after the Exxon Valdez oil spill in river otters close to spill site (Peakall and McBee, 2001).

2.4 Soil function indicators

The following biomonitoring tools provide evidence of the impacts of pollution on ecosystems through changes in their functioning. Such changes may result in negative outcomes, in that changes in the microbial community may alter the environment for soil-dwelling invertebrates and possibly conditions for vegetation. These, in turn, may change the diversity of the ecosystem for above-ground invertebrates and higher trophic level organisms.

The breakdown of organic matter is a crucial process in terrestrial systems. It regulates nutrient availability for plants and maintains soil fertility in sustainable agriculture. Organic matter breakdown consists of interacting processes carried out by soil micro-organisms and animals. Furthermore, in many systems the decomposing plant litter provides habitats and food for a wide range of organisms.

A number of assays have been used to estimate the rate of organic matter breakdown in soil systems, including:

- measurement of carbon and nitrogen mineralisation rates;
- the use of litterbags (Paulus *et al.,* 1999)
- mini-container decomposition assays (Eisenbeis *et al.,* 1999);
- the bait lamina test (von Törne, 1990; Kratz, 1998).

2.4.1 Bait lamina strips

These strips measure the overall feeding activity of soil-dwelling organisms (including invertebrates and micro-organisms). They are a fast and inexpensive measure of soil function and can provide an overall indication of the health of an area, though they are not pollutant-specific. The rationale is that disturbed areas will have fewer soil-dwelling organisms and so the feeding rate will be low compared to undisturbed areas (which should have a high feeding activity rate). Soil properties (such as pH) must be taken into account when analysing the results from bait lamina, and so it is important to have a site control to ensure that differences in feeding rates are not due to soil properties but to the stressor.

At certain times of the year it is not suitable to carry out bait lamina studies, for example in summer when the ground is dry and soil-dwelling organisms do not tend to reside in the top layer of soil (which is measured in bait lamina studies). Spring and autumn are suitable, but it would be advisable to avoid sampling in winter as the ground is hard and the strips are more likely to snap.

Field studies have shown that bait lamina strips are a useful means of biomonitoring. Filzek *et al.* (2004) investigated the impact of a large zinc smelter on soil organisms using bait lamina strips to determine effects on the feeding rate of soil invertebrates. The study found greater feeding activity at sites furthest from the factory and lowest feeding activity at sites closest to the factory. The study suggested that differences in bait removal could be attributed to the direct effects of metals on invertebrate abundance and diversity. Bait lamina strips were also used to measure the ecological risks of oil-polluted soils (van Gestel *et al.,*

2001), where the study found a reduction in feeding activity in polluted soils. Bait lamina could be useful for determining the effectiveness of bioremediation measures in organic-chemical polluted areas.

Table 2.12: Characteristics of bait lamina strips method

Target organism or community	Soil dwelling invertebrates and micro-organisms.
Measurement	Plastic strips with 16 holes filled with bait are placed in the soil and the number of pierced bait holes is recorded.
Field-based (*in situ)* or lab-based (sampling required)	Field-based.
Background knowledge	Used in the academic sector, and is being trialled at the moment for use in the tiered framework for environmental risk assessments in the UK.
Level of response	Community level.
Mechanism of action	The bait lamina test measures the feeding activity of soil organisms by assessing the removal of a series of bait material pellets embedded in plastic strips. Studies have shown that the lower the feeding activity (high numbers of unpierced holes), the fewer organisms are present, which may be due to contamination.
Sampling requirements	It is preferable to carry out sampling between autumn and end of spring, otherwise the soil is too dry. At least three replicates per sampling plot are needed, with 12-16 strips per replicate.
Skill requirements- field	An implement to make a small hole in the ground.
Skill requirements - lab	A light source to be able to determine number of holes pierced.
Calibration information	A site control is necessary to be able to determine that changes in feeding activity are due to pollutants and not soil properties.
Indicative costs	Fieldwork – low. Just requires deploying the bait lamina strips at field sites, with no specialist expertise required. Laboratory costs – low, as can prepare own bait and re-use strips. Interpretation – low.
Existing use for monitoring	Larink and Lübben (1991) used the bait lamina test to study the influence of heavy metals in arable fields amended with sewage sludge. Reduced feeding activity was found in the more contaminated plots. Kula and Römbke (1998) used bait lamina to assess the effects of diflubenzuron in a deciduous woodland. Results indicated strong effects of the pesticide on invertebrate feeding activity measured using bait lamina. These changes were in accordance with changes in the abundance of different springtail species, confirming the ability of the assay to detect community-level changes.

2.4.2 Community-level physiological profiling (CLPP) using BIOLOG plates

This method determines the impact of soil disturbance on the microbial community. The method was used to evaluate the effects of heavy metals (Ellis *et al.,* 2001) on soil microbial communities and found that copper and zinc did affect the microbial community. However, other soil parameters such as pH also affect microbes. Changes in soil microbial communities may have large impacts on the functioning and biodiversity of the ecosystem, including impacts on soil invertebrates, vegetation and ultimately organisms at higher trophic levels as the soil function changes. CLPP is not pollutant-specific, but it can reveal changes in the soil microbial community profile. Some factors can influence the results; complex mixtures of pollutants can contain chemicals that increase or inhibit the

community, making analysis difficult. Other factors include seasonal effects, soil type, moisture and pH. The response occurs within days and is reversible. Therefore, CLPP may be useful in determining the success of remediation techniques and control measures.

Table 2.13: Characteristics of community-level physiological profiling (CLPP) using BIOLOG plates method

Target organism or community	Soil bacterial community.
Measurement	Bacterial community profiling.
Field-based (*in situ)* or lab-based (sampling required)	Lab-based assay. Need to collect soil samples from the field and return to laboratory for analysis.
Background knowledge	Used in academic and regulatory sectors.
Level of response	Community level.
Mechanism of action	Measure carbon substrate use with multi-well plates. Pollution-induced community tolerance (PICT) is the process by which exposure to a chemical alters the structure of microbial assemblages through adaptation and extinction, so that the resulting community becomes more resilient to the effects of that chemical.
Sampling requirements	Collect soil from contaminated areas and return to the laboratory for analysis.
Skill requirements - field	Soil corers, fork, spades.
Skill requirements - lab	Requires specialist knowledge for microbial assemblage identification.
Calibration information	Information about community profiles in clean reference areas is required to compare with results from impacted sites.
Indicative costs	Fieldwork – low; collect soil and return to laboratory. Laboratory costs – medium; sample preparation and running the analysis Interpretation – medium.
Existing use for monitoring	Rutgers *et al.* (1998) used the PICT approach to study the effects of zinc in 2.5-year old contaminated field plots. For most substrates, the metabolic activities showed an increased community tolerance with increasing zinc concentration, indicating that PICT had evolved. The method proved sensitive, as PICT could be demonstrated at soil zinc concentrations close to the Dutch soil protection guideline value.

2.4.3 Mini-containers and litterbags

The rationale behind litterbags and mini-containers is similar to bait lamina, in that they are filled with organic matter and placed in a disturbed site; after a set time, the bags are removed and the amount of organic matter remaining is calculated. These techniques provide a measure of the effectiveness of below-ground decomposers. The greater the organic matter remaining, the lower the number of decomposers present in the soil. Several studies have investigated the effects of chemicals on decomposers (Au *et al.,* 1992; Bressan and Paoletti, 1997; Laskowski *et al.,* 1995), where increased chemical disturbance resulted in a greater amount of litter remaining at the end of the study. Litterbags and mini-

containers are not pollutant-specific, providing a measure of the general health of an ecosystem. However, one study (Bressan and Paoletti, 1997) collected the remaining litter and analysed the residues to determine which air pollutants were affecting the ecosystem. Factors which can influence results are soil type and soil properties; season also has an effect. The timescale of response is days and is reversible, once the decomposer community returns to the area. Thus, litterbags and mini-containers may be useful for measuring the success of remediation schemes.

Table 2.14: Characteristics of mini-container and litterbag methods

Target organism or community	Detritivorous soil fauna.
Measurement	Litterbags are constructed at least in part from mesh, the size of which can be selected to allow the activity of particular invertebrate groups to be assessed. Mini-containers have been developed from litterbags. Each mini-container has a central body over which gauze discs of variable mesh sizes are placed. The mesh allows access to selected soil fauna that facilitate litter breakdown.
Field-based (*in situ)* or lab-based (sampling required)	Field-based assay.
Background knowledge	Litterbags have been used extensively to investigate the effects of land use and soil type on soil ecology. In use in academic sectors.
Level of response	Community level.
Mechanism of action	If known – evidence of direct cause-effect relationships with pollutants.
Sampling requirements	At least three replicates per sample plot.
Skill requirements - field	None necessary, as only need to dig holes to put litterbags or mini-containers in.
Skill requirements - lab	No specialist skills required.
Calibration information	Litter bags and mini-containers must be deployed at clean reference sites for comparison with polluted sites.
Indicative costs	Fieldwork – low; just need to dig holes and place containers in the holes. Laboratory costs – low (unless carrying out chemical analyses, which will increase the cost). Interpretation – low.
Existing use for monitoring	Some studies have found a clear negative effect of exposure, for example for carbendazim (Forster *et al*., 1996) and captan (De Jong, 1998). By contrast, Huuselaveistola *et al*. (1994) found no effect of two pyrethroids on the decomposition rate, indicating that the method could be insensitive to some exposures (or alternatively that the approach was accurately predicting a minimal effect).

2.4.4 Life-cycle bioassays

These methods monitor the effects of soil pollutants on various life-cycle parameters of a chosen species (usually a soil-dwelling invertebrate such as an earthworm or springtail); parameters include growth, survival and reproduction, which are important for the community. Naïve adult organisms are placed in the soil so that previous exposure will not affect results and all organisms will be at the same developmental stage. After a designated time period (between four and eight weeks), the animals are removed. The numbers and weights of survivors are recorded. Reproductive capacity is determined by measuring the number of cocoons remaining in the soil.

Several studies have used these methods (van Gestel *et al.,* 2001; Spurgeon *et al.,* 2003) and the Organisation for Economic Cooperation and Development (OECD) has developed guidelines for conducting these tests on earthworms. Responses are not pollutant-specific, but are usually measured alongside chemical analysis of the soil. The responses provide evidence of the effects of complex mixtures of pollutants, but cannot determine which pollutant is responsible for the changes observed. Other factors that may affect responses include species differences, disease, season, water moisture and temperature; however, these last can be controlled in the laboratory. The timescale of response is days to weeks. Life-cycle bioassays may be useful for determining the effectiveness of remediation techniques and control measures.

Table 2.15: Characteristics of life-cycle bioassay method

Target organism or community	Invertebrates.
Measurement	Used to predict the long-term consequences of chemical exposure, by considering the effects of pollutants on a number of life-cycle parameters such as growth, reproduction and development in invertebrate species.
Field-based (*in situ)* or lab-based (sampling required)	Lab-based assay. Collect soil samples from the field and return to laboratory to conduct assays.
Background knowledge	In use in academic sector.
Level of response	Individual level.
Mechanism of action	See 'Measurement' section.
Sampling requirements	At least three replicates should be taken at each sampling plot. Soil should be stored at 4°C upon return to the lab.
Skill requirements - field	No specialist skills are needed as the only activity in the field is to collect soil.
Skill requirements - lab	No specialist skills are needed.
Calibration information	There are guidelines and criteria as to the numbers of control animals that survive for the test to be valid; these are reported in OECD guidelines.
Indicative costs	Fieldwork – medium. Requires large quantities of soil for the tests (kgs). Laboratory costs – medium. Preparing the soil for the tests, counting the worms (usually counted during the test as well as at the beginning and end) and ending the tests are time-consuming. Interpretation – low. The results are usually straightforward to interpret.

Existing use for monitoring	For soil invertebrates, Kammenga *et al.* (1996) optimised a life cycle test for *P. acuminatus* that could be adapted for other species, such as *C. elegans*. Crommentuijn *et al*. (1997) also developed a life cycle-based approach with the springtail *F. candida*. For longer-lived invertebrates, practical difficulties mean that demographic studies are rare. Laskowski and Hopkin (1996) combined data from a three-month metal exposure study with the snail *Helix aspersa* and information on age-specific survival in the field, to construct transition matrices. The approach proved successful, suggesting that with further development, this targeted testing approach could prove useful (Spurgeon *et al.,* 2003).

2.5 Conclusions

No single biological measurement is in itself likely to provide clear evidence of an air quality outcome associated with industrial emissions. The lack of specificity of most responses tends to result from a generalised response to stress. Even where clear spatial and temporal patterns of response correlate with the pattern of emissions, biological measurements in themselves may not provide direct causal evidence that the response is related to emissions and not some other factor.

An integrated approach should therefore be adopted when using biological tests and chemical analyses in biomonitoring schemes to assess the extent of contamination and potential effects on habitats. All measurements should be considered complementary rather than stand-alone. A suite of specific and non-specific biomarkers covering a range of biological responses from molecular to whole organism changes should be used as screening-level indicators of widespread contamination, as well as community-level responses, chemical analyses and soil function tests.

Baseline variations in responses should be well characterised to ensure that deviations from the baseline demonstrate effects from stressors. This should also be useful for establishing whether remediation measures have been effective, as normal responses and variations will be known.

Some programmes would benefit from the use of non-specific tests while others, including those monitoring the success of remediation measures, should use tests targeted at specific pollutants. Ecosystems should continue to be monitored to measure the success of remedial action and for early warning of potential adverse effects before more severe, irreversible community-level changes occur.

3 Review of technical advances and developments (community analysis, demography and ecotoxicogenomics)

3.1 Background

The techniques described in this section are at various stages of development. Some may still be at the laboratory evaluation stage, some may be well recognised for laboratory but not field use, and some may have been tried under field conditions, but may not yet be fully recognised by the scientific community.

Publications first describing the original ideas and initial applications may be from a decade ago and may be published in fields other than environmental sciences. Within environmental sciences the techniques have all been applied in laboratory studies, but many have not yet reached maturity for use in the field. Indeed, in some cases, the methods are unlikely to reach this stage. Most techniques make use of fairly new technological or analytical developments which have permitted new ways of studying biological responses to chemical stress. Some are based on modifications to well-established methods that offer significant advances in understanding mechanisms of action of the chemical involved, or as diagnostic tools under field conditions.

3.2 Summary of available assays

Several approaches have been adopted from molecular ecology, population biology, toxicology and biotechnology to produce tools to better understand the responses of species living in contaminated ecosystems. These encompass methods that investigate responses at levels of biological organisation ranging from changes in population dynamics and community structures to differences in the expression of individual genes and the proteins they encode. Areas with particular potential include:

- lipid and nucleic acid analysis for assessing changes in the composition of the soil microbial community;
- use of life-cycle theory and population modelling to predict the long-term fate of exposed populations (Kammenga and Laskowski, 2000);
- measurement of changes in the genetic structure of an exposed population or the expression of genes linked to pollutant exposure and/or effect (Kille *et al.,* 2003; Snape *et al.,* 2004);
- measurement of changes in the expression of proteins linked to pollutant exposure and/or effect (Pennington and Dunn, 2001);
- profiling changes in activities of all metabolites (Lindon *et al.,* 2003a; Nicholson and Wilson, 2003);

- transgenic organism biosensors .

These areas are developing relatively quickly, and are thus at the forefront of many research programmes in environmental biology and risk assessment, such as the sixth framework programme of the European Union and a range of UK, European and international science programmes. Initial research has already produced several techniques that could have future uses in soil, air and water pollution monitoring. In many cases the techniques are still in development, although a few have already demonstrated clear potential to improve our understanding of the responses of species to chemically derived stress.

3.3 Community-based methods

3.3.1 Community census analysis using macrofauna/flora

The pollution-induced absence of species from contaminated areas where they would normally be expected to occur is strong evidence of effects on ecosystem integrity. Community studies of contaminated ecosystems have been carried out in a range of habitats. Macro-invertebrates are the best studied group as they are present in a range of diverse habitats, are relatively easy to collect and identify (when good keys are available), are functionally important as ecosystem engineers and have relatively low mobility, meaning they are representative of the local environment. These benefits outweigh disadvantages such as the uncertain taxonomic status of selected groups, the limitations of some sampling methods to provide a representative sample and the time demands for sample processing and identification. Another group with potential for community-based analysis are plants. Plants are sedentary, have established taxonomy, can be readily sampled and are functionally important. Despite these advantages, far fewer studies have used plant community structures to assess contaminated sites compared to macro-invertebrates.

3.3.2 Community census analysis with macro-invertebrates

Many studies have assessed the effects of chemical pollutants on soil communities, by measuring community changes in earthworms, springtails, ants, ground beetles, molluscs, oribatid mites and spiders along contamination or land use gradients. The changes observed included reduced community size (including extinctions), less diversity, altered composition and changes in species dominance/structure.

A large body of work has been conducted around the Avonmouth smelter in South West England to explore the use of invertebrate communities to detect pollution effects. This site has a long and defined contamination gradient across semi-natural land and pasture. Studies undertaken include assessing the distribution of major invertebrate groups (Hopkin and Martin, 1985; Martin *et al.,* 1982), a carabid beetle study (Read *et al.,* 1987) and earthworm community profiling (Spurgeon and Hopkin, 1996, 1999). By far the most detailed study of the effects of metal deposition on macro-arthropods was conducted at this site by Sandifer (1996). Results indicated severe impacts on decomposers such as earthworms, isopods, molluscs, myriapods, springtails and mites. These groups were all absent or reduced in abundance and/or diversity at the two sites closest

to the smelter and in some cases, also at more distant sites. Currently, this work is being repeated to establish its reproducibility.

The feasibility of using community census to monitor the impact of chemical contamination and land use on soil ecosystems has been explored in two studies. The SOILPACS study (Weeks, 1997) investigated the feasibility of the community census approach for soils. The study concluded that a system could be constructed, but substantial development was needed. In particular, full faunistic, physical, geological and chemical surveys of many (diverse) reference sites would be needed in order to develop a system with predictive capability. For development of the UK river invertebrate prediction and classification scheme (RIVPACS), over 400 sites were sampled in this manner. The fact that this number would need to be matched for SOILPACS presents a substantial hurdle to development.

The second study, the BBSK project, was launched in Germany as an initiative to develop a soil community-based monitoring scheme, but currently the method is not in day-to-day use. This is for a number of reasons, some of which are political, but scientific issues hindering development include a lack of data from which to establish the normal biotic community (or biocoenosis), taxonomic problems for some groups and the absence of internationally standardised sampling methods.

An invertebrate-based system worth considering is the nematode maturity index (MI) (Bongers, 1990). The system is based on the fact that nematode families adapted for rapid colonisation dominate soil nematode communities in disturbed ecosystems. At any site, recording of the families of nematodes present can be used to derive an MI value, with high values indicating undisturbed soils and low values those subject to perturbation. Reduced MI values have been found after nitrogen addition (Sarathchandra *et al.,* 2001) and pesticide use (Neher and Olson, 1999; Ruess *et al.,* 2001). For metals, Korthals *et al,* (1996) found a lower MI in copper, nickel and zinc, but not cadmium, spiked soils. Not all uses of the nematode MI have shown clear responses to contamination. Nagy *et al,* (1999) found that of 13 metals, only selenium and chromium consistently reduced MI; Fuller *et al.* (1997) found no effect of toluene on MI in two soils and an effect of trichloroethylene in one soil only. Despite these occasional anomalies, the vast majority of published work supports the fact that pollution or land use change decreases MI, whereas recovery coincides with MI increase. This approach is, at present, probably the best invertebrate community-based approach for soil quality assessment.

3.3.3 Community census analysis with plants

One recognised use of community census analysis for vegetation is to establish the effects of nitrogen and acidifying substances on plants. However, community-based surveys of the effects of more generalised chemical contamination on plants are relatively limited. Salemaa *et al,* (2001) recorded under-storey vegetation along a gradient from a copper/nickel smelter, and studies with sewage sludge and mine wastes have also been conducted (Stoughton and

Marcus, 2000; Vasseur *et al.,* 2000). These all showed clear effects on coverage, community composition and biodiversity. Overall, however, considering the frequency with which plant surveys are used to analyse long-term change in habitat structure and land use, full plant community surveys appear to have been underused for evaluating chemical effects.

3.3.4 Method evaluation

Macro-invertebrate and plant community profiling have both been widely used to assess the effects of pollutants on the ecological status of terrestrial ecosystems. Further, systematic methods based on fauna analysis (such as RIVPACS) have already been developed to assess the quality of stream and river waters. For these reasons, it may be surprising that we choose to include such surveys in this section.

To date, no systematic system for using soil faunal characteristics to classify the ecological status of sites has been established in any group except for nematodes. Three problems in particular are preventing the development of such a system. The first is the sheer volume of work needed to obtain the full census data and site characterisations for the many hundred of sites needed to be able to predict normal biocoenosis. Such work, whether for macro-invertebrates, plants or microbes, requires a major investment in resources, particularly as the composition of soil fauna and flora varies markedly through the seasons. The second problem is that, assuming a stage is reached at which the fauna for a site can be predicted with some confidence, full faunal characterisation of single study sites will require extensive sampling, processing and identification. Conducting such work will require extensive taxonomic expertise to commit to this routine task. Finally, there are technical limitations to the development of community monitoring for soil, such as the absence of standardised sampling methods, good keys and increasingly, the required taxonomic expertise.

The idea of developing a soil invertebrate or plant community-based scheme for assessing the ecological status of perturbed or contaminated soils has existed for many years. With the potential advent of the EU Soil Framework Directive, the need for suitable tools to assess effects on soil quality is becoming ever more apparent. It is not clear whether community-based methods will ever be a viable option for terrestrial systems, given the time-consuming nature of such studies and their demand for taxonomic expetise. Future research may need to focus on other multivariate methods capable of identifying deviations from normal in their subjects, such as DNA or fatty acid profiling in microbes, or transcription/metabolic profiling in sentinel species, as alternatives to detailed community assessment.

3.4 Microbial community profiling

3.4.1 Microbial community profiling: signature lipid biomarker analysis

Signature lipid biomarker analysis uses phospholipid fatty acid (PLFA), glycolipids, esterified lipids or lipopolysaccharide hydroxy fatty acid to identify the species and strain composition of a microbial community. Total lipid is extracted

directly from a soil sample using mixed organic solvent, from which lipid classes (neutral, glycolipid, polar) are separated out. In the most commonly used PLFA methd, the phospholipid containing polar fractions is subjected to mild alkaline methanolysis and the resulting fatty acid methyl esters (FAME) analysed by gas chromatography and mass spectrometry. The PLFA pattern is then analysed by multivariate methods. Although developments in nucleic acid technology have provided alternatives to PLFA for microbial community analysis, PLFA is still considered one of the most reliable methods for assessing the structural composition of microbial communities (Bell *et al.,* 2005a, 2005b). The reliability of PLFA means that the technique has now become one of the most widely applied and routine methods in environmental microbiology.

Thompson *et al.* (1993) were among the first researchers to use PLFA in a study of the composition of microbial communities associated with plant seeds and leaves, and also in soil samples. As well as bacteria, PLFA methods can also be used to study the composition of fungi and actinomycetes. Since its development, PLFA has become widely used in studies of the effects of environmental perturbations. Changes in phospholipid fatty acids, indicating a shift in the bacterial species composition, have been found in soil polluted by alkaline dust (Bååth *et al.,* 1998), sewage sludge (Witter *et al.,* 2000), metals (Kelly *et al.,* 1999; Khan and Scullion, 2000) and organic compounds (Fuller and Manning, 1998; Thompson *et al.,* 1999).

Most studies have been conducted in spiked soils, although a number have worked on contaminated field sites. Hinojosa *et al.* (2005) found lower microbial diversity in soils polluted by metals following the Aznalcollar acid mine waste spill (Meharg *et al.,* 1999). Bundy *et al.* (2004a) used the method to track the return of bioremediated oil-contaminated soils to a status approaching that of controls, while Ramsey *et al,* (2005) used both PLFA and broad functional measures for microbial communities in mine waste to provide insight into the importance of functional redundancy in preventing pollution-derived effects on ecosystem function.

These studies suggest that the method could be developed for contaminated soil assessment, although suitable baseline data must be available or alternatively, a similar site-specific control soil against which to compare profiles from polluted sites. In ongoing work to develop an ecological risk assessment method in support of Part IIa of the Environmental Protection Act (1990), conducted by CEH and other institutes on behalf of the Environment Agency, the availability and selection of site-specific control soils has been identified as one of the most important and potentially problematic issues for biological tests as part of risk assessment of contaminated land.

3.4.2 Microbial community profiling: nucleic acid analysis

Methods that analyse the microbial community from nucleic acid composition are based on use of the polymerase chain reaction (PCR). Universal forward and reverse primers are used in combination with PCR to amplify species-specific DNA fragments (usually 16S subunit of ribosomal DNA) from nucleic acid samples isolated from soil. Samples are then separated according to sequence

by a range of methods such as denaturing and temperature gradient gel electrophoresis (DGGE/TGGE); single strand confirmation polymorphism (SSCP); amplified ribosomal DNA restriction analysis (ARDRA); or terminal-restriction fragment length polymorphism (T-RFLP). Separation is followed by analysis, using methods such as image capture of gel banding patterns with subsequent image analysis of this data. If needed, the PCR products can also be collected for sequencing and thereby for species identification through bioinformatic analysis against public databases.

DGGE and TGGE have both been used to examine soil bacterial community responses to pollutants. For metals, Kozdroj and Van Elsas (2001) found that the microbial community in an industrially contaminated soil differed in richness and structure from clean soils. Muller *et al.* (2001) found altered community structure and decreased diversity along a mercury gradient, and Rasmussen and Sorensen (2001) and Gremion *et al.* (2004) also found a negative effect of metal exposure on both the species richness and diversity of the microbial community. For organic compounds, Duarte (2001) found a reduction in the numbers of detected bands with increasing soil hydrocarbon content and Andreoni *et al.* (2004) found lower diversity in heavily PAH-polluted soils. In a field study, Mouser *et al.* (2005) used community analysis by DGGE to classify pollutant levels in groundwater samples. This survey indicated that sites of the same contamination level could be grouped by principal component analysis, even when sites were distantly located. In contrast to these positive results, Kandeler (2000) found that addition of high concentrations of zinc, copper, nickel and cadmium did not change DGGE profiles, indicating potential differences in the sensitivity to change of different microbial communities.

SSCP, ARDRA and T-RFLP have not been as widely applied as DDGE for assessing microbial community change in contaminated soils. Cho and Kim (2000) used SSCP to identify shifts in bacterial communities in soils subject to two remediation treatments. Similarly, Junca and Pieper (2004) found a shift in the microbial community present in BTEX-contaminated soils using the SSCP approach. Again, not all studies using SSCP have provided positive evidence of an effect of contamination on communities. Beaulieu *et al.* (2000) showed reduced complexity of SSCP profiles in a pentachlorophenol-treated soil slurry, but no change in a combined pentachlorophenol and hydrocarbon wood preservative-amended soil.

