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Developing a new method for soil pollution monitoring using molecular genetic biomarkers

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Physiological responses to environmental stressors may induce changes in gene expression as part of an organism's homeostatic mechanisms. Thus molecular genetic biomarkers have the potential to be used for monitoring sublethal chemical exposure in ecosystems. This paper describes a methodological assessment of the suitability of a protocol to monitor selected biomarkers. The TaqMan® real-time quantitative polymerase chain reaction was used to measure gene transcription in earthworms (*Lumbricus rubellus*) maintained on control or cadmium- or copper-spiked soil. Changes in the expression of two target genes, that encoding metallothionein isoform 2 (MT-2) and that encoding the mitochondrial large ribosomal subunit (MLRS), were quantified against the internal control gene β-actin. The protocol used produced reliable and reproducible results. Transcript levels displayed qualitative and quantitative differences in the responses to the two metal ions. MLRS gene levels were unaffected by exposure to cadmium but displayed a response to high levels of copper. Conversely, cadmium greatly induced MT-2 gene expression, but copper only altered transcription of this gene at high exposure concentrations. This study demonstrates that it is now technically feasible to use gene expression as an index of pollution exposure in environmentally relevant organisms.

Keywords: earthworm, metallothionein, large ribsomal subunit, cadmium, copper, biomarker.

Introduction

A problem presented by contaminated land is that the majority of sites are tainted by a cocktail of toxicants whose interactive effects are poorly understood. Managing these sites with regard to environmental impact and human health requires robust monitoring tools. Historically, pollution monitoring has relied heavily on chemical measurements. However, this approach has fundamental limitations. Factors such as toxicant bioavailability and toxico-kinetic interactions between individual chemicals are frequently not established. In addition, there may be technical difficulties and high costs involved in quantifying all the compounds and their metabolites in complex mixtures. Perhaps the most important disadvantage of chemistry-based monitoring is an inability to predict with certainty the relationship between generated data and environmental or human health. This is exactly the strength of biological monitoring.

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Of the bioassays currently available, two approved and standardized laboratory acute toxicity tests exploit earthworms as sentinels (Organisation for Economic Co-operation and Development 1984, European Economic Community 1995). These tests are based on measuring the short-term survival of adult earthworms, but do not take into account chronic effects on sublethal parameters such as reproductive output, which have important impacts for population stability (Spurgeon and Weeks 1998). On the basis of these considerations, a number of sublethal bioassays measuring the life-cycle response of earthworms after exposure to pollutants have also been developed (Van Gestel 1992, Van Gestel *et al*. 1992, Kula and Larink 1997).

An alternative approach to standard toxicity tests that can provide more information about the organism's response to individual toxicants and mixtures is the use of novel biomarkers (Kammenga *et al*. 2000, Scott-Fordsmand and Weeks 2000). Such an approach, although not without practical challenges, offers a number of attractive features, including the potential for detection of low levels of exposure, the potential to link molecular or cellular responses to ecologically relevant life-cycle traits (such as reproduction and growth), the ability to tease out toxicant-specific effects, and the evaluation of subtle non-lethal effects on laboratory and field populations of contaminant mixtures. The expanding functional genomic database provides a huge resource from which candidate molecular biomarkers can be identified, and subsequently exploited, to address these issues. To date, individual genes have been assessed within small-scale studies but the techniques used were not compatible with the high throughput required to deal with ecologically relevant questions (Stürzenbaum *et al*. 1998a,b, Morgan *et al*. 1999, Stürzenbaum *et al*. 2001a). The present study describes the application of a real-time quantitative polymerase chain reaction (PCR) to yield a high throughput, fully quantitative molecular assay, giving examples of how it enables the exploitation of specific molecular biomarker indices to assess exposure and differentiate between the effects of different chemical challenges. This study focuses on measuring changes in the expression of two genes – that encoding metallothionein isoform 2 (MT-2) and that encoding the mitochondrial large ribosomal subunit (MLRS).

