Title: Use of transgenic GFP reporter strains of the nematode *Caenorhabditis elegans* to investigate the patterns of stress responses induced by pesticides and by organic extracts from agricultural soils.

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Abstract

As a free-living nematode, *C. elegans* is exposed to various pesticides used in agriculture, as well as to persistent organic residues which may contaminate the soil for long periods. Following on from our previous study of metal effects on 24 GFP-reporter strains representing four different stress-response pathways in *C. elegans* (Anbalagan et al. 2012), we now present parallel data on the responses of these same strains to several commonly used pesticides. Some of these, like dichlorvos, induced multiple stress genes in a concentration-dependent manner. Unusually, endosulfan induced only one gene (*cyp*-34A9) to very high levels (8-10-fold) even at the lowest test concentration, with a clear plateau at higher doses. Other pesticides, like diuron, did not alter reporter gene expression detectably even at the highest test concentration attainable, while others (such as glyphosate) did so only at very high concentrations. We have also used five responsive GFP reporters to investigate the toxicity of soil pore water from two agricultural sites in south-east Spain, designated P74 (used for cauliflower production, but significantly metal contaminated) and P73 (used for growing lettuce, but with only background levels of metals). Both soil pore water samples induced all five test genes to varying extents, yet artificial mixtures containing all major metals present had essentially no effect on these same transgenes. Soluble organic contaminants present in the pore water were extracted with acetone and dichloromethane, then after evaporation of the solvents, the organic residues were redissolved in ultrapure water to reconstitute the soluble organic components of the original soil pore water. These organic extracts induced transgene expression at similar or higher levels than the original pore water. Addition of the corresponding metal mixtures had either no effect, or reduced transgene expression towards the levels seen with soil pore water only. We conclude that the main toxicants present in these soil pore water samples are organic rather than metallic in nature. Organic extracts from a control standard soil (Lufa 2.2) had negligible effects on expression of these genes, and similarly several pesticides had little effect on the expression of a constitutive *myo*-3::GFP transgene. Both the P73 and P74 sites have been treated regularly with (undisclosed) pesticides, as permitted under EU regulations, though other (e.g. industrial) organic residues may also be present.

Introduction.

The nematode *Caenorahbditis elegans* has been utilised widely for toxicity testing of field samples (Mutwakil et al. 1997; Traunspurger et al. 1997; Power and de Pomerai , 2001), as well as single and mixed toxicants (Leung et al. 2008), including pesticides ranging from the fungicide captan (Candido and Jones, 1996) to a series of organophosphates (Rajini et al. 2008). Several different types of assay are available, including whole-organism endpoints such as growth, reproduction and motility (Dhawan et al. 1999; Thompson and de Pomerai 2005; Boyd et al. 2010) or feeding inhibition (Jones and Candido 1999), as well as molecular assays measuring the induction of stress-responsive genes – often via GFP or *lacZ* reporter transgenes (Stringham and Candido 1994; Guven et al. 1994, 1995; Cioci et al. 2000; Chu and Chow 2002; Swain et al. 2004; Ma et al. 2009). *C. elegans* is convenient for such studies because of its small size (c. 1 mm as adults), transparency, short life-cycle (3.5 days at 25°C), hermaphroditic self-fertilisation (allowing easy maintenance of mutant or transgenic stocks), fully characterised somatic cell lineage (Sulston et al. 1983), complete genome sequence (*C. elegans* Sequencing Consortium 1998), and the possibility of genome-wide RNA interference by feeding (Kamath et al. 2003). These powerful methods provide the investigator with an armoury of genetic tools for probing the mechanisms underlying toxicity.   On the down side, *C. elegans* is considerably less sensitive to many common environmental toxicants than are rival test species (Sochova et al. 2007). This may be due in part to the opportunistic lifestyle of *C. elegans* in soil and compost, where it faces a variety of heavy metals, organic compounds and bacterial toxins.

In our previous study (Anbalagan et al. 2012), we documented expression changes for 24 stress-inducible GFP-reporter transgenes in *C. elegans* in response to 10 toxic metals (Al, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Zn) and a metalloid (As). The genes represented included representatives of the heat-shock, metal-sequestering, xenobiotic and oxidative stress pathways, along with their core transcription factors, plus *cep*-1 (the worm p53 orthologue) and *daf*-16 (the master regulator of stress and ageing; Kenyon 2010). Diverse toxic chemicals can exacerbate protein misfolding and/or aggregation, and in consequence activate the heat shock response. The resultant heat-shock proteins (HSPs) act both to prevent/reverse protein aggregation and to unfold/refold/reactivate damaged but salvageable proteins in the cell (de Pomerai, 1996). Different heat-shock proteins operate in different compartments of the cell; the cytosolic pathway controlled by HSF-1 is the best characterised of these (including *C. elegans hsp*-70, *hsp*-16.1/-16.2 and *hsf*-1 genes), but formally similar unfolded protein responses (UPRs) operate in both the mitochondria (*hsp*-6 and *hsp*-60) and endoplasmic reticulum (*hsp*-3) (Haynes and Ron 2010). The metal-sequestering pathway involves just two metallothionein genes (*mtl*-1 and *mtl*-2; Freedman et al, 1993), regulated by the general gut-specific ELT-2 transcription factor and a putative metal-sensitive repressor (Moilanen et al. 1999). Oxidative stress responses are inducible by reactive oxygen species (ROS), which activate

genes encoding superoxide dismutases (e.g. *sod*-1/-3/-4), catalases (e.g. *ctl*-2) and several recently curated glutathione peroxidises (including T09A12.2 now designated *gpx*-6, and C11E4.2 designated *gpx*-4); many of these are activated by the global stress regulator SKN-1 (An and Blackwell 2003). Xenobiotic stress responses involve various combinations of genes from several large families, including >80 phase I cytochrome P450 (*cyp*) genes and >40 phase II glutathione-S-transferase (*gst*) genes. We have selected a few members of these families which are known to be strongly inducible by xenobiotics, namely *cyp*-29A2, *cyp*-34A9 and *cyp*-35A2 (by PCBs; Menzel et al. 2001), plus *gst*-1 and *gst*-4 (by organic compounds such as acrylamide; Hasegawa et al. 2008). The *cyp*-35A2 gene is also inducible by fenitrothion (Roh and Choi 2011). However, we accept that this is merely scratching the surface – and that apparently ineffective toxicants might well induce different members of these extensive gene families. The transcription factors (TFs) involved in up-regulating xenobiotic-responsive genes remain obscure, although there may be some involvement of the NHR-8 orphan nuclear receptor (Lindblom et al. 2001). Finally, we have included among our test genes both the p53 orthologue *cep*-1 (Derry et al. 2001) and the master regulator of stress and ageing pathways, *daf*-16 (Kenyon 2010). Together, this panel of GFP reporters covers most of the stress-responsive pathways and their core TFs, apart from those genes involved in dealing with genotoxic stressors (with the sole exception of *cep*-1/p53).

Our previous study of metal-induced stress responses also included mathematical modelling of the underlying gene circuits, together with experimental verification and testing of predictions arising from those models in regard to both simple and complex mixtures of metals (Anbalagan et al. 2012). In this paper we report the patterns of response to a range of widely used pesticides for the same panel of 24 stress-responsive GFP-reporter strains. However, we have not yet applied mathematical modelling to predict the likely effects of pesticide mixtures, although the broad principles for each gene circuit are likely to be similar – since the regulatory module remains the same even if it is being perturbed by a completely different type of stressor. In collaboration with the ECOMETRISK project (CG and JRM), we have further tested samples of soil pore water from two agricultural soils from south east Spain – one of which (designated P74) is significantly metal-contaminated while the other (P73) is not (Table 2 below). Both pore-water samples strongly induced a variety of stress-responsive genes, yet equivalent mixtures of their metal constituents had no significant effect. By contrast, organic extracts prepared from each soil pore-water sample – after redissolving and diluting to the same final concentration – provoked similar or stronger induction of these GFP reporters than did the soil pore water; addition of the corresponding metal mixtures tended to reduce the effects of these organic extracts. Thus the major water-soluble toxic constituents in these agricultural soils appear to be organic rather than metallic in nature.

Materials and Methods.

