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Global alterations in gene expression during organophosphate pesticide intoxication and recovery: Interim report

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14. ABSTRACT
The effects of low-level, transient exposures to organophosphate pesticides (OPP) and chemically related nerve agents (e.g., sarin, soman, tabun) remain difficult to detect. Because *Caenorhabditis elega* is relatively resistant to OPP lethality, studies in this nematode provide an opportunity to observe alterations in global gene expression following OPP exposure that cannot be readily observed in less resistant organisms. It may be possible to highlight changes in gene expression that might be important in low-level, transient exposures. Conventional culture techniques for *C. elegans* use bacteria as food source. The presence of the bacteria may confound interpreting the effects of a test substance if the substance is metabolized by the bacteria. Further, it can be difficult to purify worm nucleic acids away from those of the bacteria for analysis. We developed a liquid medium, CeHR medium, to facilitate the propagation of worms in the absence of bacteria. We exposed cultures of worms in CeHR medium to 2 doses of dichlorvos under 3 exposure protocols. In the first, worms were exposed continuously throughout the experiment. In the second and third, worms were exposed for 2 or 8 h, the dichlorvos was washed out of the culture, and the worms were allowed to recover. We then harvested RNA for global gene expression studies.

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Authors' contributions:

MS designed and performed experiments, analyzed data and wrote the paper.
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JL conceived, designed and performed experiments.
EC conceived and designed experiments and analyzed data.
DAJ conceived and designed experiments, analyzed data, and wrote the paper.

Abstract

Threats to Soldier performance and health can occur from commercially available organophosphate pesticides (OPP) and from chemically related weaponized nerve agents (e.g., sarin, soman, tabun). The effects of low-level and transient exposures to these compounds remain controversial and difficult to detect. Because the genomic model organism, *Caenorhabditis elegans,* is relatively resistant to OPP lethality, studies in this nematode provide an opportunity to observe alterations in global gene expression following OPP exposure that cannot be readily observed in less resistant organisms. Thus, it may be possible to highlight changes in gene expression that are difficult to discern but that might be important in low level and transient exposures. Conventional culture techniques for *C. elegans* use bacteria as food source. But the presence of the bacteria may confound interpreting the effects of a test substance on the worms if the substance is metabolized by the bacteria. Further, it can be difficult to purify worm nucleic acids away from those of the bacteria for analysis. Therefore, we developed a liquid medium, CeHR medium, to facilitate the propagation of large numbers of worms in the absence of bacteria. We exposed cultures of worms in CeHR medium to 2 doses of dichlorvos under 3 exposure protocols. In the first, worms were exposed continuously throughout the experiment. In the second and third, the worms were exposed for either 2 or 8 h, the dichlorvos was washed out of the culture, and the worms were allowed to recover. We then harvested RNA for global gene expression studies.

Introduction

Threats to Soldier performance and health can occur from weaponized nerve agents (e.g., sarin, soman, tabun) and from commercially available organophosphate pesticides (OPPs) chemically related to them. All of these compounds are classed as, and exert their primary effects as, acetylcholinesterase (AChE) inhibitors (antiacetylcholinesterases). Both short-term and long-term adverse effects of AChE inhibitor exposure have been described. In general, acute and short-term effects are well documented, but the consequences of low-level exposure and the nature and mechanism of persistent effects are relatively poorly understood and, in many cases, controversial (see below). The principal diagnostic tool for assessing exposure, the blood cholinesterase assay, displays substantial variation across individuals, and the changes in enzyme activity after exposure tend to be very persistent (reviewed in Brown and Brix, 1998; Munro *et al.*, 1994). Consequently, baseline levels of cholinesterase activity must be established for each individual, and the baseline value may still be incorrect if the individual has been exposed to OPPs or nerve agents with or without his/her knowledge up to several months before the test is performed. Understanding the mechanisms, targets, and consequences of low-dose and transient OPP toxicity should result in (1) improved methods for protecting Soldiers from intoxication due to AChE inhibitor exposure, (2) better intervention strategies for treating individuals who have already been exposed, and (3) the availability of improved biomolecular indicators of exposure and effect (biomarkers) for assessing the severity of an AChE inhibitor exposure on an individual basis.

Without intervention, high-level, acute exposures to OPPs and other AChE inhibitors result in death from respiratory failure; less severe exposures may cause salivation, lacrimation, incontinence, and convulsions followed by paralysis—among other symptoms (reviewed in Weinbroum, 2005). People and animals that recover from acute exposures to many of these compounds may present with a delayed syndrome, organophosphate-induced delayed polyneuropathy (OPIDP), characterized by the appearance of numbness, weakness, and parathesia in the limbs, and degeneration of peripheral nerve and central nervous system myelin sheaths 7-21 days after exposure (reviewed in Costa, 2005; Jokanovic *et al.*, 2002; Brown and Brix, 1998). A so-called intermediate syndrome, which is characterized by weakness of the neck, proximal limb, and respiratory musculature and presents 24-96 h after exposure, has also been described. This syndrome is believed to result from acetylcholine receptor desensitization (reviewed in Jokanovic *et al.*, 2002; Costa, 2005). The occurrence of chronic neurological and neuropsychiatric effects—some of which might persist for years—after low-level exposure and of developmental neuro-behavioral effects has also been described (Raveh *et al.*, 2003; Sarin and Gill, 1998; reviewed in Kamel and Hoppin, 2004). These effects remain controversial (Jamal *et al.*, 2002; reviewed in Costa, 2005 and Kamel and Hoppin, 2004) because it is difficult to obtain persuasive evidence of low-level and transient exposures to these compounds (reviewed in Worek *et al.*, 2005).

In an effort to resolve some of the issues regarding low-level, acute, and transient exposures to OPPs, we have undertaken studies tracking both development of and recovery from OPP intoxication at the global gene expression level using the genomic model organism *Caenorhabditis elegans* and Affymetrix whole genome *C. elegans* GeneChip microarrays.

