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The use of biomonitoring tools to detect ecosystem response following exposure to contaminants emitted from a regulated industrial source

Science Report – SC030175/SR3

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Steve Killeen

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Executive summary

Until now, regulation to protect the environment has relied mainly on chemical and physical measurements of air pollutant concentrations. Such methods have worked well historically when pollutant concentrations were high enough for their impacts to be inferred unambiguously. However, they ultimately offer only a measure of exposure, rather than of the actual impact on living organisms and the ecosystem. In the current climate of much-reduced pollution concentrations more direct evidence is frequently called for in order to demonstrate a link between changing exposure and actual environmental outcome.

Biomonitoring measures the actual response of an organism to a pollutant. At the very least, bioaccumulation of pollutants in living organisms provides an early warning system of potential impacts on the ecosystem, and shows how pollutants are being transferred through the food chain. At best, biomonitoring captures the accumulated response to pollution in one 'snapshot', taking into account the full circumstances of the ecosystem under consideration.

In order to explore the practicality of an 'outcomes' approach to pollution control, as described in two associated reports (*Air quality outcomes in pollution regulation: strengths, limitations and potential* and *Review of biomonitoring for assessment of air quality outcomes*), a case study was designed to assess the feasibility of using a selection of techniques (chemical analysis of soil, animal and plant tissue; plant growth tests; bait lamina strips; biochemical assays of biota tissue) to detect a signal in the landscape arising from pollutants emitted from a well-characterised source. An elevated point source was chosen for the study since such a source would typically give rise to gradients of ground concentrations extending over several kilometres, against which the performance of the biomonitoring tools could be assessed.

The specific test site, an area surrounding such a source, was selected because the site was thought to be relatively free of the influence of other sources which might interfere with any spatial signal from the point source. Data and model outputs were available from a review of air quality which showed locations likely to be exposed to a range of concentrations of the air pollutants emitted from the stack.

The required sample and replicate numbers for the study were estimated using a statistical power analysis based on data from a contaminated industrial site.

The suite of test media included soil, nettles, earthworms and small mammals as passive accumulators of pollutants. The study focused on polycyclic aromatic hydrocarbons (PAHs, determined by extraction followed by GC-MS) and metals (Cd, Cr, Cu, Ni, Pb, V, Zn, determined by ICP-AES).

Functional biological tests included feeding activity assays using bait lamina strips, seed germination and growth rate in sampled soils, metallothionein mRNA expression in earthworms as an indicator of exposure to metals and increased cytochrome P450 activity as a result of exposure to planar organic molecules such as PAHs.

All of the suite of metals tested for were found in the soil, earthworms and shrew livers, and all but vanadium and lead in nettles. There was no clear spatial pattern

except for chromium and nickel, which showed an initial increase followed by a decrease in concentration with distance from the source , consistent with a source dispersing from an elevated stack (a similar pattern was not observed, however, for other metals generally regarded as co-emitted with chromium and nickel). Expression of metallothionein mRNA was observed in earthworms. This correlated with levels of cadmium in the earthworms and also, though more weakly, with lead concentrations.

Several sites showed elevated PAH levels, with some exceeding international soil guideline values. Levels were sometimes, but not always, high in earthworms sampled from sites with high soil PAH, suggesting that bioavailability of PAH may vary. Soil organic matter content was found to vary between the two transects taken; the presence of organic matter is known to reduce bioavailability of PAH and so should be recorded. Elevated cytochrome P450 activity was also seen in the livers of shrews taken from sites with raised PAH levels in the soil.

Seed germination and feeding activity varied from site to site, but no clear pattern emerged linking the variation to other factors such as metal or PAH levels The non-specific nature of both tests makes them susceptible to confounding factors such as temperature and moisture variation, which might mask more subtle responses to contaminants.

All of the functional biomonitoring tests proved practicable. They responded in varying degrees to local conditions, but did not show patterns of spatial variability that might be associated predominantly with emissions from any single source This general lack of a clear spatial pattern in the data may arise as a consequence of multiple sources influencing the test area, or of natural variations in natural or anthropogenic background which are at least as great as local source contributions

One of the sampling sites (a children's playing field) chosen within this area showed levels of PAH that were elevated compared with other sites in the study. It transpired to have once been a landfill site. Another historical influence may have been coal-fired train emissions from a local marshalling yard. This highlighted the importance of carrying out a detailed survey prior to site selection to ensure that any historical or current factors which may influence the results are accounted for. Use of biota in conjunction with soil analysis offers a rapid screening approach to assess pollutant bioavailability, which could indicate the likelihood of a contaminant entering the food web. The cytochrome p450 activity test is relatively inexpensive and so might serve as a useful screening method to identify areas contaminated by PAH or certain pesticides, before the more expensive technique of extraction followed by GC-MS is used. Earthworms can be collected easily, but capturing small mammals is labour-intensive and so potentially costly.

When planning long-term biomonitoring the future, as well as the history of potential sampling should be considered. For example planning consents around the site should be investigated, to ensure there are no planned changes during the project which would impact upon the site.

This study was designed primarily to establish the feasibility of using these tests in the field. When designing biomonitoring schemes in which the primary aim is to establish pollutant levels and patterns, considerable effort should be devoted to sampling design to ensure the required level of statistical power can be achieved.

Contents

1. Introduction

1.1 Background

Until now, regulation to protect the environment has relied on chemical and physical measurements, such as ambient air concentrations, to provide information on emissions or atmospheric chemistry. Measurements of this type can also be used to model impacts upon the environment, where a pollutant has a known level of toxicity or critical load for a given organism or ecosystem that can be compared with a measured ambient concentration. However, this method can only show that exposure has occurred; it cannot describe the actual impact on the biota or ecosystem. Biomonitoring, on the other hand, measures an actual response in biota to a pollutant and shows that pollutants are biologically available to the organism. It provides a first level assessment of an ecosystem's health, and makes use of a diverse range of techniques measuring responses of individual organisms (or parts of organisms, such as at the cellular level), and of communities (ecosystem level).

Accumulation of pollutants in organisms provides an account of exposure over time, with or without visible injury symptoms. In the case of no visible symptoms, accumulation offers an early warning system of potential impacts and shows the transfer of pollutants within the biological chain. In other words, bioaccumulation is an effect which may have no impact or outcome on the organism. However, responses at the community level (such as changes in species composition) which often occur over years can be described as an outcome, in that the effect and impacts have already occurred. Both types of biomonitoring have the advantage over physical and chemical measurements of measuring the actual response of a community or organism.

While physical monitoring is susceptible to temporal differences over daily, monthly and seasonal scales, biomonitoring can capture the integrated response to these changes. Therefore, useful information about the long term effects of a pollutant can be captured by measurements taken in the field on a single occasion.

In order to demonstrate the potential of an 'outcomes' approach to pollution control, as described in associated reports *(Air quality outcomes in pollution regulation: strengths, limitations and potential* and *Review and implementation study of biomonitoring for assessment of air quality outcomes*), a case study was identified in consultation with Environment Agency staff in which the principles of, and tools for, 'outcomes' monitoring could be tested. This report describes the case study, and the results obtained.

1.2 Aims

The aim of the field study was to test the effectiveness and practicality of a suite of biomonitoring tools for measuring responses to exposure to contaminants emitted from a pollution source. Tests chosen ranged from the "passive collector" type, such

as chemical analysis of animal and plant tissue, to the functional, using plant growth and feeding activity tests and biochemical assays of biota tissue.

1.3 Site selection

An elevated point source was chosen as the type of study site. Such sources in the UK are well characterised in terms both of emissions and local dispersion patterns. Their height allows dispersal and subsequent deposition to take place over distances of a few kilometres, and in the near-field the expected pattern of deposition is expected to give rise to spatial variations of concentration along the proposed transects which would offer a range of conditions against which to test the biomonitoring tools. Several sites were discussed in the scoping phase of the project, and the source which was eventually selected combined a minimum of potential confounders with relatively simple local topography and a good range of access to potential sampling points. Detailed emission records were available, together with dispersion modelling indicating what patterns of ground-level pollutant concentrations might be expected to have occurred over time, including a contour plot which modelled ground level SO_2 concentrations arising from emissions from the source. This gave an indication of locations likely to be subject to the highest concentrations of air pollutants emitted from the source stack, and showed concentration variation along the proposed sampling transects.

No other large industries were close to the site, and there was approximately 20 miles of open country between it and the nearest motorway. Suitable sampling sites were available on two transects. Potentially confounding influences around the source included the motorway and a number of landfill sites, as well as a mainline train line with a spur for delivery of coal to the source and a marshalling yard which historically saw much activity by coal-fuelled locomotives.

2. Methods

2.1 Fieldwork

2.1.1 Experimental Design

Given the time constraints of the project, it was decided to use spatial (geographical) changes in biological and chemical responses as a surrogate for changes that might occur following implementation of measures to further reduce ambient pollution levels. To a first approximation, samples collected further away from the pollution source might be expected to reflect the 'after' response, being from relatively clean areas, whereas samples collected close to the source would reflect the 'before' response, being from more polluted areas.

An initial survey of the area was carried out to identify suitable sampling sites and any permission needed for access to the sites. Two orthogonal transects running from the source were chosen. One transect ran east-north-east from the source. Samples collected from this transect would potentially be the most affected by emissions because of the prevailing south-westerly wind. This was reflected in modelling and monitoring of pollutant concentrations carried out by the site operator. One of the sites chosen was close to an air quality monitoring station and this station showed elevated concentrations of pollutants compared with a site directly north of the source. The second transect ran north as this was thought to be a direction less likely to be influenced by the source.

Power analysis
A power analysis was conducted using previously collected field data from an unrelated site. The power analysis was used to estimate the number of sample sites and replicates that would be required to detect a measurable change in soil metal concentration; see Appendix 1 for details of the power analysis.

The chosen sampling scheme involved replicate sampling at sites along both transects. Site locations are given in Table 2.1.

Table 2.1: Site locations

	Distance from source (km)	
Site	Transect 1 (East Transect)	Transect 2 (North Transect)
1	0.5	0.5
2	1.5	1.5
3	3.0	2.5
4	4.0	(4.0)
5	(7.0)	6.0
6	10.5	9.5

The figures shown in brackets are suggested extra locations to augment the original proposed scheme of five locations. To offset the greater number of sites, the number

of replicate samples at each site is reduced from five to four, reducing the total number of samples from 50 to 48. Following the power analysis, it was decided to sample at six locations per transect and reduce the replicate number to four at each location. Appendix 1 shows the steps used in the power analysis to determine sampling locations and replicate numbers.

2.1.2 Small mammal trapping

A licence was obtained from English Nature to trap common shrews (*Sorex araneus*) from the sites. Shrews were captured in Longworth live traps (Maddox *et al.,* 1998) in August 2005 from six out of the 12 sites. Shrews were transported to a laboratory where they were humanely killed. Livers and kidneys were dissected, snap frozen in liquid nitrogen and stored at -80^{o}C until analysis.