Materials. Strain PC161 (*hsp*-16.1::GFP:*lacZ*) was developed in-house (David et al. 2003); Joel Rothman supplied JR2474 (*cep*-1::GFP; Derry et al. 2001)), Chris Link CL2070 (*hsp*-16.2::GFP; Link et al. 1999), Cynthia Kenyon CF1553 (*sod*-3::GFP), Ralph Menzel the pPD97 87-35A2prIII-GFP strain (*cyp*-35A2::GFP; Menzel et al. 2001), and the *Caenorhabditis* Genetics Center (University of Minnesota, funded by NIH National Center for Research Resources) TJ356 (*daf*-16::GFP). Other strains were supplied as integrated promoter::GFP fusions by the Baillie Genome GFP Project (Simon Fraser University, Burnaby, Vancouver, Canada; Hunt-Newbury et al. 2007), each containing c. 3 kb of upstream promoter sequence (apart from BC20306 with only 250 bp of promoter, located between the *cyp*-34A9 gene and its upstream neighbour *cyp*-34A10). These are:- BC17553 (T09A12.2 glutathione peroxidase now designated *gpx*-6::GFP), BC20303 (*hsp*-6::GFP), BC20305 (C11E4.1 glutathione peroxidise designated *gpx*-4::GFP), BC20306 (*cyp*-34A9::GFP), BC20308 (*hsp*-3::GFP), BC20309 (*mtl*-1::GFP), BC20314 (*elt*-2::GFP), BC20316 (*gst*-1::GFP), BC20329 (*skn*-1::GFP), BC20330 (*gst*-4::GFP), BC20333 (*sod*-4::GFP), BC20334 (*cyp*-29A2::GFP), BC20336 (*ctl*-2::GFP), BC20337 (*hsf*-1::GFP), BC20342 (*mtl*-2::GFP), BC20343 (*hsp*-60::GFP), BC20349 (*hsp*-70::GFP) and BC20350 (*sod*-1::GFP). Fusion gene arrays were integrated by X irradiation, and stable transgenic lines were outcrossed 4x. Expression data for these genes are available at www.wormbase.org. Strain PD4251 was used as a control strain that should be unresponsive to chemical stress, and was obtained from the *Caenorhabditis* Genetics Center (details above). PD4251 contains 3 transgenes:- pSAK2 comprising a *myo*-3 muscle myosin promoter driving a nuclear-targeted *lacZ* reporter, pSAK4 comprising a *myo*-3 promoter driving a mitochondria-targeted GFP reporter, and a wild-type *dpy*-20 gene rescuing the *dpy*-20 mutation in the parental strain. For our purposes in this study, PD4251 worms constitutively express a strong GFP signal in their mitochondria throughout the body wall muscles. The Lufa 2.2 standard soil (a loamy sand, pH 6.1)) used as a negative control for the organic extractions was obtained from Bezirks Verband (Speyer, Germany).

Determination of metals and organic contaminants in soil pore water.   Composite soil samples were taken from agricultural sites P73 (pH 8.3) and P74 (pH 8.1) (Rodriguez et al. 2006), following the method of Lopez Arias and Rodriguez (2005); these are located 6.3 and 7.0 km, respectively, from the abandoned mine site (P79) described previously (Anbalagan et al. 2012). Soil samples were sieved (2 mm mesh) and air-dried prior to analysis. Samples of these soils sent to Nottingham were reconstituted with 15 ml of ultrapure (UW) water per 60 g dried soil (20% by weight), stirred thoroughly with a glass rod, and kept overnight at 4°C in sealed beakers. Soil pore water was extracted by mild centrifugation (4,500 x g), as described previously (Power and de Pomerai 1999;

Anbalagan et al. 2012), and 0.5 ml aliquots were stored frozen for analysis or toxicity testing. Exactly the same procedure was applied to the Lufa 2.2 standard soil, though in this case the soil pre water extract was yellowish-brown in colour and fluoresced strongly in the GFP range (see text). Metal analysis was conducted by Dr Liz Bailey (Environmental Sciences Division, School of Biosciences, University of Nottingham), using soil pore-water samples diluted 10-fold in a final concentration of 2.5% (v/v) Analar grade HNO_3, as described previously (Anbalagan et al. 2012). Equivalent metal mixtures at the same final concentrations were prepared using soluble salts of the principal metals detected (Anbalagan et al. 2012), though notably our method does not allow the determination of Hg, and hence this metal was excluded. Organic extracts were prepared from 10 g of dried soil by extraction for 18 h with 250 ml of a 1:1 mixture of acetone and dichloromethane in a Soxhlet extractor according to EPA Standard Method 3540C (USEPA, 1996). After extraction, the organic solvents were removed as completely as possible by evaporation in a fume hood, leaving a gummy residue. For toxicity testing, water-soluble components from these organic residues were reconstituted with ultrapure water (2 x 1.0 ml and 1 x 0.5 ml successively for each extract from 10 g soil, stirred overnight at room temperature), although it was impossible to redissolve all of the material present. As a negative control, exactly the same procedure was applied to organic extracts prepared in the same way from 10 g samples of Lufa 2.2 soil.

Toxicity testing of pesticides, soil pore water and organic extracts. Worms were grown on NGM agar plates and washed off using ice-cold K medium (53 mM NaCl, 32 mM KCl; Williams and Dusenbery 1990). Equal aliquots of mixed-stage worms from each GFP-reporter strain were then dispensed into 24-well plates along with the test samples and appropriate controls (see below), with four replicates in different plates for each test condition. Plates were incubated at 20°C, and the contents of each well were transferred at early (4-6 hr), intermediate (8-20 hr) and late (24-40 hr) time points into a black non-fluorescent U-bottomed 96-well microplate. After standing on ice for 15 min, GFP expression was quantified in the worm pellet using a Perkin-Elmer Victor 1420 Multilabel plate reader using narrow band-pass filters at 485 nm (excitation) and 525 nm (emission), as described previously (Anbalagan et al. 2012). After reading, worm suspensions were returned to their parent 24-well plates for further incubation, or examined briefly under a low-power inverted microscope to estimate mortality before disposal. All pesticides tested were Pestanal grade from Sigma/Aldrich Ltd (Poole, Dorset, UK). Glyphosate, paraquat (methyl viologen) and 2,4-dichlorophenoxyacetic acid (2,4-D) were dissolved in ultrapure water (UW), but most other pesticides were dissolved in dimethyl sulphoxide (DMSO) at or near the limit of their solubility, generating stock solutions containing between 2and 50 mg ml^{-1}; endosulfan, cypermethrin and carbendazim were dissolved in ethanol rather than DMSO, chlorpyrifos in methanol, and diuron in a 1:1 (v/v) mixture of DMSO and ethanol. The highest test concentration

of each pesticide was in all cases a 500 or 1,000-fold dilution of the stock solution, such that the maximal concentration of solvent present was 0.2% (v/v). In all cases apart from dichlorvos and the water-soluble herbicides, two sets of controls were set up for each pesticide – one using ultrapure water (UW) and the other the maximal concentration of solvent (0.1 or 0.2% v/v). All test results were normalised against the solvent (or UW) control as 1.0 at each time point, but the differences between UW and solvent controls were negligible (< 10%) and non-significant for all 24 test genes. For soil pore water extracts, 5 test conditions were routinely compared (each in quadruplicate):- ultrapure water control (UW), freshly extracted soil pore water (P73 or P74), the corresponding metal mixture (MM), the corresponding organic extract (OE), and a mixture of OE + MM to mimic the original soil pore water in terms of both metals and water-soluble organic contaminants. For Lufa 2.2 control organic extracts, OE and soil pore water were compared against the UW control.

Feeding inhibition assays. In order to assess the general toxicity of these pesticides to *C. elegans*, we used feeding inhibition assays on wild-type N2 worms (Jones and Candido 1999). Worms were washed extensively with K medium, and equal aliquots (1000 worms per well in 6-well plates) were mixed with a dense suspension of food bacteria (*E. coli* OP50, at an initial optical density at 550 nm [OD_{550}] of 1.0) plus a full set of test concentrations and solvent and/or ultrapure water controls for each pesticide, in a final volume of 1250 µl. All assays were performed in quadruplicate. Plates were placed in a sealed humidified box and incubated at 20°C for 28 hr. The contents of each well were transferred to Eppendorf tubes and kept on ice to allow worms and faecal pellets to settle; 1.0 ml of each supernatant was withdrawn carefully and the OD measured at 550 nm in a plastic cuvette. Controls without worms present were also included. Percentage feeding inhibition (Table 1) was calculated as follows, where FI = feeding inhibition, NWC = OD_{550} of no-worms control at the highest pesticide concentration (no feeding), HPC = OD_{550} after 28 hr at the highest pesticide concentration, and ZC = OD_{550} after 28 hr of the solvent /ultrapure water control (maximal feeding).

$$FI\ (\%) = \frac{[HPC - ZC] \times 100}{[NWC - ZC]}$$

Statistical analysis. For dose response data, mean fluorescence measurements (in arbitrary relative fluorescence units, RFU ± SEM) were analysed by one-way ANOVA with Dunnett's *post hoc* multiple comparisons test against solvent controls (no pesticide) at the same time point. Throughout the Figures, Tables and Supplementary Material, all fluorescence measurements have been normalised against the corresponding zero (ultrapure water or solvent) controls at the same time point, giving an expression ratio which is always 1.0 for the controls Other comparisons within data sets were made using one-way ANOVA with Bonferroni's *post hoc* multiple comparisons test. Straightforward comparisons between control and test conditions used a two-tailed Student's t test.