Using *C. elegans* for toxicological studies can provide many potential benefits since the organism is transparent, has a very simple body plan, and has a completely sequenced genome with commercially available microarrays. However, a drawback to using *C. elegans* is that the worms are usually propagated on agar plates with bacteria as food source (see for example, Lewis and Fleming, 1995). However, we were able to surmount the complications of performing toxicological tests with two organisms in the experiment and the inconvenience of harvesting relatively small numbers of worms from plates by developing a liquid sterile medium in which *C. elegans* could be stably propagated (CeHR medium).

C. elegans shows substantial similarity to mammals in relevant biochemistry and genomics. The acute toxicity of the commercial OPPs, including dichlorvos, results from inhibition of acetylcholinesterase (AChE) in vertebrates (reviewed in Pope, 1999) and appears to do so in worms as well (Cole *et al.*, 2004 and references therein). The nematode possesses 4 AChE genes rather than a single alternatively spliced gene as in mammals (Combes *et al.*, 2003). Mutations in at least 18 *C. elegans* genes have been shown to confer resistance to the OPP-like carbamate pesticide aldicarb, and all these resistance genes are known —or plausibly believed—to be involved in acetylcholine metabolism, secretion, or recycling (Nguyen *et al.*, 1995). The *C. elegans* genome also contains a close homolog of the vertebrate secondary OPP target, neuropathy target esterase (NTE; NCBI RefSeq NM_066525, 19 DEC 2005; reviewed in Glynn, 2005). Impairment of NTE function is thought to underlie OPIDP (reviewed in Glynn, 2005, Jokanovic *et al.*, 2002, and Brown and Brix, 1998).

In other work, we observed that *C. elegans* is relatively resistant to OPP lethality (in preparation); therefore, we were able to observe developmental abnormalities in worms treated for 24 h with high doses of the AChE inhibitors aldicarb (a carbamate), fenamiphos, and dichlorvos (OPPs); the abnormalities included malformed cuticles, severe anatomical disorganization, and protruding vulvae, suggesting that the OPPs also target molecules other than AChE. A large number of non-AChE and non-NTE targets have been proposed in humans and other vertebrates, including nicotinic, muscarinic, and cannabinoid receptors, kinases, and carboxylesterases in addition to AChE and NTE (reviewed in Jokanovic *et al.*, 2002, Costa, 2005, and Pope, 1990). The high level of neurotoxicity of the OPPs has, however, made it difficult to study “off-target” effects in the past.

Since we had utilized dichlorvos to compare the effects of different OPPs on *C. elegans* global gene expression, we chose to use it in the experiments described here as well in order to maintain comparability with previous work. Furthermore, since dichlorvos reversibly inactivates AChE (ATSDR, 1997; see also Results and Discussion), we reasoned that it might be possible to readily wash it out of the worm culture so that the worms’ recovery from acute intoxication could be followed (see Results and Discussion). While it is unknown whether *C. elegans* experiences a syndrome like OPIDP, the syndrome has been described in humans following dichlorvos exposure (reviewed in Lotti and Moretto, 2005), raising the possibility that dichlorvos might be a suitable compound for investigating OPIDP-like phenomena in *C. elegans*. As noted above, we have observed developmental abnormalities in worms exposed to dichlorvos suggesting that there could be hitherto undiscovered targets for OPPs in *C. elegans.*

Because *C. elegans* is relatively resistant to OPP lethality, yet shows substantial similarity to mammals in the principal biochemical systems targeted by OPP, we reasoned that by using *C. elegans* to study the effects of dichlorvos, it might be possible to highlight changes in gene expression due to transient and low-level OPP exposures that are difficult to discern using classical methods or animal models less resistant to OPPs. We exposed synchronized *C. elegans* cultures in a sterile, axenic, liquid medium (CeHR medium) to two doses of dichlorvos. Based on prior work and preliminary experiments investigating the recovery of worms from dichlorvos exposure (see Materials and Methods), we exposed L4 worms to 15.0 µM and 0.6 µM dichlorvos, for either 2 or 8 h, washed the dichlorvos out of the worm culture, and followed the recovery of the worms from dichlorvos intoxication (Figure 1). We also followed the course of intoxication in worms that were continuously exposed over the course of the experiment (26 h) and the normal development of unexposed worms. At the time points indicated in Figure 2, we harvested worms and prepared RNA for global gene expression analysis using Affymetrix GeneChips.

We anticipate that these studies will lead to a comprehensive understanding of toxic, adaptive and maladaptive consequences of OPP exposure. It is likely that these benefits will also be applicable to understanding exposures to similar weaponized compounds.

Materials and Methods

Culture

CeHR medium

CeHR medium was developed at the US Army Center for Environmental Health Research (USACEHR) as a sterile, defined medium, supplemented with 20% (v/v) ultrapasteurized (HT) organic, fat-free milk for the axenic propagation of *C. elegans*. A detailed description of the preparation of the medium is available from USACEHR on request. The composition of the medium is presented in Table 1. The sterility of the milk is tested by culturing a sample of complete medium for 72 h at 37º C. Batches of the same lot of milk that have passed the sterility test are frozen and stored in convenient quantities at -80º C.

Developmental synchronization

Worms were synchronized using a modification of the method described by Hope (1999). Worms in established cultures in CeHR medium were harvested by centrifugation, and the CeHR medium supernatant was removed. The worms were then suspended in 100 mM NaCl and allowed to settle on ice. The supernatant is aspirated, fresh 100 mM NaCL is added followed by one half volume of 67% bleach (Clorox brand, germicidal grade)/1.7 M NaOH, and the mixture is incubated with periodic vortexing to dissolve all the worms except embryos. After several washes in sterile water, the embryos were suspended in M9 Salts (6.0g Na_2HPO_4, 3.0g KH_2PO_4, 5.0g NaCl, 0.25g $MgSO_4 \cdot 7 H_2O$ per L) and placed into an incubator at 22.5° C for 24 h to hatch and arrest in the L1 stage. The timing of the worm life cycle in CeHR medium along with some important events is shown in Figure 2.

