

# Effects of polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans on phagocytic response of *Eisenia andrei* coelomocytes

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## Abstract

The immunotoxicological effects of polychlorinated dibenzo-*p*-dioxins/dibenzofurans (PCDDs/Fs) mixtures on *Eisenia andrei* earthworms have never been studied. In this work we investigated these effects both for *in vitro* and *in vivo* exposure, using the viability and the phagocytic activity of coelomocytes as immunological biomarkers and the flow cytometry was used for analysis. The *in vitro* exposure revealed a cytotoxic effect of PCDD/Fs mixture (C2) containing  $50 \times 10^{-3}$  ng/mL of 2, 3, 7, 8-TCDD and an induction of the phagocytic capacity at the mixture (C1) containing  $25 \times 10^{-3}$  ng/mL of 2, 3, 7, 8-TCDD. In the *in vivo* filter paper exposure, the immunocompetence of earthworms was assessed after 3 h-exposure to mixtures of PCDD/Fs at the levels of C1, C2, C3 and C4 containing about; 0.05, 0.3, 0.5 and 0.83 ng of 2, 3, 7, 8-TCDD/cm<sup>2</sup>, respectively. Morphological observations showed an excessive secretion of mucus and body surface lesions in worms exposed to higher concentrations (C3 and C4), which revealed that these organisms were affected by PCDD/Fs either through skin and/or by feeding. The levels of the extruded cell yield decreased significantly at all the concentrations tested. However, the cell viability was shown to be unaffected by PCDD/Fs concentrations. It was also shown, that exposure to the highest PCDD/Fs concentrations; C2, C3 and C4 inhibited both phagocytic activity and efficiency.

## Introduction

Earthworms have been extensively used as standard test organisms in soil toxicity testing<sup>1</sup>. They have been used to assess environ-

mental impact from heavy metals,<sup>2,4</sup> pesticides<sup>5</sup> and some persistent organic pollutants (POPs) such as; PCBs, dioxin-like PCBs and PAHs.<sup>6-10</sup> However, the knowledge on toxic effects of persistent organic pollutants (POPs) upon the worms is still very limited.<sup>11,12</sup> For example, there is paucity in the literature describing the toxicity of polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) in earthworms which play an important role in soil formation processes and are considered as interesting bio-indicators for soil contamination. It is well recognized that PCDDs and PCDFs represent the most toxic anthropogenic chemicals in the environment where they enter as by-products of combustion processes. Their toxicity is associated to their stability, lipophilicity, bioaccumulation and high persistence. In addition, previous studies have reported that prolonged exposure to TCDD presents adverse health effects, including immunotoxicity, neurotoxicity, hepatotoxicity and carcinogenesis.<sup>13-17</sup> Moreover, the international Agency for Research on Cancer (IARC) evaluated TCDD as a Group 1 carcinogen *i.e.*, a human carcinogen.<sup>18,19,20</sup> Environmental exposure concentrations of 1 ng 2378-TCDD/g soil were considered *a level of concern* for causing cancer.<sup>21</sup> Thus, the presence of these toxic contaminants in soils may have direct harmful effects to the terrestrial ecosystem. Previous studies have reviewed a large battery of biomarkers to measure the sublethal effects of chemicals in earthworms, such as the immunological responses. Since the immune responses are important host defense mechanisms, their modulation may result in increased incidence of infections that could influence the survival of individuals and their populations.<sup>22</sup> It has been demonstrated that many chemicals, including organic and inorganic trace elements, may adversely affect the immune system of earthworms.<sup>23</sup> In fact, it has been shown that the exposure to mercury, cadmium or zinc was toxic and affected cell viability and phagocytosis.<sup>23-26</sup> In addition, Goven *et al.* and Ville *et al.* showed that the phagocytic competence was also inhibited by polychlorinated biphenyls (PCBs).<sup>6,7</sup> Thus, the suppression of the phagocytic activity or reduction of coelomocytes viability decreases animal resistance to infection.<sup>23</sup> This deficiency in immune functions is considered as a sign of toxic effects of environmental pollutants.<sup>23</sup>

To date, the immunotoxicological effects of PCDD/Fs on earthworms have not been studied. Thus, the objective of the present study was to investigate, *in vitro* and *in vivo*, the effects of PCDD/Fs mixtures on the immunocompetence of *oligochaete Eisenia andrei* using the phagocytosis assay as immunological biomarker.