Results.

Figure 1 shows the patterns of induction for 4 stress-responsive genes (out of 24) by 4 of the tested pesticides (out of 12) after 28 hours; complete concentration-response data for all genes, pesticides, doses and time-points are provided in the Supplementary Material. Dichlorvos (Figure 1A) is unusual in that most of the genes tested (though not *cep*-1) showed concentration-dependent induction after 24 hours (some heat-shock protein genes were up-regulated as early as 4 hr). Although a solvent control (0.1% v/v DMSO) was not included in the data-set shown, parallel experiments using rotenone dissolved in the same solvent (Figure 1B) confirmed that 0.1% DMSO on its own did not alter expression for any of the 4 genes tested, and this held broadly true for all of the solvents used with all of the genes tested (Supplementary Material set 1). Lethality experiments on *C. elegans* have not been published for most pesticides tested in our study, but feeding inhibition studies (top row of Table 1 and Supplementary data set 2) suggested that the concentrations tested were almost all sublethal (feeding inhibition is a good predictor of starvation and subsequent lethality; Jones and Candido 1999). Only in the case of dichlorvos did feeding inhibition approach 50% at the highest test concentration (400 mg l^{-1}), while for chlorpyrifos, paraquat and glyphosate feeding inhibition was between 20 and 25% at the highest test concentration (Table 1; complete data set shown in Supplementary Material set 2). We conclude that the gene-expression responses described in this paper were also elicited at mostly sublethal concentrations. The limited solubility of the natural insecticide rotenone precluded testing at high concentrations (Figure 1B), but two of the genes (*cyp*-34A9 and *hsp*-16.1) showed significant ($p < 0.05$) up-regulation at 6.7 mg l^{-1} and above, whereas two others (*cep*-1 and *gpx*-4) did not. A third pesticide, the popular herbicide glyphosate (Figure 1C), up-regulated both *cep*-1 and *hsp*-16.1 at lvery high concentrations (1,000 and 10,000 mg l^{-1}), whereas expression of *cyp*-34A9 did not increase even at the highest concentration ($p > 0.05$). By contrast, the *gpx*-4 glutathione peroxidase gene showed concentration-dependent up-regulation, which was significant ($p < 0.05$) at 10 mg l^{-1} and above – well within the range of application doses recommended for spraying on garden weeds. Perhaps the most unusual pattern of response was that seen for endosulfan (Figure 1D), where a single gene (*cyp*-34A9) showed 8-10 fold induction even at the lowest concentration tested (0.2 mg l^{-1}), and this was already evident by 4 hours (Supplementary Material set 1); by contrast, none of the other 23 genes showed any sign of altered expression even at the highest (but still sublethal; top row, Table 1) test concentration of 200 mg l^{-1}.

The rest of Table 1 summarises the outcome of these tests for all 12 pesticides and all 24 stress-response genes. Only the maximal response is shown in Table 1, irrespective of the time-point or

concentration at which that effect was noted.   For several pesticides, effects were confined to one or a very few genes, such as the up-regulation of *cyp*-34A9 at the highest concentration of cypermethrin, or of *cyp*-35A2 at the highest concentration of DDT. Similarly, deltamethrin only up-regulated the mitochondrial superoxide dismutase gene *sod*-3, albeit at low rather than high concentrations. These limited effects are consistent with the fact that these 3 insecticides specifically target insect sodium channels, for which there are no *C. elegans* orthologues (Bargmann 2006). In the case of the herbicide diuron, we were unable to detect any expression changes for any of our 24 test genes – although we cannot exclude the possibility that other stress genes (e.g. other members of the extensive *cyp* and/or *gst* gene families) may respond to this agent, or that responses might only become apparent at higher concentrations (there was no sign of feeding inhibition by the highest achievable test concentration of diuron; Table 1). However, since the target for diuron action is plant photosynthesis, this agent could be non-toxic to *C. elegans*. For the other herbicides tested (glyphosate, 2, 4-D, paraquat), reporter gene induction was almost always observed at high concentrations (> 1,000 mg l^{-1}), although several oxidative stress-response genes were up-regulated by paraquat at lower concentrations, as was *sod*-3 in response to 2, 4-D (see Supplementary Material set 1). A range of different genes showed up-regulation by dichlorvos and rotenone, whereas endosulfan and chlorpyrifos both gave strong induction of *cyp*-34A9, even though the former is an organochlorine and the latter an organophosphate pesticide. The fungicide carbendazim, by contrast, down-regulated several heat-shock genes, but also up-regulated *sod*-3.

In this study we have also tested soil pore water samples from two agricultural sites in the Murcia region of S.E. Spain – one (P73) used for lettuce production and the other (P74) for growing cauliflowers. Table 2 shows the concentrations of soluble metals present in soil pore water extracted (as detailed in Materials and Methods) from both soils. P74 is more heavily contaminated with metals than P73, with the exception of V (much higher in P73), Ni (slightly higher in P73) and Cu (very similar in both). In particular, the most toxic metals present were far more abundant in P74 as compared to P73; thus Cd was 10-fold higher, Zn 7-fold higher and Pb nearly 20-fold higher, whereas most other metals were only 2-3 fold higher. Overall, P74 soil is regarded as metal-contaminated whereas P73 is not (Rodriguez et al. 2006). However, the concentration of each metal even in P74 soil pore water (μg l^{-1}) was far below the range needed to induce stress gene expression (of the order of mg l^{-1}) with this same panel of GFP reporter strains (Anbalagan et al. 2012).

Organic contaminants were also extracted from both P73 and P74 soils, and the water-soluble fractions of these organic extracts (OE) have been tested (as whole mixtures) both independently and in combination with mixtures of metal ions (MM) at concentrations corresponding to the original soil pore water (Table 2), samples of which were also tested within the same experiment.

Results are shown for two heat-shock genes (cytosolic *hsp*-16.2 and mitochondrial *hsp*-6) in Figure 2, for *gpx*-6 and *gst*-1 in Figure 3, and for *cyp*-34A9 in Figure 4. In only one case was gene expression induced above UW control levels by an MM metal mixture (*gst*-1 at 6 hr for P73 in Figure 3C; p > 0.05). Soil pore water from both P73 and P74 soils up-regulated the expression of all 5 GFP reporter genes, as did the corresponding OE organic extracts (often more strongly) and the OE + MM mixtures. All of these differences from UW controls were significant (p < 0.05) or highly significant (p < 0.01) using the Bonferroni test, whereas those for the MM mixtures alone were not (p > 0.05). For *hsp*-16.2, P74 pore water and especially its organic constituents induced a much stronger response (up to 4-fold) than did P73 (just over 2-fold; Figure 2A and 2B). By contrast, P73 pore water and its organic constituents induced *hsp*-6 expression strongly (2-3 fold after 6 hours), whereas the responses to P74 pore water, OE and OE + MM were modest (< 2-fold), though both declined over time (Figure 2C and 2D). A different pattern was apparent for *gpx*-6 in Figure 3A and 3B, where gene induction by P73 pore water and its mixtures was modest (c. 2-fold) and increased slightly over time, whereas the corresponding responses for P74 were much stronger (3-5 fold) and were sustained at least up to 36 hours. As for *gst*-1, induction was stronger with P74 and organic mixtures than with P73, although in both cases there was a marked decline over time (Figure 3C and 3D). There were interesting differences between the effects of soil pore water and of the OE and OE + MM mixtures; for *gpx*-6, all 3 were very similar in the tests with P73 (Figure 3A; p > 0.05), but differed greatly in the tests with P74 (Figure 3B; p < 0.01). This pattern was also seen for *hsp*-16.2 and *hsp*-6 with P74 (Figure 2B and2 D) and for *hsp*-6 with P73 at 6 hours only (Figure 2C). In all such cases, the response for the organic extract (OE) was always much stronger (sometimes by as much as 2-fold – e.g. for *gpx*-6 with P74 at 6 hr in Figure 3B; p < 0.01) than that given by the soil pore water, and in most cases addition of MM reduced the OE response down towards the levels seen with the corresponding soil pore water (though this reduction was not always significant). This was especially marked for *hsp*-16.2 with P73 (Figure 2A; p < 0.05 only at 18 hr) and for *gst*-1 with P74 (Figure 3D; p < 0.05 at 6 hr), although similar trends were also apparent elsewhere (e.g. Figure 2B and 2C, Figure 3C; p > 0.05 in all cases).   In a minority of cases, the response to OE was comparable to or slightly lower than that for soil pore water, and was further reduced by addition of the MM metal mixture (e.g. *hsp*-6 with P74 in Figure 2D, and for *gst*-1 with P73 in Figure 3D; p < 0.05 between soil water and OE+MM in both cases). Broadly speaking, similar trends were seen for *cyp*-34A9 responses to P73 and P74 soil pore water samples and the corresponding organic extracts (Figure 4A and 4B). In this instance (Figure 4B), the response to P74 organic extract was far greater (around 7-fold) than to the original P74 pore water sample (just over 2-fold; p < 0.01). This 3-fold disparity is difficult to explain, unless the organic extraction of P74 released pesticide or other organic residues that were tightly bound to soil particles and thus poorly soluble in soil pore water. It is noteworthy that both of the other large disparities between soil pore water samples and the corresponding organic

