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Potential New Method of Mixture Effects Testing Using Metabolomics and *Caenorhabditis elegans*

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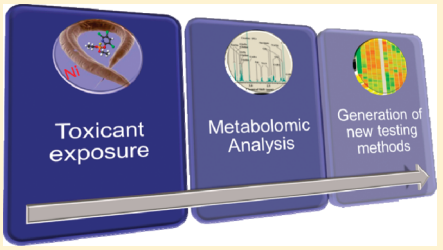
Supporting Information

ABSTRACT: The development of superior tools for molecular and computational biology in recent years has provided an opportunity for the creation of faster toxicological screens that are relevant for, but do not rely on, mammalian systems. In this study, NMR spectroscopy and GC−MS based metabolomics have been used in conjunction with multivariate statistics to examine the metabolic changes in the nematode *Caenorhabditis elegans* following exposure to different concentrations of the heavy metal nickel, the pesticide chlorpyrifos, and their mixture. Novel metabolic profiles were associated with both exposure and dose level. The biochemical responses were more closely matched when exposure was at the same effect level, even for different chemicals, than when exposure was for different levels of the same chemical (e.g., low versus high dose). Responses to the mixture reflected the contribution of the chemicals to the overall exposure. In common with the metabolic responses of several other species exposed to the same chemicals, we observed changes in branch chain amino acids and tricarboxylic acid cycle intermediates. These results form the basis for a rapid and economically viable toxicity test that defines the molecular effects of pollution/toxicant exposure in a manner that is relevant to higher vertebrates.



KEYWORDS: *NMR, metabonomics, mixture effects, pollution, systems toxicology*

INTRODUCTION

Organisms in the environment are routinely exposed to a multitude of chemicals including pesticides, pharmaceuticals, polycyclic aromatic hydrocarbons, and many other pollutants.[1] Although the majority of these substances cause no overt toxicity at environmentally relevant concentrations in short-term tests, undesirable and often unforeseen effects may occur following extended (chronic) exposure.[2] When exposure is to several toxicants in a mixture, responses can be additive (or even synergistic), with these effects further exacerbated if the organisms are already stressed by other factors such as low food levels and/or suboptimal climatic conditions.

In order to understand the interactions between chemicals and with other stressors, it is necessary to understand how individual and combinations of xenobiotics affect normal cellular biochemical processes that govern higher organization level traits. This is difficult with standard test organisms such as rats (*Rattus norvegicus*) and mice (*Mus domestica*) since to give useful results for a multitude of mixtures, extensive experimental designs are needed that require the use of a large number of test organisms. Such large-scale testing is challenging even for single chemicals and is most likely impossible for mixtures.[3] However, toxicologists face increasing demands to assess more chemicals and mixtures of chemicals with greater speed and accuracy and to do so using fewer resources and experimental animals.[4] Cell culture techniques, while able to make a useful contribution, are of only limited use since they do not capture the functional complexity of whole organism biology.

The nematode worm *Caenorhabditis elegans* is an excellent, alternative model organism to higher vertebrates for functional genomic studies into the molecular effects of toxicants for several reasons. Its morphology, histology, and physiology have been studied in detail. It has a short generation time (3−5 days) and is easily grown and manipulated in the laboratory in a similar manner to cell cultures but is a fully differentiated multicellular organism, with a nervous system, specialized muscles, and digestive and reproductive systems and is capable of exhibiting complex behavior.[5] Finally, the *C. elegans* genome has been sequenced, and this has revealed that there is a high degree of evolutionary conservation between *C. elegans* and higher organisms in many of the stress-response genes and cognate signal transduction pathways that are affected by toxicant exposure.[6] These facts suggest that if a metabolic response for a specific toxicant can be identified in *C. elegans*, then it is likely that these mechanism(s) could be extended to higher vertebrates.[7,8] Therefore *C. elegans* could play an

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important role in the current worldwide effort to reduce, replace, or refine the use of bioassays, which use mammals and higher vertebrates (particularly when studied with high throughput methods such as metabolomics and other -omic approaches).

In this study, we have examined the metabolome of *C. elegans* using a combination of ^{1}H nuclear magnetic resonance (NMR) spectroscopy and gas chromatography mass spectrometry (GC−MS) to assess biochemical responses in the metabolic profile of *C. elegans* following exposure to the heavy metal nickel (Ni) and the organophosphorus insecticide chlorpyrifos (Chlp), both singly and as a mixture. Both compounds occur in the environment and have previously been investigated as a model pair of dissimilarly acting chemicals.[9,10] Metabolomics, the analysis of the small molecules (metabolites) that are the products of cellular metabolism,[11] has been widely applied in toxicology research. For instance, the molecular responses of several mammal species to heavy metals have been studied, yielding new insights into the biochemical pathways involved in the response to toxicant exposure.[12]