Materials and methods

Earthworm preparation and exposure protocol

Earthworm exposures were conducted in a commercially available loam (Broughton Loams, Kettering, UK) amended with 3% (dry weight) of composted bark. This gave a medium with a pH of 7.7 and a 10% loss on ignition. For the tests, 1.25 kg dry weight of soil medium was placed into plastic containers ($180 \times 180 \times 93$ mm). Four concentrations of copper and cadmium were tested. In the control, the soil was amended with distilled water (475 ml) to give a water content of 50–60% of its water-holding capacity. In the metal-spiked soils, the same volume of an aqueous solutions of copper (purity $>99\%$, Sigma) or cadmium chloride (purity $>98\%$, Sigma) was added to give the required water-holding capacity and nominal metal concentrations of 0.16, 0.63, 2.52 and 10.07 μmol Cu g^{-1} and 0.111, 0.45, 1.78 and 7.12 μmol Cd g^{-1}. These doses were selected to represent the full potential sublethal range for the selected earthworm species on the basis of previous work (Spurgeon and Hopkin 1995) and to cover a range of environmental contamination levels ranging from low/moderate to severe. Four replicates were used for all treatments.

After the addition of water or metal solutions, the soils were left to stabilize for 2 weeks at 15°C. Adult *Lumbricus rubellus* (weighing 0.548–2.164 g; average 1.111 g) were collected from cultures and

eight individuals randomly assigned to each soil replicate. Containers were covered with a perforated lid to minimize water loss, and kept for 42 days at $15 \pm 1.5^{\circ}C$ in a 16 h dark:8 h light regime. During the exposure period, 5 g dry weight of air-dried horse manure was added weekly to the soil surface of each container to optimize earthworm survival. At the end of each week, any remaining food was removed from the soil and replaced with fresh material.

To validate the nominal metal concentrations, the total cadmium and copper levels in the test soils were assessed by digesting soils in boiling nitric acid as described by Hopkin (1989). Solutions were analysed by flame atomic absorption spectrometry (Varian Spectra 30, GTA 95). Treatment blanks and standard reference materials were included in all analyses. For spiked soils, good agreement (within 15%) between the actual and the nominal values were found in all cases except the two lowest concentrations of copper, where the presence of residues in unamended medium meant that measured concentrations exceeded nominal values (data not shown). For simplicity, nominal concentrations are referred to throughout the paper.

Utilization of TaqMan® technology for measuring molecular genetic responses

Two worms were taken from each of the four replicates for every treatment (total of eight worms) if available (see below). In containers with an excess of worms, two animals representative of the mean weight of the survivors were selected. Total RNA was isolated from approximately 300 mg of earthworm tissue removed by direct caudal amputation using the Tri-reagent protocol of Chomczynski and Sacchi (1987). Total RNA was quantified spectroscopically and its integrity assessed by ethidium bromide visualization of undegraded ribosomal bands after resolution on a 1% agarose gel. The PolyAttract® Series 9600™ mRNA isolation procedure (Promega Ltd, Southampton, UK) was used to isolate mRNA from 2 μg of total RNA for all worms. This was immediately transcribed to cDNA using the PolyAttract Series 9600™ cDNA synthesis system (Promega). After synthesis of separate cDNAs, equal volumes from all the eight worms for each treatment were pooled to give a single sample for each soil concentration. The exception to this was in the soil spiked with the highest dose of copper, where only one worm survived. To avoid over-consideration of this one individual within the dataset, transcript measurements were not made for this worm.

Expression profiles of genes were determined by quantitative real-time PCR using a fluorogenic 5′ nuclease assay performed with the TaqMan® PCR reagent system (PE Biosystems, Warrington, UK) on an ABI PRISM® 7700 Sequence Detection System (PE Biosystems), according to the manufacturer's recommendations. Three genes were selected for full analysis: the β-actin gene, the MLRS gene and the MT-2 gene. The β-actin gene was selected as an internal control (housekeeping gene) to account for variation in sample handling and reaction efficiency, as this gene has previously been shown to be relatively invariant under metal exposure (Stürzenbaum *et al*. 1998a, 1999a). The primer and probe sets (Table 1) required for the fluorogenic 5′ nuclease assay were designed using the Probe Administrator® system (PE Biosystems) from sequence information obtained during previous work in this laboratory (Stürzenbaum *et al*. 1998a, 2001b). In cases where specific isoforms are to be measured, such as with the MT-1 and MT-2 genes, probe and primer sets are located in unconserved areas of the sequence and are