extracts were also seen with P74 (Figures 2B and 3B).

Two sets of controls were utilised in this study. Firstly, soil pore water and organic extracts were prepared from a widely-used uncontaminated standard soil (Lufa 2.2), using the same methodology as for P73 and P74. Surprisingly, the pore water extracted from Lufa 2.2 soil was yellowish brown in colour and showed strong fluorescence in the GFP range. We therefore washed the worms extensively (5 times in fresh K medium; Williams and Dusenbery 1990) after the final exposure period (20 hr), then re-measured the fluorescence signal present within the worms themselves, without interference from fluorescent contaminants in the medium surrounding them. A similar procedure was applied to the worms treated with Lufa 2.2 organic extract, although the interfering fluorescent material was largely absent from this fraction. As shown in Figure 4C, GFP reporter induction (as judged by the residual fluorescence after washing) was 50% or less in all cases (expression ratios of between 0.95 and 1.51), although this differed significantly ($p < 0.05$) from the UW control in 3 instances (for *hsp*-16.2 with Lufa 2.2 pore water only, but for *hsp*-6 with both pore water and organic extract). We conclude that soil pore water from a non-contaminated site did not induce reporter expression strongly (in contrast to both P73 and P74), and there was similarly no evidence that solvent residues in the organic extracts had any such effect.

A separate set of pesticide controls is shown in Figure 4D; here, high concentrations of the 4 pesticides studied in Figure 1 were applied to a constitutively expressed *myo*-3::GFP reporter strain (PD4251), as well as to a stress-responsive strain already known to be induced by that pesticide (see Figure 1 and Table 2). In all cases, there was only a small increase or reduction in *myo*-3::GFP expression – and although these changes sometimes attained statistical significance (as indicated), none of them altered the expression ratio by as much as 50%. In contrast, the same doses of all 4 pesticides strongly induced expression of the selected stress reporter strain (by as much as 8-fold in the case of *cyp*-34A9 and endosulfan; Figure 4D). The case of glyphosate is worthy of comment; although all of our stress reporter strains showed >75% survival at 10,000 mg l^{-1} glyphosate (see top row in Table 1), the PD4251 *myo*-3::GFP strain was far more sensitive to this agent, with signs of mortality (c. 30% immobile) already apparent at 6 hr, extending to most worms (>75%) at 20 hr. Although high concentrations of glyphosate induced *gst*-1::GFP expression by only 1.8 fold at 6 hr (Figure 4D), this increased to > 2-fold after 20 hr; however, over this longer time-frame expression of *myo*-3::GFP decreased by nearly 50% due to extensive worm mortality.

Discussion.

In our previous paper (Anbalagan et al. 2012), we documented the effects of 10 metals plus a metalloid on the expression of this same panel of 24 stress-responsive GFP reporter strains in *C. elegans*. With the exception of Mn, Pb, and to some extent Ni, all of these metals up-regulated the expression of a broad range of genes – including members of the heat-shock, oxidative stress and xenobiotic stress gene families, as well as those encoding metal-binding proteins. Similar broad-spectrum activation of stress-response genes was seen for a minority of the pesticides tested here – namely dichlorvos, rotenone, very high concentrations of glyphosate (Figure 1 and Table 1) and of paraquat (Table 1). These findings are consistent with the general toxicity of these agents, as evinced by our feeding inhibition data (top row of Table 1 and Supplementary Material set 2), although both herbicides have minimal effects on animals at field application doses, thanks to their plant-specific modes of action. Among the other herbicides tested, 2, 4-D showed limited effects on a handful of genes (most notably the p53 orthologue *cep*-1), while diuron had no detectable effects within the concentration range achievable. Many of the pesticides studied here had to be dissolved in organic solvents (methanol, dimethyl sulphoxide and/or ethanol) prior to dilution in K medium for testing; using a maximum final solvent concentration of 0.2% v/v averted confounding solvent effects on gene expression (Figure 1B and 1D; Supplementary Material), but also imposed a maximum limit on the test concentration achievable for each such pesticide (ranging from 67 to 400 mg l^{-1}). Several pesticides induced one or a restricted subset of stress-responsive genes, usually at the highest dose tested (Table 1); these included DDT (*cyp*-35A2), carbendazim and deltamethrin (both *sod*-3), as well as cypermethrin, endosulfan and chlorpyrifos (all *cyp*-34A9). Given the solubility issues outlined above, it was impossible to determine whether a broader range of genes might have been affected at still higher concentrations. Dichlorvos, and to some extent chlorpyrifos, significantly (p < 0.05) inhibited feeding at the highest concentrations tested (by nearly 50% and 25%, respectively; Table 1), and this was also true for two of the water-soluble herbicides tested (glyphosate and paraquat). For these 4 agents, at least, genes induced only at the highest test concentration may represent emergency responses in moribund or dying worms. However, the example of endosulfan suggests that this is not necessarily the case; here, *cyp*-34A9 was very strongly induced even at the lowest concentration tested (0.2 mg l^{-1}; Figure 1D) and from the earliest time point (Supplementary Material set 1), yet all of the other test genes were unaffected even at a 1,000-fold higher concentration (200 mg l^{-1}), where feeding was only 1% inhibited (Table 1). It is interesting that this same gene is also induced strongly by chlorpyrifos and dichlorvos, as well as more weakly by cypermethrin and rotenone, whereas DDT induced a different member of the large cytochrome P450 gene family (*cyp*-35A2). It might be worth developing pathway-specific gene arrays for inducible *cyp* and *gst* genes, since different pesticides may well induce different members

of these large gene families. Literature reports suggest that moderate doses of chlorpyrifos (0.1 and 0.5 mg l^{-1}) can up-regulate both *cyp*-35A2 (albeit marginally; Roh and Choi 2008) and *cyp*-35A3 (Vinuela et al. 2010); however, in the present study, our *cyp*-35A2 reporter was not detectably induced across the test concentration range (0.3-300 mg l^{-1}; Supplementary Material set 1), whereas *cyp*-34A9 was induced 7-fold at 300 mg l^{-1}. This does not reflect a general lack of responsiveness by the *cyp*-35A2::GFP fusion strain (Menzel et al. 2001), which was inducible by DDT, dichlorvos and rotenone (Table 1), but key regulatory elements required for the chlorpyrifos response might lie outside the promoter region driving GFP expression. The organophosphorus pesticide fenitrothion also up-regulates *cyp*-35A2 expression (Roh and Choi 2011). Lastly, very high concentrations of cypermethrin (5 mM = 2080 mg l^{-1}) have been reported to up-regulate *hsp*-16.1 expression in a *lacZ* reporter strain (Shashikumar and Rajini 2010). This gene was not detectably up-regulated in our study (see Supplementary Material), but our maximum test concentration was only 100 mg l^{-1}.