Figure 2.1: Longworth traps for trapping small mammals.

2.1.3 Soil, nettle and earthworm collection

Soil was collected from all sites in August 2005, with four replicates sampled per site. Each replicate was sampled as follows: soil was collected from each corner of a one metre square and placed in a polypropylene box with a lid. Soil was returned to the laboratory and stored at 4^{o}C until analysis.

Nettle leaves were collected at each site in August 2005, with four replicates sampled per site. The nettle leaves were placed in sealed plastic bags, returned to the laboratory and stored at 4^{o}C until analysis.

Lumbricus species were collected from all sites in October 2005. Earthworms were separated into four replicates (two to three earthworms per replicate) and placed into plastic boxes with damp tissue paper. Earthworms were returned to the laboratory and placed in an incubator at 12^{o}C for a further 24 hours to allow evacuation of gut contents before being snap frozen in liquid nitrogen. The frozen earthworms were stored at -80^{o}C until analysis.

2.1.4 Bait lamina test

To measure soil organism feeding, bait lamina strips purchased from Terra Protecta GmbH, Berlin, Germany were deployed (see Figure 2.2). Bait material made from a mixture of cellulose, activated charcoal and bran was inserted into holes (16 holes per strip, five mm apart) in the test strips and deployed vertically into the soil. Bait lamina strips were deployed at all sites in October 2005. Three replicates of 12 strips per replicate were deployed at each site. Strips were inserted with the aid of a stainless steel insertion tool and were deployed two cm apart in a square configuration, with four strips per row and three rows per replicate.

Strips were also placed at Monks Wood and were removed on a daily basis to determine the optimum time to return to the site to remove the test strips; this is when approximately 50 per cent of the bait has been eaten, usually three to four weeks after deployment. Strips were removed, wrapped in cellophane wrap and stored at 4^{o}C until analysis. Strips were gently rinsed in water to remove soil and scored according to Filzek *et al.* (2004). Briefly, each strip was given a score out of 32 according to the amount of bait remaining in each hole: zero for no consumption, all bait remaining; one for partial consumption; and two for no bait remaining, completely pierced. The per cent overall feeding activity (OFA) was recorded per strip. Mean percent OFA per replicate was arcsine square root transformed prior to statistical analysis (Zar, 1999).

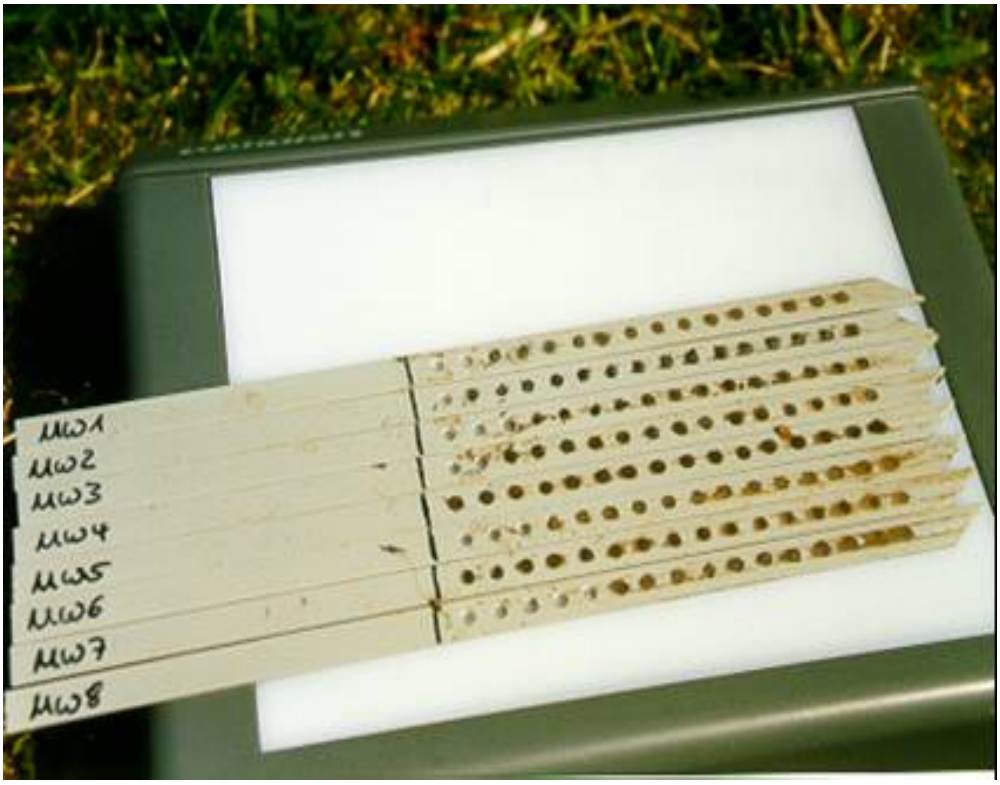

Figure 2.2: Bait lamina strips following *in situ* exposure

2.2 Laboratory work

2.2.1 Soil parameter analysis

Methods for measuring soil pH and organic matter are described in Kalra and Maynard (1991).

Soil pH was measured potentiometrically in a saturated paste or a supernatant liquid that was in equilibrium with a soil suspension of a 1:2 soil mass to liquid volume mixture (1:4 for organic soils). Ten grams of 2mm-screened air-dried soil were weighed into a beaker and 20 ml 0.01M $CaCl_2$ solution were added. The soil was allowed to absorb $CaCl_2$ solution without stirring, and then was thoroughly stirred for 10 seconds. This was repeated four or five times over the next 30 minutes. The suspension was allowed to settle for 30 minutes and the pH of the solution was measured and recorded.

Organic matter is oxidised by heating at 375^{o}C and is estimated by weight loss. Approximately 5 g of soil were weighed into each crucible and dried at 80^{o}C overnight. The crucibles were re-weighed and then placed in a muffle furnace and dried at 375^{o}C for eight hours. Once the crucibles had cooled, the weights were recorded.

2.2.2 Residue analysis

Soil and earthworm polycyclic aromatic hydrocarbon (PAH) analyses were only carried out on six of the sites, three sites per transect, on the same sites used for small mammal trapping.

Soil extraction

A sub-sample of soil (25g) was taken by quartering the soil. Two handfuls of soil were placed onto a metal tray and divided into four quarters. Two opposite quarters were discarded and the remaining soil was mixed together. This process was repeated until the desired sample size was obtained, ensuring that the subsample would be representative of the whole soil sample.

This sample was then placed into ounce universal containers. A sub-sample (5g) was dried using Kieselguhr and placed into an accelerated solvent extraction (ASE) cell and spiked with a deuterated PAH surrogate standard. For quality assurance, sample blanks and a certified reference material (CRM) were run with each batch. Samples were extracted in the ASE cell using dichloromethane as the solvent. The extract was filtered through glass wool and anhydrous sodium sulphate to remove any water and then reduced in volume to 1 ml using a Turbovap set at 40^{o}C under a stream of nitrogen gas. The concentrated extract was cleaned up by open column chromatography using activated alumina prior to analysis by gas chromatography mass spectrometry (GC-MS), using an Agilent 6890 GC-MS.

Earthworm tissue extraction

Two grams of earthworm tissue were placed in the ASE cells with Kieselguhr and sand and spiked with a deuterated PAH surrogate standard. For quality assurance, sample blanks, spiked recovery and a recovery control sample were run with each batch. PAHs were extracted in the ASE cell using dichloromethane as the solvent. The extract was filtered through glass wool and anhydrous sodium sulphate to remove any water and was reduced to 0.5 ml using a Turbovap at 40^{o}C under a stream of nitrogen gas. Approximately 5 ml of n-hexane were added to the concentrated extract and concentrated down to dryness. N-hexane was added to the dried down sample and 1 ml of this solution was cleaned up by open column chromatography using glass wool and 5% deactivated alumina; n-hexane was used as the solvent. One ml of the cleaned up solution was transferred to a chromatography vial for analysis by GC-MS.

GC-MS

The following parameters were used for the GC-MS analysis: initial oven temperature 50^{o}C, initial time 2.5 minutes, maximum oven temperature 360^{o}C, equilibration time 0.5 minutes. Column type was SGE HT8 50 m x 0.22 mm, df 0.25 µm fitted with a 5 m x 0.25 mm id retention gap (Thames Restek) using a Vu2 column connector (Thames Restek). Column nominal length was 55.4 m, nominal diameter was 220 µm and nominal film thickness was 0.30 µm. The mode was constant flow, with an initial flow rate of 2.0 ml/min and initial pressure of 40.69 psi; the average velocity was 38 cm/sec.

2.2.3 Metal analysis

This was carried out on soil, nettle leaves, earthworm tissue and shrew livers.

Digestion of soil and nettles

Sub-samples (1g) of air-dried soil were placed into boiling tubes ready for cold digestion. For quality assurance a sample blank, spike control, spike recovery and two CRMs were run with each batch of samples. Concentrated hydrochloric acid (7.5 ml) was added to each sample followed by 2.5 ml concentrated nitric acid (HNO_3). The tubes were sealed and left in the fume cupboard to cold digest for a minimum of 12 hours. After this time, the boiling tubes were placed in hot blocks at 90^{o}C for 20 minutes followed by two hours at 120^{o}C. The condenser was rinsed with approx 5 ml 12.5% HNO_3 and washings were allowed to collect in the boiling tube. The digestate was filtered (Whatman No 6 filter paper) and made up to 25 ml with 12.5% HNO_3.

Analysis by ICP-AES

Soil and nettle samples were received as *aqua regia* digests. Calibration standards and calibration verification standards were made up in 12.5% Aristar HNO_3. One sample from each batch was analysed in duplicate. Results for calibration verification standard and duplicate samples were within expected ranges.

Spiked samples were diluted tenfold prior to analysis and the results corrected afterwards.

Samples were analysed on PerkinElmer 4300DV Inductively Coupled Plasma – Atomic Emission Spectrometer (ICP-AES) with the following conditions:

- Gemcone nebuliser;
- Scott double-pass spray chamber;
- Radial viewing mode.

Detection limits based on 4.65 x standard deviation of 10 consecutive blank readings to give 99% confidence.

Table 2.2: Wavelengths and limits of detection for nettle and soil metal analyses using *aqua regia* digests

	Cd	Cr	Cu	Ni	Pb	V	Zn
Wavelength/nm (nettles)	228.8	267.7	324.7	231.6	220.3	290.8	206.2
Detection limit mg/l (nettles)	0.001	0.001	0.002	0.009	0.006	0.002	0.003
Wavelength/nm (soil)	228.8	357.8	327.4	231.6	220.3	292.4	206.2
Detection limit mg/l (soil)	0.002	0.008	0.003	0.034	0.061	0.008	0.002

Liver and earthworm tissue

Tissue samples (approximately 0.5 g) were weighed into a boiling tube and dried at 80°C for at least 24 hours. For quality assurance a sample blank, spike control, spike recovery and CRM were run with each batch of samples. Concentrated nitric acid (0.5 ml) was added to the dried sample and left to cold digest overnight. After this time, the boiling tubes were placed in hot blocks at 90°C for 20 minutes followed by two hours at 120°C. Hydrogen peroxide (0.2 ml) was added just prior to the end of hot digestion to aid digestion of fats. The digestate was transferred into ounce universals and made up to 5 ml using distilled deionised water (final concentration of solution was 10% nitric acid).