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Key words: PCDDs/Fs, earthworms, *Eisenia andrei*, immunosuppression, phagocytosis, coelomocytes.

Acknowledgments: this work was conducted in the environmental immunotoxicology laboratory at Institut Armand Frappier (IAF-INRS). The financial support has been provided by the Canada Research Chair in Environmental Immunotoxicology (Dr. Michel Fournier). The authors are thankful to Marlene Fortier and also appreciate her excellent technical assistance. We wish to thank Dr Pierre Yves Robidoux (CNRC-IRB), Louis Martel and Elloise Veilleux (CEAEQuebec) for earthworms donations.

Received for publication: 8 June 2011.

Accepted for publication: 6 October 2011.

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Journal of Xenobiotics 2011; 1:e6  
doi:10.4081/xeno.2011.e6

## Materials and Methods

### Chemicals

The mixture of PCDDs/Fs in nonane solution was obtained from Wellington laboratories (Ontario, Canada) and stored at 1°C. Dimethyl sulfoxide 99.5% (DMSO), Formaldehyde 37% and phosphate buffer solution (PBS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA) was purchased from MP Biomedicals, LLC (Cleveland, Ohio, USA) and sodium azide (NaN<sub>3</sub>) from FisherChemical (Pittsburgh, PA, USA). Purified water was obtained from a Milli-Q water purification system.

### Earthworms

The earthworms, *Eisenia andrei* were purchased from the Carolina Biological Supply (Burlington, NC, USA). Prior to the experiment, the animals were maintained in earthworm bedding (Magic Products, Amherst, Jct, WI) at 20±1°C, 70-80% (w/w) moisture and 12:12 h light/dark cycle, and fed once a week with cereals (Magic Worm Food, Magic Products Inc., Amherst Junction, WI, USA).

### Cell extrusion

Coelomocytes were extracted by inserting a

single worm into a 15-mL tube containing 3 mL of *Lumbricus* balanced salt solution (LBSS) composed of 1.5 mM NaCl, 4.8 mM KCl, 1.1 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, 0.3 mM Na<sub>2</sub>PO<sub>4</sub>·7H<sub>2</sub>O, 3.8 mM CaCl<sub>2</sub>, and 4.3 mM NaHCO<sub>3</sub>.<sup>27,28,24</sup> The liquid medium in the tube was submitted to a 6 V current for 20-30 s using aluminium wires.<sup>25</sup> Following the electric shock, a higher turbidity was observed in the solution. Then, the worm was removed from the tube and the solution was gently shaken. Total number of cells and initial cell viability were determined by diluting 20 µL of cell suspension with 20 µL of 0.4% trypan blue (Sigma-Aldrich) and this cell suspension was placed into an improved Neubauer haemocytometer which was microscopically observed.<sup>24,25</sup>

### The phagocytosis assay

The phagocytic activity of cœlomocytes was measured in each worm, based on the protocol of Brousseau *et al.*<sup>28</sup> For each cell suspension, 1.716-µm fluorescent latex beads (Polysciences Inc., Warrington, PA, USA) were added in a bead: cell ratio of 100:1. The beads were previously sonicated for 5 min at room temperature to get rid of doublet and triplet beads. The cells (with beads) were then incubated for 18 h at room temperature. To remove the beads that were not phagocytosed, the cell suspension was layered over a 3% bovine serum albumin (BSA) and centrifuged at 150 g, 4°C and for 8 min. The cells collected in the pellet were then resuspended in 500 µL of hematal (Fisher Scientific, Ottawa, ON, Canada) containing 0.185% formaldehyde and 0.2% sodium azide. Then, the phagocytosis was measured by flow cytometry in FL1 (Becton Dickinson, San José, CA, USA)<sup>28</sup> and for each sample, the fluorescence of 5000 events was recorded. Approximate instrument settings were presented on Table 1. Results were analysed with the Cell Quest Pro software (Becton Dickinson) to determine the percentage of cells that engulfed one bead and more (phagocytic activity) or three beads and more (phagocytic efficiency). The results were expressed as the percentage of phagocytosis.

