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Ecotoxicological evaluation of chlorpyrifos exposure on the nematode *Caenorhabditis elegans*

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Abstract

To investigate the effects of chlorpyrifos (CP), an organophosphorus insecticide, on the soil nematode *Caenorhabditis elegans*, the toxicity of the insecticide on the molecular, biochemical, and physiological levels were investigated upon sublethal exposure, and an acute toxicity test was conducted using lethality as an endpoint. To assess the molecular-level effect, stress-related gene expression was investigated, and the neurotoxicity indicator, acetylcholinesterase (AChE) activity was assessed as the biochemical-level response. Growth, reproduction and development were also studied as physiological-level responses. The overall results indicate that CP exposure leads to the alteration of the expression of some stress genes, such as of heat shock protein, metallothionein, vitellogenin and *C. elegans* p53-like protein genes; the inhibition of AChE activity; and the retardation of development. These data suggest that the toxicity of CP on *C. elegans* occurred in multiple biological organizations; nevertheless this is not sufficient to conclude that there is a casual relationship between them. Thus, direct experimental demonstrations of the wider relationships between the molecular/biochemical effects of CP exposure and their consequences at higher levels of biological organization are needed to fully understand the effects of this compound on *C. elegans*.

Keywords: *Caenorhabditis elegans*; Chlorpyrifos; Ecotoxicity; Multiple biological-level effect

1. Introduction

Caenorhabditis elegans, a free-living nematode that lives mainly in the liquid phase of soils, is a good animal model for the study of ecotoxicology. Due to its abundance in soil ecosystems, its convenient handling in the laboratory, and its sensitivity to different kinds of stresses, *C. elegans* is frequently used in ecotoxicological studies utilizing various exposure media, including soil and water (Peredney and Williams, 2000; Willams et al., 2000; Boyd and Williams, 2003). Much ecotoxicity testing on environmental species, including, *C. elegans*, has worked to the endpoint of lethality. However, there has been much debate about more appropriate endpoints than lethality in ecotoxicity testing. Viewed holistically, ecotoxicology is a subject that progresses through different, and increasingly complex, levels of biological organization. In the first place, an active molecule interacts with its site of action in the cell. Which causes consequent disturbances at the cellular level. These can lead to effects at the level of the whole organism, which may then be translated to further effects at the levels of population, community and ecosystem. In this study, ecotoxicity of environmental contaminant on *C. elegans* was investigated using multiple toxicological endpoints.

Chlorpyrifos (*O*,*O*-diethyl-*O*-[3,5,6-trichloro-2-pyridinol] phosphorothionate; CP), an organophosphorus (OP) insecticide, was selected as a model chemical, since this compound is widely used in agriculture and in house because of its safety relative to comparable OP compounds. It is used to control a wide range of pests, such as cutworms, corn rootworms, cockroaches, grubs, flea beetles, flies, termites, fire ants, and lice. This compound is applied to different crops including cotton, nuts, vegetables, and ornamental plants (Extoxnet, 1996), and may enter surface water by runoff, spraydrift, or accidental spills (Cowgill et al., 1991). It exhibits moderate persistence in natural systems and low solubility in water (Worthing and Walker, 1987; Van Wijngaarden et al., 1993). CP has been reported to be toxic to freshwater fish,

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aquatic invertebrates, and estuarine organisms (Extoxnet, 1996). The effects of CP to aquatic organisms are relatively well-known and risk-assessment studies have shown that invertebrates, especially crustaceans and larval insects, are the most affected (Giesy et al., 1999). However, few studies have been conducted on soil organisms.

In this study, in order to investigate the ecotoxicological effects of CP on *C. elegans*, an acute toxicity test was performed estimating 24 h median lethal concentration (LC_{50}). Responses on molecular, biochemical, and physiological levels were subsequently investigated upon sublethal exposure. Stress-related gene expression analysis was conducted as molecular-level marker, while, the neurotoxicity indicator, acetylcholinesterase (AChE) activity was assessed as biochemical-level response. As physiological-level responses, growth, reproduction, and development were studied. A full life cycle assay was applied to assess effect of CP on the development of *C. elegans*.

