SUPPRESSION OF IMMUNE FUNCTIONS BY PCBs IN THE EARTHWORM

LUMBRICUS TERRESTRIS

DISSERTATION

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By

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This research is part of an effort to develop non-
mammalian surrogate immunoassays with the earthworm
<u>Lumbricus</u> <u>terrestris</u> to assess immunotoxic potential of
xenobiotics to mammals. The objective was to determine if
earthworm immunoassays, namely E- and S- rosette formation
and phagocytosis, are sensitive to a known mammalian
immunotoxin, the PCB Aroclor 1254. Results are presented in
terms of PCB exposure and tissue concentrations during
uptake/depuration. Using 5-d filter paper contact exposure,
$LC_{50}$ and $LD_{50}$ were 300 $\mu g/cm^2$ and 1140 $\mu g/g$ dry mass.
Nominal PCB exposure concentration of 5 $\mu g/cm^2$ and actual
tissue levels of 76 $\mu g/g$ dry mass were sufficient to
suppress S-rosette formation, a humoral response. S-rosette
suppression and return of function followed PCB levels in
coelomic leukocytes, the presumed site of toxicity.
Phagocytic response also showed suppressive effects but only
at a nominal PCB exposure level of 10 $\mu g/cm^2$, which
corresponds to an actual tissue level of 185 $\mu g/g$ dry mass.
However, PCBs had no effect on E-rosette formation with

rabbit red blood cells, a component of immunity associated with surface receptor molecules.  Compared to published mammalian data on PCB suppression of immunoglobulin production, the earthworm S-rosette immunoassay appeared to be more sensitive.

# TABLE OF CONTENTS

# LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

A non-controversial and cost effective system of
laboratory and in situ bioassays capable of integrating
variables of environmental concentration, route of exposure
and bioavailability with a broadly applicable suite of toxic
endpoints is needed to assess biological risks of
environmental pollutants from Superfund waste sites before
and after clean-up. The system would also be useful in
screening or categorizing wastes, such as industrial and
municipal solids, combustion residues from incinerated
solids and refuse-derived fuels, sewage treatment sludge and
dredged sediments for appropriate landfill disposal (i.e.
sanitary vs hazardous). An extensive literature on the basic
biology and ecology of earthworms [1-4] and from laboratory
and in situ toxicity and/or bioaccumulation studies [5-22]
supports several earthworm species as ideal organisms with
which to develop standardized protocols [13-15, 21, 23-26]
for evaluating biological risks of terrestrial pollutants,
even to mammals [21, 27]. Most toxicity studies and
protocols are for acute or $LC_{50}$ tests. Only a few have
focused on more subtle chronic or sublethal effects [15, 18,
28-35]. Of those, the giant axon conduction velocity

protocol shows promise as a reliable companion to mortality tests, especially for neurotoxins [15, 30]. However, additional tests using earthworms are needed to assess a wide range of sublethal toxic endpoints, specifically those which can be used to predict effects in mammals [26, 27], including humans.

Among potential subacute toxic endpoints that could be assayed by using earthworms, few are of greater contemporary relevance to public health than those concerning immune function. Traditionally, assessment of potential damage to the human immune system by xenobiotics involves the use of mammals (e.g. mice, rats and guinea-pigs). However, mammals are costly, require expensive maintenance and in many cases have been the target of controversy over animal cruelty. Literature in earthworm immunobiology is extensive enough [36-56] to support development of a suite of in vivo and in vitro sublethal assays for sreening chemicals, complex mixtures and materials for their immunotoxic potential in mammals.

There is an on-going research program at the University of North Texas (UNT) to develop a model system of immunoassays in the earthworms, Eisenia foetida and Lumbricus terrestris, for use with standardized filter paper contact and artificial media or soil exposure protocols (14, 24-26) in evaluating risks from environmental pollutants, and for understanding mechanisms by which xenobiotics

interfere with recognition, processing and effector phases of immune function. This program is divided into three principal phases: Phase 1, is to determine which of the specific immune parameters (or endpoints) in the earthworms are sufficiently sensitive for inclusion in an integrative assay protocol to assess immunotoxicity of xenobiotics; Phase 2, involves xenobiotics from a variety of chemical classes, and the objective is to select appropriate xenobiotics as standard or reference toxicants for each immunoassay and determine their environmental exposure concentration/dose-response profiles using standardized filter paper contact and soil exposure protocols; Phase 3, will compare actual dose-responses for reference immunotoxicants between earthworm species and laboratory mice, with the objective of determining "earthworm dose-equivalents" of the reference toxicants necessary to produce analogous immunosuppressive effects in laboratory mice.

Since most of the immune functions (Table 1) selected for model development are analogous or perhaps homologous, in the case of phagocytosis, to those in mammals, the resultant immunoassays have potential for predicting toxicity of xenobiotics to phagocytosis, and cellular and humoral immunity in mammals. Use of L. terrestris to develop a model for screening immunotoxicity of xenobiotics is supported by several factors: (1) The basic biology and immunology of L. terrestris are well known (more than 2,000

TABLE 1. Relation between immune function of
earthworms and mammals

| Immune Function | Earthworm | Mammals |
|---|---|---|
| Phagocytosis | | |
| Cells: | Coelomocytes | Macrophages<br>Neutrophils<br>Eosinophils |
| Function: | Phagocytosis<br>Inflamatory Response | |
| Common<br>  Characteristics: | Engulfment of Small Particles<br>Encapsulation of Large Particles<br>Digestion of Organic Material<br>Bactericidal / Bacteriostatic Effects | |
| Cell Mediated Immunity | | |
| Cells: | Coelomocytes | T-Lymphocytes |
| Function: | Recognition of self<br>Rejection of Non-Self<br>Immune Regulation (earthworm ?) | |
| Common Characteristics: | Xenograft Rejection<br>Allograft Rejection<br>Alloimmune Memory<br>PHA Mitogen Stimulation | |
| Humoral Immunity | | |
| Cells: | Coelomocytes | B-Lymphocytes |
| Molecules: | Antisomes:<br>Lytic/Agglutination<br>    Factors | Antibodies |
| Function: | Agglutinate Particular Antigen<br>Lyse Cells (antibody + complement) | |
| Common Characteristics: | Proteins<br>Specific Induction by Antigen<br>$1^o$ and $2^o$ Specific Response | |

publications during 1930-1980, [1-4]); (2) it is easy and
inexpensive to maintain and conduct immunological
experiments with; (3) its large size facilitates harvesting
of large numbers of coelomocytes; (4) the animals can be
obtained from a reliable source of healthy specimens taken
from a specific geographic location; (5) the earthworm is an
invertebrate, a non-controversial research organism; (6) L.
terrestris is virtually ubiquitous and an important
component of terrestrial ecosystems having potential as an
in situ bio-indicator or sentinel of harm to humans and
other animals portending from terrestrial pollution from
toxic and hazardous waste sites; (7) its immune system is
sufficiently analogous to those in mammals, including humans
for use with established mammalian immunoassays to screen
xenobiotics for potential immunotoxicity in humans [36-56];
and (8) there are no currently established protocols for use
of invertebrates for immunotoxicity, the earthworm model may
well serve this need.

Aroclor 1254, a PCB mixture, was selected for model
development in phase 1 because of its known
immunosuppressive effects in mammals [57-63]. In addition,
the choice of Aroclor 1254 directly relates to the
following: (1) PCBs are widely distributed, and Aroclor
1254 was one of the mixtures most frequently used; (2) as
are any other PCBs, it is very persistent, and thus,
frequently found in the environment; (3) methodology for

tissue extraction of PCBs is well established [64]; (4) analytical procedures involving gas chromatography/electron capture detection and/or gas chromatography/mass spectrometry techniques are equally well-established and available for research at UNT; and (5) due to its persistence and highly lipophilic character, there should be considerable uptake of Aroclor 1254 by the earthworm, and a slow rate of metabolism to allow long-term monitoring of this PCB mixture during tissue analyses.

The work presented herein is within Phase 1 of the overall program, and specifically centers on the effects of Aroclor 1254 (referred to as PCB hereon), on erythrocyte (E) and secretory (S) rosette formation, and phagocytosys by L. terrestris coelomocytes (coelomic leukocytes). Determination of the sensitivity of these immune parameters for inclusion in an integrated immunobioassay model is central to this research. Therefore, the study included exposure-uptake/depuration dynamics of PCB and dose-response profiles for each immune parameter under consideration. Specific tasks/objectives of this work were to:

(1) Determine the 5-d $LC_{50}$ and $LD_{50}$ from data obtained from an acute definitive test (preceded by a range finding test).

(2) Determine uptake/depuration curves and depuration rates for a sub-lethal exposure concentration.

(3) Determine effects of sub-lethal PCB concentrations on the immune parameters: phagocytosis, and E- and S-rosette formation.

(4) Determine effects of PCBs on mass-specific oxygen consumption of _L. terrestris_ to establish whether sublethal PCB exposures had an overall effect on the earthworm's general physiology.

(5) Combine the information above to determine the sensitivity of those earthworm immunoassays for screening xenobiotics.

## PCBs: What they are and Their Effects on Biological Systems

The term polychlorinated biphenyls (PCBs) is assigned
to a group of organic compounds which are produced by
chlorination of a biphenyl. Theoretically, 209 individual
isomers of chlorinated biphenyls can be formed, but only 100
are likely to occur in commercial products [65]. The extent
of chlorination specified by the number of chlorine
substitutions, determines which isomer is produced. Thus,
chemical nomenclature refers to dichlorobiphenyl,
trichlorobiphenyl, etc., as it corresponds to the number of
chlorine substitutions (x) present in the following general
formula for PCBs [66]:
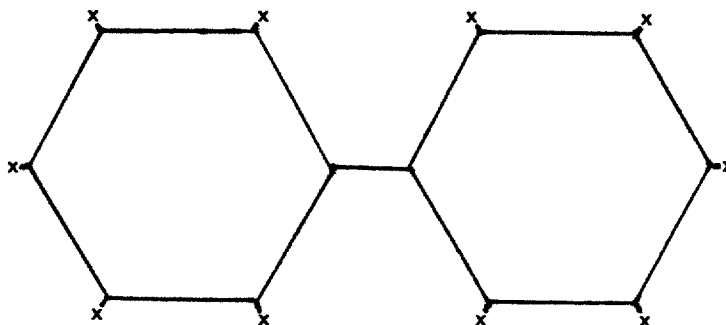


Polychlorinated byphenyls were first manufactured in
1929. Since then, 600,000 metric-tons have been used in the
United States alone, and it is estimated that worldwide
production totaled about 1,200,000 metric-tons. Of these
estimates, one-third of the worldwide production of PCBs has

been released into the global environment [67]. Because of excellent coolant/dielectric properties, their primary use was in industrial capacitors and transformers. Subsequently, use of PCBs was extended by applications as plasticizers, hydraulic fluids and lubricants, inks, adhesives, pesticide extenders, and microencapsulation of dyes for carbonless duplicating paper [68].

Despite their industrial and domestic importance, PCB production in the United States ended in 1977, when the Monsanto Industrial Chemical Co., the only major U.S. producer of PCBs, stopped their manufacture. At present, the Toxic Substances Control Act (TSCA), Public Law 94-469, prohibits production of PCBs within the United States, regulates disposal of materials contaminated by PCBs, and restricts use of any such materials already in service [69].

Potential hazards from exposure to PCBs were initially unknown by producers and users as well. In 1937, occupationally exposed workers showed toxic effects, indicating the need to impose a threshold limit at manufacturing sites. However, environmental effects of PCBs were not noticed until 36 years after their introduction into the world market when fish taken from the Baltic Sea were discovered to have high concentrations of PCBs accumulated in their tissues [69]. After, several countries began monitoring PCBs, the problem was recognized to be one of global environmental contamination [70]. Today, PCB

contamination is documented by reports that show accumulation in the food chains of many animals producing acute and chronic effects on reproduction, growth behavior and immune function [57-63, 69-77].

Immunotoxicity of PCBs has been reported in vertebrates. In general, chronic PCB exposure causes atrophy of the lymphoid tissues in most vertebrates. Vos and Koeman [72] reported decreased spleen and bursa weights in chickens. The same effects were observed in the progeny of chickens fed with 10 ppm PCBs [73]. In another study, increased suceptibility to hepatitis duck virus was reported in ducklings experimentally exposed to PCBs, although no other signs of toxicity were observed [74]. In addition, there are reports of immunotoxicity in guinea pigs, rabbits, mice, rats, and monkeys [58, 60, 75-77]. The mechanism of PCB immunotoxicity is not clear, however, some evidence suggests that the activity may be associated with the more toxic components of these mixtures (although dioxins are more toxic organic compounds than PCB, PCB isostereomers of 2,3,7,8-Tetrachloro-p-di-Benzodioxin are generally regarded as the most toxic PCB components, [78]).

In human populations, there have been two major outbreaks of poisoning by PCB-contaminated rice bran oil. More than 1600 victims were reported to be involved in the 1968 incident which occured in Japan. The Japanese outbreak was given the name "Yusho", to designate the symptoms and

disease associated with PCB-poisoning [79]. The second
incident occurred in Taiwan (1979), where more than 2000
victims were found to suffered from "Yu-cheng" (meaning "oil
disease"), the chinese name given to the Taiwanese outbreak
[62]. PCB concentrations in contaminated rice reached
2000-3000 ppm and 50-100 ppm in the Japanese and Taiwanese
samples respectively [62]. Among many other health effects,
immunosuppression occurred in the Yusho and Yu-cheng
patients chronically exposed to PCBs. Moderate decrease in
immunoglobulin concentrations were detected, especially in
IgM and IgA levels [79, 80].

Vos et al. [81] suggested three possible mechanisms by
which immune function may be altered because of exposure to
PCBs and related compounds: a) chemically induced
alterations in normal hormone levels which indirectly alter
immune function; b) chemically induced functional defects of
cells involved during immune response; c) chemically induced
alterations in numbers of responding cell types. The third
option was regarded to be the main cause in some animal
studies in which either a decrease in B-cell numbers [81] or
a decrease in T-helper cell counts [62] were involved.
Although Yu-cheng patients showed no significant effects on
B-cells, there is no conclusive evidence to disregard the
effects of PCBs on either lymphocyte cell population.
Moreover, effects of PCBs and related compounds on immune
function must be viewed as a complex one, and thus, all

three mechanisms proposed by Vos et al. [81] are likely to interact during immunesuppression.

## Immune System of Earthworms

In earthworms, the immune system is housed essentially within the coelomic cavity. Two major components are the coelomic fluid, which is functionally similar to serum, and a cellular component which is composed of a complex group of leukocytes called coelomocytes. Coelomic fluid, the humoral component of earthworms, contains proteins that can agglutinate erythrocytes of most vertebrates [49]. According to Wojdani et al. [82], hemagglutinins appear in increased amounts within 24 h after injecting erythrocytes and organic components, but not inert material (eg. saline and carbon particles). Agglutinins are inhibited by several glycoproteins and polysaccharides and the eight-carbon sugar 2-keto-3-deoxyoctonate [83]. In addition to agglutinins, lytic substances (notably in Eisenia) are also present [84]. The lytic factor is one of the five protein components which lyse sheep erythrocytes but whose activity is inhibited by 15 min heating at 56°C. The greater occurrence of lytic factors in Eisenia may be related to the inhibition of growth of some bacteria isolated from manure where it lives [49].

Coelomocytes which contribute to the cellular immune component mediate a number of responses, although a great

variety of non-immune functions have been attributed to them, such as nutrition, excretion and reactions to injuries including clotting, wound-healing and regeneration [85]. The first important role of coelomocytes is the ability to distinguish self from non-self (immune recognition), which is a fundamental attribute of most, if not all, animals and is the first step in the initiation of every immune response [86]. Immune recognition is mediated in many defense functions of earthworm coelomocyte responses that have been analyzed both in vivo and in vitro.

Coelomocyte phagocytic ("A", Appendix I) response is essential in the defense against invading microorganisms. Most of the five major coelomocyte types are capable of phagocytosis and the efficacy of this non-specific response is dependent upon interactions with the coelomic fluid. Encapsulation similar to granuloma formation in humans is mediated by coelomocytes against foreign objects too large to be phagocytized such as protozoa or nematodes [85]. When transplants are exchanged between earthworms to demonstrate histo-compatible responses, coelomocytes are known to be the major effectors in the recognition and ultimate rejection of a foreign graft [86]. By means of light and electron microscopy coelomocytes have been shown to infiltrate and to effect the rejection by mechanisms which are not yet clear [85, 86]. Rejection is progressive, occuring earliest in those muscle layers closest to the coelomic cavity. The

number of coelomocytes increases after first and second set grafting with a higher response after a second graft. Coupled with adoptive transfer data, counts of incorporated tritiated thymidine suggest a memory component as in higher forms, that is mediated by a population of memory cells that divided in response to the original transplantation antigens used in the first immunization. This is confirmed by the demonstration that coelomocytes can be stimulated by mitogens such as Con A, PHA, and LPS which could cause the incorporation of tritiated thymidine [85].

Another response, rosette formation, shows characteristics of both cellular and humoral immunity, presumably by means of agglutinins and surface receptors for the antigens on erythrocytes. There is measurable and consistent response to erythrocytes, some binding to surfaces to form with the coelomocyte a secretory rosette (S) while others bind to form erythrocyte (E) rosettes [43]. S-, E- and non-rosetting cells may be defined as follows: S-rosetting cells are those leukocytes surrounded with two or more layers of adhering erythrocytes ("B", Appendix I), E-rosetting cells are leukocytes with a single layer of at least four or more erythrocytes adhering to the cell surface ("C", Appendix I), and non-rosetting cells consist of those leukocytes not adhering to erythrocytes. E-rosettes are presumed to be formed by binding of antigen (RRBC) through leukocyte surface receptors, while S-rosettes are formed by

leukocyte secretion of agglutinating substances which produce adherance of RRBCs to coelomocytes [87]. Phagocytic, S- and E-rosette assays were used in the present work for testing the immune response against RRBCs after exposure to PCBs.