### Exposure protocol

In our study, we carried out two experiments. The first one consists to study the effects of the PCDDs/Fs dose on viability and phagocytic activity of cœlomocytes, after an *in vitro* exposure. In the second experiment, we used a filter paper-contact assay to evaluate the *in vivo* response (viability and phagocytic activity) of the worms.

### *In vitro* exposure to polychlorinated dibenzo-*p*-dioxins/dibenzofurans

Before contaminant exposure, the cell solu-

tion of each worm was adjusted to 0.5x10<sup>6</sup> cells/mL. To establish the *in vitro* dose-response curves, two series of glass tubes containing the cell suspension were exposed to dilutions of a PCDDs/Fs mixture; C1 and C2 (Table 2). These dilutions were done in DMSO (final concentration 0.004% per tube). This DMSO concentration showed no effects on function cell. So, a set of tubes with DMSO (vehicle) was added as negative control and another set of tubes containing untreated cœlomocytes was added to monitor their viability during the experiments. Cells were exposed for 3 h to PCDDs/Fs and then the latex beads were added to quantify phagocytosis. The preincubation time of 3 h was previously optimized (data not shown). PCDDs/Fs-incubated cell viability was also monitored in the same time of phagocytosis. It was determined by adding 5 µL of PI (1 mg/L) to the cell suspensions (PI final concentration 10 µg/L) and measuring fluorescence by flow cytometry in FL3 using a FACSCalibur cytometer (Becton Dickinson, San José, USA).<sup>27,28</sup> Data are expressed as a percentage of viable cells. Six worms were used and the experiment was repeated two times.

### *In vivo* exposure to polychlorinated dibenzo-*p*-dioxins/dibenzofurans

The contact toxicity assay was performed following the method described in the OECD guidelines for the testing of chemicals.<sup>1,29</sup> Three h prior to the exposure, the worms were removed from the culture, rinsed with water, dampened on filter paper and kept in the dark at 20±1°C in order to allow the removal of the gut content.

Then, volumes of 10, 50, 100 and 150 µL of initial mixture solution of PCDDs/Fs (diluted in DMSO < 0.01%) containing; 0.625 ng (2378-TCDD)/µL, 0.625 ng (2378-TCDF)/µL, 1.562 ng (12378-PeCDD)/µL, 1.562 ng (12378-PeCDF)/µL, 1.562 ng (123678-HxCDD)/µL, 1.562 ng (123678-HxCDF)/µL, 1.562 ng (1234678-HpCDD)/µL, 1.562 ng (1234678-

HpCDF)/µL, 3.125 ng OCDD/µL and 3.125 ng OCDF/µL, were added to 6-cm diameter plastic Petri dishes containing a No. 3 Whatman filter paper. The resulting exposure concentrations for each volume were illustrated in Table 3. Negative controls received just deionized water (200 µL) were included along the test with controls received DMSO (without PCDDs/Fs) to test for any effect due to the solvent (vehicle). Then, the worms were individually rinsed with distilled water, blotted dry on a paper towel, weighed (between 0.3 and 0.6 g), and placed in the dish. Four replicates per treatment were used, consisting of one earthworm per Petri dish. All experiments were real-

**Table 1. Approximate parameters used for acquisition of phagocytosis Data by flow cytometry.**

Parameters	Value
Forward scatter (FSC Lin)	1.00
FSC threshold	52
Side scatter (SSC log)	227
Fluorescence (FL1 log)	273
Fluorescence (FL3 log)	650

**Table 2. Polychlorinated dibenzo-*p*-dioxins/dibenzofurans concentrations used for dose-response curves: test *in vitro*.**

PCDDs/Fs	C1 (ng/mL) x10 <sup>-3</sup>	C2 (ng/mL) x10 <sup>-3</sup>
2378-TCDD	25	50
2378-TCDF	25	50
12378-PeCDD	62.5	125
12378-PeCDF	62.5	125
123678-HxCDD	62.5	125
123678-HxCDF	62.5	125
1234678-HpCDD	62.5	125
1234678-HpCDF	62.5	125
OCDD	125	250
OCDF	125	250

PCDDs/Fs, polychlorinated dibenzo-*p*-dioxins/dibenzofurans.