Using ARDRA, Sandaa *et al.* (2001) found greater bacterial diversity in a high-sludge/high-metal amended soil compared with low-sludge, low-metal treatments. This study showed that soil microbial diversity may not always be linked to soil contaminants, but may instead depend on other soil factors (such as organic content).

Turpeinen *et al.* (2004) used T-RFLP to show that, while the glucose mineralisation capacity of metal-polluted and unpolluted soils was similar, contaminated communities were distinct from those in clean soils. Overall, the studies outlined above suggest that there is potential for the use of microbial

community profiling based on nucleic acid analysis to assess species richness and microbial diversity in contaminated soils. As alluded to above, care must be taken because profiles can be influenced by a range of soil factors as well as exposure to contaminants. Examples include land use variables; soil properties such as texture, pH, nutrient status and organic carbon content; and biological variables such as the nature of the resident vegetation and indigenous soil invertebrate community structure. These potential confounding influences again emphasise the importance of the choice of a site-specific control soil when designing a site-specific risk assessment programme.

A final method for analysis of microbial community characteristics is the use of DNA microarray technology. DNA microarrays have been used primarily to investigate gene transcription and regulation, for example in detecting the up- or down-regulation of metabolic pathways in the presence of pollutants. Recently, a small number of laboratories have begun to investigate the potential application of microarry-based methods for characterisng microbial communities (Wu *et al.,* 2004; Zhou, 2003). Arrays have been developed either as whole sections of genomic DNA or small oligonuceotides that relate to the different 16S rDNA at different taxonomic levels (such as kingdom, phyla, family, genus, species, strains) of the bacterial community. Sampling the community in the soil of interest gives an initial qualitative measure of the relative abundance of each of the taxonomic levels included on the microarray.

3.4.3 Method evaluation

Both PLFA analysis and the various methods based on nucleic acid analysis (except community microarrays) have been used extensively in laboratory studies and in the field on a number of occasions. These methods are well suited to standardisation and accreditation, and increasingly skilled staff are becoming available with the capability to undertake such assessments. For both of these methods, the major problems with their application are not technical, but rather ecological. Soil conditions unrelated to the presence or absence of pollutants (such as pH, organic content, mineralogy, season, climate, land use, soil management regime and changing plant cover) all have a pronounced influence on results from individual PLFA or nucleic acid composition analyses. Because some of these parameters vary even over small spatial scales (such as plant cover, local soil conditions) and others with time (weather, season), this makes it difficult to link any observed changes to changes in the concentration of a chemical stressor. If the widespread adoption of microbial profiling were to be proposed, the issue of what is ‘normal’ could be resolved through the collection of many hundreds of profiles from spatially and temporally referenced samples and the use of multivariate data methods to classify profiles based on these collection characteristics. Such work would, however, require time and money and could not be done ‘piecemeal’.

3.5 Life-cycle theory and models to predict the fate of exposed populations

3.5.1 Demographic modelling

Toxicity tests have been standardised for plants and a number of soil invertebrates. The prevailing view is that estimates of critical effect levels for a single sensitive trait can predict population effects. In reality, this is not the case. It is only possible to predict the long-term consequences of chemical exposure by considering the effects of pollutants on all life-cycle parameters (Forbes and Calow, 2002; Van Straalen and Kammenga, 1998). This is the principle that underlies demographic methods.

Because demographic modelling studies work best when data are available on the effects of chemicals on different vital rates (age-specific survival, maturation time, fecundity) over the full duration of the life cycle, it is unsurprising that most studies have used short-lived species (Van Straalen and Kammenga, 1998). In invertebrates, favourites are cladocerans and copeopods in freshwater, and springtails and nematodes in soil. For the latter group, Kammenga *et al.* (1996) optimised a life-cycle test for *P. acuminatus* that can be adapted for other species, such as *C. elegans* (Jager *et al.,* 2005; Jonker *et al.,* 2004). Crommentuijn *et al.* (1997) have also developed a life-cycle based approach with the springtail *F. candida*. For longer-lived invertebrates, practical difficulties mean that demographic studies are less common.

In aquatic systems, species such as the oligochaete *Capitella* and the larval stage of the midge *Chironomus* have been successfully used to model chemical exposure (Forbes and Cold, 2005; Forbes *et al.,* 2003). In soil species, Laskowski and Hopkin (1996) combined data from a three-month metal exposure with the snail *Helix aspersa* and information on age-specific survival in the field to construct transition matrices. With further development, this targeted testing approach could prove useful (Spurgeon *et al.,* 2003). Data collected from life-cycle tests can be analysed using structured population models to predict the effects of exposure on population growth rate and the relative contribution of changes in individual traits to this overall effect (Kammenga *et al.,* 2001).

Plants also have the potential for demographic testing. Sheppard *et al.* (1993) carried out a 35-day test with *Brassica rapa*. Comparison of the results with a suite of assays in metal-spiked soils indicated that bloom initiation was more sensitive than lettuce emergence or earthworm survival for zinc, but not for mercury. Saterbak *et al.* (1999) used this test on hydrocarbon-contaminated soil. The study, which used *Brassica rapa* selectively bred to reduce generation time, proved unsuccessful due to low germination rates in the control soil, suggesting that further development is needed. .

3.5.2 Dynamic energy budget model

A further approach to analyse the potential population effects of exposure, also based on measurement of changes in life-cycle traits, is the dynamic energy

budget (DEB) model (Kooijman, 1993). According to the principle of allocation, energy resources in an organism are partitioned between reproduction, somatic maintenance and growth. These traits are inextricably linked in the competition for finite energy resources. The DEB model makes a number of assumptions: that growth and maintenance compete more closely with each other for available energy than reproduction; that energy requirements take precedence over growth and reproduction; and that food intake is proportional to surface area of the individual, with growth and reproduction proportional to the wet weight of the individual.

DEBtox, a modelling program developed by Kooijman (1993), can be used to evaluate how toxic substances alter the allocation of resources, where chemicals with different modes of action may change the allocation patterns. Jager *et al.* (2004) have developed scripts in DEB to model the potential effects of toxicity through different modes of action (reduced energy acquisition, increased maintenance, toxicity for growth, toxicity for reproduction, hazard to embryo) and compare these against measured data. This approach can provide a more mechanistic insight into the possible energetic effects of chemical exposure that underpin ecological effects.

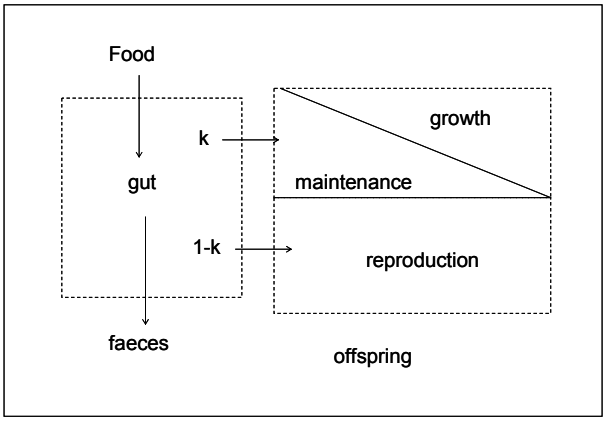

Figure 3.1: Energy channelling, taken from Kooijman and Metz (1984)

A major issue in demographic analysis is translating the effects of chemical stress on vital rates, and ultimately on population growth rate, to effects on the dynamics of populations in contaminated environments. To address the role of density-dependent mechanisms, joint effects can be measured in studies of the combined effects of population density on population growth rate (Hooper *et al.,* 2003; Linke-Gamenick *et al.,* 1999). Alternatively, density effects can be modelled by assuming that the population density tends towards an equilibrium value or other kind of attractor intrinsic to the life history and vital rates.

A number of methods can be used to develop population models. Many experts use generic modelling software, the use of which requires some effort to become familiar with the particular programming. Some specialist packages and add-ins targeted specifically at those interested in demographic modelling can be easier

to use. These can simplify the modelling process by providing menu-driven options and a help menu on the basics of population modelling and use of the software. As with all modelling, the outputs of such models depend on reliable estimates of inputs.

3.5.3 Method evaluation

Like macro-invertebrate and plant community profiling, population viability analysis through life-cycle theory and demographic effect assessment has been around for some time. Its use in ecotoxicology has become widespread, with a number of theoretical predictions already made on the likely population-level impacts of pollutants (such as lower population growth, smaller equilibrium population size). Demographic studies work best on short-lived species, although the feasibility of the approach for long-lived organisms has also been explored. The principal advantage of the demographic approach is that it provides information directly relevant to long-term population fates. As the principal aim of environmental management is to preserve populations within the boundaries of their natural variation, this information is particularly useful to outcomes-based monitoring and regulation. The trade-off is that the tests take longer and are more complex than single-parameter assays.

For long-lived species, the length of exposure needed means that the method is only suited to analysis of priority pollutants. For short-lived species (such as soil dwelling nematodes and some springtails), the method has potential for soil quality assessment, but to date this has not been systematically tested in the field. Overall, population viability analysis through modelling is founded on powerful theory, and could become a useful tool for comparing potential effects on populations.

3.6 Airborne remote sensing of plant physiological stress

3.6.1 Development of methods

Chemical emissions and accidents at industrial installations can spread pollutants over a wide area (many square kilometres). This can make the assessment of effects on individual plants or animals difficult, requiring carefully designed sampling strategies. Methods that can help to highlight areas of greatest potential concern (hotspots or highest points on contamination gradients) can be valuable here. Analysis of chemical concentrations in the environment and in exposed organisms can help to establish the initial distribution of contaminants, and thereby assist the design of a sampling plan. Indeed, if the distribution of chemicals in an area is not considered, the choice of sampling sites is unlikely to optimise the amount of information gained for the level of resource expended. Techniques that give some insight into the possible distribution of contaminants on a large scale (extent of the whole potentially contaminated area) could therefore be of great value. One method that could be used for a first-pass assessment is a remote sensing method capable of detecting plant stress.

Several studies have linked responses in leaf spectral reflectance, transmittance or absorption to physiological stress in plants. A variety of stressors including dehydration, flooding, freezing, exposure to ozone or herbicides, competition, disease, insect damage, deficiencies in ectomycorrhizal development and N fertilization have been imposed on species ranging from grasses to conifers and deciduous trees (Carter and Knapp, 2001; Lichtenthaler, 1988). By monitoring changes in the reflectance of leaves within the 400-850 nm wavelength range (particularly around 700 nm) (Carter and Knapp, 2001), remote sensing methods are able to detect varying chlorophyll concentrations in plants leaves. Such changes in chlorophyll content have been linked to physiological symptoms of stress, such as chlorosis. In particular, cases have been linked to the presence of toxic chemicals such as metals, as well as to changes in the availability of essential nutrients (Lei, 2001; Odasz-Albrigtsen *et al.,* 2000; Rosso *et al.,* 2005; Schuerger *et al.,* 2003).

Initial chlorophyll fluorescence monitoring as a measure of plant stress made use of handheld devices, but more recently instruments have been mounted on airborne systems. These systems can scan the whole receiving ecosystem to identify areas of apparent plant stress. The systems thus act as a first scan to target more detailed investigation into the cause of any observed stress effects

3.6.2 Method evaluation

Airborne remote sensing is currently used to detect stress-induced changes in plant leaf chlorophyll content. The value of this approach lies in targeting more detailed chemical and biological measurements on areas of greatest potential concern, as well as providing an initial indication of any chemically-induced stress effects on plants. One issue in interpreting remotely-sensed (or *in situ*) chlorophyll fluorescence data is that the method does not differentiate betwen causes of stress. Changes in leaf chlorophyll fluorescence can be caused by water stress or disease, as well as by pollutants or nutrient availability. Similarly, the absence of an effect on leaf fluorescence does not necessarily imply an absence of chemical effect. At chronically or historically polluted sites, adaptation may have resulted in plants more able to survive in soil that would affect naïve plants without evident physiological symptoms. For these reasons, remote sensing data need to be interpreted in conjunction with other background data on site characteristics and possibly alongside further chemical analysis or biological effect assessments.

3.7 Measurement of changes in the genetic structure or gene expression of exposed populations

3.7.1 Molecular genetic assays: genome mutation analysis using PCR-based methods

Exposure to genotoxins can lead to the formation of DNA adducts, the faulty repair of which can result in point sequence mutations. Two molecular genetic techniques, randomly amplified polymorphic DNA (RAPD) and arbitrarily primed PCR (AP-PCR) can be used to detect point mutations. RAPD has been used to

investigate DNA mutation in parallel with life-cycle responses in *Daphnia magna* exposed to B[a]P (Atienzar *et al.,* 1999). Results showed that RAPD was more sensitive than co-measured fitness parameters. In barley, Liu *et al.* (2005) used the method to differentiate cadmium-exposed and unexposed plants. Savva (2000) used AP-PCR to detect DNA damage in rats and shore crabs exposed to B[a]P in the laboratory and in crabs from control and polluted areas. Results indicated differences in fingerprints between treatment in both the laboratory and field samples.

One issue with this technique is that the choice of mutation detection method would be based on initial screening of the potential presence of known genotoxins. A strong conceptual model of the site and, if possible, good analytical data on the contaminants present would thus be a prerequisite to any decision to apply RAPD or AP-PCR. Should such evidence be available, RAPD could prove a useful tool for ecological (and even human) health effect assessment, given that the mechanisms leading to point sequence mutation may be conserved between species.

3.7.2 Molecular genetic assays: single gene transcript quantification methods

A central paradigm in (eco)toxicology is that there is a tiered cascade in biological responses to chemical exposure. The first responses to stress will occur at low levels of biological organisation (such as changes in gene and protein expression). If the severity of the toxic stress increases, these molecular effects will combine to induce cellular effects and then tissue-level dysfunction. Further increases in toxic stress will lead to changes in the life cycle of the organism and ultimately to population and community changes.

Given that changes in the expression of genes and proteins will be the first indications of an impact on an organism, it has been widely recommended that such parameters be used as sensitive measures of pollutant exposure (Kammenga *et al.,* 2000; Peakall, 1992; Snape *et al.,* 2004). A suitable method for assessing changes in the expression of any gene (quantification of the amount of specific mRNA present in cells) is the reverse transcriptase polymerase chain reaction (RT-PCR). Quantitative analysis of gene expression using RT-PCR requires a representative sample of RNA from the test organism which is converted to complementary DNA (cDNA) via the reverse transcription reaction. This cDNA sample can then be used for each gene-specific quantification via PCR using electrophoretic or fluorescence detection. As the measurement of changes in the expression of potentially pollutant-responsive genes can be undertaken for a vast range of sequences using a variety of protocols, it is not possible to outline all possible targets and approaches here. Instead, two aspects will be summarised: firstly, details of sequences that can potentially be analysed; secondly, a brief description of measurement techniques.

3.7.3 Genes for analysis

Over the past 15 years, extensive research has aimed to identify genes differentially expressed in organisms exposed to toxicants. Initially, studies

focused on human or rodent models. More recently, the field of environmental genomics has emerged, meaning that homologues of pollutant-responsive sequences have been identified in several species (Cossins and Crawford, 2005; Stürzenbaum *et al.,* 2004). Sequencing programmes, expressed sequence tag (EST) projects and techniques to identify differentially expressed genes have all helped to identify, clone and sequence pollutant-responsive genes (Gershon, 2002; Neumann and Werner, 2000; Steiner *et al.,* 2004; Sultan *et al.,* 2000) and are starting to become widely applied in the identification of pollutant-responsive genes in wildlife (Dallinger, 2004; Filby and Tyler, 2005; Galay-Burgos *et al.,* 2003; Miracle *et al.,* 2003; Snell *et al.,* 2003).

When choosing molecular targets to quantify gene expression, the influence of a range of factors on the target sequence should be established, including:

- the degree, magnitude and direction of the transcriptional change;
- the range of pollutants for which the gene response is elicited;
- influences of environmental condition and biological factors on gene expression;
- temporal stability of the transcriptional response.

In reality, the range and complexity of contaminants present in soil and water means that even for a single species, a single gene suitable for quantitative assessment of the mode of action of a contaminant is unlikely to be identified. Measurement of a suite of responsive genes, including both generic and chemical-specific sequences, is likely to offer a more viable approach. As gene microarrays that can simultaneously assess the responses of multiple genes become cheaper, transcriptomic platforms may become the preferred option for expression quantification. Until this time, large-scale studies are likely to use microarray data to indicate potential mechanisms, followed by detailed measurements and investigations of the activities of single genes.

Among known sequences, three primary groups of genes are commonly found to be differentially expressed following exposure to toxic chemicals. These can loosely be grouped into biomarkers of exposure, physiological compensation and effect. Examples within each of these three categories are given below.

The ‘exposure’ category includes genes encoding proteins known to be involved in pollutant handling and detoxification pathways, and a number of established biomarkers are represented. For metals, well-known examples are the genes encoding for metallothioneins in microbes, animals and plants (Dallinger *et al.,* 2004; Kille *et al.,* 1999; Maywald and Weigel, 1997; Stürzenbaum *et al.,* 1998), phytochelatin synthase in plants and animals (di Toppi and Gabbrielli, 1999; Heiss *et al.,* 2003; Maywald and Weigel, 1997) and ferritin in plants and *Daphnia magna* (Chiancone *et al.,* 2004; Kumar and Prasad, 1999; Richards *et al.,* 2000). In addition to these genes, which may exhibit differential expression in a range of species, a number of genes encoding metal chaperones have also been identified in genetic models. These include a suite of intracellular copper chaperones in yeast (Culotta *et al.,* 1999), arsenic-responsive genes that form

the *ars* operon (Sarkar, 1999) and mercury-responsive genes in the *mer* operon (Kiyono *et al.,* 2000).

Several pathways are involved in the detoxification of organic compounds and a number of genes that encode for these have been found to be upregulated. Among the most widely distributed and responsive are cytochrome P450s, particular the 1a isoform (Baumgart *et al.,* 2005; Meneses-Lorente *et al.,* 2003; Menzel *et al.,* 2001), and glutathione-S-transferases (Gallagher and Sheehy, 2000; Hodgson and George, 1998; Pathiratne and George, 1998; Shen *et al.,* 2003).

The 'physiological compensation' category includes a range of genes involved in adaptive biochemical pathways. Best known are the heat shock family of protein chaperones involved in protein folding, which have been found to respond to a range of metals and organic chemicals (Ait-Aissa *et al.,* 2003; Mukhopadhyay *et al.,* 2003). Other genes found to be responsive to pollutants include mitochondrial genes (Galay-Burgos *et al.,* 2003), where responses increase the level of the mitochondrial message either by an increase in energy demand or via toxic effects on the organelle. A further group of compensatory genes are linked to lysosomal function (Kille *et al.,* 1999; Liao *et al.,* 2002). Upregulation of these represents an increase in lysosomal activity in pollutant-exposed cells. Metal-containing enzymes can also be upregulated in response to pollutants, notably metals that compete for binding in the active site. Finally, genes encoding proteins in the antioxidant system, such as superoxide dismutase, catalase and peroxidase, are also commonly found to respond to chemical and other stresses in a diverse range of species. Indeed, effects on this system are some of the most widely observed physiological responses to chemical stress in plants and animals (De Coen and Janssen, 2003; Guecheva *et al.,* 2003; Vezina *et al.,* 2004).

In the third category, response-linked biomarkers, rather than being directly linked to pollutant handling or compensatory systems, are instead involved in physiological effects. The most well-known example is vitellogenin, particular where exposure to environmental oestrogens is known or suspected (Brion *et al.,* 2004; Celius *et al.,* 2000; Denslow *et al.,* 2001b). This protein, whose major function is to provide nutrition for the developing embryo, has been isolated and fully characterised from a large range of egg-laying vertebrates and invertebrates. Other potential reproductively linked genes quantified as indictors of pollutant exposure are the zona pellucida proteins (Arukwe *et al.,* 2001a, 2001b; Denslow *et al.,* 2001a), which maintain the species specificity of fertilisation. Annetocin, an oxytocin-related peptide isolated from the earthworm *Eisenia fetida* and known to induce a series of egg-laying related behaviours, is another potential target in this group (Inomata *et al.,* 2000; Oumi *et al.,* 1994; Ricketts *et al.,* 2004). A further group of genes linked to the effects of exposure are those involved in the apoptotic pathway responsible for programmed cell death. To date, relatively few studies have been carried out to specifically link the expression of these genes to pollutant exposure.

3.7.4 Development of a RT-PCR method for a gene of interest

Quantitative analysis of gene expression in any biological system by RT-PCR requires the progression of samples through a series of experimental steps.

First, based on leads from expression studies, the required gene product should be cloned and sequenced. Sequences can be identified using bioinformatic methods by comparison with existing databases or using functional approaches. Probes and primers to detect the gene target can be designed following sequencing. A series of quality control checks is then carried out, to confirm that primers amplify only the single targeted sequence, that is, only the expressed gene product and not the genomic DNA region from which it is derived. This can be done either bioinformatically or by resolving gene product on a gel to confirm the presence of only a single PCR product of the correct amplicon size. Assuming the primers are specific, optimisation of the assay should follow. For quantitative analysis, a set of standards for each sequence should be prepared based on a dilution series of known concentrations of either a plasmid preparation containing the gene of interest or a quantified sample of PCR product. These standards are used to generate a standard curve that describes the amplification patterns of different concentrations of the initial starting transcript. This standard curve can then be used to assign gene transcript concentrations within the unknown samples (see section B4.16 for a full description of this method).

Along with reagents and standardised and custom-made probes to measure transcript levels for particular genes, manufacturers can supply analytical instruments for PCR and detection of fluorescence over the course of the reaction. Different instruments have different approaches to measuring fluorescence. Some are multi-well plate based, allowing many samples to be run simultaneously, while others use capillaries which can be heated and cooled very quickly. Analyses can thus be run more rapidly than systems using heated blocks for temperature control.

Quantitative RT-PCR studies allow the expression of the gene of interest to be measured quantitatively as well as qualitatively. Quantification of the target gene should be normalised to account for differences in the amount of template present. For this, parallel measurement of a control (non-responsive) gene (or genes) can be employed (Bustin, 2000; Stürzenbaum and Kille, 2001). An alternative approach is to normalise the amount of RNA added to the reverse transcription, using the concentration measurements made for the total RNA extract.

3.7.5 Transcriptomics using oligonucleotide and cDNA microarrays

With the increasing volume of sequence information available in public databases, new technologies have been developed to measure changes in the expression of multiple genes simultaneously, the best known of which are microarrays (Schena, 1999). Microarrays consist of hundreds to thousands of oligonucleotide sequences, cloned fragments or whole cDNAs printed onto a solid support (slide or membrane). By sampling all the expressed genes in an

organism (the total RNA complement) and hybridising these to multiple sequences of known gene identity printed on the microarray, a measurement of the expression of multiple genes can be made.

In practice, gene expression is analysed by fluorescently labelling the total mRNA (during the reverse transcription phase) from organisms exposed to different treatments. The labelled material is placed on the array either as individual (one colour) or paired reference and treatment (two colour) samples, one being labelled with a red dye and the other with a green dye. In the one-colour system, expression is measured as the total amount of fluorescent mRNA hybridised to each spot on the array. In the two-colour system, the labelled cDNA is mixed and hybridised to the DNA spots on the array slide. If the gene is differentially expressed between the two conditions, one colour dominates and the spot appears red or green. If there is no difference, colours merge and the spot appears yellow.

The first published studies of microarrays used to analyse environmentally-induced changes in global gene expression were limited to applications in functional genomics, including analysis of expression in different life stages (Jiang *et al.,* 2001; Wang and Kim, 2003), control of longevity (McCarroll *et al.,* 2004; Murphy *et al.,* 2003) and detailed mechanistic understanding of biological processes (Schnable *et al.,* 2004; Zhang *et al.,* 2002). The method was then adopted by medical research for disease and cancer diagnostic investigations (Jiang *et al.,* 2004; Smith *et al.,* 2004).

The potential power of the microarray (and its commercial potential for new drug discovery) quickly established it as a core research method in toxicology. This led to a surge in publications in toxicogenomics, where studies assessed responses to single compounds to improve our understanding of possible modes of action and effects of individual substances (Andrew *et al.,* 2003; Terasaka *et al.,* 2004; Yamada and Koizumi, 2002). Microarrays have also been used for clustering and separation of toxicants with the same and different modes of action (Parker *et al.,* 2003; Steiner *et al.,* 2004). One of the most comprehensive applications of microarrays in toxicology and drug discovery has been the categorisation of chemicals that cause hepatic and renal toxicity. Based on a database of thousands of separate microarray analyses, in combination with associated histological data, Natsoulis *et al.* (2005) and Fielden *et al.* (2005) used a supervised multivariate data analysis method (support vector machines) to identify a minimal transcript signature of early stage liver and renal tissue damage. These signatures can be used to categorise unknown compounds for their potential toxicity to these organs. The power of this approach, based on identification of the signature of chemically-induced disruption of normal physiology, has clear parallels in environmental biology and pollution monitoring.

Environmental sciences are also making use of microarrays. To date, microarray studies have been published for bacteria, yeast, a range of crop and non-crop vascular plants, protozoa, nematodes, molluscs, crustaceans, insects from many orders, fish, amphibians, birds and mammals such as mouse, rat and man. Ongoing research projects mean that many further microarray systems will

become available for studies in air, soil and fresh, brackish and salt water. Projects have already shown that microarrays can be used to identify stressed organisms (Matsuyama *et al.,* 2002) and common responses to stress (Becher *et al.,* 2004). This approach has potential, but will only be realised through systematic studies that separate transcriptional changes linked to exposure and chemically-derived biological effects from the normal variability of physiological responses in species living under normal (and at times sub-optimal) climatic and food availability conditions. If such chemical effect signatures can be identified, these will not only offer fundamental information on responses to stress, but may also lead to reliable diagnostics of pollution effects.

3.7.6 Method evaluation

The last 30 years have witnessed the development of methods to analyse the activities of genes encoded by an organism's DNA. The point has now been reached where the development of assays for single genes in a particular species are comparatively routine. The power of oligonucleotide and cDNA arrays to monitor changing expression in thousands of genes has brought optimism that this technology will become one of the dominant methods in biology. Recent research investments have extended the use of microarray techniques into the environmental field, including ecotoxicology. The potential power of gene-based assessments lies in the fact that they operate at the lowest level of biological organisation, giving them the potential to be sensitive. In the case of microarrays, a further advantage is that they characterise responses across the whole transcriptome, meaning there is a high likelihood of detecting gene expression changes.

One of the many problems with array studies, however, is that simultaneous analysis of so many genes creates particular problems in interpretation. To date, transcriptional studies (especially with microarrays) have been largely laboratory-based. For field monitoring, the challenge lies in identifying the pollution signal amongst the noise of transcription change driven by diurnal and seasonal fluctuations and by environmental variations unrelated to pollution. In effect, the problem with microarrays is the same as that for microbial and community methods, namely establishing the boundaries of what is normal and developing computational methods to identify deviations based on the obtained transcriptional data. As with community studies, the solution lies in a database of sufficient breadth to allow such response categorisation to be made. The nature of microarray work demands that a single species (or possibly closely related group of species) be selected as the bioindicator(s) of choice for such studies.