Analysis by ICP-AES

Samples received as 10% HNO_3 digests. Calibration standards and calibration verification standards made up in 10% Aristar HNO_3 plus 0.1 mg/l yttrium. One sample from each batch was analysed in duplicate. Results for calibration verification standard and duplicate samples were within expected ranges.

Samples were analysed on PerkinElmer 4300DV ICP-AES with the following conditions:

- Meinhardt nebuliser;
- Cyclonic spray chamber;
- Axial viewing mode;
- 0.1 mg/l yttrium internal standard.

Samples were centrifuged for 20 minutes at 3,000 rpm to remove sediment. Five ml were then removed from each sample and added to an acid-washed test tube before pipetting in 50 µl of 10 ppm yttrium and shaking.

Detection limits based on 4.65* standard deviation of 10 consecutive blank readings to give 99% confidence.

Table 2.3: Wavelengths and limits of detection for whole earthworm and shrew liver tissue digests

	Cd	**Cr**	**Cu**	**Ni**	**Pb**	**V**	**Zn**
Wavelength/ nm	228.8	267.7	324.4	231.6	220.3	290.8	206.2
Detection limit mg/l	0.0001	0.0002	0.0003	0.0003	0.0014	0.0015	0.0004

Cytochrome P450 analysis

The catalytic activity of cytochrome P4501A was measured in shrew liver tissue using the method described by Kennedy and Jones (1994), Trudeau and Maisonneuve (2001) and Long *et al.* (2006). Tissue was homogenised in 0.15 M KCl and the microsomal fraction collected after centrifugation at 9,000 g followed by 100,000 g. The microsomal fraction was resuspended, snap frozen in liquid nitrogen and stored at -80°C until enzyme analysis (no more than one week after microsome preparation).

Ethoxyresorufin-*O*-deethylase (EROD) was measured on a Synergy HT fluorescent microplate reader using resorufin as the standard and an increase in fluorescence

was observed over time. Samples were run in triplicate and an internal standard was used on every plate. A Lowry assay (Lowry *et al.,* 1951) was conducted on the microsomal fraction to measure the total protein content and to allow EROD activity to be expressed as pmol resorufin formed per minute per mg protein.

Plant growth tests

The method used for the plant growth test is described in detail in Environment Canada (2005). Briefly, soil was sieved through a 10 mm mesh to remove debris and the water holding capacity (WHC) was determined. The soil was placed into plastic plant pots (10 cm diameter), five replicates per site, and wetted to 80 per cent WHC using distilled water. Positive (artificial soil spiked with 600 mg/kg boric acid) and negative controls ran alongside the sample soil.

Five lettuce seeds (*Lactuca sativa*) were placed at the top of each pot and lightly covered with soil. This species grows in a variety of soil types including sandy loams and clay soils and requires a pH of between 6 and 8 (Canada, 2005). Plant pots were covered with cellophane film and placed randomly in a constant temperature room (12^{o}C) for seven days. After seven days, the number of emerged seedlings (greater than 2 mm in height) was recorded for each pot and the pots were moved to a glasshouse for the remainder of the test. Fourteen days after the beginning of the test, the number of emerged seedlings was recorded and the shoots carefully removed, measured in length and dried at 80^{o}C for 48 hours and the dry weight recorded.

Metallothionein gene expression in earthworm tissue

The method used is described in Spurgeon *et al.* (2004). Briefly, earthworm tissue was crushed under liquid nitrogen and homogenised in a phenol buffer (TRI-reagent). The resultant homogenate was centrifuged at 10,000 rpm and the RNA extracted using chloroform. The pellet was then cleaned up using RNeasy spin columns and the total RNA quantified on a UV-Vis spectrophotometer. RNA was reverse transcribed to complementary DNA by reverse transcription polymerase chain reaction (RT-PCR). Primers specifically designed to recognise *Lumbricus terrestris* metallothionein sequences were used to quantify the PCR products and determine the quantity of metallothionein mRNA present in earthworms from each site.

2.2.4 Data analysis

Analysis of variance was used to check for differences between the sites ($P < 0.05$). Tukey's *post hoc* test was then used to determine where the differences were. Prior to analysis, datasets were checked to ensure that they conformed to the assumption of homogeneity of variance between groups and normal distribution of residuals. Correlation and regression were carried out on the chemical residue data to determine whether the concentration of contaminants changed with distance from the source and whether contaminants within the same chemical class co-occurred.

3. Results and discussion

3.1. Fieldwork

3.1.1. Site access

Permission was obtained for access to all sites except the 10 km site on the North Transect; this site was excluded from fieldwork, resulting in a total of 11 sites. Soil, earthworms and nettles were collected and bait lamina strips placed at all sites. Small mammals were trapped at three sites on both transects, a total of six sites.

3.1.2. Small mammal trapping

Longworth traps were set on four trapping nights over a two-week period. Fifteen traps were set at each site in the evening and were checked twice during the day, once in the morning and again in the evening. The species, numbers and weights of small mammals trapped were recorded (see Appendix 2); all species except *Sorex araneus* (common shrew) were released. Because of the timing of trapping (mid-summer), juvenile shrews were trapped rather than adults; therefore the organs (liver and kidney) were relatively small and few analyses could be done on them. No shrews were collected at two sites in the East Transect. It was thought the sites were suitable habitat for shrews and so it was surprising that no shrews were collected. Moreover, no small mammals were collected at Site 6 throughout the study. This site was close to a bridleway, which may have had an impact on the presence of small mammals.

There are limitations to sampling small mammals. It is time-consuming, in that it took eight person days to sample the sites, and the numbers of organisms collected were small, which makes it an expensive biomonitoring tool in labour terms. Timing is also a factor: to trap adults, sampling should ideally take place in early summer, as the young will be distinguishable from the adults and there are likely to be more adults. It is preferable to trap adults for bioaccumulation purposes because they will have been exposed for longer. To sample shrews requires a licence from English Nature. Nevertheless, shrews are ideal small mammals to sample because they are insectivores and will consume invertebrates, indicating effects/impacts at higher levels of the food chain. They also have small territories, and so are unlikely to roam from one sampling site to another.

3.1.3. Earthworm collection

Lumbricus species of earthworm were collected from most sites in October 2005. No earthworms could be found at Site 4 on the North Transect, possibly because the soil was very dry, despite recent rainfall. *Lumbricus rubellus* and *L. terrestris* earthworms were collected from the other sites.

3.2. Laboratory work

3.2.1. Soil parameters

There were no significant differences in soil pH at the sites (see Appendix 3) ($P = 0.444$). However, there were differences in percent organic matter ($P < 0.001$); soil from the North Transect tended to have less organic matter than soil from the East Transect.

3.2.2. Chemical analyses

PAH analysis of soil and earthworm tissue

Varying levels of polycyclic aromatic hydrocarbons (PAHs) were present in the soil and in earthworms collected from various distances from the source (Figure 3.1).

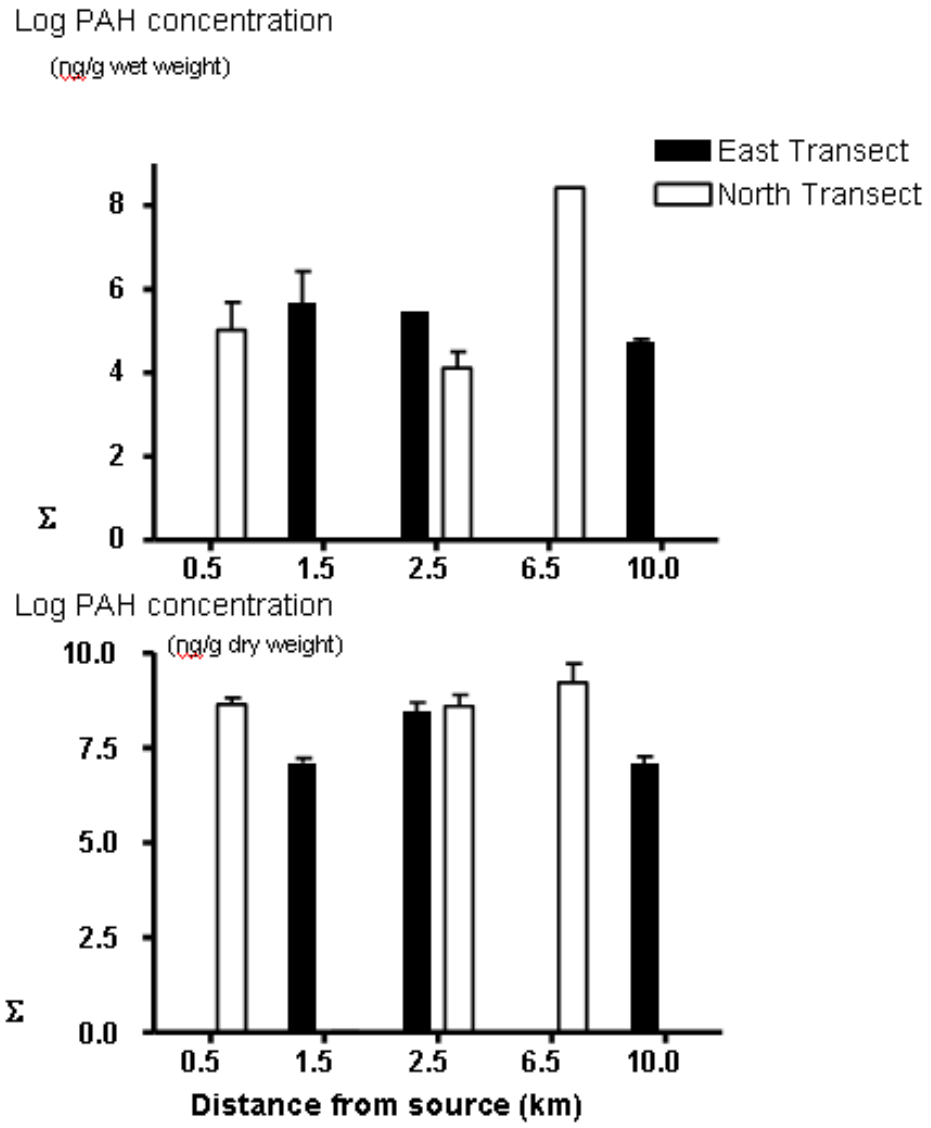

Figure 3.1: Graph showing log concentrations of the sum of 15 PAHs (as recommended for analysis by the US Environmental Protection Agency) present in earthworm (top graph) and soil (lower graph) from six sites at various distances from the source. Bars represent the mean +/- SE of four replicates for the soil analysis and at least two replicates for the earthworm analysis.

