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Application of sublethal ecotoxicological tests for measuring harm in terrestrial ecosystems

R&D Technical Report P5-063/TR2

CEH Centre for Ecology & Hydrology
NATURAL ENVIRONMENT RESEARCH COUNCIL

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Statement of Use
This report is for information purposes only. The Environment Agency will consider the research findings for uptake into its ecological risk assessment (ERA) framework. The policies, tools and techniques recommended in the ERA framework will be considered by regulators, landowners, their advisors and other stakeholders through public consultation in 2004. One of the aims of this consultation is to agree a list of recommended toxicity tests for use in assessing risks to terrestrial ecosystems in the UK by mid-2005.

This report describes the results from the application of novel and standardised sublethal biological tests. The tests have been applied in the field and in the laboratory using soils from two potentially contaminated sites in the UK. The tests may be used to determine the health of an individual organism, a population or whole soil ecosystem. The tests will also help determine the future health of soils and predict how soils will respond to stress resulting from point source or diffuse pollution events, including agrochemical use.

Keywords
Contaminated land, sublethal, bioassays, biomarkers, soil processes, invertebrates, microbes, soil pollution, field tests, laboratory assays, molecular markers.

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Executive Summary

Background

In order to judge the effects that chemicals have on soil ecosystems, scientists need to use monitoring frameworks and agreed indicators. This meets new statutory requirements placed on the Environment Agency regarding how scientists assess land that has been contaminated by industrial activity, as well as other policy initiatives concerning terrestrial ecosystem sustainability. In a previous Environment Agency and Scotland and Northern Ireland Forum For Environmental Research (SNIFFER) research project, tools and processes for assessing risks to ecosystems were reviewed to:

- recommend a potential ERA framework (Byrns & Crane, 2002);
- recommend standardised toxicity tests for potential application within the framework (Crane & Byrns, 2002).

This proposed framework and set of tests are being 'trialled' in another Environment Agency Project (P5-069). As a supplement to this work, the Environment Agency also commissioned forward-looking collaborative work with the Centre for Ecology and Hydrology. This aims to fill a knowledge gap within the Agency, concerning approaches being developed at the academic research horizon that could be used for sublethal assessment of the effects of contaminants in terrestrial ecosystems.

Project aim

We had two aims. The first was to review and recommend currently available and emerging methods of biological tests that could be used to assess the sublethal effects of soil contamination. This work was reported in 2002 in the *Review of sublethal ecotoxicological tests for measuring harm in terrestrial ecosystems* (Spurgeon et al., 2002). Our second aim was to trial the recommended tests using potentially contaminated soils and to assess their suitability for use in regulatory risk assessment. We present here the results from initial field and laboratory trials conducted to investigate the utility of a sub-set of these assays. The biological tests we investigated were:

- bait lamina test for *in-situ* assessment of soil invertebrate community feeding;
- a laboratory based chronic earthworm reproduction test;
- an instantaneous rate of population increase test with a rapidly reproducing invertebrate such as the springtails *Folsomia candida* or *Folsomia fimetaria*;
- measurement of lysosomal membrane stability using the neutral red retention time (NRR-T) assay in earthworm coelomocyte cells using indigenous or naïve earthworms;
- measurement of the expression of potentially contaminant responsive genes using a sensitivity detection system;
- measurement of metallothionein using sensitivity detection systems for quantification of gene transcripts in a suitable soil species (in this study earthworms have been used);
- a bioluminescence assay using a *lux*-based bacterial biosensor exposed to soil pore-water samples collected by centrifugation or rhizon sampler.

We conducted trials at two sites. Site A is around a closed primary smelting works. This area is contaminated with high concentrations of metals (mainly cadmium, lead and zinc) and has been well characterised and studied in national and international research. Site B is a former (demolished) tank farm area where crude oil and refined petroleum products used to be stored. At each site, we selected a series of spatially separated (but geologically similar) areas ('patches') for detailed study. These patches encompass a range of contamination levels. We used these for detailed laboratory and field based investigations on selected biological responses. That is, mesocosm tests using all patch soils, a temperature and pH amendment study using selected site soils, and field assessments of responses in different seasons.

Results

In the studies undertaken at Site A, many of the biological tests (eg. bait lamina, chronic earthworm toxicity test, lysosomal membrane stability, gene expression measurement, *lux* based biosensor) differentiated responses at patches where gross effects on invertebrate diversity and function have been reported in published literature. At Site B, the biological tests we conducted in the laboratory and field identified probable differences in the extent of actual exposure. We saw stronger biological effects in the laboratory where, unlike in the field, the organisms could not avoid the contamination.

In summary, we found that the tests that measure higher ecological organisation level parameters (eg. the bait lamina test and the earthworm reproduction bioassay) generally gave results with lower variability and were not affected by other factors (eg. season). The biomarkers (such as the measurement of lysosomal membrane stability using NRR-T and measurement of gene expression) were more sensitive: they often indicated a difference from controls in the less contaminated patches. But, despite their inherent sensitivities, we could not always establish significant differences in biomarker responses between patches with different contaminant levels. We therefore recommend the use of these assays as indicators of exposure. As biomarkers, life-cycle and functional assays were often measured together, their respective responsiveness and robustness increased the probability of identifying and diagnosing the impacts of a contaminant stress. The combination of assays used had a much higher diagnostic power than the use of any single biological assay in isolation. The tests proved they would be useful in a 'weight of evidence' approach to ERA.

This project met our aim of recommending biological assays for use in the risk assessment of potentially contaminated soils. A comprehensive review of tests for measuring sublethal effects was reported in 2002. This report (and previous work) proves the suitability of the selected tests through the laboratory and field trials. To make full use of the clear potential of using a suite of biological responses, our recommendations include:

- linking current project data with the developing ERA framework;
- using earthworms as preferred organisms for the biological assessment of soils;
- establishing baselines for each biological assay;
- multiple biological assessment;
- an awareness of the rapid development occurring in molecular genetics and the potential value of this for environmental diagnostics;
- the best approach for finalising the ERA framework.

Keywords

Ecological risk assessment, soil sustainability, biomarker, bioassay, ecological indicator, earthworm reproduction, bait lamina, lysosomal membrane stability, metallothionein, mitochondrial large ribosomal subunit, molecular genetics, temperature, pH, seasonality.

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Introduction

1.1 Background to the selection of the biological assays used in the project

During its review of the state of UK soil, the Royal Commission (1996) recognised a number of pressures that could reduce soil quality. Among the most important were erosion, loss of organic content and chemical contamination through past and present industrial activity, agriculture, waste processing and diffuse chemical use. The Royal Commission's report has led to the development of soil strategies for England (Department of the Environment, 2001), Wales (Stevens et al., 2002) and Scotland (Adderley et al., 2001). Concern regarding the status of soil has also been reflected at the European level. This is reflected in the European soil protection strategy (Commission of the European Communities, 2002). Within all of these soil strategies, there is recognition that contamination (both point source and diffuse) can affect the quality of soil ecosystems. In the UK, statutory requirements have been introduced under Part IIA of the Environmental Protection Act 1990 (Department of Environment Transport and the Regions, 2000). These are concerned specifically with assessing the risks of land contamination from past industrial activities that would or might cause significant possibility of significant harm to land, or pollution of controlled waters is occurring, or is likely to occur. Part IIA specifies that a risk assessment must be performed when a linkage between a contaminant and a receptor is identified. Part IIA of the EPA 1990 defines eight types of protected site that are to be regarded as *eco-receptors* for the purposes of the Act. Local Authorities and the Environment Agency are the enforcing authorities for contaminated land in England and Wales. In addition to Part IIA, other policy drivers, such as the European Union Habitats Directive, also require that the 'favourable condition' of Special Protected Areas and Special Areas of Conservation is not compromised by industrial, agrochemical and domestic chemical use.

Two factors now require the Environment Agency to improve its knowledge of chemicals and their effects on the terrestrial environment. First, the new statutory requirements placed on it regarding the assessment of land contaminated by past industrial activity in Part IIA.

Second, an increased awareness of the continued potential threat that industrially derived point sources, diffuse chemical use and agrochemical use may have for soil sustainability and the condition of habitats. To monitor the ecological impacts of these sources, investigators need monitoring frameworks and indicators that allow them to judge the effects of chemicals on soil ecosystems. A previous Environment Agency research project (completed in 2002) delivered two reports. These were (i) Technical Report P299, *Assessing risks to ecosystems from land contamination* (Byrns & Crane, 2002), and (ii) Technical Report P300, *Review of ecotoxicological and biological test methods for the assessment of contaminated land* (Crane & Byrns, 2002). These recommended both a framework for risk assessment and also an initial series of seven 'off the shelf' or standardised (ie. already internationally accepted) biological tests that could be used for the assessment of contaminated sites. These tests were selected by Crane and Byrns (2002) as they "are relatively rapid, easy to perform and cheap; there are national standards and quality control procedures available, and the measured endpoints have proved sensitive to several different toxicants". The recommended assays were:

- acute lethal earthworm test with *Eisenia fetida* or *E. andrei*;
- acute springtail test with *Folsomia candida*;
- germination and growth test with a monocotyledon crop plant such as barley;
- germination and growth test with a dicotyledon crop plant such as cress;
- microbial toxicity tests with *Vibrio fischeri* eg. Microtox and Mutatox;
- microbial nitrogen mineralisation test;
- microbial carbon mineralisation test.

These tests are currently being 'trialled' in Environment Agency Project P5-069 'Ecotoxicological And Biological Test Methods For The Assessment Of Contaminated Land: Stage 2: A Demonstration Of The Use Of A Framework For The Ecological Risk Assessment of Land Contamination'. Further work is also being undertaken within this project concerning

how best to include this collected data in the tiered risk assessment framework.

In parallel to Project P5-069, the Agency also commissioned a forward-looking collaborative project with the Centre for Ecology and Hydrology (CEH). This is Project P5-063, *Review and application of sublethal ecotoxicological test for measuring harm in terrestrial ecosystems*. The was to inform the Agency of approaches being developed at the academic research horizon that could have potential for the sensitive sublethal assessment of the effects of contaminants in soils on terrestrial fauna and flora. The first phase of Project P5-063 reviewed sublethal biological techniques available for current soil quality assessments. It also reviewed rapidly evolving research areas that may yield future biologically-based assessment methods (Spurgeon et al., 2002). In its conclusions, this first report listed a series of methods with potential. The second phase of the project, reported here, evaluated the application of the tests with potential. A series of laboratory and field based studies have been trialled in order to assess the usefulness of the tests for soil quality assessment.

These are listed below with the assays that have been assessed in this project highlighted in **bold**. The assays that are being trialled in project P5-069 are highlighted in *italics*.

Screening methods suitable for initial site spatial characterisation

Highly recommended

- **A bioluminescence assay using a *lux*-based bacterial biosensor exposed to soil pore-water samples collected by centrifugation or rhizosampler;**
- **Bait lamina test for *in situ* assessment of soil invertebrate community feeding.**

Recommended

- *A microbial nitrogen mineralisation test;*
- **Measurement of lysosomal membrane stability using the neutral red retention time (NRR-T) assay in earthworm coelomocyte using indigenous or naïve animals.**

Promising

- A bioluminescence assay using a transgenic strain of the nematode *Caenorhabditis elegans* in which exposure is in the solid phase;
- Use of the BIOLOG test system for community level physiological profiling of microbial community;
- A solid phase genotoxicity test using the Ames test or similar, to be used only when site history strongly suggests presence of mutagenic contaminants.

Established method suited to detailed assessment of contaminated soil

Highly recommended

- *A germination and growth test with dicotyledonous and/or moncotyledonous plants. Currently, selection of cultivars such as barley and/or cress is recommended; there is, though, also the potential to use non-crop species;*
- **A laboratory chronic earthworm reproduction test with *Eisenia fetida*, or *Eisenia andrei*;**
- ***A laboratory reproduction test with the springtail*** Folsomia candida.

Recommended

- A laboratory chronic enchytraeid reproduction test with *Enchytraeus albidus*. This test may be of greatest value in low pH soils in which an earthworm test is not suitable;
- **An instantaneous rate of population increase test with a rapidly reproducing invertebrate such as the springtails *Folsomia candida* or *Folsomia fimetaria*;**
- Characterisation of microbial diversity using PLFA profiles or a nucleic acid based method such as 16S rDNA based DGGE or T-RFLP.

Promising

- A full life-cycle test with the nematode *Caenorhabditis elegans*, from which results are incorporated in a suitable demographic model;
- A life-cycle based toxicity test using a plant species with rapid life-cycle;
- **Measurement of metallothionein or phytochelatins using sensitivity detection systems;**
- Measurement of induction of the 1A isoform of cytochrome P450. This can be conducted by either EROD assay or gene expression measurements;
- Quantification of cholinesterase inhibition. Most organisms suitable for this assay are surface-active invertebrates;
- Use of the ALAD assay in a species with haemoglobin based blood to detect the extent of exposure to lead.

The assays selected for detailed study include a 'functional' parameter (bait lamina feeding by soil-dwelling detritivores), individual life-cycle effects (earthworm bioassay), cellular biomarkers (earthworm lysosomal membrane stability), molecular genetic biomarkers (earthworm metallothionein and other gene expression measures) and biosensor (*lux*-marked bacteria) methods. Assays span the three categories used to group methods in the initial review (Spurgeon et al., 2002). There are: standardised procedures (earthworm bioassay), academically established methods (bait lamina, lysosomal membrane stability) and blue sky approaches (gene expression quantification, *lux*-based eukaryotic biosensor). As well as methods already well established within the scientific literature (eg. earthworm reproduction and springtail reproduction) the assays selected also include methods based on developing technologies (eg. *lux*-based biosensors and quantitative PCR methodologies). Finally, the selected methods encompass approaches suitable for initial site screening (eg. *lux*-based biosensors, bait lamina, earthworm lysosomal membrane stability), established methods suited to detailed assessment (eg. earthworm reproduction test), and promising methods in early development (eg. quantitative PCR methodologies).

1.2 Harmonising the results with the standardised tests (in R&D Project P5-069)

For the field and laboratory based trials of the selected biological assays and biomarkers, we needed to identify suitable sites for detailed investigation. To harmonise the work being conducted in this project with that concerning the standardised assays being undertaken in Project P5-069, we decided at an early stage to use the same sites and patches for both projects. The two projects are thus directly complementary. Project P5-069 focuses on risk assessment using modifications of the standardised test that measure severe effects of contaminants in soils on primary producers (plants), decomposers (earthworms, springtails) and on the functional status of soil microbial communities (nitrogen and carbon mineralisation). This project focuses on the use of innovative (and potentially more sensitive) sublethal bioassays, biomarkers and functional assessments.

1.3 Overview of site selection and design of experimental studies

We designed the work programme to trial the tests to complement the work to investigate the performance of the seven standardised tests being undertaken in Project P5-069. It focused on two contaminated field sites:

Site A: an area contaminated by aerial deposition from a primary cadmium/lead/zinc smelter located at Avonmouth in the South West of England;

Site B: a former (demolished) tank farm area where crude oil and refined petroleum products used to be stored.

Full details of these sites, including the rationale for selecting patches for detailed study, are set out in chapter 3.

On each site, we analysed a number of patches (spatially distinct areas within the site) separately in order to give an overview of the impact of soil heterogeneity on biological responses. The patches were all low to moderate contamination, so that any harmful effects seen in the organisms are present, but sublethal. We selected patches by considering the historic use of the sites and any results from past chemical characterisations. (Data for Site A was taken from: NERC-supported work of Jones 1991, BBSRC-supported work of Spurgeon 1994; Danish Research Council-supported work of Svendsen 2000; and work completed in the EU-funded project BIOPRINT (ENV4-CT96-0222). We thank the site-owners for providing data about Site B).

At Site A, we initially selected five patches (patch 1-5). Three of these were then used for more detailed study (patches 1, 3, 4). At Site B, we initially intended to use nine patches, spanning a range of contaminant concentrations. Initial chemical characterisation of the site indicated, though, that soils contained either near background or elevated levels of hydrocarbon contamination, with no intermediate concentrations (see Fig. 3.8 a-c and 3.9). So we created an artificial concentration gradient for testing dose-response relationships for the laboratory assays, using one of the highly contaminated patches as stock to generate a series of intermediate concentrations. The highly contaminated and scarcely contaminated patches were retained (intact) for the ends of the concentration gradient (see Section 3.3.4 for full details). We performed field studies at Site B only at particular patches – those that corresponded to soil used in the laboratory experiments without artificially generated concentrations.

Patches at each of the two sites encompassed a range of contamination. We could thus use them as a basis from which to examine the ability of the test to produce meaningful results in contaminated field soils and the relative sensitivities of the selected biological assays. Additionally, as Site A had been used previously in a series of ecological surveys to establish the effects of metals on ecological communities, we could compare published data with that from this

project. This helped us gauge the ability of the biological tests to indicate the long-term effects of contamination on biological communities.

Once the sites and patches had been selected, we conducted an initial characterisation of the soils. This included assessing soil texture, water-holding capacity, pH and soil organic matter content. We then made detailed chemical analyses of the patch soils. At Site A, we analysed for a suite of six metals (As, Cd, Cu, Hg, Pb, Zn). At Site B, analyses were performed for the same six metals, total petroleum hydrocarbons (TPH), specific analyses of benzene and toluene, and an extensive suite of unsubstituted and substituted polycyclic aromatic hydrocarbons (PAHs). The results appear in Section 3.2.5. Completing this chemical analysis furnished us with the vital information we needed to interpret the results of the biological assays conducted at each site.

At Site A, which was contaminated mainly by metals (see Section 3.1.4), we undertook experimental and field based studies to establish response profiles for the selected series of biological assays in relation to the metal concentration present in each patch soils (Chapter 5). Next, we investigated the influence of two environmental factors (temperature and soil pH change) on each biological parameter in laboratory tests. (Chapter 6). Finally, a series of seasonal studies was undertaken to establish the effects of sample seasonal on measured biological responses (Section 6.6).

At Site B, which was contaminated mainly by aliphatic and aromatic hydrocarbons, we undertook both laboratory and field based studies in order to establish the response profile of each biological test to the mixture of organic contaminants present in this soil (Chapter 6). Outlines of the experimental designs for these appear in Table 2.1.

1.4 Structure of this report

In **Chapter 2**, we introduce the biological tests selected for study in this project. For each test, we summarise the aim and method. A full review of each test appears in the *'Review And Application Of Sublethal Ecotoxicological Test For Measuring Harm In Terrestrial Ecosystems'* (Spurgeon et al., 2002).

We provide a detailed description of the sites used in this project and the rationale behind their selection in **Chapter 3**.

Chapter 4 describes the biological testing regime used at each site. During the study, we made seven separate evaluations on the performances of the selected biological tests. This chapter provides a simple reference for which biological test was used in which experimental study. Summary tables appear at the end of the chapter.

In **Chapter 5**, we present the results of biological profiling. At Site A, we carried out biological testing in order to establish response profiles for each of the biological tests. The chapter is split into two sections. The first section details the outcome of a gene expression profiling study conducted using indigenous worms collected from all patches along the Site A gradient (with the exception of patch 5 where worms could not be collected). On the basis of these expression profiles, we selected sequences for further analysis during the seasonal studies using indigenous worms and also in the mesocosm and temperature and pH amendment bioassays conducted using Site A soils. The second section outlines results from a semi-field mesocosm study used to establish the profile of a range of biological response measurements in the earthworm *L. rubellus* to soils collected from all Site A patches and a control soil (a sterilised Kettering loam).

Chapter 6 details the results obtained in the rigorous studies that we conducted to assess the performance of biological testing at each site. This chapter is also split into a series of sections. Each covers one specific portion of the experimental work. The details of each experimental study include a brief description of the aims of the work, the principal methods used for the exposure and the biological responses measured.

In **Chapter 7**, we evaluate the performance of each test. We base this on the same evaluation used in the review of sublethal tests, i.e. the criteria of the 5Rs outlined by Hopkin (1993), as developed from Rapport (1990). We use the 5Rs to judge if a test is relevant, robust, reproducible, representative, and responsive.

We present our recommendations from this research in **Chapter 8**.

Biological tests

One of the aims of this project is to assess how well a series of sublethal biological measurements (which quantify the responses of individuals to chemical exposure) can indicate the potential effects of chemical contaminants on ecosystems. We selected six assays for detailed assessment. The specific aims of each assay and the method used in this study to conduct each assessment are outlined below (for full details see Spurgeon et al., 2002). We outline the application of each assay within the project in Chapter 5 and 6. Each assay's performance is then discussed in detail in Chapter 8. Full methods used for the assessments are set out as standard operating procedures in R&D Project Record P5-063/PR.

2.1 Feeding activity using bait lamina strip

Aim

The bait lamina test measures the feeding activity of soil organisms. It does this by assessing how quickly these organisms remove bait pellets from small holes in engineered plastic strips. The method introduced by Törne (1990a; 1990b) is an integrating method, investigating a soil process that involves soil invertebrates as well as soil microorganisms (Kratz, 1998). Soil fauna are responsible for the majority of the feeding activity. Decomposition by microorganisms comprises only a small share (< 4 %, Helling et al., 1998).

Method

For the assay, three groups of 16 strips containing a series of baits were placed vertically into the soil in a grid pattern at each patch. This gave a three dimensional picture of feeding activity.

2.2 The ISO and OECD Draft Earthworm Reproduction Test

Aim

The test assesses the impact of soil contaminants on sublethal parameters in earthworms. A range of life-cycle based endpoints, such as weight change and various reproduction parameters, can be measured.

Method

When the test is used for testing single chemicals (ie. as described in the ISO and OECD Draft protocols), adult mature worms are exposed to the test substance in a standard test soil in a similar way to the acute earthworm toxicity test (see Crane & Byrns, 2002). The range of test concentrations is selected to encompass levels likely to cause both sub-lethal and lethal effects over a period of eight weeks. In (non-standardized) adaptations for application to field soils, homogenised and air-dried soil samples are sieved and added to the test chamber to a constant volume rather than constant weight (due to different soil bulk densities). They are then brought to a water-holding capacity of approximately 60 per cent. In our studies, we added groups of adult *Lumbricus rubellus* (eight in laboratory tests, 15 in the Site A mesocosm study) to each vessel containing the selected soil. Mortality and growth effects on the adult worms were determined after six weeks' exposure. Adults were then removed from the soil. By wet-sieving the soil by hand to collect and count the cocoons, we could then also assess the effects on reproduction. We could thus compare the reproductive output of the worms exposed to the test substance to that of worms in a reference soil (or preferably an on-site control soil), in order to determine the significance of any differences observed.

2.3 An instantaneous rate of population increase study using toxicity data collected for the springtail *Folsomia candida* in Project P5-069

Aim

To predict the potential consequences on springtail populations of prolonged exposure to contaminated soils.

Method

The prevailing view in short-term single species toxicity tests is that estimating the critical effect levels for a single (sensitive) trait is of high ecological relevance. In population terms, though, this is not always the case. In fact, the only way to predict the long-term consequences of chemical exposure is to consider the effects of pollutants on all life-cycle parameters. (Van Straalen & Kammenga, 1998). To translate individual changes to population effects, researchers have proposed using demographic models based on life-tables (Kammenga & Laskowski, 2000; Sibly & Calow, 1989). Such calculations, though, require a lot of experimental work. An alternative method for estimating population effects is to assess the instantaneous rate of population increase (IRPI). This is simpler to estimate and is quantified by counting actual population size in toxicological exposures and calculating according to Equation 1. Because IRPI is based on measurement of actual population growth, this test best suits species with short generation times. In this study, we applied the statistical approach to data on springtail reproduction collected in a Collembola test conducted following the ISO (1999) protocol during the course of project P5-069.

Equation 1

IRPI = LogN (final number of animals/initial number of animals) / experiment length (days).

2.4 Lysosomal membrane stability

Aim

This assay assesses increases in lysosomal membrane fragility caused by pollutant exposure in earthworm coelomocytes present in collected samples of coelomic fluid. At the subcellular level, the lysosomal system is a particular target for the toxic effects of contaminants (Moore, 1990). Pathological alterations in lysosomes have been used to identify adverse environmental impacts on marine organisms (Giamberini & Pihan, 1997; Moore, 1980; Moore et al., 1996). One of the characteristic changes is the increased fragility of the lysosomal membrane (Moore, 1988b). The mechanism causing this alteration in membrane stability is not well understood. It may involve direct effects of chemicals on the membrane or the increased frequency of secondary lysosomes in toxicant stressed cells (Mayer et al., 1992).

Method

The neutral red retention assay to measure cell damage is technically much simpler than both of the traditional cytochemical and biochemical techniques. It uses the fact that only lysosomes in healthy cells permanently retain the cationic dye after initial uptake. Coelomocytes cells were isolated from either body fluids or tissues and placed on microscope slides suspended in earthworm physiological Ringer solution (Lockwood, 1963). A dilute solution of neutral red dye was then added to the cell suspension and covered with a cover slip. The slide was then scanned for one-minute intervals using a light microscope at 400 times magnification. During each interval, the number of cells with leaked lysosomes (stained red) and the number of cells remaining unstained was counted. When 50 per cent of the total number of cells were stained, the time since the dye was added was noted. We took this time as the neutral red retention time (NRR-T).

2.5 RT-PCR for measurement of gene expression (including metallothionein)

Aim

To measure the expression of target genes by isolating mRNAs and using quantitative RT-PCR. In this study, we measured the expression of a number of sequences in order to establish metal responsive sequences. Further analyses of these were then conducted.

Method

In the past, the nature of amplification was a major obstacle to use of PCR for gene expression quantification. This amplification is characterised by a logarithmic increase to a plateau (Kochanowski & Reischl, 1999). Because the plateau is caused by limitations not related to the quantity of template, parallel reactions with vastly different template inoculations result in near-identical final product levels. Comparisons of product levels at the end of amplification would therefore indicate no differences between samples with different initial transcript levels (Kille et al., 1999). To overcome the problems of PCR for gene quantification, new techniques now take a snapshot of product levels during reaction. At first, these quantitative reverse transcriptase PCR (Q-RT-PCR) methods used either amplification for a limited number of cycle, followed by gel electrophoresis and image analysis or quantitative competitive PCR (Evans et al., 2001). Such methods have now been supplanted by fluorescence *in situ* monitoring. This uses specifically

designed instruments such as the PE Biosystem GeneAmp® and Roche Lightcycler™ systems. These monitor PCR progress using fluorescence detection.

In this study, we undertook detection using the fluorogenic 5' nuclease assay (for full details see Spurgeon et al., 2002). To assess the relationship between transcript frequency and the number of cycles required to obtain a specific threshold, a series of calibration standards containing cloned copies of the target gene at known concentrations can be used. This standard curve is then used to determine gene concentration in samples. In Q-RT-PCR, the usual practice is to normalise quantification of the target gene in order to account for differences in the amount of template that may be present. To do this, a control (non-responsive) gene(s) can be measured in parallel. As long as the factor being studied does not affect this control gene, the concentrations of this gene should be directly related to the amount of RNA that has been successfully isolated and transcribed to cDNA. A number of invariant 'housekeeping genes' have been identified for this purpose (Stürzenbaum & Kille, 2001). In this study, we used the gene ß-actin for all normalisations, as a sequence was available within the genetic database for the mRNA transcript of this gene in *L. rubellus*. Previous work has established that the gene is not sensitive to metal exposure in *L. rubellus* (Galay Burgos et al., 2003). By measuring ß-actin expression, we could remove the effect of differences in sample quantity on the expression of potentially responsive genes by simply dividing the measured concentration of that gene by the measured concentration of ß-actin in the same sample (Equation 2). For each gene, we refer to this value as 'relative gene expression'.

Equation 2

Relative gene expression =

Potentially responsive gene (e.g. *cyc-B, rrnL, MT-2, AmOx*) expression / ß-actin expression.

Measured genes

Spurgeon et al. (2002) identified a number of criteria for selecting molecular targets for gene expression quantification. The first was that in initial selection, the degree, magnitude and direction of the transcriptional change should be quantified. In this study, we decided to focus on establishing proof-of-concept for gene-based environmental monitoring for potentially metal responsive sequences. A number of such sequences have been identified in previous work by the group of Dr Peter Kille and Dr Stephen Stürzenbaum, at the School of Biosciences, Cardiff University, UK. They identified genes as potentially metal responsive by using methods such as directed differential display and subtractive library construction and screening (Stürzenbaum et al., 1998a, b, c; Stürzenbaum et al., 1999). The genes we selected for our initial survey were:

- cyclophilin-B (*cyc-B*): a signal sequence from the family of peptidyl- proline cis-trans-isomerases, the *cyc-B* isoform has been shown to be metal responsive;
- mitochondrial large ribosomal sub-unit (*rrnL*): a commonly expressed gene located on the mitochondrial genome that encodes one of the proteins of the mitochondrial ribosome system;
- metallothionein-2 (*MT-2*): a cystein-rich low molecular weight protein involved in cadmium detoxification;
- amine oxidase (*AmOx*): a copper-containing enzyme that catalyses the substrate oxidation of tyramine and tryptamine.

We initially trialled the expression of these genes using indigenous worms collected from most patches along the Site A contamination gradient. On this basis, we selected sequences that showed a clear and directional change in expression for more detailed analysis in the remainder of the project. These sequences would allow us to assess the effects of changes in temperature, soil pH and season on the nature of the genetic response.

2.6 Bacterial biosensors

Aim

To detect contaminants or specific contaminant classes using transgenic bacteria engineered to contain bioluminescent gene constructs.

Method

No naturally luminescent soil bacteria are known. So one solution is to fuse the genes responsible for bioluminescence in aquatic bacteria into soil-dwelling strains (Paton et al., 1997). Using recombinant technology, naturally non-luminescent microbes can be marked constitutively with these *lux*-genes. In an approach analogous to Microtox™, light output can be linked to metabolic activity. Any chemical that disrupts bacterial metabolism decreases light output. In this study, we recorded the effects of exposure on soil bacterial metabolism using *Berkholdaria sp.* RASC strain engineered to contain the *lux* construct (Shaw et al., 1999). We added microlitre volumes of bacterial culture to soil pore water extracts and immediately detected light output by scintillation counter (Shaw et al., 2000a).

2.7 Chemical measurement in soils and earthworm tissues

Aim

To quantify the concentrations of a range of contaminant chemicals present in soil samples taken from all patches at both sites. Also to determine the uptake of these by exposed animals during the biological assays.

Methods

We used applicable analytical approaches to measure different chemical groups. We measured metals in soils in two extracts: a hot acid digestion and a weak calcium chloride extract, and also in hot acid digests of earthworm tissue. Cadmium, copper, lead and zinc concentrations were measured by using flame atomic absorption spectroscopy, mercury by cold vapour atomic adsorption spectroscopy and arsenic by inductively coupled plasma-optical emission spectrometry. We also measured concentrations of major ions in soils by using high performance ion chromatography. Using this data, we modelled metal speciation in the soils in the pH study. At Site B, a full ICRCL suite analysis of soils was made in a single soil sample per patch as part of Project P5-069 by WRc-NSF. This included a measurement of total petroleum hydrocarbon. To support this preliminary analysis, our study measured the concentrations of over 50 polycyclic aromatic hydrocarbons (PAHs) in soils and exposed earthworm following solvent extract and clean up by GC-MS at CEH Monks Wood. Concentrations of benzene and toluene in soils were also measured by GC-MS by WRc-NSF.

Site histories and patch selection

3.1 Site A – The Avonmouth primary cadmium/lead/zinc smelter

3.1.1 Site description and history

The area around Site A has been subject to high levels of aerial metal input, principally from a primary Cd, Pb and Zn smelter which ceased operation in December 2002 (see below). It was the largest smelter of its type in the world and was the major source of metal pollution to an area that includes parts of Avon, North Somerset, South Gloucestershire and the Greater Bristol conurbation (see photo in Fig. 3.3). The smelter was commissioned in 1920 and began full operation in 1929. In 1969, Imperial Smelting Furnace No. 4 was commissioned at the same location. The new addition to the plant used a revolutionary smelting process. It significantly increased the production capacity of lead and zinc, and it meant that cadmium could also be recovered (Martin & Bullock, 1994).

Despite the enforcement of emission controls legislation during the latter years of operation (Environment Agency authorisation AS 7396), total emissions from the smelter have been continually high. Total release figures to air published for 2001 were: 830kg Cd $year^{-1}$, 8,300kg Pb $year^{-1}$, 16,675kg Zn $year^{-1}$ and 89kg Cu $year^{-1}$. In addition to these four metals, the factory also released large quantities of other pollutants, including 722kg of mercury, 31kg antimony, 232kg arsenic, 2,388,913kg of sulphur dioxide and 280,801,000kg of carbon dioxide during 2001 (www.environment-agency.gov.uk).

Though the annual metal output from the smelter has reduced over recent years, this does not negate the effects of the past 70 years of activity. Previous studies of soil contamination in the Avonmouth area have indicated that there is an elevation of metal concentrations in soils at least 15km downwind of the smelter. The degree of metal contamination decreases exponentially with distance from the source (Hopkin et al., 1986; Spurgeon & Hopkin, 1995, 1996a). Organic material collected from a location within 200m of the perimeter fence of the factory contains almost 10 per cent total metal content on a dry weight basis (373mg Cd g^{-1}, 2,640mg Cu g^{-1}, 52,267mg Pb g^{-1} and 33,887mg Zn g^{-1}) (Sandifer, 1996). The fact that these concentrations are higher than for some of the ores used for the smelting process illustrates the severity of the contamination in the locality. Dispersion modelling based on these emission data, discharge conditions and local meteorology demonstrates the gradient of deposition of these metals to soils surrounding the factory (see Fig. 3.1).

The smelter is the main of source of metal pollution in the Avonmouth area. But the region is also home to a number of other industries (see www.environment-agency.gov.uk and follow links from 'your environment' to 'what's in your backyard?' for permitted activities). Among these are a fertiliser manufacturer (Terra Nitrogen (UK) Ltd), a solvent processing plant (Chemical Recoveries Ltd), a pharmaceutical manufacturer (Zeneca Ltd), a carbon black works (Sevalco Ltd), an organic chemical manufacturer (Albright and Wilson) and a bulk acid production plant (associated with the smelter). In addition to these potential industrial sources of pollution, there is also a refuse disposal tip, a sewage works and the now closed Bristol municipal incinerator. The incinerator (which ceased operation in 1996) produced significant particulate emissions of metal from a single stack (Scott, 1987). The most recent major change to the area was the building of the Second Severn Crossing. Completion of this major project entailed construction of three new sections of motorway (M49, M48 and M4) that link the existing M4 and M5 roads with the new bridge (Fig. 3.2). Thus, in addition to the smelter, the Avonmouth area is subject to considerable anthropogenic disturbances, including the inputs of pollutants from a range of point and diffuse sources.

Topologically and geologically, the area immediately surrounding the Avonmouth smelter (within a 2.5 - 5km wide strip along the river Severn) is characterised by flatland less than 10m above sea-level, with the uppermost stratum being estuarine alluvium. An elevated zone adjoins this plain in the East. This area reaches up to 80m within the investigation area. Despite the heavy

industrialisation around the Avonmouth smelter, much of the surrounding land remains as managed (and abandoned) pasture or semi-natural woodland. In particular, the area to the north east of the factory consists of a mixture of permanent pasture in the flat, low-lying areas, and mixed oak woodland and hedgerow in the steeply sloped zones. This diversity of habitat means that the area is ideal for collecting soils and analysing the status of soil ecosystems.

3.1.2 Justification for using Site A

There is a long history of research into the effects of metals on soil fauna in the Avonmouth area. Studies have addressed the effects of these metals on populations and communities of invertebrates such as woodlice (Hopkin & Hames, 1994; Jones & Hopkin, 1998), spiders (Spurgeon et al., 1996) and earthworms (Spurgeon & Hopkin, 1999a). In addition to evidence of direct effects of metals on soil invertebrate communities, there is also indirect evidence of effects of metals on soil function (Coughtrey et al., 1979; Hopkin & Martin, 1985; Martin et al., 1982). The body of work that has been conducted at Avonmouth (at least 30 Institute of Scientific Information listed papers since 1982) provides a basis against which to gauge the performance of our laboratory tests. For example, if a particular test were to suggest no exposure/effect at patches where previous work had shown substantive effects on the diversity of certain faunal groups, this would suggest our test was failing to predict potential ecological harm.