**Table 3. Initial concentrations of polychlorinated dibenzo-*p*-dioxin/dibenzofuran in Petri dishes: *in vivo* exposure.**

PCDD/F	C1 (ng/cm <sup>2</sup> )	C2 (ng/cm <sup>2</sup> )	C3 (ng/cm <sup>2</sup> )	C4 (ng/cm <sup>2</sup> )
2378- TCDD	0.055	0.276	0.553	0.829
2378-TCDF	0.055	0.276	0.553	0.829
12378- PeCDD	0.138	0.691	1.382	2.073
12378- PeCDF	0.138	0.691	1.382	2.073
123678- HxCDD	0.138	0.691	1.382	2.073
123678- HxCDF	0.138	0.691	1.382	2.073
1234678-HpCDD	0.138	0.691	1.382	2.073
1234678-HpCDF	0.138	0.691	1.382	2.073
OCDD	0.276	1.382	2.765	4.147
OCDF	0.276	1.382	2.765	4.147

PCDD/F, polychlorinated dibenzo-*p*-dioxin/dibenzofuran.

ized in the dark at  $20 \pm 1^\circ\text{C}$  for 3 h. The assay was done in duplicate. After 3 h-exposure duration, the worms were again rinsed with distilled water, blotted and individually weighed. Cell extrusion was carried out and the cell yield, viability and phagocytosis were then determined as described above.

### Statistical analysis

The data were expressed as arithmetic means  $\pm$  standard deviations. Statistical significances of differences among treatments were determined by ANOVA one-way followed by Tukey HSD post-hoc test for specific comparison of means ( $P < 0.05$ ), significantly different from the vehicle control: \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ . The software Statistica, Version 6.0 (StatSoft, Tulsa, OK, USA) was used.

## Results

To distinguish coelomocyte populations by flow cytometry, based on a dot plot of complexity versus the size of the cells, two gates were drawn around coelomocytes. We observed a population of smaller, complex cells following the SSC axis, corresponding to chloragocytes (G1), and population of larger, simple cells following the FSC axis corresponding to amœbocytes (G2) (Figure 1A). Debris represent the part not gated in Figure 1A. Furthermore, it was also shown that G1 cells do not represent phagocytic activity (Figure 1B) but G2 represents the delimitation of phagocytic cell population (Figure 1C).

### In vitro exposure

In this experiment, cells were exposed to two concentrations of PCDDs/Fs mixture (C1 and C2) during 3 h before the incubation with beads. The effects of selected concentrations were evaluated by determining cell viability and phagocytic activity. Figures 2 and 3 showed

that an increase of PCDDs/Fs concentration can induce changes in viability and phagocytosis profiles, respectively. In fact, a decrease of 10% in viability of treated cells with C1, compared to vehicle control, was shown in Figure 2. However, a significant decrease ( $P < 0.01$ ), greater than 50% in viability of treated cells with C2 compared to vehicle control was also observed in Figure 2. A significant induction ( $P < 0.05$ ) of phagocytic activity was shown, in Figure 3, for cells exposed to C2.

### In vivo exposure

Acute toxicity of PCDD/Fs on *E. andrei* earthworms was carried out by paper contact method. In the first minutes of exposure some observations were noticed about worms compartmental as lifting the body and getting longer. After 3 h-exposure duration, no mortality of earthworms was observed in controls and in dishes contaminated with PCDD/Fs. However, mucus secretion was noted in all PCDD/Fs - exposed worms and was excessive for worms exposed to C2, C3 and C4 (Table 3). Also, about 80% of exposed worms to C3 and C4 exhibited surface lesions and other morphological abnormalities like coiling and curling.

The extruded cell yields of worms exposed to the various PCDD/Fs concentrations tested (C1, C2, C3 and C4) were significantly ( $P < 0.05$ ) lower from the vehicle controls (Figure 4). While, the cell viability (Figure 5) was shown to be unaffected by PCDD/Fs concentrations used in this experiment.