A correlation matrix showed that sites with high soil concentrations of total PAHs also had high soil concentrations of individual PAH compounds, indicating they were released together (Figure 3.2).

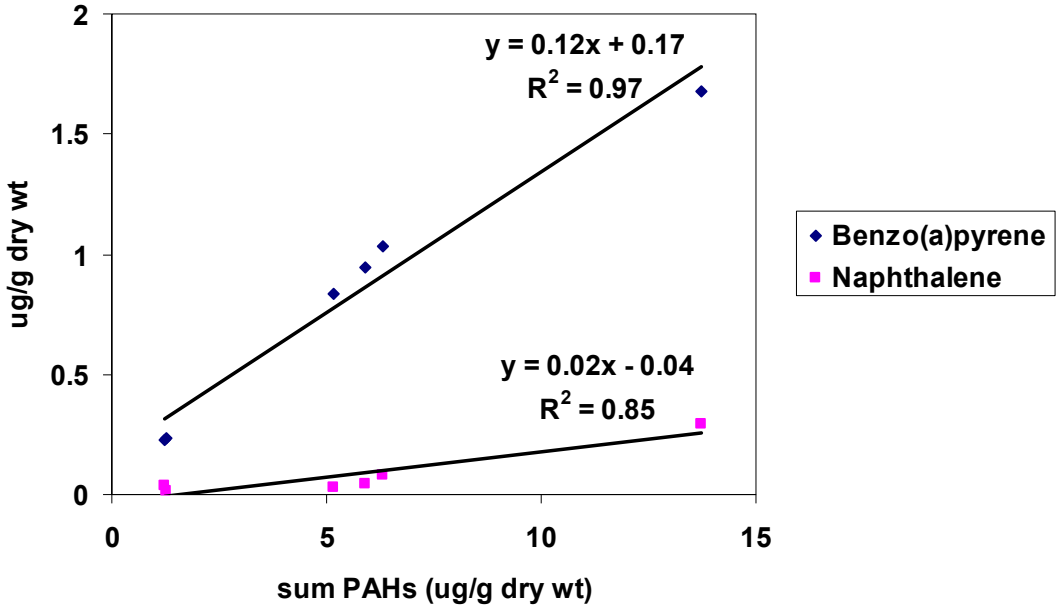

Figure 3.2: Graph showing the correlation between total and individual PAHs in soil collected at various distances from the source

PAH concentrations in worms from Site 5 on the North Transect were elevated compared with other sites, which fits with the soil PAH data. However, PAH concentrations in earthworms at the other sites were low. This may have been due to low concentrations in the soil but, given that Sites 1 and 3 in the North Transect and Site 3 in the East Transect showed elevated soil PAH concentrations, higher concentrations in the corresponding earthworms would have been expected. An alternative possibility is the bioavailability of PAHs. Soils from the North Transect had less organic matter compared with soils from the East Transect. PAHs are known to bind strongly to organic matter so they may have bound more strongly to East Transect soil, reducing their bioavailability and accumulation in earthworms.

In most cases, only a small proportion of PAH compounds in a sample is measured. Chemical analysis alone does not reveal the total effect of PAHs compounds (this is dependent on availability) or whether the total PAH load of soil is being measured.

A number of international soil quality guidelines (Canadian Council of Ministers of the Environment (CCME), Netherlands (Revised Dutch List) soil remediation guidelines (VROM) and the US Department of Energy (DoE) Standards) provide information on soil contaminant concentrations regarded as acceptable at a specific receptor (Weeks *et al.,* 2004) (see Appendix 8 for soil quality guideline concentrations for contaminants measured in this study). CCME and US DoE standards were derived using toxicological data to determine threshold levels of effects for ecological receptors. The New Dutch List has two values: the first is the target value to ensure sustainable soil quality (allowing the functional properties of the soil to fully recover for plant, animal and human life). The target value also indicates the benchmark for quality in the long term. The second value is the intervention value which, if exceeded, entails serious potential risk to biota and the functional properties of the soil. A preliminary set of soil quality guidelines for UK soils is currently being developed, but only published guidelines were used for this report.

A number of sites exceeded soil quality guidelines for individual PAHs and sum PAH concentrations, (Table 3.1). However, although sum PAH levels exceeded New Dutch List target values at all sites, they were below the intervention value, meaning the functional properties of the soil would not be seriously diminished. Nevertheless, monitoring of the soil might be needed to ensure that concentrations do not exceed the intervention value.

Since bioavailability is an important determinant of whether effects will occur, measurements that also integrate this parameter (such as solid phase micro-extraction (SPME) or biota monitoring) can be used as an add-on to measurements of solvent-extractable PAHs.

All congeners measured were positively correlated ($r \geq 0.473$, $P < 0.05$) with each other. The majority of correlations (96 out of 105) were highly significant ($P < 0.001$); see Appendix 5 for the correlation matrix table. When results from Site 5 from the North Transect were omitted from the regression analysis (because the high PAH concentration was probably due to contaminated land), the PAH concentration declined with increasing distance from the source (Figure 3.3). The sum soil concentration of PAHs decreased with distance from the source (log [sum PAH] = 36.5 - 0.0544 x distance; $R^2 = 0.271$, $P < 0.05$), though the R^2 value suggests that this association, while statistically significant, accounts for only a small part of the variance in PAH concentration over the transects. Concentrations of all individual congeners also showed statistically significant, but generally weak, trend of declining concentration with distance from the source ($P < 0.05$), with the exception of fluoranthene (log [fluoranthene] = 2.80 = - 0.0406 x distance; $R^2 = 0.178$, $P = 0.064$); see Appendix 6 for the regression table. The data were skewed with outliers in the analysis, therefore the logarithm function was used to normalise the statistical distribution.

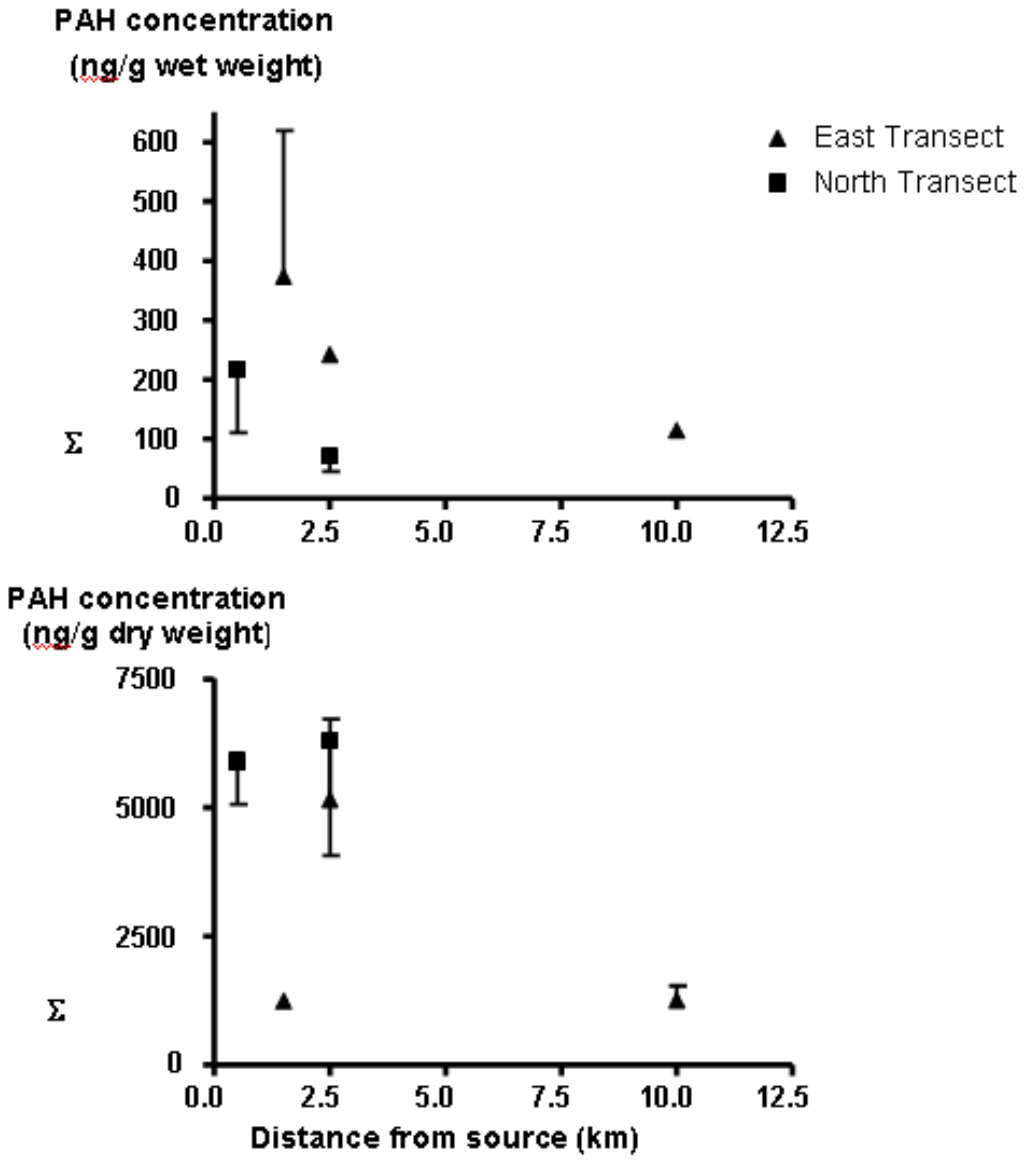

Figure 3.3: PAH concentrations in earthworms (top graph) and soil (bottom graph) from sites at various distances from the source. Data points from Site 5 along the North Transect has been excluded.

The highest concentration of PAHs in earthworms was at Site 5 on the North Transect. Soil concentrations were only slightly higher at this site than at some other sites, but the earthworm concentration was considerably higher than at those same sites. This implies a difference in uptake or bioavailability, which might arise from differences in congeners present or in local soil properties. It may be significant that this site was on a restored landfill site. There was high variability in PAH concentrations between the sites (see Appendix 4). However, the results from the certified reference material were consistent and so this variability is due to variation in soil concentration rather than analytical variation. Soil was collected within a 10 m square; however, each replicate was within a one m square so there could have been variation within this small area.

Table 3.1: Soil concentrations (µg/g dry weight) of naphthalene, benzo(a)pyrene and total PAHs at six sites at various distances from the source. Values are the mean of four replicates per site.

Contaminant	Distance from source (km)					
	East Transect			North Transect		
	1.5	2.75	10	0.5	2.75	6.5
Naphthalene [1]	0.033	0.029	0.015	0.044	0.084	0.296
Benzo(a)pyrene [1]	0.227	0.835	0.232	0.943	1.035	1.675
Sum PAHs [2]	1.240	5.158	1.262	5.893	6.306	13.721

[1] Cells shaded in grey are sites where soil concentrations exceed CCME soil quality guidelines for residential, park, commercial and industrial sites for naphthalene and benzo(a)pyrene.
[2] Cells shaded in grey are sites where soil concentrations exceed New Dutch List soil quality guidelines for total PAHs.