Maintenance culture

For maintenance culture, 5×10^5 developmentally synchronized L1 stage *C. elegans* [N2 wild type, DR subclone of CB original (Tcl pattern I), obtained from Caenorhabditis Genetics

Center] larvae were inoculated into 40 mL sterile CeHR medium in a T-75 tissue culture flask and grown at 22.5° C on a platform shaker at 70 rpm in an incubator. All solutions and containers were sterile, and all worm manipulations were performed in a laminar flow hood.

C. elegans development studies

Synchronized cultures were started at 8,000 worms/mL in 10 mL CeHR medium and allowed to grow to gravid adult stage with shaking in T-25 flasks as above. Worm development was observed, and the times for >50% of the population to reach the following molts were recorded: L1/L2 molt, L2/L3 molt, L3/L4 molt and L4/Adult molt. Observations of the times of appearance of embryos *in situ* and *ex situ* were also taken.

Fecundity experiments

Synchronized worms at a concentration of 8,000 worms/mL in 10 mL CeHR medium were allowed to grow to the L4 larval stage at 22.5° C. At the L4 larval stage they were transferred into multiple wells of sterile 96-well plates and diluted to 1 worm per well in 100 µL of CeHR medium. The plates were incubated on a Labline shaker (speed setting #3) at 22.5° C. The worms were observed daily and the number of offspring counted; after counting, the adults and/or offspring were transferred by pipette into fresh wells containing 100 µL medium/well. The adults were not transferred more than twice per day to avoid stress. Once past reproductive age, longevity was determined by allowing the adults to age to death in the 96-well plates.

Rangefinding studies

In other experiments, we observed that continuous exposure of worms to a concentration of 15 µM dichlorvos in CeHR medium was sufficient for reducing the number of early L4 worms (46 h after synchronization; see Figure 2) that progressed to the early gravid adult stage in 24 h of exposure to 10% of the number of untreated controls that matured. Under these conditions, essentially all the untreated worms matured. To maintain consistency with this work while investigating the effects of lower doses of dichlorvos on *C. elegans*, we set 15 µM as our maximum for this experiment. The effects of a series of continuous exposures on worm development and mobility with 5-fold dilutions of dichlorvos (15.0, 3.0, 0.6, 0.12 µM) were monitored by microscopy over 24 h beginning at the early L4 stage (46 h), and both still and video images were recorded. In addition, we exposed 10 mL cultures of worms to dichlorvos at the four concentrations above for 4 h, pelleted the worms, removed the medium, washed the worm pellet three times with 15 mL of medium without dichlorvos or milk, and, finally, resuspended the pellets in 10 mL of fresh medium. Similarly treated unexposed cultures and continuously exposed cultures were used as controls. Observations on the exposed cultures were made at 2, 3, 5, 6, 7 and 24 h after dosing, and observations were made on the washed out cultures at 1, 2, 3 and 20 h after removal of the dichlorvos; the course of intoxication and recovery was recorded using digital video.

Exposure studies

The experimental time course is diagrammed in Figure 1. Cultures of 2.5 X 10^5 synchronized worms were grown in 30 mL CeHR medium on a rotating shaker (70 rpm) at 22.5° C in an incubator for 41 h to the late L3 or very early L4 stage. At this point, 50% of the worms have passed the L3/L4 molt. The molt coincides with early turning of the gonadal arms and the appearance of a vulvar slit approximately one cell width wide. The developmental stage of

several representative flasks of worms was confirmed microscopically before commencing the experiment. One flask of worms was harvested prior to beginning the exposure as the 0 h/0 dichlorvos dose base line. Equal volumes of water, low (0.6 µM final) or high (15.0 µM final) dichlorvos stock as appropriate were added to the remaining flasks, which were then returned to the incubator. The flasks were treated according to one of three washout protocols. One set of flasks, consisting of an untreated control and low and high dichlorvos doses, was incubated without interruption for the duration of the experiment with flasks being harvested at designated intervals during the exposure (see Figure 1). A second set, consisting of an untreated control (sham) and low and high doses of dichlorvos, was incubated for 2 h, at which time the worms were centrifuged out of the exposure medium, washed 3 times with Washout Buffer (see below), resuspended in fresh CeHR medium without dichlorvos, and returned to the incubator. Flasks were harvested by centrifugation at designated intervals (see Figure 1). The third set was treated essentially as the second except that the dichlorvos exposure period was 8 h rather than 2 h (see Figure 1). Each condition was represented by an individual culture. The whole set of exposures was repeated four times.

The washout procedure required approximately 30-45 m, and we wished to perturb normal worm culture conditions as little as possible in effort to maintain normal development. Since CeHR medium contains 20% milk, repeated washes with whole medium would have resulted in the accumulation of milk solids during centrifugation. Therefore, the washouts were performed with a modified CeHR medium (Washout Buffer). Fat-free HT organic milk was repeatedly centrifuged and then sterile filtered to produce clarified milk. In addition to 20% (v/v) clarified milk, Washout Buffer contains the flowing components at the same concentrations as CeHR medium (Table 1): lactalbumin, essential amino acids, non-essential amino acids, KH_2PO_4, HEPES, glucose. The osmolarity and pH of Washout Buffer are similar to CeHR medium.

Chemistry

Stock solutions of dichlorvos {99.4% [CASRN: 62-73-7 (Chem Service)]} in water were prepared weekly and filter sterilized, and the concentration was verified (see below). Reported half-lives of dichlorvos in water vary depending on pH, but are typically in the day to week range (ATSDR, 1997). We tested the stability of dichlorvos in CeHR medium and observed less than a 10% decline in concentration over 24 h on two occasions (data not shown). This observation is consistent with a reported loss of 8% over 24 h in water at pH=6.2 (National Library of Medicine Hazardous Substances Database); the pH of CeHR medium is typically 6.0 – 6.5. Dichlorvos concentrations were determined using a variation of EPA method 8141Aa and a Hewlett-Packard model 6890 gas chromatograph equipped with an electron capture detector and a Hewlett-Packard model 7673 auto sampler.