A further concern with any molecular method is the perceived cost. While chips from manufacturers are currently expensive, new methods for printing are being developed with the potential to reduce the costs of required hardware greatly, making the use of microarrays for routine studies a realistic possibility. Indeed, compared to the cost of some other methods (such as those that need extensive taxonomist or ecotoxicologist time), it could be argued that transcriptional studies already offer good value.

3.8 Proteomics

3.8.1 Method and application

Proteomics has been made possible by the advent of techniques to separate proteins based on some physical characteristic (such as size, charge or both), and for protein identification using time-of-flight mass spectrometry. The field of proteomics is developing rapidly, with a number of new separation techniques becoming available. Since many methods are in the early stages of development, and often focus on detecting an increased percentage of protein in the proteome, a technical discussion is not appropriate here (for a review of these, see Ferguson and Smith, 2003). Instead, two of the simpler and more widely applied methods are discussed here.

The most traditional approach to proteomic analysis is by separation of proteins using two-dimension electrophoresis (2-DE). This method separates proteins on a sodium dodecyl sulphate (SDS) polyacrylamide gel. First-dimension separation is by charge using isoelectric focusing, and second-dimension separation is by size. Following separation, gels are visualised and image analysis is used to identify differentially expressed proteins. These can then be excised from the gel for characterisation and identification by time-of-flight mass spectrometry, following a tryptic digest to form a mix of peptides. Excised protein can be most rapidly identified when the complete genome sequence of the organism is available, where the peptide patterns detected for each protein can be matched against those predicted from known open-read frames in the genome. When a full sequence is not available, it may be possible to use data from ESTs for protein identification (Wasmuth and Blaxter, 2004). Alternatively, more time-consuming tandem mass spectrometry methods can be used.

The 2-DE and mass spectrometry approach to proteomics has been applied in human health research, including toxicology, although studies of proteome responses to environmental chemicals have only been conducted relatively recently. Kuperman *et al.* (2004) used the approach to identify differentially expressed proteins in earthworms exposed to chemical warfare agents. Toxicological studies have also been undertaken, where Vido *et al.* (2001) analysed yeast cells exposed to an acute cadmium stress; 54 proteins were induced and 43 repressed. Bradley (2000) used 2-DE to demonstrate differences in protein expression signatures from oysters collected along a chemical gradient. While preliminary, these results suggest that proteomics may become a valued tool in future ecotoxicological assessment.

A more recent proteomics approach that is superseding the traditional 2-DE method is the protein chip technology known as surface enhanced laser desorption/ionization (SELDI) technology. These protein chips allow researchers to capture, separate and quantitatively analyse proteins directly on the chip from crude biological samples. The arrays are read (protein molecular weights) directly without radioactive or fluorescent labels or genetically-engineered tags that may interfere with the protein. Coupled with the detection system is a pattern recognition program that accelerates the search for diagnostic or prognostic

patterns and automates the recognition of multivariate protein patterns associated with particular biological treatments such as chemical exposure or disease. Overall, the SELDI approach could have several advantages over traditional proteomics including higher throughput, versatility, ease of use and comparatively low cost.

Using a SELDI analysis, Tong *et al.* (2004) demonstrated a high (above 98 per cent) sensitivity and specificity for detecting and segregating cancerous and non-cancerous samples. The technique has also been used to identify new biomarkers of disease in man. Pampanin (2004) analysed hemolymph (blood) samples from marine organisms (*Mytilus edulis*, *Carcinus maenas* and *Strongylocentrotus droebachiensis*) and showed that the technology was able to clearly distinguish exposed animals from non-exposed animals. The approach has also been used to study the effects of toxicant and endocrine active compounds on marine mussels, where the method detected a range of proteins upregulated following exposure to dispersed oil and oil spiked with alkylated phenols and nonylphenol (Bjørnstad *et al.,* in press). A similar study of protein signatures in mussels exposed in cages at three sites showed that multivariate and neural net analysis of protein patterns could distinguish the sites based on a suite of proteins, with highly significant differences in peak intensities (Knigge *et al.,* 2004).

These results support the idea that SELDI protein profiling can identify contaminated sites and aid discovery of new biomarkers. As with transcriptional studies, the capacity to reliably discriminate between contaminant-stressed and unstressed animals subject to normal environmental variability will improve as the database of protein signature information and associated toxicological effects expands.

3.8.2 Method evaluation

Proteomics remains probably the most challenging of techniques for systems biology, with a number of problems still to be overcome. Sample throughput is lower than for transcriptional studies and metabolic profiling. Current techniques are capable only of detecting the more abundant proteins and not the rarer ones, such as transcription factors. For biological assessments of chemical effects, the latter issue may be less of a problem, given that chemical effects are mediated through major pathways such as energy management; as a result, many of the proteins chemically induced or repressed are abundant. Compared to transcriptomics, proteomics measures actual changes in the biologically active protein complement of cells, rather than inferring this through transcripts. Currently, however, proteomics remains a method being developed in the laboratory, with little testing in the field. When this changes, the major problem to overcome will be the same as for transcriptomics, namely identifying the boundaries of normal physiology under naturally fluctuating conditions.

3.9 Metabolomics using nuclear magnetic resonance spectroscopy and mass spectrometry

3.9.1 Method and application

Metabolites are small molecules or non-polymers that represent the substrates and products of chemical reactions occurring within a cell. These include well-known compounds such as sugars, lipids, amino acids and nucleotides and more novel structures, sometimes of unknown function (particularly in less well-studied taxa). Metabolomics is the study of the levels and composition of these metabolites within a cell. In terms of nomenclature, both metabolomics and metabonomics are used. Although absolute definitions are still being discussed, it is widely accepted that metabolomics describes the levels of native metabolites in a cellular system, whilst metabonomics describes a systemic response profile in a sample collected from a complex, multi-cellular organism. In parallel with proteomics, many chromatography, mass spectrometry and spectroscopy techniques are available to analyse a discreet class of metabolites. Two methods which are widely used are discussed here, namely ^{1}H nuclear magnetic resonance spectroscopy and mass spectrometry.

Nuclear magnetic resonance (NMR) spectroscopy is probably the most widely used for metabolomics. The technique is based on the fact that atomic nuclei orientated by a strong magnetic field absorb radiation at characteristic frequencies. In different atomic environments, nuclei of the same element give rise to distinct spectral lines. This makes it possible to observe and measure signals from individual atoms in complex macromolecules and from these, to interpret molecular structure. There are a number of NMR active nuclei: ^{1}H is the most sensitive and stable nucleus; ^{13}C may also be used, but has low sensitivity and abundance; and ^{31}P and ^{19}F can be used for specific applications.

The use of NMR spectroscopy in environmental sciences is comparatively undeveloped compared with toxicology and drug discovery (Lindon *et al.,* 2003b; Nicholson *et al.,* 2002; Nicholson and Wilson, 2003). In soil science, NMR has been used in studies of the nature of soil organic matter and pollutant binding to soil constituents (Kohl *et al.,* 2000). The method has also been used in environmental epidemiology to classify diseased shellfish and identify the mechanisms and putative early disease biomarkers of the disease phenotype (Viant *et al.,* 2003a, 2003b). In ecotoxicology, ^{1}H-NMR has been used for initial characterisation of metabolic profiles in a range of invertebrates (Gibb *et al.,* 1997). In earthworms, the method has been used to identify increasing free histidine in tissue extracts of *L. rubellus* (Bundy *et al.,* 2001) and model compound (substituted anilines) effects on metabolite profiles in *E. veneta* (Bundy *et al.,* 2001; Warne *et al.,* 2000).

The NMR method has also been used to classify the biochemical effects of a range of fluorinated organic compounds, where the study separated compounds based on their biochemical effects (Bundy *et al.,* 2002). In a field study, NMR-based metabolomics was used to differentiate between earthworms at different sites on a metal pollution gradient and to identify putative biomarkers based on

sugar and histidine metabolism (Bundy *et al.,* 2004b). NMR spectroscopy is also a well-established tool in plant biology and has been used to study physiological stress (Fan *et al.,* 1992). It would be easy to extend these studies to investigate toxic effects at contaminated sites.

Literature on the use of mass spectrometry to assess metabolite profile changes is also well populated, including the use of liquid and gas chromatography-based separation techniques prior to mass spectrometry. Only a relatively small number of studies have measured endogenous metabolites, although mass spectrometry has been used in a variety of toxicological applications (Griffin and Bollard, 2004; Lenz *et al.,* 2005).

3.9.2 Method evaluation

Metabolomics has shown its value as a tool for assessing the mode of toxic effects in human toxicology. Sample throughput can be high using the NMR method and the consumable cost is low. For both NMR and mass spectrometry, the expense is in the purchase of equipment to undertake analyses. Machine costs run to several hundred thousands of pounds and so are beyond the reach of many small laboratories. Increasingly, universities are building facilities dedicated to metabolic profiling for drug discovery and disease research. While access to the best machine at a site may be restricted for environmental scientists, it is increasingly possible to secure time on less advanced equipment, and this is usually more than adequate for the task.

As for transcriptomics and proteomics, metabolomic studies have been largely laboratory-based, although field studies to identify pollutant and disease effects have been conducted. For field monitoring, the major challenge lies in identifying the pollution signal within the noise of normal environmentally-driven metabolic variation. High throughput makes this technique well suited to conducting the number of analyses required to build up baselines in a suitable environmental sentinel species; funding of these essential baseline studies is needed.

3.10 Biosensors

3.10.1 Transgenic bacterial biosensors

Systems such as the Microtox™ assay use the marine species *Vibrio fischeri* as the sensor. Because it uses a marine bacterium, Microtox™ must be conducted in saline solution which is ecologically irrelevant for most soils. As no naturally luminescent soil bacteria alternatives are available, one solution is to fuse the genes responsible for bioluminescence into soil-dwelling strains using recombinant technology (Paton *et al.,* 1997). Two approaches can be used:

- in an approach analogous to Microtox™, light output can be linked to metabolic activity so that any chemical that disrupts metabolism decreases light output;

- the *lux* gene can be fused to a functional gene linked to pollutant homeostasis.

Soil-dwelling bacteria marked with the *lux* gene cassette include *Pseudomonas fluorescens*, *R. leguminosarum bv trifolii*, *Escherichia coli* and *Burkholderia* sp. Of these, *P. fluorescens* has been most widely used for the metabolic system. Light output has been found to be reduced by heavy metals (singly and in combination) (Chaudri *et al.,* 1999; Palmer *et al.,* 1998; Paton *et al.,* 1995), organometals (Bundy *et al.,* 1997), benzene (Boyd *et al.,* 1997b) and chlorophenols both singly (Boyd *et al.,* 2001; Shaw *et al.,* 2000) and in combination (Tiensing *et al.,* 2002). There are a few examples in which a change in bioluminescence has not been found, including for PAHs phenanthrene, pyrene and B[a]P (Reid *et al.,* 1998). Turner *et al.* (2001) used the *P. fluorescens* marked strain in a temporal study to separate calibration pollutants, with response patterns then used to identify unknown environmental samples. An 83 per cent success in identification was achieved.

The increased ecological relevance of engineered soil bacteria over Microtox™ is clear. However, comparative studies of the two systems are relatively rare. Boyd *et al.* (1997a) screened contaminated groundwater using both biosensors. Results indicated the reproducibility of *lux*-marked *P. fluorescens* and Microtox™ was similar. For chlorobenzenes and chlorophenols, response profiles for the *lux* system agreed with fathead minnow, cilliate and diatom toxicity test results, but not with Microtox™ (Boyd *et al.,* 1998, 2001). Bundy (1997) also found different response profiles between the two systems for organotins, with Microtox™ more sensitive to tri-organotins and *P. fluorescens* to di-organotins.

An alternative to linking *lux* to metabolism is fusion to the promoters for functionally active genes such as HSPs, and enzymes involved in degradation or resistance. Van Dyk *et al.* (1994) fused *lux* and heat shock genes in *E. coli*. This system was used to establish the toxicity of a range of organic and inorganic pollutants. A further application of the *lux* gene by Heitzer *et al.* (1992) involved insertion into the genes responsible for naphthalene and salicylate catabolism in *P. fluorescens*. This approach could be used for *in situ* monitoring of hydrocarbon remediation.

3.10.2 Transgenic biosensors in complex eukaryotes

A number of transgenic strains of the nematode *C. elegans* have been developed, in which a fluorescence or *lac-Z* reporter gene has been linked to the HSP16 promoter (David *et al.,* 2003; Guven *et al.,* 1999; Power *et al.,* 1998). Because of the relative novelty of transgenic nematode technology, the system has not yet been widely applied in contaminated soil assessment. Power and De Pomerai (1999) examined responses to cadmium, copper and zinc. High concentrations (250 mg/kg) of cadmium induced a stress response, but the response to copper and zinc was minimal. Another study found that the surfactant Pluronic F-127 enhanced the stress response of transgenic nematodes to cadmium, mercury, copper, manganese and zinc (Dennis *et al.,* 1997). David *et al.* (2003) investigated the response of the green fluorescent protein-*lac-Z* reporter gene strain to a range of stressors. Heat shock, cadmium

and exposure to microwave radiation all showed a strong potential to induce both reporter genes. As an alternative to HSP, Cioci *et al.* (2000) linked a beta-galactosidase reporter to the *C. elegans* MT-2 promoter. A comparative study showed that the system was more sensitive for cadmium, mercury, zinc and nickel than either a 24-hour LC50 assay or the HSP strain.

In *Drosophila,* a system based on fusion of *lac-Z* to the HSP 70 gene promoter has also been developed. This system was used to compare the relative sensitivity of heat shock system induction and life-cycle parameters to the pollutant captan. The study demonstrated the greater sensitivity of HSP induction compared to effects on survival and fecundity (Nazir *et al.,* 2003). The HSP 70 linked *lac-Z* strain has also been used to show time and dose-dependent activation of the heat shock system by an organophosphate pesticide (Gupta *et al.,* 2005).

3.10.3 Method evaluation

The use of terrestrial bacteria modified to contain the *lux* gene cassette has all the advantages offered by the Microtox™ system (simple to use, rapid, economical), with the added benefit of greater ecological relevance. Commercialisation of support for the system is currently improving the technique and identifying applications. As a result, routine use is now a real possibility.

One area which requires further investigation is the generality of the response. In particular, the absence of a response to PAHs in some studies suggests that the assay may not be suitable for these compounds. Furthermore, the *lux* biosensor assay measures a response not directly related to a higher ecological effect. A further concern is that the test is usually conducted in a soil extract, with a variety of methods available to extract the 'bioavailable' fraction. Transgenic nematodes clearly have potential, but this emerging area needs additional work before any system can be recommended. Most studies using transgenic organisms of this type have been limited to the laboratory, raising issues about sensitivity that need further investigation.

3.11 Summary

The techniques evaluated in this chapter are in a range of developmental stages. For most, there is a history of a least three years application in biological sciences and for some, much longer. Currently, the best developed and/or most promising techniques for routine soil quality assessment are:

- **Use of transgenic bacterial biosensor**. This could replace initial screening pioneered by Microtox™ systems that exploit natural bioluminescence in the marine bacterium *V. fischeri*. The principal advantage of transgenic soil species over Microtox™ is the increased ecological relevance for soil ecosystems.

- **Molecular-based methods for analysis of community structure.** In nematodes, the maturity index has potential application, particularly if this can be coupled to multiplex PCR to allow more rapid quantification. For microbes, a range of signature lipid and PCR approaches for assessing the diversity

and composition of the soil microbial community are now available for potential use.

- **The application of transcript or metabolite profiling** to identify the toxicological effects of exposure to particular pollutant or pollutant groups in a set of biomonitoring species or closely related species groups. Such species should be ubiquitous, with the required tools being available. Common plant species (such as nettle, grasses), earthworms, springtails and snails are all candidates.

- **Use of ecological modelling approaches** to predict the fate of populations of priority species of key conservation interest in their own right (for example, as rare species), or of value as indicators of the status of terrestrial ecosystems.

When considering the potential of these emerging methods, some common threads emerge. The most obvious is the need for a database on the range of response profiles that can be considered 'normal'. In the development of the RIVPACS system that is now a standard tool for assessing river water quality, a large number of samples were collected for community characterisation, in conjunction with data describing the characteristics of each collection site. In all, over 500 of reference sites were characterised. From this extensive dataset, RIVPACS developers can use multivariate data analysis to predict the expected composition of the river community at any uncharacterised site. Comparing the observed community at this site to the predicted community enables the quality of water at the study site of interest to be determined. In the laboratory, many studies have demonstrated chemical effects on soil microbial composition (though PLFA or DNA analysis) or on organism physiological status through measurement of transcript, protein or metabolite profiles. To become broadly applicable monitoring tools for field assessment, similar campaigns to that organised for RIVPACS would be needed to generate reference profiles against which response profiles could be compared. Being such a large commitment, a campaign of this type could not be approved without a proof of concept study that systematically and without bias investigated the diagnostic power of each of these techniques.

A second common thread is that technical developments are often imminent to speed up and improve performance. That means that although some methods are currently time-consuming and/or expensive, this is likely to change. Exceptions may be for plant and invertebrate community profiles, where, because of changes in biological sciences teaching in the UK, there is currently a danger of losing the taxonomic expertise needed to conduct such assessments. This issue reflects general concerns of the wider loss of taxonomic skills from universities and research institutes. However, despite the difficulties outlined here, these techniques would be well suited to an outcomes-based approach to environmental protection, and therefore worth pursuing.

4 Guidance on techniques and sampling strategies - Technical worksheets

4.1 Introduction

This section provides details of some of the methods described in Section B2, with guidance on how best to implement and evaluate a particular method. Where first-hand experience of techniques is available, a detailed protocol is provided. Otherwise, guidance outlines the steps that need to be followed and the effort involved.

Common to all procedures is the need for careful design of the sampling strategy, and an awareness of factors which might compromise the use of biomonitors. The following paragraphs describe the key elements of design of a biomonitoring scheme.

4.2 Sampling and statistical design

Biomonitoring relies on the ability to distinguish the pollutant signal from background levels and noise from other sources. The ability to resolve the confounding effects of multiple sources can only be achieved with good spatial coverage, which is a further advantage of biomonitoring over physical methods, because biological responses are used to estimate the spatial extent of any effects.

4.2.1 Sampling strategy

Sampling strategy and design are often overlooked in biomonitoring studies. Sampling strategy will very much depend on information available on the pollutant being emitted and the natural distribution of sampled species and vegetation landscape (such as forest or grassland). For biomonitoring local point sources, the key is to identify the area affected by the pollutant plume. Air dispersion models can help to identify the likely dispersion and concentration fields for the plume from a source. Most regulated sources have monitoring data going back many years, together with air dispersion modelling. This information should be readily at hand and used in the design of a biomonitoring scheme. Moreover, to identify suitable near-source environments for biomonitoring, including sensitive ecosystems, it is desirable to carry out a site visit to establish the presence of suitable vegetation and animal matter for sampling.

When monitoring the impacts of a point source, samples should be chosen in a pattern corresponding to the expected distribution of pollutants, taking into account distance from source, prevailing wind direction and other source characteristics (Figure 4.1). Other methods use a geographical area and grid to ensure regular distribution of sampling units. The sampling strategy (linear

transect, grid and so on) will depend on the scale of the study and the degree of variability in the area surrounding the source. The number of sampling sites along a transect, or the extent and frequency of sampling within a grid, will also depend on the scale of the study, but will be influenced by (natural) variability in the biological response under background or control conditions (see below).

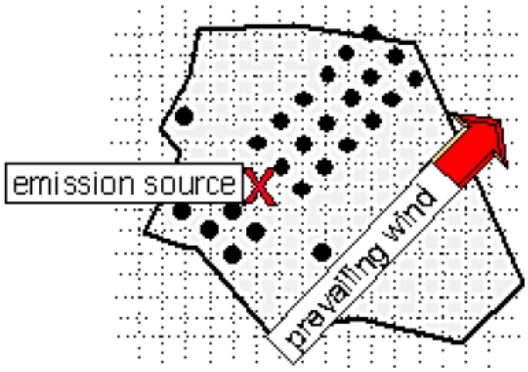

Figure 4.1: Sampling strategy around a point source (Asta *et al.,* 2002a)

The number of sampling locations for a grid-based approach may be large, and may be too expensive to be practicable. Use of linear transects is often an appropriate compromise between gathering two-dimensional spatial information and sampling costs. It can work effectively where there is a single prevailing wind direction at the site, ideally supported by local wind rose data. Consideration should also be given to factors such as topography and the presence of large buildings, which can interfere with the pattern of dispersion and deposition of contaminents.

Care must be taken in any transect to ensure that observed responses are related to the pollution, and not to some other coincident factor. An example of problems that may be encountered is outlined in Figure 4.2, which shows the possible interaction of environmental conditions and prevailing wind direction. Measurements of biological response along the transect, in the absence of other information, could be used (wrongly) to attribute the cause of damage to the pollutant. In this case, interaction with the environment would be detected by a second transect perpendicular to the prevailing wind direction (dashed line in Figure 4.2); no change would be observed on crossing the centre-line of the plume – see Figure 4.3.

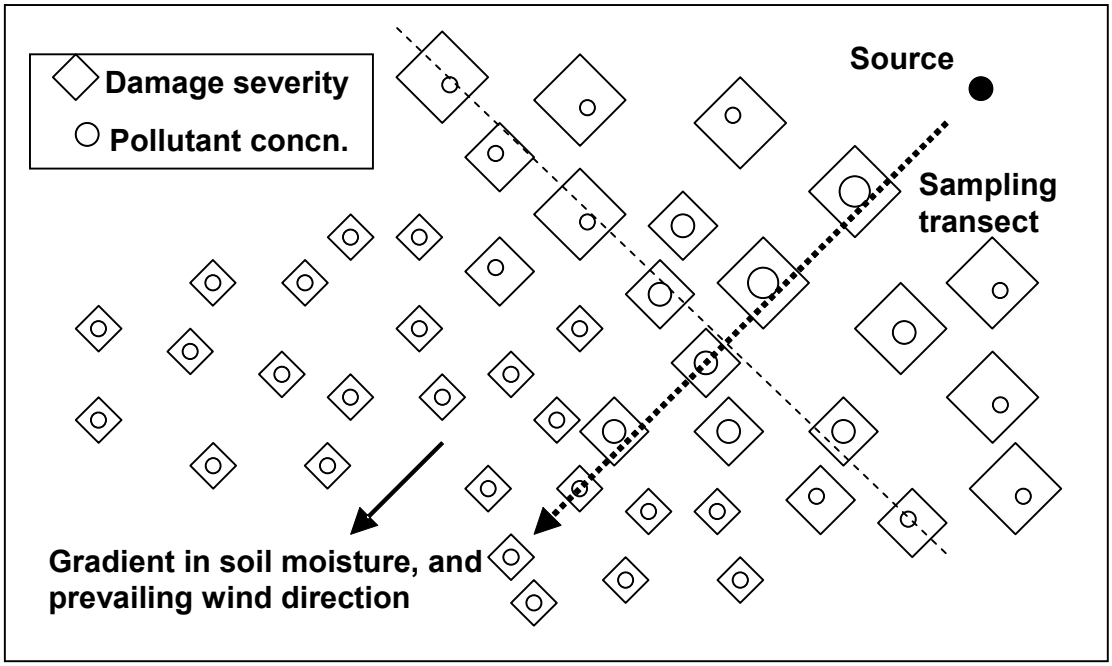

Figure 4.2: Schematic representation of the danger of interpreting the results of a transect study in terms of cause-effect relationships.

The variation in visible damage (diamonds) is caused by an underlying change in soil properties that makes plants more sensitive to drought towards the top right of the diagram. This variation is correlated with the dispersion of pollutants from the source, as measured by foliar accumulation (circles). Measurements along the transect, in the absence of other information, could be used (wrongly) to attribute the cause of damage to the pollutant.

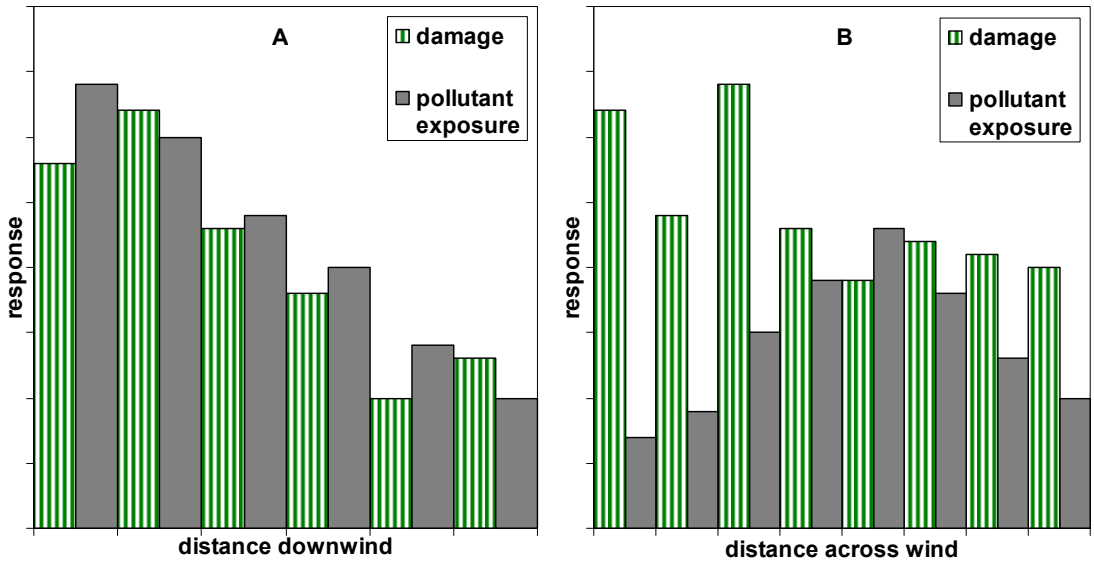

Figure 4.3: Variation in plant damage (response) and pollutant exposure along the transect in Figure 4.2 (A) and perpendicular to the transect (dashed line in Figure 4.2) (B).

This shows how a correlation between damage and pollutant exposure along a transect (A) can be wrongly interpreted as indicative of a causal relationship.

The distance downwind from the source is critical and will vary depending on the size of the plume and the pollutants emitted. Characteristics of the emission source should be taken into account including transport distance, which depends on the size of emission, the effective height of the stack, and the size of the particles. For example, in a biomonitoring study around a chicken farm (where emissions were close to ground level) three replicate samples to measure nitrogen in tissue were taken at five distances of 5, 20, 40, 100, and 250 metres away from the poultry unit (Leith *et al.,* 2005). Goodman and Roberts (1971) monitored heavy metals around industrial (high-level) sources using the moss *Hypnum cupressiforme* along transects at 8 and 25 km downwind of the site. The concentration in tissue at 25 km downwind corresponded with that at 18 km upwind, indicating a return to background levels.

Sampling strategies may use material *in situ* or use transplanted material. In situations where suitable sampling material or species are not present, transplants are an alternative option (see transplant methods).