Ni toxicity is the result of a range of effects involving interactions with a wide range of enzymes and cofactors. Chlorpyrifos toxicity occurs via the specific targeting and inactivation of acetylcholinesterase at nerve junctions. Both compounds when combined have relevance as model substances that exert toxicity through multiple and specific mechanisms but should not induce synergistic or antagonistic effects. By characterizing effects in *C. elegans*, the aims of the work were (1) to ascertain how the metabolic profile of *C. elegans* changes in response to exposure to Ni and Chlp and to assess the size of any metabolic changes against brood size, (2) to characterize the joint effects of mixture exposure in comparison to the effects of single substance exposure, and (3) to assess the potential of *C. elegans*-based functional genomics as a screening tool in toxicological studies.

EXPERIMENTAL SECTION

Nematode Growth and Chemical Exposure

C. elegans wild-type (Bristol, N_2) were obtained from the *Caenorhabditis* Genetics Centre (University of Minnesota). Worms were grown at 20 °C on plates seeded with *Escherichia coli* strain OP50 under the conditions outlined by Strange et al.[13]

All chemicals (Chlorpyrifos: CAS No. 2921-88-2, 98% pure) and $NiCl_2$ were purchased from Sigma-Aldrich Ltd. (Dorset, U. K.). The mixture experiment was designed to investigate whether the observed chemical exposure effect on reproduction could be correlated to changes in metabolic profiles using *C. elegans* as a model organism. Brood size (reproductive output) was chosen as relevant higher tier biological end point of exposure. The six treatments each consisted of five independent biological replicates of a control (no exposure), two concentrations of single chemical exposures (nominally equivalent to EC_{29} and EC_{50} (for brood size) for each toxicant, and a mixture exposure comprising the EC_{29} of both chemical. The test concentrations (μM) used were as follows: control (0 Ni, 0 Chlp), EC_{50} Ni (157.8 Ni, 0 Chlp), EC_{29} Ni (139.9 Ni, 0 Chlp), EC_{50} Chlp (0 Ni, 0.643 Chlp), EC_{29} Chlp (0 Ni, 0.385 Chlp), and the EC_{29} + EC_{29} mix (139.9 Ni, 0.385 Chlp).

To ensure that all individuals were at the same developmental stage, a synchronized population was obtained using the egg preparation technique outlined in Strange et al.[13] by bleach-prepping gravid adults to obtain L1 larvae. Aliquots of approximately 2000 L1 larvae were transferred to appropriately dosed nematode growth medium (NGM) agar plates. L1 larvae were allowed to grow adult at 20 °C and then synchronized again by bleaching. The resultant larvae (derived from lifetime exposed parents) were then transferred to freshly dosed 90 mm diameter NGM plates and allowed to grow to the L4 stage. At this point, worms were harvested in M9 buffer (3 g of KH2PO4, 6 g of Na2HPO4, 5 g of NaCl, 1 mL of 1 M MgSO4, H_2O to 1 L)[13] and stored at −80 °C prior to metabolomic analysis. Simultaneously, 24 individual worms were taken at random from the five replicate plates per treatment and individually transferred to separate wells of 12-well plates dosed to the same test concentration. The reproductive output was assayed at 20 °C, and worms were transferred daily to freshly dosed plates. At each transfer, the number of offspring (hatched eggs) was determined the following day. This allowed the quantification of cumulative and total brood size.

Metabolite Extraction

Extraction strategies for comprehensive metabolome coverage in *C. elegans* were previously assessed by Geier et al.[14] Since we intended to use NMR and GC−MS analysis, we utilized the well-established methanol−chloroform-based approach to give good overall metabolomic coverage.[5,15] Worms collected from the large plates (approximately 2000 per plate) were pelleted by centrifugation (400*g*) and then mixed with 600 μL of a methanol−chloroform mixture (2:1 v/v) and sonicated for 15 min. Two aliquots (200 μL) of both chloroform and water were then added, and the samples were centrifuged for a further 20 min. This resulted in the formation of aqueous and organic layers, which were transferred to separate microcentrifuge tubes. The aqueous layer was dried overnight in a Concentrator 5301 evacuated centrifuge (Eppendorf, Histon, U. K.) and analyzed via NMR and GC−MS. The lipid fraction was dried overnight in air and analyzed using GC−MS only because NMR does not detect lipids well.

High Resolution ^{1}H NMR Spectroscopy

The dried extracts from the extraction step were first rehydrated in 500 μL of D_2O (Goss Scientific Instruments, Great Baddow, U. K.) in order to provide a deuterium lock for the NMR spectrometer. Since NMR chemical shifts are sensitive to pH, 100 μL of D_2O buffered in 240 mM sodium phosphate, pH 7.0, containing 0.25 mM sodium (3-trimethylsilyl)-2,2,3,3-tetradeuteriopropionate (TSP) (Cambridge Isotope Laboratories, Inc., Hook, U. K.) was also added.