RNA extraction, processing, and labeling

Worms were harvested by centrifugation at 800 X G for 3 m. The worm pellets were washed with 100 mM NaCl and re-centrifuged, after which the NaCl solution was aspirated. The worm pellet was suspended in the residual wash solution and frozen by dropwise addition to liquid N_2 in a ceramic mortar. Frozen worm droplets were transferred to 15 mL conical tubes and stored at -80º C until use.

The frozen worm droplets were pulverized under liquid N_2 in a Spex 6750 Freezer Mill using two 2 m cycles of 15 Hz milling separated by a 1.0 m cooling intermission. The pulverized worms were transferred to a 15 mL conical tube and vortexed in Trizol RNA extraction solution (Invitrogen); total RNA was isolated essentially as described by the manufacturer. An additional purification step was performed using the RNEasy Midi Kit (Qiagen) according to the manufacturer's directions. The quality of the preparation was assessed throughout processing and labeling using an Agilent Bioanalyzer, and the mass yield of some samples was confirmed using a Nanodrop Technologies spectrophotometer.

Poly (A)+ RNA was isolated from the total RNA using OligoTex (Qiagen) essentially as described by the manufacturer. Two µg of poly (A)+ RNA (adjusted for rRNA contamination) was used as the template for cDNA synthesis using the SuperScript Choice kit (Invitrogen) as recommended by the manufacturer except that (1) an HPLC-purifed $T_{24}T7$ primer (Integrated DNA Technologies) was used to initiate first strand synthesis as recommended by Affymetrix; (2) the second strand synthesis was not terminated using EDTA; we found that EDTA carryover interfered with subsequent enzymatic manipulations; (3) and PelletPaint (Novagen) was used in place of glycogen for precipitation. Biotin labeled cRNA was synthesized from the T7 promoter incorporated in the cDNA using the Enzo BioArray High Yield kit essentially as described by the manufacturer; approximately 1 µg of cDNA is used for synthesis. cRNA was purified from unincorporated nucleotides and other reaction components using the RNEasy Mini kit (Qiagen).

cRNA samples were hybridized to Affymetrix *C. elegans* whole genome GeneChips, processed, and scanned by the laboratory of Dr. Maryanne Vahey, Division of Retrovirology, Walter Reed Army Institute of Research essentially as recommended by Affymetrix.

Standard Affymetrix-recommended quality control parameters were used. In addition, replicate samples were compared after RMA normalization (Irizarry *et al.*, 2002) using the Pearson correlation coefficient, and replicate vs. replicate dot plots. Replicates were accepted if they passed Affymetrix recommended standards, had an $R^2 \geq 0.93$, and displayed no gross deviations from linearity on the dot plot.

Statistical analysis and data processing (including RMA) were performed using the Partek Pro Genomics Solution 6.2 and Affymetrix MAS 5.0 to recover present absent calls from GeneChip scans.

Results and Discussion

CeHR Medium

Under standard culture conditions, *C. elegans* is propagated on agar plates with lawns of *E. coli* as a food source (see for example, Lewis and Fleming, 1995). However, this method has several undesirable characteristics. In toxicological experiments, the metabolism of the test substance by the bacteria may confound interpreting the effect of the toxicant on the worm. Separating bacterial nucleic acids and proteins from those of the worm can also be problematic, and it is relatively inconvenient to harvest the large numbers of worms needed for genomic and proteomic experiments from agar plates. To surmount these difficulties, we developed a liquid, axenic medium in which *C. elegans* can be stably propagated. Although we typically refresh our

cultures every three to six months from frozen stocks, we have maintained continuously passaged worm cultures for a year.

Initial tests of liquid media described in the literature produced slow-growing cultures with reduced fecundity. By trial and error, we developed a nutrient-rich medium (Table 1) based on several media described in the literature [Tomlinson and Rothstein (1963; use of lactalbumin); Sayre *et al.*, (1963; nucleic acids, salts, amino acids, lipids, energy sources); Lu and Goetsch (1993; nucleic acids, salts, amino acids, lipids, energy sources); Hieb *et al.* (1970), Vanfleteren (1970), and Brockelman and Jackson (1978) (hemin, cytochrome C)]. The medium contains 20% (v/v) commercial ultra-pasteurized (HT), fat free, organic bovine milk. The milk appears to provide critical nutrients for maintaining the growth rate of the worms. Several brands of organic milk have been tried; all proved satisfactory. The use of organic milk is intended to reduce or eliminate nematicides that may be present in commercial milk. The presence of fat globules interferes with microscopy. Sterilization of the milk by filtration or heat appears to eliminate materials required for successful worm culture.

The growth and lifespan (see Materials and Methods) of worms in CeHR medium is substantially similar to that of worms grown on lawns of *E. coli* (not shown). The lifespan of the worm is approximately 12 d; and by 63 h, approximately 50% of worms have reached the Early Gravid Adult stage. These values do not differ markedly from those described in the literature for worms grown on bacterial lawns at 22º C (Lewis and Fleming, 1995, pp. 4-9; Riddle *et al.*, 1997); likewise Rao *et al.* (2005), using a variant of CeHR medium, reported that worm growth, mobility, development, generation time were similar under CeHR and bacterial culture conditions. The fecundity of the worms does, however, appear to be somewhat reduced in CeHR medium from around 300 embryos per worm under bacterial growth conditions to about 100; a similar phenomenon has been observed in another liquid, sterile medium (Szewczyk *et al.*, 2003).

Effects of dichlorvos on worm motility

Prior to beginning the large-scale experiment to investigate the time course of dichlorvos intoxication and recovery, we performed rangefinding experiments to ensure that the doses we selected produced observable effects and that the worms could recover following washout as expected. Since impairment of worm movement is a significant and readily observable consequence of OPP exposure in worms (Cole *et al.*, 2004; Nguyen *et al.*, 1995; Opperman and Chang, 1991), we used reduction in worm movement as the endpoint for rangefinding.