4.2.2 Sampling design

Sampling design will include a number of important considerations, such as to:

- identify the objective of the study, including specific pollutants if known;
- define the time period of monitoring – permanent, temporary or a snapshot – where snapshots can be used to assess health, but detecting changes over time requires a monitoring programme;
- identify the area or domain of monitoring;
- choose the indicators and methods;
- select the distribution of sites for sampling;
- select the sampling density and number of replicates to be taken.

Ideally, a power analysis would be conducted to determine the optimum number of sample sites and replicates needed to obtain statistically reliable results.

The process requires quality assurance and quality control. Training in field techniques and species identification is a necessity, as are user guidance notes. The timing of sampling and subsequent analysis is important, to avoid potential changes in samples during storage. Contamination during collection should be avoided, and measurement units should be consistent. Location of sampling units is important, where biased sampling should be avoided (for example, near a road or track).

4.2.3 Statistical design

Biomonitoring programmes around point sources will always need to detect and attribute the pollutant signal. Many statistical methods include a correlation or regression technique to compare measurements (such as lichen diversity or

tissue concentration) along a gradient away from a source. Furthermore, to eliminate noise and other confounding factors, multivariate analysis can be used to investigate patterns of variation and relationships with external variables. Comparison between plots or monitoring sites involves the use of two-way ANOVA or similar techniques.

Fundamental to any biomonitoring approach is the detection of an attributable signal significantly different from the noise or natural variability in the parameter being measured. Even with a careful sampling design, the number of samples required to detect a significant response is generally not known, because there are few data on the natural variation of biological responses to climate, soil type, water availability and so on. Some data may be available for similar situations, and can be used to guide a power analysis, but in general this cannot guarantee that a given level of precision will be achieved in the field. Ideally, the area of interest should be studied before the source begins operation, or an area nearby with similar land use that is known to be unaffected by the source.

Although care must be taken to ensure analytical precision when examining sampled material, the greatest variability is likely to arise from temporal and spatial hetrogeneity. Paradoxically, this is the area to which least attention tends to be given when designing a statistical approach. Many low-cost and relatively imprecise measurements may deliver more useful information than a few high-cost and precise measurements, if the major source of variation arises in the field. This is one of the strengths of many biomonitoring approaches which sample across a wide area.

A further problem is the requirement to show a null effect. In order to demonstrate that a pollution source has been regulated effectively, there should be no measurable response that can be attributed to the source. Bad statistical design, however, can easily lead to the conclusion that there is ‘no response’ if the variability is too high. Results should always be expressed in terms of the size of effect that would be detected, if it were present, with a defined probability − that is, a sampling exercise should use data from control areas to estimate the size of effect that could be detected with the given number of samples, with a probability of (say) 95 per cent. Such a calculation also shows the number of samples required to determine whether a control strategy is effective. If, for example, a two-fold change in a measured parameter is known to lead to an unfavourable outcome, the sampling design should be able to detect that size of difference as statistically significant at a pre-agreed level. This need not, however, be set at the conventional level of 95 per cent; less stringent criteria may be more appropriate, depending on the decisions that will be made on the basis of information obtained.

An example of the difficulties faced in this process is found in unpublished data gathered at Whim bog, in eastern Scotland, by Sheppard and co-workers at CEH Edinburgh. Prior to the start of an experiment to test the effects of nitrogen additions to an ombrotrophic bog, vegetation samples were taken from each of the 54 designated experimental plots (all within an area of 0.5 ha). The bog had had no management for at least a century, as far as could be established. An example of the scale of variation is shown in Figure 4.4, which shows the

distribution of N% dry weight for *Sphagnum* moss. The natural variability of this population means that 90% of measurements fall within ± 20% of the mean value of 0.80 N% (assuming a normal distribution).

If we wished to test for a (positive) effect of the experimental treatment (N addition) on the N content of the plant tissue, and could assume that the variance of affected plants was the same as for the original population, we would need to take a sample size, *n,* that satisfied the condition ($\sqrt{n}.(x-\mu)/s \geq t_{\alpha,n-1}$) where *t* is Student's t-statistic with a one-sided probability of α, x is the mean of the sample data, s is the standard deviation, and μ is the mean of the control population. For a sample size of $n = 3$, this inequality requires that $(x-\mu)/s \geq 1.69$ for $\alpha = 0.05$. This means that for this example, with a statistical uncertainty of five per cent and assuming that the standard deviation remained constant, the mean N content of triplicate samples of 'affected' foliage would need to be at least 0.97 N% to be demonstrably larger than would be expected from natural variability. For $n = 5$ the threshold falls to 0.90 N%. This calculation, however, makes the unwarranted assumption that the variance in the affected population is the same as for the control, whereas in reality it is likely to be somewhat greater if the affected population responds in a non-uniform way to the pollutant.

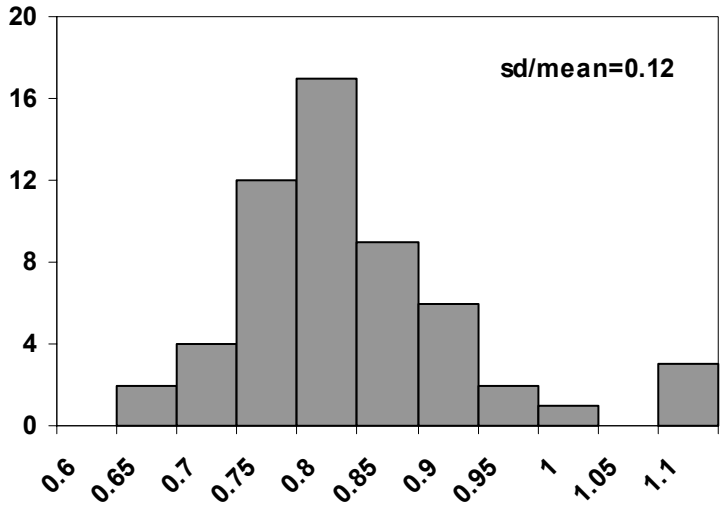

Figure 4.4: Variation in N content of a moss, Sphagnum capillifolium at Whim bog, Scotland. (L. J. Sheppard, personal communication). Vertical axis shows frequency of occurrence. Horizontal axes show N content as % dry weight.

This example, apart from demonstrating the degree of natural variability even in near pristine conditions, shows the type of approach required if biomonitoring is to successfully determine 'outcomes' of pollution exposure. In this case, the direction of the pollutant effect was hypothesised in advance; more usually, the statistical test may need to be two-sided (to show significantly smaller *or* larger results than the control) and would use the two-sided t-statistic which is more demanding than the single-sided test. However, as noted above, this type of analysis only shows whether there is a significant difference from the control population. The apparent lack of such a difference cannot be safely construed as showing that there is no effect – only that the size of any effect is smaller than

the variation detectable with the statistical design used. This may, or may not, be at a level where unfavourable outcomes would occur.

Where a linear transect is used, and providing that checks have been put in place to show there are no confounding factors on the same spatial gradient, a more powerful statistical technique may be regression analysis of the measured response against distance. Appropriate transformation of variables may be required; for example, if the measured response (y) varied with distance (x) according to an exponential decay ($y = y_0 + a.e^{-b.x}$), where y_0 is the background level, the logarithm of ($y – y_0$) would show a linear relationship with distance from source (x). The statistical significance of the correlation coefficient between log($y-y_0$) and x can be established – this shows whether there is a spatial correlation which might be attributable to emissions from the source. Because this test uses spatial information, rather than relying on data from a single sampling point, it is intrinsically more powerful in assessing the data. More complex multivariate analyses are possible, but are beyond the scope of this review.

4.3 Measuring pollutant levels in plant tissue (Method 2.2.1)

Vegetation is an important part of the ecosystem and thus of a biomonitoring study, particularly for analysis of accumulated pollutants. Pollutants may be present on the surface of the vegetation, demonstrating recent aerial deposition, and may also be present in the tissues from accumulation through root or leaf uptake. Vegetation is relatively easy to collect and process.

Lichens, bryophytes and higher plants have long been used to measure pollutant content in tissue. Techniques have been used to measure exposure to heavy metals and nitrogen, sulphur and persistent organic compounds, as well as temporal and spatial trends in pollutant deposition. Mosses, in particular pleurocarpous species or feather mosses, are probably the most frequently used organisms for monitoring heavy metals, nitrogen and sulphur (Burton, 1986). They are widely distributed, are good at accumulating pollutants, and are easy to collect, prepare and analyse.

4.3.1 Sampling strategy

If collected as part of a study on the effects of pollutants on different compartments in the ecosystem, vegetation should be collected at the same sampling sites as the other samples. Typically, three to five replicates should be collected at each site and analysed separately. The amount of vegetation sampled depends on the analytical techniques; typically two grams of tissue is required for PAH and metal analysis, so four grams per replicate will be required if both analyses are conducted.

The timing of sampling is important. For example, nitrogen is an important nutrient and constituent of plant growth, and its concentration in plant tissue varies throughout the growing season. For coniferous tree species, the best time for sampling is generally after growth has stopped in the autumn. Deciduous

trees, however, can be sampled during the growing season and some time after bud burst. For moss species, the best time is in the spring or autumn when nitrogen uptake is at its greatest. Sampling mosses to measure heavy metals should be carried out during the growing season and the actively growing green shoots should be sampled. A detailed analysis of the difficulties inherent in the timing of sampling is provided by Cape (2007). The pattern of accumulation of a pollutant may vary depending on the route of uptake and any processes by which the material is eliminated from the tissue. Figure 4.5 shows a hypothetical example where vegetation is exposed to a constant input, or where uptake follows a sine curve, increasing during the growth phase and decreasing with leaf senescence. Where plants are exposed to intermittent exposures, or to a single acute exposure, very different patterns of accumulation can be seen (Figure 4.6).

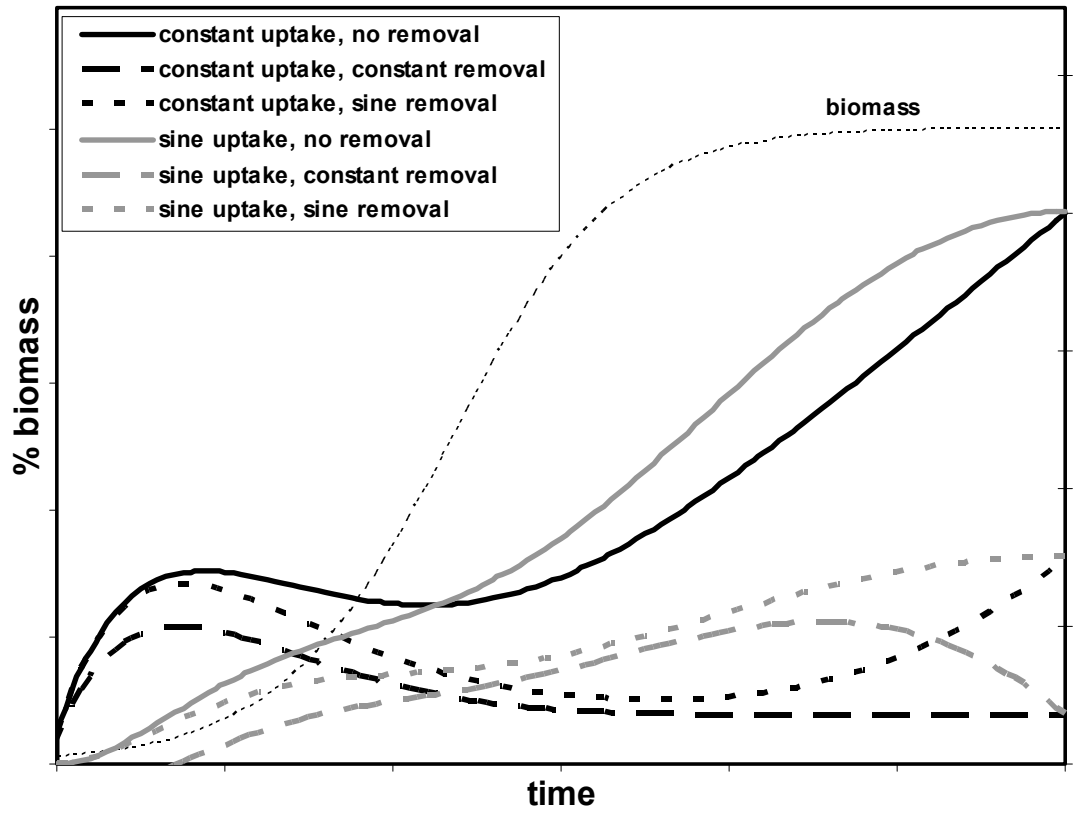

Figure 4.5: Simple model of variation in accumulation of a pollutant in a leaf throughout the growing season (change in biomass is shown by the dotted line) under different scenarios. The six scenarios are;

1. rate of pollutant uptake is proportional to the surface area only (simulating constant exposure to the pollutant), and no removal processes operate;
2. as (1), but removal rate within the leaf is proportional to the product of leaf biomass (simulating metabolising or export capacity) and amount of pollutant present;
3. as (2), but the removal rate is weighted by a sine function that peaks midway through the growth period, simulating varying metabolic or export capacity throughout the leaf's life;

4. as (1), but the rate of pollutant uptake is weighted throughout the period by a sine function, simulating varying pollutant exposure;
5. as (4), but with removal rate calculated as (2);
6. as (4), but with removal rate also weighted by a sine function, as (3).

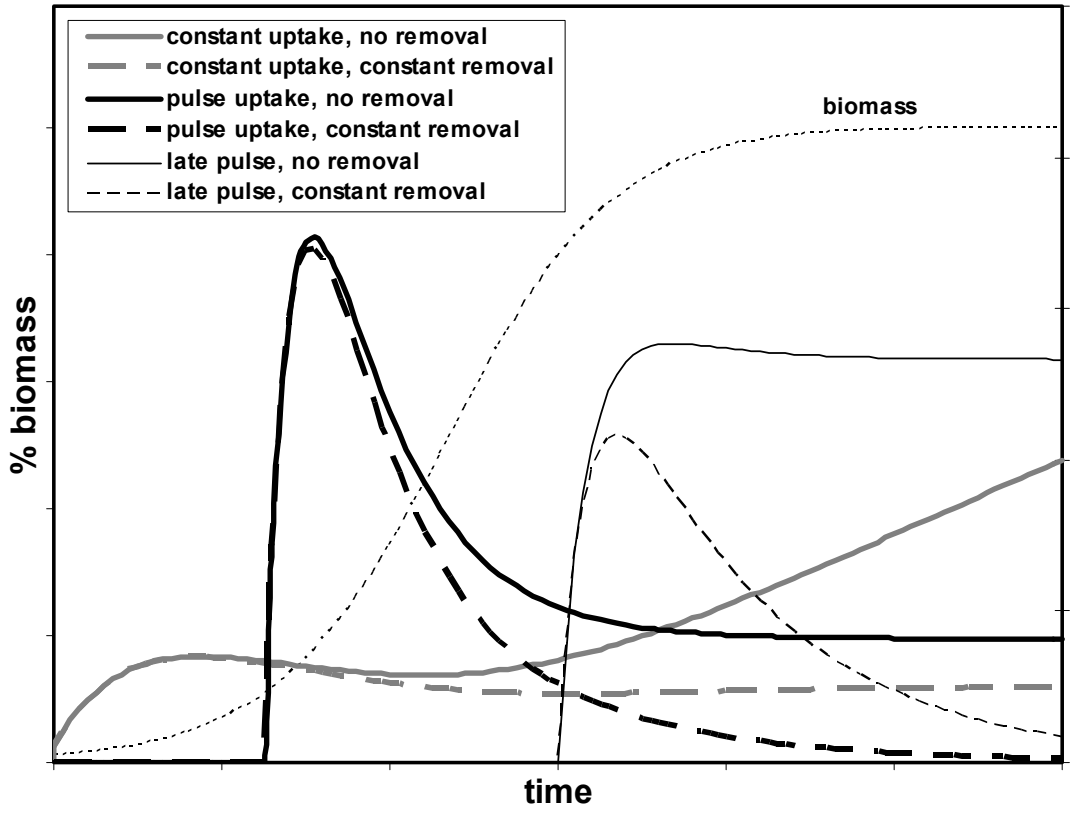

Figure 4.6: Variation on Figure 4.5, showing the effect on plant concentrations of a single large exposure to a pollutant, in this case simulating the experimental treatment of soil with fertilizer.

Total exposure is matched to the reference case of constant exposure in Figure 4.5 (grey lines), with a single pulse decaying exponentially over 30 days (simulating removal/dispersion processes in soil), applied at the start of the growing season or towards the end of leaf expansion. The dashed lines show the effect of including a removal process (similar to case 2 in Figure 4.5).

Such temporal variations are also seen in practice. Figure 4.7 (Cape, 2007) shows how accumulation of a heavy metal can vary across sites, even for a single element and the same plant species.

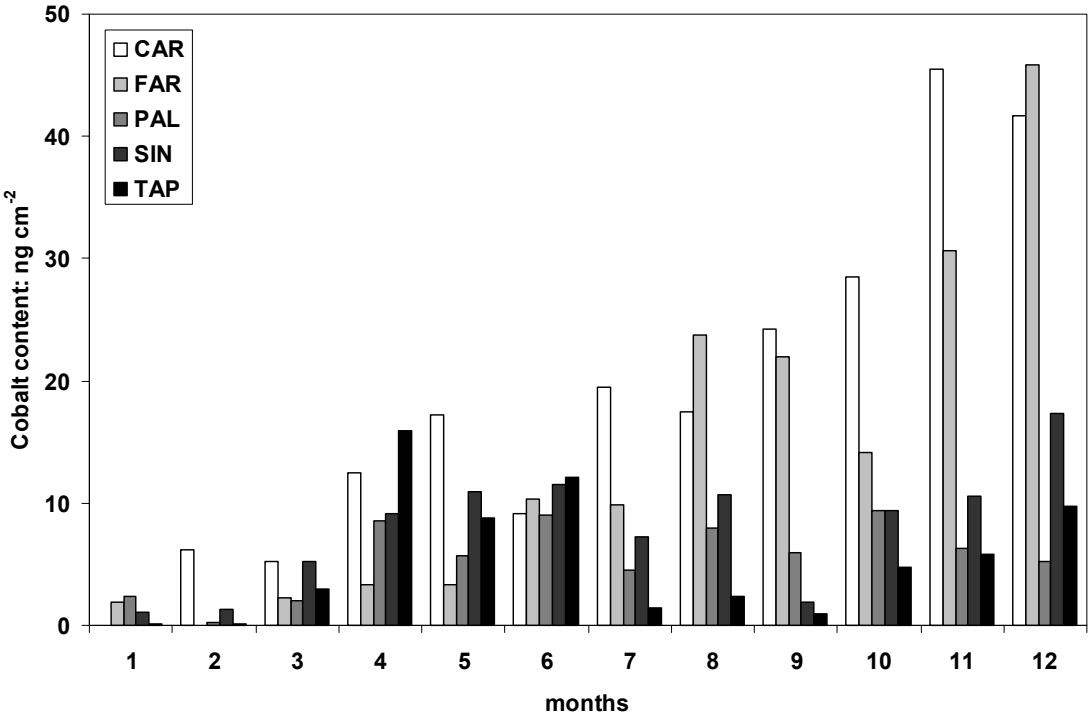

Figure 4.7: Illustration of temporal variation in plant accumulation of a pollutant and consequences for interpretation of data sampled at different times of year.

Data are plotted from Freitas *et al.* (2004) and show the accumulation of cobalt in the lichen *Parmelia sulcata* at five sites in Portugal over the course of a year. The letter codes indicate the site names.

4.3.2 Sample collection

There are a number of guidelines and precautions for collecting plant material in the field:

- Contamination should be minimised during sampling. Trace element-free vinyl gloves could be used to minimise contamination.
- When sampling in forests or woodland, samples should be taken from canopy openings and not from areas exposed to throughfall. Effects of stemflow should be avoided by sampling several metres from tree stems.
- Three composite samples should be collected for each species.
- Actively growing green material should be sampled using a fixed length of shoot. Samples of spruce or pine needles should be taken from the upper crown during the dormant period of October or November, while the sampling of deciduous species should be carried out during the growing season. Leaves are again taken from the crown area.
- Collected material should be stored in polythene or paper bags and kept cool while being transported to the laboratory for analysis.

4.3.3 Preparation and analysis

Analysis of tissue content for heavy metals will normally involve the use of atomic absorption spectrometry (ASS) or inductively coupled plasma atomic emission spectroscopy or mass spectroscopy (ICP-AES, ICP-MS). For measuring compounds of nitrogen or sulphur, an elemental analyser is used to determine amounts of individual elements. Analysis for persistent organic compounds will require solvent extraction prior to chromatographic analysis.

Preparation

- Samples, particularly lichen and moss samples, are checked for correct species identification. Unwanted species are discarded. At the same time, samples are hand-cleaned for dead material and attached litter.
- Samples are then dried for 48 hours at around 40-50°C.
- When analysing for nitrogen, samples are rinsed with deionised water to remove any possible surface nitrogen contamination, soil particles or debris.
- Samples can then be ground down to a powder or sieved and diluted with deionised water before analysis.

Analysis

- For heavy metal analysis, samples are mineralised with concentrated HNO_3 and H_2O_2 in a microwave. Digests are then analysed, typically by ICP-AES. See Appendix A1.1 for detailed protocols for digestion of tissue samples.
- When assessing nitrogen, samples may be analysed for % N content by CN analysis using an elemental analyser which converts the tissue N into gaseous form for quantification.

Individual protocols for chemical analysis will depend on the equipment used and normal practices of the analytical laboratory, which should include appropriate use of certified reference materials and quality control and assurance procedures.

4.3.4 Data interpretation

Data are conventionally expressed per unit dry weight of tissue, although for pollutants predominantly accumulated on the exterior of leaves, data may be expressed per unit leaf area. This requires the measurement of leaf area before samples are prepared for analysis. Background values are obtained for each element from control sites. Bioaccumulation in relation to distance typically follows a standard decay curve of form: $y = y_0 + a.e^{-bx}$, where a concentration (y) is dependent on the size of emission (a) and distance from the source (x), with rate of decay (b) per unit distance, and where y_0 represents the background value. Correlations between different elements can be assessed by Pearson correlation coefficients. Analysis of variance (ANOVA) can be used to assess any significant differences between means.

Spatial patterns may be more clearly recognised by using ratios of one component to another, for example, the (content) ratio of a trace element to nutrient. Element ratios provide better correlations with visible injury symptoms than absolute concentrations of individual elements (Cape *et al.,* 1990). Multivariate techniques using data from several components (such as a range of

heavy metals) may help to identify patterns of similar behaviour related to potential emission sources.

4.4 Vegetation community change (Method 2.2.2)

Spatial or temporal changes in species composition provide a useful tool for assessing outcomes from exposure to pollutants, as the actual biological responses are being manifested. By gathering information on the presence or absence of species and their abundance, ecological indicators can be used to assess impacts of air pollution. Other characteristics of visible impacts or damage can also be recorded. Indices can be used for different communities of species, such as mosses, lichens or higher plants, and combinations of these can be used in any one biomonitoring scheme. Although these methods are relatively straightforward, the correct identification of species is essential and good botanical knowledge is required.

The following method offers an example of a strategy to assess community change by measuring lichen diversity values (LDV), using lichens on tree bark as the community change test. This method has been developed out of around 20 different methods (Asta *et al.,* 2002a,b), and can be used to assess the existence and impact of air pollution from an emission source. Repeat monitoring at the same sites enables assessment of the effects of environmental change, and long-term changes over time. Some principles and techniques can be applied using other community change methods. A number of recommendations from other studies are also offered.

4.4.1 Sampling strategy

The aim of the study is to define an area for sampling and record the species present, along with their abundance. Monitoring sites and sample trees should be selected after a preliminary survey to assess the availability of suitable trees (species and size) and to rule out areas with no trees. When monitoring the impacts of a point source, sampling units should be chosen in a pattern corresponding to the expected distribution of pollutants, taking into account distance from source, prevailing wind direction and other source characteristics. The next requirement is to establish the number of trees to be sampled for each unit. A higher number of samples offers greater precision, but four to eight trees are often used to avoid the study becoming too costly. If there are more than enough trees in a sampling unit, they should be chosen randomly. The unit can be divided into four subsectors and the nearest tree(s) in each sector sampled (for example, two trees for an eight-tree sampling unit). Where too few or no trees are present in a subsector, the nearest tree from the next sector (clockwise) is sampled instead. All trees should be georeferenced so they can be sampled at a later date.

4.4.2 Fieldwork/identification

Trees of the same species should be selected, preferably with the same bark properties (such as pH) and degree of exposure. Trees in contact with livestock or any form of disturbance should be avoided. Four sampling ladders are attached to the trunk of each tree at the cardinal points of the compass (north, south, east, and west), and at about 1.5 metres above the ground (at the upper

edge) (see Figure 4.8). Each sampling ladder contains five 10 cm x 10 cm contiguous quadrats. Certain areas of the trunk should be avoided, including damaged parts, parts with knots, and parts with more than 25 per cent moss cover. A maximum shift of 20° clockwise is permitted for shifting ladders to avoid these damaged parts and at least three ladders should be able to be placed on the trunk or the tree should be discarded. All lichen species and their frequency should be recorded in each of the five quadrats in each ladder.

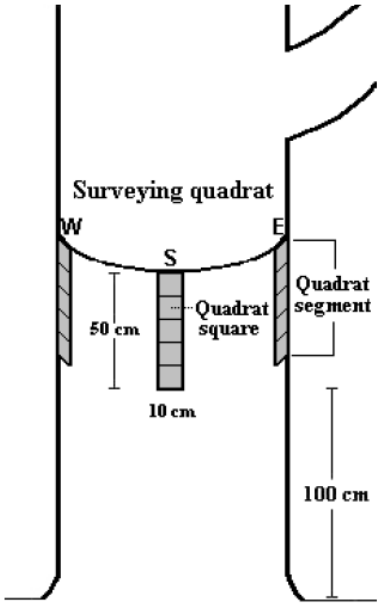

Figure 4.8: Ladder sampling for lichens diversity (Asta *et al.,* 2002a)

4.4.3 Interpretation and scoring

Lichen density value (LDV) is calculated as follows:

1. For each sampling unit, sum the frequencies of all lichens species at each cardinal point on each tree. Therefore, for each tree there are four sums of frequency (SF): north (SF_{iN}), south (SF_{iS}), east (SF_{iE}), and west (SF_{iW}).
2. For each cardinal point, the arithmetic mean of the sums of frequencies (MSF) for each sampling unit are calculated:

   $$MSF_{Nj} = (SF_{1Nj} + SF_{2Nj} + SF_{3Nj} + SF_{4Nj} \ldots_{+} SF_{nNj})/n$$

   where:

   _MSF is the mean of the sums of frequencies of all trees of unit j at a given cardinal point;

   _SF is the sum of frequencies of all species found at one cardinal point of tree i;

   _N is the number of ladders with a given exposure at sampling unit j.
3. The LDV can be sorted into classes and used to define zones of environmental quality. Classes should be wide enough to reflect statistical and environmentally significant variation among sampling units. Multivariate techniques can be used to further investigate local sensitivity of lichens to air pollution, to identify indicator species or to assess the effect of aspect.