2. Materials and methods

2.1. Organisms

The wild-type *C. elegans* Bristol strain N2 was used in this study. *C. elegans* were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* strain OP50, at 20 °C, using the standard method previously described by Brenner (1974).

2.2. Sample preparation

Five types of endpoints (mortality, growth, reproduction, neuroenzyme activity, and stress-related gene expression) were assessed to investigate the multi-level effects of CP on *C. elegans* (overview of experiment scheme was presented in Supplementary Table 1). Young adult from synchronized culture (3 days after hatching) were incubated with CP for 24 h and the experiments were conducted for mortality, gene expression, enzyme activity, growth, and reproduction analyses. As for the analysis of development, one L4 stage worm from synchronized culture was incubated with CP for 96 h, and the status of worm development was investigated at regular time intervals (i.e., every 12 h), until the end of treatment (96 h). Nematodes were exposed to CP (pure analytical-grade, Sigma-Aldrich Chemical, St. Louis, MO, USA) prepared in a K-medium (0.032 M KCl, 0.051 M NaCl; Williams and Dusenbery, 1990). Acetone was used as a solvent and at the beginning of the experiment, 3 μl of an acetonic solution of CP were added to the 3 ml of experimental plates (K-medium). Three replicates for each concentration and a control were conducted for all the test types. The CP concentrations in a K-medium were nominal values.

2.3. Lethal toxicity tests

Each test consisted of four concentrations and a control, in which 10 ± 1 of young *C. elegans* adults were transferred onto 24-well tissue culture plates containing 1 ml of the test solution for each of the five wells. The worms were exposed for 24 h at 20 °C. After the 24 h, the numbers of live and dead worms were determined through visual inspection and by probing the worms with a platinum wire under a dissecting microscope.

2.4. Semi-quantitative reverse transcription-polymerase chain reaction

Following the 24 h incubation with exposure to sublethal concentrations of CP, nematodes were harvested for the preparation of RNA. Total RNA was prepared by phenol–chloroform extraction, according to the manufacturer's standard protocol (Hope, 1999). RNA concentrations were determined by the absorbance at 260 nm. The quality of total RNA was estimated based on the ratio of the optical densities from RNA samples measured at 260 and 280 nm. The two-step reverse transcription-polymerase chain reaction (RT-PCR) method was used with RT and PCR Premix kits (Bioneer Co., Seoul, Korea), using a PTC-100 thermal cycler (MJ Research, Lincoln, MA, USA). The primers were designed on the basis of the sequences retrieved from the *C. elegans* database (www.wormbase.org; Supplementary Table 2). Actin mRNA was used for expression-level normalization of the studied genes. The PCR products were separated through electrophoresis on 1.5% agarose gel (Promega, Madison, WI, USA) and were visualized with ethidium bromide (Bioneer). All the tests were replicated at least three times, and the relative densities of each band were determined with the use of a Kodak EDAS 290 image analyzer (Kodak, Rochester, NY, USA), with a TFX-20.M UV transilluminator (Vilber Lourmat, Marne la Vallee, France).

2.5. Acetylcholinesterase activity

Young adults were collected 24 h after treatment from control and experimental plates, were pooled and were homogenized in 2.5 ml of Tris–EDTA buffer (40 mM, pH 7.8; Sigma-Aldrich Chemical, St. Louis, MO, USA) for AChE activity measurements. Crude homogenate was centrifuged for 15 min at $500 \times g$ (4 °C) and supernatant was centrifuged for 30 min at $12{,}000 \times g$ (4 °C). The resulting supernatant (post-mitochondrial fraction) was used to measure the activity of AChE. AChE activity was measured using the method of Ellman (1961). Enzymatic activities were calculated relative to the protein content of the extracts, measured by the method of Bradford (1976).