The limited changes in gene expression seen with pesticides that target the insect sodium channel (pyrethroids and DDT) are consistent with their limited toxicity towards non-target organisms; the notoriety of DDT stems from its environmental persistence and biomagnification, rather than high toxicity *per se* (Casida, 2009). Notably, *C. elegans* does not possess direct orthologues of the insect or vertebrate sodium channel genes (Bargmann 2006; Vinogradova et al. 2006). Previous studies of pesticide effects on *C. elegans* (reviewed in Leung et al. 2008) have measured whole-organism endpoints (Rajini et al. 2008, for organophosphorus compounds; Boyd et al. 2010, for neurotoxins), or else specific gene responses such as *hsp*-16.1::*lacZ* in response to captans (Candido and Jones 1996), *gst*-4 in response to acrylamide (Hasegawa et al. 2008), or cytochrome P450s in response to PCBs and other xenobiotics (Menzel et al. 2001, 2007; Roh and Choi 2011). Another very sensitive biochemical assay for the toxicity of organophosphorus insecticides (OPIs) is provided by the inhibition of acetylcholinesterase (AChE) activity in *C. elegans* (Melstrom et al. 2007; Rajini et al. 2008; Roh and Choi 2008; Jadhav and Rajini 2009; Roh and Choi 2011), and more recently a mathematical model has been developed to describe the effects of one such OPI (chlorpyrifos) on the growth and development of *C. elegans* (Boyd et al. 2009).

Overall, the data presented here (Figure 1, Table 1 and Supplementary Material set 1) demonstrate the utility of this wide-ranging panel of transgenic GFP reporter strains for exploring patterns of stress-gene expression in response to pesticides. Certain genes (such as *cyp*-34A9) seem particularly useful for this purpose, whereas others (e.g. *ctl*-2, *hsf*-1) did not respond to any of the agents tested. Several recent reports have begun to explore the effects of two-component pesticide mixtures on *C. elegans* (Gomez-Eyles et al. 2008; Vinuela et al. 2010; Negga et al. 2011), using a variety of different approaches. Our GFP panel provides a simple high-throughput approach to monitoring pesticide

effects – not only for the pure active agents, but also for their commercial formulations, and for both simple and complex mixtures of pesticides, as well as their degradation products and residues present in environmental samples.

Turning to two such environmental samples, soil pore water from the P73 and P74 agricultural sites in Murcia (SE Spain) strongly up-regulated several stress-response genes, of which 5 representative examples are shown in Figures 2 (for *hsp*-16.2 and *hsp*-60), 3 (for *gpx*-6 and *gst*-1) and 4 (for *cyp*-34A9). Induction ranged from around 1.5 fold to >7-fold, which is comparable to the responses seen at very high doses of single metals (Anbalagan et al. 2012) or pesticides (this paper). Initially, P73 and P74 soil samples were selected because the latter contains much higher levels of soluble metals in extracted pore water (Table 2); however, equivalent mixtures of these metals were unable to induce any of the transgenes tested to a significant extent ($p > 0.05$; 2-tailed Student's t test), as shown by the MM (metal mixture) bars relative to UW controls in Figures 2, 3, 4A and 4B. Despite this evidence for high levels of toxicity, >80% of test worms (all strains) remained motile in the soil pore water and organic extracts from both soils, even after 40+ hours. Broadly speaking, it was the organic extracts (OE) from these soils that induced elevated GFP expression in several GFP reporter strains (Figures 2, 3, 4A, 4B). In many cases, expression levels were higher for OE than for the original soil water sample, and often the effects of OE on gene expression were moderated by addition of the corresponding metal mixture (OE+MM; Figures 2A, 2C, 3B, 3C, 3D, 4A and 4B). This may be explicable in terms of interfering effects between different toxicants acting antagonistically on the same gene expression module, as suggested previously by mathematical modelling of stress-gene regulatory circuits (Anbalagan et al. 2012). However, in several instances the effects of organic extracts (OE) on reporter-gene expression were dramatically greater than those elicited by the corresponding soil pore-water sample, and in such cases the moderating effect of the metal mixture (OE+MM) did not reduce gene expression back down to the levels seen with the original soil pore water.   Examples of this pattern were seen for P74 with *hsp*-16.2 (Figure 2B), with *gpx*-6 (Figure 3B) and most notably with *cyp*-34A9 (Figure 4B), whereas for P73 this behaviour could only be discerned for *hsp*-6 (and possibly *hsp*-16.2) at 6 hours (Figure 3A and 3C).

In regard to the control experiments reported in this study, despite the unanticipated fluorescence interference from Lufa 2.2 soil pore water, neither this nor the corresponding organic extract could induce reporter transgene expression to the high levels seen with P73 or P74 samples.   This implies that the stress reporter inductions observed were not artefacts of the organic extraction procedure. Since the corresponding metal mixtures (MM) were unable to induce stress-gene expression, these findings confirm that the strong inducing effects of P73 and P74 soil pore water were caused largely by water-soluble organic constituents. Similarly, none of the pesticides tested in Figure 1 elicited any

major induction of the constitutively expressed *myo*-3::GFP transgene in PD4251 worms (Figure 4D).

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Tables.

Pesticide concentration range →	Gly 0-10⁴ mg/l	2,4D 0-5000 mg/l	Pq 0-2500 mg/l	Diur 0-67 mg/l	Carb 0-100 mg/l	DDT 0-100 mg/l	Cyp 0-100 mg/l	Del 0-100 mg/l	End 0-200 mg/l	Dc 0-400 mg/l	Clp 0-300 mg/l	Rot 0-20 mg/l
Percentage Feeding Inhibition at highest test concentration	24.6 ± 2.20 %	0 ± 0.51 %	22.1 ± 0.31 %	0 ± 0.24 %	0 ± 0.71 %	5.84 ± 0.38 %	2.34 ± 0.187 %	6.39 ± 0.58 %	1.086 ± 0.121 %	48.75 ± 0.26 %	19.67 ± 0.136 %	8.20 ± 1.37 %
Heat-shock (Proteotoxicity Response) Genes												
hsp-16.1	++									+++		++
hsp-16.2	-				-					+++		++
hsp-3	+									++		
hsp-6	+									+++	+	+
hsp-60	-		+		-					++		+
hsp-70										++		
hsf-1												
Metal Response Genes.												
mtl-1			+									+
mtl-2		+								++		++
elt-2*	++		++									+
Xenobiotic Response Genes.												
gst-1	-				-					+		+
gst-4	-	-								+		
cyp-29A2	+		+							+		++
cyp-34A9							+		++++	+++	++++	+
cyp-35A2						+				+		++
Oxidative Stress Response Genes.												
sod-1	+									+		++
sod-3	- -	+			++			+		-		
sod-4	++		+							+		
ctl-2	-									-		
gpx-6 T09A12.2	++		+							+		+
gpx-4 C11E4.1	+++		+			-				++		
skn-1	+		+					-		+		++
Core Stress-Responsive Transcription Factors.												
cep-1	++	++								+		
daf-16			+							++		

Table 1. Summary of feeding inhibition and strongest responses for 12 pesticides in 24 GFP reporter strains. Gly = glyphosate; 2,4D = 2,4 dichlorophenoxyacetic acid; Pq = paraquat (methyl viologen); Diur = diuron; Carb = carbendazim; DDT = dichlorodiphenyl-trichloroethane; Cyp = cypermethrin; Del = Deltamethrin; End = endosulfan; Dc = dichlorvos; Clp = chlorpyrifos; Rot = rotenone. The top row shows the percentage feeding inhibition (calculated as described in Materials and Methods) for the highest test dose of each pesticide, measured over a 28 hour period with wild-type N2 worms (mean ± SEM). The remaining rows show the relative strength of response for each gene group: -- = >2 fold down-regulated; - = 1.5 to 2 fold down-regulated; no entry = no change(>45% up or down from control); + = 1.5 to 2 fold up-regulated; ++ = 2 to 3 fold up-regulated; +++ = 3 to 4 fold up-regulated; +++++ = >4 fold up-regulated. Complete data for all pesticide concentrations and controls is provided in the Supplementary Material (set 1 for GFP reporter responses, set 2 for the feeding inhibition data). *Note that *elt*-2 encodes a gut-specific transcription factor, which is involved in but not confined to the metal response group.