4.4.4 Other community change methods

The Ellenberg N index method relies on the preclassification of different vascular plant species preferences to N availability, which were modified by Hill *et al.* (1999) for British conditions. The overall community is scored on a scale of one (nutrient poor) to 10 (nutrient rich). Ellenberg indices have also been applied using the lichen diversity approach, based on the preference of species as nitrophytes or acidophytes. This has been incorporated into an empirical scale of one to seven, with nitrophyte species having the highest scores.

A similar method to the above is the lichen acidophyte-nitrophyte (Dutch) method (van Herk, 1999), which assesses the lichen diversity and cover on trunks of trees classified as nitrophytes (preferring nitrogen-enriched bark) and acidophytes (preferring naturally acidic clean bark). Ten acid-barked trees (one to 2.5 m girth) are selected at each site and all lichen species are recorded up to two metres up the trunk. Abundance of lichens at each site is recorded using a six-point scale from "one thallus present" to "present on six out of 10 trees with more than 10 cm^2 per tree". Species are then scored as nitrophytes or acidophytes and the mean number of species found per tree is expressed as NIW and AIW values. This method is particularly good at monitoring spatial patterns of ammonia pollution.

The 'lichen on twig' approach uses the diversity of lichens growing on twigs around an exposed site. Lichens are recorded annually on five to 10 trees to identify any shift in species communities. This method has been simplified with an easy-to-use identification key for lichens on twigs (Wolseley *et al.,* 2002) and can be found at www.nhm.ac.uk/botany/lichen/twig. This method can also be used with the nitrophyte-acidophyte method to indicate nitrogen pollution.

4.5 Visible symptoms on vegetation (Method 2.2.3)

While chemical analysis of pollutant accumulation in tissue only indicates a potential impact, visible injury assessment can be used in conjunction with accumulation methods to provide a clearer picture of impacts and outcomes. Although visible symptoms on native vegetation may be useful in assessing possible effects around a pollutant source, in general the non-specific nature of visible injury means that confident attribution of cause and effect is not possible using visible injury alone. Of greater value is the exposure of standardised plant material that is well-characterised in terms of its specific response to pollutants.

The EuroBionet (Klumpp *et al.,* 2004a) project used poplar *Populus nigra* clone *Brandaris* as a biomonitor to assess visible symptoms from air pollution. Poplar has the advantage of being an easy plant to propagate and by means of shoot propagation, sample material can be genetically homogeneous (Ballach, 1997). Some hybrids are also specific enough to be used for detecting air pollutants like SO_2, NO_x and O_3.

4.5.1 Sampling strategy

The duration of exposure is 12 weeks from June to August. It is acceptable, with unfavourable climatic conditions, to extend the trial into mid-September. Four plants per site are exposed over the period at varying distances downwind from

the source. A further four plants are placed away from the pollutant footprint and are used as a background comparison, with another left in the greenhouse as a control.

4.5.2 Cultivation and exposure

Two glass fibre wicks are fitted into the plastic pots as described in the *Lolium multiflorum* method. Poplar cuttings are placed in the pots, with two buds of each cutting remaining above the soil surface. The poplar cutting can then form two shoots. At least another 50 per cent of plants should be cultivated to account for any losses during establishment. The cuttings are then watered. During the first weeks, the pots are placed into water reserve basins and are watered manually. After four weeks, the plants should be established to receive their water supply through the wicks. The cultivation time is around eight weeks.

For exposure, plants should be transported to the site in a closed vehicle. Pots are placed in pre-constructed racks – a system of square tubes connected together. The racks are placed in a north-south direction. Water reserve basins are placed at the bottom of the frame bedded in a polystyrene block and the pots are then placed in ready cut holes in the polystyrene. The top and three sides (east, south, and west) are covered in green shading fabric. The water basins need to be regularly topped up (weekly). Pest and diseases should be checked for and controlled. Pesticides should, however, be avoided and if used, must be reported for injury assessment.

4.5.3 Assessment of foliar injury and growth parameters

The first assessment is carried out immediately before exposure. Further assessments are carried out every 14 days. Each plant is assessed for impact criteria by examining leaves and shoots as follows:

1. Count the number of leaves present per plant.
2. Determine the number of dropped leaves by counting the empty leaf attachments.
3. Determine the number of damaged and undamaged leaves.
4. Document any visible symptoms of injury by photos or by writing down the symptoms. A classification of leaf injury can be used to record symptoms – extended yellowing/chlorosis, spot chloroses/necroses, extended necroses and intermediates.
5. Measure shoot length.

If tissue analyses are done in conjunction with visible symptoms, the *Lolium multiflorum* method described for measuring pollutant content in tissue can be used.

4.5.4 Interpretation

Very high concentrations of pollutant can irreversibly damage leaves without accumulating any pollutant (Taylor *et al.,* 1986). Many similar-looking symptoms appear on leaves due to viruses, fungal diseases and insects in addition to

pollutant exposure. Thorough training may be necessary for individuals to confidently recognise the difference and rule these confounding factors out.

4.6 Native transplantation (Method 2.2.4)

Native transplants are plants, together with their substrate, that are moved from an unpolluted site to a known or suspected polluted site or *vice versa*. After a set time period, the responses of transplants can be compared with those of the control. While this method can be used for higher plants, lichens and mosses attached to twigs, bark or stone are more easily transplanted without disturbing the native plant. Native transplants are very useful for assessing the negative impacts of a pollutant source, and positive recovery from a recently abated pollutant source.

4.6.1 Sampling strategy

When designing a native transplant study, environmental and physical characteristics of the clean and polluted site should be the same. One strategy to assess this is to transplant any material back into a similar site to that from which it was collected. Control samples should also be transplanted *in situ* so that the effects of transplanting and disturbance are normalised across both the test and control samples. In this way, confounding factors can be accounted for. Monitoring around a point source may involve positioning transplanted material at increasing distances from the source.

4.6.2 Collection and transplantation

Collecting native samples for transplant should follow a few sensible rules. The substrate of the plant should be included in any native transplant, except where lichen or moss bags are employed. The bag method does not take into account adaptation, and in this case the moss or lichen is simply used as a bioaccumulator. Collecting material with substrate may require digging around and under plants. Epiphytic lichens that grow on twigs and bark are easier to collect. In general samples should be collected from the same substrate, as changes in chemical composition of the substrate can introduce unwanted variation. Enough material should be collected to carry out necessary replications, and material should be kept in bags to avoid contamination.

Transplantation should be carried out as soon as possible after collecting the native material. Epiphytic lichens can be attached to trees or shrubs or even to artificial posts. Every attempt should be made to position the transplants at similar heights, substrate and aspect to the original location. This is particularly useful if no suitable host species are present (for a study site with no trees).

For material collected without any substrate, such as mosses or lichen in the bag method, the plant material is placed in plastic net bags to be hung from trees or posts.

For both methods, samples should also be transplanted back into their original site to estimate the effect of the transplantation process.

4.6.3 Exposure period and analyses

Exposure for bag transplants is normally one to three months, as they do not contain substrate. Longer periods of up to one year may be necessary for transplants that have sufficient substrate and require a time to establish. Allowing sufficient time for transplants to become established at the new site ensures that the effects of transplanting are decreased relative to the effects of the pollutant. Some studies have taken part of the transplant for analysis on a monthly basis after transplanting (Pilegaard, 1979). Samples are dried and prepared in the usual way before using chemical analysis suitable for the pollutant being measured.

4.6.4 Interpretation

Chemical analysis of transplants under background conditions should show no sign of increased pollutant levels or damage. If they do, further investigation is necessary to ascertain the underlying causes. These may arise from contamination during the transplant procedure, or from changes in factors like precipitation or aspect. Some transplants experience memory effects and time lags, so a longer exposure time may be necessary to counteract any lag effect and seasonal differences. The downside of a longer study is that other factors start to obscure any pollutant effect.

4.7 Standardised transplant method (Method 2.2.4)

4.7.1 Sampling strategy

This technique is best described by reference to a well-established method for monitoring environmental change. The standard transplant technique described here is for the grass culture *Lolium multiflorum LAM spp* VDI-Guideline 3957/2 (VDI, 2001) used in the EuroBionet study (Klumpp *et al.,* 2004a). The method is useful for measuring the accumulation in tissue of sulphur and nitrogen compounds, fluorine compounds, heavy metals and trace elements. Analysis of foliar biomass can also be used for assessing nutrient nitrogen pollution. This grass culture was the first standard bioindication method established under the VDI-Guideline (VDI 1978). *Lolium multiflorum* does not exhibit any visible damage symptoms and is a true bioindicator.

Sampling units are usually placed at various intervals along a gradient away from the source. One or more control transplants are placed to avoid exposure to the source.

4.7.2 Cultivation and exposure

Cultivation normally starts at the beginning of the growing season, in March/April, and takes around five to six weeks before exposure. Therefore, about five series can be grown and cultivated for an experimental period from mid-May to mid-October. Exposure duration is for 28 days ± 1 day per series.

Size 14 cm plant pots, with four drainage holes, are used to grow the culture using a standard soil. Two glass fibre wicks are fitted into the plastic pots,

threading them diagonally through opposite drainage holes of the pot. The wicks should extend at least 10 cm into the substrate. All free ends must be cut to the same length. The pots are then filled with the substrate and are levelled off at the top, with the soil pressed down to form a seed bed two cm below the edge of the pot. The substrate is then fertilised with 100 ml nutrient solution.

One grass culture pot per exposure site and three to four reserves are prepared for each series. Around 0.6 g of grass seed is evenly sown in each plant pot and covered with a thin layer of sifted standard soil. The surface is sprayed with water and fertilised with 100 ml nutrient solution. Plant pots are placed on a grating above water basins and the water is supplied automatically through the wicks. As soon as plants have reached a height of 8-10 cm, the grass cultures are cut back to four cm in order to improve tillering. Further cutback can take place every 8-10 days. The cultivation period is normally five to six weeks. Three weeks after sowing and one week before exposure, plants pots are fertilised again with 100 ml of nutrient solution. One day before exposure, the plants are cut back to four cm and final fertilisation is carried out.

At the exposure sites, the rods are rammed into the ground. Steel baskets, equipped with water reserve containers, are fixed to the rods. Plants are transported to the site in a closed vehicle and at least one pot is left in the greenhouse as a control on the cultivation method. The plant pot cultures are fixed to the top of the rod at a height of 1.5 m. The water supply to the plants should be checked regularly.

4.7.3 Collection and analysis

After an exposure period of 28 days ± 1 day, grass cultures are sampled and the next series is put in place. Sampling can be carried out on site or back in the greenhouse. Grass cultures are cut back to a four cm stubble and are wrapped in thin paper, unwashed, and placed in paper bags. They must be stored in a dry place before being dispatched for analysis.

For measuring elemental content, samples are dried at 80°C and ground with an agate mill. Sulphur and nitrogen concentrations in tissue are measured using a CS or CN analyser. To measure heavy metal content, the samples are digested in a microwave oven and analysed in an atomic absorption spectrometer. Trace elements like antimony (Sb), arsenic (As) and vanadium (V) are measured using ICP-MS.

4.7.4 Interpretation

The relationship between distance from the source and concentration in tissue is determined. A log-linear relationship with tissue content and distance from the pollutant source is often the best way of plotting the data.

4.8 Stable isotopes in vegetation (Method 2.2.5)

Lichens are good indicators of air pollution, as they absorb pollutants largely from the atmosphere. The method described here for analysis of S isotopes in lichens comes from Spiro *et al.* (2002).

4.8.1 Sampling strategy

Each lichen thallus should be sampled randomly from the total population of the species. The same species should be sampled to avoid interspecific variation amongst samples. Fruticose or foliose species are recommended, with epiphytes sampled whenever possible. Common European species used are *Xanthoria parietina, Parmelia spp., Hypogymnia physodes, Pseudevernia furfuracea and Parmotrema spp.* Samples can be collected downwind from the source at varying distances.

4.8.2 Collection

Collect samples of at least one gram of more than six individual thalli from at least three different trees. Samples should be collected from trees which satisfy a number of conditions (Bargagli and Nimis, 2002): trunks with an inclination not higher than 10°; no signs of disturbance; surfaces with no stem-flow and away from wounds in the bark; growth of bryophytes not higher than 25 per cent. Samples should be taken at a height of more than one metre above the ground. Samples should be kept dry during transport back to the laboratory.

4.8.3 Sample preparation and analysis

Samples are air-dried for around a week and are then cleaned with tweezers to remove detritus and other lichen species. Samples are then crushed to a fine powder using a tungsten carbide puck mill.

The analysis is carried out in three parts:

1. Preparation of analyte ($BaSO_4$).
2. Preparation of SO_2 for analysis.
3. Mass spectrometry analysis.

Two methods exist for preparing the analyte ($BaSO_4$) from the lichen samples:

a) Ground lichen material is placed in a suspended boat of a cylindrical oxygen bomb. Distilled water and H_2O_2 are poured onto the bottom of the bomb. $BaCl_2$ solution is added. The bomb is closed and filled with oxygen to operate. The lower part of the bomb contains the aqueous solution with $BaSO_4$ precipitate.
b) The lichen is oxidised with Eschka mixture (Na_2:MgO 1:2) followed by combustion at 900°C. Precipitation of $BaSO_4$ is completed by the addition of $BaCl_2$ solution.

One the analyte is ready, a mixture of $BaSO_4$ and either CuO or V_2O_5 along with quartz powder are reacted at 1150°C in a tube furnace attached to a vacuum line. SO_2 is also prepared from laboratory standards. The combustion is followed in sequence by cryogenic transfer of the SO_2 gas through the vacuum line, reduction of minor SO_3 to SO_2 in a copper furnace, cryogenic trapping of H_2O, separation of CO_2, purification of SO_2 and final collection for mass spectrometry analysis.

Analysis of SO_2 can be carried out by dual inlet mass spectrometry alternating between the sample and reference gases, or by continuous flow isotope ratio mass spectrometry.

4.8.4 Interpretation

The method examines the fractionation of $\delta^{34}S$ in lichen tissues and is defined as the ratio of the number of ^{34}S atoms to ^{32}S in the samples according to the ratio:

$$\delta^{34}S = ([^{34}S/^{32}S] \text{ sample} / [^{34}S/^{32}S] \text{ standard}) -1 \times 10^3$$

Replicate analyses can be carried out to define the spatial distribution of atmospheric sulphur. A good understanding of potential sources will aid interpretation of the isotope results. Knowledge of the meteorological regime which can affect chemical reactions in the atmosphere is also important.

4.9 Tissue analysis of small mammals (Method 2.3)

4.9.1 Introduction

Small mammals have been used to measure pollutant content in tissue, specifically liver, kidneys and fat. Techniques using small mammals have been used to measure heavy metals, radionuclides, organochlorine pesticides (OCs) and POPs (persistent organic pollutants). Small mammals are widely distributed across the UK and bioaccumulate heavy metals, halogenated aromatic hydrocarbons (such as PCBs and dioxins) and OCs. It is not advisable to measure PAHs because small mammals can actively metabolise and excrete these compounds, resulting in low concentrations in the liver. Alternative methods can determine exposure to PAHs (such as induction of cytochrome P450 enzymes, which will be discussed later).

4.9.2 Sampling strategy

Before trapping, a site survey should be carried out to ensure that there are suitable areas for small mammal trapping, such as hedgerows, undergrowth in wooded areas and tall swards of grass/vegetation (small mammals are less likely to be found in exposed areas). Access should be limited (where the general public are excluded), as this will ensure traps are not lost or disturbed.

Before trapping begins, a general licence to collect shrews should be obtained from English Nature (even if shrews are not the target species, it is possible that shrews will enter the traps). The licence can be downloaded from http://www.english-nature.org.uk/science/licensing/pdf/shrew_general_licence.pdf. If the study requires capturing and killing shrews for tissue analysis, a licence must be obtained (see http://www.english-nature.org.uk/science/licensing/application_forms.asp). Similar restrictions apply in Scotland.

Longworth traps should be placed 10 m apart, with between 10 and 20 traps per site. However, if more than 50-60 per cent of the traps are occupied at any one

time, then more traps should be placed at each site (Gurnell and Flowerdew, 1994). When monitoring the impacts of a point source, sampling units should be chosen in a pattern corresponding to the expected distribution of pollutants, taking into account distance from source, prevailing wind direction and other source characteristics. If possible, a power analysis should be carried out prior to the start of sampling, to determine the number of sites and replicates needed to obtain statistically reliable results. The number of animals to be caught depends on the objectives of the study; for example, one gram of tissue is required for a chemical or biochemical analysis, therefore two grams of tissue per replicate is required if both chemical and biochemical analyses are to be carried out. If other samples are to be included, these should be collected or surveyed from the same area.

4.9.3 Collection

The number of days for trapping depends on the objectives of the study and the time available. Trapping should ideally be over more than one night. Traps should be checked at least twice every 24-hour period to ensure animals are not in distress, and bait should be placed in the traps along with bedding material, such as hay or non-absorbent cotton wool (Gurnell and Flowerdew, 1994). Upon retrieval of the traps, the small mammals should be weighed and either marked and released or retained in suitable cages until they are sacrificed.

4.9.4 Preparation, analysis and interpretation

Tissue should be snap frozen in liquid nitrogen immediately after removal from the body and stored at -80^{o}C until analysis. Further preparation, analysis and interpretation depend on the biomonitoring programme; see Appendix 1 for detailed protocols and techniques for heavy metal (Section A1.1) and PAH (Section A1.2 and A1.4) analysis.

4.10 Collection of earthworms from contaminated areas (Method 2.3)

4.10.1 Introduction

Earthworms play a vital role in terrestrial ecosystems, as they are involved in decomposition of organic matter in soil. Earthworms collected *in situ* can be used to determine contaminant residue at that time and provide an indication of the effects of exposure. Responses will demonstrate whether exposure to the pollutant has had an effect on the organisms.

4.10.2 Sampling strategy

Earthworm sampling should be carried out when the ground is moist, from autumn to the end of spring; this must be taken into account when designing a sampling strategy for earthworm collection. The number of earthworms collected depends on the aims of the study and therefore the amount of tissue required for analysis; typically two grams of tissue is required for PAH and metal analysis and approximately three grams for physiological assays (such as gene expression,

cytochrome P450, antioxidant assays and phase II detoxification assays) for each replicate. Earthworms should be collected from sites close to soil sampling sites and placed in labelled containers with lids, containing wet tissue paper. Earthworms should be returned to the laboratory and placed in incubators at 12^{o}C.

4.10.3 Sample preparation, analysis and interpretation

Earthworms should be allowed to depurate (expel impurities) for at least 24 hours after collection, depending on species, to ensure all the gut contents have been eliminated. Following depuration, earthworms should be snap frozen in liquid nitrogen and stored at -80^{o}C until analysis. See Appendix A1.1 and A1.4 for detailed protocols of analytical techniques for heavy metals and PAHs respectively.

4.11 Determination of cytochrome P450 activity (total P450 and EROD activity) (Method 2.3.1)

Vertebrates can metabolise certain organic compounds (such as PAHs) easily. Measuring levels of P450 enzymes (compared to the chemical itself, which will have been metabolised) provides evidence of exposure, as these enzymes are elevated for a number of days after exposure.

4.11.1 Preparation of microsomes

Microsomes should be prepared as follows (though they should not be stored for more than two to three days, as they are fragile):

- Remove tissue from -80^{o}C freezer and allow it to thaw on ice for 30 minutes.
- Homogenise one to two grams of liver in four volumes v/w homogenisation buffer (0.15 M KCl).
- Centrifuge the homogenate at 9,000 g for 15 minutes.
- Pour off the supernatant into another centrifuge tube and spin it at 100,000 g for 60 minutes. All the centrifugation steps should be performed at 4^{o}C.
- The final pellet should be resuspended in microsomal buffer (50 mM Tris-HCl pH 7.4, 20% v/v glycerol, 1 mM EDTA, 1 mM Dithiothreitol) to give a total protein concentration of approximately 20 mg/ml (equivalent to a resuspension ratio of microsomal material from 1 g wet weight of tissue into 0.5 ml buffer).
- Microsomes should be divided into two aliquots and immediately frozen in liquid nitrogen and stored at -80 ^{o}C until EROD and total P450 analysis.

4.11.2 EROD activity determination

The method used to measure ethoxyresorufin O-deethylase (EROD) activity is as described by Kennedy and Jones (1994).

Solutions required:

Assay buffer (50 mM phosphate buffer, pH 8.0)
Ethoxyresorufin (ER)
NADPH
Resorufin (standard curve)

Preparation of solutions:

Assay buffer
50 mM Na_2HPO_4 1.42 g in 200 ml ddH_2O
50 mM NaH_2PO_4 0.6 g in 100 ml H_2O

Incubate at 37 ^oC in a water bath; once temperature in beakers reaches approximately 37 ^oC, bring solutions to pH 8.0 by slowly adding NaH_2PO_4 to Na_2HPO_4.

Ethoxyresorufin (ER)
Put some ethoxyresorufin crystals into an eppendorf (preferably a coloured one as ER is light sensitive) and add 1 ml methanol; mix well. Take out 60 µl and add to 2.94 ml methanol, read on spectrophotometer at 470 nm (perform a wavelength scan from 400 to 600 nm and measure absorbance at 470 nm). To calculate the concentration of ER in the stock solution, divide absorbance by 16 and multiply by 50. If the concentration is less than 1000 µM, add more ER crystals; if it is greater than 1000 µM, add more methanol. Continue until a final concentration in stock of 1000 µM +/- 100 µM is reached. Absorbance max of approximately 0.34. Store stock solution at 4 ^oC, make fresh daily. The working solution concentration is 47 µM, so dilute with buffer immediately prior to use, where the final concentration in wells should be 10 µM (note that this solution is very unstable so use immediately after preparation).

Resorufin (Res) (for the standard curve)
Weigh out approximately 0.25 mg resorufin, add 5 ml methanol and mix well. Take out 200 µl and add to 2.8 ml phosphate buffer. Perform a wavelength scan from 800 to 500 nm and measure absorbance maximum at 572 nm. To determine concentration of resorufin in the stock solution, divide absorbance by the extinction coefficient (to be determined by each laboratory, as this depends on conditions such as pH and temperature) and multiply by 15. The working solution concentration for standards is 3 µM (in buffer), so dilute accordingly to obtain this concentration.

NADPH
Dissolve 8 mg in 1 ml so that final concentration in reaction mixture is 1 mM (note that this solution is unstable so only prepare it immediately prior to use).

Volumes to be pipetted into wells (µl):

Total volume in each well is 235 µl.

Standards using 3 µM stock resorufin solution (concentrations of standards used in the standard curve should be determined by each laboratory, as concentrations of unknown samples should be within the range of standards):

Standard number	1	2	3	4	5	6
Buffer (µl)	160	155	150	140	135	120
Standard (µl)	0	5	10	20	25	40
NADPH (µl)	25	25	25	25	25	25
ER (µl)	50	50	50	50	50	50

Samples:

	Blank	Sample
Buffer (µl)	175	150
Microsomes (µl)	10	10
ER (µl)	50	50
NADPH (µl)	0	25

Protocol:

1. Switch on plate reader and computer, open protocol and pre-heat instrument to 37^{o}C.
2. Weigh NADPH in a centrifuge tube and keep powder on ice.
3. Prepare stock solutions of ER and resorufin and determine concentrations using spectrophotometer.
4. Add buffer to the wells in the plates.
5. Dilute resorufin stock and add to the standard wells.
6. Mix microsomes and add 10 µl to each well (not standards).
7. Dilute ER stock solution and immediately add 50 µl to wells.
8. Incubate plate in reader set at 37 ^{o}C, for five minutes.
9. After incubation, add 25 µl NADPH to each of the sample and standard wells.
10. Insert plate into reader and press run. Perform a kinetic run using excitation wavelength 530 nm and emission of 590 nm, set sensitivity to 50 and shake before first run (intensity is 1) for 15 seconds. Read for 10 minutes using top probe.

All standards should be run in duplicate and samples run in triplicate. Microsome samples may need to be diluted (with assay buffer) so that the reaction is linear throughout the 10 minute scan.

4.11.3 Total cytochrome P-450 determination

The method used to determine total cytochrome P450 concentration in tissue samples is as described by Omura and Sato (1964):

- Total cytochrome P-450 is determined using a dual beam spectrophotometer from 500-400 nm at 25^{o}C.
- The final reaction volume of 2 ml is as follows: 100 mM Tris-HCl, pH 7.6, 20% v/v glycerol, dd H_2O and 150 µl microsomes.
- The solutions are mixed and divided between two semi-microcuvettes.
- The baseline is recorded.
- Carbon monoxide is gently bubbled through the sample cuvette for approximately 30 seconds, and a corrected baseline run.
- A volume of 10 µl sodium dithionite (0.19848 g in 1 ml of distilled water) is added to each cuvette and the spectra scanned several times over a 10-minute period.
- The scanning time is determined experimentally prior to commencement of assay. The assays are run in duplicate.

Following EROD and cytochrome P450 assays, a Lowry assay is performed (see below) on each of the samples to determine total protein concentration and allow results to be expressed as per mg microsomal protein.

4.12 Bait lamina assay of invertebrate feeding activity (Method 2.4.1)

4.12.1 Introduction

The bait lamina test measures the disappearance of standardised substrate material from holes in small plastic strips inserted into the soil. It scores highly compared to other methods in terms of realistic exposure, low variation, flexibility, and usability of results (Kula and Rombke, 1998). An increasing number of studies have made use of this technique, including those examining:

- its diagnostic power;
- the parameters under which it can be used;
- how the standard protocol can be adapted to operational requirements.

The test is much more efficient than others and makes minimal use of resources.

The aim of the test is to measure the feeding activity of soil organisms, by assessing the removal of bait material pellets held in small plastic strips inserted into soil. Bait removal gives an integrated measure of a soil process that involves invertebrates and micro-organisms.

4.12.2 Test methodology

This test differs from others in the ERA Framework in that it is carried out *in situ*. Plastic strips 160 x 5 x 1 mm (length x width x depth) with 16 holes (two mm diameter; three mm between holes) drilled into the lower nine centimetres are used.

These holes are filled with bait, a food substance attractive to the organisms of concern. This food consists of cellulose, bran flakes and activated charcoal. Bait sticks and dried bait are obtainable from Terra Protecta GmbH, Berlin (http://www.terra-protecta.de/englisch/frame-en.htm). The suppliers have a general operating protocol which can be used as the standard upon which study methods can be based.

The ideal duration of exposure is the time in which 40 to 60 per cent of the bait holes are pierced by soil organisms. Depending on the investigation site (soil type, moisture content and temperature), an exposure of 10 to 20 days is needed in temperate zones.

At the reference site, several strips should be removed carefully every few days to establish the appropriate amount and duration of feeding activity.

4.12.3 Sampling strategy

Because of the inherent variability in invertebrate distributions even at apparently homogenous sites and typical patchy distribution of contamination, a minimum of

10 bait laminae per sample point should be used, with an optimum of 16. Three such plots (30 to 48 sticks) should be deployed at any patch being investigated. At each plot, the distance between strips should be approximately four cm (roughly the width of two fingers). When choosing exposure sites, pay attention to a homogenous microrelief and soil consistency, as well as the peculiarities of covering vegetation.