Phagocytic activity (Figure 6) and efficiency (Figure 7) were significantly inhibited, when worms were exposed to C2, C3 and C4 which contained 0.276, 0.553 and 0.829 ng 2378-TCDD/cm<sup>2</sup>, respectively, compared to vehicle controls.

## Discussion

In *Eisenia andrei* earthworms, two populations are distinguished; chloragocytes and

amœbocytes (small and large or granular and hyaline).<sup>30</sup> The gates used to delimitate the phagocytic cell populations showed that only G2 (amœbocytes) which present a phagocytic activity (Figure 1). These results confirms the reported data which showed that in *Oligochaeta* (*Lumbricus terrestris*, *Eisenia foetida*, *Eisenia andrei*), all coelomocyte types, with the exception of chloragocytes (chloragogen cells), produce pseudopodia and are capable of phagocytosis.<sup>31</sup> However, no peaks of fluorescence by cells engulfing beads were obtained for G1 (chloragocytes), the autofluorescence obtained in Figure 1B was due to riboflavin<sup>32,33</sup> so the chloragocytes had been excluded from analysis in all our experiments.

The results of the *in vitro* exposure showed that C2 presents a cytotoxic effect on coelomocytes (mortality was greater than 50%) (Figure 2). In addition, the evaluation of phagocytosis in cells exposed to C1 showed an induction of the phagocytic activity (capacity of cells to engulf one bead and more) compared to vehicle control (Figure 3). The PCDD/Fs-associated immuno-stimulation observed in our study

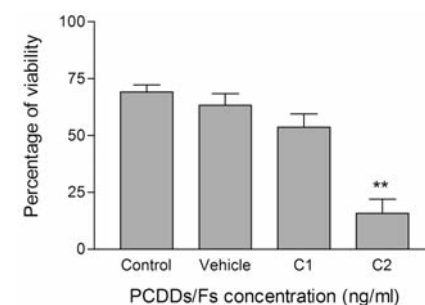


Figure 2. Viability of coelomocytes following 3 h of preincubation with polychlorinated dibenzo-*p*-dioxins/dibenzofurans (PCDDs/Fs) and 18 h with beads. Data are presented as mean  $\pm$  SD (n=6), asterisks indicate significant difference ( $P < 0.01$ ) from vehicle.

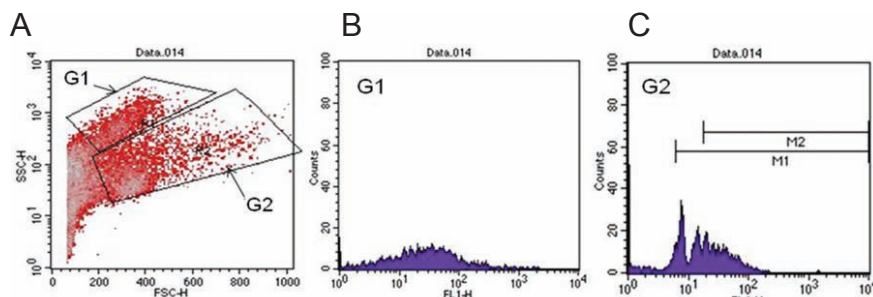


Figure 1. Pattern (A) and fluorescence histograms of *Eisenia andrei* coelomocytes after the phagocytosis assay (B: Chloragocytes, C: amœbocytes, M1: cells have engulfed one bead and more, M2: cells have engulfed three beads and more).