Since we decided to use the Avonmouth locality for this project, the smelter owners have closed the site with effect from the 1 March 2003. The change to the operational status has potential implications for our work and indeed for previous work, also. While operational, the factory was regulated within the Integrated Pollution Control regime. It was due to be included within the Integrated Pollution Prevention and Control (IPCC) regulations. With the closure and potential change of land use, the site may now be eligible for regulation under the Environmental Protection Act, Part IIA. The site was chosen on the basis that it provides an ideal test bed for the assays. The results could ultimately be of direct relevance to the future management of the site (under Part IIA) should there be a requirement to assess the potential and actual long-term ecological harm caused by metal deposition derived from the years of smelter operations.

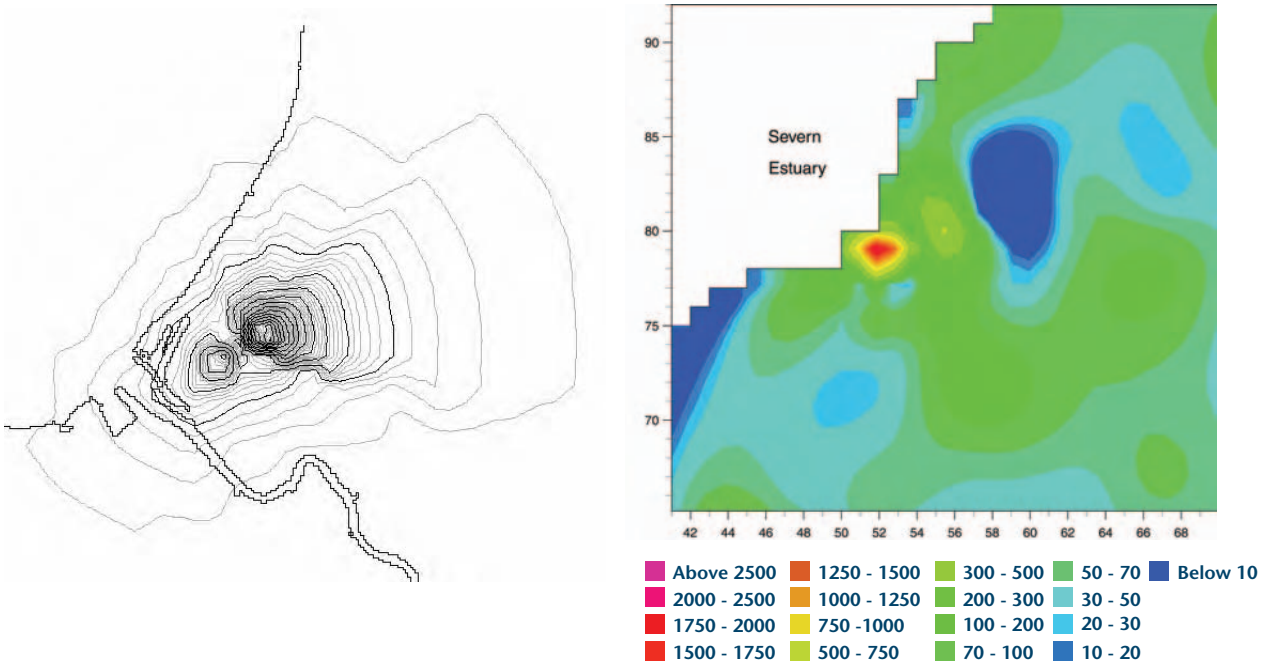


Fig. 3.1. Profile of metal contamination around Site A. a) predicted deposition of zinc modelled using the Air Dispersion Modelling System model, stack properties and assuming emissions are identical to 2000 authorised rates. Contours are at 1 and 5kg/ha/50 year. b) Contours of measured soil zinc concentration (all values in mg/g) in the soil interpolated using kriging in a geographic information system. Dispersion modelling data provided by Dr R. A. Wadsworth (CEH Monks Wood) with thanks. Soil metal data provided by Dr S. P. Hopkin (Reading University) and Dr D. J. Jones (Natural History Museum, London) with kind thanks.

3.1.3 *Patch selection and initial characterisation*

Following our decision to focus work at Avonmouth, we needed to identify a transect across the contamination gradient along which we would place the patches to be used during the study. It is fortunate that a great deal of work to characterise the spatial distribution of metals in soils around the Avonmouth site has already been done. Deposition modelling based on 2000 emission data and the physical characteristics of the smelter stack used an 'off-the-shelf' atmospheric dispersal model (the Air Dispersion Modelling System version 2.02). This suggests that, after a rapid increase within the first few metres from the stack, there is a progressive and exponential decrease in deposition (Colgan et al., 2003). This is indicated clearly by the fact that contours are initially close together, but subsequently increase in spread with distance from the stack.

The spatial distribution of metals in soils around the smelter has previously been established by Jones (1991) using a kriging-based method in a geographical information system to interpolate concentrations of metals measured at 96 sites (see Fig. 3.1b for the map of soil zinc concentrations). Outputs again showed an exponential reduction in soil zinc concentration, as indicated by the increase in the separation of the contours with distance away from the source. Comparing predicted deposition with soil metal levels confirms that, though there are small differences in the shape of the contours, there is a generally good agreement between the predicted pattern of deposition and the actual distribution of metals in soils (compare Fig. 3.1 a and b). This correlation between the dispersion modelling and the kriged soil data thus provides compelling evidence that the principal cause of the zinc contamination gradient present in the Avonmouth area is aerial deposition from the smelter stack

To align the work conducted here as closely as possible with previous ecological and biomarker survey studies, we selected a transect that had previously been used in an EU Framework IV project (BIOPRINT) for our project (see Fig. 3.2). This transect runs for approximately nine km in a north easterly direction from the factory (direction of the prevailing wind). We chose five patches along the transect. All were on managed or abandoned grassland (see Table 3.1 for full descriptor of patch locations).

All patches were used in a study of earthworm responses in mesocosms and for an initial series of gene expression measurement in indigenous earthworms (see Section 5.1 and 5.2). Three patches (1, 3, 4) were then used for more detailed study. Fig. 3.3 shows photos of each of these three patches. Schematic maps showing the location of individual sampling plots used in the bait lamina work are included in Appendix 1. Details of these three patches were:

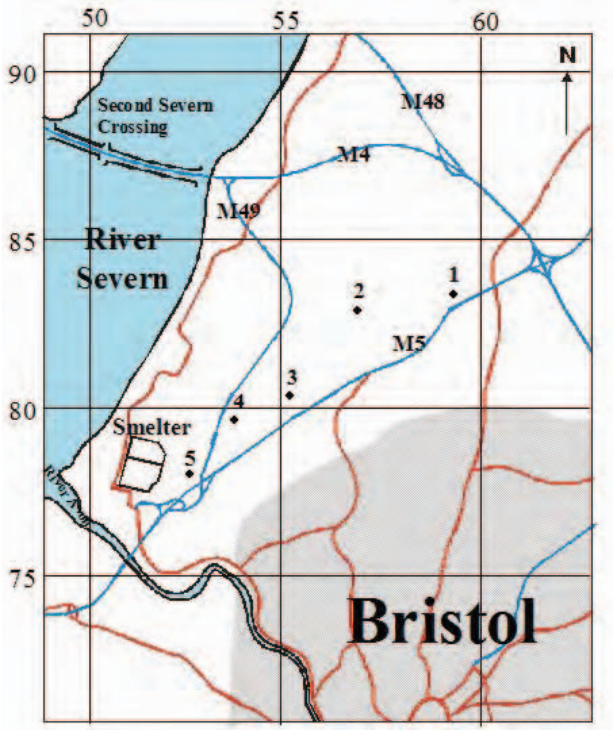


Fig. 3.2 Location of five patches situated along a transect running to the north east of a primary cadmium/lead/zinc smelter. Axes give Ordnance Survey grid reference values in km. Shaded areas indicate the extent of major urban zone of Bristol. Major motorways (M4, M5, M48 and M49) are indicated.

- patch 1 was located 8.1km north east of the factory, on raised ground close to a steep oak-wooded slope;
- patch 3 was located 3.2km north east of the factory, on a grass slope approximately 50m from Hallen Wood (a mixed pine and deciduous stand);
- patch 4 was located on low-lying ground close to the minor road near the now abandoned refuse incinerator.

From our initial survey, the local geographical factors of note included: the main habitat type present, the proximity of point pollution sources in addition to the smelter, the presence of major and minor roads and agricultural practices associated with the patch. The extent and nature of other anthropogenic disturbances were assessed (see Table 3.1).

Fig 3.3a

Fig 3.3b

Fig 3.3c

Fig 3.3d

Fig. 3.3. Photos of the smelter source at Site A and the three patches used for collection of soils for the pH and temperature experiments and for the seasonal field work (bait lamina deployment, earthworm NRR-T and gene expression quantification), a) the Avonmouth smelter taken from the north east, b) patch 1 (collecting samples), c) patch 3, d) patch 4.

All patches were on grassed areas. Three (patches 1, 2 and 4) were on unmanaged grassland adjacent to a minor roads (patches 2, 4) or a public footpath (patch 1). Patch 3 was a managed grassland area used in the past for grazing. Patch 5 was grassland overgrown by a sparse oak plantation. Geologically, three patches (1, 2, 5) were on low-lying land primarily of alluvium or mixed alluvium (head material) origin. Two patches (1, 3) were on elevated areas of Jurassic origin. Both were situated on, or close to, a slope.

The survey indicated that the Avonmouth area has a complex mixture of light and heavy industry, domestic sewage works and local waste management sites (including landfill sites and a now disused refuse incinerator). These all represent potential sources of local contamination that could influence the results of bioassays conducted in soils at these sites. The severity and length of the gradient of metal pollution from the smelter, though, represents the dominant contamination influence in the area.

3.1.4 Soil chemical characterisation – trends and relationships to ecological effects

After collecting soils for laboratory analysis, we measured the basic physiochemical soil characteristics, pH, organic C content and moisture content. We also recorded the concentration of metal (arsenic, cadmium, copper, lead, mercury, zinc) in soil samples, as required for specific experimental studies (see Tables 3.2). The exact measured values of metal concentrations varied as different batches of soil were collected for different studies. (For example, the soil batch used in the mesocosm study (see Section 5.2), was different for that used in the pH and temperature (see Section 6.1 and 6.2) studies). Concentrations,

Patch	OSGR	Brief description	Underlying geology	Distance from smelter	Altitiude (m above sea level)	Other local sources of contamination and disturbances
1	**ST 595827**	**Grass verge of footpath on (west) crest of north-west facing slope bordered by oak woodland and managed pasture**	**Jurassic**	**8.1**	**80**	**Approximately 100 m from M5 motorway**
2	ST 568826	Grass verge (20 m wide) of minor road bordered by road and field hedgerow. Heavily rutted in places	Head	5.6	7	Cattle disturbance. Possible agrochemical input and motor vehicle emissions
3	**ST 554802**	**Steep south-west facing slope of managed pasture with adjacent oak woodland**	**Jurassic/ Keupar marl**	**3.2**	**60**	**Horse disturbance. Possible agrochemical input. Also 200 m from M5 motorway.**
4	**ST 535797**	**Densely grassed verge of minor road (20 m wide) with occasional hawthorn. Bordered by pasture, road and a ditch**	**Alluvium**	**1.5**	**6**	**Close to site of a disused refuse incinerator. Subject to fly tipping.**
5	ST 534790	Sparsely planted oak stand with dense grass cover as field layer, surrounded by abandoned pasture. Approximately from a well used local road	Alluvium	1.0	7	Sulphuric acid input from nearby plant. Motor vehicle emissions from road.

Table 3.1. Ordnance Survey Grid Reference (OSGR), descriptions and geographical locations of five patches in the vicinity of the Avonmouth primary cadmium/lead/zinc smelter. We used all sites in the mesocosm experiment and initial gene expression profiling (Chapter 5). Patches 1, 3 and 4 highlighted in **bold** were used in the laboratory pH and temperature experiments (Sections 6.1 and 6.2) and in the seasonal monitoring studies (bait lamina, earthworm lysosomal membrane stability by NRR-T, earthworm gene expression quantification for *MT-2* and *rrnL*) detailed in Section 6.6.

Patch	Soil metal concentration µg g^{-1}			
	Cadmium	Copper	Lead	Zinc
Control	0.05 ± 0	12.4 ± 0.7	17.2 ± 4.2	47 ± 2
Patch 1	0.95 ± 0.07	25.7 ± 0.4	146 ± 3	499 ± 11
Patch 2	3.53 ± 0.14	38.8 ± 0.6	153 ± 3	519 ± 8
Patch 3	18 ± 0.4	80.5 ± 2.1	411 ± 12	1526 ± 79
Patch 4	66.6 ± 1.1	326 ± 11	2783 ± 95	4990 ± 111
Patch 5	177 ± 11	947 ± 62	5962 ± 499	11427 ± 855

Table 3.2. Concentration of cadmium, copper, lead and zinc in the control and Site A patch soils.

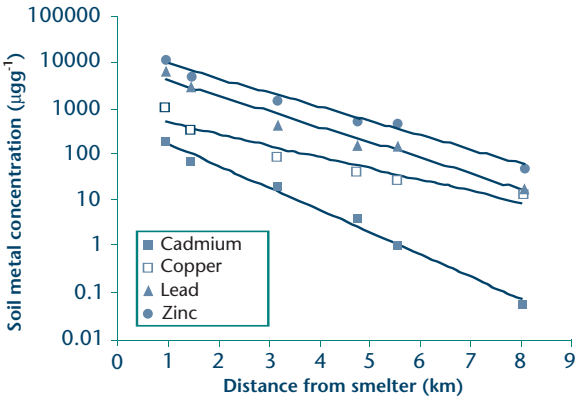


Fig. 3.4. Trends of cadmium, copper, lead and zinc concentrations at Site A, patches 1-5 plotted against distance from the point source. Lines indicate fit of negative exponential regression of soil metal concentration against distance.

though, were generally similar, with the same trends apparent. These trends for cadmium, copper, lead and zinc can be summarised with reference to the measurements in soil from all patches and clean reference soil (a sterilised Kettering loam from Broughton Loams Ltd, Kettering, UK).

Concentrations of cadmium, copper, lead and zinc increased with increasing proximity to the smelter. Concentrations were highest for zinc, then lead, copper and finally cadmium. At patch 5, total cadmium, copper, lead and zinc comprised almost two per cent of the total soil weight. A comparison of the relationship between concentrations of each of the four metals and distance from the smelter indicated a good fit of a negative exponential model. This pattern of metal deposition is typical of that for contamination point sources (Davies, 1989). A general linear model (GLM) was used to compare linear regressions between log metal concentration and distance from the source. This indicated that the slopes of the regressions were not significantly different for copper, lead and zinc, but was significantly steeper for cadmium. Intercepts did, though, differ between all metals, indicating difference in metal concentrations present in the uncontaminated soil. These differences also appeared in the contaminated soils. A comparison of the slopes of the regression lines and the median of relative concentrations of the four metals in the measured soil showed that the average ratios of cadmium : copper : lead : zinc in site soils were 1 : 5 : 40 : 80. The fact that the negative exponential regressions were parallel (on a log axis) indicate that these ratio were typical for soils sampled at all patches along the transect.

To gauge the extent of the metal contamination in the Site A soils, we compared measured cadmium, copper, lead and zinc concentrations with existing soil protection values (New Dutch list values). The New Dutch list gives environmental quality standards (EQSs) for soil at two protection levels. The lowest and most protective are termed 'target concentrations'. These are optimal values. Any soil with concentrations below these values can be considered clean. The values are: cadmium 0.8µg g^{-1}, copper 36µg g^{-1}, lead 85µg g^{-1} and zinc 140µg g^{-1} soil.

In all cases, the control soils contained metal concentrations below the Dutch target values. The most distant Site A patch, though, contained metal levels marginally above the target for cadmium, below for copper, and in excess of the target by a factor of 1.8 and 4.5 for lead and zinc respectively. Patch 2 soils exceeded targets by factors of more than 4 for cadmium, 2 for lead and 4.5 for zinc, and by a small factor for copper. All four metals at all other patches exceeded targets by large factors.

Dutch list 'intervention values' are the concentrations which trigger remedial action. These are: cadmium 12µg g^{-1}, copper 190µg g^{-1}, lead 530µg g^{-1} and zinc 720µg g^{-1}. Patch soil concentrations did not exceed these values at patches 1 and 2. At patch 3, cadmium and zinc did exceed these intervention values. And all four metals exceeded the intervention values at patches 4 and 5. In summary, when compared with Dutch EQSs for soil, patches 1 and 2 can be considered moderately contaminated and patches 3, 4 and 5 highly to very highly contaminated.

Data from ecological surveys conducted at the Site A patches in previous work was collated and reviewed in light of measured metal concentrations. The conclusions support the assumptions made from the comparison with the Dutch EQSs. Community surveys have suggested that deteritvorous groups (such as earthworms, molluscs, millipedes, woodlice and springtails) are those most heavily affected by the metals present. At a location equivalent to patch 3, Spurgeon and Hopkin (Spurgeon & Hopkin, 1999a) and Sandifer (1996) found an effect on earthworm diversity. At patch 4, as well as effects on earthworms, reduced millipede and woodlouse diversity was found. At patch 5, earthworm, molluscs, woodlice and millipedes were absent and springtail, mites and harvestmen diversity was reduced. Limited, but detectable, effects on community composition occur at patch 3 (where only some metals exceed intervention values). Gross effects on community composition are seen at patch 4 and 5 (where metal levels greatly exceed intervention values). This shows that the presence of high to very high metal

Class	Order	Family	Patch 5	Patch 4	Patch 3 (equivalent)
	Araneida				
		Lycosidae	√√	√	√
		Linyphiidae	√√	√	√
	Opiliones		X	√	√
	Acari		X	√	√
Insecta					
	Collembola		X	√	√
	Orthoptera		√	√	√
	Hemiptera		√	√	√
	Coleoptera				
		Staphylinidae	√√	√	√
		Carabidae	√	√	√
	Hymenoptera				
		Formicidae	√√	√	√√
Crustacea	Isopoda		XX	X	√√
Myriapoda					
	Chilopoda	Diplopoda	XX	X	√
Mollusca					
	Slugs		XX	√	√
	Snails		XX	√	√
	Oligochaeta		XX	X	X/√

Table 3.3. Invertebrate groups represented in pitfall trap samples from patches 5, 4 and a location to the north of the smelter at an equivalent distance to sites 3. √ = members of group present at site, √√ = members of group present in high numbers, X = members of group found in low numbers or with reduced diversity, XX = group absent from site. Data summarised for Oligochaeta from Spurgeon and Hopkin (1999) for molluscs from a range of sources and for all other groups from Sandifer (1996).

contamination at patches 3, 4 and 5 causes 'significant harm' to these ecosystems.

3.1.5 Collection and treatment of Site A patch soils for use in laboratory bioassays

At each patch, we marked out areas and excavated soil (by spade) from the four corners of a marked central square. In each corner, 0.5m^2 was excavated to a maximum depth of 25cm, providing four samples of 20l volume (large stones etc were removed at this point). If turf was present at the sampling point, we removed this (making sure to collect the soils from the root mat) and the soil excavated below the root-mat further removed. We mixed collected samples on site to ensure homogeneity. Finally soils were bagged and individually marked with unique sample point codes and then taken to the laboratory in refrigerated containers.

As soon as they arrived at the laboratory, sub-samples for use in chemical analysis were immediately stored at -20°C. This effectively removed the indigenous soil macroinvertebrate fauna. Site A is contaminated principally by metals, which are less likely to be volatilised (with the possible exception of mercury). So we dried the soils and broke up aggregates prior to use. After they had defrosted, soil aggregates were broken up further while they were still damp and placed in an oven at 60°C until dry. Dried soils were then crushed in a mechanical soils crusher, and the equipment carefully cleaned after each patch soil. Next, soils were sieved through a two millimetre mesh. At this point, we took soil samples for analysis of pH, percentage loss on ignition (%LOI), and maximum water holding capacity and field capacity. Finally, soils destined to be used in the bioassays were placed in the correct volume into the experimental containers and re-wetted to approximately 60 per cent of their moisture retention capacity (Spurgeon & Hopkin, 1995), as determined using an established method (Kalra & Maynard, 1991). Soils were then left to stabilise for one week before the test animals were added.

3.2 A former (demolished) tank farm area where crude oil and refined petroleum products used to be stored.

3.2.1 Site description and history

Site B comprises a discrete area of approximately 150m by 150m, currently covered by rough grassland. It is at the edge of (though still within) the boundary of an operational petrochemical facility. A full timeline of operation at the site is given in Fig. 3.5.

The site is approximately 20m above sea level with minimal slope. Between 1967 and 1972, six oil storage tanks were built on the site and used until the mid 1980s. During their time of operation, these tanks contained crude oil or refined petroleum products. By 1986, the storage tanks had become surplus to requirement and were removed. Following demolition, the resulting rubble from the tank bases was covered with between 25 and 50cms of top-soil. A fairly uniform grass cover interspersed with a range of shrub and herb species has since appeared.

Since the tanks were demolished and the site 'beautified' with soil coverage, it has been surveyed twice by consultants. A plan of the site with the locations of these sampling points is presented in Fig. 3.6.

In 1990, 12 trial pits were dug in an even-spaced grid. This survey concluded that while some hotspots of hydrocarbon concentration existed, the mean site Toluene Extractable Material (TEM) concentration was 3400 mg/kg. Site mean pH and concentrations of

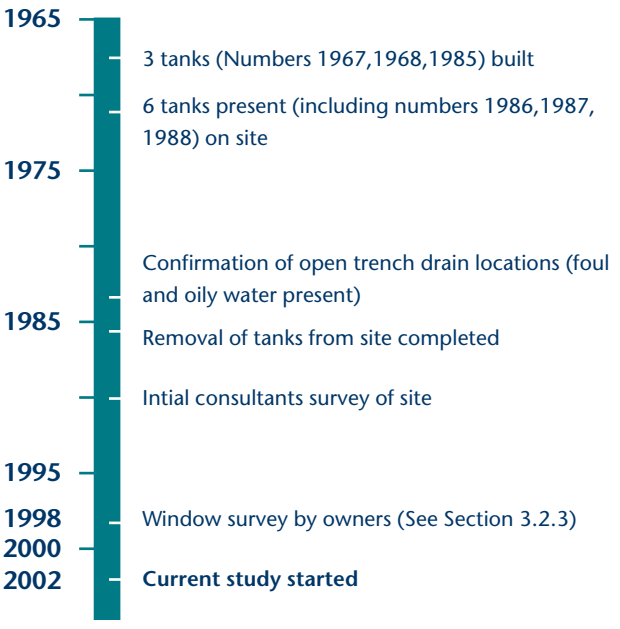


Fig. 3.5. Time-line of operations at Site B including timing of recent site surveys.

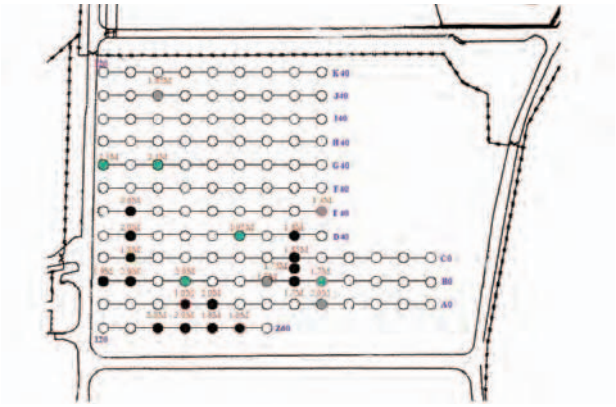


Fig. 3.6. Location of window samples conducted by owners of Site B in 1998/99 and total petroleum hydrocarbon concentrations (µg TPH g^{-1} soil). Site map and TPH data provided by site owners with thanks.

phenols, cyanide and sulphate were below the now withdrawn Interdepartmental Committee on the Redevelopment of Contaminated Land (ICRCL) threshold concentrations.

One hot spot for metals was identified, though site metal concentrations in general were not identified as being as of concern by reference to ICRCL threshold concentrations. In 1998 and 1999, a second investigation was undertaken in-house for research purposes. This took window-bore samples across the site in a 10m-spaced grid to a depth of two metres. These samples were analysed for total petroleum hydrocarbon (TPH) concentrations. The patches that have been used for previous sampling of the site are shown in Fig. 3.6. In this figure, the sample sites have been superimposed on to the site plan of the area to indicate the position of samples. A number of samples from this window sampling campaign indicated that soils at specific depths from a limited number of areas within the site contain elevated concentrations of either fresh or weathered crude oil. Most of these areas of hydrocarbon contamination were associated either with the previous location of tank number 1986, or with the drainage channels that surrounded the site. Based on the TPH data (provided by the site owner), we selected locations that we hoped would represent a range of TPH in top one metre of soil (for further details see Section 3.2.3). The location of these patches and the TPH concentrations from the consultant data there are shown in Fig. 3.8a.

3.2.2 *Justification for using Site B*

We chose Site B because we consider it to be typical of sites that will be encountered during to the enforcement of the Part IIA contaminated land regime. The site is within the boundary of a working industrial facility. But it is also close to the perimeter, which means that it would be possible for there to be a significant exposure pathway, either as a result of horizontal pollutant movement, or possibly due to visits by more active species such as surface invertebrates, mammals or birds. For example, if the site were located immediately adjacent to a cited Part IIA receptor such as a Site of Special Scientific Interest (SSSI), such pathways could mean that the site could become subject to formal investigation under the Part IIA regime. This is in fact not the case at Site B, but it is a probable scenario for similar sites in the UK.

3.2.3 *Patch selection and details of soil characterisations*

At the start of the project, we undertook an initial site visit (see Fig. 3.7a for view of the site). The purpose of this was twofold. First, to conduct a visual inspection of the sites. Second, to collect soils sample to confirm the presence of the TPH gradient between patches indicated in the consultant data. During this initial visit, we noted several discrete pools of crude oil on the soil surface. This oil seemed to originate from sources below 0.5m (see Fig. 3.7b).

We postulated that the crude oil was seeping to the surface through the holes bored during the window sampling. In any case, it seemed that significant pools of hydrocarbon remained at some depth below the currently existing overfills. We collected soil samples for TPH analysis from each of the specified locations selected to represent the potential TPH concentration gradient (dug to depth of 20cm). Additionally, soil from a further site at which surface oil contamination was observed was also collected. The samples were returned and stored at 3°C, with a sub-sample sent for TPH measurement. At the same time, we collected large soil volumes for use in the earthworm bioassay (for details of soil handling see Section 3.2.4, for experimental results see Section 6.3).

Chemistry data for the 11 sampled points at Site B showed that the concentration gradient anticipated from the analytical data supplied by the owner was not in fact present between the patches in the samples collected for this study (Fig. 3.8b). This is probably for two reasons:

- The original samples were taken from deeper down in the soil profile (0.25 – 2.0m) whereas the samples for this project were taken from the top 0.25cm. The analysis conducted in this project would have picked up only that contamination which had migrated into the once previously clean top-soil.
- Even if sampling for this project had been done at the same depth as the original sampling, it is unlikely the same pattern of contamination would have been found due to the heterogeneity in contaminant distribution in soil.

The TPH soil values included two very high concentrations (14,300 and 34,400µg TPH g^{-1} soil, both located at patches with visible surface oil

Fig 3.7a

Fig 3.7b

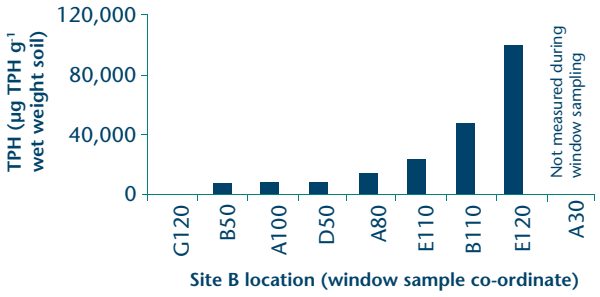
Fig. 3.7. Photos of Site B. a) area of site showing general aspect and vegetation cover, b) one of the surface oil contamination spots present at a limited number of patches on the site.

contamination), two low intermediates (160 and 320µg TPH g^{-1}), and a range of low to very low values (between 8.7 and 36.3µg TPH g^{-1}). Because the collected samples did not span a continuum of exposure concentrations, we decided to manipulate the soils to create a range of TPH concentrations suitable for investigation of effects on the utilised biological responses in the laboratory bioassay. For this, we made a dilution series by mixing soils from the highest concentration (34,400 µg TPH g^{-1}) with a blend of the low-ranged soils (after drying and hand-sieving them through a two-mm mesh). Such approaches have been used successfully in published studies (e.g. Schaefer, 2001). By creating a dilution series (keeping five of the original soils), we obtained a range of soil TPH concentrations. These ranged from low to high values 8 (control), 12, 160, 320, 700, 1600, 3500, 7250, 14,300µg TPH g^{-1} wet wt soil. These patches were designated patch B1 – patch B9 (Fig. 3.8c). The soil was prepared using a large mixer to ensure homogenous mixing. This comprehensive mixing was likely to result in the loss of substantial amounts of some of the most volatile compounds. In the fieldwork phase, only those sites included within this modified concentration series (B1-B4 and B9) were used for biological response assessment.

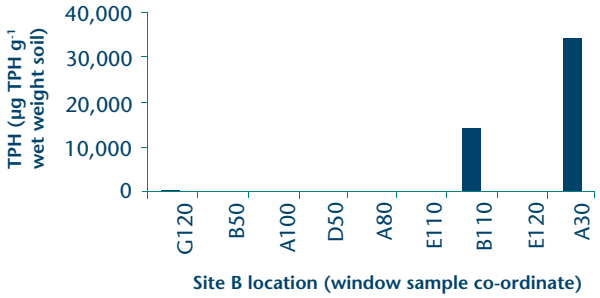
3.2.4 *Collection of Site B soils and creation of the dilution series for use in bioassays*

Soil sampling for the bioassay was conducted in a similar way to Site A. At each designated sampling point, we marked out areas of one square metre and excavated soil from the four corners of a marked central square (reserved for *in situ* testing). In each corner, 0.5m^2 was excavated to a maximum depth of 25cm, providing four samples of 20l volume. Any large stones and turf present were removed at this point (making sure to collect the soils from the root mat) and the soil excavated below the root-mat further removed. We mixed collected samples on-site, to ensure homogeneity. Finally, soils were bagged and individually marked with unique sample point codes ready to be taken to the laboratory in refrigerated containers.

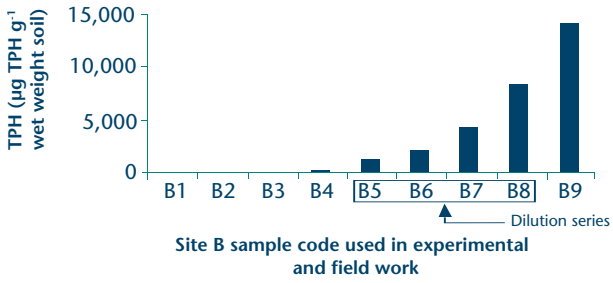
Upon return, sub-samples for use in chemical analysis were immediately collected and stored at -20°C to prevent compound volatilisation and degradation. We expected Site B soils to contain more volatile organic compounds (such as gasoline range hydrocarbons), and so we adopted a different approach to preparing samples. Instead of being dried, soil was instead screened thorough a 10mm mesh while still damp. It was then remixed and used directly for the tests. This approach ensured that biological and chemical tests conducted on sub-samples of the soil from each



3.8a



3.8b



3.8c

Fig. 3.8. a) predicted TPH in top one metre of soil based on historical data from 1998 window sampling; b) measured TPH in soils collected from a selection of Site B locations within the identified potential gradient; c) modified TPH concentrations of Site B patch soils based on measured concentrations for five patches (B1-B4, B9) and four dilutions of the most contaminated soil (34,400 µg TPH g^{-1}) (B5-B8). All values are in µg TPH g^{-1} wet weight soil.

sampling point were comparable. We acknowledge that even during this more limited preparation, a large proportion of some of the more volatile fractions (ie. up to C_{10}) may have been lost from the samples. Within the constraints of the project (ie. to evaluate the potential of the chosen assay to contaminated field soils), though, this approach represented the most practical option to testing in soils where the

contaminant profile suggested the presence of volatile compounds (or indeed if the profile is unknown). As discussed above (Section 3.2.3), an initial measurement of TPH concentrations in the soils showed that a contamination gradient was not present. To overcome this problem (and so allow assessment of biological responses across a concentration range), we made a dilution series of soils from one of the highly contaminated soils.

3.2.5 Soil chemical characterisation trends among major contaminant groups

Once we had agreed a putative oil contamination concentration series with the project board, and once we had collected, mixed and if necessary diluted the soils, we then carried out detailed chemical characterisations of the nine site soils. We conducted analyses at two points. Firstly we analysed TPH, metals and cyanides as part of a standard ICRCL analysis. In addition, we also measured concentrations of 54 separate PAHs and selected BTEX. These analyses were conducted in a single replicate of each patch soil only. The reason for this was that because soil batches had been prepared as a single batch, analysis of more than one sample from each batch would have created pseudo-replicate data unsuitable for use with the analysis of variance method. In addition, unreplicated analytical data is often encountered during site risk assessment. To link this study with project P5-069, it was considered useful for this level of chemical analysis to mimic a real site survey as closely as possible.

We conducted a second set of soil analyses immediately after earthworms were removed, after 42 days of the exposure. Soil samples were taken separately from each experimental replicate, providing truly replicated samples. We used these for a detailed analysis of the concentrations of the 54 PAHs.

Fig. 3.9 Actual TPH concentrations of Site B patch soils based on measured concentrations for five patches (B1-B4, B9) and four dilutions of the most contaminated soil (34,400µg TPH g^{-1}) (B5-B8). All values are in µg TPH g^{-1} wet weight soil.

Measured TPH concentrations indicated that the concentration in the B1 soil was at below-detection level (Fig. 3.9). This indicates that this soil is largely clean of oil contamination. It is thus suitable as an on-site reference (control). Site B2 also had a very low level of TPH (5.0µg TPH g^{-1} wet weight soil). The concentrations at both B3 and B4 were also low (between 40 and 50µg TPH g^{-1} wet weight soil in both cases). In patch B9, where there was visible oil contamination, we found a very high concentration of TPH. This exceeded the expected concentration by almost an order of magnitude, suggesting that worms exposed to this soil would be exposed to a concentration above presumed concentration. It is, though, important to point out that this analysis is based on only a single sample and that the soil sent for analysis was notably heterogeneous in oil distribution. In the dilution series (B5-B8), measured TPH concentrations were all lower than anticipated. The reason for this is likely to be volatalisation of the short chain hydrocarbon during the mixing process. Despite this, there was a clear increase in the concentration of TPH increasing in the order B5 < B6 < B7 < B8, with concentration roughly doubling between each treatment.

Comparisons of the concentrations of measured TPH with the EQSs for soils set out in the New Dutch list indicated that patches B1 – B4 contained TPH concentrations below the target value for mineral oil (sum of total alkanes) of 50µg g^{-1}. These soils can be considered to be clean. Soils within the dilution series (B5-B8) all contained measured TPH concentrations that were higher that the target, but below the New Dutch list intervention value of 5000µg TPH g^{-1}. These soils can be considered moderately contaminated. Soil B9 contained TPH concentrations many times the intervention value. B9 soil can therefore be considered to be very polluted, containing TPH concentrations likely to cause harm to the soil ecosystem there.

BTEX analysis found concentrations close to or below the detection limit in all soils except B7 and B9 (which were within a factor of two of the detection limit). As the detection limit represents the New Dutch list target values, these compounds may be a minor contaminant stress in these soils. No soil contained BTEX concentrations above the New Dutch list intervention value. Measurement of BTEX levels in soils at the end of the earthworm bioassay found no compounds present above the detection level.