2.6. Growth and reproduction

Following the 24 h incubation with exposure to sublethal concentrations of CP, growth and reproduction were evaluated. Growth was investigated by measuring the worms that had been killed by heat through microscopy, with a scaled lens in each sample. The average length of the unexposed control worm was in the range of 1.0–1.2 mm. Reproduction was investigated by counting the eggs of each worm through the microscopic inspection of the transparent *C. elegans* body in each sample. Although this procedure differs from more commonly used reproduction tests of offspring counting from an age-synchronized single worm, this simple detection method seems appropriate for the rapid screening of the reproduction effect. The average number of eggs per worm in the unexposed controls was in the range of 15–25. One hundred worms were examined per treatment for growth and reproduction experiments.

2.7. Development

The effects of CP on the development of nematodes were investigated for 96 h by identifying the life stage of *C. elegans*, such as an egg, 1st–3rd stage larva (L1–L3), 4th stage larva (L4), and an adult, at the regular time intervals (every 12 h). To obtain the F1 generation, one worm in the L4 stage from an age-synchronized culture (P generation) was introduced in the test medium. The total number of worms at each stage was determined for the F1 and F2 generations, by observing the *C. elegans* under an optical microscope. The results were expressed as the percentage of each stage and as the actual number of individuals at each stage.

2.8. Data analysis

Median lethal concentration (LC_{50}) was derived through Probits analysis. The statistical differences between the control and treated worms were determined with the aid of the parametric *t*-test.

3. Results

Prior to the main experiment, a solvent control test was conducted to compare the response of the non-solvent control group and the solvent control group, which indicated that acetone did not provoke any significant effect in all the experiments (data not shown). The controls presented in the Results section of this paper were solvent controls.

Acute toxicity was studied using LC_{50} derived through Probit analysis (Table 1). The 24 h LC_{50} of CP in *C. elegans* was 0.966 mg l^{-1}. Based on the results of the acute toxicity test, three concentrations—corresponding to 1/1000, 1/100, and 1/10 of the 24 h LC_{50}—were selected for the sublethal exposure conditions, which were 1, 10, and 100 μg l^{-1}.

Stress-related gene expression profiling analysis was conducted on the selected genes, which were based on the result from the *C. elegans* microarray (unpublished data) and on our previous studies (Roh et al., 2006, 2007). We investigated alteration on the gene expression of heat shock proteins (*hsp-16.1*, *hsp-16.2*, *hsp-16.48*, *hsp-70*), metallothioneins (*mtl-1*, *mtl-2*), vitellogenins (*vit-2*, *vit-6*), xenobiotic metabolism enzyme (*cyp35a2*), tumor suppressor and apoptosis proteins (*cep-1*, *ape-1*), and antioxidant enzymes (*sod-1*, *ctl-2*) in CP exposed *C. elegans* (Fig. 1). An increase in the expression of *hsp16-2*, *mtl-2*, *cep-1*, and *vit-6* occurred, whereas a decrease in the expression of the *mtl-1* and *ape-1* genes was also observed in the *C. elegans* exposed to CP. Decrease in the expression of antioxidant enzymes genes (*sod-1*, *ctl-2*) was observed in the worm exposed to low level of CP (1 μg l^{-1}). Expression of *cyp35a2* gene was not changed by CP exposure.

AChE was studied in the *C. elegans* exposed to CP, to verify the exposure level of the larvae to this compound, as the inhibition of AChE is a mode of action of OP compounds (Fig. 2). About 50% of the enzyme activity was inhibited in the *C. elegans* that was exposed to 10 and 100 μg l^{-1} of CP.

As growth and reproduction indicators, the changes in the worms' body lengths and the number of eggs per worm after CP exposure were presented in Fig. 3. No significant effect of CP exposure on the body length or on the number of eggs per worm was observed.

The effect of CP on the development of nematodes was investigated by identifying the life stage of *C. elegans*, such as an egg, L1–L3, L4, and an adult, for 96 h (Fig. 4). A statistically more significant decrease than that in the control group occurred in the total number of eggs and worms in the L1–L3 stage and worms in the L4 stage, which were counted after 96 h of exposure to 10 and 100 mg l^{-1} of CP (Fig. 4a). In the control group, 48 h after the beginning of the experiment, about half of the population was in the L1–L3 stage and L4 stage (F1); and at 96 h, the number of eggs from the F2 generation

Table 1
24 h LC_{10}, LC_{50}, and LC_{90} of chlorpyrifos in *C. elegans*

	24 h LC (mg l^{-1})		95 % Confidence interval
Chlorpyrifos	LC_{10}	0.298	$0.068 < LC_{10} < 0.520$
	LC_{50}	0.966	$0.570 < LC_{50} < 1.508$
	LC_{90}	3.135	$1.898 < LC_{90} < 10.981$

LC, lethal concentration.