Table 1. Primer and probe sets for the real-time quantitative PCR assays. Unmodified oligonucleotides were used for both forward and reverse primers, whilst TaqMan® probes incorporate the fluorescent molecule 6-carboxyfluorescein (FAM) at the 5′ end and the complementary quencher 6-carboxy-tetramethyl-rhodamine (TAMRA) at the 3′ end.

Gene	Primer/probe	
β-Actin	Forward primer	5′-CGTCGACAATGGATCTGGAA-3′
	Reverse primer	5′-CGACCATCACACCCTGATGA-3′
	TaqMan® probe	5′-TTTCGCCGGTGATGACGCTCC-3′
MT-1	Forward primer	5′-ACCAACTGCAGATGCCTGAAA-3′
	Reverse primer	5′-CTTGGGAATCAGCACAGCAA-3′
	TaqMan® probe	5′-CTTCCTGCAGTTTGGCGAACATTCACT-3′
MT-2	Forward primer	5′-CGATTTGATACATTTTTATATATGGACGA-3′
	Reverse primer	5′-TGAATTTAGCTCTGAATGTTTCTTGC-3′
	TaqMan® probe	5′-ACTTTACCACATACATCTTTTGATTTCGTTTGCCA-3′
MRLS	Forward primer	5′-GGGTAACTCCTGCCCAGTGA-3′
	Reverse primer	5′-GCAAGTGATTATGCTACCTTTGCA-3′
	TaqMan® probe	5′-TTCAACGGCCGCGGTATCCTAACC-3′

thus isoform-specific. This specificity was validated by analysis of the cross-reactivity of the primer and probe sets for the MT-1 and MT-2 gene targets.

Calibration standards (target genes cloned into the Promega pGEM-T vector system) were prepared by diluting to a series of concentrations in the range of 100 pg to 1 fg plasmid per microlitre (3×10^7 to 3×10^2 molecules μl^{-1}). These standards were included in each series of PCR reactions and used to quantify gene expression in unknown samples by computer-generated regression analysis. Gene expression in the worms were assessed by eight repeat analyses of a pooled cDNA sample. This approach was taken in order to separate procedural measurement variability from any biological variability inherent within the exposed worm.

Results and discussion

The evaluation and detection of the biological effects of toxic compounds at low, environmentally relevant concentrations has posed difficulties for ecotoxicological assessment. By exploiting genomic advances we are able to quantify potential molecular indices that may aid environmental risk assessment. The key to tapping this genomic potential has been the development of assay technologies that do not require the same degree of technical expertise as established procedures, such as Northern blotting, whilst also delivering quantitative and specific analyses that allow differentiation between very similar gene products.

Real-time quantitative PCR

The major obstacle in exploiting the enormous power of the PCR for quantitative assay procedures has been that parallel reactions with vastly different template inoculations result in near-identical final product levels (see Figure 1). This occurs because reaction constituents are exhausted, causing an amplification plateau. Real-time analysis has shown that the number of cycles required to attain a specific concentration within the logarithmic phase of the reaction is directly related to the log of the starting amount of the template (see Figure 1). Initial techniques that tried to harness this relationship by taking a 'snap-shot' of the amplification reaction, such as competitive PCR, exhibited a limited dynamic range. However, recent advances in fluorescence *in situ* monitoring have led to the development of a number of platforms that allow amplicon production to be monitored throughout the reaction across a wide range of initial template concentrations.