We summarise the results from the detailed analysis of the concentrations of 54 PAH compounds in all the Site B patch soils in Tables 3.4a and 3.4b. These present measured concentrations in soils collected both at the start of the earthworm bioassay (Table

Patch	Total PAH ng PAH g $^{-1}$	Total EPA 16 ng PAH g $^{-1}$	Total non EPA 16 ng PAH g $^{-1}$	% non EPA 16 ng PAH g $^{-1}$
B1	633	315	318	50.2
B2	1591	825	766	48.2
B3	3275	1690	1585	48.4
B4	1516	770	746	49.2
B5	5761	1687	4074	70.7
B6	6074	1649	4425	72.8
B7	15917	3576	12341	77.5
B8	25271	2742	22529	89.1
B9	36915	3270	33645	91.1
Reference soil	858	474	384	44.8

Table 3.4a

Patch	Total PAH ng PAH g $^{-1}$	Total EPA 16 ng PAH g $^{-1}$	Total non EPA 16 ng PAH g $^{-1}$	% non EPA 16 ng PAH g $^{-1}$
B1	838 ± 424	392	446	53.2
B2	1255 ± 262	610	646	51.4
B3	2770 ± 181	1319	1452	52.4
B4	1339 ± 243	617	723	54.0
B5	2635 ± 255	903	1733	65.7
B6	5152 ± 2575	2314	2838	55.1
B7	6457 ± 1928	1786	4672	72.3
B8	11536 ± 5222	2089	9448	81.9
B9	28886 ± 4581	3727	25160	87.1
Reference soil	688 ± 116	353	336	48.7

Table 3.4b

Table 3.4. Measured concentrations of the sum of 54 PAH compounds in Site B patch soils field soils (B1-B4, B9) and four dilutions of the most contaminated soil (34,400µg TPH g^{-1}) (B5-B8). a) Concentrations measure in a single replicate sample taken before use of each patch soil in the earthworm bioassay; b) means of four samples per patch ± SD, each taken from individual replicates at the termination of the earthworm bioassay.

3.4a) and upon termination (Table 3.4b). We give detailed data for the individual compounds measured at both the time points in Table 3.5.

The PAH concentration measured in soils at the start of the bioassay indicated the presence of a clear concentration series within the selected Site B patch soils. This ranged from very low levels (patches B1 and B2) to high concentrations in the most contaminated soils within the dilution series (patches B7 and B8), and very high concentrations the field soil at which visible oil contamination was present (patch B9). Though there were differences between individual compounds, the trend for increases in concentration from patch B1 – B9 were reflected for all 54 of the measured compounds (see Table 3.5), as well as the sum of PAHs (Table 3.4).

We confirmed the presence of a concentration series of PAHs within the Site B patches in the more detailed analyses at the end of the 42-day earthworm bioassay. Again, as in the initial samples, the trend for concentrations of individual compounds increased from low in B1 soil to very high in the B9 soil (Table 3.5), and also for the sum of total PAHs (Table 3.4). The presence of a clear trend of increasing PAH concentrations for both individual compounds and total PAHs in samples taken both before and after the earthworm exposure demonstrates unequivocally the presence of a concentration series relating to the contamination of Site B patch soils.

A comparison of sum PAH concentration (in the sample collected before the bioassay) with the EQSs set out in the New Dutch list indicated that patches B1, B2 and B3 had sum PAH concentrations below the target value of 1µg PAH g^{-1}. At all the other patches, sum PAH concentrations fell between this target and the intervention value of 40µg TPH g^{-1}, though these concentrations ranged from just above the target value in B3 to just below the intervention value in soil B9.

Comparisons of the initial PAH concentrations with values measured at the end of the earthworm bioassay indicated a decrease in concentration for individual compounds during this exposure. This ranged from less than two per cent in soil B3 to up to around 50 per cent in three soils from the dilution series (B5, B7 and B8). Compounds of higher molecular weight had the greatest losses over the duration of the earthworms' exposure, comparisons of the degradation of individual PAH compounds revealed. For compounds of low molecular weight, analysis in some cases indicated the presence of increased concentration at bioassay termination (see Table 3.4). The change in profile for different PAH compounds reflects the likely degradation of the high weight compounds by soil bacteria during the earthworm bioassay. This evidently results in the formation of compounds of low molecular weight.

One interesting point arose as a result of analysis of the extended suite of PAHs. This was that the usual practice of analysing soils for only the 16 individual PAHs (recommended by the US EPA for site investigations) gave a rather poor picture of PAH contamination. The percentage of total PAHs represented by the US EPA standard suite was 50 per cent in soil B1-B4, but decreased steadily through the dilution series up to only approximately 10 per cent in soil B9. In these more contaminated soils, analysis of the restricted US EPA PAH suite would thus have underestimated PAH exposure by a factor of 10.

Table 3.5. Measured concentrations of 54 PAH compounds in Site B soils (patches B1-B4, B9) and in four dilutions of the most contaminated soil (B5-B8). The values in the top half of the table are for a single replicate of each soil taken from a single sample collected before the start of the earthworm bioassay (see Section 3.3.4). Values in the bottom half are means of four samples ± SD, each taken from individual replicates at bioassay termination.

Patch	Naphthalene	2-Methylnaphthalene	3-Methylnaphthalene	1-Ethylnaphthalene	2-Ethylnaphthalene	2,6-Dimethylnaphthalene	1,3-Dimethylnaphthalene	1,6-Dimethylnaphthalene	2,3-Dimethylnaphthalene
Start (day 0)									
B1	7.8	8.6	1.8	ND	ND	5.1	5.3	3.1	2.9
B2	17.6	27.8	7.7	ND	0.6	20.0	18.3	10.1	18.6
B3	23.5	36.1	9.0	0.3	0.7	16.9	16.3	11.2	13.6
B4	14.5	26.2	7.2	0.1	0.3	13.0	14.2	9.3	9.0
B5	27.5	69.6	29.7	9.4	8.9	110.1	141.3	141.1	107.9
B6	19.6	50.1	22.6	6.7	8.0	92.8	129.7	129.3	91.7
B7	43.9	136.7	80.2	28.7	32.5	340.7	481.4	514.7	332.0
B8	40.9	84.1	59.8	26.7	31.4	407.6	607.6	764.9	567.0
B9	64.4	480.6	261.3	98.8	91.5	1007.2	1461.6	1631.7	1086.3
Reference soil	9.4	3.5	ND	ND	ND	1.0	2.2	1.4	ND
End (day 42)									
B1	13.9 ± 3.5	16.4 ± 9.8	3.0 ± 2.4	ND	ND	3.8 ± 6.2	7.9 ± 4.5	6.4 ± 3.5	11.1
B2	18.7 ± 1.6	25.0 ± 2.4	13.1 ± 13.0	ND	ND	15.1 ± 5.8	16.8 ± 3.5	11.5 ± 1.2	14.0 ± 7.7
B3	49.4 ± 13.5	70.0 ± 11.1	19.7 ± 0.5	1.7 ± 1.7	2.7 ± 3.1	26.5 ± 3.4	28.7 ± 3.6	22.1 ± 1.8	18.3 ± 1.9
B4	23.1 ± 4.4	43.1 ± 9.3	13.5 ± 2.8	ND	2.5 ± 0.3	17.5 ± 1.1	19.2 ± 2.5	13.8 ± 2.9	10.5 ± 1.3
B5	25.1 ± 2.1	44.5 ± 3.8	15.8 ± 4.1	0.7 ± 0.5	0.7 ± 0.5	38.2 ± 6.1	39.9 ± 2.6	36.9 ± 3.1	32.9 ± 5.2
B6	43.3 ± 21.2	81.5 ± 28.3	26.7 ± 11.7	2.9 ± 2.7	3.3 ± 2.1	47.1 ± 22.5	56.5 ± 30.8	49.7 ± 26.5	46.0 ± 26.3
B7	38.2 ± 5.8	63.1 ± 12.9	17.6 ± 5.2	2.6 ± 3.1	2.7 ± 2.7	50.2 ± 23.3	52.7 ± 28.4	48.9 ± 29.0	45.0 ± 24.6
B8	38.9 ± 5.6	80.0 ± 14.4	34.2 ± 7.8	5.2 ± 3.0	6.9 ± 4.7	86.8 ± 43.9	115 ± 72.1	131.4 ± 90.2	118 ± 74
B9	61.8 ± 11.5	294.2 ± 53.6	150.0 ± 24	56.0 ± 5.4	58.5 ± 6.0	585.9 ± 66.7	858 ± 125	1016.1 ± 77.4	683.3 ± 90
Reference soil	14.3 ± 2.4	11.1 ± 5.5	6.9 ± 3.0	0.4	ND	1.8 ± 2.1	2.4 ± 1.1	2.5 ± 1.4	ND

Table 3.5 cont. | Measured concentrations of 54 PAH compounds in Site B soils. For full details, see previous page.

Patch	1,5-Dimethylnaphthalene	Acenaphthylene	1,2-Dimethylnaphthalene	1,8-Dimethylnaphthalene	Acenaphthene	2,3,5-Trimethylnaphthalene	Fluorene	Dibenzothiophene	Phenanthrene
Start (day 0)									
B1	1.9	6.9	ND	ND	8.0	ND	13.1	1.0	66.6
B2	0.1	110.5	ND	ND	8.6	7.2	10.7	3.2	153.7
B3	ND	108.5	ND	ND	18.2	3.1	20.2	9.0	320.7
B4	ND	100.0	ND	ND	4.9	ND	8.0	5.0	116.8
B5	61.0	57.5	27.4	0.2	14.0	292.3	67.5	79.6	378.6
B6	53.1	36.2	22.5	0.1	10.2	274.1	66.6	93.4	345.4
B7	225.3	23.7	165.0	1.6	31.2	940.9	218.7	254.8	744.7
B8	362.1	37.3	199.4	4.2	56.7	2316.0	345.0	387.8	861.3
B9	699.2	72.1	430.2	8.8	88.9	3369.1	668.9	368.5	1171.5
Reference soil	ND	119.4	ND	0.5	ND	ND	2.7	0.8	40.0
End (day 42)									
B1	ND	6.6 ± 5.5	ND	4.4	7.5 ± 12.2	101.3	12.5 ± 16.8	4.6 ± 5.0	55.8 ± 5.9
B2	ND	7.7 ± 10.5	ND	0.3	7.9 ± 4.9	8.0 ± 7.5	8.2 ± 1.1	4.1 ± 0.9	121.6 ± 24.5
B3	ND	4.8 ± 3.8	ND	ND	28.9 ± 8.5	12.5 ± 7.9	36.8 ± 13.8	19.0 ± 6.8	264.0 ± 39.5
B4	ND	3.1 ± 1.7	ND	ND	7.7 ± 5.5	23.4 ± 10.3	17.4 ± 10.8	12.3 ± 6.4	104.0 ± 11.3
B5	5.6 ± 1.6	8.3 ± 10.0	ND	ND	9.4 ± 5.8	51.6 ± 8.9	29.2 ± 1.8	30.3 ± 3.5	173.5 ± 21.9
B6	12.2 ± 12.4	31.8 ± 35.2	111.2 ± 151.7	1.5 ± 1.1	41.4 ± 45.3	326.9 ± 443.7	116.0 ± 131.1	119.4 ± 137.0	175.9 ± 26.9
B7	12.3 ± 14.4	35.5 ± 28.4	45.5 ± 24.1	2.0 ± 0.2	28.0 ± 24.3	300.7 ± 334.6	80.3 ± 69.3	98.4 ± 83.8	168.1 ± 12.7
B8	57.8 ± 48.3	37.5 ± 17.5	87.8	2.0 ± 0.9	29.5 ± 12.4	431.6 ± 349.6	59.9 ± 70.1	126.4 ± 91.6	201.3 ± 50.2
B9	458.4 ± 29.8	81.1 ± 14.7	277.6 ± 118.8	8.7 ± 3.4	63.6 ± 16.2	2284.5 ± 705.6	397.0 ± 160.1	273.9 ± 100.6	441.5 ± 37.8
Reference soil	ND	4.3 ± 5.2	ND	1.5	2.5 ± 2.1	ND	4.2 ± 2.3	4.1 ± 3.8	40.8 ± 14.7

Table 3.5 cont. | Measured concentrations of 54 PAH compounds in Site B soils. For full details, see previous page.

Patch	Anthracene	2-Methylphenanthrene	1-Methylphenanthrene	3,6-Dimethylphenanthrene	Fluoranthene	9,10-Dimethylphenanthrene	Pyrene	2-Methylfluoranthene	1-Methylfluoranthene
Start (day 0)									
B1	10.2	11.8	9.8	2.2	77.7	1.7	68.6	3.9	25.7
B2	22.2	33.2	27.1	5.9	179.8	ND	154.6	17.8	7.3
B3	54.7	52.7	38.5	9.6	432.0	1.8	362.1	17.1	12.7
B4	16.3	27.8	22.2	6.3	130.5	2.2	114.9	14.6	5.9
B5	31.3	283.3	546.1	245.2	216.2	21.0	298.3	68.8	23.9
B6	23.4	315.1	635.6	289.3	170.2	24.0	284.1	76.9	312.7
B7	44.2	799.8	1728.1	786.8	189.2	51.9	586.5	181.0	892.7
B8	47.3	1223.5	2727.7	1449.0	174.5	76.4	928.4	318.0	1810.2
B9	50.9	999.3	2756.8	1666.4	167.7	126.7	1827.7	606.5	3260.0
Reference soil	5.4	9.9	7.3	2.3	81.0	2.1	69.3	10.8	0.6
End (day 42)									
B1	8.8 ± 2.3	11.8 ± 3.1	9.3 ± 3.9	2.9 ± 1.5	77.3 ± 19.1	ND	72.6 ± 24.0	9.8 ± 6.0	11.7 ± 13.3
B2	18.5 ± 3.9	27.9 ± 10.9	23.2 ± 10.2	4.3	141.3 ± 19.6	ND	127.5 ± 20.1	15.3 ± 3.5	11.1 ± 9.5
B3	47.0 ± 10.9	42.9 ± 3.2	29.1 ± 2.5	5.4 ± 3.5	327.0 ± 38.8	ND	283.4 ± 24.5	25.1 ± 6.7	54.2 ± 28.4
B4	16.0 ± 1.9	23.4 ± 3.0	17.2 ± 2.2	4.6 ± 0.4	116.3 ± 12.4	ND	111.0 ± 18.0	14.5 ± 3.3	20.5 ± 18.9
B5	22.9 ± 3.8	65.0 ± 2.1	93.1 ± 11.2	51.0 ± 9.5	127.3 ± 19.4	1.8 ± 1.0	188.6 ± 15.2	32.2 ± 1.4	103.2 ± 11.3
B6	21.7 ± 3.2	69.4 ± 20.0	108.9 ± 41.0	70.4 ± 23.3	105.3 ± 19.3	7.1 ± 2.1	234.1 ± 38.4	35.9 ± 27.0	136.1 ± 105.2
B7	25.6 ± 2.0	78.2 ± 34.7	159.7 ± 94.5	191.3 ± 64.9	127.1 ± 48.3	32.4 ± 28.2	317.4 ± 14.6	81.9 ± 20.3	268.3 ± 205.4
B8	32.9 ± 3.6	164.0 ± 104.8	403.8 ± 256.5	415.3 ± 191.9	212.0 ± 179.0	127.4 ± 111.4	475.3 ± 286.6	144.7 ± 97.2	341.2 ± 392.4
B9	44.1 ± 0.4	360.2 ± 59.2	1089.0 ± 116.5	749.1 ± 70.4	227.5 ± 102.3	200.1 ± 42.9	2223.5 ± 655.4	607.3 ± 172.6	991.1 ± 1596.4
Reference soil	6.3 ± 3.8	8.9 ± 1.4	6.0 ± 1.1	1.9 ± 0.6	81.3 ± 14.6	ND	74.4 ± 11.9	8.8 ± 1.3	11.0 ± 4.7

Table 3.5 cont. | Measured concentrations of 54 PAH compounds in Site B soils. For full details, see previous page.

Patch	Benzo[b]fluorene	Benzo[a]fluorene	1-Methylpyrene	Benzo[c]phenanthrene, Benzo[g,h,i]fluoranthene	Cyclopenta[cd]pyrene	Benz[a]anthracene	Chrysene, Triphenylene	3-Methylchrysene	2-Methylchrysene
Start (day 0)									
B1	20.6	9.7	2.5	7.0	0.8	47.8	51.6	12.6	8.3
B2	42.7	24.4	6.2	16.7	1.8	115.3	117.2	31.7	21.1
B3	91.6	69.6	11.7	34.7	3.2	360.4	254.3	73.2	40.8
B4	36.4	21.6	6.3	13.9	1.8	144.6	103.2	43.5	22.5
B5	215.7	109.6	46.9	29.2	6.8	612.8	410.2	124.0	147.3
B6	285.0	76.1	57.0	30.8	7.1	714.8	453.4	135.4	193.2
B7	815.7	352.0	154.5	51.6	18.5	1628.2	1055.2	331.7	473.3
B8	1640.1	758.9	287.3	70.8	35.4	277.3	1842.5	615.9	878.4
B9	2924.5	1426.6	701.0	168.6	188.1	593.3	3255.3	1062.6	1515.1
Reference soil	32.5	12.8	5.4	8.6	3.0	93.1	64.2	9.4	14.0
End (day 42)									
B1	10.1 ± 5.6	10.1 ± 4.4	2.5 ± 1.1	9.0 ± 4.9	0.9 ± 0.3	51.5 ± 20.7	63.6 ± 36.9	15.8 ± 9.1	9.9 ± 7.0
B2	26.6 ± 15.3	19.4 ± 4.4	4.5 ± 1.9	14.7 ± 3.1	1.4 ± 0.7	84.8 ± 35.6	102.2 ± 20.4	23.6 ± 7.5	16.4 ± 6.9
B3	76.1 ± 7.9	54.8 ± 7.8	10.3 ± 2.0	32.2 ± 5.8	3.7 ± 1.8	195.5 ± 61.1	232.7 ± 47.2	48.0 ± 16.0	36.4 ± 7.0
B4	35.4 ± 6.5	22.9 ± 4.5	5.6 ± 1.0	14.5 ± 4.2	1.7 ± 0.2	89.8 ± 34.7	110.2 ± 24.8	22.0 ± 17.2	22.1 ± 3.0
B5	114.9 ± 13.7	46.8 ± 5.7	26.9 ± 1.7	22.7 ± 3.0	4.0 ± 0.2	309.5 ± 204.3	271.7 ± 19.0	63.4 ± 10.5	89.2 ± 14.8
B6	143.2 ± 108.6	51.5 ± 34.1	51.8 ± 13.9	26.3 ± 13.0	5.4 ± 1.5	472.3 ± 483.2	471.3 ± 137.7	68.5 ± 45.1	102.1 ± 68.9
B7	357.5 ± 110.5	134.2 ± 45.1	105.6 ± 18.6	35.4 ± 7.8	10.7 ± 2.5	779.8 ± 795.7	790.3 ± 153.9	683.7 ± 561.5	258.4 ± 78.8
B8	571.2 ± 658.7	280.2 ± 252.8	370.2 ± 242.9	56.9 ± 10.6	280.3 ± 491.7	458.0 ± 556.0	394.1 ± 552.6	190.4 ± 193.0	634.7 ± 230.4
B9	1000.7 ± 1603.6	280.7 ± 512.8	872.5 ± 243.6	150.2 ± 58.3	115.2 ± 38.7	205.0 ± 290.9	1693.5 ± 2345.6	382.7 ± 513.3	1654.0 ± 530.0
Reference soil	13.2 ± 3.7	4.1 ± 1.3	1.5 ± 0.3	9.5 ± 2.1	3.1 ± 1.3	33.7 ± 10.5	62.2 ± 11.6	10.5 ± 7.2	9.1 ± 1.8

Table 3.5 cont. | Measured concentrations of 54 PAH compounds in Site B soils. For full details, see previous page.

Patch	5-Methylchrysene	4-Methylchrysene	1-Methylchrysene	Benzo[b]fluoranthene, Benzo[j]fluoranthene,	Benzo[e]pyrene	Benzo[a]pyrene	Perylene	Ideno[1,2,3-cd]pyrene	Dibenz[a,h]anthracene
Start (day 0)									
B1	4.3	2.6	3.6	35.9	14.5	37.8	7.7	32.2	4.7
B2	11.6	6.8	9.5	79.9	32.9	87.6	18.8	63.4	10.5
B3	20.9	12.1	18.4	206.7	84.1	237.4	61.1	169.4	26.8
B4	12.4	7.4	10.9	81.6	41.6	89.6	22.9	70.5	12.6
B5	149.9	87.2	93.4	197.4	88.5	120.5	31.2	89.6	6.7
B6	171.7	107.7	114.6	190.2	95.1	109.3	26.2	73.3	18.8
B7	448.4	283.5	290.4	470.4	202.6	141.4	45.4	14.0	31.1
B8	841.6	525.8	547.4	656.7	347.5	814.1	57.9	94.7	51.5
B9	1939.7	1215.6	995.6	10.5	691.8	362.8	96.6	9.1	81.4
Reference soil	2.3	5.6	6.4	58.2	19.9	48.9	11.5	42.4	6.8
End (day 42)									
B1	2.6 ± 1.7	2.9 ± 2.8	4.2 ± 3.2	68.6 ± 61.7	17.3 ± 7.5	30.5 ± 4.0	9.0 ± 1.9	56.7 ± 46.1	8.3 ± 8.2
B2	2.3 ± 3.2	5.4 ± 2.1	6.1 ± 2.0	78.5 ± 21.0	28.1 ± 5.8	64.3 ± 20.7	15.7 ± 3.7	67.4 ± 12.7	10.6 ± 3.7
B3	8.4 ± 2.5	14.4 ± 5.7	15.2 ± 3.5	194.2 ± 41.2	73.7 ± 9.7	160.7 ± 27.6	44.1 ± 8.0	151.6 ± 13.9	14.5 ± 5.9
B4	3.3 ± 2.2	7.4 ± 2.0	9.4 ± 1.8	96.7 ± 26.1	42.1 ± 8.0	70.8 ± 12.7	18.4 ± 3.5	74.5 ± 12.8	7.8 ± 4.1
B5	49.2 ± 29.8	40.4 ± 4.9	47.0 ± 4.8	141.1 ± 34.6	73.2 ± 5.5	99.3 ± 14.2	25.7 ± 2.5	77.8 ± 9.7	10.9 ± 7.1
B6	74.4 ± 77.0	78.1 ± 30.7	83.6 ± 28.1	255.7 ± 104.9	90.8 ± 66.4	156.7 ± 145.9	375.6 ± 692.1	721.6 ± 1227.0	250.4 ± 441.5
B7	125.2 ± 141.9	147.6 ± 44.8	169.2 ± 52.1	396.0 ± 29.2	149.1 ± 102.5	240.6 ± 219.5	33.9 ± 6.1	85.0 ± 19.4	31.9 ± 6.1
B8	852.6 ± 762.6	1681.4 ± 1552.6	336.5 ± 250.5	503.7 ± 328.7	197.9 ± 229.2	621.6 ± 576.1	121.9 ± 111.0	224.2 ± 226.5	131.0 ± 117.9
B9	1019.4 ± 971.2	2040.6 ± 1944.3	1235.8 ± 385.6	1567.9 ± 490.8	794.9 ± 699.5	1692.6 ± 1555.7	220.6 ± 143.1	265.1 ± 182.2	200.9 ± 119.4
Reference soil	1.3 ± 0.4	3.0 ± 0.7	4.0 ± 1.1	70.1 ± 44.1	22.1 ± 4.2	39.5 ± 6.8	13.5 ± 2.1	75.2 ± 23.1	7.2 ± 3.7

Table 3.5 cont. | Measured concentrations of 54 PAH compounds in Site B soils. For full details, see previous page.

Patch	Benzo[g,h,i]perylene	Anthanthrene	Dibenzo[a,i]pyrene	Coronene	Dibenzo[a,e]pyrene	Dibenzo[a,h]pyrene
Start (day 0)						
B1	25.3	4.8	7.8	3.7	3.3	1.8
B2	54.7	12.4	18.6	8.4	7.2	3.9
B3	160.3	45.6	56.5	24.0	22.8	12.3
B4	80.2	17.1	25.3	13.9	11.7	6.3
B5	99.0	23.6	35.1	17.5	15.5	8.4
B6	92.9	18.9	28.8	16.7	13.2	7.1
B7	159.1	20.0	49.9	30.4	91.9	49.5
B8	209.0	20.8	70.5	47.1	151.0	81.3
B9	293.4	13.3	39.4	93.8	223.4	120.3
Reference soil	36.7	11.4	17.5	6.9	6.3	3.4
End (day 42)						
B1	25.7 ± 8.4	3.8 ± 1.9	5.0 ± 0.6	4.8 ± 2.5	4.2 ± 3.7	ND
B2	44.6 ± 6.4	7.7 ± 4.4	8.6 ± 4.1	7.9 ± 1.2	6.2 ± 3.1	3.1 ± 3.8
B3	115.1 ± 6.4	19.2 ± 11.3	22.2 ± 8.2	20.0 ± 2.7	20.6 ± 7.8	16.1 ± 32.2
B4	62.4 ± 8.0	8.0 ± 5.9	10.1 ± 2.2	11.0 ± 2.4	19.6 ± 5.6	ND
B5	68.9 ± 3.5	14.5 ± 3.4	16.1 ± 3.5	13.1 ± 3.2	16.8 ± 7.2	ND
B6	90.2 ± 8.7	7.9 ± 6.7	15.8 ± 4.1	15.5 ± 4.3	35.2 ± 18.9	ND
B7	120.0 ± 5.8	9.0 ± 5.6	21.6 ± 5.7	19.3 ± 2.6	59.7 ± 16.2	ND
B8	201.2 ± 28.8	10.0 ± 3.5	28.3 ± 4.8	43.2 ± 12.6	144.8 ± 35.5	0.4 ± 0.8
B9	273.1 ± 35.0	5.8 ± 0.8	49.0 ± 3.5	77.6 ± 17.9	140.5 ± 94.6	1.7 ± 1.3
Reference soil	34.3 ± 3.2	3.2 ± 1.4	5.3 ± 1.1	7.3 ± 0.7	5.7 ± 1.0	ND

4

Testing regime used in the project

We performed a total of seven separate evaluations to assess the performances of the selected biological tests. These have been listed below. They are also summarised in Tables 4.1 and 4.2.

4.1 Feeding activity using bait lamina strip

Use within the project: The bait lamina test was used to measure feeding activity of soil organisms (predominantly invertebrates) by assessing the removal of a series of bait pellets. As the bait lamina method is conducted *in-situ*, it was used only in the fieldwork phases of the study. These were:

- measurement at selected Site A patches in spring, autumn and winter;
- measurement at all Site B field patches (B1, B2, B3, B4, B9) in autumn.

4.2 The ISO and OECD Draft Earthworm Reproduction Test

Use within the project: To assess the impact of soil contaminants on a set of life-cycle parameters in earthworms. The assay was used to assess earthworm (*Lumbricus rubellus*) responses in a number of studies. These were:

- responses in all Site A patch soils in a semi-field mesocosm exposure;
- responses in all Site B patch soils in a laboratory exposure;
- responses in selected Site A patch soils under different temperature regimes (10, 15, 20°C) in a laboratory exposure;
- responses in selected Site A patch soils under different soil pH amendment regimes (unamended, lower by one unit, raised by one unit) in a laboratory exposure.

The species used in all exposures was the epigeic soil dwelling earthworm *L. rubellus*. We favoured this species over the more commonly used compost dwelling species *Eisenia fetida*, because it was more relevant to the sites under study (*L. rubellus* was common at both sites, while (perhaps not surprisingly) *E. fetida* could not be found). The endpoints measured during the earthworm exposures were: survival, weight change, and reproductive rate (recorded as the production of cocoons). The exposures also generated worm samples that could be used for subsequent biomarker measurements.

4.3 An instantaneous rate of population increase study using toxicity data collected for the springtail *Folsomia candida* in Project P5-069

Use in the project: The IRPI model was used to evaluate possible population consequences for springtails exposed to each patch soil. We took data for the interpretation from the toxicity test being conducted as part of Project P5-069.

4.4 Lysosomal membrane stability

Use within the project: This biomarker measurement assessed the effects of soil contaminants on the membrane fragility of the lysosomes of earthworm coelomocyte cells. As lysosome membrane stability is affected by exposure to both metals and organic chemicals, the NRR-T assay was conducted on exposed earthworms at both sites (Eason et al., 1999; Hankard et al., 1999; Scott-Fordsmand, 1998). The assay included:

- responses in naïve *L. rubellus* exposed to soils from all Site A patches in the semi-field mesocosm exposure;
- responses in naïve *L. rubellus* exposed to selected Site A patch soils under the different temperature regimes;

- responses in naïve *L. rubellus* exposed to selected Site A patch soils under the different soil pH amendment regimes;
- responses in naïve *L. rubellus* exposed to all Site B patch soils;
- responses in field-collected indigenous *L. rubellus* from three Site A patches in spring, autumn and winter and all Site B patches in one season (autumn).

4.5 RT-PCR for measurement of gene expression

Use within the project: The previous work conducted on earthworms identified genes that respond to metal exposure. For this reason, gene expression quantification focused on the earthworms exposed to Site A patch soils.

Initially, we measured expression in field-collected *L. rubellus* from all patches along the Site A patches where worms occurred (this excluded patch 5 where all worms are known to be absent Spurgeon & Hopkin, 1999a). This was to identify metal responsive sequences.

Based on this profiling, we selected two sequences for further analysis in field-collected *L. rubellus* from three Site A patches in spring, autumn and winter. These sequences were an isoform of metallothionein-2 (*MT-2*) known to be upregulated by cadmium (Stürzenbaum et al., 2001), and the mitochondrial large ribosomal sub-unit protein of the mitochondrial ribosomal system (*rrnL*), that is upregulated by copper (Galay Burgos et al., 2003).

We then studied further the expression of just the *MT-2* transcript in naive *L. rubellus* exposed to soils from all Site A patches (in a mesocosm exposure) and from selected Site A patches under different temperature and pH amendment regimes.

4.6 Bacterial biosensors

Use within the project: Such *lux*-marked bacterial biosensors respond to both organic and metal contaminants (Boyd et al., 1997; Paton et al., 1995; Shaw et al., 2000b). The assay was thus used to detect the presence of contaminant in soil sampled from all patches, at both sites.

Table 4.1. | Summary of experimental design and analyses used to evaluate the suitability of the selected biological assays for the evaluation of the status of contaminated soils at the two study sites. For full details of procedures used for exposures and responses analyses, see R&D Project Record P5-063/PR.

Experimental Regime	Results chapter	Sites/patches used	Soil collection/treatments	Experimental design and measurement parameters
Laboratory based reproduction test exposing the earthworm *L. rubellus* to soil from three Site A soil at three temperatures	6.1	Site A – 1, 3, 4	Collect 10 kg site soil collected at each site (sampled at the same time as for the pH modification assay). Soil air dried, ground and 2 mm sieved prior to use in tests.	1.26 kg soil place into 12 containers for each site. 4 replicates maintained at 10°C, 4 at 15°C and 4 at 20°C. Measure survival, weight change, reproduction, NRR-T, gene expression and body metal residue levels.
Laboratory based reproduction test exposing the earthworm *L. rubellus* to pH modified soil from three Site A patches	6.2	Site A – 1, 3, 4	Collect 10 kg site soil (from top 5 cm) at each site. Soil air dried, ground and 2 mm sieved prior to use in tests. Soil either left unamended at current pH or pH raised by one unit by addition of powdered calcium carbonate or lowered one unit by addition of 0.125 M H_2SO_4	1.26 kg soil place into 12 containers for each site. 4 replicates maintained at ambient pH, 4 at pH + 1 unit and 4 at pH -1 unit. 8 *L. rubellus* added to each replicate. Measure survival, weight change, reproduction, NRR-T, metallothionein expression and body chemical residue levels. Also conduct full soil chemical analysis and WHAM modeling of metal speciation.
Laboratory based reproduction test exposing the earthworm *L. rubellus* to all site B patch soils	6.3	Kettering loam control and Site B patch soils 1-9	Sample of 10 kg of site soil or soil mix screened for large stones and the stored at 3°C prior to start of test	1.25 kg soil placed into each of 4 replicate containers per treatment. 8 *L. rubellus* added to each replicate. Measure survival, weight change, reproduction, NRR-T, metallothionein expression and body chemical residue levels.
Laboratory based test on the effects of soils from Sites A and B on demographic parameters of the springtail *Folsomia candida*.	6.4	Kettering loam control, Site A – 1,3,4 and Site B patches 1-9, Positive control	Site A – Soil air dried, ground and 2 mm sieved prior to use in tests. Site B – sample of site soil or soil mix screened for large stones and the stored at 3°C prior to start of test	30g (wet weight) of soil placed into each of 5 replicate jars for each patch. 30g control soil and 30g soil dosed with KCl at the LC_{50} concentration. Surviving adults and number of juveniles produced recorded after 28 days.
Use of a luminescence-marked bacterial biosensor exposed to extracts from soils at Sites A and B.	6.5	Kettering loam control, Site A - 1,3,4 and Site B patches 1-9, Positive control	Site A – Soil air dried, ground and 2 mm sieved prior to use in tests. Site B – sample of site soil or soil mix screened for large stones and the stored at 3°C prior to start of test	Sample of 5g of site soil shaken with distilled water for 10 minutes. Bacterial biosensor added and luminescence detected in scintillation counter. Reduction in luminescence from pure culture recorded.
Seasonal analysis of soil invertebrate feeding and earthworm *L. rubellus* biomarker responses at three Site A patches	6.6	Site A – 1, 3, 4	Sampled in three seasons spring (April), autumn (October), winter (December). At each time collected *L. rubellus* direct from the field, returned on own soil and used immediately for NRR-T and RNA extraction. Then snap freeze for chemical analysis. Also direct deployment of 3 bait lamina sets at each site.	For each seasonal, 10 individual stage synchronised (adult) *L. rubellus* collected from each site and analysed for NRR-T and RNA extraction, and metallothionein and mitochondrial ribosomal large sub-unit expression and chemical residue analysis. Soil invertebrate feeding of bait lamina recorded.
Field assessment soil invertebrate feeding and earthworm *L. rubellus* biomarker responses at five Site B patches	6.7	Site B 1, 2, 3, 4, 9	Direct deployment of 3 bait lamina sets (16 strips) at each patch. Collection of worms direct from the field. Return on own soil and use immediately for NRR-T and RNA extraction. Then snap freeze for chemical analysis.	Up to 8 individual *L. rubellus* analysed for NRR-T and RNA extraction, expression and chemical residue analysis. Soil invertebrate feeding of bait lamina recorded.

Table 4.2 | Summary table of assays used within the experiment and field analyses set out in Table 4.1. For full details of the procedures used for measuring exposures and responses see R&D Project Record P5-063/PR.

Experimental Regime	Earthworm survival	Earthworm reproduction	Earthworm weight change	Earthworm NRR-T	Earthworm *MT-2*	Earthworm *rrnL*	Earthworm other genes	Bait lamina	Lux	Metal analysis	PAH analysis	Soil metal speciation
Laboratory assay of Site A soil at 3 temperatures with *L. rubellus*	√	√	√	√	√					√		
Laboratory assay of pH modified Site A soil using *L. rubellus*	√	√	√	√	√					√		√
Laboratory assay of site B soil using the earthworm *L. rubellus*	√	√	√	√						√	√	
Seasonal analysis of bait lamina and earthworm biomarkers at Site A				√	√	√		√				
Field assessment of Site B soil using bait lamina and earthworm biomarkers				√				√	√		√	
Mesocosms exposure of earthworm to soil from a control and five Site A soils	√	√	√	√	√							
Expression profiling of earthorm genes along the Site A transect					√	√	√			√		

5

Initial response profiling of biological responses at site A

This chapter details the results obtained during the biological testing conducted at Site A in order to establish response profiles for each test. The chapter is split into two sections:

Section 5.1 details the outcome of gene expression profiling study conducted using indigenous worms collected from all patches along the Site A gradient (with the exception of patch 5, where worms could not be collected). On the basis of these expression profiles, we selected sequences for further analysis during the seasonal studies using indigenous worms (see Section 6.6) and also in the mesocosm and temperature and pH amendment bioassays conducted using Site A patch soils (see Sections 5.2, 6.1, 6.2).