The samples were analyzed using an AVANCE II NMR spectrometer operating at 500.13 MHz for the ^{1}H frequency (Bruker, Germany) using a 5 mm Broadband TXI inverse automatic tuning and matching (ATMA) probe. Spectra were collected using a solvent suppression pulse sequence based on a one-dimensional NOESY pulse sequence to saturate the residual ^{1}H water signal (relaxation delay = 2 s, t_1= 3 μs, mixing time = 150 ms). Solvent presaturation was applied during the relaxation time, and the mixing time and 128 transients were collected into 16 000 data points over a spectral width of 10 ppm at 27 °C.

NMR spectra were processed using ACD SpecManager 1D NMR processor (Version 8, Advanced Chemistry Development Inc., Toronto, Canada). Free induction decays were multiplied by an exponential weighting function equivalent to a 1 Hz line, after which they were Fourier transformed from the time to the frequency domain and referenced to the TSP single peak at 0.0

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ppm. All spectra were then phased and baseline corrected manually and integrated using 0.04 ppm integral regions (bins). Areas of the spectrum less than 0 ppm and greater than 9.5 ppm were removed because they contained no data of interest. The region between 4.5 and 5 ppm was also excluded in order to avoid the residual H_2O region. Prior to multivariate analysis, the spectra were converted into numerical vectors, representing the individual metabolites, by integrating across the spectrum using 0.04 ppm integral regions (bins). To account for any concentration differences between samples, the binned intensities were normalized to the total integral region. Individual integrals were thereby standardized to the total integral of all low weight molecular metabolites. Individual metabolites were identified in conjunction with reference to the chemical shifts detailed in the literature,[16] with NMR Suite Professional, version 5.1 (Chenomx, Alberta, Canada), the online Madison Metabolomics Consortium NMR Database (http://mmcd.nmrfam.wisc.edu/index.html), and Madison Biological Magnetic Resonance Data Bank (http://www.bmrb.wisc.edu/metabolomics/). Representative samples of nematodes were also examined via two-dimensional NMR techniques, namely, correlation spectroscopy (COSY) and heteronuclear single quantum coherence (HSQC) experiments.

GC−MS of Aqueous Fraction

Since ^{1}H NMR is not as sensitive as MS-based technologies, all NMR samples were also analyzed via GC−MS. For this, the aqueous metabolites were derivatized via methoximation and trimethylsilylation prior to analysis.[17] A 150 μL sample of the aqueous extract previously analyzed by ^{1}H NMR spectroscopy was evaporated to dryness in an evacuated centrifuge. A 30 μL aliquot of methoxyamine hydrochloride (20 mg/mL in pyridine, Sigma-Aldrich, Gillingham, U. K.) was then added to the dried extract, which was then vortex-mixed for 30 s and then left for 17 h at room temperature. After this time, 30 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA; Sigma, U. K.) was added. The samples were again vortex mixed for 30 s and then left to react for 1 h. Samples were then made up to a volume of 500 μL with hexane prior to analysis via GC−MS.

GC−MS of Lipid Fraction

The lipid content of the cell pellets was analyzed via GC−MS following the method of Atherton et al.[5] For this, the dried organic fraction was first dissolved in 0.25 mL of chloroform/methanol (1:1 v/v;). A 100 μL aliquot of a solution of 10% BF_3/methanol (Sigma-Aldrich) was added, and the vials were incubated at 80 °C for 90 min. The samples were then left to cool for 10 min, after which 0.3 mL of H_2O and 0.6 mL of hexane were added and each vial vortex was mixed for 1 min. The aqueous layer was discarded, and the remaining organic layer was evaporated to dryness before being reconstituted in 200 μL of hexane for analysis.[18]

GC−MS Parameters

A Finnegan Trace GC ultra (Thermo Fisher Scientific, Hemel Hampstead, U. K.) coupled to a Finnegan Trace DSQ mass spectrometer (operated in the EI^+ mode) was utilized for metabolite analysis. Helium (1.2 mL/min) was used as the carrier gas. For the aqueous metabolites, a ZB-5MS column (Phenomenex Macclesfield, Cheshire; 30 m × 0.25 mm ID × 0.25 μm) was used. The oven temperature program started at 70 °C and was then increased at 10 °C per minute to 130 °C, then by 5 °C a minute to a temperature of 230 °C, and then by 20 °C a minute to a final temperature of 310 °C, which was held for 5 min. The total run time was ∼34 min per sample.