Limitations of PAH analysis

In general, as the PAH concentration in the sample decreases, the cost of analysis increases, making PAH analysis of areas around emission sources expensive. PAH analysis requires access to instruments such as GC-MS and HPLC and analytical staff with a good level of training and experience. All methods involve extraction of PAHs using a solvent, removal of any compounds that may interfere with analysis (clean-up) and measurement of the concentration of individual PAHs in the cleaned-up extract.

Except in cases where PAH levels are very high (such as at coal gas sites) and interfering compounds are relatively low (organic matter, petroleum and sulphur), some form of clean-up of the sample extract is required. In the simplest cases, removal of sulphur by copper followed by dilution is all that is required. In extreme cases (such as bird eggs and peaty soils), a multiple step clean-up stage involving alumina/silica column chromatography, size exclusion chromatography and fractionation by HPLC is required. In general, the lower the level of PAHs in relation to interferences, the greater the effort required for clean-up. In particular cases such as seabird eggs, interferences are extremely difficult to remove and clean-up is not always successful. Even in cases where PAH levels are high and interferences are low, some simple form of clean-up is prudent to protect instrumentation and maintain good quality control. Analysis of low levels of PAHs also carries higher costs, due to higher instrument specifications and maintenance and higher levels of staff expertise.

Analysis of tissue is particularly difficult because levels of PAHs are generally low and levels of compounds that interfere are high. The added problems of lack of certified reference materials and availability of staff with the required experience, means that few laboratories are prepared to take on analysis of this sample type. That said, ignoring the PAH content of soil and biota can lead to incorrect interpretation of the potential impacts of a pollution source.

Heavy metal analysis of soil, nettle, earthworm and shrew liver tissue

A suite of seven metals, namely cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb), vanadium (V) and zinc (Zn), was analysed in four different environmental media from sites at various distances from the source. All seven metals were detected in the soil, earthworm and shrew liver samples but vanadium and lead were not detected in the vegetation samples. Sites 3 and 5 (2.75 and 6 km from the source) along the North Transect exceeded soil intervention values for all metals. Sites 3 and 6 (2.75 and 9.5 km from the source) along the North Transect had the highest soil, vegetation and earthworm concentrations of most metals. These high metal concentrations correlate with high PAH concentrations at these sites, potentially indicating additional sources of pollution obscuring the spatial pattern and in some cases exceeding soil quality guideline values.

In general, there was no obvious spatial pattern to variations in metal concentrations, and no significant correlations between deposited concentrations and distance along the transects. Chromium and nickel were the only metals where soil concentration showed any inverse correlation with distance from the source, though no such trend was seen for other metals emitted in similar concentrations from the source. Differences in sensitivity and background levels arising from other natural and/or anthropogenic sources might account for this.

A number of sites exceeded New Dutch List intervention values for one metal in earthworms or several metals in soils. Site 3 along the North Transect exceeded the soil intervention value for all metal, in nettles and in soils. The only biomarker response which reflected elevated metal concentrations was earthworm metallothionein mRNA expression. Elevated levels of metallothionein are a response to metal exposure and have been shown in previous studies to rise following exposure to cadmium (Spurgeon *et al.,* 2005).

Table 3.2a: Metal concentrations (µg/g dry weight) in soil collected from sites at various distances from the source**.** Values are the mean of at least three replicates per site. Cells shaded in pale grey exceed the Dutch target value and cells shaded in dark grey exceed the Dutch intervention value.

Contaminant	Distance from source (km)										
	East Transect						North Transect				
	0.5	1.5	2.75	4	6.5	10	0.5	1.5	2.75	4	6.5
Cadmium	3.1	3.2	8.5	3.3	5.7	6.0	2.2	4.1	15.1	3.4	8.7
Chromium	564.6	652.9	487.4	499.9	580.7	368.7	556.6	586.7	596.6	401.6	597.8
Copper	2246.5	242.1	320.1	240.7	437.4	343.2	293.1	285.2	405.5	187.7	830.7
Nickel	540.0	465.6	418.3	316.9	500.3	554.8	393.7	443.4	404.4	289.9	522.8
Lead	1097.0	465.6	462.8	412.6	2007.8	424.8	839.2	319.3	950.5	569.7	3199.4
Vanadium [1]	756.5	936.4	661.9	674.7	1065.1	620.8	914.9	953.0	798.8	843.6	983.6
Zinc	2439.9	1344.3	1259.1	1185.3	3382.8	822.9	1488.1	1308.8	2124.6	1605.0	3812.2

[1] There is no target or intervention value for vanadium on the Dutch list.

Table 3.2b: Metal concentrations (µg/g dry weight) in vegetation collected from sites at various distances along the transects. Values are the mean of four

replicates per site. Cells shaded in pale grey exceed the US Department of Energy (US DoE) plant benchmark values.

Contaminant	Distance from source (km)										
	East Transect						North Transect				
	0.5	1.5	2.75	4	6.5	10	0.5	1.5	2.75	4	6.5
Cadmium	2.7	0.3	0.3	1.2	0.3	0.2	0.3	0.5	0.5	0.3	0.3
Chromium	0.7	1.4	1.1	1.1	1.0	0.9	0.9	1.7	1.3	1.6	1.1
Copper	9.0	10.3	8.0	9.5	10.7	7.0	10.9	8.3	9.4	7.2	10.2
Nickel	1.3	2.4	4.3	2.2	1.5	1.9	1.9	2.4	2.7	3.3	1.5
Lead	nd	nd	nd	1.1	1.0	nd	0.9	0.7	Nd	1.7	nd
Vanadium	nd	nd	nd	nd	nd	nd	nd	nd	Nd	0.9	nd
Zinc	23.4	25.9	21.9	24.1	27.4	18.9	29.8	26.4	23.4	22.7	31.9

nd – concentrations below limits of detection

Table 3.2c: Metal concentrations (µg/g dry weight) in earthworms *Lumbricus terrestris* collected from sites at various distances from the source. Values are the mean of at least three replicates per site, except for the site along the East Transect four km from the source where there was only one replicate. Cells shaded in pale grey exceed US DoE earthworm benchmark values.

Contaminant	Distance from source (km)									
	East Transect						North Transect			
	0.5	1.5	2.75	4	6.5	10	0.5	1.5	2.75	6.5
Cadmium	3.7	2.9	1.6	1.3	7.0	2.2	4.2	1.4	6.2	3.3
Chromium	2.0	2.9	2.8	2.6	3.8	2.3	2.4	2.9	3.0	10.9
Copper	11.1	4.9	8.5	9.8	11.3	7.5	7.8	6.9	8.4	15.5
Nickel	2.9	3.4	4.0	2.4	3.0	3.8	2.3	3.8	2.7	4.0
Lead	3.4	3.2	2.4	2.7	6.9	2.4	4.5	2.1	5.8	21.1
Vanadium	2.5	6.2	3.4	3.1	5.0	3.0	4.4	5.9	4.0	6.0
Zinc	293.9	140.4	289.6	1022.2	463.2	216.1	270.2	172.5	511.2	490.6

Table 3.2d: Metal concentrations (µg/g dry weight) in shrew (*Sorex araneus*) liver tissue collected from sites at various distances from the source. Values are the mean of between two and five replicates per site. There are no published quality guidelines for concentrations in small mammal tissue.

Contaminant	Distance from source (km)			
	East Transect	North Transect		
	2.75	0.5	1.5	2.75
Cadmium	2.2	3.3	5.6	12.4
Chromium	0.3	0.5	0.4	0.5
Copper	18.1	23.2	23.5	26.8
Nickel	1.6	2.0	2.0	2.0
Lead	0.6	0.8	0.9	1.1
Vanadium	0.1	0.1	0.1	0.1
Zinc	74.3	98.1	103.3	108.3

3.2.3. Bait lamina test

Bait lamina strips were deployed in October 2005 and retrieved in November 2005. There was feeding activity at all sites (Figure 3.4), but with a significant difference between sites ($P < 0.05$). Site 5 on the East Transect was significantly lower than Site 4 on the North Transect. However, these sites were not selected for PAH analysis, so there is no evidence of this effect being due to contaminant exposure. Sites with high concentrations of PAHs (Site 5 on the North Transect and Site 4 on the East Transect) did not have lower feeding activity compared to other sites.

Bait lamina strips have successfully been used to show the effects of heavy metal emissions from a zinc smelter on feeding activity of soil biota in previous studies (Paulus *et al.,* 1999; Filzek *et al.,* 2004; Spurgeon *et al.,* 2004). These studies showed low feeding activity at sites with higher levels of contamination, and feeding activity increased at sites where metal concentration decreased. Pesticide exposure has also been shown to affect soil organism feeding activity, using the bait lamina test (Paulus *et al.,* 1999).

Limitations in the use of bait lamina strips include confounding factors which may influence the results, such as water moisture, pH and temperature. There were no significant differences in pH between sites studied here. The bait lamina test is inexpensive and does not require technical skills to deploy the strips or analyse the results. However, the fact that the test measures a parameter (bait removal) related to a functional endpoint (litter removal) means it has quite low sensitivity, since large effects on total activity of the decomposer community are needed to see an effect. Bait lamina is currently being evaluated by the Environment Agency as a screening tool to assess contaminated land.

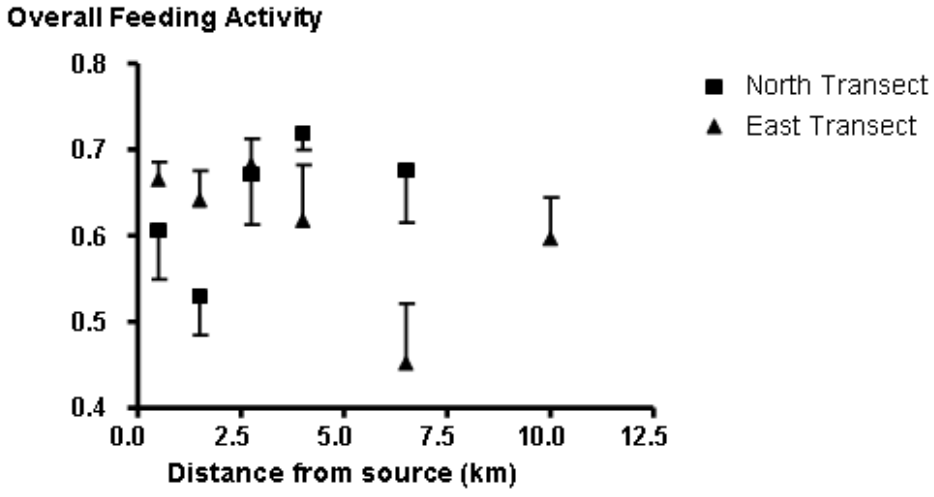

Figure 3.4: Feeding activity of soil organisms as a proportion of pierced bait obtained by the bait lamina test in October 2005. Data points represent mean (+/- SEM) of three replicates per site.