CHAPTER III

MATERIALS AND METHODS

## Source and Maintenance of Earthworms

Earthworms were purchased from Carolina Biological
Supply (Burlington, N.C.) which acquires them through a
dealer from sites near Hamburg, New York; thus obviating
geographic variation. The collection sites are rural and
distant from industries, and produce large and healthy
worms. During experimentation, control earthworms were
assayed for background levels of PCBs.

Stock earthworms were maintained in media of peat moss
reconstituted with water within plastic containers (70x40x15
cm$^3$) kept in continuous darkness at 10°C in environmental
chambers. Earthworms were acclimated for 2 wk prior to
experimentation. Commercial dry powdered baby food layered
on the surface proved to be an excellent feeding method.
Worms were checked on daily, and supplemented with food if
required. Fresh moistened peat moss was prepared upon
arrival of every new batch of worms. Unhealthy individuals
were immediately discarded. Usually, worms were all used in
experiments within 3-4 wk after arrival. Sexually mature
adults with well-developed clitella and masses of 2-6 g were
used for experiments.

## Exposure and Uptake/Depuration of PCBs

Gross Exposure of Whole Earthworms to PCBs.

Sublethal concentrations of PCBs were determined by estimating the contact exposure $LC_{50}$ and $LD_{50}$. Exposure of earthworms was performed by running a 5-d contact test on filter paper previously treated with PCBs at various nominal exposure levels. The protocol, based on methods described by Roberts and Dorough [12] for earthworms and general standardized bioassay techniques recommended by EPA [88], has been shown to be effective and reproducible with several advantages over alternatives (see section on "uptake/depuration analysis of PCB") Both range finding and definitive contact tests were conducted in 0.47 L glass jars. A 9 cm diameter filter paper (Whatman No. 1) was placed at the bottom of each jar. A constant volume of 1 ml of acetone containing the appropiate amount of PCB was spread evenly on the filter paper, and then completely evaporated. Prior to exposure, each jar was moistened with 1 ml deionized water. Twelve replicates per exposure concentration were used, with an individual earthworm per jar. The jars were sealed with a lid and ventilated every 24 h when worms were checked for survivorship. Exposure was conducted under continuous darkness at $10\pm1^{\circ}C$ throughout the 5-d exposure period. Lethality end point followed criteria described by Roberts and Dorough [12], in which individuals were regarded dead if no response was observed following

gentle touch.

## Range Finding Test

A range finding test using a wide range of concentrations with five replicates per exposure level, established an exposure range for the definitive test. Three range finding tests were performed with different concentrations and time intervals. Subsequently, a 5-d exposure with concentrations of 10.0, 100.0 and 1000.0 $\mu g/cm^2$ was found to yield reasonable results. The earthworms were washed with deionized water, weighed and placed individually in the exposure jars. Handling of worms followed the same procedures as stated before. A control group was included in the range finding test to detect effects produced by factors other than the toxicant actions.

## Definitive Contact Test

Based on results of the range finding test , five concentrations were selected as a definitive exposure range: 50.0, 100.0, 200.0, 400.0 and 800.0 $\mu g/cm^2$. A total of 12 replicates were used for each exposure level and control group. Handling of the earthworms followed the previously mentioned range-finding procedures. Each replicate jar contained only one worm. Results of this test were used to calculate a 5-d $LC_{50}$ and $LD_{50}$ values.

## Analysis of Results ($LC_{50}$/$LD_{50}$ calculations)

Results of the definitive contact test were analyzed statistically to estimate the 5-d $LC_{50}$ and $LD_{50}$ and their

corresponding 95% confidence limits. These values were
determined by an $LC_{50}$ computer program developed by Stephan
et al. [89] and currently available from EPA.  The program
estimates the $LC_{50}$ and/or $LD_{50}$ by probit, moving average and
binomial techniques . Also, probit analysis results were
confirmed with a SAS computer program [90].

Tissue Analysis For PCB.

Quantification of PCBs in worm tissues followed
procedures described by Plumb [64] for analysis of
pesticides and PCBs in biological tissues, modified for
micro-extraction. The method involves an initial solvent
(50/50 hexane/benzene) extraction followed by tissue
disruption using an ultrasonic probe.  Before extraction,
worms were oven-dried at $30^{\circ}C$, pulverized with mortar and
pestle, and transfered into test tubes for extraction.  The
tissues were free from interferences at the PCB
concentrations used in these experiments, therefore, extract
clean-up was not required.

Extract concentrations were estimated by quantification
against an internal standard (decachlorobiphenyl) added to
all preparations. A standard curve based on 5 concentrations
related the ratio of the sum of the principal PCB peak
heights to that of the internal standard as a function of ng
of PCB injected (sample chromatographs are presented in
Appendix II: "A" represents a 50 ppm Aroclor 1254 standard,
"B" represents a typically PCB exposed earthworm extract,

and "C" represents a control earthworm extract; the last
eluting peak, of approximate retention time of 34.0 min
corresponds to the internal standard).  Tissue
concentrations were expressed as μg total PCB per g dry
mass. The following were the general chromatographic
conditions:

a) Column: 30 m Glass capillary (0.75 mm I.D.,

   SPB 1, film thickness 1.0 μm).

b) Detector: Electron capture.

c) Carrier gas: 95% argon/5% methane, at 5 ml/min.

d) Detector temperature: 300°C.

e) Injector temperature: 300°C.

f) Initial oven temperature: 200°C.

g) Final oven temperature: 275°C.

h) Ramp rate: 5°C/min.

i) Initial hold time: 20 min.

j) Final hold time: 15 min.

k) Injection volume: 0.2-0.4 μL.

Efficiency of extraction was evaluated in an experiment
where known doses of PCB were directly injected into the
coelomic cavity of worms. The animals were immediately
killed, and PCBs were extracted following the above
procedures. Extraction efficiency was later evaluated by
comparing PCB recovery from the worm extracts containing the
doses injected. Two doses were administered: 500 μL
injection from a preparation of 655 ng/μL and 500 μL

injection from a preparation 360 ng/μL. Extraction efficiency was thus evaluated at two different concentrations approximating the range used for immunoassay experiments. Four individual worms were examined for each dose used (fresh mass range: 2-4 g).

Uptake/Depuration Analysis of PCB.

A critical step in examining effects of chemicals on the immune system is consistent exposure and dosing. Based on previous experiments, the contact method used here appears to be the most effective exposure technique for non-volatile, nonpolar compounds. The contact method avoids bioavailability problems associated with soil exposure, and inflamation and leakage problems associated with injection. Tissue accumulation and loss of PCB were described using standard techniques [91], which allowed estimation of the depuration rate constant and half-life (t1/2) of PCB in entire earthworms and their coelomocytes.

To determine uptake distribution between carcass and coelomic material, earthworms (n=4-5) exposed on filter paper to PCB were removed at 1, 4, 8, 16, 32, 64 and 120 h and each compartment (coelomic material and carcass) analyzed. Coelomic material was collected by inserting a sharpened Pasteur pipette into the coelomic cavity; then, PCBs were extracted as decribed previously. Loss or depuration of PCB post exposure was determined weekly for 5 months.

In addition, a small experiment was conducted to determine the total percent body fluids in a "typical" earthworm. This was done, because it became important to have a gross estimate of the mass relation between carcass and total fluids, in order to explain the relative abundance of PCBs in carcass and coelomic fluid compartments by virtue of PCBs hydrophobic nature. Thus, 51 earthworms were initially weighted and oven-dried at 30°C for 48 h, and then, their final dry mass was determined. Their initial fresh mass minus final dry mass allows to estimate the percent mass contributed by total body fluids.

Potential for bioaccumulation usually is evaluated by comparing the equilibrium concentration of a given chemical in animal tissues with a constant exposure concentration in the surrounding medium. The equilibrium or steady-state concentration is reached after an uptake or accumulation period, and may be followed by an elimination or depuration stage if the organism is returned to a pollutant-free medium. Thus, it is usually possible to calculate rates of tissue uptake, ratio of concentrations in the tissue and the environment and rate of loss from tissues. However, because of the nature of exposure, it was not possible to determine an environmental concentration (expressed here as PCB mass/ square area of filter paper) which could be meaningfully rationed to the tissue concentration (expressed as PCB mass/ tissue mass). Consequently, a bioconcentration and $k_1$ were

not estimated. Filter paper contact test essentially constitutes a bidimensional space, and thus, there is no equivalency in terms of concentration units (e.g. ppm, ppb); therefore, it is not possible to associate exposure with an actual environmental concentration. As a result, only depuration rates were calculated here using the following regression model [91]:

$$Ln(Ct) = Ln(Co) + (k_2)t$$

where $Ln(Ct)$ is the natural log of tissue PCB concentration at time t, $Ln(Co)$ is the natural log of the initial (t = 0) PCB concentration, $k_2$ the depuration rate constant and t is time in days. The PCB t1/2 may be calculated with the following formula:

$$t1/2 = Ln(0.5)/k_2$$

Persistent non-polar compounds with high n-octanol partition coefficients (Log Poct for PCBs is in the range of 5-7), such as PCBs, readily bioconcentrate. The analysis of uptake/depuration dynamics allowed a formal description of the relation between initial exposure to PCBs and resulting body burden, and subsequent correlations with immuno-competence.

## Immunoassays

Two sets of immunological experiments were conducted. The first kind are called from hereon "time series immunoassays", in which worms were analyzed at 2, 3 and 5-d

of exposure and at 3, 7, 14, 21, 28 and 35-d of depuration (after a maximum exposure of 5-d) at a fixed nominal filter paper concentration of 10.0 $\mu$g/cm$^2$. In the second experiments, called from hereon "concentration series immunoassays", worms were exposed for 5-d at different nominal exposure concentrations (2.5 $\mu$g/cm$^2$, 5.0 $\mu$g/cm$^2$, 10.0 $\mu$g/cm$^2$ and 40.0 $\mu$g/cm$^2$). A new batch of experimental and control worms (14-20 individuals) was always used for each assay period. A sub-sample of two-four experimental and control worms were individually analyzed for tissue PCB levels after each assay period. Collection of coelomic leukocytes followed the same procedures on both types of experiments, time and concentration series immunoassays.

Earthworms were exposed to PCBs by spreading 1 ml of the corresponding concentration preparation on a filter paper disk of 63.6 cm$^2$ (i.e time series immunoassays: 1 ml from a PCB solution of 636 ppm, which yielded a nominal exposure of 10.0 $\mu$g/cm$^2$). PCBs were directly applied to the filter paper disks that were already on the bottom of 0.47 L glass jars used as exposure chambers. During exposure, jars were mantained at 10$^o$C, without light in an environmental chamber. PCB was diluted in acetone to the desired concentration, evaporated and replaced with 1 ml of deionized water. During each assay period, coelomic leukocytes from experimental and control earthworms were obtained by a non-invasive protocol developed for chronic

immunotoxicity studies [92]. Essentially, collection of coelomocytes required bathing individual earthworms in 2 ml of an extrusion solution of 5% ethanol containing a mucolytic agent (10 mg/ml guaicol glyceryl ether), 2.5 mg/ml EDTA and q. s. with 0.85% saline. Ethanol stimulated them to extrude mucous which contained immunoactive cells. The mucolytic agent freed cells by dissolving the mucous. EDTA prevented clumping of cells by chelating calcium ions. Extruded cells were later mixed gently with cold 0.85% saline and centrifuged at 150 xg for 10 min. The supernatant was removed, cells washed once with and resuspended in 0.2 ml saline, then mixed with 0.8 ml _Lumbricus_ balance salt solution (LBSS) adjusted to 300 mOsm (LBSS-300). By washing leukocytes initially in saline, then re-suspending them later in LBSS, clumping and cell damage were reduced. Cell density was adjusted to $1 \times 10^6$ cell/ml by adding additional LBSS-300 at 4°C. Leukocytes from each worm were collected and processed individually for all assays.

Rabbit red blood cells (RRBC, 2% suspensions) in LBSS-300 were added as 0.1 ml aliquots to an equal volume of leukocytes in LBSS-300 at $1 \times 10^6$ cells/ml. Leukocytes and erythrocytes were mixed briefly, centrifuged at 100 x g for 5 min, then kept at 4°C for 24 h. Just before rosettes were counted, 0.1 ml of a solution of 0.1% crystal violet in LBSS-300 was added to the cell suspension to stain nuclei. Tubes were shaken gently to resuspend the pellet, and a sub-

sample was withdrawn and placed in a hemocytometer chamber. One hundred cells were counted for each sample at each time-point. Fourteen to 20 worms were used for each set of assays, with each providing cells for all time-points. Cells were scored either as S-rosettes, E-rosettes ("A" and "B", Appendix) or non-rosetting; also phagocytic coelomocytes ("C", Appendix) were identified, by the presence of ingested RRBCs, and included in the cell counts.

In addition to assaying rosette formation and phagocytosis, coelomocyte viability was determined within eight sub-samples on each assay using trypan blue stain. Viable cells were those not able to take up the dye, whereas dead cells stained blue. This procedure allowed evaluation of the effects of PCBs and extrusion media on cell viability.

### Respirometry

Mass-specific $O_2$ comsumption rate ($\dot{V}O_2$) as a function of body mass was determined with 53 earthworms (2.6-5.6 g) at 10°C with a Gilson Differential Respirometer under dark conditions (see Davis and Slater [93], for effects of light on respiration on L. terrestris). $\dot{V}O_2$ was measured on control and experimental worms. Experimental earthworms were exposed for 5-d to nominal filter paper concentrations of 10.0 μg $PCB/cm^2$. Two sets of controls were used: one directly from peat moss cultures and second, treated identically to the experimentals except for PCB exposure.

Earthworms were transfered from the 10°C environmental chambers to specially-constructed respiration vessels 2 h prior to measurements. Vessels, covered with black electrical tape to exclude light, contained several ml $H_2O$ to provide lubrication between the earthworms and glass walls, and 100% humidity.  Carbon dioxide ($CO_2$) was absorbed with 20% KOH.  Measurements were taken every 15 min between 0900 and 1300 h CST, and converted to $\mu$L/g/h STPD. Data were subjected to a one-way analysis of variance (ANOVA).

CHAPTER IV

RESULTS

## Acute Toxicity Tests

Injection of PCBs into the coelomic cavity of worms with extract concentrations of 655 and 360 ppm yielded mean % recoveries of 144 and 107% respectively (Table 2). Thus,

TABLE 2. Efficiency of the hexane/benzene extraction method used for obtaining the dose of PCB present in Lumbricus terrestris

| Sample Description | Concentration (mg/L) | % Efficiency |
|---|---|---|
| Original Extract | 654.9 | -- |
| Worm 1 | 779.8 | 119.07 |
| Worm 2 | 1101.2 | 168.15 |
| Worm 3 | 860.6 | 131.41 |
| Worm 4 | 1023.8 | 156.33 |
| $\bar{x}$ | 941.4 | 143.74 |
| sd | 147.2 | 22.47 |
| Original Extract | 360.4 | -- |
| Worm 5 | 418.0 | 115.98 |
| Worm 6 | 216.7 | 60.13 |
| Worm 7 | 505.0 | 140.12 |
| Worm 8 | 410.3 | 113.85 |
| $\bar{x}$ | 387.5 | 107.52 |
| sd | 121.7 | 33.76 |

an overall mean % extraction efficiency of 125.6% was
obtained. Analyses of compounds involving multiple peaks
such as PCBs (see chromatographs of Appendix II), usually
yield deviations from 100% recovery as high as those
presented here. Deviations from the original extract
concentrations could have been produced from inaccuracies at
the moment of injection (e.g. inaccurate injection volumes,
spilling caused by worm movement, differences in injection
pressure). PCB concentrations in control earthworms were
found to be below the method detection limits (ca. 0.5 $\mu$g/g
erthworm fresh mass).