Section 5.2 outlines results from a semi-field mesocosm study. This established the profile of a range of biological response measurements in the earthworm *L. rubellus* to soils collected from all Site A patches and a control soil (a sterilised Kettering loam). Exposures were undertaken in mesocosm units. These consisted of 40cm sections of plastic pipes of 30cm internal diameter, sealed at the bottom. A layer of black nylon was sandwiched firmly between layers of medium density polyethylene of mesh size 4mm and 64g m^{-2} phormisol and secured with a silicone sealant, so that even small earthworms could not enter or escape. Each mesocosm was filled to eight centimetres from the top with dried two millimetre screened site soil. Adult and juvenile *L. rubellus* were then added, and the mesocosm kept outdoors for 70 days. During the exposure, detailed measurements of the prevailing climatic conditions were made. At the end of the exposure, the biological responses measured were the effects on the life-cycle parameters: survival, adult and juvenile weight change and cocoon production rate. We also measured the biomarker responses of NRR-T and *MT-2* expression.

To present data in an understandable and concise format, only noteworthy results are detailed on separate pages, accompanied by a brief discussion of the significance of the results. In Chapter 7 we give a detailed overview of the performance of each biological test within each of the experimental phases.

5.1 Gene expression profiling using indigenous worms collected from all patches along the site a gradient

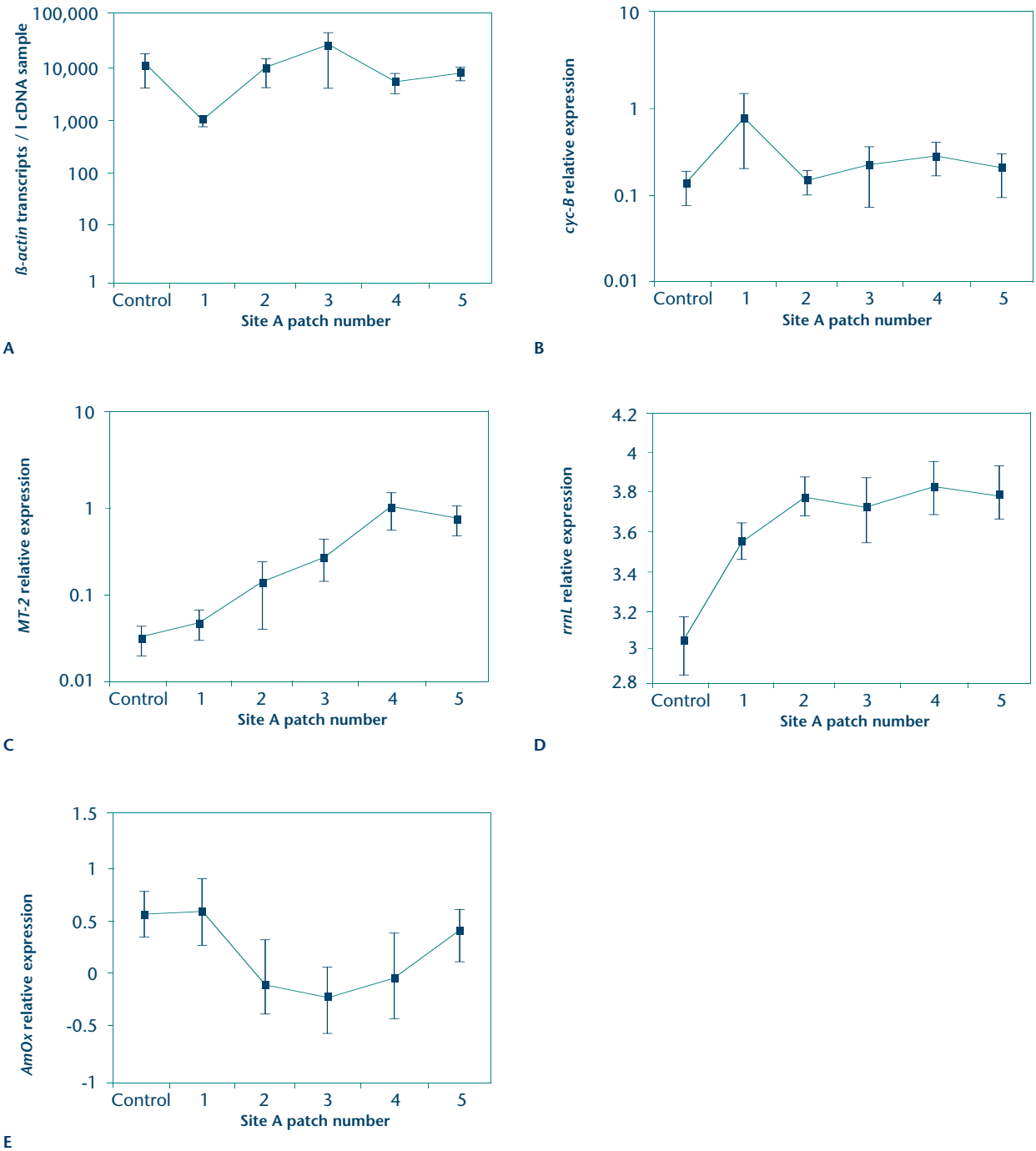
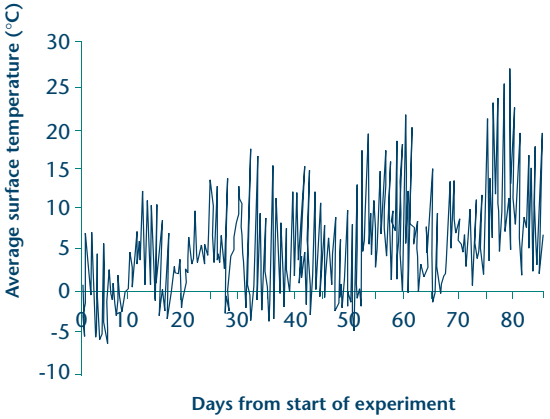


Fig. 5.1.1. Expression of five gene transcripts measured using the 5' nuclease (Taqman™) assay based quantitative RT-PCR protocol for five sequences in *L. rubellus* collected from all patches along the Site A gradient. Bars are standard errors. A – *ß-actin (ß-act)* transcripts. B – cyclophilin (*cyc-B*) relative expression. C – metallothionein isoform 2 (*MT-2*) relative expression. D – mitochondrial large ribosomal sub-unit (*rrnL*) relative expression. E – Amine oxidase (*AmOx*) relative expression.

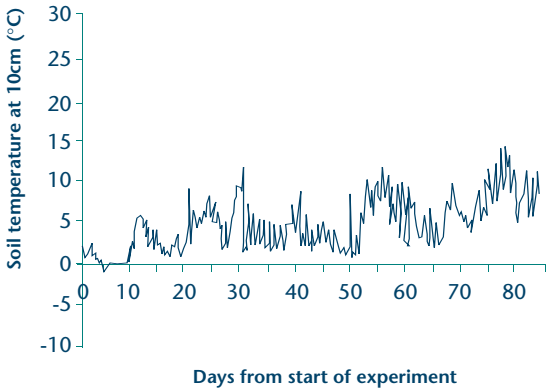
Summary of results

- Primer and probe sequences for quantification by real time PCR using Taqman™ were designed from *L. rubellus* sequence data held within NCBI databases (www.ncbi.nlm.nih.gov). The volume of this sequence data is rapidly expanding as part of ongoing projects being conducted at major sequencing centres and in universities and institutes. Already, within the database, a series of further potentially chemically responsive sequences have been identified in *L. rubellus* (see www.earthworms.org). Primer and probe sets that can be used for quantification of these are being developed in ongoing work being funded within the NERC Environmental Genomics Thematic Programme (see www.nerc.ac.uk/funding/thematics/envgen).
- Three sequences showed no changes in expression across the transect. This was confirmed by the absence of a significant effect of patch in a one-way ANOVA of log transformed raw expression values (*ß-act*) or relative expression (*cyc-B*, *AmOx*). Two sequences, *MT-2* and *rrnL*, were upregulated along the gradient. For both, one-way ANOVA of log transformed expression ratio indicated a significant effect of patch ($p<0.001$). For *MT-2*, post-hoc comparison indicated that the mean expression in control worms (taken from culture) and worms from patch 1 were significantly lower than in patch 4 worms. For *rrnL*, post-hoc comparison indicated expression in the control was lower than in worms collected from all Site A patches which could not be separated.
- For *MT-2*, the functional link between metal contamination and the change in expression is well established. *Lumbricus rubellus MT-2* binds metals such as cadmium *in vivo* (Stürzenbaum et al., 2001) and so probably acts as a detoxification mechanism. The functions of changes in *rrnL* expression in metal contaminated soils are less well established. This gene encodes a protein associated with the mitochondria. Changes in the transcription of this gene could represent either a direct change in mitochondrial status resulting from the toxic effects of the metals, or an indirect response of energy metabolism.
- The changes in *MT-2* and *rrnL* expression seen along the gradient meant that these sequences were selected for seasonal expression analysis in worms collected from three Site A patches (see Section 6.6). We also investigated *MT-2* expression in laboratory studies using Site A soils under different temperature and pH regimes (see Section 6.1 and 6.2).

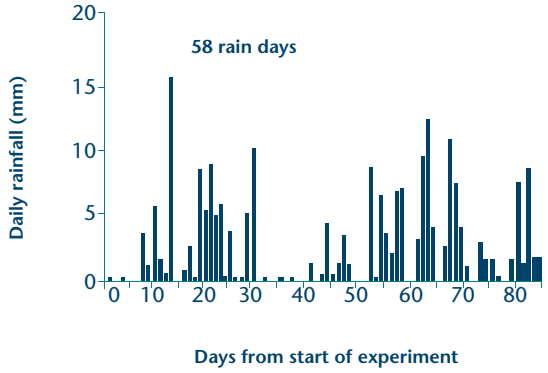
5.2 Life-cycle and biomarker responses of earthworms exposed to all site A patch soils in semi-field mesocosms.



5.2.1a



5.2.1b

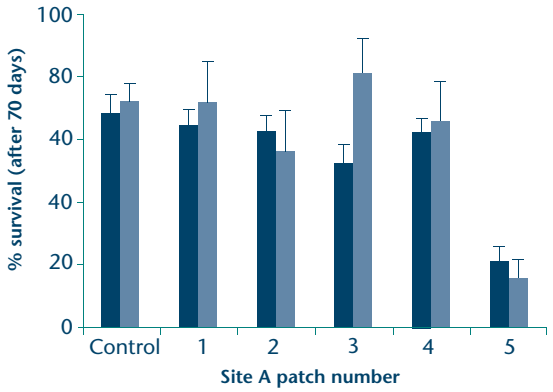


5.2.1c

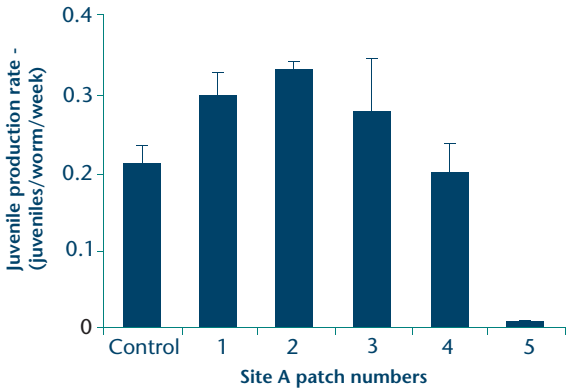
Fig. 5.2.1. Soil temperature (one minute average each hour) at surface (a) and 10cm depth (b) and daily rainfall during exposure of *L. rubellus* to a control and soils from all Site A patches in a mesocosm experiment for 70 days.

Summary

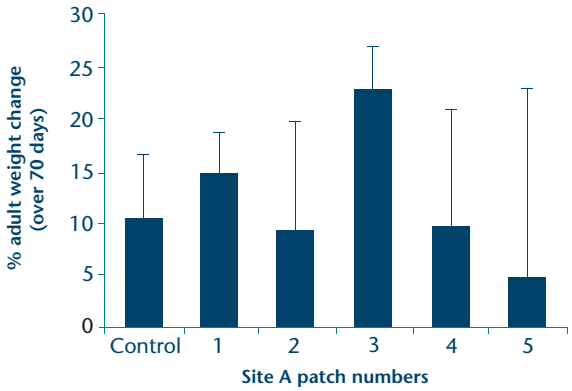
- We began the exposure in January, when soil temperatures were low (regularly below 0°C at surface and below 0°C at 10cm for approximately 24 hours). Temperatures rose steadily throughout, though at no point did the soil temperature at 10cm reach 15°C (the constant temperature usually in later laboratory tests with *L. rubellus*).
- There was heavy rainfall during the exposure (58 days), but no waterlogging of the mesocosms was seen.



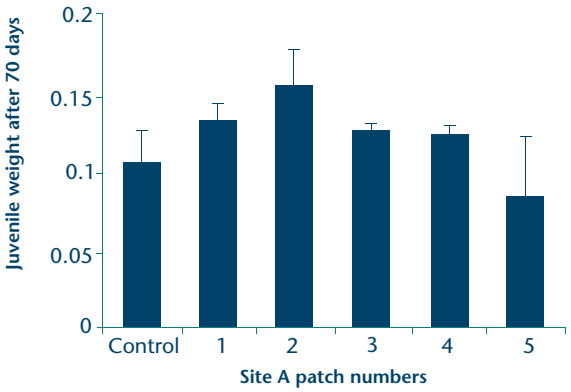
5.2.2a



5.2.2b



5.2.2c



5.2.2d

Fig. 5.2.2. Performance of the earthworm (*L. rubellus*) exposed to a control and soils from all Site A patches in a mesocosm experiment for 70 days a) survival of adults (solid) and juveniles (hatched); b) reproduction as juvenile production rate; c) adult weight change (start of exposure compared to end); d) weight of newly hatched juveniles grown for 70 days. All values are based on a mean of four replicated per treatment + SE.

Summary of results

- Metals present in Site A soils significantly altered the survival of both adults and juveniles (ANOVA, $p<0.001$). Survival of both life-stages was significantly reduced at patch 5 (Tukey's test $p<0.05$).
- Reproduction in the control soil was lower than in patches 1, 2 and 3. It is therefore better to use the most distant Site A patch (patch 1) as the basis for comparison of trends in reproduction.
- Within the Site A soils, there is a typical hormesis-type response of juvenile production, with marginally higher reproduction at patch 2 than at patch 1. After that, there is a progressive reduction in reproduction at patches approaching the smelter. ANOVA indicates a significant effect of patch on juvenile production ($p<0.001$). Only at patch 5, though, is reproduction rate significantly lower than at patch 1 (Tukey's test $p<0.05$).
- There was no clear trend in the adult weight change over the exposure, or in the weight of juveniles after 70 days. ANOVA confirmed no significant effect of patch on either parameter.

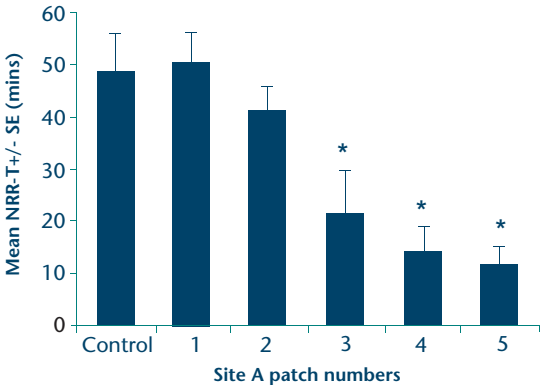


Fig. 5.2.3. Measured NRR-T for earthworms (*L. rubellus*) exposed to a control and soils from all Site A patches in a mesocosm experiment for 70 days. All values are based on a mean of four replicates per treatment + SD. Treatments marked with an asterisk are significantly different from the controls and patch 1 at $P < 0.05$.

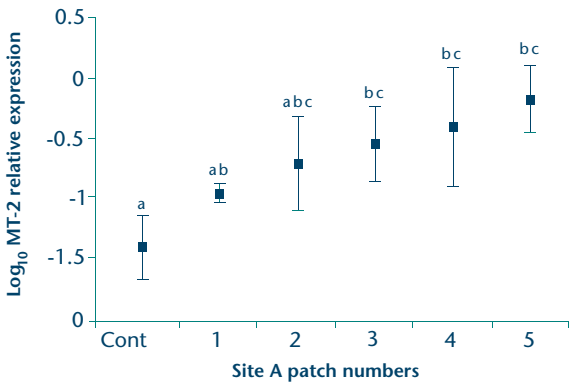


Fig. 5.2.4. *MT-2* relative expression in earthworms (*L. rubellus*) exposed to a control and soils from all Site A patches in a mesocosm experiment for 70 days. All values are based on a mean of four replicates per treatment + SD. Treatments not sharing the same letter are significantly different at $P < 0.05$.

Summary of results

- There was an excellent performance of this assay in both the control and Avonmouth patch 1 soil-exposed worms, with mean retention times approaching the maximum value of 60 minutes.
- Despite differences in ecological performance (reproduction) between the control and patch 1 soil, there is no clear or significant difference in NRR-T for worms kept in these two soils. Either could therefore be used as a basis for comparison of NRR-T between remaining patches (where patch 1 soil is used).
- There is a clear trend of lower NRR-T in worms kept in patch soils collected from close to the smelter. ANOVA indicated a highly significant effect of patch on NRR-T ($p<0.001$), with NRR-T being significantly lower in soil collected from patches 3, 4 and 5 than at patch 1 (Tukey's test $p<0.05$).

Summary of results

- We rigorously analysed *ß-act* and *MT-2* expression, to allow for a simple but accurate calculation of normalised expression. Three worms from each mesocosm were each analysed three times for both genes. The mean expression of *MT-2* and *ß-act* was calculated for each worm. The *MT-2* mean was then normalised against the total amount of RNA used for reverse transcription using the housekeeping gene *ß-act* as a putative invariant transcript (ie. measured concentrations of this latter gene represents the sample quantity – see Section 2.5). Based on individual ratios, we calculated a mean replicate expression value. This value was then used for the statistical analyses.
- Relative *MT-2* expression increased in worms exposed to soils from all Site A patches (ratio at least 2.56) when compared to worms kept in the control soils. This effect, ANOVA indicated, was significant. *Post-hoc* comparison indicates that relative *MT-2* expression increased significantly compared to controls in soils from patches 3, 4 and 5.
- Despite the rigorous sample analysis protocol that we used, a large variation in mean replicate expression was apparent. The ratio of expression between individual worms – even within the same replicate – varied, in some cases by over one order of magnitude. Averaging between replicates reduced this variation. This high variation reduced our ability to discriminate between patches. For example, worms exposed to soils from patches 2

and 5 could not be separated, despite a near five-fold difference in log mean expression ratio.

- Metal exposure resulted in a very large upregulation of *MT-2* expression. As a result, large differences in *MT-2* expression appear between control worms and exposed worms. This means that despite within-sample variation, we had no problem differentiating the worms exposed to control soil from those exposed to soil from the three most contaminated sites (patches 3, 4 and 5). These three patches are the ones at which metal concentrations exceed New Dutch list 'intervention' values and where ecological effects have already been observed (see Section 3.1.4). Our results demonstrate that earthworm *MT-2* expression measurement can be used to indicate exposure to potentially harmful concentrations of metals.

Detailed experimental studies at sites A and B

This chapter gives the results obtained in rigorous studies that assessed the biological tests performed at each site. Each section of the chapter covers one specific portion of the experimental work. Within each section, we detail the noteworthy results on a separate page, and briefly discuss their significance. A summary appears below.

Section 6.1 discusses the results of an earthworm reproduction test and subsequent biomarker measurements for earthworm (*L. rubellus*) exposed to soils from three Site A patches (1, 3 and 4) at three different temperatures (10°C, 15°C and 20°C). Exposure in all treatments was undertaken with four replicates boxes. Each box contained 1.26kg of dried two-millimetre screened soil, to which we added eight mature *L. rubellus*. The aim was to evaluate whether the biological response measurements were equally valid when the tests were conducted at different temperatures. Biological responses measured were effects on these life-cycle parameters: survival, weight changes and cocoon production rate. We also measured the biomarker responses of NRR-T and *MT-2* expression.

Section 6.2 gives the results of earthworm reproduction test and biomarker measurements for *L. rubellus* exposed to soils from three Site A patches (1, 3 and 4) under three pH treatment regimes. These were either unamended pH (0), pH lowered by one unit (-1) by the addition of the required amount of 0.125 M H_2SO_4, and pH increased by one unit (1) by the addition of the required quantity of powdered calcium carbonate. The aim was to evaluate whether the biological response measurement would vary at different soil pHs in soils with the same concentration of metals. In particular, we intended to investigate the relationship between metal bioavailability and biological response. Measurements made during the test were these life-cycle parameters: survival, weight change and cocoon production rate. We also noted the biomarker responses of NRR-T and *MT-2* expression. Measurements of weak calcium chloride extractable and also predicted free ion metal concentrations (based on soil solution chemistry modelling using WHAM) were also made, to assess the effects of pH treatment on metal availability.

Section 6.3 gives the data from an earthworm reproduction test and biomarker assay with the earthworm *L. rubellus* exposed to soils collected from all Site B patches. We measured these life-cycle responses: survival, weight changes and cocoon production rate, and also the biomarker responses NRR-T. The aim was to evaluate whether these biological responses could be used to assess the effects of exposure to the complex mixture of organic and inorganic contaminants present in oil and its derivatives.

Section 6.4 presents a preliminary analysis of the effects of soils from all Site A and B patches on a demographic parameter: the instantaneous rate of population increase in the springtail *Folsomia candida*. Data on the effects of each patch soil on springtail reproduction and survival were gathered as part of project P5-069. A full analysis of the data collected from this test will be presented in the final report for this project (Environment Agency R&D Technical Report P5-069/TR1). It will include a detailed assessment of the performance of the original test.

Section 6.5 gives the data produced by exposing distilled water extracts from soils collected from the sites to a bacterial luminescence bioassay. The aim was to establish whether the contaminants extracted from the soils were causing harm to the normal metabolic functions of a modified bacterium *Berkholdaria sp.*

Section 6.6 gives results from the measurement of biological response conducted in three seasons (spring, autumn, winter) at three Site A patches (1, 3 and 4). The aim was to assess the effects of seasonal environmental conditions on measured biological responses. The assessments made at each site, in each season, were soil invertebrate feeding activity (using the bait lamina test) and also NRR-T, *MT-2* expression and *rrnL* expression in indigenous *L. rubellus*.

Section 6.7 outlines results of the measurement of biological response conducted *in-situ* at all field Site B patches. The aim was to establish the extent of exposure and effect of soils invertebrate to the crude oil and refined petroleum products at the site. Responses measured were soil invertebrate feeding activity (using the bait lamina test) and NRR-T in indigenous *L. rubellus*.

6.1 Lifecycle and biomarker responses of earthworms to three Site A patch soils at three temperatures (10 °C, 15 °C, 20 °C).

	Hg			Cd			As			Cu			Zn			Pb		
	Mean		SE	Mean	±	SE	Mean	±	SE	Mean	±	SE	Mean	±	SE	Mean	±	SE
Patch 1	0.12	±	0.02	5.6	±	0	12.5	±	1.3	27.1	±	1.1	752	±	59	106	±	2
Patch 3	0.16	±	0.07	24.2	±	2.8	13.5	±	2	44.6	±	5.3	212	±	229	514	±	53
Patch 4	0.15	±	0.06	29.9	±	4.7	12.0	±	2.6	38.1	±	9.7	3280	±	435	309	±	77

Table 6.1.1a

	pH		% loss on ignition
	Mean	range	Mean
Patch 1	5.30	5.17-5.54	6.25
Patch 3	6.55	6.31-6.73	2.68
Patch 4	6.50	6.09-6.58	4.53

Table 6.1.1b

Table 6.1.1 Measured metal concentrations and soil properties for soils from the three Site A patches (1, 3 and 4). Soils were collected and prepared for use in the laboratory toxicity studies to examine the effects of temperature and pH amendment on measured biological responses. Table 6.1.1a shows concentrations of mercury, cadmium, arsenic, copper zinc and lead in Site A patch soils. Table 6.1.1b shows the soil organic content (as loss on ignition) and soil PH.

Summary of results

- There was no clear elevation of soil mercury, copper or arsenic concentrations in patches 3 and 4 soils compared to patch 1. For cadmium, copper and zinc, concentrations were already elevated above expected backgrounds in patch 1 soil, and further elevated at patches 3 and 4.
- The higher metal levels in patch 1 soils represented direct deposition of particulate metals from the smelter (10km away) and also deposition from local sources (in particular, the nearby M5 motorway). The elevation of cadmium, lead and zinc in patch 3 and 4 soils represented particulate metal deposition from the smelter.
- We found similar pHs in patch 3 and 4 soils, with the pH of patch 1 soil being lower by one unit. Measured loss on ignition was lower than has previously been found at Site A (Spurgeon, 1994). This is probably due to differences in how site soils were collected. We took samples from the top10cm of the soil profile, as compared to the top two centimetres for Spurgeon's study. Soil percentage loss on ignition was still, though, within the range expected of lowland soils.

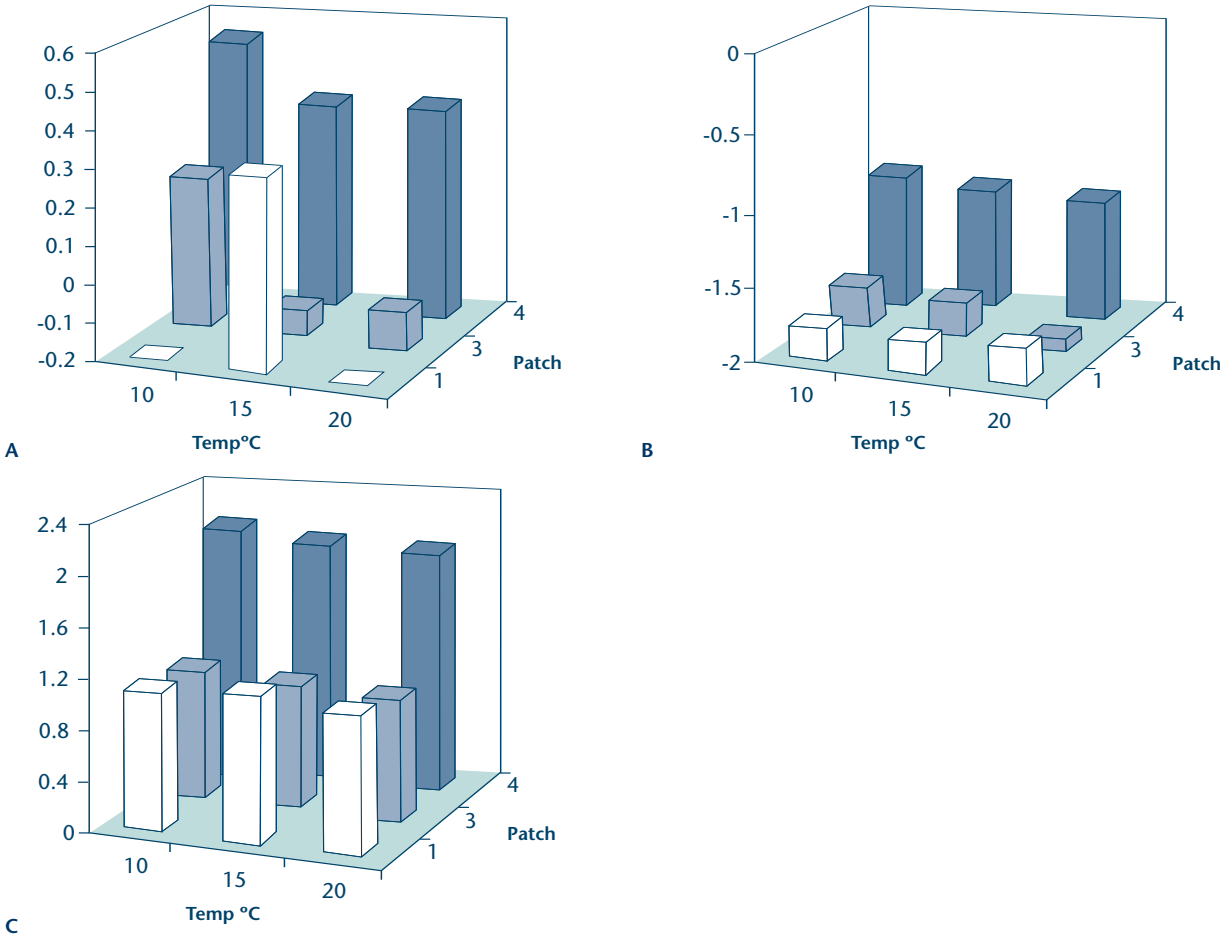


Fig. 6.1.1. Log_{10} concentrations (in $\mu g g^{-1}$ dry weight soil) on the Y-axis of A) arsenic; B) cadmium; C) zinc in soils collected at termination of the exposure of earthworms (*L. rubellus*) to soils from Site A patches, 1 (dark shading), 3 (pale shading) and 4 (not shaded)) at three different temperatures (10°C, 15°C and 20°C). All values are log_{10} values of the mean treatment metal concentrations based on four replicates per treatment.

Summary of results

- We present data for arsenic, cadmium and zinc only, since these metals were always present at detectable concentrations within the calcium chloride extract. Of the other metals measured, mercury, copper and lead were present in some treatments at close to or below the detection limit. This meant that a full statistical analysis of the trend of concentrations between treatments was not possible.
- We analysed the influence of patch and temperature on extractable metal concentrations by two-way ANOVA. Results were similar, with a significant effect of patch, but not of temperature, found for all three metals. *Post-hoc* comparisons for each metal indicated higher extractable metal concentration in patch 4 soil than for either patch 1 or 3.
- Our analysis of extractable metal levels in soils confirmed that concentrations were higher in patch 4 than at patch 1 soil. Higher concentrations were, though, not found in patch 3 soils, despite the fact that these soils contained approximately three times the concentration of zinc, 50 per cent more arsenic and more than 20 times the cadmium concentration of the patch 1 soil. The reason for the higher than anticipated extractable zinc levels in patch 1 soils (or the lower than expected patch 3 extractable metal levels) is probably associated with differences in pH between the two soils. Patch 1 soil had a pH lower than that of patch 3, by 0.5-1 pH unit.
- The extractable metal levels in the calcium chloride extracts explain variations in cocoon production by worms exposed to the different patch soils. They do not, though, explain variations in cocoon production by worms exposed at different temperatures. Differences in metal accumulation and cocoon production between temperatures are therefore due to the effects of ambient conditions on the worms' biological responses.

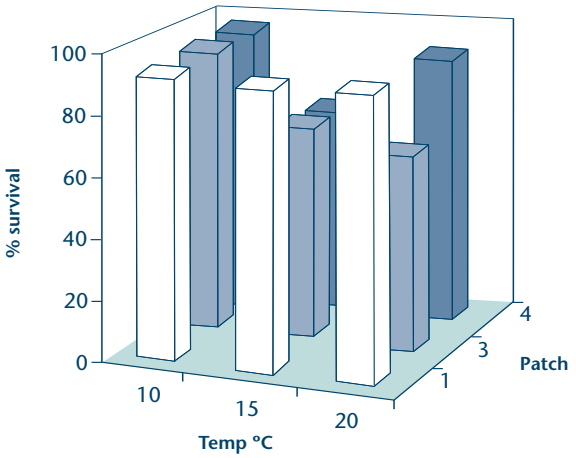


Fig. 6.1.2. Survival of earthworms (*L. rubellus*) exposed to soils from Site A patches, 1, 3 and 4 for 42 days at three different temperatures (10°C, 15°C and 20°C). All values are based on the mean of four replicates per treatment.

Summary of results

- There was good survival of the worms in the majority of the treatments. This demonstrates that it is possible to use a soil-dwelling species such as *L. rubellus*, rather than the standard test species *Eisenia fetida* in toxicity tests with contaminated field soils.
- Two-way ANOVA indicated no significant effect of either patch or temperature on survival of the exposed worms.

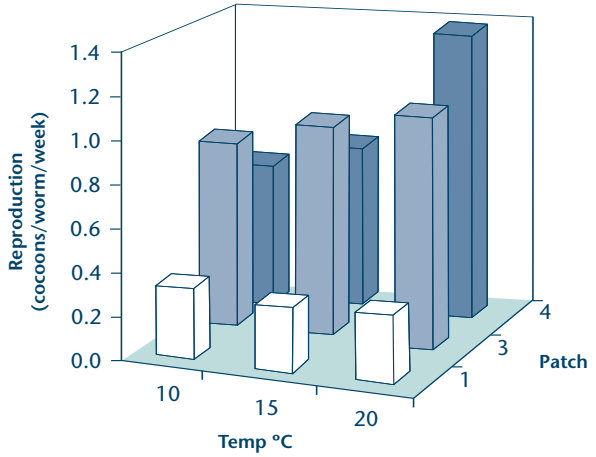


Fig. 6.1.3. Cocoon production of earthworms (*L. rubellus*) exposed to soils from Site A patches, 1, 3 and 4 for 42 days at three different temperatures (10°C, 15°C and 20°C). All values are based on a mean of four replicates per treatment.

Summary of results

- We measured high rates of reproduction (mostly in the less contaminated soil), with cocoon production rates close to the optimum reported in previous laboratory studies conducted with *L. rubellus* (Lowe & Butt, 1999; Spurgeon et al., 2000).
- Two-way ANOVA indicated a significant effect of patch. A post-hoc comparison showed that cocoon production at patch 4 was significantly lower than at both patches 1 and 3 (which could not be separated). Our observation of a significant reduction in cocoon production at patch 4 only related well with previous observations of a lower diversity of earthworms at patch 4 in relation to patch 1 (Spurgeon & Hopkin, 1996a, 1999a).
- Increasing temperature generally increased rates of reproduction. The two-way ANOVA indicated that this effect was significant with a post-hoc test identifying that cocoon production was significantly higher at 20°C compared to 10°C and 15°C. Analysis of the combination of patch and temperature within the ANOVA indicated a significant interaction. The effect of this was the tendency for higher temperatures to increase cocoon production was progressively removed at the more contaminated patches, being absent at patch 4. This suggests that earthworms in polluted soils are less able to take advantage of optimum conditions (in terms of reproduction) than those in uncontaminated soils.

	Patch 1		Patch 3		Patch 4	
	mean NRR-T	±SE	mean NRR-T	±SE	mean NRR-T	±SE
10°C	19.8	8.4	7.5	3.2	5.3	1.3
15°C	5.8	1.6	15.3	7.5	11.5	3.9
20°C	8.5	4.2	4.0	0.9	6.3	1.0

Table. 6.1.2. NRR-T of earthworms *(L. rubellus)* exposed to soils from Site A patches, 1, 3 and 4 for 42 days at three different temperatures (10°C, 15°C and 20°C). All values are based on a mean of four replicates per treatment with SE also given.

Summary of results

- NRR-T measurements in this study showed retention times well below the normal levels seen in unstressed worms (approximately 20 minutes c.f. Svendsen et al., 2004) in all the patch soils at all temperatures.
- A two-way ANOVA found no significant effect of either patch or temperature on NRR-T. This assay could not detect differences in NRR-T between worms exposed to cleaner soils and those exposed to soils more contaminated with metals. This is a direct result of the low retention time found.
- Measurements in worms taken from cultures (conducted at the same times as the measurements of NRR-T made in worms exposed to Site A patch soils) showed good retention time (up to 60 minutes). The presence of high retention times in these unexposed worms shows that the low retention times in all the Site A patch soils are not a result of some failure of the assay. Instead, it is likely that the worms exposed to all patch soils are subject to a significant stress. Measurements of metal concentrations in the patch 1 soil revealed concentrations of cadmium, lead and zinc above background concentrations (see Section 3.1.4). This exposure stress is probably the principal cause of the low retention times observed in this least contaminated soil. These results suggest therefore that this laboratory assay is highly sensitive to metal exposure.