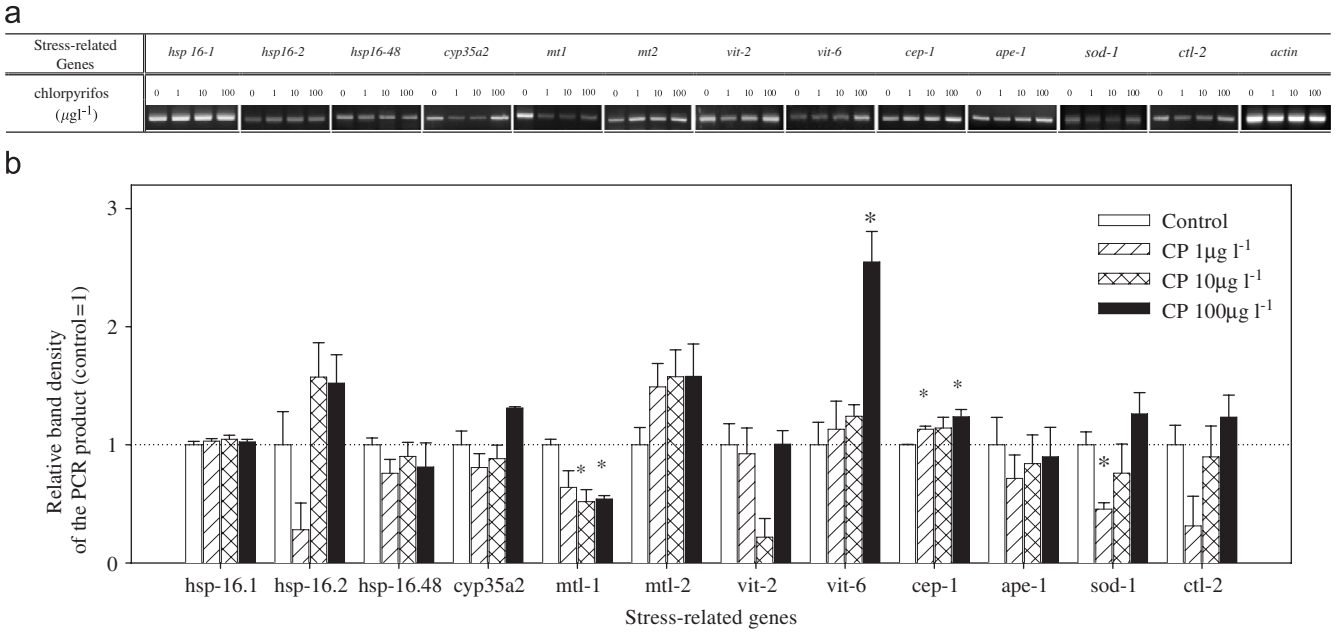

Fig. 1. Stress-related gene expression profiling in the young adult of *C. elegans* exposed to chlorpyrifos for 24 h (a). Densitometric values of stress-related gene expression, normalized using actin mRNA. Data are presented in arbitrary units compared to control (b; control = 1, number = 3; mean ± standard error of mean; $^{*}p < 0.05$).

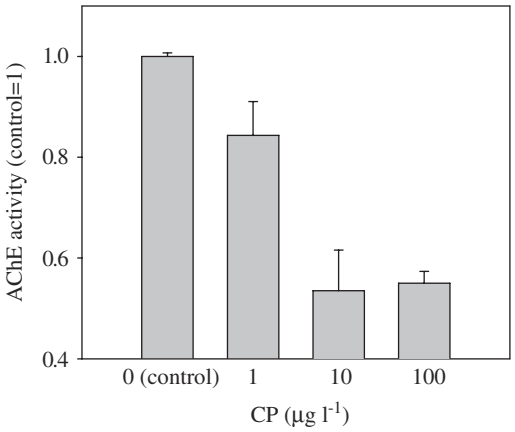

Fig. 2. Acetylcholinesterase activity in *C. elegans* exposed to chlorpyrifos for 24 h (control = 1, number = 3; mean ± standard error of mean; $^{*}p<0.05$).