Acute toxicity of PCB to earthworms was evidenced by a
well defined dose-response curve (Figs. 1 and 2) within a
range of nominal concentrations of 50-800 $\mu$g/cm$^2$ (body
burden concentrations of 780-1426 $\mu$g/g dry mass). Control
body burden PCB concentrations were found to be below the
method detection limits throughout all experiments. Probit
analyses applied to the data in Table 3 yielded a 5-d $LC_{50}$
of 300 $\mu$g/cm$^2$ (95% confidence interval: 180-590) and an
$LD_{50}$ of 1139 $\mu$g/g dry mass (C.I.: 1000-1368). These values
allowed the establishment of a range of sublethal
concentrations (Table 3) for use in immunoassay experiments.
The lowest nominal exposure concentration used in
immunoassays was 2.5 $\mu$g/cm$^2$ (body burden: 56 $\mu$g/g dry mass),
and the highest was 40 $\mu$g/cm$^2$ (221 ug/g dry mass). Thus,
immunoassay experiments were conducted using exposure levels

Figure 1.   Relation between mortality and nominal PCB exposure concentration showing the $LC_{50}$ for Lumbricus terrestris as predicted by probit analysis.
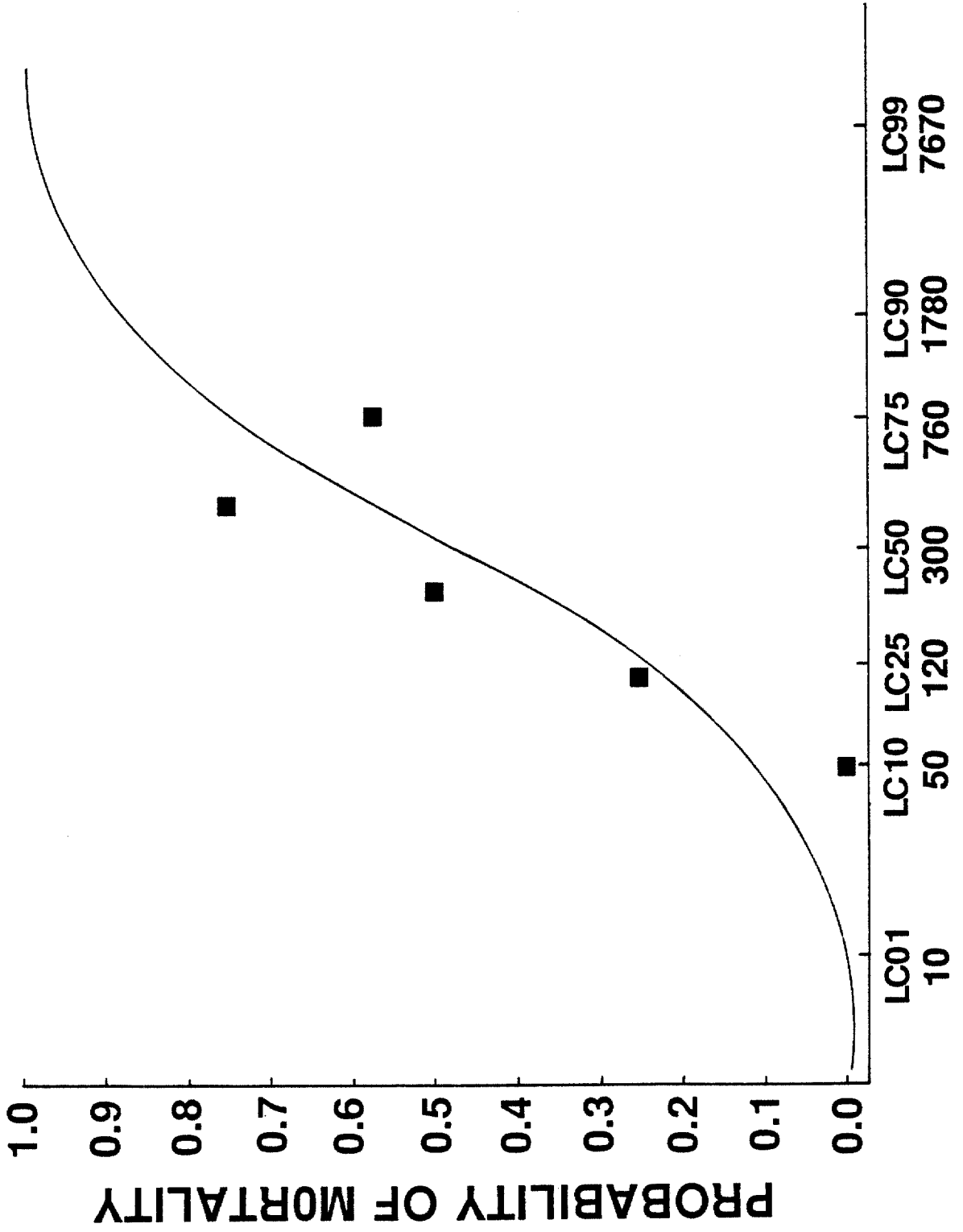
Figure 2. Relation between mortality and PCBs body concentration showing the $LD_{50}$ for $\underline{L}$. $\underline{terrestris}$ as predicted by probit analysis.
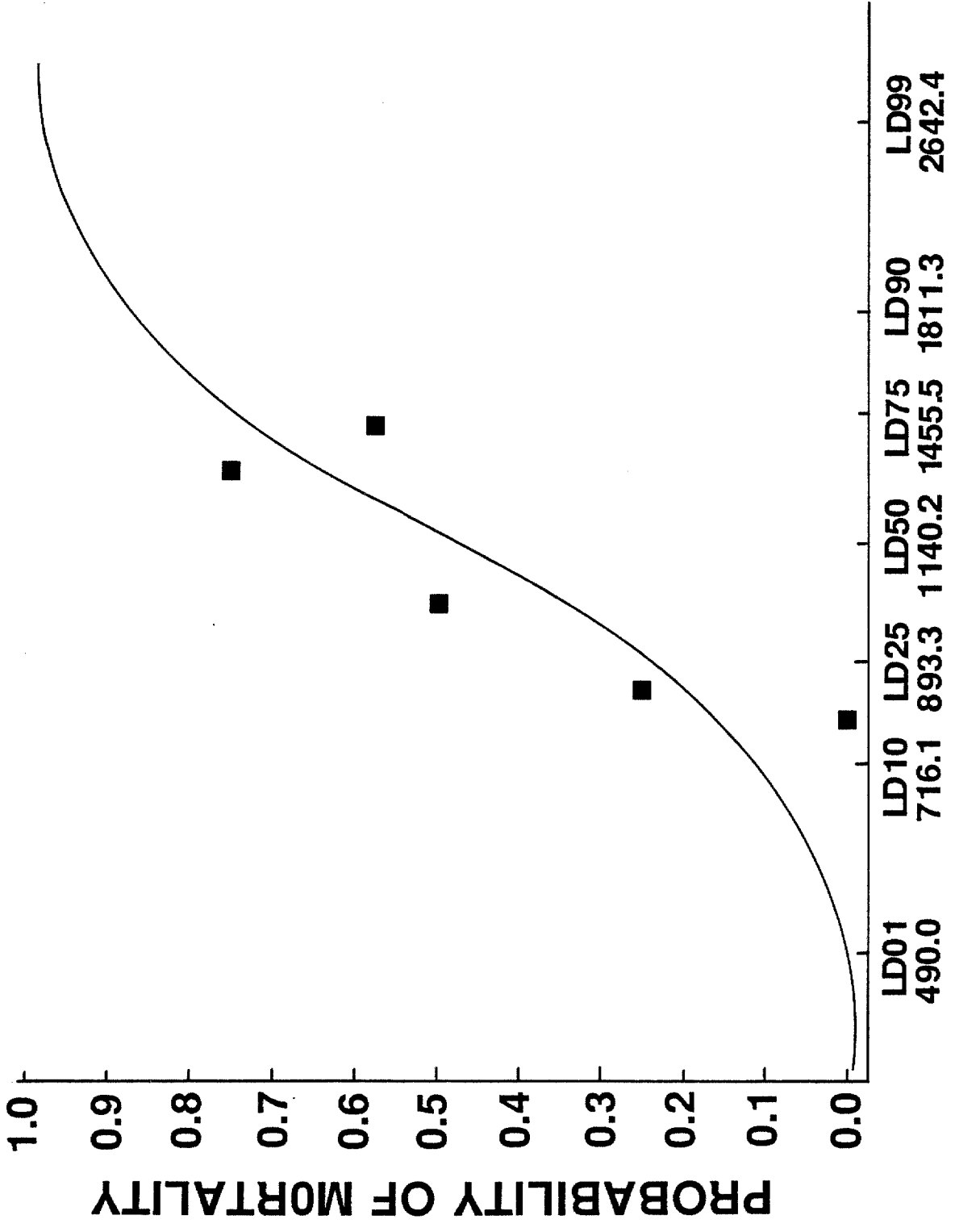
which yielded body burden concentrations 50% the $LD_1$ levels during a maximum 5 d exposure period. Use of those concentrations for immunoassays allowed a 100% survival throughout the experiments. The overall health of experimental worms was comparable to controls at the end of exposure based on gross behavioral observations.

TABLE 3. Nominal filter paper exposure and entire body concentrations in L. terrestris exposed to PCBs for 5-d, and respective mortalities.

| Nominal Exposure Concentration ($\mu g/cm^2$) | Body Burden Concentration $\bar{x} \pm sd$ ($\mu g/g$ dry mass)[1] | Number of Worms | |
| --- | --- | --- | --- |
| | | Exposed | Surviving |
| 2.5 | 56.1 ± 9.9 | 15 | Sublethal |
| 5.0 | 76.5 ± 4.7 | 18 | 100% |
| 10.0 | 184.9 ± 66.4 | 26 | survivors |
| 40.0 | 221.0 ± 51.3 | 18 | |
| 50.0 | 779.6 ± 218.7 | 12 | 12 |
| 100.0 | 834.2 ± 337.3 | 12 | 9 |
| 200.0 | 982.4 ± 414.2 | 12 | 6 |
| 400.0 | 1311.0 ± 436.5 | 12 | 3 |
| 800.0 | 1425.9 ± 467.1 | 12 | 5 |

[1] For conversion to fresh mass multiply by 0.22

In addition, there was a highly significant correlation ($r = 0.67$, $p < 0.0001$) between log-transformed nominal filter paper and entire-body PCB concentrations in earthworms exposed for 5-d (Table 3). Exposure and body concentrations at and below 50.0 $\mu g/cm^2$ and 779.6 $\mu g/g$ dry

mass, respectively produced no mortality.

## Uptake/Depuration Experiments

Mean PCB concentration in the carcass compartment of
L. terrestris was estimated to be 182 µg/g dry mass at the
maximum exposure time of 5-d to a filter paper having a
nominal concentration of 10 µg/cm² (Table 4; Fig. 3).

TABLE 4. PCB carcass concentration in L. terrestris exposed
to a nominal concentration of 10 µg/cm²

| Time | Treatment | x̄ ± sd (µg/g dry mass) | N | Range |
|---|---|---|---|---|
| 1h | Uptake | 18.78 ± 7.33 | 4 | 12.3-26.9 |
| 4 | | 17.68 ± 3.11 | 4 | 14.2-21.7 |
| 8 | | 34.16 ± 10.22 | 4 | 27.9-49.4 |
| 16 | | 75.20 ± 8.74 | 4 | 63.9-85.2 |
| 32 | | 82.05 ± 13.92 | 3 | 66.2-92.4 |
| 64 | | 147.44 ± 36.94 | 5 | 101.6-186.9 |
| 120 | | 182.84 ± 35.19 | 5 | 152.4-223.6 |
| 3d | Depuration | 134.69 ± 47.45 | 5 | 91.8-193.8 |
| 7 | | 148.89 ± 60.28 | 5 | 95.4-243.5 |
| 14 | | 152.56 ± 32.98 | 5 | 104.5-194.5 |
| 21 | | 93.75 ± 39.17 | 5 | 57.3-157.7 |
| 28 | | 77.44 ± 21.07 | 5 | 43.2-93.2 |
| 35 | | 78.36 ± 21.62 | 5 | 54.7-104.4 |
| 49 | | 61.85 ± 26.09 | 5 | 33.0-93.1 |
| 63 | | 30.50 ± 15.08 | 5 | 12.2-50.1 |
| 91 | | 15.12 ± 8.00 | 5 | 5.0-25.4 |
| 119 | | 5.23 ± 2.13 | 5 | 3.5-8.9 |
| 147 | | 6.24 ± 2.05 | 4 | 4.0-8.9 |

During depuration, elimination of PCBs was slow for the
first 2 wk, with about 75% of the maximum PCB levels still
remaining in this compartment. However, begining at 3 wk of
depuration, a slow but steady decrease occurred, reaching

Figure 3.    Mean PCB concentration in carcass compartment of
earthworms L. terrestris during uptake and depuration
phases. Indicated is the mean (horizontal line), wide
rectangle is ± standard error, and vertical lines ± 95%
confidence interval.    Sample sizes are given above the
symbols.
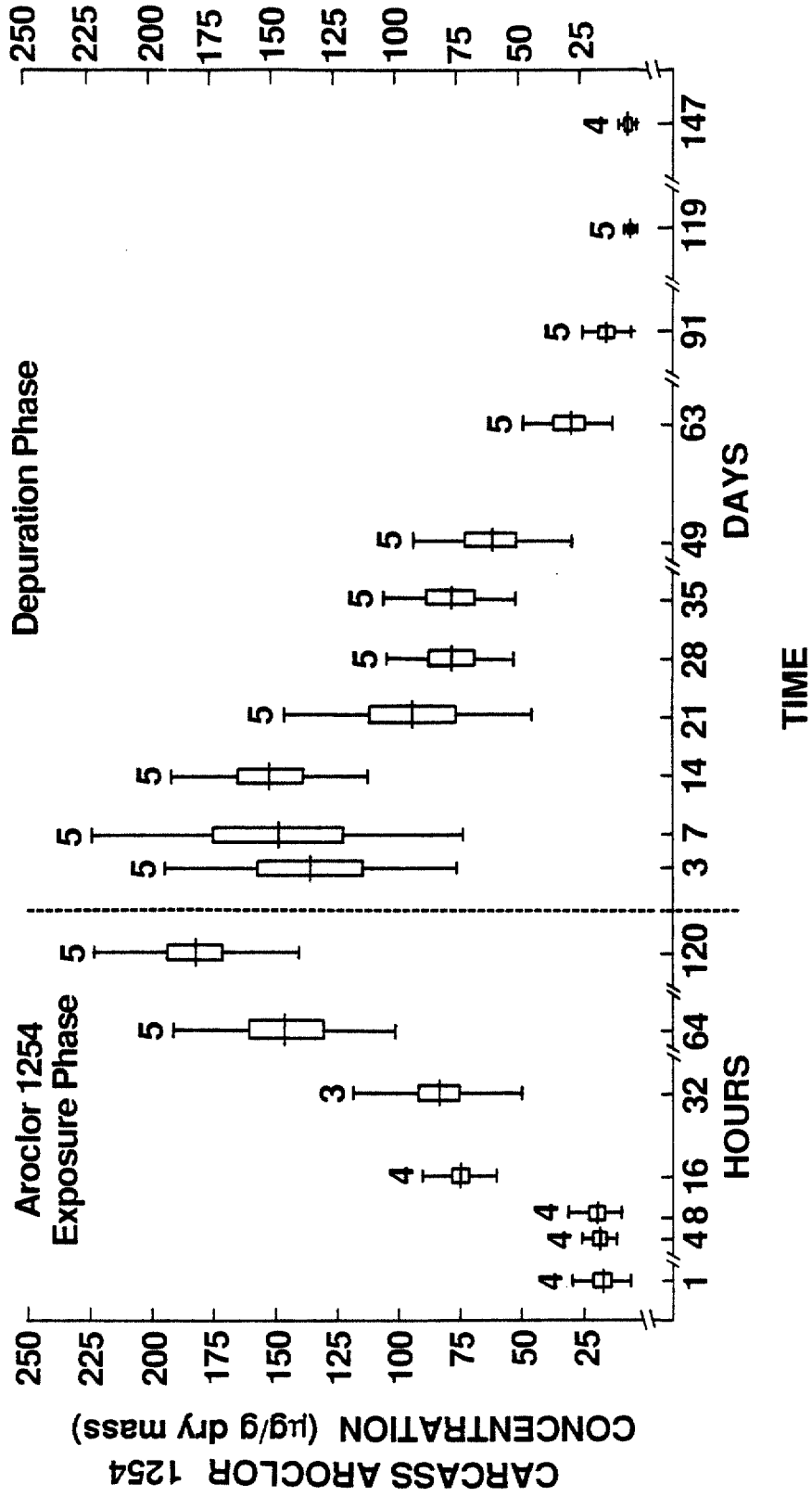
mean levels of about 2 $\mu$g/g dry mass after 21 wk of depuration; that is, only about 1% of the compound still remained in this compartment at the end of the experiment.

The following regression equation (Fig. 4) describes the depuration data in Table 4, which allowed the calculation of a carcass depuration rate constant:

$$Ln(Dose) = 5.117 + (-0.026)t$$

where Ln(Dose) refers to the natural log of the carcass concentration, and t refers to time of depuration. The regression model has an $r^2$ of 0.8854, which indicates that a significant amount (89%) of the variability is explained by the regression model. The slope of the regression curve (Figure 4) corresponds to the depuration rate constant [91] "$k_2$" ($k_2$= -0.026/day). The PCB t1/2 for the carcass compartment was estimated as 26.7 days.

PCB concentrations in coelomic fluid (Table 5 and Figure 5) were below the method detection limits at 1-32h of the uptake phase, but reached a mean level of 115.93 $\mu$g/g dry mass at 64 h. This indicates that uptake was rapid between 32 and 64 h. As with carcass, maximal uptake occurred at 5-d (182.92 $\mu$g/g dry mass). Elimination was slow, reaching 67.55 $\mu$g/g at 9 wk, and then dropping below detection limits.

As with carcass, the depuration phase of coelomic fluid was also analyzed by a regression model (Fig. 6), which yielded the following:

Figure 4. Relation between carcass concentration of PCBs in
L. terrestris and depuration time.