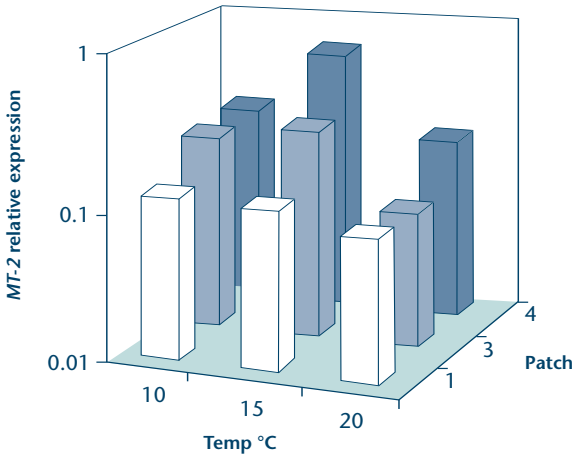


Fig. 6.1.4. *MT-2* relative expression in earthworms (*L. rubellus*) exposed to soils from Site A patches, 1, 3 and 4 for 42 days at three different temperatures (10°C, 15°C and 20°C). All values are based on a mean of four replicates per treatment.

Summary of results

- We measured *MT-2* relative expression in each of three worms per treatment (if available), for each of the four replicate containers used for each experimental treatment. Analysis of the mean values suggested no apparent trend for the effect of temperature on expression. Between patch, expression was, though, always highest in worms that had been exposed to patch 4 soil. It was lowest in patch 1 soil worms.
- Our comparisons of MT–2 relative expression in worms exposed to soil from all Site A patches with worms collected from a known clean culture indicated significantly higher expression for the Site A patch soil worms, in all cases. The upregulation of *MT-2* in worms exposed to all of the Site A soils reflects the fact that these soils all contain metals level that are substantively elevated above typical background concentrations (see Section 3.1.4). Therefore, measuring *MT-2* in *L. rubellus* using the Q-RT-PCR protocol adopted here appears a suitable method of demonstrating substantive metal exposure.
- A two-way ANOVA based on expression values for worms exposed to the Site A patch soils only indicated no significant effect of either patch or temperature on *MT-2* expression. Despite the apparent trends in the data (for example, the suggestion of increased expression in patch 4 soils), the presence of high variation between replicates meant that no significant effects of either patch or temperature could be identified.
- The replication used within each treatment would have to be increased in order to overcome the issue of variation within replicates and to allow significant separation of *MT-2* relative expression in worms exposed to the different patch soils. In the near future, technical improvements in gene expression quantification protocols (in particular the development of higher throughput sample preparation methods) are likely to ease sample preparation significantly. It will soon be technically and financially feasible to run a greatly increased number of samples within each analysis.

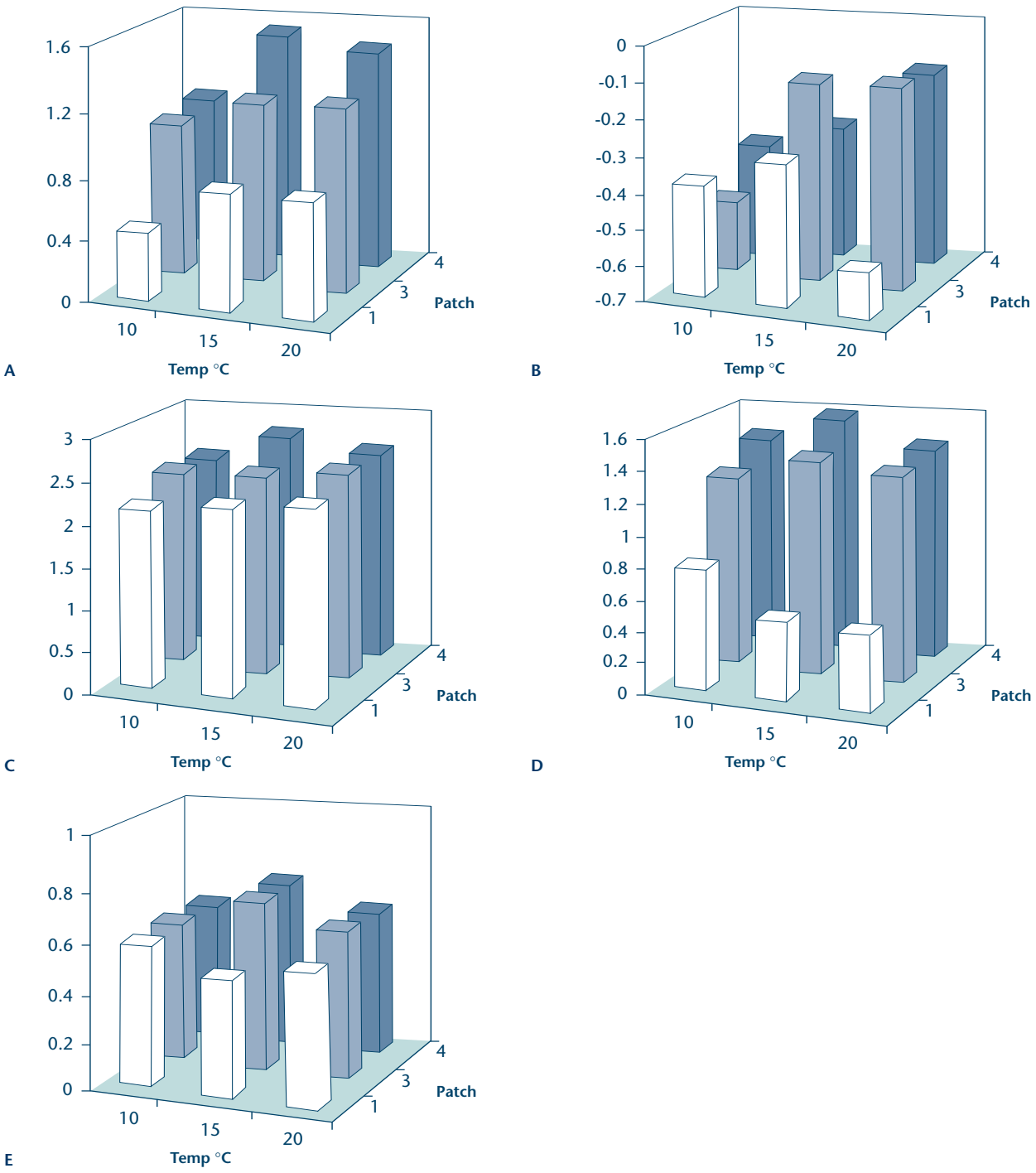


Fig. 6.1.5. Log_{10} concentrations of A) arsenic, B) cadmium, C) copper, D) lead and E) zinc in the tissues of earthworms (*L. rubellus*) exposed to soils from Site A patches, 1 (dark shading), 3 (pale shading) and 4 (not shaded) for 42 days at three different temperatures (10°C, 15°C and 20°C). All values (scale on the Y axis) are log_{10} values of the mean treatment metal concentrations ($\mu g g^{-1}$ dry weight soil) based on four replicates per treatment.

Summary of results

- We found detectable residues of five of the six metals measured in the study (As, Cd, Cu, Hg, Pb, Zn) in most samples. Only mercury was not accumulated to measurable levels in worms exposed to all patch soils.
- We analysed the influence of patch and temperature on tissue metal burden using two-way ANOVA for each metal. Results were as follows: Arsenic: significant effect of patch, but not of temperature, and a significant patch-temperature interaction. Cadmium: significant effect of patch and temperature, but interaction between the two not significant. Copper: no significant effect of patch, temperature, or the interaction. Lead: significant effect of patch, but not of either temperature or the interaction. Zinc: significant effect of both patch and temperature, but interaction not significant.
- There was a significant effect of patch on tissue concentrations for all metals except copper. *Post-hoc* comparisons for As, Cd, Pb and Zn all indicated that concentrations at patch 1 were significantly lower than at patches 3 and 4. These latter two patches could, however, not be separated.
- Temperature significantly influenced tissue concentrations of cadmium and zinc. *Post-hoc* comparisons in both cases indicated that tissue concentrations were significantly lower at 10°C than either 15°C or 20°C. The latter two temperatures could not be differentiated from each other.

6.2 Lifecycle and biomarker responses of earthworms exposed to three Site A soils at either unamended pH(0), with pH lowered one unit (-1), or pH increased one unit (1).

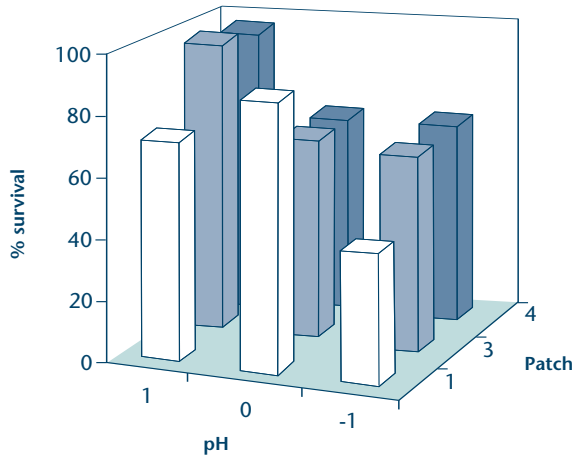


Fig. 6.2.1. Survival of earthworms (*L. rubellus*) exposed to soils from Site A patches, 1, 3 and 4 for 42 days at either unamended pH (0), with pH lowered by one unit (-1), or increased by one unit (1). All values are based on a mean of four replicates per treatment.

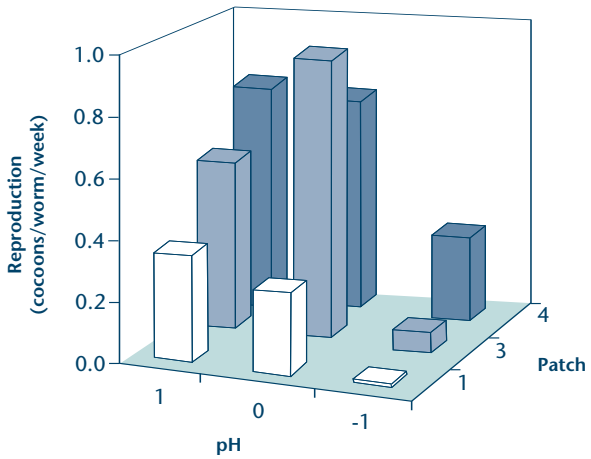


Fig. 6.2.2. Cocoon production of earthworms (*L. rubellus*) exposed to soils from Site A patches, 1, 3 and 4 for 42 days at either unamended pH (0), with pH lowered by one unit (-1), or increased by one unit (1). All values are based on a mean of four replicates per treatment.

Summary of results

- As in the temperature study, there was good survival of the worms in most soils. Again, as in the temperature experiment, this demonstrates the feasibility of using *L. rubellus* in laboratory toxicity tests with field soils.
- Two-way ANOVA indicated no significant effects of patch on survival. For pH, the effect was close to significant ($p = 0.07$) and a *post-hoc* test indicated significantly lowered survival in pH –1 soil compared to the pH + 1. *Lumbricus rubellus*, thus, appears to be intolerant of low pH soils (or at least of the pH amendment regime used in this study).

Summary of results

- We found high rates of cocoon production in less contaminated soils under some conditions. These were consistent both with previous work (eg. Lowe & Butt, 1999; Spurgeon et al., 2000) and also with the temperature study.
- Two-way ANOVA indicated a significant ($p < 0.001$) effect of patch on reproduction. A *post-hoc* comparison showed that cocoon production at patch 4 was significantly lower than at both patches 1 and 3, which could not be separated. This result was in agreement with the results of the temperature study (see Fig. 6.1.3).
- The two-way ANOVA indicated a significant ($p < 0.001$) effect of pH on cocoon production. A *post-hoc* comparison indicated that rates were significantly lower at pH –1 than and pH 0 and pH +1, which could not be separated. As well as this direct effect of reducing pH on the fecundity of earthworms, the two-way ANOVA indicated that there was also a significant interaction between pH and patch. This interaction resulted in a reduction in cocoon production at patch 3 to a level more comparable to that at patch 4 than patch 1 in the pH –1 soil. This effect is probably a result of the enhanced mobilisation of metals in the patch 3 soils at the lower pH values.

	Patch 1		Patch 3		Patch 4	
	mean NRR-T	±SE	mean NRR-T	±SE	mean NRR-T	±SE
pH-1 soil	11.0	3.9	5.0	1.7	3.3	0.3
normal pH	5.8	1.6	15.3	7.5	11.5	3.9
ph+1 soil	4.0	1.0	11.5	6.0	4.0	0.7

Table. 6.2.1. NRR-T of earthworms (*L. rubellus*) exposed to soils from Site A patches, 1, 3 and 4 for 42 days at either unamended pH (0), with pH lowered by one unit (-1), or increased by one unit (1). All values are based on a mean of four replicates per treatment with SE also given.

Summary of results

- In agreement with the results of the temperature study (see Table 6.1.2) there was a low retention time in worms exposed to all soils at each of the tested pHs. Even in worms exposed to patch 1 soil, NRR-T assays were well below normal unstressed levels (up to 60 minutes).
- A two-way ANOVA indicated no significant effect either of patch or pH treatment on NRR-T. As in the temperature study (see Table 6.1.2), the assay could not be used to discriminate between soils, as a result of the low retention time found across the experiment.
- As in the temperature study, measurements in worms taken from cultures conducted at the same times as the measurements of NRR-T made in worms exposed to Site A patch soils showed good retention time (up to 60 minutes). The presence of high retention times in these unexposed worms indicates that the low retention times are not a failure of the assay, but that they may reflect the exposure of worms in all patches (including patch 1) to metal levels that are substantially above background (see Section 3.1.4).

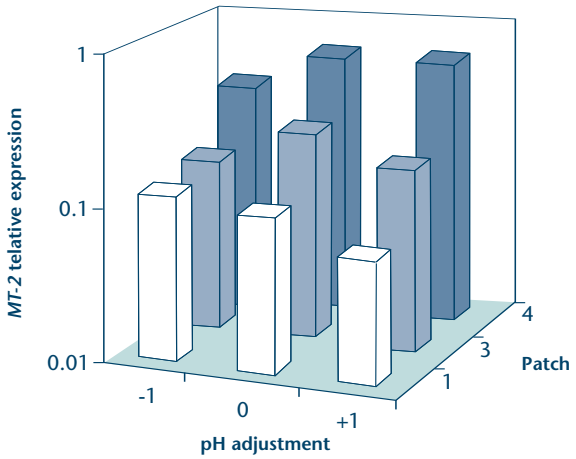


Fig. 6.2.3. *MT-2* relative expression in earthworms (*L. rubellus*) exposed to soils from Site A patches, 1, 3 and 4 for 42 days at either unamended pH (0), with pH lowered by one unit (-1), or increased by one unit (1). All values are based on a mean of four replicates per treatment.

Summary of results

- As in the temperature study, we measured *MT-2* relative expression in each of three worms per treatment (if available), for each of the four replicate containers used for each treatment.
- Comparisons of MT–2 relative expression with that for worms collected from a known clean culture indicated significantly higher expression in all Site A patches. This result was the same as for the temperature experiment (see Fig 6.1.4). The upregulation of *MT-2* in worms exposed to all Site A soils reflects the fact that these all patches contain metals level that are substantively elevated above typical background concentrations. This finding confirms that measurement of *MT-2* in *L. rubellus* is a suitable way to demonstrate substantive metal exposure.
- As in the temperature study, an analysis of means suggested that higher expression exists in worms exposed to soils from patch 3 and 4 than to those exposed to soils from patch 1 (except in the pH –1 treatment). There was no apparent trend for the effects of pH on *MT-2* relative expression.
- Two-way ANOVA confirmed no significant effect of pH on *MT-2* expression. The absence of a significant effect of pH on *MT-2* expression was unsurprising, since there is little evidence of a link or mechanistic involvement of metallothionein in direct pH tolerance.
- From the two-way ANOVA, we found a significant effect of patch. A *post-hoc* comparison indicated that *MT-2* expression was significantly higher in worms exposed to soil from patch 4 than for those exposed to patch 1 soil. In this case, the measurement of *MT-2* relative expression was able to distinguish clearly between the least and most polluted patches (unlike the temperature experiment). The role of this increased *MT-2* expression is likely to be in metal detoxification.

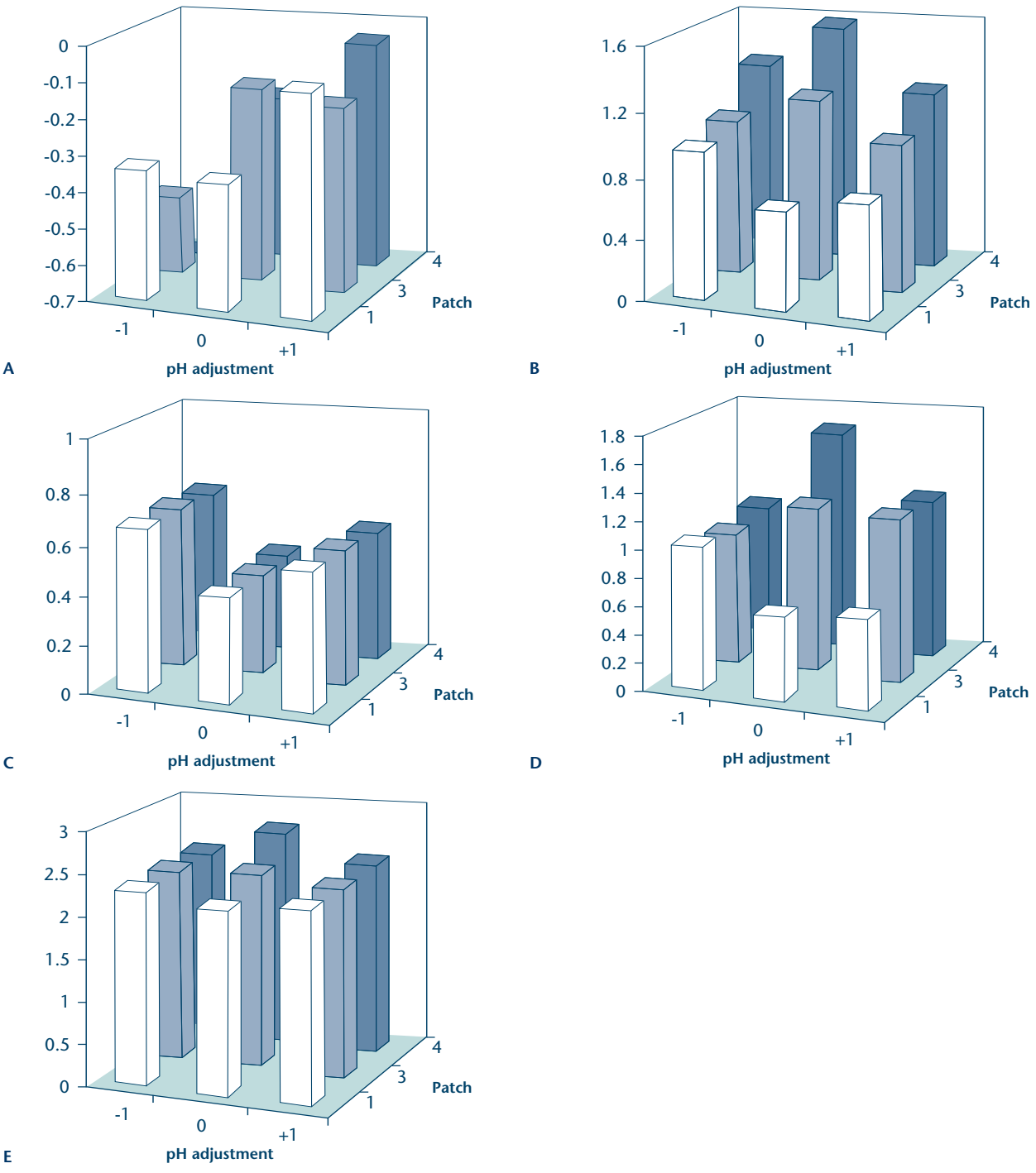


Fig. 6.2.4. Concentrations of A) arsenic, B) cadmium, C) copper, D) lead and E) zinc in earthworms (*L. rubellus*). The worms were exposed to soils from Site A patches 1 (dark shading), 3 (pale shading) and 4 (not shaded) for 42 days at either unamended pH (0), with pH lowered by one unit (-1), or increased by one unit (+1). All values are $\log_{10}$ of the mean treatment metal concentrations (in µg/g dry weight tissue) on the Y-axis based on four replicates.

Summary of results

- Of the six measured metals, only mercury was not present at detectable concentrations in the majority of soils. At patches 1 and 3 in particular, its concentrations were below detection. The absence of mercury from the soils reflects the low deposition of this metal at these patches when the smelter was in operation.
- We analysed the influence of patch and pH on tissue metal burden and their interaction by two-way ANOVA. Results were as follows: Arsenic: effect of patch close to significance ($p = 0.058$), no effect of pH, but a significant interaction between the two, with greater accumulation in more polluted patches at pH 0 and +1. Cadmium: significant effect of patch and pH, but the interaction was not significant. Copper: no significant effect of patch, pH or the interaction between them. Lead: a significant effect of patch, but not of pH, with the interaction between the two being significant. Zinc: significant effect of patch but not pH, interaction just significance ($p=0.05$).
- To summarise, there was a significant effect of patch on tissue concentrations for all metals except copper and arsenic (which was close to significance). This is comparable to the results found in the temperature study (see Fig. 6.1.5). *Post-hoc* comparisons indicated that for As, Cd, Pb and Zn, the concentrations of metals in the body tissues of worms exposed to patch 1 soil were significantly different from those exposed to patch 4 soil. Patch 1 could be separated from patch 3 for Cd, Pb and Zn. Patches 3 and 4 could not be separated for any metal.
- The pH treatment significantly influenced tissue concentrations for cadmium only. *Post-hoc* comparison indicated that pH +1 could be separated from both pH 0 and –1, which could not be separated.

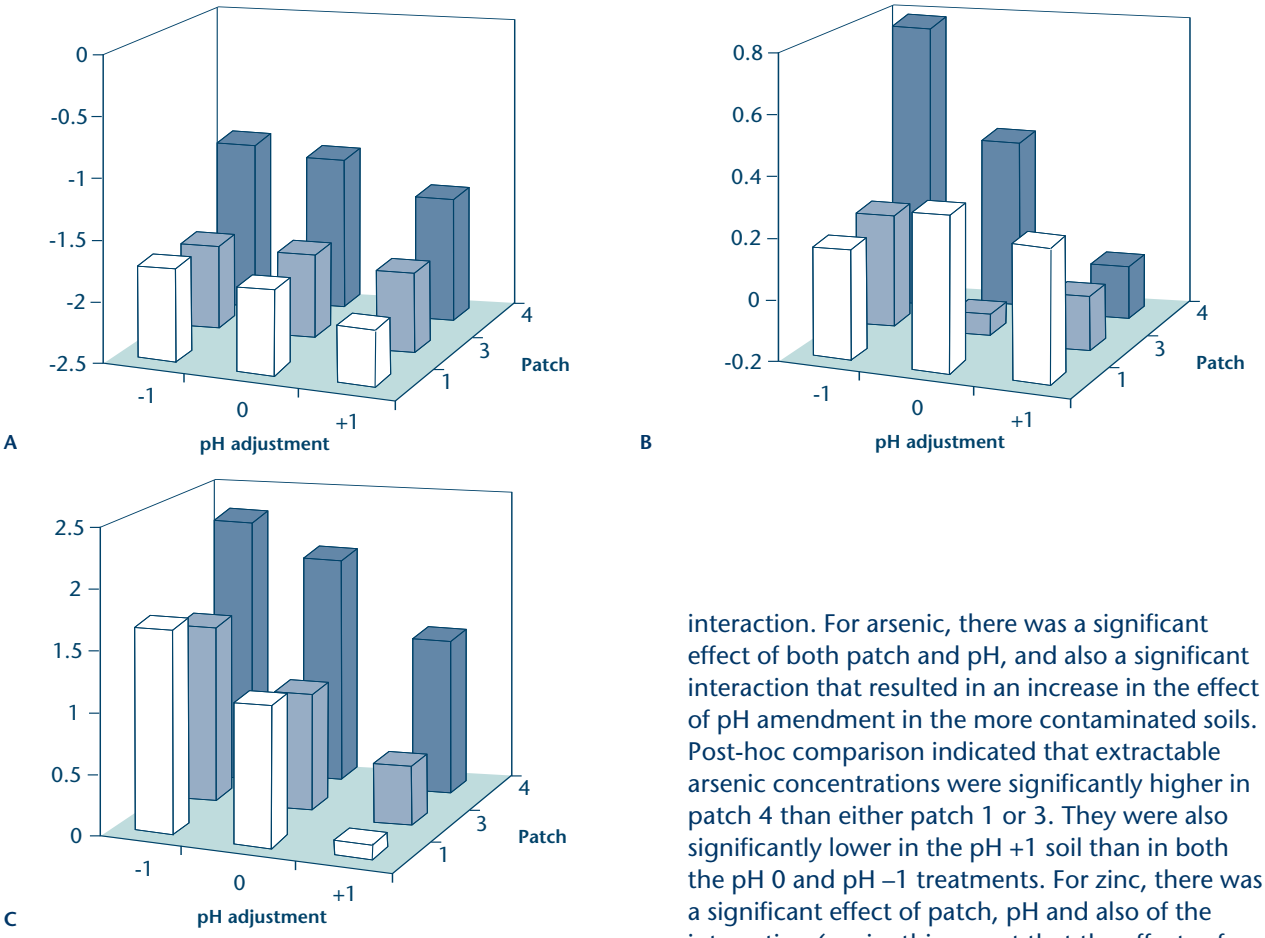
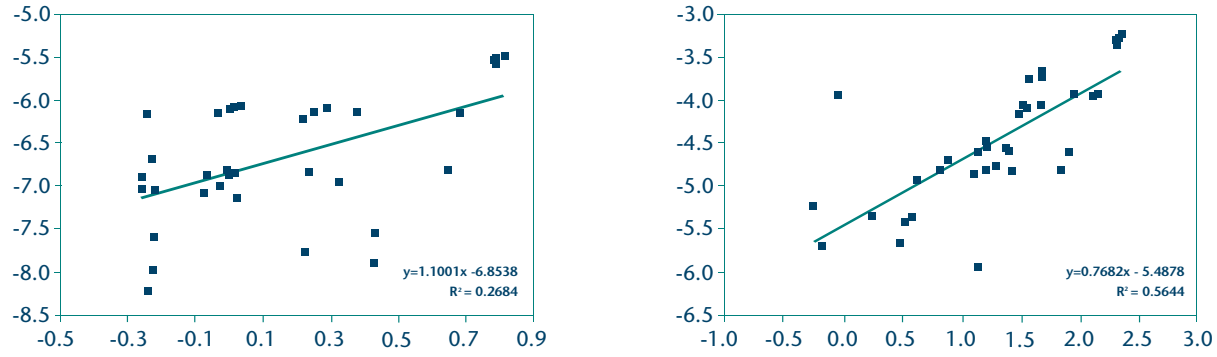


Fig. 6.2.5. Log_{10} concentrations (in $\mu g g^{-1}$ dry weight soil) on the Y-axis of calcium chloride extractable A) arsenic, B) cadmium, C) zinc in soils collected at termination of the exposure of earthworms (*L. rubellus*) to soils from Site A patches. Patches are 1 (dark shading), 3 (pale shading) and 4 (not shaded) at either unamended pH (0), with pH lowered by one unit (-1), or increased by one unit (+1). All values are log_{10} values of the mean treatment metal concentrations based on four replicates per treatment.

Summary of results

- We present data for arsenic, cadmium and zinc only here, since these metals were always present at detectable concentrations within the calcium chloride extract. Mercury, copper and lead were present in some treatments at close to or below the detection limit. Our interpretation of the data for these metals was therefore inhibited by the absence of suitable values for some treatments.
- We analysed the influence of patch, pH and their interaction on extractable metal concentration by two-way ANOVA. For cadmium, there was no significant effect of patch, pH treatment or the interaction. For arsenic, there was a significant effect of both patch and pH, and also a significant interaction that resulted in an increase in the effect of pH amendment in the more contaminated soils. Post-hoc comparison indicated that extractable arsenic concentrations were significantly higher in patch 4 than either patch 1 or 3. They were also significantly lower in the pH +1 soil than in both the pH 0 and pH –1 treatments. For zinc, there was a significant effect of patch, pH and also of the interaction (again, this meant that the effects of pH were more pronounced in the more contaminated soils). Comparison between sites indicated that extractable zinc concentrations were significantly higher in patch 4 than either patch 1 or 3, and that they significantly different from each other for all the pH regimes.
- Analysis of the levels of extractable metals in soils indicated that there were (as would be expected) higher 'bioavailable' concentrations in the more contaminated soils (eg. significantly higher in patch 4 for arsenic and zinc than either patch 1 or 3). The soil pH amendment regime also affected extractable metal. Concentrations were higher in the pH –1 soil and lower at pH +1. An interaction meant the effects of pH were greatest in the most contaminated soils.
- The effect of pH amendments seen in the analysis of calcium chloride extracts could have been responsible for the toxic effects seen in the biological tests, for example earthworm cocoon production. The reduction of pH in the treated soils could have resulted in the mobilisation of metal into solution where the metals became more bioavailable to the exposed earthworms, resulting in the greater toxic effect observed.

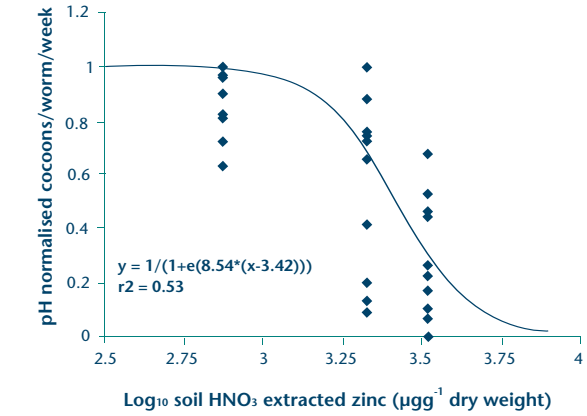


A B

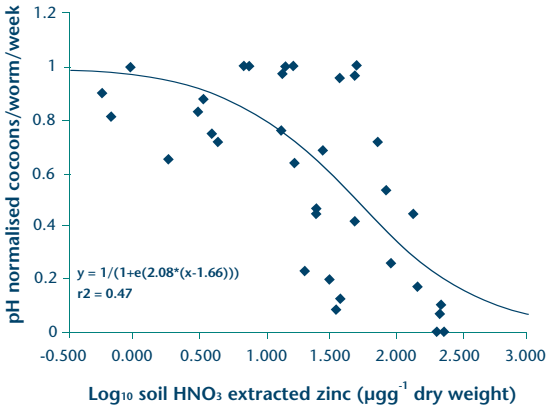
Fig. 6.2.6. Linear regression comparison of log_{10} calcium chloride extractable metal concentrations (in $\mu g g^{-1}$ dry weight soil) on X-axis and Log_{10} soil Me^{2+} concentration ($\mu g ml^{-1}$ soil porewater) modelled from a range of measured soils characteristics using the Windermere Humic Aqueous Model on Y-axis for A) cadmium (NB. one outlier removed) and B) zinc.

Summary of results

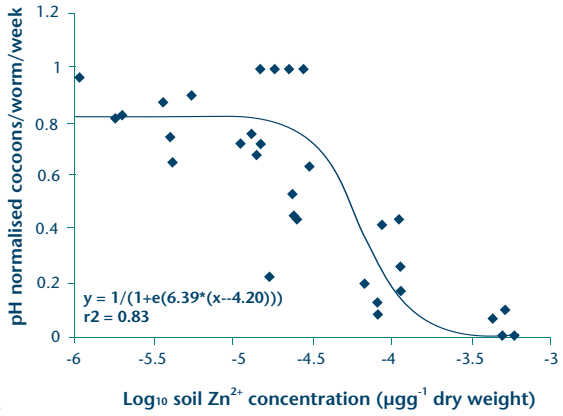
- It has been hypothesised that the toxic effects of metal on soils invertebrates are derived primarily by uptake of the metal as the free ion (Di Toro et al., 2001; Oste et al., 2001). Tipping (1994) measured a suite of the soil chemical parameters (concentrations of Na, Mg, Al, K, Ca, total Cu, total Zn, total Cd, total Pb, Cl, NO3, SO4 and porewater pH) needed to calculate free metal ion concentrations in soil using the Windermere Humic Acid Model (WHAM). This work was conducted only in the pH experiment where it was known (from the measurement of calcium chloride extracts) that the treatment had a direct effect on metal extractability.
- We used the model to calculate metal speciation in the differently treated patch soils. WHAM calculates equilibrium chemical speciation in surface and ground waters, sediments and soils. It combines a Humic-Ion-Binding Model with a simple inorganic speciation code for aqueous solutions. WHAM takes into account the precipitation of aluminium and iron oxides, cation exchange on an idealised clay mineral, and the adsorption-desorption reactions of fluvic acids. The model was initially developed by Tipping (1994). It has been widely used to study the speciation under a range of water and soil conditions. The model is also suitable for model speciation in aquatic and terrestrial ecotoxicity studies (Tipping et al., 2003).
- We calculated linear regressions between log_{10} transformed extractable and free ion concentrations. This was to assess the relationship between free ion and extractable cadmium and zinc within the different patch and pH treatment combinations. We focused particularly on cadmium and zinc. The reason for this was that our previous analysis of the comparative toxicity of the principal contaminant metals at Site A with measured soils concentrations had suggested that the observed toxic effects were most likely to be caused by zinc. In addition, our study found that cadmium was strongly accumulated by exposed worms (Fig. 6.1.5, 6.2.4).
- Comparisons of log soil calcium chloride extractable metal concentrations with log modelled free ion concentration indicated a significant relationship for both metals. The strongest relationship was for zinc. This confirmed that the WHAM modelling approach can predict the speciation and extractability of these two metals in pH amended soils from the different patches.



A



B



C

Fig. 6.2.7. Relationship between cocoon production rate (normalised against the patch 1 mean cocoon production rate at each pH to remove the direct effect of pH) plotted against zinc concentration in three soil fractions. A) 'total' soil in a hot nitric acid digestion; b) zinc extracted in a weak (0.01M) calcium chloride solution; c) predicted free zinc ion concentration modelled from a range of measured soils characteristics using the Windermere Humic Aqueous Model.

Summary of results

- The relationships for zinc are examined here in detail, to evaluate the ability of different metal extractions to predict the toxic effects observed in each patch soil under the different pH amendment regimes. Previous analysis of the comparative toxicity of the principal contaminant metals at Site A, with measured soils concentrations suggests that zinc is most likely to cause the toxic effects observed in previous studies (Spurgeon & Hopkin, 1995).
- Measurement of cocoon production in this study provided a robust and consistent indication of toxic effect. So we chose this test to investigate which zinc species was exerting the greatest toxic effect. We expected the strongest correlation between zinc species and the toxic endpoint (cocoon production) to indicate this.
- Cocoon production is a key toxicological parameter governing the effects of chemicals on the population stability of earthworms (Spurgeon et al., 2003). To assess the ability of the different soil zinc fractions to predict effects on the exposed earthworms, we compared measured and modelled zinc concentrations with the effects on cocoon production. We removed the influence of the direct effect of pH on cocoon production (see Fig. 6.2.2) by normalising rates found in each replicate against the patch 1 mean value for that pH, setting any value above this level at one. We then used least squared fitting of separate logistic regressions between normalised cocoon production rate and the different zinc concentrations to gauge which soil fraction provide the best prediction of the toxicity effects in the Site A patch soil and pH treatment combinations.
- A logistic regression could be fitted between all three soil zinc fractions and pH normalised cocoon production. Compared to the fit for total zinc, the regression for calcium chloride extractable zinc was not improved. This meant that the measurement of zinc in the calcium chloride extract was a relatively poor indication of the earthworms' exposure to this metal. In contrast, fitting the pH normalised cocoon production rate against modelled free zinc ion concentration did improve the correlation of the relationship. This suggests that free zinc ion concentration provides a better prediction of toxicity than either total or extractable metal.
- The strongest relation between any zinc concentration and cocoon production rate was found for predicted free metal ion concentration. This suggests that measuring this fraction in soils provides a sounder basis for estimating earthworm exposure to zinc than measuring either total concentrations or calcium chloride extractable concentrations. A number of authors have identified that metal toxicity and accumulation for soil invertebrates can be explained with respect to portioning between the solid and the liquid phase (Janssen et al., 1997; Peijnenburg et al., 1999; Spurgeon & Hopkin, 1996b). Their work suggests that the toxicity of metals results either directly from the pore water, or indirectly through an uptake route closely related to pore water. This fits with our findings here, that toxicity is mediated most strongly by the concentrations of free metal ion in solution.