The specificity of the 5′ assay is demonstrated in the present study by its ability to distinguish between the genes for the two MT isoforms with 74.4% sequence homology. By carefully designing probes and primer sets within the most variable regions, we were able to show isoform-specific amplification. This is illustrated for the MT-1 gene primer/probe set, which exhibited a dynamic range of between 3×10^7 and 3×10^3 molecules when tested against an MT-1 gene template, but extremely low amplification when tested against 3×10^7 molecules of the MT-2 gene (Figure 1A). For the MT-2 gene primer/probe set, opposite specificity was shown; a similar dynamic range could be observed when tested against the MT-2 gene template, but no amplification was found for the MT-1 gene template (Figure 1B).

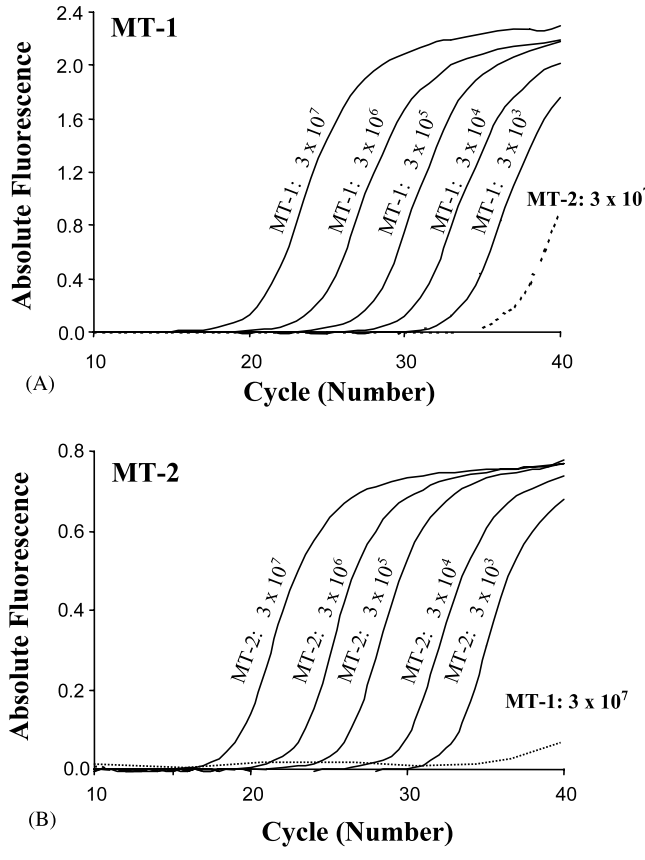

Figure 1. Isoform-specific quantitative amplification assays designed against the metallothionein genes isolated from the earthworm *L. rubellus*. (A) Exponential amplification using the primer and probe set designed against MT-1. The reaction template was either a dilution series (3×10^7 to 3×10^2 molecules μl^{-1}) of the cloned MT-1 standard (solid lines) or 3×10^7 molecules μl^{-1} of the cloned MT-2 standard (dashed line) (ΔRn). (B) Exponential amplification using the primer and probe set designed against MT-2. The reaction template was either a dilution series (3×10^7 to 3×10^2 molecules μl^{-1}) of the cloned MT-2 standard (solid lines) or 3×10^7 molecules μl^{-1} of the cloned MT-1 standard (dashed line).

Assay reliability and repeatability

To be a useful pollution monitoring tool, a gene-specific assay must be able to give reproducible results. In this study, which was set out to assess exposure to copper and cadmium in a terrestrial invertebrate, repeat measurements of pooled samples of cDNAs from worms exposed to each soil treatment were made. The reliability of the assay was demonstrated by the fact that there were no failed reactions of any of the measured transcripts (all 216 reactions gave successful amplification). This was independent of the range of initial starting template concentrations present within the respective pooled samples. Assay reproducibility was also good for all the transcripts (Table 2). The measurement variance for of all three genes was low in all cases and no obvious changes in measurement repeatability at different mean transcript concentrations were observed (Table 2).

Table 2. Transcript levels of genes for β-actin, MLRS and MT-2 in *L. rubellus* exposed to a control soil and four concentrations of copper or cadmium. Values are means, SE and coefficient of variance (CV) for eight analyses of a pooled cDNA sample consisting of eight equal volumes from the eight individual cDNA samples.