$Ln(C_T) = Ln(C_0) + (k_2)$ Time
$Ln(C_T) = 5.117 + (-0.026)$ Time

LEGEND: ■ = 1 OBS
● = 2 OBS
□ = 3 OBS

LNPCB

Ln Aroclor 1254 Carcass Concentration
(μg/g dry mass)

DEPURATION PHASE (DAYS)
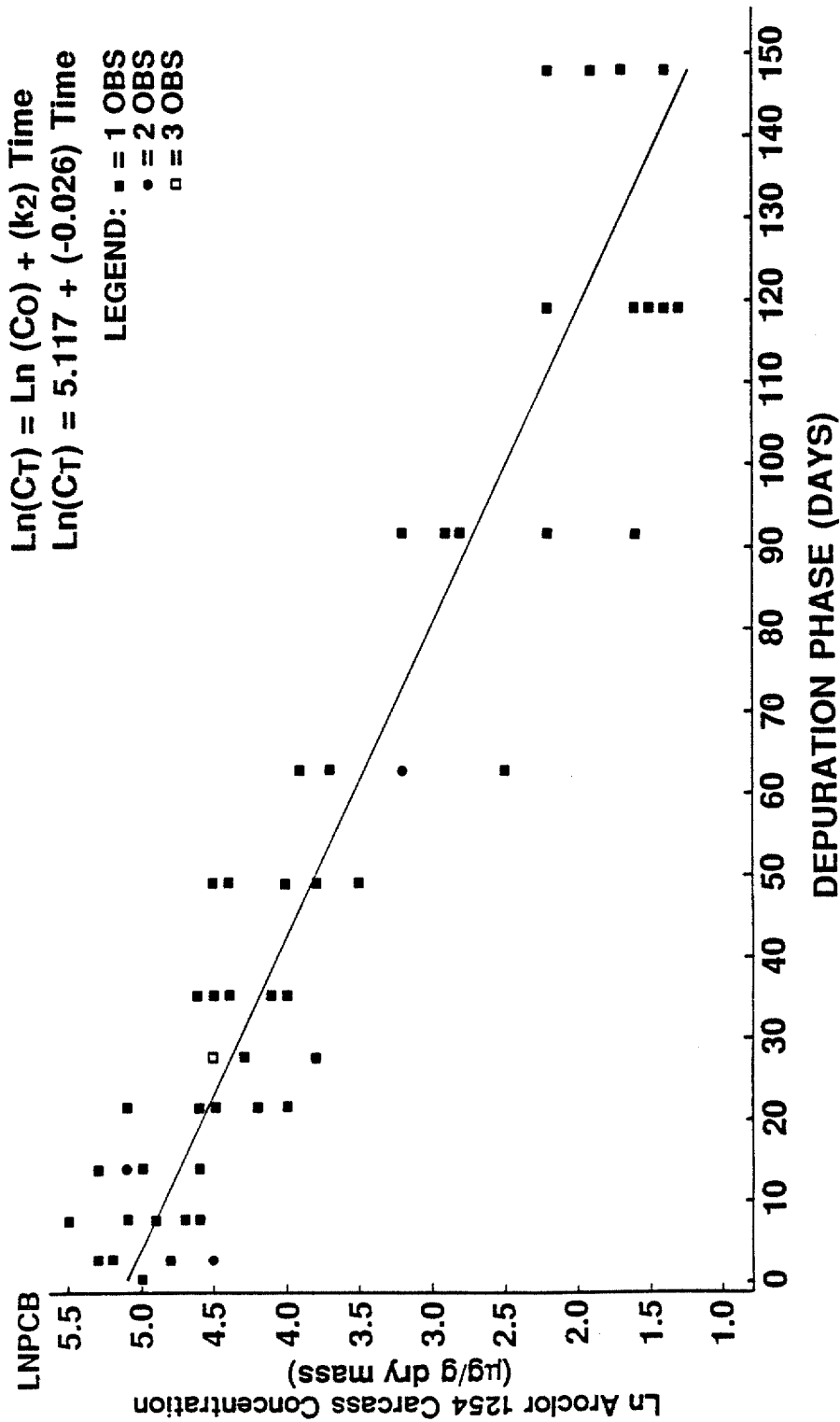
TABLE 5. PCB concentration in coelomic material of
L. terrestris exposed to a nominal
concentration of 10 $\mu g/cm^2$

| Time | Treatment | $\bar{x} \pm sd$ (µg/g dry mass) | N | Range |
|------|-----------|------------------|---|-------|
| 32h | Uptake | below detection limits[1] | | |
| 64 | | 115.93 ± 27.52 | 4 | 89.4-154.5 |
| 120 | | 182.92 ± 105.35 | 11 | 83.0-402.7 |
| 3d | Depuration | 174.59 ± 46.78 | 5 | 112.3-229.0 |
| 7 | | 171.50 ± 40.27 | 4 | 147.5-231.4 |
| 14 | | 97.33 ± 45.36 | 5 | 25.7-137.8 |
| 21 | | 59.54 ± 34.05 | 3 | 34.1-74.9 |
| 28 | | 67.80 ± 10.07 | 4 | 62.3-82.8 |
| 49 | | 105.43 ± 64.09 | 4 | 50.4-197.1 |
| 63 | | 67.55 ± 41.90 | 4 | 5.0-92.6 |
| 91 | | below detection limits[1] | | |

[1] detection limits = 2 µg/g earthworm dry mass

$$Ln(Dose) = 5.003 + (-0.019)t$$

the low $r^2$ for this model (0.2819) was due in part to the

high variability of the individual estimates (Table 5). The

estimated depuration rate constant ($k_2$) for coelomic fluid

was -0.019/d with a corresponding t1/2 of 36.5-d.

Results of an experiment to determine the partitioning

of PCBs between coelomocytes and the fluid portion of the

coelomic compartment are shown in Table 6 (a 5-d exposure

and a nominal concentration of 10.0 µg/cm² were mantained).

This experiment showed PCB levels below detection limits in

the fluid portion of the coelomic compartment, while

coelomocyte PCB concentration reached a mean of 156.4 µg/g

dry mass after 5-d. In addition, an experiment conducted to

Figure 5.   Mean PCB concentration in coelomic material
(fluid + cells) of L. terrestris during uptake and
depuration phases. Symbols same as Figure 3.
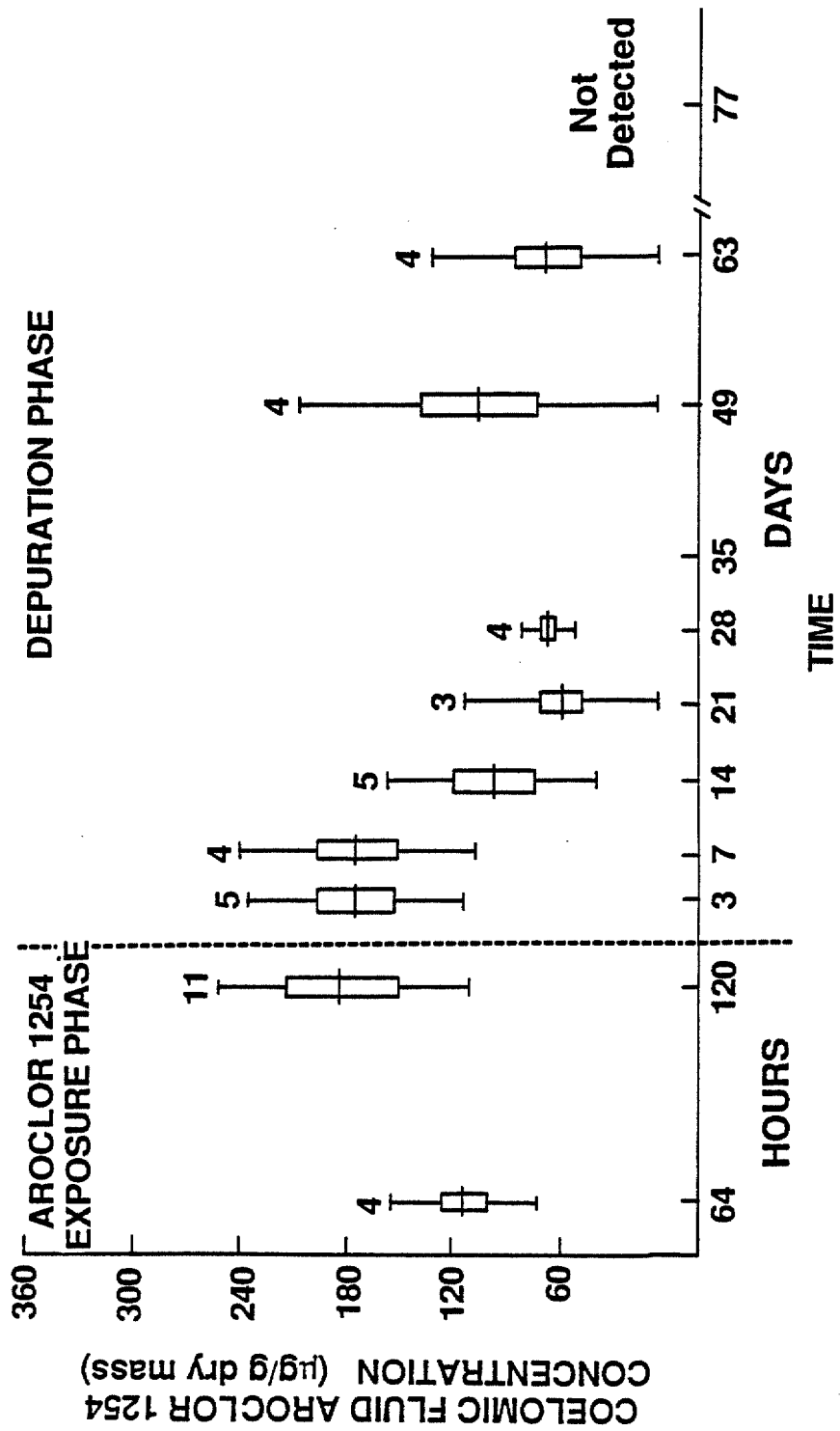
Figure 6.    Relation between coelomic material
concentration of PCBs in L. terrestris and depuration time.

$$Ln(C_T) = Ln(C_0) + (k_2) \text{ Time}$$

$$Ln(C_T) = 5.003 + (-0.019) \text{ Time}$$

LEGEND: ■ = 1 OBS
● = 2 OBS

LNPCB

Ln Aroclor 1254 Coelomic Fluid Concentration
(µg/g dry mass)

DEPURATION PHASE (DAYS)
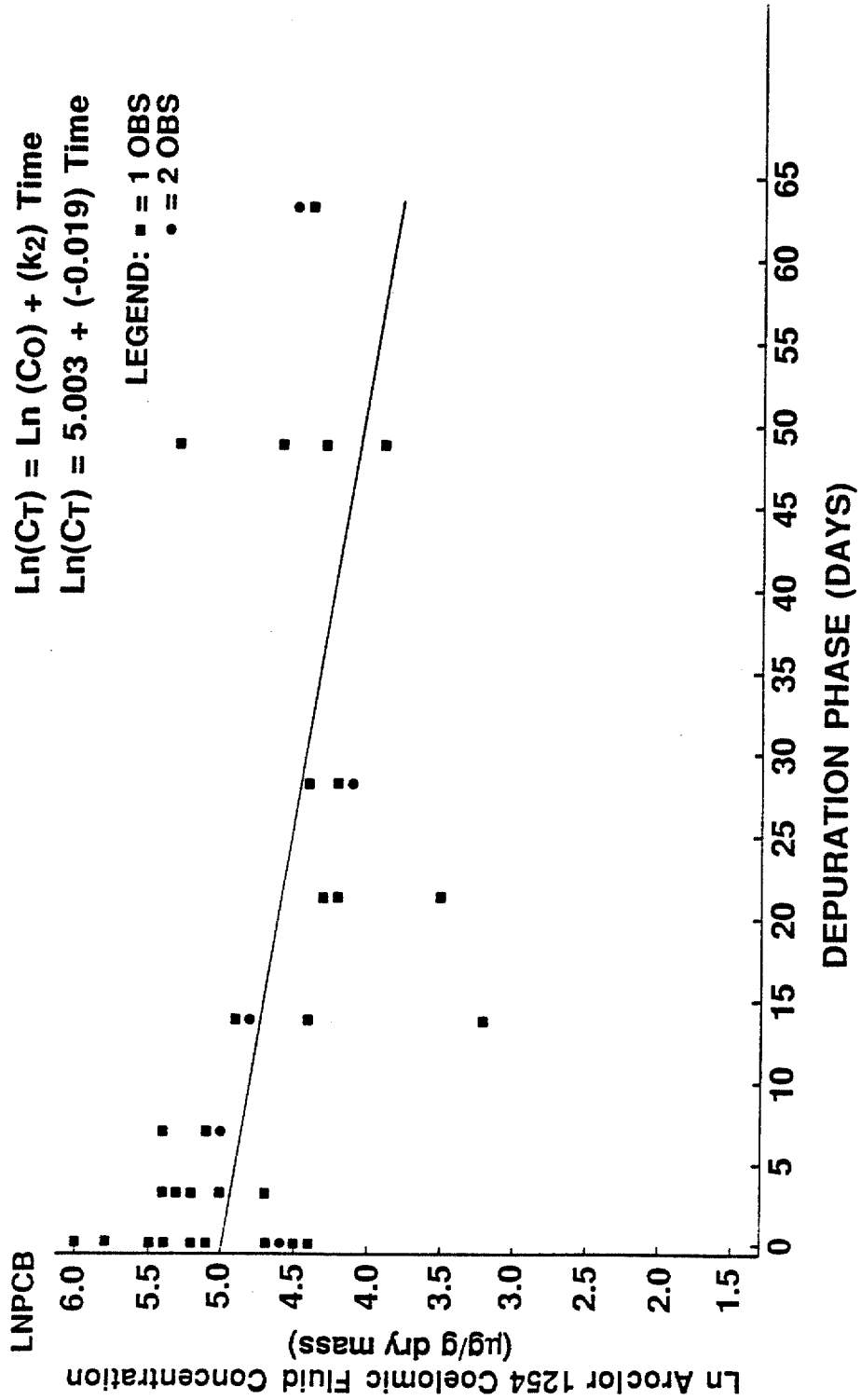
analyse the mass relations between carcass and total fluids
shows that an estimated mean of 80% (N=51; sd=0.62) of
earthworm fresh mass is contributed by total body fluids;
this, and PCBs known hydrophobic charater helps to explain
the slow initial uptake of PCBs in the coelomic compartment.

TABLE 6. Compartmentalization of PCB among coelomocytes,
coelomic fluid and carcass in L. terrestris

| Compartment | $\bar{x} \pm sd$ ($\mu$g/g dry mass) | N | Range |
|---|---|---|---|
| Coelomic Cells | 156.4 ± 70.4 | 6 | 91.9-248.9 |
| Coelomic Fluid | Not detected[1] | 6 | -- |
| Carcass | 139.6 ± 54.0 | 6 | 89.7-230.8 |

[1]Detection limit = 2 $\mu$g/g dry mass

Combining the PCB concentrations of coelomic fluid and
carcass compartments allowed estimation of whole body-burden
of PCBs (Table 7; Fig. 7). Because of smaller amounts of
PCBs in the coelomic compartment, whole body PCBs followed
an uptake/depuration pattern similar to that of carcass
(Fig. 7). In addition, the regression equation for the
depuration phase of whole body compartment (Fig. 8) yields a
high $r^2$ of 0.8865, despite of the high variability of
coelomic fluid estimates. The corresponding regression
equation was:

Figure 7.    Body-Burden PCB concentration in earthworms L.
terrestris during uptake and depuration phases. Symbols same
as Figure 3.
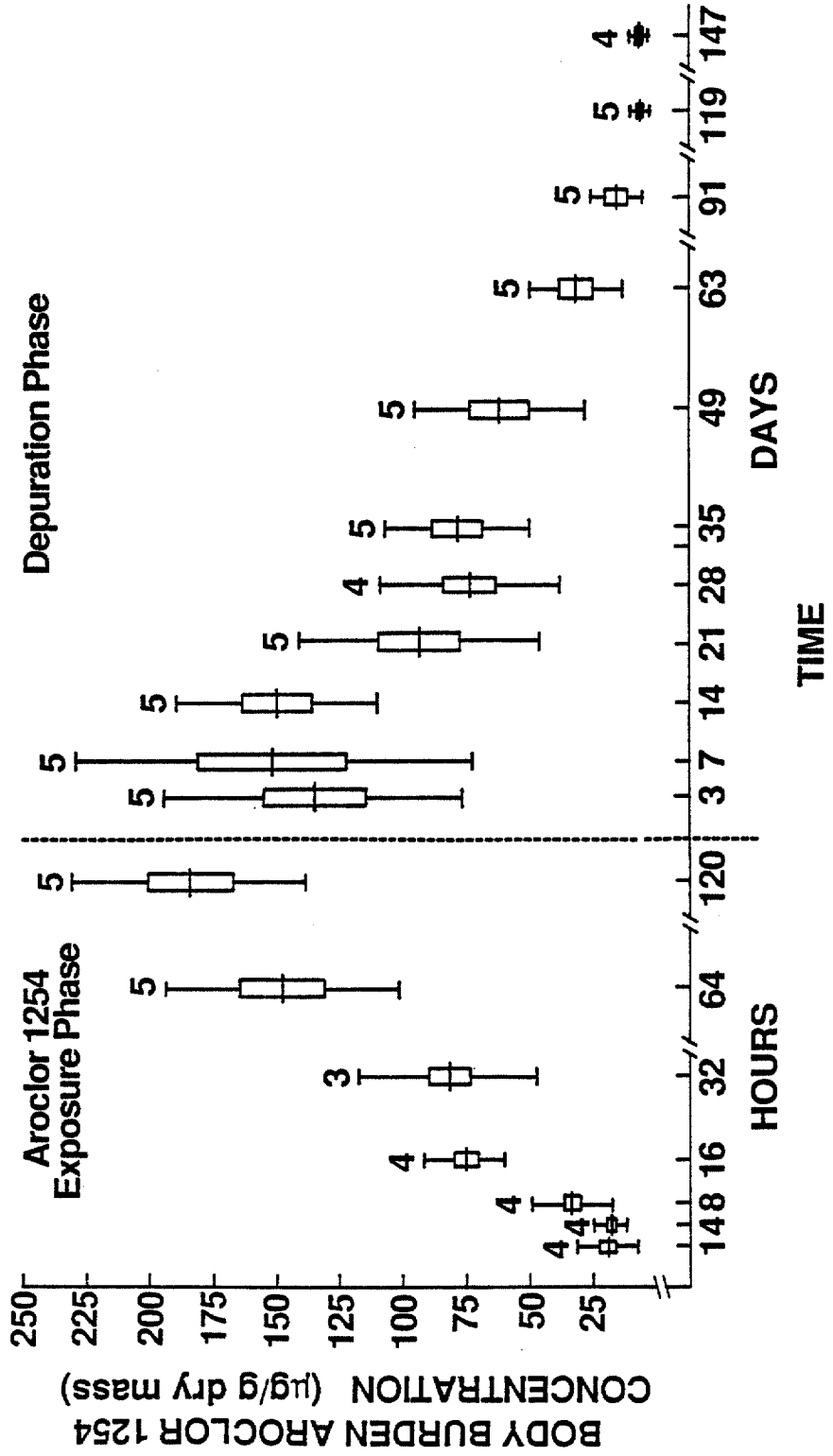
Figure 8. Relation between body-burden PCBs in earthworms L. terrestris and depuration time.