Metals	Al	V	Cr	Mn	Fe	Ni	Cu	Zn	As	Cd	Ba	Pb	U
Soil sample	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l
P73 n = 3	360 ± 129	19.8 ± 0.35	1.19 ± 0.16	106 ± 2.5	188.5 ± 71.4	21.4 ± 0.48	65.0 ± 0.98	26.4 ± 4.1	21.6 ± 0.72	0.45 ± 0.03	205.8 ± 2.28	4.21 ± 0.96	2.78 ± 0.05
P74 n = 3	693 ± 115	6.91 ± 0.12	2.65 ± 0.19	217 ± 2.9	432.3 ± 56.2	15.2 ± 0.09	62.0 ± 1.2	182.6 ± 29.8	32.1 ± 0.33	4.41 ± 0.12	211.0 ± 1.65	79.02 ± 12.2	4.45 ± 0.03

Table 2 . Metal contents of soil-water samples from the P73 and P74 test sites.

Soil samples from the agricultural sites P73 and P74 were obtained fresh in November 2009; both samples were air-dried to constant weight and mixed with 25% w/w ultrapure water, then soil water was extracted centrifugally and diluted in 2.5% (v/v) HNO_3 for subsequent metal analysis by ICP-MS, as described in Materials and Methods. Table 2 shows mean and standard deviation for each metal concentration (in µg l^{-1} or ppb present in undiluted soil water) derived from 3 replicate samples. Note that it was not possible to determine the concentration of Hg using this analysis method.

Figure Legends.

Figure 1. Dose-response patterns at the late time point for four pesticides using four transgenes.

Each panel shows the dose responses for four transgenic GFP reporter strains, namely *cyp*-34A9 (solid line, filled circles), *hsp*-16.1 (dashed line, filled squares), *gpx*-4 (dotted line, open triangles) and *cep*-1 (alternating dash and dot line, filled inverted triangles). All data have been normalised to the relevant solvent control as 1.00. Panel A shows the responses to dichlorvos dissolved in a maximum of 0.1% (v/v) DMSO (0.04 to 400 mg l^{-1}); panel B shows responses to rotenone in 0.1% DMSO maximum (0.74 to 20 mg l^{-1}); panel C shows responses to glyphosate in ultrapure water (1-10,000 mg l^{-1}); and panel D shows responses to endosulfan in 0.1% ethanol maximum (0.2 to 200 mg l^{-1}). The statistical significance of differences from the solvent control is indicated for each group (right) of test genes by asterisks: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. Brackets indicate p values that approach but do not quite reach the stated significance.

Figure 2. Response of heat-shock reporters to soil pore water, metal mixtures and organic extracts.

Both P73 (parts A and C) and P74 (parts B and D) agricultural soils were used to prepare soil pore water (P73 or P74) and whole-soil organic extracts (OE), as described in Materials and Methods. Metal mixtures (MM) were prepared from soluble salts, at the final concentrations measured in soil pore water (see Table 2). Expression ratios have been calculated by normalising all data against the corresponding ultrapure water control within each group (UW = 1.00). Each group of 5 bars shows (from left to right):- UW control, MM alone, P73 (A, C) or P74 (B, D) soil pore water, OE alone, and OE+MM (reconstituting the water-soluble organic and metal components of the corresponding pore water sample). The three groups of bars in each panel show (from left to right):- early responses (6 hr), intermediate responses (18 hr) and late responses (36 hr) for two heat shock genes – *hsp*-16.2 (cytosolic small HSP) in parts A and B, and *hsp*-6 (mitochondrial HSP70) in parts C and D. All bars show the mean and SEM derived from 4 replicates; statistical significance is discussed in the text.

Figure 3. Response of stress reporters to soil pore water, metal mixtures and organic extracts.

P73 and P74 pore water, organic extracts (OE) and metal mixtures (MM) were prepared exactly as described in the legend to Figure 2, and expression ratios have been calculated in the same way from the normalised data (UW control = 1.00 throughout). Each group of 5 bars shows (from left to right):- UW control, MM alone, P73 (A, C) or P74 (B, D) pore water, OE alone, and OE+MM (as above). The three groups of bars in each panel show (from left to right):- early responses (6 hr), intermediate responses (18 hr) and late responses (36 hr) for two oxidative/xenobiotic stress genes, namely *gpx*-6 in parts A and B, and *gst*-1 in parts C and D. All bars show the mean and SEM derived from 4 replicates; statistical significance is discussed in the text.

Figure 4. Responses of constitutive and stress reporters to soil pore water and pesticides.
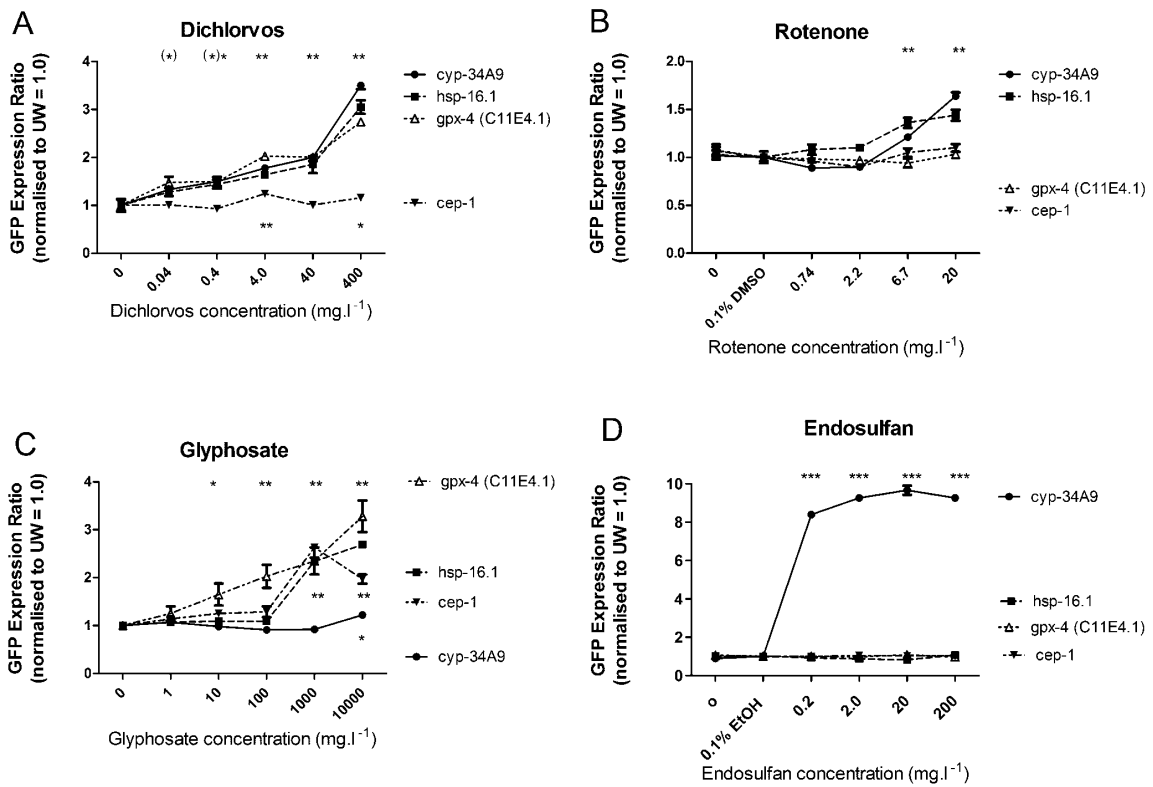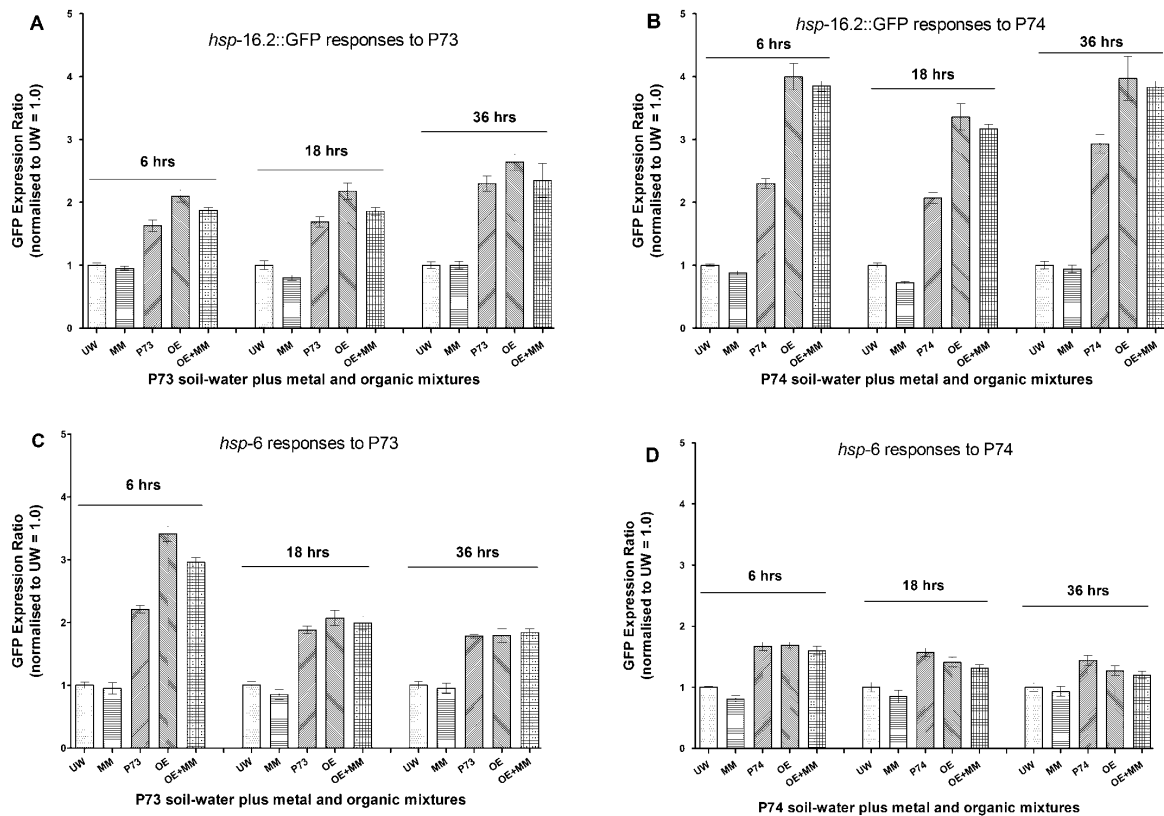
In the upper half of this figure, P73 (part A) and P74 (part B) soil pore water, organic extracts (OE) and metal mixtures (MM) were prepared exactly as described in the legend to Figure 2, and expression ratios have been calculated in the same way from the normalised data (UW control = 1.00 throughout). Each group of 5 bars shows (from left to right):- UW control, MM alone, P73 (A) or P74 (B) pore water, OE alone, and OE+MM (as above). The two groups of bars in each panel show intermediate (12 hr; left) and late responses (24 hr; right) for the xenobiotic stress gene *cyp*-34A9 (parts A and B).   All bars show the mean and SEM derived from 4 replicates; statistical significance is discussed in the text. Part C shows the responses of four test reporters (*cyp*-34A9, *gpx*-6, *hsp*-16.2 and *hsp*-6) to soil pore water (L 2.2)and redissolved organic extract (L 2.2 OE) prepared from the Lufa 2.2 control soil, as described in Materials and Methods. Only the late responses seen after 20 hr are shown here, for reasons explained in the text. Each group of 3 bars shows the mean and SEM from 4 replicate assays; the left-hand bar in each group is UW control, the central bar is Lufa 2.2. soil pore water, and the right-hand bar is the organic extract from Lufa 2.2 soil. The reporter gene tested is indicated above each group of bars.   Part D shows the responses of the constitutively expressed *myo*-3::GFP reporter to the highest test doses of 4 pesticides, alongside parallel data for one stress reporter gene that is strongly induced by that pesticide (i.e. *cyp*-34A9::GFP for both chlorpyrifos and endosulfan, *hsp*-16.2::GFP for rotenone, and *gst*-1 for glyphosate). For each group of 4 bars, the left-hand pair show the response of the *myo*-3::GFP reporter to UW (left) and pesticide (right) respectively, whereas the right-hand pair show the response of one selected stress-reporter to the same conditions (UW on left, pesticide on right). Late responses at 20 hrs are shown, apart from glyphosate where early 6 hr responses are shown (see text). In parts C and D, data were normalised to the UW control in each group (expression ratio of 1.00), and the statistical significance of any differences from UW controls is indicated by asterisks: * = p < 0.05; ** = p < 0.01; *** = p < 0.001.