For the organic phase metabolites, a TR-FAME stationary phase type column (Thermo Fisher Scientific; 30 m × 0.25 mm ID × 0.25 μm, cyanopropyl polysilphenyl-siloxane) was used with a splitless injection. The injector temperature was set to 230 °C, and the GC oven temperature program was 60 °C held for 2 min, then increased at 15 °C per minute to 150 °C, and then 4 °C a minute to a final temperature of 230 °C, which was held for 7 min. The total run time was ∼28 min per sample.

In both cases, the column eluent was introduced into a Thermo Fisher Scientific DSQ quadrupole mass spectrometer (transfer line temperature 340 °C for aqueous metabolites, 240 °C for FAMEs), with ion source temperature = 250 °C, electron beam = 70 eV, source current = 100 μA, and injection volume = 2 μL in all cases. The detector was turned on after a solvent delay of 4 min, and data was collected in full scan mode using 3 scans s^{-1} across a mass range of 50−650 m/z.

GC−MS chromatograms were analyzed using Xcalibur, version 2.0 (Thermo Fisher Scientific), integrating each peak individually. Identification of individual peaks was performed by comparing the full mass spectrum for each compound with those in the inbuilt National Institute of Standards and Technology (NIST) database (2002 edition) in conjunction with retention time matching to known standards (Supelco 37 Component FAME Mix; Sigma Aldrich). Deconvolution of overlapping peaks was achieved by traces of single ions. A 0.1 min threshold window was used for the deviation of peaks away from the predicted retention time and each integrated peak was normalized to the total peak area prior to analysis.

Multivariate Statistical Analysis

Data sets from both analytical methods were imported into SIMCA-P version 11.0 (Umetrics, Umeå, Sweden) for processing. GC−MS data was scaled to unit variance. The ^{1}H NMR spectroscopy data were Pareto-scaled.[5]

Samples were analyzed using principal component analysis (PCA) and partial least squares-discriminate analysis (PLS-DA). Identification of major metabolic perturbations within the pattern recognition models was achieved by analysis of the corresponding loading plots. All models were further validated by resampling the model 99 times under the null hypothesis (meaning generating models with a randomly permuted *y*-matrix not related to the factors of interest).[19] Models that failed validation, i.e., where no difference between the randomly generated and the real data was observed, were not included in further analysis. For all models, the control/exposed classification was also tested using Coomans plots created from the NMR data. In this type of analysis, two independent PCA models are calculated that define two separate classes of samples. Mapping of unknown samples onto the calculated models can then provide the class identity based on the similarity between the unknown samples and the samples in the predefined class models.[9,20]

RESULTS

Reproduction

Figure 1 shows the effects of the various treatments on worm brood size. Exposure to a putative EC_{29} resulted in actual effects on brood size that were close to expectation (27% reduction) for Ni but lower than anticipated (16% reduction) for chlorpyrifos. EC_{50} exposure led to effects on brood size only marginally lower than anticipated for both chemicals (43%

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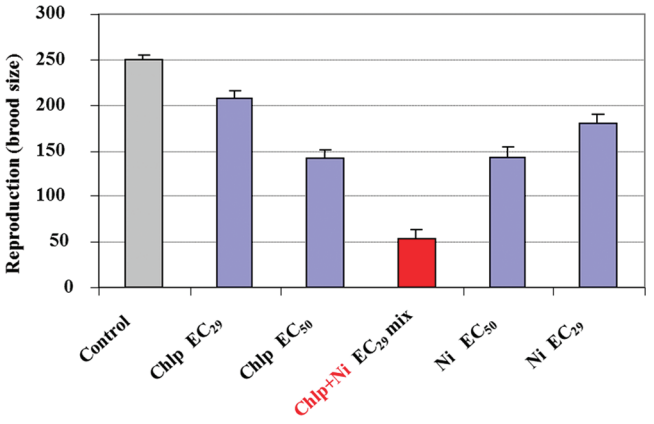

Figure 1. Mean (± SE) brood size of individual *C. elegans* (n = 12) exposed to low (EC_{29}) and high (EC_{50}) predicted effect levels of Ni and Chlp and a mixture of the EC_{29} of both chemicals predicted (according to independent action) to result in an effect equivalent to an EC_{50}.

reduction for Ni, 42% reduction for chlorpyrifos). On the basis of these measured effects, exposure to a combination of the EC_{29} of both chemicals would be expected to result in a 40% reduction in brood size, assuming that both chemicals exert effects independently, according to the principals of independent action. This smaller magnitude predicted effect is due mainly to the lower than anticipated toxicity of chlorpyrifos at the EC_{29} level used in the mixture test. In fact, the mixture exposure resulted in an observed 78% reduction in brood size. This suggests there is a greater than additive (synergistic) effect of this mixture.