$Ln(C_T) = Ln(C_0) + (k_2)$ Time

$Ln(C_T) = 5.125 + (-0.026)$ Time

LEGEND: ■ = 1 OBS
● = 2 OBS

DEPURATION PHASE (DAYS)

LNPCB

Ln Aroclor 1254 Body Burden Concentration
(μg/g dry mass)
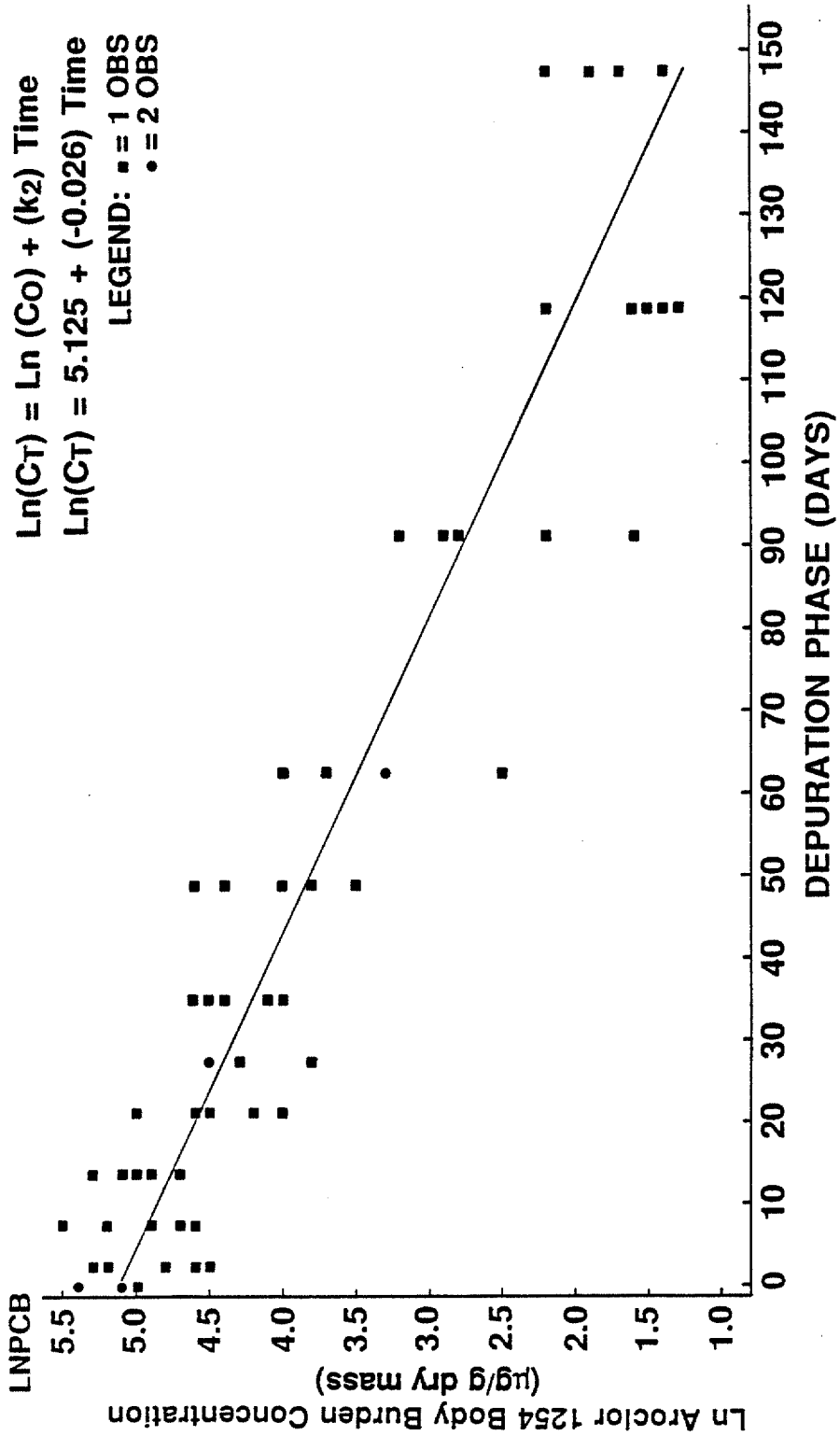
$$Ln(Dose) = 5.12 + (-0.026)t$$

which indicates an equal depuration rate constant and t1/2

as that of carcass ($k_2 = 0.026$/day; t1/2 = 26.7).

TABLE 7. Whole body-burden concentration of PCBs in
L. terrestris exposed to a nominal
concentration of 10 $\mu$g/cm$^2$

| Time | Phase | $\bar{x} \pm$ sd ($\mu$g/g dry mass) | N | Range |
|------|-------|-----------------|---|-------|
| 1h | Uptake | 18.78 ± 7.33 | 4 | 12.3-26.9 |
| 4 | | 17.68 ± 3.11 | 4 | 14.2-21.7 |
| 8 | | 34.16 ± 10.22 | 4 | 27.9-49.4 |
| 16 | | 75.20 ± 8.74 | 4 | 63.9-85.2 |
| 32 | | 82.05 ± 13.92 | 3 | 66.2-92.4 |
| 64 | | 147.96 ± 36.92 | 5 | 104.2-187.8 |
| 120 | | 184.65 ± 36.89 | 5 | 152.7-227.8 |
| 3d | Depuration | 136.18 ± 46.92 | 5 | 94.5-195.8 |
| 7 | | 152.54 ± 63.75 | 5 | 97.0-254.0 |
| 14 | | 150.42 ± 32.12 | 5 | 105.0-192.5 |
| 21 | | 94.20 ± 38.83 | 5 | 56.5-156.0 |
| 28 | | 75.00 ± 22.93 | 5 | 43.9-92.4 |
| 35 | | 78.36 ± 21.62 | 5 | 54.7-104.4 |
| 49 | | 62.85 ± 27.07 | 5 | 34.2-97.0 |
| 63 | | 31.70 ± 14.91 | 5 | 12.5-52.1 |
| 91 | | 15.12 ± 8.00 | 5 | 5.0-25.4 |
| 119 | | 5.23 ± 2.13 | 5 | 3.5-8.9 |
| 147 | | 6.24 ± 2.05 | 4 | 4.0-8.9 |

## Chronic Immunotoxicity Tests

### Cell Counts and Percent Viability

Throughout the immunoassay experiments, cell counts and

percent viability assessments were made to assure

consistency between the number of viable coelomocytes and

rabbit red blood cells (RRBCs) used as antigen. Percent

viability of coelomocytes, as indicated by the percent live

cells, was determined as a precaution for detection of

possible cell damage in the extrusion media and/or

differential reaction of controls and experimentals to the

media (Table 8). Adjustment of coelomocyte numbers to

TABLE 8. Total cell count obtained during immunoassay
time and concentration series experiments

### Immunoassay Time Series Experiments

| | | G r o u p | |
|---|---|---|---|
| Time (days) | Phase | Control ($\bar{x} \pm$ sd x $10^4$/ml) | Experimental ($\bar{x} \pm$ sd x $10^4$/ml) |
| 2 | Uptake | 60.31 ± 34.83 | 67.59 ± 39.38 |
| 3 | | 57.81 ± 48.03 | 62.50 ± 22.54 |
| 5 | | 81.98 ± 36.86 | 52.13 ± 37.20 |
| 3 | Depuration | 52.25 ± 24.82 | 57.22 ± 20.65 |
| 7 | | 95.69 ± 45.53 | 106.06 ± 61.43 |

### Concentration Series Experiments

| | G r o u p | |
|---|---|---|
| Nominal Conc ($\mu$g/cm$^2$) | Control ($\bar{x} \pm$ sd x $10^4$/ml) | Experimental ($\bar{x} \pm$ sd x $10^4$/ml) |
| 2.5 | 74.56 ± 37.55 | 43.53 ± 22.03 |
| 5.0 | 95.03 ± 47.34 | 64.66 ± 26.97 |
| 10.0 | 81.98 ± 36.86 | 52.13 ± 37.20 |
| 40.0 | 62.25 ± 54.37 | 72.00 ± 52.56 |

reasonably similar counts for controls and experimentals

(Table 9) was also considered. Although no attempt was made

to test the effect of increasing coelomocyte density on

rosette or phagocytosing leukocyte counts, I believe it is
crucial to maintain a coelomocyte density within a similar
range if comparisons between and within treatments are
desired.

TABLE 9. Total viability as represented by percent live
coelomocytes during time and concentration
series experiments

Immunoassay Time Series Experiments

|  |  | G r o u p | |
|  |  | Control | Experimental |
| Time (days) | Phase | ($\bar{x} \pm$ sd) | ($\bar{x} \pm$ sd) |
| 2 | Uptake | 91.56 ± 6.11 | 91.12 ± 5.43 |
| 3 |  | 89.02 ± 4.95 | 90.68 ± 3.54 |
| 5 |  | 94.06 ± 1.89 | 92.78 ± 4.09 |
| 3 | Depuration | 88.81 ± 3.78 | 93.07 ± 4.77 |
| 7 |  | 91.37 ± 5.18 | 92.39 ± 5.09 |

Concentration Series Experiments

| Nominal Conc ($\mu$g/cm$^2$) | Control (x ± sd) | Experimental (x ± sd) |
| 2.5 | 2.71 ± 2.14 | 3.91 ± 1.84 |
| 5.0 | 2.27 ± 1.77 | 2.28 ± 1.81 |
| 10.0 | 5.94 ± 1.89 | 7.22 ± 4.09 |
| 40.0 | 6.44 ± 2.56 | 7.18 ± 4.09 |

The overall cell counts ($\bar{x} \pm$ sd x 10$^4$/ml) during
immunoassay time-series experiments (Table 8) for controls

and experimentals were 69.6± 38.0 x $10^4$/ml and 69.1 ± 36.24 x $10^4$/ml respectively. For the concentration series experiments (Table 8), the overall cell counts ($\bar{x}$ ± sd x $10^4$/ml) were 78.5 ± 44.03 x $10^4$/ml and 53.2 ± 34.7 x $10^4$/ml for controls and experimentals respectively. Data in Table 8 were further examined by Nested Analyses of Variance (Nested ANOVA),which showed no significant differences between control and experimental mean cell counts in both time series (F= 0.58, p= 0.7169) and concentration series experiments (F= 1.72, p= 0.1575).

The viability of coelomocytes as indicated by the overall mean percent live cells (Table 9) during time series experiments for controls and experimentals were 91 and 92 % respectively. Nested ANOVA showed no significant differences in mean cell mortality between control and experimental groups within these experiments (F= 0.89, p= 0.4930).

Viability tests for the concentration series experiments (Table 9) resulted in an overall mean percent coelomocyte viability of 96 and 95 % for control and experimental groups respectively. Results of nested ANOVA showed no significant differences in mean cell mortality between controls and experimentals for these experiments (F=0.55, p= 0.6999).

<div align="center">Immunoassay Results</div>

E-Rosette Immunoassay

Occurrence of E-rosettes (number of E-rosettes per 100

randomly counted coelomocytes) in control and experimental

(10 $\mu$g/cm$^2$ nominal exposure) earthworms during a sequential

uptake or exposure phase, lasting 5-d, and a depuration or

elimination phase of up to 35-d (time series immunoassay

experiments) yielded overall means of 15.7% (sd= 1.98) and

14.3% (sd= 1.10) for controls and experimentals

respectively (Table 10). Though E-rosettes were slightly

lower in experimentals than controls (Fig. 9), nested ANOVA

showed no significant differences in mean E-rosettes between

controls and experimentals (F= 0.90, p= 0.5282).

TABLE 10. Descriptive statistics of E-rosette formation
in control and experimental worms during
time series immunoassay experiments

| Time (Days) | Treatment | Control ($\bar{x} \pm$ sd) | N | Experimental ($\bar{x} \pm$ sd) | N |
|---|---|---|---|---|---|
| 2 | Uptake | 14.50 ± 5.91 | 16 | 14.47 ± 5.93 | 15 |
| 3 | | 13.00 ± 5.15 | 13 | 13.38 ± 3.73 | 13 |
| 5 | | 16.41 ± 4.85 | 29 | 14.38 ± 5.38 | 26 |
| 3 | Depuration | 15.07 ± 5.13 | 15 | 13.75 ± 4.29 | 12 |
| 7 | | 13.82 ± 4.94 | 28 | 12.85 ± 4.84 | 26 |
| 14 | | 14.40 ± 6.06 | 15 | 13.67 ± 6.84 | 15 |
| 21 | | 16.75 ± 4.75 | 12 | 14.07 ± 5.43 | 14 |
| 28 | | 18.46 ± 4.75 | 13 | 15.60 ± 4.53 | 15 |
| 35 | | 18.50 ± 5.95 | 12 | 16.38 ± 5.03 | 16 |

Similar results (Table 11; Fig. 10) were found during

concentration series experiments in which slightly lower

Figure 9.   Comparison of E-rosettes produced by coelomocytes from experimental (open symbols) L. terrestris exposed to PCBs and controls (shaded symbols) during time series experiments. Mean is horizontal line, wide rectangle is ± standard error, norrow rectangle is ± 95% confidence interval and vertical line is the range. Sample sizes are given above and below the symbols.
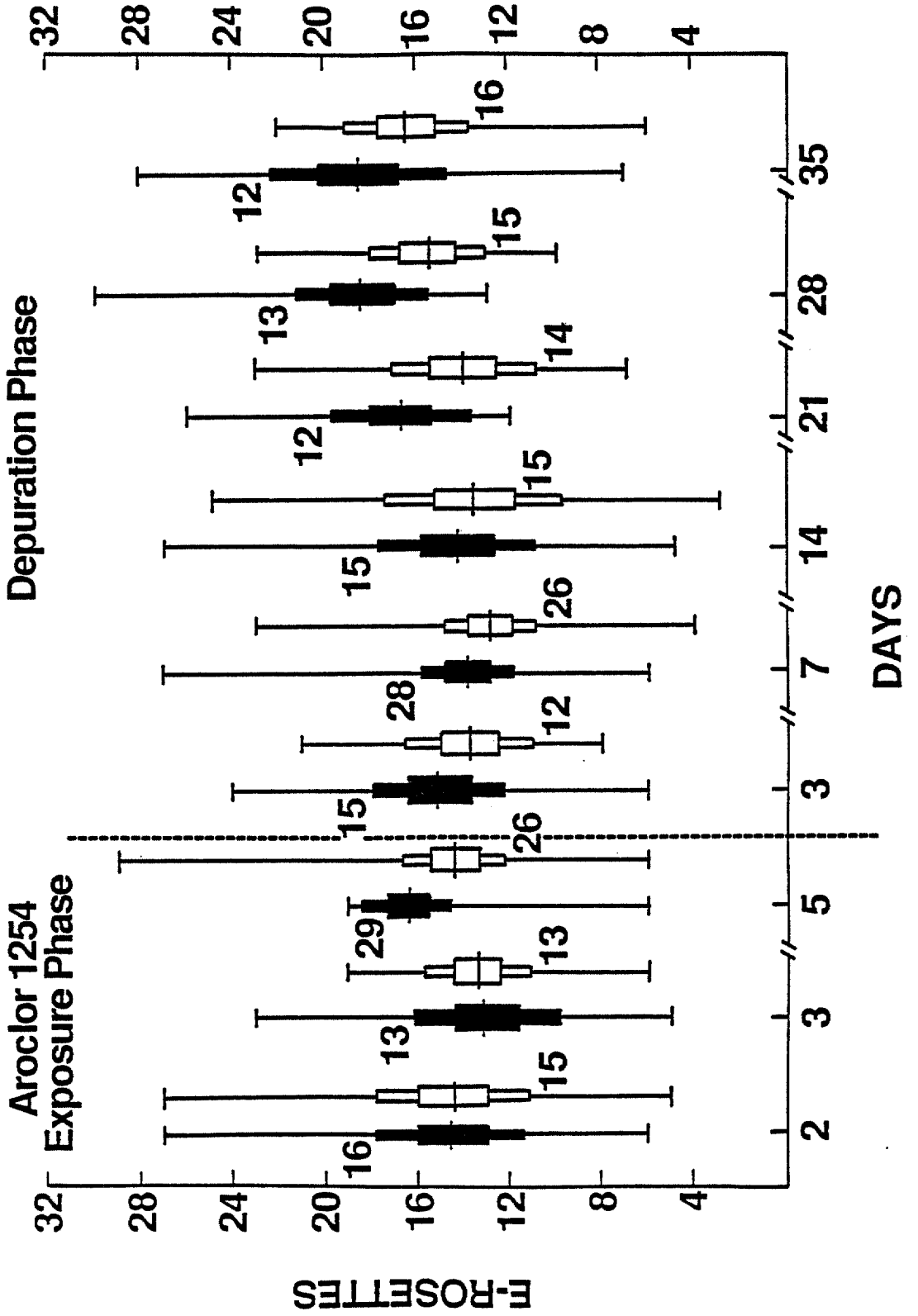
Figure 10.   Comparison of E-rosettes produced by coelomocytes during concentration series experiments for earthworms L. terrestris in a 5-d exposure period and no depuration allowed. Symbols same as previous Figure.
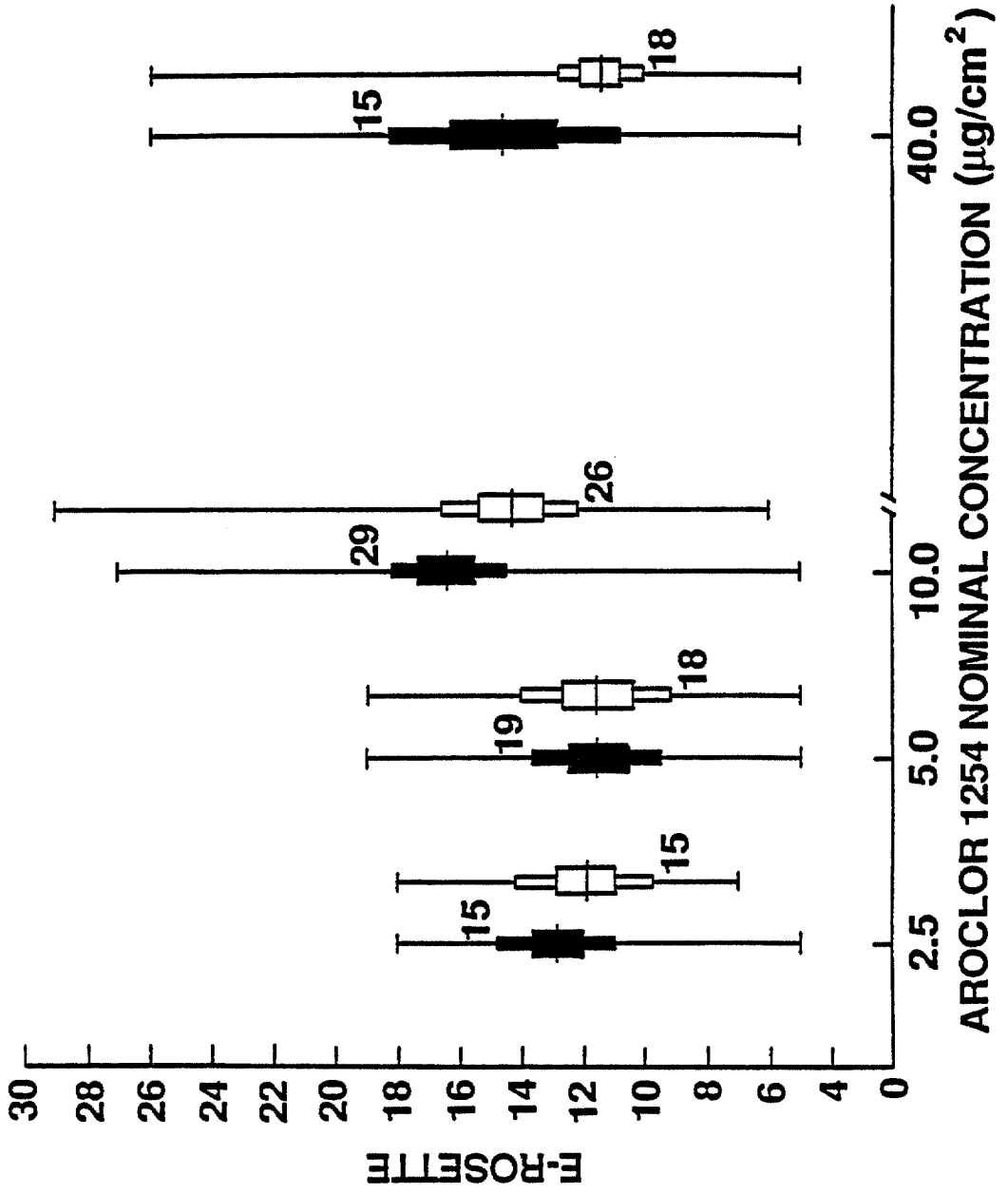
E-rosette counts in experimental worms (overall $\bar{x}$= 12.4%; sd= 1.36%) than in controls (overall $\bar{x}$= 13.9%; sd= 2.05%) were obtained. A nested ANOVA demonstrated no significant differences between control and experimental groups among the individual exposure levels of the concentration series experiments (F= 1.61, p= 0.1743).