Figure 1

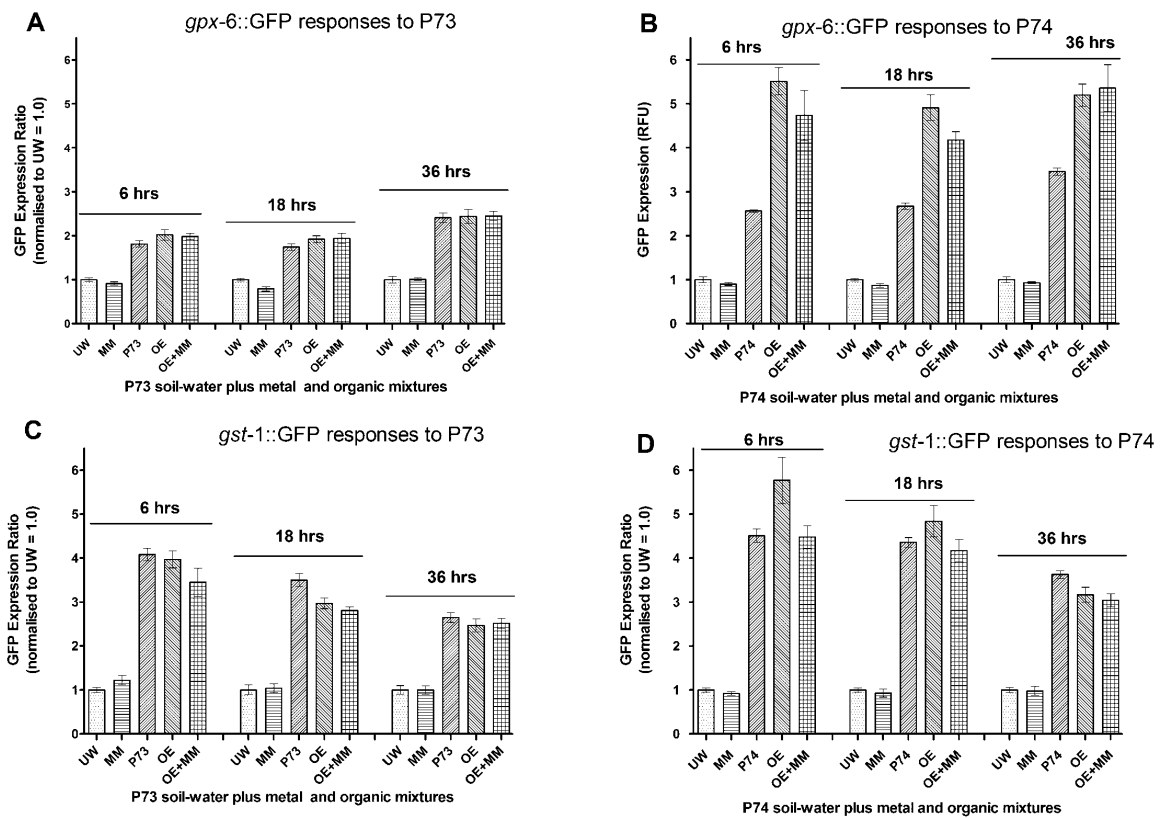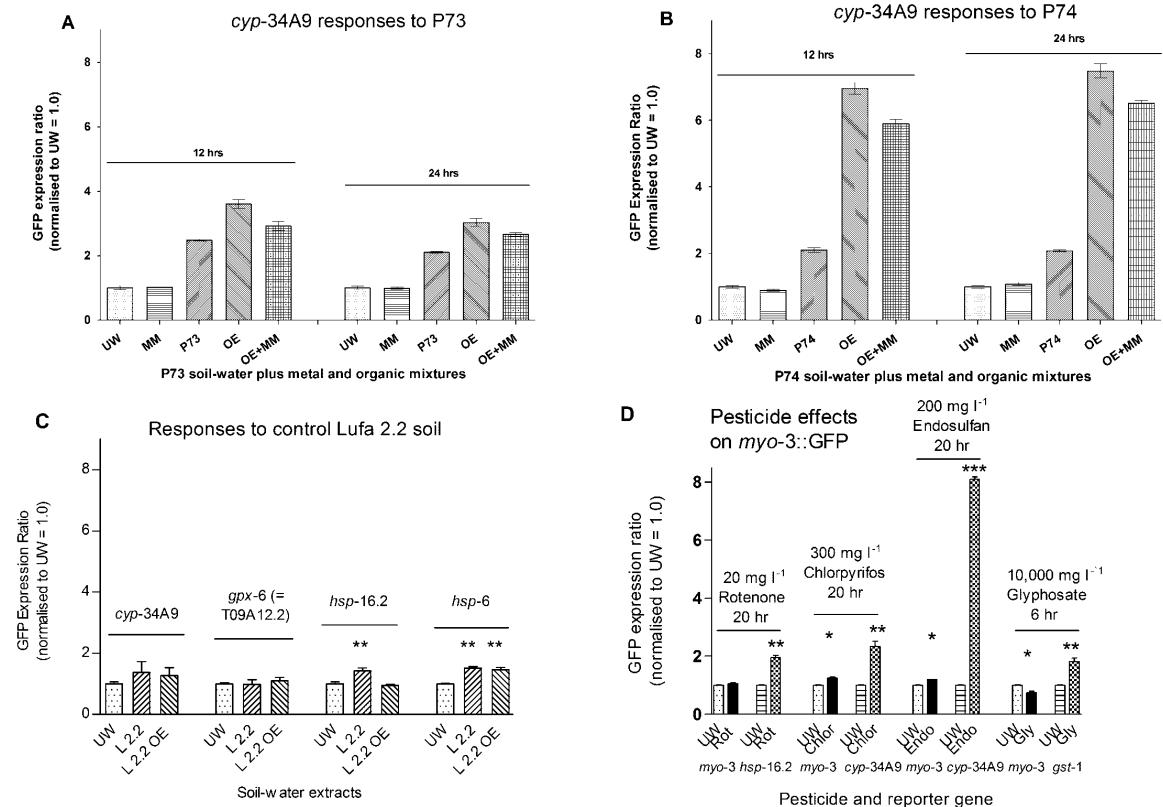

A Dichlorvos

B Rotenone

C Glyphosate

D Endosulfan

Figure 2.

A *hsp*-16.2::GFP responses to P73

B *hsp*-16.2::GFP responses to P74

C *hsp*-6 responses to P73

D *hsp*-6 responses to P74

Figure 3.

A

gpx-6::GFP responses to P73

GFP Expression Ratio (normalised to UW = 1.0)

6 hrs   18 hrs   36 hrs

UW MM P73 OE OE+MM (×3)

P73 soil-water plus metal and organic mixtures

B

gpx-6::GFP responses to P74

GFP Expression (RFU)

6 hrs   18 hrs   36 hrs

UW MM P74 OE OE+MM (×3)

P74 soil-water plus metal and organic mixtures

C

gst-1::GFP responses to P73

GFP Expression Ratio (normalised to UW = 1.0)

6 hrs   18 hrs   36 hrs

UW MM P73 OE OE+MM (×3)

P73 soil-water plus metal and organic mixtures

D

gst-1::GFP responses to P74

GFP Expression Ratio (normalised to UW = 1.0)

6 hrs   18 hrs   36 hrs

UW MM P74 OE OE+MM (×3)

P74 soil-water plus metal and organic mixtures

Figure 4.

A

cyp-34A9 responses to P73

GFP expression ratio (normalised to UW = 1.0)

12 hrs   24 hrs

UW MM P73 OE OE+MM (×2)