Metabolic Profile

The metabolic content of *C. elegans* is comprehensively covered in the literature.[5,14,21−28] In this study, a total of 68 aqueous phase metabolites were identified via the combination of NMR and GC−MS, with prominent compounds including lactate, alanine, and succinate. In the organic phase, a total of 30 compounds including steric acid, oleic acid, and linoleic acid were positively identified. These results confirmed the feasibility of using *C. elegans* in metabolomic analyses. A representative NMR spectra and GC−MS chromatogram are shown in Supporting Information Figures S1 and S2, respectively, and full lists of metabolites are given in Supporting Information Tables S1 and S2.

Changes in the *C. elegans* Metabolome as a Result of Toxicant Exposure

Figure 2 shows a PCA plot of all exposure groups using the aqueous phase NMR (panel A) and GC−MS organic phase (panel B) data. In panel A, it can be seen that the EC_{50} Ni samples separate from the controls toward the top left of the chart along PC 1, whereas the EC_{50} Chlp samples are clustered

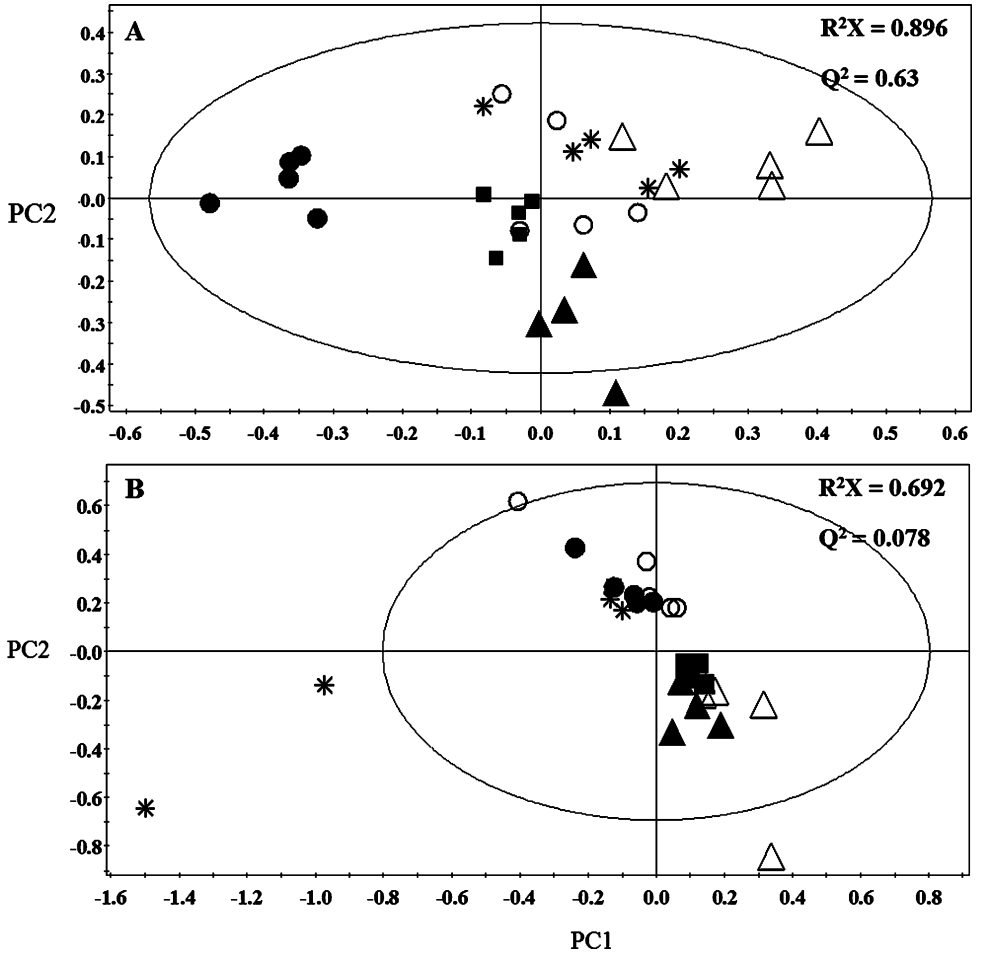

Figure 2. PCA models showing separation of the control samples (■) from those exposed to EC_{50} Chlp (▲), EC_{50} Ni (●), EC_{29} Chlp (Δ), EC_{29} Ni (○), and a mix of EC_{29} Chlp and EC_{29} Ni (∗) using the aqueous phase (NMR) data (A) and the organic (GC−MS) data (B).