We had already established doses of dichlorvos sufficient for inhibiting the development of 10% (15 µM) , 50% (70 µM) and 90% (225 µM) of early L4 worms (46 h; see Figure 2) into adults 24 h later as part of another study (not shown). The effect of the 50% and 90% inhibition doses was quite severe, and large changes in the pattern of global gene expression could be observed even 4 or 8 h after exposure; however, the effects of the 10% inhibitory exposure were more modest (not shown). Since we had already performed a number of experiments comparing various OPPs at the level of global gene expression using developmental inhibition as an end point, we chose to use 15.0 µM dichlorvos as the high dose for this series of experiments in order to maintain consistency. We believe it is likely that 15 µM dichlorvos is a sufficiently high dose to cause significant changes in the gross development of the worms based on the foregoing experiments. Figure 3 displays representative images of worms exposed for 24 h beginning at 46

h (Figure 2) to a slightly higher dose of dichlorvos than we used for the current experiments (20 vs. 15 µM). Abnormalities of the reproductive system are apparent, especially reduced numbers of embryos and apparent failure of gonadal development, indicating that dichlorvos doses in the range under consideration here are capable of producing off-target effects.

We exposed worms beginning at the early L4 stage (46 h) to a series of 5-fold reductions in dichlorvos concentration beginning with 15 µM continuously for 24 h. At the end of the exposure, the worms cultured with 15.0 and 3.0 µM dichlorvos were immobile and had laid few if any embryos. The movement of worms exposed to 0.6 µM was sluggish, and fewer embryos were present than in control cultures. There was no obvious difference by inspection between control cultures and cultures exposed to 0.12 µM.

Reduction in the rate at which the worms writhed was observed as early as 10 m after dosing at 15.0 µM dichlorvos, and by 40 m at the 3.0 µM dose. By the time that reduced movement was apparent, many worms assumed a static form with multiple sharp bends (Figure 4), suggesting severe disruption of cholinergic motor control. The rate at which worms exposed to 0.6 µM dichlorvos writhed was clearly slowed by 4 h post-exposure, yet few if any worms became sharply bent or completely immobile even after 24 h of exposure. As noted above, the 0.12 µM dose did not obviously affect the worms compared with control even after 24 h of exposure. These observations are consistent with those of Cole and coworkers (2004), who developed a software package to quantify worm mobility. They reported that a 4-h exposure to 0.7µM dichlorvos reduced worm motion by 50%.

To verify the reversibility of the effects of dichlorvos exposure, we performed time course experiments, in which we exposed early L4 (46 h) worms to varying concentrations of dichlorvos for 4 h and then diluted the dichlorvos out of the culture medium by washing and pelleting the worms three times (see Materials and Methods). The control worms were subjected to the same washing and pelleting regimen. By inspection, worms exposed to 15.0 µM dichlorvos showed a gradual but incomplete recovery over 3 h; however, by 20 h they were not readily distinguishable from control worms with respect to motion or number of embryos present in the culture. Similarly, worms exposed to 3.0 µM dichlorvos recovered rapidly over 3 h, but were still somewhat impaired in movement; by 20 h, the cultures were not readily distinguishable from control. In contrast, worms exposed to 0.6 µM dichlorvos appeared to be fully recovered by 3 h after exposure and were indistinguishable from control by 20 h. There was no discernible effect of 0.12 µM dichlorvos on the worms under any condition.

The results are consistent with the reversibility of dichlorvos inhibition of AChE and may also reflect a short biological half-life. We are unaware of work investigating the biological half-life or bioaccumulation of dichlorvos in nematodes. At least, in mammals, there is no known bioaccumulation of the pesticide, and there is extremely rapid metabolism (minutes) of dichlorvos by a number of carboxypeptidases (ATSDR, 1997), a highly conserved and broadly distributed family of enzymes (Oakeshott *et al.*, 1999).

However, when Opperman and Chang (1991) studied the recovery of adult *C. elegans* from a 24-h exposure to the AChE inhibitory pesticides aldicarb, carbofuran, oxamyl and fenamiphos, they observed that even when behavioral evidence indicated complete recovery from OPP

exposure, AChE activity could still be inhibited. The 24-h exposures resulted in immobility and essentially complete inhibition of AChE activity, but 24 h after the pesticides were washed out, the worms showed apparently complete restoration of motility. Surprisingly, in the cases of carbofuran and fenamiphos, the recovery of AChE activity was incomplete; less than 15% of untreated control AChE activity was observed in normally moving worms even 24 h after fenamiphos had been washed out. Experiments with a number of nematode genera suggest that nematodes may exhibit robust recovery from AChE inhibitory pesticide exposure in general (Kimpinski *et al.*, 1983; Pree *et al.*, 1989; Opperman and Chang, 1991 and references in the foregoing), although the reasons are not clear at present.

Effects of dichlorvos on global gene expression during intoxication and recovery.

Based on the preliminary work already described, we chose 15.0 μM dichlorvos as a high dose and 0.6 μM as a low dose. The high dose tied this work into experiments already performed, and the low dose produced readily observable effects from which the worms rapidly recovered. To extend the scope of the experiment, we elected to use two exposure durations, 2 and 8 h, bracketing the 4-h period used in the pilot work.

Based on the pilot experiments above and on work in the literature (see discussion above), we attempted to balance the length of the recovery period and the time course of the worm's development to increase the likelihood that we could identify the molecular mechanisms underlying abnormalities in worm morphology and gene expression pattern. Therefore, we chose to set the total length of the experiment at 26 h, which permitted a full 24-h recovery period for the 2-h exposure and 18 h for the 8-h exposure (Figure 1). The rapid development of *C. elegans* can complicate attempting to observe alterations in *C. elegans* gene expression over time since marked changes in gene expression can occur because of the normal development of the worm. During the course of the experiment proposed here, for example, the hermaphrodite worm undergoes sexual maturation, with spermatogenesis, oogenesis, internal fertilization, and embryogenesis (see Figure 2). A 26-h experiment begun at 46 h easily extends into the egglaying period (~ 72 h) for many of the worms in the experiment. Therefore, we shifted the beginning of the wash out experiments to the very beginning of the L4 larval stage at the boundary of the L3/L4 stage cuticular molt (41 h; see Materials and Methods and Figure 2).