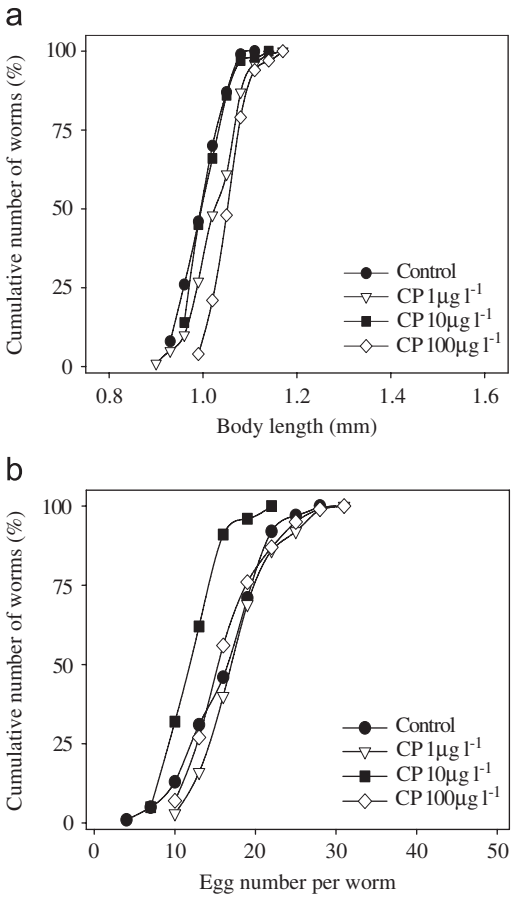

Fig. 3. Growth (a) and reproduction indicators (b) examined in the young adult of *C. elegans* exposed to chlorpyrifos for 24 h.

reached about 70% of the total population (Fig. 4b). The percentage of the egg population observed 96 h after the beginning of the experiment (F2) was 50% after exposure to 10 and 100 $\mu g l^{-1}$ of CP, whereas in the control group and after 1 $\mu g l^{-1}$ of CP treatment, the percentage of the egg population reached about 70%.

4. Discussions

With advances in biochemical toxicology and associated technology, it is possible to get a clearer insight into the operation of toxic mechanisms initiated by chemicals acting upon organisms, mechanisms that can have adverse effects at the population level. The nematode *C. elegans* is an excellent model organism for the mechanistic biomarker approach. Comprehensive knowledge on the genome of *C. elegans* allows the development of a combination of tests on different organizational levels, such as the genome, proteome, organ, organism, and population levels. This allows the assessment of toxicity on the basis of specific modes of action and ecologically relevant parameters. Up- or down-regulation of the genes that are involved in chemical toxicity can be a good indicator of chemical exposure. The basic premise that changes in gene expression can be harnessed to diagnose the exposure and effects of environmental chemicals is currently receiving significant attention (Custodia et al., 2001; Aït-Aïssa et al., 2003; Reichert and Menzel, 2005; Stürzenbaum et al., 2005; Roh et al., 2006, 2007; Cui et al., 2007; Kim and Sun, 2007; Menzel et al., 2007). As shown in Fig. 1, differential gene induction or repression was detectable in response to CP exposure; they were *hsp*, *mtl*, *vit*, and *cep*. In the case of some genes, such as *mtl-1* and *mtl-2*, the physiological meaning of the over- or under-expression is unclear. Although, the nematode *C. elegans* is an excellent model organism for the mechanistic biomarker approach, our data sets are not sufficient enough to provide a clear explanation for this phenomenon. If more sub-cellular parameters (molecular/biochemical markers) had been tested with longer exposure period, involvement of observed CP-induced altered gene expression in physiological pathway could probably be better evaluated and explained.