	Nominal soil concentration		Transcript levels of quantified gene (molecules μl^{-1})								
			β-Actin			MLRS			MT-2		
Metal	$\mu mol\ g^{-1}$	$\mu g\ g^{-1}$	Mean	SE	CV	Mean	SE	CV	Mean	SE	CV
Copper	0	0	2.63×10^6	3.27×10^5	0.353	1.08×10^8	1.11×10^7	0.29	6.98×10^4	1.4×10^4	0.2
	0.16	10	6.7×10^6	5.24×10^5	0.221	1.08×10^8	9.1×10^6	0.238	2.78×10^4	5.67×10^3	0.202
	0.63	40	6.36×10^6	4.36×10^5	0.194	4.31×10^7	1.07×10^6	0.07	9.73×10^4	1.52×10^4	0.153
	2.52	160	2.4×10^6	1.68×10^5	0.198	3.87×10^8	7.56×10^6	0.055	1.96×10^5	1.8×10^4	0.096
	10.07	640									
Cadmium	0	0	1.82×10^6	1.74×10^5	0.271	9.06×10^7	3.24×10^6	0.101	7.66×10^4	1.06×10^4	0.138
	0.111	12.5	1.64×10^6	9.54×10^4	0.164	2.83×10^7	1.08×10^6	0.109	8.9×10^5	1.95×10^5	0.219
	0.45	50	8.11×10^6	6.41×10^4	0.224	1.09×10^8	7.6×10^6	0.198	1.41×10^6	2.03×10^5	0.144
	1.78	200	3.07×10^6	1.93×10^5	0.178	8.72×10^7	2.5×10^6	0.081	3.26×10^6	5.13×10^5	0.157
	7.12	800	1.19×10^6	5.15×10^4	0.123	1.14×10^8	7.93×10^6	0.196	7.13×10^6	1.54×10^6	0.216

Internal standardization

Normalization of cDNA template concentrations are required before accurate sample comparison can be made. Parallel measurements of an invariant control or housekeeping gene can be used to adjust expression levels for transcription efficiency. In *L. rubellus*, a number of potential housekeeping genes have been isolated and sequenced (Stürzenbaum *et al*. 1999b). Of these, the gene encoding for the muscle fibre and cell cytoskeletal structural protein β-actin was selected for this study, since quantitative PCR using SyBr® green technology has already indicated that it is invariant in worms exposed to metals under different exposure regimes (Stürzenbaum *et al*. 1998a, 1999a,b).

Quantified β-actin gene expression for the pooled samples ranged from 8.11×10^5 molecules μl^{-1} for worms exposed to 0.45 μmol Cd g^{-1} to 1.19×10^6 molecules μl^{-1} for the surviving worm exposed to 7.12 μmol Cd g^{-1} (Table 2). Comparison of β-actin gene levels indicated no clear dose-related trend across the dose range for the two metals. This was confirmed by calculation of exposed to control worm β-actin gene ratios in both the copper and the cadmium tests.

MLRS gene expression in response to copper and cadmium

Comparison of transcript levels indicated that the MLRS gene was approximately two orders of magnitude more abundant than the β-actin gene (Table 2). Concentrations of this gene ranged from 3.88×10^8 at 2.52 μmol Cu g^{-1} to 2.82×10^7 at 0.111 μmol Cd g^{-1}. For worms exposed to cadmium, pooled cDNA samples showed no clear exposure-dependent changes in the expression of the MLRS gene either in the raw values or when calculated relative to the β-actin gene (Figure 2A). For copper, complex effects of exposure concentration on MLRS gene transcript levels relative to β-actin gene levels were detected in pooled samples (Figure 2A). Analysis of transcript values indicated that relative expression was lower than controls within the earthworm exposed to the two lowest copper concentrations, but was upregulated by a factor of 4 above controls at 2.52 μmol Cu g^{-1}. The observed expression for the MLRS gene reflects known patterns of copper toxicity for earthworms. For example, Spurgeon *et al.* (1994) found that copper had no negative effect on, or even enhanced, the reproduction of *Eisenia fetida* at low concentrations (hormesis) but was toxic at higher levels (50% lethal concentration = 10.76 μmol Cu g^{-1}). This complex response pattern may be linked to the fact that copper is an essential nutrient for earthworms.