TABLE 11. Descriptive statistics of E-rosette formation between controls and experimentals in concentration series immunoassays

| Nominal Conc ($\mu$g/cm$^2$) | Control ($\bar{x} \pm$ sd) | N | Experimental ($\bar{x} \pm$ sd) | N |
|---|---|---|---|---|
| 2.5 | 12.93 ± 3.43 | 15 | 11.93 ± 3.75 | 15 |
| 5.0 | 11.68 ± 4.40 | 19 | 11.61 ± 4.84 | 18 |
| 10.0 | 16.41 ± 4.85 | 29 | 14.38 ± 5.38 | 26 |
| 40.0 | 14.60 ± 6.77 | 15 | 11.50 ± 2.64 | 18 |

S-rosette Immunoassays

Mean S-rosette counts for control and experimental worms in the time series immunoassay experiments (Table 12; Fig. 11) were highly significantly different (Nested ANOVA; F= 6.80, p<0.0001). Since multiple range tests were not appropriate for testing significance between each treatment pair (controls and experimentals), a series of two tailed Student t-tests were conducted. These showed that differences in mean S-rosette counts for experimentals and

controls were highly significant at 72 and 120 h of the uptake period (t= 3.93, p= 0.0006; t= 5.87, p<0.0001, respectively) and at 3-d of the depuration phase (t= 4.63, p<0.0001). Immune response, as indicated by S-rosetting was unaffected at 48 h of exposure and after 1 wk of depuration.

TABLE 12. Descriptive statistics of S-rosette formation betweeen controls and experimentals in time series immunoassay experiments

| | | Group | | | |
|---|---|---|---|---|---|
| Time (Days) | Phase | Control ($\bar{x} \pm sd$) | N | Experimental ($\bar{x} \pm sd$) | N |
| 2 | Uptake | 13.44 ± 5.12 | 16 | 12.00 ± 5.17 | 15 |
| 3 | | 13.57 ± 4.93 | 14 | 7.36 ± 3.27 | 14 |
| 5 | | 13.79 ± 5.11 | 29 | 6.23 ± 4.36 | 26 |
| 3 | Depuration | 14.53 ± 5.08 | 15 | 6.42 ± 3.68 | 12 |
| 7 | | 11.50 ± 5.80 | 28 | 9.96 ± 5.28 | 26 |
| 14 | | 12.53 ± 6.56 | 15 | 15.27 ± 5.93 | 15 |
| 21 | | 12.83 ± 5.18 | 12 | 13.29 ± 5.90 | 14 |
| 28 | | 16.85 ± 6.05 | 13 | 13.87 ± 4.49 | 15 |
| 35 | | 13.17 ± 5.01 | 12 | 12.75 ± 5.57 | 16 |

A highly significant difference between control and experimental mean S-rosettes (Table 13, Fig. 12) was found during concentration series experiments (Nested ANOVA; F= 18.94, p<0.0001). Two tailed t-tests showed highly significant differences between controls and experimentals exposed to 10 and 40 $\mu g/cm^2$ (t=5.87, p<0.0001; t=4.61, p<0.0001, respectively), and significant differences

Figure 11.  Comparison of S-rosettes produced from
experimental L. terrestris exposed to PCBs and controls
during time series experiments. Symbols same as Figure 9.
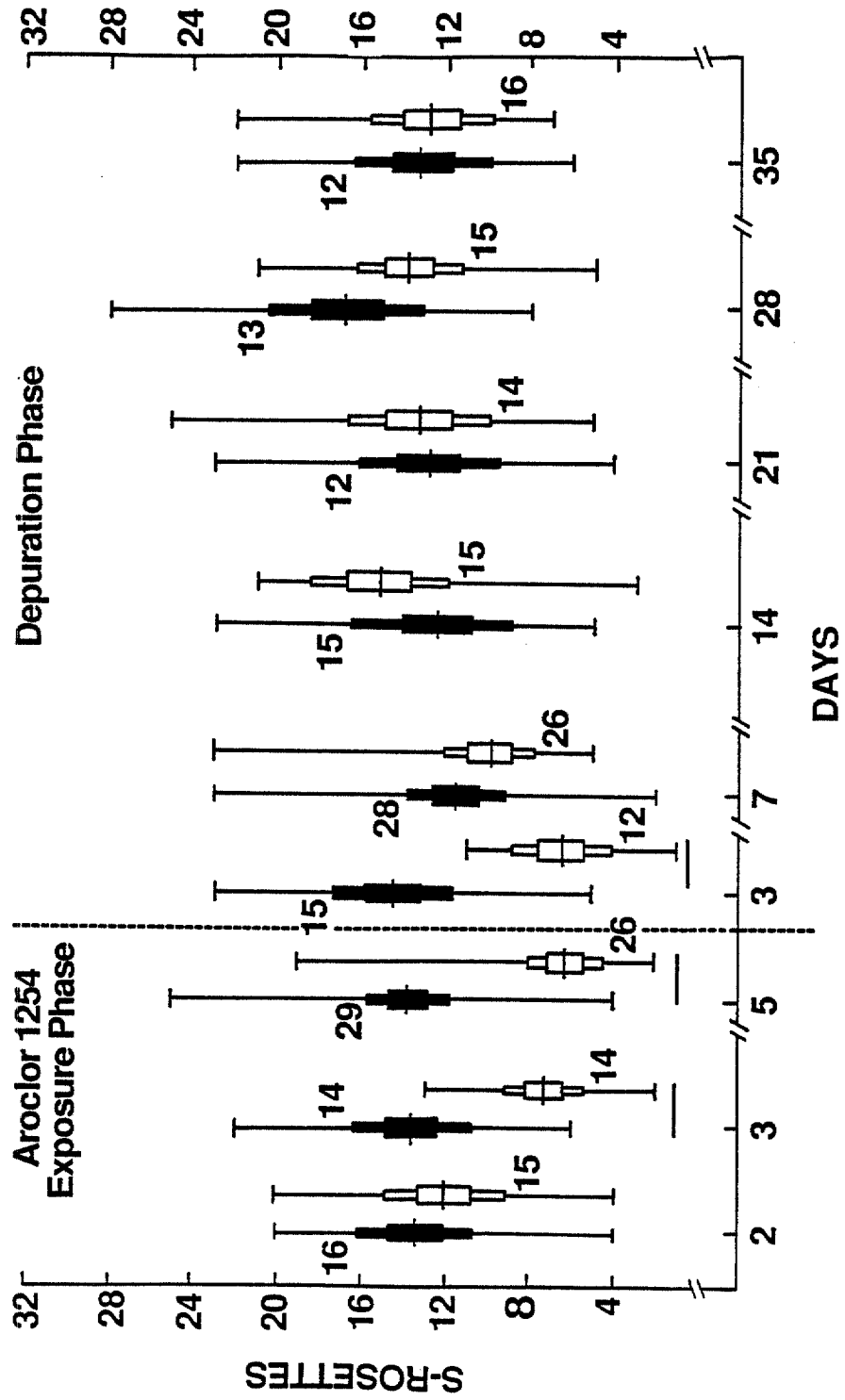
(t=2.63, p= 0.0125) between controls and experimentals

exposed to 5 $\mu$g/cm². In addition, a highly significant

correlation (r= -0.59, p < 0.001) was found between nominal

concentrations and experimental % S-rosettes indicating an

actual dose-response for the range 2.5-10 $\mu$g/cm² in which

subsequent increased effects of PCBs were observed (Fig 12).

Figure 13 shows that the general pattern of S-rosette

formation by coelomic leukocytes from PCB-exposed earthworms

during uptake/ depuration (Fig. 11), followed actual PCB

concentrations in coelomic cells (Fig. 5). Although PCBs

were below detection at 48 h of exposure, S-rosette

formation was 88% normal. By 64 h, when PCB was first

detected, S-rosettes were only 55%. Maximal suppression (45%

normal) occurred at 5-d, coincident with the highest PCB

TABLE 13. Descriptive statistics of S-rosette formation
between controls and experimentals in
concentration series immunoassays

| Nominal Conc ($\mu$g/cm²) | Group | | | |
|---|---|---|---|---|
| | Control ($\bar{x}$ ± sd) | N | Experimental ($\bar{x}$ ± sd) | N |
| 2.5 | 11.73 ± 3.61 | 15 | 12.93 ± 2.99 | 15 |
| 5.0 | 12.32 ± 3.59 | 19 | 9.44 ± 2.99 | 18 |
| 10.0 | 13.79 ± 5.11 | 29 | 6.23 ± 4.36 | 26 |
| 40.0 | 12.67 ± 5.56 | 15 | 5.39 ± 3.43 | 18 |

level in the coelomic leukocytes. Recovery of the humoral

Figure 12.    Comparison of S-rosettes produced by
coelomocytes from experimental Lumbricus terrestris exposed
to PCBs in a 5-d contact test with no depuration and
controls, during concentration series experiments.    Symbols
same as Figure 9.

S - ROSETTES

AROCLOR 1254 NOMINAL CONCENTRATION ($\mu g/cm^2$)
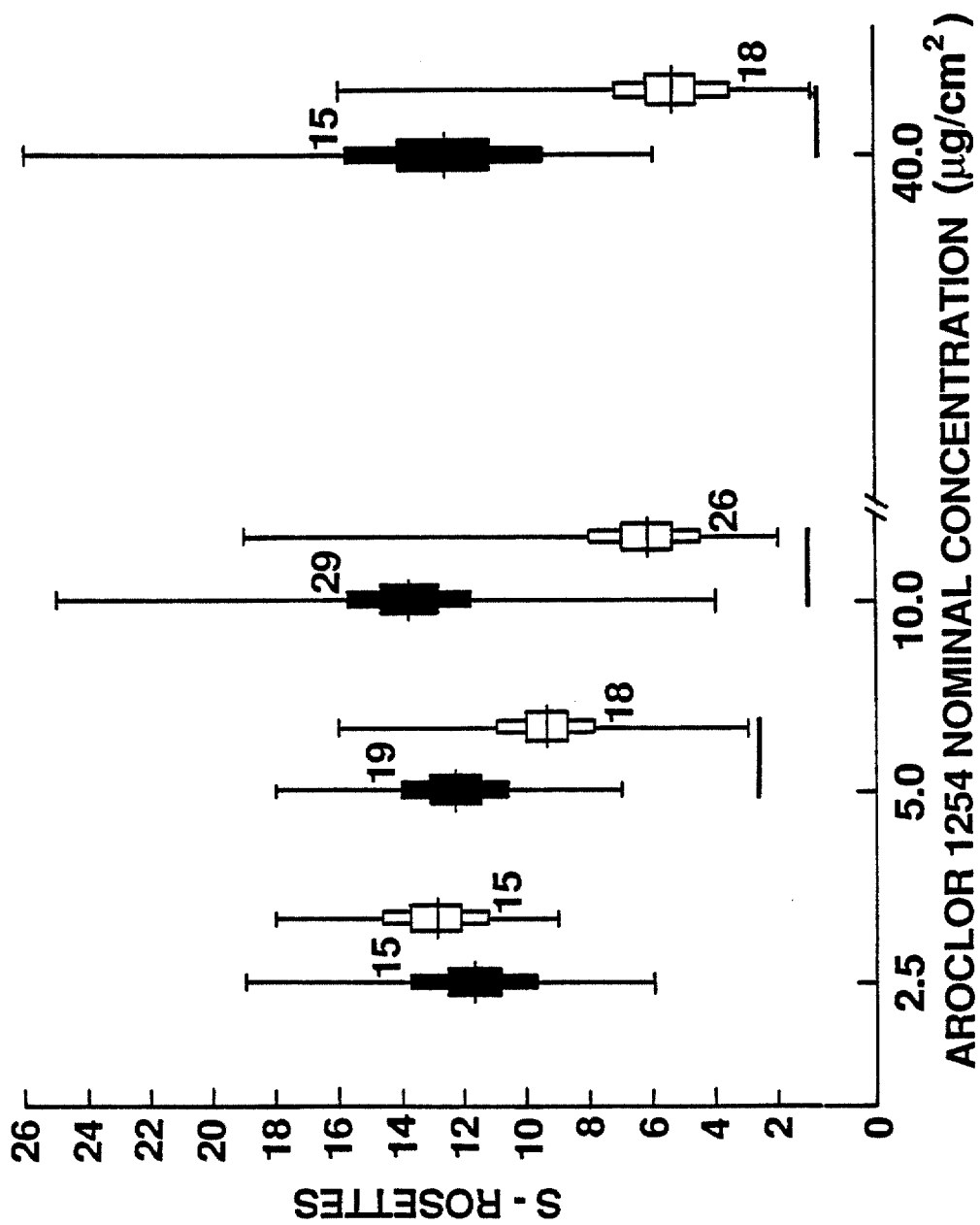
Figure 13. General relation between humoral immune function
as demonstrated by S-rosette (shaded circles) and levels of
PCBs (open circles) in coelomic leukocytes from earthworms
L. terrestris. S-rosettes are expressed as % normal rosette
formation by unexposed/control earthworms (i.e. 14 per 100
randomly - counted coelomic leukocytes; N= 154 controls).
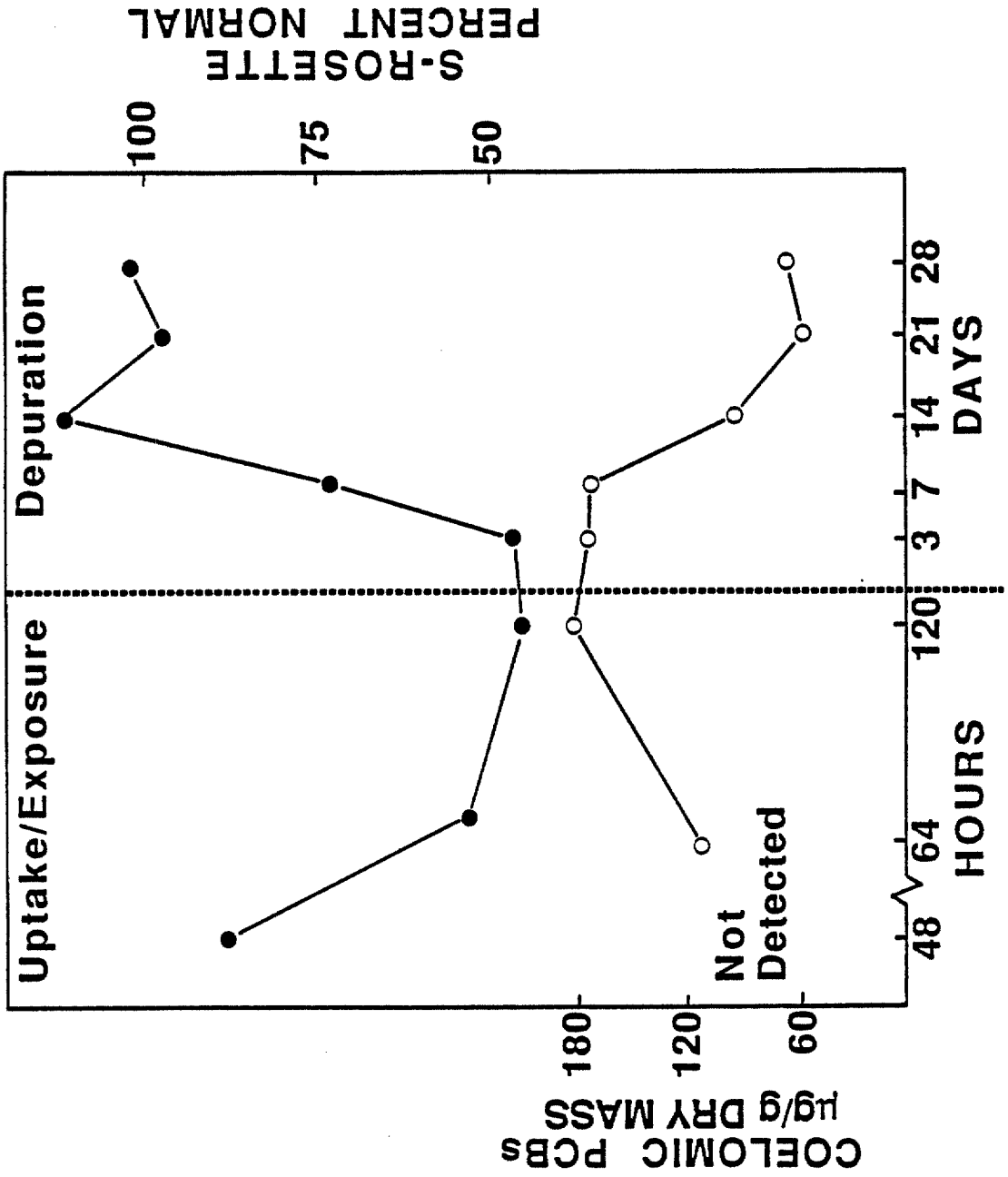
immune function was evident at 7-d post exposure (73%

normal) and complete by the second wk.