In a small scale effort to evaluate whether shifting the timing would cause results markedly different from those we had seen in earlier work, we sampled developing worms close to the L3/L4 molt (40 h), at the time used in prior experiments (46 h), and at two time points close to the L4/adult molt (64 and 68 h), we prepared prepared RNA from the worms and examined global gene expression using Affymetrix GeneChips in two independent experiments with a principal components analysis (PCA). Previous observations showed that the most marked difference in clustering in PCAs was related to the developmental stage of the worm (not shown) regardless of the treatment we had employed. Consistent with those results, we observed two clusters, early L4 and L4/adult (Figure 5). Approximately, 58% of the variance in the experiment is explained by the life stage of the worm; early and late L4 stage worms cluster separately (first component, horizontal axis). An additional, 14% is explained by the second component (vertical axis), which seems to reflect the differences between the replicate experiments at the late time points. A further ~10% of the variance is explained by the third component (not shown); it also seems to arise from differences between replicates. Even when we considered only those probe

sets with present calls in all samples, we observed clear clustering of the early and late time points along the first component, indicating that the distinctions between the early and late worms are not due to large binary differences in the genes expressed as maturation progresses (not shown). There appears to be little variability in the patterns of gene expression at the early time points, and when differences between replicates are taken into account, differences at the late times are likewise not extensive. At present it is not clear why the variability at the late times is so much greater than at the earlier times. We concluded that altering the timing of the experiment slightly was unlikely to markedly alter the results we observed in the pilot wash out and rangefinding experiments.

Therefore, we performed four replicate experiments in which 41 h L4 larvae were treated with dichlorvos as depicted in Figure 1 at either 15.0 or 0.6 µM. Each experimental condition was represented by a single culture; while it is theoretically possible to sample a given culture repeatedly, we have observed that the growth rate of the worms is highly dependent on the volume and surface area of the medium in the culture flask. To eliminate variability over the course of the experiment, we set up each condition in its own flask.

Cultures were harvested and RNA was prepared up to the point of poly(A)+ RNA purification as indicated in Materials and Methods and stored at -80° C.

Conclusion

We developed an axenic, liquid medium (CeHR medium) for eliminating confounding effects in experiments due to the presence of *E. coli* as a food source in *C. elegans* cultures. In CeHR medium the worms propagate and develop at rates similar to worms grown on bacteria. The medium also facilitates the handling and production of large numbers of worms for genomic and proteomic experiments.

We laid the foundations for performing experiments in CeHR medium to investigate the course of intoxication by dichlorvos and recovery from exposure by *C. elegans.* Under the conditions that we defined, we observed both effects on the motility of the worms which are likely due to the classical mechanism of OPP toxicity, the inhibition of AChE activity, as well as developmental abnormalities that are presumed to result from “off target” effects. We confirmed that dichlorvos intoxication is reversible in *C. elegans* at least over short exposures, and we identified a No Observed Response Effect (0.12 µM) for dichlorvos.

We performed a large scale experiment to investigate how dichlorvos intoxication develops in *C. elegans* over time, and what alterations in gene expression may occur as the worm recovers from exposure. On completion of global analysis of gene expression in this experiment, we expect that it will contribute to the acquisition of a comprehensive understanding of toxic, adaptive and maladaptive consequences of OPP exposure. Understanding these phenomena should lead to 1) the identification of new biological markers, or biomarkers, of exposure and effect; 2) new targets for interventions following exposure; and 3) an understanding of potential long-term effects of OPP exposure. It is likely that these benefits will also be applicable to understanding exposures to similar weaponized compounds.

Table 1: CeHR Medium Components: Table 1 presents the concentrations of the components of CeHR medium

Chemical	Concentration	Chemical	Concentration
KH_2PO_4	9 mM	Fe $(NH_4)_2(SO4)_2$	0.15 mM
Choline di-acid citrate	590 mg/L	$CaCl_2$	0.14 mM
i-Inositol	432 mg/L	N-Acetylglucosamine	15 mg/L
d-Glucose	7 mM	DL-Alanine	15 mg/L
Cytochrome C	50 mg/L	p-Aminobenzoic Acid	7.5 mg/L
Hemin chloride (RT)	10 mg/L	Biotin	3.75 mg/L
HEPES	10 mM	Cyanocobalamine (B-12)	3.75 mg/L
Lactalbumin Hydrolysate	3.4 g/L	Folinate (Ca)	3.75 mg/L
Cholesterol	5 mg/L	Nicotinic Acid (Niacin)	7.5 mg/L
Adenosine 2'- & 3'-PO_4	1mM	Niacinamide	7.5 mg/L
Cytidine 5'-PO_4	1mM	Pantetheine	3.75 mg/L
Guanosine 5'-PO_4	1mM	Pantothenate (Ca)	7.5 mg/L
Uridine 5'-PO_4	1mM	Pteroylglutamic Acid (Folic Acid)	7.5 mg/L
Thymine	1mM	Pyridoxal 5'-phosphate	3.75 mg/L
$MgCl_2$	2 mM	Pyridoxamine.2HCl	3.75 mg/L
$ZnCl_2$	0.075 mM	Pyridoxine.HCl	7.5 mg/mL
$MnCl_2$	0.113 mM	Riboflavin 5-PO_4(Na)	7.5 mg/L
$CuCl_2$	.04 mM	Thiamine.HCl	7.5 mg/L
HT organic fat free milk	20%	DL-6,8-Thioctic Acid	3.75 mg/L
10 ml Non-essential Amino Acid Mix (GIBCO #11140-050)	10 mL/L	Sodium Citrate	1mM
20 ml Essential Amino Acid Mix (GIBCO #11130-051)	20 mL/L	Potassium Citrate	1.5 mM