3.2.4. Cytochrome P450 enzyme assays

Cytochrome P450 activity (determined by measuring EROD activity) was detected in all shrew liver samples analysed (Figure 3.5). However, due to the small size of livers in the shrews, there was not enough tissue to measure BROD activity. There was no significant difference in EROD activity between the sites (P = 0.505); however, higher activity was found at sites with high concentrations of PAHs (Site 5 from the North Transect and Site 3 from the East Transect).

A study in the Netherlands measured EROD activity in *S. araneus* collected from a site contaminated with PCBs and found elevated levels in shrews (Van den Brink and Bosveld, 2005). Hepatic EROD activity in the present study at the two sites with elevated PAHs was comparable with those in the Dutch study (256 +/- 96 and 271 +/- 159 pmol/min/mg protein for Site East 3 and Site North 5, compared to 200-300 pmol/min/mg protein for the Dutch study). This shows that shrews in our study were exposed to organic compounds (including PAHs) which elevated EROD activity.

Vertebrates rapidly metabolise PAHs and so hepatic PAH concentration are likely to be at or below the limit of detection; measuring EROD activity instead provides evidence of exposure to organic compounds. EROD activity has been used previously as a biomarker of exposure to organic compounds in terrestrial and aquatic environments. Elevated EROD activity may be due to consumption of contaminated prey such as earthworms. However, elevated earthworm PAH concentrations were only observed at Site 5 on the North Transect so elevated EROD activity at Site 3 on the East Transect must be due to consumption of other prey or exposure to other contaminants not measured in the present study, including pesticides.

Although the technique is inexpensive and relatively easy to learn, one of the limitations of this biomonitoring tool is that induction of EROD activity is not specific to a particular compound. Induction of cytochrome P450 enzymes is also not well characterised in invertebrates. Studies have investigated the effects of exposure to PAHs on *L. rubellus* cytochrome P450 activity (Brown *et al.* 2004), but preliminary results did not show induction.

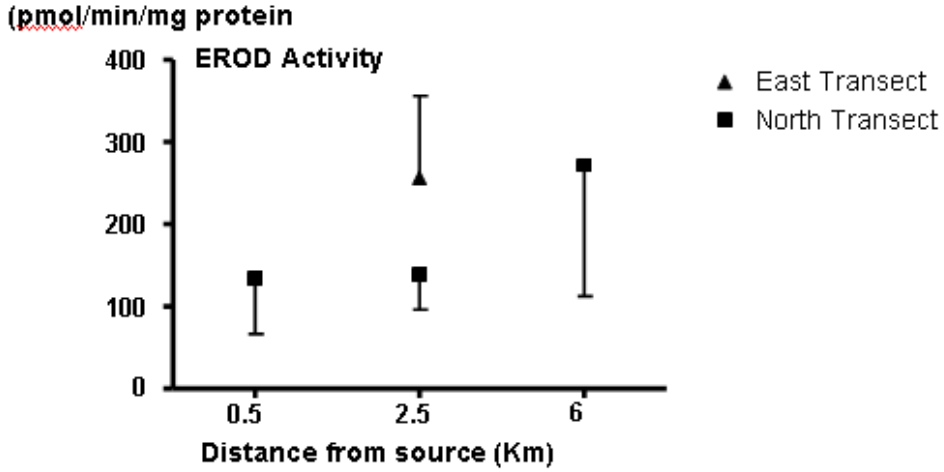

Figure 3.5: EROD activity in liver tissue from Sorex araneus collected from sites at various distances from the source

Plant growth tests

Soil collected from the 11 sites in this study was used in the plant growth tests. The test was deemed to be valid because it passed the following criteria, as described in Environment Canada (2005):

- greater than 70 per cent emergence from the negative control;
- mean percent survival for the negative control was greater than 90 per cent ;
- mean percentage of seedlings grown in the negative soil that exhibited phytotoxicity and/or developmental anomalies was less than 10 per cent .

Lettuce seedlings emerged in all the soils tested, and inhibition of emergence was low in some sites (Figure 3.6). However, inhibition decreased with increasing distance from the source. There were no significant differences in mean shoot length between the sites (P = 0.098) or in mean shoot dry weight (P = 0.731), see Figure 3.7.

Limitations with this biomonitoring tool include factors that influence the responses of seedlings such as pH, temperature and soil moisture. The method is inexpensive but requires specialist facilities such as a constant temperature room and glasshouses. The method is being trialled by the Environment Agency to monitor contaminated land.

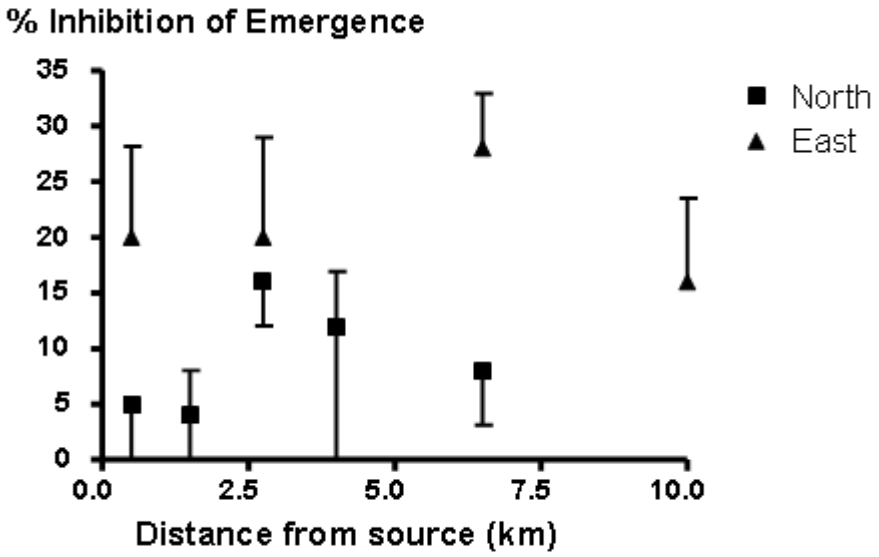

Figure 3.6: Graph showing percent inhibition of emergence of lettuce (*Lactuca sativa*) seedlings exposed for 14 days to soil collected from sites at various distances from the source

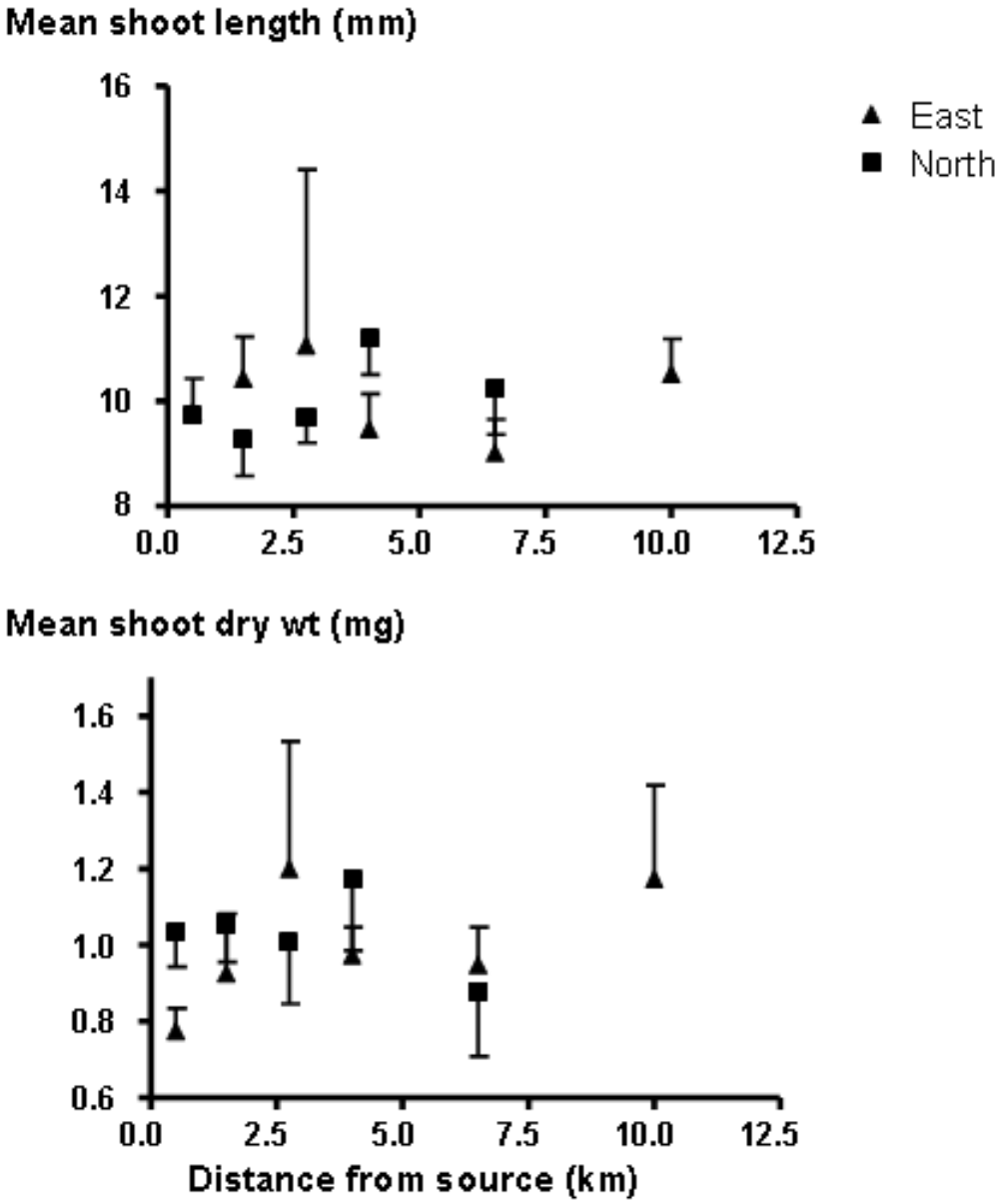

Figure 3.7: Graph showing 14-day mean shoot length (top graph) and mean shoot dry weight (bottom graph) of lettuce seedlings planted in soil from sites at various distances from the source. Data points represent mean (+/- SEM) of five replicates per site.

Earthworm metallothionein gene expression

All earthworms expressed mRNA for metallothionein (Figure 3.8). MT responses in earthworms at Sites 3 and 5 along the North Transect were more elevated than the other North Transect sites; these sites had higher soil and earthworm metal concentrations so this result is not surprising. There was no significant difference between metallothionein mRNA expression at the sites ($F_{(9,24)}$= 1.14, P = 0.374).

Other studies using gene expression of earthworm metallothionein have shown this biomarker to be more sensitive to metal contamination in the field than higher organisational level parameters such as the bait lamina (Spurgeon *et al.,* 2004). As with our study, the authors could not show a statistically significant difference between contaminated and control sites and so suggested that measuring MT mRNA and other biomarkers should be used as indicators of exposure. However, there was a positive relationship between cadmium concentration in earthworms and metallothionein expression ($P < 0.001$) and there was a weaker relationship between earthworm lead concentration and metallothionein expression ($P = 0.067$).