6.3 Lifecycle and biomarker responses of earthworms exposed to all Site B patch soils from in a laboratory bioassay.

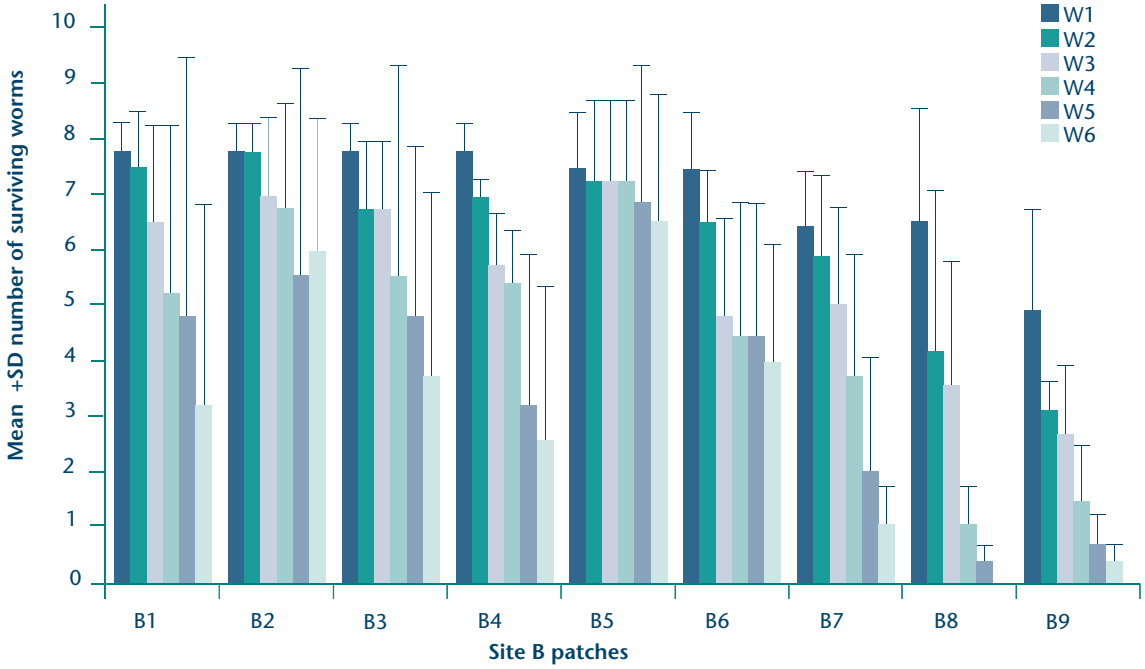


Fig. 6.3.1. Mean number of surviving worms after 1, 2, 3, 4, 5 and 6 weeks (W1-W6) of earthworms (*L. rubellus*) exposed to control and soils from all Site B patches for 42 days. All values are based on a mean of four replicates per treatment + SD with eight worms initially added to each.

Summary of results

- Worms in several soils had high rates of mortality. This was true not only for worms in the soils most polluted with oil (eg. B7, B8 and B9), but also those in some of the less polluted soils (eg. B1 and B4). A number of the earthworms had dorsal lesions typical of a disease previously seen but currently uncharacterised. This disease was probably the reason for the high mortality.
- The poor survival of worms in the Site B reference soils would have led to the termination of the experiment if this had been a trial with, for example, a plant protection product. We could not terminate this experiment, though, since it was not possible to return to Site B to collect further soil. We could not have re-used the existing soils because the oil-derived organic compounds would probably have degraded over the initial weeks of the study. Instead, we decided to continue with the existing trial, recording survival on a weekly basis and logging the number of worms with the dorsal lesions characteristic of the disease.
- At the end of the experiment, a two-way ANOVA indicated a significant effect of both patch and time on earthworm survival. Though this analysis had limited value as there was autocorrelation within the dataset, it does show that survival was increasingly affected (by both the disease agent and contaminants) over the duration of the exposure.
- To remove time-dependent effects on survival, we conducted a one-way ANOVA of the dataset using survival data for weeks two and six. The ANOVA for week two indicated a significant patch effect: survival in B9 soil was significantly lower than in patches B1-B5, and survival in patch B8 was significantly lower than in patches B1 and B2. The ANOVA at week six also indicated a significant effect of patch on survival. A post-hoc comparison at this time indicated significantly lower survival in B7, B8, B9 compared to B5 and B2. In both weeks, therefore, earthworm survival rates were lower in the most hydrocarbon polluted soils.

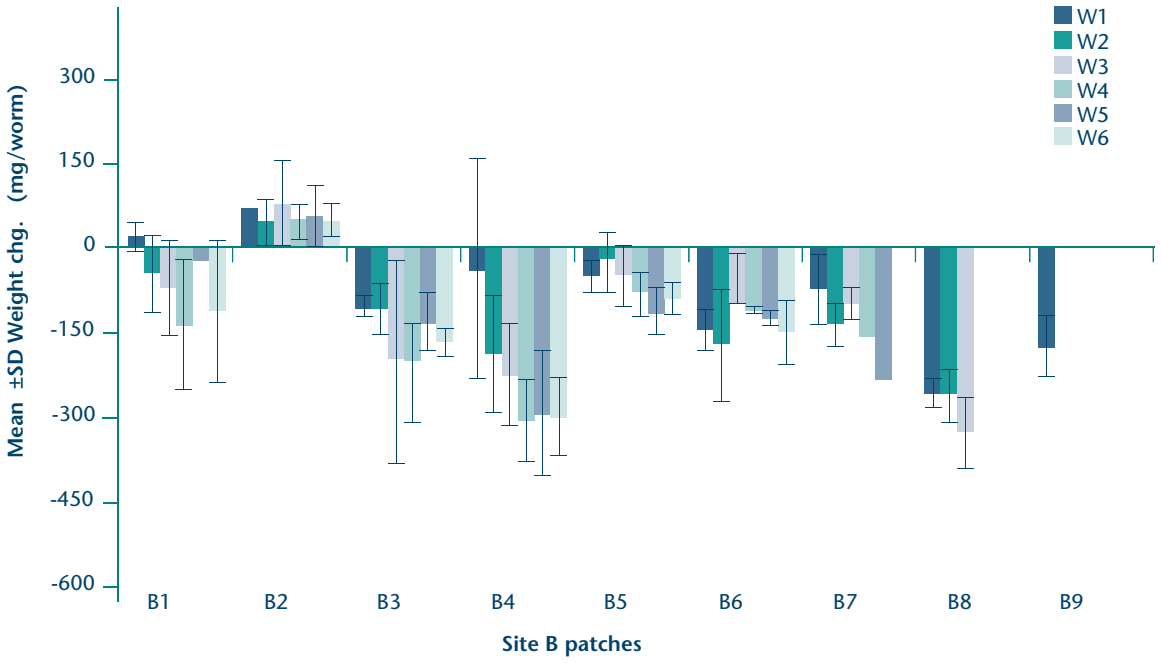


Fig. 6.3.2. Weight change of earthworms (*L. rubellus*) exposed to control and soils from all Site B patches for 6 weeks (W1-W6). All values are based on a mean of four replicates per treatment ± SD.

Summary of results

- The primary effect on *L. rubellus* of exposure to all Site B soil except B2 was a mean weight loss. This was despite the fact that soil moisture was held constant during the exposure and an adequate supply of food (rewetted horse manure) was provided. In many soils, we saw a time-dependent and progressive weight loss over the six weeks of the exposure.
- Two-way ANOVA was not possible in the data set due to the number of missing data points (eg. B8 and B9). A series of one-way ANOVAs conducted for the separate weeks indicated that the soils from patch B3, B4, B8 and B9 all caused significant weight loss compared to B1 and B2.

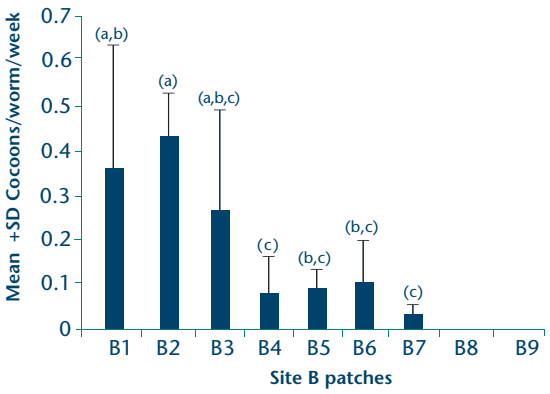


Fig. 6.3.3. Cocoon production rate (cocoons/worm/week) of earthworms (*L. rubellus*) exposed to control and soils from all Site B patches for six weeks. All values are based on a mean of four replicates per treatment + SD. Columns not sharing the same letter are significantly different at $P < 0.05$.

Summary of results

- Despite high earthworm mortality in many of the containers (see Fig 6.3.1), cocoon production rates were within the ranges typically found in studies with *L. rubellus*, particularly in the on-site control (B1) and less contaminated patches (B2, B3). The good rates of reproduction found there indicate that the earthworm reproduction test (when conducted with *L. rubellus*) is robust. They also show that the observed mortality probably resulted from an infectious disease rather than exposure to a sustained (chemical) stress, since worms were healthy and reproducing well until they died.
- Cocoon production rates split basically into three groups. One group showed 'normal' reproductive output (patches B1-B3), the second showed a strongly reduced reproduction (patches B4 – B7) and the third showed no reproduction (patches B8 and B9). One-way ANOVA indicated there was a significant effect of patch on cocoon production. Post-hoc comparisons showed that reproduction was significantly lower in sites B4-B9 than in either B1 or B2.
- The soils in which we found lower reproduction encompass all those in the dilution series made from a heavily oil polluted soil (B5-B8), as well as the soil collected from an area on the site contaminated by a surface oil patch (B9). All of these patches contained total petroleum hydrocarbon (TPH) concentrations substantively above background, and all are contaminated with a range of PAHs (see Section 3.2.5 for details). Unlike these established contaminated soils, soil from patch B4 did not contain TPH or PAH levels elevated above background (see Section 3.2.5). Nevertheless, our results suggest that *L. rubellus* may be exposed to as yet unmeasured compounds in these soils.
- The fact that a significant effect of oil-derived organic exposure on earthworms could be identified at patch B5-B9 in this study, despite the difficulties in completing the test, bears out the robustness of the earthworm chronic toxicity test protocol. We also demonstrated that the test can measure the effects of complex mixtures of organic compounds on earthworm ecology.

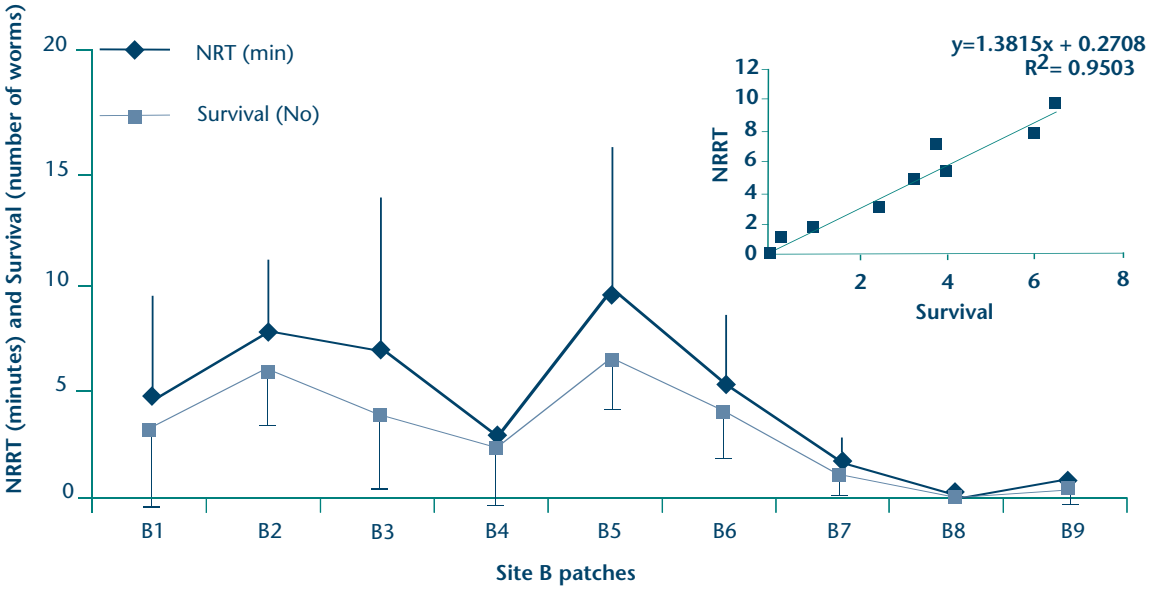


Fig. 6.3.4. NRR-T (diamonds) and number of survivors after six weeks (squares) for earthworms (*L. rubellus*) exposed to Site B patch soils for six weeks. All values are based on a mean of four replicates per treatment + SD with eight worms initially added to each. Inset graph shows the linear regression of the relationship between survival and NRR-T.

Summary of results

- Retention times for the NRR-T assay in the worms exposed to the on-site reference soil (B1) were low – well below the normal levels seen in unstressed worms. Low retention times were also found in worms exposed to the other less polluted patches (B2-B4), as well as in worms exposed to soil from the hydrocarbon contaminated patch (B9) and the dilution series (B5-B8).
- Though the three lowest mean retention times were for the most oil-polluted soils (B7-B9), a one-way ANOVA did not indicate a significant effect of patch on NRR-T. This is due mainly to the scarcity of surviving worms. Only a few treatments had survivors in all replicates to provide statistically strong data.
- Comparison of NRR-T and survival, weight change and reproduction data indicated that the trend in lowered NRR-T closely matches the trends seen for all other parameters, both sublethal and lethal. This is illustrated here by plotting the correlation between NRR-T and survival in week six. NRR-T times in this study thus provides a correlative indication of potential stress of the complex organic contaminant mix present in the Site B soil (see inset graph). The presence of this correlation indicates that the NRR-T assay can derive a quantitative indication of stress, even when retention times are relatively short.

6.4 Measurement of instantaneous rate of population increase in springtails *(Folsomia candida)* exposed to all soils from Sites A and B.

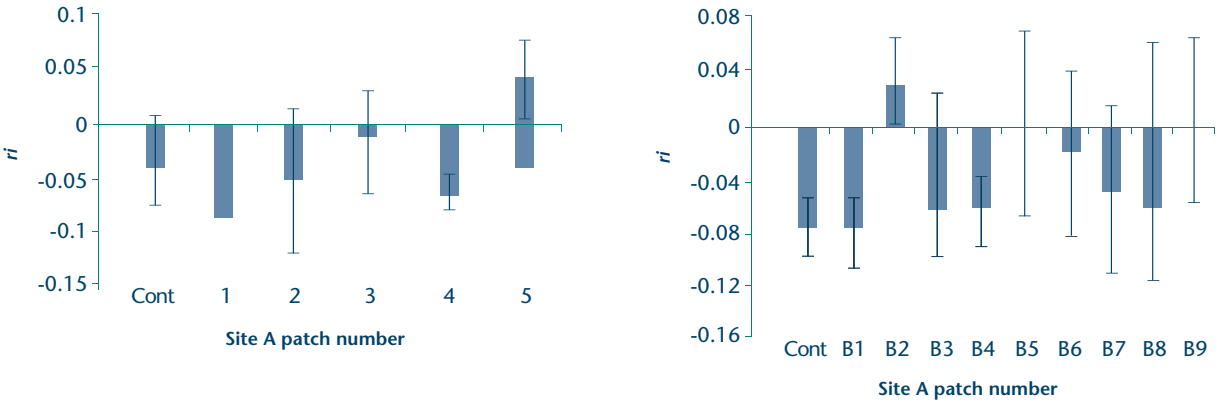


Fig 6.4.1. Instantaneous rate of population increase in springtails *Folsomia candida* exposed to soils from all Site A and Site B patches. Values are the mean of five replicates. Error bars represent standard deviations.

Summary of results

- We calculated the instantaneous rate of population increase using a modified form of the classic equation ($ri = \ln(n_f/n_0)/\Delta T$) similar to that used by Krebs and Boonstra (1978) and Mallorie and Flowerdew (1994) such that:

  $$ri = \ln(n_f+1/n_0+1)/\Delta T.$$

  where nf is the final number of animals, n_0 the initial number of animals and ΔT the difference in time (number of days the experiment was run). Solving ri creates values between –1 and +1, where positive values represent a growing population, zero a stable population and negative values show a population in decline and heading toward extinction.

- For both soils there was, unfortunately, the springtail tests performed poorly, with high adult mortality and low reproduction found in many of the patch soils. Both adult survival and juvenile production were frequently below the validation criteria applied for studies conducted with the ISO (1999) test; that is, adult mortality was greater than 20 per cent in all but two replicates, and less than 100 juveniles were produced in all but one replicate.

- We attribute the poor performance of this test particularly to the proliferation of fungi seen in many of the test soils during the exposure. These fungal hyphae covered the whole surface of the soil, preventing movement of the springtails at the soil-air interface. Fountain (pers. comm.) has also previously observed blanketing of the soil by fungi during toxicity tests and found that this growth inhibited springtail survival and reproduction in urban contaminated soils. Fountain was, though, able to successfully complete the springtail test after sterilising the soils to stop fungal growth. If the soils in this study were suitably modified, we would expect to complete the springtail tests successfully. A full discussion of the cause of this fungal growth is beyond the scope of this report, but will be included in the final report of associated project P5-069.

- Pending further data, we used the adult survival and juvenile production data found in the initial test to calculate a value for instantaneous rate of population increase in each replicate. The low values found for instantaneous rate of population increase and the absence of any trend in the value across the transect meant that we could not use these values in a formal statistical analyses.

- Instantaneous rate of population increase is a relatively simple parameter to calculate. It is also a potentially powerful endpoint for use in risk assessment, as it integrates the effect of a pollutant on a series of life-cycle traits. In this study, the use of this approach was undermined by the poor performance of the springtail reproduction test.

6.5 Detection of the change in luminescence of a *lux*-marker bacterial biosensor exposed to soil extracts collected from Site A and B.

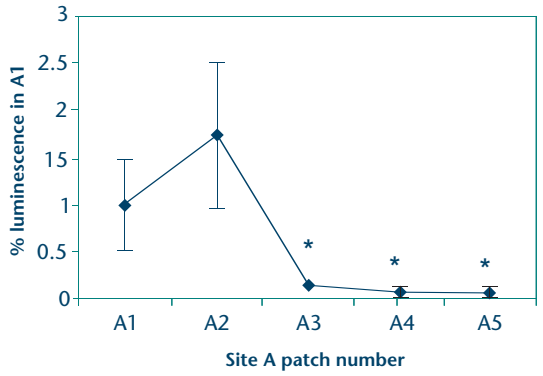
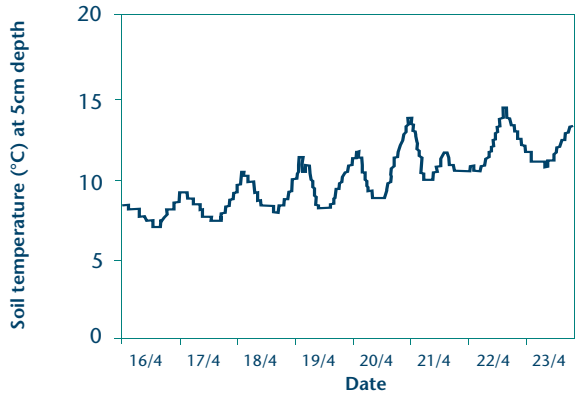


Fig 6.5.1. Relative luminescence of *lux*-marked bacteria in soil extracts collected by water shaking and centrifugation for all Site A patch soils. Site A values are expressed as a percentage of the luminescence found in patch A1 soil.

Summary of results

- We found a significant effect (ANOVA, $p < 0.001$) of patch on the luminescence of *Berkholdaria sp.* RASC strain engineered to contain the *lux* construct (Shaw et al., 1999). *Post-hoc* comparisons indicated that, after a non-significant increase in luminescent for A2 soil, luminescence was significantly reduced in bacteria that were exposed to water extracts taken from soils from the three patches close to the factory (A3-A5). These are sites at which previous ecological surveys have found significant effects on the structure and function of soil communities. Our result therefore offers some support for the use of the bacterial biosensor to indicate the severity of exposure at site contaminated by metals.
- The soil at Site B is dominated by hydrocarbons, many of which are lipophilic. We did not conduct *lux* assays with these soils. This is because a simple water extraction at this site may have collected an unrepresentative fraction of the contaminants present in patch soils for this site. Several workers have reported that, at sites contaminated by hydrophobic compounds, a solvent extract elicited a greater toxic response in microbial biosensors (NB. in both examples, the Microtox™ system) than did an aqueous extract or elutriate (Demuth et al., 1993; Guzzella, 1998). The problems of obtaining a suitable extract for soil bacterial exposure from soils that may be contaminated lipophilic compounds can, though, be overcome. This can be done by using, for example, improved extraction methods (Reid et al., 1998), co-solvents (Bundy et al., 2003) or a solid phase assay (Shaw et al., 2000a). Such work was, though, outside the scope of this project.

6.6 Comparison of biological responses in earthworms *(L. rubellus)* collected in three seasons (spring, autumn, winter) from three Site A patches (1,3 and 4).



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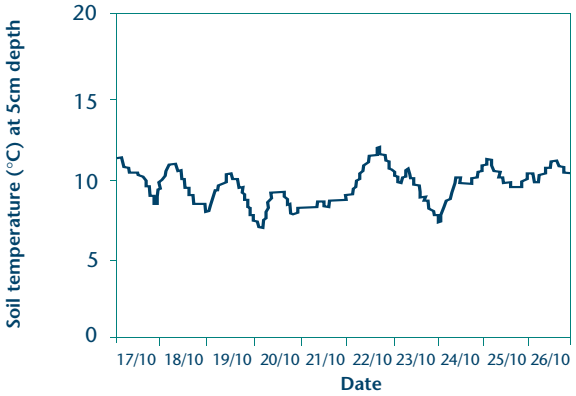
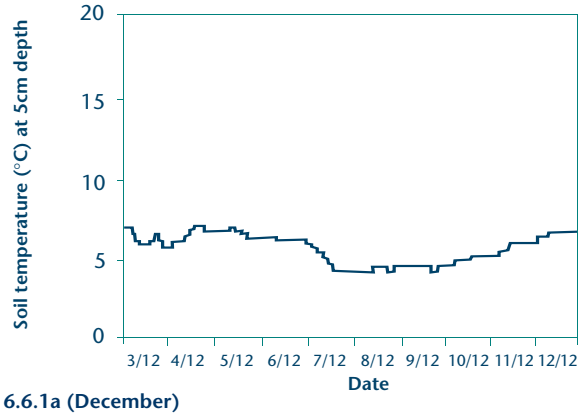


6.6.1a (October)



6.6.1a (December)

Fig. 6.6.1. Soil temperature (one minute average each hour) at 10cm depth during seasonal bait lamina deployments at patch 1 of Site A in a) spring, b) autumn, c) winter.

Summary of results

- As expected, temperatures were lower in winter than in autumn and spring by approximately 5°C. In the latter two seasons, temperatures were comparable.
- More pronounced diurnal fluctuations were found when day temperatures were higher (eg. autumn and particularly spring).

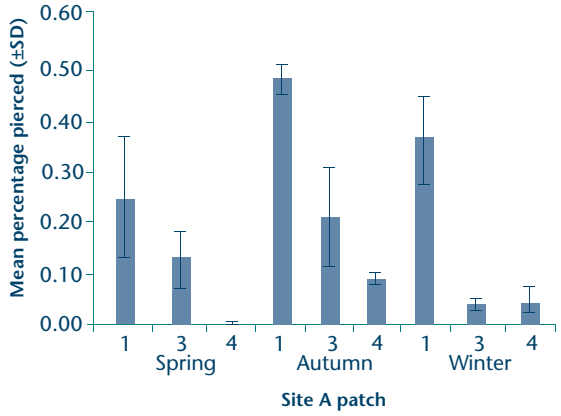


Fig. 6.6.2. Feeding activity of soil invertebrates organisms as proportion of pierced baits in bait lamina deployments conducted at three Site A patches (1, 3, 4) in three seasonal samples (spring: April 2002; autumn: October 2002; winter: December 2002). Site values are means ± SD of three bait lamina sets each consisting of 16 vertical inserted strips.

Summary of results

- We found the highest overall feeding in the autumn sample interval. Previous seasonal analyses of the abundance of both earthworms (in 1997) and springtails (in 1995) at Site A showed no clear trend in the total abundance of these key detritivorous groups between spring and autumn (Sandifer, 1996; Spurgeon & Hopkin, 1999a). Both these studies were, though, conducted in years of very dry summers (1995 and 1997). The summer of our study (2002) had been fairly wet, creating more favourable conditions. As a result, there may have been a higher survival and recruitment of soil invertebrates than in either of the previous years. This may have lead to high population sizes and, thus, higher feeding in the autumn of 2002. In addition to possible stoichastic differences between years, it may have been that local soil temperature and moisture conditions at the time of the three samplings had an affect on the activities of individuals, causing the higher autumn feeding seen.
- Comparison of feeding in each of the three seasons using a separate X^2 test indicated a significant effect of patch on feeding in all three cases. Further analysis by two-way ANOVA (after arcsine transformation of the percentage data to ensure the assumptions of normality were met) indicated a significant effect of both patch and season, but no significant interaction. *Post-hoc* comparison between patches indicated a significantly higher feeding at patch 1 compared to patch 3 and 4. Patches 3 and 4 could not be separated. Between seasons, feeding was significantly higher in autumn than in spring. Differences in feeding between autumn and winter were different, though not significantly so ($p=0.067$).
- To analyse between-site differences further, we analysed arcsine-transformed data for each season by separate one-way ANOVAs. We found a significant effect of patch in spring, with feeding significantly lower at patch 4 than at patch 1. A significant effect of patch was also present in autumn and winter. *Post-hoc* comparisons for these seasons indicated that feeding was significantly lower at patches 3 and 4 than patch 1. Patches 3 and 4 could not, though, be separated. These results indicate that the bait lamina test identified lower feeding when compared to patch 1 at the most contaminated patch independently of season, and at patch 3 in two seasons. The ability of the assay to discriminate between patches was good, but depended to an extent on the level of variation within the dataset.

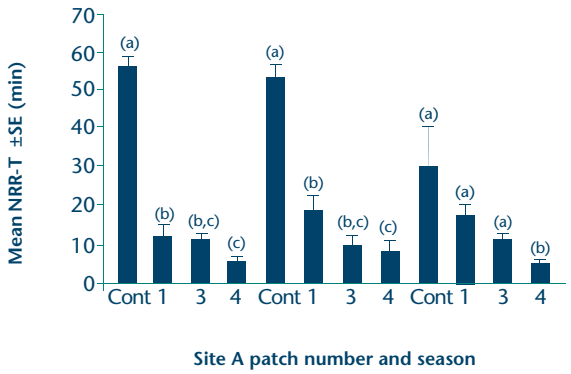


Fig. 6.6.3. NRR-T of earthworms (*L. rubellus*) collected either from outdoor maintained culture beds (cont) or from 3 Site A patches (1, 3, 4) in three seasons (spring: April 2002; autumn: October 2002; winter: December 2002). Values are mean of 10 adults ± SE.

Summary of results

- The NRR-T assay performed well in the control worms for both the spring and autumn sample interval, with values approaching the 60-minute maximum. In winter, NRR-T in the control worms was lower, indicating a potential effect of season on retention. In these worms, though, NRR-T still remained above the 20-minute threshold found by Svendsen et al. (2004) to indicate a potential detrimental effect of environmental conditions on earthworm ecological (eg. reproductive) performance.
- NRR-T in worms collected at all the Site A patches were below the control retention times for all three sample intervals. In all seasons, NRR-T in Site A worms was highest at patch 1 and lowest at patch 4. Only in winter was there a clear, stepwise pattern of retention times in relation to the proximity of the patch to the point source. The presence of low retention times in worms even at the less contaminated patches around Site A confirmed Svendsen's (2000) previous findings.
- Two-way ANOVA indicated a significant effect of patch, no effect of season and a significant interaction (caused by a lowered control time in winter, due to some seasonal 'base stress' on this parameter). The result of interaction is that in winter the assay does not identify either patch 1 and 3 as stressed, though at patch 4 (where earthworm communities are known to be impacted) a stress effect was still clearly detected. In the two other seasons, all Site A patches could be separated from the control.
- Separate one-way ANOVAs conducted for each season indicated a significant effect of patch on NRR-T in all cases. *Post-hoc* comparisons indicated that there were similar trends in spring and autumn, with controls differing significantly from all Site A patches, and a significant difference between worms from patches 1 and 4. In winter, the relationship between sites was different with NRR-T in patch 4, with worms having significantly lower retention time than in all other patches.

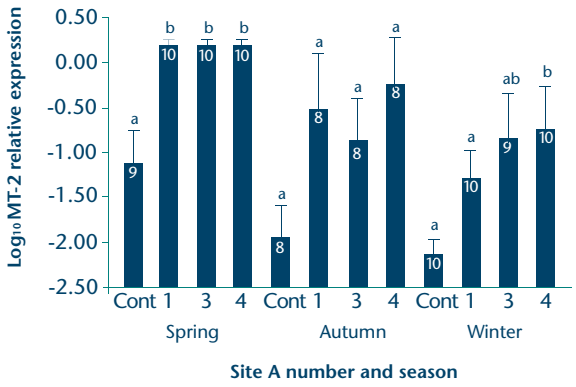


Fig 6.6.4. *MT-2* relative expression in earthworms (*L. rubellus*) collected either from outdoor maintained culture beds (cont) or from 3 Site A patches (1, 3, 4) in three seasons (spring: April 2002; autumn: October 2002; winter: December 2002). Values are means ± SE. Columns not sharing the same letter are significantly different from each other ($p < 0.05$). The white numbers on each bar indicated the number of worms collected and analysed.

Summary of results

- Measurements of the ratio of *MT-2* transcripts relative to *ß-act* in up to 10 worms per patch in the three seasons showed large variances, even between individuals collected from the same patch in the same season (bars on graph are standard errors). Despite the variance within the data set, the potentially large upregulation of *MT-2* that can occur in earthworms exposed to metals meant that we saw consistent trends in expression. *MT-2* relative expression was lowest in the control worms. It was highest in worms collected from patch 4 in all three seasons.
- We analysed *MT-2* expression data by a two-way ANOVA. Results indicated a significant effect of both patch and season, but not of the interaction. A *post-hoc* comparison indicated that *MT-2* relative expression in the control worms was significantly lower than for all three Site A patches, which could not be separated. Expression in spring was significantly different from autumn and winter, which could not be distinguished.
- For further analysis of the data, we analysed expression ratios for each season using a one-way ANOVA. In spring, there was a significant effect of patch, with expression in the control significantly different from all Site A patches, which could themselves not be separated. In autumn, no patch effect was present. In winter, a patch effect was present, with expression in the control worm significantly different from patch 4 worms. These results indicate that the conclusions from a study of *MT-2* expression could differ depending on the time of sampling. In particular, we could not distinguish a significant increase in expression in autumn, though comparisons of mean values showed that the same trends in *MT-2* expression could be seen.
- We observed a consistent upregulation of *MT-2* at all Site A patches and in all seasons. This supports the conclusions drawn in studies of the effects of temperature and pH on earthworm (including *MT-2* expression) responses (see Fig. 6.1.4 and 6.2.3), ie. that the measurement of *MT-2* can identify whether earthworms have been substantively exposed to metal.

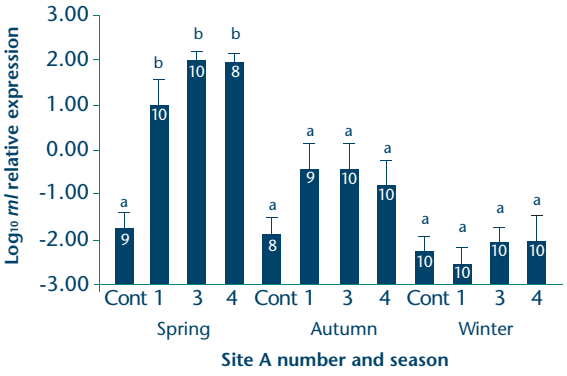


Fig 6.6.5. *rrnL* relative expression in earthworms (*L. rubellus*) collected from either outdoor maintained culture beds (cont) or from 3 Site A patches (1, 3, 4) in three seasons (spring: April 2002; autumn: October 2002; winter: December 2002). Values are means ± SE. Columns not sharing the same letter are significantly different from one another ($p < 0.05$). Numbers in white on column indicate the number of worms collected and analysed.

Summary of results

- Our measurements of the ratio of relative *rrnL* expression in up to 10 worms per patch in the three seasons showed that, as for *MT-2*, there was a large variance between individuals collected from the same patch in the same season.
- Our analysis of *rrnL* expression using a two-way ANOVA indicated a significant effect of patch and season, and also a significant interaction. *Post-hoc* comparisons indicated that expression ratio in the control worms was significantly lower than for all three Site A patches, which could not be separated. The seasonal comparisons indicated that expression in spring was significantly different from either autumn or winter, which could not be separated.
- A one-way ANOVA that we conducted for autumn only indicated a significant effect of patch, with expression in the control significantly lower than at all the Site A patches. In contrast, an analysis for both the autumn and winter sample intervals failed to show a significant effect of patch. These results show that, as for *MT-2*, the conclusions from a study of *rrnL* expression could differ depending on the sampling season. The biological reasons for the difference in *rrnL* expression observed between seasons arose probably because, as a mitochondrial gene, the gene has a direct link to metabolism. As earthworms are poikilothermic, their metabolism (and thus mitochondrial activity) is likely to be dependent on prevailing condition (especially temperature). The lower expression of *rrnL* transcripts observed, particularly in winter, could thus represent a lower rate of metabolism in worms during the colder conditions that prevailed at this time (see Fig. 6.6.1 for plots of temperature at the time the worms were collected).

6.7 Biological responses at all Site B field patches.

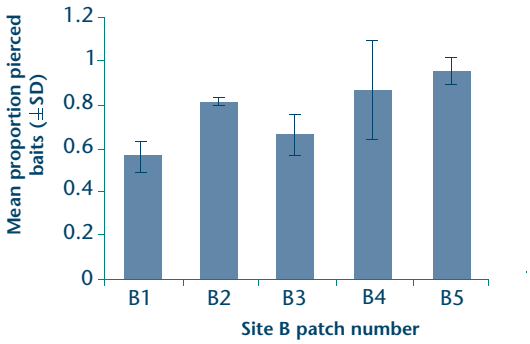


Table 6.7.1. Feeding activity of soil organisms as proportion of pierced baits as obtained by the bait lamina test conducted at 5 Site B patches in Autumn 2002. Patch values are means ± SD of three bait lamina sets each consisting of 16 vertical inserted strips.

Summary of results

- We found the highest overall feeding in patch B9, even though this patch was near one of the spots of visible surface oil contamination. Initially, we suspected that this higher rate of bait removal was due to a direct effect of oil on the retention of the baits within the strip. To assess this, we conducted trials to see if oil could reduce the number of retained baits. No effect was seen. This indicated that the higher feeding observed was due to the action of members of the soil community. The lowest mean feeding was in patch 1 soil, even though this was established as a clean control.
- Our analysis to identify between-site differences by one-way ANOVA of arcsine transformed percentage data (conducted to ensure that the conditions of normality were met) indicated a significant effect of patch. *Post-hoc* comparison between patches identified that feeding was significantly lower at patch 1 than patch 9. We didn't find any other statistical differences.
- One explanation of the high bait removal in the most polluted patch (B9) when compared to the on-site control is that the area around the oil patch may support a very active bacterial community. Bacteria could be using organic material derived from oil as carbon sources. An increase in bacteria numbers or bacterial activity could also contribute to bait loss.
- Whether the increased bait removal found at patch B9 was due to increased bacterial activity or invertebrate feeding, the bait lamina test appears, at first sight, to fail to identify the impacts of the contamination. In fact, this may not be the case. With the exception of two sticks, bait lamina at patch B9 was not put directly in the area affected by pooled oil – but nearby (within 0.1 – 2 metres of the oil). If the oil's toxic effect (see for example Fig. 6.3.3 for effects on earthworm reproduction) are localised to the area of the surface pool (ie., if there is no horizontal movement), then soil fauna that do not come into direct contact with the surface oil may not be affected.