4.12.4 Inserting the strips into the soil

Before inserting the strips, check for cracks and shrinkages in the bait. Lamina strips are inserted into the soil by pushing them gently straight into the upper soil profile. It is possible that, in some soils, bait can be removed from the strips by friction. Therefore, it is advisable to insert a single control strip into the soil and immediately withdraw it to determine if this is likely and, if so, what correction factor to apply.

Make sure that all strips are exposed to the same soil depth by inserting the strips until the top of the uppermost bait is level with the soil surface. If resistance to penetration is high (such as in heavy clays or loams), push the strips into prepared slits. A specialised tool can be bought from terra protecta GmbH (see above), but barbecue skewers can serve equally well. Ensure that the slit is not larger than the strip, as soil organisms might otherwise not be able to overcome the gap between soil and bait. If it becomes necessary to prepare slits at one of the investigation sites, it is advisable to do so at every site to ensure comparability. In the case of a dry, heavy soil that barely allows the insertion of strips, water the site to soften the soil.

4.12.5 Examination of bait lamina strips

At the end of the exposure period, remove the strips gently and wrap them in cling film to prevent baits drying out and producing false positives by cracking or being displaced from the holes. Place the wrapped strips in cool, dark conditions (such as a cool box) to limit further degradation. Laminae can be stored under these conditions in the laboratory, but it is essential to carry out recording within five days of removal from the soil.

Before recording, rinse the laminae under cold water and wipe them gently with tissue to remove soil. Once this has been carried out, it is necessary to record laminae immediately (within four hours) to prevent drying out of the bait.

Examine baits individually by eye under bright light conditions, for example using a portable or bench top light box, or low-powered magnifying equipment. Remove any soil remaining in fully or partially removed holes.

Classify each bait hole into one of three categories:

- completely removed;
- untouched;
- those where some feeding activity has occurred (where light is detected through a proportion of the hole).

Score the bait holes as follows:

0 = hole not pierced;
1 = hole partially pierced (light passes through a proportion of the bait);
2 = hole completely pierced.

4.12.6 Data handling and reporting

The test report should include the following information:

- soil type (texture or type of soil, physical and chemical properties including pH and percentage organic matter);
- bait (composition, date of acquisition);
- duration of the test;
- overall feeding activity (see below);
- information on the bait lamina depth profile (see below).

4.12.7 Overall feeding activity

Express overall feeding activity (OFA) as follows. The maximum score (all baits on a single lamina strip) is 16 x 2 = 32 (when all 16 holes are completely pierced). This is converted to a percentage value to allow for missing or broken lamina strips. Figure 4.9 shows a sample spreadsheet record.

	Top															Bottom
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
2	2	2	1	1	0	0	1	0	1	0	0	1	0	0	1	0
3	0	2	1	1	2	1	0	0	2	2	0	0	0	0	0	0
4	2	2	2	2	2	2	2	2	1	0	2	2	2	2	2	2
5	2	2	2	0	0	0	1	0	1	0	0	0	0	0	0	0
6	2	2	2	1	2	2	2	2	2	2	1	2	2	2	2	2
7	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
8	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	0
9	2	2	2	2	2	2	2	0	1	1	0	0	1	0	1	0
10	2	2	2	0	1	0	0	0	0	0	1	2	0	2	0	1
11	2	2	2	2	2	2	2	2	2	2	2	1	1	1	0	0
12	2	2	0	0	0	1	2	0	2	2	0	0	0	1	0	0
%	75	92	75	54	63	58	67	42	67	54	42	50	38	46	38	29

Figure 4.9: Example sample record showing scores and percentage values for a single plot of 12 bait lamina sticks, each with 16 baits

4.12.8 Bait lamina depth profiles

As stated earlier, one advantage of the bait lamina test is the ability to compare the amount of feeding at different soil depths. Soil invertebrates usually decrease with depth, particularly when this is accompanied by decreasing temperature and moisture. This has been shown in several studies, with a declining proportion of baits removed lower down the depth profile (Larink and Sommer, 2002; Filzek *et al.,* 2004).

Balanced multivariate analysis of variance (MANOVA) on arcsine-square root transformed bait lamina feeding data can be used to confirm that the proportion

of bait lamina consumed is significantly different from control patches. MANOVA can also be used to look for interactions between patch and depth.

Removal of bait correlates directly with the activity of soil invertebrates, particularly earthworms, Collembola, mites and enchytraeids. This represents greater individual numbers of the groups mentioned above (biomass or richness), rather than greater numbers of decomposer groups present (diversity).

4.13 Earthworm survival and reproduction tests (Method 2.4.4)

4.13.1 Introduction

Earthworms play a key role in terrestrial ecosystems and were the first soil invertebrate group for which toxicity tests were approved by OECD (1984, 2000). The two species recommended for use by the OECD are the compost-dwelling worms *Eisenia fetida* and *Eisenia andrei*. Although separate species (Bundy *et al.,* 2002), these two earthworms are thought to be closely related and, as a result, are considered equivalent in toxicity tests. But because they are separate species, their sensitivity to different toxicants may differ. Studies comparing toxicity across a dose range or between contaminated sites should therefore use worms from one species only, with all individuals preferably taken from a single culture population.

The soil-dwelling species *Lumbricus rubellus* has been used in the development of the ERA Framework. This species is commonly used in toxicity testing (Spurgeon and Hopkin, 1996; Svendsen and Weeks, 1997; Stürzenbaum *et al.,* 1998), and will improve the sensitivity and ecological relevance of earthworm toxicity tests using field soils.

This guideline recommends the use of *Eisenia* spp. or *Lumbricus rubellus* in the ERA Framework. Where a risk assessment includes the collection of earthworms from the site being studied (for example, for body burden analysis), the use of *L. rubellus* in comparable toxicity testing of site soils is preferred.

The aim of the test is to assess the impact of soil contaminants on survival and sublethal parameters in earthworms. Endpoints that can be measured include reproductive rate, weight change and mortality. The earthworm test can also generate worm samples for other biomarker measurements, such as gene-based assays.

4.13.2 Culture conditions: *Eisenia fetida/Eisenia andrei*

These two closely related yet distinct species can both be held and cultured in the laboratory. Both species have fast reproductive rates (up to eight juveniles per worm per week under ideal conditions) and a relatively short generation time (15 weeks under ideal conditions). All culturing work should be conducted under temperature stable conditions, such as in a controlled temperature room.

Temperature
Maintain a temperature of 20 ± 5°C for culturing. Keep cultures either under constant light or in a regime of 18-hours light, six-hours dark to reduce the possibility of earthworms escaping from the culture boxes.

Medium
Use a medium for culturing that consists of a mixture of 33 per cent loamy soil, 33 per cent peat or peat alternative and 33 per cent separated horse or cattle manure (by volume). The manure used for the culturing should be collected from animals known to be grazing uncontaminated pasture. It is also important to check that the animals supplying the manure have not been subject to recent worming medication that could result in the presence of residues of these compounds in the manure. A number of antihelminthic drugs given to horses and cows are known to be toxic to earthworms. Before adding the manure, it should be thoroughly frozen and defrosted to ensure that other earthworm species or harmful invertebrates do not become part of the culture.

Mix the medium thoroughly, either by hand or using a clean mechanical mixer, before adding water to increase its moisture content to around 80 per cent of its water holding capacity. This equates to a medium in which water is retained, but can easily be released by squeezing.

Once wetted, place the medium into the culture tanks. These should consist of specially constructed wooden boxes lined with a permeable material such as Phormisol (available, for example, from LBS Horticulture Ltd, Colne; http://www.lbshorticulture.com) or similar rot-resistant, heavy sacking material. Wooden boxes should allow drainage of excess water from the soil, thus preventing the development of anoxic conditions. After adding the medium to the boxes, leave it for at least a week to settle. Turn it manually several times during this period.

4.13.3 Culture method: *Eisenia fetida/Eisenia andrei*

To set up a culture, add 120 to 150 commercially supplied adult (fully clitellate) *Eisenia fetida* or *Eisenia andrei* (but not a mixed population) to the medium and leave for four weeks. During this time, cover the medium surface with a layer of suitable air permeable material (such as Phormisol) to avoid excess water loss from culture boxes.

Once a fortnight, add a quantity of (previously frozen and defrosted) horse or cow manure as food to the cultures. Spread the manure over the medium surface to a depth of approximately one cm.

Water the cultures regularly to maintain the moisture content at around 80 per cent of the water holding capacity (the amount and regularity of watering will depend on the holding facility characteristics) and turn the soil gently by hand to prevent the development of anoxic conditions.

After four weeks, remove all adult worms from the culture boxes. The worms can be used for another culture, but 25 per cent should be discarded and replaced

with new adults to prevent a decline in reproduction occurring in the seeding adult worms.

To obtain the test organisms, leave the cocoons produced by the adults in the culture box for 12 to 16 weeks (three to four months), during which time they should grow to full adult size. Feed the worms throughout this time with increments in food to match growth (retain a one-cm layer of manure on the surface). Once the worms have reached adulthood, top up the cultures with manure until they are required for testing. Use the worms within three to four months.

4.13.4 Holding conditions: *Lumbricus rubellus*

Collect adult worms from a suitable (uncontaminated) field site or buy them from a recognised earthworm supplier. Worms can be purchased as adults in the UK or as cocoons from international suppliers. Purchased adult worms can be kept for a maximum of six months for use in tests.

Holding units

The holding units should be 40 cm deep and have a cross-sectional area of at least 1.5 x 1.5 m. Such a unit can hold 2,000 worms. Line the units, which can be constructed from wooden pallets, with a water-permeable material (Phormisol or similar) to prevent earthworm escape.

Use a medium for long-term maintenance consisting of 33 per cent loamy topsoil, 33 per cent composted bark and 33 per cent peat or peat alternative (by volume).

Medium

Keep the holding units outdoors, preferably in a covered area such as a large shed or barn. However, they must not be allowed to freeze. Placing carpet on the surface or use of soil-warming cables will prevent freezing of the medium, which would be fatal for the worms during winter.

Mix the medium *in situ* within the units, which should be filled to within 10 cm of the rim. Once mixed, water the medium thoroughly to around 80 per cent of its water holding capacity by adding water (this equates to a medium in which water is retained, but can easily be released by squeezing) and leave it at least three days to settle. *Lumbricus rubellus* can then be added to the medium at a loading rate of up to 1,000 worms per m^2 of medium.

Cover the units completely with a layer of air-permeable membrane to avoid excessive water loss.

Feeding

Feed the worms with horse manure (frozen and defrosted) at a rate of approximately 1.5 kg wet weight manure per 1,000 worms per week. In addition, fresh vegetable scraps (brassica, root vegetables and so on) can be incorporated in the top layer of the medium, as the availability of fresh food appears to improve the condition of the worms. To prevent the build-up of rotting material, do not add excessive amounts – around a bucket per 2,000 worms per fortnight.

Water the holding units at least fortnightly. Make sure that water percolates fully through the full depth of the holding medium to ensure that the water content remains as it was when the worms were added.

Lumbricus rubellus can be held in good reproductive health for at least six months. Beyond this time, individuals are likely to show signs of senescence (clitellum scarring, weight loss) and should not be used for test exposures. At this point, start a new culture based on new media and newly purchased earthworms.

4.13.5 Test method: *Eisenia fetida/Eisenia andrei*

The test procedure described here is the OECD earthworm chronic toxicity test (OECD, 2000) with *Eisenia fetida* or *Eisenia andrei* adapted for use with contaminated soils.

Collect and prepare soils from each site to be investigated as described in Guideline 4.10, *Collection of field soils and preparation for use in biological assays*. It is also necessary to prepare a reference soil and a positive control soil (see below).

Preparation of the soil

For each field and reference soil, fill at least four replicate jars (Kilner-type jars are particularly suitable) with 750 ml of soil. It is necessary to fill the jars with a constant volume of soil, rather than a constant weight, because use of a constant weight of soils with different bulk densities would result in worms being stocked at different densities. Controlling the stocking density of the worms within test vessels is important, as worms maintained at different soil volumes may show effects due to changing density rather than the presence of contamination.

For each soil exposure, record the dry weight of soil that constitutes the 750 ml for each site. This value can be used subsequently to calculate the total number of contaminant molecules within the closed system, based on the concentration data from chemical analyses.

Once added to the jars, re-wet the soils to approximately 60 per cent of their moisture retention capacity as determined using an established method and leave them to stabilise for at least 24 hours. After stabilisation, tip out the soil from each jar, mix it thoroughly by hand and return it to its respective jar before adding the worms.

Preparation of the test organism

Seven days before the start of the test, collect clitellate adult *Eisenia fetida* or *Eisenia andrei* (weighing 300 to 600 mg) from a culture and place them on manure in a controlled environment at 20 ± 2°C and subject to an 18-hours light, six-hours dark regime to acclimatise to the test conditions. The volume of manure should be in proportion to the holding culture.

Collect groups of 10 (fully clitellate) adult worms from the available stock and weigh them either individually or as a group. Based on the group weights, assign

worms to replicates to ensure an even distribution of mean weights between treatments.

Once assigned, add the worms to the surface of the soil. Healthy worms will burrow below the soil surface within 15 minutes. Remove any worms that have not entered the soil within this timeframe and record these worms as damaged. Replace them with new worms, remembering to weigh them prior to substitution so that the start weight can be recorded.

In some severely contaminated soils, an avoidance response can mean that a number of the worms may fail to burrow into the soil. If this is the situation after two to three days, the worms will die on the soil surface. Record this fact as it is a measurable endpoint for the test.

Monitoring test conditions and feeding regime
Monitor the jars weekly for moisture loss by recording total jar weights. Add distilled water as required to maintain the start weight.

Provide food during the exposure as follows:

- dry uncontaminated horse manure in an oven at 60°C overnight;
- re-wet 4.5 g (dry weight) to 80 per cent moisture content for 24 hours (but not longer than 48 hours);
- scatter it on the soil surface of each jar;
- seal the jars with an air-permeable covering (such as gauze mesh).

Incubate the jars at 20 ± 2°C and under an 18-hours light, six-hours dark regime.

On day 14, remove uneaten manure and empty each jar. Remove surviving adults from the soil. Rinse them in distilled water before counting and weighing them (blot dry on tissue paper before weighing). Then return the original soil, the worms and a fresh 4.5 g (dry weight) of wetted manure to their respective jars.

Test duration and endpoints
On day 28, remove the adult worms and weigh them to allow the mean weight change over the test duration to be determined. Do not return the worms to the test vessel.

The contents of the jars (without adults) can now be sieved to allow reproduction to be assessed in terms of cocoon production, or returned to controlled environmental conditions to allow cocoon development.

After 56 days from the introduction of adults, assess the number of juveniles and/or cocoons. This can be done in one of two ways:

- by wet-sieving the soil through a two mm and then a one mm mesh, and counting the number of hatched and unhatched cocoons and juveniles present;

- by placing the jars of soil in a heated water bath at 60°C and collecting juveniles as they come to the soil surface.

If the latter method is used, sieve the soils to collect cocoon shells if total productivity is to be determined.

In both cases, juveniles may also be weighed to assess total productivity by mass.

4.13.6 Test method: *Lumbricus rubellus*

This test is based on the above procedure for *Eisenia fetida*/*Eisenia andrei*, but includes a number of modifications to account for differences between the species (such as the larger size and slower reproduction rate of *Lumbricus rubellus)*.

Preparation of the soil

Four replicate test vessels consisting of glass or plastic containers with dimensions of approximately 180 mm x 180 mm x 90 mm (such as ice cream containers) are recommended. Add 1,400 ml of soil (a fixed volume is used to avoid any changes in density that could arise from the use of a constant weight of soil) to each test vessel.

Re-wet the soils to approximately 50 to 60 per cent of their moisture retention capacity as descried above for the test with *Eisenia*. Mix the soils thoroughly and leave to stabilise for at least 24 hours, but not more than 48 hours. After stabilisation, tip the soils from the containers. Mix them thoroughly by hand and return them to their respective containers before adding the worms.

Preparation of the test organism

The *Lumbricus rubellus* selected for use in the assay should be adult (fully clitellate) and weigh 600 to 1,500 mg. One week before the test, recover sufficient adult worms from the holding units and transfer them to plastic boxes containing culture medium. About 200 worms can be kept in a 0.5 m^2 box containing a 20-cm depth of culture soil.

For each test replicate, select eight suitable adult worms and weigh them individually or in batches. Add the batches to the test replicates, ensuring an even distribution of batch mean weights between the sites. Place the adult worms on the surface of the soil in each test container.

At the same time as the worms are added, add food as follows:

- dry manure in an oven at 60°c overnight;
- re-wet the dry manure to 80 per cent water content;
- add 10g (dry weight, 40 g wet weight) horse manure to the test vessels.

Incubate the vessels at 12 ± 2°C under an 18-hours light, six-hours dark regime.

As for *Eisenia*, assess the ability of the worm to enter the test soil by noting any repellent effect on burrowing after the first 24 hours.

Monitoring conditions and feeding regime
Monitor the jars weekly for moisture loss by recording total jar weights. Add distilled water as required to maintain the start weight.

After 14 days, remove uneaten manure and empty each jar. Remove surviving adults from the soil. Rinse them in distilled water before counting and weighing them (blot dry on tissue paper before weighing). Then return the original soil and adults to their respective jars and add fresh wetted manure (10 g dry weight re-wetted as above).

Test duration and endpoints
The minimum duration of the test is 28 days; a longer exposure can be used if desired. After at least 28 days, remove the adult worms and weigh them to allow mean weight change to be determined. Do not return the worms to the jar. Excess food can also be collected and weighed. Record adult earthworm survival for each vessel.

Two approaches can be used to measure reproduction rate in *Lumbricus rubellus*.

Firstly, after removing the adults, sieve the soils through a two mm and then a one mm mesh, and collect and count any cocoons present. This allows reproduction to be assessed in terms of cocoon production rate. This approach is likely to measure the most significant effects on earthworm reproduction in industrial contaminated soils where toxicity is due to effects on the energy dynamic and, as a result, the ability of the worm to produce cocoons (Spurgeon *et al.,* 2003, 2004b).

Alternatively, in soils where endocrine-disrupting or teratogenic compounds are suspected, it may be necessary to measure effects on cocoon viability. The approach used for *Eisenia* of incubating cocoons in the main experimental soil is not recommended, due to the long incubation time required for this species. Juveniles that hatch early could die and remain uncounted, cocoon shells could rot and disappear, and there could be implications for degradation of the contaminant profile.

Instead, sieve the cocoons from the soil at the same time as removing the adults (see above). Then expose them for at least 16 weeks to a new sample (at least 250 g) of the field soil (from the same batch) in which they were previously laid. Add food to the surface of the container as above, removing and replacing it every 14 days.

After this second incubation, sieve the soil as above and count the number of hatched and unhatched cocoons and juveniles. Juveniles can also be weighed.

After the tests

Following the tests with both species, surviving worms can be depurated by placing them on wetted filter paper for 36 hours (changing the paper every 12 hours). Worms can then be stored at –20°C for subsequent chemical analysis and/or –80°C for other biochemical or molecular biomarker analysis.

4.13.7 Selection of appropriate control soils

Full evaluation of contaminated soils requires quality control criteria to be met. This includes three control soils with each batch of earthworm toxicity tests, as follows:

- a **negative control** soil to assess the performance (survival, weight change, reproduction rate) of the earthworms under normal (uncontaminated) conditions;

- a **positive control** to demonstrate that the population of worms used in the study is typically sensitive to species reported in previous toxicity studies;

- a **site-specific control** that evaluates the performance of the earthworms in an uncontaminated soil with the same physicochemical properties as the potentially contaminated soils.

In any experiment, the total number of control vessels should be at least 10 per cent of the total number of test jars, with a minimum of four replicates per control.

4.13.8 Data handling and reporting

The report for the earthworm test should include the following information:

- location of sites and specific sample locations in the study;
- physiochemical details of the tested soils including pH, soil organic matter content, bulk density, soil texture and particle size distribution;
- concentrations of any chemical measured within the test soils;
- details of soil collection and handling procedures to prepare the soil for bioassay;
- nature and location of negative, site-specific and positive controls;
- test species, age, source of supply, keeping and breeding conditions;
- test conditions – description and details of any variation in test materials and recommended conditions, including information on the preparation of the test medium and any method used for soil sterilisation;
- food supplied during exposure, including its source and treatment before being added to test replicates;
- number of adults added to each replicate and the number recovered per treatment;
- counts of the number of cocoons collected and, in tests with *Eisenia fetida*/*Eisenia andrei*, the number of hatched and unhatched cocoons and the number and weight of hatched juveniles recovered at the end of the post-exposure incubation period;

- description of obvious physical or pathological symptoms or distinct changes in behaviour observed in the test organisms.

4.13.9 Data analysis

Risk assessors will need to know the following results for these endpoints:

- number of surviving adults at day 14 and day 28 expressed as a percentage in each replicate (test container);
- total mass of alive adults per replicate at the beginning of the test;
- total mass of surviving worms per replicate after 28 days;
- changes in body weight of live adults (percentage of initial weight) in each replicate after 28 days;
- total number of cocoons counted in each replicate;
- normalised reproduction rate per replicate accounting for adult mortality (see Equation 1).

Equation 1:

$$\text{Cocoon production rate} = \frac{\text{total cocoons}}{(\text{worms day 0} + \text{worms day 14}/2) + (\text{worms day 14} + \text{worms day 28}/2)}.$$

These results can be used by risk assessors to compare survival, weight change and reproduction between treatments (including the site-specific reference) and against the negative control. The mean number of adults surviving can be compared between treatments and with the negative control using analysis of variance (ANOVA) methods. A confidence level of 95 per cent is appropriate.

This provides a comparison of mean adult survival in contaminated soils versus control or site reference soils. If there is a significant difference at the 95 per cent confidence level ($P < 0.05$), this is deemed an effect worthy of further consideration.

ANOVA may be performed in a number of modern statistical or general software packages and includes General Linear Model, Student's t-test and Dunnett's test.

4.14 Use of field soils in biological assays (Method 2.5)

In studies assessing the effects of contaminants in field soils (for example, using biological assays selected by the Environment Agency for the risk assessment of contaminated land in projects SRP050/0611, P5-029, P5-063 and P5-069), it is necessary to visit the field site and collect a soil sample representative of the area. Because field-collected soils may differ greatly in the basic soil characteristics that influence pollutant availability (such as mineral and organic matter content, water holding capacity), as well as possible chemical content, some alterations are necessary to standardised protocols to allow them to be used for toxicity assessment.

4.14.1 Overall strategy and considerations in soil collection

Any site investigation as part of the management of contaminated land must be based on a conceptual site model. This should identify possible contaminants that could be present in the soil, along with where they might occur. In practice, however, prior knowledge of a site is likely to be incomplete. To ensure the sample collected is representative of the location, the collection of a number of subsamples, which are subsequently pooled and mixed, is recommended.

Most soil organisms occupy the upper soil layers (including the litter). To ensure realistic exposure, collect soil from the upper layers (zero to 15 cm after removal of the litter), although deeper sampling may be required if there is deep contamination and/or burrowing or deep-rooted receptors (refer to conceptual site model).

In practice, anthropogenic disturbance around potential contaminated sites may mean that there is considerable heterogeneity in soil types around any site. Thus, it may be necessary to collect samples from a range of possible control patches and send these to the laboratory for initial soil physicochemical analysis (pH, percentage loss on ignition and soil texture) and an initial set of chemical analyses. The most suitable patch to act as the site control can be selected on the basis of these analyses.

4.14.2 Collection vessels

For chemical analysis, various specialist containers are recommended. The type should be agreed with, and if possible supplied by, the laboratory carrying out the analysis.

Given the larger volumes needed for biological testing, plastic sealable (such as snap-lid) containers should be used to retain volatiles. They should be large enough to accommodate the volumes required.

4.14.3 Sample volumes and collection procedure

The quantities of ‘test-ready’ soil required for biological test protocols recommended in the ERA Framework are given in

Table 4.1; the values are an approximation of the weight of dried (and wet) soil required. Because the tests need to be set up using a constant volume of soil rather than a constant weight (to avoid any effects on organism performance due to density-dependent regulation), these values should be treated as approximate.

Table 4.1: Approximate weights of soil required for biological assays for contaminated site assessment and soil weights recommended for collection from the field for each assay*

Assay	Per replicate (grams)	Number of replicates	Per sample point (kg)
Earthworm physiology	1,500	4	6
Plant growth assays	400	5	2
Chemical analysis	50	4	0.2
Total dry weight	2,050		~8
Total wet weight – coarse sorted in field	~4,000		~16

* Weights are approximate and can be varied depending on soil bulk density and water content (more needed in dense and/or wet soils; less in low density and/or dry soils). Number of replicates is given according to standard guidelines for the individual tests.

Where the soil in the study area has a high bulk density or high water content, it is advisable to collect a greater weight of field soil. Do not carry out soil collection when the soils are saturated (for example, following heavy rainfall). When the soil has a low bulk density and/or is very dry, a lower weight of soil may be sufficient.

Because the tests require the use of homogenous coarse-sieved soil, free from building material, root matter, large stones and so on, the weight of field-moist soil collected must equate to approximately twice that required for the combined set of assays.

At each sampling patch, mark out a one m^2 area and excavate soil using a spade from the four corners of this marked central square (see Figure 4.10). In each corner, prepare an area of 0.5 m^2 for excavation by removing the turf to the base of the grass root area; take care to shake off as much of the root associated soil as possible. Place the turf to one side for later replacement (after back-filling the excavations with topsoil).

Excavate underlying soil to a maximum depth of 15 cm using a spade, though if it is suspected that contamination is principally in the surface layers (for example, as a result of aerial deposition of particulates), it may be possible to restrict soil collection to the upper layers. During collection, remove and discard any large items of debris, large stones and root material.

Soil samples taken from the four corners of the patch are pooled and mixed to provide a homogenous soil sample that is representative of the patch. Initial mixing of the soil from each of the four corners takes place on-site, to create one sample per patch for transport to the laboratories.