Metallothionein expression in response to copper and cadmium

Quantification of pooled sample MT-2 gene expression indicated that transcript levels were approximately an order of magnitude lower than β-actin gene levels in the control worms, although higher levels were found in cadmium-amended treatments (Table 2). For copper, no consistent trend in MT-2 gene expression was found across the lower part of the dose range (Figure 2D). Expression was reduced at 0.16 μmol Cu g^{-1} and unchanged compared with controls at 0.63 μmol Cu g^{-1}. At 2.52 μmol Cu g^{-1}, MT-2 gene transcript levels were, however, increased three-fold, indicating upregulation at higher concentrations of copper. In the worms exposed to cadmium, a clear exposure-dependent increase in MT-2 gene expression

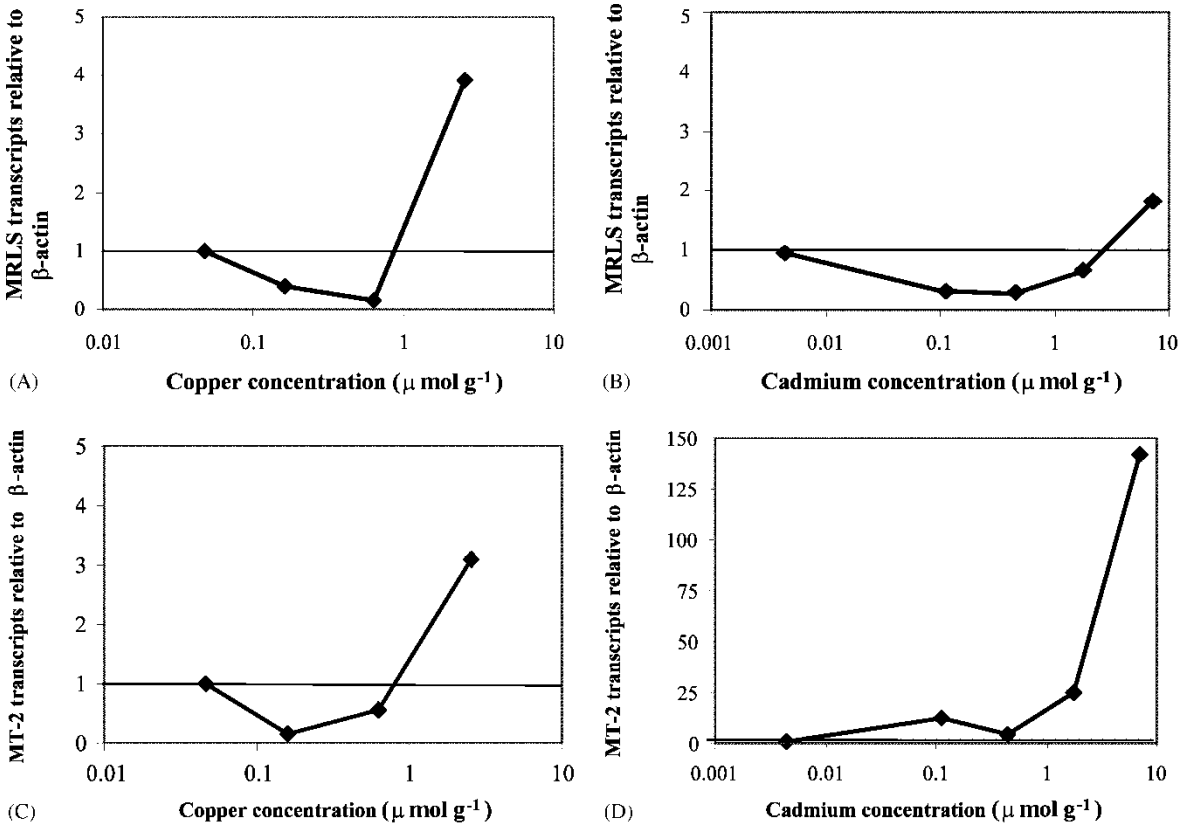

Figure 2. Relative expression of the three target sequences in *L. rubellus* measured using the described quantitative PCR protocol. (A) and (B) show MLRS gene transcript levels relative to β-actin gene levels as a ratio to the control sample value in worms exposed to copper (A) or cadmium (B). (C) and (D) show MT-2 gene transcript levels relative to β-actin gene levels as a ratio to the control sample value in worms exposed to copper (C) or cadmium (D). SE values are given in Table 2.

was apparent (Figure 2F). Comparisons of the product level of this gene indicated substantial differences in expression, with gene copy numbers being increased 12-fold at 0.111 μmol Cd g^{-1}, 25-fold at 1.78 μmol Cd g^{-1} and 142-fold in the sample from the five surviving worms exposed to 7.12 μmol Cd g^{-1}. These results indicated that transcription of the MT-2 gene was highly increased under low/moderate cadmium exposure, even though concentrations were well within the sublethal range.

Conclusions

The current focus on the assessment and remediation of contaminated land has led to increased interest in the use of biological tools as a means to conduct risk-based assessments. The use of living organisms for pollution monitoring has advantages in that they integrate in time and space both the bioavailability and toxicity of potentially interactive chemicals. Different organisms with distinct lifestyles and activities do, of course, 'sample' and respond to different aspects of their surroundings. However, the principle of using sublethal responses in these organisms is a better basis for risk assessment than chemical analysis alone. Most contaminated soils are complex, variable matrices, where there are many factors (e.g. contaminant–contaminant interactions, particle size, and organic matter content influencing contaminant mobility) that modulate the bioavailability, and potential toxicity, to biota of the chemicals present. In reality, these two approaches are complementary rather than exclusive options.