Phagocytosis Immunoassays

There were significant differences (Nested ANOVA: F=

2.29, p= 0.0226) in mean number of phagocytes between

experimentals and controls during time series experiments

(Table 14 and Fig. 14). A series of independent two tailed

t-tests confirmed that only the 120 h uptake control and

experimental groups were actually contributing to the

overall significant difference (120 h groups: t= 4.75,

p<0.0001).

TABLE 14. Descriptive statistics of phagocytosis
between controls and experimentals in
time series immunoassays

| Time (Days) | Phase | Group | | | |
|---|---|---|---|---|---|
| | | Control ($\bar{x} \pm sd$) | N | Experimental ($\bar{x} \pm sd$) | N |
| 2 | Uptake | 7.79 ± 3.83 | 14 | 6.23 ± 4.17 | 13 |
| 3 | | 6.64 ± 3.71 | 14 | 6.64 ± 3.13 | 14 |
| 5 | | 6.77 ± 2.97 | 26 | 3.30 ± 1.96 | 23 |
| 3 | Depuration | 4.27 ± 2.52 | 15 | 3.75 ± 2.01 | 12 |
| 7 | | 3.08 ± 2.07 | 12 | 3.83 ± 2.04 | 12 |
| 14 | | 5.33 ± 3.24 | 15 | 5.80 ± 3.93 | 15 |
| 21 | | 3.75 ± 2.86 | 12 | 4.43 ± 4.22 | 14 |
| 35 | | 5.80 ± 2.94 | 10 | 5.58 ± 2.11 | 12 |

Experimental and control worms had an overall mean

phagocytosing coelomocytes of 2.8 and 4.0% respectively

Figure 14. Comparison of phagocytic coelomocytes from
experimental <u>L</u>. <u>terrestris</u> exposed to PCB and controls
during time series experiments.  Symbols same as Figure 9.
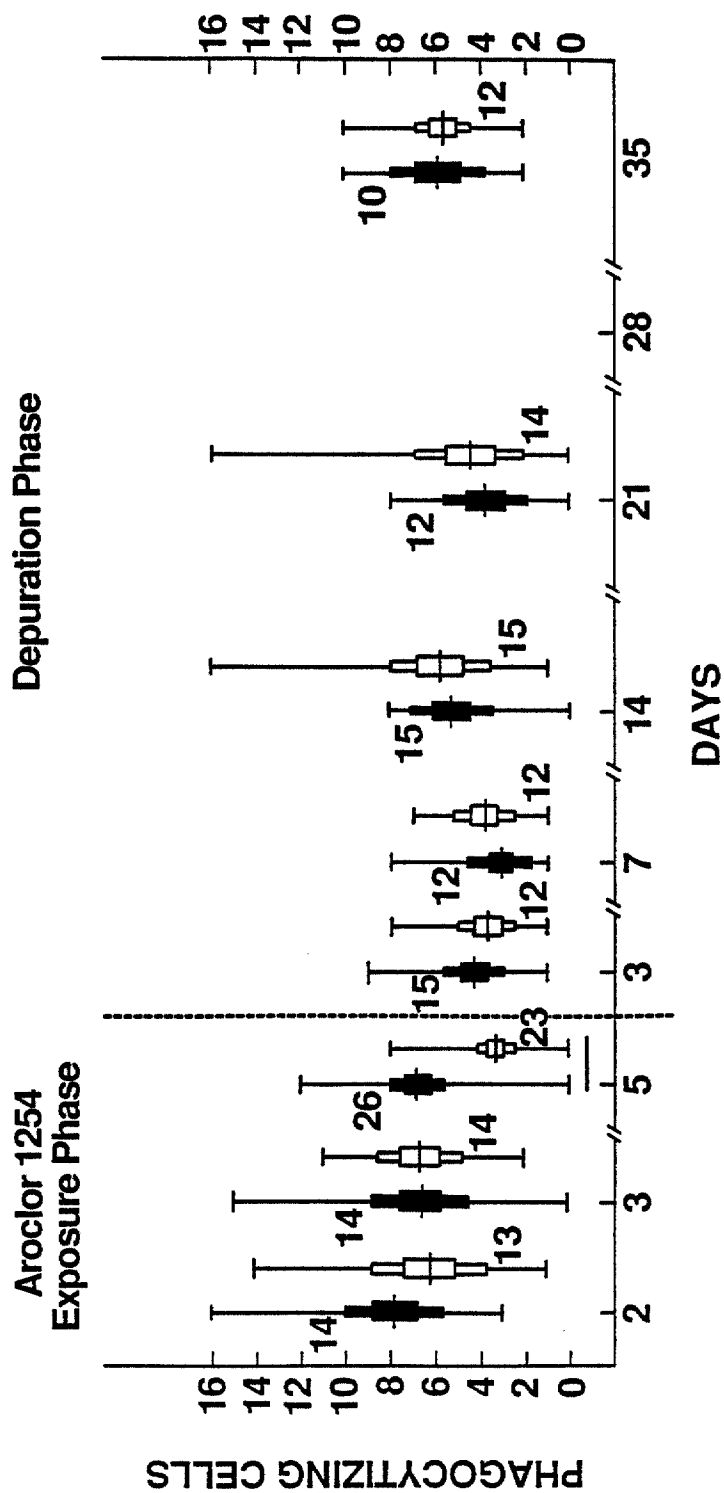
Figure 15. Comparison of phagocytic coelomocytes from experimental L. terrestris exposed to PCB for 5-d with no depuration and controls during concentration series experiments. Symbols same as Figure 9.
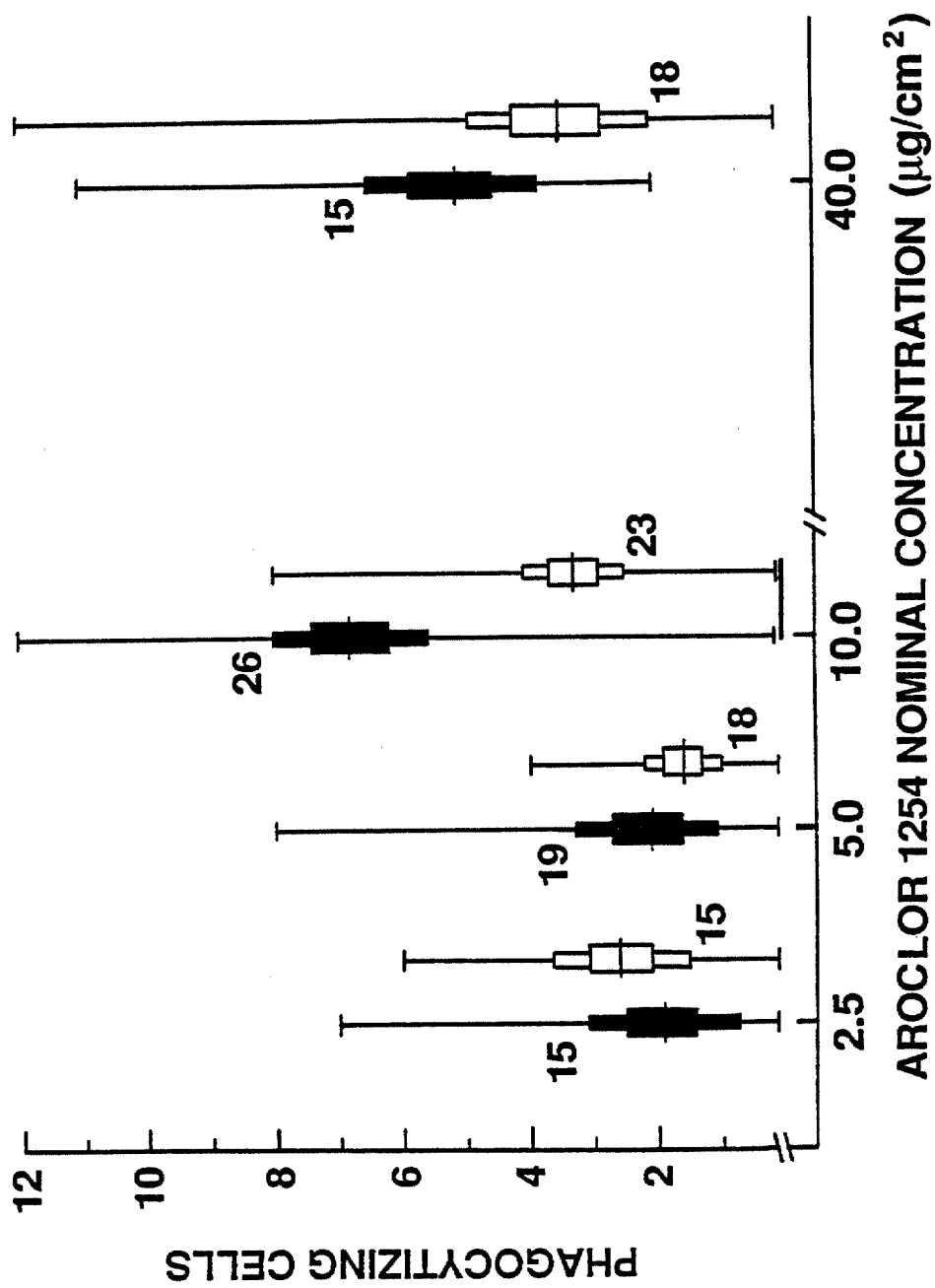
during concentration series experiments (Table 15; Fig. 15).

Differences in mean numbers of phagocytozing cells between

controls and experimentals were highly significant (Nested

ANOVA; F= 7.85, p<0.0001). Two tailed t-tests applied to

each exposure concentration showed that highly significant

differences occurred between controls and experimentals

exposed to 10.0 $\mu g/cm^2$ (t= 4.75, p<0.0001). However two

tailed t-tests for the groups exposed to 40.0 $\mu g/cm^2$, a

higher concentration, indicated no significant difference

TABLE 15. Results of descriptive statistics for phagocyte
counts during concentration series immunoassays

| Nominal Conc ($\mu g/cm^2$) | Group | | | |
|---|---|---|---|---|
| | Control ($\bar{x} \pm$ sd) | N | Experimental ($\bar{x} \pm$ sd) | N |
| 2.5 | 1.93 ± 2.15 | 15 | 2.60 ± 1.92 | 15 |
| 5.0 | 2.21 ± 2.42 | 19 | 1.61 ± 1.29 | 18 |
| 10.0 | 6.77 ± 2.97 | 26 | 3.30 ± 1.96 | 23 |
| 40.0 | 5.13 ± 2.53 | 15 | 3.50 ± 2.87 | 18 |

between experimentals and controls (t= 1.71, p= 0.0964). If

the 40.0 $\mu g/cm^2$ treatment is tested using a one tailed t-

test the resulting gain in significance would indicate a

significantly lower mean in experimentals than in controls

(0.05>p>0.025).

## Respirometry Results

Mean mass-specific $O_2$ consumption rates ($\dot{V}O_2$) were
determined (Table 16 and Fig. 16) for earthworms handled in
three different treatments: (1) Those taken directly from
the culture chambers; (2) controls taken through a 5 day
contact test but not exposed to the toxicant; and (3)
experimental worms exposed to 10.0 $\mu g/cm^2$ of PCB. The $\dot{V}O_2$
means (Table 16) did not differ significantly from each
other (One-Way ANOVA: F= 1.03, p= 0.3644). Since these
results could have been influenced by mass differences among
groups, the mean weights (Table 16) of the three groups were
statistically compared (One Way ANOVA). Results of these

TABLE 16. Mass-specific oxygen consumption ($VO_2$) for
L. terrestries showing the results for
culture, control and experimental worms

| Group | $\dot{V}O_2$ ($\bar{x} \pm$ sd) ($\mu L/g/h$) | N | Live Mass (g) ($\bar{x} \pm$ sd) |
|---|---|---|---|
| Culture | 31.94 ± 8.64 | 12 | 3.76 ± 0.43 |
| Control | 29.15 ± 3.83 | 18 | 3.91 ± 0.76 |
| Experimental | 31.13 ± 4.46 | 18 | 3.91 ± 0.90 |

comparisons showed there were no significant differences
among culture, control and experimental worms (F= 0.17, p=
0.8420).

Figure 16. Mass specific $O_2$ consumption rates ($\dot{V}O_2$) in earthworms $\underline{L}$. $\underline{terrestris}$ exposed to 10 µg/cm² nominal concentration (experimentals), control worms used during the experiments, and culture worms not subjected to a contact test. Symbols same as Figure 9.
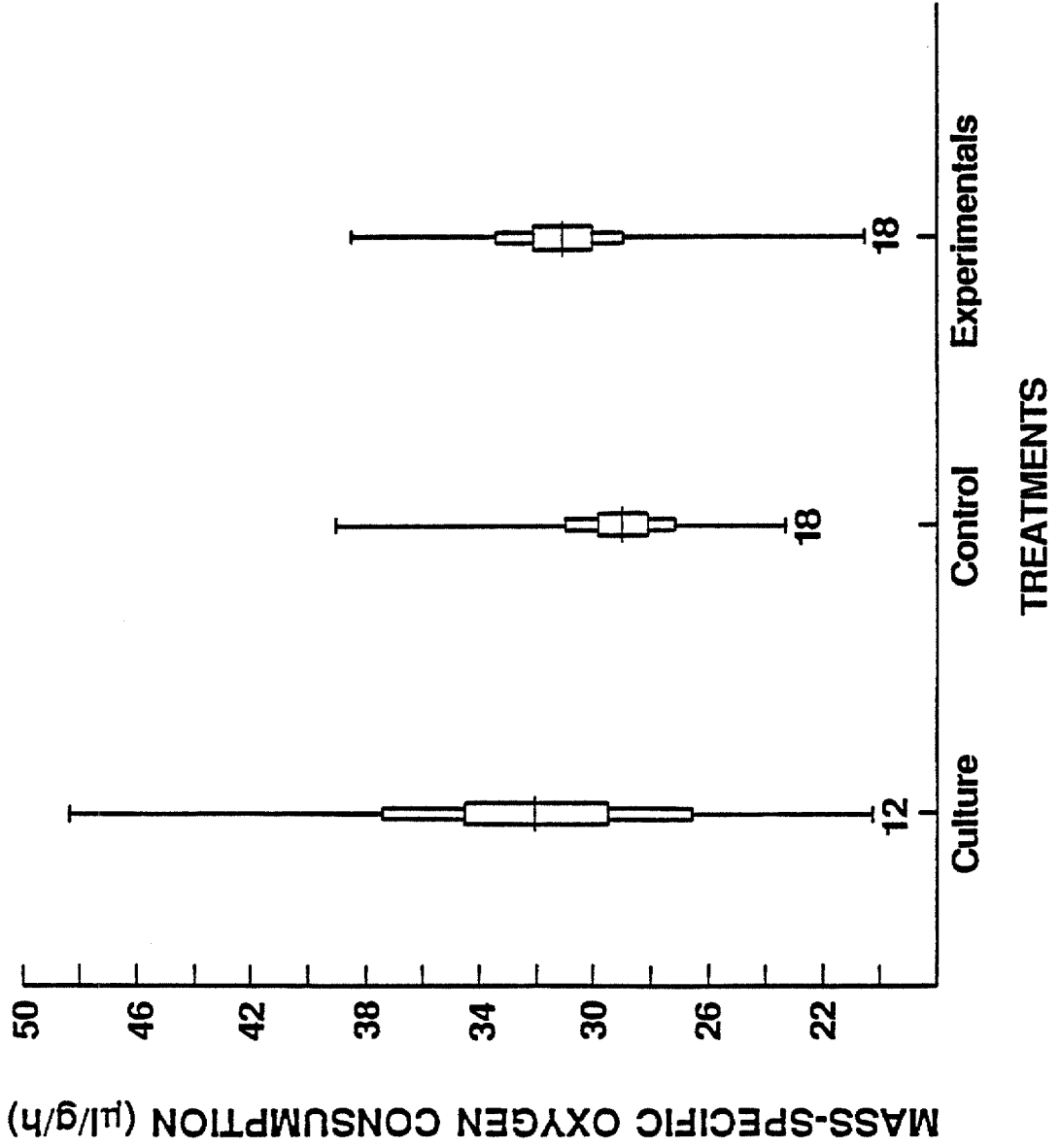
CHAPTER V

DISCUSSION

Filter paper exposure resulted in consistent body
burdens of PCBs. However, because exposure concentrations
were nominal, only the depuration rate constant could be
calculated. Both the constant and half-life presented here
(-0.026/d and 27-d, respectively) compare closely with those
for PCBs (-0.022/d and 31-d) reported for the polychaete
Capitella capitata [94]. PCBs absorbed by earthworm
carcasses were readily transferred into the coelomic
leukocytes, presumably where immunotoxic effects occurred.
Although, mass-specific PCB levels were equivalent in the
coelomic cellular fraction and carcass tissue, the absolute
amount in the coelomic leukocytes was only ca. 1% of the
total body burden at 5-d exposure.  Coelomic cells represent
a minor portion of an earthworm's dry mass, while total
fluids in L. terrestris represent approximately 80% the
total fresh mass; of this, the great majority is coelomic
fluid. Undetectable levels of PCB in the fluid portion of
coelomic material compartment, and distribution within it
being essentially restricted to coelomocytes, can be
explained by the lipophilic nature of PCBs. They are highly
hydrophobic, and thus, their disposition and compartment

bioavaliability is dependent upon lipid distribution. Since in coelomic material the cellular component constitutes the major lipid source (cellular membranes, vacuoles and granules), cellular distribution of PCB should be expected to take place.