The inhibition of the AChE activity after exposure to CP confirmed that the nematode was actually exposed to CP, and the toxic effects observed were due to CP exposure, as this parameter is a typical exposure biomarker of the OP compound. The responses of these molecular/biochemical parameters could be used as an 'early warning system' for ecotoxicity monitoring. Pollutant-induced molecular/biochemical effects may have consequences at higher levels of biological organization, such as, changes in population dynamics and in biological diversity at both the intra- and inter-specific levels (Walker, 1998; Hyne and Maher, 2003; Roh et al., 2006; Sarkar et al., 2006). However, molecular/biochemical endpoints alone do not seem to be sufficient to diagnose environmental quality. Multi-level and multi-biomarker approaches would be more prudent for useful ecotoxicity monitoring (Neuparth et al., 2005; Lee and Choi, 2006; Pereira et al., 2007; Roh et al., 2007).

Physiological-level alterations, such as growth, reproduction, feeding, movement, and behavior, have been used as endpoints for chemical-induced toxicity testing in

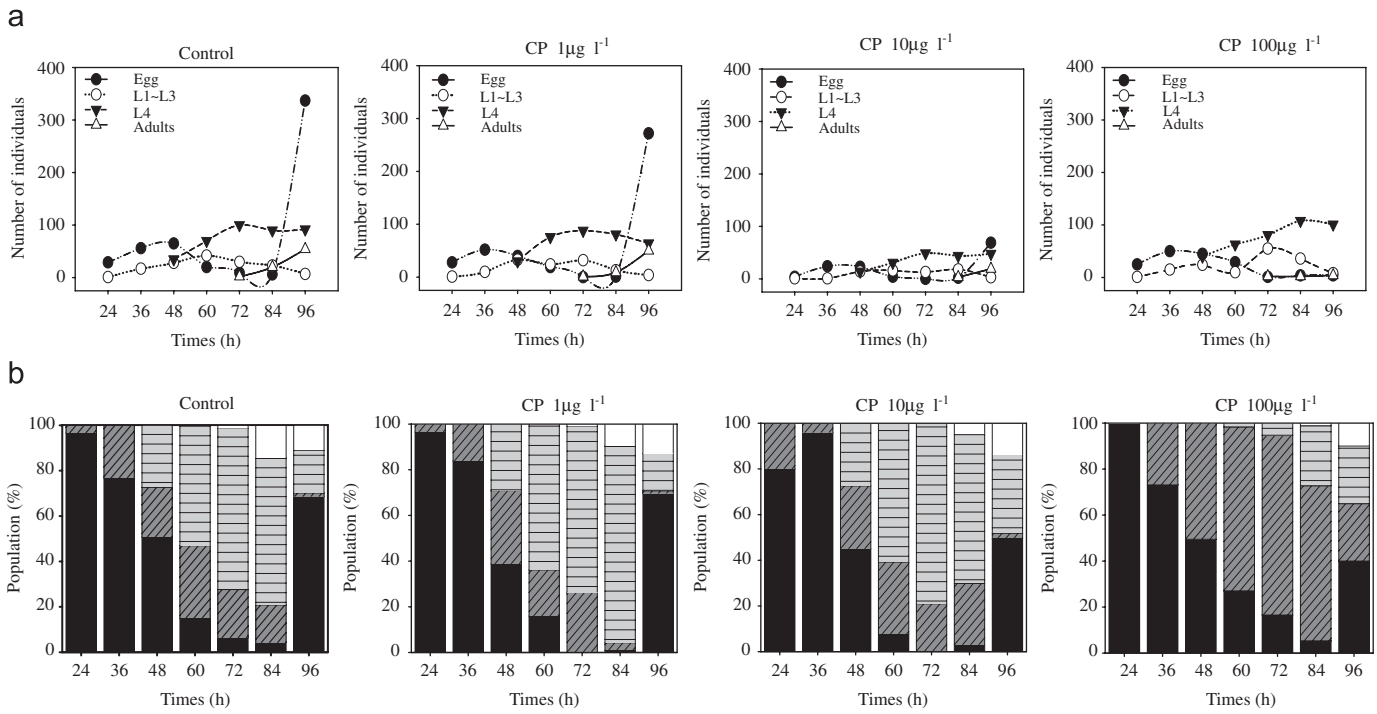

Fig. 4. Development parameters in *C. elegans* exposed to chlorpyrifos for 96 h. Number of individuals (a) and kinetics of population (b) of *C. elegans* exposed to sublethal concentration of chlorpyrifos for 96 h (■ egg, ▨ L1~L3, ▤ L4, □ adult).