In the present study, a technique initially developed for the assessment of molecular responses in clinical applications was applied to address an ecotoxicological issue. This technique has been shown to have a low measurement variability and high signal specificity, thus allowing the identification and measurement of closely related isoforms. Among the genes measured, marked variability in expression levels were detected in the samples studied. The extent to which gene expression is altered by pollutants (e.g. MT-2 gene expression in cadmium-exposed worms) illustrates that gene-based techniques have potential as reliable, repeatable and responsive methods to indicate sublethal biological effects. Further, because in this work we have analysed gene expression at a range of soil metal concentrations, we have been able to demonstrate exposure-related changes in the expression of particular genes within the range of cadmium and copper contamination levels that may be encountered at low/moderate to highly contaminated industrial sites. While MLRS gene expression was only altered by copper, an extreme upregulation of the MT-2 gene was specific to exposure to cadmium. The ability to identify the pollutants within the complex cocktail of chemicals that is often present at active and abandoned industrial sites may be of use to site managers, as it will identify the processes that have caused or are causing greatest environmental harm. Furthermore, the potential to screen for the presence of potentially toxic substances is possible using micro-array technology (see, for example, De Benedetti *et al*. 2000). This technology is capable of the simultaneous measurement of the expression profile of a large number of genes ('high throughput'), which may include invariant control genes as well as pollution-responsive genes. After an initial 'global' genetic

screen, any indication of the presence of bioactive toxicants can then be further investigated using specific gene assays of the type outlined in this study.

Within the design of any environmental monitoring regime using biomarkers, a particular challenge is to link the observed molecular and cellular differences in response to environmental quality to whole-organism life-cycle parameters and also to ecological-level changes. A significant step towards this goal would be provided by molecular biomarkers that have explicit mechanistic links to parameters such as growth or reproduction. In this respect, both metallothionein (Vasak and Hasler 2000) and certain mitochondrial components (Green and Beere 2000) have been linked with the fundamental biological process of regulated cell deletion known as apoptosis. This link means that the molecular changes observed here are not only correlated with contaminant exposure, but may also reflect wider physiological changes in the higher tiers of biological organization.

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