However, two aspects that remain unanswered in the present work are the distribution of PCBs among the different cell populations and translocation of PCB from carcass to the different populations or sub-populations of coelomic leukocytes. Liebmann [95] recognized two distinct types of coelomocytes: the eleocytes or chloragogen cells and true leukocytes (mostly basophils and acidophils according to Cooper and Stein [85]). Chloragogen cells lack immune function, they are involved principally in excretory and nutritive functions, and contain considerable quantities of lipids (61%), proteins and glycogen [85]. For PCBs to cause any immunological effect, sufficient bioconcentration would have to take place within leukocytic cells which perform essential immunological functions. Concentrations of PCBs, approximating those found in carcass tissue, were observed in the coelomic material cellular fraction and is assumed to be associated with coelomocytes. However, this "coelomocyte" concentration may have been overestimated due to the presence of high-lipid-content chloragogen cells which appear to be over-represented when coelomic fluid is collected by puncture. Although a formal comparison of the

abundance of chloragogen cells present in puncture and extrusion techniques was not made, preliminary observations indicate that the % chloragogen cells obtained by extrusion is much lower than that obtained by puncture (5% vs. 15%, respectively).

Chloragogen cells may also play an important role in PCB translocation, the second aspect mentioned above. A possible mechanism of translocation could be transfer of PCBs from previous accumulation in lipid reserves of chloragogen cells to leukocytes. Chloragogen tissue is formed in the visceral peritoneum which surrounds the intestine, most of the dorsal blood vessels and smaller vessels leading from the intestine to the dorsal vessel [85]. Translocation of PCBs from carcass to chloragogens may initially take place as a result of migration of maturing chloragogen cells. When mature, some chloragogens gain access to the coelomic fluid, and once there, are frequently called eleocytes [95]. Subsequently, eleocytes undergo degeneration, thus releasing their granules into the coelomic cavity [95]. Some free chloragogen granules are known to be incorporated into leukocytic coelomocytes [85], which might explain bioconcentration of PCBs in those cells. Excretion of PCBs could ultimately occur by direct removal of granules by the nephridia and subsequently discharged through the dorsal pores, a route of detoxification known to take place during metal contamination of earthworms [96].

There is need for further research in this area to clarify the translocation routes and distribution of PCBs within the cell components of coelomic fluid. A promising approach would be to trace PCB pathways with radio-labeled mixtures or congeners.

Based on Roberts and Dorough's [12] classification for acute toxicity in the manure worm Eisenia foetida, PCBs were moderately toxic in L. terrestris. Differences in exposure and/or dosing protocols, and general absence of actual tissue or lesion-site concentration data in the literature precludes direct comparisons of PCB toxicity between L. terrestris and other organisms. Direct comparisons and clear interpretation of toxic effects of xenobiotics are best made when actual tissue doses are known. In general, $LD_{50}$ values (oral route) for PCBs range between 1000-10,000 ppm for rats, rabbits and mice, with toxicity directly related to the degree of chlorination of PCBs [78]. The actual entire-body PCB $LD_{50}$ in L. terrestris, when converted to fresh mass (1140 ug/g dry mass x 0.22 = 251 ug/g fresh mass), is considerably lower than oral (not tissue-level) Aroclor 1254 $LD_{50}$ values reported for pheasant (1100 ppm), bobwhite quail (600 ppm), Japanese quail (2900 ppm) and rats (10,000 ppm) [68]. However, it is quite similar to the actual tissue $LD_{50}$ (280 ppm Aroclor 1254) reported for bobwhite quail [78]. Greater acute sensitivity than laboratory rats to a variety of toxic compounds has also been reported for E. foetida

[97].

PCB effect on humoral immune function of $\underline{L}$. terrestris appears to have been direct as indicated by the parallel fluctuations of decrease/increase of S-rosette formation by coelomic leukocytes with a corresponding increase/decrease in coelomic PCB levels (Fig. 13). Cell viability and general physiology, as measured by $\dot{V}O_2$, were not affected by sublethal levels of PCB. $\dot{V}O_2$ values were not only nearly equivalent among control and experimental groups, they were consistent with 10°C rates reported previously by Fitzpatrick et al. [98] for unexposed healthy worms. S-rosette formation indicates the ability of coelomic leukocytes to produce an agglutination factor in response to foreign challenge such as bacterial or fungal infection. This humoral factor serves to aggregate particular antigen and acts as an opsonin to facilitate phagocytosis. As such, it is functionally analogous to immunoglobulin in mammals. Thus, PCB effects on S-rosetting in $\underline{L}$. terrestris may be considered analogous to those reported for Aroclor 1254, 1260 and 1248 on immunoglobulin production in rabbits [59], guinea pigs [58, 99] and, monkeys and mice [60], respectively.

Threshold humoral immunosensitivity (76 ug/g dry mass) to PCBs was much lower than that of acute toxicity (834.2 ug/g dry mass). In terms of fresh tissue mass, the threshold level (ca. 17 ug/g) is within the same order of magnitude

(1-10 ppm) of PCB level reported for a variety of
invertebrate and vertebrate wildlife from contaminated sites
[100]. Although, as with acute toxicity, direct comparison
of humoral immuno-sensitivity between S-rosette formation in
L. terrestris and immunoglobulin synthesis in mammals
requires knowing lesion-site tissue levels, indirect
comparison with Rhesus monkeys and laboratory mice [60]
suggests that earthworms may be more sensitive to PCBs.
Monkeys and mice required PCB levels, as measured in
subcutaneuos and inguinal fat tissue, of 100 and 3760 ppm to
suppress immunoglobulin production. Thus, it appears that S-
rosette formation in L. terrestris is a potentially
sensitive assay for screening  xenobiotics for their humoral
immunotoxicity to mammals. Validation of this and other
immunoassays, using several different compounds, and
comparing their toxicities between earthworms and laboratory
rodents (i.e. earthworm dose-equivalents) must be carried
out in future laboratory work. If immune functions in
earthworms should prove to be more sensitive than those in
mammals, earthworm immunoassays may produce more false
positives than false negatives. However, as McLain et al.
[97] pointed out for using E. foetida to predict acute
toxicity in mammals, it is better to err in the conservative
direction.

PCBs apparently had no effect on cell-surface receptors
for RRBCs, as determined by the ability of coelomic

leukocytes to consistently form E-rosettes throughout the experiments. E-rosette formation is a response that has been described for mice and human lymphocytes [101, 102]. In mammals, E-rosette formation is used for identification of T-lymphocytes [103]; the ability of lymphocytes to form rosettes with sheep red blood cells is a unique function of T-cells. Immunological studies on chronically exposed humans, conducted after the 1979 PCB poisoning in Taiwan ("Yu-cheng" disease), showed a significant reduction in T-lymphocyte numbers as indicated by a decrease in E-rosettes [80]. This could represent an indirect evaluation of the percent E-rosettes being formed, an analogous approach to the one presented here. However, a direct comparison of Yu-cheng results with L. terrestris data is not possible because the mechanisms for rosette formation in mammals and earthworms may be entirely different. In addition, it is not clear if a decrease in E-rosette formation on Yu-cheng patients was due to an alteration of cellular function or an actual decrease in cell numbers [62, 80]. In mammals, it has been shown that E-rosette formation requires T-cells to display intact metabolic pathways for glycolysis, and protein and nucleic acid synthesis [103]; also, hormonal regulation of intracellular cyclic nucleotide levels influences E-rosette formation [103, 104]. Although it is suspected that E- and S-rosette formation in earthworms might be associated with specific populations or sub-

populations of coelomocytes, this has not been proved. The
inability of Aroclor 1254 to suppress L. terrestris E-
rosettes could be viewed as an indirect proof that the
mechamisms of E-rosette formation is different from that of
mammals. If this is not the case, the cause for unaffected
E-rosette formation could be related to inadequate test
conditions (see Mendes, et al. [105] for technical aspects
of rosette tests in humans), an actual lack of dose-response
effect for this immune parameter at the given PCB
concentrations, or, a characteristic inherent to PCBs which
make this chemical unable to specifically alter surface
receptors of coelomocytes to RRBCs.

Phagocytic response also showed suppressive effects by
a reduction in mean number of phagocytosing leukocytes in
experimental worms. However, throughout the experiments the
phagocyte numbers were unusually low compared to published
work [53]. In addition, while significant differences were
only found at 5-d exposure during time series experiments,
there were other control values during depuration (Fig. 14)
showing similarly low phagocytosing leukocyte counts as the
lowest levels found for experimental worms. Thus, these
inconsistencies do not support a demonstration of suppresive
effects of PCBs on phagocytosis. Rather than assuming the
inadequacy of phagocytosis as a suitable immune response for
assessing immunotoxicity, it could well be an indication for
the need to improve the assay conditions to enhance the

phagocytic response. Throughout this work, the experimental
conditions for assaying rosettes and phagocytosis were
essentially the same (incubation at 4°C and 24 h of
incubation period). By doing so, I was able to conduct
rosette and phagocytic counts simultaneously. However,
optimal conditions for rosette formation and phagocytosis
may be different. An incubation period of 24 h is probably
enough to allow significant phagocytosis to take place,
although longer incubation time facilitates better contact
between leukocytes and RRBCs thus increasing the chances of
phagocytosis and rosette formation. Temperature of
incubation, however, plays a more important role in
improving conditions for a phagocytic response. There has to
be an optimal temperature at which phagocyte pseudopodia
mobility is enhanced. In addition, the use of glass test
tubes during the collection of coelomocytes could have also
influenced the variability of phagocytosing cell counts
observed. One feature of phagocytic cells is their ability
to adhere to glass [53]. Although efforts were made to keep
the test tubes at a temperature of 4°C at all times to
reduce this effect, temperature differences often occur
during the transfer of cells which could cause cell
adherance, and thus differential loss of phagocytic cells
throughout the different assays periods. More work in these
technical aspects is needed to improve the assay conditions
to enhance phagocytic response. Further research should

also focus on measuring other associated phagocytic response such as baterial killing and phagocytic spreading. Inclusion of other phagocytic measurements and improvement of assay conditions may prove phagocytosis to be as a suitable a response as S-rosette formation was in the present work.

Another important area for future research is congener-specific immunotoxicity. Aroclor 1254 is a specific mixture of isomers and congeners of PCBs. According to Sawhney [106], it is known that components of Aroclor 1254 are essentially tetrachlorobiphenyl 11% (molecular weight 292 g/mol), pentachlorobiphenyl 49% (326 g/mol), hexachlorobiphenyl 34% (361 g/mol), and heptachlorobiphenyl 6% (395 g/mol). Further research should center on investigating the immunotoxic effects of those PCB species for hypothesis testing of increase and/or decrease of percent chlorination/molecular weight on immune competence. However, such an approach will be highly complicated by recognized structure-specific relations of PCBs to toxicity [107]. Different congeners display differential toxic effects according to the degree of chlorination and positions of chlorine atoms in their ring structures [108]. In particular, according to Hansen [66] most toxicity work has focused on the effects of PCB congeners which are approximate isostereomers of 2,3,7,8-Tetrachloro-p-di-Benzodioxin (TCDD). TCDD is generally regarded as the most potent xenobiotic, and thus is used as standard for

comparison for other organic toxicants that are more or less isosteric [108]. As a result, PCB congeners regarded as the most toxic and potent mimics of TCDD are those having no ortho substitution, 2 para and at least 2 meta chlorines [66]. Although most of the work on structure-activity related toxicity has been accomplished by using mixed function oxidase (MFO) activity end points [107, 109-111], such relative toxicity ranking can be helpful in establishing criteria for more closely analyzing the immunocompetence of earthworms exposed to potentially immunotoxic PCB congeners and isomers. This is an important area of research which should reveal the extent to which suppression of MFO activety and immunosuppression may share common mechanisms.

In addition to being sensitive and having certain immune functions analogous to those in mammals, earthworms possess other advantages for studying immunotoxicity of terrestrial pollutants. Their large surface area to volume and feeding behavior facilitate rapid uptake and tissue distribution of chemicals. Tissues are easily separated, prepared and extracted, allowing for rapid determination of actual xenobiotic doses for the corresponding toxic responses. This is especially true for immunoactive coelomic leukocytes, which can be obtained without harm to individual earthworms by extrusion method [92]. Also, earthworm immunoassays should demonstrate direct effects of chemicals

on immunity without the confounding influence of endocrine-related stress, as occurs in vertebrates.

The significance of the observed suppresive effects on L. terrestris is the potential of predicting immunotoxic effects of xenobiotics in mammalian systems. As far as I know, the present work constitutes the first attempt to use an invertebrate model for assessing xenobiotic immunotoxicity. Thus, being able to relate these results with published mammalian studies is a major step towards model development for using earthworms in immunotoxic studies as a predictive tool.

APPENDIX I

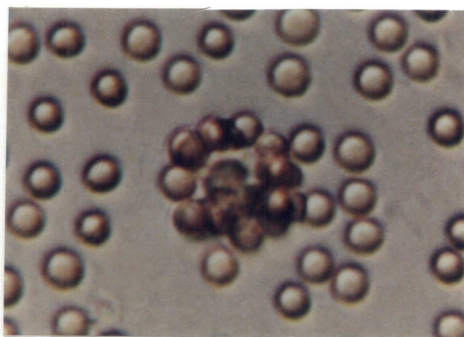(A) Phagocytic Coelomocyte
(B) S-Rosetting Coelomocyte
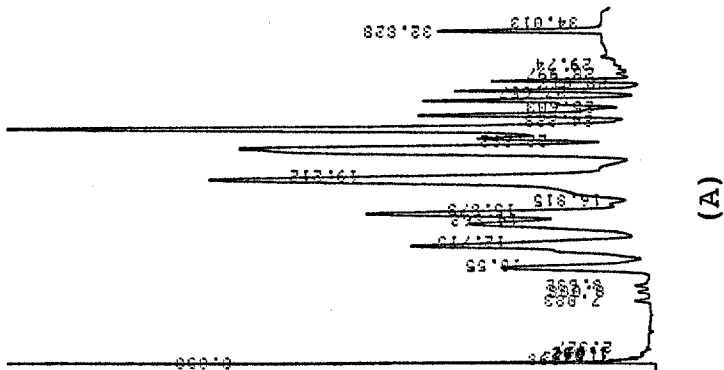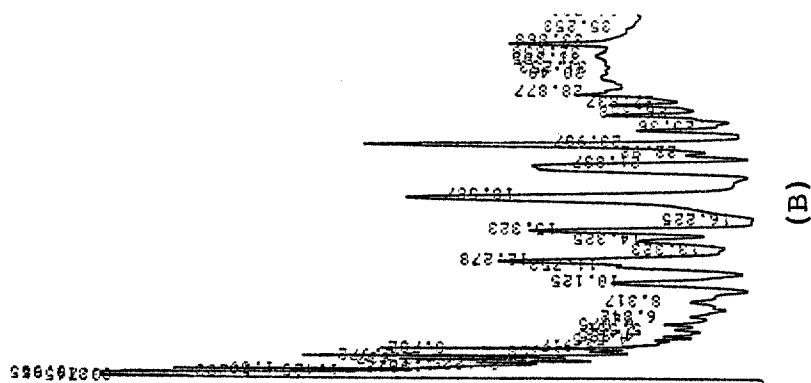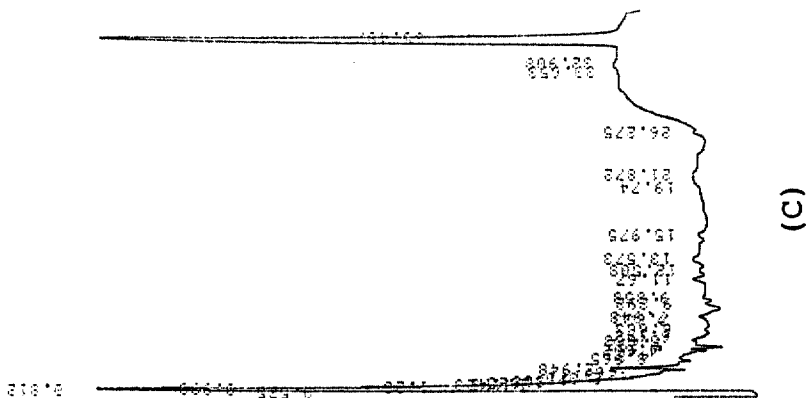(C) E-Rosetting Coelomocyte
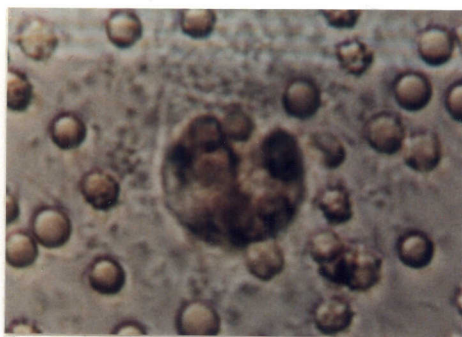
(A)



(B)



(C)

APPENDIX II

(A) Chromatograph of a 50 ppm Aroclor 1254
    Standard (Attenuation: 128)
(B) Chromatograph of an Extract from an Earthworm
    Exposed to 50 $\mu$g/cm$^2$ of Aroclor 1254
    (Attenuation: 128)
(C) Chromatograph of an Extract from a Control
    Earthworm (Attenuation: 64)

(C)

(B)

(A)

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    Earthworms. Chapman and Hill, London.

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