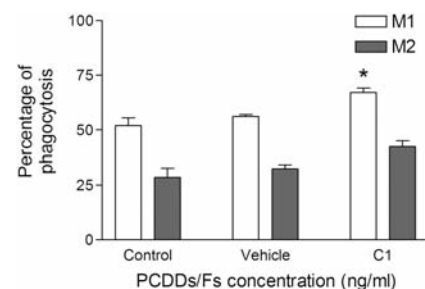


Figure 3. Phagocytic response of coelomocytes following 3 h of preincubation with polychlorinated dibenzo-*p*-dioxins/dibenzofurans (PCDDs/Fs) and 18 h with beads (M1: cells engulfed one bead and more, M2: cells engulfed three beads and more). Data are presented as mean  $\pm$  SD (n=6) and asterisk indicates significant difference ( $P < 0.05$ ) from vehicle.

was considered as an immunotoxicological effect, since it may result in the loss of regulation within the immune system and can lead to adverse outcomes including autoimmune disease, anergy, and cancer.<sup>34,35</sup> Also, these stimulatory effects caused by normally inhibitory and toxic substances such as PCDD/Fs may be interpreted as hormesis phenomenon. In fact, hormesis was previously observed in mammalian laboratory models exposed to dioxins.<sup>36,37</sup> For example; in female Sprague-Dawley rats exposed to single and multiple doses of 1, 2, 3, 4, 6, 7, 8-heptachlorodibenzo-*p*-dioxin (HpCDD) to study its chronic toxicity and carcinogenicity in lifetime experiments, it was noted that the lowest doses prolonged the life of rats, while higher doses resulted in a shortening of their life.<sup>36</sup>

To the best of our knowledge, no other studies examined the effects of PCDD/Fs on phagocytic activity using an invertebrate model, but using just mammalian model. Also, few data of the PCDDs/Fs effects on the phagocytosis are available. Omara *et al.* have exposed leucocytes from male Fischer rats to PCDDs/Fs mixtures (1-15 µg/mL) to study the effects on phagocytosis.<sup>38</sup> Their results indicate that *in vitro* exposure to this range of concentrations had no suppressive effects on the immune functions assayed, and thus produced no additive immunotoxicity.

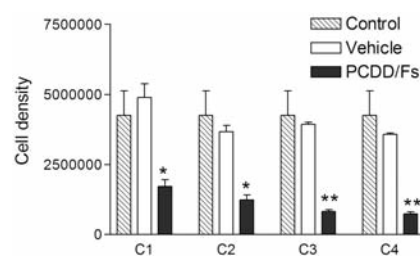
The impacts of PCDDs/Fs on vertebrates are known to be mediated through the ligation and activation of the aryl hydrocarbon receptor (AHR). The specific effects of AHR activation by PCDDs/Fs on an immune response are determined by the cell types involved, their activation status, and the type of antigenic stimulus.<sup>39</sup> However, for the invertebrates, few data are available on the presence of AHR-like and their TCDD dependence. Indeed, AHR-homologs have recently been shown for nematodes (*Caenorhabditis elegans*),<sup>40</sup> mollusks (*Mya arenaria*)<sup>41</sup> and arthropods (*Drosophila melanogaster*).<sup>42</sup> Concerning earthworms, some efforts to identify an AHR-homolog in *Eisenia fetida* with RT-PCR analysis were not successful.<sup>43</sup>

The filter paper exposure assay showed that earthworms exposed to higher concentrations of PCDD/Fs (C3 and C4) exhibited surface lesions; this reflected that worms were affected by dioxins either through skin and/or by feeding. Previous studies have demonstrated that earthworm skin has direct contact with the contaminated soils and is considered as a significant route to uptake of toxicants.<sup>44,45</sup> It was also suggested that these toxicants passing through the skin reach the coelomic fluid and were then transported into all the body.<sup>46</sup> In our study, the observable morphological abnormalities like coiling and mucus secretion revealed that the contact toxicity of PCDD/Fs through skin of *E. andrei*, increased with

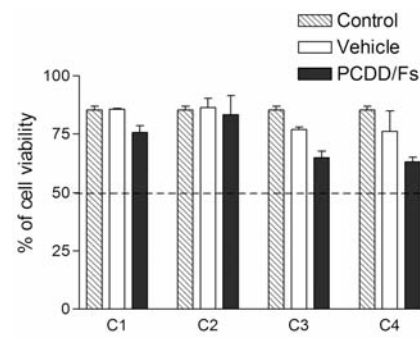
increasing concentration. Our results were consistent with the work of Chakra Reddy *et al.* on the contact toxicity of profenofos pesticide (PFF) through skin of *E. fetida*, in which, it was found that the toxicity of PFF increased with increasing concentration, and the same observations were noticed.