Figure 1

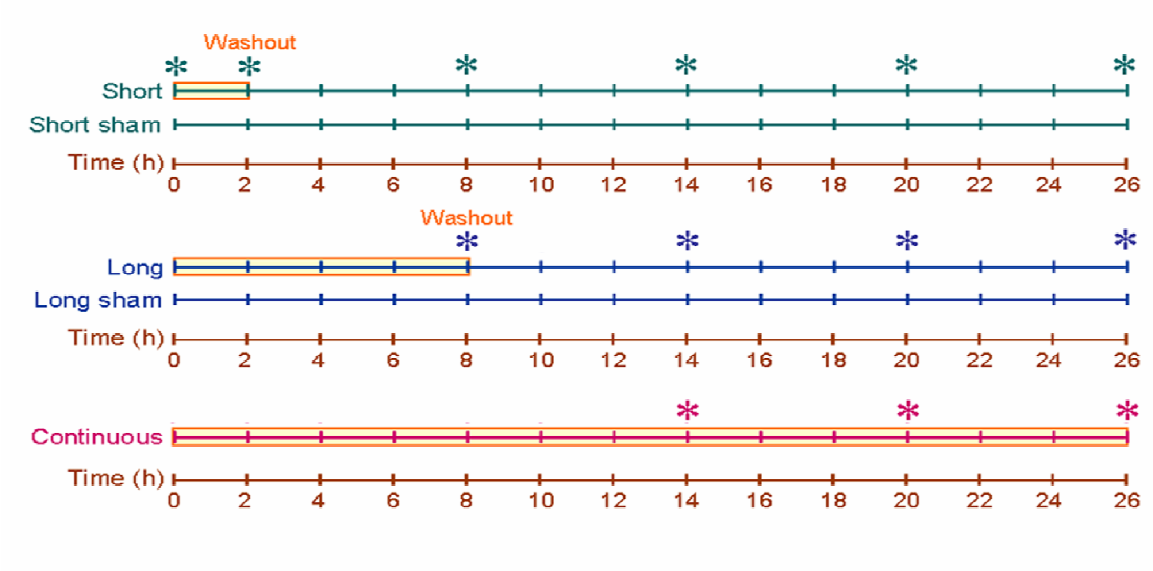

Figure 1: Experimental design for OPP acute time course experiment. Asterisks indicate when samples were taken; orange bars indicate the duration of exposures. Times at which dichlorvos was washed out are indicated. Each of the protocols (short, long, and continuous) was performed using a set of unexposed cultures along with a set exposed to 15.0 or 0.6µM dichlorvos. Each condition was represented by an individual culture. Sham exposures are untreated controls that have been subjected to the washout procedure.

Figure 2

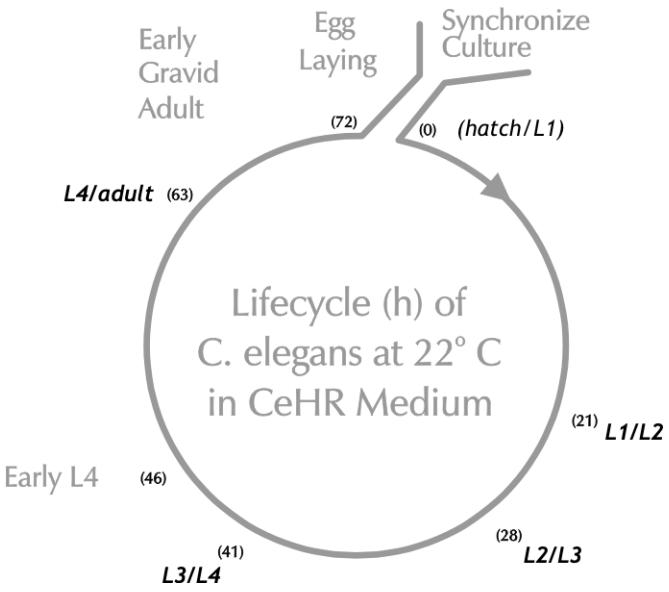

Figure 2: Significant developmental markers and times for synchronized C. elegans cultures. Cuticular molts between the 4 larval stages (L1-L4) and the adult worm stage are shown in italics. Hours post release from the synchronization procedure are shown in parenthesis. Some significant events and periods are also indicated.