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Table 1. List of Identified Aqueous Phase Metabolites from NMR and GC−MS Data

EC_{50} Chlp		EC_{29} CHLP		EC_{29} Mix		EC_{29} Ni		EC_{50} Ni	
alanine	↑	alanine	↑	alanine	↑	alanine	↓	alanine	↓
betaine	↑	betaine	↑	betaine	↓	arginine	↑	α-hydroxybuterate	↑
choline	↓	choline	↓	carnosine	↓	choline	↑	arginine	↑
glutamate	↓	glutamate	↓	choline	↓	glutamine	↑	betaine	↑
glycine	↓	glycine	↓	creatine	↑	glycine	↓	choline	↓
isoleucine	↓	isoleucine	↓	glycine	↓	isoleucine	↑	glutamine	↑
lactate	↓	lactate	↓	histadine	↑	lactate	↑	glycine	↓
lysine	↓	*n*-buterate	↓	isoleucine	↓	isoleucine	↓	isoleucine	↓
n-buterate	↓	taurine	↓	lactate	↑	succinate	↑	lactate	↓
phenyl acetyl-glycine	↓	ornithine	↑	leucine	↓	valine	↓	lysine	↑
succinate	↓			lysine	↓			malonate	↓
taurine	↑			valine	↓			phenyl acetyl-glycine	↓
ornitheine	↑							proline	↑

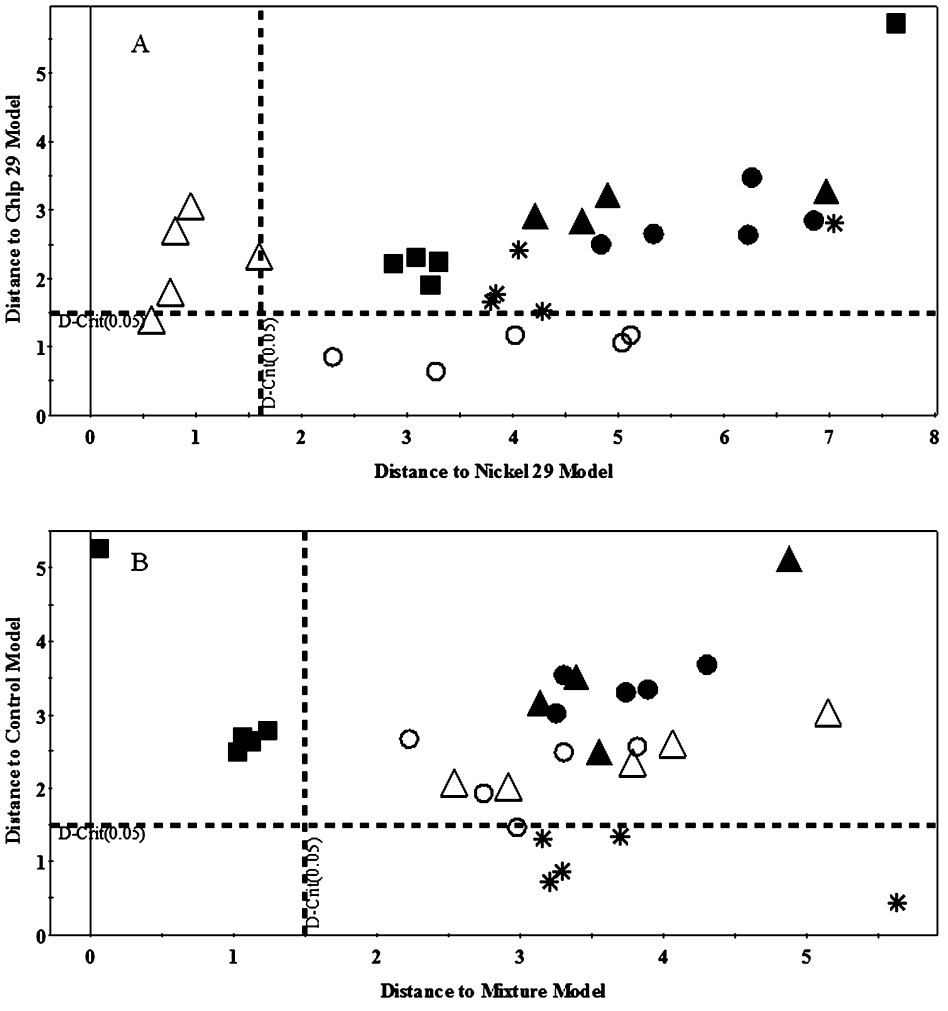

Figure 3. Coomans plots created from *C. elegans* exposed to 29-Ni (○ *x*-axis) and those exposed to 29 Chlp (Δ *y*-axis) (A) and control *C. elegans* samples (∗ *x*-axis) and those exposed to the equitoxic (29) mixture (■ *y*-axis) (B). The 95% confidence limit is used to define class membership in both cases.