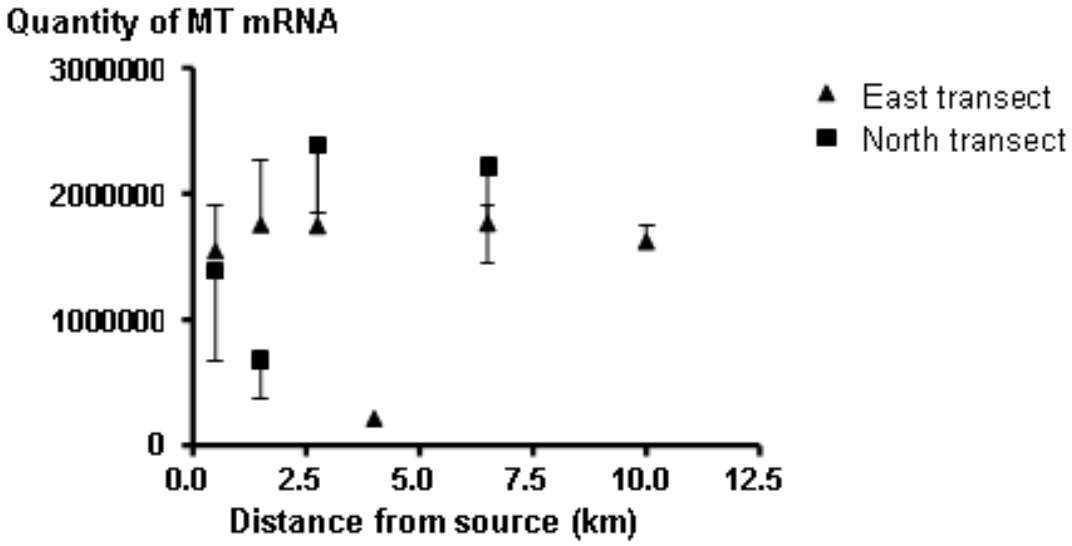

Figure 3.8: Graph showing field-collected *Lumbricus terrestris* metallothionein mRNA expression from sites at various distances from the source. Bars represent mean (+/- SEM) of up to four replicates per site. No earthworms were collected from Site 4 in the North Transect.

4. Conclusions

A selection of biomonitoring techniques was tested in the vicinity of an elevated industrial point source in the UK, to assess their practicality and ease of use.

The test site was chosen because data and information were available from an annual review of air quality for the source which indicated a pattern of pollutant emission and dispersion that would provide a range of test conditions. The test site was also thought to be relatively free of interference from other pollutant sources which might have similar emission profiles.

Site 5 on the North Transect, which was a children's playing field, transpired to have once been a landfill site. This highlights the need for a detailed survey prior to site selection to ensure that any historical or current factors which may influence the results are accounted for. A further consideration when planning long-term biomonitoring campaigns is to investigate planning consents around the site, to ensure there are no planned changes during the project which would result in future loss of the site.

The sample and replicate numbers needed to give the required sensitivity were estimated through a power analysis based on data from an unrelated contaminated industrial site.

The suite of tests using natural materials as passive collectors/accumulators of pollutants included use of soil, vegetation (nettles), earthworms and small mammals. The study concentrated on PAHs (determined by extraction followed by GC-MS) and metals (Cd, Cr, Cu, Ni, Pb, V, Zn, determined by ICP-AES).

Functional biological tests included feeding activity assay using bait lamellae, seed germination and growth rate in sampled soil, metallothionein mRNA expression in earthworms as an indicator of exposure to metals, and level of cytochrome P450 activity as an indicator of exposure to planar organic molecules such as PAHs.

Elevated levels of all metals were found in the soil, earthworms and shrew livers, and of all but V and Pb in vegetation. There was no clear spatial pattern except for Cr and Ni, which showed some correlation with distance from the source. Other metals known to be co-emitted with Cr and Ni did not show such a correlation. Expression of metallothionein mRNA was observed in earthworms. This correlated with levels of Cd in the earthworms, and also, though more weakly, with Pb concentrations.

Several sites showed elevated PAH concentrations, with some exceeding international soil guideline values. Concentrations were not always high in earthworms sampled from sites with high soil PAH, suggesting that bioavailability of the PAH may vary. Soil from the North Transect was found to be higher in organic matter than that in the East Transect; the presence of organic matter is known to reduce bioavailability of PAH. Elevated cytochrome P450 activity was seen in the livers of shrews sampled from sites with raised PAH concentrations in the soil.

Both seed germination and feeding activity varied from site to site, but no clear pattern emerged linking the variation to other factors such as metal or PAH concentration.

All of the functional biomonitoring tests proved practicable. They did not show significant patterns of spatial variation that could be linked with emissions from any single source, but both the metallothionein expression and cytochrome P450 activity assays responded to elevated levels of metal and PAH respectively. The lack of any obvious pattern of response in feeding activity and plant growth tests may be a result of the relatively low levels of pollutants at the test sites. The non-specific nature of both tests makes them susceptible to confounding factors such as temperature and moisture variation, which might mask more subtle responses to contaminants.

Use of biota in conjunction with soil or artificial sample media offers a rapid screening approach to assess contaminant bioavailability, which could indicate the likelihood of a contaminant entering the food web. The cytochrome P450 activity test is inexpensive and so might serve as a useful screening method for identifying areas contaminated by PAH or other planar organic molecules (such as certain pesticides) before the more expensive technique of extraction followed by GC-MS is used. However, capturing small mammals is labour-intensive and so potentially costly.

There was no pattern in the spatial variation of the metal and PAH concentrations measured that indicated a strong association with, or dominant contribution from, any one source. This may be a consequence of multiple sources influencing the test area, or of natural variations in natural or anthropogenic background which are at least as great as local source contributions.

The primary purpose of this study was to test the efficacy and practicality of a suite of biomonitoring techniques. When designing biomonitoring campaigns in which the primary aim is to determine pollutant levels and patterns, even greater effort should be devoted to sampling design to ensure the required level of statistical power can be achieved.

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Appendix 1: Power analysis

Results from a power analysis using previous fieldwork data from soil cadmium concentrations along a pollution gradient from an industrial site.

1. We consider power for detecting a linear trend in log transformed Cd levels with distance from source, that is, an exponential decline in Cd levels. Analysis of existing data shows that working with log transformed values helps to stabilize the variability between replicate samples at sites, an assumption of the test using linear regression.

The analysis partitions the total variation as follows.

ANOVA

Source	*d.f.*
Between transects	1
Linear trend	1
Trend × transect interaction	1
Residual:	
Deviations about trend	8
Between replicates within sites	36
Total	47

The standard error of the estimated linear trend is given by:

$$\text{se}\,[b] = \sqrt{[(V_D + V_R / 4)/(SSD1 + SSD2)]}$$

where V_D is the variance of the deviations about the trend, V_R is the variance between replicates at a site. $SSD1$ and $SSD2$ are the sums of squares about the mean of the distances at each site, where $SSD1 = 69.70$, $SSD2 = 53.91$.

Using existing data, estimates of the variances are as follows: $V_D = 0.285$, $V_R = 0.077$.

This gives the standard error of the estimated slope as:

$$\text{se}\,[b] = \sqrt{[(0.285 + 0.077 / 4)/(69.70 + 53.91)]} = 0.050$$

2. The *t*-test for trend uses $t = b/\text{se}\,[b]$ with eight degrees of freedom. The statistical power of the test (two-tailed, five per cent level) for detecting a trend of magnitude B is calculated as:

$$\textit{Power} = \Phi(B/\text{se}\,[b] - t_{8,0.05})$$

where Φ is the cumulative distribution of the standardized normal distribution and $t_{8,0.05}$ is the two-tailed five per cent of Student's *t*-distribution with eight degrees of freedom (Barker Bausell and Li, 2002).

The slope which can be detected with 80 percent power is obtained from the above as:

$se[b][\Phi^{-1}(0.80) + t_{8,0.05}] = se[b][0.842 + 2.31] = 3.15se[b]$

3. Substituting for the standard error gives the slope which can be detected with 80 per cent power as $3.15 \times 0.05 = 0.16$ per unit distance. A decrease of 0.16 in log cadmium level per kilometre corresponds to a 15 per cent decrease in cadmium levels. Over a distance of 10 km, the decrease in log levels is -1.60, that is, an 80 per cent decrease in levels. In other words, cadmium levels of 100 at source are reduced to 20 at 10 km.

Appendix 2: Small mammal trapping data

Site (distance from source)	Trapping date	Species	Sex	Weight (grams)
N1	09/08/2005	*Microtus agrestis* [a]	f	31.5
(0.5 km)		*Microtus agrestis*	f	19.5
		Microtus agrestis	f	30
		Mustela nivalis [b]		
		Sorex araneus [c]	juvenile	6.9
	10/08/2005	*Microtus agrestis*	f	17
		Microtus agrestis	f	16
		Microtus agrestis	f	14
		Microtus agrestis	m	19
	16/08/2005	*Sorex araneus*	juvenile	6.5
	17/08/2005	*Apodemus sylvaticus* [d]	m	18
		Apodemus sylvaticus	m (juvenile)	13
		Apodemus sylvaticus	m (juvenile)	15.5
		Sorex araneus	juvenile	7
N3	09/08/2005	*Clethrionomys glareolus* [e]	m	21.5
(1.5 km)		*Clethrionomys glareolus*	f	13.5
		Clethrionomys glareolus	m	17.5
		Sorex araneus	juvenile	7
		Sorex araneus	juvenile	7
		Sorex araneus	juvenile	7.9
	10/08/2005	*Clethrionomys glareolus*	m	14.5
		Clethrionomys glareolus	m	21
		Sorex araneus	juvenile	7.2
		Sorex araneus	juvenile	6.5
		Sorex araneus	juvenile	6.9
N5	09/08/2005	*Sorex araneus*	juvenile	7.2
(6 km)		*Microtus agrestis*	f	32
		Apodemus sylvaticus	f	13.5
		Clethrionomys glareolus	f	13.5
	10/08/2005	*Sorex araneus*	juvenile	6.9
		Microtus agrestis	f	
		Microtus agrestis	f	
		Apodemus sylvaticus	f	13.5
		Apodemus sylvaticus	m	18.5
		Apodemus sylvaticus	f	13.5
	16/08/2005	*Microtus agrestis*	f	27
		Microtus agrestis	m	23
		Microtus agrestis	f	25
		Microtus agrestis	f	22
		Microtus agrestis	m	
		Clethrionomys glareolus	f	21.5
		Apodemus sylvaticus	f	13.5
		Apodemus sylvaticus	m	22
		Sorex araneus	juvenile	7

Site	Date	*Species*	Sex	Weight (grams)
N5 (contd.)	17/08/05	*Microtus agrestis*	m	23
		Microtus agrestis	f	26.5
		Apodemus sylvaticus	f	22
		Apodemus sylvaticus	f	23
		Apodemus sylvaticus	f	15
E2	16/08/2005	*Clethrionomys glareolus*	f	28
(1.5 km)	17/08/2005	*Clethrionomys glareolus*	m	17
E3	09/08/2005	*Apodemus sylvaticus*	m	23.5
(2.5 km)	10/08/2005	*Sorex araneus*	juvenile	7
		Apodemus sylvaticus	m	15.5
	17/08/2005	*Sorex araneus*	juvenile	6
		Apodemus sylvaticus	m	18
		Apodemus sylvaticus	f	15
		Apodemus sylvaticus	m	14.5
		Apodemus sylvaticus	m	12
		Apodemus sylvaticus	m	17

a – field vole, b – weasel, c – common shrew, d – wood mouse, e – bank vole

Appendix 3: Soil properties

Table shows the mean percentage organic matter and mean pH measured from two replicates per site.