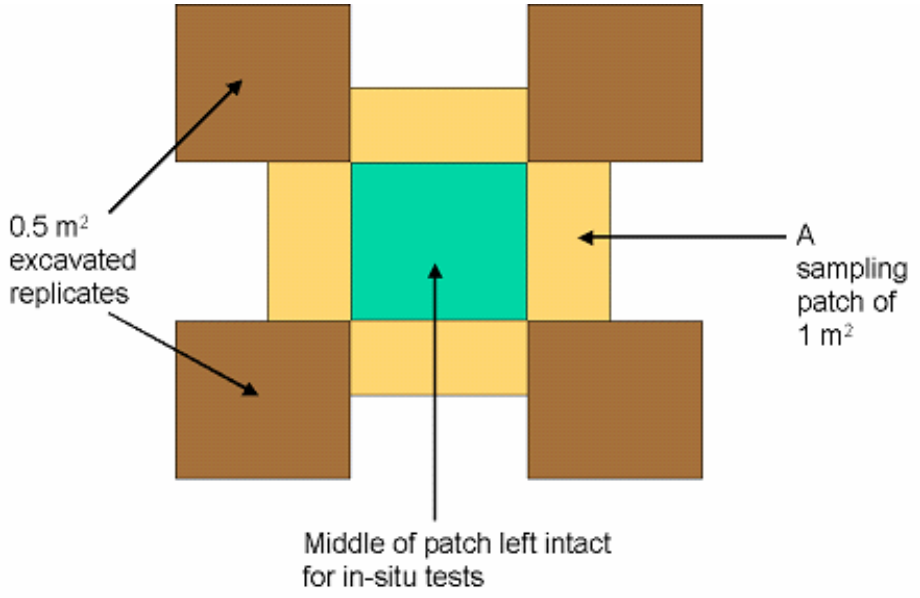

Figure 4.10: Sampling patch illustrating where replicates are excavated

Sampling soil from only the patch corners allows the central area of the patch to be left undisturbed. This area can then be used for any required *in situ* testing (such as the bait lamina assay), though it is preferable to conduct such assays before removing any of the patch soil.

To roughly homogenise the sample, place all the dug soil on a strong canvas or plastic tarpaulin, and mix it thoroughly using a garden fork or spade. Then place the mixed soils in the collection vessels. Label all the containers used both internally and externally (for example, using permanent marker pen on plastic cards).

Once collected, all samples must be kept cool and in the dark and transferred to the laboratory within 12 hours.

The weight of soil collected from any site (particularly if there are a number of areas at which collection could take place) will make it necessary to use a vehicle to transport the soil off-site. Make sure that this vehicle is capable of carrying the weight of soil collected safely. This applies particularly to cars when loading the collected soil in the boot compartment.

4.14.4 Preparation, analysis and interpretation

On arrival at the laboratory, examine the soils visually and record any observations. Store all collected material in the dark at 4°C, where these cool conditions should limit the disappearance of organics. Despite these measures, any disturbance of the soil (including that needed for chemical analysis) will inevitably cause loss of, for example, C_1–C_4 hydrocarbon compounds. For this reason, it may be advisable to conduct an initial analysis of the contaminant profile immediately after collection. This can then be repeated at the start and

end of individual bioassays, to allow quantification of the loss of volatile or fragile contaminants during storage and over the course of the exposure.

For contaminated soil (where the exact composition of contaminants may not be known), soil preparation should minimise the loss of volatile and unstable compounds and maintain the integrity of the soil for some biological tests (such as the nitrogen mineralisation test).

Soil preparation for laboratory bioassays should be completed as follows.

For soils that will be used in toxicity tests for invertebrates, it is necessary to sterilise samples to eliminate indigenous fauna and reduce the size of the seed-bank. This can be done by freezing samples to -20°C for at least 24 hours and preferably for 72 hours (over a weekend). Soils should then be defrosted ready for preparation for each assay.

Soils for the remaining assays (such as plant growth tests) are used directly from the field and should not be sterilised. Store these samples at 4°C to await use in the assay.

1. Following freeze sterilisation or prior to starting plant tests, roughly screen the soil that will be used in each assay (for example, pass it through a 10 mm mesh while still damp). Drying the soil is not recommended as this will result in the loss of a large quantity of the more volatile organic compounds. After screening, return the soil to the sealed containers and place them in the 4°C facility again.

2. To initially characterise each soil, remove a subsample of the screened samples for physicochemical characterisation. The conducted analyses should include (at least):
   - water holding capacity
   - pH
   - percentage loss on ignition
   - soil texture.

3. Once the physicochemical characterisation is complete, soils for use in each of the specific assays can be separated. To ensure maximum comparability between the results of different assays, carry out the separation at the time at which soils are needed. This will prevent divergence of contaminant profiles during storage of soils.

Further divide the soil sample to provide the soil needed in each of the biological replicates for that location.

To prepare the soil, add water as required until the soil reaches the required water holding capacity for each assay. In some cases, the soil may be wetter than needed for the biological test. If particularly heavily waterlogged, move the soils into water-permeable sacking to allow excess water to run out of the saturated soil before it is used. If soils are only slightly too wet, use them directly because any errors relating to the effects of water content on organism

performance are likely to be less than those arising from loss of specific volatile compounds during an extended drying procedure.

Further preparation, analysis and interpretation depends on the biomonitoring programme; see Appendix A1.3 for an example protocol for an analytical technique.

4.15 Plant seedling emergence, growth and vegetative vigour tests: monocotyledons and dicotyledons (Method 2.2.6)

4.15.1 Introduction

Toxicity testing with plants has been carried out for many years, mainly to help prevent damage to crops by pesticides and other plant protection products. These tests are used routinely in the approval of agrochemicals, but many data are not publicly available due to product confidentiality agreements. The procedure recommended in this guideline is based on the method described by Environment Canada (Environment Canada, 2005) with modifications described by Fountain *et al.* (2005).

The aim of the test is to assess the impact of soil contaminants on the reduction in seedling emergence, growth and vigour in common monocotyledonous and dicotyledonous crop plants.

4.15.2 Test method

Selecting species and obtaining suitable seeds

Unlike other guidelines, many species are acceptable in international guidelines for plant tests.

In general, plants are selected according to the following criteria:

- accessibility;
- amenability to testing in the laboratory, to allow reproducible results within and across testing facilities;
- plant uniformity;
- economic importance as food, forage or ornamental crops;
- distribution, abundance and taxonomic representation suggesting broad applicability of results to other plant species;
- sensitivity to many toxic compounds, as indicated by previous bioassays;
- compatibility with growth conditions and time constraints of the test method;
- no need for pre-treatment of seeds (such as soaking, chilling pre-washing, exposure to light, or scarification).

Although these criteria are not equally applicable in studies of the ecological effects of contaminated land and associated pollution, practical realities currently necessitate the use of the same species as those used for crop protection work.

For crop protection products, the number of species typically varies from six to ten species representing two monocotyledonous and four to six dicotyledonous families, with a species ratio of approximately 1:2 monocotyledonous-to-dicotyledonous. This list may be extended to include non-crop species if a suitable seed source, culturing conditions and information on the amount of variability within the species and desired endpoints are available.

For general chemical assessment and testing (including to assess contaminated soils), the use of two or three species is recommended: one monocotyledon and two dicotyledons (representing three families). Species should be selected on the environmental conditions suitable for the species based on soil characteristics such as pH, soil type and so on.

All seeds to be used in the assay should be purchased less than one month before the test begins, to ensure maximum possible emergence in all treatments. Details of the supplier should be recorded and reported.

Test pot size and seeding density: modifications to the OECD protocol
A number of modifications to the Environment Canada (Environment Canada, 2005) method are suggested, to make the procedure more relevant to contaminated land assessment. These involve the test pot size. As part of Environment Agency Project P5-069, Weeks *et al.* (2004) assessed the effects of using smaller test pots (10 cm diameter) when examining the emergence and growth of pea and wheat (comparisons were made on identical sowing densities in each pot size). Their results showed no significant difference in the emergence or dry weight of either species grown in 10 cm pots compared with 15 cm pots. Therefore, this guideline recommends the use of 10 cm diameter pots.

Use a plant pot that is 10 cm in diameter and 10 cm high, made of non-porous plastic or glazed with individual trays (such as a Petri dish) underneath. Five replicates per site should be used when using field-collected soil.

Fill the pots with approximately 400 g of soil. It is important to use a constant volume of soil because use of a constant weight of soils with different bulk densities would result in plants being present in different amounts of soil and thus different volume densities. Use of a constant soil volume should avoid density effects if competition for root space means that crowding affects plant growth. The total amount of nutrients within each test replicate may be lower (depending on concentration) in less dense soils. This could influence growth, but the extent of this effect is currently unclear.

Before soils are added to the pots, they should be re-wetted to approximately 60 per cent of their moisture retention capacity, as determined using an established method (Environment Canada, 2005; Kalra and Maynard 1991). Wet the soil in a separate container before transferring it after one day to the test pot. After this time, plant seeds can be sown into each pot. Place the seeds according to the

supplier's instructions and in a defined pattern, to allow distinction between test and non-test species. Treat the reference and site-specific control groups in the same way as the test soils.

Growing the seedlings
Having placed the recommended number of seeds within each pot, record the weight of all replicates. Use these data to monitor water loss from the soil during the test. Monitor water loss by weight change twice a week and add water to the pots as necessary to compensate for this loss. Do this by base, rather than surface, watering.

Place pots with planted seeds in a random arrangement within a controlled chamber, phytotron or greenhouse. In practice, glasshouse lighting and temperatures can be difficult to maintain and enclosed controlled environment rooms/chambers are preferred for the long-term maintenance and growth of plants. However, the facilities must provide conditions suitable for the species selected (particularly lighting).

The addition of nutrients to soils is not currently recommended, as it is not clear how these might interact with the contaminants present.

Day zero starts when the seeds are placed into the soil. The tests run for 14 or 21 days (depending on the species used, see Environment Canada (2005) for details). For this test, emergence is defined as when seedlings have achieved a height of two mm above the soil surface. Once this has occurred, manually remove any seedlings of other species that emerge; non-test species can be recognised by their position outside the pattern in which the test species were planted.

Record seedling emergence and visual effects (such as chlorosis, development abnormalities) weekly and compare them to the plants in the positive, negative and site-specific control soils.

At the end of the test, tip out the soil and extract the plant material carefully. Record the wet weight of the plant material and, following drying at 60ºC for 24 hours, record the dry weight. Record the root and shoot length and the number of emerged seedlings at the end of the test.

Selection of control soils
When selecting control soils, the same principles apply as in other guidelines.

The use of three control soils is recommended as follows:

- a **negative control** soil to validate the performance (emergence, growth) of the plants during the test;

- a **positive control** to demonstrate that the seeds have a sensitivity typical of that reported in previous toxicity studies;

- a **site-specific control** to evaluate the performance of the test seeds in a clean soil of a type similar to that in the potentially contaminated patches.

Following selection, control soils should be treated in the same way as the potentially contaminated soils.

Performance criteria for validation of the test
Validity criteria for the Environment Canada plant test are based on control emergence (above 65 per cent emergence required) and visual condition of the emerged plants. No formal criteria for acceptance of test results are available, but percentage emergence should be as high as possible (near 100 per cent) in control soils.

4.15.3 Data handling and reporting

The report for the plant test should present:

- the results of the studies;
- a detailed description of test conditions;
- a thorough discussion of the results, analysis of the data and conclusions drawn from this analysis.

The report should include the following information:

General
- location of sites and specific patches within the study;
- physiochemical details of the tested soils including pH, soil organic content, bulk density, soil texture and particle composition, and the levels of any chemical measured within the test soils;
- details of collection and handling procedures to prepare soils for bioassay;
- nature and location of negative, site-specific and positive controls.

Test species
- species and variety, source and history of the seed (name of the supplier, batch or lot number);
- number of monocotyledon and dicotyledon species tested;
- description of seed storage, treatment and maintenance.

Test conditions
- testing facility (growth chamber, phytotron, greenhouse);
- description of test system (pot dimensions, pot material, amount of soil);
- soil characteristics (texture or type of soil, physical and chemical properties including pH and percentage organic matter);
- number of seeds per pot;
- number of replicates (pots) per sample point;
- duration of test;
- growth conditions: light intensity, photoperiod, day/night temperatures, watering schedule.

Results

- table of all endpoints for each replicate and species;
- number and percentage emergence compared with controls;
- biomass measurements, fresh and dry weight as a percentage of the controls;
- percentage visual injury (chlorosis, necrosis, wilting, leaf and stem deformation, as well as any lack of effects) compared with control plants.

Express results as:

- the number of seedlings emerging per replicate;
- the quantity of biomass (wet and dry weight) at the end of the test.

These numbers can be compared between treatments using ANOVA (analysis of variance) methods to establish which patches give a significantly lower survival compared with controls. Juvenile counts give a measure of total reproduction during the exposure. These can be compared between treatments by ANOVA and *post hoc* testing to establish significant differences with the controls. Assuming that there is adequate germination and growth in the site-specific control, data for this soil should be used for all statistical comparisons.

4.16 Quantitative RT-PCR for chemical effect assessment in species: selection of biomarker gene targets and initial approach to quantification (Method 3.7)

New detection methods for assessing the expression of individual genes have opened up opportunities to use gene transcription quantification to assess the status of organisms at potentially contaminated sites. Changes in the expression of genes and proteins will be the first indications of an impact of a chemical on organism biology. At present, the most suitable and simple methods in molecular biology for assessing changes in the expression of any individual gene (by quantifying the amount of specific messenger RNA (mRNA) present in cells) are based on the use of the reverse transcriptase-polymerase chain reaction (RT-PCR).

To undertake quantitative analysis of gene expression using RT-PCR, it is first necessary to collect a representative sample of RNA from the test organism. This is then converted to complementary DNA (cDNA) via the reverse transcription reaction. This cDNA sample can then be used for gene-specific quantification. During all RNA handling steps, however, great care is essential to protect the sample from degradation by widespread RNA-degrading enzymes known as ribonucleases (RNAses).

Changes in the expression of potentially pollutant-responsive genes can be measured for a vast range of sequences using a variety of protocols. It is

therefore not possible to describe fully all possible targets and approaches in this guideline. Instead, details of two aspects are given:

- sequences that can potentially be used for analysis;
- the use of a specific technique for a non-model terrestrial invertebrate species.

The aim of the test is to assess the impact of soil contaminants on the expression of target genes by isolating mRNA and using quantitative RT-PCR.

4.16.1 Single gene transcript quantification methods: scope, performance and adaptation for non-model species

To use RT-PCR for a quantitative analysis of gene expression in any biological system requires progression through a series of steps. These stages are detailed below.

Knowledge of the gene sequence is vital for RT-PCR in order to design the primer oligonucleotides that will be used to amplify and detect the target. The first step is to clone and sequence the required gene product based on global expression studies, targeted comparative analysis or detailed gene hunting. Sequences can be identified using bioinformatic methods with existing databases or functional approaches.

Following sequencing, probes and primers to detect the gene target can be designed. Quality control checks are then conducted to confirm that:

- the primers amplify only the single targeted sequence;
- the primers amplify the expressed gene product and not the genomic DNA region from which it is derived.

This can be done either bioinformatically or by resolving the gene product using gel electrophoresis to confirm the presence of only a single PCR product of the correct amplicon size. The amplified genomic DNA product will be larger that the expressed transcript, due to the presence of introns in the amplified region.

Assuming the primers are gene-specific, the next stage is to optimise the assay. For quantitative analysis, a set of standards should be prepared for each sequence based on a dilution series of known concentrations of either a plasmid preparation containing the gene of interest or a quantified sample of PCR product. These standards are used to generate a curve describing the amplification patterns of different concentrations of the initial starting transcript. This curve can be used to assign gene transcript concentration within the unknown samples (see Figure 4.11).

4.16.2 Method for quantitative RT-PCR measurement in earthworms

RNA isolation and handling

After preparation of reagents and consumables for RNA handling (ensuring RNase free), RNA can be collected from the samples. A range of reagents (such

as TRI Reagent®, RNAzol) can be used to collect total RNA from eukaryote tissue.

The sample is first homogenised in an acid guanidinium thiocyanate phenol reagent. This is then centrifuged to remove any remaining cellular debris and chloroform is added. Collection of the RNA sample proceeds in a two-stage process. In the first stage, chloroform is added to the sample and, after shaking, the sample is centrifuged. This separates the reagent into two phases: an upper aqueous phase that contains the RNA and a lower organic phase that contains the DNA. Proteins collect at the interface between the two phases. In the second stage, the aqueous phase is collected and the RNA precipitated by addition of 2-propanol (also known as isopropanol, *iso*-propyl alcohol, IPA); when spun at high speed, the RNA collects as a pellet at the bottom of the tube. After removing the 2-propanol and washing with 75 per cent ethanol, the pellet of RNA can be redissolved in RNAse-free water, ready for further purification and use.

In some cases, a purification step may be required to remove impurities from the total RNA sample which can potentially block reverse transcription. One relatively simple purification step is a phenol/chloroform clean-up. Alternatively, commercially available RNA separation columns can be used.

Following purification, the RNA should be examined using spectroscopic techniques to determine the total concentration of RNA present and also its purity. Next, the total RNA should be assessed qualitatively using agarose gel electrophoresis. High quality, non-degraded RNA samples should show clearly defined bands of ribosomal RNA, the exact molecular weight of which will vary depending on the species being studied.

A number of suppliers also provide kits to purify mRNA from total RNA samples.

Reverse transcription

The next stage is to reverse transcribe the RNA samples. This involves the use of the mRNA as a template for the synthesis of a complementary strand that changes the transcript to a cDNA.

Reverse transcription is catalysed by the enzyme reverse transcriptase. This enzyme can be obtained from all major molecular biology reagent suppliers at a number of purity levels. The exact protocol to be used varies between the available reverse transcriptases, with manufacturers supplying protocols with each enzyme type.

To help normalise the data, the quantity of starting RNA present within the sample should be similar in all RNA samples used for the reverse transcription reaction.

Quantitative RT-PCR detection of gene copy numbers

Once reverse transcription has been performed, the samples are ready for quantitative gene analysis based on RT-PCR. The power of PCR makes the procedure ideal for detecting gene-level responses of organisms exposed to pollutant stress.

All instrument platforms for conducting quantitative RT-PCR analysis are based on the principle of fluorescence detection to monitor the production of the product during PCR cycles. Two approaches are commonly used to produce fluorescence. The first is based on a fluorescent dye (SYBR® Green I), while the second uses specifically designed oligonucleotide probes that contain a fluorescent and a quencher molecule. Reagent and probe sets can be bought from major analytical equipment suppliers.

The two methods are as follows:

- **SYBR® Green I dye.** This fluoresces when bound inside the double helix of DNA. Because the fluorescence is proportional to the amount of DNA, repeated monitoring after completion of each PCR cycle allows product quantification to be assessed in real time over the duration of the PCR reaction.

- **The fluorogenic 5' nuclease assay.** This uses the 5′ nuclease activity inherent as a secondary function of *Taq* DNA polymerase. An oligonucleotide probe complementary to the target is included within the reaction. The 3′ end of the probe is labelled with a fluorescent molecule such as FAM (6-carboxy-fluorescein) and the 5′ end with a complementary quencher such as TAMRA (6-carboxy-tetramethyl-rhodamine). When both are present, the quencher suppresses the fluorescent molecule and no light can be detected. In PCR during primer annealing, the probe hybridises to the target between the two primer sites. On primer extension, the 5′ nuclease activity of the *Taq* DNA polymerase cleaves the probe, liberating the fluor from the quencher. This results in detectable fluorescence. A schematic illustration of this detection method (commonly referred to as TaqMan®) is shown in Figure 4.11. Inclusion of the TaqMan probe in the PCR reaction allows production of an amplicon to be monitored at each cycle.

As well as supplying reagents and standardised and custom-made probes to measure transcript levels for particular genes, major manufacturers also supply analytical instruments for PCR and the detection of fluorescence over the course of the reaction.

A series of calibration standards containing cloned copies of the target gene at known concentrations can be used to establish the relationship between transcript frequency and the number of cycles required to obtain a specific threshold (Figure 4.11). This standard curve is then used to determine gene concentrations in samples.

Quantitative RT-PCR measurements should be conducted on 1 µl of either standard plasmid DNA or sample cDNA. To this, add 4 µl sterile water and 20 µl TaqMan® master-mix, with the required primer and probes included at optimised concentrations.

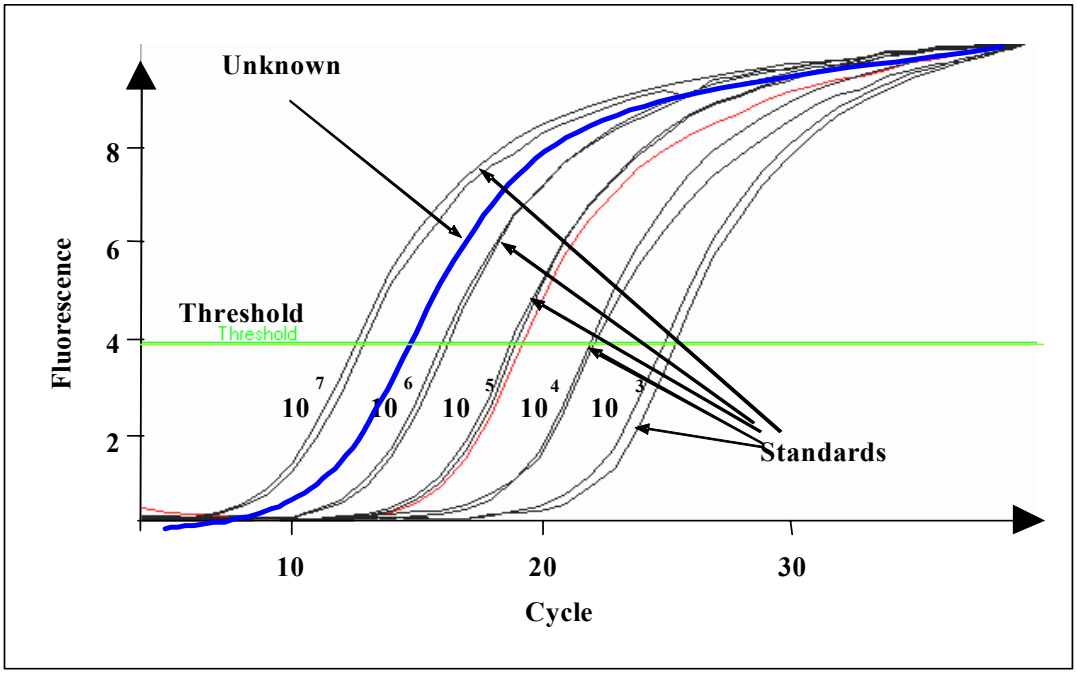

Figure 4.11: Relationship between number of cycles and fluorescence detection of PCR products

In quantitative RT-PCR studies, it is usual practice to normalise quantification of the target gene to account for differences in the amount of template present. For this, parallel measurement of a control (non-responsive) gene(s) can be employed (Bustin, 2000; Stürzenbaum and Kille, 2001). A number of invariant 'housekeeping genes' including actin, glyceraldehyde-3-phosphate dehydrogenase (GAPD), ribosomal genes, cyclophilin and elongation factor 1-alpha have been identified for this purpose (Bustin, 2000). When selecting a suitable invariant gene, two major properties are needed:

- the gene should be essential for function and viability and, as a result, ubiquitously expressed in all tissues;
- transcription should not be affected within the experimental context being investigated.

The issue of which gene is most suitable as an internal control for quantitative RT-PCR has been the subject of considerable debate. The outcome appears to be that no one gene can be employed reliably to account for differences in the quantity of initial starting template within the sample. For this reason, the simplest strategy is to normalise analyses at the outset using the same quantity of total RNA for the reverse transcription in each sample. Use of a single batch of reagent in sample preparation and random placement of different treatment samples across the PCR plate used for reverse transcription should minimise systematic treatment-dependent bias in samples.

4.16.3 Data handling

In gene expression studies, the usual aim is to compare gene expression in two tissues:

- a tissue subjected to an experimental condition (treated), such as a toxicant;
- a reference tissue (untreated).

In order to correct for differences in tissue amounts, expression of the experimental gene is analysed relative to the expression of a 'housekeeping' gene, here called the control gene. Expression of the control gene is assumed not to vary in the tissues or cells under investigation, or in response to the experimental treatment.

This guideline uses the terms 'gene', 'target' and 'amplicon' loosely. However, the terms 'target' and 'amplicon' are used to refer to that part of the gene 'between the primers' that is actually amplified, rather than the whole gene sequence.

The standard curve of a single gene target

The amplification efficiency (*E*) of the polymerase chain reaction, is important, as it indicates whether the amount of amplicon product perfectly doubled with each cycle. Equation 2 relates the amount of amplicon as a function of cycling conditions:

Equation 2

$$N = N_0(1+E)^n$$

where N is the amount of amplicon, N_0 is the initial amount of amplicon (the concentration of target template), n is the number of cycles and E is the efficiency.

From Equation 2, it can be seen that if the efficiency is one (100 per cent), the amount of amplicon doubles after every cycle. The amplification efficiency is often below 100 per cent (80 to 95 per cent); this can be deduced from the slope of the standard curve. The standard curve is a straight line on a plot: on the *x*-axis, the logarithmic value for the starting amount of amplicon [$\log(N_0)$] and on the *y*-axis, the threshold cycle (Ct).

The standard curve takes the form y = *a* + *b*x, which is a straight line; in this example, Ct = 40.3 – 3.28 × $\log(N_0)$. The amplification efficiency can be calculated using Equation 3:

Equation 3

$$E = 10^{-1/b} - 1$$

where *b* is the slope of the standard curve. However, this MT-2 dataset is not a good example to illustrate the consequences of poor amplification efficiency because the efficiency was superb: $E = 10^{-1/-3.28} = 1$, in other words, 100 per cent.

5 Potential sources of archived material for retrospective studies of ecosystem change

In order to evaluate potential diagnostic measurements that could indicate outcomes, or an increased risk of effects leading to outcomes, it may be useful to apply techniques to material sampled before the particular chemical stress was applied. For widespread pollutants (such as deposited sulphur or nitrogen from coal combustion) samples may need to be investigated from the early years of the last century or earlier. In some cases, it may be possible to use historical samples to indicate where environmental conditions have improved. More generally, the use of archived samples could provide a reference against which current-day records could be compared – to establish what constitutes normal behaviour of biota in response to a particular stress.

In a particular situation, for example where an industrial source was established several decades ago, it may be instructive to investigate how the environment (as measured by the chemical composition or morphology of biota nearby) has changed. If no biological sampling was carried out before construction of the source, this would be impossible. However, samples may have been taken for some other reason, and their use would enable a *post hoc* analysis of any environmental effects.

The type of biological samples that would be useful in any retrospective analysis would depend on the question being asked. Consequently, the following information does not provide great detail on what is available, or a catalogue of holdings (which is beyond the scope of this study), but suggests lines of enquiry for those embarking on a retrospective study. Where possible, a contact name has been provided, often of a researcher involved in biological monitoring who may have an appreciation of what may be available within his or her organisation. In other cases, examples of a type of source are provided, such as a local natural history society. We have not researched where such organisations exist throughout the UK or what (if any) biological material they may have archived; their inclusion is to provide suggestions for the type of body (often voluntary) that could hold detailed local knowledge in a particular case.