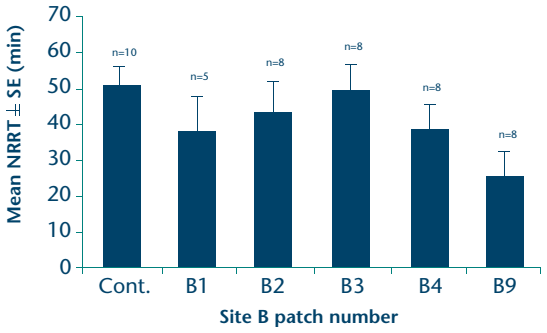


Fig. 6.7.2. NRR-T of earthworms (*L. rubellus*) collected from outdoor maintained culture beds (cont) or from 5 Site B patches (1, 2, 3, 4, 9) in autumn (October) 2002, winter. Values are mean of adults ± SE.

Summary of results

- We could collect worms of the correct species (*L. rubellus*) from all patches. In all cases, we found enough worms (at least five) to give some statistical robustness to the analysis.
- The NRR-T method performed well in the control, with retention times close to the maximum value of 60 minutes.
- In the Site B patch worms, we found the highest mean retention times in the on-site reference patch (B1) worms. Worms collected from patch B9 (close to one of the spots of visible surface oil contamination) had the lowest retention times.
- Despite differences in mean values, a one-way ANOVA indicated no significant effects of the Site B patch on the NRR-T of the indigenous worms.
- The absence of a significant effect on NRR-T could represent a failure of the assay. It is, though, more likely that there is a low exposure of indigenous worms to the oil contamination. Another possibility is that earthworms living close to the oil have acclimatised to the presence of hydrocarbons and associated contaminants. Such acclimatisation is, though, unlikely. There is limited evidence of acclimatisation in earthworms from previous studies (Posthuma & Van Straalen, 1993; Spurgeon & Hopkin, 1999b) and we know that oil had been present on the surface at this patch for only a short period of time (see Section 3.2).

7

Evaluation of the performance of the biological tests used during the project

The tests that we selected for appraisal were all chosen because in previous studies they had been shown to be useful for chemical assessment. Our selection was based on a comprehensive review of all available biological tests that could potentially be used to assess contaminated soil. We also took into account the specific skills of members of the project team. The review (conducted by Spurgeon et al., 2002) was based on published literature and discussions with method developers (if applicable). From this information, we evaluated each procedure according to the criteria of the 5Rs outlined by Hopkin (1993), as developed from Rapport (1990). These were that the methods should be:

Reproducible	The assay should produce similar responses to the same level of pollution after repetition of the assay.
Representative	It should be possible to use the assay at a range of potentially contaminated sites to facilitate comparisons between spatially separated locations. In this respect, the ecological compartment, community or species used for the test should be present at each site.
Responsive	The biological response should exhibit a measurable response after exposure to pollutants when compared to the results of assays conducted in uncontaminated soils.
Robust	The assay should be suitable for use with naturally contaminated field soils and should not respond to environmental factors that are not related to pollution or environmental degradation.
Relevant	The assay should provide data that is ecologically meaningful or can be related directly, preferably in a mechanistic way, to effects at higher levels of organisation (population, community, ecosystem).

In addition to assessing each technique with regard to 5Rs, we also considered the practicality of performing each test in routine soil assessment. We took into account the technical difficulty of each procedure, the speed with which it could be undertaken, the technical expertise needed to perform the assay, the ease of interpretation of results, and the costs in terms of both capital equipment and consumables.

All of the assays that we selected for this detailed experimental evaluation had performed well in the literature review (Spurgeon et al., 2002). The aim of this report is not to repeat the review, but to conduct an evaluation based solely on the outcomes of the laboratory and field experimental phases of this project. We evaluate each test, including the data generated in each experimental phase of the project.

7.1 Feeding activity using the bait lamina strip

Use within the project

We used the bait lamina test in the fieldwork phases of the project. The strips were deployed three times (in spring, autumn and winter) at three Site A patches (1, 3, 4) and once at five patches at Site B (B1, B2, B3, B4, B9).

Method used

At each patch, we set out three sets of 16 strips in a 4 x 4 grid (see Appendix 1 for locations at Site A). We inserted the strips vertically into the soil and left them there for about two weeks. Using a lightbox, we then scored the baits as either 'fully pierced' or 'partially pierced' by soil fauna. For all statistical analyses, we grouped these two categories and analysed the total numbers of pierced baits between sites. During all applications, we recorded soil temperature at 10cm depth using electronic data-loggers.

Table and figure pages presenting principal results

Fig. 6.6.2. Feeding activity of soil invertebrates organisms at three Site A patches in three seasons.

Fig. 6.7.1. Feeding activity of soil organisms obtained by the bait lamina test conducted at five Site B patches.

Discussion of principal results

This test performed very well in the project. At Site A, feeding rates reduced with increasing proximity to the smelter in spring and autumn. In winter, feeding at patch 1 was higher than that at both patches 3 and 4, which were comparable. Comparison between the seasons indicated that there was a seasonal effect on feeding. Bait removal was highest in autumn, lower in spring and particularly low in winter. This corresponds with soil temperatures which were higher in autumn than in winter, and to a lesser extent higher than spring. Additionally, as the summer of 2002 was wet, it was possible that more of the main detritivorous soil invertebrates (earthworms, springtails, isopods and oribatid mites) survived than usual before the autumn part of the test. This would explain the increased feeding on the baits found. The detection of an effect of the metals present in soils at patches close to the point source at Site A agrees with a previous study of invertebrate feeding activity conducted along a transect at Site A (Filzek, 2000). Our comparisons of the results of the bait lamina studies to previous ecological survey work conducted in the area (see Table 3.3) shows good agreement between the data. Previously, a notable effect on soil invertebrate diversity (particularly of detritivorous groups) was found at patch 3, and a more substantial effect at patch 4. The bait lamina assay was able consistently to identify reduction in bait removal at these two sites. The assay is, therefore, an excellent means of identifying the effects of metal on soil communities.

At Site B, we did not find a significant effect of patch on bait lamina feeding. This included not only the control and putative low oil compound contaminated patches, but also patch B9, at which surface oil contamination was present. The absence of an effect of the surface oil on bait removal at patch B9 could represent a failure of the bait lamina test to identify the impacts of petroleum hydrocarbon contamination on key soil groups. Alternatively, as the small oil patches are contained, the results of the bait lamina study could correctly be identifying the fact that the surface oil affects only the soil it is in direct contact with, but not the soil immediately surrounding it. Only a detailed characterisation of the structure of detritivore communities in the patches used for bait lamina deployment would have allowed us to assess this. One thing to note, though, was collecting worms to use in the NRR-T assay (see Fig. 6.7.2) was no more difficult at patch B9 than at any of the other patches. This offers some support to the theory that the bait lamina test was in fact correctly identifying a limited spatial effect of the surface oil on the abundance and activity of the soil fauna.

Method evaluation based on the results

***Reproducible**: Bait lamina have successfully been used in a number of studies to examine feeding rates at contaminated land sites (Filzek, 2000; Gestel et al., 2003). The power of the method resides in the fact that, though there are large variations in feeding on individual sticks, differences in feeding rates can still be identified. This is because a great many baits can be deployed at each patch. (In this study, 768 baits were set out on 16 sticks in three separate plots at each patch.)*

***Representative:** At Site A, there was agreement between 1) seasonal temperature patterns, probable soil invertebrate abundances and observed feeding rate and 2) also between feeding rates in the different patches and past studies of the abundance of key detritivore groups (earthworms, springtails) (Sandifer, 1996; Spurgeon & Hopkin, 1999a). This confirms that the assay is a suitable tool for measuring the abundance and activity of the soil fauna. On the basis of available data, it is not clear whether the absence of an effect at Site B represents the status of the soil fauna. The fact that earthworms could be as easily collected for use the NRR-T study as at any other patch suggests, though, that the effects of surface oil may indeed be spatially limited.*

***Responsive:** At Site A, the assay was good as a measure of overall invertebrate feeding activity. In particular, the agreement between changes in measured feeding at Site A and the results from previous ecological surveys is clear evidence that the assay can identify major*

ecological effects of contaminants on soil communities. At Site B, no effect of hydrocarbons was detected. As discussed, though, this probably reflects the limited spatial toxicological effect of the surface oil rather than a failure of the assay.

Robust: *Environmental conditions may have influenced feeding, which could represent a weakness of the test. In the seasonal comparison at Site A, we found that weather conditions altered feeding. Advantageous conditions during both the assay period and possibly in the months before could have increased the size of the detritivorous invertebrate community. Despite these effects, we reached similar conclusions about the impacts of metals on feeding within each temporal sample interval. So, though it is not possible to compare bait lamina data collected at different sample times, climate and season should not greatly influence the conclusions drawn, as long as temporal controls are included within any study.*

Relevant: *Previous work at Site A has identified an accumulation of un-decomposed organic matter at patch 4 and, to a lesser extent, at patch 3 (Filzek, 2000; Martin & Bullock, 1994). It was suggested that the presence of this thatch was a result of the effects of metals on the detritivorous soil fauna (Hopkin, 1989). Surveys have borne this out, finding changes in the abundance and diversity of major detritivorous groups present in soils at these locations (see Section 3.1.4). The bait lamina in our test detected a reduction in feeding of the soil fauna at both patch 3 and patch 4, when compared to patch 1. These results correspond to the direct effects of the metals present on litter decomposition seen in the previous studies. At Site B, previous research has found no evidence of the failure of decomposition, even in areas near the surface oil. The fact that we found no reduction in feeding rate at this site is in agreement with this observation.*

Practicalities: *Like any field-based assay, the bait lamina test can be influenced by the weather. For example, in this study, we did not plan an assay for summer, because it was likely that drought conditions would severely limit invertebrate feeding. The need for a series of pre-deployments can also mean a large time commitment (particularly if the site is distant). If, though, a suitable local site could be found to act as a surrogate for the on-site control, (to compare with potentially contaminated plots), this could reduce the time needed. Overall, despite these issues, the bait lamina test is relatively rapid and requires only limited manpower commitment. The assay is also cheap, particularly if sticks are refilled and reused. The statistical methods used to interpret results are also relatively simple to grasp.*

Recommendations for future use within an ecological risk assessment (ERA) framework for contaminated soils

The bait lamina test (Törne, 1990a) proved to be a sensitive means of detecting the effects of chemical exposure on the feeding activity of the soil community. The results obtained were entirely consistent with on-site observations and previous knowledge about the ecological and chemical status of patches on both sites. The fact that the assay is relatively simple and cheap means that it can be used within the early tiers of an ERA framework. This would include initial site screening and also more detail site-specific risk assessment. A key task in bait lamina studies conducted at potentially contaminated sites will be to identify a suitable on-site control for use as a comparison – a key issue with the application of any biological method.

7.2 The OECD draft earthworm reproduction test with *L. rubellus*

Use within the project

We assessed earthworm reproduction in the laboratory following a procedure based on the OECD (2000) earthworm reproduction test, in three studies, to measure the effects of:

1. soils from three Site A patches (1, 3, 4) on the survival and reproduction (cocoon production) of the earthworm *L. rubellus* at three different temperatures;
2. changes in pH (unamended, +1 unit or -1 unit) in soils from the same three Site A patches (1, 3, 4) on the survival and reproduction of *L. rubellus*;
3. soils from all Site B patches on survival, weight change and reproduction of *L. rubellus*.

To support these studies (and the results from later biological tests) we performed several chemical analyses. First, we measured the total and calcium chloride extractable metal levels in soils and worms in both the temperature and the pH experiments for Site A. Second, we measured soil pore water chemistry properties (to allow metal speciation calculation) in soils in the Site A pH experiment. Finally, we determined the concentrations of unsubstituted and substituted PAHs in soils in the Site B exposure. An additional benefit of the earthworm reproduction test was that it generated earthworm tissue samples that could be used as control exposures in the biomarker (NRR-T, gene expression) studies.

Method used

We prepared soil samples for all earthworm reproduction assays as described in Section 3.1.5. We added soil to the

test containers (clean plastic one-litre 'ice cream' containers). We filled soil within the replicates to a constant volume (800ml), rather than to a constant weight, because soils had different bulk densities, so the use of a constant soil weight would have resulted in worms being present at different densities. Before being exposed to test conditions, all the worms were maintained in culture. Adult worms (fully clitellate, weight >500mg) were then sorted from the soil, washed, weighed individually, and eight worms added to each replicate. We then covered the containers to limit water loss. The containers were then kept at 15 °C ± 1.5°C, in a 16-hour light/eight-hour dark regime for 42 days in all cases, except the temperature experiment where a set of four replicates of each patch soils were kept at 10 °C ± 1°C and 20°C ± 1.5°C. Throughout the exposures, we added suitable food (dried horse manure wetted to 80 per cent moisture content).

Earthworm mortality was assessed every seven days. We assessed the worms' weight change by comparing mean weights at the start and end of the exposure. The rate of cocoon production (cocoons/worm/week) was determined by wet-sieving the soil, collecting all cocoons and comparing this number to survival data. Immediately after the experiment, worms were put on filter paper for 48 hours to allow them to void any soil present in the gut. These purged worms could then be used for cellular and molecular biomarker measurements and chemical residue analysis. Where necessary, tissue samples were store at -80°C to await processing.

Table and figure pages presenting principal results

Fig. 5.2.2. Effects of Site A soils on earthworm (*L. rubellus*) life-cycle traits in the mesocosm exposure.

Fig. 6.1.2. Survival of earthworms exposed to three Site A patch soils at three temperatures.

Fig. 6.1.3. Cocoon production of earthworms exposed to three Site A patch soils at three temperatures.

Fig. 6.2.1. Survival of earthworms exposed to three Site A patch soils under three pH regimes.

Fig. 6.2.2. Cocoon production of earthworms exposed to three Site A patch soils under three pH regimes.

Fig. 6.2.7. Relationship between cocoon production rate of earthworm exposed to Site A patch soils under three pH regimes and zinc concentration in three soil fractions a) 'total'; b) calcium chloride extractable; c) predicted free zinc ion concentration.

Fig. 6.3.1. Mean number of surviving worms after 1, 2, 3, 4, 5 and 6 weeks (W1-W6) exposure to Site B patch soils.

Fig. 6.3.2. Weight change of earthworms exposed to Site B patch soils.

Fig. 6.3.3. Cocoon production rate (cocoons/worm/week) of earthworms exposed to Site B patch soils.

Discussion of principal results

In the temperature experiment, we did not find an effect of either temperature or patch on the survival of exposed worms. For cocoon production, we found significant effects of soils (cocoon production at patches 1 and 3 was higher than at patch 4), temperature (cocoon production was lower at 10 °C and 15°C than at 20°C) and also a significant interaction (lower temperatures reduced the effect of patch). The presence of a significant effect on earthworm reproduction, but not survival, at patch 4 indicates that, in earthworm bioassays, measuring reproduction is likely to be more useful in determining the effects of exposure than measuring survival. The temperature effect, and in particular the presence of an interaction, indicates that temperature greatly influences both the performance and the reproductive potential of earthworms. The significant interaction shows that a greater separation in reproduction occurs between patches at higher temperatures. This reflects the fact that the metals present can limit reproduction only when the reproductive output is high. As recruitment is likely to take place when reproduction rates are high, any toxic effects are likely to reduce population growth rates and thus affect the sustainability of earthworm population in these soils.

We found no statistically significant effect of patch on the survival of exposed worms in the pH experiment. We did find a difference between survival in the +1 and –1 pH treatments. For cocoon production, we found a significant effect of patch soils (cocoon production at 1 and 3 was higher than at patch 4). This was the same as in the temperature study. A highly significant effect of pH was found, with cocoon production in the +1 and 0 pH treatments being higher than in the –1 treatment. And there was a significant interaction between patch and pH treatment. This meant that the effects of patch were greater in the acidified than in the unamended or neutralised soils. To investigate this interaction further, we used two approaches to analyse metal bioaccessability/bioavailability and speciation. The first was the direct measurement of metals in a calcium chloride extracts. The second involved analysing the chemistry of soil pore water to allow metal speciation modelling using WHAM. We compared measured extractable zinc concentrations and modelled free ion zinc concentration with cocoon production rates normalised to remove the direct toxic effect of pH. This indicated that only calculation to free

ion could increase the prediction of the relationship over total metal concentration. Calculating free ion concentrations therefore predicts metal toxicity better than other soil chemical measurements.

In the two experiments conducted with Site A soils, survival in all soils was high (at least 50 per cent in all soils except the patch 4, pH –1 soil). But the survival of *L. rubellus* in the Site B soils was poor. For example, in soils B1 – B6, which had lower TPH contents, survival ranged from only 30 to 80 per cent. Survival in B8 and B9 (the soils most contaminated by TPH) was very low (less than 20 per cent). The fact that there was high mortality in the on-site reference soil (B1) and also in the other soils less contaminated by oil suggests that the low survival rates during this bioassay were not linked to contaminant concentrations. Skin lesions found during post-mortems on some of the worms revealed skin lesions typical of a disease previously seen, but currently uncharacterised.

Despite the high mortality of the worms in the Site B bioassay, the pattern in cocoon production rates broadly reflected measured TPH and PAH concentrations. ANOVA indicated significant effect of patch on reproduction. Cocoon production was significantly lower in soils from patches B4-B9 (where TPH and PAH concentrations were highest) than in soil from patch B1. The reproduction assay was able to demonstrate a pattern of change in reproduction that is in accordance with the extent of oil derived contamination, in spite of the presence of the disease and the resulting poor survival of the worms. This is evidence of the robustness of the reproduction bioassay. This is particularly important for its use in the risk assessment of industrial sites, where soil conditions even in less polluted areas will not always be favourable for earthworms.

Method evaluation based on project data:

Reproducible: *Differences between replicates in the reproduction rate of worms meant that values had appreciable standard deviations. This did not, though, prevent the identification of significant differences between sites (eg. Site A patch 4 soil was always higher than patch 1; Site B patches B4-B9 were significantly lower than B1). The use of a rate value (cocoons produced/total number of worm-weeks) minimised the influence of mortality.*

Responsive: *The effects on earthworm reproduction were seen after exposure to contaminated soils from both sites. This confirms that earthworm reproduction was sensitive to both metals and complex organic chemical mixtures.*

Representative: *Here, we successfully used a soil species to identify potential toxic effects. (A mineral soil dwelling species such as Aporrectodea caliginosa could also be used, eg. Khalil et al., 1996.) The use of such soil dwelling species means that the assay has greater representative value than assays that use compost dwelling earthworms such as Eisenia fetida, E. andrei or E. veneta.*

Robust: *We encountered some problems (such as the disease effect in the Site B study) when conducting this assay in the selected field soils. But the fact that we obtained useful results in all experiments (even with the range of manipulations used and problems encountered) confirms that the approach is very robust for data collection.*

Relevant: *In the Site A studies, we always demonstrated significantly reduced reproduction at patch 4. The effects of metal on the diversity of earthworm communities has been demonstrated for both patches 3 and 4 (Spurgeon & Hopkin, 1999a). This suggests that the earthworm assay has a good potential for predicting the ecological consequences of pollutant exposure. It could, though, miss subtle effects that might threaten the survival of some of the most sensitive soil-dwelling invertebrates. Measurements of survival alone would have missed the ecological effects present at Site A.*

Practicalities: *The test is relatively simple to carry out and requires simple equipment. In adapting the test to field soils, a number of practical issues need to be addressed, such as the maintenance of soil conditions and choice of reference soils. One of the values of the test is that a number of parameters (survival, weight change, cocoon production, cocoon viability and feeding rate) can be measured. The test is also well suited for the controlled generation of tissue samples for biomarker and/or chemical analysis.*

Recommendations for future use within an ecological risk assessment (ERA) framework for contaminated soils

The OECD draft earthworm reproduction test (OECD, 2000) conducted with L. rubellus proved to be a robust method that is sensitive enough to identify ecological effects. Because of the greater inherent sensitivity of measuring reproduction, this assay should be favoured over the OECD acute earthworm test (OECD, 1984). The assay is relatively simple and cheap. It is therefore suitable for wide application and can be used with the earlier tiers of an ERA framework. The test is also appropriate for detailed site-specific risk assessment where, on the basis of initial chemical and biological screens, patches of concern have been identified as having the potential to cause significant harm.

7.3 Instantaneous rate of population increase (IRPI) using toxicity data collected for the springtail *Folsomia candida* in Project P5-069

Use within the project

We applied the simple IRPI equation to data collected using the ISO collembolan reproduction protocol, in order to equate these findings in terms of the potential growth of springtail populations. The approach is simple and has proved to be valuable in past work (Stark & Banks, 2000; Walthall & Stark, 1997).

Method used

We translated data from the ISO collembolan test (conducted in all patch soils to measure the reproduction of the soil dwelling springtail *Folsomia candida*) to a measurement of population growth rate. To do this we used the equation $r_i = \ln(n_f+1 / n_0+1) / \Delta T$ where n_f is the final number of animals, n_0 the initial number of animals and ΔT the difference in time (number of days the experiment was run). Solving this equation for r_i creates values between –1 and +1. Positive values represent a growing population, zero represents a stable population, and negative values show a population in decline and heading towards extinction.

Table and figure pages presenting principal results

Fig 6.4.1. Instantaneous rate of population increase in springtails *Folsomia candida* exposed to all Site A and Site B patch soils.

Discussion of principal results

Risk managers and policy-makers would greatly value a test that could predict the critical concentration of chemicals that would result in the long-term decline to extinction of exposed natural populations. Unfortunately, in this study, we could not assess the value of the additional interpretation given to data from the springtail reproduction test by the use of the IRPI method. This was because the standard ISO (1999) Collembola tests failed to produce useful results with these field soils. The principal problem in the test was that extensive fungal growth blanketed the surface of the soils. This growth evidently restricted the survival and reproduction of the exposed springtails. Solutions to the problems of fungal growth have already been reported in the literature (eg. pre-sterilisation of the test soil). These modifications have meant that the springtail reproduction assay can be used to assess contaminated field soils (Smit & Van Gestel, 1996; Vijver et al., 2001). So it is possible to adapt the test to produce usable results. Because the aim of this project (and project P5-069) was specifically to trial each test by adhering as closely as possible to the standard test protocol, we did not apply these adaptations. The failure of the springtail test to produce useable results on this occasion should not, therefore, be seen as a bar on the use of the test (with the IRPI demographic approaches) for assessing the effects of contaminated soils.

Recommendations for future use within an ecological risk assessment framework for contaminated soils

With modification, it would certainly be possible to collect the data that would be required to apply the IRPI method. The value of the test is that it can be used to gain an overview of the potential population consequences of exposure. This would be greatly valued by regulators, as the protection of long-term population status is usually an aim within risk assessment.

7.4 Lysosomal membrane stability

Use within the project

We used the NRR-T assay extensively throughout the project to investigate earthworm lysosomal membrane stability. We carried out the following assays on both indigenous worms collected directly from patches at both sites, and also on naïve worms exposed to the two field soils during earthworm laboratory bioassays.

Site A

1. Responses in naïve *L. rubellus* after exposure to soils from all patches along the metal gradient at Site A in semi-field mesocosms.
2. Measurement in indigenous *L. rubellus* collected from three Site A patches (1, 3, 4) in three seasons (spring, autumn and winter).
3. The effects of soils from three Site A patches (1, 3, 4) on lysosomal membrane stability of naïve *L. rubellus* and the influences of incubation temperature on the observed response.
4. The effects of changes in soil pH (+ or - 1 unit) on lysosomal membrane stability of naïve *L. rubellus* exposed to soils from three Site A patches (1, 3 and 4).

Site B

1. Responses in indigenous *L. rubellus* collected from five Site B patches (B1, B2, B3, B4, B9) in one sample interval (autumn).
2. The effects of soils from all Site B patches (including the dilution series) on lysosomal membrane stability of naïve *L. rubellus*.

Method used

The neutral red retention assay measures cell damage. It makes use of the fact that only lysosomes in healthy cells permanently retain the cationic dye after initial uptake. All earthworms used for the assay were starved for at least 24 hours after either termination of the laboratory bioassay or collection from the field. Cells were isolated from coelomic fluid and placed on microscope slides suspended in earthworm physiological Ringer solution. A dilute solution of neutral red dye was added to the cell suspension and covered with a cover slip. The slide was then scanned for successive two-minute intervals using a light microscope at 400 times magnification. During each interval, the number of cells with leaked lysosomes (stained red) and the number of cells remaining unstained were counted. When 50 per cent of the total number of cells were stained, the time since the dye was added was noted. This was the neutral red retention time.

Table and figure pages presenting principal results

Fig. 5.2.3. NRR-T for earthworms (*L. rubellus*) exposed to Site A patch soils in mesocosms.

Table. 6.1.2. NRR-T of earthworms exposed to Site A patch soils at three temperatures.

Table. 6.2.1. NRR-T of earthworms exposed to Site A patch soils at three temperatures under three pH regimes.

Fig. 6.3.4. NRR-T and number of survivors after six weeks for earthworms exposed to Site B patch soils.

Fig. 6.6.3. NRR-T of earthworms collected from either culture beds maintained outdoors or from 3 Site A patches in three seasons.

Fig. 6.7.2. NRR-T of earthworms collected from outdoor maintained culture beds or five Site B patches.

Discussion of principal results

Site A

The mesocosm test with naïve worms demonstrated the potential for the NRR-T assay to identify exposure. Good retention times were found after exposure to the control and Site A patch 1 soils. A progressive and significant reduction in retention time was found in soils collected at patches approaching the smelter. ANOVA indicates a highly significant effect of site on NRR-T ($p<0.001$), with NRR-T significantly lower in soil collected from patches 3, 4 and 5 than from patch 1, and lower in patch 4 and 5 soils than in patch 2 (Tukey's test $p<0.05$). In a previous study of the composition of earthworm communities at Site A, Spurgeon and Hopkin (1999a) found lower community sizes and diversity in patches 3 and 4 than in patches 1 and 2; while at patch 5 no earthworms could be collected. The fact that NRR-T response also separates patches 1 and 2 from patches 3, 4 and 5 is evidence that the changes in lysosomal membrane stability measured in this study can be linked to changes at higher tiers of biological organisation (community effects).

In the laboratory tests to assess the effects of temperature and pH amendment, retention times were well below normal unstressed levels (up to 60 minutes), even in worms exposed to patch 1 soil. To confirm that these low retention times were not due to a technical failing of the assay, we also measured NRR-T in worms taken from culture at the same time as the Site A soil exposed worms were collected. In these control worms, we found long retention times. This suggests that the low retention times found in all Site A worms represent a real physiological response to exposure. These low retention times are likely to be due to the fact that the patch 1 soil, even though it was collected more than 10km from the smelter, still contained metal levels that were elevated above background concentrations (derived from both smelter deposition and local sources). This suggests that the NRR-T assay is highly sensitive to metals and can detect significant exposure even at sites where previous ecological surveys have found a normal earthworm fauna.

For our final investigation using the NRR-T assay for Site A soils, we examined the effects of season on NRR-T in indigenous *L. rubellus* collected from a subset of patches (1, 3 and 4). Here, there was also good performance of the assay in control worms, with NRR-T values approaching the 60 minute maximum in spring and autumn. Worms from all Site A patches had retention times below controls in all seasons. NRR-T was always highest at patch 1 and lowest at patch 4. Two-way ANOVA indicated a significant effect of patch, no effect of season, but a significant interaction. Separate one-way ANOVAs for each season indicated a significant effect of patch on NRR-T in all cases. We also compared the NRR-T of worms collected from the contaminated soils with the relevant seasonal controls. This indicated significantly reduced retention times at all Site A patches in spring and autumn, but only at patch 4 in winter. It appears that to benefit most from the sensitivity inherent in the NRR-T assay, it is better to analyse worms in autumn and spring, rather than winter. Notwithstanding this minor seasonal effect, the field application of the NRR-T assay confirmed the ability to discriminate exposure, even to low-moderate metal concentrations. It supported the use of this approach as a workable tool for assessing contaminated sites.

Site B

We found low NRR-T in the laboratory exposure to Site B soils, even in the soil with low TPH concentrations. These low retention times were probably related to the presence of the pathogenic agent that caused the unusually high earthworm mortalities in many patch soils. A significant correlation between NRR-T and earthworm survival during the bioassay supports the theory of a link between low observed retention times and the disease. Though the three lowest mean retention times were for the most soils most polluted with oil, a one-way ANOVA did not indicate a significant effect of patch on NRR-T. This was because there were not enough surviving worms to ensure a robust dataset. The absence of a clear, statistically significant difference for the exposed worms reflects the fact that low retention times in the control made it difficult to separate effects due to exposure from those caused by the pathogenic bacteria. Svendsen (2000) found an interaction between a disease-causing agent and the performance of cellular assays in a study with *Eisenia veneta*. In our study, we found that a bacterial infection changed the earthworms' immune systems, though, in this case, the NRR-T assay was unaffected.

In the worms collected from the Site B field patches, the highest (> 40 minute) retention times were found in those from the on-site control patch (B1). The lowest retention times were found in worms collected from patch B9, which was close to one of the spots of visible surface oil contamination. One-way ANOVA, though, indicated that there was no significant difference in NRR-T between worms collected from the different patches, including patch B9. The absence of a significant effect of the pooled oil present at B9 on earthworm NRR-T could be for two reasons. First, it is not yet known whether oil or oil derivatives have an effect on NRR-T. If they do not, it may be that exposure is occurring, but that it does not cause a change in NRR-T. This possibility is, though, unlikely, given that significant NRR-T effects have been found in earthworms following exposure to the complex organic chemical mixture in soils from a coal gasification plant (Eason et al., 1999). A second explanation is that, though there is oil both on the surface and through the fissure from which it is released at B9 (see Section 3.2.), the contamination is in fact extremely localised. It may not move horizontally into the surrounding soil from which the earthworms were collected (nb. all worms were taken from within one metre of the oil patch, but none were taken from the visually the oiled soil). This is the more likely explanation, especially in light of the bait lamina data. If this is the case, then the NRR-T results confirm that worms have not been exposed to the oil.

Method evaluation based on project data

Reproducible: *Within experiments, the reproducibility of the results was good. NRR-Ts were within the expected ranges of natural variation based on previous results. Differences were, though, found in comparative NRR-T between experiments, depending on the exposure protocol used. This is exemplified by comparing the results of the mesocosm, laboratory and seasonally collected field worm studies for Site A. In the mesocosm, long NRR-T was found in the control and less polluted soils. Times reduced in the soils from the three closest patches to the smelter. In the field study, long retention times were found in the controls (worms kept in culture). Times were significantly reduced at patch 1, 3 and 4 in spring and autumn and patch 4 in winter. In the laboratory pH amendment and temperature studies, long retention times were again found in controls, but short retention times were found in all three Site A soils, and there were no discernible differences between the Site A soils. The differences observed in NRR-T between different exposure systems are likely to result from a combination of factors acting on worms to alter retention times. These include exposure time (longer in the field worms than mesocosm exposed worms) and the intensity of exposure (likely to be higher in the laboratory tests than in the mesocosm and field, where there may be greater heterogeneity). Other factors include the influences of season (in the mesocosm and field) and soil types and incubation conditions such as density (low in field and mesocosm, high in the laboratory test). These variations between studies can mostly be overcome by including appropriate controls, for example a seasonal field study. Importantly, despite these differences, we always identified lowered retention times in worms exposed to soils in which metals have previously been shown to affect earthworm communities (see below). At Site B, there was a clear difference between the laboratory based and field assessment. Two factors are likely to have caused this. First, the presence of the disease in the Site B laboratory test may have reduced retention times. Second, worms in the field may be able to avoid exposure – they can't do that in the laboratory. If this is the case, then the differences in NRR-T for the field and laboratory exposed worms represents a real difference in the extent of chemical exposure.*

Responsive: *For Site A, there was a significant effect of NRR-T at the three patches closet to the point source in the mesocosm study. In all other studies (laboratory temperature and pH amendment studies, seasonal field measurements), we found low retention times for all patch A soils (1, 3 and 4). Possible reasons for this, such as differences in the duration and intensity of the exposure, are outlined above. In all cases, though, a*

reduction in NRR-T was noted in soils from patches 3, 4 and 5, at which changes in earthworm community composition and bait lamina feeding have been observed in previous work (Filzek et al., in press; Spurgeon & Hopkin, 1996a, 1999a). In some cases, lower retention times were also observed at patches 1 and 2. The NRR-T assay was thus always able to indicate a potential for harmful chemical exposure for soil invertebrates. And in some cases, the assay could identify significant exposure at the less contaminated sites.

Representative: *In the field phase of the study, we managed to collect enough suitable earthworms (in this study, adult L. rubellus) from all patches at both sites. This confirms that earthworms can be found in industrial contaminated soils, assuming that concentrations are not so high as to impact directly on populations. Earthworms live in close association with the soil matrix. This means that measured biomarkers in these species are likely to be closely linked to the nature of exposure and its subsequent effects.*

Robust: *We encountered some problems with use of the assay, such as the probable effect of the disease outbreak in the Site B laboratory study. These problems contributed to the fact that some differences in the conclusions between different studies at Site B were drawn. At both sites, the NRR-T assay clearly worked well with field collected indigenous worms and worms exposed in large outdoor semi-field mesocosm, but didn't work so well when worms were exposed in the laboratory (eg. for Site B). This suggests the greatest value for the NRR-T assay will probably be gained by using field-collected worms. There was also an apparent effect of season on control retention times. This meant that the conclusions drawn from studies conducted in each season differed. The use of appropriate seasonal control can overcome much of this effect.*

Relevant: *Previous work has demonstrated a correlation between chemical exposure, lysosomal membrane stability and higher organisation level (reproductive) effects (Svendsen & Weeks, 1997a, b). Though the relationship between NRR-T and specific life-cycle parameters was not investigated in this study, the relationship between NRR-T and high tiers of biological organisation (life-cycle, population, community) can be inferred by comparing our results with previous work. At Site A, low retention times were always found in worms collected at, or exposed to, soils from patch 3, 4 and 5. These patches are known to have a reduced earthworm fauna (Spurgeon & Hopkin, 1999a). They were also identified as having lower bait lamina feeding in this study (Fig. 6.6.2) and in previous work (Filzek, 2000). In some cases, low retention times were also found at patch 1, where the earthworms were unaffected. In conclusion, the NRR-T assay always showed a reduced retention at the same patches where there was a higher biological organisation effect (bait lamina). The greater sensitivity of the NNR-T assay meant that the test was able to show substantive exposure at some sites where effects at higher level have not to date occurred (but may do so in the future). At Site B, NRR-T was low in the laboratory tests. This reflected the presence of a probable combined stress due to disease and the complex organic chemical mix present. In the field, no effect on NRR-T was found. Though the earthworm fauna was not measured at the Site B patches, bait lamina removal was assessed. The bait lamina tests also showed no effect of the oil present. This supports the conclusion that the NRR-T assay correctly identified the fact that horizontal movement of oil-associated contaminants into the surrounding soil was limited.*

Practicalities: *The neutral red retention assay can be undertaken with a simple light microscope, and by relatively unskilled staff given appropriate training. Our results suggest that the best results may be gained using field collected worms. Nonetheless, if worms cannot be collected on site (either due the extent of contamination or other ecological factors) the assay can be a useful add-on to sub-lethal earthworm toxicity tests. Currently, the assay is based on a real-time analysis of lysosomal membrane stability, using cells collected from live worms at the time they are removed from experimental soils. This presents two disadvantages. First, the assay is relatively low throughput (maximum 30 worms can be analysed per day). Second, the real-time nature of the assay means that careful resource planning is needed. These issues aside, the assay is sensitive. It can be conducted with both field collected and laboratory exposed worms. In, this study, it always gave low retention times at patches where higher organisation effects have previously been observed. These facts suggest that the assay can form a useful component of any biological testing toolkit for contaminated soil.*

Recommendations for future use within an ecological risk assessment framework for contaminated soils

The neutral red retention time assay is sensitive to metals and is likely also to be sensitive to organic compounds (Eason et al., 1999; Hankard et al., in press). This inherent sensitivity means that the assay is useful for both site screening and more detailed risk assessment. The link between assay results and higher tier biological responses confirms the value of the NRR-T assay for predicting significant ecological effects.