C. elegans (Dhawan et al., 2000; Anderson et al., 2001, 2004; Kohra et al., 2002; Tominaga et al., 2003). They are broadly accepted reliable test parameters and were found to be more sensitive indicators of toxicity than lethality.

As shown in Figs. 3 and 4, growth, reproduction and development seem to be appropriate indicators of the toxicity of CP on *C. elegans*. Short-term (24 h) growth and reproduction experiments were conducted to compare the sensitivity of physiological-level response with that of the molecular-level response (i.e., gene expression) toward CP exposure. Twenty-four hour reproduction test only provides a snapshot of reproductive output and may not fully reflect embryonic lethality and delays in reproductive peaks; however, such information can be obtained from the 96 h-long development experiment (Fig. 4). Exposure to CP may have serious consequences on the *C. elegans* population, as this compound induced significant disturbance on development, such as, a decrease in the total population number, egg generation (F2), etc. The development study also suggests that full life cycle assays using *C. elegans* can generate robust and population-relevant endpoints, which can provide an integrated approach to predicting population responses of nematodes to pesticides, including CP.

The overall results of this study showed that alteration of the expression of some stress genes, AChE inhibition, and development retardation occurred following CP exposure. From the ecotoxicological point of view, the establishment of a causal relationship between molecular and organism/population indicators is important, although it is difficult to perform. The experimental evidence generated in this study, however, are not sufficient to elucidate any casual relationship between responses across biological levels. Thus, direct experimental demonstrations of the wider relationships between the molecular/biochemical effects of CP exposure and their consequences at higher levels of biological organization, are needed to fully understand the effects of this compound on *C. elegans*. The characterization of the causal relationships between the biomarker responses of *C. elegans* and the effects of the CP exposure of *C. elegans* at higher biological levels will help defining the sublethal hazards of chemicals in this animal.

The data obtained from this study may serve as an important contribution to knowledge on the ecotoxicology of, a widely used pesticide, CP in *C. elegans*, on which few data are available. Besides ecotoxicology, the data provided herein may also provide an insight into the mammalian toxicity of CP. Biomonitoring organisms have long been used as a means of warning people of unsafe environments. The role of *C. elegans* is particularly valuable in this regard. *C. elegans* is considered an ideal system for understanding mammalian pathology, including toxicity, as the genome of *C. elegans* shows an unexpectedly high level of conservation with the vertebrate genome (Brown and Botstein, 1999; Wilson, 1999). Indeed, the use of *C. elegans* as a model for mammalian neurotoxicity was already investigated by comparing the relative order of toxicity of OP substances in *C. elegans* to the order of their toxicity in mammals (Cole et al., 2004). Their results

suggest that *C. elegans* may react to neurologically active chemicals with enough similarity to mammals to be useful as a first-round screening agent for neurotoxicity. *C. elegans* seems to have a high potential to be a good candidate for an alternative animal model for mammalian toxicity screening study.

5. Conclusions

The ecotoxicity of CP were investigated on the soil nematode, *C. elegans*, using stress-related gene expression, AChE activity, growth, reproduction, and development, as endpoints. The overall results indicate that CP exposure leads to the alteration of *hsp*, *mtl*, *vit*, and *cep* genes expression; the inhibition of AChE activity; and the developmental retardation, which suggest that the toxicity of CP on *C. elegans* occurred in multiple biological organizations and further study on the relationships between the molecular/biochemical effects of CP exposure and their consequences at higher levels of biological organization may be needed to fully understand the effects of CP on *C. elegans*.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2007.11.007.

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