Institution	Contact name	E-mail/telephone website address	Position	Specimens held
Botany				
Welsh Plant Breeding Station		Aberystwyth		Temperate forage plants of agricultural value, such as species of *Lolium, Festuca, Dactylis* and *Trifolium*. Maintains a seed bank with medium-term storage capacity, containing 6,688 accessions, representing 464 species.
British Antarctic Survey	Helen Peat	hjpe@bas.ac.uk		40,000 plant species from Antarctica and sub-Antarctica. Mosses, liverworts, lichens and a small collection of vascular plants.
Defra National Focus Point on Access to Genetic Resources and Benefit Sharing		genetic.resources @defra.gsi.gov.uk Branch Research Policy and International Division. www.defra.gov.uk/Science/GeneticResources/Access/Ex-Situ/Botanic Gardens.asp		Lists UK botanical gardens. Each garden entry states whether there is a herbarium and summarises specimen numbers and main collections. Some have been listed in this table. Links to PlantNet (see below).
Herbaria (Cambridge)	Gina Murrell	gina.murrell@plantsci.cam.ac.uk www.plantsci.cam.ac.uk	Ass. Curator	50,000 plant specimens.
National Museum of Wales	Sally Whyman	Sally Whyman		32,480 specimens of mosses and hepatics.

The British Bryological Society Herbarium (BBSUK)		www.britishbryologicalsociety.org.uk/Resources/BBS Herbarium Tel. 02920 397951		
PlantNet	Judith Cheney	jc151@cam.ac.uk www.defra.gov.uk/Science/GeneticResources/Access/Ex-Situ/PlantNet.asp	PlantNet Administrator	PlantNet is the national network of botanic gardens, arboreta and other documented plant collections in Britain and Ireland.
Centre for Ecology & Hydrology. Moorhouse SSSI records	John Adamson	jka@ceh.ac.uk		Herbarium specimens; mainly bryophytes, lichens and liverworts from 1950s onwards.
Oxford University Herbaria	Stephen Harris	stephen.harris@plants.ox.ac.uk www.herbaria.plants.ox.ac.uk	Curator	800,000 angiosperms, gymnosperms, mosses, lichens, ferns, algae and fungi.
Reading University Herbarium RNG & Plant Identification Service	Dr S. Jury	s.i.jury@reading.ac.uk www.herbarium.reading.ac.uk	Curator	264,000 phanerograms, pteridophytes, bryophytes and lichens.
Personal Herbaria of Prof. M.R.D. Seaward	M.R.D. Seaward	m.r.d.seaward@bradford.ac.uk Tel. 01274 234212		25,000 specimens of botanical material; bryophytes, flowering plants, ferns, lichens, fungi, microfungi and seeds.
Natural History Museum	Rob Huxley	r.huxley@nhm.ac.uk Botany Department http://www.nhm.ac.uk/researchcuration/collections/departmental-collections/botany-collections/index.html	Head of Botany Collection	5.2 million seed plants, pteridophytes, lichens, myomycetes and algae (including substantial diatom collection).
The Perthshire Herbarium		Perth Museum and Art Gallery www.museum@pkc.gov.uk		12,000 vascular plant specimens. Many from Perthshire.
Rothamsted Archive Rothamsted Research	Paul Poulton	paul.poulton@bbsrc.ac.uk www.rothamsted.bbsrc.ac.uk/res		200,000 crops and soils from agricultural field experiments. Also see soils section below.

		ources/The RothamstedArchive		
The Linnaean Collection	Dr C.E. Jarvis	c.jarvis@nhm.ac.uk	Curator (Botany)	14,000 plant specimens.
The Linnaean Collection	Ms Kathie Way	kmwa@nhm.ac.uk	Curator (Zoology)	158 specimens of fish.
The Linnaean Collection	Dr M.G. Fitton	m.fitton@nhm.ac.uk	Curator (Insects)	3,198 specimens of insects.
Royal Horticultural Society		RHS Garden Wisley Woking, Surrey www.rhs.org.uk/research/standard_loans	The keeper of the herbarium	Horticultural specimens. No. of taxa: 23,000 (9,000 spp., 1,616 genera, 36,900 accessions, 42,500 plants). Special conservation collections: *Calluna* (24 spp., 1,060 taxa), *Hosta*, *Crocus* (115 spp.), *Colchicum*, *Daboecia* (2 spp.), *Rheum* (culinary - 1 spp., 100 accessions), *Daphne*, *Pulmonaria*, *Epimedium* (22 spp.), *Galanthus* (14 spp.).
Royal Botanic Gardens (Edinburgh)		Rbg-web2.rbge.org.uk/vherb/vherb2.php		Link to online RBGE Herbarium Catalogue.
Royal Botanic Gardens (Edinburgh)	Sally Rae	herbarium@rbge.org.uk		General. No. of taxa: 19,243 (including 3,044 genera, 15,550 spp., 34,551 accessions and 37,478 plants). A wide range of rare and endangered plants listed in the IUCN international Red Data Book. Special conservation collections: native Scottish flora.
Royal Botanic Gardens Kew		herbarium@kew.org Tel. 02083325212	The keeper of the herbarium	Approx. no. of herbarium specimens: 7,000,000, including 250,000 types, 200,000 specimens of cultivated plants and 80,000 artefacts of plant and fungal origin. A wide range of rare and endangered plants listed in

				the IUCN international Red Data Book.
University of Aberdeen Herbarium	Dr Chris Wilcock	bioscience@abdn.ac.uk Tel. 01224 272696 www.abdn.ac.uk/pss/research/herbarium	Keeper of the herbarium	120,000 plant specimens with special emphasis on British (Scottish) material; 55,000 specimens. Upland plants.
Ulster Museum Herbarium	Catherine Tyrie	catherine.tyrie@magni.org.uk www.ulstermuseum.org.uk Tel. 02890 938 3152	Curator	110,000 specimens of mostly plant collected from the wild (especially NI).
Worcester City Museums	Garston Phillips	Worcester City Museum (Natural History Collection)	Collections officer	Plants, mosses, algae, lichens and ferns.
York Museum Gardens		Yorkshire Museum, YORK YO1 2DR, Yorkshire.		44,500 specimens. Ex situ collections: *Acer, Aesculus, Acuba, Fagus, Fraxinus, Ilex, Narcissus, Rosa, Pyrus, Taxus, Tilia*. No. of taxa: 500 (870 accessions, 4,500 plants)

Entomology				
Natural History Museum	Dr M.G. Fitton	m.fitton@nhm.ac.uk www.nhm.ac.uk/research-curation/collections/entomology	Head of collections Entomology	28 million specimens of prepared insects and other terrestrial and freshwater arthropods (10 per cent from the British Isles).
Zoology				
Natural History Museum	Clare Valentine	c.valentine@nhm.ac.uk www.nhm.ac.uk/research-curation/collections/zoology	Head of collections (Zoology)	28 million: higher invertebrates, lower invertebrates and invertebrates.
PBMS (Predatory Bird Monitoring Scheme)	Dr Richard Shore	CEH Monks Wood.		Tissue samples (liver, kidney, brain, muscle and fat) of birds of prey (kestrels, sparrow hawks, barn owls, tawny owls, buzzards and heron) from the past 40 years. They also hold egg contents from the same period and the species are merlin, gannet (North Sea and Irish Sea) and golden eagle. Recently they have been storing feathers and bone, but only since 2005. There are replicate samples which are deep frozen.
CEH Monks Wood	Vic Simpson	CEH Monks Wood. (Contact via Richard Shore)		Otter samples. There has been long-term monitoring since the

CEH Banchory	Dave Carss	rfs@ceh.ac.uk dnc@ceh.ac.uk		early 1990s. Vic Simpson holds otter samples from the South and SW England. The University of Cardiff carries out postmortems on otters from the rest of England and Wales. Dave Carrs at CEH Banchory holds otter samples from Scotland, but it is not a formal archive and has been going since 1990.
CSL (York)	Dr Mark Fletcher	Wildlife Incident Investigation Scheme (WIIS)		Possible source of tissue material.
SEERAD	Ken Hunter			Possible source of tissue material.
Veterinary Laboratory Agency		Weybridge		Do rabies testing on bats and may have archive material.
University of Bristol	Stephen Harris			Holds fox and possibly badger samples.
Oxford University	Dr David McDonald	Wildlife Conservation Research Unit (Wild CRU)		Tissue samples from foxes and badgers.
National Museums of Scotland & Vincent Wildlife Trust	Dr Andrew Kitchener Dr Johnny Birks	Based in Edinburgh Ledbury, Herfordshire		Polecat tissues

Soils				
CEH	Dr Helena Black	CEH Lancaster hbl@ceh.ac.uk		Soil samples from countryside surveys
British Geological Survey		Murchison House, Edinburgh		Soil samples for UK.
The Macaulay Institute		The Macaulay Institute Aberdeen www.mluri.sari.ac.uk Tel. 01224 311556	Database manager	Approx 12,000 archived soil samples for 1950s onwards from Scotland.
National Soils Resources Centre	Richard Andrews	www.silsoe.cranfield.ac.uk/nsri Tel. 01525 863000 nsri@cranfield.ac.uk		Approx 8,000 reference soil samples from the UK.
National Soils Resources Centre	Timothy Fairwell	www.silsoe.cranfield.ac.uk/nsri Tel. 01525 863000 nsri@cranfield.ac.uk		Map information on soils.

Useful contacts

CEH Bangor	Lawrence Jones	lj@ceh.ac.uk		Don Perkin's bryophyte samples from 1970s Anglesey, N.Wales.
CEH Lancaster	Rick Stuart	rsu@cewh.ac.uk		Plant specimens collected from countryside surveys

General information on botanical gardens:
Total no. of botanic gardens recorded in the United Kingdom: 80 (plus other non-botanic gardens with major *ex situ* plant collections).
Number of gardens with herbarium: 36
Approx. no. of living plant accessions recorded in these botanic gardens: 600,000 to 700,000.
Approx. no. of taxa in these collections: 70,000 - 80,000 (around 50,000 spp).
Source: http://www.defra.gov.uk/Science/GeneticResources/Access/Ex-Situ/Botanic_Gardens.asp

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A1. Appendix 1

A1.1 Nitric acid digestion for metal determination in tissue samples

A1.1.1 Procedure: sample preparation and cold digestion

- Weigh up to 2 g wet sample into pre-weighed 50 ml boiling tube.
- Place samples in a drying oven at 80°C for 48 hours.
- Samples should be weighed at several intervals until they reach a constant weight.
- Add 2 ml concentrated nitric acid to the dried samples.
- Leave the samples in a fume cupboard fitted with scrubbers to cold digest for a minimum of 12 hours.

A1.1.2 Hot digestion

- Place the tubes in the heating blocks and heat at 90°C, gradually raising the temperature until the sample boils to a maximum of 120° C.
- Boil the sample until no more brown fumes are evolved and the digestate has become clear.
- If the sample boils too vigorously and starts to froth, remove the tube from the block and put it on the Whirlimixer for a few minutes; this should also be situated in the fume cupboard.
- Once the sample has stopped producing brown fumes, add 0.5 ml hydrogen peroxide. Allow the sample to heat for a further 15 minutes.
- Add an aliquot of deionised water to the samples and allow to cool to room temperature. This allows the undigested fat to form into large globules.
- Pour the contents of the tube into a labelled plastic ounce universal and make up to the desired volume with deionised water.
- The batch number must be clearly labelled on every tube for ease of identification and to allow the samples to be traced.

A1.2 The determination of PAHs in soil and tissue: Analysis by GCMS

A1.2.1 Scope

This method is suitable for the analysis of soil and animal tissue samples that have been extracted with DCM and cleaned up by alumina column chromatography.

A1.2.2 Surrogate standards

The following deuterated PAHs are added before sample extraction:

Acenaphthylene D8
Benzo[a]pyrene D12
Benzo[g,h,i]perylene D12
Fluoranthene D10
Phenanthrene D10
Pyrene D10

They are used to calculate the concentration of analytes in the sample by the isotope dilution method, and as retention time references for the identification of peaks in the GCMS chromatograms.

A1.2.3 Instrument operation

Running samples
Samples, standards and instrument blanks must not be transferred to chromatography vials until just before analysis by GCMS: evaporation of solvent from vials cannot be prevented, detected or corrected for once the sample/standard is in the vial. Immediately after transfer, weigh and record the new weights of all samples and standards.

Working standards must be run immediately after samples to check that the GCMS has maintained its performance during the batch run. Instrument blanks must be run between samples and standards in order to detect carryover. In between running batches, the *Standby* method should be loaded into the instrument to prevent the build-up of contaminants.

A1.2.4 Calibration

The solvent used for all standards must be the same as that used for the cleaned-up sample extracts. Calibration is by the internal standard method. The instrument response factors must be calculated from the average instrument responses (peak area) of standards run immediately before and after the samples.

A1.2.5 Multilevel calibration

A multilevel calibration should be carried out:

- Whenever there is a significant change in the instrument (such as a change in instrument parameters or replacement of the analytical column).
- When the instrument has not been used for more than a month.
- At the beginning of each major project.

Each multilevel calibration must be given a batch number. All data pertaining to the MLC should be recorded in the relevant batch directory. The range of MLC standard concentrations must:

- Enable determination of the linear range of the detector: a minimum of six concentrations are required that range from just above the instrument LoD, to above the linear range of the detector.
- Include, in addition to the above, a standard of zero concentration.

A1.2.6 Co-elutions

The following analytes are not separated using this method:

- 2,6-dimethylnaphthalene and 2,7-dimethylnaphthalene.
- 1,3-dimethylnaphthalene and 1,7-dimethylnaphthalene.
- 1,4-dimethylnaphthalene and 2,3-dimethylnaphthalene.
- Benzo[b]fluoranthene, benzo[j]fluoranthene and benzo[k]fluoranthene.

A1.2.7 Identification of analytes and calculation of results

Check all peak identities and peak integration by visual inspection of the chromatograms. For a peak to be identified as the target analyte:

- The ion ratios must be within 20 per cent of the standard.
- The relative retention time (using nearest isotopically labelled standard) of the peak must be within three times of the standard deviation of the relative retention time. The standard deviation of the relative retention time is calculated from the repeat analysis of a sample spiked with the analytes and deuterated standards.
- The retention times of the individual ions in a peak must be within one data collection point of each other.

Calculate the calibration factors for a sample using the average responses of the standards bracketing the sample. Calculate the concentration of analytes in the sample using the isotope dilution method. Subtract the total mass of the analyte in the sample blank from the total mass of the analyte in the sample.

The analyte is reported as not detected (ND) if:

The total amount of analyte in the sample is less than the method LoD.

Or

The total amount of analyte in the sample is less than the amount of analyte in the sample blank.

A1.2.8 Calculation of limit of detection (LoD)

The method LoD is defined as: the average amount of analyte in the sample blank plus three times the standard deviation of the amount of analyte in the sample blank. The sample blanks used for the calculation of LoD may either be those in each batch of samples analysed or a single batch of samples prepared specifically for the determination of LoD. In either case, a minimum of seven blanks must be used.

A1.3 Determination of PAHs in soil by GCMS: Extraction and clean up

A1.3.1 Scope

This document describes the standard method for the extraction and clean up of soil samples, for the determination of high levels of PAHs by GCMS.

A1.3.2 Procedures: Prevention of contamination and degradation

The analytes determined by this method are contaminants of the atmosphere. Any exposure to the atmosphere of the sample or reagents or surfaces that will contact the samples or reagents may result in contamination of the samples. Exposure to the atmosphere cannot be eliminated, but can be kept to a minimum.

A1.3.3 To limit contamination:

- Solvent rinse glassware immediately prior to use.
- Use vessels that can be closed with a ground glass jointed closure or a Teflon coated closure.
- If a vessel does not have a proper closure, cover tightly with aluminium foil and transfer the vessel contents to another vessel as soon as possible.
- Where possible do not prepare or store standards in the same room as samples.

A1.3.4 To limit carryover from one sample to the next:

- Do not re-use septa or seals.
- Do not analyse samples containing high levels of analytes with samples containing low levels.

A1.3.5 To reduce sample degradation:

- Do not leave samples or standards exposed to light.
- Store samples at -20°C in the dark.

- Store standards in the dark in the chemical store.
- Use amber coloured glassware where possible.
- Store sample extracts in a spark proof fridge.
- Time between extraction and instrumental analysis should be kept to the absolute minimum.

A1.3.6 Quality assurance

Batches of samples should consist of a maximum of 14 samples. In addition to the samples, each batch must contain a sample blank and a certified reference material.

A1.3.7 Sample blank

Follow all procedures (weighing, extraction, lipid determination, moisture determination and column clean up) in this document, but without using a sample.

A1.3.8 Certified reference material

Follow all the procedures (weighing, extraction, lipid determination, moisture determination and column clea-nup) in this document, using 1 g +/- 0.1 g of the CRM.

A1.3.9 Subsampling

The amount of time the samples are out of the freezer should be kept to the absolute minimum. Samples must be defrosted on the day that they are subsampled and returned to the freezer as soon as they have been subsampled. Samples should be exposed to the atmosphere for as short a time as possible.

The amount of wet soil taken for analysis should be equivalent to a dry weight of 5 g +/- 0.25g. Where there is insufficient sample, the whole sample must be used (after removing approx. 1 g for the determination of water content). If the water content of the sample is over 50 per cent, take 10 g wet weight for analysis.

Rinse a 100ml beaker with 5 x 5 ml of DCM. Allow the DCM to evaporate from the beaker, then label it and record the weight. Cover the beaker with aluminium foil. Weigh the subsample in the beaker and record the weight of beaker plus subsample.

A1.3.10 Moisture determination

All weighing should be carried out on the four-place balance using the dry-weight template. Label and weigh an ounce universal. Record the weight. Transfer up to 1 g of sample into the ounce universal. Weigh the ounce universal and sample. Record the weight. Place the ounce universal and sample in the drying oven at 80°C for 24 hours. Allow the ounce universal and dried sample to cool to room temperature in a desiccator and then weigh it.

A1.3.11 Extraction by automated solvent extraction (ASE*)*

Rinse a glass rod with 3 x 2 ml of DCM. Dry the subsample with Kiesselguhr. Add the dried sample to the cell and tamp it down with the filter insertion tool. Using a 500 ul gastight syringe, spike every cell (samples and QC samples) with 500 ul of deuterated PAH surrogate standard. Fill the cell to within approx. 1 mm of the top sand. Cover the sand with a cleaned filter, push the filter down with the filter insertion tool and screw on the cell end cap.

Set up the Turbovap to the following:

Temperature	40ºC
Nitrogen supply	70 psi
Cell pressure	9 psi
Endpoint	Manual

Add two spatulas of anhydrous sodium sulphate to each ASE sample bottle, shake the contents and allow them to settle. Weigh an ASE collection bottle with its top and record the weight. Rinse the bottle with 5 x 10 ml of DCM.

Insert a small plug of glass wool into a drying funnel and push down gently with a wooden stick to the point where the funnel narrows. Rinse the funnel with 5 x 10 ml of DCM. Add anhydrous sodium sulphate to the funnel to a depth of approximately 1 cm and tap to compact. Place the funnel in a burette stand with the ASE collection bottle underneath as the collecting vessel and then pour the extract into the funnel. Once the extract has passed through the anhydrous sodium sulphate, rinse the ASE collection bottle with 10 ml of DCM and transfer the DCM into the drying funnel. Repeat this procedure three more times.

Replace the lid of the ASE collection bottle containing the filtered extract and reweigh the bottle. Calculate the weight of the filtered extract. Transfer to a Turbovap tube the mass of extract required for analysis. Record the weight of the extract remaining in the ASE collection bottle. Reduce the volume of the extract in the Turbovap to approx 1 ml using the settings above. Remove the tube from the Turbovap, rinse any dried residue from the side of the tube with approximately 2 ml of DCM and then return the tube to the Turbovap. When the volume of the extract has reduced to approximately 1 ml, remove the Turbovap tube, cover it with aluminium foil and allow to cool. Quantitatively transfer the extract to a 2 ml volumetric flask and make up to the mark with DCM.

A1.3.12 Clean up by open column chromatography

Insert a small plug of glass wool into the top of a 150 mm Pasteur pipette and push it down gently with a wooden stick, until the plug sits neatly at the point where the pipette narrows. Ensure there are no loose wisps of glass wool protruding from the pipette.

Place the pipette into the column stand. Rinse the inside of the Pasteur pipette three times with 1 ml DCM. Add 0.8 g of deactivated alumina to the

pipette column using a calibrated measure. Gently tap the side of the column until the alumina has been compacted. Insert a tiny piece of glass wool into the pipette to where it narrows. Place a 2 ml volumetric flask below the column to collect the eluate.

Transfer 200 µl of the concentrated extract to the column using a 250 µl gastight syringe. Once the meniscus reaches the top of the alumina, add 200 µl of DCM. Continue to add DCM in 1 ml lots until 2 ml of eluate has been collected in the volumetric. Do not allow the alumina column to dry out. Seal with the glass stopper and store in a dark, well ventilated location.

A1.3.13 Transfer to chromatography vials for analysis by GCMS

The samples should not be transferred to chromatography vials until just before analysis by GCMS. Prior to transfer, any loss of solvent due to evaporation must be made up with DCM. After transfer, re-weigh the volumetric (without top), recording the weight and store it as described above. Transfer the cleaned up extract (approx 200 µl) into an amber, 0.3 ml crimp top, fixed insert vial and seal with a blue Teflon septum. Transfer the remaining extract into a 2 ml vial as a reference and seal with a blue Teflon septum.

A1.4 Determination of PAHs in earthworms by GCMS: Extraction and clean up

A1.4.1 Scope

This document describes the standard method for the extraction and clean up of earthworm tissue, for the determination of PAHs by GCMS.

A1.4.2 Procedures

Prevention of contamination and degradation
The analytes determined by this method are contaminants of the atmosphere. Any exposure to the atmosphere of the sample or reagents, or surfaces that will contact the samples or reagents, may result in contamination of the samples. Exposure to the atmosphere cannot be eliminated, but can be kept to a minimum.

To limit contamination:

- Solvent rinse glassware immediately prior to use.
- Use vessels that can be closed with a ground glass jointed closure or a Teflon coated closure.
- If a vessel does not have a proper closure, cover tightly with aluminium foil and transfer the vessel contents to more suitable vessel as soon as possible.
- Where possible do not prepare or store standards in the same room as samples.

To limit carryover from one sample to the next:

- Do not re-use septa or seals.
- Do not analyse samples containing high levels of analytes with samples containing low levels.

To reduce sample degradation:

- Do not leave samples or standards exposed to light.
- Store samples at -20°C in the dark.
- Store standards in the dark in the chemical store.
- Use amber coloured glassware where possible.
- Store sample extracts in a spark proof fridge.
- Time between extraction and instrumental analysis should be kept to the absolute minimum.

A1.4.3 Quality assurance

Batches of samples should consist of a maximum of 14 samples. In addition to the samples, each batch must contain a sample blank, a spiked recovery and a recovery control sample.

A1.4.4 Sample blank

Follow all the procedures (weighing, extraction, lipid determination, moisture determination and column clean up) in this document, but without using a sample.

A1.4.5 Recovery

Follow all the procedures (weighing, extraction, lipid determination, moisture determination and column clean up) described in this document, using 1 g +/- 0.1 g of the control worm tissue.

A1.4.6 Subsampling

The amount of time the samples are out of the freezer should be kept to the absolute minimum. Samples must be defrosted on the day that they are subsampled and returned to the freezer as soon as they have been subsampled. Samples should be exposed to the atmosphere for as short a time as possible.

The ideal sample weight is 2 g, but this weight must not be exceeded. If there is insufficient sample to take 2 g for this analysis due to the requirements of other analyses on this sample, then the sample must be divided proportionally between the various methods.

Rinse a 100 ml beaker with 5 x 5 ml of DCM. Allow the DCM to evaporate from the beaker, then label it and record the weight. Cover the beaker with aluminium foil.
Weigh the subsample into the beaker and record the weight of beaker plus subsample.

A1.4.7 Moisture determination

All weighing should be carried out on the four-place balance using the dry-weight template. Label and weigh an ounce universal. Record the weight. Transfer up to 1 g of sample into the ounce universal. Weigh the ounce universal and sample. Record the weight. Place the ounce universal and sample in the drying oven at 80°C for 24 hours. Allow the ounce universal and dried sample to cool to room temperature in a desiccator and then weigh it.

A1.4.8 Extraction by automated solvent extraction

Rinse a glass rod with 3 x 2 ml of DCM. Dry the subsample with Kiesselguhr. Add the dried sample to the cell and tamp it down with the filter insertion tool. Using a 500 ul gastight syringe, spike every cell (samples and QC samples) with 500 ul of deuterated PAH surrogate standard. Fill the cell to within approx. 1 mm of the top sand. Cover the sand with a cleaned filter, push the filter down with the filter insertion tool and screw on the cell end cap.

Setup the Turbovap to the following:

Temperature	40ºC
Nitrogen supply	70 psi
Cell pressure	9 psi
Endpoint	Manual

Add two spatulas of anhydrous sodium sulphate to each ASE sample bottle, shake the contents and allow them to settle. Insert a small plug of glass wool into a drying funnel and push down gently with a wooden stick to the point where the funnel narrows. Rinse the funnel with 5 x 10 ml of DCM.

Add anhydrous sodium sulphate to the funnel to a depth of approximately 1 cm and tap to compact. Place the funnel in a burette stand with a Turbovap tube underneath as the collecting vessel and then pour the extract into the funnel. Once the extract has passed through the anhydrous sodium sulphate, rinse the ASE collection bottle with 10 ml of DCM and transfer the DCM into the drying funnel. Repeat this procedure three more times.

Reduce the volume of the extract in the Turbovap to approx 0.5 ml using the settings above. Remove the tube from the Turbovap, rinse any dried residue from the side of the tube with approximately 5 ml of n-Hexane and then return the tube to the Turbovap. When the extract has been reduced to near dryness, remove the Turbovap tube, cover it with aluminium foil and allow to cool.

A1.4.9 Clean up by open column chromatography

Using a 5 ml positive displacement pipette, add 5 ml of n-Hexane to the dried extract in the Turbovap tube. Gently swirl the contents to reconstitute the extract. Insert a small plug of glass wool into the top of a 150 mm Pasteur pipette and push it down gently with a wooden stick, until the plug sits neatly

at the point where the pipette narrows. Ensure there are no loose wisps of glass wool protruding from the pipette.

Place the pipette into the column stand. Rinse the inside of the Pasteur pipette three times with 1 ml of DCM, followed by three 1 ml lots of n-Hexane. Add 0.8 g of 5 per cent deactivated alumina to the pipette column using a calibrated measure. Gently tap the side of the column until the alumina has been compacted.

Transfer 1 ml of the concentrated extract to the column using a 1000 µl gastight syringe. Once the meniscus reaches the top of the alumina, add 1 ml of n-Hexane. Continue to add n-Hexane in 1 ml lots until 5 ml of eluate has been collected in the volumetric. Do not allow the alumina column to dry out. Seal with the glass stopper and store in a dark, well-ventilated location.

A1.4.10 Transfer to chromatography vials for analysis by GCMS

The samples should not be transferred to chromatography vials until just before analysis (e.g. by GC-ECD or GC-MS). Prior to transfer, any loss of solvent due to evaporation must be made up with n-Hexane.

After transfer, re-weigh the volumetric (without top), recording the weight and store it as described above. Transfer the cleaned up extract (approx 200 µl) into an amber, 0.3 ml crimp top, fixed insert vial and seal with a blue Teflon septum. Transfer the remaining extract into a 2 ml vial as a reference and seal with a blue Teflon septum.

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