toward the lower center/right along PC 2. In contrast, the low (EC_{29}) exposures separate from controls along PC 1 but toward the right of the chart. This latter trend is also seen in the mixture (EC_{29}/EC_{29}) samples. The clustering of the mixture samples within the range of EC_{29}-exposed samples for each chemical indicate that the mixture's effect on metabolism reflects the level of exposure (i.e., the concentrations of Chlp and Ni used in the EC_{29} and mixture tests) rather than the effect level of the mixture in the brood size tests, which is more similar to the individual EC_{50}s. The PCA of the NMR data resulted in successful classification in all cases with high Q^2 values and successful model validation. The data were further assessed by comparing only two groups at a time using PLS-DA (Supporting Information Figure S3). In these cases, the R^2 values varied between 0.5 and 0.99 (but were mostly toward the higher end of this range). The associated Q^2 values were all

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over 0.7 (and in the majority of cases over 0.9), indicating high quality models.

Applying PCA and PLS-DA to the integrated peaks of the chromatograms of the aqueous GC−MS data sets, perhaps unsurprisingly, resulted in very similar results to the NMR analysis, given that similar metabolites were measured. Highly reliable models with robust Q^2 and R^2 values were also found for the GC−MS data (Supporting Information Figure S4). The metabolites responsible for the separation (95% confidence limits) between control and toxicant-exposed models in the NMR and GC−MS data (summarized in Table 1) were mainly those involved in energy metabolism. We observed increases in levels of lactate, alanine, and leucine with a concomitant decrease in lysine. These changes are similar to those previously observed in other species exposed to the same compounds.[9,10] This demonstrates the similarity in the mechanisms of toxicity of the selected chemical pair across taxa.

In contrast to the aqueous phase, analysis of organic phase metabolites did not change significantly in response to toxicant exposure. PCA (Figure 2B) models built using the organic phase data, while initially looking promising, proved to be of low predictive power, with Q^2 values indicating that results were little better than chance. These models also failed validation with a random data matrix. Further analysis was therefore not undertaken.

Coomans plots are an extremely useful tool to visualize principal groupings, in which the two axes represent the distance of individual model.[20] To generate these models, two independent PCA models are calculated, defining two separate classes of samples, and the residual distances of samples to each of the two models are plotted against each other. Mapping of unknown samples onto these models will classify those samples as belonging to one of the two predefined classes, to both classes, or to neither of the two classes.[20] In Figure 3, the PCA models used were either the EC_{50} Ni group (y-axis) and the EC_{50} Chlp group (x-axis) in panel A or the EC_{29} mixture (y-axis) and no dose control (x-axis) groups in panel B.

In each case, data from the groups used to build the model were used to calculate the critical distance (D-Crit) value, representing the multivariate 95% confidence limit for each group (dashed line in each figure). Samples below the horizontal D-Crit line belong to the Ni exposed group in panel A and the equitoxic mixture samples in panel B, whereas samples to the left of the vertical D-Crit value belong to the 29-Chlp group in panel A and the control group in panel B. In both figures, any samples within the bottom left square are classified as part of both groups, while those in the top right box are classified as belonging to neither model. That the mixture-exposed worms in Figure 3A fall outside both the bottom left corner and indeed the D-Crit of both models indicates that the mixture effect represents a unique metabolic phenotype.

DISCUSSION

The effects of each exposure level on reproduction were more clearly marked than the effects on metabolism. Exposure to a putative EC_{29} resulted in actual effects on brood size that were close to expectation (27% reduction) for Ni but lower than anticipated (16% reduction) for chlorpyrifos. An EC_{50} exposure led to effects only marginally lower than anticipated for both chemicals (43% reduction for Ni, 42% reduction for chlorpyrifos). At first glance, it might seem that these results are counterintuitive, but this is not necessarily the case. Organisms encountering a suboptimum environment may exhibit behavioral and/or physiological adaptations that allow them to better cope with the stressor. Such phenotypic changes could mean the activation of chemical efflux pumps or detoxification enzymes or sensing and/or moving away from the most contaminated microsites in order to reduce exposure. Since the profiling of metabolites offers a snapshot of all physiological responses occurring at any one time, the observed metabolic changes underlying these phenotypic effects could represent general metabolic disturbance as a response to toxicity, as well as the more specific metabolomic changes associated with the mechanisms of action of the chemical in question.

Multivariate statistical analysis of the aqueous metabolite data showed clear differences between the metabolic profiles of control groups of *C. elegans* from any of the groups exposed to either chemical at both the effect levels and also to the mixture of the lower concentration of the two differentially acting toxicants. High exposure concentrations give a different effect from the low dose exposures, although certain changes were common across all groups (see Table 1). The highest (EC_{50}) doses of each compound both introduced similar (though distinct) changes in thirteen metabolites, mainly amino acids and TCA cycle intermediates. The lower (EC_{29}) single exposure doses induced changes in ten metabolites in the case of nickel and nine in the case of Chlp. The metabolomics approach, thus, makes it possible to both identify and assess biological end points of toxicological concern and to delineate the detailed basis of mixture effect profiles, thereby of mixtures, as well as to increase knowledge of the effects of cumulative pollution exposure on organism health.