Coelomocyte cell yield, quantified using a light microscope, showed significant low levels at all the various PCDD/Fs concentrations tested compared to controls (Figure 4). Numerous studies have demonstrated the relationship between cell yield and worm weight and proved that cell yield is a linear function of body weight.<sup>26</sup> However, in the present experiments, the time of exposure was very short (3 h) and the weight lost was very negligible and this parameter (body weight) was not considered (data not shown). In a recent study on mammalian laboratory model, it was found that TCDD exposure of mice immunized with ovalbumin (OVA) decreased the splenocyte numbers and also decreased the production of cytokines by splenocytes at lower doses of TCDD.<sup>47</sup>

The viability of extruded coelomocytes seems to be not affected by the PCDD/Fs con-



**Figure 4.** Effects of various concentrations of polychlorinated dibenzo-*p*-dioxins/dibenzofurans (PCDDs/Fs) on extruded cell yield of *E. andrei* earthworms. Asterisks represent significant difference compared with vehicle controls (\*  $P < 0.05$ ; \*\*  $P < 0.01$ ).

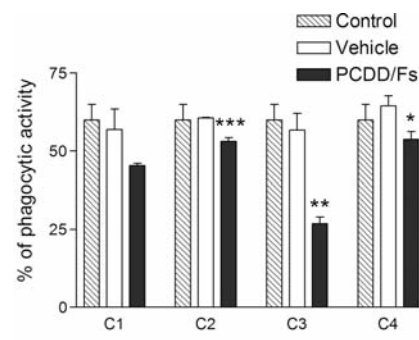


**Figure 5.** Effects of various concentrations of polychlorinated dibenzo-*p*-dioxins/dibenzofurans (PCDDs/Fs) on the viability of the extruded cells of *E. andrei* earthworms.

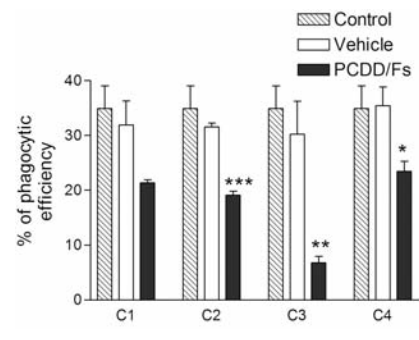
centrations (Figure 5). This indicated that the adverse effects noted were not the result of decreased cell viability.

The measurement of the phagocytic response of earthworm's coelomocytes revealed that PCDD/Fs inhibited the phagocytic activity (Figure 6) and efficiency (Figure 7) at C2, C3 and C4 containing 0.276, 0.553 and 0.829 ng 2378-TCDD/cm<sup>2</sup>, respectively. Since PCDDs/Fs and PCBs belong to persistent organic pollutants (POPs) class, our results may be consistent with two published reports on the toxicity of PCBs<sup>6,7</sup> which showed that phagocytic competence was inhibited by polychlorinated biphenyls (PCBs). A decrease in immune function may have adverse effects on the survival of organisms. Furthermore, chemical-induced immune suppression may increase susceptibility, incidence and severity of infectious diseases.<sup>23,34,35</sup>

In summary, our study showed that the effects of PCDD/Fs mixtures on phagocytosis tested *in vitro* were somewhat difficult to quantify because of hormesis and cell mortality. However, the *in vivo* effects include not only the inhibition of the phagocytosis, but also



**Figure 6.** Effects of various concentrations of polychlorinated dibenzo-*p*-dioxins/dibenzofurans (PCDDs/Fs) on the phagocytic activity of the extruded cells of *E. andrei* earthworms. Asterisks represent significant difference compared with vehicle controls (\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ).



**Figure 7.** Effects of various concentrations of polychlorinated dibenzo-*p*-dioxins/dibenzofurans (PCDDs/Fs) on the phagocytic efficiency of the extruded cells of *E. andrei*. Asterisks represent significant difference compared with vehicle controls (\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ).

exhibited significant morphological changes in the body wall which suggested that further histopathological study is necessary to develop our understanding of morphological alterations and histological responses caused by PCDD/Fs-exposure.

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