Figure 3

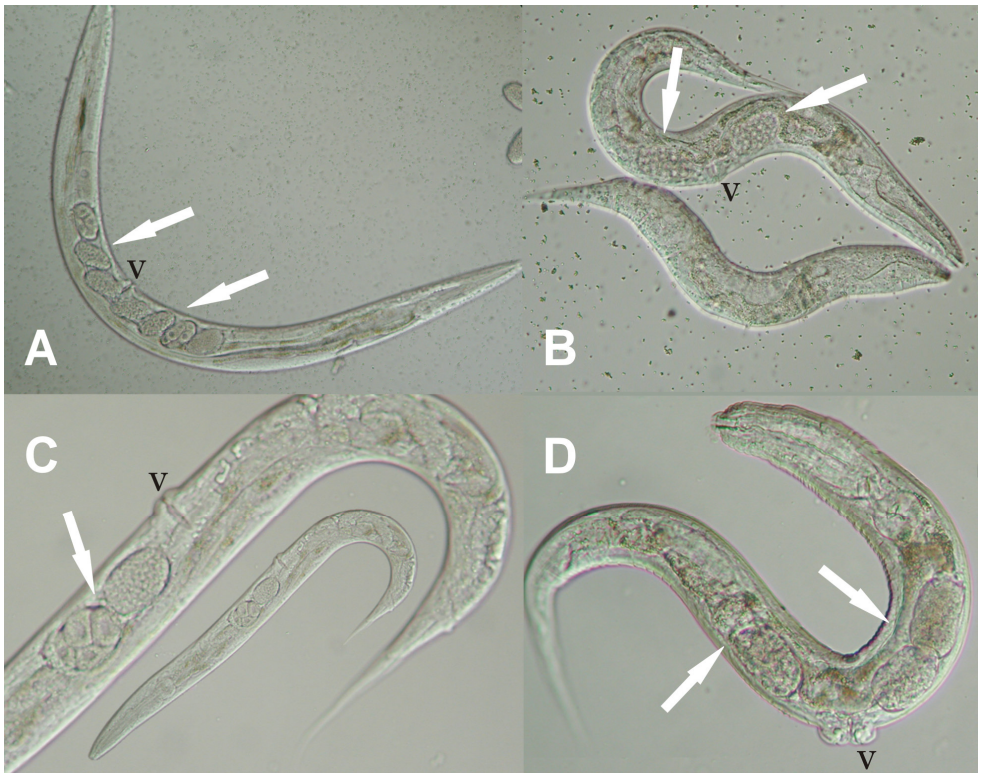

Figure 3: Developmental abnormalities observed in adult *C. elegans* nematodes exposed to 20 µM dichlorvos for 24 h beginning at the early L4 larval stage and extending through the early gravid adult (EGA) stage. **A**: Unexposed EGA worm. Note the presence of developing embryos (arrows) along both the anterior and posterior arms of the gonad flanking the vulva (V). **B.** Exposed worms. Note the reduced number of embryos (arrows) flanking the vulva (V) in the upper worm. No embryos are discernable in the lower worm, and a vulva does not seem to be present. **C.** Expanded view of exposed worm. Embryos (arrow) appear to be present only on the anterior arm of the gonad though a vulva (V) is clearly present. An image of the whole worm is inset. **D.** Exposed worm with reduced number of embryos (arrows) and a protruding vulva (V).

Figure 4

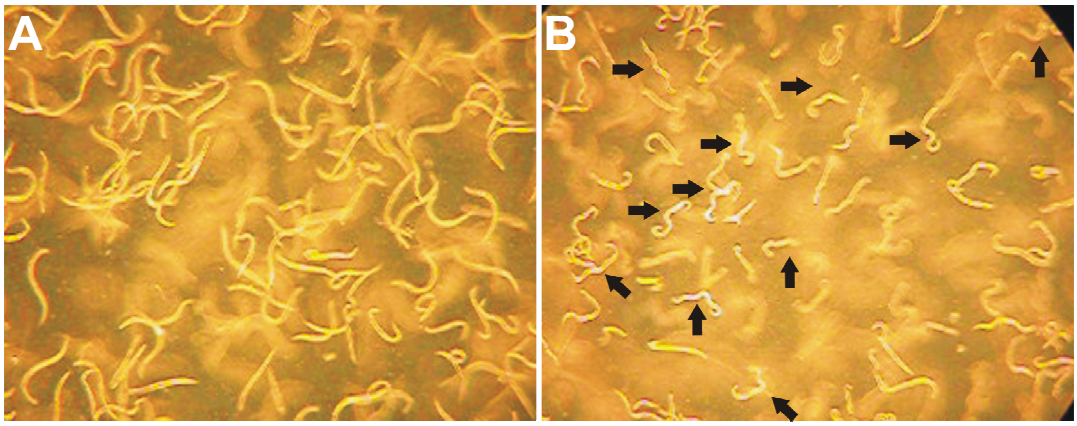

Figure 4: Effect of 1 h exposure to 15 µM dichlorvos on *C. elegans* body conformation. **A.** Unexposed stage L4 worms. **B.** L4 worms exposed to 15 µM dichlorvos for 1 h. Note the sharp bends in the in the worms (arrows). Screen captures from digital video.

Figure 5

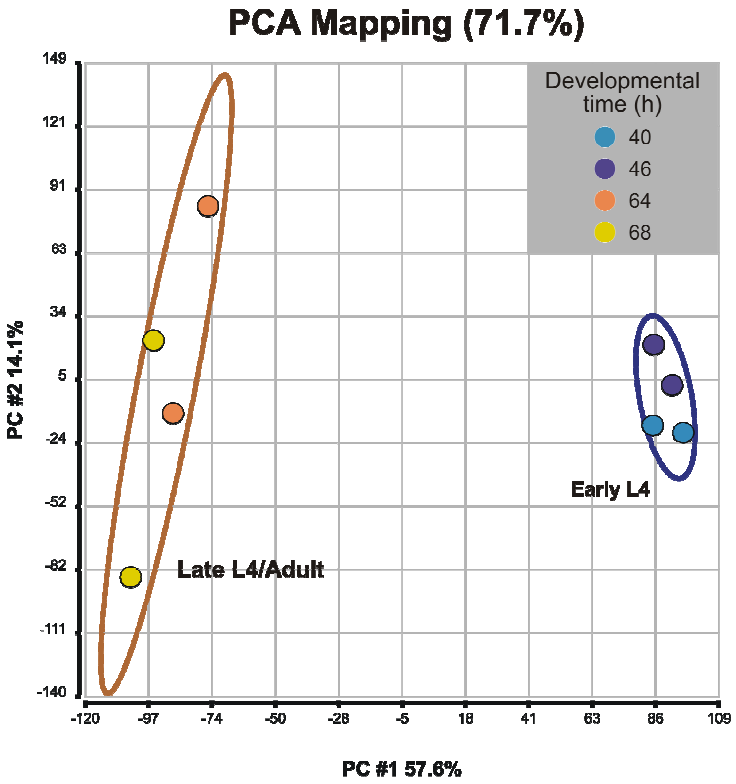

Figure 5: Principal components analysis of genes expressed by *C. elegans* near the beginning and the end of the L4 stage. A principal components analysis was performed using ~16,000 Affymetrix probe sets with no absent calls on the C. elegans GeneChip array. Gene expression was compared for early L4 (41 and 46 h) and L4/adult molt (64 and 68 h) worms in two replicate experiments. Times are measured from the release of L1 worms from the synchronization procedure. The radii of the ellipses equal one standard deviation.

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