P73 soil-water plus metal and organic mixtures

B

cyp-34A9 responses to P74

GFP Expression Ratio (normalised to UW = 1.0)

12 hrs   24 hrs

UW MM P74 OE OE+MM (×2)

P74 soil-water plus metal and organic mixtures

C

Responses to control Lufa 2.2 soil

GFP Expression Ratio (normalised to UW = 1.0)

cyp-34A9   gpx-6 (= T09A12.2)   hsp-16.2 **   hsp-6 ** **

UW L 2.2 L 2.2 OE (×4)

Soil-water extracts

D

Pesticide effects on myo-3::GFP

GFP expression ratio (normalised to UW = 1.0)

20 mg l⁻¹ Rotenone 20 hr   300 mg l⁻¹ Chlorpyrifos 20 hr   200 mg l⁻¹ Endosulfan 20 hr ***   10,000 mg l⁻¹ Glyphosate 6 hr

** * ** * *

UW Rot UW Rot | UW Chlor UW Chlor | UW Endo UW Endo | UW Gly UW Gly

myo-3 hsp-16.2   myo-3 cyp-34A9   myo-3 cyp-34A9   myo-3 gst-1
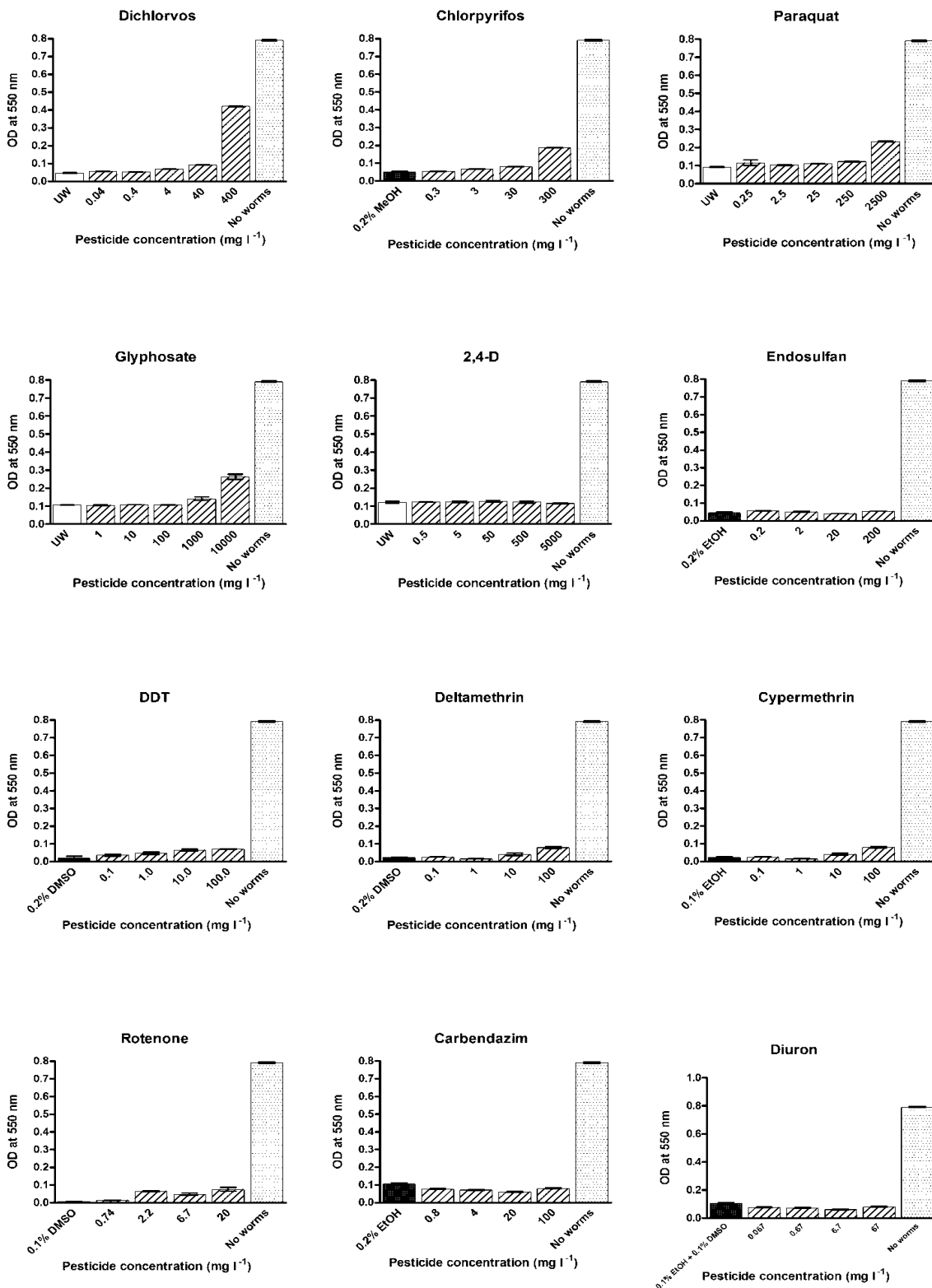
Pesticide and reporter gene

Supplementary Figure 1.   Feeding inhibition of N2 *C. elegans* by test pesticides .

Final column

First column shows extent of feeding for a standard aliquot of worms with no toxicant present; final column shows original optical density of bacteria plus highest toxicant dose with no worms.