7.5 Single gene transcript quantification

Use within the project

We undertook single gene quantifications in a number of studies within the project. These were all focused on Site A because, at the outset of the project, only putative metal responsive sequences were available for the designers of primers and probes sets (nb. see discussion below under Site B). We made an initial investigation of expression profiles using cDNAs transcribed from mRNA samples isolated from worms collected at all patches along the Site A gradient. The aim of the pre-testing was to confirm the identity of potentially responsive genes. Once a metal effect on gene expression had been established, the usefulness of these genes for the identification of exposure and effect was then identified in mesocosm and laboratory toxicity tests and in field collected worms. The specific investigations conducted were:

Site A

- the expression of a series of putative metal responsive genes in adult *L. rubellus* collected from all Site A patches;
- the quantification of established metal responsive transcripts (*rrnL* and *MT-2*) at three Site A patches (1, 3, 4) in three seasons (spring, autumn and winter).
- the response of the *MT-2* gene in *L. rubellus* exposed to soils from three of the Site A patches (1, 3, 4) at different temperatures
- the effects of changes in soil pH (+ or - 1 unit) on *L. rubellus MT-2* expression in Site A patch 1, 3 and 4 soil.

Site B

At the outset, we intended to develop assays to measure the gene expression and/or enzyme activity for enzymes involved in the detoxification of polycyclic aromatic compounds (P450s, glutathione-S-transferases, catalases). Despite our best efforts, we have not been able to do this. We continue to work on this area and will report our results elsewhere (in a scientific journal paper). A burgeoning amount of sequence information is now available (including expressed sequence tag project with a series of environmentally relevant soils species including nematodes, earthworms and springtails). This could aid the development of further high-throughput, gene-specific assays. It could also facilitate the development of microarray based systems for measuring the expression of multiple (1000s) gene products.

Method used – sample preparation

Thanks to advances in the handling of nucleic acid, a number of methods are now available for detecting gene activity via messenger RNA. In this study, we used the following method. First, earthworm tail samples (50mg) were homogenised into one millilitre of target capture system buffer (Molecular Light Technology, Cardiff, UK). We collected messenger RNA directly from the tissue sample using a polythiaminated magnetic bead system. After conversion to cDNA by reverse transcription, the 5' nuclease assay was then used to measure the expression of specific target gene sequences. This assay relies on the 5´ nuclease activity of the Taq DNA polymerase to cleave a fluorescent labelled oligonucleotide probe complementary to the target sequence. This results in detectable fluorescence. Including the TaqMan probe in the PCR reaction allows the production of an amplicon to be monitored at the end of each cycle. We included a series of calibration standards containing cloned copies of the target gene at known concentrations. This enabled us to obtain the relationship between transcript frequency and the number of cycles required to reach a specific threshold. We then used this standard curve to determine transcript concentration in samples. It is standard practice in Q-RT-PCR to normalise cDNA template concentrations to account for differences in sample handling, transcription efficiency and sample loading (Stürzenbaum & Kille, 2001). Parallel measurement of a control (non-responsive) gene(s) can be employed for this. We used *ß-act*.

Table and figure pages presenting principal results

Fig. 5.1.1. Expression of five gene transcripts measured using 5' nuclease assay based quantitative RT-PCR for five sequences in earthworm collected from all patches along the Site A gradient.

Fig. 5.2.4. *MT-2* relative expression in earthworms (*L. rubellus*) exposed to Site A patch soils in mesocosms.

Fig. 6.1.4. *MT-2* relative expression in earthworms exposed to three Site A patch soils at three temperatures.

Fig. 6.2.3. *MT-2* relative expression in earthworms exposed to three Site A patch soils under three pH regimes.

Fig. 6.6.4. *MT-2* relative expression in earthworms collected either from outdoor-maintained culture beds or from 3 Site A patches in three seasons.

Fig. 6.6.5. *rrnL* relative expression in earthworms collected either from outdoor maintained culture beds or from 3 Site A patches in three seasons.

Discussion of principal results

Initial characterisation of five sequences along the contamination gradient at Site A showed different response patterns. Some sequences (*ß-act*, *cyc-B*,

AmOx) showed no apparent alteration of expression in worms collected from different sites along the gradient. We had expected this for the *ß-act* sequence, which is why we had selected it as an invariant control against which to compare the expression data for all other transcripts. In the case of *cyc-B* and *AmOx*, we had anticipated a change in relative expression resulting from metal contamination, but did not find one. For *rrnL* and *MT-2*, a significant upregulation of expression was found at patches approaching the smelter. At a number of the patches, this upregulation was significant. One notable feature that was common in all earthworm molecular genetic measurements was the presence of a high inter-individual variation in the expression measurement. In some case (eg. *rrnL*), this variation was high enough to preclude full statistical separation of the contaminated patches.

The long history of metal contamination at Site A meant that the invertebrate populations in the area may have developed resistance. It is thus possible that the changes in gene expression observed in the field-collected worms represent an adaptive rather than a transient response. To investigate this further, we measured the expression of the *MT-2* gene in naïve worms exposed to soils from all Site A patches in mesocosms. Our findings indicated that the expression in naïve worms was comparable to the indigenous worms. This suggested that any adaptation had not altered the expression pattern in the indigenous animals. Either indigenous worms or naïve worms could therefore be used for gene expression profiling. There was good agreement in comparisons between *MT-2* expression and the results of other biological assessments. In the mesocosm study, we found significant *MT-2* upregulation at Site A in patches 3-5. These are the same patches where a reduction in NRR-T was found in the mesocosm study, and also where changes in earthworm communities and bait lamina feeding have been found in this work (Fig. 6.6.2) and in previous work (Filzek, 2000; Spurgeon & Hopkin, 1999a). This confirmed that there is a link between *MT-2* upregulation and higher organisation effects.

Seasonal changes in expression of two metal responsive genes, *rrnL* and *MT-2*, were investigated in worms collected from three Site A patches (1, 3 and 4) in spring, autumn and winter.

Analysis of *rrnL* relative expression by two-way ANOVA indicated a significant effect of patch and season. It also showed a significant interaction. Expression in control worms was significantly lower than at all three Site A patches and between seasons, and expression in spring was significantly higher than in either autumn or winter. One-way ANOVA conducted for each season indicated a significant effect of patch in spring, but not in autumn and winter. These results show that the conclusions drawn from a study of *rrnL* expression would differ depending on the time of year that sampling took place. The biological reason for the difference in *rrnL* expression observed between seasons is probably that, as a mitochondrial gene, *rrnL* is directly linked to metabolism. Earthworms are poikilothermic. Their metabolism (and thus mitochondrial activity) therefore depends on the prevailing conditions (especially temperature). The lower expression of *rrnL* transcripts that we observed in autumn and especially in winter could reflect a lower rate of metabolism in worms during colder weather (see Fig. 6.6.1 for plots of temperature at the time the worms were collected).

In all three seasons, *MT-2* relative expression was lowest in the control worms and highest in worms collected from patch 4. Two-way ANOVA indicated a significant effect of both patch and season, but not of the interaction. Expression in the control worms was significantly lower than all three Site A patches, which could not be separated. Expression in spring was significantly different from autumn and winter. For further analyses, we analysed expression ratios for each season by one-way ANOVA. In spring, there was a significant effect of patch, with the controls being significantly lower than all Site A patches. In autumn, a patch effect was not present. In winter, a patch effect was present, with expression in the control worms significantly different from that in worms from patch 4. These results indicated that the conclusions from a study of *MT-2* expression could differ depending on the timing of sampling. In autumn in particular, a significant increase in expression could not be distinguished. Unlike *rrnL*, there is no simple biological explanation of the observed differences in expression between seasons. The absence of a significant patch effect in autumn was a result of the especially high variability between individuals in this season. Cocoons laid in the spring and early summer begin to hatch in autumn. This season therefore represents a major time for recruitment. So one possible explanation for the higher variation in autumn could be that worms collected then comprise a mixture of individuals at various stages of growth. In winter and spring, worms are more likely to be fully mature. Such differences in the biological status of collected worms could alter the genetic responses of individuals to environmental cues and stresses. This would further increase the variation of our genetic measurements.

We undertook a further evaluation of *MT-2* expression in adult *L. rubellus* exposed to Site A soils under different temperature and pH amendment regimes.

Mean expression was always highest in worms exposed to patch 4 soil and lowest in patch 1 soil. But a two-way ANOVA could discern a significant effect of patch only in the pH amendment study. No effect of either pH or temperature was seen. Again, a major issue within both the temperature and pH amendment studies was the high inter-individual variation. Some of this variation is likely to be due to the methods used for the quantification of gene expression. The procedures that we adopted for analysing the data should have removed some of the sample handling effects. Indeed, a detailed appraisal of the performance of the 5' nuclease assay for measuring the expression of both *rrnL* and *MT-2* in earthworms (Galay Burgos et al., 2003) demonstrated low variance between repeat measurements of the same sample, with coefficients of variation between 0.055 and 0.353. This suggests that the high variances seen here were unlikely to be accounted for by analytical issues. Instead, they must reflect real biology. Currently, we can only speculate on the causes of this variation. Relevant factors could include age difference, parasite loads, disease states, reproductive status and microhabitat quality. Because variation is evidently relatively high in any study of expression responses of earthworm to pollutants, we recommended that assays be designed to maximise replication in studies that aim toidentify significant differences in gene expression between samples.

Method evaluation

Reproducible: *This work indicated that it is possible to measure gene expression in environmentally relevant species for the purposes of the detection of pollutant exposure. The suitability of the method depends on the target chosen. The metal binding protein MT-2 gives the clearest and most consistent response of the sequences investigated here. The high variability between individual measurements presents one of the principal challenges regarding the interpretation of gene transcript measurement in risk assessment. Some of this variation is likely to be related to methodological issues regarding RNA handling, reverse transcription and the 5' nuclease assay. The prevalence of the variation in many of the studies suggests, though, that much of the variation may be related to individual worm biology. The development of higher throughput methods ongoing in the medical bioscience could help in the future, both in terms of improving the method's repeatability and increasing sample throughput. In the short term, practitioners need to be aware of the issue of variability and design studies accordingly (ie. focus on maximising replication).*

Representative: *The choice of transcripts is of key importance. MT-2 was demonstrated here as suitable for detecting exposure to metals. But this sequence is unlikely to respond to selected pesticides, aromatic compounds or hydrocarbons. To improve the potential for representative measurement, it may be more useful to measure expression of a suite of transcripts from the same cDNA sample. This should be possible within the next five years, thanks to the increasing availability of sequence information for environmentally relevant species. Furthermore, as sequence information and clone libraries develop, it may be feasible to use tools such as microarrays to simultaneously measure many thousands of sequences. These methods should prove to be powerful tools for environmental diagnostics.*

Responsive: *If suitable transcripts for quantification are identified, it is likely that they will prove to be sensitive for the detection of chemical exposure. Here, the measurement of both MT-2 and rrnL showed upregulation at many of the patches at Site A. In microarray experiments, upregulations of a twofold increase are considered to be biologically important. In this study, MT-2 was upregulated at least two-fold compared to control measurements in all of the patch soils used. This shows how sensitive these transcripts are to metals. The main concern in exploiting the inherent sensitivity of gene expression measurement is the repeatability of the assay and the limits that this places on establishing statistical differences. For now, the only way to overcome this is to optimise the assays further and increase sample throughput.*

Robust: *The outcome of gene expression measurements varied depending on season. This was the case both for rrnL and MT-2. In the case of rrnL, which is linked to metabolism, a potential mechanism underpinning the observed response can be identified (the effects of seasonal temperature on metabolic rate). This mechanism would account for the reduction in the expression of this gene in the autumn and, especially, the winter samples. For MT-2, the assessment was characterised by higher variability in the autumn sample. No clear mechanism can be identified for this response, though the timing of recruitment could play a part. The presence of significant variations between seasons means that studies conducted at different times of the year cannot directly be compared.*

Relevant: *A change in gene expression can be one of the most sensitive biological response measurements. This was seen in this study. In microarray experiments, a change in gene expression by a factor of two is considered to be functionally important. In the gradient study conducted with field-collected worms and mesocosm exposed worms, the increase in mean expression was well above this value for all the*

patches, though the difference was significant for patches 3, 4 and 5 only. This indicates that functionally important changes in gene expression can be linked to low metal concentrations, while statistically significant changes occur at higher concentrations. The inherent sensitivity and variation of gene measurement mean that these assessments are not as relevant as functional assays such as bait lamina, or life-cycle toxicity tests. The best use of these assays therefore appears to be in the diagnostic detection of pollutant exposure. Higher organisation assessments will provide more robust information about the significance of associated biological effects.

Practicalities: *The initial identification of pollutant responsive genes and the development of reagent sets and protocols for their expression quantification is a specialist task. The level of expertise needed to conduct individual assays is, though, no greater than for traditional chemical analyses. Equipment costs are also comparable with traditional chemical methods. Consumable costs, though, are at present probably higher than for organic analysis, and certainly higher than for atomic absorption spectrophotometry. As mentioned above, the key to optimising the value of molecular genetic characterisation is to improve sample throughput. This is a challenge shared by both medical and environmental diagnostics. In the former, field methods have been developed that will allow transcript measurement for many thousands of genes simultaneously. These methods (microarray, serial analysis of gene expression) are powerful tools for identifying the profiles of response to specific environmental stimuli. When coupled to multivariate data handling and pattern recognition methods, these techniques will offer a real possibility for developing methods that can give a complete picture of the effects of exposure to mixtures of stressors on the health of organisms. Currently, a series of research initiatives in the UK, Europe and the USA are developing the application of gene profiling techniques for assessing the effects of environmental change (including pollutant exposure) on both sequenced and un-sequenced species (including plants, earthworms, springtails, Daphnia and nematodes). Research initiatives of this type will, in the near future, yield new tools that can be used for characterising biological responses to contaminants. The use of these profiling techniques was recently endorsed by the Royal Commission for Environmental Pollution (Royal Commission on Environmental Pollution, 2003).*

Recommendations for future use within an ecological risk assessment framework for contaminated soils:

Trials using molecular genetic biomarkers to assess the exposure of organisms to metal contaminated soils prove that such assays can be used in the biological assessment of multiple contaminated patches. Technically, the work is no more challenging than traditional chemical assessment. Current developments in medical diagnostics mean that the range and throughput of techniques available is improving all the time. One of the major issues for the use of molecular genetic methods is the selection of a suitable target for each analysis. Here, both rrnL and particularly MT-2 could be used to identify earthworms' exposure to metal contaminants. These genes may not, though, be effective for organic chemical exposure. To address this, molecular biologists have in the past three years been developing methods that can be used to measure the expression of large number of genes simultaneously. These methods (eg. microarrays) offer the possibility of measuring many genes that respond to multiple environmental stresses within a single analysis. Such methods are still in the early phases of development. But the pace of current research suggests that this technology will become one of the dominant methods in environmental biology, offering the real possibility of assessing the effects of multiple chemical exposure on a range of aspects of organism health.

7.6 Bacterial biosensors

Use within the project

We recorded the effects of exposure on soil bacterial metabolism using *Berkholdaria* sp. RASC strain engineered to contain the *lux*-construct (Shaw et al., 1999) for soil water extract taken from all Site A patch soils. As the soil at Site B is dominated by hydrocarbons, many of them lipophilic, we did not conduct *lux* assays there. This is because using a simple water extraction at this site may have collected an unrepresentative fraction of the contaminants present in patch soils for this site (see Discussion below).

Method used

The effects of exposure to a water extract of each Site A patch on soil was recorded using *Berkholdaria* sp. RASC strain engineered to contain the *lux* construct (Shaw et al., 1999). We added microlitre volumes of bacterial culture to soil pore water extracts and immediately detected light output using a scintillation counter (Shaw et al., 2000a).

Table and figure pages presenting principal results

Fig 6.5.1. Relative luminescence of *lux*-marked bacteria in soil extracts collected by shaking and centrifugation for all Site A and B patch soils.

Discussion of principal results

The luminescence of the *lux* construct marked *Berkholdaria* sp. RASC strain was significantly different after exposure to water extracts from the Site A patch soils. Compared to patch A1, luminescence in soil extracts from the three patches closest to the smelter (A3-A5) was significantly reduced. The results found in this initial *lux* survey are broadly in agreement with those for Microtox™ conducted as part of Environment Agency project P5-069. This suggests that the two biosensors have a similar sensitivity to the mixture of metals present at Site A. We compared the bacterial biosensor results with previous ecological survey work. Significantly reduced luminescence was found at the three Site A patches at which soil invertebrate community alteration have been observed in previous work (see Section 3.1.4 for references). This shows that the sensitivity of luminescence response is within the range of exposure concentrations that have a deleterious effect on native soil fauna at Site A. We must stress here, though, that these are preliminary findings. A great deal of further work will be required to establish the comparative sensitivity of the luminescence response to soil communities' structural and function endpoints under different exposure scenarios.

We did not use the *lux* system for Site B. This was because contamination of this site is dominated by hydrocarbons, many of which are lipophilic. We were concerned about whether a simple water extract would be suitable for the analysis of these soils. Problems of obtaining a suitable soil extract for biosensor analysis of lipophilic soil contamination can be overcome by using targeted extraction methods (Reid et al., 1998), co-solvents (Bundy et al., 2003) or a solid phase assay (Shaw et al., 2000a). Indeed, in work conducted in Environment Agency project P5-069, this latter approach has proved effective for use with the marine bacterial based biosensor Microtox™ when applied to soil from the Site B patches (Weeks, pers. comm.). The issue about preparing a suitable soil extract is not specific to the use of transgenic soil bacterial biosensors. A similar issue would need to be addressed if the marine bacteria based Microtox™ biosensor system was used. The work conducted with Microtox™ in Environment Agency project P5-069 and previous literature studies conducted with *lux*-marked soil bacterial strains suggests that, with suitable modification of the exposure method, bacterial biosensors can become useful tools in the analysis of soils contaminated by hydrocarbons.

Recommendations for future use within an ecological risk assessment framework for contaminated soils:

Soil bacteria engineered to contain the lux gene cassette offer a realistic alternative to the Microtox™ system. The limited work conducted in this project suggests that, for sites dominated by metal contamination, there is a similar sensitivity of the two bacterial based biosensors. The lux marker Berkholdaria sp. RASC strain based system, though, has an added advantage: because it does not use a marine bacteria, this Tsystem has higher ecologically relevance. The method is rapid and relatively easy to conduct. As a result, it has the potential to act as a screening assessment that can be applied in the initial tiers of an ecological risk assessment framework. Because of concerns over the suitability of a simple water extract for analysis of Site B, bacterial biosensor assays were not conducted for these patch soils. Past and ongoing work, though, suggests that, with suitable modifications, transgenic bacterial biosensors could be used to assess the contamination status of soils or similar character to those at Site B.

8

Recommendations

This project demonstrates that it is possible to use biological tests to detect the presence of environmental contaminants and gain an overview of their potential ecological effects. Some methods include those adapted from standardised procedures used in chemical risk assessment, such as the OECD earthworm reproduction test (OECD, 2000). Others are methods now established within the scientific literature, such as the bait lamina (Törne, 1990a) and earthworm lysosomal membrane stability assay (Weeks & Svendsen, 1996). And, at the research horizon, methods such as molecular genetic measurements (Galay Burgos et al., 2003), may also be used.

Responses from all the studies undertaken at Site A – that is, mesocosm tests (all patch soils), temperature and pH amendment studies (selected patch soils), and field assessments (including a seasonal evaluation) – reflected responses found in previous research. At least one biological test was able to differentiate control soils (and, in some cases, less contaminated patches) from patches where gross effects on the diversity and biological function of invertebrate communities had been established in previous work. At Site B, the biological tests conducted in the laboratory and in the field identified likely differences in the extent of exposure. Stronger biological effects were seen in the laboratory when, unlike in the field, soil organisms could not avoid the oil contaminant.

As a summary of the performance of the assays, the tests that measured higher organisation level function (eg. the bait lamina test and the earthworm reproduction bioassay) gave the most robust results. In these, only limited effect of season and exposure scenario was seen. The more sensitive assays, such as the measurement of lysosomal membrane stability using NRR-T and measurement of gene expression, often indicated differences from the control in some of the less contaminated patches. Despite the evident inherent sensitivity, though, we could not always establish the significance of such responses, due to inter-individual variations and, in some cases, differences between exposure scenarios. The responsiveness of the biomarker and the robustness of the life-cycle and functional assays, though, complement each other. Together, they increase the chances of identifying the presence of a potential (contaminant) stress and in then diagnosing the significance of that stress for soil organisms living in the affected patch. To make full use of potential inherent in the use of a suite of biological response measurements for the detection an assessment of contaminants in soils, the results of this project need to be integrated with ongoing work. This will enable the development of risk assessment frameworks and, beyond that, move these methods forward into formal risk assessment. Recommendations to achieve this are set out below.

1. *The risk assessment framework needs to be completed in conjunction with a clarification of the overall goal of soil sustainability. Work to develop the required framework for contaminated land is underway in Environment Agency Project P5-069. To gain the best value the results should be aligned as closely as possible with the outcomes of our project. In the wider context of soil sustainability, setting key protection aims – including environmental quality standards for chemicals, and functional standards for soil – remains a priority.*

2. *This project focussed on measuring the effects of contaminant on soil invertebrates, earthworms in particular. While this focus, in part, represents the specific skills of the team, it does recognise that, in soil ecotoxicology, studies on invertebrates are more common than for microorganisms and then plants. As*

stated in the recommendation from the review conducted earlier in this project, the development and use of biological tests with plants should be encouraged. The reality, though, is that among soil species, earthworms are by far the most extensively studied (there have been three international workshops of earthworm ecotoxicology since 1990). This body of knowledge provides essential background information on earthworms: their basic biology, chemical toxicity and bioavailability, exposure routes, biochemical function, immunology, molecular ecology and life-cycles. This knowledge gives greater power to studies with earthworms, as it makes it easier to interpret the results from any specific assessment. For this reason, earthworms should remain a preferred (but not the only) organism used in the risk assessment of contaminated soils.

3. *For many tests, the interpretation and use would be easier if data on baseline responses were available. Currently, the quantity and quality of baseline data varies between tests. In the cases of standardised tests, such as the earthworm reproduction bioassay, existing performance criteria can discern whether an observed effect is significant. For other assays, information of this type is not available. One area that the work should focus on in particular is the interplay between soil factor, ecotypes and measured responses. This will give baselines for variation within the test. It will also help to derive performance criteria that can be used within the risk assessment framework as part of an algorithm to discern the status of a given location. In the case of gene expression measurements, for example, the microarray community has set a standard whereby a difference in the expression of a gene is considered to be functionally significant.*

4. *The work conducted here demonstrates the value of using multiple assays in any assessment, particularly when the sensitivity and robustness vary. For example, biomarker responses often identified exposure, while the bioassay and functional assessment established the ecological significance of the effect. Many of the assays used in this study can be conducted in a complementary manner. The earthworm reproduction test, for example, can also generate worms for biomarker assessment. Indigenous worms can be collected when setting out bait lamina. The extra effort required to measure multiple responses is, usually, rewarded by a proportionately greater increase in the value of the data obtained.*

5. *In this study, the potential to use molecular genetic assays for detecting contaminant exposure and effects has been trialled. This field is developing rapidly. Medical diagnostics is the principal driving force. In their wake, there is opportunity to use of some the high throughput gene profiling methods being developed to assess the effects of a variety of environmental cues and stresses on relevant species, including soil fauna. The pace of development in this field means that the Agency should remain informed on current developments (Kille et al., 2003). The Agency should also be prepared to support specific initiatives that will move developing technologies forward into application. This support should be rewarded by the development of some extremely powerful diagnostic tools.*

6. *After finalising the framework and final agreement on the biological tests that should be included, a full road-testing of the finalised risk assessment procedure is recommended. This should be conducted in real time. Its aim should be to publish a dossier relating to the status of the site of the type that would be required by the Agency prior to initiation of appropriate action. The collaboration of contaminated land officers and other Agency staff in this initiative is to be encouraged. Best value will be gained if this study is conducted at previously characterised sites that are representative of the locations the Agency would be most likely to encounter during the implementation of policy directives.*

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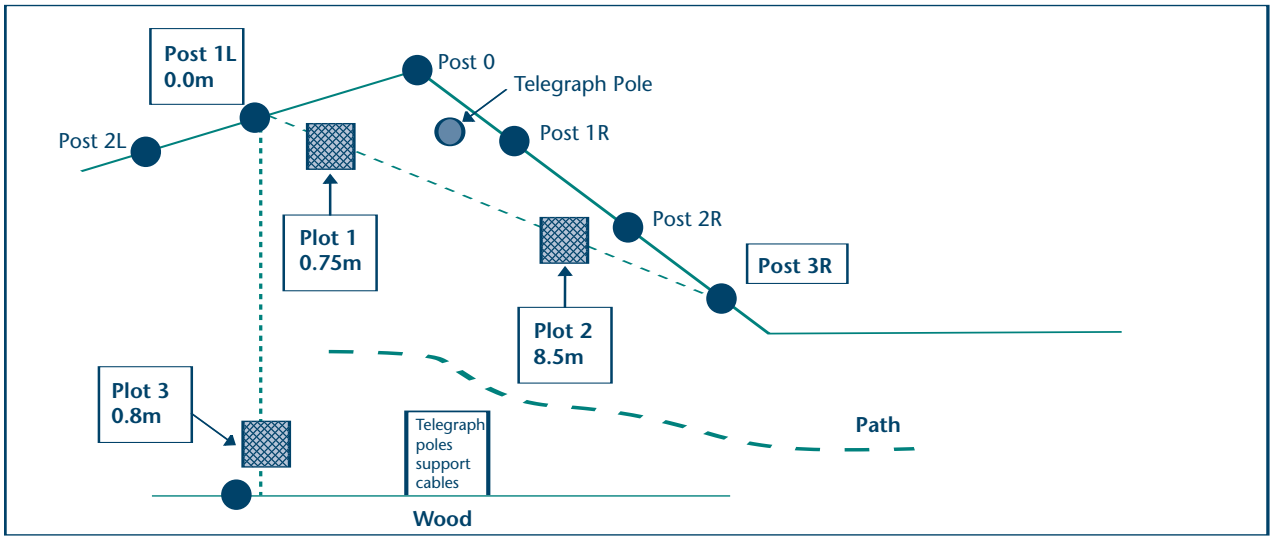
Appendix 1

Location plans used in setting out bait lamina strips at Site A, patches 1, 3 and 4

Site A, Patch 1



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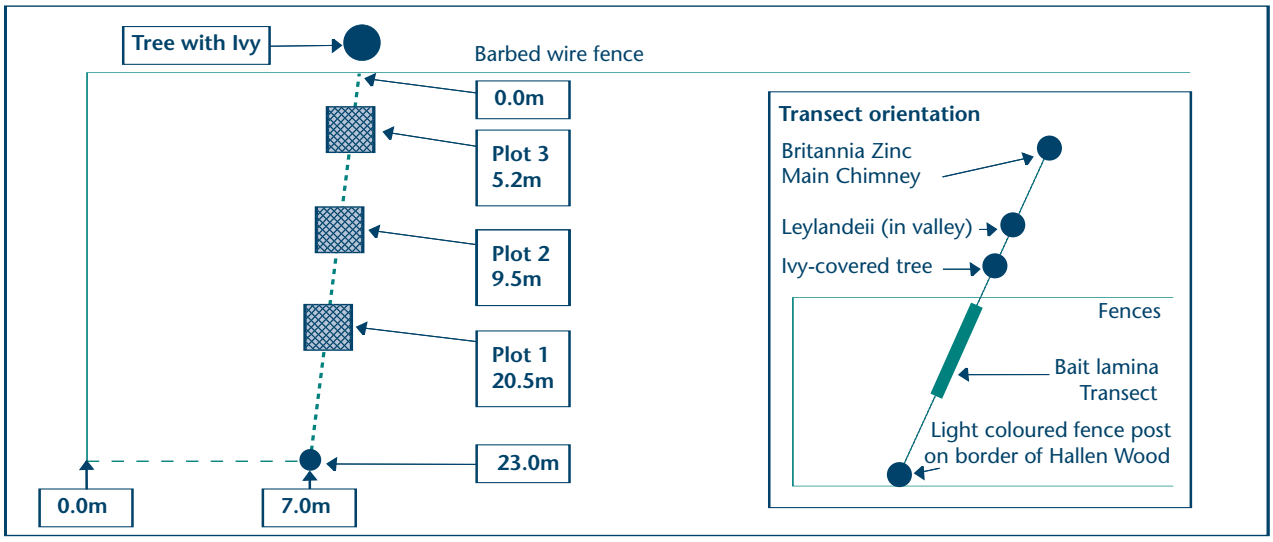
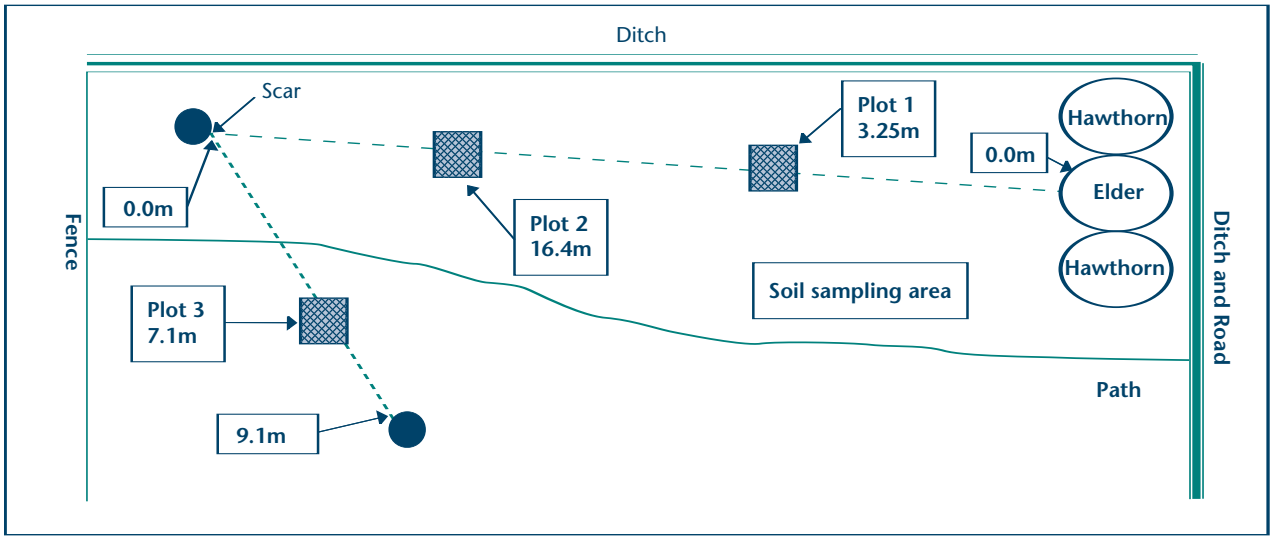


Site A, Patch 4



List of abbreviations

Abbreviation	Meaning thereof
AAS	Atomic absorption spectroscopy
ALAD	Aminolevulinic acid dehydratase
ANOVA	Analysis of variance
ß-act	ß-actin, a eukaryotic protein important in cytoskeleton and muscles
BTEX	Benzene, toluene, ethylbenzene, xylenes
cDNA	Complimentary DNA
ChE	Cholinesterase
DDT	2,2, bis (p-chlorophenyl)-1,1,1-trichloroethane
DGGE	Denaturing gradient gel electrophoresis
EQS	Environmental Quality Standard
EROD	Ethoxyresorufin-o-dealkylase
GLM	General Linear Model
GC-MS	Gas Chromatography Mass Spectrometry
GST	Glutathione S-transferase
ICP-OES	Inductively Coupled Plasma Optical Emission Spectroscopy
ICRCL	Interdepartmental Committee on the Redevelopment of Contaminated Land
IRPI	Instantaneous Rate of Population Increase
ISO	International Organisation for Standardisation
LOI	Loss on Ignition
mRNA	messenger RNA
MT	Metallothionein
NRRT	Neutral red retention time
OECD	Organisation for Economic Cooperation and Development
P450	Cytochrome P450 monooxygenases
PAH	Polycyclic aromatic hydrocarbon
PCR	Polymerase chain reaction
PLFA	Phospholipid fatty acid
Q-PCR	Quantitative Polymerase Chain Reaction
Q-RT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
rDNA	Ribosomal Deoxyribose Nucleic Acid
RNA	Ribonucleic acid
rrnL	Large sub-unit of the mitrochondrial ribosomal system
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SSSI	Site of Special Scientific Interest
TEM	Toluene Extractable Material
TPH	Total Petroleum Hydrocarbons
T-RLFP	Restriction Fragment Length Polymorphism
WHAM	Windermere Humic-Aqueous Model

Glossary

Bioassay A test in which the toxicity of a contaminant or environmental sample is measured by exposing a specific organism and measuring a life-cycle (e.g. survival, reproduction, development, growth) parameter.

Bioavailability The degree to which a chemical can be taken into the tissues of an exposed organism.

Biomarker Any biological response to an environmental chemical at the individual level or below demonstrating a departure from the normal status.

Biosensor An analytical device composed of a biological recognition element directly interfaced to a signal transducer, which together relate the concentration of a pollutant to a measurable response.

Chronic Characterised by a time period that represents a substantial portion of a life span of an organism (e.g. chronic toxicity is the characteristic of a chemical to produce a toxic response when an organism is exposed over a long period of time).

Coelomocyte Descriptive of cells found within the fluid contained within the main body cavity of many invertebrates (including earthworms).

Concentration The amount of a chemical substance expressed relative to the amount of environmental medium (e.g. micrograms of chemical per gram of soil).

Demography The study of organisms in a population and their variations over time.

Detritivore Organisms that feed on dead plant and animal matter, breaking it down physically and chemically and recycling elements and organic compounds to the environment, and which include chiefly microorganisms and small animals.

Dicotyledon Plant with two primary leaves (e.g. lettuce and carrot).

Epigeic Organisms that live close to the soil surface.

Eukaryotes Organisms with cells possessing a membrane bounded nucleus in which the DNA is complexed with histones and organised into chromosomes.

Expression (of a gene) The method by which codes within a gene are translated to their end product.

Hormesis The phenomenon whereby toxins have a positive effect on the health of organisms at low doses.

Kriging A method of spatial data analysis that allows interpolation to surfaces or isotherms from point sample data.

Lysosomes Sub-cellular particles that contain enzymes used for digestion and removal of dead cells and other particles.

Mesocosm Medium-sized multi-species test system for assessing toxicity.

Microarray An orderly arrangement of DNA samples on a “gene chip” that provides a medium for matching known and unknown DNA samples based on base-pairing rules and automating the process of identifying the unknowns.

Monocotyledon Plant with a single primary leaf (e.g. wheat and rice).

PCR Polymerase chain reaction is a reaction in which specific DNA sequences are multiplied by sequential splitting of double stranded DNA molecules, annealing of specially designed complimentary oligonucleotide primers and extension to form a complimentary strand under the action of heat stable DNA.

Pore water Water present within the interstitial spaces between soil inorganic and organic particulate matter.

Reverse transcription The process by which single-stranded RNA is converted to a double-stranded DNA copy.

Rhizon sampler A soil solution extractor consisting of a porous polymer tube connected to a PVC tube and a connector vacuum tube or syringe. Pore water samples are collected under vacuum.

Ringer solution A solution of inorganic salts that mimic the physiological conditions surrounding cells in the body.

Sequence The order of base pairs within a strand of nucleic acid.

Speciation Refers to the various forms in which chemicals occur (e.g. metals, ions complexes).

Transcription The process by which DNA is read and used to construct molecules of RNA.

WHAM Windermere Humic-Aqueous Model, designed to calculate equilibrium chemical speciation in surface and ground waters, sediments and soils.

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