The aqueous phase data indicate a similar pattern of metabolite changes, independent of the analysis method used. Here it appears that the exposure level is most important in determining metabolite patterns (with the mixture that was a combination of the two EC_{29}s looking similar to the single EC_{29} level treatments). For the organic phase metabolites, samples originating from the same chemical exposure cluster more closely. In this case, the mixture exposed samples separate and cluster with Ni exposed worms, possibly reflecting the greater systemic effects of this substance. However, since models based on the organic phase data did not pass validation, it is not possible to state this conclusively.

The Coomans plots were found to accurately describe the class boundaries for each set of samples. The analysis correctly identified that control samples did not belong to either of the single classes used to build the model. The results also indicated that samples exposed to the same chemicals, or to the mixture, did not produce profiles that overlap with the samples used to build each model. This indicates unique, effect-level associated phenotypes for each chemical and also a mixture phenotype that is separate from the response to exposure of constituent chemicals at a putative similar effect level. Building a model using the mixture further demonstrates that mixture effect is distinct from those induced by either compound alone. The fact that the mixture effect was unique and not dominated by any single chemical (or combination) may indicate that this particular combination results in a higher toxicity (synergism) for effect on brood size, which may be underpinned by unique mechanisms of action relevant only for the mixture.[29]

Changes in individual metabolites indicate separate system effects of exposure for each chemical. Ni is known to have multiple effects on enzymes, cells, and membranes (cofactor substitution, reactive oxygen species production, etc.). In

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contrast, chlorpyrifos (which inhibits acetylcholinesterase activity, blocking neuronal activity and causing paralysis) affects only one receptor. However, it is of note that in contrast to mammals, *C. elegans* express several acetylcholinesterase genes (mammals only express one) and these are all potential targets of chlorpyrifos.[30] In addition, chlorpyrifos can have system effects that affect reproduction and possibly feeding/digestion (since these require muscle activity). *C. elegans* is also known to respond to a variety of stressors by reducing the rate of pharyngeal pumping.[31] This may result in both a reduction in intake of the stressor but also a cessation of feeding, which may induce its own metabolic fingerprint.[32] This may also explain the changes in levels of branched chain amino acids, lactate, and other energy generation related metabolites because these have previously been observed to change in response to reduced feeding rate. The extent of this effect can be assessed and controlled for from a metabolic prospective and assayed by monitoring the decrease in the density of the bacterial lawn in liquid cultures of nematodes or by direct measurement of pumping rates.[31,32]

Mixtures of chemicals constitute a highly important issue in toxicology. Risk assessors face increasing demands to assess more chemicals and mixtures of chemicals with greater speed and accuracy. Thus, the development of new methods to reduce the uncertainties associated with their risk assessment without vastly increasing the number of experiments or experimental animals is called for.[33] New approaches in biological and computational sciences may also be able to generate mechanistic information that could help in meeting these challenges. However, to use mechanistic data to support chemical assessments, there is a need for effective translation of this information into biologically relevant end points.[4]

The use of *C. elegans* in combination with metabolomic analysis represents a potentially powerful approach that can be used to assess the toxicity of mixtures of compounds. Nematodes are well-established model organisms with a rapid life-cycle and are easily kept in the laboratory. They have a nervous system, specialized muscles, digestive and reproductive systems, and a fully sequenced genome. The latter has confirmed that *C. elegans* possesses homologous genes with humans, which allows the use of functional genetic tools such as knockout, knock-in, green fluorescent protein (GFP)-tagged strains, and RNAi microarray studies. *C. elegans*-based work therefore has the potential to generate the amount of data needed to elucidate useful hypotheses for toxicity testing in a way not possible with vertebrate systems.

CONCLUSIONS

Even though in its infancy in regard to applications in *C. elegans*, metabolomics is a high throughput technique that facilitates the analysis of a wide range of small molecules relevant to metabolism. By using this combined toxicological and metabolomics approach using such a well-established biological model, it is possible to both identify and assess end points of regulatory concern and delineate detailed toxicological and toxicokinetic profiles of mixtures, as well as to increase knowledge of the effects of cumulative pollution exposure on ecosystem health. This study demonstrates the versatility of a combination of *C. elegans* and metabolomics as a functional genomic tool that could form the basis for a rapid and economically viable toxicity test for the molecular effects of pollution/toxicant exposure.

ASSOCIATED CONTENT

Supporting Information

Supplementary tables and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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