Site	Distance from source (km)	% organic matter	pH
N1	0.5	5.46	5.93
N2	1.5	3.29	6.31
N3	2.5	9.81	6.20
N4	4	8.89	6.32
N5	6	6.10	6.27
E1	0.5	15.92	6.36
E2	1.5	6.42	6.19
E3	2.5	12.41	6.24
E4	4	12.00	6.21
E5	6	14.17	6.13
E6	10	7.34	6.33

Appendix 4: Sum PAH in soil

Sum PAH concentration in soil collected from six sites at various distances from the source. The PAH compounds measured were priority compounds determined by the US EPA: naphthalene, acenaphthylene, acenaphthene, fluorene, anthracene, fluoranthene, phenanthrene, pyrene, benzo(a)anthracene, chrysene, benzofluoranthene, benzo(a)pyrene, indeno(1,2,3-cd)pyrene, dibenz(a,h)anthracene and benzo(ghi)perylene.

Site	Distance from source (km)	Sum PAH concentration (µg/g dry weight)
N1	0.5	3.45
N1	0.5	6.34
N1	0.5	6.78
N1	0.5	7.01
N3	2.5	4.09
N3	2.5	3.25
N3	2.5	12.95
N3	2.5	4.93
N5	6	2.68
N5	6	2.46
N5	6	19.37
N5	6	8.24
E2	1.5	1.37
E2	1.5	0.88
E2	1.5	1.45
E2	1.5	1.27
E3	2.5	2.93
E3	2.5	4.00
E3	2.5	9.78
E3	2.5	3.93
E6	10	2.05
E6	10	1.10
E6	10	1.08
E6	10	0.82
CRM		47.29
CRM		43.08

Appendix 5: PAH correlation matrix

A5: Pearson correlation matrix for logarithm of soil concentrations of individual PAH compounds over all sites. For each correlation upper value is Pearson r-value, lower value is P-value.

	Naphthalene	Acenaphthalene	Acenaphthene	Fluorene	Phenanthrene	Anthracene	Fluoranthene
Acenaphthylene	0.981						
	<0.001						
Acenaphthene	0.964	0.966					
	<0.001	<0.001					
Fluorene	0.960	0.935	0.973				
	<0.001	<0.001	<0.001				
Phenanthrene	0.916	0.888	0.908	0.962			
	<0.001	<0.001	<0.001	<0.001			
Anthracene	0.960	0.966	0.954	0.958	0.940		
	<0.001	<0.001	<0.001	<0.001	<0.001		
Fluoranthene	0.742	0.745	0.678	0.711	0.827	0.801	
	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	
Pyrene	0.786	0.757	0.739	0.810	0.922	0.832	0.956
	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Benzo[a]anthracene	0.962	0.994	0.962	0.914	0.867	0.963	0.752
	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Chrysene	0.607	0.541	0.548	0.687	0.842	0.662	0.811
	0.001	0.003	0.003	<0.001	<0.001	<0.001	<0.001
Benzofluoranthene	0.638	0.600	0.584	0.679	0.819	0.708	0.926
	<0.001	0.001	0.001	<0.001	<0.001	<0.001	<0.001
Benzo(a)pyrene	0.740	0.691	0.717	0.793	0.875	0.784	0.882
	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Indeno(123-cd)pyrene	0.957	0.949	0.947	0.958	0.969	0.954	0.835
	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Dibenz(ah)anthracene	0.952	0.987	0.959	0.898	0.846	0.948	0.736
	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Benzo(ghi)perylene	0.966	0.967	0.960	0.956	0.950	0.960	0.821
	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

A5 (continued): Pearson correlation matrix for logarithm of soil concentrations of individual PAH compounds over all sites. For each correlation upper value is Pearson r-value, lower value is P-value.

	Pyrene	Benzo[a]anthracene	Chrysene	Benzofluoranthene	Benzo(a)pyrene	Indeno(123-cd)pyrene	Benzo(ghi)perylene
Benzo[a]anthracene	0.745						
	<0.001						
Chrysene	0.926	0.506					
	<0.001	0.006					
Benzofluoranthene	0.962	0.591	0.928				
	<0.001	0.001	<0.001				
Benzo(a)pyrene	0.945	0.680	0.883	0.931			
	<0.001	<0.001	<0.001	<0.001			
Indeno(123-cd)pyrene	0.895	0.937	0.746	0.775	0.838		
	<0.001	<0.001	<0.001	<0.001	<0.001		
Dibenz(ah)anthracene	0.724	0.997	0.473	0.569	0.662	0.929	
	<0.001	<0.001	0.011	0.002	<0.001	<0.001	
Benzo(ghi)perylene	0.867	0.959	0.693	0.738	0.809	0.997	0.954
	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Appendix 6: Regression analysis

A6: Summary of regression analysis of distance versus the logarithm of soil concentration of individual PAH congeners and their sum.

	Parameters				
	Y-intercept	Slope	R^2	P-value	Significance[1]
Naphthalene	1.71	-0.0542	0.444	0.001	**
Acenaphthylene	1.35	-0.0971	0.503	<0.001	***
Acenaphthene	1.21	-0.0779	0.377	0.004	**
Fluorene	1.30	-0.0774	0.381	0.004	**
Anthracene	1.76	-0.0926	0.279	0.017	*
Fluoranthene	2.80	-0.0406	0.178	0.064	ns
Phenanthrene	2.43	-0.0696	0.349	0.006	**
Pyrene	2.78	-0.0543	0.294	0.013	*
Benzo(a)anthracene	1.26	-0.0618	0.305	0.012	*
Chrysene	2.64	-0.0629	0.272	0.018	*
Benzofluoranthene	2.76	-0.0512	0.253	0.024	*
Benzo(a)pyrene	2.86	-0.0504	0.25	0.025	*
Indeno(123-cd)pyrene	2.67	-0.0619	0.286	0.015	*
Dibenz(ah)anthracene	1.80	-0.0600	0.257	0.023	*
Benzo(ghi)perylene	2.65	-0.0596	0.306	0.011	*
Sum	3.65	-0.0544	0.271	0.019	*

[1] ns = not significant, * = significant, ** = highly significant, *** = very highly significant

Appendix 7: Metal correlation matrix

Pearson correlation matrix for logarithm of concentrations of individual metals in vegetation (a), soil (b), earthworm tissue (c) and shrew liver tissue (d) over all the sites. Pearson correlation matrix for logarithm concentrations of individual metals and metallothionein gene expression in earthworm tissue is also presented (A7c). For each correlation upper value is Pearson r-value, lower value is P-value.

A7a: Vegetation

	Cadmium	Chromium	Copper	Nickel	Lead	Vanadium
Chromium	-0.164					
	0.288					
Copper	0.096	0.109				
	0.534	0.481				
Nickel	-0.181	0.599	0.257			
	0.321	0.000	0.155			
Lead	0.192	0.004	0.080	0.544		
	0.679	0.993	0.865	0.206		
Vanadium	-0.059	0.748	0.238	1.00	a	
	0.963	0.462	0.847	a	a	
Zinc	0.086	0.129	0.710	0.104	0.131	0.086
	0.577	0.403	0.000	0.570	0.779	0.945

a – denotes not enough data in column for analysis

A7b: Soil

	Cadmium	Chromium	Copper	Nickel	Lead	Vanadium
Chromium	0.124					
	0.423					
Copper	0.266	0.615				
	0.081	0.000				
Nickel	0.078	0.364	0.373			
	0.617	0.015	0.013			
Lead	0.179	0.582	0.833	0.293		
	0.246	0.000	0.000	0.054		
Vanadium	-0.226	0.446	-0.093	0.292	0.195	
	0.141	0.002	0.547	0.055	0.205	

Zinc	0.237	0.698	0.875	0.233	0.931	0.189
	0.121	0.000	0.000	0.129	0.000	0.220

A7c: Earthworm tissue

	Metallothionein	Cadmium	Chromium	Copper	Nickel	Lead	Vanadium
Cadmium	0.516						
	0.002						
Chromium	0.120	0.050					
	0.498	0.780					
Copper	0.157	0.368	0.498				
	0.374	0.032	0.003				
Nickel	-0.069	-0.216	0.573	0.161			
	0.697	0.220	0.000	0.364			
Lead	0.239	0.333	0.747	0.676	0.149		
	0.174	0.054	0.000	0.000	0.402		
Vanadium	-0.175	-0.023	0.632	0.027	0.505	0.456	
	0.323	0.895	0.000	0.877	0.002	0.007	
Zinc	0.221	0.418	-0.038	0.564	-0.223	0.351	-0.206
	0.202	0.014	0.832	0.001	0.205	0.042	0.243

A7d: Shrew liver tissue

	Cadmium	Chromium	Copper	Nickel	Lead	Vanadium
Chromium	0.327					
	0.275					
Copper	0.474	0.900				
	0.102	0.000				
Nickel	0.244	0.759	0.860			
	0.422	0.003	0.000			
Lead	0.565	0.523	0.715	0.737		
	0.044	0.066	0.006	0.004		
Vanadium	0.033	0.277	0.384	0.584	0.327	
	0.916	0.359	0.195	0.036	0.275	
Zinc	0.480	0.845	0.955	0.899	0.682	0.510
	0.097	0.000	0.000	0.000	0.010	0.075

Appendix 8: Soil quality guidelines

International soil quality guidelines for metal and selected PAHs, taken from Weeks *et al.* (2004).

Contaminant	**CCME**		**New Dutch List**		**US DoE**	
	Soil		Soil sediment		Earthworm	Plant
	Agri	Res/Park	mg/kg dry weight		Benchmark	benchmark
	mg/kg	mg/kg	Target value	Intervention value	mg/kg	mg/kg
Cd	1.4	10	0.8	12	20	4
Cr	64	64	100	380	0.4	1
Cu			36	190	50	100
Ni	50	50	35	210	200	30
Pb	70	140	85	530	500	50
V	130	130				2
Zn	200	200	140	720	200	50
Naphthalene [1]	0.1	0.6				
Benzo(a)pyrene [1]	0.1	0.7				
Total PAH			1	40		

[1] denotes PAHs where there are values for soil.

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